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JAMES COOK UNIVERSITY
AUSTRALIA

The effects of climate change on the *Acropora aspera* holobiont

Thesis submitted by

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January 2014

for the degree of Doctor of Philosophy in Biochemistry

within the Department of Pharmacy and Molecular Sciences and the

ARC Centre of Excellence for Coral Reef Studies

James Cook University

Townsville Queensland Australia

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Statement on the contribution of others

Some of the chapters of this thesis are also manuscripts that have been submitted for publication in peer-reviewed journals or are in preparation for submission. Several researchers have made contributions to these manuscripts and it is necessary to recognise their contribution.

Chapter 2 is a manuscript currently in preparation for submission, co-authored by W. Leggat and P. Fromm. All experiments, sample analysis, and statistical analyses were performed by DO except flow cytometry, which was assisted by P. Fromm. WL provided comments and editing of the manuscript. DO and WL contributed intellectual input into the manuscript.

Chapter 3 was published in the journal Coral Reefs in Dec 2013 (Ogawa 2013). It is co-authored by T. Bobeszko, T. Ainsworth, and W. Leggat. Experiments for this chapter were performed by DO. Sample processing, mRNA isolation, cDNA synthesis, and qPCR were performed by TB. Assistance with histochemistry provided by TA. DO performed qPCR and all other assays and all statistical analyses included in the manuscript. TB contributed methodology section for sample processing, mRNA isolation, cDNA synthesis, and qPCR. WL provided comments and editing of the manuscript. Intellectual input into manuscript by DO and WL.

Chapter 4 is a manuscript currently in review at Applied and Environmental Microbiology, co-authored by D. Bourne, T. Ainsworth, and W. Leggat. Experiments and DNA isolation for this manuscript were performed by DO. A. Muirhead (AIMS) performed PCR, sequencing was done at ACE, and QIIME pipeline sequence analysis performed by DB and DO. B. Gordon assisted with PCA statistical analysis. GLM

statistical analysis by DO. TA, DB, and WL provided comments and editing of the manuscript. All authors provided intellectual input into this manuscript.

Chapter 5 is a manuscript currently in review at Proceedings of the Royal Society B: Biological Sciences and is co-authored by T. Ainsworth, G. Torda, and W. Leggat. TA and WL performed the experiment and sample collection. GT processed samples, isolated mRNA, and performed cDNA synthesis. DO performed all qPCR. Statistical analyses were performed by DO and WL. TA and WL provided comments and editing of the manuscript. DO, TA, and WL contributed intellectual input into this manuscript.

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Publications

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Ogawa, D., Bourne, D., Ainsworth, T., Leggat, W. *Symbiodinium* loss during a simulated bleaching event under elevated pCO_2 is not associated with shifts in bacterial community of *Acropora aspera.*
In review: Applied and Environmental Microbiology.

Ogawa, D., Ainsworth, T., Torda, G., Leggat, W. Sub-lethal pre-stress leads to acclimation response of *Acropora aspera* and *Symbiodinium* during a simulated bleaching event.
In review: Proceedings of the Royal Society B: Biological Sciences.

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Abbreviations

ACE	Australian Centre for Ecogenomics
ADP	advanced digital processing
BLAST	basic local alignment search tool
CA	carbonic anhydrase
CCM	carbon-concentrating mechanism
cDNA	complementary deoxyribonucleic acid
Chl *a*	chlorophyll *a*
Chl *c*	chlorophyll *c*
CNRQ	calibrated normalised relative quantity
CO_2	carbon dioxide
CoCA	coral carbonic anhydrase
CRM	certified reference material
DAB	3,3'-diaminobenzadine
ddH_2O	double-distilled water
DEPC	diethylpyrocarbonate
DGGE	denaturing gradient gel electrophoresis
DIC	dissolved inorganic carbon
DNA	deoxyribunucleic acid
EDTA	ethylenediaminetetraacetic acid
F_m	maximum chlorophyll fluorescence yield
F_o	minimal chlorophyll fluorescence yield
F_v/F_m	dark-adapted yield of chlorophyll fluorescence
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gb	gigabase
GBR	Great Barrier Reef
gDNA	genomic deoxyribonucleic acid
GLM	generalised linear model
GOI	gene of interest
H_2O_2	hydrogen peroxide
HCO_3^-	bicarbonate
$HgCl_2$	mercuric chloride
HSP	heat shock protein
ICG	internal control gene
iPAM	imaging pulse-amplitude modulation
IPCC	Intergovernmental Panel on Climate Change
ITS1	internal transcribed spacer region 1
LSD	least significant difference
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NBS	National Bureau of Standards
NCBI	National Center for Biotechnology Information
NP_c	net production of organic carbon
NPQ	non-photochemical quenching
NTC	no-template control

O_2	oxygen
OA	ocean acidification
OTU	organisational taxonomic unit
PAM	pulse-amplitude modulation
PAR	photosynthetically active radiation
PBS	phosphate-buffered saline
PCA	principal components analysis
PCNA	proliferating cell nuclear antigen
pCO_2	partial pressure of carbon dioxide
PFA	paraformaldehyde
P_n	net photosynthesis
ppm	parts per million
PSII	photosystem II
QIIME	Quantitative Insights into Microbial Ecology
qN	non-photochemical quenching
qP	photochemical quenching
qPCR	quantitative polymerase chain reaction
rDNA	ribosomoal deoxyribunucleic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SD	symbiont density
SST	sea surface temperature
Y(II)	Maximum quantum yield of photosystem II

Abstract

Corals host a community of microbes, comprised of endosymbiotic algae, *Symbiodinium*, bacteria, Archaea, and viruses. This coral/microbe assemblage is known as the coral holobiont. The mutualistic nutrient exchange between coral host and *Symbiodinium* is arguably the most critical and therefore most studied of these associations; energy supplied by the algae contributes the majority of corals' daily energy requirements. The loss of *Symbiodinium* during coral bleaching can potentially be fatal to the host. Unfortunately, mass bleaching episodes have become more frequent with intensifying global climate change. Anthropogenic CO_2 emissions are increasing the concentration of atmospheric CO_2, which causes a greenhouse effect resulting in elevated sea surface temperature. These anomalously high temperatures cause the holobiont to become stressed and triggers expulsion of *Symbiodinium*. Concomitant with temperature rise is the acidification of the surface ocean, caused by uptake of atmospheric CO_2. The interactive effects of these two factors on coral holobiont physiology and community dynamics are largely unknown.

Symbiodinium in vitro and *in hospite* exhibit significant photosynthetic dysfunction above 34 °C, which coincides with the bleaching temperature of *Acropora aspera*. Expression of targeted key photosynthetic and stress response genes of *Symbiodinium* (measured using quantitative PCR) remains unchanged by heat stress, irrespective of symbiosis status (isolated or *in hospite*) and pCO_2. The stress response of *Symbiodinium*, therefore, likely relies on some other mechanism, possibly post-translational modification of transcripts or shifts in the metabolome. Conversely, transcript levels of

key carbon metabolism genes of *A. aspera* are significantly altered by a synergistic effect of pCO_2 and bleaching stress, despite relatively small increases in treatment pCO_2 compared to the diurnal fluctuations experienced by these corals in the reef flat environment. This suggests the physiological effects of ocean acidification will be felt by mid-century, much sooner than predictions made for calcifying processes. Although 34 °C bleaching stress resulted in mass expulsion of *Symbiodinium* in both ambient pCO_2 and elevated pCO_2 treatments, there was no apparent change in the bacterial community. This contradicts many studies that have found an increase in *Vibrio* in bleached corals. These results suggest a relatively static nature of the *A. aspera* bacterial community, implicating that shifts in the bacterial assemblage do not contribute to the environmental response of this holobiont. The ability of corals to acclimate to changing temperature regimes was demonstrated in *A. aspera*. Exposure to sub-lethal temperature stress prior to a bleaching-level stress conferred an acclimatory response in *Symbiodinium* non-photochemical quenching of chlorophyll fluorescence and *A. aspera* antioxidant and heat shock protein gene expression, which was not observed in repeated bleaching-level stresses.

The results of this thesis suggest *Acropora aspera* transcriptional regulation and *Symbiodinium* photophysiology are the key factors in environmental response and *Symbiodinium* transcriptional regulation and bacterial community dynamics play a limited role in temperature and acidification response.

Chapter 1: General Introduction

Introduction

Coral reefs are in global decline (Hughes et al. 2010). The key reef-builders, scleractinian corals, are holobionts, defined as the coral host and its microbial community; these coral holobionts are increasingly being damaged by anthropogenic climate change (Hoegh-Guldberg 2011). Elevated sea surface temperature (SST) and increased acidity of the surface ocean, both caused by a rise in atmospheric CO_2, are two major global threats and have been shown to adversely affect coral holobiont physiology (Kaniewska et al. 2012b). However, the study of synergistic effects of these two environmental factors on corals is still in its infancy. In particular, the transcriptomics of host and endosymbiotic algae and the bacterial community dynamics under high SST and decreased pH are largely uncharacterised. This thesis aims to address this knowledge gap by studying key members of the *Acropora aspera* holobiont: the coral animal, *Symbiodinium* algae, and bacterial consort under future climate change scenarios.

The coral holobiont

The organisms commonly referred to as simply "corals" are actually an assemblage of organisms from several different taxa: the coral animal, endosymbiotic algae *Symbiodinium*, bacteria, fungi, Archaea, and viruses, collectively named the coral "holobiont" (Rohwer et al. 2002). The obligate association between the coral animal and *Symbiodinium* is arguably the cornerstone of the holobiont and is the most well defined of these relationships. Knowledge of the bacterial community has expanded greatly since the application of culture-independent methods of identification, such as

clone libraries, DGGE and high throughput sequencing. There remains a dearth of information about the other holobiont constituents, although recent studies have worked to fill this knowledge gap (Wegley et al. 2004; Wegley et al. 2007; Littman et al. 2011).

Coral host

Coral colonies are comprised of individual polyps that are connected by live tissue (coenosarc). Long thought to be genetically identical, a recent study has shown genetic variation within a single colony of *Seriatopora hystrix* (Maier et al. 2012b). Coral polyps have a relatively simple body structure made up of 2 cell layers, the epithelium and the gastroderm, which are connected by an acellular layer (mesoglea) (Figure 1.1). The calicoblastic epithelium secretes an organic matrix and calcium and bicarbonate ions to the site of calcification where calcium carbonate is formed, a process known as biomineralisation (Allemand et al. 1998) (Figure 1.1). This creates the calcium carbonate skeleton that is the structural basis for coral reef formation (Allemand et al. 2004). Although coral polyps are capable of feeding heterotrophically, generally, most of their nutrition is derived from their algal symbionts, *Symbiodinium* (Muscatine 1990).

Symbiodinium

Once thought to be a single species, *Symbiodinium microadriaticum* (Taylor 1969), it has since been discovered that *Symbiodinium* comprise several species (Trench and Blank 1987), which are phylogenetically diverse, spanning 9 clades (designated A-I and include numerous sub-clades, or types) (LaJeunesse 2001; Pochon and Gates 2010). In the Caribbean and Indo-Pacific regions, most coral/*Symbiodinium* associations are dominated by clade C; Caribbean corals also commonly associate with clades A and B (LaJeunesse 2001; LaJeunesse et al. 2003). Many corals harbour background

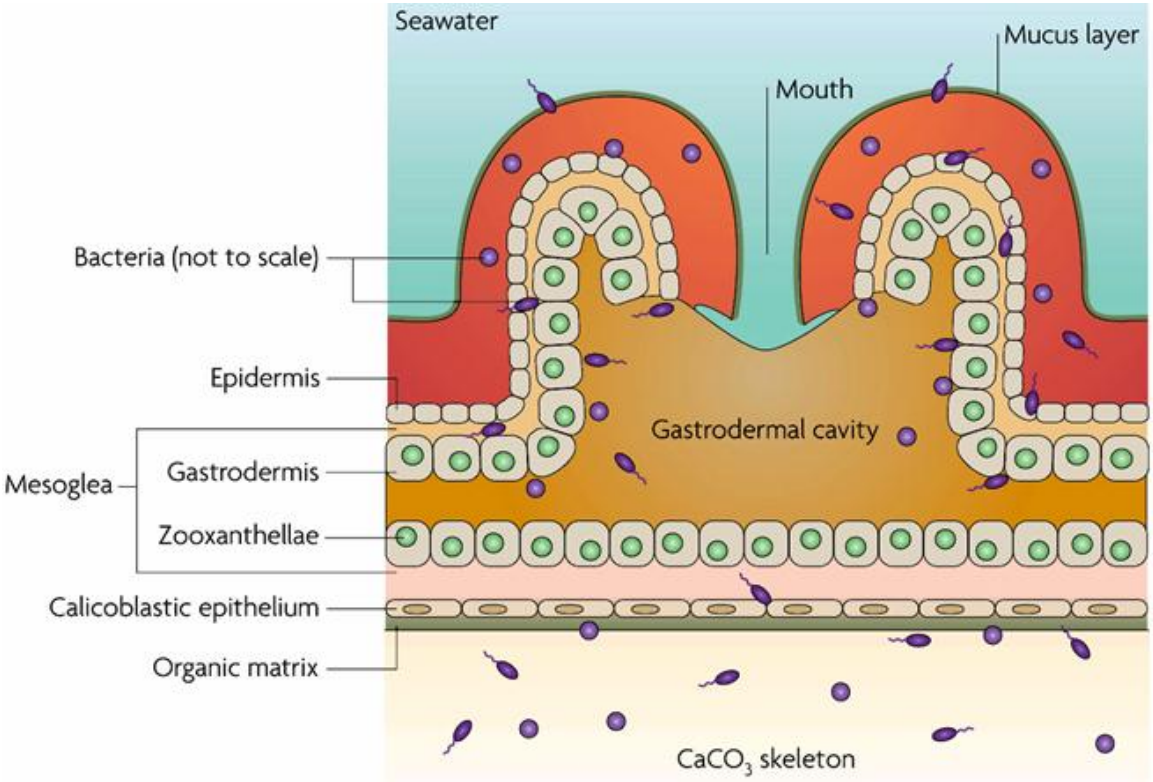


Figure 1.1. Organisation of the coral holobiont showing two cell layers of the coral animal (epidermis and gastrodermis) connected by the acellular mesoglea with associated microbial partners, the *Symbiodinium* (zooxanthellae) located in the gastrodermis, and bacteria throughout. The organic matrix secreted by the calicoblastic epithelium and the aragonite skeleton is also shown (Rosenberg et al. 2007).

populations of *Symbiodinium* in addition to the dominant clade (Berkelmans and van Oppen 2006; Mieog et al. 2007; McGinley et al. 2012). This has led to the adaptive bleaching hypothesis (ABH), which posits that expulsion of *Symbiodinium* during heating (or other stress) events will allow more tolerant types in low abundance to proliferate, or be acquired from the water column, thus conferring greater stress resistance to the host (Buddemeier and Fautin 1993; Buddemeier et al. 2004).

Symbiodinium photosynthesis

The role of *Symbiodinium* within the context of the coral holobiont is better characterized than the other constituents; the intracellular algae, which reside within the coral gastroderm, supply up to 95% of the coral host's daily energy requirements in the

form of exported photosynthates (products of photosynthesis) (Muscatine 1990). Recent evidence suggests that glucose is the main photosynthate transferred from endosymbiont to host (Burriesci et al. 2012), although it was long maintained that glycerol was the primary export (Muscatine 1967). In return, the algae is supplied with inorganic carbon to fuel photosynthesis, as well as nutrients in the form of ammonium and phosphate from the host (Davies 1984). In order to maintain high levels of *Symbiodinium* photosynthesis, the host employs a carbon-concentrating mechanism (CCM) to ensure adequate intracellular inorganic carbon (C_i) supply (Allemand et al. 1998).

Symbiodinium require efficient CCMs because of the presence of a form II Rubisco, generally found only in anaerobes because of its poor discrimination between CO_2 and O_2 substrates (Whitney and Andrews 1998). The relative specificity factor of Rubisco for CO_2 over O_2 (S_{rel}, a unitless measurement) is approximately 100 in C_3 and C_4 plants, from 41 to over 100 in algal Rubiscos, and just 12 in the anaerobic proteobacteria *Rhodospirillum rubrum* (Jordan and Ogren 1981; Read and Tabita 1994). The Rubisco found in *Symbiodinium* is structurally similar to the homomeric form II enzyme in *Rhodospirillum* (Whitney et al. 1995). *Amphidinium carterae*, a dinoflagellate, possesses a form II Rubisco that has an S_{rel} roughly twice that of *Rhodospirillum*, suggesting *Symbiodinium* may have a higher S_{rel} than previously thought (Whitney and Andrews 1998). In order to overcome the relatively low S_{rel} and high K_m, the algae must increase the intracellular concentration of CO_2 around Rubisco.

One of the key enzymes of coral/*Symbiodinium* CCM is carbonic anhydrase (CA) (Figure 1.2). CA, a polyphyletic group consisting of 5 classes, α-ζ (Deutsch 1987; Lane et al. 2005; Supuran and Scozzafava 2007; Xu et al. 2008), increases the relatively

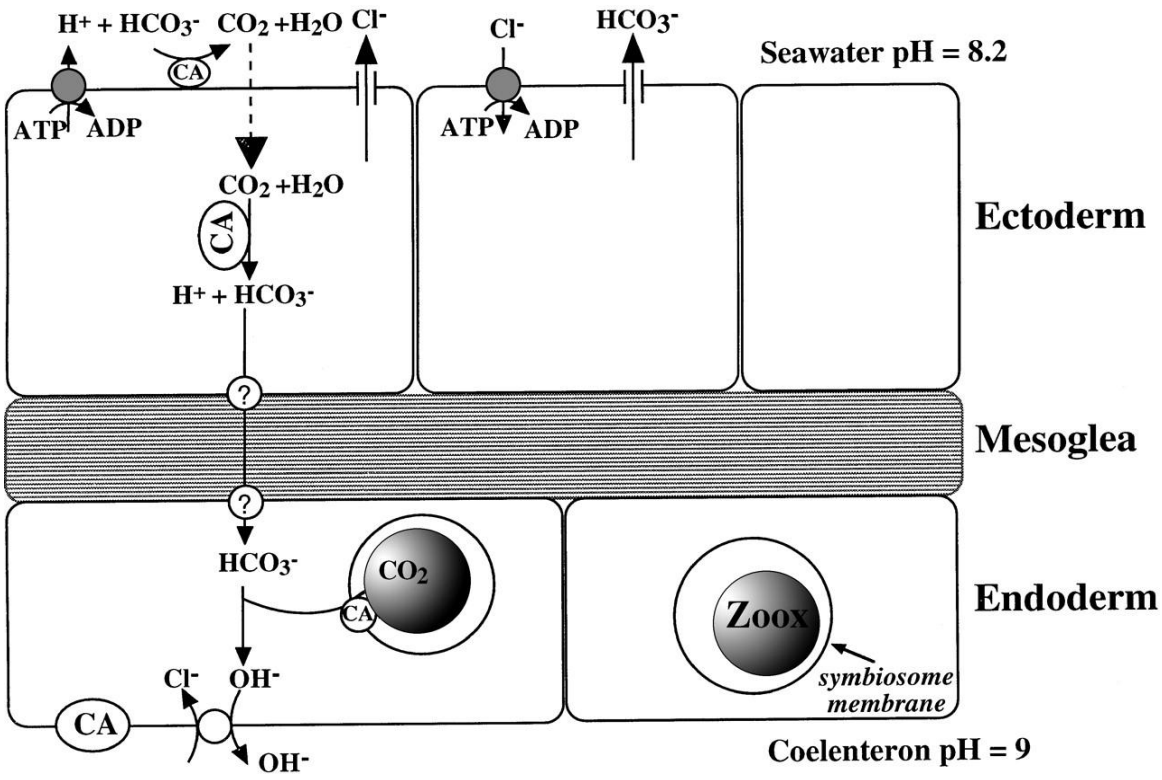


Figure 1.2. Carbon-concentrating mechanism in a cnidarian. Zoox= zooxanthellae (*Symbiodinium*); CA= carbonic anhydrase. (Furla et al. 2000a)

slow dehydration of HCO_3^-, allowing rapid equilibration of HCO_3^- and CO_2 (Khalifah and Silverman 1991). As the bulk of the CO_2 required for photosynthesis originates from the surrounding seawater, both the coral host and *Symbiodinium* are involved in the supply of CO_2 for photosynthesis and both utilize CA for this function.

The coral CCM

There are a number of locations that CA has been found, or hypothesized to be, in the coral host. Much of the information on coral CCM has been inferred from work with sea anemones as a model symbiotic cnidarian (Furla et al. 2000a; Furla et al. 2005). It has been suggested that the host likely possesses an external carbonic anhydrase located on the epithelium facing the seawater, increasing the concentration of CO_2 in the extracellular space (Furla et al. 2000a) (Figure 1.2). The CA inhibitor acetazolamide

(AZ), which has low membrane permeability (Palmqvist et al. 1988), shows non-additive effects on epithelium and gastroderm in net photosynthesis, indicating a similar amount of CA activity in both cell layers (Benazet-Tambutte et al. 1996; Furla et al. 2000a). Upon entering the cell, CO_2 is converted back to HCO_3^- by cytoplasmic CA to prevent leakage from the cell (Lucas and Berry 1985; Furla et al. 2000a) (Figure 1.2). Studies using anti-bodies for human CA show the enzyme on or near, but not bound to, the perisymbiotic membrane in the sea anemone *Aiptasia pulchella* (Weis 1993). Likewise, it has shown uniform distribution around the periphery of the zooxanthellar membrane in the coral *Galaxea fascicularis* (Al-Moghrabi et al. 1996). Up to 90% of photosynthesis is inhibited by ethoxyzolamide (EZ), a membrane-permeable CA inhibitor (Palmqvist et al. 1988), in microcolonies of *G. fascicularis*, and 60% in freshly isolated and cultured zooxanthellae (Al-Moghrabi et al. 1996). The differing results from localization/inhibition studies suggest that at least 2 isoforms of CA exist: one linked to symbionts, located on/around the perisymbiotic/algal membrane, and one specific to the cnidarian cell (Weis et al. 1989; Weis 1993; Al-Moghrabi et al. 1996). The currently accepted pathway for carbon sequestration is that initial efflux of H^+ via H^+/ATPase in the host membrane protonates HCO_3^- to H_2CO_3 in the extracellular space, which is quickly dehydrated to CO_2 by a membrane-bound CA, increasing concentration of CO_2, which in turn increases the rate of passive diffusion of CO_2 into the cell (Allemand et al. 1998; Furla et al. 2005).

CA is also commonly studied in corals for its role in biomineralisation (calcification) (Allemand et al. 2004). Involvement of CA in coral calcification has been verified by pharmacological studies in which CA inhibitors have been shown to significantly decrease the levels of calcification compared to controls which receive no CA inhibitors (Benazet-Tambutte et al. 1996; Furla et al. 2000b) and histochemical

localisation, which shows CA associated with the calicoblastic epithelium where calcification occurs (Moya et al. 2008).

The *Symbiodinium* CCM

Evidence for a *Symbiodinium* CCM was first proposed by Leggat et al. (1999), who found intracellular DIC was significantly higher than can be accounted for by passive diffusion alone in *Symbiodinium* isolated from giant clams (genus *Tridacna*). *Symbiodinium* have been shown to possess both intracellular and extracellular CAs, which belong to the α-class (Yellowlees et al. 1993; Leggat et al. 2000). Recent studies have also found β and δ-CAs in *Symbiodinium* in culture (Bobeszko, pers comm). Light-stimulated CA activity, inhibited by EZ, also indicates active C_i uptake by *Symbiodinium* (Leggat et al. 1999). Rubisco has been localised to the pyrenoid in algal CCMs (Badger et al. 1998) and *Symbiodinium* (E. Marendy unpub data), suggesting that C_i need only be elevated within the pyrenoid to sustain photosynthesis which, in *Symbiodinium*, may explain the relatively low intracellular C_i compared to other marine microalgae despite its inefficient form II Rubisco (Leggat et al. 1999).

Experiments have shown decreased expression of CCMs in algae in the presence of elevated CO_2, with some isoforms of CA upregulated by decreased CO_2 while others are downregulated (Allemand et al. 1998; Leggat et al. 2000; Moroney et al. 2001). Whether these changes in CCM activity directly correlate to changes in photosynthetic rate has not yet been characterized. Zimmerman (1997) found elevated pCO_2 stimulates photosynthesis only in those species which utilize CO_2, such as seagrasses, as opposed to those that rely on bicarbonate; it is unclear whether corals have the capacity to increase CO_2 use as its proportion in the DIC pool increases (Gattuso et al. 1999).

Coral bleaching

Due to the coral hosts' dependence on their intracellular symbionts for nutrition, the loss of *Symbiodinium* and/or associated pigments during bleaching events, so-called because of the visible paling of coral tissue during symbiont/pigment loss and resultant white appearance of corals arising from the white calcium carbonate skeleton showing through transparent coral tissue, can be highly detrimental to coral health (Hoegh-Guldberg 1999). Though numerous factors can trigger a bleaching event, the most wide-scale and prevalent causes are thermal stress and high solar radiation (Brown 1997; Hoegh-Guldberg 1999). The initial lesion point for bleaching has been an area of intense study and mounting evidence suggests dysfunction within algal photosystems is the cause of symbiont dissociation (Warner et al. 1996; Jones et al. 1998; Warner et al. 1999; Takahashi et al. 2004; Takahashi et al. 2009) (see chapter 2 for more detail). Loss of *Symbiodinium* from the host can occur via: degradation of algal cell *in situ*, exocytosis of algae from host cell, release of algae from mesenterial filaments, apoptosis/necrosis of gastrodermal cells, and/or host-cell detachment (Gates et al. 1992; Brown et al. 1995). As global sea surface temperatures (SST) continue to rise due to anthropogenic climate change, models predict mass bleaching will become an annual occurrence within 50 years unless coral holobionts can acclimate/adapt at an adequate pace (Hoegh-Guldberg 1999; Donner et al. 2005).

Microbial partners

The role of bacteria and other microbial consort within the context of the holobiont is still largely unknown. Recent evidence from sequencing of the 16S rDNA of prokaryotic DNA (used to phylotype bacteria) suggests a wide diversity of bacteria types living in association with corals (Rohwer et al. 2002). However, the identification

of bacterial ribotypes does not necessarily inform of the functions of these associated bacteria. Ascribing function to these microbes, the vast majority of which are uncultivable, relies on analogy to other systems. For example, the presence of nitrogen-fixing bacteria Rhizobia suggests a mutualistic association, as Rhizobia in association with terrestrial plants allows these plants to grow in nitrogen-poor soils (Long 1989; Lema et al. 2012). Recently, the role of dimethylsulfoniopropionate (DMSP) metabolism by certain coral-associated bacteria (e.g. Alteronomonas, *Roseobacter*, Spongiobacter, and *Vibrio*) has shown sulfur cycling within the holobiont is also likely influencing the microbial community composition (Raina et al. 2009; Raina et al. 2010). These findings are significant because of the role of dimethyl sulfide (DMS, a product of DMSP cleavage) in cloud formation (Malin 1996). DMS released into the ocean by corals, which equilibrates with the atmosphere above, produces sulfate aerosols which act as cloud nucleation sites (Liss et al. 1993; Jones and Trevena 2005). This cloud cover has the potential to reduce solar irradiation over reefs, thereby alleviating high light stress over reefs and more broadly, the albedo effect could lower global temperature by reflecting solar radiation back into space (Charlson et al. 1987; Ayers and Gillett 2000; Jones and Trevena 2005; Deschaseaux et al. 2012). Therefore, DMSP metabolism by marine bacteria is an area of immense interest with wide-ranging ecological implications.

The association between corals and bacteria is not passive, as it seems both the host and some microbes can modify the bacterial community. Coral mucus and mucus-associated bacteria exhibit antimicrobial properties, suggesting that coral hosts exert some level of control in structuring their bacterial assemblages (Ritchie 2006; Nissimov et al. 2009; Shnit-Orland and Kushmaro 2009; Vidal-Dupiol et al. 2011). This is further evidenced by species-specific microbial assemblages found within coral hosts (Rohwer

et al. 2001; Bourne and Munn 2005; Littman et al. 2009). Antimicrobial properties of the mucus and associated beneficial bacteria are lost during thermal stress events, which may allow proliferation of opportunistic pathogens (Ritchie 2006). Bleaching events have indeed been linked with a rise in *Vibrio sp*. in several coral species across geographic locations (Ben-Haim and Rosenberg 2002; Ritchie 2006; Bourne et al. 2007). Interestingly, although Koch's postulates were tested and confirmed for *Vibrio*-induced bleaching of *Oculina patagonica* in the Mediterranean (Kushmaro et al. 1997), subsequent studies failed to find a correlation between *O. patagonica* bleaching and increased *Vibrio* populations (Ainsworth et al. 2007).

The role of bacterial populations in directly triggering *Symbiodinium* expulsion remains unclear, however, bacteria are known to cause disease outbreaks on coral reefs. In recent decades, the incidence of coral diseases has increased worldwide, especially in the Caribbean, prompting closer scrutiny of the microbial community associated with coral reefs as potential etiological agents (Bruno et al. 2007; Harvell et al. 2007). Defining etiological agents of disease for corals has proved problematic, as symptoms can vary and description relies heavily on macroscopic observations, thereby confusing the exact differentiation between similarly presenting diseases (for review see Richardson 1998; Willis et al. 2004). About 20 diseases have been described world-wide (some estimates put this number closer to 30; see Weil et al. 2004), of which the pathogen has successfully been identified for 5 using Koch's postulates (Harvell et al. 2007). The increased prevalence of disease observed in recent decades has been attributed to environmental changes.

Importance of coral reefs

As the keystone species of coral reefs, the health of the coral holobiont is vitally

important to maintain this ecosystem. Decline in coral health and subsequent loss of reef habitat will have a devastating impact on the vast multitude of organisms associated with reefs as well as the people who depend on them for their livelihoods. Coral reefs are known as the "rainforests of the sea" because of the high biodiversity associated with them; estimates of species associated with reefs vary hugely, from 1 million up to 9 million (Knowlton 2001). Coral reefs and associated biota provide a vast array of ecological, economical, and social goods and services, which have a combined estimated global value of approximately $29.8 billion dollars (USD) per year (Conservation International 2008). Many coral reefs are located offshore of lesser-developed nations, which rely on them not only for economic services in the form of tourism and recreation, but also for fisheries which supply these communities with their primary source of protein (for review see Moberg and Folke 1999). Unfortunately, the health of reefs is deteriorating worldwide, driven by local and global-scale stressors (Hoegh-Guldberg 1999; Knowlton 2001; Hughes et al. 2003).

Anthropogenic climate change: A threat to coral reef health

The most widespread and potentially devastating threat to coral reefs on a global scale is anthropogenic climate change. Atmospheric CO_2 concentration has risen from the pre-industrial era level of 280 parts per million (ppm) (Houghton 2002) to 392 ppm currently (Tans and Keeling 2012), and is projected to rise to nearly 1000 ppm by the end of this century according to 'worst-case-scenario' predictions of the Intergovernmental Panel on Climate Change (IPCC 2007). Future CO_2 emissions are represented by 6 possible scenarios as outlined by the IPCC in the fourth assessment report (FAR) of 2007 and range from approximately 500 ppm to 1000 ppm atmospheric CO_2 at the end of this century (IPCC 2007). These emissions scenarios vary in

projections of population expansion, technological advances, and economic growth. For example, the lowest emissions scenario (B1) corresponds with a population that peaks mid-century, accompanied by rapid changes towards a service and information-based economy, while the worst-case-scenario (A1F1) assumes little technological change and continued intensive fossil fuel use (IPCC 2007). This A1F1 scenario will result in approximately 140 gigatons of CO_2-equivalent greenhouse gases (including methane, CH_4, and dinitrogen oxide, N_2O) per year released to the atmosphere. Notably, even the lowest emissions scenario results in atmospheric CO_2 concentrations well above the proposed "safe" level of CO_2 of 350 ppm (Veron et al. 2009). Increased atmospheric CO_2 concentrations have a two-fold impact on the world's oceans: 1) increased sea surface temperature (SST) from the "greenhouse" effect of CO_2 and equivalent gases and 2) ocean acidification, a reduction of pH caused by uptake of CO_2 by the surface ocean. Sea surface temperature (SST) has increased 0.74 °C since the 20th century and is predicted to increase another 1.1-6.4 °C in the next century (Hoegh-Guldberg et al. 2007). Surface ocean pH has decreased by 0.1 unit since pre-industrial times, which corresponds to a 30% increase in the concentration of H^+ ions via the chemical equation:

$$CO_{2\,(atm)} = CO_{2\,(aq)} + H_2O = H_2CO_3 = HCO_3^- + H^+ = CO_3^{2-} + 2H^+ \quad (1)$$

(Cao and Caldeira 2008). These factors, especially in conjunction with local stressors, can bring about coral bleaching. The link between elevated temperature and mass coral bleaching has been well documented over several decades (for review see Brown 1997). Degree-heating weeks (DHW), or weeks when SST exceeds average summertime temperature by 1 °C, have been linked to subsequent bleaching events and has

successfully been used to predict bleaching events (Gleeson and Strong 1995). However, the effect of lowered ocean pH on coral reefs is still uncertain.

Ocean acidification (OA) research, compared to thermal bleaching studies, is still in its infancy. It has only been in the last 5-10 years that researchers have focused on this "other CO_2 problem" (Doney et al. 2009). The majority of research on OA is concerned with the effect of elevated CO_2 (alone or in synergy with elevated temperature) on coral calcification because of the critical role of coral biomineralisation in reef and ecosystem creation (Houlbreque et al. 2012; Kline et al. 2012; Maier et al. 2012a; Moya et al. 2012). Aragonite is the form of calcium carbonate ($CaCO_3$) secreted by corals; lowered pH decreases the aragonite saturation state ($\Omega_{aragonite}$) due to the decreased availability of the carbonate ion (CO_3^{2-}) (Ca^{2+} concentration in seawater is stable), creating an environment where it is more difficult for coral to accrete their skeleton, and in extreme cases, may cause dissolution of existing skeleton (Orr et al. 2005). Although the field is rapidly expanding, relatively few studies have thus far examined the effects of CO_2 on coral physiology (Reynaud et al. 2003; Langdon and Atkinson 2005; Schneider and Erez 2006; Anthony et al. 2008; Suwa et al. 2010; Nakamura et al. 2011; Kaniewska et al. 2012a) and even fewer have assessed transcriptomic response to acidification (that is not related to calcification) (Kaniewska et al. 2012a; Moya et al. 2012). Reported effects of OA on coral vary widely from study to study and are influenced by species of interest, level of acidification, and duration of study. Overall, there appears to be little/no effect of increased pCO_2 on colony dark respiration (Reynaud et al. 2003; Langdon and Atkinson 2005; Schneider and Erez 2006; Anthony et al. 2008). Effects on *Symbiodinium* photosynthesis are mixed, with some coral species decreasing in net photosynthesis at high levels of pCO_2

Figure 1.3. Photograph of *Acropora aspera*, taken on Heron Island reef flat.

(Anthony et al. 2008), some increasing (Langdon and Atkinson 2005), and others being relatively unaffected (Reynaud et al. 2003; Schneider and Erez 2006). Direct comparison of results is difficult and must be done cautiously due to the different parameters being measured and the difference in pCO_2 treatment levels.

