Characterisation of carbonic anhydrase in the symbiotic dinoflagellate *Symbiodinium*

Thesis submitted by

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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 and staff have made contributions to these manuscripts and I am sincerely thankful to the following people for providing resources, intellectual support, editorial assistance and scientific assistance to me during my candidature:

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Abbreviations

acPCPs	peridinin chlorophyll a-c binding proteins
AGRF	Australian Genome Research Facility
AMTs	ammonium transporters
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
CA	carbonic anhydrase
Cab	chlorophyll a/b- binding protein
$CaCl_2.2H_2O$	calcium chloride dihydrate
Cal	calmodulin
CCM	carbon-concentrating mechanism
CCMP	Centre for Culture of Marine Phytoplankton
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
Chl *a*	chlorophyll a
Chl *c*	chlorophyll c
Ci	inorganic carbon
CNRQ	calibrated normalised relative quantities
CO_2	carbon dioxide
CO_3^{2-}	carbonate
$CoSO_4.7H_2O$	cobalt (II) sulphate heptahydrate
cpDNA	chloroplast deoxyribonucleic acid
CSD	cold shock domain
CSPs	cold shock proteins
Cyc	cyclophilin
Cys	cycteine
ddH_2O	double-distilled water

DEGs	differentially expressed genes
DEPC	diethylpyrocarbonate
DIC	dissolved inorganic carbon
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EST	Expressed Sequence Tag
FBP	fructose-1,6-bisphosphatase
Fd	ferredoxin
FDR	false discovery rate
$FeCl_3.6H_2O$	iron(III) chloride hexahydrate
FNR	ferredoxin-NADP reductase activity
F_v/F_m	dark-adapted yield of chlorophyll fluorescence
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gb	gigabase
GBRMPA	Great Barrier Reef Marine Park Authority
gDNA	genomic deoxyribonucleic acid
GLM	generalised linear model
Gln/Q	glutamine
GO	gene ontology
GOI	gene of interest
H_3BO_3	boric acid
HATS	high-affinity transporter system
HCO_3^-	bicarbonate
His	histidine
HL	high-light
HSPs	heat shock proteins
ICG	internal control gene
iPAM	imaging pulse-amplitude modulation

IPCC	Intergovernmental Panel on Climate Change
ITS	internal transcribed spacer
ITS1	internal transcribed spacer 1
ITS2	internal transcribed spacer 2
KCl	potassium chloride
LATS	low-affinity transporter system
LB	Luria broth
LEF	linear electron flow
LHC	light-harvesting complex
LHCI	light-harvesting complex 1
LHCII	light-harvesting complex 2
LL	low-light
LogFC	log-fold change
LSU	large subunit
MAFFT	multiple alignment using fast fourier transform
Mb	megabase
Mbp	megabase pair
$MgSO_4.7H_2O$	magnesium sulfate heptahydrate
ML	medium-light
$MnSO_4.4H_2O$	manganese(II) sulphate tetrahydrate
mRNA	messenger ribonucleic acid
MTCA	*Methanobacterium thermoautotrophicum*
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
$NaNO_3$	sodium nitrate
NCBI	National Center for Biotechnology Information
NH_4NO_3	ammonium nitrate
Nt	nucleotide

NTA	nitrilotriacetic acid
NTC	non-template control
NPQ	non-photochemical quenching
O_2	oxygen
OA	ocean acidification
OPP	oxidative pentose phosphate
PAM	pulse amplitude modulated
PAR	photosynthetically active radiation
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCP	peridinin-chlorophyll a-protein
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
pCO_2	partial pressure of carbon dioxide
PCP	peridinin-chlorophyll a-protein
PCR	polymerase chain reaction
POR	protochlorophylide reductase
PORB	protochlorophylide reductase B
PGPase	phosphoglycolate phosphatase
ppm	parts per million
PSI	photosystem I
PSII	photosystem II
qPCR	quantitative polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
Rp-S4	S4 ribosomal protein
Rubisco	ribulose-1,5-bisphosphte carboxylase/oxygenase

SAM	S-adenosyl methionine synthetase
SEM	standard error of mean
SL	spliced leader
SST	sea surface temperature
TMM	trimmed mean of M values
tRNA	transfer ribonucleic acid
Y(II)	Maximum quantum yield of photosystem II
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

Abstract

Photosynthesis by *Symbiodinium* plays a central role in the coral-algal symbiosis as the majority (around 95%) of the hosts' metabolic demand is derived from photosynthetically fixed carbon. Photosynthesis in *Symbiodinium* is augmented by the use of a carbon-concentrating mechansism (CCM), of which the enzyme carbonic anhydrase (CA) plays a significant role in the accumulation, transportation and interconversion of inorganic carbon (Ci) forms to ultimately provide CO_2 for the carbon-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Environmental changes associated with climate change, such as ocean acidification and warming, represent key threats to coral reef ecosystems and are the major causes of the decline and deterioration of coral reefs worldwide and have prompted a major research focus on how climate related stressors affect coral-algal symbioses. Given the hosts' dependency on the symbionts ability to perform photosynthesis, how climate change will affect *Symbiodinium* photosynthesis is therefore an area that needs to be investigated.

Current understanding of eukaryotic CCM expression is predominately derived from the green alga *Chlamydomonas reinhardtii*. While it has been demonstrated that *Symbiodinium* possess a CCM, the signals that trigger the expression of the CCM and subsequent genes involved have not been precisely defined in *Symbiodinium.* Therefore, the aims of this research were to use sequence tag data for *Symbiodinium* sp. Clade C3 to characterise the genes encoding CAs involved in the *Symbiodinium* CCM and to determine if *Symbiodinium* CAs were modified by external CO_2 concentrations as in other photosynthetic algae; to determine what the combined effects of elevated CO_2 and temperature were on

Symbiodinium photosynthesis and CA expression; to examine varying light intensities on the regulation of CA; and to investigate possible long-term effects of CO_2 enrichment on the *Symbiodinium* transcriptome.

To achieve these aims a sequencing project was performed. Bioinformatic analyses including analysis of conserved amino acid residues of CA sequences and homology of translated CA protein sequences with other known CA genes from algae and higher plants was performed. Phylogenetic comparison of the translated protein sequences of CAs with CA sequences from a variety of organisms was also undertaken. Quantitative PCR was used for RNA transcript analysis of the identified CAs, Rubisco and phosphoglycolate phosphatase (PGPase) under the various environmental stressors and Illumina RNA-seq was used to investigate long-term CO_2 effects on the *Symbiodinium* transcriptome.

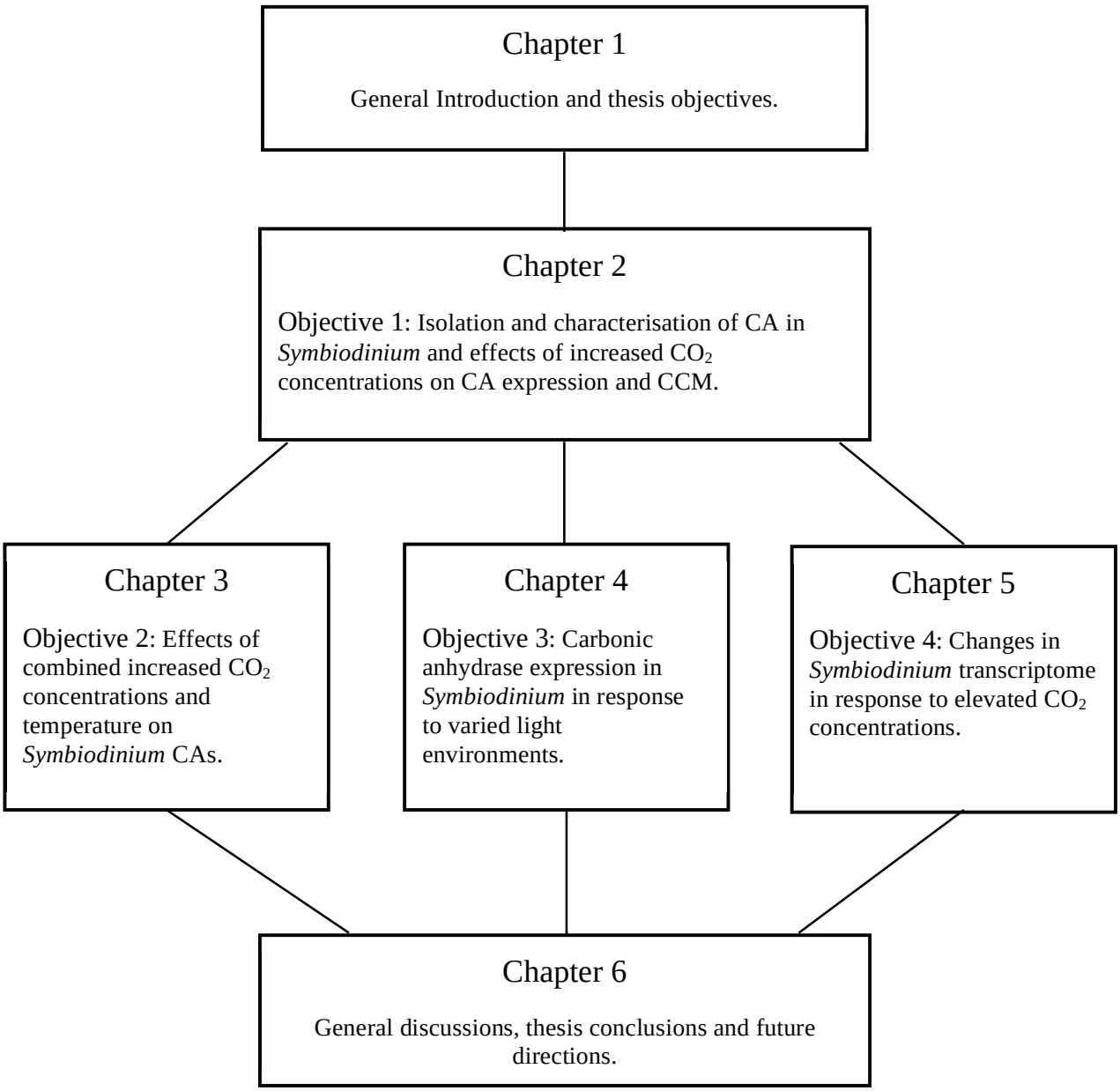
Two distinct β-CAs and one δ-CA protein were identified and characterised in this study. Both β-CAs were encoded as polyproteins and, were presumably localised to the cytosol while the δ-CA protein is likely localised to the plasma membrane. Phylogenetic analysis revealed that the dinoflagellate β-CAs form a novel group within this gene family, illustrating the diversity that exists within the β-CA class. Expression analysis of CAs in *Symbiodinium* sp. clade C1 under elevated CO_2 concentrations revealed that CAs are down-regulated by elevated CO_2 conditions as seen in other algae however, expression patterns differ between different phylotypes of *Symbiodinium*.

Exposure to combined elevated CO_2 and temperature illustrated that thermal stress was the main driver of changes in both transcript levels and physiological parameters of *Symbiodinium* sp. clade F, while CO_2 concentrations relevant to current through to projected future levels of CO_2 had little significant effect overall.

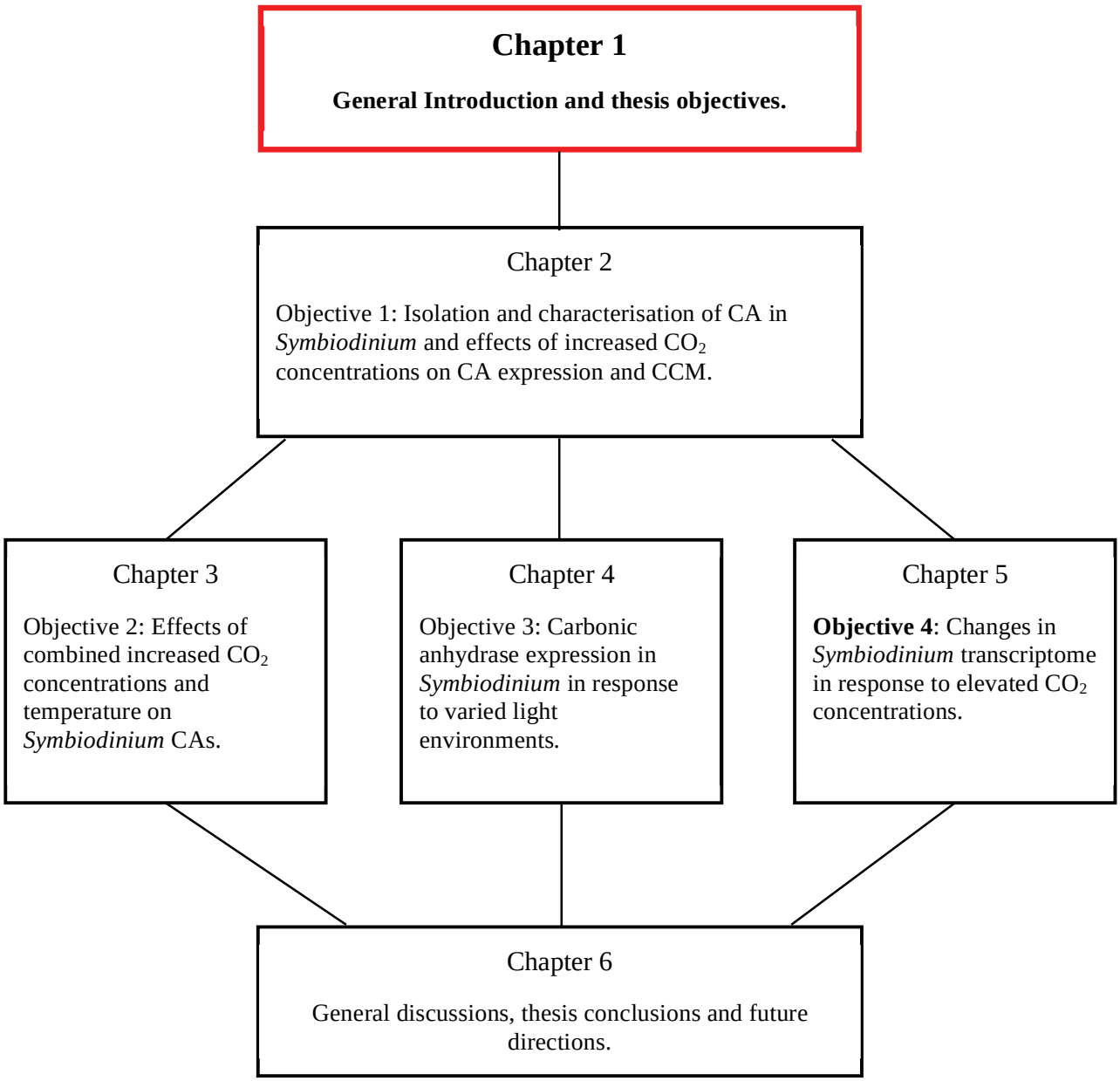
Transcript abundance of *Symbiodinium* CAs under varied light intensities was also examined. High-light environments caused both a decrease in *Symbiodinium* CA transcripts and photosynthetic efficiency. Lastly, the response of *Symbiodinium* clade F to long-term elevated CO_2 concentrations highlighted the transcriptome wide changes with elevated CO_2 significantly enhancing processes such as photosynthesis, energy and ATP metabolism and CA transcript abundance while processes such as transmembrane transport and protein phosphorylation were significantly down-regulated.

The information resulting from this research therefore provides a basis for future investigations into the role and functioning of CAs in *Symbiodinium*; a means to compare the expression of CAs between stress tolerant and susceptible *Symbiodinium* species; and a platform to understand how *Symbiodinium* photosynthesis and therefore the coral-algal symbiosis may be affected by future climate change conditions.

Schematic representation of thesis outline



Schematic representation of thesis outline



Chapter 1. General Introduction

Introduction

Photosynthetic dinoflagellates belonging to the genus *Symbiodinium* are well known for their endosymbiotic role in a variety of marine invertebrates and are largely responsible for the photosynthetic productivity that underlies the vast growth of tropical coral reefs (Falkowski et al. 1984). The effectiveness of photosynthesis in *Symbiodinium* is largely dependant on the availability, assimilation and transportation of inorganic carbon (Ci) from the external seawater medium to the carbon-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Mechanisms such as a carbon-concentrating mechanism (CCM) are employed by dinoflagellates to increase the internal concentration of CO_2 at the active site of Rubisco, of which the enzyme carbonic anhydrase (CA) plays a major role by the interconversion of the Ci forms (Moroney et al. 2001). Despite the evolution of CCMs, changes in the availability or concentration of Ci in the external seawater medium may have the potential to affect the operation of the CCM, CA enzymes and therefore the photosynthetic capabilities of *Symbiodinium*. One of the major concerns is that the rapidity of the current environmental change could exceed the capacity of *Symbiodinium* to acquire and fix carbon under elevated atmospheric CO_2 conditions. Therefore, this thesis addressed some of the potential effects of global climate change conditions, such as increases in atmospheric CO_2, increases in sea surface temperature (SST) and light availability, on the operation of the *Symbiodinium* CCM through the expression of CA enzymes.

Dinoflagellates

Dinoflagellates (phylum Dinoflagellata) are unicellular eukaryotes belonging to the infrakingdom Alveolata that also comprises the phyla Ciliophora and Apicomplexa (Burki et al. 2007). Most dinoflagellates are 10 -100 µm in diameter and are characterised by two flagella that facilitate motility of cells, and a unique cell covering referred to as the theca. Dinoflagellates form a monophyletic group that are closest to apicomplexans and can be found in both the marine and freshwater ecosystems (Hackett et al. 2004). The vast majority of dinoflagellates exist as free-living cells, with autotrophic and heterotrophic lifestyles, but can also form symbiosis with a wide range of marine invertebrates. The dinoflagellates are most notable for their symbioses with reef-building corals, the production of toxic algal blooms, termed red tides, and their spectacular bioluminescence. Apart from diatoms, the dinoflagellates are the most important eukaryotic primary producers in the oceans and as a group, are responsible for a high proportion of net global primary productivity that play a major role in the drawdown of atmospheric CO_2 via the process of photosynthesis (Taylor and Pollingher 1987).

As a group, dinoflagellates are characterised by many unusual and unique genetic characteristics that distinguish them from other eukaryotes. As with other photoautotrophs, the dinoflagellate consists of three distinct genomes, the nuclear genome, the mitochondrial and the plastid genome. Perhaps the most striking genetic feature of dinoflagellates is their large nuclear genome of 1,500 – 245,000 megabase pair (Mbp) (Lin 2011, Wisecaver and Hackett 2011). This is in contrast to other eukaryotic algae whose genome sizes typically range from tens to hundreds of million Mbp (Veldhuis et al. 1997). The large genome size in dinoflagellates is thought to be due to high gene copy numbers, with several genes such as Rubisco (Rowan et al.

(1996), actin (Bachvaroff and Place 2008), peridinin-chlorophyll a-protein (PCP) (Reichman et al. 2003), a variety of chlorophyll *a*-chlorophyll c_2 – peridinin (acpPCP) binding proteins (Boldt et al. 2012), transmembrane transporters such as bicarbonate and ammonium related transporters and CAs (Aranda et al. 2016) all shown to be present in multiple copies.

Structurally, the nuclear genome is unconventional in that the genome lacks histones (the proteins involved in DNA packaging and ordering) and the DNA is not arranged into nucleosomes (the basic method of DNA packaging in eukaryotes), although small, basic, histone-like proteins that may play a role in DNA packaging have been identified (Okamoto and Hastings 2003, Hackett et al. 2005, Lin 2011). Although the dinoflagellate nuclear genome displays high gene copy numbers, approximately 98 – 99 % of the genome is mostly non-coding DNA (Hou and Lin 2009, Lin 2011, Bayer et al. 2012). The genomic DNA also occurs in a liquid crystalline state (Bouligand and Norris 2001), is permanently condensed and is extensively methylated with, 5-hydroxymethyluracil replacing 12 – 70 % of thymine bases, and varying levels of cytosine methylation also being observed (Blank et al. 1988). Spliced leader (SL) *trans*-splicing also appears to be ubiquitous in dinoflagellates (Zhang et al. 2007, Zhang and Lin 2008). In this phenomenon a highly conserved 22 nt SL sequence is transferred to the 5' end of mRNA molecules, which can serve a variety of roles and may also play a role in regulating gene expression (Zhang et al. 2007). The dinoflagellate SL is unique from SL in other *trans*-splicing organisms in that the 'T'-rich Sm-binding motif occurs in the exon instead of the intron region (Zhang et al. 2007, Zhang and Lin 2009). Given the uniqueness of the dinoflagellate SL it can therefore be used as a selective primer to obtain full-length transcripts and analyse the dinoflagellate transcriptome.

In contrast to their large nuclear genomes both the mitochondrial and plastid genomes of dinoflagellates are highly reduced which appears to be the result of extensive horizontal gene transfer to the nuclear genome. Although there is little information on the structure of the mitochondrial genome, such as whether it is circular or linear and or even the number of genomes (Waller and Jackson 2009), to date the mitochondrial genome of dinoflagellates has been shown to carry genes for only three essential components of the electron transport chain (*Cob*, *cox1* and *cox3*) plus two fragmented rRNA genes (Norman and Gray 2001, Chaput et al. 2002, Jackson et al. 2007, Nash et al. 2007, Jackson et al. 2012).

The plastid genomes of dinoflagellates are extremely unique both structurally and evolutionarily. The plastid (commonly referred to as the chloroplast) in all eukaryotic photoautotrophs is thought to have arisen from an initial endosymbiotic event in which a eukaryote engulfed a photosynthetic cyanobacterium (Figure 1.1). Divergence of this primary endosymbiosis gave rise to the green lineage, resulting in chlorophytes including plants and green algae, the red lineage resulting in the red algae or rhodophyta and the glaucophytes (Delwiche 1999, Gould et al. 2008). The uptake of a green alga or red alga by a second eukaryote resulted in secondarily acquired plastids. Independent uptake of green algae lead to the euglenoids and chlorarachniophyte, but it was the uptake of red algae, and the integration of the engulfed red alga plastid with the host cell, that lead to a much greater diversity of organisms (Keeling 2009).Dinoflagellates, haptophytes, heterokontophytes and cryptophytes contain red algal plastids acquired via secondary or tertiary endosymbiotic events. Organisms with a plastid of red algal origin may have acquired the plastid via a single endosymbiosis of a red alga or via several endosymbiotic events in which the organism engulfed another organism that had

already integrated a plastid of red algal origin. As a consequence of these multiple symbiotic events, dinoflagellates have plastids with at least three surrounding membranes and pigmented with chlorophyll *a* (chl *a*) and chlorophyll *c* (chl c) and the unique carotenoid peridinin.

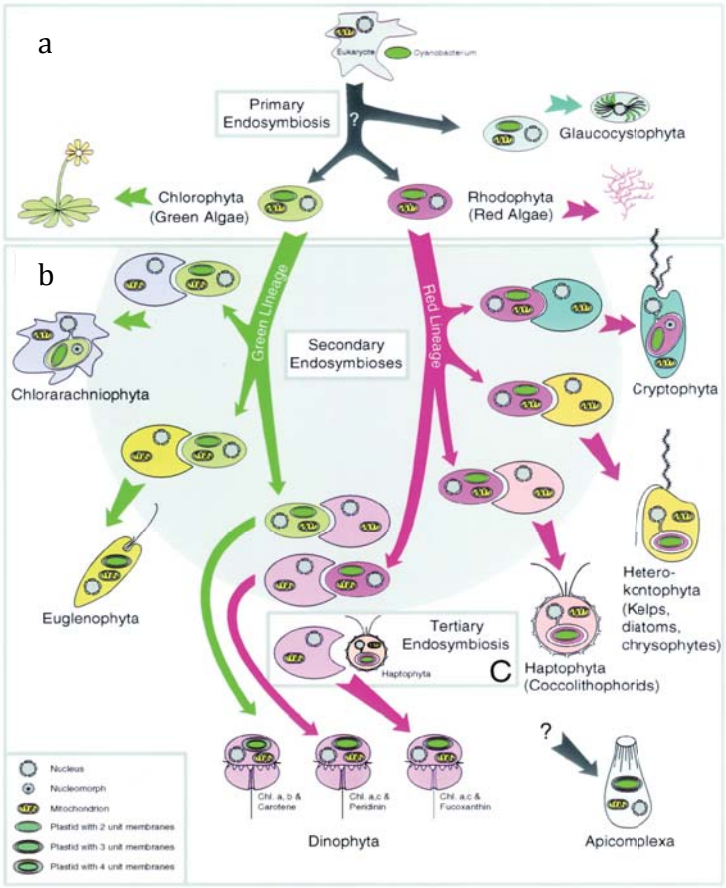


Figure 1.1. Schematic representation of the evolution of plastids through a series of endosymbiotic events. (a) Three algal lineages with primary plastids are illustrated: the red algae (rhodophyta), green algae (chlorophyta) and glaucocystophyta, (b) The occurrence of a secondary endosymbiotic event in which a eukaryote already equipped with plastids engulfed both red and green algae, which resulted in euglenophyta, chlorarachniophyta and dinophyta in the green lineage and cryptohyta, heterokontophyta, haptophyta and dinophyta in the red lineage. The Dinophyta lineage shows plastids of several different types with the plastids from the red lineage

and includes the unique pigment peridinin. In most photosynthetic dinoflagellates, the plastids are derived from the red linage (c) A tertiary endosymbiotic event where species have acquired fucoxanthin-pigmented plastids from the haptophyte lineage (from Delwiche 1999).

Structurally, the plastid genome of dinoflagellates is also unconventional. Chloroplast genomes are typically composed of a single circular genome of approximately 70 – 200 kb which encodes between 42 – 251 chloroplast proteins (Wolfe et al. 1992) with the remaining chloroplast genes found in the nuclear genome. In contrast, the dinoflagellate plastid genome has been fragmented and encodes no more than 20 core genes of the photosynthetic electron transport chain plus two rRNAs and several tRNA genes (Zhang et al. 1999, Howe et al. 2008, Green 2011). The genes are organised on small minicircles (Zhang et al. 1999, Barbrook and Howe 2000, Barbrook et al. 2001, Moore et al. 2003) of between 2-10 kb (Nisbet et al. 2008) with these mincircle genes also displaying a number of anomalous features such as, non-traditional translation start codons, (for example GUA in *Amphidinium* and AUA in *Heterocapsa* (Zhang et al. 1999, Barbrook and Howe 2000, Barbrook et al. 2001), accelerated rate of evolution compared to other chloroplast genes of other organisms (Zhang et al. 2000) and several small deletions (Barbrook and Howe 2000). The fact that dinoflagellates have a highly reduced plastid genome has meant that nearly all plastid proteins are nuclear encoded with a number of these nuclear encoded plastid proteins, such as Rubisco (Rowan et al. 1996), PCP (Norris and Miller 1994, Reichman et al. 2003) and acpPCP binding proteins (Boldt et al. 2012), occurring as polyproteins. These multi-copy proteins are translated as one mRNA, translated as one protein and are directed through the triple membrane chloroplast via

a N-terminal targeting sequence (Patron et al. 2005, Yoon et al. 2005). Once inside the chloroplast the N-terminal peptide is typically cleaved and proteins may be subsequently processed to their final mature state.

Yet another distinguishing feature of the dinoflagellates is that they are currently the only eukaryotic taxon to utilise a Form II Rubisco of proteobacterial origin as the carbon-fixing enzyme (Whitney et al. 1995). As a bi-functional enzyme fixing both CO_2 and O_2, the Form II Rubisco is known to have the lowest carboxylation:oxygenation specificity factor among eukaryotic phytoplankton (Badger et al. 1998, Whitney and Andrews 1998) and is therefore less able to discriminate between CO_2 and O_2 (Jordan and Ogren 1983, Morse 1995, Whitney et al. 1995, Whitney and Andrews 1998). This has given dinoflagellates a disadvantage with respect to carbon acquisition and fixation and has therefore led to the evolution of carbon-concentrating mechanisms (CCMs) that serve to increase the concentration of CO_2 at the active site of Rubisco (Leggat et al. 1999).

Symbiodinium

Symbiodinium (or commonly referred to as zooxanthellae), are primarily known for their role as mutualistic endosymbionts with reef-building corals and a wide range of other marine invertebrates (Muscatine et al. 1981), but are also found free-living. As symbionts, *Symbiodinium* are essential as a large proportion of the hosts' energy requirements (ca. 95%) are met by the translocation of photosynthetically fixed carbon from the symbiont (Muscatine 1990). Initially considered a single pandemic species and classified *Symbiodinium microadriaticum* (Freudenthal 1962), morphological and physiological studies have revealed that *Symbiodinium* is an extremely functionally diverse genus exhibiting much higher sequence diversity than

that observed between genera of non-symbiotic dinoflagellates (LaJeunesse 2001, Coffroth and Santos 2005, LaJeunesse et al. 2010). Analyses of *Symbiodinium* nuclear (rDNA) and chloroplast ribosomal DNA (cpDNA) phylogenies have revealed variation in genotypes and the varying genotypes have been arbitrarily designated using an alpha-numeric system (Rowan and Powers, 1991a, Coffroth and Santos, 2005, Pochon et al., 2006). *Symbiodinium* are currently divided into nine highly divergent clades designated A-I (Figure 1.2), which in turn contain multiple subclades (phylotypes) based on small differences in internal transcribed spacers (ITS), partial large subunit (LSU), and plastid gene sequences (Stat et al. 2006). Each of these clades can also exhibit distinct host taxon, geographic distribution (LaJeunesse 2001, Pawlowski et al. 2001, Santos et al. 2002), and display variations in tolerance to abiotic factors such as temperature and irradiance (Warner et al. 1999, Savage et al. 2002).

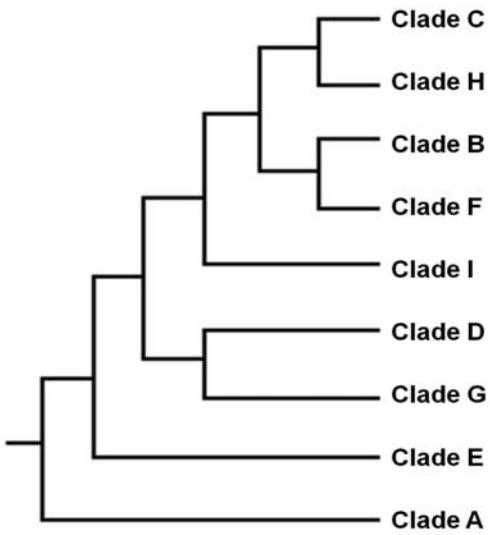


Figure 1.2. Phylogenetic relationship between the nine *Symbiodinium* clades (A-I).

Many coral hosts' can harbour multiple *Symbiodinium* clades and the dominance or distribution of the different clades can vary within the same coral species. Reef-building corals have been shown to associate with symbionts from clades A, B, C, D or F. Clades A and B are more common in Caribbean scleractinians (LaJeunesse 2001). Symbionts from clade D appear to be thermally tolerant with their abundance known to increase in reefs that have suffered episodes of severe bleaching (Baker et al. 2004, Rowan 2004, Berkelmans and Van Oppen 2006). The most prevalent *Symbiodinium* clade, clade C, comprises the greatest number of ecologically distinct species and dominates scleractinian corals in the Pacific and Indian Oceans (Lien et al. 2012).

Cultured verse symbiotic *Symbiodinium*

There are numerous variations between cultured and symbiotic cells in morphology, size, biochemistry, gene expression, motility and, growth rates (Shoenberg and Trench 1980). For example, *Symbiodinium* cells in culture possess a thick prominent pellicle, which in non-cultured cells is reduced (Shoenberg and Trench 1980b). Cell doubling in cultured *Symbiodinium* can range between 2.7 to 33 days (McBride et al 2009) while in symbiotic *Symbiodinium* cell doubling can range between 53-73 days (Muscatine et al 1984). In addition, the immediate environment surrounding the cell is very different in symbiosis and in culture. In symbiosis the algae is surrounded by a highly acidic symbiosome membrane complex that most likely has a high pCO_2 (Barott et al. 2015).

Morphological and physiological descriptions for *Symbiodinium* have primarily arisen due to studies involving cultured *Symbiodinium* (Trench and Blank, 1987, Iglesias-Prieto et al., 1991, Iglesias-Prieto et al., 1992, Iglesias-Prieto et al.,

1993, Iglesias- Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a, Iglesias-Prieto and Trench, 1997b). A variety of *Symbiodinium* clades and sub-clades have been successfully cultured (Santos et al., 2001, Coffroth et al., 2006) however, *Symbiodinium* can demonstrate differential culturability. For example, clade A cells are generally quite easily cultured, but specific sub-clades, such as C3, have been challenging to culture suggesting particular clades or sub-clades may not be able to exist as free- living organisms outside their host (Pochon et al., 2010). It has also been recognised that culture conditions may select one genotype of *Symbiodinium* over another (Rowan 1998).

While the response of cultured *Symbiodinium* to experimental parameters may not reflect that of cells in symbiosis, research with symbiotic *Symbiodinium* poses difficulties, such as the complex interaction between the host organism, *Symbiodinium* and associated bacterial communities, not applicable to cultured *Symbiodinium*. Benefits of working with cultured *Symbiodinium* include: the ability to directly compare specific genotypes, accurately measure growth parameters and record responses to manipulated and controlled environmental factors without host and bacterial interference. Cultured *Symbiodinium* also provide an unlimited supply of cells of a known molecular type from which information can be extracted.

Photosynthesis

Algal photosynthesis accounts for a large proportion of global primary productivity (Falkowski and Raven 2013). Approximately half of the dinoflagellates are photosynthetic (Keeling 2009) and play a major role in the drawdown of atmospheric CO_2 via this process. Photosynthesis in eukaryotes is carried out by plastids (chloroplasts) and is the process of converting light energy and CO_2 into chemical

energy. Numerous physical and chemical reactions are involved resulting in the assimilation of carbon from atmospheric CO_2 for the production of carbohydrates and oxygen from water (Figure 1.3).

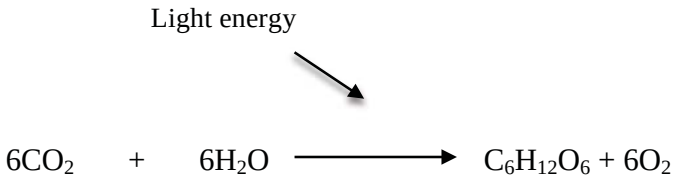


Figure 1.3. Overall reaction of photosynthesis, showing conversion of carbon dioxide to carbohydrates using water and light energy from the sun while producing oxygen.