Study species: *Acropora aspera*

The study species of this thesis work, *Acropora aspera*, is a tropical scleractinian coral found throughout the Indo-Pacific on upper reef slopes and shallow (0-5 m) lagoons (Veron and Stafford-Smith 2000; Aeby et al. 2008) (Figure 1.3). Blue, light blue, and tan morphs of *A. aspera* occur on Heron Island Reef flat where study samples for this work were collected (Dove 2004). Experiments were conducted at Heron Island

Research Station (University of Queensland) in the southern Great Barrier Reef, 60 km offshore from the Australian mainland.

Thesis objectives

There is currently a paucity of research concerning the potentially synergistic effect of increased SST and OA on coral physiology, in particular where bleaching and nutrient exchange within the holobiont is concerned. As climate change progresses, temperature and CO_2 concentration will be increasing in concert, i.e. the rise in temperature will be concomitant with increased acidity, therefore it is vital to address this current knowledge gap. This thesis focuses on addressing this knowledge gap by studying the effects of increased temperature and CO_2 on a key reef-building coral of the GBR, *Acropora aspera*.

In the first data chapter (Chapter 2), the photophysiology of *Symbiodinium in vitro* will be measured to assess the extent of photodamage and photoinhibition under differing levels of thermal stress concordant with sub-lethal and bleaching thresholds of *Symbiodinium in hospite*. This will provide the context for interpretation of expression of targeted key photosynthesis and heat shock protein genes. Gene expression quantification was conducted throughout the thesis using the highly sensitive method of quantitative PCR (qPCR). This chapter served as a pilot study for an experiment with the intact coral-algal association *in situ*.

Chapters 3 and 4 characterised the response of the *A. aspera* holobiont to elevated temperature and increased pCO_2. *A. aspera* were subjected to a simulated bleaching event under CO_2 levels equivalent to mid-century predictions (about 50-100 ppm above current ambient). Expression of carbon metabolism genes was assessed from both coral and algal partners in order to ascertain potential impacts on nutrient exchange and usage in the holobiont. Localisation of 2 isoforms of the key CCM enzyme, CA, provided

additional evidence of the synergistic effects of temperature and CO_2. *Symbiodinium* photophysiology and cell density (per surface area coral tissue) was measured. Bacterial community dynamics were investigated and represented a novel study into climate change influence on coral microbiota.

To test models that predict annual bleaching by mid-century, a repeated stress event was applied to *A. aspera* and expression of targeted stress-mitigating protein genes were assessed for potential acclimatory response in Chapter 5. This was once again done using qPCR. Specifically, the potential for corals to increase their thermal tolerance and therefore their bleaching threshold was explored. Due to the relatively complex nature of the experimental design, only one factor (temperature) was tested.

Finally, the findings of these experiments are summarised in Chapter 6 and directions for future studies discussed.

Chapter 2: Elevated temperature decreases *Symbiodinium* Rubisco expression

Abstract

Intracellular algae (genus *Symbiodinium*) and reef-building corals form a symbiosis based on mutualistic nutrient exchange. Photosynthetic processes in *Symbiodinium* have been proposed as the weak point in the coral holobiont when it is exposed to elevated temperature; this *Symbiodinium* dysfunction eventually leads to coral bleaching (loss of algae or algal pigment from host tissue). This experiment exposed cultured *Symbiodinium* (C1-ITS2) to heat stress with the aim of identifying gene expression changes that may be driving observed physiological changes. Cultures were exposed to 30 °C, 32 °C, and 34 °C treatments as well as a 26 °C control for 4 days and pulse-amplitude modulation (PAM) measurements of chlorophyll fluorescence and quantitative PCR (qPCR) were performed. Photosynthetic efficiency of photosystem II (PSII), measured as dark-adapted quantum yield of PSII by PAM fluorometry, declined significantly at 34 °C over the experimental period from 0.412 to 0.227 ($n = 3$; $p<0.001$). Concurrent measurement of expression of a suite of genes involved in photosynthesis was analysed using qPCR. It was found that in cultures exposed to 32 °C and 34 °C treatment the gene encoding ribulose 1,5-bisphosphate carboxylase/ oxygenase (Rubisco) was down regulated. Relative expression on day 1 was 0.73, which decreased significantly to 0.66 at day 2 and 0.30 fold of controls on day 3 at 34 °C ($p<0.05$). Rubisco expression was also found to decrease at 32°C treatments at days 2 and 3 (relative expression = 0.58 and 0.46, respectively). Interestingly, there was an up-regulation at day 4 in the 30 °C treatment (= 2.83; $p<0.05$). A sugar transporter gene was significantly up-regulated at 32 °C on day 4 (= 1.78; $p<0.05$) and at 34 °C on days

3 and 4 (= 2.06 and 2.16, respectively). Analysis of a variety of other genes encoding proteins involved in photosynthesis as well as heat shock proteins found no significant pattern of changes in expression under elevated temperature. These results suggest that Rubisco gene expression is sensitive to elevated temperatures in *Symbiodinium* clade C1.

Introduction

Single-celled microalgae of the genus *Symbiodinium* form symbioses with a variety of hosts including giant clams, sea anemones, hydras, and scleractinian (reef-building) corals (Yellowlees et al. 2008). The partnership between corals and these dinoflagellates (also known generically as zooxanthellae) is particularly of interest because it is the foundation upon which the coral reef ecosystem is built. Intracellular algae residing within gastrodermal cells of the coral host export large amounts of photosynthate, providing the coral with approximately 95% of its energy needs (Muscatine 1990). In return, the algae are provided with nitrogen from host waste products, inorganic carbon from ambient seawater, and protection from predation (Davies 1984).

The association between coral and *Symbiodinium* is vulnerable to heat stress and therefore under threat from increasing sea surface temperatures. Elevated temperature above long term average summertime maxima have been identified as one of the primary factors that induces coral bleaching (visible loss of endosymbionts and/or associated pigments) (Jokiel and Coles 1977; Hoegh-Guldberg 1999; Fitt et al. 2001). While it was initially believed that sensitivity of the host initiated the bleaching process (Gates et al. 1992), studies have suggested that lesions to photosynthetic processes in the symbiont may be the initial site of damage under heat stress (Iglesias-Prieto et al. 1992; Lesser 1996; Warner et al. 1996; Warner et al. 1999; Jones et al. 2000; Takahashi et al. 2004). This has led to the hypothesis that the algal partner is the weak point in the symbiosis and thus the underlying cause of bleaching.

Numerous studies of isolated *Symbiodinium*, employing a variety of assays have attempted to reveal the mechanism by which photosynthesis is disrupted at elevated temperatures. The initial site of damage to photosynthesis has been postulated to occur at the oxygen-evolving complex of PSII reaction centre (Warner et al. 1996), D1 protein

in PSII (Warner et al. 1999), repair machinery of photosynthesis (Takahashi et al. 2004; Fitt et al. 2009), the light harvesting complexes (Takahashi et al. 2008), or the dark reactions (Jones et al. 1998). Using O_2 evolution measurement and whole cell fluorescence techniques it has been shown that *Symbiodinium* photosynthesis is impaired at 30 °C and oxygen evolution ceases altogether at 34-36 °C (Iglesias-Prieto et al. 1992). It was suggested that this disruption of photosynthesis was likely due to a change in the thylakoid membrane resulting from the elevated temperatures (Iglesias-Prieto et al. 1992). Subsequently, it has been shown the thylakoid membrane is damaged by reactive oxygen species (ROS) that build up in the chloroplast during periods of oxidative stress, overwhelming the protective cellular mechanisms of more thermally sensitive types (Tchernov et al. 2004).

Jones et al. (1998) has suggested that damage to PSII and decrease in maximum quantum yield observed by others is actually a secondary effect of thermal stress in *Symbiodinium*. The increase of non-photochemical quenching (NPQ) in the absence of a decrease in photochemical quenching combined with the oxygen-dependent nature of NPQ in *Stylophora pistillata* led them to postulate that the initial site of disruption is within the carbon-fixing stage of photosynthesis (Jones et al. 1998). This disruption of carboxylation may then lead to an upstream build-up of electrons (sink limitation) that eventually overwhelms protective mechanisms of the oxygen-dependent Mehler-Ascorbate Peroxidase cycle and subsequently cause damage to PSII (Lesser 1996). A decrease in Rubisco enzyme activity has been found at elevated temperature, lending credence to this theory (Leggat et al. 2004; Lilley et al. 2010).

This study seeks to uncover responses at the level of gene expression that may be driving physiological changes that are observed when *Symbiodinium* are exposed to heat stress. Cultures of ITS2 type C1 *Symbiodinium*, a basal strain of the C clade that

dominates the Indo-Pacific region, was exposed to various heat treatments *in vitro* (LaJeunesse 2001; LaJeunesse et al. 2003). Changes in quantum yield of photosynthesis was measured via PAM fluorometry and compared to changes of expression of selected genes assessed using real-time quantitative PCR (qPCR). This study is one of the first to assess responses of photosynthetic processes of *Symbiodinium* at the level of gene expression and how it relates to overall photosynthetic efficiency of PSII.

Materials and methods

Culture maintenance

Cultures of clade C1 (ITS2) of *Symbiodinium sp.* were grown in ASP- 8A media (McLaughlin and Zahl 1957) in 80-100 μmol photons m^2s^{-1} light on a 12:12 light: dark cycle. Doubling time for cells was approximately five to seven days. The experiment started with cells in log-phase growth to avoid self-shading and age-dependent responses. Culture stock flasks were pooled and divided evenly amongst 56 flasks, which were randomly assigned to an incubator (14 flasks per incubator). Treatments were: 26 °C (control), 30 °C, 32 °C, and 34 °C. Flasks were placed in incubator cupboards and temperature was increased gradually from 26 ^oC in 3 equal increments over 16 hours, until they reached the desired temperature. Three replicate flasks were taken for sampling daily at apparent midday (i.e. 6 hours into the 12 hour light cycle) for 4 consecutive days. Flasks were scraped with a cell scraper to suspend cells settled on the bottom of the flask and aliquots were taken for RNA extraction, flow cytometry, and PAM fluorometry.

Pulse-amplitude modulated (PAM) fluorometry

A small aliquot of cells (approximately 300 μl) was aspirated from each sample flask

into a 96 well plate for use in an imaging PAM (iPAM) chlorophyll fluorescence system (MAXI Imaging PAM, Walz, Effeltrich, Germany). Samples were taken at apparent mid-day and dark-adapted for 30 minutes prior to measurements. The minimum fluorescence, F_o, was measured with a weak pulse of light, followed by a saturating pulse of 2700 μmol quanta m^2s^{-1} of photosynthetically active radiation (PAR) for 800 ms to ascertain the maximal fluorescence, F_m. The maximum quantum yield of PSII, F_v/F_m, was calculated using these parameters using the Genty equation (Genty et al. 1989):

$$F_v/F_m = (F_m-F_o)/ F_m$$

Change in F_v/F_m was used as a proxy to determine the effect of elevated temperature on photosynthetic efficiency of PSII.

Flow cytometry

Approximately 1 x 10^6 cells were aliquoted for staining and live/dead count via flow cytometry. A serial dilution was carried out to determine a suitable concentration of SYTOX green (Invitrogen, Mulgrave, Vic, Australia), a nucleic acid stain that cannot penetrate the membrane of live cells, thus allowing a count of dead cells and calculation of the proportion of dead cells to live cells. Cells microwaved for 3 minutes on high power to induce high levels of mortality were used as positive control. A final concentration of 100 nM of SYTOX green in 1x phosphate-buffered saline (PBS) buffer was used to stain cells in darkness for 20 minutes. Cells were then washed twice in 10x volume of PBS buffer before resuspending in 500 μL of 1x PBS and analysed on a

CyAn™ ADP analyzer flow cytometer (Beckman Coulter, Fullerton, Ca, USA) set to acquire SYTOX (488 nm laser).

RNA isolation and cDNA synthesis

Approximately 6-10 x 10^6 cells were aliquoted for total RNA extraction for each sample period. Cells were pelleted by centrifugation at 4500 *g* for 2 minutes. Excess media was poured off and the pellet resuspended and pipetted into RNA lysing matrix D tubes containing 1.4 mm ceramic spheres (Qbiogene, Seven Hills, NSW, Australia), and immediately snap-frozen in liquid nitrogen and stored at -80 °C for future use. Cell disruption was carried out by adding RLT buffer and β-mercaptoethanol as per the protocol (RNeasy Plant Mini Kit, Qiagen, Australia) to the frozen cells in lysing matrix tubes and vortexed on a MP fastprep ® -24 for 40 seconds (MP Biomedicals, Seven Hills, NSW, Australia). Total RNA extraction was then performed using RNeasy plant mini kit (Qiagen, Australia) following manufacturer's protocol. RNA yield was quantified using a NanoDrop-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, De, USA). Following removal of genomic DNA contamination using gDNA wipeout buffer (Qiagen, Doncaster, Vic, Australia), 500 ng of total RNA was used for complementary DNA (cDNA) synthesis reaction. Reverse transcription was carried out using Quantitect Reverse transcription kit (Qiagen, Doncaster, Vic, Australia) following manufacturer's protocol (20 μL total reaction volume).

Quantitative PCR

Gene-specific primers were designed using Lasergene Primer select program (DNASTAR, Madison, Wi, USA) (Table 2.1). These genes were chosen in attempt to capture a wide array of genes involved in a variety of processes to ascertain what effect

temperature stress might have at different stages of energy production and transfer from algal symbiont to host. Bacl-1 was found to be one of the dominant transcripts, composing up to 3% of the transcriptome in an expressed sequence tag (EST) library of stressed C3 *Symbiodinium* (Leggat et al. 2007), and plays an as-yet unknown role in *Symbiodinium* and was therefore included in this study as a possible stress response gene. Potential genes for normalization of data (i.e. internal control genes, ICG) were considered from Boldt et al. (2008).

A serial dilution of cDNA was used to calculate the amplification efficiency of primers and appropriate concentration of template for qPCR. A 1:40 dilution was determined to be suitable for all primers; efficiency was between .90 and 1.1 with r^2 greater than 0.96 for all primers (most were >0.99). SYBR GreenER qPCR SuperMix Universal (Invitrogen, Australia) was used to quantify amplification of product in a reaction containing: 7.5 μl of SYBR Greener qPCR Supermix Universal, 0.3 μl Gibco Ultrapure RNase/Dnase-free water (Invitrogen, Australia), 3.2 μl of primer mix for each gene of interest (GOI) (final concentration of 100 nM for each primer), and 4 μl 1:40 diluted template cDNA was pipetted by Corbett CAS-1200 pipetting robot (Qiagen, Australia). No-template controls (NTC) used 4 μL Ultra-pure GIBCO water instead of template cDNA. Amplification and quantification were performed on Rotor-gene™ 6000 (Qiagen, Australia) with the following temperature profile: initial holding step of 50 °C for 2 minutes, activation step of 95 °C for 10 minutes, 35 cycles of a denaturing step of 95 °C for 15 seconds and annealing and elongation step of 66 °C for 60 seconds, followed by a melt of product from 56 °C to 95 °C. A single, distinct peak in the melt curve indicated amplification of a single product.

β-actin, PCNA, and both glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) isoforms 1 and 4 were considered for use as reference genes to compare relative

expression of GOI. GeNorm (Biogazelle) analysis showed stable expression of GAPDH isoform 4 (GAP4; *M* = 0.329) and β-actin (*M* = 0.374) and they were therefore used as ICG for relative expression analysis (*V2/3* = 0.129, below the recommended 0.15 threshold) (Vandesompele et al. 2002).

Statistical analysis

Calibrated normalised relative quantities (CNRQ), the relative gene expression values for GOI, were calculated using qBASEplus software package (Biogazelle). All expression values are represented as the CNRQ of GOI in treatment, relative to control-level expression for that GOI for each day. All statistical analyses were carried out using SPSS statistical software package v.18 (IBM) using a generalized linear model with a standard ANOVA and sequential Bonferroni post-hoc ($\alpha<0.05$). Temperature and time were set as fixed factors; flasks were treated as a random factor.

Results

Photosynthetic efficiency of PSII under elevated temperature

To assess the effect of heat stress on photosynthetic efficiency of PSII, cultures were subjected to temperatures from 26 °C to 34°C and measurements of dark-adapted quantum yield of PSII, F_v/F_m (a proxy for photosynthetic efficiency), were taken at apparent mid-day for 4 consecutive days. There was a slight decline from 0.44 to 0.37 in F_v/F_m for control samples; however, only day 4 was significantly lower than day one (Figure 2.1; $p<0.05$). Samples at 30 °C remained steady between approximately 0.44 and 0.45. The significant difference of this treatment to control is due to the decline in control measurement, not in the treatment itself ($p<0.05$). F_v/F_m had no change at 32 °C over the 4 days, ranging from 0.44 to 0.41. Samples taken from 34 °C treatment

Table 2.1. Gene name, function, GenBank accession number or source paper, and primer sequence used for genes of interest for quantitative PCR to assay differential gene expression in response to thermal stress of isolated *Symbiodinium sp*. Genes considered for use as internal control gene (ICG) are indicated with an asterisk.

Gene	Function	GenBank accession no. or citation	Primer sequence Forward (F) Reverse (R)
Ribulose-bisphosphate carboxylase/oxygenase (Rubisco)	Carbon fixation in dark reactions of photosynthesis	AAG37859.1	F: CTGTATGATGGCCCCTCCGTGAAC R: GCTTTGGCTTGATGATGGTG
Carbohydrate transporters (ID: sugar transporter, sucrose transporter)	Transport of carbohydrates	Sugar: ABO47823.1	Sugar transporter F:TACTGCCAGGGTTAGCGGATTTGAG R: CTGCGGGTAGCGACTGATGAGC Sucrose transporter F:CACGGACTCCCTTGCCACCTTGAACA R: GCCGCCGCCCGAAACGAGAG
Heat shock proteins (isoforms 70 and 90)	Molecular chaperones aiding in folding and transport of proteins under cellular stress	HSP70: AAM02973.2 HSP90: ADV03069.1	HSP70 F:AGGAGACCGCTGAGGCATACTTG R: CTTGGTGGCCTGACGCTGAGAATC HSP90 F: ATTCCGAGGATCTGCCACTGA R: TCTCTGCGATCTCTGCGAACAT
Chloroplast acyl	Fatty acid synthesis	AAW79290.1	F: CTTTGCTCTGCTGCCTCGTGTTCCTG R: CATGGTGTCGTCGGTGGCTGTGG
Bacteria-like 1	Unknown	ABO47821.1	F: TGCCTTCAGCCAGCCATTTCAA R: GCTGCCTGCACACTCTTCACTTCC
Glyceraldehyde-3-phosphate dehydrogenase (chloroplastic isoform, GAP1; cytoplasmic isoform, GAP4)*	Enzyme involved in glycolysis; putative house-keeping gene	GAP1: AAP83170.1 GAP4: BAC87920.1	GAP1 F: CCGTGCTGGCCCTGGAAACATCAT R: GCCGGTCAGCTTGCCCTTCACTTC GAP4 F: CCGGCGCCAAGAAGGTCATCATC R: TTGGCCTTGTCGTACTCCGTGTGGTT
Beta actin*	Cell structure	Boldt et al. 2008	F: TGGACAACGGAAGCGGAATG R: GCCAACAATGGATGGGAAAACT
Proliferating cell nuclear antigen (PCNA)*	DNA synthesis	Boldt et al. 2008	F: GAGTTTCAGAAGATTTGCCGAGAT R: ACATTGCCACTGCCGAGGTC

experienced a significant decline in F_v/F_m compared to control, dropping steadily from 0.42 on day 1 to 0.23 on day 4 (Figure 2.1; $p<0.05$).

Assessment of culture death at elevated temperature

Temperature treatment did not have a significant impact on the proportion of dead cells in cultures compared to live cells (Figure 2.2; $p>0.05$). However, time was a significant factor affecting live/dead ratio; the percentage of dead cells decreased slightly but statistically significantly over the 4 day period ($p<0.05$). At 34 °C there was a significantly higher percentage of dead cells on day 3 compared to control (Figure 2.2). Overall, there was a very low percentage of dead cells compared to live cells (less than 1% to 3%) for all treatments/days.

Differential gene expression under heat stress

Changes in expression of GOI were on a relatively small scale (largest fold-change was Rubisco with a 2.83-fold increase compared to control). Rubisco exhibited the most notable changes in expression under treatments, with a pattern of down-regulation on days 2 and 3 at 32 °C (= 0.58 and 0.46, respectively) and 34 °C (= 0.66 and 0.30, respectively) (Figure 2.3a; $p<0.05$). Interestingly, day 4 showed significant up-regulation at 30 °C, which was the largest change observed for any gene in any treatment during the experiment (= 2.83; $p<0.05$). Bacl-1 was significantly down-regulated on day 3 at 32 °C and 34 °C (= 0.50 and 0.48, respectively) (Figure 2.3b; $p<0.05$), which returned to control level on day 4. Sugar transporter expression was significantly up-regulated at 34 °C on days 3 and 4 compared to control (= 2.06 and 2.16, respectively) and at 32 °C on day 4 (= 2.06) (Figure 2.3c; $p<0.05$). There was no

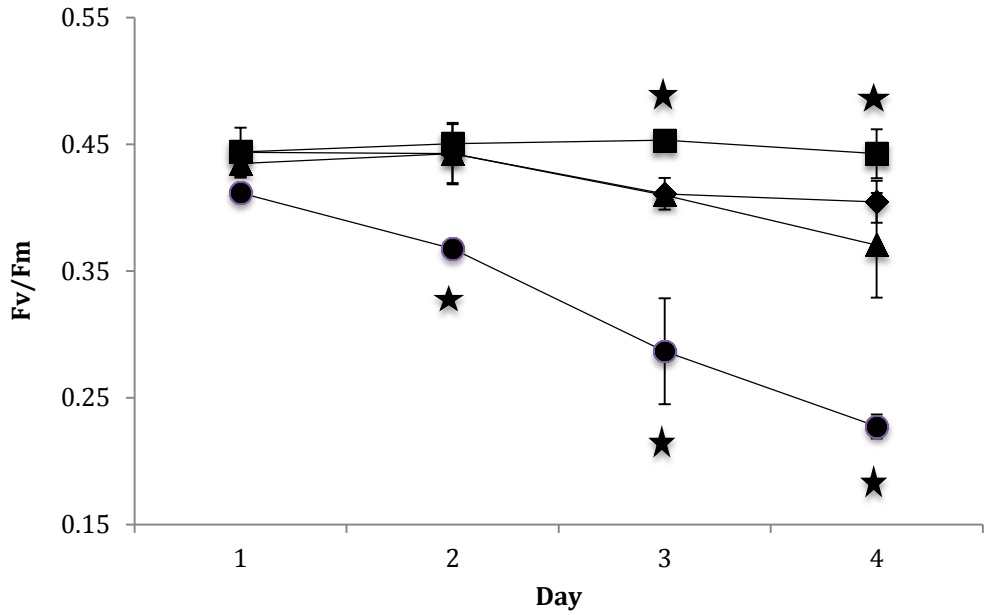


Figure 2.1. Photosynthetic efficiency of PSII, measured via dark-adapted quantum yield of PSII (F_v/F_m) of *Symbiodinium* chlorophyll fluorescence at: 26 °C (triangle), 30 °C (square), 32 °C (diamond) and 34 °C (circle) over 4 day experimental period. Error bars represent standard error of the mean (n = 3); some error bars are obscured by data points. Stars indicate significant difference of treatment from control (sequential Bonferroni; $p<0.05$).

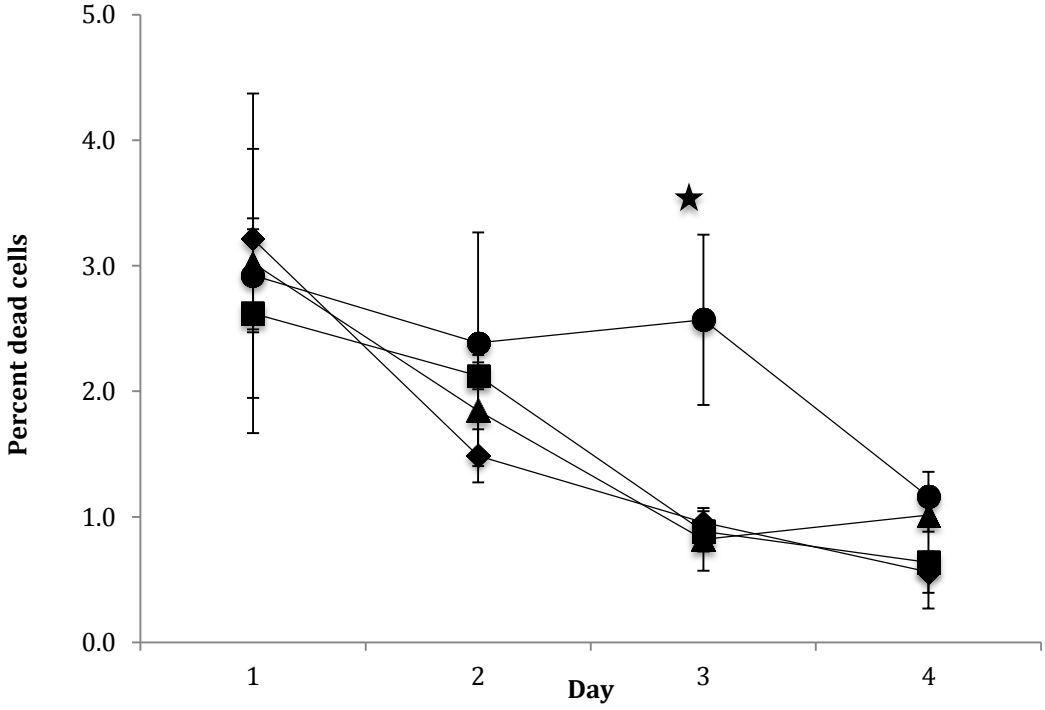


Figure 2.2. Percent of dead cells (out of total population) for 26 °C (triangle), 30 °C (square), 32 °C (diamond) and 34 °C (circle) treatments as a ratio to live cells over 4 day experimental period. Error bars represent standard error of the mean (n = 3). Stars indicate significant difference of treatment from control (sequential Bonferroni; $p<0.05$).

significantly different expression on any day for any treatment for: sucrose transporter, HSP70, HSP90, and chloroplast acyl carrier genes (Figure 2.3d-g; $p>0.05$).

Discussion

Effect of elevated temperature on photosynthetic efficiency of PSII and mortality

Many studies have demonstrated that *Symbiodinium* photosystem II is damaged or otherwise interrupted during periods of heat stress. The reduction of photosynthetic efficiency of PSII (F_v/F_m) at highly elevated temperature has been well-documented in many strains of *Symbiodinium*, both *in vitro* and in symbiosis on the scale from minutes to days (Iglesias-Prieto et al. 1992; Warner et al. 1996; Jones et al. 1998; Warner et al. 1999; Takahashi et al. 2004; Tchernov et al. 2004). This study, likewise, has shown a significant decrease in maximum quantum yield at 34 °C after 4 days (decrease of almost half; Figure 2.1). Yield was significantly different from control (26 °C) at 30 °C due to a slight decrease in the control F_v/F_m; yield at 30 °C did not change significantly during the course of the experiment. *Symbiodinium* C1 PSII appears fairly tolerant of thermal stress, as mild thermal stress (+4-6 °C above ambient) caused no significant decreases in photosynthetic efficiency of PSII. The extent of decrease of F_v/F_m during thermal stress can vary with *Symbiodinium* type; large differences in thermal susceptibility are observed within clades, making general attributions of thermal tolerance about one clade compared to another problematic (Robison and Warner 2006). These differential thermal susceptibilities of *Symbiodinium* types have been implicated in thermal tolerance of hosts (most notably reef-building corals) harbouring these algae (Rowan 2004). Sytox staining for dead cells demonstrated that there was not a significant increase in dead *Symbiodinium* cells at 34 ºC, even after 4 days, indicating that while this temperature inhibited photosynthesis, it was not lethal. The overall

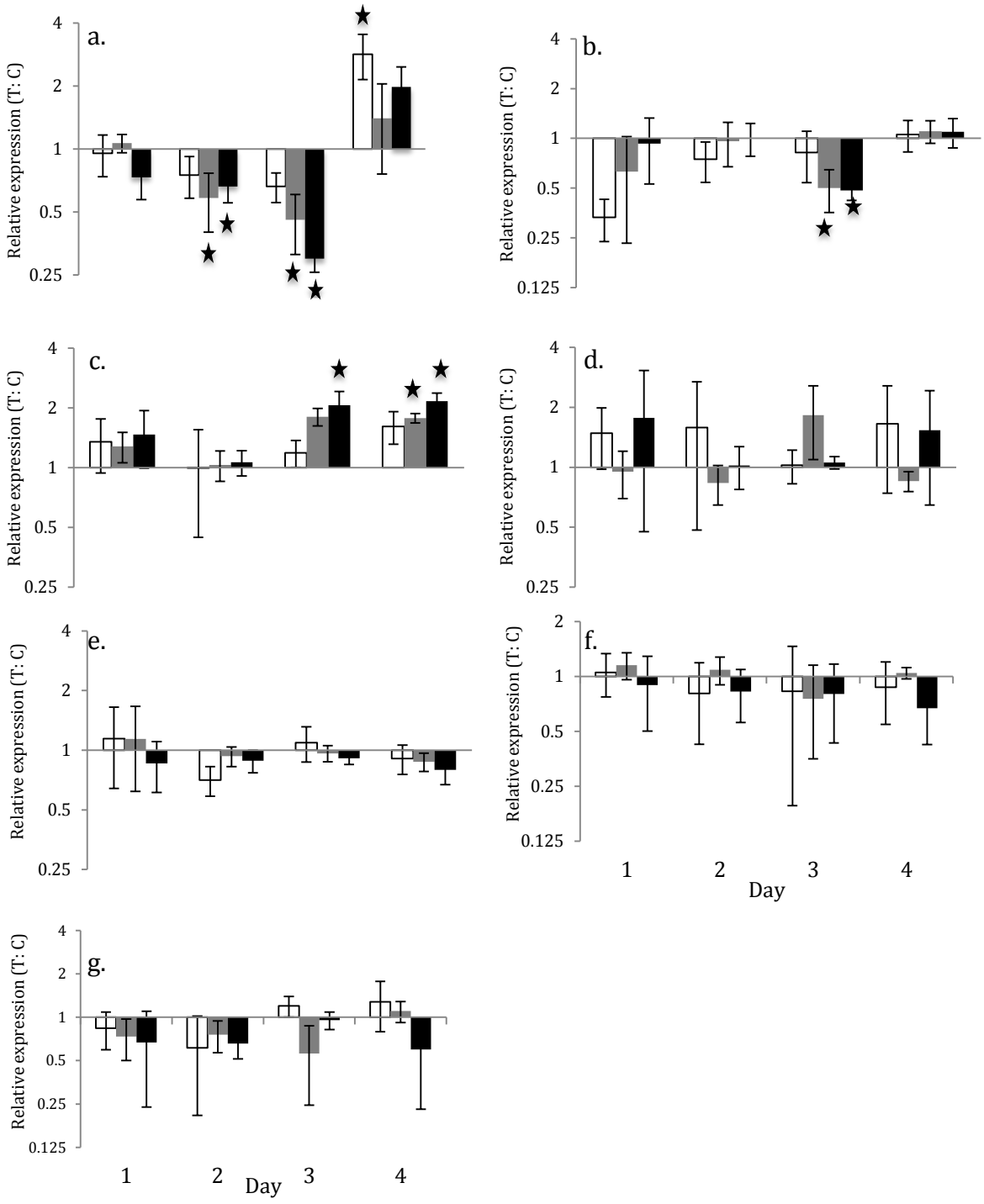


Figure 2.3. Relative expression of: a. Rubisco, b. Bacl-1, c. sugar transporter, d. sucrose transporter, e. HSP70, f. HSP90, g. chloroplast acyl transporter genes of *Symbiodinium sp.* exposed to 30 °C (white bars), 32 °C (gray bars), and 34 °C (black bars) for 4 days. Expression values are represented relative to control (26 °C) level expression for each day. Error bars represent standard error of the mean (n = 3); stars represent significant difference to control (sequential Bonferroni post-hoc, $p<0.05$).

decrease in percentage of dead cells is attributed to cells in culture being in log growth phase, therefore number of dead cells compared to live cells would decrease over time as cells multiply and mortality remains low. Absence of *Symbiodinium* mortality at elevated temperature has also been noted by Iglesias-Prieto et al. (1992), who found that although photosynthesis ceased, cell respiration continued, suggesting the cells were still alive but photosynthetically compromised. To further investigate the response of *Symbiodinium* the expression of a variety of genes was also measured.

Molecular chaperones

The role of the molecular chaperones (heat shock proteins, HSP) is to aid in proper protein folding and prevent inappropriate protein aggregation at elevated temperature and under other stressors (Sørensen et al. 2003). Thus, they are generally found to be up-regulated by organisms under stress (see Feder and Hofmann 1999 for review). It is therefore unusual that a pattern of down-regulation of HSP70 and 90 gene expression was observed during this study. However, this may represent the norm for *Symbiodinium*, as Rosic et al. (2010a) found significant down-regulation of *Symbiodinium* HSP70 and HSP90 at elevated temperature (+7 °C above ambient) *in vitro*. Those results must be compared with caution, as the light levels used for culture maintenance of that study were approximately 10% of that used in this study, and irradiance is known to have an interactive effect with heat in causing *Symbiodinium* stress (Robison and Warner 2006). Rosic et al. (2010a) also found a slight increase of HSP70 expression after 24 hours of a gradual stress (+3 °C above ambient), followed by a significant decrease (69% of controls) at 120 hours (temperature = 32 °C, 9 °C above ambient) of *Symbiodinium in hospite* with *Acropora millepora* . HSP90 was consistently down-regulated in the same experiment (Rosic et al. 2010a). Leggat et al.

(2011a) found a similar decrease of *A. aspera Symbiodinium* HSP90 expression when exposed to a simulated bleaching event (34 °C). Unlike most organisms, *Symbiodinium* do not appear to up-regulate HSPs in response to external stressors. Whether this is due to constituently high expression or the relatively static nature of the *Symbiodinium* transcriptome in general (Leggat et al. 2011b) is unknown. Lack of differential up-regulation of molecular chaperones HSP70 and HSP90 could help explain the decrease in photosynthetic efficiency of PSII at highly elevated temperature. Given the lack of transcriptional up-regulation of *Symbiodinium* HSP it would be interesting to further investigate changes in the protein levels of these HSPs. If this lack of up-regulation is also seen in protein content it may explain at least partially the thermal susceptibility of *Symbiodinium* as they are unable to effectively increase the HSP levels in response to a thermal challenge. Alternatively, an increase in HSP protein content would support the idea that much of the regulation of *Symbiodinium* protein levels occurs post-translationally (Leggat et al 2011). A recent study of another dinoflagellate (*Prorocentrum minimum*) found that when thermally challenged, HSP90 transcripts increased by up to 2.4-fold (Guo and Ki 2012), suggesting that the lack of HSP up-regulation may be found only in *Symbiodinium* and is not dioflagellate-wide.

Disruption of Calvin cycle at elevated temperature

Significant changes in Rubisco expression levels were found in all temperature treatments (Figure 2.3a). A decrease in photosynthetic efficiency of PSII at 34 °C was accompanied by a significant decrease in Rubisco expression during days 2 and 3 of the experiment (Figure 2.3a). However, a similar decrease in Rubisco expression at 32 °C was not associated with a drop in F_v/F_m (Figure 2.1). These results indicate relative expression measurements are a more sensitive indicator of cellular responses than

chlorophyll fluorescence. It was initially suggested that disturbance in the carboxylation mechanisms of Rubisco is the product of decreased availability of CO_2, a consequence of disruption in the carbon-concentrating mechanism (CCM) (Jones et al. 1998). While the CCM likely remains functional, Rubisco was seen to have a significant decrease in activity at elevated temperature under illumination and heat stress (Leggat et al. 2004). These findings were later verified by a similar but independent study in darkness, showing the mechanism by which Rubisco activity is disrupted is likely not directly caused by photoinhibition but heat stress (Lilley et al. 2010). Decrease in Rubisco activity is observed within hours of heat exposure (Leggat et al. 2004; Lilley et al. 2010). In higher plants, Rubisco activase is compromised by elevated temperatures and thus loses its ability to maintain Rubisco in a catalytically viable conformation (Crafts-Brandner and Salvucci 2000; Salvucci and Crafts-Brandner 2004). It is possible a homologue of Rubisco activase exists in *Symbiodinium*, which may be affected by heat stress in a similar way, but this has not yet been investigated. If it was present the function and/or structure of Rubisco activase in *Symbiodinium* would likely differ to that of higher plants because the algae contain form II Rubisco, a type consisting of only large subunits (form I have both large and small subunits) (Badger et al. 1998).

The observed decrease in Rubisco expression found in this study may be a precursor to decreases in Rubisco content of *Symbiodinium* at elevated temperature. In *Stylophora pistillata* and *Turbinaria reniformis*-associated *Symbiodinium*, Rubisco protein content was lower in heated treatments (+6 °C above ambient) than in ambient conditions (Hoogenboom et al. 2012). We suggest disruption in the dark reactions of *Symbiodinium* photosynthesis under heat stress is two-fold: a decrease in expression of Rubisco, which ultimately leads to less Rubisco protein in the cell, and a decline in

activity of remaining Rubisco. These two phenomena in concert would significantly diminish the ability of the algae to perform carbon fixation.

The ability of *Symbiodinium* to maintain high rates of photosynthetic efficiency of PSII at +6 °C above control but not +8 °C, when both treatments exhibit significant down-regulation of Rubisco, suggests a protective mechanism is functioning at intermediate temperatures that is insufficient at higher temperature, or these higher temperatures also affect other areas of *Symbiodinium* photosynthesis not examined here. Decrease in Rubisco activity/protein content would lead to an accumulation of excess NADPH and ATP. This would, in turn, cause an upstream build-up of electrons that would be diverted to non-assimilatory pathways such as the Mehler-Ascorbate peroxidase (MAP) cycle. Non-photochemical quenching in *Symbiodinium* is O_2-dependent, demonstrating the MAP cycle is most likely the photoprotective mechanism at work (Jones et al. 1998). The MAP cycle alleviates excess electron build-up by converting harmful reactive oxygen species (ROS) in the form of superoxide radicals to harmless water via ascorbate-peroxidase and super-oxide dismutase (see Miyake 2010 for review). At intermediate temperatures, this non-assimilatory electron flow is most likely sufficient to prevent high levels of ROS accumulation. Prolonged or severe decrease in electron acceptors (sink limitation) would eventually lead to MAP cycle being overwhelmed and excessive ROS formation would occur, ultimately leading to cellular damage and subsequent reduction in quantum yield of PSII (Lesser 1996). Furthermore, at elevated temperatures, the repair mechanisms of PSII are impaired, thus exacerbating the damaging effects of ROS (Takahashi et al. 2009). The lack of HSP upregulation may also contribute to a buildup of damage or denatured proteins further exacerbating thermal stress.

Carbohydrate transfer

The significant decrease in Rubisco expression was followed by a pattern of increased expression of a carbohydrate transporter gene. The increase of the 'sugar' transporter on days 3 and 4 (at 32 °C and 34 °C) may indicate that a study on a longer time scale may have shown more significant differences. Given the nature of their symbiosis, the transfer of photosynthate from algae to coral host cells during periods of stress would be an important factor impacting host health. To date there have been no studies elucidating these mechanisms under heat stress in reef-building corals, arguably the most well studied *Symbiodinium*-host interaction. In symbiosis with the giant clam, *Tridacna gigas*, *Symbiodinium* remaining in host after a bleaching event were exporting the same amount of photosynthate (per cell) as they were prior to bleaching (Leggat et al. 2003). An increase in expression of carbohydrate transporter gene may offset the down-regulation of Rubisco, thus enabling *Symbiodinium* to maintain photosynthate export during thermal stress.

Fatty acid metabolism

One target of ROS in cells are lipid membranes (Lesser 2006). Differences in composition of thylakoid membrane lipids affect the thermal tolerance of different *Symbiodinium* strains (Tchernov et al. 2004). Damage to the membrane causes "leakiness" and loss of proton gradient, which suppresses protein synthesis-independent repair of PSII (Takahashi et al. 2009). This study did not find significant changes in the expression of a chloroplast acyl synthesizer, which synthesizes precursors of membrane lipids, suggesting that differential expression of this gene at highly elevated temperature is not the mechanism by which membrane integrity is restored.

Conclusions

This study is among the first to assess transcriptional responses of genes involved in photosynthetic processes in *Symbiodinium* exposed to heat stress (see also McGinley et al 2012). These expression changes were then interpreted using photophysiological responses. In general, there were few perturbations seen at 30 °C compared to 32- 34 °C in gene expression, while photophysiology was severely impaired at 34 °C. Results of this study, which assessed the transcriptional responses of 7 GOI, suggest that during thermal stress *Symbiodinium* do not rely heavily on differential gene expression to adjust to the changing environmental conditions. Several other studies have found similarly minor transcriptional changes (less than 5-fold difference to controls in nearly all cases) in *Symbiodinium*, both *in vitro* and *in hospite* (Rosic et al. 2010a; Rosic et al. 2010c; Leggat et al. 2011a) (for a review of Symbiodinium transcriptomics see Leggat et al. 2011b), compared to corals, where fold-changes can be from 10- to 100s-fold different to control under various conditions (Csaszar et al. 2009; Meyer et al. 2009; DeSalvo et al. 2010; Meyer et al. 2011). This may be due to the relatively low number of transcription factor domains found in *Symbiodinium* (Bayer et al. 2012) or the permanently condensed condition of the chromosomes (Diaz de la Espina et al. 2005). As with all transcript studies, caution must be taken when interpreting results. Post-transcriptional and translational regulation can have a significant impact on protein abundance in cells, ergo transcript abundance does not necessarily correlate directly with protein content. Future research should therefore also focus on proteomics and metabolomics when *Symbiodinium* are exposed to heat stress (Gordon and Leggat 2010).

A study of *Symbiodinium* HSP expression *in hospite* and *in vitro* during thermal stress revealed that changes were relatively similar, regardless of symbiotic state (Rosic et al. 2010a). This is important, since *in vitro* studies are relatively easier to perform and

cell cultures easier to manipulate than corals or other *Symbiodinium*-harbouring organisms. Therefore, although *in hospite* studies are indispensable from an ecological point of view, there is utility of isolated *Symbiodinium* experiments in attempting to elucidate effects of environmental perturbation on *Symbiodinium*-associated symbioses in natural settings.

Chapter 3: Small changes in pCO_2 lead to altered gene expressions in *Acropora aspera*

Abstract

Anthropogenic CO_2 emissions are fundamentally altering the world's oceans by increasing sea surface temperature and decreasing pH (ocean acidification). By mid-century, tropical corals will be threatened with annual mass bleaching events, coupled with a 50-100 ppm increase in the partial pressure of CO_2 (pCO_2) in surface waters. This study explored the interactive effects of near-term CO_2 increases (40-90 ppm above current ambient) during a simulated bleaching event (34 °C for 5 days) of *Acropora aspera*. *Symbiodinium* photosynthetic efficiency of PSII (F_v/F_m) was significantly depressed by the bleaching event, which did not recover during the 9 day recovery period (+1.7 °C above ambient). Interestingly, elevated pCO_2 increased F_v/F_m in the heated treatment above heated treatment alone during the final 4 days of the recovery period, indicating a slight mitigating effect. The loss of *Symbiodinium* from host tissue was not recovered following the bleaching event (significant decrease in symbiont density from day 5 to 14 in heated treatments, regardless of pCO_2). Effects of CO_2 were only seen in synergy with elevated temperature; elevated pCO_2 alone had no effect on F_v/F_m or symbiont density. Expression of targeted *Symbiodinium* genes involved in carbon metabolism and heat stress response was not significantly altered by any treatment on any day, indicating a relatively unresponsive transcriptome. Targeted *A. aspera* carbon sequestering genes carbonic anhydrase (CA) isoforms 2 and 3, conversely, exhibited significant expression changes, most notably in crossed bleaching and elevated pCO_2 treatments. CA2 was significantly down-regulated on day 14 in all

treatments, with the greatest decrease in the crossed treatment (relative expression compared to control = 0.16; $p<0.05$); CA3 showed a similar trend, with crossed treatment expression 0.20-fold of controls on day 14 ($p<0.05$). Interactive effects of CO_2 and temperature also altered the immunolocalisation of two coral carbonic anhydrase proteins, which appeared to be less abundant in tissue sections taken from elevated pCO_2 and heated corals than control or single factor treatments. The synergistic effects of ocean acidification and bleaching were evident during this study and demonstrate that increased pCO_2 in surface waters will impact corals much sooner than studies utilising end-of-century pCO_2 concentrations would indicate.

Introduction

Anthropogenic CO_2 emissions are creating an environment that is becoming increasingly inhospitable to tropical reef-building coral. The problem is two-fold: increasing atmospheric CO_2 levels contribute to rising sea-surface temperature (SST) as well as cause ocean acidification (OA) via direct uptake of CO_2 by surface ocean water (Caldeira and Wickett 2003; Doney et al. 2009). The Intergovernmental Panel on Climate Change (IPCC) projects that within 50 years, atmospheric CO_2 will rise by 50-100 ppm, depending on the emissions scenario (IPCC 2007), leading to increasing SST on the Great Barrier Reef (GBR) of more than 1 °C and decreasing ocean pH by more than 0.15 units (Johnson and Marshall 2007). While coral reefs in shallow lagoons along the GBR experience daily fluctuation in pH (for example, from 8.0-8.4 on Heron Island reef flat (Kline et al. 2012)) and temperature fluctuations far greater than the changes predicted by the IPCC and the GBR Marine Park Authority (GBRMPA) for the open ocean, the influx of warmer, more acidic water coming over reef flats may push these resilient coral past their limits (Anthony et al. 2011; Kleypas et al. 2011) leading to increased coral mortality.

A well-studied consequence of higher-than-average SST is coral bleaching, or the loss of endosymbiotic algae, *Symbiodinium* (or their pigments). Recently, increases in pCO2 to between 520 and 700 ppm have also been linked to coral bleaching (Anthony et al. 2008) and a number of papers have suggested that pCO_2 levels above 500 ppm will lead to significant coral loss (Hoegh-Guldberg 2005, Veron 2009). Although corals are capable of heterotrophic feeding, some may obtain up to 95% of their daily energy from photosynthate (products of photosynthesis) translocated from their algal endosymbionts (Muscatine et al. 1984). Thus, prolonged loss of *Symbiodinium* during a bleaching event can lead to coral mortality (Glynn 1984; Glynn

1990; Brown 1997; Baird and Marshall 2002). *Symbiodinium*, residing within the gastroderm of the host, export excess photosynthates to the coral. The host can use this organic carbon input for mucus production, growth, reproduction, or it can use it for energy storage within cells as glycogen or lipids (Yellowlees et al. 2008). In order to maintain high levels of photosynthesis of intracellular *Symbiodinium*, the coral host must supply their endosymbionts with inorganic carbon from ambient seawater (Benazet-Tambutte et al 1996). This is accomplished by employing a carbon-concentrating mechanism (CCM), the primary enzyme of which is carbonic anhydrase (CA) (Al-Moghrabi et al. 1996; Goiran et al. 1996; Allemand et al. 1998). CA reversibly converts bicarbonate (HCO_3^-) and CO_2 at the epithelium, greatly increasing the concentration of CO_2 that can freely diffuse across the membrane (Weis et al. 1989; Goiran et al. 1996). CA associated with inorganic carbon supply for photosynthesis are purportedly found on the gastrodermal membrane of host cells and within *Symbiodinium* cells (Al-Moghrabi 1996), as well as the perisymbiotic membrane (Weis 1993). Inorganic carbon in the coral can be used for calcification as well as photosynthesis by *Symbiodinium*. A variety of studies have shown that CA is involved in the supply of inorganic carbon for both purposes, however it is possible to distinguish the roles of the various CAs by their location; CA on the calicoblastic epithelium, for example, most likely contributes to calcification (Moya et al. 2008), while CA on the perisymbiotic membrane contributes to carbon supply for photosynthesis (Weis 1993).

The synergistic effects of increased pCO_2 and elevated temperature on the physiology of the symbiosis are not well characterised. One study has shown an increase in net productivity with a moderate increase of pCO_2 (520-700 ppm) and elevated temperature, but a precipitous drop with further increase of pCO_2 (1000-1300 ppm) at both ambient and elevated temperature (Anthony et al. 2008). Similarly, net

photosynthesis (P_n) of *Stylophora pistillata* was enhanced by increased temperature at ambient pCO_2 but declined when crossed with elevated pCO_2 (~800 ppm) (Reynaud et al. 2003). Primary productivity increased in a coral assemblage with increasing pCO_2, but there was no correlation with temperature increase (Langdon and Atkinson 2005). A significant decrease in calcification is seen in *Porites* at a CO_2 seep when temperature increases, but not with decreased pH alone (8.0-7.8), demonstrating a synergistic effect of the two factors. (Fabricius et al. 2011). Recently, a microarray study by Kaniewska et al. (2012a) found differential gene expression under high pCO_2 (1010- 1360 ppm) indicating metabolic suppression, increased oxidative stress, apoptosis, and loss of *Symbiodinium* from *Acropora millepora*. These changes were observed before changes in calcification became evident (Kaniewska et al. 2012a).

This study simulated conditions using predicted near-future ocean conditions in order to explore potential synergistic effects of elevated pCO_2 and temperature stress on carbon assimilation of a tropical reef-building coral, *Acropora aspera*. Branches of *A. aspera* were subjected to a simulated bleaching event (4 days at +4-6 °C above ambient mid-day maxima) in aquaria supplied with water at pCO_2 levels projected by the IPCC for mid-century following the "business-as-usual" scenario of carbon emissions (approximately +50-100ppm pCO_2 above current ambient CO_2 level; IPCC FAR 2007). Both physiological and transcriptomic responses of host and symbiont were monitored during a 2-week experiment during the austral summer on Heron Island on the southern Great Barrier Reef.

Materials and methods

Experimental design

Sample coral nubbins, approximately 6-8 centimeters long each, (n = 250) were

collected (under Great Barrier Marine Park Authority permit G09/32575.1 and G08/26873.1) from 3 colonies of *A. aspera* (tan morph) on the reef flat of Heron Island at low tide in January 2011. Nubbins were taken to Heron Island Research Station and placed upright in test tube racks and placed in a holding tub with continuous flow of sand-filtered water pumped from the reef flat (salinity = 32.8). Nubbins were left to recover for 14 days in ambient conditions; tissue regrowth was observed on the cut end of nubbins at the end of recovery.

Following recovery, racks were randomly assigned to one of four treatments: control (ambient CO_2/ambient temperature), heated (at ambient pCO_2), elevated pCO_2 (at ambient temperature), and elevated CO_2/heated. Each treatment aquarium consisted of a 250 L sump (mixing) tank, which supplied water to two 65 L replicate tanks containing nubbin racks. Water was pumped in a semi-closed system. 300W Eheim Jager (Eheim, Deisizou, Germany) heaters were used in each heated sump tank and temperature was recorded every 5 minutes throughout the experiment with Hobo data loggers (Onset, Massachusetts, USA). Temperature of the heated treatments were increased to simulate a bleaching event (approximately 34 °C daily maximum, +3-5 °C ambient midday maxima) for the first five days, then decreased to a mild heat stress of approximately +2 °C above ambient for the remaining portion of the experiment (Table 3.1; Figure 3.1a).

A CO_2-mixing system was built based on the design of Munday et al. (2009). Pure gaseous CO_2 was mixed with ambient air in the mixing chamber and the resulting CO_2-enriched air was bubbled into both sump and replicate tanks. pH was monitored 4-5 times daily with a calibrated YSI 600QS Sonde (YSI, Ohio, USA). Approximately 0.1 unit decrease in pH was maintained throughout the entire experiment for elevated CO_2 treatments (Table 3.1; Figure 3.1b). Natural fluctuations in pH and temperature were

retained in control tanks and treatments were imposed on top of ambient fluctuations, resulting in a dynamic CO_2/temperature regime throughout the diurnal cycle. In order to differentiate effects of CO_2 and temperature stress from high irradiance stress, tanks were covered with 70% shade cloth.

Water samples were taken throughout the experiment for water chemistry analysis from replicate tanks, poisoned with 0.2% saturated $HgCl_2$ solution, and stored at 4 °C for later analysis. Alkalinity was determined by potentiometric titration using a Metrohm titrator (MEP Instruments, Australia) and 0.05M HCl acid using certified reference material (CRM) supplied by Andrew Dickson (2003) as the reference standard. Alkalinity, pH, and water temperature were entered into CO2CALC (Robbins et al. 2010) to calculate pCO_2 of each sample (Table 3.1).

Pulse-amplitude modulated fluorometry (PAM)

Measurements of chlorophyll fluorescence of *A. aspera Symbiodinium* were taken using imaging PAM fluorometry (MAXI Imaging PAM, Waltz, Effeltrich, Germany) every second day 30 minutes after sunset, starting on day 1 of the experiment. See Chapter 2 methodology for dark-adapted maximum quantum yield measurements and calculations. Five replicate nubbins from each treatment replicate tank were designated for PAM measurements and used throughout the experiment. Nubbins were transported to the lab and measured in dishes filled with respective treatment water. Nubbins were returned to treatments immediately following measurement.

Sample processing

On days 1, 4, 6, 9, and 14, three replicate nubbins were taken from each replicate tank (total 6 nubbins per treatment) and snap-frozen immediately in liquid nitrogen and

stored at -80 °C for later mRNA isolation. On days 5, 10, and 14, three nubbins were taken from each replicate tank and snap-frozen immediately in liquid nitrogen and stored at -80 °C for pigment quantification and *Symbiodinium* cell count. Two nubbins per replicate tank were fixed in 4% PFA in 3x PBS on day 14 for immunohistochemistry.

Pigment quantification and *Symbiodinium* cell count

Nubbins frozen for *Symbiodinium* cell counts were stripped of tissue using a dental irrigator (Waterpik™) filled with 0.22 μm filtered seawater. The blastate volume was recorded, then homogenized with an immersion blender for 20 seconds and centrifuged at 3220 g for 5 minutes to pellet algal cells. An aliquot was taken for cell counts and another for chlorophyll *a* and *c* quantification, both stored at -20 °C. Chlorophyll was extracted in 90% acetone for 48 hours in the dark at 4 °C, then measured on a DU-650 spectrophotometer (Beckman, USA) at 630 and 664 nm light wavelength. Chlorophyll *a* and *c* content of the sample were calculated using the equations of Jeffrey and Humphrey (1975). Replicate cell counts were performed on a haemocytometer (n = 6-10). Surface area of Waterpiked nubbins was determined using the 3D modeling method of Jones et al. (2008a) with modification of grid to 10 x 10 cm. *Symbiodinium* density and pigment content were normalised to skeleton surface area.

Gene expression analysis

Coral branches that were snap frozen in liquid nitrogen were crushed with a hydraulic press before transfer to a mortar chilled with liquid nitrogen and ground finely with a chilled pestle. The resulting powder was used for mRNA purification using the Dynabeads mRNA DIRECT kit (Invitrogen, Australia). Approximately 100-200 mg of

powder was added to 400 µl of Dynabeads lysis buffer and vortexed for 5 min then centrifuged for 2 min at 1200 g. The supernatant was then added to 100 µl of pre-washed Dynabeads and vortexed for 7 min. The sample and beads were placed on a magnetic column for 8 min, supernatant discarded and washed twice with 400µl of wash buffer A and twice with 300 µl of wash buffer B. mRNA was eluted from the beads by adding 27 µl of 10mM Tris-HCl and heated to 80 °C for 2 min and placed on ice to chill. mRNA was then removed from the tube and all samples quantified spectrophotometrically on a NanoDrop-1000 (Thermo Scientific, USA).
Following mRNA purification, DNase treatment of all samples was performed to eliminate genomic DNA contamination. Approximately 100 ng of mRNA was treated with RQ1 RNase-Free DNase (Promega, USA) as per manufacturer's protocol then added to 10 µl 2X RT reaction mix and 2 µl SuperScript III. cDNA was synthesised by incubating samples at 25 °C for 10 min, 50 º C for 30 min and 85 ºC for 5 min. Reactions were then chilled on ice before 1 µl of RNase H was added and incubated at 37 ºC for 20 min.

Several genes for both *A. aspera* and *Symbiodinium* were selected as genes of interest (GOI, Table 3.2). Coral host primers for glycogen phosphorylase, glycogen synthase, and glyceraldehyde-3-phospahte dehydrogenase (GAPDH), and *Symbiodinium* heat shock proteins (HSP70 and HSP90; same as in chapter 2), were taken from Leggat et al (2011a). Primers were designed for genes involved in carbon uptake and fixation, including two coral α-carbonic anhydrases (designated CoCA2 and CoCA3), one *Symbiodinium* β-carbonic anhydrase (SymBCA1) and Rubisco (same primer as in chapter 2), and for photorespiration (phosphoglycolate phosphatase, PGPase) using the software Primer Select (Lasergene 8) (Table 3.2). Candidate internal control genes (ICG) primers tested were: AdoHcyase and ribosomal protein S7 (RPS7)

(Seneca et al. 2010), unidentified contig 1913 (Ctg1913) and ribosomal protein L9 (RPL9) (Souter et al. 2011), and RNA polymerase II (RPII) (Brady et al. 2011) for coral genes (Table 3.2). Candidate *Symbiodinium* ICG were*:* cyclophin (Cyc) and GAPDH (Rosic et al. 2010b) and proliferating cell nuclear antigen (PCNA) (Boldt et al. 2008). Validation for use as ICG in this experiment was performed using GeNorm in the qBASE plus software package (Biogazelle).

The amplification efficiency for each gene-specific primer for both coral host and *Symbiodinium* was determined by serial dilutions of cDNA from 1:10 to 1:160. A 1:40 cDNA concentration was optimal for all primer sets. qPCR was then performed; see Chapter 2 methodology for detail on reaction mix and qPCR parameters. Modifications of Chapter 2 methodology for this experiment were: 66 °C elongation step during qPCR for *Symbiodinium* primers and 62 °C for *A. aspera* primers, and the melt curve analysis ranged from 60 °C to 95 °C. Relative expression analysis was performed using qBASE plus (Biogazelle).

Characterisation of carbonic anhydrase

Two full length carbonic anhydrase sequences were obtained from a λ-Zap II directional cDNA library (Stratagene, La Jolla, CA, USA) constructed from *A. aspera* and using the PCR protocol of Kvennefors et al. (2008) with degenerate primers designed to conserved carbonic anhydrase domains; these forward and reverse primers were used to generate overlapping PCR products using primers from the λ-Zap II library. Using this method, two complete sequences were obtained (CoCA2 and CoCA3). These sequences were then aligned against representative carbonic anhydrase sequences and phylogenies constructed using the core carbonic anhydrase domain corresponding to between ^{6}Trp and ^{255}Arg of human CA1 using Mr Bayes.

Table 3.1. Summary of physical and chemical parameters of experimental treatments.

	Temp (°C)	Average ΔT °C from control (treatment-control)	pH	Average ΔpH (control-treatment)	pCO_2 (μatm)	Average ΔpCO_2 from control (treatment-control)	TA (μmol kg^{-1})
Control	25.4-30.8	-	8.1-8.4	-	142-435	-	2045-2538
Ambient CO_2/ heated	26.7-35.2	Bleaching: 4.24 ±0.91 Stress: 1.28 ±0.54	8.1-8.4	-0.04 ±0.05	141-350	-11 ±26	2042-2365
Elevated CO_2/ ambient temperature	25.3-31.2	Bleaching: 0.35 ±1.0 Stress: 0.31 ±0.69	7.9-8.4	0.11±0.07	194-756	70 ±50	2036-2688
Elevated CO_2/ heated	25.9-35.3	Bleaching: 3.77 ±1.54 Stress: 1.86 ±1.05	7.9-8.4	0.08±0.05	204-827	59 ±28	1998-2422

Values of parameters ±SD. Ranges indicate differences within treatments due to diurnal fluctuations in incoming reef water supplying mixing tanks. Total alkalinity (TA) and pH were measured directly and pCO_2 was calculated using CO2CALC.

Table 3.2. Primer sequences used for quantitative PCR for coral host *A. aspera* and *Symbiodinium.*

	Gene name	Forward Primer	Reverse Primer	Reaction efficiency	Accession number/ citation
Symbiodi-nium	SymBCA1	GCCCATCCGCACAACAACT	GTGTGGTGCCTGTCCCTCTTT	1.03	Bobeszko
	PGPase	TCACTGACCGATCCCGCATTTG	CCTTTCTTTCTGGGCACCTTGTCC	0.99	Bobeszko
	Rubisco	AACGAGTGCATTCCTGAAGTGGTGA	GGCAGTGATGTTGGCGGAGAAGA	1.01	Ogawa
	HSP70	AGGAGACCGCTGAGGCATACTTG	CTTGGTGGCCTGACGCTGAGAATC	0.99	Ogawa
	HSP90	ATTCCGAGGATCTGCCACTGA	TCTCTGCGATCTCTGCGAACAT	1.01	Ogawa
	PCNA*	GAGTTTCAGAAGATTTGCCGAGAT	ACATTGCCACTGCCGAGGTC	1.02	(Boldt et al. 2008)
	GAPDH*	CCGGCGCCAAGAAGGTCATCATC	TTGGCCTTGTCGTACTCCGTGTGGTT	0.99	EH036392.1
	CYC*	ATGTGCCAGGGTGGAGACTT	CCTGTGTGCTTCAGGGTGAA	0.98	EH037450
A. aspera	Glycogen synthase	TCAAATCTGCCGAGGAATCAATCAA	AGTCGCTCCGTTCTGTTTCTCTGG	1.13	GW213759.1
	Glycogen phosphorylase	CTCAGGAAAGCCATCAATCAAATCAGG	TGCATCAAAGTCGGCCATAAGAAGG	0.94	EZ031953.1
	GAPDH	GAGGCTGGTGCAGATTTTGT	TGACTTTCTTGGCTCCACCT	1.01	EZ026309.1
	CoCA2	CATCTTCCCGCTGGCTCCTCTTCT	GCTTCGTTGCAGGTTGGGGTGGTAA	1.01	Leggat

CoCA3	TGCCGCCCAGCTGGAAGC	TGGACACGGTGGGCGGTAGTTT	1.05	Leggat
RPL9*	CCCTTGTGCGGAATTTTAGA	CACCAATGTGCCATTCTCAG	1.01	EZ026324
Ctg1913*	GATTTAACCACCGGCAGTGT	ATGGTAGGGAGGAGGCTGTT	0.90	EZ040581
RPII*	CCAAACTCCAATCCACCTTG	AAGACCTAAATAGTCATCCATGAGG	1.03	EZ031385
RPS7*	AGCAAAGGAGGTTGATGTGG	GACGGGTCTGGATCTTTTGA	1.04	(Seneca et al. 2010)
AdoHcyase*	AAGAAGACAAACATCAAGCCTCA	CACATCCAAGGTTCACAAGACG	1.05	(Seneca et al. 2010)

*denotes putative internal control genes (ICG)

Localisation of coral carbonic anhydrase

Immunolocalisation was performed using commercially generated polyclonal anti-coral carbonic anhydrase antibodies (GenScript, USA). Synthetic 15 residue peptides for two coral carbonic anhydrases (CoCA2 peptide (TTPSQKFKSSNNKHC) and CoCA3 peptide (CMLPKVKKAGSSEPL)) were injected into rabbits to induce an immune response and production of CA-specific antibodies. Over a period of 6 weeks rabbits were injected 4 times, and when high antibodies titres were obtained the rabbits were sacrificed and bleed. After bleeding antibodies were affinity purified and lyophilised. Lyophilised antibodies were resuspended in ultra-pure water (Invitrogen, Australia), aliquoted, and stored at -80 °C until use. Polyclonal swine anti-rabbit immunoglobin conjugated to horseradish peroxidase (Dako, Australia) was used as the secondary antibody. Western blotting using coral extracts indicated that only one band of the appropriate size was detected for each antibody (see Figures A1-A2 in Appendices).

At the conclusion of the 2 week experiment, 2 sample nubbins from each replicate tank were fixed in 4% paraformaldehyde (PFA) in 3x PBS (4 °C, overnight), then stored in fresh 3x PBS at 4 °C. Fixed nubbins were cut into segments and decalcified in 25% (w/v) ethylenediaminetetraacetic acid (EDTA) in DEPC-treated water then dehydrated in an ethanol series and embedded in paraffin wax (as per Ainsworth et al. 2007). Five μm thick tissue sections were cut and collected onto Superfrost Plus glass slides (Thermo Scientific, Australia). Slides were dewaxed in xylene and dehydrated in an ethanol series then blocked with 0.05% Roche blocking solution (Roche Diagnostics Australia Pty Ltd, Australia) for 2 hours at 4 °C. After optimization primary antibody concentrations of 1:100 for CoCA3 and 1:50 for CoCA2 were used and incubated overnight at 4 °C without agitation. Slides were washed in Tris-buffered saline with 0.1% Tween-20 (TBS-T; Sigma-Aldrich Pty Ltd, Australia)

before incubating in 1:500 concentration of secondary antibody in blocking solution for 1.5 hours (room temperature). Slides were washed in TBS-T prior to staining with SigmaFAST 3,3'-diaminobenzadine (DAB) tablets (Sigma-Aldrich Pty Ltd, Australia) for 30 minutes. DAB dye was thoroughly rinsed off in double-distilled H_2O and finally slides were counterstained with Harris's hematoxylin stain (Sigma-Aldrich Pty Ltd, Australia) for visualisation of tissue. Slides were visualised on a Zeiss microscope with Axiom image processing camera/software.

Data analyses

Statistical software package SPSS (IBM) was used for all statistical analyses. A generalised linear model (GLM) was employed for pairwise comparison of F_v/F_m, chlorophyll *a* and *c*, and cell density with sequential Bonferroni post-hoc test to determine significant differences between treatments compared to control. qBASE PLUS (Biogazelle) was used to calculate calibrated normalised relative quantities (CNRQ) of genes of interest in qPCR using validated ICG determined by GeNorm (Biogazelle). These values were also analysed in SPSS for statistical differences (GLM; sequential Bonferroni post-hoc).

Results

Experimental set-up

In order to mimic future climate change conditions in an ecologically relevant manner, diurnal fluctuations in temperature and pH of incoming water from the reef flat were preserved and treatments imposed on top of these natural changes. Treatment tanks were heated an average 4.39 °C (range: 3.69-5.67 °C) above ambient midday maxima for five days, followed by nine days of recovery slightly above ambient but below bleaching threshold (average Δ1.72 °C above ambient midday maxima; Figure 3.1a,

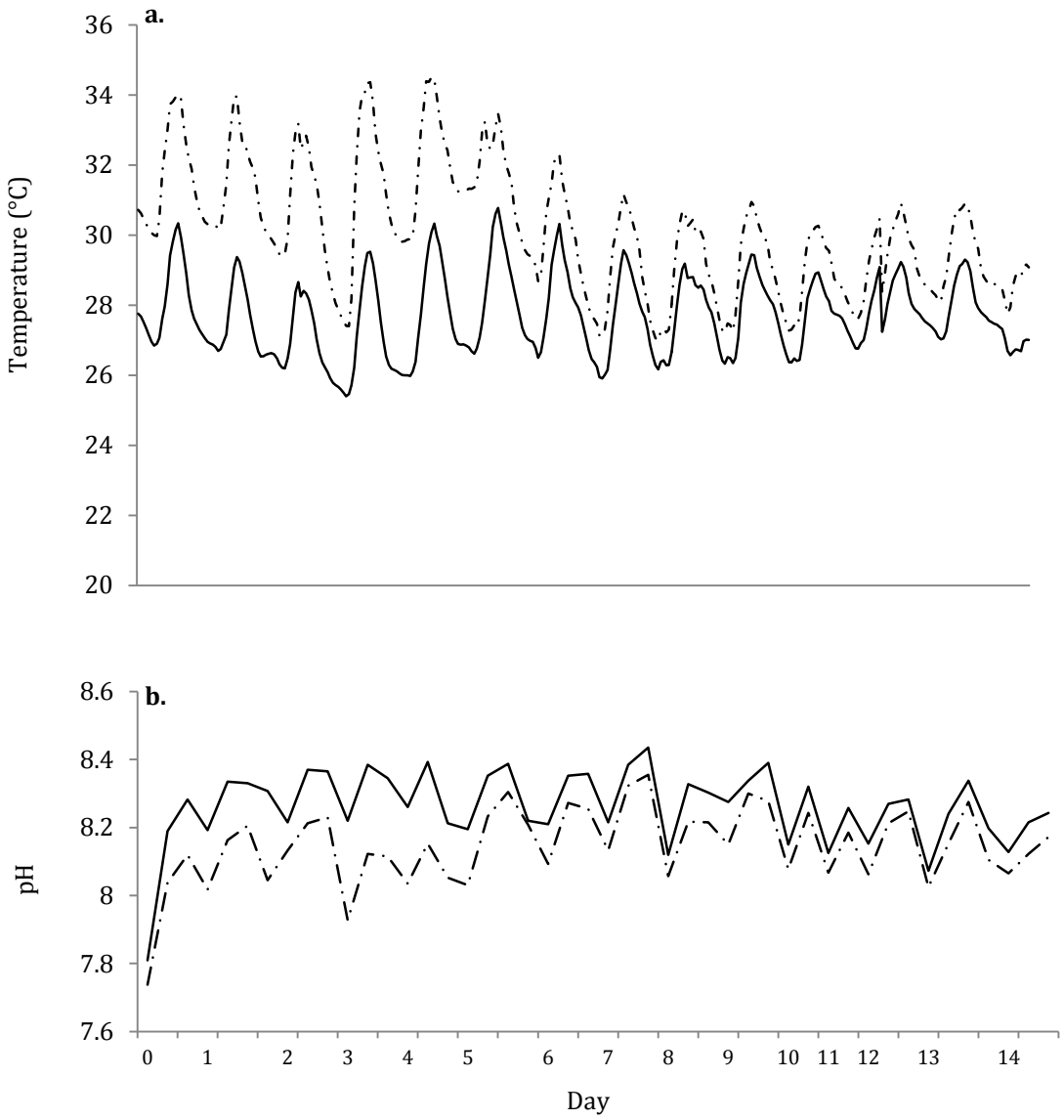


Figure 3.1. a) Temperature of ambient (solid line) and heated treatments (dotted line) during the 2-week experiment. Values represent the average of 4 replicate tanks at each temperature (2 replicate tanks at ambient CO_2, 2 replicate tanks at elevated pCO_2 for each temperature); b) pH of tanks for ambient (solid line) and elevated pCO_2 (dotted line) treatments during the 2-week experiment. Values represent the average of 4 replicate tanks at each pH (2 replicate tanks at ambient temperature, 2 heated replicate tanks for each pH).