The photosynthetic process occurs in two stages: the light-dependant reactions that occur in the thylakoid membranes and the light-independent, or dark reactions, that occur in the stroma. The light reactions are based around two photosystems (PS), PSI and PSII, where the primary photochemistry occurs which involves firstly the capture of light via chlorophyll molecules, that are attached to light harvesting complexes (LHCs) (Figure 1.4) (Larkum and Howe 1997), and the subsequent transfer of the absorbed energy to the photosystem reaction centres (Horton et al. 1996). The transfer of electrons resulting from photochemistry by PSII to PSI generates a pH gradient in the thylakoid membrane reducing $NADP^+$ to NADPH (Horton et al. 1996), with the transferred electrons from PSII replaced by the photolysis of water resulting in the release of oxygen. This in turn results in the

generation of a proton gradient across the thylakoid membrane resulting in the generation of adenosine tri-phosphate (ATP) by ATP synthase (Mitchell 1961). The light-independent or dark reaction is where carbon fixation occurs and is where the enzyme Rubisco catalyses the first step of carbon fixation via the capture of atmospheric CO_2 through the Calvin-Benson cycle that uses the NADPH and ATP, generated from the light-reactions, to generate 3-phosphoglyceraldehyde (GAPDH) which is then exported to the cytosol and used to generate organic compounds. The resulting compounds are further reduced to form carbohydrates such as glucose or glycerol that is then used to meet the alga's own energy requirements, if free-living, or is translocated to the host if in symbiosis.

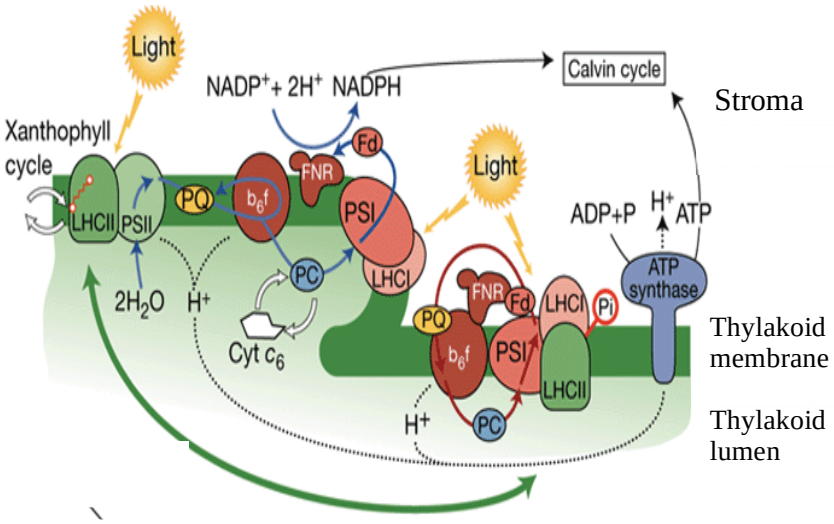


Figure 1.4. Schematic representation of the photosynthetic process illustrating the domains of the thylakoid membrane and the electron-flow throughout the light reactions. The light reactions involve the capture of light via chlorophyll molecules that are attached to the light harvesting complexes (LCHII and LCHI) and the transfer

of energy to the photosystem reaction centres (PSII and PSI). Any excess light is dissipated by the xanthophyll cycle. Energy transfer to PSII and PSI then generates electron transport in the thylakoid membrane. Red arrows illustrate cyclic electron flow which results in generation of H^+ gradient and ATP synthesis. Blue arrows illustrate electron flow resulting in production of ATP and NADPH used for carbon fixation in the dark reactions. Cyt c_6, cytochrome c6; Fd, ferredoxin; FNR, Fd:$NADP^+$ reductase; PQ, plastoquinone (Adapted from Finazzi et al 2003).

Inorganic carbon supply for *Symbiodinium* photosynthesis

Inorganic carbon (Ci) is required for photosynthesis in *Symbiodinium*. However the supply of Ci in the surrounding seawater medium is one of the major factors affecting photosynthesis. Ci in seawater exists in three forms: CO_2, HCO_3^- and CO_3^{2-} and although free-living *Symbiodininium* live in an environment with a relatively large and constant pool of Ci (~2.2 mM), the relative concentrations of the individual carbon species vary depending upon seawater pH. At ambient pH of open ocean seawater (pH 8.2) greater than 99% of the Ci content is in the form of membrane impermeable HCO_3^-, while CO_2, the carbon species required by Rubisco, constitutes less than 1%. The CO_2 availability is further reduced owing to a slow diffusion rate and slow conversion rate between HCO_3^- and CO_2 to support maximal photosynthetic rates (Cook et al. 1986). In addition, the Form II Rubisco of dinoflagellates imposes restrictions to photosynthesis. Although there are a number of structurally distinct forms of Rubisco (Delwiche and Palmer 1996, Horken and Tabita 1999), all are capable of both a carboxylation reaction, which leads to the net fixation of carbon, and an energetically wasteful oxygenase reaction (Jordan and Ogren 1983). As a

consequence of the oxygenation reaction, one molecule of 3-phosphoglycerate and one 2-phosphoglycolate is formed. Phosphoglycolate represents a potent inhibitor to photosynthesis and loss of fixed carbon if not converted to glycolate by the enzyme phosphoglycolate phosphatase (PGPase) via the photorespiratory pathway. The extent to which the two reactions occur depends on both the concentration of CO_2 and O_2 at the active site of Rubisco and affinity for each species. Dinoflagellate Form II Rubisco is known to have the lowest carboxylation:oxygenation specificity factor among eukaryotic phytoplankton (Whitney and Andrews 1998) and is therefore less able to discriminate between CO_2 and O_2.

In contrast to free-living *Symbiodinium*, those that exist in symbiosis have additional limitations in Ci supply. Due to intracellular location within the animal host, the symbiont does not have immediate access to the Ci pool in seawater. Although the symbiont is able to fix host metabolically produced CO_2, when photosynthetic rates exceed respiratory rates of the association *Symbiodinium* must rely on the Ci transport systems of their host to mediate the flux of Ci to support photosynthesis. To avoid such carbon limitations and the inefficiencies of the Form II Rubisco dinoflagellates, including *Symbiodinium*, have evolved CCMs that function to increase the intracellular concentration of CO_2 at the active site of Rubisco, thereby allowing maximal photosynthetic rates.

Carbon-concentrating mechanisms

To avoid the limitations of carbon supply and the kinetic inefficiencies of Rubisco, algae have developed CCMs. The primary function of algal CCMs is to augment photosynthetic productivity by increasing intracellular levels of CO_2 at the active site of Rubisco. The CCM also simultaneously serves to minimise the energetically

wasteful oxygenation reaction and CO_2 loss via leakage (a risk that increases with increasing accumulation of Ci). To date, CCMs have been identified in a wide range of symbiotic and free-living marine photolithotrophs including cyanobacteria, microalgae (Badger and Price 1992) and macrophytes (Johnston 1991) and also in marine invertebrates such as corals (Al-Moghrabi et al. 1996, Furla et al. 2000) and sea anemones (Bénazet-Tambutté et al. 1996). A wide diversity of Ci acquisition mechanisms therefore exist (Badger et al. 2002, Colman et al. 2002) and while components of the CCM can vary among different species, most CCMs primarily involve one or more of the following processes: (1) the active transport of CO_2 into the cell via Ci transporters; (2) the dehydration of HCO_3^- to CO_2 at the cell surface via the enzyme carbonic anhydrase (CA) and/or the subsequent transfer of CO_2 into the cell; (3) the active transport of HCO_3^- directly into the cell and the subsequent conversion to CO_2 via CA and (4) the localisation of Rubisco to a specialised microcompartment (carboxysomes in prokaryotic CCMs and the pyrenoid in most eukaryotic CCMs), for the delivery of CO_2, suggesting that Ci need only be elevated within these specialized microcompartments to sustain photosynthesis.

The most well studied CCMs are those from the freshwater cyanobacteria and the green alga *Chlamydomonas reinhardtii*. *C. reinhardtii* was the first eukaryotic alga in which a CCM was demonstrated (Badger et al. 1980) and has since become a model organism with one of the better understood CCMs in eukaryotes. In *C. reinhardtii*, three components are seen to be essential for CCM activity: (1) Ci transporters at the plasma membrane and chloroplast envelope (Spalding 2007); (2) CAs which facilitate the conversion of HCO_3^- and CO_2 (Moroney et al. 2011) and (3) the localisation of Rubisco to a microcompartment within the chloroplast known as the pyrenoid that serves to minimise CO_2 leakage (Ma et al. 2011, Meyer et al. 2012)

(Figure 1.5). In addition, it has also been demonstrated that the CCM of *C. reinhardtii* is inducible (Badger et al. 1980, Somanchi et al. 1998) with components of the CCM not detected in cells that have been cultured at high CO_2 concentrations (>1% CO_2).

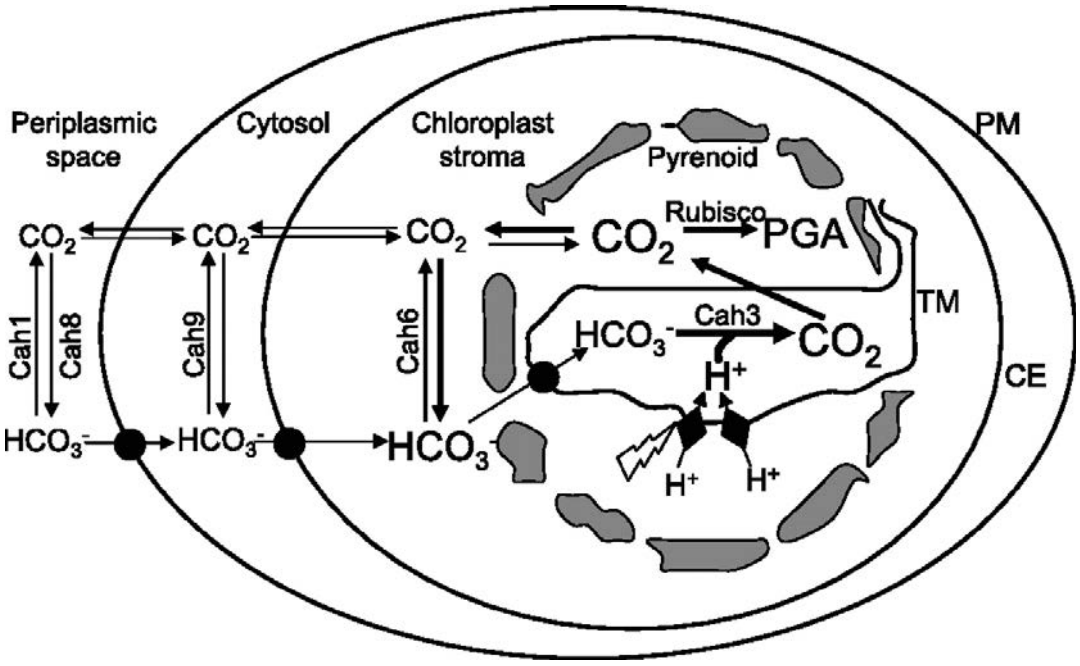


Figure 1.5. Model of the CCM of *Chlamydomonas reinhardtii* depicting an algal cell with a single chloroplast and a single pyrenoid. The relative concentrations of bicarbonate and carbon dioxide are indicated by the size of the lettering. Cah1, Cah3, Cah6, Cah8, and Cah9 are all specific CA isoforms localised to specific cell compartments. Possible bicarbonate transporters are indicated by filled circles and closed diamonds represent the photosynthetic electron transport chain. CE,

chloroplast envelope; PGA, 3-phosphoglyceric acid; PM, plasma membrane; TM, thylakoid membrane (Moroney and Ynalvez 2007).

While most of the present knowledge about algal CCMs has been elucidated from cyanobacteria and *C. reinhardtii*, the CCMs of *Symbiodinium* are far less understood. Evidence for a CCM in *Symbiodinium* was first proposed by Leggat et al (1999) who found intracellular dissolved inorganic carbon DIC was significantly higher than can be accounted for by passive diffusion alone in *Symbiodinium* isolated from the giant clam *Tridacna gigas*. Although the exact mechanisms and identity of the components involved have not been fully established, a number of CCM components have been identified such as, the utilisation of both the HCO_3^- and CO_2 pool of Ci, H^+-ATPase and Na^+/HCO_3^- co-transporters (Yellowlees et al. 1993, Leggat et al. 1999), the presence of both intra-cellular and extra-cellular CA enzymes for the interconversion of HCO_3^- and CO_2, and the localisation of Rubisco to the pyrenoid (Leggat et al. 2002). As with the CCM of *C. reinhardtii*, it is also apparent that the expression of the *Symbiodinium* CCM differs depending on whether *Symbiodinium* is free-living or in symbiosis and if in symbiosis can depend on host type, such as corals verse clams (Al-Moghrabi et al. 1996, Leggat et al. 1999, Bertucci et al. 2009). Recent studies have also shown that CCM expression in *Symbiodinium* can also be phylotype-specific with different clades and sub-clades of *Symbiodinium* showing differential regulation in CCM activity when cultured under differing CO_2 concentrations (Brading et al. 2013).

Carbonic anhydrase

CA is a metalloenzyme that increases the relatively slow dehydration of HCO_3^-,

allowing rapid equilibration of HCO_3^- and CO_2 (Khalifah and Silverman 1991) in a reversible reaction that can be summarized as follows:

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$$

The enzyme is involved in a range of biological functions, including carboxylation reactions, acid-base balance, ion exchange and in a variety of biochemical processes such as respiration and photosynthesis (Tashian 1992). Since its first discovery in bovine red blood cells by Meldrum and Roughton (1933), CA has been found in virtually all animals as well as plants, algae and bacteria (Burnell et al. 1990, Fukuzawa et al. 1992, Funke et al. 1997, Karlsson et al. 1998, Satoh et al. 2001). Six different CA gene families (α, β, γ, δ, ζ and η) have been identified to date (Table 1.1), all of which have evolved independently and show little sequence homology (Hewett-Emmett and Tashian 1996, Roberts et al. 1997, Lane et al. 2005, Soto et al. 2006). It is believed that the α, β and γ- CAs are convergently evolved, whereas the δ, ζ and η CAs appear to be highly divergent variants of the β- or α-class CAs (Hewett-Emmett and Tashian 1996, Liljas and Laurberg 2000, Supuran 2016). Recent studies on the phylogenetic and kinetic properties of the CA of the protozoan parasite *Plasmodium falciparum*, (*Pf*CA) have suggested that these protozoan CAs do not encode a CA belonging to the α-class as once thought, but instead encode for a new, yet undisclosed, genetic family of CAs termed η-CAs (Del Prete et al. 2014). Based on further bioinformatic analyses, these studies have also hypothesised that a possible seventh class of CAs may also exist (proposed to be denominated è-CA), however the confirmation or refutation of this class needs to be further investigated.

	α- CA	β - CA	γ - CA	δ - CA	ζ - CA
Animals	+	+	−	−	−
Eukaryotic algae	+	+	+	+	+
Higher plants	+	+	+	−	−
Archaebacteria	−	+	+	−	−
Eubacteria	+	+	+	−	−

Table 1.1. Known distribution of CA genes in the five CA families. Adapted from Hewett-Emmett and Tashian (1996); Smith et al (1999) and Tiwari et al (2005).

All known CA proteins require a metal co-factor for activity that is coordinated by specific amino acid residues. In most CAs zinc is the metal co-factor, however cadmium and cobalt have also been shown to substitute for zinc and promote catalysis in certain CA proteins.

Alpha CA gene family

The α-CAs are the most extensively studied CA class to date. The α-CAs can be found in vertebrates (Meldrum and Roughton 1933), algae (Fujiwara et al. 1990, Fukuzawa et al. 1990) higher plants (Moroney et al. 2001) and eubacteria (Soltes-Rak et al. 1997, Chirica et al. 2001). Based on sequence similarity the α-CA family can be sub-divided into two broad groups, one containing those CAs from algae and higher plants and the second containing CAs from animals, viruses and bacteria. It is best

studied in humans where it occurs in a number of isozymic forms (numbered I-XV), eleven of which are active and the remaining three devoid of catalytic activity due to a change in the amino acids of the active site (Vullo et al. 2005). Among the most well characterised isozymes is α-CAII, which has one of the most rapid turnover rates known for an enzyme with a k_{cat} of 1.4 x 10^6 s^{-1} (Silverman 1991). Most α-CAs are active as monomers of approximately 30 kDa with the zinc ion required for catalysis coordinated by three conserved histidine residues and a water molecule (Moroney et al. 2001).

Beta CA gene family

Initially the β-class was thought to be expressed only in the chloroplasts of higher plants, however β-CAs have now been identified in a variety of organisms from bacteria and archaea to invertebrates, missing only from vertebrates and most chordates (Eriksson et al. 1996, Hewett-Emmett and Tashian 1996, Syrjänen et al. 2010). The β-class appears to be more diverse in sequence and distribution than any of the other CA classes (Smith and Ferry 2000, Zimmerman and Ferry 2008). The active site of β-CAs is composed of a zinc atom that is coordinated by one histidine and two cysteine residues, unlike that of the three histidine residues conserved in the α, γ- and δ- classes. β-CAs also contain a highly conserved dyad consisting of an aspartate and arginine residue that is crucial for catalysis with mutations of these residues causing reduced catalytic activity (Smith et al. 2002). Instead of functioning as monomers, like most α-CAs or trimers like γ-CAs, β-CAs are usually active as dimers or multimers (Kimber and Pai 2000, Smith et al. 2000, Strop et al. 2001). An exception to this is the β-CA from the red alga *Porphryidium purpureum* in which there are two catalytic CA domains within the one protein (Mitsuhashi and Miyachi

1996) that fold upon themselves to form a pseudo-dimer. This particular β-CA is suggested to be a product of gene duplication with the N- and C-terminus halves displaying 72% similarity.

Based on sequence identity, β-CAs can be further divided into six different subgroups (Figure 1.6). The plant sequences form two groups representing dicotyledonous and monocotyledonous plants (Smith et al. 1999) while the remaining non-plant sequences separate into four distinct subgroups (A – D). The enzymes of eukarya and bacteria form two of these subgroups while the other two subgroups are solely prokaryotic with one group including enzymes from gram-negative bacteria only and the second group consisting of sequences from archaea and gram-positive bacteria.

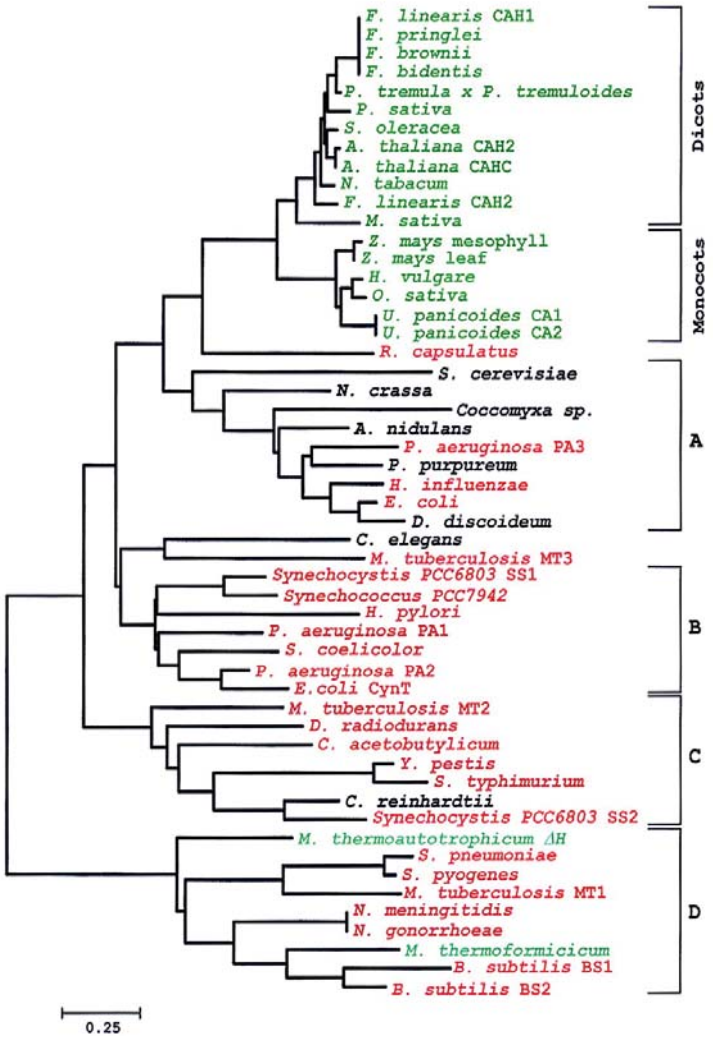


Figure 1.6. Phylogenetic analysis of the beta-CA gene family depicting the different sub-groups (monocots, dicots and groups A – D). The eukarya plant sequences are in green, lower eukaryotes are in black and bacteria in red (Smith et al 1999).

Gamma CA gene family

An active γ-CA was first identified in the archaebacterium *Methanosarcina thermophila* (Alber and Ferry 1994). Since then, γ-CAs have been identified in eubacteria and plants (Newman 1994) but have apparently been secondarily lost in animals and fungi (Elleuche and Poggeler 2009). γ-CAs have been shown to function

as trimers with the zinc ion coordinated by three histidine residues (Alber et al. 1999). Studies have indicated that γ-CAs are part of Complex I of the mitochondrial electron transport chain in plants and algae (Sunderhaus et al. 2006, Klodmann et al. 2010). While the specific role of these γ-CAs is still not fully understood, it has been proposed that the CA domain of complex I is involved in a CO_2 recycling mechanism that mediates the efficient transport of CO_2 from the mitochondria to the chloroplast for photosynthetic fixation (Braun and Zabaleta 2007, Zabaleta et al. 2012). This proposed mechanism firstly involves the conversion of CO_2 to HCO_3^- in the mitochondria, transportation of HCO_3^- from the mitochondria to the chloroplast where it is converted back to CO_2 by chloroplastic CAs and finally fixation of CO_2 by Rubisco (Braun and Zabaleta 2007; Zabaleta et al 2012).

Delta CA gene family

To date, this family is widespread in the marine phytoplankton, being present in haptophytes, dinoflagellates, diatoms, and chlorophytic prasinophytes. It was first characterised in the diatom *Thalassiosira weissflogii* (Roberts et al. 2007), in which the CA sequence showed no significant homology to previously sequenced CAs. The active site has been examined by X-ray absorption spectroscopy and has been found to be similar to that of α- and γ-CAs (Cox et al. 2000) in which the zinc ion is coordinated by three histidine residues and a water molecule. In addition, studies have demonstrated that cobalt can be substituted for zinc with no significant affect on CA activity (Lane and Morel 2000), suggesting an adaptive mechanism employed to maintain activity in waters that are low in zinc concentrations.

Zeta CA gene family

The ζ-gene family currently appears to be limited to marine protists. The geometry of the active site has been shown to resemble the β-CAs (Lane and Morel 2000, Park et al. 2007) with some similarity in the fold structure that has led to the suggestion that this gene family may represent a distantly diverged subtype of β -CAs (Xu et al. 2008). This particular enzyme has also been shown to contain cadmium or cobalt in the active site that can substitute for zinc, when zinc concentrations are low, without a significant loss of the catalytic activity (Lane and Morel 2000; Lane et al 2005; Park et al 2007).

Eta CA gene family

Recent studies on the phylogenetic and kinetic properties of the CA of the protozoan parasite *Plasmodium falciparum*, (*Pf*CA) cloned over ten years ago (Reungprapavut et al. 2004), have suggested that these protozoa do not encode a CA belonging to the α-class as once thought, but instead encode CAs belonging to a new family termed η-CAs (Del Prete et al 2014). To date η-CAs are only represented in the genus *Plasmodium* data on the structure of these proteins are currently unavailable.

CAs in photosynthetic eukaryotes

It is well known that CA in photosynthetic organisms plays a major role in the operation of the CCM via the concentration of Ci for efficient photosynthetic activity (Badger 2003, Badger and Price 2003, Moroney et al. 2011, Wang et al. 2011). Early studies have demonstrated that photosynthesis in *C. reinhardtii* was severely impaired and the affinity for Ci was reduced when the alga was exposed to sulfonamides (strong inhibitors of CA), providing evidence that CAs directly participated in the

CCM of *C. reinhradtii*. Subsequently studies have shown CA to participate in all stages of the algal CCM from Ci entry, prevention of CO_2 leakage from the cell and intra-cellular conversions of the Ci forms. The enzyme can be located externally and/ or internally, where its function differs depending on locality. In general, internal CAs are considered constitutive and are used to facilitate Ci transport within the cell (Merrett et al. 1996) via the rapid interconversion of CO_2 and HCO_3^-. External CAs, can be located external to the plasma membrane where they are used to facilitate the diffusion of Ci to the cell surface by rapid dehydration thereby maintaining the Ci species equilibrium at the site of transport for diffusion into the cell (Bozzo and Colman 2000).

There is wide variation in the number of CA gene families found among eukaryotic microorganisms (Table 1.2). Simultaneously, the same organism may have a different number of genes for the representatives of each individual CA class, which suggests that the specific function of one kind of CA gene may be different (Xiao et al. 2016). But even within the same class, CAs may be represented by a number of isoenzymes that show different molecular features, oligometric arrangement, cellular localisation and responses to different classes of inhibitors (Alterio et al. 2012). Such variation may either be the result of genome or cell size and or the environment in which these organisms are found. Again, much of the current knowledge about photosynthetic eukaryotic CAs has been elucidated from plants and the green alga *C. reinhardtii* (Mitra et al. 2005, Spalding 2007) with little knowledge on the CAs present in the symbiotic dinoflagellate *Symbiodinium*. In *C. reinhardtii* there are at least 12 CAs identified, including three α-CAs, six β-CAs, and three γ-CAs. The α-CAs have been localised to the periplasm (Fujiwara et al. 1990, Rawat and Moroney 1991) and thylakoid lumen (Karlsson et al. 1995), β-CAs to the mitochondria

(Eriksson et al. 1996), chloroplast stroma (Mitra et al. 2004), periplasm (Ynalvez et al. 2008) and the cytoplasm, while all three γ-CAs have been localised to the mitochondria (Cardol et al. 2005). In comparison, while *Symbiodinium* have also been shown to possess both intracellular and extracellular CAs (Yellowlees et al. 1993, Leggat et al. 2000), initial protein studies indicate that the symbiotic dinoflagellate possesses at least three α-CAs, which can be found in the thylakoid, pyrenoid, and membrane bound (Marendy, unpublished data). Analysis of an established expressed sequence tag (EST) library of clade C3 *Symbiodinium* has also identified partial gene sequences with some homology to the β-CA gene family (Leggat et al. 2007). With advances in sequencing technologies, recent genome data from *Symbiodinium minutum* clade B1 (Shoguchi et al. 2013) and *Symbiodinium kawagutii* clade F (Lin et al. 2015) have now begun to reveal the multiplicity of CA isoforms in *Symbiodinium*. For example, analysis of the *S. minutum* genome revealed the presence of 14 genes encoding CAs belonging to the α, β, and ζ-CA families. In *S. kawagutii* 49 CA genes have been identified (Lin et al. 2015) most of which are cytoplasmic with only two δ-CAs predicted to be localised to the plasma membrane and one β-CA localised to the thylakoids (Lin et al. 2015).

Organism	Classification	Number of CAs				
		α	β	γ	δ	ζ
Chlamydomonas reinhardtii	Green algae	3	6	3	ND	ND
Micromonas pusilla CCMP1545		1	1	2	1	ND
Ostreococcus lucimarinus		ND	1	ND	1	ND
Chlorella sp. NC64A		4	3	3	ND	ND
Thalassiosira pseudonana	Diatom	2	ND	4	4	1
Emiliania huxleyi	Coccolith	4	1	2	2	ND
Symbiodinium	Dinoflagellate	3	5	ND	2	1
Heterocapsa triquetra		ND	1	ND	ND	ND
Lingulodinium polyedrum		ND	ND	ND	1	ND

Table 1.2. Known distribution of CA isoforms among selected eukaryotes. Gene families that have not been determined in a particular species are indicated by the letters ND. Adapted from Moroney et al. (2011); Leggat et al. (2007); Shoguchi et al. (2013) and Lin et al. (2015).

Environmental regulation of CCMs and CAs

Although *Symbiodinium* and other algae have evolved mechanisms to increase the

supply of Ci that supports photosynthesis, the capacity of many microalgal cells to express CCMs and CA is regulated by several environmental factors, including but not exclusively the concentration of Ci in the surrounding seawater medium (Beardall et al. 1998). It has become well known that many microalgae are able to adapt various components of their CCM in response to external Ci concentrations (Coleman 1991, Kaplan and Reinhold 1999). In particular, the CCM activity of cells grown under air levels of CO_2 often leads to the induction of high affinity CCMs and an increase expression of external CA while growth under enriched CO_2 concentrations causes a decrease in CCM and external CA activity (Shiraiwa and Miyachi 1985). It has also been demonstrated that in at least some eukaryotic algae there is a complete loss of CCM expression when cells are grown under extreme CO_2 concentrations of > 5% resulting in the reliance on diffusive CO_2 transport from the surrounding media to Rubisco (Giordano et al. 2005, Falkowski and Raven 2007). In several dinoflagellates, CCMs have also been shown to respond to such changes in carbonate chemistry (pH values of 8.0 – 9.1 and CO_2 concentrations of 370 ppm and 5000 ppm CO_2) by lowered photosynthetic affinities for CO_2 (Rost et al. 2006, Ratti et al. 2007) and/or by the transcriptional regulation of CA, with some isoforms of CA up-regulated by decreased CO_2 concentrations while others are down-regulated (Leggat et al. 2000, Moroney et al. 2001). The response of CCMs to external Ci concentrations can also be dependent on a particular carbon species, for example, HCO_3^- concentration has been suggested to be the controlling factor governing CCM expression in cyanobacteria (Mayo et al. 1986) while in some eukaryotes such as *Peridinium gatunense* (Berman-Frank et al. 1995) and *Chlorella ellipsoidea* (Matsuda and Colman 1995), CO_2 appears to be the Ci species that regulates CCM activity.

Aside from external CO_2 concentrations, other environmental factors such as, light intensity and temperature have also been known to control the expression of various algal CCMs and CAs (Coles and Jokiel 1978, Hoegh-Guldberg and Smith 1989, Al-Khatib and Paulsen 1999, Demmig-Adams and Adams 2000). For example, it has been demonstrated in *C. reinhardtii* that light can exert its control on CA transcript abundance through a photosynthesis-dependant process (Dionisio et al. 1989) and that the operation of a complete photosynthetic apparatus is necessary for CA and CCM induction (Spencer et al. 1983). One of the major reasons light has this effect on CCMs is because the operation of the CCM is an active process, driven by the ATP derived from photosynthesis (Beardall and Giordano 2002). Elevated temperature has also been shown to inhibit CO_2 fixation, either through inactivation of the carbon fixing enzyme Rubisco or by the impairment of the CCM, with excess ATP causing a backup in the photosynthetic transport electron transport chain that increases oxidative stress and can damage photosynthetic proteins (Jones et al. 1998, Wooldridge 2009, Buxton et al. 2012).

Significance and thesis objectives

Given that it has been well documented that CO_2 concentrations affect the activity of algal CCMs and CAs (Kaplan et al. 1980, Tsuzuki and Miyachi 1989, Raven 1991, Matsuda et al. 2001), a major concern is that the rapidity of current environmental change could exceed the evolutionary and or acclimatory capacity of photosynthetic algal species (Hughes et al. 2003). While the planet has experienced significant variations in climate in the geological past, the rate at which the present changes are occurring is extraordinary. Currently, the concentration of atmospheric CO_2 is approximately 400 parts per million (ppm) and is expected to rise to reach between

480 and greater than 1000 ppm by the year 2100, depending on the climate model used for future projections (IPCC 2013). As atmospheric CO_2 is in equilibrium with CO_2 in the surface ocean waters, the higher the concentration of atmospheric CO_2, the greater the amount of CO_2 dissolved in the surface ocean. These changes in seawater carbonate chemistry result in lowered pH levels a phenomenon known as ocean acidification (OA). In addition to increasing dissolved CO_2 in surface ocean waters, increases in atmospheric CO_2 are predicted to result in the rise of sea surface temperature (SST) with predicted increases of 1 - 3°C likely to occur on the Great Barrier Reef by the year 2100 (GBRMPA 2011). Elevated SST will in turn cause a decrease in CO_2 solubility and may have important repercussions for photosynthetic marine algae, including *Symbiodinium*, to acquire and fix carbon under elevated global CO_2.

Although there is an extensive body of literature on the repression of CCMs by elevated CO_2 concentrations in cyanobacteria and *C. reinhardtii* (Price and Badger 1989, Sültemeyer et al. 1989, Coleman 1991, Matsuda and Colman 1995, Kaplan and Reinhold 1999, Giordano et al. 2005, Falkowski and Raven 2007), CCMs and CAs in *Symbiodinium* are far less understood. Despite CAs also being identified in *Symbiodinium*, there is still a substantial amount of knowledge lacking on how many CAs *Symbiodinium* possess, what gene families are represented, their roles in the CCM and how they are regulated under varied environmental stressors. Given the importance of algal photosynthesis in the global carbon budget (Field et al. 1998, Falkowski and Raven 2007) and, in terms of symbiotic relationships, the hosts' dependency on *Symbiodinium* for their energy supply (Falkowski et al. 1984), it is necessary to understand how *Symbiodinium* CAs and CCMs respond to environmental changes. The fundamental aim of this thesis is therefore to establish a basis for

understanding what affect global climate change, including increasing atmospheric CO_2 levels, temperature and light availability, will have on the functioning of *Symbiodinium* photosynthesis. This was achieved by several objectives:

Objective 1 - The first objective was to identify additional CA enzymes in *Symbiodinium.* Emphasis was placed on isolating and characterising these CA isoforms and examining the expression of these CAs, along with other genes involved in the photosynthetic pathway, under elevated CO_2 concentrations (Chapter 2) to see if the *Symbiodinium* CAs identified respond in the same manner as other photosynthetic algae with CCMs.