Table 3.1) in order to mimic future baseline temperatures, these heating values are similar to previous bleaching events seen on Heron Island. A ΔpH of approximately 0.11 units (NBS scale; range: 0.038-0.29 units) was maintained between elevated CO_2 treatments and ambient CO_2 treatments, a value equivalent to mid-century levels following IPCC worst-case-scenario projections (Figure 3.1b, Table 3.1) (IPCC 2007).

This difference translated to an average +40-90 ppm aqueous pCO_2 as calculated by CO2CALC (Robbins et al. 2010). To explore possible synergistic effects of CO_2 stress and temperature stress, CO_2 enrichment and heat treatments were crossed.

Photosynthetic efficiency of PSII under elevated temperature and pCO_2

Maximum quantum yield of photosynthesis, F_v/F_m, was measured every 2nd day during the experiment with an iPAM fluorometer. Generally, chlorophyll fluorescence was more influenced by temperature than CO_2 concentration in the treatments. F_v/F_m was significantly depressed in both heated treatments compared to ambient temperature treatments every day except day 5 ($p<0.05$; Figure 3.2). F_v/F_m of elevated CO_2/heated treatment was slightly, but statistically significantly, greater than ambient CO_2/heated treatment on days 9-13 ($p<0.05$; Figure 3.2).

***Symbiodinium* density and pigment content**

Over the course of the experiment, the heated treatments experienced significant declines in symbiont density (SD) cm^{-2} ($p<0.001$) and were significantly lower than control treatment SD for all days measured ($p<0.001$; Figure 3.3). SD declined to 7.2 x 10^4 cells cm^{-2} in heated treatment and 1.4 x 10^5 cells cm^{-2} in elevated pCO_2/heated treatment by day 14. Although SD was still 58-66% of control in heated treatments on day 5, the nubbins appeared visibly paler to the naked eye. By day 14 nubbins in heated treatments appeared very pale with little to no colouration. CO_2 concentration did not have an effect on algal cell density within temperature treatments ($p>0.05$; Figure 3.3). Despite severe bleaching, polyps in heated treatments were observed to be feeding throughout the experimental period, until the termination of the experiment at day 14 (9 days after first visible signs of bleaching were observed). SD in ambient temperature treatments remained unchanged throughout the experiment.

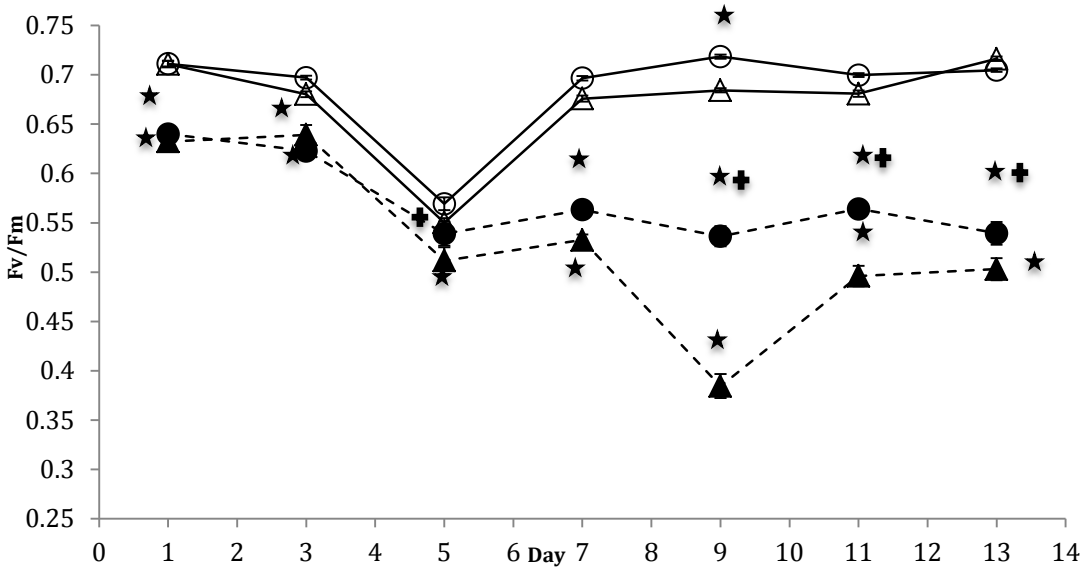


Figure 3.2. Photosynthetic efficiency of PSII of *Acropora aspera*, measured as dark-adapted yield, F_v/F_m. Data represented are: control (open triangles), heated treatment (filled triangles), elevated pCO_2 (open circles), and elevated pCO_2/heated (filled circles). Error bars represent the standard error (n = 9-10, some error bars obscured by data points). Stars represent significant difference to control on that day ($p<0.05$). Crosses indicate difference of temperature treatments ($p<0.05$).

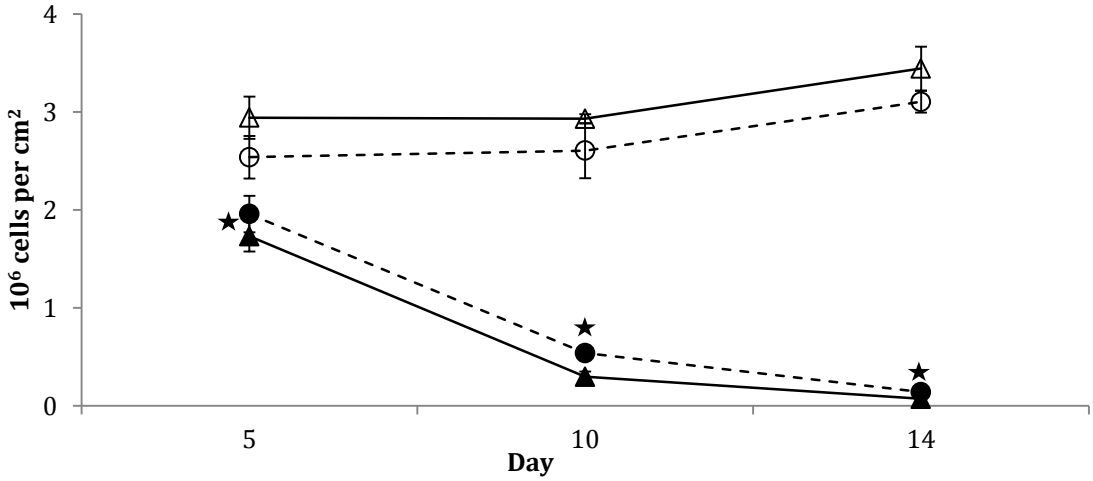


Figure 3.3. *Symbiodinium* cell density per cm^2 in *Acropora aspera* nubbins exposed to control (open triangles), heated treatment (filled triangles), elevated pCO_2 (open circles), and elevated pCO_2/heated (filled circles). Error bars represent the standard error (some error bars obscured by data points; n = 6-10). Stars indicate significant difference to control ($p<0.05$).

Chlorophyll *a* content per *Symbiodinium* cell was unchanged for ambient temperature treatments (at ambient CO_2 and elevated CO_2) over the experimental period

(Figure 3.4a). There was no significant difference between treatments and control on day 5 and 10 ($p>0.05$; Figure 3.4a), however, chlorophyll *a* per algal cell increased in the heated treatments at day 14 ($p<0.001$; Figure 3.4a). There was a significant rise in chlorophyll *c* per cell in both heated treatments at days 10 and 14 compared to control ($p<0.05$; Figure 3.4b). Elevated pCO_2/heated chlorophyll *c* values were significantly higher than ambient pCO_2/heated treatment on days 10 and 14 ($p<0.05$; Figure 3.2b). The ratio of chl *c* to chl *a* in heated treatments was significantly lower than control at day 14 (Figure 3.5c).

Gene expression analysis

Several candidate internal control genes (ICG) were considered for use in qBASE plus analysis (Hellemans et al. 2007; Table 3.2). GeNorm analysis determined ribosomal protein L9 (RPL9; $M = 0.49$), unidentified contig 1913 (Ctg1913; $M = 0.052$), and ribosomal protein subunit 7 (RPS7; $M = 0.54$) were the most stably expressed putative ICG in *A. aspera*. Although the *V 2/3* value of these three genes was 0.187, higher than the 0.15 threshold recommended by GeNorm (Vandesompele et al. 2002; Hellemans et al. 2007) , the next most-stable candidate ICG gene was RPII, at $M = 0.647$. We deemed this unacceptably high, as it was greater than some GOI, therefore it was not included as an ICG. GAPDH ($M = 0.44$) and cyclophilin (Cyc; $M = 0.52$) were used for *Symbiodinium* gene expression normalisation (*V 2/3* = 0.14).

SPSS GLM analysis of qBASE plus-generated calibrated normalised relative quantities (CNRQ) revealed few statistically significant changes in expression of *A. aspera* GOI during the experiment, despite large physiological responses. The biggest fold changes (compared as a ratio to control samples for the same day) were in the coral carbonic anhydrases, CoCA3 and CoCA2. On day 14, CoCA3 experienced significant

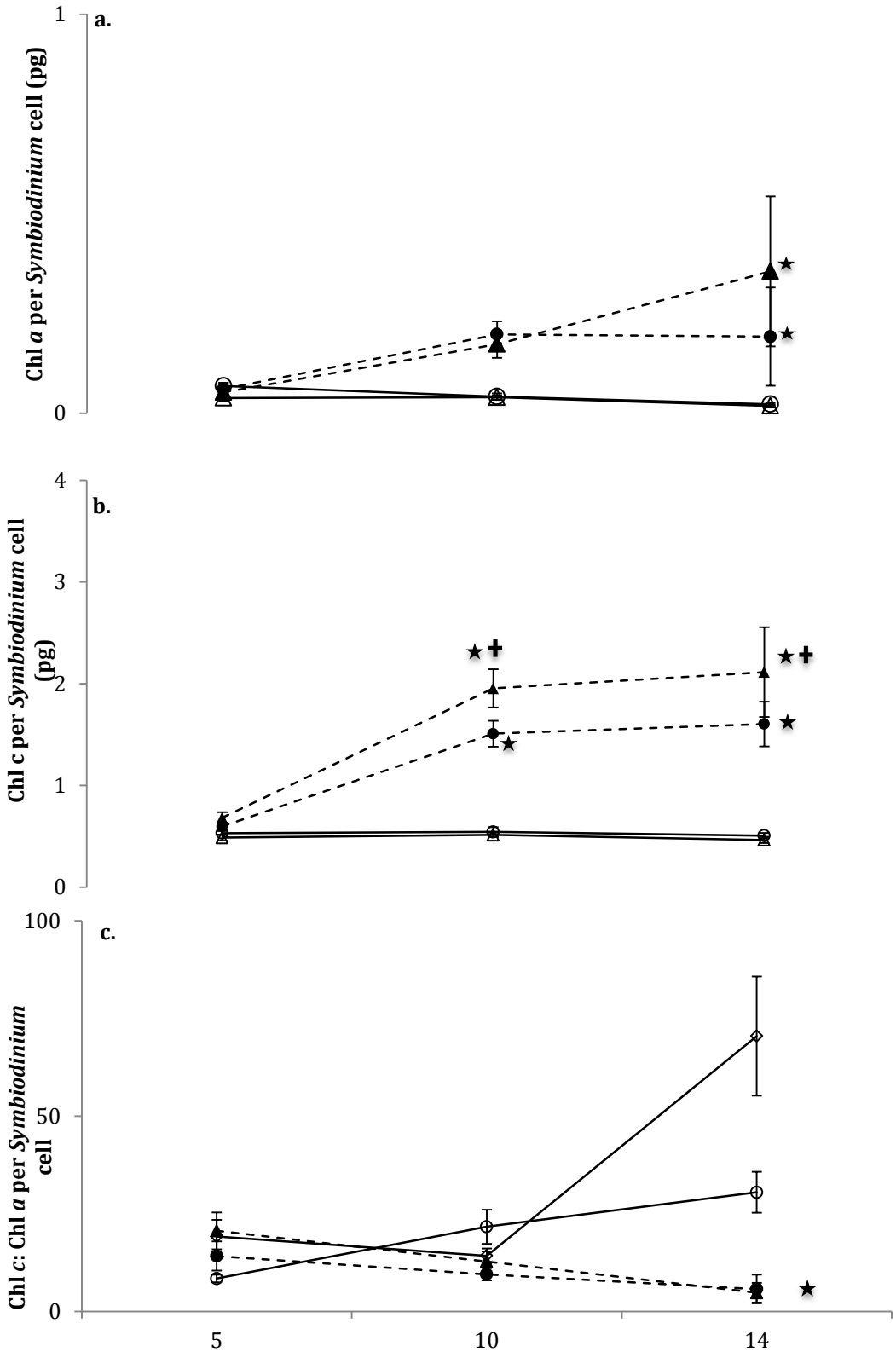


Figure 3.4. *Symbiodinium* pigment concentrations in *Acropora aspera*. (a) Chlorophyll *a* per *Symbiodinium* cell, (b) chlorophyll c per *Symbiodinium* and (c) ratio of chl a to chl c per *Symbiodinium* of nubbins subjected to: control (open triangles), heated (filled triangles), elevated CO_2 (open circles), and elevated CO_2/heated treatments (filled circles). Error bars represent the standard error (some error bars obscured by data points; n = 6). Stars represent significant difference to control on that day ($p<0.05$). Crosses indicate difference within temperature treatments ($p<0.05$).

down-regulation in elevated pCO_2/heated treatment (relative expression 0.20; $p<0.001$; Figure 3.5a). CoCA2 was down-regulated in all treatments on day 14, in heated treatment expression was 0.60 of control ($p<0.005$), in elevated pCO_2 0.49 ($p<0.001$), and in elevated pCO_2/heated treatment 0.16 ($p<0.001$; Figure 3.5b). There was simultaneous up-regulation of glycogen synthase (relative expression = 1.6), glycogen phosphorylase (= 2.1), and GAPDH (= 2.4) in elevated pCO_2/heated treatment on day 4, near the end of the bleaching period (Figure 3.5c-e). While glycogen synthase and GAPDH had no other times/treatments that exhibited differential regulation, glycogen phosphorylase was significantly down-regulated in elevated pCO_2 at day 1 (= 0.53) and at day 14 (= 0.65), and in elevated pCO_2/heated treatment on day 1 (= 0.67) (Figure 3.5d). All *Symbiodinium* GOI (HSP70, HSP90, PGPase, Rubisco, GAPDH and β-CA) were unchanged throughout the course of the experiment (Figure 3.6) despite the large physiological differences seen in dark adapted yield, chlorophyll and cell density (Figures 2,3,4).

Bioinformatic analysis of coral carbonic anhydrases

Two complete coral carbonic anhydrase cDNA sequences were obtained and named CoCA2 and CoCA3. CoCA2 is a predicted membrane-associated protein of 307 amino acids with a predicted protein mass of 33.7 kDa, containing a 22 amino acid signal sequence (Petersen et al. 2011) and a putative glycophosphatidylinositol anchor site at ^{284}Ala (Eisenhaber et al. 1999). CoCA3 encoded a protein of 260 amino acids with a predicted mass of 29.2 kDa (Figure 3.7a). Both CAs contain conserved histidine residues required for zinc binding and activity (Figure 3.7a).

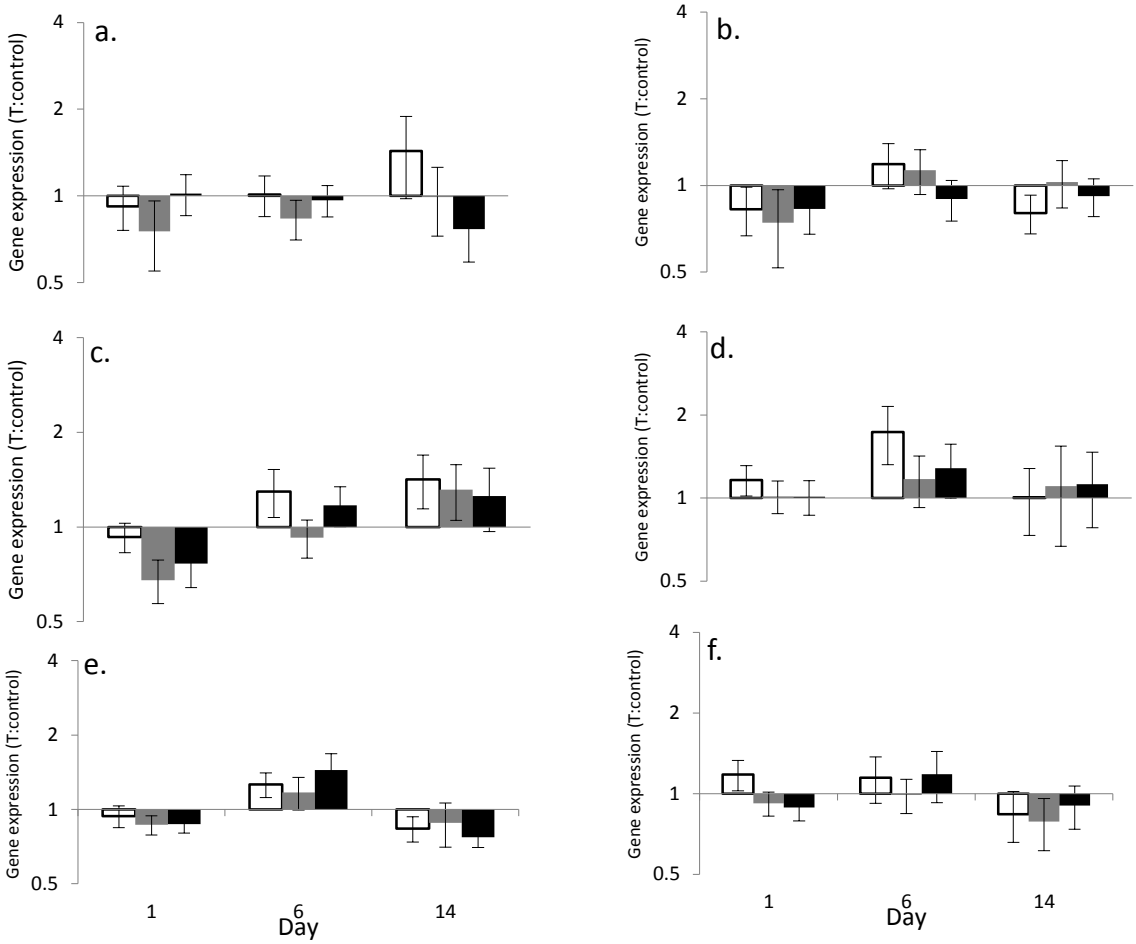


Figure 3.6. Relative expression of genes of interest in *Symbiodinium* when exposed to heated (white bars), elevated pCO_2 (gray bars), and elevated pCO_2/heated (black bars) treatments. Values expressed as ratios of treatment to control (T:control) for each day: a. HSP70, b. HSP90, c. PGPase, d. Rubisco, e. GAPDH, and f. β-CA. Error bars are standard error (n = 6). No significant differences were found for any gene compared to control.

Phylogenetic analysis shows that CoCA2 is closely related to a previously described carbonic anhydrase from *Acropora millepora* sequence (C007-E7) and forms a group with other membrane associated CA from vertebrates (Figure 3.7b), in addition this protein forms a distinct grouping but is separate to three other previously described coral membrane-associated CAs from *Stylophora pistillata* and *Fungia scutaria* and *Nematostella*, which form a cnidarian-specific grouping. In contrast, CoCA3 groups with other cytosolic CAs (Figure 3.7b).

Immunohistochemistry

Immunohistochemistry was used to give qualitative indications of changes in carbonic anhydrase expression under experimental conditions. Coral carbonic anhydrase CoCA2 and CoCA3 were localised in coral tissue sections using antibodies specific for each protein. CoCA2 exhibited consistent staining in a specific, unidentified cell type in the epithelium (Figure 3.8). These cells appear basal in the coenosarc epithelium (adjacent the mesoglea) and staining is largely absent in other areas of the epithelium, including polyps. In control treatment sections, staining was pervasive throughout the coenosarc epithelium; in all other treatments there appeared to be fewer of the unidentified cells stained.

CoCA3 exhibited staining throughout the mesoglea of mesenteries and polyps of control sections. In heated treatment sections, staining was visualised on the epithelium as well as the mesoglea, although there appeared to be less staining in the mesoglea than in control sections, particularly within polyps (Figure 3.9). In elevated pCO_2 samples there was little signal visualised, most of which was in the mesoglea of mesenterial filaments. In elevated pCO_2/heated sections, very little signal developed in any cell type.

Discussion

Response of *Symbiodinium* physiology to increased temperature and pCO_2

This study explores the impact of elevated pCO_2 during a simulated bleaching event of *A. aspera* on the gene expression of target genes in both the coral host and their dinoflagellate symbiont. The experimental parameters (temperature and CO_2 concentrations) were chosen to reflect values likely to be found within the next 50 years, e.g. pCO_2 levels 50-90 ppm above current levels. In contrast to a number of previous

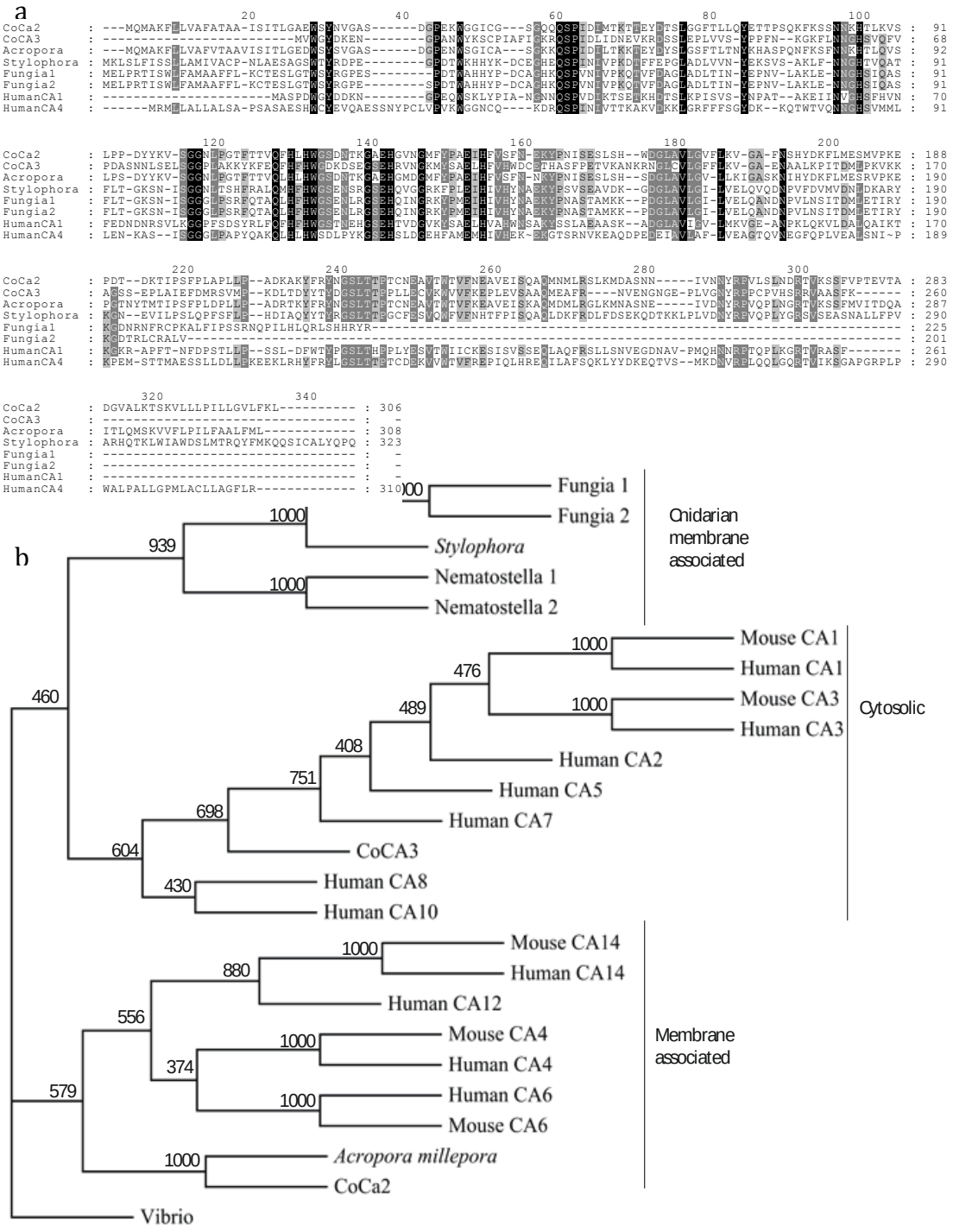


Figure 3.7. a). Alignment of two carbonic anhydrase isoforms (CoCA2 and CoCA3) isolated from *Acropora aspera*. CoCA2 has a 22 amino acid predicted signal sequences (^{1}Met-^{22}Ala) and a putative GPI anchor site at ^{284}Ala. Histidines involved in catalysis and zinc binding are found at numbered position ^{130}His, ^{132}His and ^{155}His. Black shading represent 100% identical, grey 80% identical and light grey 60% identical. b) Bayesian phylogenetic analysis of coral CAs and other representative CAs demonstrating three distinct clades, cytosolic, membrane associated and cnidarian membrane associated which contains some, but not all, cnidarian sequences. CoCA2 is found in the membrane associated clade while CoCA3 is found in the cytosolic

grouping. Numbers at branches represent the number of times this node was found in 1000 bootstraps.

studies (Langdon and Atkinson 2005; Anthony et al. 2008; Kaniewska et al. 2012a) there were no significant physiological changes detected in *Symbiodinium* solely in response to increased pCO_2 levels, while they were found in response to temperature.

Despite no significant changes in *Symbiodinium* gene expression patterns of key genes of interest, there were significant changes to its physiology (in contrast to the results in the previous chapter, where there were GOI expression changes but no change in physiology), as evidenced by the large change in *Symbiodinium* densities, pigment concentrations, and photosynthetic efficiency of PSII in response to temperature and temperature crossed with elevated pCO_2. However, the main driver of these changes is thermal stress and CO_2 alone had no significant effect. Photosynthetic efficiency of PSII was significantly depressed under both heated treatments, with elevated pCO_2 during heating slightly mitigating the effects on F_v/F_m over heat alone (Figure 3.2). Maximum quantum yield of photosynthesis (F_v/F_m) is a standard measure of photosynthetic efficiency of PSII and an indicator for physiological stress in photoautotrophs. During this two week experiment, F_v/F_m of *A. aspera Symbiodinium* was not significantly altered by elevated pCO_2 alone. Houlbreque et al (2012) found pH had no effect on F_v/F_m of *Stylophora pistillata* under greater pH differences than used in this study (7.5, 7.8, and 8.1), even after 5 weeks. Several studies have found that elevated pCO_2 has no effect on net photosynthesis (P_n), measured as O_2 evolution, even after weeks in treatment (Schneider and Erez 2006). Although this is a comparison between two different parameters (F_v/F_m and P_n), a correlation between the two at elevated temperatures has been observed and provides a basis for comparison (Jones et al. 1998).

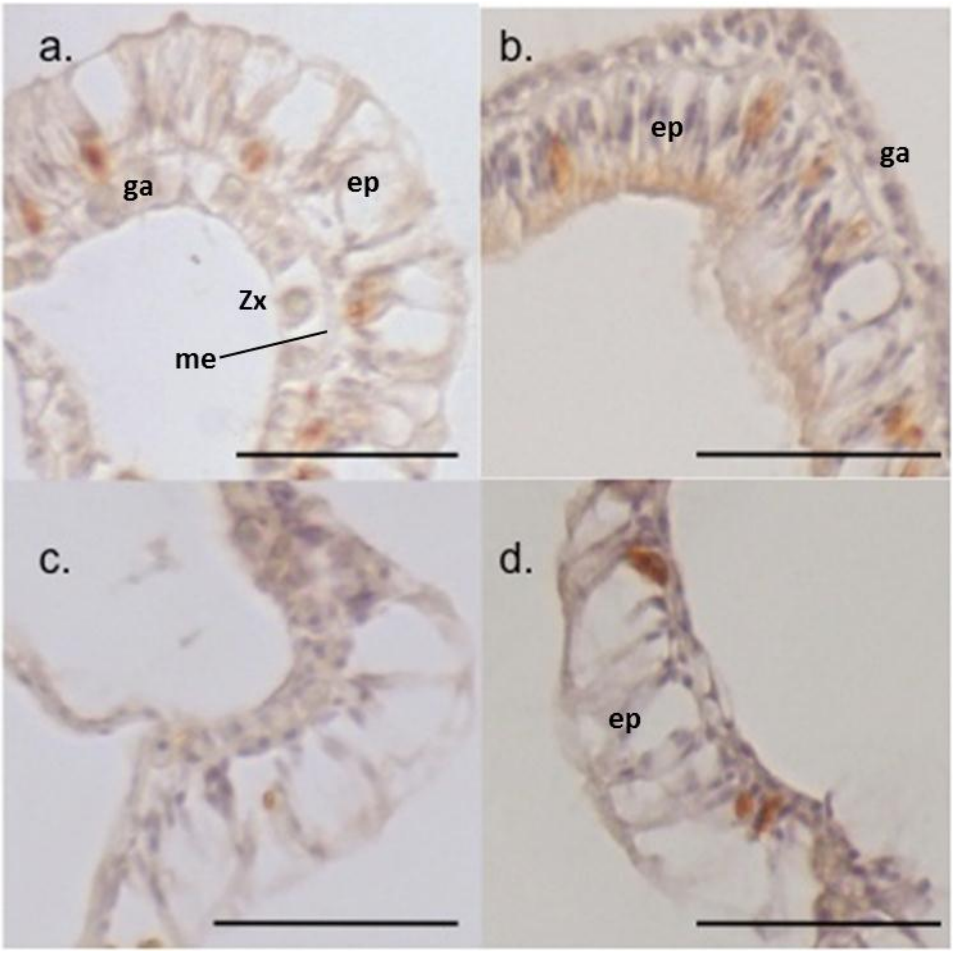


Figure 3.8. Localisation of coral carbonic anhydrase isoform 2 using specific monoclonal antibodies on *Acropora aspera* samples (5 μm cross sections) exposed to: a. ambient conditions (control), b. bleaching stress, c. elevated pCO_2, and d. heat and elevated pCO_2. Antibody visualization performed with SigmaFAST 3,3'-diaminobenzadine tablets, tissue stained with Harris's hematoxylin stain (Sigma-Aldrich Pty Ltd, Australia). Epidermis *ep*; gastroderm *ga*; zooxanthellae *zx*; mesoglea *me*. Scale bar, 50 μm.

There are few studies that have found pCO_2 to affect photosynthesis directly. One study of a coral assemblage under increased pCO_2 showed an increase in net production of organic carbon (NP_c) (Langdon and Atkinson 2005) while another observed decreasing F_v/F_m with increased acidity in *Porites australiensis* (Iguchi et al. 2012). Other studies have also noted the synergistic effect of heat stress and increased pCO_2 (Reynaud et al. 2003; Anthony et al. 2008). Anthony et al. (2008) observed an increase in P_n in *A. intermedia* and *Porites lobata* under a moderate temperature stress (+3 °C above

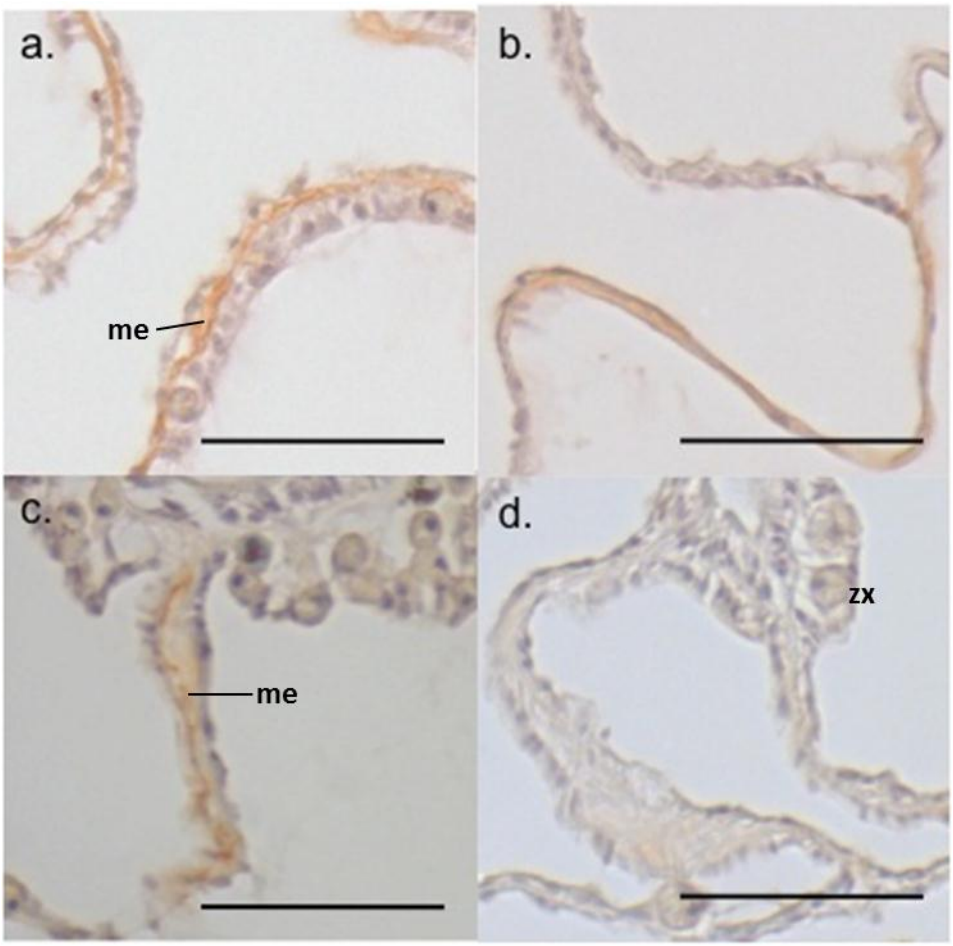


Figure 3.9. Localisation of coral carbonic anhydrase isoform 3 using specific monoclonal antibodies on *Acropora aspera* samples (5 μm cross sections) exposed to: a. ambient conditions (control), b. bleaching stress, c. elevated pCO_2, and d. heat and elevated pCO_2. Antibody visualization performed with SigmaFAST 3,3'-diaminobenzadine tablets, tissue stained with Harris's hematoxylin stain (Sigma-Aldrich Pty Ltd, Australia). Zooxanthellae *zx*; mesoglea *me*. Scale bar, 50 μm.

ambient), but when crossed with increased pCO_2 (pH 7.85-7.95), P_n in *A. intermedia* increased, whereas it dropped significantly in *P. lobata*. This once again demonstrates the variable susceptibility to acidification based on genera/species. In *S. pistillata*, elevated temperature has been shown to significantly increase P_n compared to control (+3 °C), while elevated pCO_2 (pH 7.83) at the same temperature did not change P_n compared to control (Reynaud et al. 2003). The increases in P_n under mild heat stress (+3 °C) most likely do not reflect the coral response during a bleaching event, rather a result of the Q_{10} effect. In severely thermally stressed corals F_v/F_m has been shown to

decline significantly above 30-33 °C (Warner et al. 1996; Jones et al. 1998; Warner et al. 1999; Middlebrook et al. 2010), depending on the rate of heating (Middlebrook et al. 2010).

Despite large losses of *Symbiodinium*, F_v/F_m levels of ~0.4 in the bleached nubbins of *A. aspera* indicate that the remnant *Symbiodinium* cells are still capable of photosynthesis. Additionally, chlorophyll *a* and *c* concentrations were significantly higher per *Symbiodinium* cell than controls, also potentially contributing to the relatively high level of quantum yield. Heat-related increase in chl *a* has been previously observed in other coral (Fitt et al. 1993; Jones 1997); however, in most instances, heated *Symbiodinium* lose pigmentation (Brown et al. 2002a; Dove et al. 2006; Venn et al. 2006). Remnant photocompetent cells may eventually recover and repopulate the coral host when suitable conditions return. It has been suggested that background populations of more thermally tolerant *Symbiodinium* clades could repopulate the coral after the dominant type is expelled, thus conferring increased thermal tolerance to the host (Fautin and Buddemeier 2004). This was not tested here, as the time scale of the experiment did not allow for full recovery of *Symbiodinium* population in bleached corals. The specific amplification of genes of interest in qPCR indicates extracted mRNA was C3 in all samples across days, although this does not preclude the presence of background populations of other clades (Mieog et al. 2007).

Symbiodinium gene expression response

The sustained photocompetency of remaining *Symbiodinium* may reflect relatively stable expression of key genes involved in photosynthesis and heat shock response. qPCR data show that none of the genes of interest (PGPase, Rubisco, β-CA, GAPDH, HSP70, and HSP90) showed any pattern of significant differential regulation in

response to any treatment, again, in contrast to results in Chapter 2 (Figure 3.6). As the environment for free-living and *in hospite Symbiodinium* is markedly different, a direct A previous study also reported similarly few transcriptomic responses to a simulated bleaching event in *A. aspera*, where there was also a significant decrease in quantum yield by the end of the experiment (all changes in *Symbiodinium* gene expression observed were less than 2-fold) (Leggat et al. 2011a). In another thermal stress study of *A. millepora*, Rosic et al. (2010) found significant differential regulation of *Symbiodinium* HSP70 and HSP90, although fold-change was still less than 2. This study and Leggat et al. (2011) were carried out in austral summer, whereas Rosic and colleagues performed their experiment in the winter. The absence of large expression changes compared to control in our studies may be due to a baseline shift in HSP70 and HSP90 in warmer, more highly illuminated summer waters. Overall, during summertime months it appears transcriptomic regulation constitutes a small or negligible part of the stress response in endosymbiotic *Symbiodinium*. Examining proteomic and metabolomic responses (Gordon and Leggat 2010) in future studies of stress response in *Symbiodinium* may be more informative than transcription-based studies (Leggat et al. 2011b).

A. aspera gene expression response

The coral host responded with more instances of differential expression in GOI than their endosymbionts during the experiment (Figure 3.5). For metabolic genes most of the significant differences came in the first part of the experiment (pre-bleaching), while CoCA2 and CoCA3, exhibited more differential regulation in the second half (post-bleaching, mostly on day 14). The expression of glycogen synthase and glycogen phosphorylase, key enzymes in the synthesis and catabolism of glycogen, respectively,

experienced changes in the first 4 days. Glycogen phosphorylase exhibited significant down-regulation in both elevated pCO_2 treatments on day 1. Then, in elevated pCO_2/heated treatment on day 4, samples showed up-regulation of both glycogen synthase and glycogen phosphorylase, as did GAPDH, which is very similar to results previously found (temperature had been approximately +4-6 °C above ambient for 4 days) (Leggat et al. 2011a). It is unknown if expression of glycogen synthase and glycogen phosphorylase would have followed the same pattern as seen in this study had their study continued beyond day 8. This concomitant up-regulation of opposing pathways suggests there is a general up-regulation of metabolism resulting in greater flux of carbon in metabolism.

In the latter half of the experiment, glycogen synthase and glycogen phosphorylase were slightly depressed in treatments while CoCA2 and CoCA3 were significantly down-regulated. CA is a key enzyme of the CCM of cnidarians and is involved in the uptake and transport of inorganic carbon to *Symbiodinium* (Weis et al. 1989; Allemand et al. 1998). On day 14 both were down-regulated in heated and elevated pCO_2 treatments (CoCA2 significantly so) and both exhibited expression 3-4 fold lower in the elevated pCO_2/heated treatment than just heated or elevated pCO_2 treatment (significantly lower than control for both genes). This decrease in CA could lead to a decrease of DIC supply to photosynthesis, potentially resulting in a decrease in fixed carbon exported by *Symbiodinium* as the algae become DIC-limited. The decrease and/or unchanged expression in the carbon-cycle related genes studied here (CoCA2, CoCA3, glycogen synthase, glycogen phosphorylase, and GAPDH in the host and β-CA, Rubisco, PGPase, and GAPDH in *Symbiodinium*) in the second week of the experiment suggests there may be a different energy source being utilised by the host after the first few days of thermal stress. It is possible that after the first few days of heat stress, corals

switch to lipid stores for energy, indeed, concentration and composition of lipids have been shown to change substantially in corals post-bleaching (Fitt et al. 1993; Middlebrook et al. 2010; Imbs and Yakovleva 2012).

The down-regulation of CoCA2 when symbiont density was still high (as seen in the elevated pCO_2 treatment on day 14) is an unusual occurrence for a cnidarian CA. In other cnidarian-dinoflagellate symbioses, CA is positively correlated with *Symbiodinium* density (Weis et al. 1989; Weis and Reynolds 1999; Estes et al. 2003; Leggat et al. 2003). However, in many plant and algae, CA decreases (seen at both transcript and protein level) at high CO_2 levels (Fukuzawa et al. 1990; Majeau and Coleman 1996; Lane and Morel 2000), therefore it is possible that at least one mechanism regulating CoCA2 is similar to plant CA. This mechanism is evidently sensitive to slight changes in pCO_2, as the increase here was 40-90 ppm, far lower than most experiments with other CA (Fukuzawa et al. 1990; Majeau and Coleman 1996; Lane and Morel 2000). This corroborates our finding that genes encoding CoCA2 and CoCA3 are significantly down-regulated under acidified conditions. This down-regulation, then, is likely leading to an actual decrease in the amount of CA in the cell. The decrease at elevated temperature could be related to the loss of endosymbionts, as in most symbiotic cnidarians, but it cannot be precluded that temperature itself has a direct effect on the expression of CoCA2. Although caution must be taken when quantifying an essentially qualitative assay, the substantial decrease in staining in all treatment samples compared to control samples in CoCA immunolocalisation (Figure 3.8) tentatively supports our qPCR data.

A key finding of note is the synergistic effect of elevated temperature and elevated pCO_2 on the expression of CoCAs. The significant down-regulation of these CoCAs under heat and increased pCO_2, and even greater decrease in elevated

CO_2/heated, indicate these two factors affect CA transcription by different mechanisms which are additive. The IPCC projects surface ocean temperature will rise by 2.4-6.4 °C and pH will drop by 0.14-0.35 by the end of the century (IPCC 2007). Therefore, studies of the effects of future climate change on the coral holobiont must manipulate both temperature and CO_2 in tandem in order to achieve ecologically relevant results.

Specific localisation of CoCA

Immunolocalisation studies have shown CA presence on the calicoblastic epithelium of coral (Tambutte et al. 2007; Moya et al. 2008; Bertucci et al. 2011), thus confirming its role in biomineralisation (Goreau and Goreau 1959). Two CAs have been localised in *A. millepora* larvae, one in the aboral end of metamorphosing larvae and another in the septa of older polyps (postsettlement stage), again consistent with location of calcification (Grasso et al. 2008). The localisation of both coral carbonic anhydrases studied here, and their phylogenetic relationship to other coral CA, suggests that both are involved in CO_2 supply to the *Symbiodinium* as opposed to directly being involved in calcification. To our knowledge this is the first immunolocalisation study in coral corroborating the role of CA in DIC supply for photosynthesis, although CA locality has previously been inferred from a pharmacological study (Al-Horani et al. 2003). Weis and colleagues have previously localised a CA to the gastroderm of the sea anemone, *Aiptasia pulchella,* on the animal "vacuolar membrane" surrounding the algae (Weis 1993). A CA inhibitor, acetazolamide, significantly decreased the photosynthetic rate of *A. pulchella,* confirming its link to photosynthesis (Weis 1993). The localisation of CoCA3 to the mesoglea is conducive to the CCM model of Allemand et al. (1998). In their model they propose a Cl^-/HCO_3^- antiporter at the epithelium/mesoglea interface transports bicarbonate ions into the mesoglea, which reacts with protons transported

there by an H^+/ATPase on the gastrodermal membrane to form CO_2, which is free to diffuse into the gastroderm. Based on our localisation study we suggest a CA within the mesoglea actively converts HCO_3^- to CO_2. CA catalyses the dehydration of bicarbonate at rate constants of approximately 10^5 to 10^6 s^{-1} (Lindskog and Coleman 1973). The presence of a CA in the mesoglea would prevent DIC limitation to photosynthesis by dehydrating HCO_3^- at high rates and creating a large CO_2 gradient that flows in the gastroderm where CO_2 is at a low concentration due to utilisation by Rubisco (see Badger and Price 1994 for review of CA in photosynthesis).

The function of CoCA2 localised to specific, yet unidentified, cells basal in the coenosarc epithelium is more enigmatic. It may be a part of the CCM despite being localised in the coenosarc epithelium and not in the polyp. It was not located on or near the calicoblastic epithelium either so a role in calcification is also unlikely. Without identifying the cell type it is difficult to interpret the function of this particular CA and it is possible that it plays another role in corals not associated with either calcification or inorganic carbon supply for photosynthesis. It is interesting to note the specificity of the dye to a particular cell type in the epithelium. This indicates the cellular differentiation in coral may not be as simple as two cell layers connected by mesoglea, which is the paradigm for coral morphology, and that distinct cellular specialization exists within coral cells.

Conclusions

This study is the first to explore the effects of near-term ocean acidification (pCO_2 50-90 ppm above current ambient) and elevated temperature on coral physiology. These results suggest that elevated SST will have a more significant impact on *Symbiodinium* photophysiology than OA in the near future. The effects of OA may manifest relatively

soon for *A. aspera*, as evidenced by the synergistic effect with temperature on gene expression of key metabolic proteins. Down-regulation of gene expression and apparent decrease in protein abundance of two CAs at elevated pCO_2 and temperature may impact negatively on inorganic carbon supply to *Symbiodinium*, as this enzyme is critical in the CCM. This has implications for the coupled nutrient exchange that is critical to the symbiosis, and, by extension, coral reefs. These differences, noted primarily in crossed treatments, highlights the necessity of studying OA and temperature stress together, as these factors will be changing concomitantly in future climate change scenarios. Additionally, studies of OA should include more subtle, near-term CO_2 levels as well as worst case scenario end-of-century predictions to determine how soon OA is likely to impact coral reefs as significant differences can be seen even with small changes in CO_2 concentrations.

Chapter 4: *Symbiodinium* loss during a simulated bleaching event under elevated pCO_2 is not associated with shifts in bacterial community of *Acropora aspera*

Abstract

This study is the first to explore the interactive effects of elevated pCO_2 and thermally induced bleaching stress on the bacterial community of a key reef-building coral, *Acropora aspera*. By directly pyrosequencing PCR amplicons of the 16S rDNA region, we were able to comprehensively sample the bacterial population with high temporal resolution from the onset and throughout a 14 day bleaching event. When exposed to either a near-future projected increase in pCO_2 of between 50-100 ppm and/or thermal stress, principal components analysis showed no significant shifts in community composition between CO_2 or temperature treatments on any day, despite a 20-fold decrease in *Symbiodinium* densities in the thermal stress treatments. An additional analysis of each individual dominant operational taxonomic unit (OTU) showed no patterns in significant shifts in abundance in any treatments. In all samples the population was dominated by γ-proteobacteria (accounting for 40-96% of sequences returned), mostly of the order Oceanospirillales. Most notably, while *Symbiodinium* density decreased dramatically in thermally stressed corals, there was no concomitant rise in *Vibrio spp*. as has been noted in several other studies. These results indicate that bacterial population changes are not necessarily concomitant with bleaching in corals over short time scales.

Introduction

The diversity of coral-associated microbes was first explored by Rohwer et al. (2002), using a culture-independent method of bacteria identification. By sequencing the 16S rDNA region, they hypothesised that up to 6000 bacterial ribotypes could be associated with coral (Rohwer et al. 2002). Since then, the constituents of the coral microbial assemblage have been analysed for a variety of coral species, and oftentimes community structure was found to be species-specific to the host (Rohwer et al. 2001; Rohwer et al. 2002; Ritchie and Smith 2004; Bourne and Munn 2005; Littman et al. 2009; Meron et al. 2012; Morrow et al. 2012). It has also been well established that the coral tissue-associated bacterial assemblage is distinctly different from that found in seawater (Rohwer et al. 2001; Frias-Lopez et al. 2002; Bourne and Munn 2005), however, the mucus and water fraction are more similar than mucus and tissue fractions (Bourne and Munn 2005). Many studies of coral bacterial associations have found that the bacterial population is dominated by γ-proteobacteria assemblages (Rohwer et al. 2002; Wegley et al. 2007; Littman et al. 2009; Kvennefors et al. 2010). In particular, the genera Oceanospirillales are found in high abundance in many coral species (Rohwer et al. 2002; Kvennefors et al. 2010; Morrow et al. 2012; Speck and Donachie 2012). This commonality suggests characteristics of the coral host, such as anti-microbial properties of healthy corals' mucus, are shaping the associated microbial community structure (Kim 1994; Ritchie 2006; Nissimov et al. 2009; Shnit-Orland and Kushmaro 2009).

Sea surface temperature (SST), water depth, water quality, proximity to human populations, and geographic location have all been documented to influence the bacterial community structure of coral (Kline et al. 2006; Bourne et al. 2007; Harvell et al. 2007; Klaus et al. 2007; Littman et al. 2009). But, while the bacterial consortia shifts between healthy, diseased, and dead states (Frias-Lopez et al. 2002; Mouchka et al.

2010), notably not all changes in holobiont status or physiological state are reflected in the bacterial community (Littman et al. 2010; Bellantuono et al. 2012; Ceh et al. 2012). The rise in incidence of coral diseases in recent decades, primarily attributed to increased SST, highlights the immediate need to understand how climate change will affect the bacterial community and the health of the coral holobiont (Harvell et al. 1999; Lafferty et al. 2004; Bruno et al. 2007; Harvell et al. 2007). For example, several studies have shown a correlation between the increase of coral-associated *Vibrio spp.* and coral bleaching, and the incidence of disease during thermal stress events (Kushmaro et al. 1996; Ben-Haim and Rosenberg 2002; Cervino et al. 2004; Sutherland et al. 2004; Bourne et al. 2007). The interactive effect of elevated SST and decreased pH on the coral microbial assemblage remains unknown. This represents a critical knowledge gap, as these two factors will be changing in concert during future climate change scenarios (Hoegh-Guldberg et al. 2007).

Rising CO_2 emissions, due in part to continued rise in human use of fossil fuels, has led to several problems: the so-called "greenhouse" effect of CO_2, which contributes to SST rise, and the "other CO_2 problem" is ocean acidification (OA, decreased surface ocean pH), caused by the direct uptake of CO_2 by surface ocean water (Caldeira and Wickett 2003; Orr et al. 2005; Doney et al. 2009). The Intergovernmental Panel on Climate Change (IPCC) projects that within 50 years, atmospheric CO_2 will rise by 50-100 ppm depending on the CO_2 emissions scenario, corresponding to an approximately 1-2 °C rise in SST and 0.1-0.15 drop in pH (depending on location) (Hoegh-Guldberg 1999; IPCC 2007). These predictions have led to models predicting annual mass bleaching events by 2050 (Hoegh-Guldberg 1999). How these environmental changes will impact coral-associated bacterial communities is unknown.

The present study aims to determine if changes in bacterial community structure of *Acropora aspera* occur under a near-term climate change scenario by introducing a low level CO_2 increase, 50-100 ppm concentration above current ambient CO_2 concentration, during a 5 day simulated bleaching event in experimental aquaria. DNA samples were isolated during the onset of the coral bleaching response, throughout the bleaching process, and from post-bleached coral tissue. Direct pyrosequencing of 16S rDNA PCR amplicons enabled detection of rare and low-abundant phylotypes. Changes in bacterial assemblages throughout the bleaching stress are compared to the *Symbiodinium* density.

Materials and methods

Experimental design

See Chapter 3 methods for experimental design.

Sample collection and preparation

On experiment days 4, 6, 9, and 14 3 replicate nubbins were taken from each replicate tank (n = 6 nubbins per treatment), immediately snap-frozen in liquid nitrogen, and stored at -80 °C until DNA isolation. Coral branches were crushed with a hydraulic press before transfer to a mortar chilled with liquid nitrogen and ground finely with a chilled pestle. Approximately 50-80 mg of resultant powder was used for gDNA isolation using the PowerPlant DNA isolation kit (MoBio laboratories, California, USA) following a modification of manufacturer's protocol from Sunagawa et al. (2009). Quality and concentration of isolated DNA were quantified spectrophotometrically using a NanoDrop-1000 (Thermo Scientific, USA). DNA was stored at -20°C until PCR amplification.

Symbiodinium density

See Chapter 3 methods.

PCR amplification and 454 tag sequencing

Four hundred ng of template DNA from 96 samples (6 replicate nubbins from each of 4 treatments from 4 days) were used with bacteria-specific non-barcoded primers SSU926F (AAACTYAAAKGAATTGRCGG) and SSU1392wR (ACGGGCGGTGTGRC) to amplify 16S rDNA in a round of PCR (55 °C annealing temperature for 30 cycles). Another round of PCR was performed at 55 °C for 10 cycles with barcoded primers pyroSSU803F and pyroSSU1392wR, assigning each sample a unique MID (on the reverse primer). 454 A adaptor sequence (CCATCTCATCCCTGCGTGTCTCCGAC) was added to the primers, with the sequencing key TCAG. Tagged PCR products were pooled in equimolar amounts for 454 pyrosequencing (Roche GS-FLX) at the Australian Centre for Ecogenomics (ACE).

Sequence analyses

Individual nubbins were pooled within replicate tanks for each treatment (total 32 samples). Sequence analysis was performed based on the methods of Ceh et al (2012), except using Green Genes 4th Feb 2011 updated release to check for chimeric sequences. Read number was normalised to 1100 reads per sample for pooled replicate nubbins within tanks to allow for comparison between samples and account for uneven numbers of sequences from different samples. Sequences for pooled samples were clustered using uclust (Edgar 2010) to obtain groups at the 80% and 90% similarity level, equating to operational taxonomic units (OTUs) at the class and genus levels, respectively. A representative sequence for each OTU was chosen using the QIIME pipeline, again following the protocol of Ceh et al (2012), for constructing OTU

diversity tables. Population distribution was analysed via principal component analysis (PCA) using The Unscrambler X software package (version 10.2, CAMO software; Oslo, Norway). Additionally, generalised linear models (GLM) with sequential Bonferroni post-hoc test ($\alpha = 0.05$) for the 8 most abundant OTUs were performed using SPSS statistical software package with temperature, pCO_2, and day as factors.

Results

Symbiodinium density

See Chapter 3 results and Figure 3.3. Briefly, over the course of the experiment, *A. aspera Symbiodinium* density declined significantly in both heated treatments compared to control corals; symbiont density was reduced to 7.2×10^4 cells cm^{-2} in heated treatment and 1.4×10^5 cells cm^{-2} in elevated pCO_2/heated treatment by day 14. Elevated pCO_2 had no effect on *Symbiodinium* density within temperature treatments.

Bacterial community

Class level analysis

Nubbins collected during and after the simulated bleaching event under elevated pCO_2 and temperature were analysed for bacterial community structure and composition via pyrosequencing of PCR amplicons of the 16S rDNA region. Initial analysis of raw data showed 196 765 high-quality sequence reads returned in 32 samples (grouped at tank level from 96 coral nubbins for clarity). Sequence reads were normalised to 1100 reads per sample to allow for comparison between samples. Thirty-four OTUs were identified at the class level, of which the dominant classes (OTUs with greater than 5% of all sequences) in all samples were the γ-proteobacteria, followed by δ-, β-, and α-proteobacteria, respectively (Figure 4.1a). γ-proteobacteria alone constituted from 40-

96% of sequences from each sample. Chlamydiae also represented greater than 5% of sequences in many samples, but not all (Figure 4.1a). These 5 OTUs together represent greater than 94% of sequence reads returned for all samples. Despite coral being stressed to 34 ^{o}C with increases in pCO_2 and significant *Symbiodinium* loss, treatment had no effect on the microbial assemblage. Principal components analysis (PCA) indicated no significant grouping of samples by any factor- day, temperature, or CO_2 at the class level (Figure 4.2a); removal of dominant γ-proteobacteria, to allow for the investigation of other phylotypes did not reveal any significant grouping of the remaining OTUs in a subsequent PCA (Figure 4.2b). GLM with sequential Bonferroni post-hoc at significance level of $p = 0.05$ run on each of the 8 dominant OTU individually likewise did not reveal any patterns of significant difference within or between days, temperature, or CO_2 treatments.

Genus level analysis

Assigning taxonomy at the genus level showed 260 OTUs with at least one sequence read returned, of which 24 had greater than 1% of sequence reads from at least one sample. Eight OTUs were dominant (i.e., had greater than 1% of sequences in nearly all samples) (Figure 4.1b, Table 4.1). These dominant groups mostly belonged to γ-proteobacteria (Enterobacteriaceae, *Endozoicimonas spp.*, Endozoicimonaceae genus SGUS388, *Pseudomonas spp.*, and *Stenotrophomonas spp*), as would be expected from data at the class level. *Endozoicimonas spp.* and Endozoicimonaceae uncharacterised genus SGUS388 constituted up to 86% of sequences from samples (Figure 4.1b). The remaining dominant OTU belonged to: α-proteobacteria *Ochrobactrum spp*., β-proteobacteria *Delftia spp.*, and δ-proteobacteria order Myxococcales (Figure 4.1b,c). The presence of *Ochrobactrum spp.* in high frequency is notable due to its classification

within Rhizobiales, the nitrogen-fixing bacteria, however, denitrifying *Pseudomonas spp.* were also highly abundant. Endozoicimonaceae uncharacterised family HOC21 (order: Oceanospirillales), α-proteobacteria Brevundimonas, β-proteobacteria *Achromobacter spp*. (another member of Rhizobia), and Chlamydiae family Simkaniaceae were also in high abundance (dominant OTUs in 72, 50, 50, and 44, % of samples, respectively). PCA plots revealed no distinct grouping at the genus level by any factor: day, temperature, or CO_2 (Figure 4.3a). The top 2 dominant genera (*Endozoicimonas spp.* and Endozoicimonaceae uncharacterised genus SGUS388) were excluded and PCA run again to determine if the dominant groups were masking subtle changes in less abundant groups, however, no differential grouping pattern was seen (Figure 4.3b). Similarly, GLM of individual OTU at the genus level did not reveal any patterns of significant differences arising from day, temperature, or CO_2 treatments ($p = 0.05$).

Diversity shifted only slightly between heated and control treatments. In 44% of control samples, *Dokdonella spp.* composed at least 1% of sequence reads, whereas in heated treatments it disappeared almost entirely. 56% of control samples had Oceanospirillales genus HOC21 as a dominant OTU, which increased by a third to 88% of samples in heated treatments. There was a notable absence of *Vibrio spp*. dominance throughout the experiments (Figure 4.1b,c). *Vibrio* species were only evident in three of the eight treatment timepoints and none of the controls. On day 4 and day 9 of heated treatment less than 1% of the total OTUs were identified as belonging to *Vibrio* genus, and CO_2 enrichment did not affect *Vibrio spp* occurrence.

Discussion

This study focused on the effects of increased pCO_2 in isolation and in

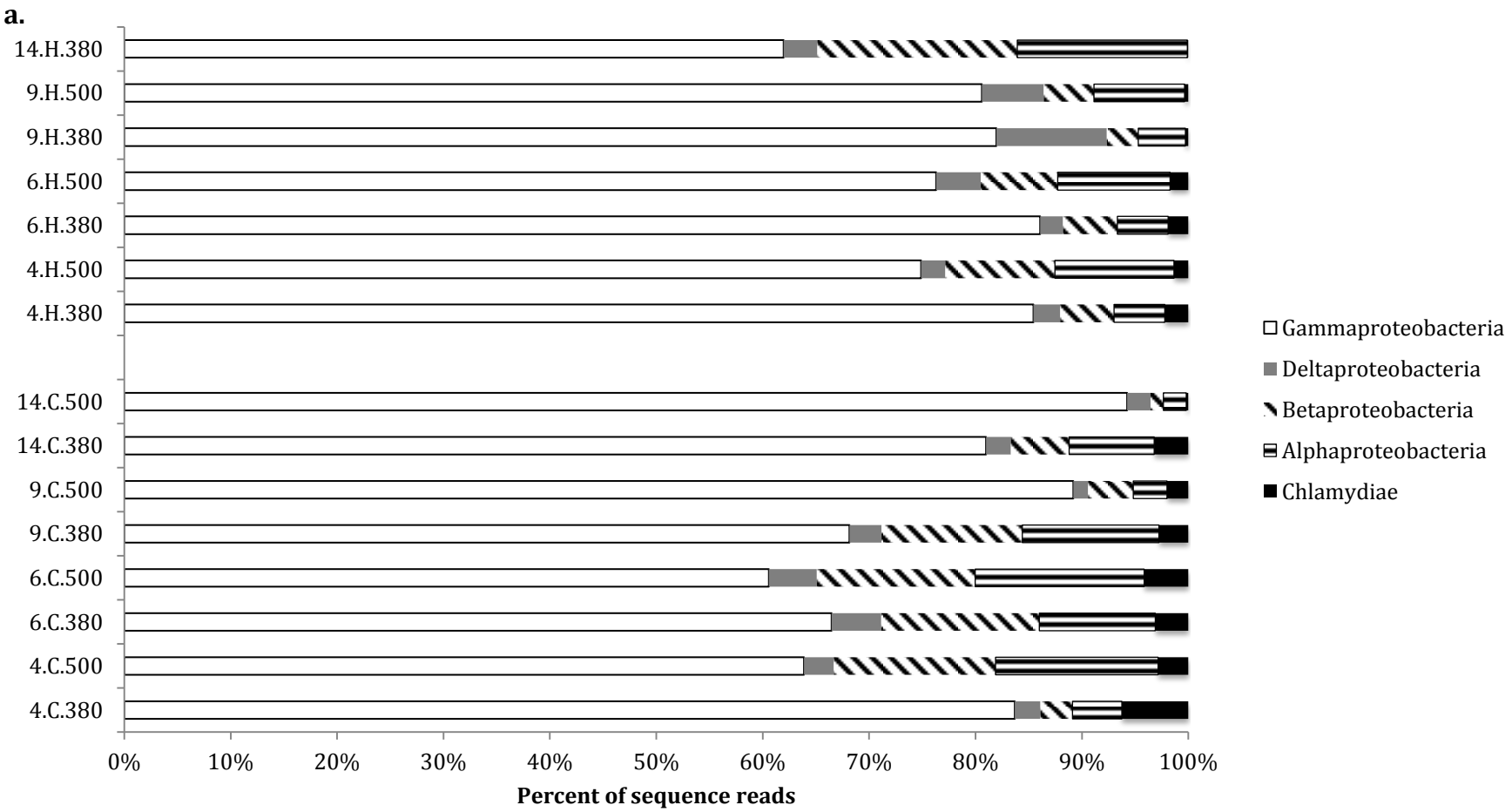
Table 4.1. Number of sequence reads from dominant OTUs (greater than 1% of sequences for a sample) grouped at 90% identity, equating to genus level classification. Sample IDs read as: Day.C (control temperature) or H (heated).380 ppm or 500 ppm pCO_2. Samples are the average of 2 tanks for each treatment/day, data represent number of sequences (out of 1100) per sample.

	Ochrobactrum sp.	*Delftia sp.*	Myxococcales	Enterobact-eriaceae	*Endozoicimonas sp.*	Endozoicimonaceae SGU388	*Pseudomonas sp.*	*Stenotropho-monas sp.*	*Vibrio sp.*
4.C.380	32	24	26	27	356	431	39	22	0
4.C.500	99	135	27	78	159	187	129	88	2
6.C.380	62	125	51	62	132	275	124	91	0
6.C.500	134	120	46	85	142	199	136	95	0
9.C.380	96	111	32	81	169	254	136	79	0
9.C.500	25	33	15	20	429	416	41	20	0
14.C.380	63	42	25	28	365	343	59	38	0
14.C.500	9	10	24	4	448	495	6	7	1
4.H.380	38	45	26	27	363	383	44	33	14
4.H.500	86	86	25	47	171	420	103	50	0
6.H.380	41	33	24	17	481	353	31	21	0
6.H.500	73	62	41	44	426	213	78	42	0
9.H.380	29	24	110	13	536	243	31	13	10
9.H.500	40	35	59	19	324	408	35	16	7
14.H.380	128	151	35	98	206	126	151	80	0
14.H.500	83	66	35	40	287	329	85	57	0

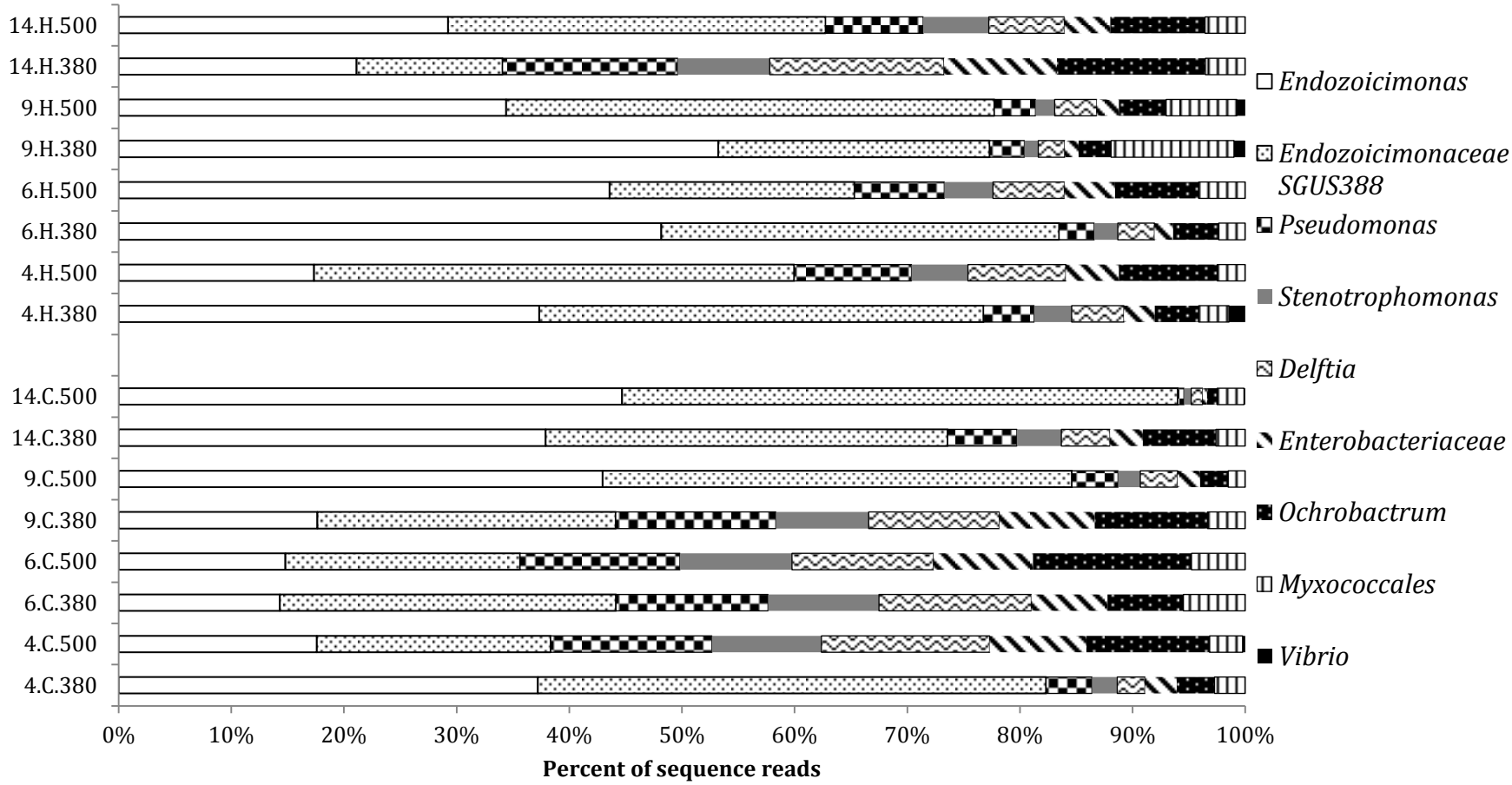
combination with increased temperature on the bacterial community of *Acropora aspera*. A high diversity (260 OTU at the genus level) was found associated with *A. aspera* during this study. The ability to directly sequence PCR products via massive parallel pyrosequencing has enabled researchers to identify low-abundance bacteria in samples that may otherwise have been overlooked by the DGGE/ clone library-based sequencing methods, which may not be sensitive enough to detect bacteria in low numbers (Sogin et al. 2006). Very few sequences from this study resulted in no BLAST hits (only one sample had greater than 1% of sequences with no BLAST hit), suggesting the bacteria associated with *A. aspera* have been well characterised in previous studies.

Influence of aquaria setting

A fine-scale study of temporal changes in the coral bacterial assemblage during subtle stages of bleaching and under altered CO_2 conditions has not been examined until this study. This necessitated the use of a controlled aquarium system to ensure capture of all stages of physiological changes in the host symbiosis associated with bleaching. Given that this was not a natural bleaching event it is important to note that aquaria conditions may influence the bacterial communities. However, despite potential aquaria effects, the large scale patterns of the bacterial communities, including the dominance of γ-proteobacteria sequences found in this study, are similar to that identified for field-collected *Acropora spp.* from the Great Barrier Reef in general (Littman et al. 2009), Heron Island in particular (Kvennefors et al. 2010), as well as *Acropora spp.* from geographically distinct locations (Ceh et al. 2011; Nithyanand et al. 2011; Liu et al. 2012; McKew et al. 2012; Wilson et al. 2012).


a.
14.H.380
9.H.500
9.H.380
6.H.500
6.H.380
4.H.500
4.H.380
14.C.500
14.C.380
9.C.500
9.C.380
6.C.500
6.C.380
4.C.500
4.C.380
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Percent of sequence reads
Gammaproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Chlamydiae

b.