Objective 2 - Given that anthropogenic CO_2 emissions are leading to rising SSTs, the combined effects of increased CO_2 and temperature on the expression of CA genes identified from the first chapter were investigated (Chapter 3) along with other selected genes involved in carbon metabolism. Physiological parameters such as photosynthetic efficiency, chlorophyll content and cell density were also examined to link any physiological changes with gene expression patterns.

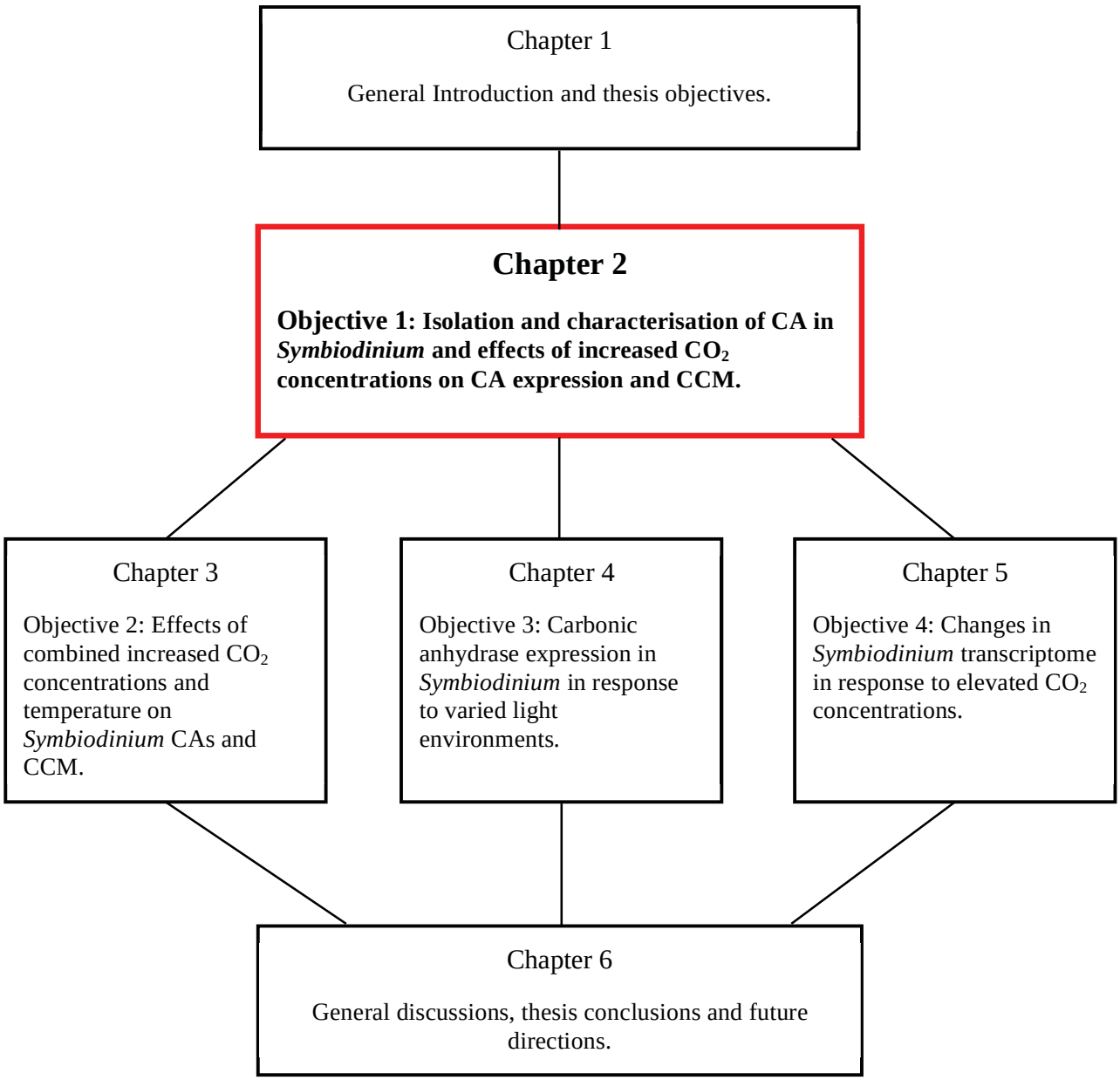
Objective 3 - As the operation of the CCM and CA activity in some algal species has been shown to be light-driven, the effect of varied light irradiances on CA expression and physiological parameters in *Symbiodinium* were also examined (Chapter 4).

Objective 4 - Lastly, to establish a basis for what processes may be affected by long-term exposure to elevated CO_2 levels in *Symbiodinium*, the effects of increased pCO_2 concentrations on cultured *Symbiodinium* cells were investigated at the transcriptome level (Chapter 5).

The findings from these objectives enable a sound basis for global climate change research on this important alga and will be imperative in understanding the

photosynthetic response of *Symbiodinium* CAs that are crucial in carbon acquisition and fixation to a changing global environment.

Schematic representation of thesis outline



Chapter 2: Identification of β- and δ-class carbonic anhydrase in the symbiotic dinoflagellate *Symbiodinium* and response to elevated CO_2 concentrations

Statement of purpose

Carbonic anhydrases (CA) are metalloenymes that play key roles in photosynthesis and the carbon concentrating mechanisms (CCMs) of algae by increasing the concentration of CO_2 around the carbon-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in a low CO_2 environment. CAs belong to various distinct evolutionary classes, which share no significant amino acid similarity and are thought to be the result of convergent evolution. It has become well known that the expression of some algal CCMs and CA is regulated by external CO_2 concentrations, with activity of CA increasing when cells are grown under air levels of CO_2 and decreasing when exposed to 1-5 % partial pressure of CO_2 (pCO_2) (Price and Badger 1989, Sültemeyer et al. 1989, Matsuda and Colman 1995, Giordano et al. 2005, Falkowski and Raven 2007). Chapter 2 reports the identification and phylogenetic characterisation of two novel β-class CA proteins (SymBCA1 and SymBCA2) and a δ-class CA protein (SymDCA1) from the symbiotic dinoflagellate *Symbiodinium*. To elucidate the relationship between the effects of elevated external CO_2 concentrations on *Symbiodinium* CAs, cells of *Symbiodinium* clade C1 were exposed to elevated CO_2 conditions for a short period of nine days where CA transcript abundance and other photosynthetic parameters were measured. In addition, transcript abundance of phosphoglycolate phosphatase (PGPase) and Rubisco were also examined to see if *Symbiodinium* CCM responds to changes in external CO_2 concentrations as seen in other algae.

Introduction

Dinoflagellates are a diverse group of eukaryotic algae that occur in both the marine and freshwater environments, either free-living or in symbiosis with a wide range of marine protists and invertebrates. Compared to other eukaryotes, dinoflagellates have several unique characteristics (Morse 1995, Zhang et al. 2007, Hou and Lin 2009): for example they posses some of the largest known nuclear genomes with many genes present in multiple, divergent copies and often arrayed in tandemly repeated subunits (Hou and Lin 2009). In contrast to their large nuclear genomes, the mitochondrial and plastid genomes are highly reduced which appears to be the result of extensive gene transfer to the nuclear genome. The nuclear genes of dinoflagellates are also unique in that a conserved 22-bp spliced leader (SL) sequence of non coding RNA is added to the 5' end of each monocistronic mRNA through *trans*-splicing, which has been suggested is used to regulate gene expression (Zhang et al. 2007). Dinoflagellates are also the only oxygenic photoautotrophs with a form II Rubisco (Morse et al. 1995) which has the lowest known carboxylation:oxygenation specificity factor among eukaryotic phytoplankton (Whitney and Andrews 1998).

Dinoflagellates belonging to the genus *Symbiodinium* are primarily known for their role as mutualistic endosymbionts and are the dominant symbiont found in corals. When in symbiosis these unicellular algae reside in the gastroderm of the cnidarian host where they translocate photosynthetically fixed products to the host that can account for up to 95% of the host's energy requirements (Falkowski et al. 1993). However, whether in symbiosis or free-living, one of the major factors effecting photosynthesis in *Symbiodinium* is the supply of inorganic carbon (Ci). Ci in seawater exists in three forms: CO_2, HCO_3^- and CO_3^{2-} and although free-living *Symbiodininium* live in an environment with a relatively large and constant pool of Ci

(~2.2 mM), the relative concentrations of the individual carbon species depends on the seawater pH. At ambient pH (pH 8.2) greater than 99% of the Ci content is in the form of non-membrane permeable HCO_3^- while CO_2, the carbon species required by Rubisco, constitutes less than 1%. The CO_2 availability in seawater required to support photosynthesis is further reduced because of the low CO_2 diffusion rate and slow conversion rate between HCO_3^- and CO_2. To avoid carbon limitation in photosynthesis many photosynthetic algae, including *Symbiodinium*, induce a carbon-concentrating mechanism (CCM) (Leggat et al. 2002) that functions to increase the concentration of CO_2 at the active site of Rubisco. Carbonic anhydrases (CA) are metalloenzymes that are ubiquitous in CCMs and rapidly catalyse the interconversion of CO_2 and HCO_3^-. The enzyme can be found extra- or intra-cellularly where its function differs depending on localisation. Generally, external CAs have been shown to prevent CO_2 limitation by ensuring a high rate of CO_2 supply to the cell while internal CAs ensure high rate CO_2 supply to Rubisco by rapid dehydration of the HCO_3^- pool accumulated in the chloroplast (Kaplan and Reinhold 1999).

Currently six different CA gene families have been characterised (α, β, γ, δ, ζ, and η), all of which have evolved independently and show little sequence homology (HewettEmmett and Tashian 1996). The three major families (α, β and γ) are present in higher plants, algae and cyanobacteria. The δ-CAs have been found in diatoms and in the dinoflagellate *Lingulodinium polyedrum*a (Roberts et al. 1997, Lapointe et al. 2008). The ζ-class appears to be confined to diatoms and currently the η-class is restricted to the genus *Plasmodium*. The β-CAs however appear to be more diverse in sequence, distribution and quaternary structure than any of the other classes (Smith et al. 2000, Zimmerman and Ferry 2008). The active site of β-CAs is composed of a zinc atom that is coordinated to one histidine and two cysteine residues and also

contain a highly conserved dyad consisting of an aspartate and arginine residue that is crucial for catalysis (Smith et al. 2002). The β-CA's are usually active as dimers or multimers (Kimber and Pai 2000, Smith et al. 2000, Strop et al. 2001). Based on sequence identity, β-CAs can be further divided into six different subgroups with the plant sequences forming two groups representing dicotyledonous and monocotyledonous plants (Smith and Ferry 1999, Smith et al. 1999) while the remaining non-plant sequences separating into four distinct clades (A – D).

CAs have been reported in many photosynthetic organisms (Fukuzawa et al. 1992, Funke et al. 1997, Karlsson et al. 1998, Satoh et al. 2001) where the enzymes' primary function in the CCM is to facilitate the acquisition and fixation of Ci under low CO_2 conditions for efficient photosynthesis (Badger 2003). It is also well documented that the expression of the majority of algal CCMs is regulated by external partial pressure of CO_2 (pCO_2) concentrations (Coleman 1991, Kaplan and Reinhold 1999), with activity of CA increasing when cells are grown under air levels of CO_2 and decreasing when exposed to 1-5 % pCO_2 (Price and Badger 1989, Sültemeyer et al. 1989, Matsuda and Colman 1995, Giordano et al. 2005, Falkowski and Raven 2007).

Initial studies on *Symbiodinium* have identified both external and internal CAs (Yellowlees et al. 1993, Leggat et al. 1999), with protein studies identifying at least three α-class CAs that were localised to the thylakoid, pyrenoid and membrane bound (Marendy, unpublished data). However, the full diversity of *Symbiodinium* CAs and their role in the CCM are currently not entirely known. While there are also numerous studies on the response of alga CCMs to external CO_2 concentrations, most of the current understanding has been elucidated primarily from freshwater cyanobacteria (Badger and Price 1992, Badger and Price 2003) and the green alga *Chlamydomonas*

reinhardtii (Moroney and Ynalvez 2007, Spalding 2007, Yamano and Fukuzawa 2009, Wang et al. 2011). To date there is no substantial data on the effects of CO_2 concentrations on CA gene expression in *Symbiodinium*.

Since elevated CO_2 concentrations have been shown to reduce the activity of CCMs and related enzymes such as CAs, it is expected to down-regulate the capacity of the CCM if cells are acclimated to high CO_2 levels. This may have important repercussions for the ability of *Symbiodinium* to acquire Ci under elevated atmospheric CO_2 (Beardall and Raven 2004), which is predicted to increase 50 – 200 ppm within 50 years depending on the emissions scenario (IPCC 2013) . Therefore the present study identified and characterised CAs from the β- and δ-class and investigate through real-time quantitative polymerase chain reaction (RT-qPCR) CA transcript abundance of *Symbiodinium* in response to elevated CO_2 concentrations.

Material and Methods

Symbiodinium genotype

Symbiodinium genotype was confirmed through sequencing of the internal transcribed spacer 2 region (ITS2). Genomic DNA was isolated using a DNeasy Plant Mini kit (Qiagen, Valencia USA). Harvested cells re-suspended in 400 µL buffer AP1 with 4 µL RNase A stock solution were transferred to a Lysing Matrix A tube (MP Biomedicals, Australia) and lysed twice for 20 s at 4.0 ms^{-1} on a FastPrep®-24 Instrument (MP Biomedicals, Australia). The lysate, including cell debris, was transferred to a 1.5 µL microcentrifuge tube and DNA extracted according to manufacturer's protocol. Elution with 50 µL of buffer AE was performed rather than 100 µL to increase DNA concentration. The ITS 2 region was amplified using the

forward primer ITSintfor2 (5'GAATTGCAGAACTCCGTG-3') (LaJeunesse and Trench 2000) and reverse primer ITS2-Reverse (5'GGGATCCATATGCTTAAGTTCAGCGGGT-3') (Coleman et al. 1994) following the protocol of LaJeunesse (LaJeunesse 2002). Purified reaction product was directly ligated into pGEM-T Vector (Promega, USA) and transformed into NM522 cells (see Appendices for methods on NM522 cell preparation, ligation and transformation protocols) and multiple clones sequenced at the Australian Genome Research Facility (AGRF) using the forward primer M13F (5'GTTTTCCCAGTCACGAC-3') and reverse primer M13R (5'CAGGAAACAGCTATGAC-3').

CA sequencing

Two partial β-CA sequences were obtained from a *Symbiodinium* sp. clade C3 Expressed Sequence Tag (EST) library (Leggat et al. 2007). Specific primers were designed to theses putative β-CA sequences using the software primer select (Lasergene 8) and were tested for compatibility against complimentary DNA (cDNA) isolated from cultured *Symbiodinium* C1. cDNA was prepared as follows: approximately 6-10 x 10^6 cultured cells were harvested for total RNA extraction. Cells were pelleted by centrifugation at 4500 g for 2 minutes and excess media was discarded. The resulting pellet was then re-suspended by adding 450 µl ice cold RLT buffer containing β-mercaptoethanol (RNeasy Plant Mini Kit, Qiagen, Australia) and pipetted into RNA lysing matrix D tubes (MP Biomedicals, Australia) to be lysed on a MP fastprep ® -24 for 40 seconds at 4.0 ms^{-1} (MP Biomedicals, Seven Hills, NSW, Australia). The resulting lysate and cell debri were transferred to a Qiashredder spin column and RNA extracted using RNeasy plant mini kit (Qiagen, Australia) protocol

including any additional centrifuge and elution steps. RNA yield was quantified using a NanoDrop-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, De, USA). cDNA amplification and genomic DNA (gDNA) elimination were performed with QuantiTect Reverse Transcription Kit (Qiagen, USA) in a 20 μL reaction using 500 ng of total RNA as template and a RT primer mix consisting of random primers and oligo-dT. Negative controls prepared without total RNA as template were included for each series of reaction.

DNA amplification was performed by polymerase chain reaction (PCR) with Platinum® Taq DNA polymerase (Invitrogen, Australia) using the following conditions: an initial cycle of 2 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at a gradient range of 50-62°C, 2 min at 72°C with a final holding temperature of 4°C. Resulting products were ligated into pGEM®-T Vector System (Promega, USA) and used to transform NM522 cells (see Appendices for methods). Plasmids were isolated using Macherey – Nagel Nucleospin® Plasmid kit (Scientifix, Australia) and were sequenced at Macrogen Inc. Korea. The resulting nucleotide sequences were trimmed and assembled using Lasergene® v8.0 (DNAStar Inc, Madison, USA). *Symbiodinium* CA sequences were identified based on transcript (blastN) and protein (blastX) similarity searches using NCBI databases and gene specific primers designed to the resulting consensus sequences using DNAStar Lasergene® v8.0 software PrimerSelect (USA).

To obtain the remaining 5' region, nested PCR was performed using internal primers and the 22 nucleotide 5' *trans*-spliced leader (SL) sequence common to all dinoflagellate mRNA (Zhang et al. 2007). All ligations and sequencing were performed as previously described. In addition to the β-CA sequences, a δ-CA sequence was obtained from an illumina transcriptome (Chapter 5) and was used to

design primers for RT-qPCR analysis. CBS prediction server (Nielsen et al. 1997, Emanuelsson et al. 2000) was used to predict cellular locations of the *Symbiodinium* CAs.

Phylogenetic analysis

Multiple sequence alignment of SymBCA1, SymBCA2 and SymDCA1 was constructed using the multiple sequence alignment program MAFFT (Katoh and Standley 2013) and edited using the multiple alignment editor Jalview 2.5.1 (Waterhouse 2009). Each domain of SymBCA1 and SymBCA2 was treated as individual sequences. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura et al. 2011), maximum likelihood and bootstrapped 10 000 times.

Cell culture and experimental conditions

Cultures of *Symbiodinium* C1 (CCMP2466) were obtained from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), Bigelow Laboratory for Ocean Sciences (Maine USA). This particular phylotype was chosen based on previous studies identifying clade C to dominate zooxanthellate corals (Lien et al. 2012), with subclade C1 being the most commonly found species amongst many host species (Wong et al. 2016). Cells were grown in a controlled refrigerated incubator (Thermoline Scientific, Australia) on a 12:12 h light/dark photoperiod cycle at 25 ºC under a photon flux density of 90-100 µE m^{-2} s^{-1} in tissue culture flasks (Sarstedt, NC, USA) containing ASP-8A medium (Blank 1987) (see Appendices Tables 1-3). For experimental purposes, cells were divided equally into a total of three flasks for each of the four experimental treatments: (1) 400 parts per million CO_2

(ppm; control), (2) 550 ppm, CO_2 (3) 750 ppm CO_2 and (4) 10 000 ppm CO_2. The experiment started with cells in log-phase growth to avoid age-dependent response. All treatments were subjected to a constant temperature of 25 ºC. Modified CO_2 levels were achieved by a CO_2 mixing system based on the design of Munday et al (2009) where pure CO_2 was mixed with air and pumped into each incubator. CO_2 was measured using a datalogging CO_2 meter (Vaisala GM70D4A1M0A0B). Experimental conditions were maintained and monitored for 9 days with samples taken on day 0, 3, 6, 9. pH measurements were also taken on each sampling day prior to harvesting of cells.

mRNA isolation and cDNA synthesis

Approximately 6 x 10^6 cells were collected and centrifuged at 5000 g for 2 min, supernatant discarded and snap frozen in lysing matrix D tubes (MP Biomedicals, Australia) containing 400 µl of ice cold Dynabead lysis buffer (Dynabeads ® mRNA DIRECT^{TM} kit, Invitrogen, Norway) for later mRNA extraction. After brief thawing on ice cells were lysed using a MP Fastprep®-24 Instrument (MP Biomedicals, Australia) at 4.5 M s^{-1} for 3 x 45 seconds and resulting lysate transferred to a microcentrifuge tube containing 100 µl of pre-washed Dynabeads oligo $(dT)_{25}$ and allowed to anneal for 5 min on a rotation vortex at approximately 900 rpm. The tube containing the lysate was then placed on a magnetic column for 10 min, supernatant discarded and the resulting pellet washed twice with 600 µl of wash buffer A, returning the tube to the magnetic column for 30 sec after each wash to remove supernatant. The sample and beads were then resuspended in 300 µl of washing buffer B and transferred to a new RNase-free microcentrifuge tube, returned to the magnet for 2 min before repeating the wash step. mRNA was eluted from the beads by

adding 22 µl of ice cold 10 mM Tris-HCl (pH 7.5) and transferred to a 0.6 ml tube and heated for 2 min at 80 °C in a PTC-200 DNA engine, programmable thermal cycler (MJ Research, Inc) then placed on ice to chill. The tube was then placed on the magnetic column for 30 sec and eluted mRNA transferred to a new tube and quantified spectrophotometrically RNA-40 setting on a NanoDrop-1000 (NanoDrop Technologies, Wilmington USA).

Prior to reverse transcription, DNase treatment of all mRNA was performed to eliminate genomic DNA contamination. Approximately 100 ng of mRNA was treated with RQ1 RNase-Free DNase (Promega) in a total volume of 9 µl (see Appendices Table 4). Following incubation of the DNase digestion reaction at 37 ºC for 30 min, 1 µl of RQ1 DNase Stop Solution was added to each digest and incubated further at 65 ºC for 10 min to inactivate the DNase and terminate the reaction.

cDNA was reversed transcribed from DNase treated mRNA using the SuperScript^{TM} III First-Strand Synthesis SuperMix for qPCR (Invitrogen). The 2X RT Reaction Mix contained the following components of oligo (dT)20 (2.5 µM), random hexamers (2.5 ng/µl), 10 nM $MgCl_2$, and dNTPs (1 mM each) (see Appendices Table 5 for reaction volumes). The cDNA synthesis reaction was then incubated at 25 ºC for 10 min followed by a second incubation step at 50 ºC for 30 min before being terminated at 85 ºC for 5 min. The cDNA synthesis reaction was then chilled on ice before 1 µl of *Escherichia coli* RNase H (2U) was added and incubated at 37 ºC for 20 min. The resulting cDNA was then either used immediately for RT-qPCR or stored at -20 ºC for later use.

RNA transcript analysis by quantitative-PCR

Quantitative PCR (qPCR) was used to measure RNA transcription levels of various genes of interest (GOI). Primers were designed for the two β-CA *Symbiodinium* genes along with one δ-CA gene, phosphoglycolate phosphatase (PGPase) and Rubisco using the software primer select (Lasergene 8) (Table 2.1). In addition, candidate *Symbiodinium* normalisation genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Jurca et al 2008), cyclophilin (Cyc) and S4 ribosomal protein (Rp-S4) (Rosic et al. 2011) were considered for use as reference genes to compare relative expression of GOI. Validation for use as reference genes in this experiment was performed across all treatments using GeNorm in the qBase plus software package (Biogazelle) with M values of 0.5 or lower deemed acceptable.

The efficiency of each gene-specific primer was determined by serial dilutions ranging from 1:10 to 1:160. A 1:80 cDNA dilution was optimal for all primer sets. A total volume of 15 µl containing 7.5 µl SYBR® GreenER^{TM} qPCR SuperMix Universal (Invitrogen, USA), 3.5 µL of a 1 µM primer mix containing forward and reverse primers for each GOI, and 4 µL of 1:80 diluted cDNA template were used. Non-template controls (NTC) were also used and included 4 µL Ultra-pure GIBCO water instead of template cDNA. All reactions were pipetted by a CAS-1200 robot (Corbett Life Science, Australia) in triplicate and quantification performed by Rotor-gene™ 6000 (Corbett Life Science, Australia) with the following temperature profile: a 2 minute holding step at 50 °C, denaturation step at 95 °C for 10 minutes, 35 cycles of 95 °C for 15 s and 66 °C for 60 s, followed by a melt curve from 60 °C to 95°C to check reaction specificity. A single distinct peak in the melt curve indicated amplification of a single product.

Calibrated normalised relative quantities (CNRQ), the relative gene expression values for GOIs, were calculated using qBASEplus software package (Biogazelle). All expression values were represented as the CNRQ of GOI in treatment, relative to control level expression for that GOI for each day.

Gene name	Forward Primer (5’ to 3’)	Reverse Primer (5’ to 3’)	Reaction efficiency
SymBCA1	AGGCTGAATTGCTCCGCTCTG	AAGGGGCCATCAGTTGTTGTGC	1.00
SymBCA2	TGAAGGTAGCATGGTTGGCAGT	GCTTGTGCTGCCTAACTGTGTC	1.00
SymDCA1	CCCCGGCACCTCCACGCTATC	GGGCTTCACTTTCGGAGGCAACA	1.14
PGPase	TCACTGACCGATCCCGCATTTG	CCTTTCTTTCTGGGCACCTTGTCC	0.93
Rubisco	AACGAGTGCATTCCTGAAGTGGTGA	GGCAGTGATGTTGGCGGAGAAGA	1.02
CYC*	ATGTGCCAGGGTGGAGACTT	CCTGTGTGCTTCAGGGTGAA	1.04
GAPDH*	CCGGCGCCAAGAAGGTCATCATC	TTGGCCTTGTCGTACTCCGTGTGGTT	1.06
Rp-S4*	CCGCACAAACTGCGTGAGT	CGCTGCATGACGATCATCTT	0.98

Table 2.1. *Symbiodinium* clade C1 primer sequences used for quantitative PCR. Asterisks indicate reference genes. CYC (cyclophilin), GAPDH (glyceraldehyde-3-phosphate dehydrogenase, PGPase (phosphoglycolate phosphatase), Rp-S4 (S4 ribosomal protein).

Imaging – pulse amplitude modulated fluorometry

Imaging – pulse amplitude modulated (iPAM) fluorometry (MAXI Imaging – PAM, Walz, Germany) was used to analyse the photosynthetic efficiency of *Symbiodinium* cells. Three replicate samples from each treatment were used, except on day 6 in the 550 ppm CO_2 treatment where one sample was omitted to due to a failed reading on the PAM instrument. *Symbiodinium* cells (≈1.6 x 10^6) were pelleted for 2 min at 4500 g then resuspended in 300 µl of ASP-8A media, transferred to a 96 well plate and dark adapted for 30 min. Dark-adapted yield and maximal fluorescence were determined using a weak pulse of light, followed by a saturating pulse of 2,700 µmol quanta m^{-2} s^{-1} of photosynthetically active radiation (PAR) for 800 ms. Induction recovery curves were performed by exposing cells to fifteen saturation pulses of photosynthetically active radiation (2800 µmol quanta m^{-2} s^{-1}) over a 5 min period with a pulse – modulated actinic light set to 111 µmol quanta m^{-2} s^{-1}. The induction phase was then followed by a dark recovery period of 14 min consisting of 16 measuring light pulses, which were used to determine photo-kinetic parameters such as effective quantum yield. Data from induction/recovery curves were used to determine photokinetic parameters, such as effective quantum yield (F_v/F_m). F_v/F_m was used as a proxy to determine the effect of increased CO_2 concentrations on photosynthetic efficiency of photosystem II (PSII) of *Symbiodinium*.

Statistical analysis

Data analysis was performed using statistical software package SPSS (IBM) version 19.0. A generalised linear model (GLM) was applied with a standard ANOVA and sequential Bonferroni post-hoc ($\alpha<0.05$) to determine significant differences between

treatments and control. CO_2 and day were set as fixed factors and CO_2 X day as an interaction. Flasks were treated as a random factor.

Results

Identification of SymBCA1 and SymBCA2 β-class *Symbiodinium* CAs

From the partial gene sequences obtained from the *Symbiodinium* clade C3 EST library, two β-CA genes were identified in *Symbiodinium.* These sequences were named SymBCA 1 and 2. SymBCA1 encodes a protein of 744 amino acids with a molecular mass of 78 kDa while SymBCA2 encodes a 483 amino acid product with a molecular weight of 51 kDa. Based on NCBI database searches both translated protein sequences were found to encode polyproteins containing multiple subunits within the one transcript (Figure 2.1a and b). The full-length transcript containing a start codon preceded by a 5' untranslated region was obtained for SymBCA1 and shown to contain three subunits within the one transcript (Figure 2.1). Each sub-unit of SymBCA1 was separated by a spacer region, which contained a conserved LPPS motif (Figure 2.2). Subunits two and three showed the highest similarity at the amino acid level (63%) followed by subunits one and two and sub-units three and one (61% and 59% respectively).

SymBCA2 was found to contain two subunits also separated by a spacer region (Figure 2.1b) however the conserved LPPS motif seen in the spacer regions of SymBCA1 was not identified for SymBCA2 (Figure 2.2). The two domains of SymBCA2 displayed a 52% identity at the protein level. Although no start codon was identified for SymBCA2, a 10 amino acid putative leader sequence was identified which may either be part of a N-terminal signal sequence or the 3' end of a third

domain (Figure 2.2). Based on CBS prediction server (Nielsen et al. 1997, Emanuelsson et al. 2000), both SymBCA1 and SymBCA2 protein sequences did not contain any transit peptide sequence and predicted that both proteins were located in the cytosol.

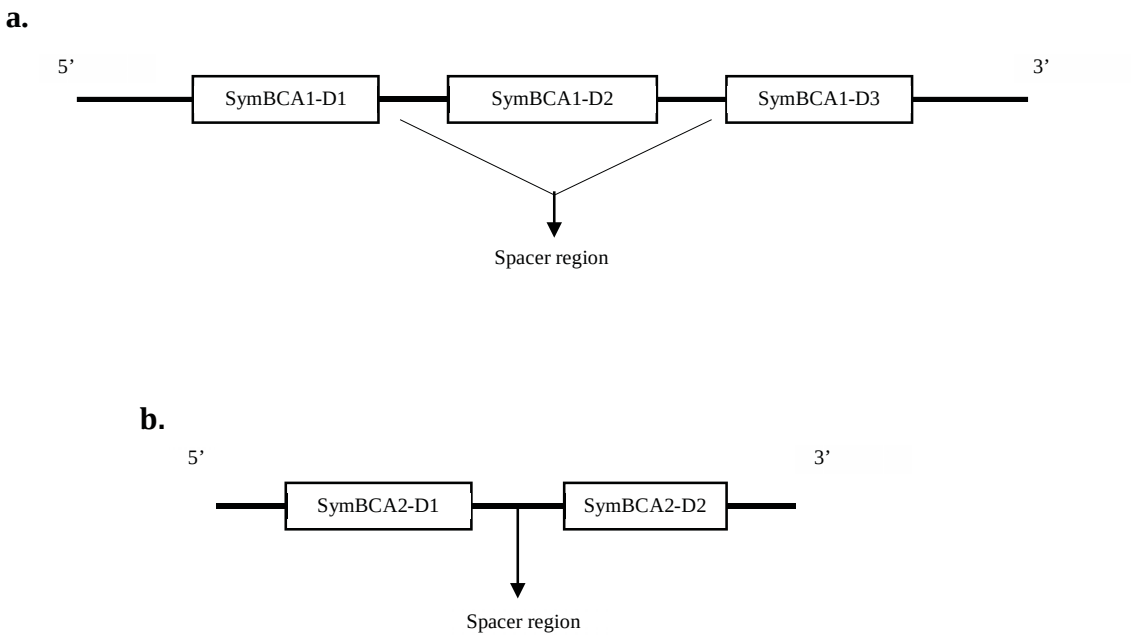


Figure 2.1. Schematic Representation of SymBCA1 and SymBCA2 polyproteins. (a) SymBCA1 protein showing the three sub-units (SymBCA1-D1, SymBCA1-D2 and SymBCA1-D3) separated by spacer regions and, (b) SymBCA2 protein showing dual domains (SymBCA2-D1 and SymBCA2-D2) separated by a spacer region.

Multiple sequence alignment of all sub-units of SymBCA1 and SymBCA2 with other known β-type CAs indicates the conservation of the two cysteine and one histidine residue required for zinc coordination in the β-class (Figure 2.2). Interestingly, although the conserved aspartate and arginine dyad is present in all sub-

units of SymBCA1, this dyad is only found in the C-terminal sub-unit of SymBCA2 with the arginine residue substituted with glutamine (Gln/Q) in the N-terminal domain of SymBCA2. All sub-units of the two *Symbiodinium* β-CA sequences were shown to have the greatest homology to the dinoflagellate *Heterocapsa triquetra*.

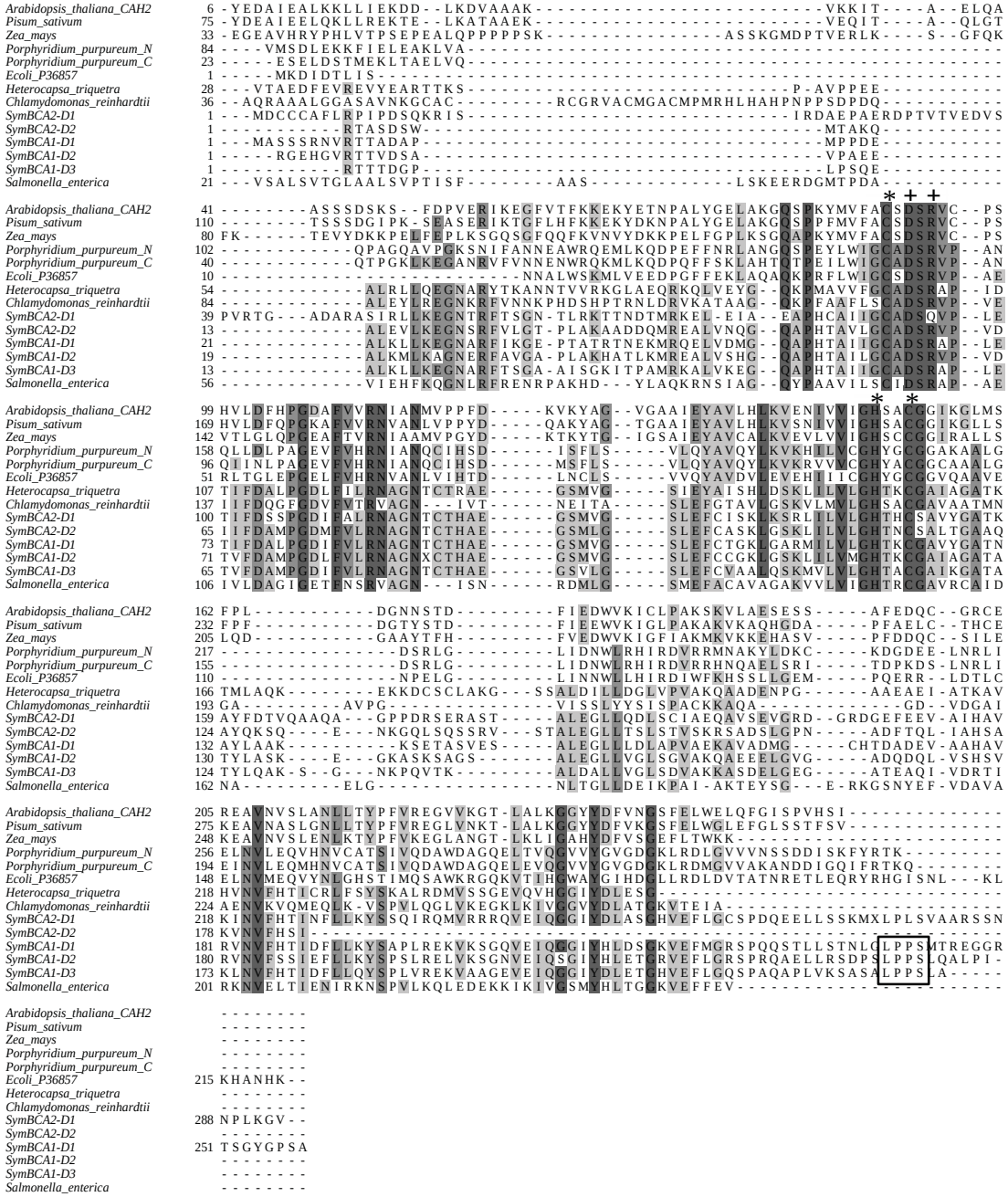


Figure 2.2. Alignment of SymBCA1 and SymBCA2 domains with other known β-CA proteins. Black shading represents >80% conservation, dark grey >60% and light grey >40%. Active site residues are indicated with asterisks and the aspartic and arginine pair conserved in all sequences except SymBCA2-D1 indicated with a cross (+). The conserved LPPS motif in the spacer regions of all domains of SymBCA1 is boxed.

The phylogenetic comparison of all sub-units of SymBCA1 and SymBCA2 protein sequences with other known β-CA proteins indicate the distinctive subgroups found within the β-CA class (Figure 2.3). The plant sequences form two monophyletic groups representing monocotyledons and dicotyledons while all other non-plant sequences separate into four sub-groups (A-D). Distinct from all other sequences are the dinoflagellates that do not conform to any of the existing subgroups and instead form a novel subgroup of β-CAs (Figure 2.3).

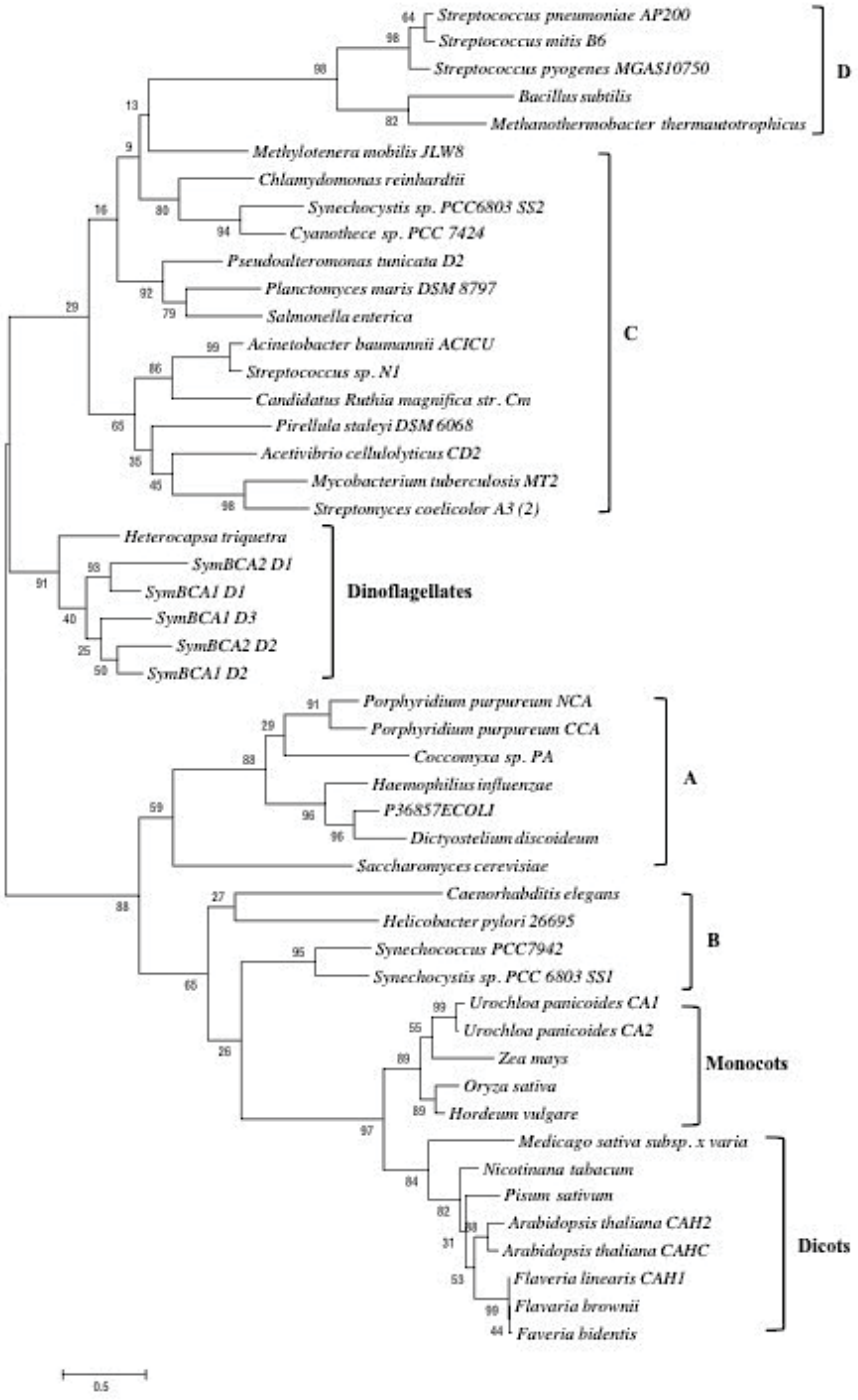


Figure 2.3. Phylogenetic analysis of *Symbiodinium* β-carbonic anhydrase domains with other β-class carbonic anhydrase sequences from plants, bacteria, eukaryotes and representative groups (A-D, monocots, dicots, and the novel dinoflagellate grouping). Phylogenetic and molecular evolutionary analyses were conducted using maximum

likelihood and bootstrapped 10 000 times. Numbers at branches represent the number of times this node was found in 10 000 bootstraps.

Identification of δ-class CA SymDCA1

In addition to the two β-CA proteins, a δ-CA sequence was obtained from an Illumina transcriptome (Chapter 5) and named SymDCA1. SymDCA1 encodes a protein of 341 amino acids with a molecular mass of 38 kDa. The CBS prediction server TargetP1.1 identified an N-terminal signal peptide followed by a cleavage site between amino acid positions 16 and 17 in the deduced amino acid sequence of SymDCA1 (Emanuelsson et al. 2000). Although no predicted trans-membrane domains were found in the N-terminal region of SymDCA1, TargetP1.1 predicts that SymDCA1 is most likely directed to the secretory pathway (Figure 2.4).

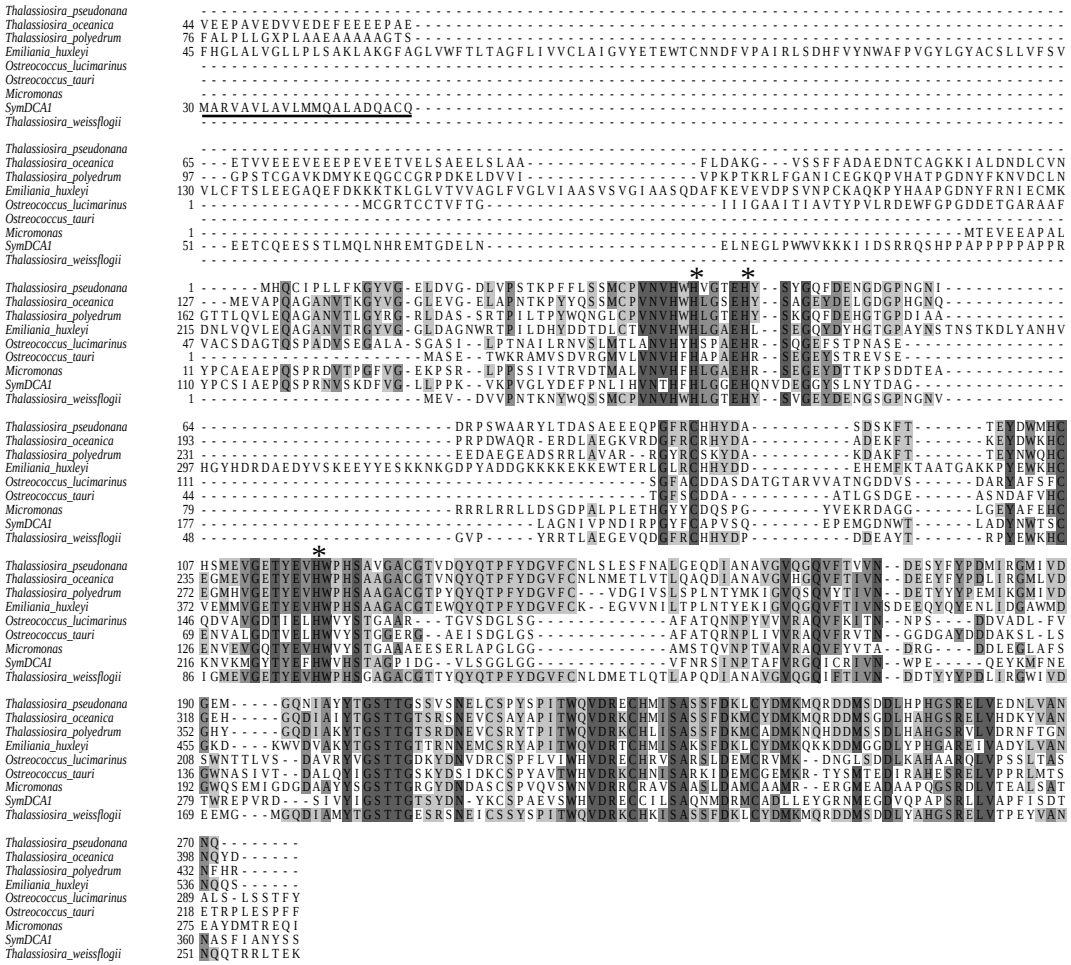


Figure 2.4. Alignment of SymDCA1 with other δ-CA proteins. Black shading represents >80% conservation, dark grey >60% and light grey >40%. Possible active site residues are indicated with asterisks and the predicted N-terminal signal peptide sequence in SymDCA1 is underlined.

Multiple sequence alignment of SymDCA1 indicates the three histidine residues conserved in metal coordination at the active site (Figure 2.4). Phylogenetic analysis of SymDCA1 with other known δ-CA sequences from diatoms, green algae, haptophyta and other dinoflagellates illustrates that SymDCA1 does not group with other dinoflagellate sequences (Figure 2.5).

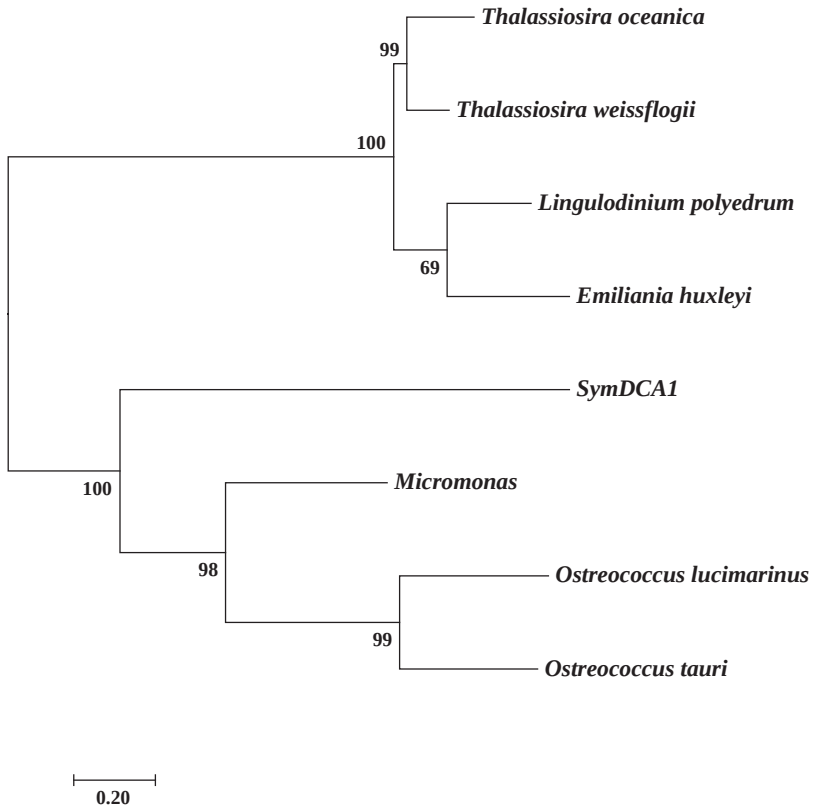


Figure 2.5. Phylogenetic analysis of *Symbiodinium* sp. C1 δ-CA domains with other δ-class carbonic anhydrase sequences. Phylogenetic and molecular evolutionary analyses were conducted using maximum likelihood and bootstrapped 10 000 times. Numbers at branches represent the number of times this node was found in 10 000 bootstraps.

Imaging – pulse amplitude modulated fluorometry

Analysis of dark-adapted quantum yield of PSII, F_v/F_m (a proxy for photosynthetic efficiency) found that there were significant differences in CO_2 ($p<0.001$) and CO_2 x day ($p<0.001$) (Figure 2.6a). F_v/F_m measured on day 3, 6 and 9 was significantly

lower in all elevated CO_2 treatments 550 ppm ($p<0.001$), 750 ppm ($p<0.001$) and 10 000 ppm ($p<0.001$) on all three sample days (Figure 2.6).

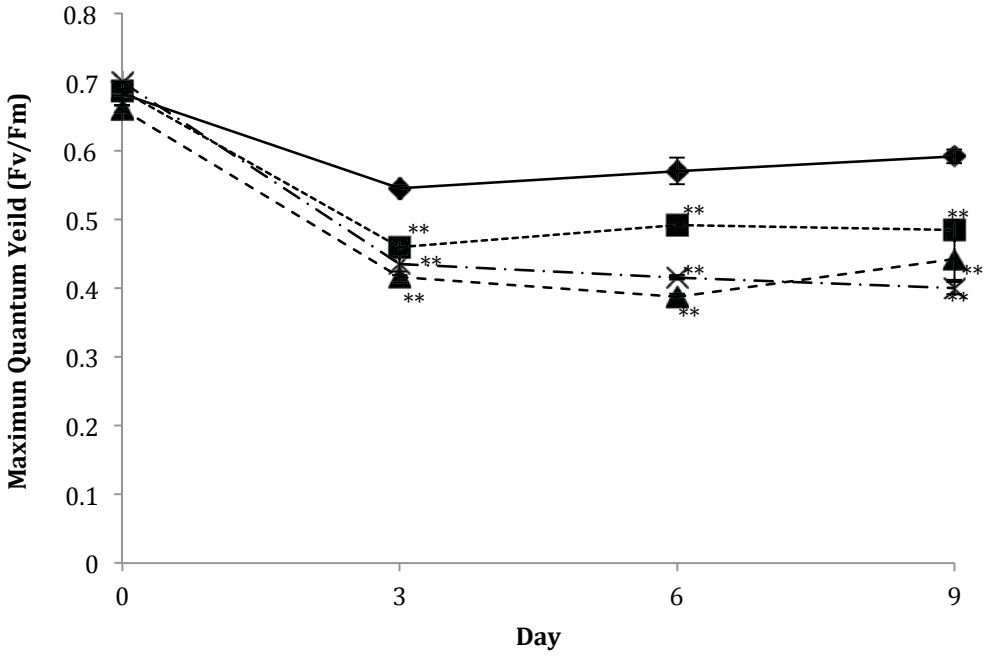


Figure 2.6. Photosynthetic efficiency of *Symbiodinium* cells measured as dark-adapted yield (F_v/F_m) for control (filled diamonds), 550 ppm (filled squares), 750ppm (filled triangles) and 10 00 ppm (×). Error bars represent SEM (some error bars obscured by data points; n = 3, except on day 6 in 550 ppm where n = 2) with ** indicating significant differences between control and treatments ($p<0.001$).

RNA transcript analysis

RNA transcript abundance of SymBCA1, SymBCA2, SymDCA1, PGPase and Rubisco were determined for the duration of the experiment. SymBCA1, SymBCA2 and SymDCA1 transcripts significantly decreased when exposed to elevated CO_2

concentrations (Figure 2.7 a-e). Both SymBCA1 and SymBCA2 transcripts were lowered in the 10 000 ppm CO_2 treatment conditions on day 6 (10-fold and 9-fold respectively) (Figure 2.7a and b). SymBCA2 transcript also decreased on day 9 when exposed to 10 000 ppm CO_2 conditions (10-fold; $p < 0.003$; Figure 2.7b). SymDCA1 transcript was significantly decreased on day 6 in both the 750 ppm CO_2 treatment by 2-fold ($p<0.001$) and 10 000 ppm CO_2 treatment by 20-fold ($p<0.001$; Figure 2.7c).

In addition to the *Symbiodinium* CA genes, transcript abundance of PGPase and Rubisco were also determined. PGPase decreased 12-fold on day 6 ($p<0.001$) and 25-fold on day 9 ($p<0.002$; Figure 2.7d) in the 10 000 ppm CO_2 treatment while transcript abundance of Rubisco increased on day 3 in the 750 ppm CO_2 by 0.5-fold ($p<0.001$) and 10 000 ppm CO_2 treatment by 0.6-fold ($p < 0.001$) and also on day 6 in the 10 000 ppm CO_2 treatment by 1.66 fold ($p<0.012$; figure 2.7e).

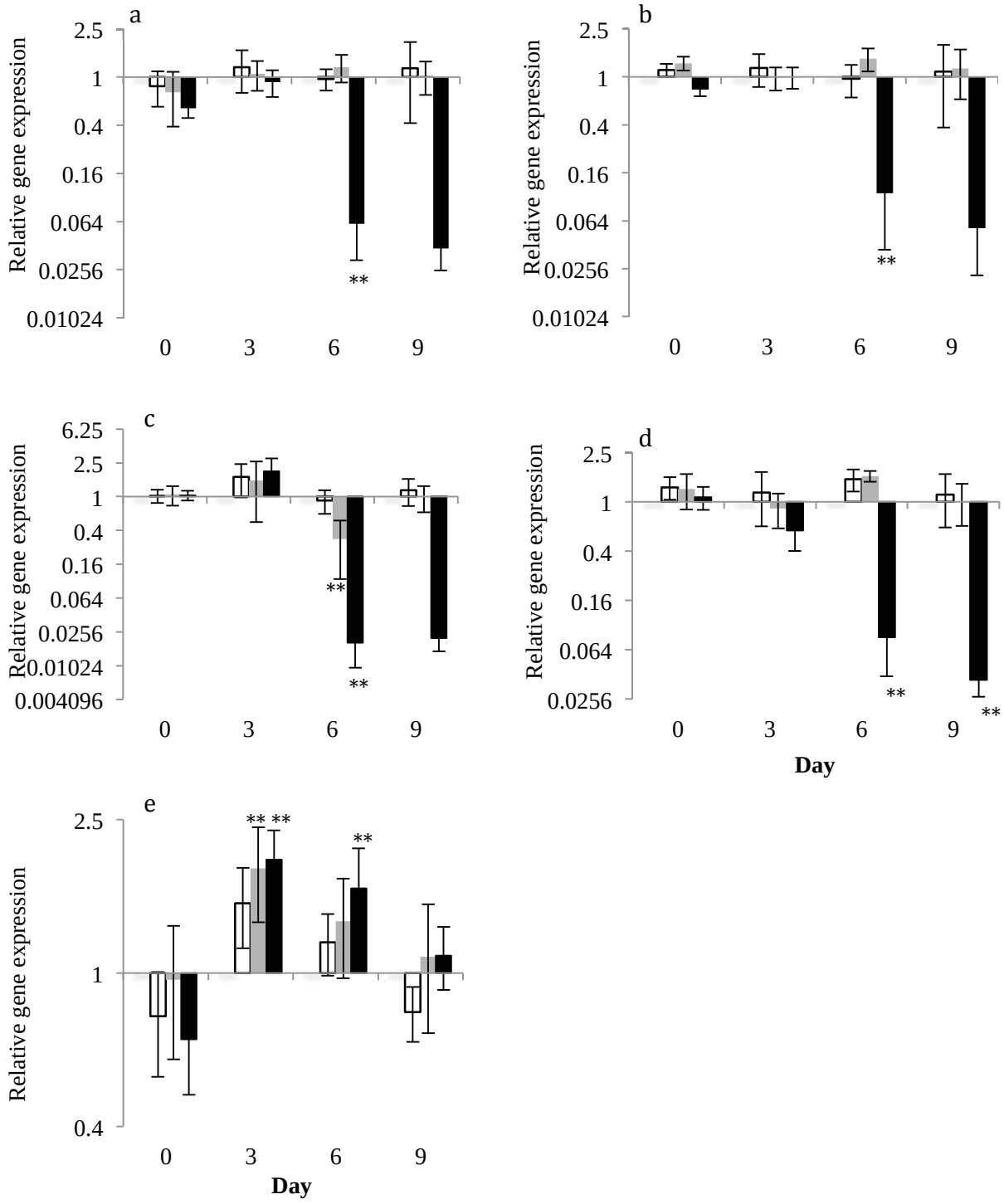


Figure 2.7. Transcript abundance of *Symbiodinium* to elevated CO_2 conditions of 550 ppm CO_2 (white bars), 750 ppm CO_2 (grey bars) and, 10 000 ppm CO_2 (black bars). (a) SymBCA1, (b) SymBCA2, (c) SymDCA, (d) PGPase and, (e) Rubisco. Gene expression values expressed as ratios of treatment to control (400 ppm CO_2). Error

bars are SEM (some error bars obscured by data points; n=3 except for data adapted yield except on day 6 for the 550 ppm CO_2 where n=2). Asterisks indicate significant differences between treatment and control ($p < 0.001$).

Discussion

The present study identified and characterised for the first time, two β-class and a δ-class CA from *Symbiodinium* and examined the response of these CAs to enhanced CO_2 concentrations. The initial survey of *Symbiodinium* β-class CAs, through both EST and PCR analysis (Bobeszko et al, unpublished data) identified that there is a large diversity of sequences found within the one strain, suggesting multiple copies within the genome. The study subsequently characterised two of these β-class CAs along with one δ-class CA and found their transcript abundance is altered under high CO_2 concentrations (10 000 ppm CO_2), while at lower concentrations (550 ppm CO_2 and 750 ppm CO_2) gene expression is unaltered.

Both SymBCA1 and SymBCA2 and the δ-CA SymDCA1 contained the three conserved amino acid residues (Cys-His-Cys for β-CA gene family and His-His-His for the δ-CA gene family) required for zinc co-ordination. While the highly conserved dyad of the aspartate and arginine residues of the β-CA gene family was identified in all sub-units of SymBCA1, the arginine residue in the N-terminal of SymBCA2 was replaced with glutamine (Figure 2.2). This is the first time to our knowledge that this particular mutation has been found in the β-CA class. The role of the arginine residue in the conserved dyad has been suggested to hold the aspartate residue in the active site through two hydrogen bonds, with amino acid substitutions influencing catalytic activity (Smith et al. 2002). For example, previous studies

conducted on the structure and catalytic mechanism of the archaeal β-CA *Methanobacterium thermoautotrophicum* (MTCA) identified that the replacement of the conserved arginine residue with lysine and alanine produced variants with substantial decreases in k_{cat} and k_{cat}/K_m for CO_2 hydration by 2–3 orders of magnitude (Smith et al. 2002). Whether the mutation seen in SymBCA2 also influences catalytic activity is uncertain and would have to be further investigated.

In addition to amino acid substitution, a β-CA lacking entire conserved amino acid residues required for activity has also been identified (Sawaya et al. 2006). The β-CA of the bacteria *Halothiobacillus neapolitanus* (CsoSCA) contains three domains of which its C-terminal domain is completely devoid of all catalytic metal ions (Sawaya et al. 2006). Interestingly, the crystal structure of CsoSCA reveals that it is a representative member of a new subclass of β-CAs that is distinguished by the lack of active site pairing. The function or physiological role of this CA protein lacking activity remains ambiguous, however it has been suggested that these diminished domains may have evolved a new function specific to its particular environment (Sawaya et al. 2006).

While the significance of the amino acid substitution observed in SymBCA2 in the present study is unclear, it is interesting to note that despite the mutation transcript abundance was altered in the same manner as SymBCA1, showing a decrease in abundance of 9-fold on day 6 when exposed to 10 000 ppm CO_2 (Figure 2.7b). This could suggest that not all of the active sites in SymBCA2 are required for the protein to function. Alternatively both sub-units may be functional however the N-terminus sub-unit missing the arginine residue does not operate with the same catalytic efficiency. Further attempt to gain full-length coverage of SymBCA2, future

protein expression studies and localisation studies are needed to assess the functionality of this particular CA in *Symbiodinium*.

Interestingly the phylogenetic analysis of SymBCA1 and SymBCA2 indicated that the dinoflagellates form a novel seventh clade within the β-CA class (Figure 2.3). A separation can also be seen in SymDCA1 where it does not group with other dinoflagellates in the δ-class (Figure 2.5).

Symbiodinium β-CA polyproteins

The polyprotein structure identified in SymBCA1 and SymBCA2 (Figure 2.1) was not surprising and has been identified in several other dinoflagellate genes, for example, Rubisco (Rowan et al. 1996), peridinin-chlorophyll a-protein (PCP) (Norris and Miller 1994, Reichman et al. 2003) and peridinin chlorophyll a-c binding proteins (acPCPs) (Boldt et al. 2012). The polyproteins of Rubisco, PCP and acPCPs are localised to the chloroplast and are directed through the multiple chloroplast membrane via a N-terminal targeting sequence (Patron et al. 2005, Yoon et al. 2005) containing a signal peptide and/or a plastid transit peptide (Nassoury et al. 2003). Once in the chloroplast the N-terminal peptide is typically cleaved off and these proteins may be subsequently cleaved at specific cleavage points to their correct monomeric size. Given the absence of any identifiable characteristic transit peptide sequence in both SymBCA1 and SymBCA2, suggests that these proteins are localised to the cytosol. However, it must be noted again that the full-length sequence was not obtained for SymBCA2. Cytosolic β-CAs have previously been identified in plants and in unicellular green algae such as *Coccomyxa* and *C. reinhardtii* (Moroney et al. 2011). The β-CA in *Coccomyxa* (Co-CA) has been suggested to facilitate the diffusion of Ci from the plasma membrane to the chloroplast envelope (Badger and

Price 1994). The β-CA from the green algae *c. reinhardtii* (CAH9), although not fully characterised, also lacks any such transit or signal peptides and has been predicted to be located in the cytosol (Moroney et al. 2011). While the role of CAH9 in the CCM of *C. reinhardtii* is not yet fully understood, expression studies indicate that CAH9 is expressed at very low levels (Moroney et al. 2011). The exact role of SymBCA1 and SymBCA2 in the cytosol of *Symbiodinium* and their role in the CCM, if any, are also unclear at present. However, these proteins may play some role in maintaining HCO_3^- pools of Ci and may also prevent leakage of CO_2 from the cytosol by trapping CO_2 in the form of HCO_3^-. The conserved LPPS motif observed in the spacer regions of SymBCA1 (Figure 2.2) may still serve as a cleavage site where the protein could be cleaved to its correct monomeric size. An alternative is that that these polyproteins are not cleaved and instead remain as mature proteins encoding multiple domains for catalysis to occur more efficiently, as occurs in the red algae *Porphyridium purpureum* where the two catalytic CA subunits are contained within the one protein and fold upon themselves to form a pseudo-dimer (Mitsuhashi and Miyachi 1996).

Gene duplication is a well-documented event in dinoflagellates (Zhang et al. 2006, Leggat et al. 2011) that is thought to have also contributed to the rise of polyproteins. The 62 kDa β-CA in *P. purpureum* appears to be a product of gene duplication with the N- and C- terminal halves displaying 72% similarity. Surprisingly the identity between the three sub-units of SymBCA1 (63% for sub-units two and three; 61% for sub-units one and two and 51% for subunit one and three) and the two sub-units of SymBCA2 (52%) is relatively small when compared to *P. purpureum*. This may suggest that the sub-units of SymBCA1 and SymBCA2 could

have arisen through a fusion event of separate CA genes or an early duplication and fusion event.

Unlike the polyprotein structure found in SymBCA1 and SymBCA2, SymDCA1 is not encoded as a polyprotein. While the deduced amino acid sequence of SymDCA1 contains a predicted signal sequence consistent with secretion of the protein, the exact location of SymDCA1 is not yet known. Given the location of other δ-CAs in dinoflagellates, such as the δ-CA in *Lingulodinium polyedrum* (LP- δ-CA) (Lapointe et al. 2008), and in the two δ-CAs identified in *Symbiodinium kawagutii*, SymDCA1 may also be associated with the plasma membrane. However, due to difficulties in bioinformatically identifying glycophosphatidylinositol anchors it is not possible to conclusively determine if SymDCA1 is membrane anchored, as is seen in Lp- δ-CA (Lapointe et al. 2008). Future work targeting SymDCA1 to a particular subcellular location may therefore provide clues to the exact role played by this class of CAs in *Symbiodinium*. However given the down regulation of SymDCA1 in response to the 750 ppm CO_2 and 10 000 ppm CO_2 treatments (2-fold and 20-fold) (Figure 2.7d) it is plausible to say that this particular CA does have a role in the operation of the *Symbiodinium* CCM.

***Symbiodinium* CAs and photosynthetic efficiency in response to elevated CO_2**

Regulation of CA in response to CO_2 has been demonstrated in many species which has shown that decreasing CO_2 concentrations increases CA activity correlating with an increase in mRNA levels (Raines et al. 1992). In addition, elevated CO_2 has been known to increase the carboxylation reaction relative to oxygenation reaction of Rubisco leading to increased photosynthetic rates. In the present study, *Symbiodinium* were exposed to CO_2 concentrations ranging from current day levels,

through near future concentrations (550 ppm CO_2 and 750 ppm CO_2) to extreme levels (10 000 ppm CO_2). Perhaps surprisingly, with the exception of SymDCA1 and Rubisco, only at extreme CO_2 concentrations was a change in transcript abundance observed for SymBCA1 and SymBCA2 (Figure 2.7a-e). The lowered transcript abundance of *Symbiodinium* CAs in the 10 000 ppm CO_2 treatment were to be expected as there is less physiological need for these enzymes to accumulate and/or concentrate CO_2 under such conditions be due to passive diffusion of CO_2. Despite CO_2 only decreasing transcription of the β-CA transcripts at concentrations higher than those currently experienced and, well above those concentrations predicted in the near future, these results are consistent with other microalgal studies that have shown growth in 5% CO_2 represses most of the characteristics of CCMs (Price and Badger 1989, Sültemeyer et al. 1989, Matsuda and Colman 1995). Furthermore, the present results are also consistent with previous *Symbiodinium* studies showing the algas' ability to regulate the CCM (Leggat et al. 2002). The apparent absence of any regulation at the lower levels of CO_2 (550 ppm CO_2 and 750 ppm CO_2) for SymBCA1 and SymBCA2 may indicate a secondary role for these CAs or that both SymBCA1 and SymBCA2 are constitutively expressed, as seen in the chloroplastic α-CA (*Cah3*) in *C. reinhardtii* (Karlsson et al 1998).

As the substrate for photosynthesis, elevated CO_2 concentrations would be expected to increase photosynthetic rates either directly by relieving carbon limitation or indirectly by lowering the energy capacity required to concentrate CO_2 against a concentration gradient. However, enhanced photosynthesis under elevated CO_2 concentrations has been shown to be species specific (Rost et al. 2003, Chen and Gao 2004, Collins et al. 2006) and unlike other dinoflagellate studies where photosynthesis and carbon fixation are enhanced under elevated CO_2 (Burkhardt et al. 1999, Rost et

al. 2006), in the present study the photosynthetic efficiency of *Symbiodinium* was significantly depressed in cells exposed to all elevated CO_2 treatments (Figure 2.6). While most organisms are able to utilize both HCO_3^- and CO_2 to support photosynthesis there does seem to be species-specific preferences (Kaplan and Reinhold 1999). This has previously been demonstrated in *Symbiodinium* where cells freshly isolated from the giant clam initially utilised CO_2 for photosynthesis however after two days of isolation there was a shift in utilisation of the HCO_3^- pool in the media to support photosynthesis (Leggat et al. 1999). While an increase in transcript abundance of Rubisco was observed in both the 750 ppm CO_2 and 10 000 ppm CO_2 treatments on day 3 (0.5- and 0.6-fold respectively) and the 10 000 ppm CO_2 treatment on day 6 (0.6-fold; Figure 2.7e) in the present study, it is evident from the maximum quantum yield data (Figure 2.6), that this increase does not relate to a greater capacity to fix CO_2 and hence increase photosynthesis. Therefore this could suggest that: (a) the carboxylase activity of Rubisco is limited by HCO_3^- supply and that this up-regulation of Rubisco may be an acclimation process to elevated CO_2 levels, (b) the accumulation of cellular CO_2, due to elevated CO_2 conditions and passive diffusion of CO_2, has possibly caused an acidification of the cytoplasm and or the chloroplast and in turn has caused possible photoinhibition of photosystem II (PSII) or, (c) since there is a significant down-regulation of both SymBCA1 and SymBCA2 (that are assumed to be localised to the cytosol), CO_2 is lost via leakage as it is unable to be converted to HCO_3^- via CA. Further studies would have to be conducted in order to examine these possibilities.