14.H.500
14.H.380
9.H.500
9.H.380
6.H.500
6.H.380
4.H.500
4.H.380
14.C.500
14.C.380
9.C.500
9.C.380
6.C.500
6.C.380
4.C.500
4.C.380
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Percent of sequence reads
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Pseudomonas
Stenotrophomonas
Delftia
Enterobacteriaceae
Ochrobactrum
Myxococcales
Vibrio

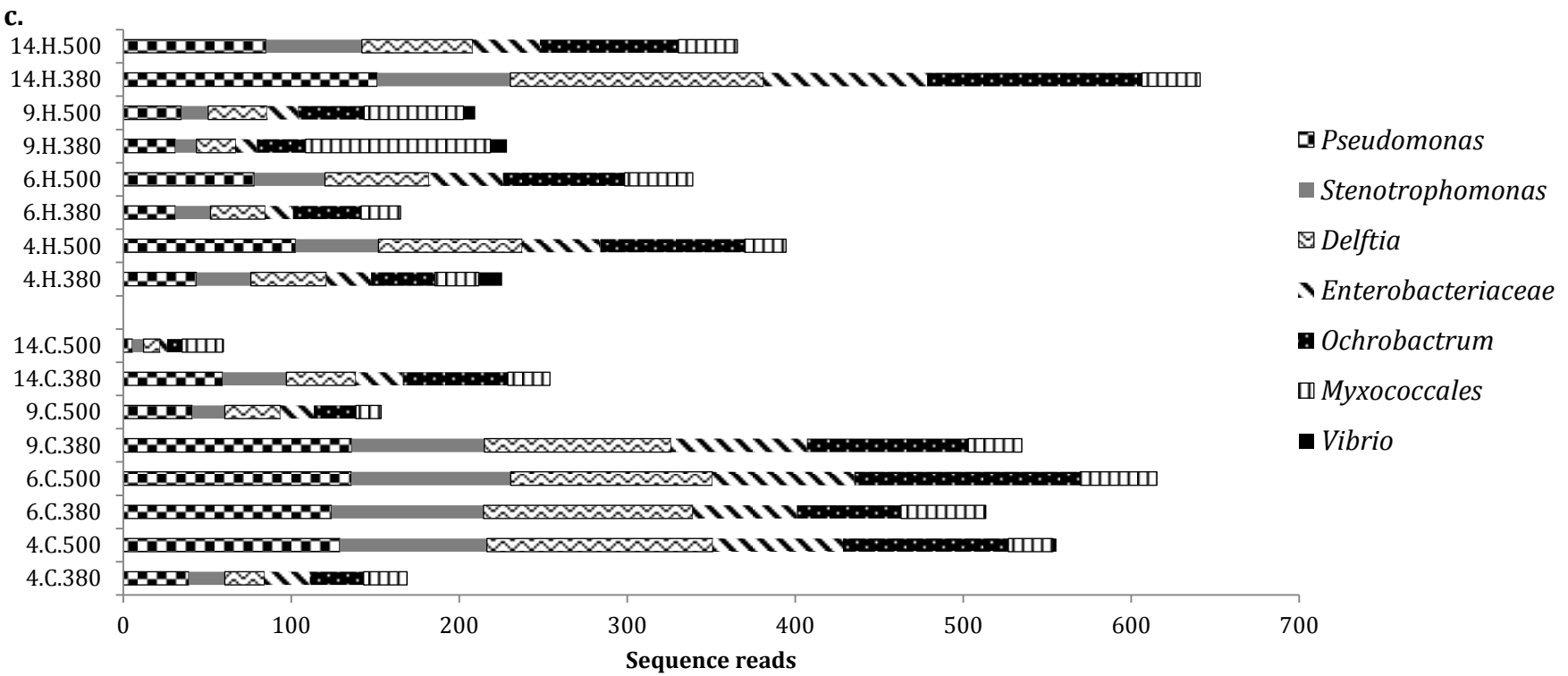
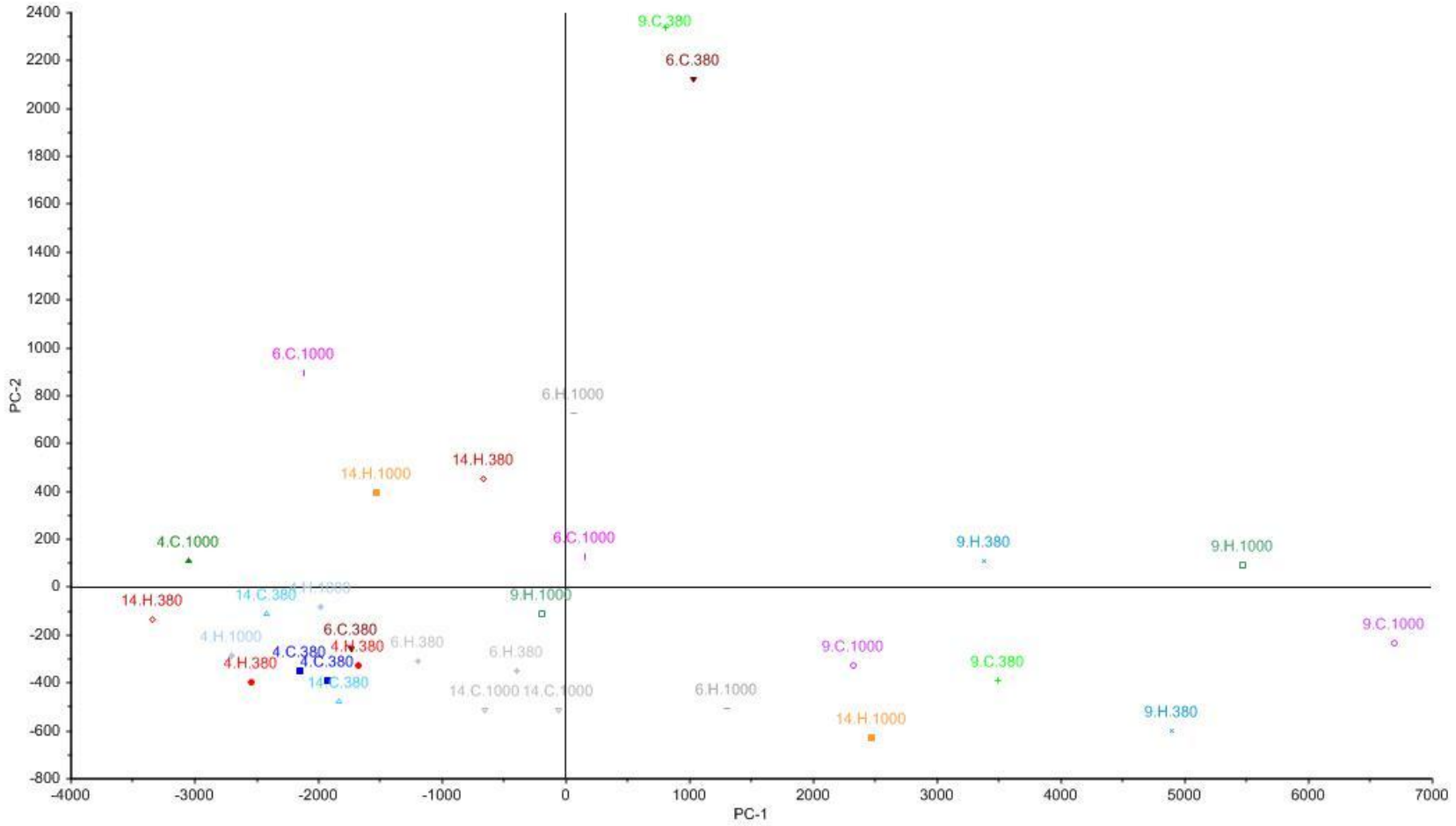


Figure 4.1. Sequence reads of prokaryote 16S rDNA amplicons from dominant organisational taxonomic unit (OTUs) of *Acropora aspera* corals subjected to a simulated bleaching event under elevated pCO_2 identified at the **a)** class level [γ- (white), δ- (gray), β- (diagonal stripes), α- proteobacteria (horizontal stripes), and Chlamydiae (black), represented as percentage of total sequences], **b)** genus level (represented as percentage of sequences, see legend for bar shading key), and **c)** genus level excluding top 2 dominant OTU (shown as absolute number of sequence reads) for each sample. Data points are labeled by treatment: Day.Temperature (Control or Heated).CO_2 concentration (380 or 500 ppm). Samples are the average of 2 tanks for each treatment/day; sequence reads normalised to 1100.

a.


PC-2
PC-1
9.C.380
6.C.380
6.C.1000
6.H.1000
14.H.1000
14.H.380
4.C.1000
6.C.1000
9.H.380
9.H.1000
14.H.380
14.C.380
9.H.1000
6.C.380
4.H.1000
6.H.380
4.C.380
4.H.380
4.H.380
4.C.380
6.H.380
9.C.1000
9.C.380
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14.C.1000
14.C.1000
6.H.1000
14.H.1000
9.H.380
9.C.1000

b.

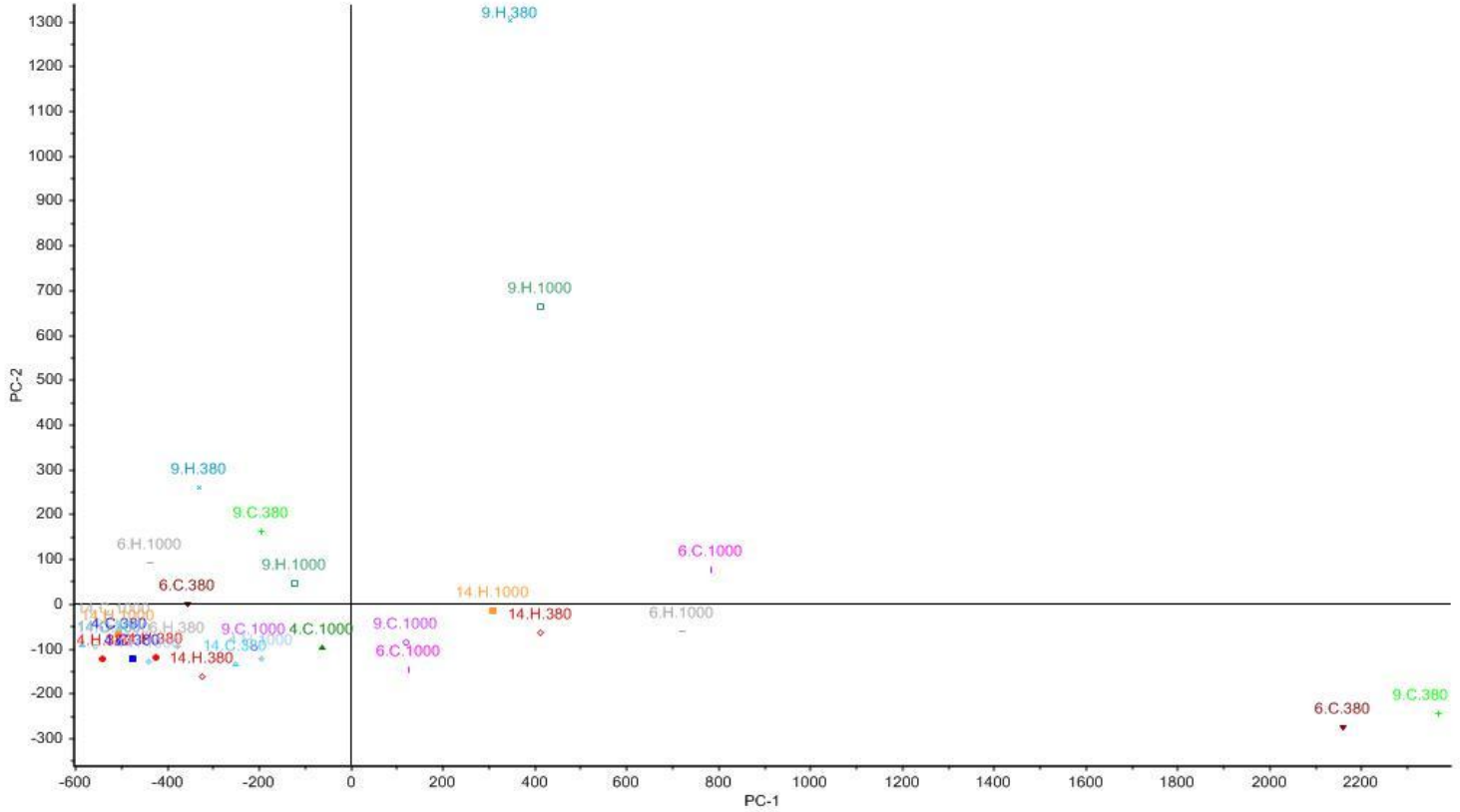
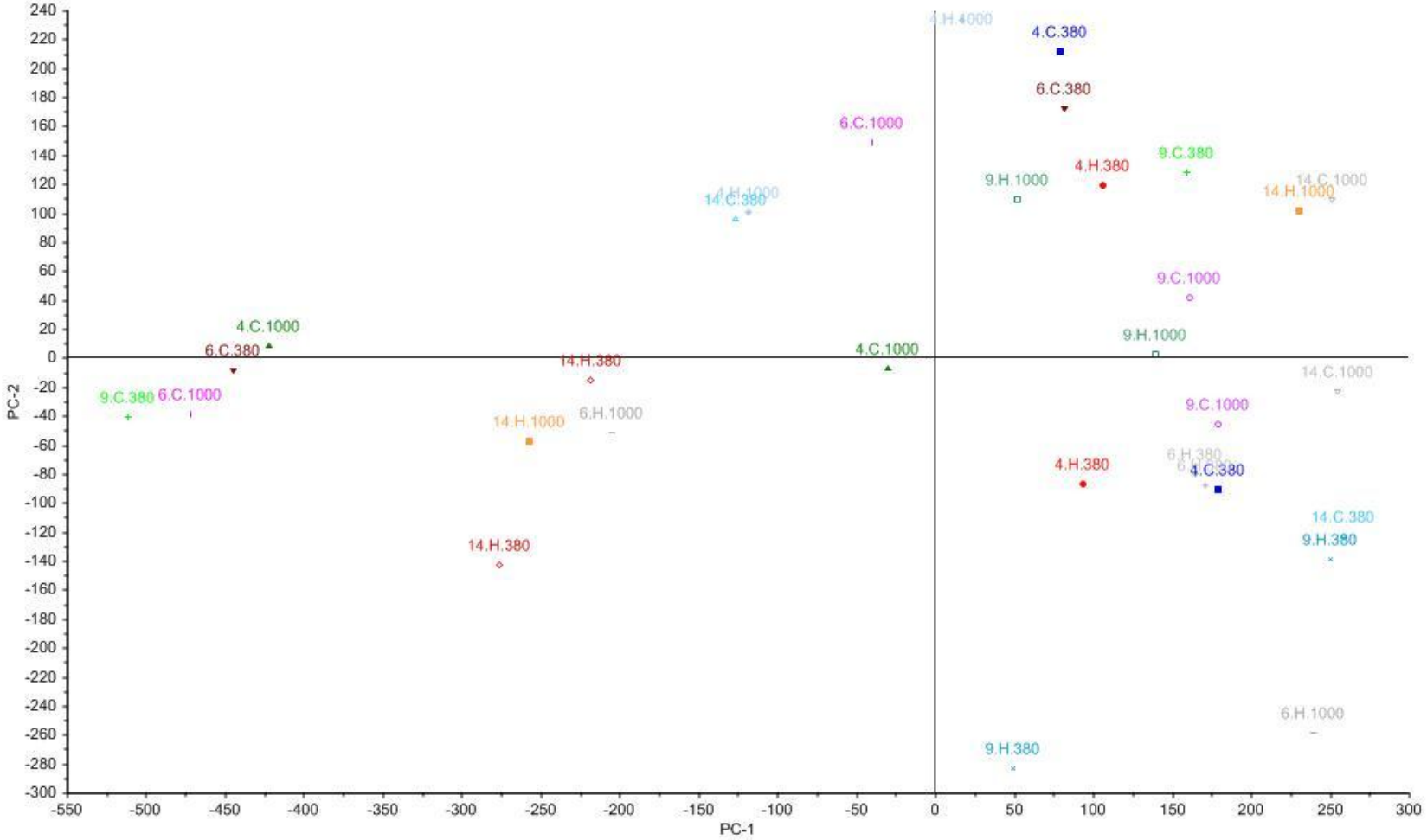


Figure 4.2. Principal component analysis (PCA) plots of bacterial ribotype diversity of *Acropora aspera* corals subjected to a simulated bleaching event under elevated pCO_2 identified at the class level of sequence similarity (80% similarity) both **a)** with all classes included for analysis and **b)** excluding top 2 dominant OTU. Variables were weighted by the inverse of the standard deviation. Data points are labeled by treatment: Day.Temperature (Control or Heated).CO_2 concentration (380 or 500 ppm). Samples are the average of 2 tanks for each treatment/day; sequence reads normalised to 1100.

a.


PC-1
PC-2
4.H.4000
4.C.380
6.C.380
6.C.1000
9.C.380
4.H.380
9.H.1000
14.C.1000
14.H.1000
14.C.380
9.C.1000
4.C.1000
6.C.380
9.H.1000
14.H.380
14.C.1000
9.C.380
6.C.1000
9.C.1000
14.H.1000
6.H.1000
4.H.380
4.C.380
14.C.380
14.H.380
9.H.380
6.H.1000
9.H.380

b.

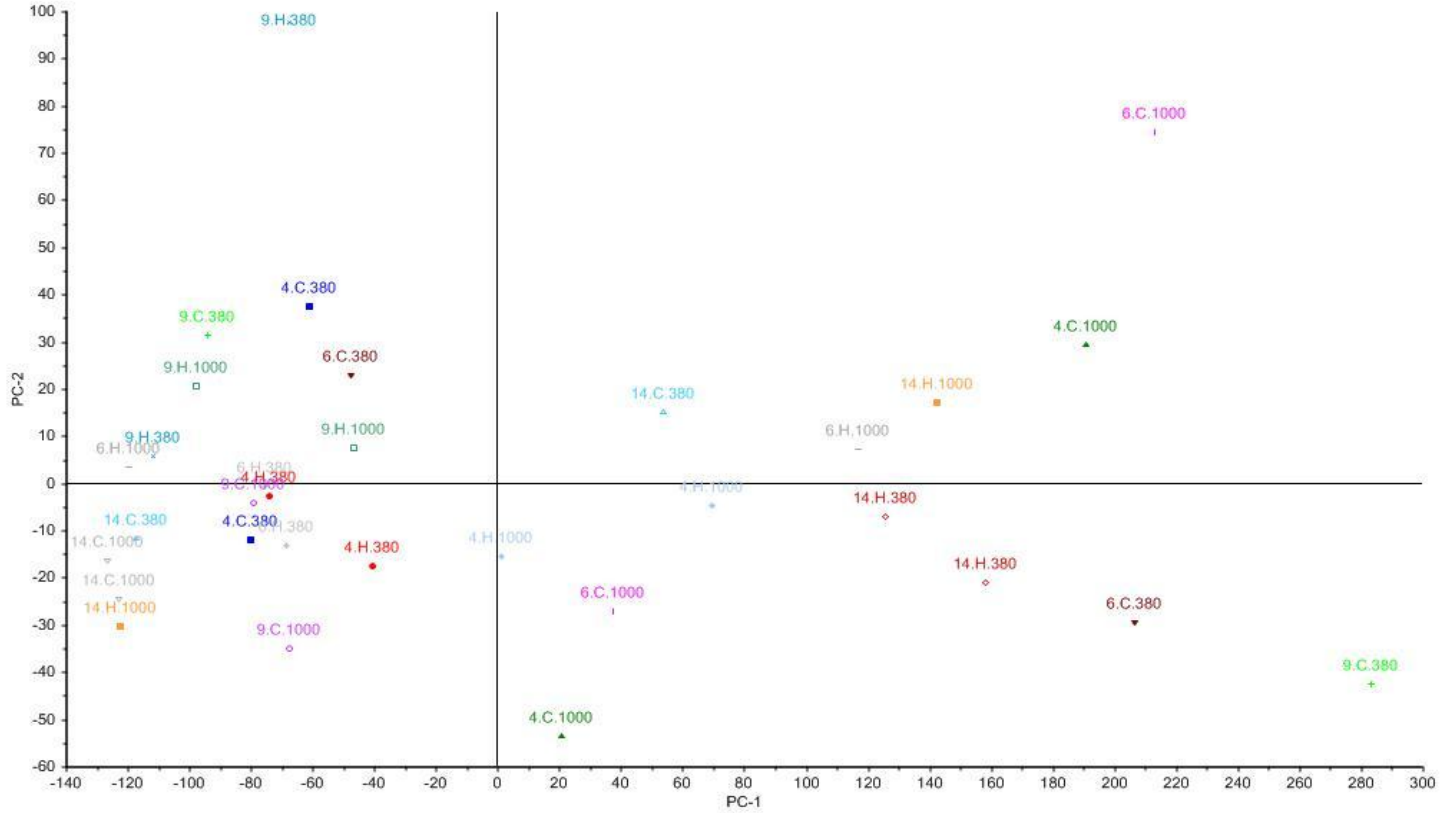


Figure 4.3. Principal component analysis (PCA) plots of bacterial ribotype diversity of *Acropora aspera* corals subjected to a simulated bleaching event under elevated pCO_2 identified at the genus level of sequence similarity (90% similarity) both **a)** with all genera included for analysis and **b)** excluding top 2 dominant OTU. Variables were weighted by the inverse of the standard deviation. Data points are labeled by treatment: Day.Temperature (Control or Heated).CO_2 concentration (380 or 500 ppm). Samples are the average of 2 tanks for each treatment/day; sequence reads normalised to 1100.

Effect of elevated pCO_2 on the *A. aspera* bacterial assemblage

Coral-associated bacterial community structure can be influenced by external factors such as pH, temperature, and nutrient load (Kline et al. 2006; Thurber et al. 2009; Meron et al. 2011). Our study manipulated CO_2 concentration and temperature of experimental aquaria using near-term climate scenarios. The pCO_2 increase had no effect on abundance of dominant bacteria genera (those accounting for more than 1% of sequence reads returned). This is perhaps not surprising, considering the modest increase in pCO_2 applied to the treatments compared to the large daily fluctuations (pH 8.0-8.4) encountered by these corals on the reef flat of Heron Island (Kline et al. 2012) (see Figure 3.1b). However, this small increase of approximately 50-100 ppm above ambient did have effects on other aspects of the *A. aspera* holobiont, such as expression of carbon metabolism-related genes of the host, and photosynthetic efficiency of PSII of *Symbiodinium* (Ogawa et al., in review) (see Figures 3.2 and 3.5). Two forms of coral carbonic anhydrase were found to be significantly down-regulated under the combined treatment of heat and elevated pCO_2 (Figure 3.5a,b). The relatively unperturbed nature of the bacterial community in response to increased pCO_2 suggests a resilient relationship between bacteria community and host, at least at pCO_2 levels predicted for the near future (mid-century). Even at late-century pH levels bacterial community changes are not necessarily inevitable, as seen in a field study of *Cladocora caespitosa* and *Balanophyllia europaea* along a volcanic seep creating a natural pH gradient from 8.1 to 7.3 (Meron et al. 2012). Meron et al. (2012) found no major shifts in bacterial composition or diversity were observed for either coral species studied, which was in contrast to a previous *in vitro* study where a shift was seen towards bacteria linked to disease and stress in *Acropora eurystoma* exposed to similar pH levels (Meron et al. 2011). These results demonstrate the highly variable effects of acidity on the bacterial

assemblage at pH levels predicted for this century under various climate change scenarios. Extreme or sudden pH changes may cause a shift in community dynamics, but near-future, smaller shifts are unlikely to alter the bacterial community. For example, a lowering of pH to 6.7 (1.4 pH units below current ambient, equivalent to over 10 000 ppm pCO_2 (Kurihara and Shirayama 2004)), increased the abundance of *Bacteroides*, *Chlorobi*, *Cyanobacteria*, and *Spirochaetes*, and decreased *Actinobacteria* significantly in *Porites compressa* (Thurber et al. 2009). Also, interestingly, severely decreased pH increased the amount of fungi and metazoans within the holobiont (Thurber et al. 2009).

Effect of temperature on the bacterial assemblage

Likewise, temperature did not have a large effect on bacterial community diversity or abundance in this study despite a significant effect on the *Symbiodinium* population. This is somewhat unexpected due to the large physiological changes experienced by the host symbiosis. Using DGGE and clone libraries Littman et al. (2010) found juvenile *A. tenuis* that hosted different *Symbiodinium* types (clade C1 and D genotyped with ITS1) contained different bacterial communities. In addition, clade C-associated juvenile *A. tenuis* maintained the same bacterial community assemblage through a 14 day heat stress event at 32 ^{o}C, whereas clade D-associated corals experienced a significant shift in the bacterial consort. In comparison to this present study it should be noted that clade C corals sustained only a 10% decrease in photochemical efficiency, indicating only a mild thermal impact on *Symbiodinium*, while clade D corals sustained a larger 44% decrease in efficiency. Given that *A. aspera* has only been found to host ITS1 type C1 (the same as ITS2 clade C3) and no bacterial population changes were seen even with a much more severe bleaching response, this may indicate that the linkage between clade

C1 *Symbiodinium* and the bacterial population is not as strong as between some clade D *Symbiodinium* and bacteria.

Absence of *Vibrio* increase

The most conspicuous lack of change in the current study was that of the *Vibrio* OTUs. A rise in *Vibrio* abundance has often been linked to the onset of coral bleaching during periods of thermal stress (Kushmaro et al. 1996; Ben-Haim et al. 2003; Rosenberg and Falkovitz 2004; Bourne et al. 2007). However, a causal link between *Vibrio* increase and *Symbiodinium* loss is uncertain, and this is particularly evident in the current study. Despite a significant loss in *Symbiodinium* there was no significant trend in the response of *Vibrio spp.* abundance, with *Vibrio* densities never exceeding 1% of the population. Previously, an 8 day experimental bleaching of *A. millepora* from Heron Island (southern GBR) also did not result in a shift in bacterial community when analysed using DGGE, although the resolution of this technique is not as high as 454 sequencing (Bellantuono et al. 2012). In contrast, Bourne et al. (2007) followed a natural bleaching of *A. millepora* on a fortnightly-monthly timescale and found a rise in *Vibrio* numbers when compared to previous months and following coral recovery during a regional mass bleaching event. The loss of algal symbionts was observed in that study following a period of 18 consecutive days where the corals experienced temperatures above their recorded bleaching threshold of 31 °C (Bourne et al. 2007). Given the different temporal sampling regime used it would be interesting to see how soon after a natural beaching event that altered *Vibrio* population can be observed. The synergistic effect of altered environmental conditions (increased temperature) following the bleaching onset and altered host physiology on community diversity also needs to be considered when determining *Vibrio*-mediated *Symbiodinium* loss in long-term ecological studies. The

compromised health of the coral host during periods of elevated temperature has the potential to decrease the hosts ability to mediate its bacterial associates, leading to unchecked replication of certain strains, such as disease-causing *Vibrio* strains (Lesser et al. 2007).

Possible functions of some coral-associated microbes

Whilst our understanding of the community make-up of coral-associated bacteria has grown rapidly in the past decade, functional description of these community members has yet to be described. This is due to the relatively uncultivable nature of the vast majority of marine microbes. However, based on sequence similarity to other members of identified taxa, it is possible to infer aspects of the function of coral-associated microbes. For example, the presence of Ochrobactrum found here, a member of α-proteobacteria group Rhizobiales, and β-proteobacteria *Achromobacter spp.*, another Rhizobium, suggests a role in nitrogen fixation. Rhizobia, a polyphyletic group, are well characterised in terrestrial plants, where they form nodules in the root systems and fix nitrogen from the soil into biologically available ammonium (Long 1989). It is this association with Rhizobia that allow certain plants, mostly legumes, to grow in nitrogen-deplete soils. Using *nifH* protein sequences, Lema et al. (2012) found distinct Rhizobia communities associated with different corals. The environmental situation of corals, which live in nutrient-poor water, parallels the situation of terrestrial plants and therefore this association with Rhizobia may be performing a similar function within the holobiont (Lema et al. 2012). Indeed, localisation of bacterial aggregates to vacuoles within the gastroderm may be synonymous to root nodules of Rhizobia-associated plants (Ainsworth and Hoegh-Guldberg 2009; Lema et al. 2012). However, this requires

further study, because the bacteria associated with the aggregates were identified as γ-, not α- or β-proteobacteria (Ainsworth and Hoegh-Guldberg 2009).

High abundance of Oceanospirillales

The high abundance of the order Oceanospirillales (here, *Endozoicimonas* and Endozoicimonaceae SGSU388, and, to a lesser extent, HOC21) is common in reef-building corals (Rohwer et al. 2002; Kvennefors et al. 2010; Morrow et al. 2012; Speck and Donachie 2012). Oceanospirillales are γ-proteobacteria that degrade complex organic compounds and are found in a wide variety of habitats, including water from oil plumes and deep ocean (Hazen et al. 2010; Swan et al. 2011), the "root" tissues of whale-bone eating *Osdex spp.* polychaete worms (Goffredi et al. 2005), and in the gills of the bivalve *Acesta excavata* (Jensen et al. 2010). Although generally considered heterotrophs, Oceanospirillales may also possess the capacity for chemolithoautotrophy (Swan et al. 2011). The presence of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) in some Oceanospirillales also indicates potential for carbon fixation of these microbes (Swan et al. 2011). Corals may acquire carbon from alternate sources when *Symbiodinium* density is low (Fine and Loya 2002). The precise function of these microbes within the coral holobiont remains unclear, particularly under periods of disturbance. The next step in determining function will be to link metagenomic analyses to coral and *Symbiodinium* responses.

Bacteria localisation

While the present study found no community differences to previously wild sampled *Acropora sp.* (Ainsworth and Hoegh-Guldberg 2009; Littman et al. 2009; Kvennefors et al. 2010), nor between control corals at the start and end of the experiment, there is the

possibility that bacterial localization altered during experimental manipulations (Kooperman et al. 2007; Ainsworth and Hoegh-Guldberg 2009; Salerno et al. 2010). Bacterial aggregates associated with *A. aspera* were localised specifically to the gastroderm of control and thermally stressed coral in experimental aquaria, but not in wild samples (Ainsworth and Hoegh-Guldberg 2009). While this indicates an aquarium impact, there were other significant differences in bacterial localization found only in thermally stressed corals, including bacterial populations evident in the mesenterial filaments in thermally stressed corals (Ainsworth and Hoegh-Guldberg 2009). Because the samples used for DNA isolation in this study were from a crushed coral (including some mucus, tissue, and skeleton), it is not possible to identify which fraction accounted for what bacterial numbers/diversity. A lack of abundance change does not, therefore, necessarily preclude shifts in localisation of the bacterial community members or shifts in diversity within niche habitats.

Conclusions

Large physiological and symbiont density changes in our experiment did not result in significant shifts in the bacterial community associated with *A. aspera* under elevated pCO_2 during a simulated bleaching event. This suggests that the bacterial community in *A. aspera* is relatively robust during the early period of a thermal stress, however, it may alter after an extended period of time. Many studies, both *in vitro* and *in vivo*, have similarly found no significant shifts in bacterial communities despite widely differing environmental and holobiont conditions (Littman et al. 2010; Bellantuono et al. 2012; Ceh et al. 2012; Meron et al. 2012). The static nature of the bacterial community in these studies is strongly suggestive of an intriguingly impervious nature of the bacteria of those corals, i.e. the community dynamics were not such that a change in

environment or health status elicited a change in structure to better suit the prevailing conditions. These findings raise questions on the coral holobiont hypothesis and the potential resilience, rather than flexibility of coral-bacterial interactions (Reshef et al. 2006). It may be that the functional relationship of bacteria in these coral is fundamental and even dramatic shifts in other aspects of the holobiont do not affect the coral-bacteria association. Alternatively, the bacterial communities may be able to functionally acclimatize to the altered holobiont and remain within niche habitats. What remains clear at this point is that coral bacterial assemblages do not always rapidly respond to environmental or host changes.

Chapter 5: Sub-lethal pre-stress leads to acclimation response of *Acropora aspera* and *Symbiodinium* during a simulated bleaching event

Abstract

Increasing sea surface temperatures (SST) have led to models predicting mass annual bleaching of corals within 50 years. However, these predictions assume a static bleaching threshold for all corals. To determine whether a pre-stress would alter the response of a coral to a subsequent bleaching temperature, *Acropora aspera* were pre-stressed to sub-bleaching 32 °C or a bleaching-level of 34 °C, then following a recovery period were exposed to a simulated bleaching event. The effect of thermal history was then assessed by examining photochemistry of *Symbiodinium* and expression of *A. aspera* anti-oxidant (catalase, Mn-SOD) and heat shock protein genes (HSP90, HSP70, HSP16). A pre-stress of 32 °C conferred thermal hardening (that is, a degree of resilience to the detrimental effects of increased temperature) to a subsequent bleaching stress. Non-photochemical quenching (NPQ) of chlorophyll fluorescence of *Symbiodinium* and expression of *A. aspera* catalase and small heat shock proteins gene (HSP16) exhibited delayed increases during the simulated bleaching event when exposed to a 32 °C pre-stress 1 week prior, as compared to samples that were exposed to 34 ^{o}C in the pre-stress and those that had no pre-stress. In contrast, in corals exposed to 34 ^{o}C, NPQ increased significantly during the pre-stress and remained significantly higher than controls, even after being kept at ambient conditions for 13 days. This is the first study to evaluate expression of stress mitigating genes during a repeated stress event. We show evidence of acclimatory ability of *A. aspera* during multiple heating events and the impact of thermal history on stress response.

Introduction

Coral reefs thrive in warm tropical waters but they are increasingly being threatened by a variety of anthropogenic stressors. The major global threat to reefs is coral bleaching, which is caused by sustained sea surface temperature (SST) increases of just 1 °C above long-term summertime averages (Hoegh-Guldberg 1999; Fitt et al. 2000). During coral bleaching, the endosymbiotic algae, *Symbiodinium* (or their pigments) are lost from the coral host. The exact mechanism which triggers the loss of algae/pigment is unknown but is widely believed to originate within the photosynthetic apparatus of the *Symbiodinium* (Jones et al. 1998; Warner et al. 1999; Takahashi et al. 2004; Tchernov et al. 2004). However, the animal host has also been shown to have a significant role in the responses of the holobiont to stress events and its capacity to recover (Baird et al. 2009; Fitt et al. 2009).

Global SST have risen 0.6 °C since the beginning of the 20th century and are forecast to rise by another 1.1-6.4 °C by 2100 (IPCC 2007). This has led to models which predict tropical corals will reach bleaching thresholds with increasing frequency (yearly, by 2050) (Hoegh-Guldberg 1999), even under the relatively moderate B2 IPCC emissions scenario (Donner et al. 2005). Annual bleaching would subsequently result in increasing colony mortality, decreased recruitment, and eventual phase shifts away from coral dominated reefs. It is predicted that for corals to acclimatise to these increasing temperatures and reducing bleaching frequency to once every five year over a 50 yr period, the holobiont's upper thermal limits would have to increase by at least 0.2-1.0 °C per decade (Donner et al. 2005). One assumption of models projecting nearly annual bleaching by mid-century is that the acclimatisation and thermal hardening of corals would be insufficient to keep pace with projected SST increases (Hoegh-Guldberg 1999). Several studies, however, have recently demonstrated the ability of corals to

naturally acclimatise (over the long term, *in vivo*) and acclimate (over the short term, experimentally) to temperatures much higher than would be expected given their thermally sensitive nature. The first such study to examine acclimation in coral was conducted by Coles and Jokiel (1978) where *Montipora verrucosa* was held at various temperatures above ambient for 56 days, then exposed to bleaching temperature stress. Corals maintained 2°C above ambient had greatest survivorship rates, indicating these samples had acclimated to higher temperature regimes and that this had conferred greater thermal tolerance during exposure to the bleaching threshold (Coles and Jokiel 1978). More recently, coral communities living in average 29 °C water in American Samoa have been documented as surviving short-term temperature fluctuations as high as 34.5 °C (Craig et al. 2001). Seasonal acclimatisation is also evident in *Pocillopora damicornis*, where corals exhibit a bleaching threshold 1 °C lower in winter than summer (Berkelmans and Willis 1999). A mass bleaching event on the Great Barrier Reef in 1998 conferred upon several coral genera (*Acropora, Pocillopora,* and *Porites*) an apparent increase in thermal tolerance, as a subsequent, more severe bleaching event in 2002 resulted in significantly less bleaching than predicted based on the 1998 event (Maynard et al. 2008). Previous exposure to high irradiance and/or temperature is therefore considered a key factor necessary for increasing thermal resistance in tropical corals (Brown et al. 2000; Brown et al. 2002a; Castillo and Helmuth 2005; Maynard et al. 2008; Middlebrook et al. 2008; Oliver and Palumbi 2011). However, the mechanisms that enable acclimatisation to occur in corals, and their endosymbiotic algae, have yet to be determined.