The carboxylase activity of Rubisco would also be inhibited by the oxygenase activity via the production of phosphoglycolate if it were not converted to glycolate by the enzyme PGPase. Accordingly, Crawley et al (2010) demonstrated that

enhanced activity of PGPase was critical to preventing the accumulation of PGP from photorespiration, which would otherwise inhibit carbon fixation. It is assumed that under ambient CO_2 the CCM still allows the oxygenase reaction to take place (Tytler and Trench 1986). In fact, it has been demonstrated that full induction of the CCM in *Synechococcus* PCC7942 requires O_2-dependant processes such as photorespiration (Woodger et al. 2005). The process of photorespiration can therefore function as a photoprotective mechanism providing an alternative pathway that diverts excess excitation energy (Niyogi 1999, Smith et al. 2005). Transcript abundance of PGPase in the present study significantly decreased on both day 6 (12-fold; $p<0.001$) and day 9 (25-fold; $p<0.002$; Figure 2.7d) in the 10 000 ppm CO_2 treatment. This decrease coincided with a decrease in both CA transcript abundance and photosynthetic efficiency. Such a decline in PGPase transcript abundance could mean a decline in the need for conversion of phosphoglycolate due to elevated affinity for Rubisco carboxylation at high CO_2 concentrations (Drake et al. 1997), suggesting either a reduced capacity to fix CO_2 and or the reduction in the oxygenase activity (via the non removal/accumulation of phosphoglycolate). Previous studies on *C. reinhardtii* and cyanobacteria have shown that there is no change to PGPase expression when cells are transferred from air to high CO_2 (Marek and Spalding 1991, Norman and Colman 1991). However, in a study conducted on *Symbiodinium* in hospite, PGPase expression was reduced by 50% when transferred to a high CO_2 environment with this reduction also coinciding with a decline in photosynthetic productivity (Crawley et al. 2010). The interpretation of results obtained in the present study on cultured *Symbiodinium* compared to those in symbiosis however must be done with caution.

In summary the data presented in this study are the first to have characterised the β- and δ-class CA gene family in *Symbiodinium* and the expression of these CAs

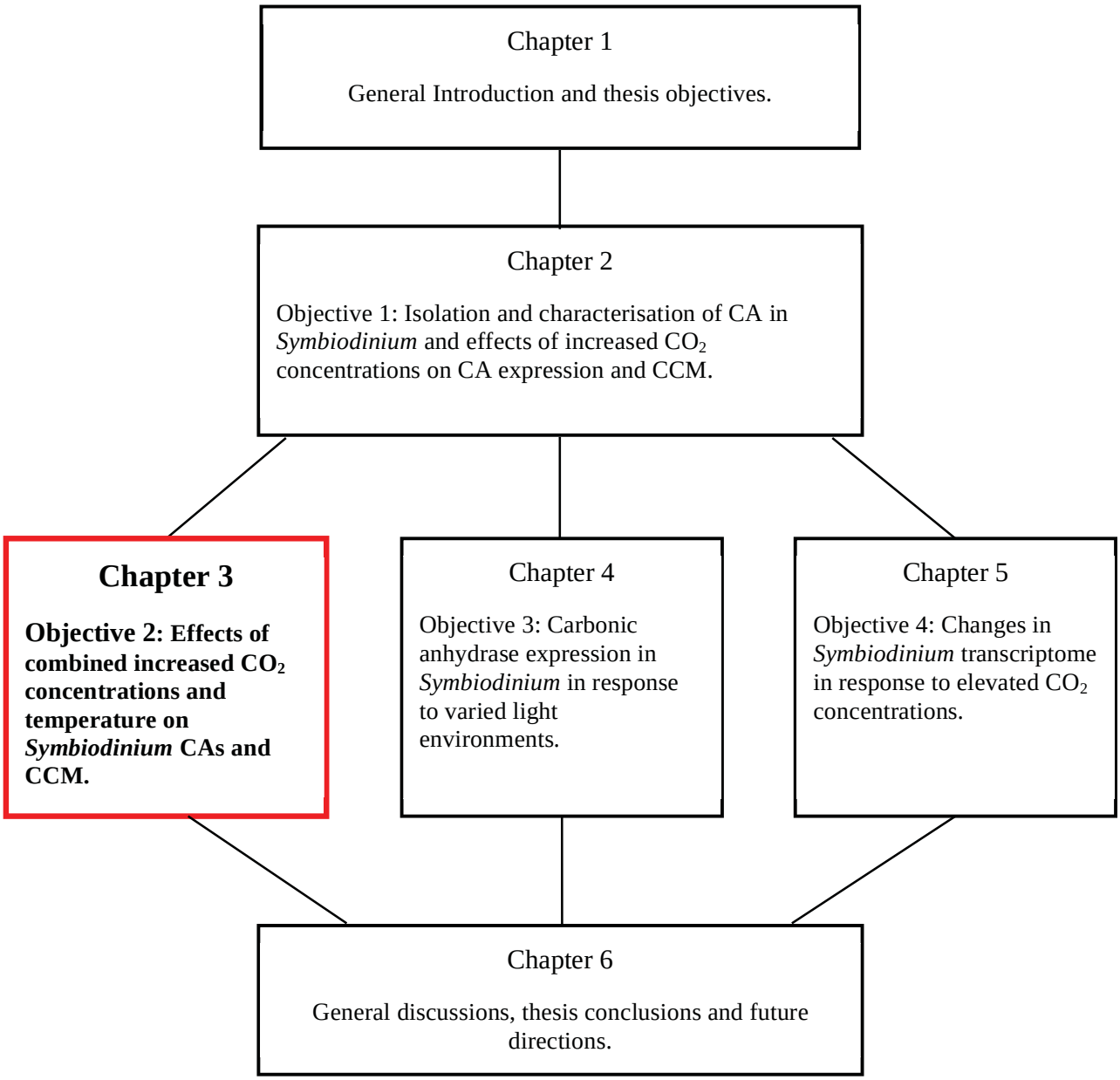
to enriched CO_2 conditions. Although transcriptional control of nuclear genes in dinoflagellates appears to be limited (Lidie et al. 2005, Moustafa et al. 2010, Leggat et al. 2011) the results of the present study indicate that *Symbiodinium* is able to modulate its CA at the transcriptional level. While elevated CO_2 concentrations can be advantageous to some microalgae and decrease the need for a CCM therefore freeing the resources and energy involved in its operation (Beardall and Giordano 2002), elevated CO_2 concentrations may not be advantageous for *Symbiodinium* clade C1 as seen in the decline of photosynthetic efficiency of *Symbiodinium* cells exposed to elevated CO_2 conditions. It is highly likely that other forms of CAs remain undiscovered and that these CAs may play some undiscovered role in the *Symbiodinium* CCM. How *Symbiodinium* controls the CCM is not yet fully understood including what signal is responsible for initiating changes. The structural complexity of the eukaryotic cell also complicates the analyses of the CCM. The results from the present study together with future work would therefore gain a better understanding of acclimation to increased atmospheric CO_2 at the molecular level within this important genus.

Chapter 2 Summary points

- Two novel β-class and one δ-class CA were identified in *Symbiodinium*.
- Both SymBCA1 and SymBCA2 are transcribed as polyproteins. Due to the absence of any characteristic transit peptide, these proteins are predicted to be localised to the cytosol.
- Phylogenetic analysis of SymBCA1 and SymBCA2 indicate dinoflagellate β-CAs are forming a novel class within the β-CA family.

- The cellular location of SymDCA1 is currently unknown. SymDCA1 was found to contain a predicted signal sequence consistent with secretion of the protein and may be associated with the plasma membrane as with other dinoflagellate δ-Class CAs.
- As with other algae, CA transcript abundance in *Symbiodinium* is modified by external CO_2 concentrations.
- Transcript analysis identified significant decline in abundance of SymBCA1, SymBCA2, and SymDCA1 when *Symbiodinium* cells were exposed to elevated CO_2 treatments.
- Photosynthetic efficiency of *Symbiodinium* cells exposed to elevated CO_2 treatments (550 ppm CO_2, 750 ppm CO_2 and 10 000 ppm CO_2) significantly declined.
- Transcript abundance of PGPase also significantly decreased in the highest CO_2 treatment while transcript abundance of Rubisco increased in elevated CO_2 treatments, however could not be linked with greater photosynthetic efficiency.

Schematic representation of thesis outline



Chapter 3: Effects of combined elevated CO_2 and temperature on gene expression patterns in the symbiotic dinoflagellate *Symbiodinium*

Statement of purpose

It is becoming well known that climate change is having profound effects on our oceans (IPCC 2013). In particular, increasing CO_2 emissions are leading to rising sea surface temperature (SST) and decreased ocean pH, a phenomenon known as ocean acidification (OA). These changes in seawater carbonate chemistry may have important repercussions for photosynthetic marine algae to acquire and fix carbon. Given that CA activity and transcript abundance is altered in *Symbiodinium* exposed to elevated partial pressure of CO_2 (pCO_2), Chapter 3 investigates the interactive effects of elevated CO_2 and temperature on the transcript abundance of the *Symbiodinium* CAs identified in Chapter 2, and on ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phosphoglycolate phosphatase (PGPase) involved in the carbon pathway. In addition, physiological parameters, such as photosynthetic efficiency, chlorophyll content and cell densities were also examined in an attempt to link any physiological changes with transcript abundance changes in *Symbiodinium*. This chapter is in preparation for publication.

Introduction

It is becoming well known that increases in atmospheric CO_2 and SST are having profound effects on our oceans (IPCC 2013). Currently, the concentration of atmospheric CO_2 is approximately 400 parts per million (ppm) and is expected to rise to reach between 550 ppm and 1200 ppm by the year 2100, depending on the emission scenarios (IPCC 2013). The chemistry of inorganic carbon (Ci) in seawater dictates that an increase in atmospheric CO_2 will lead to increases in dissolved CO_2. These changes in seawater carbonate chemistry result in lowered pH levels (Wolf-Gladrow et al. 1999), a phenomenon also known as ocean acidification (OA). In addition to increasing dissolved CO_2 in surface ocean waters, increases in atmospheric CO_2 will also result in the rise of SST with predicted increases of 1 - 3°C likely to occur on the Great Barrier Reef by the year 2100 (GBRMPA 2011). Given that photosynthesis is directly dependent on the supply and fixation of CO_2 (Badger et al 1998), such acidification of ocean waters and increases in SST may have important repercussions for *Symbiodinium* to acquire and fix carbon.

The genus *Symbiodinium* is divided into nine phylogenetically distinct clades designated A-I (Pochon and Gates 2010). The successful symbiosis between hermatypic corals and their dinoflagellate endosymbionts of the genus *Symbiodinium*, is largely based on the translocation of photosynthetically fixed carbon form the symbiont (Muscatine 1990). Photosynthesis in *Symbiodinium* is augmented by the use of a CCM. CA plays an important role in most algal CCMs by accelerating the interconversion of HCO_3^- and CO_2 and the facilitation and transportation of CO_2 and HCO_3^- across cell membranes (Badger and Price 1994; Kaplan and Reinhold 1999; Sültemeyer 1998). However, the operation of the CCM and the sensitivity of photosynthesis and associated photosynthetic proteins to anthropogenic stress such as

increased CO_2 levels and elevated seawater temperature is a crucial environmental response that underlies the resilience between host-symbiont relationships.

Prior research has demonstrated that enrichment of CO_2 can significantly influence photosynthesis, growth and CCM function in some taxonomic groups of phytoplankton for example, marine diatoms, cyanobacteria (Hutchins et al. 2007) and coccolithophores (Riebesell et al. 2000, Riebesell 2004, Rost et al. 2008). However, whether phytoplankton benefit from increases in CO_2 or are disadvantaged by such increases vary according to the particular species examined and the dependency on the particular inorganic carbon (Ci) species preferred for uptake by these species. The few studies examining photosynthetic capacity and growth of *Symbiodinium* cells exposed to enriched CO_2 conditions have also produced varied results according to the particular phylotype of *Symbiodinium* examined with certain phylotypes responding positively to enriched CO_2 conditions while other phylotypes remain unchanged (Brading et al. 2011).

In addition to physiological parameters such as photosynthetic efficiency and growth rate, it is well known that the activity and expression of CA is inhibited under increased Ci concentrations and stimulated under limited Ci concentrations (Beardall et al 1998; Giordano et al 2005). Previous research on algal CCMs and CA activity in response to enriched CO_2 conditions has demonstrated that pCO_2 concentrations projected for 2100 down-regulate some algal CCMs (Kaplan et al. 1980, Tsuzuki and Miyachi 1989, Raven 1991, Matsuda et al. 2001) by lowered photosynthetic affinity for CO_2 (Rost et al. 2006, Ratti et al. 2007) and/or by down-regulation of CA transcripts (Van de Waal et al. 2013) and presumably protein. Whether or not *Symbiodinium* photosynthesis will be enhanced by increased CO_2 due to climate change still remains controversial given; (1) the different phylotypes of *Symbiodinium*

and, (2) the varied responses in physiology, modes of Ci uptake, utilization of CAs and tolerance to environmental stressors (Brading et al. 2013).

Increases in temperature can also exert its effect on ocean carbonate chemistry and algal CCMs. Increasing SST decreases the solubility of CO_2 and favours speciation of Ci into more carbonate ions (CO_3^{2-}) and less CO_2. Experimental evidence has shown that CA activity can increase in some microalgae as temperature increases (Shiraiwa and Miyachi 1985) and that carbon acquisition and CCM function can also be enhanced in certain phylotypes of *Symbiodinium* (Oakley et al. 2014). A well-studied consequence of elevated SST is coral bleaching where *Symbiodinium* are expelled from the coral host or photosynthetic pigments are lost from the symbiont cells (Glynn 1993, Jones et al. 1998, Dove et al. 2006, Hoegh-Guldberg 2011). There is strong evidence that impairment of the *Symbiodinium* photosynthetic mechanism is involved in coral bleaching. Studies conducted on *Symbiodinium* cells in culture have shown that photosynthesis is impaired at temperatures above 30 °C and is completely disrupted at 36 °C (Iglesias-Prieto et al. 1992). While the trigger for thermal damage in *Symbiodinium* remains controversial, several possible sites of temperature sensitivity have been considered such as degradation of the Rubisco enzyme (Jones et al. 1998, Lilley et al. 2010, Buxton et al. 2012), impairment of the CCM (Leggat et al. 2004); causing disequilibrium in the photosynthetic electron transport chain leading to oxidative stress, damage to photosynthetic proteins such as the D1 protein of photosystem II (PSII) (Warner et al. 1999) and, thylakoid membrane integrity (Tchernov et al. 2004) such as light-harvesting complexes (LHCs) (Takahashi et al. 2008). However, none of these potential sites have conclusively been demonstrated as the initial site of thermal damage. In addition, it is well known that thermal tolerance and sensitivity of *Symbiodinium* varies across the many phylotypes.

Phylotypes D1, D1-4, C15, A3 are generally accepted as thermotolerant, while phylotypes C3, C7, B17 and A13 are more thermosensitive (Thornhill et al. 2006, Silverstein et al. 2011, McGinty et al. 2012, Tonk et al. 2014).

Although there have been numerous studies on the response of various phytoplankton, including *Symbiodinium*, to elevated CO_2 (Hutchins et al. 2007, Rost et al. 2008, Brading et al. 2011) or temperature (Robison and Warner 2006, Oakley et al. 2014, Gierz et al. 2016, Gierz et al. 2017) many of these studies have examined these stressors in isolation. Despite the environmental relevance of combined interactions between rising CO_2 and temperature few studies have examined these environmental stressors in combination. Given the importance of *Symbiodinium* to symbiotic relationships, the sensitivity of *Symbiodinium* photosynthesis, in particular CAs, to OA is currently limited. Therefore the present study aims to examine the response of this important species to future conditions of OA and warming focusing on growth, photosynthetic efficiency, chlorophyll content and gene expression of the two *Symbiodinium* β-CAs (SymBCA1 and SymBCA2) identified in Chapter 2. From these results we also aim to identify any possible links between *Symbiodinium* physiology and gene expression under combined pCO_2 and temperature conditions.

Material and Methods

Experimental treatments

Symbiodinium clade F cells were grown in a controlled refrigerated incubator (Thermoline Scientific refrigerated incubator, Sanyo TRISL-495-1-SD) on a 12:12 hr light/dark photoperiod cycle in tissue culture flasks (Sarstedt, NC, USA) containing ASP-8A medium (Blank 1987). This particular phylotype was chosen, as the C1

phylotype was not available at the time. This however presented an opportunity to explore if the response of *Symbiodinium* clade F would be similar to that of clade C1 used in Chapter 2. The experiment started with cells in log-phase growth to avoid age-dependent response. Cells were divided equally in triplicate flasks for each of the six experimental treatments that included three CO_2 concentrations of 390 – 400 ppm pCO_2 (control), 750 ppm pCO_2 and 1500 ppm pCO_2 at a control temperature of 25 ºC (CO_2 alone conditions) and the same three CO_2 concentrations at an elevated temperature of 31 ºC (thermally stressed conditions). A CO_2 mixing system based on the design of Munday et al (2009) was used where pure gaseous CO_2 was mixed with ambient air in a mixing chamber and pumped into each incubator. Temperatures were recorded every 30 min with HOBO® temperature/alarm pendant data loggers (Onset, MA, USA). Experimental conditions were maintained and monitored over 21 days with samples taken on days 0, 4, 7, 14 and 21 after a six-hour photoperiod.

Approximately 30 mls of ASP-8A media was taken for alkalinity measurements conducted by the Townsville City Water Council and both temperature and pH were measured daily with a calibrated YSI 600QS Sonde (YSI, Ohio, USA). Resulting alkalinity, pH and water temperature values were entered into CO2CALC (Robbins et al 2010) to calculate pCO_2 of each sample (Figure 3.1a).

Doubling time for cells was approximately 7 days therefore all flasks were split in half on day 7 and 14 after experimental measurements were taken to prevent cells entering stationary phase of growth. Splitting of *Symbiodinium* cells involved discarding the culture media and adding fresh media to the same flask. Cells were then resuspended in the fresh culture media using a disposable sterile cell scrapper (Starstedt, NC, USA) and cell counts were performed using a haemocytometer. Approximately half of the fresh media, including suspended cells, were then

transferred into new sterile flasks and filled to approximately 80 mls with fresh media. The new flasks were then used for the remainder of the experiment.

Imaging – pulse amplitude modulated fluorometry

Measurements of chlorophyll fluorescence of *Symbiodinium* cells were taken using Imaging – pulse amplitude modulated (iPAM) fluorometry (MAXI Imaging – PAM, Walz, Germany). See Chapter 2 methodology for dark-adapted maximum quantum yield measurements. Three replicate samples of *Symbiodinium* cells from each treatment were designated for PAM measurements and used throughout the experiment. Induction recovery curves were performed to examine the ability of *Symbiodinium* to dissipate excess light energy and recover from light stress after experimental conditions.

***Symbiodinium* cell density and pigment content**

Approximately 3 ml of culture were taken from all treatments in triplicate and centrifuged at 5000g for 2 min to pellet algal cells for chlorophyll *a* (chl a) and chlorophyll *c* (chl c) pigment quantification. Chlorophyll was extracted by re-suspending the algal pellets in 90 % acetone for 24 hours in the dark at 4 °C. After 24 hours cells were briefly disrupted using a MP Fastprep®-24 Instrument (MP Biomedicals, Australia) at 4.5 M s^{-1} for 3 x 45 seconds and chlorophyll measured on a DU-650 spectrophotometer (Beckman, USA) at 630 and 664 nm light wavelength. Chl *a* and chl *c* content were quantified using the equations of Jeffrey and Humphrey (1975) for 90 % acetone. A total aliquot of 1 ml from each sample was also taken for cell counts with replicate counts performed on a haemocytometer (n = 8).

mRNA isolation and cDNA synthesis

Approximately 6 x 10^6 *Symbiodinium* cells were collected for mRNA extraction and snap frozen in lysing matrix D tubes for later mRNA extraction (MP Biomedicals, Australia). See Chapter 2 methodology for details on mRNA isolation procedures. DNase treatment of all mRNA samples was performed prior to reverse transcription with approximately 100 ng of mRNA (see Appendices Table 4). All mRNA samples were treated with RQ1 RNase-Free DNase (Promega) as per Chapter 2 methodology and used as a template for SuperScript™ III First-Strand Synthesis SuperMix for qPCR kit following manufacturer's protocol (Invitrogen, California, USA). See Appendices Table 5 for reaction volumes.

RNA transcript analysis

Quantitative PCR (qPCR) was used to measure transcript levels of various genes of interest (GOI) (Table 3.1). Candidate internal control genes (ICG) used for *Symbiodinium* were: glyceraldehyde 3-phosphate dehydrogenase GAPDH (Rosic et al. 2011), proliferating cell nuclear antigen (PCNA) (Boldt et al 2008) and S-adenosyl methionine synthetase (SAM) (Rosic et al. 2011). Validation for use as ICG in this experiment was performed using GeNorm in qBASE plus software package (Biogazelle) with M values of 0.5 or lower deemed acceptable. The amplification efficiency for each gene specific primer was determined by serial dilutions ranging from 1:10 to 1:160. A 1:40 dilution was determined to be optimal for all primer sets with reaction efficiencies between 0.9 and 1.0. qPCR was then performed; see Chapter 2 methodology for details on reaction mix and qPCR parameters. Relative expression analysis was performed using qBASE plus (Biogazelle).

Gene name	Forward Primer (5’ to 3’)	Reverse Primer (5’ to 3')
SymBCA1	AGGCTGAATTGCTCCGCTCTG	AAGGGGCCATCAGTTGTTGTGC
SymBCA2	TGAAGGTAGCATGGTTGGCAGT	GCTTGTGCTGCCTAACTGTGTC
PGPase	TCACTGACCGATCCCGCATTTG	CCTTTCTTTCTGGGCACCTTGTCC
Rubisco	AACGAGTGCATTCCTGAAGTGGTGA	GGCAGTGATGTTGGCGGAGAAGA
PCNA*	GAGTTTCAGAAGATTTGCCGAGAT	ACATTGCCACTGCCGAGGTC
GAPDH*	CCGGCGCCAAGAAGGTCATCATC	TTGGCCTTGTCGTACTCCGTGTGGTT
SAM*	GCCTACATTTGCCGACAGATG	AATGGCTTGGCAACACCAAT

Table 3.1. *Symbiodinium* primer sequences used for quantitative PCR. Asterisks indicate internal control genes. GAPDH (glyceraldehyde-3-phosphate dehydrogenase, PCNA (proliferating cell nuclear antigen), PGPase (phosphoglycolate phosphatase), SAM (S-adenosyl methionine synthetase).

Statistical analysis

Data analysis was performed using statistical software package SPSS (IBM). A generalised linear model (GLM) was applied for pairwise comparison with sequential Bonferroni post hoc ($\alpha<0.05$) test to determine significant differences between treatments compared to controls where mains or interaction effects were significant.

Results

Imaging – pulse amplitude modulated fluorometry

Maximum quantum yield of photosynthesis, as measured by F_v/F_m was determined on day 0, 4, 7, 14 and 21. Elevated CO_2 concentrations alone did not have an affect on F_v/F_m in *Symbiodinium* cells (Figure 3.2) and maximum quantum yield of *Symbiodinium* cells was more influenced by thermal stress conditions. Significant decreases in F_v/F_m in *Symbiodinium* cells exposed to thermal stress of 31 °C were observed on day 4 in the 1500 ppm pCO_2 treatment ($p< 0.007$), day 14 in all pCO_2 treatments (380 ppm pCO_2, 750 ppm pCO_2 and 1500 ppm pCO_2) ($p< 0.000$), and on day 21 in the 380 ppm pCO_2 ($p<0.001$) and 750 ppm pCO_2 ($p<0.005$) treatments (Figure 3.2).

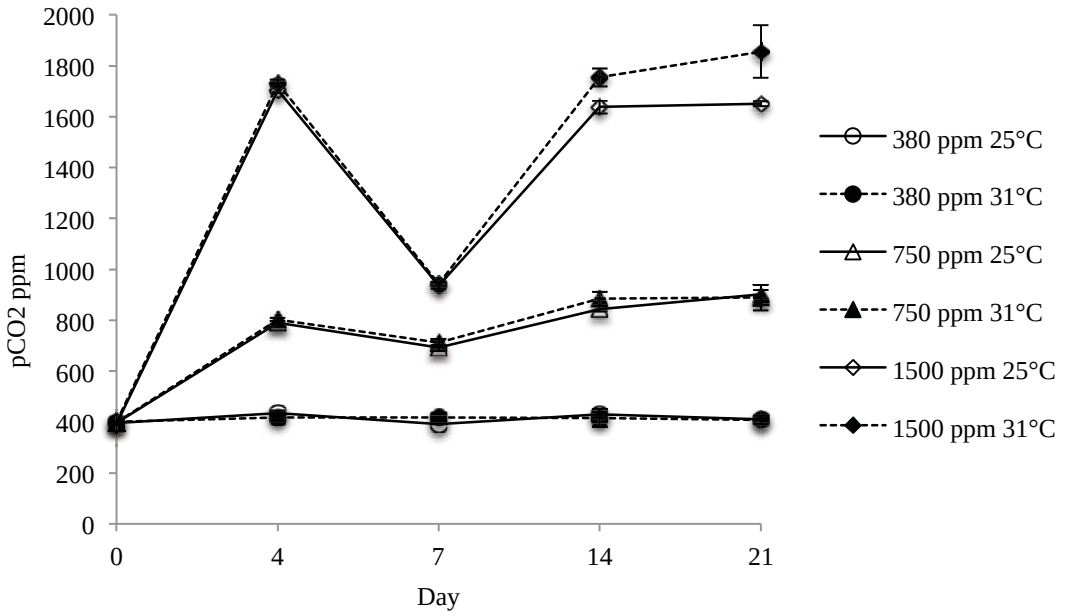


Figure 3.1. pCO_2 concentrations of culture media during the 21 day experiment. Values represent the average of 3 replicate flasks. Data represented are: control 380 ppm: 25 °C (open circle), 750 ppm: 25°C (open triangle), 1500 ppm: 25 °C (open diamond), 380 ppm: 31 °C (closed circle), 750 ppm: 31°C (closed triangle), 1500 ppm: 31°C (closed diamond). Error bars represent the standard error (some error bars obscured by data points; n=3).

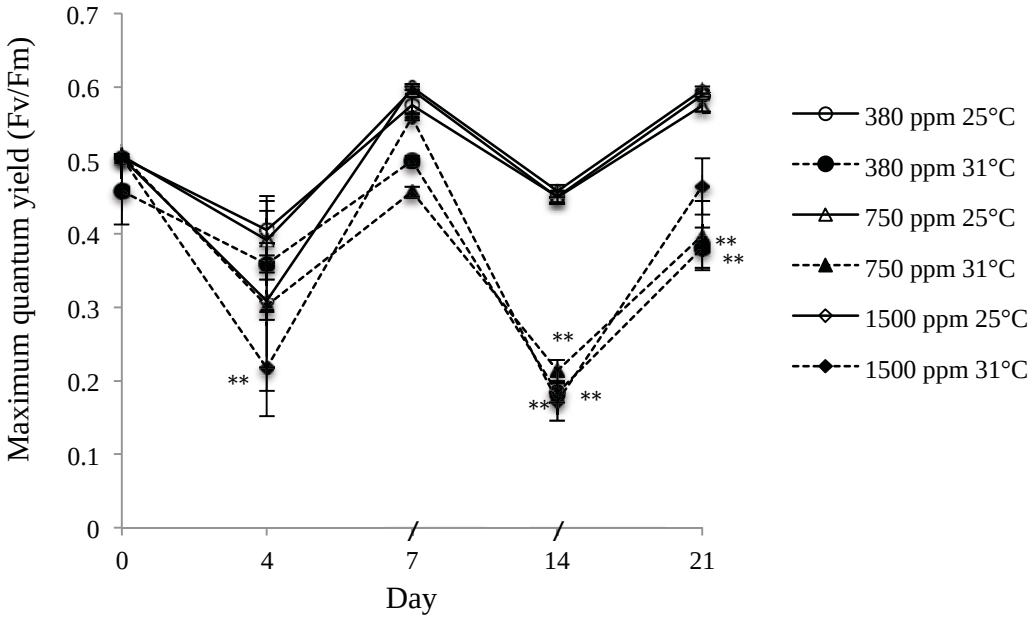


Figure 3.2. Photosynthetic efficiency of PSII of cultured *Symbiodinium* clade F, measured as dark-adapted yield, F_v/F_m. Data represented are: control – 380 ppm: 25 °C (open circle), 750 ppm: 25 °C (open triangle), 1500 ppm: 25 °C (open diamond), 380 ppm: 31 °C (closed circle), 750 ppm: 31°C (closed triangle), 1500 ppm: 31°C (closed diamond). Cells were split on days 7 and 14 to prevent stationary phase of growth (indicated on x-axis by dash). Error bars represent the standard error (some error bars obscured by data points; n = 3). Asterisks indicate significant difference to control ($p<0.05$).

Symbiodinium cell density and pigment content

Both elevated CO_2 and thermal stress had a significant effect on *Symbiodinium* cell densities throughout the course of the experiment (Figure 3.3). CO_2 alone treatments experienced a significant decline in cell density on day 7 in both the 750 ppm pCO_2 treatment ($p<0.001$) and 1500 ppm pCO_2 treatment ($p<0.001$). However on day 21 an

increase in cell density was observed in the 750 ppm pCO_2 treatment ($p<0.007$) compared to control treatment (380 pCO_2: 25 °C; Figure 3.3). From day 7 of the experiment all *Symbiodinium* cells exposed to thermal stress experienced significant declines in cell densities ($p<0.001$) and were significantly lower than cell densities in control treatments ($p<0.001$). The cell density continued to decrease over the remaining course of the experiment in all thermally stressed treatments on day 14 ($p<0.001$) and day 21 ($p<0.001$). By day 21 average cell densities of all thermal stress treatments significantly declined to approximately 8 x 10^4 cells/ml compared to average cell densities of controls 1.4 x 10^6 cells/ml. No other changes in cell densities were seen for any other pCO_2 treatment.

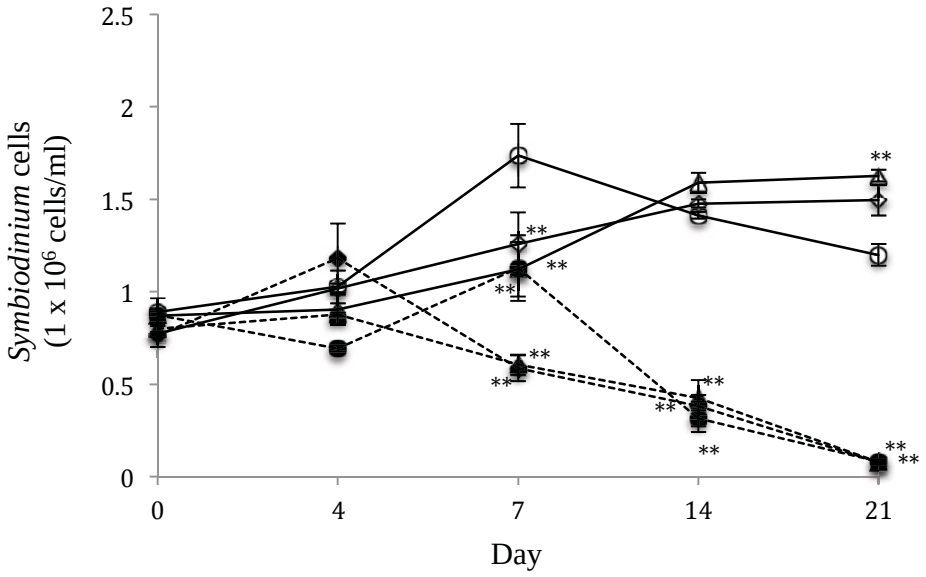


Figure 3.3. *Symbiodinium* cell density exposed to control 380 ppm: 25 °C (open circle), 750 ppm: 25 °C (open triangle), 1500 ppm: 25 °C (open diamond), 380 ppm: 31 °C (closed circle), 750 ppm: 31°C (closed triangle), 1500 ppm: 31°C (closed

diamond). Asterisks indicate significant difference to controls ($p<0.05$). Error bars represent the standard error (some bars obscured by data points; n = 8).

Elevated CO_2 alone conditions did not have any significant effect on chlorophyll pigment content of *Symbiodinium* cells. There was however a significant increase in both the chl *c* and chl *a* content per *Symbiodinium* cell for thermally stressed treatments from day 7 onwards (Fig 3.4a and b). Chl *c* per algal cell increased in thermally stressed treatments on day 14 in the 1500 pCO_2 treatment ($p<0.001$) and again on day 21 in all thermally stressed treatments (380 ppm pCO_2: 31 °C, 750 ppm pCO_2: 31 °C and 1500 ppm pCO_2: 31 °C) ($p<0.001$; Figure.3.4a). Chl *a* per algal cell also increased in thermally stressed treatments on day 7 in the 1500 ppm pCO_2 ($p<0.011$), day 14 in the 1500 ppm pCO_2 ($p<0.001$) and on day 21 in all thermally stressed treatments (380 ppm pCO_2: 31°C ($p<0.001$), 750 ppm pCO_2: 31 °C ($p<0.001$) and 1500 ppm pCO_2: 31 °C ($p<0.001$) (Figure 3.4b). The ratio of chl *c* to chl *a* was significantly increased in all thermally stressed treatments on day 21 ($p<0.05$ (Figure 3.4c). No CO_2 effect was observed for of chl *c* to chl *a* ratio.

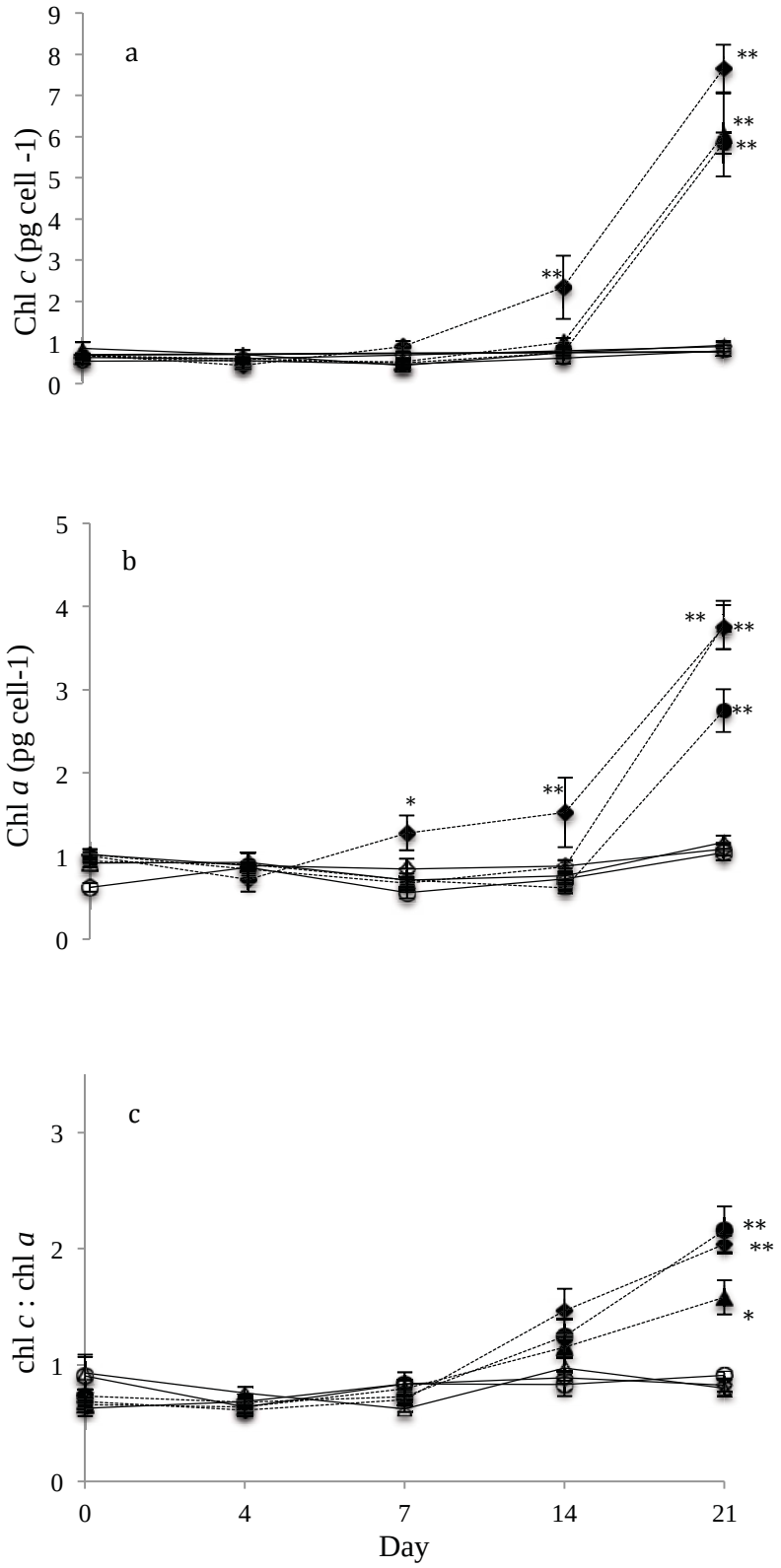


Figure 3.4. *Symbiodinium* pigment concentrations exposed to control - 380 ppm: 25 °C (open circle), 750 ppm: 25 °C (open triangle), 1500 ppm: 25 °C (open diamond),

380 ppm: 31 °C (closed circle), 750 ppm: 31°C (closed triangle), 1500 ppm: 31°C (closed diamond). (a) chl *c* per *Symbiodinium* cell, (b) chl *a* per *Symbiodinium* and, (c) ratio of chl *c* to chl *a*. Asterisks indicate significant difference to controls ($p < 0.05$). Error bars represent the standard error (some bars obscured by data points).

RNA transcript analysis

Transcript abundance of SymBCA1, SymBCA2, Rubisco and PGPase were determined for the course of the experiment. Analyses revealed few statistically significant changes in transcript abundance in the majority of *Symbiodinium* genes of interest during the course of the experiment (Figure 3.5). Both SymBCA1 and SymBCA2 transcripts increased under thermal stress. SymBCA1 transcript abundance increased by thermal stress alone on day 21 by 0.1 fold ($p<0.009$; Figure 3.5a) with no CO_2 effect observed. SymBCA2 transcript also increased by thermal stress on day 4 in the 1500 ppm pCO_2 treatment by 0.3-fold ($p< 0.022$) and on day 21 in the 750 ppm pCO_2 treatment (0.5-fold; Figure 3.5b). Rubisco transcript abundance increased on day 4 by elevated CO_2 concentration alone in the 1500 ppm pCO_2 treatment by 0.3-fold ($p< 0.035$; Figure 3.5c). No statistically significant changes were observed for PGPase throughout the course of the experiment (Figure 3.5d).

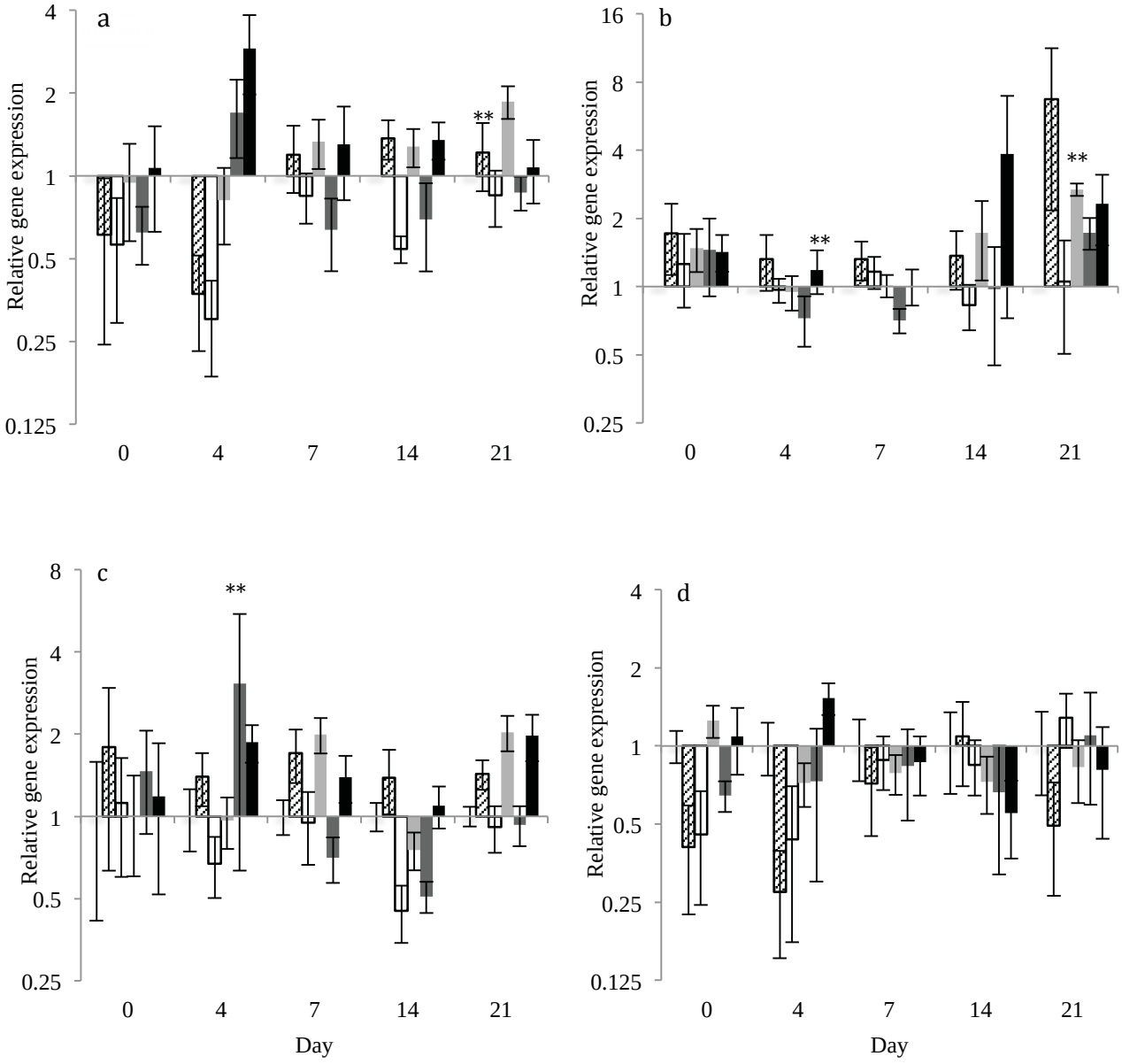


Figure 3.5. Transcript abundance of *Symbiodinium* clade F when exposed to 380 ppm: 31 °C (diagonal line bar), 750 ppm: 25 °C (white bars), 750 ppm: 31°C (light grey bar), 1500 ppm: 25 °C (dark grey bar) and, 1500 ppm: 31°C (black bar). Values expressed as ratios of treatment to control (380 ppm: 25°C) for each day. (a) SymBCA1, (b) SymBCA2, (c) Rubisco and, (d) PGPase. Error bars are standard error (n=3). Asterisks indicate significant difference to controls ($p < 0.05$).

Discussion

This study examined the effects of a prolonged 21 day experiment on cells of *Symbiodinium* clade F that were exposed to combined elevated pCO_2 and temperature conditions. The response of certain physiological parameters and transcript analyses of *Symbiodinium* β-CAs, Rubisco and PGPase were investigated to examine links between physiological responses and gene expression. The results of this study revealed that the greatest changes in *Symbiodinium* cells exposed to combined elevated pCO_2 and temperature were on physiological parameters and cell density while RNA transcript analysis of the chosen genes of interest revealed few, small significant changes. In addition, the main driver of these changes was thermal stress while CO_2 conditions that are environmentally relevant had minimal significant effects over all.

During the course of the experiment *Symbiodinium* cells exposed to thermal stress decreased to approximately half the density compared to controls (Figure 3.3), which is consistent with a variety of other studies examining thermal stress on *Symbiodinium* cell densities (Ogawa et al. 2013, Gierz et al. 2016). The CO_2 driven effect that was observed on the decline of cell densities in the 750 ppm pCO_2 and 1500 ppm pCO_2 treatments on day 7 (Figure 3.3) may be due to cell cultures being split on this particular time point to prevent stationary phase of growth, as these changes were not observed throughout the remainder of the experiment. Additionally, the only other CO_2 driven effect that was observed on cell density was an increase in density in the 750 ppm pCO_2 treatment on day 21. Growth rates in *Symbiodinium* when exposed to elevated CO_2 conditions appear to vary according to the phylotype examined. For example, the A13 phylotype of *Symbiodinium* appears to respond positively to CO_2 enrichment while phylotypes A1, A2 and B1 remain largely

unaffected by a doubling in pCO_2 from 380 ppm to 780 ppm (Brading et al. 2011). The heat related increases in *Symbiodinium* chl *a* and chl *c* were surprising and contradict coral bleaching events, given that previous studies have demonstrated that cells of *Symbiodinium* exposed to thermal stress generally lose pigmentation (Brown et al. 2002, Dove et al. 2006). However, recent studies on *Symbiodinium* cells isolated from the coral *Acropera aspera* have also shown increases in chlorophyll content under thermal stress (Ogawa et al. 2013, Gierz et al. 2016). The exact cause of such increases in chlorophyll content is not yet known but has been attributed to repackaging of chlorophylls in the chloroplast membrane (Bissett et al. 1997) and may be associated with alterations in the type of pigment-protein complexes expressed under thermal stress.

Analysis of maximum quantum yield (F_v/F_m) demonstrated that *Symbiodinium* cells exposed to thermal stress exhibited depressed photosynthetic rates (Figure 3.2), while elevated pCO_2 concentrations had no significant effect. The decline in maximum quantum yield efficiency of PSII is a hallmark of thermal stress in symbiotic dinoflagellates with severe impairment of photosynthesis at temperatures 32-34 °C (Iglesias-Prieto et al. 1992, Jones et al. 1998, Robison and Warner 2006). While these results are consistent with previous studies on *Symbiodinium* cells exposed to thermal stress, both *in vitro* and *in symbiosis* (Iglesias-Prieto et al. 1992, Warner et al. 1996, Warner et al. 1999, Tchernov et al. 2004, Warner et al. 2006, Gierz et al. 2016), they are in contrast to a study by Oakley et al (2014) that showed an increase in net and gross maximum photosynthetic rates of 32 % in *Symbiodinium* phylotype B when exposed to temperatures up to 34 °C. Oakley et al (2014) found no evidence for thermal inhibition of the CCM and concluded that CCM components are therefore not likely to be primary sites of thermal damage in *Symbiodinium* clade B.

However, it must be noted that differential thermal stress sensitivity has been observed across the diverse *Symbiodinium* species, therefore leading to different impacts on photobiology of cultured *Symbiodinium* under thermal stress (Tchernov et al 2004; Robinson and Warner 2006; Takahashi et al 2008). The response of F_v/F_m during thermal stress may therefore vary with *Symbiodinium* depending on what phylotype is examined. Despite the significant decreases observed in *Symbiodinium* cell density exposed to thermal stress (Figure 3.3), photosynthetic efficiency of remnant cells was still maintained throughout the course of the experiment (Figure 3.2). Previous studies on cultured *Symbiodinium* from phylotype A1 under thermal and light stress have identified similar differences in growth of cells and PSII response (Robison and Warner 2006, Krämer et al. 2012). Krämer et al (2012) have suggested that photosynthesis and growth become uncoupled in A1 cells and that decline in growth rates may be caused by allocating more energy to protein repair and or metabolic suppression. However, it is important to note that photochemical efficiency of PSII can remain high even when there is significant decline in PSII protein content under thermal stress (Jeans et al. 2014).

While it has previously been shown that enrichment of CO_2 can significantly increase photosynthetic efficiency of *Symbiodinium* from phylotype A2 (Brading et al 2011), in the present study no CO_2 effect was observed on the F_v/F_m in *Symbiodinium* clade F cells exposed to enriched pCO_2. This is in contrast to results obtained in Chapter 2 where F_v/F_m was depressed in *Symbiodinium* clade C1 under elevated pCO_2 conditions (Figure 2.6a). These results suggest that compared to clade C1, clade F may be more CO_2 tolerant and therefore could possibly fair better under conditions of OA.

Increases in photosynthetic efficiency not only seem to be species specific but may also depend on the particular Ci species preferred for uptake by these species. Species that rely on diffusive uptake of CO_2 are possibly far more likely to show increases in photosynthetic rates and growth than those species utilising HCO_3^- or taking up CO_2 actively via a CCM. In addition CCMs generally differ in their design and efficiency between algal species (Tortell 2000, Giordano et al. 2005), therefore it is not inconceivable that CCMs may differ in their efficiency and their sensitivity to enriched CO_2 conditions between *Symbiodinium* phylotypes. In fact CCM expression has been shown to be environmentally driven and host dependent for some phylotypes of *Symbiodinium*. For example, *Symbiodinium* isolated from corals have been shown to initially utilise HCO_3^- but then indiscriminately utilise CO_2 and HCO_3^- (Goiran et al. 1996), whereas *Symbiodinium* isolated from giant clams exhibit a preference for direct CO_2 uptake (Leggat et al. 1999). Interestingly, in the latter study, this preference changed to direct HCO_3^- uptake after 2 days of being maintained in culture (Leggat et al. 1999). Free-living *Symbiodinium* in culture have been shown to utilise HCO_3^- by a sodium dependent mechanism (Al-Moghrabi et al. 1996), which may reflect a change in physiology between symbiotic and free-living forms.

Although large changes were seen in *Symbiodinium* physiology, transcript analysis of selected photosynthetic genes revealed few significant changes in *Symbiodinium*. The sustained photocompetency of remaining *Symbiodinium* cells exposed to thermal stress seen in the present study may therefore reflect the relatively stable expression of Rubisco, PGPase and CAs. While it can be assumed that increases in CO_2 should increase the carboxylase activity of Rubisco and hence photosynthesis, the increase in transcript abundance of Rubisco (0.3-fold; $p<0.035$) (Figure 3.5c) did not correspond to any increases in photosynthetic efficiency in the

present study nor did the up-regulation continue through the remainder of the experiment. Similar transcript patterns for Rubisco were observed in Chapter 2 where an initial up regulation of Rubisco occurred in *Symbiodinium* clade C1 cells exposed to elevated CO_2 (750 ppm pCO_2; Figure 2.6f). However no other significant changes in transcript abundance were observed for the remainder of either experiment. In many plant species CO_2 concentration becomes an increasingly greater limitation on Rubisco activity as less of the Rubisco capacity is used for carboxylation (Salvucci and Crafts-Brandner 2004), due to reduced affinity for CO_2 and lower solubility of CO_2 at elevated temperatures (Berry and Bjorkman 1980).

Inactivation of Rubisco due to elevated temperature has been one of many potential sites of thermal sensitivity in *Symbiodinium* where damage results in a decrease in photosynthetic efficiency (Jones et al. 1998). While decreases in Rubisco enzyme activity have previously been observed in *Symbiodinium* exposed to thermal stress (Leggat et al. 2004; Lilley et al. 2010), no thermal effect was observed on Rubisco gene expression patterns in the present study. In addition PGPase remained unaffected by either elevated CO_2 or temperature. This is in contrast to previous studies conducted on *Symbiodinium* that have shown PGPase transcript abundance to decrease in *Symbiodinium* within corals that had been exposed to elevated pCO_2 concentrations (Crawley et al. 2010) and increase when exposed to elevated temperature treatments (Mayfield et al. 2012). The present results for PGPase transcript abundance is also in contrast to results obtained in Chapter 2 that demonstrated a decrease in PGPase transcripts of cultured *Symbiodinium* clade C1 exposed to elevated pCO_2 concentrations (Figure 2.6e). However, the CO_2 concentrations used in Chapter 2 to illicit this response was much higher than the concentrations used for the present study.

Both *Symbiodinium* β-CA transcripts examined (SymBCA1 and SymBCA2) were significantly increased by thermal stress (Figure 3.5a and b). It is interesting to note however that SymBCA1 transcript abundance was only increased by thermal stress at ambient pCO_2 concentrations while SymBCA2 transcript abundance was only increased by thermal stress combined with elevated pCO_2 concentrations of 1500 ppm pCO_2 on day 4 (0.3-fold) and on day 21 by 750 ppm pCO_2 (0.5-fold; Figure 3.5b). Given the sequence diversity that exists within the β-class of CAs it is not implausible to assume that differences in expression pattern between SymBCA1 and SymBCA2 may be due to different roles played in the *Symbiodinium* CCM of clade F. Theoretically elevated temperatures increase the need for CA due to the shifting equilibrium of Ci to less CO_2 (Beardall et al. 1998). For example, it has been demonstrated in microalgae that CA activity has been shown to increase concomitantly with temperature up to 37 °C (Shiraiwa and Miyachi 1985). Furthermore, Oakley et al. (2014) showed that overall CCM function was enhanced by temperatures up to 34 °C in strains of cultured B1, A and D1a *Symbiodinium*. While there were increases in transcript abundance of both *Symbiodinium* β-CAs in the present study, there was no evidence to suggest a shift in the equilibrium of Ci to less CO_2 as seen in the pCO_2 of culture media (Figure 3.1). Whether or not the increase in transcripts of SymBCA1 and SymBCA2 were due to decreases in CO_2 concentration due to increased temperatures would have to be further examined as no clear pattern of regulation was observed for either SymBCA1 and SymBCA2 throughout the remainder of the study and increases of these transcripts did not correspond to any changes in physiological parameters. This would be achieved by examining how much CO_2 decreases from 31 °C in comparison to 25 °C with the same total Ci content.

While it is well known that CA activity and transcripts can decrease with increasing CO_2 concentrations (Van de Waal et al. 2013), both SymBCA1 and SymBCA2 transcript abundance remained unchanged when exposed to elevated CO_2 (750 ppm pCO_2 and 1500 ppm pCO_2) without thermal stress. These results are consistent with the RNA transcripts analyses from Chapter 2 where changes in gene expression were only observed at extreme pCO_2 levels of 10 000 ppm pCO_2 (Figure 2.6a and b). As in previous targeted studies that have examined transcript levels in *Symbiodinium* exposed to environmental stressors, small to minimal changes have been observed even when significant stress such as light or temperature is applied (Boldt et al. 2010, Rosic et al. 2010, Leggat et al. 2011, Ogawa et al. 2013, Krueger et al. 2015, Gierz et al. 2016). These small changes in *Symbiodinium* transcriptome levels are most likely a result of a greater dependency on post-transcriptional regulation within the dinoflagellates (Okamoto and Hastings 2003, Bachvaroff and Place 2008) and have been hypothesized to primarily drive changes in the proteome, especially under heat stress (Barshis et al. 2014). Another possibility is that these particular β-CA genes are constitutively expressed in the CCM even at CO_2 concentrations of 750 ppm pCO_2 and 1500 ppm pCO_2.

In summary, the results of this study have demonstrated that the greatest change in *Symbiodinium* clade F exposed to combined elevated pCO_2 and temperature was on physiological parameters and cell density while minimal to no change was observed for transcript analysis of selected genes. In addition, thermal stress was the main driver of these changes while CO_2 had little effects over all. While no clear link between physiology and transcript abundance could be inferred from the present study, it does not rule out the possibility that that these changes may be independent of some CCM or photosynthetic components, such as CA, Rubisco and or PGPase.

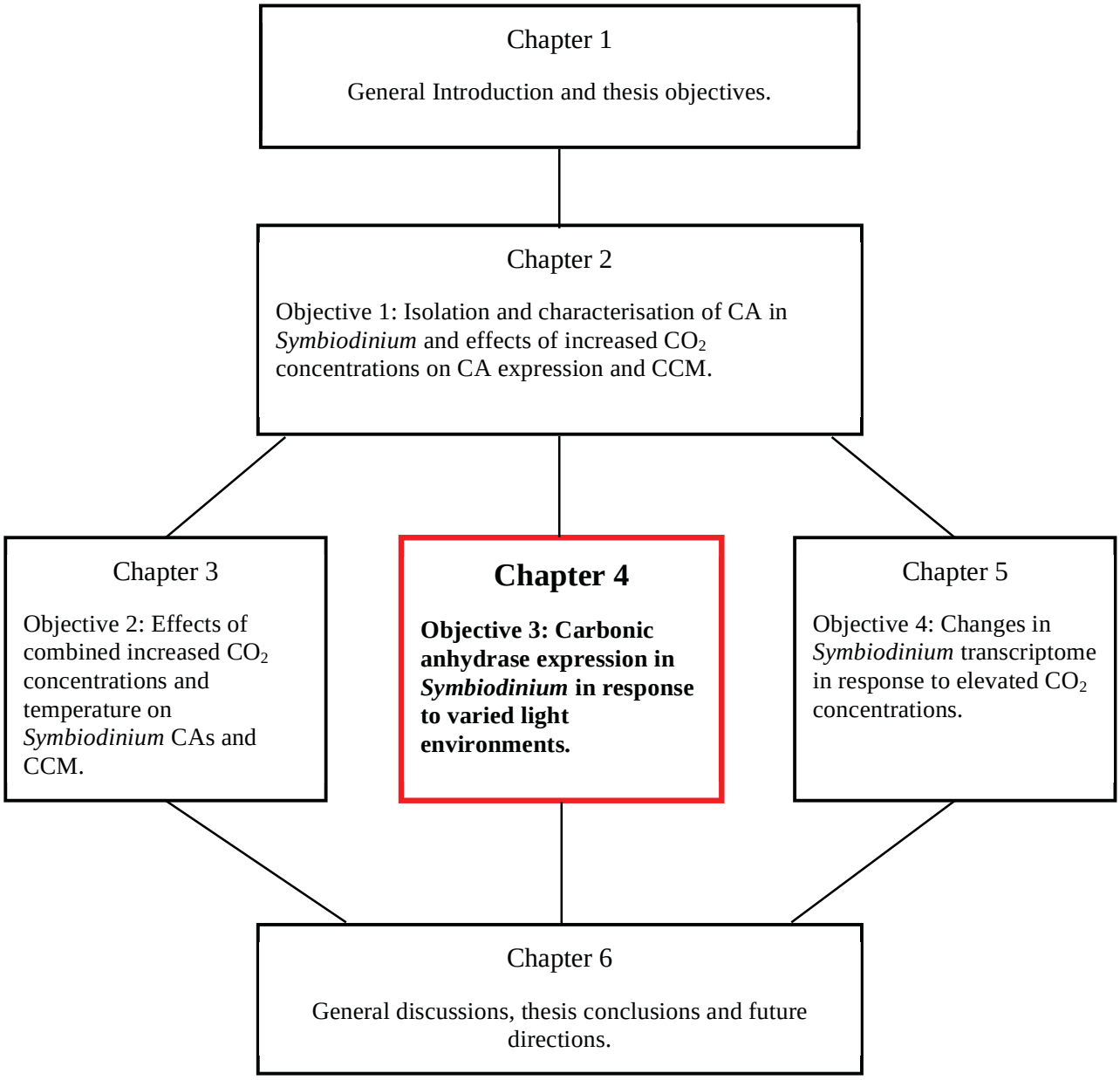
Future work should therefore focus on post-transcriptional responses of *Symbiodinium* in order to determine the response of *Symbiodinium* to combined elevated pCO_2 and temperature conditions. Unfortunately there is currently little literature evaluating the effects of temperature on *Symbiodinium* CCMs with which to compare our results therefore interpretation of results should be done with caution and until additional experiments are conducted. The results of the present study however emphasize the diversity and variability of physiological responses that can exist both between and within different phylotypes of *Symbiodinium* when exposed to significant environment stresses, and illustrate minimal transcriptional regulation under such conditions.

Chapter 3 Summary points

- The greatest change in *Symbiodinium* clade F exposed to combined elevated pCO_2 and temperature was on physiological parameters and cell density while minimal to no change was observed for transcript analysis of selected photosynthetic genes.
- Elevated temperature was the main driver of these changes.
- Both SymBCA1 and SymBCA2 transcripts increased under thermal stress however, SymBCA2 transcript levels were only increased by thermal stress if combined with elevated pCO_2 concentrations.
- Rubisco transcript abundance increased under elevated pCO_2 alone (1500 ppm pCO_2)

- No clear link between physiological responses or with genes coding for proteins involved in the physiological pathways examined were observed, with greater changes possibly observable post-transcriptionally.
- The present results support and demonstrate the variability between and within the different phylotypes of *Symbiodinium* exposed to certain environmental stressors.

Schematic representation of thesis outline



Chapter 4: Carbonic anhydrase expression in *Symbiodinium* sp. in response to varied light environments

Statement of purpose

Light is a crucial factor in photosynthesis that serves as an energy source to fuel photosynthetic fixation of CO_2. However, under certain conditions, light also has the potential to be toxic to photosynthesis and forces photoautotrophs to acclimate to maintain the efficiency of photosynthesis and avoid photodamage. Natural environments exhibit a dynamic range of light conditions, from very high intensities in full sunlight (higher than 2000 μmol photons m^{-2} s^{-1}) to heavily shaded conditions with little available light. In addition to CO_2 concentrations, light has been shown to control the extent of some algal carbon-concentrating mechanisms (CCMs), such as in the green algae *Chlamydomonas reinhardtii*, with transcriptional changes in certain photosynthetic genes involved in the CCM under varied light conditions. In the present study, quantitative PCR (qPCR) was used to determine transcript abundance of SymBCA1 and SymBCA2 and two chlorophyll *a*-chlorophyll *c* 2-peridinin-proteins in *Symbiodinium* C1 cells acclimated to high and medium-light conditions and transferred to different light conditions for nine days in air levels of CO_2. The efficiency of photosystem II (PSII) to perform photosynthesis was also measured to resolve two key issues: (1) do varied light intensities regulate transcription of *Symbiodinium* β-CAs and acpPCPs and are these changes linked to physiological parameters and, (b) are response times of *Symbiodinium* to changes in light intensity consistent with changes in the time scale observed for other organisms.

Introduction

It is well known that a key component of the *Symbiodinium*-coral relationship is the symbionts' ability to perform photosynthesis and the subsequent translocation of photosynthetic products to the host that accounts for the majority of metabolic energy required by the host (Muscatine 1990). Numerous studies have identified environmental factors that can affect the process of photosynthesis, such as temperature (Berry and Bjorkman 1980, Hoegh-Guldberg and Smith 1989, Iglesias-Prieto et al. 1992, Jones et al. 1998, Al-Khatib and Paulsen 1999), CO_2 (Beardall et al. 1998, Bhatt et al. 2010, Errera et al. 2014, Clement et al. 2017) and light availability (Hoegh-Guldberg and Smith 1989, LaRoche et al. 1991, Iwai et al. 2007, Erickson et al. 2015). However little attention has been paid to the effects varied light intensities have on transcription rates of several genes involved in the *Symbiodinium* CCM.

Photosynthesis is a light-driven process that occurs in two sequential stages, the light-dependant reactions and the light-independent (or dark) reactions. The light reactions are based around two photosystems, photosystem I (PSI) and photosystem II (PSII). PSI and PSII perform the primary photochemistry involving the absorption of light energy via chlorophyll molecules that are attached to light-harvesting complexes (LHCs) and the subsequent transfer of energy to the photosystem reaction centres, where charge separation of electrons occur (Huner et al. 1998). Light harvesting is mediated in dinoflagellates by two LHCs that bind these photosynthetic pigments; the water-soluble protein peridinin-chlorophyll *a*-binding proteins (PCPs) and the water-insoluble chlorophyll *a*-chlorophyll *c* 2-peridinin-protein complexes (acpPC), both of which are present in multiple forms in dinoflagellates (Boldt et al. 2012). The light-independent or dark reaction is where carbon fixation occurs and is where the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the first step of

carbon fixation via the capture of CO_2 through the Calvin-Benson cycle. The fixation of CO_2 by Rubisco is an active process that is driven by the adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) generated from the light-dependant stage of photosynthesis (Raven et al. 2000; Beardall and Giordano 2002). In *Symbiodinium* the process of carbon acquisition and fixation is augmented by the use of a CCM, which allows cells to concentrate Ci at the site of Rubisco. Algal CCMs involve several CA enzymes that, based on locality within the cell, are involved in CO_2 capture, CO_2 transportation and rapid inter-conversion between the carbon species (Giordano et al. 2005, Moroney and Ynalvez 2007). The acquired CO_2 then undergoes reduction in photosynthetic reactions to form carbohydrates that are either used to meet the alga's own energy requirements, if in a free-living state, or translocated to the host if in symbiosis.

In all photosynthetic organisms the balance between light flux and carbon fixation must be balanced for the photosynthetic process to be carried out efficiently. Although light is an energy source that fuels photosynthetic CO_2 fixation, limited or excess light intensities are known to stress and imbalance aspects of photosynthesis including light-utilisation, carbon acquisition and fixation (Coles and Jokiel 1978, Hoegh-Guldberg and Smith 1989, Al-Khatib and Paulsen 1999, Demmig-Adams and Adams 2000). Limited light levels have been shown to cause a decrease in affinity for inorganic carbon (Ci), suggesting that the activity of the CCM is lowered as photon flux densities decrease and that the accumulation and transportation of Ci within cells is light dependent (Spalding and Ogren 1982) and may be driven by photosynthesis (Badger and Andrews 1982, Spalding et al. 1984, Kaplan et al. 1987). Conversely, exposure to high light environments can lead to a limitation of CO_2 and result in the absorption of excess light energy by photosynthetic pigments. When

light absorption exceeds the capacity for carbon fixation, production and accumulation of reactive oxygen species (ROS) may damage cells, result in PSII excitation pressure that can affect an organism's photochemistry (Huner et al. 1998) and disrupt PSII reaction centers leading to photoinhibition (Niyogi 1999). All photoautotrophs must therefore be able to alter components of their photosynthetic apparatus and employ a range of photo-acclimation responses to maximise the efficiency of light capture and hence carbon fixation under varying environmental conditions (Iglesias-Prieto and Trench 1994, Iglesias-Prieto and Trench 1997, MacIntyre et al. 2002). Some of these photo-acclimation responses include changes in pigment-protein complexes (Demmig-Adams and Adams 1992), redistribution of excitation energy between PSI and PSII (Biggins and Bruce 1989) and lowering quantum yield of PSII in high light (HL) conditions via induction of non-photochemical quenching (NPQ) of fluorescence (Müller et al. 2001). Activation of these mechanisms in response to varying environmental factors can occur on varying time scales ranging from seconds or hours (for functional adjustments to individual proteins within the photosynthetic apparatus) to weeks or months (for developmental adjustments to the whole or parts of the organism) (Walters 2005).

Prior research has demonstrated that transcription rates of several genes involved in photosynthesis in many algal species, including *Symbiodinium*, are influenced by light intensities (Hobson et al. 1985, Xu and Tabita 1996, Im and Grossman 2002, Xiang et al. 2015). In addition, the redox state of the electron transport system may be the driving factor behind gene regulation in photosynthetic organisms and also represents a sensing or signalling system (Maxwell et al. 1995). In the green algae *C. reinhardtii* transcription rates of several Ci transporters and CAs involved in the CCM are transcriptionally up-regulated in light, with evidence

suggesting that in addition to CO_2 limitation, light and the operation of a complete photosynthetic apparatus is associated with CA and CCM induction (Spencer et al. 1983, Spalding et al. 1984, Yamano et al. 2008). As with other photosynthetic organisms, *Symbiodinium* are also able to adjust any imbalances between light absorption and utilisation to minimise induced damage to PSII, limiting photoinhibition and therefore maintaining maximum photosynthetic rates (Iglesias-Prieto and Trench 1994, Iglesias-Prieto and Trench 1997, MacIntyre et al. 2002). Although studies on cultured *Symbiodinium* indicate that the varied phylotypes differ in their capacity for photoacclimation (Chang et al. 1983, Porter et al. 1984, Iglesias-Prieto and Trench 1994), photoacclimation processes can also include cellular changes that optimise their light-harvesting capabilities such as alterations of their chlorophyll and peridinin concentrations (Dubinsky et al. 1984, Porter et al. 1984, Titlyanov et al. 2001, Mass et al. 2007) and preferentially modifying the PSII reaction centre content (Porter et al. 1984, Iglesias-Prieto and Trench 1994, Hennige et al. 2009).