The molecular mechanisms underpinning observed coral thermal acclimatisation are currently unknown. In many instances of differential thermal susceptibility, symbiont type (clade) has been considered the primary source of any observed thermal

differences (Rowan et al. 1997; Glynn et al. 2001; Berkelmans and van Oppen 2006; Abrego et al. 2008; Jones et al. 2008b). However, thermal tolerance cannot be attributed to only one aspect of the holobiont. For example, despite identical symbiont type on both sides of shallow water *Goniastera aspera* colonies in Thailand, preferential bleaching on the east faces of colonies has been observed (Brown et al. 2002b). The more highly irradiated western sides of colonies exhibited a greater abundance of antioxidant enzyme copper zinc superoxide dismutase (CuZnSOD) and heat shock proteins (molecular chaperones) HSP60 and HSP70 when experimentally heat stressed, while algal photosystem II (PSII) showed less chronic photoinhibition on western than eastern, less irradiated, sides of colonies (Brown et al. 2002b). Middlebrook et al. (2008) also demonstrated that a "pre-stress" of sub-lethal temperature increased thermotolerance by increasing photoprotective mechanisms of *Acropora aspera Symbiodinium* when exposed subsequently to a simulated bleaching event (Middlebrook et al. 2008). These studies demonstrate that both host and symbiont are capable of inferring thermal acclimation and contribute to increased heat tolerance following previous exposure. Recently, the concept of "frontloading" stress response genes, such as heat shock proteins and antioxidant enzyme genes, has been proposed to explain increased heat tolerance of some corals (Barshis et al. 2013). These frontloaded genes were found to have higher baseline expression in heat resilient corals, even without the application of heat stress, and exhibited relatively less up-regulation compared to thermally sensitive conspecifics during a simulated heat stress event (Barshis et al. 2013).

Changes in gene expression of corals and their symbionts to a single thermal stress event has recently been documented and HSPs are key to thermal stress responses (Sharp et al. 1997; Kregel 2002; Desalvo et al. 2008; DeSalvo et al. 2010). Antioxidant

genes, which are up-regulated in response to oxidative stress, are especially vital in coral, as oxidative stress is thought to be one of the main triggers of bleaching, especially under high levels of irradiance (Lesser 1997; Downs et al. 2002). Reactive oxygen species (ROS) cause direct cellular damage to lipid membranes, proteins, and DNA, and have also been linked to the induction of caspases in *Aiptasia viridis*, a sea anemone (Lesser 2006; Richier et al. 2006). Transcriptional regulation of stress mitigators, such as HSPs and antioxidant enzymes, in conjunction with the photophysiological alterations of the algal symbiont will likely play key roles in the survival of corals subjected to heat stress. The current study investigates the photochemical response of *Symbiodinium* and transcriptional responses of *A. aspera* HSPs and target antioxidant genes to a simulated heating event where the coral was subjected to moderate and severe "pre-stress" events. The ability of coral and algal responses to become "primed" to environmental stress by previous exposures potentially allow the holobiont to acclimatise to a changing environment.

Materials and methods

Experimental design

Braches of *A. aspera* (7cm) were collected from three colonies on the reef crest adjacent to Heron Island, at low tide, in January 2011 (maximum irradiance: 700-1200 µmol photons m^{-2} s^{-1} (Anthony et al. 2008)) and transported immediately to aquaria with ambient flow-through seawater. Coral branches were held upright for a period of 5 days to allow recovery from collection; recovery was determined based on re-growth of coral tissue over the branch base. Coral branches (340 nubbins) were randomly allocated to one of two replicate experimental aquaria (per treatment) designated as control (ambient temperature), 32 °C, or 34 °C treatments. Corals were exposed to daily increased

seawater temperatures during an initial 4-day heat stress (termed the "pre-stress"). Three sample branches were taken from each replicate tank of pre-stress treatments on day 4 (end of pre-stress) at 1300h, immediately snap-frozen in liquid nitrogen, and stored at -80 °C for later mRNA purification and subsequent qPCR (all samples were collected in this manner). The coral branches were then exposed to flow through ambient seawater for a 7-day recovery period, following which replicate branches (n = 6 for control and 34 °C pre-stressed treatments, n=4 for 32 °C pre-stressed treatment) were sampled (as per previous). Following recovery, branches from each pre-stress treatment were designated to ambient conditions or were exposed to a second, gradual (Δt = 1-2°C day^{-1}), heat stress to 34 °C (t = 30 °C on the 1st day of 2nd stress, 32 °C on day 2, 33 °C on day 3, then to 34 °C on day 4 for 3 days) (Figure 5.1). Replicate coral branches (n = 3) were collected every second day of the second stress exposure (experimental days 2, 4 and 6).

The treatment regimes were labeled as follows: control (ambient temperature throughout the experiment), 32-Amb (pre-stress 32 °C, remainder at ambient), 34-Amb (pre-stress 34 °C, remainder at ambient), Amb-34 (no pre-stress, 34 °C 2nd heat stress), 32-34 (32 °C pre-stress, 34 °C 2nd heat stress), and 34-34 (pre-stress at 34 °C, 34 °C 2nd heat stress) (Figure 5.1).

Pulse-amplitude modulated (PAM) fluorometry

See Chapter 3 for F_v/F_m measurement methodology.

Non-photochemical quenching (NPQ) of chlorophyll fluorescence was measured using the light induction-recovery curve of the PAM fluorometer. This Y(NPQ) value, a measure for the quantum yield of regulated energy dissipation, was taken from the end of the induction period (531 μmol m^{-2} s^{-1} for 4 mins) of the induction-recovery curve to

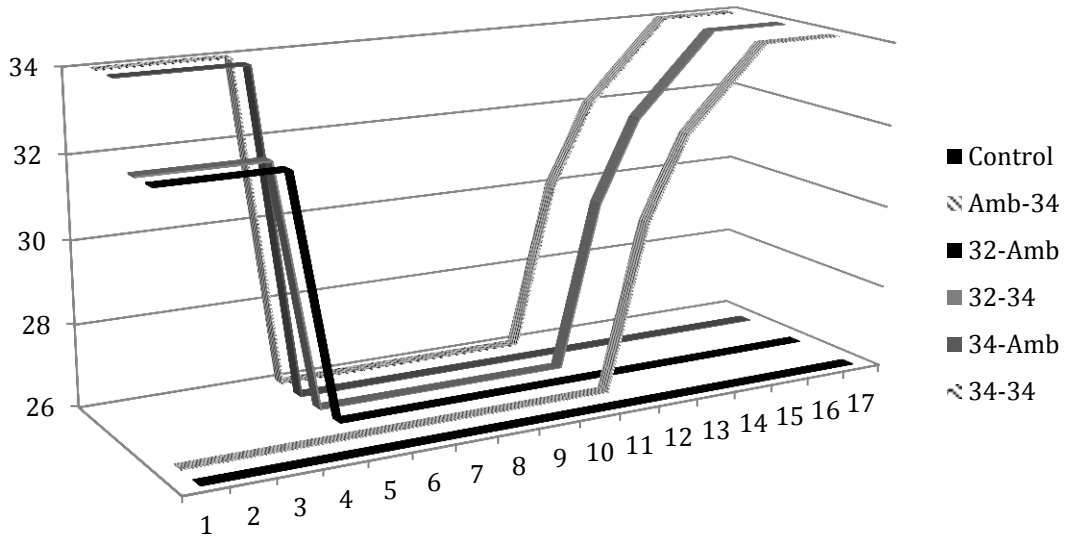


Figure 5.1. Temperature profile for replicate treatment tanks over 17 day experimental period. Front to back: control tanks (black line), ambient-34 °C (hashed line), 32 °C – ambient (dark grey line), 32 °C- 34 °C (gray line), 34 °C- ambient (light gray line), and 34 °C- 34 °C (checked line). See text for more detailed treatment explanations.

assess response of *Symbiodinium* PSII to short-term light stress. Y(NPQ) is calculated by the Kramer equation (Kramer et al. 2004):

$$Y(NPQ) = 1-Y(II)-1/(NPQ+1+qL(Fm/Fo-1))$$

Each coral branch was returned to treatments immediately following measurement; each branch was measured daily throughout the experiment period.

Quantitative PCR (qPCR)

Coral branches were snap-frozen, stored at -80 °C, crushed in a hydraulic press and finely ground with a chilled mortar and pestle and 250 mg of the resultant powder was used for mRNA isolation using the Dynabeads mRNA DIRECT kit (Invitrogen, Australia) following a modified protocol of Leggat et al. (2011a). mRNA yield were

quantified on a NanoDrop-1000 (Thermo Scientific, USA) and 150 ng of mRNA from each sample was treated with RQ1 RNase-free DNase (Promega, USA) to eliminate gDNA contamination and used in reverse transcription using SuperScript III First-strand synthesis kit (Invitrogen, Australia), as per manufacturer's recommendations, but adjusting reaction volume to 1.5 times the suggested protocol.

Target genes of interest were chosen for analysis based on their putative roles in thermal stress response. Molecular chaperones, heat shock protein (HSP) isoforms 16, 70, and 90, and antioxidant genes manganese super-oxide dismutase (MnSOD) and catalase were selected for study (Table 5.1). *Symbiodinium* gene expression was not analysed here, as previous work has indicated limited transcriptional responses of the algal symbiont (Leggat et al. 2011a; Leggat et al. 2011b).

Quantitative PCR was performed as per Chapter 3 methods. Analysis in GeNorm (Biogazelle) of a subset of samples showed Ctg1913 (M = 0.57), RPL9 (M = 0.57), and RPII (M = 0.59) to be the most stably expressed genes, with V ¾ = 0.14; these 3 genes were thus used as internal control genes (ICG) to analyse relative expression of all genes of interest (GOI). All genes (GOI and putative ICG) were included in initial GeNorm analysis. Relative expression of GOI compared to ICG was analysed by qBasePlus (Biogazelle). All results for GOI are discussed in terms of fold-change relative to control samples for each day, for each treatment, and are represented as such in figures.

Statistical analysis

All statistical analyses of relative expression quantities were performed using SPSS (IBM) software package using a mixed effects model with treatment and day as fixed effects and tank nested within both treatment and day. Significant differences were then examined using a sequential Bonferroni post-hoc test ($\alpha = 0.05$).

Table 5.1. Primer sequences, reaction efficiency, and Genbank accession numbers (or source paper) for *Acropora aspera* genes of interest and internal control genes used for qPCR.

Gene name	Forward primer	Reverse primer	Reaction efficiency	Accession number or citation
HSP16	TCCTCCTCTGAGCGTTGTT	CTCACGGGAATCAAACGAGT	0.98	(Meyer et al. 2009)
HSP70	GTCGCTCTCAATCCATCAAATACT	GTCTCCCACCTTCGCTTACG	0.94	(Leggat et al. 2011a)
HSP90	TTTCTTGTTGCTGACCGTGTAATAGTA	CCCCTGGGATCCTCTGTGA	0.99	(Leggat et al. 2011a)
Catalase	GCAAAGTAGTTGGACGCGTTAC	GGAATCCTTTCGACCTCACTAAG	1.00	(Seneca et al. 2010)
MnSOD	CGATGCCTTGAAACCTGCAA	TTTCTCTTCGGCCGCGTTAAG	1.04	(Csaszar et al. 2009)
Ctg1913	CCAATGATTGTGATTTAACCACCG	CACAGAAGCAGCAAGCAATGAT	0.87	(Seneca et al. 2010)
RNA Pol, 2	CCAAACTCCAATCCACCTTG	AAGACCTTAAATAGTCATCCATGAGG	0.99	(Brady et al. 2011)
RPL9	CCCTTGTGCGGAATTTTAGA	CACCAATGTGCCATTCTCAG	0.99	(Souter et al. 2011)

Results

Photosynthetic efficiency of PSII

Pre-stress and recovery

Chlorophyll fluorescence was measured during the pre-stress, mid-way and at the end of the 7-day recovery period, and daily during the 2nd heat stress. The 32 °C treatment had no significant effect on dark-adapted, maximum quantum yield (F_v/F_m) during the pre-stress or recovery as compared to the control (ambient temperature) (Figure 5.2; $p>0.05$). Corals exposed to 34 °C pre-stress showed a significant decline in F_v/F_m from day 1 ($F_v/F_m = 0.620$) to days 3 (= 0.541) and 4 (= 0.546), which were significantly lower than control for each respective day (control day 3 = 0.604, day 4 = 0.595) (Figure 5.2a; $p<0.05$). Photosynthetic efficiency of PSII increased during the recovery period such that by the end of the 7-day recovery (day 11 of the experiment), 34 °C pre-stressed nubbins returned to control levels (Figure 5.2a; $p>0.05$).

2nd thermal stress

Dark-adapted yields of corals that were pre-stressed at 32 °C and 34°C that were subsequently maintained at ambient temperatures were the same as control corals throughout the second half of the experiment (Figure 5.3a). F_v/F_m of 34-Amb was slightly depressed compared to control throughout the 6-day period, but only significantly so on day 4 ($p<0.05$). Corals raised to 34 °C in the 2nd stress (Amb-34, 32-34, and 34-34) showed a steady decline in F_v/F_m from approximately 0.64 at the beginning to 0.359-0.409 at the conclusion of the experiment (day 6 of 2nd stress, day 17 of experiment), regardless of pre-stress treatment (Figure 5.3a). The difference between heated and ambient treatments was statistically significant from day 4 onward, when

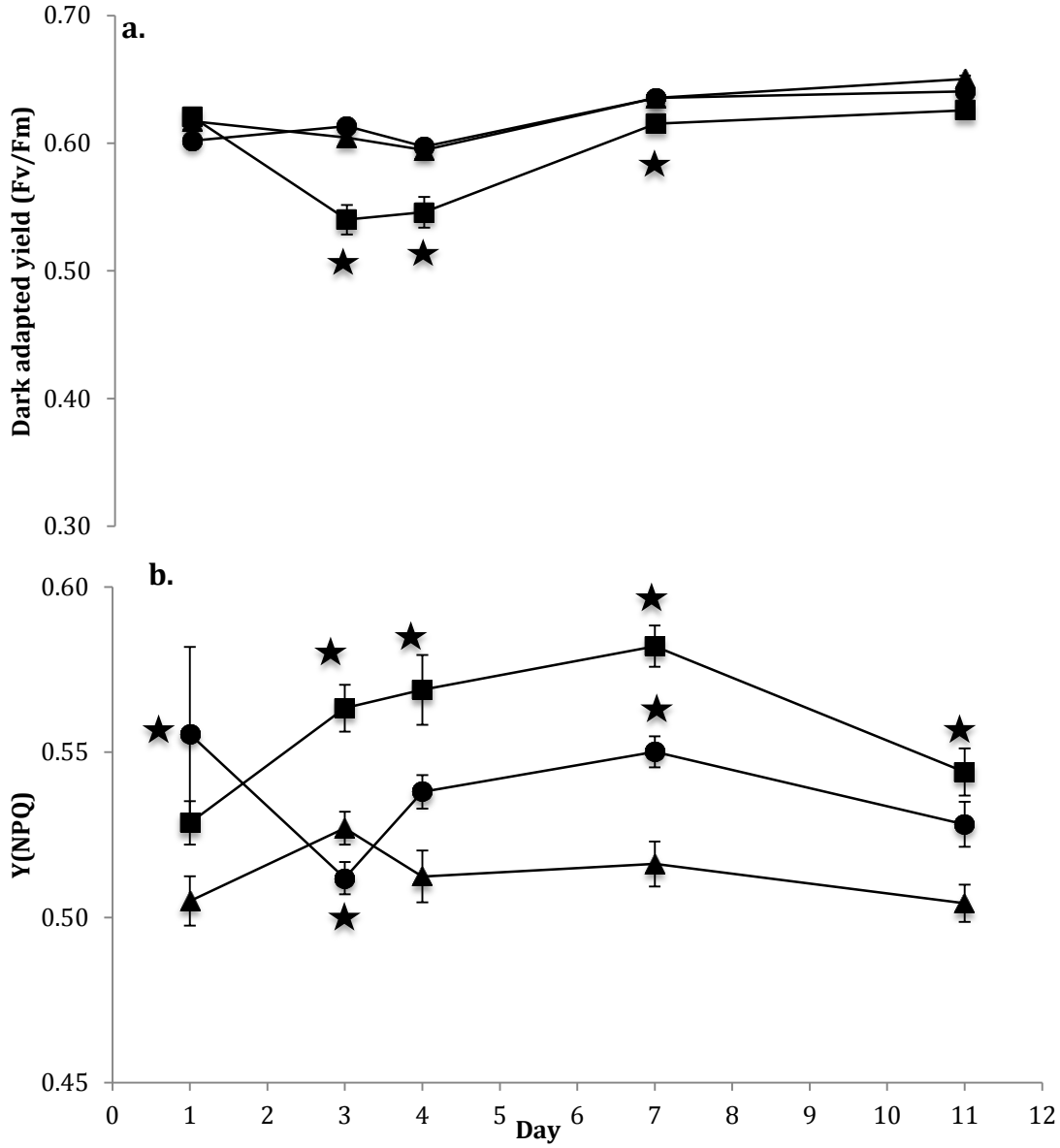


Figure 5.2. Chlorophyll fluorescence-based measurements of a) photosynthetic efficiency of PSII and b) yield of non-photochemical quenching of photosystem II of *Acropora aspera Symbiodinium* during a 4-day thermal stress followed by 7-day recovery (days 5-11) at ambient temperature. Control (ambient temperature; triangles), 32 °C stress (circles), and 34 °C stress (squares) are shown (days 1-7 n = 10 per treatment, day 14 n = 6 per treatment). Error bars represent standard error of the mean; some bars are obscured by data points. Stars indicate significant difference to control ($p<0.05$).

temperature reached 34 °C ($p<0.05$). Corals that had no pre-stress (Amb-34) consistently exhibited the lowest Fv/F_m during the 2nd stress, however the difference between Amb-34 and pre-stressed corals were not statistically significant ($p>0.05$).

Non-photochemical quenching

Pre-stress and recovery

Yield of non-photochemical quenching, Y(NPQ), was significantly higher on days 3 and 4 of the pre-stress in the 34 °C treatment (Y(NPQ) = 0.563 and 0.569, respectively) than in control (Y(NPQ) = 0.527 and 0.512, respectively) and remained significantly higher throughout the recovery period (0.06 and 0.04 higher on days 7 and 11, respectively, $p<0.05$) (Figure 5.2b). Y(NPQ) in the 32 °C pre-stress treatment was higher than control during the recovery phase (day 7 = +.034, day 11 = +.024 above control), but only significantly so on day 7 ($p<0.05$) (Figure 5.2b).

2nd stress

32 °C pre-stressed corals that remained at ambient during the 2nd phase of the experiment returned to control levels. 34 °C pre-stressed corals remained significantly higher in Y(NPQ) during the 2nd phase of the experiment at ambient temperature (ΔY(NPQ) = +0.074-0.106; $p<0.05$) compared to controls (Figure 5.3b).

Corals exposed to the 2nd heat stress all increased significantly in Y(NPQ) across the 6-day stress period. Amb-34 and 34-34 increased steadily with increasing temperature and continued to increase as temperature was held at 34 °C for the final 3 days of the 2nd stress. 32-34 held steady Y(NPQ) until day 4 (first day of 34 °C), after which it increased significantly, up to levels seen in 34-34 and amb-34 on day 6 (Figure 5.3c).

Quantitative PCR

Pre-stress and recovery

Catalase and HSP16 were significantly up-regulated (relative expression compared to

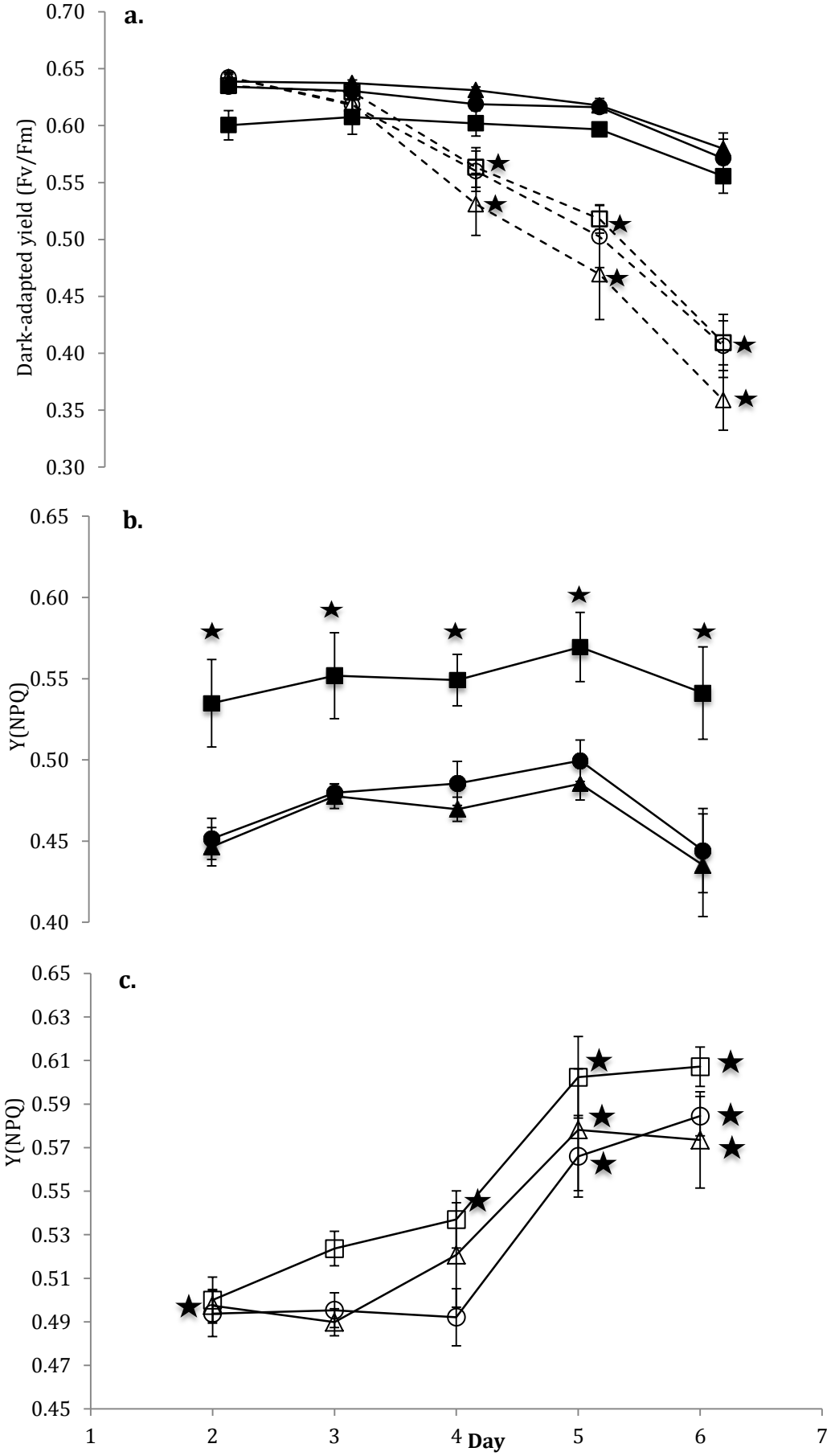

a.
Dark-adapted yield (Fv/Fm)
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
b.
Y(NPQ)
0.65
0.60
0.55
0.50
0.45
0.40
c.
Y(NPQ)
0.65
0.63
0.61
0.59
0.57
0.55
0.53
0.51
0.49
0.47
0.45
1
2
3
4
5
6
7
Day

Figure 5.3. Chlorophyll fluorescence-based measurements of a) photosynthetic efficiency of PSII and b) and c) yield of non-photochemical quenching of photosystem II of *Acropora aspera Symbiodinium* during a 2nd treatment/stress following an initial 4 day pre-stress (ambient, 32 °C or 34 °C) and recovery at ambient for 7 days. Control (closed triangles), 32°C pre-stress/ambient 2nd treatment (closed circles), 34 °C pre-stress/ambient 2nd treatment (closed squares), ambient pre-treatment/34 °C 2nd stress (open triangles), 32 °C pre-stress/34 °C 2nd stress (open circles), 34 °C pre-stress/34 °C (open squares) (n = 6 for each treatment). Error bars represent standard error of the mean; some bars are obscured by data points. Stars represent data points significantly different to control (ambient-ambient) for that day.

control = 2.7- and 3.2-fold, respectively; $p<0.05$) at 32 °C at the end of the 1st stress, while all other genes remained unchanged (Figure 5.4b,e). MnSOD (relative expression compared to control = 1.9-fold), catalase (= 5.8), HSP16 (= 5.9), and HSP90 (= 1.3) were significantly up-regulated at 34 °C pre-stress compared to control ($p<0.05$) (Figure 5.4; Table 5.2). HSP70 did not show differential expression at 32 °C or 34 °C ($p>0.05$) during the pre-stress. All genes returned to control-level expression after the one-week recovery period, regardless of pre-stress treatment (Figure 5.4).

2nd Stress

Corals pre-stressed at 32 °C and 34 °C largely maintained control-level expression when kept at ambient temperature through the 2nd half of the experiment (Figure 5.5). The exception was antioxidant gene MnSOD on day 4, which was significantly down-regulated for both 32-Amb and 34-Amb treatments ($p<0.05$; Figure 5.5a; Table 5.3). MnSOD also exhibited the unexpected down-regulation in heated treatments 32-34 and 34-34 on day 4 of the 2nd stress ($p<0.05$), after which expression returned to control level in all treatments (Figure 5.6a; Table 5.4). No other days showed any significant responses in any treatments for MnSOD during the 2nd heat stress ($p>0.05$). Catalase significantly increased expression in nearly all samples in heated treatments on days 4 and 6 of the 2nd heat stress (fold-changes from 2.7 to 5.6 of control; $p<0.05$) (Figure

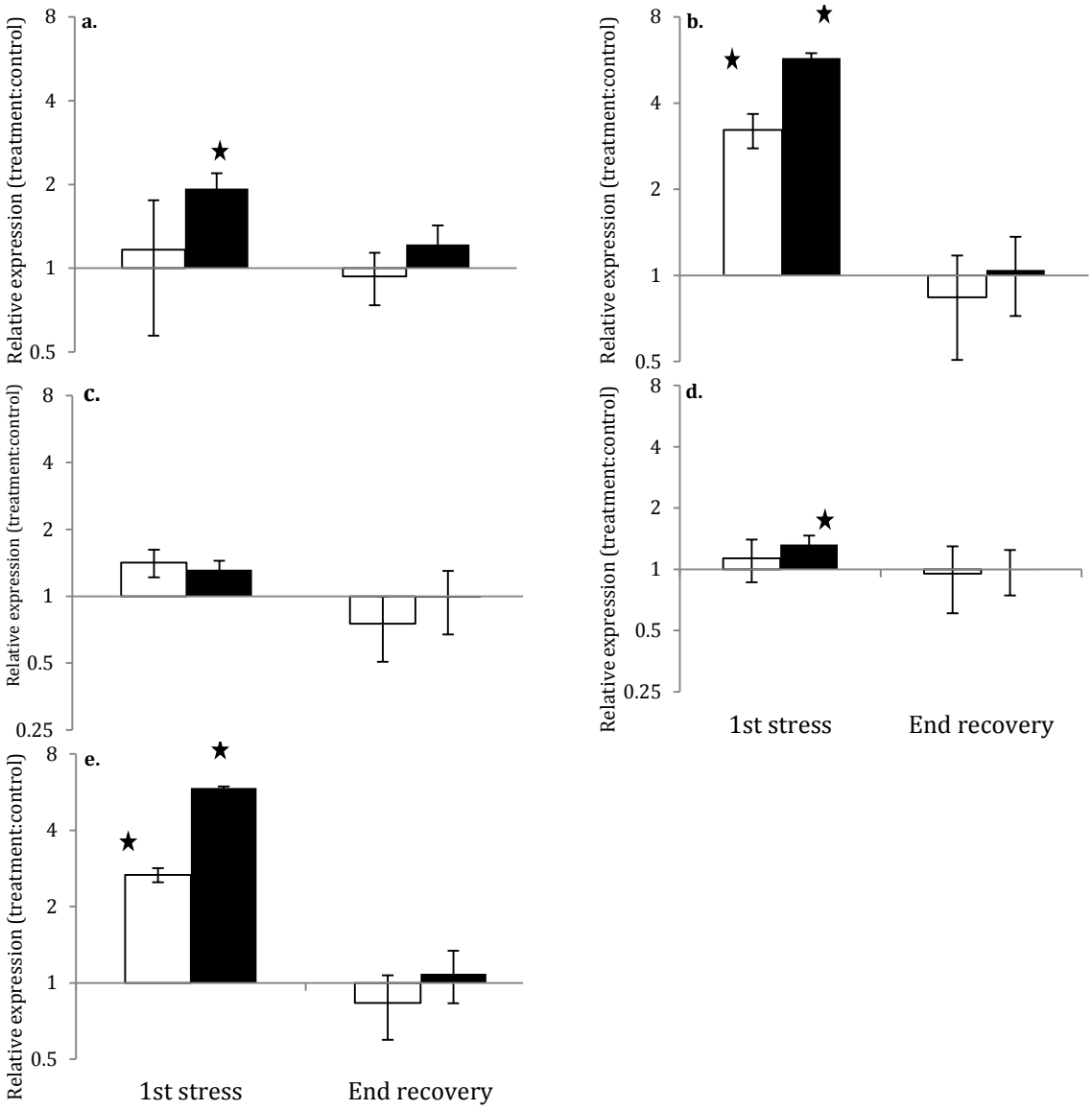


Figure 5.4. Relative expression of *Acropora aspera* a) manganese superoxide dismutase, b) catalase, c) heat shock protein (HSP) 70, d) HSP90, and e) HSP16 genes at the end of a 4 day pre-stress and end of 7 day recovery at ambient temperature. White bars represent 32 °C pre-stress expression relative to control expression; black bars represent 34 °C expression relative to control expression. Error bars represent standard error of the mean; stars indicate significant difference from control (ambient temperature) expression level ($p<0.05$, sequential Bonferroni).

5.6b; Table 5.4).

Molecular chaperones HSP70 and HSP90 showed similar patterns to each other; both were significantly up-regulated in all heated treatments on day 6, when treatment

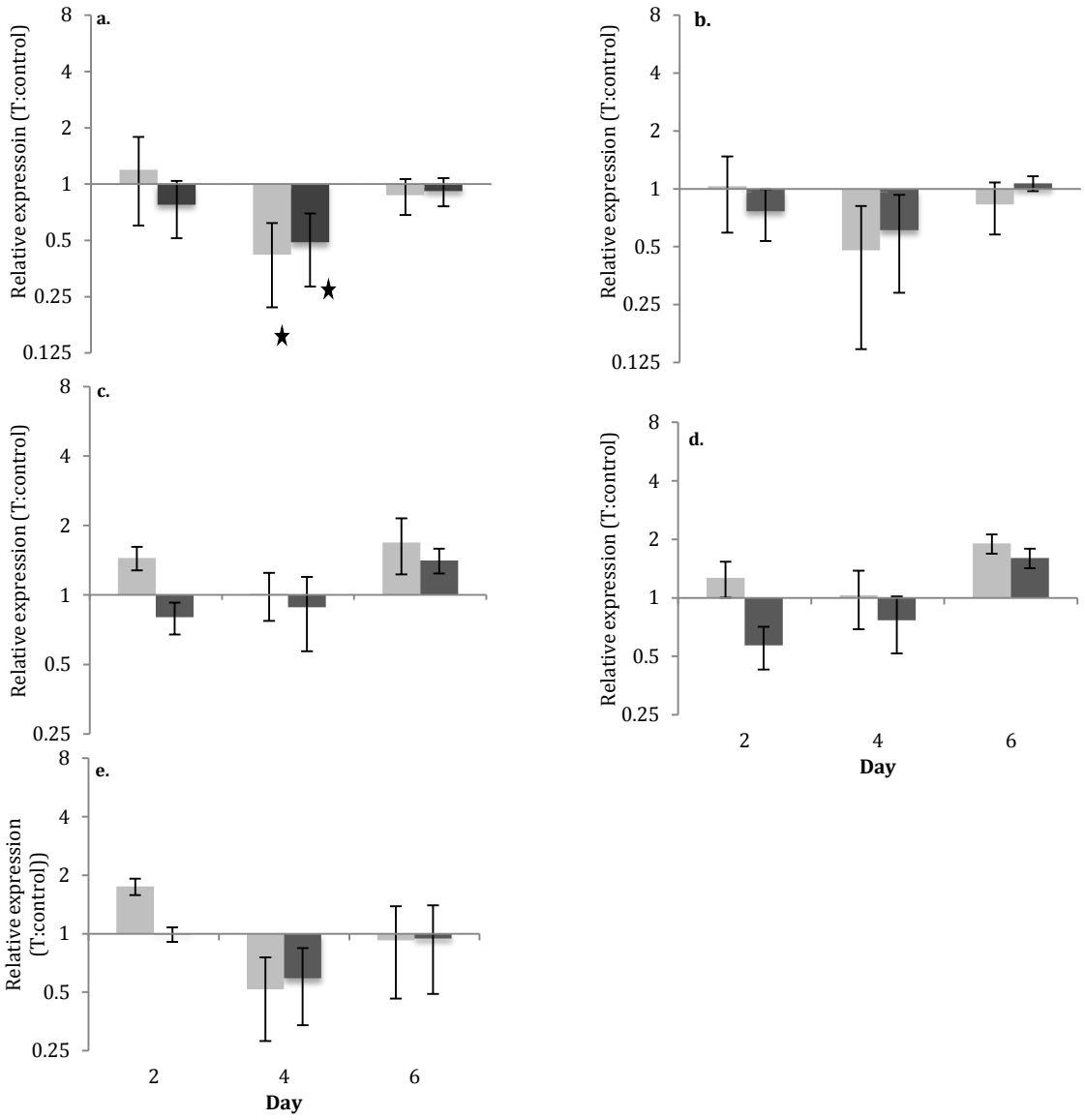


Figure 5.5. Relative expression of *Acropora aspera* a) manganese superoxide dismutase, b) catalase, c) heat shock protein (HSP) 70, d) HSP90, and e) HSP16 genes during a 2nd stress following a 4 day pre-stress at ambient (control), 32 °C (light gray bars), or 34 °C (dark gray bars) and 7 day recovery. These data represent the 2nd half of the experiment where half the corals from each pre-stress treatment were kept at ambient temperature for a further 6 days following recovery while another subset was subjected to a 2nd heat stress.

temperature had been at 34 °C for 3 days. The extent of up-regulation was greater in HSP90, with expression levels 2.9-6.3 times greater than control in day 6, whereas HSP70 increased 2.0-3.2 fold of control (Figure 5.6c-d; Table 5.4). Small HSP16 was significantly up-regulated in all heated treatments on days 4 and 6 ($p<0.05$; Figure 5.6e;

Table 5.2. Relative expression values for genes of interest (GOI) during the first 4 day "pre-stress" on *Acropora aspera* and recovery (ambient temperature for 7 days). Data are expressed as fold-changes relative to control; only significantly different expression shown ($p<0.05$).

GOI	1st stress		Recovery
	32 °C	34 °C	Amb
Catalase	3.22 ↑	5.75 ↑	
MnSOD		1.93 ↑	
HSP16	2.67 ↑	5.88 ↑	
HSP70			
HSP90		1.32 ↑	

Table 5.4). Overall, it exhibited the greatest fold changes of all GOI in response to the heat stresses (up to 20-fold greater than control in some treatments). Expression in Amb-34 treatment of HSP16 showed a more immediate response to the gradual 2nd heat stress than pre-stressed samples, significantly up-regulating expression to 5.4-fold of controls when temperature was just 32 °C (day 2; $p<0.05$). Pre-stressed corals 32-34 and 34-34 showed significant up-regulation when temperature reached 34 °C on day 4 (10.1- and 11.4-fold, respectively) and remained significantly high through day 6 (12.3- and 20.1-fold, respectively).

Effect of pre-stress on GOI expression levels

HSP16 in Amb-34 corals appeared more sensitive in expression induction than pre-stressed corals. Expression was significantly higher in Amb-34 corals on day 2 (5.4-times control; $p<0.05$) than 32-34 and 34-34 corals (4.0- and 1.0-fold of controls, respectively; $p>0.05$) (Figure 5.6e). HSP16 Amb-34 expression was also significantly higher on day 4 (16.8-fold; $p<0.05$) than 32-34 (10.1-fold) and 34-34 (11.4-fold). Amb-34 on day 6 was significantly more highly expressed (19.5-fold) than 32-34 (12.3-fold; $p<0.05$); 34-34 expression level had reached 20.1-fold of controls, similar to Amb-34.

Table 5.3. Relative expression of genes of interest from *Acropora aspera* exposed to a pre-stress at either 32 or 34 °C, then allowed to recover at ambient temperature for 13 days. Data are expressed as fold-changes relative to control; only significantly different expression shown ($p<0.05$).

GOI	Day 2 heat		Day 4 heat		Day 6 heat	
	32-Amb	34-Amb	32-Amb	34-Amb	32-Amb	34-Amb
Catalase			0.048 ↓	0.610 ↓		
MnSOD			0.419 ↓	0.489 ↓		
HSP16						
HSP70						
HSP90						

Table 5.4. Relative expression values for genes of interest for *Acropora aspera* exposed to a pre-stress at either 32 or 34 °C, recovered for 7 days at ambient temperature, and subjected to a 2nd stress which increased in temperature 1-2 °C day^{-1} to 34 °C where it remained for 3 days to simulate a bleaching event. Data are expressed as fold-changes relative to control; only significantly different expression shown ($p<0.05$).

GOI	Day 2 heat			Day 4 heat			Day 6 heat		
	Amb-34	32-34	34-34	Amb-34	32-34	34-34	Amb-34	32-34	34-34
Catalase				3.35 ↑		2.87 ↑	2.74 ↑	4.71 ↑	5.55 ↑
MnSOD					0.32 ↓	0.49 ↓			
HSP16	5.37 ↑			16.80 ↑	10.08 ↑	11.40 ↑	19.50 ↑	12.32 ↑	20.05 ↑
HSP70							1.99 ↑	3.14 ↑	3.24 ↑
HSP90							2.87 ↑	5.89 ↑	6.29 ↑

Discussion

The ability of coral hosts and their symbionts to acclimatise and adapt to climate change is an unknown factor in models predicting the response of corals to future climate change. Models predicting mass annual bleaching by mid-century assume an unchanging thermal tolerance in corals that, authors state, is evident from the increasing frequency (i.e., no apparent mitigation) of mass bleaching events in recent decades

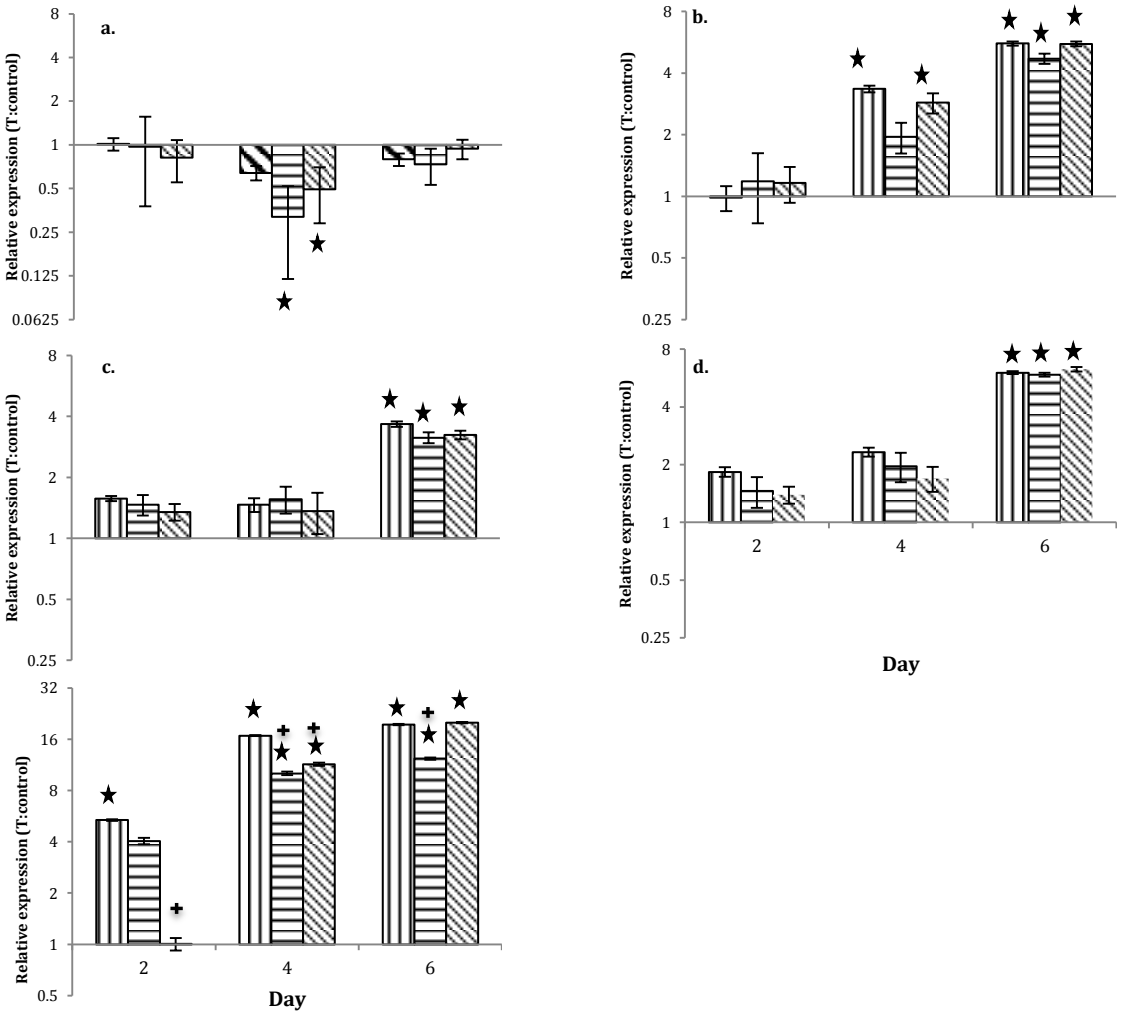


Figure 5.6. Relative expression of *Acropora aspera* a) manganese superoxide dismutase, b) catalase, c) heat shock protein (HSP) 70, d) HSP90, and e) HSP16 genes during a 2nd stress following a 4 day pre-stress: ambient (vertical striped bars), 32 °C (horizontal striped bars), or 34 °C (diagonal striped bars) and 7 day recovery. Corals were subjected to a 2nd heat stress simulating a bleaching event with gradual rise in temperature to 34 °C. Stars represent significantly different ($p<0.05$) expression from control (ambient temperature pre-treatment, ambient 2nd treatment). Crosses indicate significant difference ($p<0.05$) to ambient-34 treatment. Error bars represent standard error of the mean (some error bars are too small to see).

(Hoegh-Guldberg 1999; Donner et al. 2005). However, patchy bleaching coverage and temperature gradients across the geographic range of a species are evidence that variation in stress tolerances exist and that a single, static thermal threshold is not realistic on a reefal scale (Hughes et al. 2003). Few studies have considered that thermal acclimation needs to occur at the necessary rate, simultaneously, in both the host

organism and its endosymbiotic algae. This experiment shows differential expression of antioxidant and heat shock protein genes in the host coral and changes in photophysiology of *Symbiodinium* occur under similar thermal profiles. This study also found that the responses and acclimatory potential of *Acropora aspera* and its endosymbiotic algae may be significantly impacted by previous exposure to sub-lethal thermal stress (32^{o}C), rather than exposure to previous bleaching events, with altered gene expression patterns still observed seven days after prestress.

Thermotolerance and recovery of symbiotic algae.

Despite prior stress exposure (irrespective of the degree) resulting in no significant impact to photosynthetic efficiency of PSII during a subsequent stress there was a significant impact of prior sub-lethal stress to *Symbiodinium* photoprotection (non-photochemical quenching). When experimental temperatures reached 34 °C in the second stress event, F_v/F_m in corals previously exposed to the 32 and 34 °C pre-stress (32-34 and 34-34 treatments) did not differ from those not pre-stressed. Y(NPQ), however, differed between 32-34 and 34-34 corals. In 34-34 treatments Y(NPQ) increased with increasing temperature, whereas 32-34 treated corals only experienced increasing Y(NPQ) from day 4 of the 2nd stress onward, after temperature reached the 34 °C mark. These findings suggest that chronic photoinhibition occurs in corals exposed to prior bleaching events but, importantly, prior exposure to sub-lethal (in this case 32 °C) temperature mitigates, or delays, PSII stress as measured by Y(NPQ) (this delay did not, however, translate into mitigation in the loss of F_v/F_m during the 2nd stress). It appears that with longer exposure to 34 °C, corals not previously stressed became more photoinhibited compared to thermally acclimated corals. Similarly, Middlebrook et al (2008) found that non-pre-stressed *A. aspera* samples also had

significantly reduced photosynthetic efficiency of PSII when heat stressed, compared to control samples and samples that had been heat-stressed 1 and 2 weeks prior.

Interestingly, NPQ yields reveal chronic PSII stress in the 34 °C pre-stressed corals compared to other corals even when returned to ambient conditions for the remainder of the experiment (34-Amb, 13 days). Y(NPQ) was significantly higher in the 34 °C treatment compared to controls during the pre-stress. Photosystems in these corals clearly experienced ongoing stress after the 4-day 34 °C exposure, potentially arising from reaction center damage incurred during the heat stress. An increase of NPQ at 34 °C and above has also been observed in *Stylophora pistillata* (Jones et al. 1998) and *Siderastrea radians* (Warner et al. 1996) *Symbiodinium*.

Acclimatory potential of the coral host

The ability of coral to adapt to their changing environment in the long term also depends on the ability of the host to acclimatise and adjust their thermal stress response in the short term. Differential thermal acclimation between sub-lethal 32 °C and 34 ^{o}C pre-stress was evident through the expression of catalase expression. Expression was significantly up-regulated in Amb-34 and 34-34 samples compared to controls on day 4 of the 2nd stress (first day heat stress reached 34 °C), but not in 32 °C pre-stressed samples. Catalase detoxifies the ROS species hydrogen peroxide (H_2O_2) to water and O_2; an increase in expression of this antioxidant gene during both pre-stress and 2nd stress suggests an increased production of H_2O_2, stress of the photosynthetic apparatus, and excess photon energy absorption during these times (Asada 1999; Asada 2000; Suggett et al. 2008). The delayed up-regulation of the 32-34 samples compared to the other 2 heated treatments may be a consequence of a lower production of H_2O_2 in that treatment for day 4. Indeed, a lower Y(NPQ) was observed in 32-34 samples than 34-34

samples on days 2 and 4 of the 2nd stress, which correlates with the catalase expression data on those days. The end of the 2nd stress saw a significant increase in Y(NPQ) in the 32-34 corals, which also corresponded with a significant increase in catalase expression. While direct causation cannot be determined without direct measurements of ROS production, less NPQ is suggestive of lower ROS production in 32-34 corals, alleviating the need for an anti-oxidant enzyme such as catalase. However, after 3 days at 34 °C (day 6 of 2nd stress), there was no significant difference in expression of catalase, regardless of pre-stress exposure, indicating a similar amount of oxidative stress occurring during prolonged exposure to the bleaching threshold. While a sub-lethal heating stress may provide a degree of acclimation in terms of ROS production, this acclimatory effect is eventually overwhelmed by longer periods of thermal stress.

HSP16 is a member of the small HSP family, which includes some of the most strongly induced HSPs in response to cellular stress (for review see Haslbeck 2002; Kregel 2002). In the present study HSP16 had the greatest expression change in response to heat stress of all genes of interest (up to 20-fold greater than control expression). During the 2nd stress, Amb-34 samples were up-regulated from day 2 onward, whereas 32-34 and 34-34 samples only became significantly up-regulated from day 4 onward. This suggests that pre-stressed corals were not experiencing thermal stress at 30 °C (day 2 seawater temperature) while coral not previously stressed were responding to thermal stress. Expression of HSP16 was related to extent of heat stress; the significantly lower expression in 32 °C pre-stressed samples compared to non-pre-stressed samples on days 4 and 6 indicates an acclimatory effect of the 32 °C pre-stress. This lower pre-stress temperature may be near the thermal threshold of *A. aspera*, where thermal damage is in balance with rate of repair mechanisms. Significantly, while 34-34 exposed corals reached the expression level of Amb-34 for HSP16 on day 6,

expression in 32-34 corals remained significantly less. Similar large changes in HSP16 expression have been observed in *Porites asteroides* in response to thermal stress (Kenkel et al. 2011), where even larger changes (up to 700 fold were observed). Similar to the catalase expression, these results indicate that the sub-lethal 32°C stress exposure alters gene expression responses to a subsequent bleaching event and possibly confers greater thermal tolerance to *A. aspera* than exposure to the beaching threshold

Expression of molecular chaperones HSP70 and HSP90 were less responsive to thermal stress than HSP16, only becoming significantly up-regulated in the final day of the 2nd heat stress. There appeared to be no indication of an acclimatory effect of either pre-stress (32°C or 34°C) on expression of HSP70 and 90. During both pre-stress events and the subsequent stress, 32 °C treatment elicited no difference in transcription for either HSP70 or 90. A 34 °C stress, however, produced a significantly higher expression for HSP90 in the pre-stress, and in both HSPs after 3 days at 34 °C during the 2nd stress. These HSP results are similar to two previous studies (Kenkel et al. 2011; Leggat et al. 2011a), which found similar levels of expression change in response to thermal stress.

MnSOD showed little differential expression in any of the experimental scenarios studied here. MnSOD is located in the mitochondria of eukaryotes and is suggested to play a significant role in antioxidant capacity and prevention of bleaching in coral (Downs et al. 2000). In the sea anemone *Anemonia viridis,* isozymes of MnSOD are associated with the endosymbiotic algae and have been localised within the gastroderm and algal fraction (Richier et al. 2003). Absence of differential expression of MnSOD (upregulation was found in bleaching responses by Seneca et al. (2010)) here may indicate a lack of oxidative stress in mitochondria of *A. aspera* and/or an absence of ROS being produced by *Symbiodinium* during the heat stress. Alternatively, the

antioxidant defense of *A. aspera* may rely predominantly on CuZnSOD instead of MnSOD. CuZnSOD was found to be the dominant form of active SOD in *Anemonia viridis* (Richier et al. 2003) and is potentially also the dominant active SOD in Acroporid corals.

Conclusions

In determining acclimatory capacity of the coral holobiont during future climate change, it is necessary to determine (1) if prior thermal stress results in differential gene expression responses to subsequent stress (2) the extent to which this thermal memory can be retained, and (3) the long-term, inter-generational stability of an acquired thermal memory. The present study clearly illustrates that acclimation of the holobiont is not solely dependent upon one partner; instead, survival of the holobiont will be as a result of the interaction of the acclimatory response of both partners and their interaction during stress events. For example, we suggest that the combined effect of the antioxidant catalase with HSP16 may be the retention of a higher F_v/F_m in pre-stressed corals than non-pre-stressed corals. Higher antioxidant levels could alleviate oxidative stress in PSII, and thus prevent cellular damage caused by ROS. Subsequent studies also need to determine whether the acclimation responses seen here result in reduced partial of colony mortality, and thereby provide a link between these gene expression changes and coral resilience.

Interestingly, I also found that the level of target gene up-regulation is directly proportional to the degree of heat stress, (i.e. 34 °C samples had higher expression levels than 32 °C). The two genes that responded significantly under the 32 °C pre-stress, catalase and HSP16, also had the highest levels of up-regulation at 34 °C (roughly 5.8-fold of control). Importantly, gene expression returns to that of control

corals during recovery. These genes could therefore potentially be used as stress biomarkers as differential expression is evident at sub-lethal temperatures, early in the thermal stress response of this coral, and is sustained throughout exposure to the bleaching threshold.

In the future, corals will be subjected to increasingly higher SST due to anthropogenic climate change. This incremental rise will expose corals to temperatures that are ever closer to their respective bleaching thresholds, eventually reaching and exceeding them on a regular basis. Our results show that a thermal history of sub-lethal temperature exposures may proffer the opportunity for corals and their symbionts to become acclimatised to elevated temperature. Through this acclimatisation it may be possible for corals to increase their thermal tolerance and therefore raise their bleaching threshold. A critical distinction to note is that repeated exposures to bleaching temperatures in this study did not confer the same acclimatory effect on coral and symbiont responses as the sub-lethal pre-stress. The extent to which corals may be able to increase the bleaching threshold is unknown, as are any potentially adverse trade-offs of living at elevated temperature. Therefore, efforts to mitigate global SST rise must continue and thus reduction of CO_2 emissions remains paramount.

Chapter 6: General Discussion

Anthropogenic CO_2 emissions are creating oceanic conditions that are becomingly increasingly inhospitable to the world's tropical coral reefs. Mass bleaching events and increased prevalence of diseases have driven a worldwide decline of up to 30% in coral cover (Wilkinson 2002). Both bleaching and increased disease prevalence have been attributed to increasing SST caused by the greenhouse effect of increasing atmospheric CO_2 (Brown 1997; Harvell et al. 2002). Acidified surface ocean, also attributed to global climate change, is linked with decreased calcification in many corals (Hoegh-Guldberg et al. 2007; Orr et al. 2005), however, the impacts of hypercapnia (direct effect of increased CO_2) on the physiology of corals remains largely unknown. Even less is known about how these stressors will interact to affect the constituents of the coral holobiont, and, by extension, the coral reef ecosystem.

This thesis explored the synergistic effects of increased sea surface temperature and elevated pCO_2 on a key reef-building coral, *Acropora aspera*. The impacts of these stressors on the coral animal, endosymbiotic algae *Symbiodinium*, and bacterial population were explored. This holobiont-based approach addressed an issue that is often overlooked by other studies, which is the environmental impacts on all members of the holobiont community, from the microbes up to the animal host. As these associations form the basis for coral holobiont health it is imperative to take into account the effects of environmental changes on all aspects of the holobiont community. Additionally, previous studies have largely focused on the impacts of temperature and CO_2 on corals separately, and in the case of increased pCO_2 have focused almost exclusively on calcification. As these environmental factors will be changing in tandem under future climate change scenarios it is ecologically pertinent to employ studies that

manipulate them in concert and assess the physiological impacts on the holobiont community.

Research undertaken during this thesis depended largely on assaying expression of targeted genes involved in various physiological processes within the coral holobiont. This was done by employing the highly sensitive qPCR technique that quantifies expression of genes of interest by comparison to stably expressed internal control genes. In this way, it was possible to target the gene expression responses of important genes of *Symbiodinium* and *A. aspera* physiology. This research also employed next-generation sequencing techniques to characterise the bacterial community of *A. aspera* and how it responds to external stressors and changed physiological status of the holobiont. Use of this new technology enabled an in-depth study of rare and low-abundance bacterial ribotypes associated with coral. Looking forward, the ability of *A. aspera* to acclimate to rising water temperature was assessed by subjecting it to repeated thermal stress events and examining transcript abundance of targeted stress response genes, again via qPCR.

The precise lesion point during coral bleaching has often been sought and remains a point of contention. Many studies have pointed to *Symbiodinium* as the weaker partner during thermal stress. Impairment or damage to PSII causes production of harmful reactive oxygen species (ROS) that may be the trigger for algal expulsion from host gastroderm (Lesser 1997; Lesser 2011). Results from *in vitro* and *in hospite* experiments of *Symbiodinium* of this thesis found that photosynthetic efficiency of PSII (measured as F_v/F_m of chlorophyll fluorescence; see Chapter 2 for details) was severely impaired at 34 °C (Chapters 2, 3, 5). At 34 °C, damage to PSII was chronic and lasted

long after temperature stress was removed (evidenced in part by extended period of elevated NPQ) (Chapters 3, 5). This long-lasting damage may have been the cause of irreversible symbiont loss from coral tissue during the recovery period following a simulated bleaching event to 34 °C (Chapter 3). The temperature of dysfunction coincides with the temperature at which *A. aspera* initiates the bleaching response (Chapters 2, 3, 5). These results corroborate other findings that *Symbiodinium* photodamage may indeed be the underlying cause for coral bleaching and demonstrates that for photosynthetic processes, *in vitro* experiments may serve as a useful proxy for *in hospite* responses. Importantly, temperature of 32 °C did not impair *Symbiodinium* photosystems (Chapter 2, 5), indicating there is a fine line between tolerable and harmful temperature for these algae *in vitro* and *in hospite*.

Transcriptional regulation does not appear to play a significant role in *Symbiodinium* thermal stress response, either *in vitro* or *in hospite*. Heat shock protein (HSP) genes were unaffected in *in vitro* and *in hospite* experiments, and other targeted genes related to photosynthesis and carbon metabolism were either unaffected or had small-scale changes in transcription (largest-fold change observed was just 3-4 fold up-regulation of Rubisco after 4 days at 30 °C *in vitro*) (Chapter 2 and 3). There was likewise no effect of increased pCO_2 on *Symbiodinium* transcription of carbon metabolism or HSPs, either alone or in synergy with elevated temperature (Chapter 3). This lack of large transcriptional response has been found in other studies of *Symbiodinium* (Watanabe et al. 2006; Rosic et al. 2010c; Leggat et al. 2011a; Rosic et al. 2011). One possible explanation for this is that dinoflagellates, including *Symbiodinium*, contain very large genomes (the Symbiodinium genome is greater than 2Gb; LaJeunesse et al. 2005), which are permanently condensed (Diaz de la Espina et al. 2005). This unusual genomic

organization may mean the cell is not able to rapidly alter gene expression patterns (Leggat et al. 2011b). Lack of transcriptional changes suggests that there may be a large amount of post-translational regulation occurring. Future studies of *Symbiodinium* stress response may benefit more from proteomic or metabolomic approaches than transcriptomic (Gordon and Leggat 2010). Overall, the results of this thesis suggest that in the near-term (next 50 years), elevated SST will have a greater influence on *Symbiodinium* physiology than ocean acidification.

The synergistic effects of elevated SST and OA on coral carbon metabolism gene expression were explored for the first time in this thesis. CO_2 levels corresponding to mid-century emissions predictions (IPCC 2007) acted synergistically with elevated temperature during a simulated bleaching event to increase expression of glycogen synthesising and catabolising genes. During the subsequent recovery phase the elevated pCO_2 treatment again interacted with the temperature treatment (bleaching stress recovery at +1.7 °C above ambient) to significantly decrease CA genes, which also corresponded to an apparent decrease of CA protein in histological sections (Chapter 3). These results indicate that near-future increases in atmospheric CO_2 concentration will likely have an impact on coral metabolism and that these impacts may be felt as early as mid-century. In particular, it is interesting to note that these corals experience large daily fluctuations in pCO_2 (Kline et al 2012) and that the experimental increases used were only between 50-90 ppm. Whether these gene expression and protein abundance changes had beneficial, detrimental, or neutral impacts on holobiont physiology was not explored and requires further study. However, this does indicate that qPCR is an effective method to determine that environmental perturbations are leading to changes in the coral.

The effects of elevated pCO_2 during a simulated bleaching event on coral-associated bacteria were also examined for the first time. Previous studies have explored the effect of one factor or the other and returned conflicting results. The bacterial assemblage of the *A. aspera* holobiont was not found to change significantly during periods of severe thermal stress and CO_2 concentration had no distinguishable effect within temperature treatments. Results presented here indicate the *A. aspera* bacterial community is highly stable and can maintain their association despite significant environmental and holobiont physiological changes over the time periods examined. In contrast, Bourne et al. (2007) found a shift toward *Vibrio*-affiliated sequences during a period of elevated SST, which immediately preceded a loss in *Symbiodinium*. Bacterial community reverted to the unbleached profile coincident with *Symbiodinium* density recovery, prompting authors to propose *Vibrio* increase as a factor in *Symbiodinium* loss (Bourne et al. 2007). Because of conflicting results such as these, there has yet to be a consensus amongst microbiologists about the general nature of coral-associated bacteria structure (i.e. fixed or dynamic). Clearly, more studies are required to elucidate the factors that influence the microbial assemblage most strongly and consistently and separate primary bacterial responses from secondary changes (e.g., colonisers).

Expression of antioxidant genes of *A. aspera* during heat stress corroborate the hypothesis that *Symbiodinium* photosystems are the lesion point for coral bleaching. Catalase and manganese-super oxide dismutase (MnSOD) were significantly up-regulated compared to control corals during exposure to a 4 day bleaching (34 °C) stress (Chapter 5). This up-regulation coincided with the temperature where a significant decrease in photosynthetic efficiency of PSII (Chapter 2, 3, and 5) and increase in yield

of non-photochemical quenching (NPQ), an indicator of PSII stress, were observed (Chapter 5). Antioxidants scavenge ROS, which in *Symbiodinium* are created from thermally damaged photosystems, therefore an increase in host antioxidant gene expression suggests an increase in ROS (Lesser 1997). Heat shock protein genes were also up-regulated at 34 °C, however, unlike antioxidant genes, this stress response cannot necessarily be linked to the algal partner and may represent simply the response to the heat stress itself (Chapter 5).

One of the key findings of this thesis is the ability of *A. aspera* to alter its transcriptional responses according to thermal history. As global SST continues to rise due to anthropogenic climate change, corals will be exposed to temperatures that are approaching their thermal bleaching threshold with increasing frequency (Hoegh-Guldberg 1999; Hughes et al. 2003). Therefore, it was important to assess the transcriptional regulation of key stress mitigators, such as antioxidants and HSP genes during multiple stress events. Exposure to sub-lethal temperature followed by a bleaching-level stress led to an acclimatory response in antioxidant and HSP gene transcription. This suggested a lesser degree of stress being experienced by those corals during the 2nd thermal stress than those that experienced repeated bleaching-level stresses, although longer term studies linked to relevant measures, such as survival and growth, are required to confirm this link. This is the first time a coral has been shown to alter its transcriptional responses according to thermal history. Importantly, this work has found a subtle difference between repeated exposure to bleaching level temperature and exposure to a sub-bleaching temperature followed by a bleaching temperature; repeated exposure to bleaching temperature did not confer as great an acclimatory response. These findings must be taken into account when modeling the impacts of

climate change on corals (Donner et al. 2005; Hoegh-Guldberg 1999). Damaging effects of climate change may be mitigated by acclimatory responses of tropical corals and models showing annual bleaching by mid-century must reflect this plasticity.

This research has improved the knowledge of the impacts of global climate change on a key reef-building coral, *Acropora aspera*, and its microbial consort. Specifically, the synergistic effects of elevated temperature and ocean acidification on the *A. aspera* holobiont and the ability of the holobiont to acclimatise to ever-increasing SST were explored. Further research should address the questions:

- Do *Symbiodinium* regulate transcription? How? If not, does that limit the potential for selective adaptation?
- Do *Symbiodinium* react to stress events via post-translational modification of exiting mRNA transcripts in lieu of transcriptional regulation?
- Can chlorophyll fluorescence measurements of Y(NPQ) be used as a proxy for production of ROS in *Symbiodinium*?
- What factors (abiotic or biotic) influence *A. aspera*-associated bacteria community structure?
- Will future oceanic CO_2 increases interact synergistically with elevated SST to alter carbon metabolism of reef-building corals?
- How will high levels of irradiance interact with elevated SST and OA to affect coral holobiont physiology?
- Is there a limit to which corals can acclimatise to increased SST? What is the extent of thermal memory of corals?
- Is thermal tolerance a heritable trait in corals? If so, will selective adaptation keep pace with the rate of climate change?

In conclusion, this thesis has furthered knowledge of the impacts of global climate change on the physiology and transcriptomic responses of the *A. aspera* holobiont. *Symbiodinium* photophysiology was severely impaired at temperatures of 34 °C, coinciding with the bleaching threshold for *A. aspera*, indicating that dysfunction of the photosynthetic apparatus may be the underlying trigger for coral bleaching. The bacterial community, conversely, was unperturbed by elevated temperature and pCO_2, as well as significant declines in *Symbiodinium* population. Additionally, this research found no evidence to support *Vibrio*-mediated coral bleaching, as has been reported in several other studies. Synergistic effects of elevated SST and increased pCO_2 significantly affected transcription of several *A. aspera* genes involved in carbon metabolism, an effect that was generally absent in treatments increasing only one factor or the other. These results show that although *A. aspera* are living in a highly variable environment on reef flats and can tolerate large shifts in temperature and CO_2 levels, they do respond to increases in pCO_2 and temperature superimposed on top of natural fluctuations. These findings have particular interest for OA research, as the low-level, mid-century CO_2 increases employed here elicited differential responses of *A. aspera* genes, indicating that larger increases in pCO_2 may elicit more drastic responses. The evidence presented here for the acclimatory capacity of corals is a promising find, however, the extent to which corals can acclimatise/adapt to ever-increasing temperature is unknown and therefore steps to curtail CO_2 emissions and mitigate climate change must remain paramount.

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Appendices

Methods for protein extraction and immunoblot analysis of coral carbonic anhydrase (CoCA)

Aliquots of crushed coral nubbin were put into 1x cell lysis buffer on lysing matrix C bead tubes (Qbiogene, California, USA). Tubes were vortexed on a MP fastprep ® -24 (MP Biomedicals, NSW, Australia) for 60 seconds x 2 at 4.0 m/s to disrupt cells. Lysate was centrifuged at 12 000 g for 10 minutes to pellet debris and supernatant was aspirated into a fresh 1.5 mL eppendorf tube. An aliquot was taken for quantification of total protein content via the Bradford assay using bovine serum albumin (BSA) as the standard (Bradford 1976).

Three µg of protein from the crude extract was electrophoretically separated on 12% SDS-PAGE and transferred to a PVDF membrane (0.22 µm; Immobilon, Millipore USA). The membrane was then blocked with either 2% ECL block (GE Healthcare) for CoCA3 antibody , or 4% BSA in PBS with 0.1% Tween-20 (PBS-T) for CoCA2 antibody (Sigma-Aldrich Pty Ltd, Australia) for 2 hours before adding the primary antibody to a concentration of 1:20 000 CoCA3 and 1:200 CoCA2. Primary antibody was left to incubate overnight, with agitation at room temperature, and washed with PBS-T the following morning (3x 5 minutes for CoCA3 membranes and 3x 10 minutes for CoCA2 membranes). The secondary antibody, polyclonal swine anti-rabbit immunoglobin conjugated to HRP (Dako, Australia), was diluted with the same respective diluents as the primary antibody to a concentration of 1:20 000 for CoCA3 membranes, and 1:1 000 for CoCA2 membranes, incubated at room temperature for 1 ½ hours, and washed with PBS-T. An enhanced chemiluminescence (ECL) kit (GE Healthcare) was used to visualise protein with a charge-coupled device (CCD) camera

and the signal was quantified using Quantity-One 1-D analysis software (Bio-Rad, Australia). Protein extracted from isolated *Symbiodinium* was also immunoblotted with each antibody; there was no signal detected with the ECL, therefore it is assumed all signal comes from the coral fraction of the protein extract. A standard sample was run on each gel for inter-gel normalisation of signal intensity.

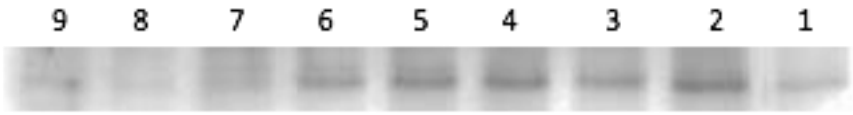

Figure A1. Example of PVDF membrane incubated with carbonic anhydrase isoform 2 antibody, imaged with enhanced chemiluminescence (ECL). Samples are, from right to left: lane 1. standard sample, 2-3. biological replicates from tank A, day 1 heated/elevated CO_2 treatment, 4-6. biological replicates from tank B, day 1 heated/elevated CO_2 treatment, 7-9. biological replicates from tank A, day 14 heated/ambient CO_2 treatment. See Chapter 3 for detailed methodology of treatments.

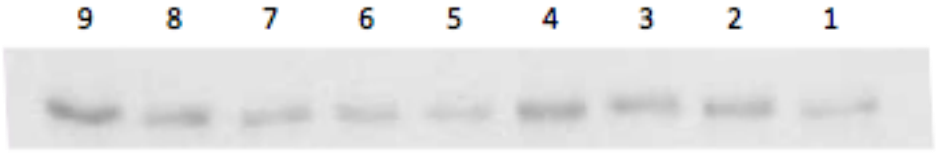

Figure A2. Example of PVDF membrane incubated with carbonic anhydrase isoform 3 antibody, imaged with enhanced chemiluminescence (ECL). Samples are, from right to left: lane 1. standard, 2-4. biological replicates from tank B, day 4 heated/ambient CO_2 treatment, 5-7. biological replicates from tank A, day 4 ambient temperature/elevated CO_2 treatment, 8-9. biological replicates from tank B, day 4 ambient temperature/elevated CO_2 treatment. See Chapter 3 for detailed methodology of treatments.

Table A1. Carbonic anhydrase isoform 3 (CoCA3) protein content expressed as ratio of treatment to control level for each day with standard error (in parentheses). *Acropora aspera* were subjected to: a bleaching event at ambient CO_2, elevated CO_2 (50-90 ppm higher than ambient), or a crossed treatment of both parameters during a 14 day experiment (see Chapter 3 for detailed methodology).

	Day 1	Day 4	Day 14
Heat	0.19 (0.11)	0.55 (0.22)	0.45 (0.17)
CO_2	1.22 (0.46)	0.57 (0.23)	0.56 (0.25)
Heat/CO_2	0.45 (0.20)	0.33 (0.17)	0.24 (0.08)

Table A2. Carbonic anhydrase isoform 2 (CoCA2) protein content expressed as ratio of treatment to control level for each day with standard error (in parentheses). *Acropora aspera* were subjected to: a bleaching event at ambient CO_2, elevated CO_2 (50-90 ppm higher than ambient), or a crossed treatment of both parameters during a 14 day experiment (see Chapter 3 for detailed methodology).

	Day 1	Day 4	Day 14
Heat	1.12 (0.27)	0.93 (0.22)	1.38 (0.38)
CO_2	1.10 (0.21)	0.71 (0.23)	0.95 (0.20)
Heat/CO_2	1.73 (0.28)	1.22 (0.32)	0.81 (0.21)