While *Symbiodinium* possess a CCM that is active in light and exhibits distinct light-activated intracellular CA activity (Leggat et al. 1999), factors regulating photosynthetic proteins still remain poorly understood in *Symbiodinium*. Little attention has been paid to the potential for light to regulate expression levels of genes involved in the CCM, especially CA, and to link any changes in gene expression to photosynthetic efficiency of *Symbiodinium* under environmental stress such as light intensity. Since Ci accumulation and transport is light dependent and requires energy, in the form of ATP and NADPH produced by the light-dependent stage of photosynthesis, and CAs are crucial for algal CCMs it is certainly feasible that fluctuations in the capacity to generate ATP by light availability may restrict the

capacity of *Symbiodinium* to accumulate and fix Ci for photosynthesis. Such changes may be evident through transcription rates of some photosynthetic proteins. Modifying the expression of genes coding for proteins in photosynthesis and the CCM may therefore be a subcellular means by which *Symbiodinium* can adjust to environmental changes such as varied light intensities. This study investigates how *Symbiodinium* C1 acclimated to medium light (ML, ~80 - 100 μmol quanta m^{-2} s^{-1}) levels or high light (HL, ~250 – 350 μmol quanta m^{-2} s^{-1}) levels, respond following transition to a different light regime. Using measures for maximum quantum yield of PSII, which is an indicator of how efficiently PSII reaction centres are able to perform photosynthesis, together with transcriptional changes of the two β-CAs, (SymBCA1 and SymBCA2) identified in Chapter 2 and acpPC proteins, this study also sought to examine whether changes in photosynthetic efficiency could be linked with changes in gene expression and if the response time of *Symbiodinium* is similar to other organisms.

Materials and Methods

Algal cultures

Symbiodinium C1 (CCMP2466) cells were grown in 75 cm^2 tissue culture flasks with PE vented caps (Sarstedt, NC, USA) containing 100 mL of ASP-8A medium (Blank 1987). This particular phylotype was chosen based on previous studies identifying clade C to dominate zooxanthellate corals (Lien et al. 2012), with subclade C1 being the most commonly found species amongst many host species (Wong et al. 2016). Flasks were maintained in a light and temperature controlled refrigerated incubator

TRISL-495-1-SD (Thermoline Scientific, Australia) on a 12 h light:dark cycle photoperiod with temperature set at 25 ^{o}C. Cells of *Symbiodinium* C1 were grown on shelves adjusted to 80 µmol - 100 µmol quanta m^{-2} s^{-1} provided by four 18 W cool white fluorescent tubes (Polylux XLR™, GE Lighting). Shade cloth was used to achieve these desired light intensities and measured using a LI-193SA Underwater Spherical Quantum Sensor with a LI-250A Light Meter (LI-COR® Biosciences, NE, USA). *Symbiodinium* genotype was confirmed through sequencing of the internal transcribed spacer 2 region (ITS 2; See Chapter 2 methodology).

Experimental design

Symbiodinium C1 cells were cultured on shelves adjusted to 80 µmol - 100 µmol quanta m^{-2} s^{-1} in replicate flasks (n=3). At week seven cells from the replicate flasks were sub-divided (n=12) and the new replicate flasks were evenly distributed (n=4) to shelves with varying light levels; high-light (HL, ~250 – 350 µmol quanta m^{-2} s^{-1}), medium-light (ML, ~80 - 100 µmol quanta m^{-2} s^{-1}), and low-light (LL, ~10 – 15 µmol quanta m^{-2} s^{-1}). Replicates were maintained at these light levels for 25 weeks. During the 25 week acclimation period cells on the HL and ML shelves were regularly divided into fresh flasks containing 100 mL of ASP-8A media. LL replicates received fresh media at the same intervals, but were not divided into new flasks due to limited cell growth. The resulting 18 flasks (HL, n=9; ML, n=9) were then used in a 10 day light manipulation experiment.

Control measurements were recorded for HL and ML cells during the mid-point of the light cycle at time point zero (Day 0). During the mid-point of the dark cycle (Day 0) three flasks from the HL shelf were moved to the ML shelf (HL to ML), three to

the LL shelf (HL to LL) and three remained on the HL shelf (HL control). The process was repeated with flasks from the ML shelf; three were moved to the HL shelf (ML to HL), three to the LL shelf (ML to LL) and three remained on the ML shelf (ML control). All 18 flasks were then sampled at 12 noon on day 1, 2, 3 and 9 with known cell numbers collected for RNA extraction to use in later qPCR analysis and to measure the photosynthetic efficiency of *Symbiodinium* cells.

RNA extraction and cDNA synthesis

All equipment used for RNA extractions was treated with RNAseZap® (Ambion Inc, USA), and any plasticware and reagents were RNAse/DNAse-free. Total RNA from cultured *Symbiodinium* was isolated using an RNeasy Plant Mini kit (Qiagen, Valencia USA). Cultures were harvested by centrifugation at 5000 x *g* for 2 min, the pellet resuspended in 450 µL RLT buffer (Qiagen kit buffer), were transferred to a Lysing Matrix D tube (MP Biomedicals, Australia) and lysed twice for 20 s at 4.0 ms^{-1} on a FastPrep®-24 Instrument (MP Biomedicals, Australia). The lysate including cell debris was transferred to a QIAshredder spin column and RNA extracted according to manufacturer's protocol, including the additional centrifuge and elution step using the initial eluate to increase RNA concentration. Total RNA integrity was assessed by formaldehyde agarose gel following the protocol of Sambrook and Russell (2001) and stained with ethidium bromide (200 µg mL^{-1}). RNA quantification and purity was determined using a NanoDrop-1000 (NanoDrop Technologies, Wilmington USA). Absorbance was measured at 260 and 280 nm with the ratio of absorbance (A_{260}/A_{280}) providing an estimation of nucleic acid purity. Only samples with an absorbance ratio of ≥1.9 were used for quantitative real time PCR (RT-PCR) applications.

cDNA amplification and genomic DNA elimination were performed with QuantiTect Reverse Transcription® Kit (Qiagen, USA) in a 20 µL reaction using 500 ng of total RNA as template and a RT primer mix consisting of random primers and oligo-dT. Negative controls prepared without total RNA as template were included for each series of reactions.

Primer Design

Normalization primers for β-actin and PCNA that were previously validated and tested for stability under varying light conditions with acpPC from *Symbiodinium* sp. clade C3 (Boldt et al., 2009) were used for quantitative PCR. Primers for both acpPCP and SymBCAs that were designed to sequences obtained from an Expressed Sequence Tag (EST) library of *Symbiodinium* sp. C3 (Leggat at al., 2007) were then tested for compatibility with cDNA isolated from cultured *Symbiodinium* clade C1. acpPC and CA primers were designed and checked as per Chapter 2 methods.

RNA transcript analysis

To optimize quantification accuracy template dilution series were prepared. After 4 - fold dilution with ddH_2O, 3 µl of diluted template was analysed using the Rotor-GeneTM 6000 (Corbett Life Science, Australia). qPCR was performed with 7.5 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen Corp, Carlsbad, USA) and gene specific primers (200 nM). The standard protocol was 95^{o}C for 2 min, followed by 45 cycles of 15 s at 95^{o}C and 30 s at 60^{o}C with the temperature increasing 1^{o}C every 5 s from 66^{o}C to 95^{o}C in a final melt stage. GeneDiscTM-100 (Corbett Life Sciences, Australia) included reactions for normalization and

Symbiodinium acpPCP and CA genes run in triplicate and non-template controls in triplicate. Standard curves for the normalization, acpPCP and CA genes were generated with four triplicates using template from ML and HL Day 0 control samples. These same controls were used as an additional PCR efficiency check and included on each GeneDiscTM-100 to ensure inter-disc comparability. Validation for use as ICG in this experiment was performed using GeNorm in qBASE plus software package (Biogazelle) with M values of 0.5 or lower deemed acceptable.

Imaging – pulse amplitude modulated fluorometry

At each time point 300 µL of cells were taken from all experimental flasks for variable chlorophyll fluorescence measurements. Cells were dark adapted for 15 min at room temperature prior to each F_v/F_m measurement using a Maxi-Imaging Pulse Amplitude Modulated Chlorophyll Fluorometer (Heinz Walz GmbH, Germany). The maximum photosystem II (PSII) efficiency of *Symbiodinium* cells exposed to different light levels was expressed as F_v/F_m. F_v/F_m results can act as an indicator of the efficiency of PSII reaction centres.

Statistical analysis

Statistical analysis was performed using statistical software package SPSS (IBM). A generalised linear model (GLM) was applied for pairwise comparison using light and day as main effects and light X day as an interaction. *Post hoc* analysis was performed on all data using sequential Bonferroni ($\alpha<0.05$) to determine significant differences between treatments and controls.

Results

Imaging – pulse amplitude modulated fluorometry

Maximum quantum yield measurements for *Symbiodinium* C1 acclimated to HL (~250 – 350 μmol quanta m^{-2} s^{-1}) and transferred to ML (~80 - 100 μmol quanta m^{-2} s^{-1}) or LL conditions (~10 – 15 μmol quanta m^{-2} s^{-1}), and ML acclimated cells transferred to HL or LL conditions, were determined for each sampling day (Figure 4.1a and b). Sampling on Day 0, the day prior to replicates being transitioned to a new light level, demonstrated that acclimation to HL or ML conditions significantly affected maximum photochemical efficiency ($p<0.001$; Figure 4.1a and b). The maximum quantum yield for *Symbiodinium* C1 grown and maintained between 250-350 μmol quanta m^{-2} s^{-1} (HL controls) did not vary significantly from day 0 to day 9 ($p=0.168$; Figure 4.1a). F_v/F_m decreased from 0.27 to 0.24 by day 3 then remained unchanged. The opposite was evident in both the HL to ML and HL to LL samples. Transition of cells from HL to ML saw an initial increase in F_v/F_m on day 1 which remained stable for the duration of the experiment (Figure 4.1a). F_v/F_m for HL to ML cells increased significantly on days 1 ($p=0.005$), 2 ($p=0.262$), 3 ($p<0.001$), and 9 ($p<0.001$) compared to HL controls. The transition of cells from HL to LL also showed significant increases in F_v/F_m for days 1 ($p=0.009$), day 2 ($p=0.0369$), day 3 ($p<0.001$) and day 9 ($p<0.001$) with PSII efficiency increasing from 0.27 on day 1 to 0.44 by day 9; $p<0.001$).

F_v/F_m for *Symbiodinium* C1 isolates grown and held at 80 – 100 μmol quanta m^{-2} s^{-1} (ML controls) remained relatively stable for the duration of the experiment decreasing from 0.37 (Day 0) to 0.35 (Day 9; $p=0.07$; Figure 4.1b). While PSII efficiency for cells transferred from ML to LL remained relatively unchanged during

the initial three days, there was a significant increase in F_v/F_m on day 9 to 0.47 ($p<0.001$; Figure 4.1b). F_v/F_m for ML to HL isolates however significantly declined on sampling days 1 ($p<0.001$), 2 ($p<0.001$) 3 ($p< 0.001$) and 9 ($p<0.001$) compared to ML controls and showed minimal signs of recovery at the end of the experimental period following an initial drop from 0.37 to 0.26 on day 1 and remained impaired at 0.28 on day 9 (Figure 4.1b). Overall, the maximum quantum yield for ML *Symbiodinium* C1 cells shifted to HL and LL differed significantly for the duration of experiment ($p<0.001$).

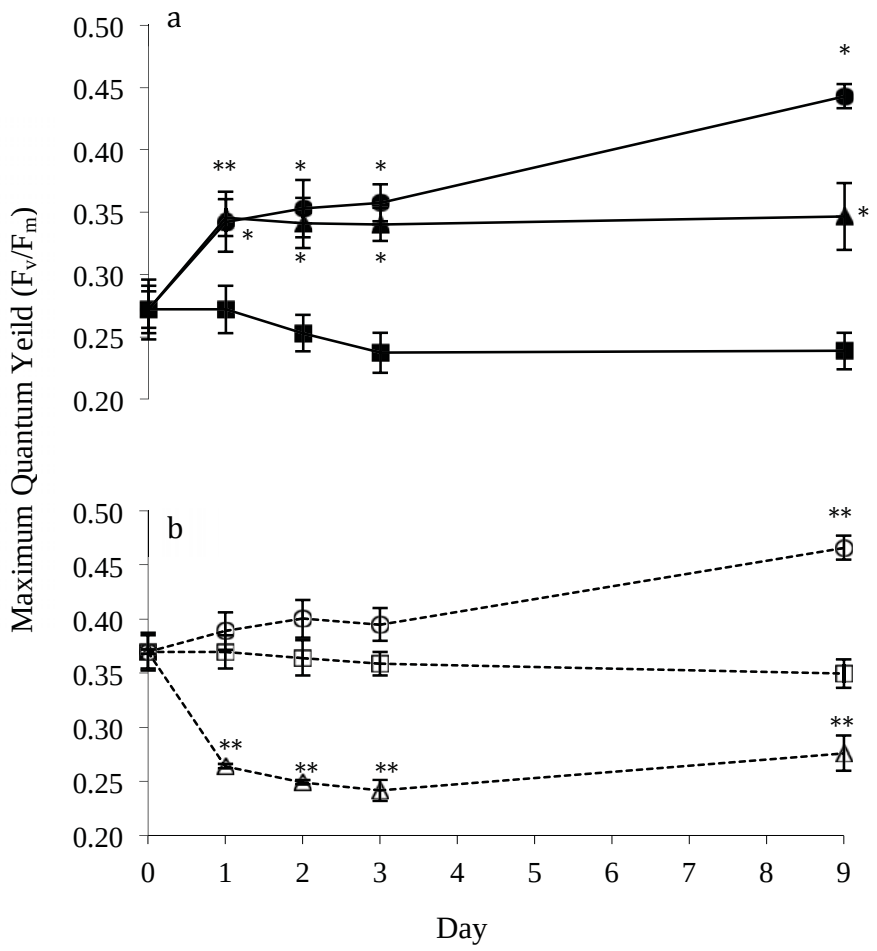


Figure 4.1. Photosystem II photochemical efficiency (F_v/F_m) of *Symbiodinium* sp. C1 acclimated to high-light (squares) or medium-light (triangles) conditions. (a) F_v/F_m for cells acclimated to HL conditions (filled squares; 250 μmol – 350 μmol quanta m^{-2} s^{-1}) then transitioned to ML (filled triangles; 80 – 100 μmol quanta m^{-2} s^{-1}) or LL (filled circles; 10-15 μmol quanta m^{-2} s^{-1}) conditions, (b) F_v/F_m for cells acclimated to ML conditions (open squares; 80 – 100 μmol quanta m^{-2} s^{-1}) then transitioned to HL (open triangles; 250 μmol – 350 μmol quanta m^{-2} s^{-1}) or LL (open circles; 10-15 μmol quanta m^{-2} s^{-1}). Control samples acclimated to HL (filled squares) and ML (open squares) were maintained at the same light condition for the duration of the experiment. Asterisks indicate statistically significant values of treatments verse

controls. Error bars represent the standard error for expression values for three biological replicates (n=3).

RNA transcript analysis

During the 9 day experimental period there were no statistically significant changes in mRNA transcript levels observed for any of the selected genes in cells of *Symbiodinium* C1 that were acclimated to HL levels and moved to either ML or LL levels (Figure 4.2).

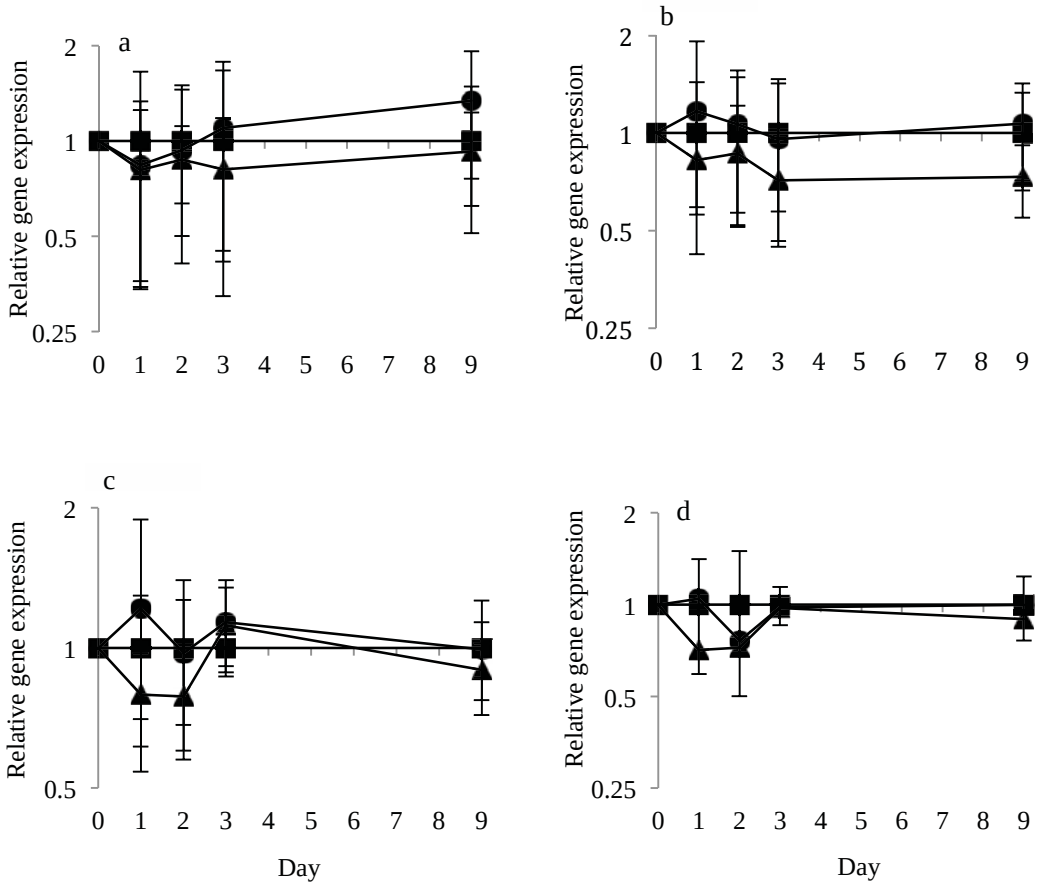


Figure 4.2. Transcript analysis of *Symbiodinium* exposed to varied light intensities. (a) SymBCA1, (b) SymBCA2, (c) acpPC_15 and, (d) acpPC_17 acclimated to high-light conditions (filled squares) prior to transitioning to medium light (filled triangles) or low-light (filled circles) conditions. Values expressed as ratios of treatment to controls (filled squares). Error bars are SEM (n = 3).

In contrast, there were statistically significant changes in transcript abundance of *Symbiodinium* C1 cells acclimated to ML conditions and moved to HL conditions (Figure 4.3a-d). mRNA transcript levels for SymBCA1, SymBCA2, acpPC_15 and acpPC_17, all experienced a significant decreases on day 9 compared to controls.

SymBCA1 and SymBCA2 transcript levels decreased by 2-fold ($p<0.001$; Figure 4.3a) and 1.9-fold respectively ($p=0.003$; Figure 4.3b), while acpPC_15 and acpPC_17 decreased by 1.8-fold ($p<0.001$; Figure 4.3c) and 1.9-fold ($p<0.001$; Figure 4.3d) respectively. There were no significant changes to transcript levels of SymBCA1, SymBCA2, acpPC_15 and acpPC_17 for cells that were acclimated to ML and moved to LL levels (Figure 4.3).

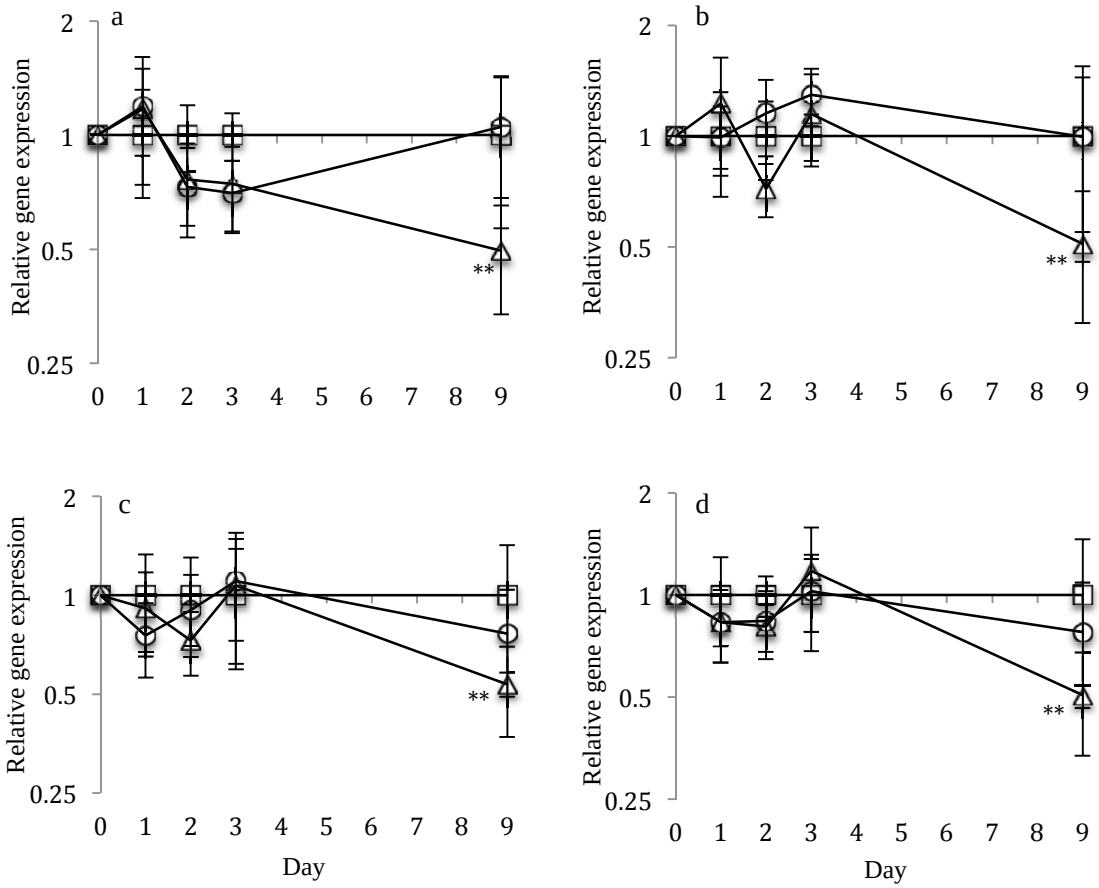


Figure 4.3. Transcript analysis of *Symbiodinium* exposed to varied light intensities. (a) SymBCA1, (b) SymBCA2, (c) acpPC_15 and, (d) acpPC_17 acclimated to medium-light conditions prior to transitioning to high-light (open triangles) or low-light (open circles) conditions. Values expressed as ratios of treatment to controls (open squares). Error bars are SEM (n = 3). Asterisks indicate significant differences between treatment and control ($p < 0.001$).

Discussion

Light is considered to be a major regulator of expression of photosynthetic proteins (Escoubas et al. 1995, Pfannschmidt et al. 1999, Pfannschmidt et al. 2008) and photoautotrophs exposed to limiting light levels demonstrate strategies that increase light adsorption while organisms exposed to HL conditions demonstrate strategies that alleviate oxidative stress (Lesser and Shick 1989). Modifying the transcription of specific genes involved in photosynthesis is therefore required for cells to adjust to a changing environment (Im and Grossman 2002). The present study examined the effects of varied light intensities on *Symbiodinium*, through transcript analysis of SymBCA1 and SymBCA2 and, on light-harvesting genes acpPC_15 and acpPC_17 involved in the capture of light energy. In addition, the photosynthetic efficiency of PSII under these varied light intensities was measured in order to determine if changes in transcript abundance are consistent with changes observed for photosynthetic efficiency and if the response time of *Symbiodinium* to these conditions occurs at the same rate as in other organisms.

In the present study, all cultures that were exposed to HL conditions showed significantly lower photochemical efficiencies compared to cultures that were exposed to either ML or LL conditions (Figure 4.1a and 4.1b), regardless of whether they were first acclimated to HL or ML conditions. A sudden rise in light intensity (or drop in CO_2 availability) increases excitation pressure at PSII (Allorent et al. 2013). Subsequently, after the onset of high excitation pressure, NPQ mechanisms are activated (Allorent et al. 2013). Decreases in F_v/F_m are characteristic of organisms exposed to HL conditions (Iwai et al. 2007). Such decreases in F_v/F_m can be associated with photoinhibition involving damage to PSII reaction centres, accumulation of photoinactive PSII (Baker 2008) or photoprotective processes

including NPQ (Brown et al. 1999). While a slight decline in F_v/F_m was observed for *Symbiodinium* acclimated to HL and left at HL conditions (HL controls), from 0.272 to 0.239 (Figure 4.1a), the decline may be due to photoacclimation, rather than photoinhibition, as after a reduction in the first 3 days, F_v/F_m stabilises at a new steady state for the remainder of the experiment. The opposite is true for cultures that were transferred to lower irradiances, with significantly higher F_v/F_m values overall, while those exposed to ML conditions maintained relatively constant F_v/F_m values throughout the experiment (Figure 4.1a and b). Despite the significant decrease in F_v/F_m at HL conditions and increases in F_v/F_m in *Symbiodinium* acclimated to HL and moved to ML or LL conditions (Figure 4.1a), significant changes in CA and or acpPCP transcript levels were not observed and therefore could not be linked to any changes in PSII for cells acclimated to HL and moved to ML or LL conditions. The light-harvesting complex II (LHCII) are one of the first detectable proteins to increase production when green algae are transferred from HL to LL conditions (LaRoche et al. 1991) and with *Dunaliella tertiolecta*, a decrease in light level corresponds with a 4 fold increase in chlorophyll a/b- binding protein (*cab*) mRNA within the first 9 hours (LaRoche et al. 1991). This initial increase is followed by a decrease in mRNA levels and a new stable state is achieved that is 2 fold higher than the *cab* mRNA levels of HL cells (LaRoche et al. 1991). The new steady state is reached within the first 18 h of being shifted to the LL conditions and is maintained for at least another 18 h (LaRoche et al. 1991). Increases in *cab* mRNA are detectable within 1.5 h of the cells being moved from HL to LL and changes in *cab* mRNA abundance correlate with increases in the amount of chlorophyll per cell (LaRoche et al. 1991). However care should be taken when comparing transcript abundance measured in the present study with protein amounts in other studies, as protein measurements were not

included in this study.

Significant changes in CA and acpPC mRNA transcript levels were however evident in *Symbiodinium* acclimated to ML and transitioned to HL conditions with significant down-regulation of both SymBCA1 and SymBCA2 and acpPC_15 and acpPC_17 on day 9, compared to controls (Figure 4.3 and 4.4). Correspondingly, the down-regulation of these transcripts coincided with decreases in F_v/F_m (Figure 4.1b). Intracellular redox levels, as set by photosynthetic electron transport rates, have previously been implicated in the regulation of nuclear-encoded genes associated with photosynthesis and has been suggested to be important for CCM induction (Escoubas et al. 1995, Pfannschmidt et al. 2001). For example, it was observed in *C. reinhardtii* that a mitochondrial CA involved in the CCM was not induced by low CO_2 conditions if cells were maintained in low light levels (<75 μmol quanta m^{-2} s^{-1}) (Villand et al. 1997). As light intensity is elevated, the imbalance between photon excitation and electron transport causes saturation of the photosynthetic rate. Photosynthesis therefore becomes incapable of using all the light energy that has been absorbed. Blocking photosynthetic electron transport has previously been shown to down-regulate expression levels of low CO_2- inducible genes, such as CAs, in *C. reinhardtii* (Dionisio-Sese et al. 1990, Im and Grossman 2002) and decreases the ability of cells to accumulate Ci for photosynthesis (Badger et al 1980).

A key observation in the present study is that while the physiological response of *Symbiodinium* moved to HL conditions is consistent with previous studies on *Symbiodinium* and other photosynthetic algae which report lower F_v/F_m values for HL conditions (Robison and Warner 2006, Hennige et al. 2009), the acclimation process of cells acclimated to ML and moved to HL takes place mostly during the first day for PSII while significant changes in transcription in both SymBCA1 and SymBCA2 and

acpPCs takes place much later on day 9 with relatively small fold changes (0.49 and 0.51 fold respectively; Figure 4.3a and b). In *C. reinhardtii* several transcripts encoding Ci transporters, mitochondrial CAs and light-harvesting proteins rapidly alter mRNA concentrations within 1-2 hours of a shift to HL conditions (Durnford et al. 2003, Tirumani et al. 2014). The abundance of mRNA encoding the PSII associated antennae proteins, LHCII and mRNA encoding LHCI complex, all decrease rapidly in response to HL conditions but recover to near starting abundance after approximately 8 hours at HL (Dunford et al. 2003). In addition, the mitochondrial CAs of *C. reinhardtii* (CAH4 and CAH5) are known to be up-regulated in light reaching maximum levels of transcript within 6 hrs of exposure to light and associated protein levels reaching maximum levels by 9-12 hours of light exposure (Tirumani et al 2014). Furthermore, transcript accumulation for the periplasmic CA (CAH1) was observed within 1-2 hours after cells of *C. reinhardtti* were exposed to HL conditions (Dionisio-Sese et al 1990). It must be noted however that these cultures were also grown in high CO_2 conditions prior to transfer to a combined low CO_2 and HL environment. There may be three possible reasons for a delay in observable mRNA transcript levels of *Symbiodinium* CAs and acpPCs in the present study. The first may relate to the mRNA stability of *Symbiodinium* under light stress. For example, a study on mRNA stability in the dinoflagellate *Karenia brevis* found dinoflagellate mRNA half-lives (the time required to degrade 50% of the mRNA) to be considerably longer than in other organisms (Morey and Van Dolah 2013). The majority of transcripts involved in the stress response, metabolism, and transcriptional regulation had half-lives over 24 h, and in some cases over four days (e.g., catalase/peroxidase, thioredoxin, and chaperone protein DnaJ) (Morey and Van Dolah 2013). Thus, detectable and significant changes in *Symbiodinium* mRNA expression

may require *Symbiodinium* to be subjected to longer experimental periods (Levin et al. 2016). The second possible reason for delayed changes in transcript abundance may be due to circadian regulation of proteins. Translational control mechanisms have previously been observed for circadian clock genes, such as bioluminescence (Morse et al. 1989), the nuclear-encoded chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fagan et al. 1999) and the water-soluble LHC protein PCP (Le et al. 2001), along with post-translational controls (Nassoury et al. 2001). It has also been demonstrated that both the periplasmic and mitochondrial CAs of *C. reinhardtii* are under the control of a circadian clock and are up-regulated in light (Rawat and Moroney 1995, Eriksson et al. 1998). In dinoflagellates, regulation of transcript levels of several photosynthetic genes have been shown to increase transcript abundance approximately 2 hours after exposure to light (Okamoto and Hastings 2003). Therefore, the possibility that more rapid changes in *Symbiodinium* CA and acpPC levels transcript levels occurs within the first few hours post transition cannot be ruled out. This however would need to be further investigated. The third possible reason may relate to the unique genomic organization and large genome size of *Symbiodinium* in which are permanently condensed (de la Espina et al. 2005). Due to this, it may mean that *Symbiodinium* are not able to rapidly alter gene expression patterns (Leggat et al 2011b).

While significant, detectable changes were observed in both *Symbiodinium* β-CAs and both acpPCs examined, these changes were once again minimal. Small (2-4 fold) differences in mRNA transcript levels for dinoflagellate genes however are not unusual, and have previously been observed in studies exposing *Symbiodinium* to a range of environmental stresses (Okamoto and Hastings 2003; Van Dolah et al 2007; Boldt et al 2009; Ogawa et al 2013; Gierz et al 2016). The results of the present study

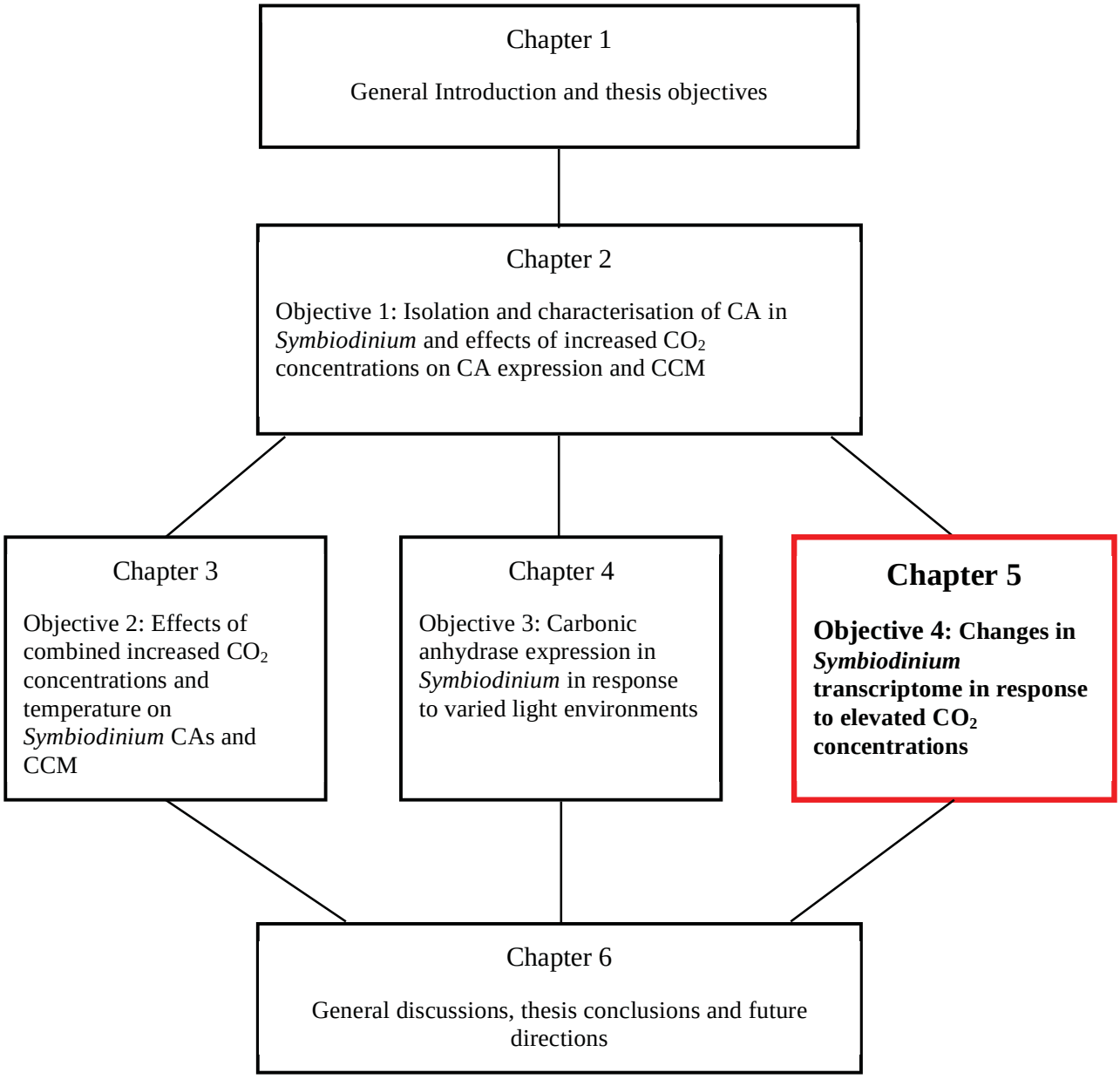
not only illustrate that dinoflagellate genes are possibly subject to translational or other controls that result in greater changes at the physiological level than those of the transcripts (Okamoto and Hastings 2003), but are also consistent with the results obtained in previous chapters of this thesis. Furthermore, the results of the present study demonstrate that like *C. reinhardtii, Symbiodinium* LHC transcripts decrease under HL conditions. In contrast, *Symbiodinium* β-CA transcript levels do not increase, as demonstrated in *C. reinhardtii*, but are instead lowered under HL conditions. This raises the possibility that CO_2 or light may not be a sole factor in regulation of *Symbiodinium* β-CAs but that an interactive effect between light and CO_2 is required. Given the diversity of CA genes identified in *Symbiodinium* so far, the question arises as to whether *Symbiodinium* CAs are regulated by the same control mechanism(s) and/or does this differ for different isoforms of CAs. These results also raise the question whether the β-CAs (SymBCA1 and SymBCA2) are regulated by the circadian clock.

Chapter 4 Summary points

- SymBCA1 and SymBCA2 transcript abundance from *Symbiodinium* sp. C1 lowered when cells were acclimated to ML and transitioned to HL. This is in contrast to CA transcripts of *C. reinhardtii* that increase under HL conditions.
- *Symbiodinium* LHC transcripts are lowered under HL conditions as in *C. reinhardtii*.
- Observed changes at the transcription level occur slower than responses evident in other algae.

- Changes to photosynthetic efficiency of PSII could not clearly be linked with mRNA expression
- The results of the present study support the notion that translational or post-translational control mechanisms potentially influence *Symbiodinium* CA and acpPC transcript abundance and are consistent with transcript changes of previous chapters of this thesis.

Schematic representation of thesis outline



Chapter 5: Changes in *Symbiodinium* transcriptome in response to elevated pCO_2 concentrations

Statement of Purpose

Endosymbioses between dinoflagellate algae (*Symbiodinium* sp.) and scleractinian coral species form the foundation of coral reef ecosystems. However in recent decades the coral-algal symbiosis has been increasingly threatened by anthropogenic disturbances such as increases in atmospheric CO_2, which are leading to elevated mean sea surface temperature (SST) and acidification of the oceans. Such changes in ocean chemistry are in turn causing increased occurrence and intensity of mass coral bleaching events on the Great Barrier Reef (GBR). Previous chapters of this thesis have employed targeted gene expression analysis (quantitative-PCR) as a method for identifying alterations of transcript abundance in *Symbiodinium* when placed under a variety of environmental stress conditions. While changes in transcription were identified, these changes were generally small, with regulation hypothesised to occur via translational or post-translational processes. Recent advances in sequencing technologies have enabled transcriptome-wide profiling allowing examination of expression response of a wide variety of genes when placed under stress. This method enables enhanced understanding of the stress biology of this important group of eukaryotes by examination of numerous molecular, cellular and biological features simultaneously. This chapter examined the transcriptome response of cultured *Symbiodinium* to elevated pCO_2 conditions using the Illumina-sequencing platform. In addition, transcriptome data was used to gain further information on the carbonic anhydrase (CA) gene families in *Symbiodinium*. Results from this experiment allow a better understanding of the response of cellular mechanisms of *Symbiodinium* under

environmental stress conditions.

Introduction

The dinoflagellates are an ecologically diverse group of eukaryotes that display many unique cellular and genetic traits. Of particular note, dinoflagellates contain some of the largest and most complex nuclear genomes ranging from 1.5 Gb to 245 Gb (Lin 2011) with the highest levels of DNA found in chromosomes that are permanently condensed and packaged into liquid crystalline forms instead of nucleosomes. Within these genomes, individual genes occur in multiple copies, are often encoded in tandem arrays (Bachvaroff and Place 2008, Lin et al. 2015) with all nuclear encoded mRNAs possessing an identical trans-spliced leader sequence (Zhang et al 2007). In addition, there appears to be a greater reliance on translational, rather than transcriptional, gene regulation in dinoflagellates (Lin 2011). While the dinoflagellates have some of the largest nuclear genomes of any eukaryote, their mitochondrial and plastid genomes are among the smallest in term of gene number with many chloroplast and mitochondrial genes transferred to the nucleus (Bachvaroff and Place 2008, Howe et al. 2008).

Members of the genus *Symbiodinium* are photosynthetic endosymbionts of stony corals and are largely responsible for the photosynthetic productivity that underlies the vast growth of tropical coral reefs (Falkowski et al 1984). Growing concerns on the effect of climate change conditions, such as warming oceans and ocean acidification, have lead to a surge in scientific research on the stress response of both the coral host and *Symbiodinium.* On a molecular level, numerous studies have focused and reported on the number and level of gene expression changes, under environmental conditions associated with bleaching such as warming oceans, predominantly in the coral host (DeSalvo et al. 2008, Voolstra et al. 2009, Polato et al. 2010, Aranda et al. 2016), while *Symbiodinium* gene expression studies has lagged

behind. The last decade however has seen a rapid growth in molecular studies for dinoflagellates, especially *Symbiodinium*, from expressed sequence tag (EST) analyses (Leggat et al 2007; Voolstra et al 2009), transcriptome studies (Leggat et al 2011; Bayer et al 2012; Rosic et al 2014; Xiang et al 2015; Levin et al 2016; Gierz et al 2017) to recent genome studies. Such genome studies include the publication of the first nuclear genome in 2013 for *Symbiodinium minutum* (clade B1; Shoguchi et al 2013), a complete chloroplast genome released in 2014 (clade C3; Barbrook et al 2014), the genome of *Symbiodinium kawagutii* released in 2015 (clade F1; Lin et al 2015) and the nuclear genome of *Symbiodinium microadriaticum* (clade A1; Aranda et al 2016). These next-generation sequencing projects have allowed for studies of specific gene families of interest and identification of specific molecular functions that may or may not be enriched in *Symbiodinium* compared to other eukaryotes. For example, comparative analyses of genomes both between the different *Symbiodinium* species and other eukaryotes have identified that, all dinoflagellates possess significantly more transmembrane transporters involved in the exchange of amino acids. In addition, there appears to be more lipids and glycerol in *Symbiodinium* than other eukaryotes and domains involved in the transport of carbon and nitrogen (bicarbonate transporters, carbonic anhydrases (CAs), and ammonium transporters) are specifically enriched in the *Symbiodinium* lineage.

The CA gene family is distinct from other families of enzymes due to the diversity that is exhibited both in cellular distribution and in the putative or biological functions of its member enzymes. These enzymes are involved in crucial physiological processes such as pH and CO_2 homeostasis and sensing, ion transport and respiration (Tashian 1989). In algae, plants and some bacteria, CAs play an important role in photosynthesis and biosynthetic reactions connected to it (Pocker

and Ng 1973, Graham et al. 1984, Smith et al. 1999, Xu et al. 2008, Capasso and Supuran 2015). Recent genome studies have provided insight into the extent of CAs present in *Symbiodinium*, which have shown CA domains to be overrepresented in the genomes of *S. microadriaticum, S. minutum* and *S. kawagutii* in comparison to other eukaryotic genomes (Aranda et al 2016). For example, 49 CAs have been identified in the genome of *S. kawagutii*, most of which are cytoplasmic with only two δ-CAs predicted to be localised to the plasma membrane and one β-CA in the thylakoids (Lin et al. 2015).

Advances in sequencing technologies have also enabled a transcriptome-wide approach to examine the stress response of *Symbiodinium* when cells are placed under environmental stress conditions (Xiong et al. 2015, Levin et al. 2016, Gierz et al. 2017). This method enables a better understanding of the stress biology of this important group of eukaryotes by examining numerous molecular, cellular and biological features simultaneously. However, despite recent advances in sequencing technologies, dinoflagellates are still one of the last major lineages of eukaryotes for which little is known about genome structure and organisation and large-scale gene expression studies in *Symbiodinium* are still lacking. While recent next-generation sequencing projects have provided large quantities of valuable data, some of these studies have lacked comparison between stress and non-stress conditions. In addition, while published transcriptional studies of cultured *Symbiodinium* under stress have explored *Symbiodinium* under conditions such as different light intensities with different growth conditions (Xiang et al 2015) and thermal stress (Levin et al 2016; Gierz et al 2017), additional studies are needed to provide insights into *Symbiodinium* transcriptomes under elevated pCO_2 conditions. Gaining further understanding of gene expression patterns in *Symbiodinium* when placed under elevated pCO_2 is a

critical step, not only to understanding the coral-algal symbiosis, but also on why the coral holobiont and hence coral reefs are susceptible to climate change.

Chapter 2 identified and characterised three CAs (two from the β-class and one from the δ-class CAs) from *Symbiodinium* and gene expression patterns when placed under elevated CO_2 (10 000 ppm) conditions. Previous chapters of this thesis have employed quantitative PCR (qPCR) as a method for gene expression analysis of *Symbiodinium* under stress conditions. These results demonstrated that alterations in gene expression generally occurred at low fold changes, with regulation hypothesised to occur via post-transcriptional mechanisms (Okamoto et al 2001; Bachvaroff et al 2008). In addition, previous chapters examining CA gene expression demonstrated that the particular CAs examined (SymBCA1 and SymBCA2) have only been altered by extreme CO_2 concentrations of 10 000 ppm CO_2 (Chapter 2)**.** This Chapter took a transcriptome-wide approach using RNA-seq, as opposed to the RT-qPCR based targeted gene expression analysis, to provide mechanistic insight into the stress response of *Symbiodinium* to high pCO_2 levels. In addition, this study identified and examined additional CA transcripts in the *Symbiodinium* transcriptome.

Material and Methods

Culture growth/Experimental conditions

Cultures of *Symbiodinium* sp. (Clade F) were grown in a controlled refrigerated incubator (Thermoline Scientific, Australia) on a 12:12 hr light/dark photoperiod cycle (80 – 100 μmol quanta m^{-2} s^{-1}) in tissue culture flasks (Sarstedt, NC, USA) containing 100 mL of ASP-8A media (Blank 1987). A control concentration of 450 ppm pCO_2 was used for growth of cells and for the experiment. During log-phase

growth approximately 10^6 cells/ml were equally divided into replicate flasks (n=10) for two experimental conditions; control pCO_2 (450 ppm pCO_2; n=5) and elevated pCO_2 (10 000 ppm pCO_2; n=5) conditions. Replicate flasks were maintained at each CO_2 concentration for a period of 35 days to induce expression of maximum number of genes. During the 35-day period all replicate flasks received fresh ASP-8A media at the same intervals and divided to maintain log-phase growth. All 10 flasks were then sampled during the mid-point of the light cycle (6 hours) on day 36. Cells of *Symbiodinium* were harvested for total RNA (tRNA) extraction to use in qPCR analysis and Illumina sequencing.

Chlorophyll analysis

A total aliquot of 3 ml of culture was taken from all 10 flasks in triplicate for pigment quantification. *Symbiodinium* cells were pelleted by centrifugation for 2 min at 5000 g for chlorophyll *a* (chl *a*) and chlorophyll *c* (chl c) quantification. Chlorophylls were extracted and measured as per Chapter 3 methodology and quantified using the equations for 90 % acetone (Jeffrey and Humphrey 1975).

Data Analysis

Statistics software package SPSS statistics (V19.0, IBM, USA) was used for all statistical analyses of physiological parameters. A generalised linear model was used for pairwise comparison of chl *a* and chl *c* with sequential Bonferroni *post hoc* test to determine significant differences between control and treatment.

Total RNA extraction and Illumina sequencing

Approximately 8 x 10^6 *Symbiodinium* cells were collected for tRNA extraction using an RNeasy Plant Mini kit (Qiagen, Valencia USA). See Chapter 3 methodology for details on extraction procedure and measurements. After extraction, concentrations of isolated tRNAs were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and approximately 100 ng of total RNA was treated with RQ1 RNase-Free DNase (Promega) as per Chapter 2 methodology. RNA-Seq libraries were prepared by the Australian Genome Research Facility (AGRF, Melbourne, Australia) using the Illumina TruSeq RNA sample preparation kit (Illumina) and standard Illumina protocols. Sequencing was performed on the 10 libraries by AGRF on an Illumina HiSeq 2500 platform on one lane, generating over 150 million 100 bp single-end reads.

mRNA extraction and quantitative PCR validation

Approximately 6 x 10^6 *Symbiodinium* cells were collected for mRNA extraction and snap frozen in lysing matrix D tubes (MP Biomedicals, Australia), for later mRNA extraction and RT-qPCR analysis. See Chapter 2 methodology for details on extraction procedures. DNase treatment of all mRNA samples was performed prior to reverse transcription with approximately 100 ng of mRNA. All mRNA samples were treated with RQ1 RNase-Free DNase (Promega) as per Chapter 2 methodology and used as a template for SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit following manufacturer's protocol (Invitrogen, California, USA). See Appendices Table 4-5 for reaction volumes.

RT-qPCR was performed to validate RNA transcription levels of selected genes of interest (GOI). Twelve GOI were chosen for qPCR validation (six up-

regulated genes and six down-regulated genes) (Table 5.1). Primers for these GOI were designed using the software DNASTAR Primer Select (Lasergene 11). Housekeeping genes used for this experiment were selected from previously established reference genes, which included S-adenosyl methionine synthetase (SAM), S4 ribosomal protein (Rp-S4), calmodulin (Cal) and cyclophin (Cyc) (Table 5.1) and validated using GeNorm in the qBASE plus software package (Biogazelle). Template cDNA dilution series were prepared to optimize quantification accuracy. For analysis, cDNA was diluted 1:40 prior to use as a template in the qPCR analysis. qPCR was performed using a Rotor-Gene™ 6000 (Corbett Life Science, Australia); see Chapter 2 methodology for detail on reaction mix and qPCR parameters. Modifications of Chapter 2 methodology for this experiment were: 62 °C elongation step during qPCR. Relative expression analysis was performed using qBASE plus 2.5 software (Biogazelle; http://www.biogazelle.com/products/qbasePLUS).

Data assembly and analysis

Sequencing reads from *Symbiodinium* were trimmed, removing sequence adaptors and low-quality regions, using libngs (https://github.com/sylvainforet/libngs) with a minimum quality of 20 bp and a minimum size of 75 bp. The trimmed reads were then assembled with Trinity (Grabherr et al. 2011) and the resulting assembly clustered using CD-HIT-EST (Fu and He 2012) using 90% similarity and a word size of 8. TransDecoder (Haas et al. 2013) and Blast2GO (Conesa et al. 2005) were used to predict protein-coding sequences. Distribution of gene ontology (GO) terms within the sequences that were differentially expressed were determined using Blast2GO (v4.1.5) (Conesa et al. 2016). Nucleotide sequences were imported into Blast2GO

and GO terms generated using default parameters for the blast (blastx), mapping and annotation steps (Conesa and Götz 2008).

To identify differentially expressed genes (DEG), a pairwise differential expression analysis was performed through Blast2GO (v4.1.5) (Conesa et al. 2016). Prior to analysis features with low counts across libraries were filtered and a Trimmed mean of M values (TMM) was used to calculate normalization factor. A pairwise comparison was used between control and treatment with exact test selected for statistical testing ($p < 0.05$). In addition, gene enrichment analysis (Fischer's exact test) of GO ID's was performed with a false discovery rate (FDR) correction (FDR ≤ 0.05) to determine the most specific GO terms for DEGs.

Results

Chlorophyll pigment quantification

Chlorophyll pigment analysis was determined for *Symbiodinium* at the completion (Day 36) of the experiment. Chl *a* content per *Symbiodinium* cell was increased in treatment cells compared to controls (Figure 5.1a). Analysis of chl *a* content found that there was a significant difference between treatment and control ($p<0.005$). Though chl *c* content increased in cells exposed to elevated pCO_2 no significant difference was found between control and treatment cells ($p < 0.214$; Figure 5.1b). In addition, no significant difference was observed in the ratio of chl *c* to chl *a* in *Symbiodinium* cells (Figure 5.1c).

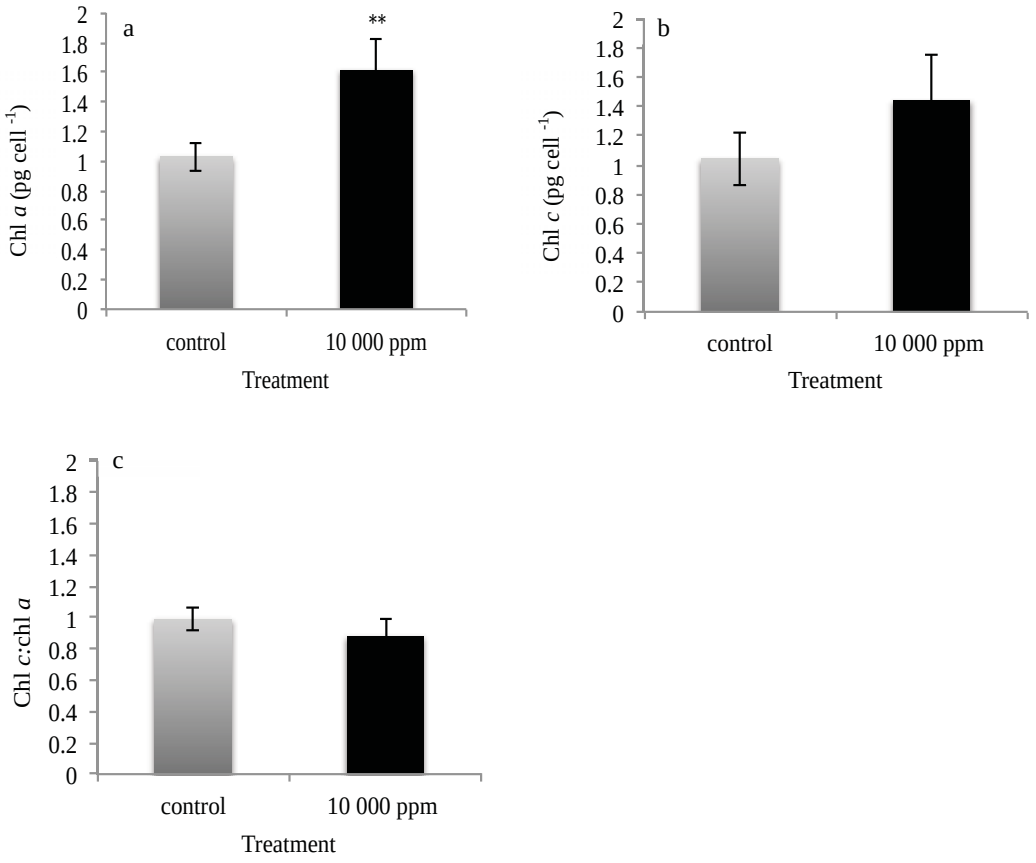


Figure 5.1. Chlorophyll pigment analysis of *Symbiodinium* clade F exposed to control (450 ppm pCO_2) and elevated pCO_2 (10 000 ppm). (a) Concentration of chlorophyll *a* (chl *a*) in *Symbiodinium* cells, (b) concentration of chlorophyll *c* (chl c) in *Symbiodinium* cells and, (c) ratio of chl *a* to chl *c* per *Symbiodinium* cells exposed to control (450 ppm pCO_2) (grey bar) and treatment (10 000 ppm pCO_2) (black bar). Error bars represent ± SEM (n = 5). Asterisks indicate significant difference to controls ($p < 0.05$).

Symbiodinium transcriptome features

Sequencing performed on the 10 libraries by AGRF on an Illumina HiSeq 2500 platform generated over 150 million 100 bp single-end reads from *Symbiodinium* clade F. Assembly of the reads yielded a total of 63 908 contigs of which 20 486 contigs (32.05%) were annotated by Blast2GO (v4.1.5; Conesa and Götz 2008; Figure 5.1a), with an average sequence length of 1023 bp (Figure 5.1b). Blast search resulted in 30 115 contigs without hits and 5667 contigs with hits (Figure 5.1a). A total of 7640 contigs were mapped with GO terms. (Figure 5.1a). A summary of features of the assembled transcripts is presented in Table 6 in Appendices.

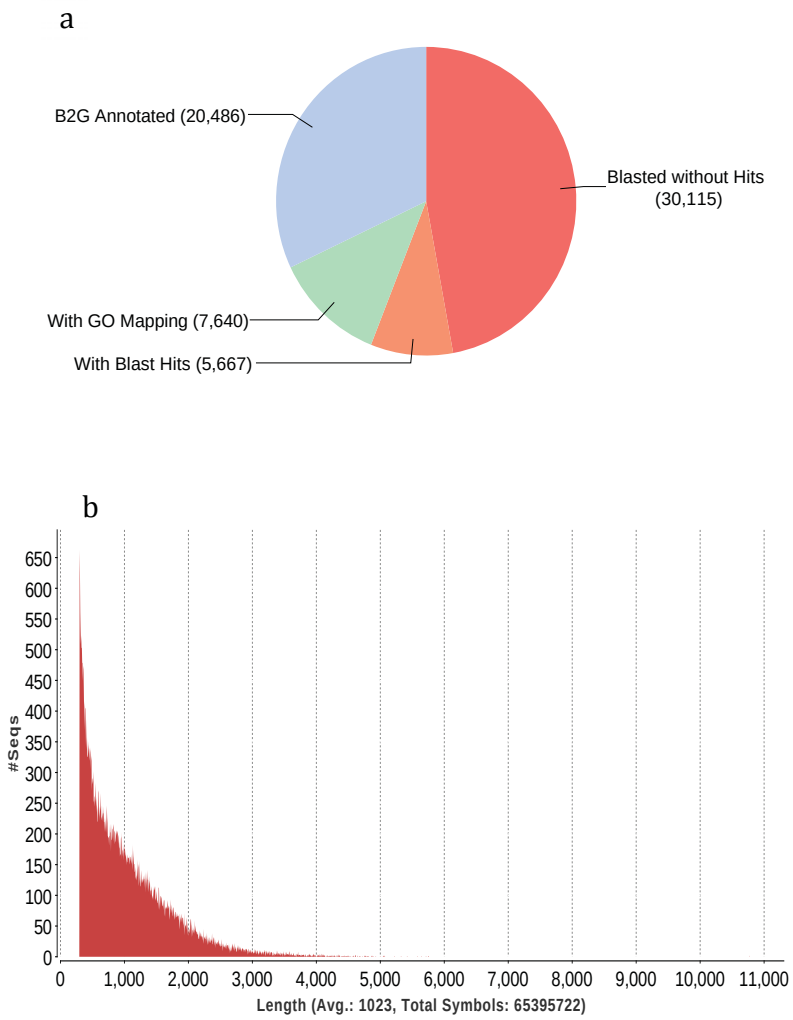


Figure 5.2. General characteristics of data distribution of *Symbiodinium* transcriptome. (a) pie chart representing the number of sequences mapped to gene ontology terms (green), number of sequences with (orange) and without hits after a blast search (red) and the number of contigs annotated by Blast2GO (blue). (b) representation of the number of sequences and corresponding sequence length.

Identification of differentially expressed genes

Differential gene expression in *Symbiodinium* in response to elevated pCO_2 concentrations for a period of 36 days was determined through Blast2GO software. A

visual summary of the pairwise differential expression analysis is presented in Figure 5.3. Analysis of sample relationship identified similarities between biological replicates and a clear separation between control and treatment samples with no outliers (Figure 5.3a). Overall, 12,728 transcripts were differentially expressed (FDR<0.05; p< 0.05; Figure 5.3b), accounting for approximately 20 % of the transcriptome. Of the 12,728 differentially expressed transcripts that were detected, 6620 significantly increased and 6108 were significantly lowered (FDR < 0,05; Figure 5.3b). The largest increase was a log-fold change (logFC) of 3.75 while the largest decrease was a logFC of 1.79 (Figure 5.3c and d). The average significant fold change of differentially expressed transcripts was < 1-fold change in expression (Figure 5.3c) with both highly and lowly expressed transcripts undergoing similar fold changes.

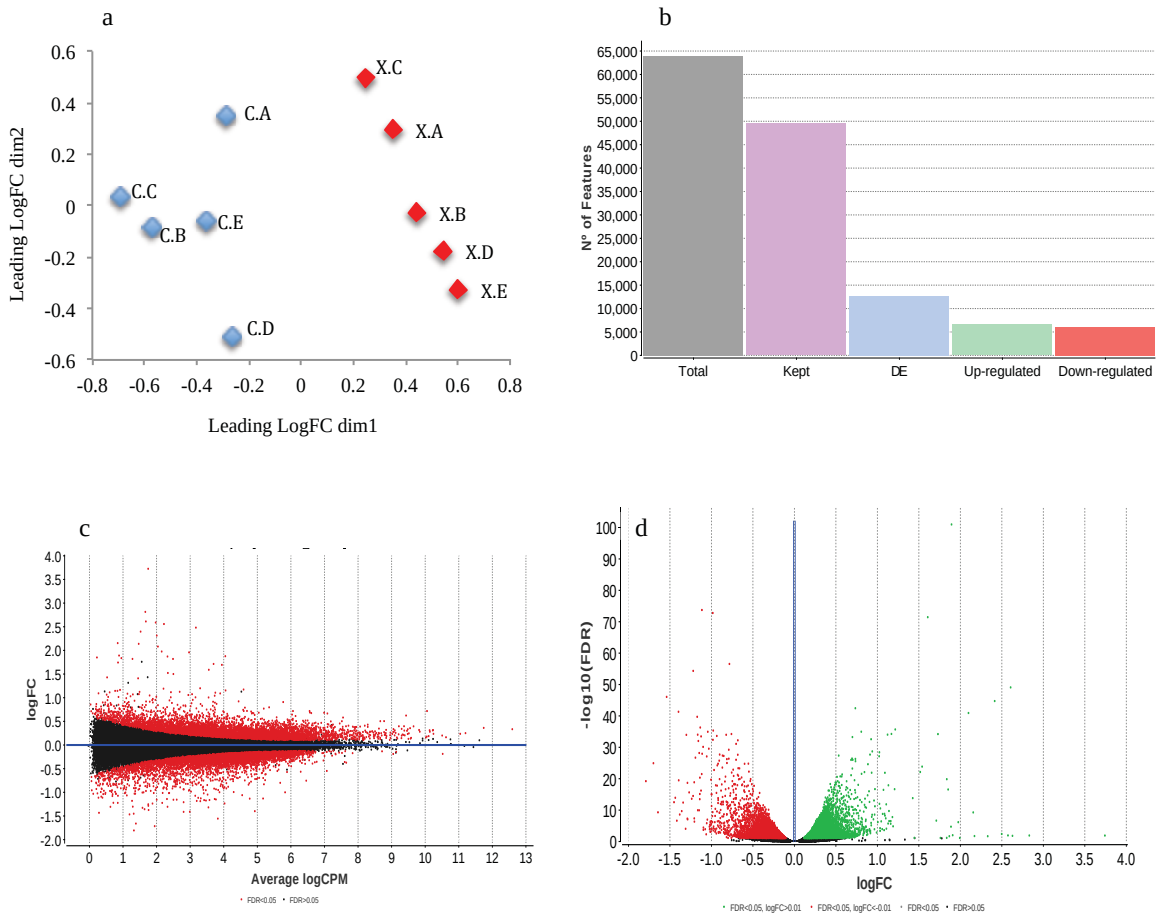


Figure 5.3. Differential expression analysis in *Symbiodinium* transcriptome when cultured cells were exposed to control (450 ppm pCO_2) and elevated CO_2 (10 000 ppm PCO_2) for 35 days. (a) multidimensional scaling (MDS) plot of experimental conditions in which the distances represent the typical log2 fold change between samples. Control samples (C. A – E) are shown in blue and elevated CO_2 samples (X.A – E) are shown in red. (b) bar chart representing a summary of differential expression results with the total amount of features in the transcriptome, the number of kept features that have passed the filtering step, the number of features differentially expressed and the number of features up-regulated and down-regulated, (c) log ratio (M) and mean average (A) plot comparing fold change and expression level with differentially expressed genes marked in red and, (d) Volcano plot

comparison of statistical significance and fold change. Up-regulated genes (green), down-regulated genes (red) and non-differentially expressed (black).

Gene ontology enrichment analysis

To further assess changes in transcript abundance affected by elevated pCO_2 conditions, GO term enrichment analysis was performed. Differentially expressed transcripts were further reduced to most specific GO terms (FDR < 0.05).

Over-represented functions in *Symbiodinium* transcriptome

GO term enrichment analysis identified several enriched functions in *Symbiodinium* cells exposed to elevated CO_2 concentrations along with the percentage of sequences annotated within each function (FDR<0.05; Figure 5.4). Biological functions involved in energy production, such as photosynthesis-light harvesting (GO:0009765), chlorophyll biosynthetic processes (GO:0015995), geranylgeranyl reductase activity (GO:0045550), PSI reaction centre (GO:0009538), ferredoxin-NADP+ reductase activity (GO:0004324), gluconeogenesis (GO:0006094), oxidative phosphorylation (GO:0006119), pentose-phosphate shunt (GO:0006098), and ATP synthesis coupled proton transport (GO:0015986) were significantly enriched in the up-regulated transcript set. The most notable enriched GO terms were DNA binding (GO:0003677), Ribosome (GO:0005840) and regulation of transcription, DNA template (GO:0006355).

Photosynthesis

Expression of the nuclear-encoded light-harvesting complex (LHC) proteins responsible for enhancing light transfer to core photosystems and photoprotection

were determined in elevated CO_2 cells. In total, fifty-seven transcripts encoding the *Symbiodinium* specific intrinsic membrane-bound LHC isoforms (acpPC), including transcripts encoding the photoprotective chlorophyll *a-b* binding protein L1818, fucoxanthin-chlorophyll *a-c* binding protein, caroteno-chlorophyll *a-c* binding protein, and LHC I LH38, were detected with significant increases under elevated CO_2 conditions.

Transcripts encoding enzymes involved in chlorophyll biosynthetic processes (GO:0015995) were also identified. One transcript encoding a magnesium-chelatase subunit H (*chlH*) was identified with significant increase in transcript abundance under elevated CO_2 conditions, along with one transcript encoding protochlorophylide reductase (POR) and one transcript encoding protochlorophylide reductase B (PORB). Six transcripts encoding geranylgeranyl diphosphate reductase, that catalyzes the reduction of geranylgeranyl diphosphate to phytyl diphosphate and provides phytol for chlorophyll synthesis, were also identified and increased.

Transcripts encoding subunits of Photosystem I (PSI) reaction center (GO:0009538) were identified within DEGs. Seven transcripts encoding polypeptide subunits of photosystem I (*psa*D, *psa*E, *psa*F, and *psa*L) were identified all with significant increases in transcript levels in *Symbiodinium* cells exposed to elevated CO_2 conditions. Five transcripts encoding for different isoforms of ferredoxin-NADP+reductase (GO:0004324), an enzyme involved in the last step of the primary phase of photosynthesis that produces NADP, were also detected with significant increases in transcript levels in cells exposed to elevated CO_2.

Carbonic anhydrases

A total of thirty-two transcripts encoding CAs were detected and annotated in the *Symbiodinium* transcriptome. Six transcripts were identified belonging to the α-CA gene family, 22 transcripts to the β-CA family, two transcripts to the γ- CA family and two transcripts to the δ-CA gene family. Of the 32 CA transcripts nine significantly increased in *Symbiodinium* exposed to elevated pCO_2 conditions (FDR <0.05; $p < 0.001$), all of which were predicted to be chloroplast targeted and belonging to the β-CA family (Table 5.2). One transcript (comp 104242_c0), showed homology to the SymBCA2 gene, previously identified in Chapter 2, and was significantly increased by elevated pCO_2 conditions in the present study (Table 5.2, shown in bold). This transcript was also predicted as chloroplast targeted, which is in contrast to the prediction of localisation of SymBCA2 in Chapter 2.

Sequence Name	LogFC	Description	CA gene family
comp104242_c0	**0.19**	**chloroplastic**	**β**
comp32265_c0	0.43	chloroplastic	β
comp58547_c0	0.32	chloroplastic	β
comp60366_c0	0.18	chloroplastic	β
comp77570_c0	0.56	chloroplastic	β
comp80831_c0	0.59	chloroplastic	β
comp80930_c0	0.73	chloroplastic	β
comp19027_c0	0.29	chloroplastic	β
comp95712_c0	0.51	chloroplastic	β

Table 5.2. Differentially expressed carbonic anhydrase genes in *Symbiodinium* clade F and their corresponding gene family and location. Log fold change (logFC) is the log-ratio of the transcripts expression value in control and treatment conditions. The transcript showing homology to SymBCA2 is comp 104242_c0 and is shown in bold.

Energy/ATP metabolism

Transcripts encoding proteins involved in energy/ATP metabolism were detected with significant increases in transcript abundance in *Symbiodinium* cells exposed to elevated CO_2 conditions. Nine transcripts encoding ATP synthases (representing F0 and F1 complex and subunits α, β, δ, and C) were identified with significant increases. Of the nine up-regulated ATP synthase transcripts, five were chloroplastic and four where identified as mitochondrial.

Transcripts encoding enzymes involved in the pentose-phosphate pathway (GO:0006098) were identified with significant increases in transcript levels. These included two transcripts encoding glucose-6-phosphate dehydrogenase (G6PD), one transcript encoding 6-phosphogluconate dehydrogenase (6PGD), one transcript encoding transketolase, and eight transcripts encoding fructose-bisphosphate aldolase.

Transcripts encoding succinate dehydrogenase complex II were identified and annotated. Succinate dehydrogenase is the only enzyme that is involved in both the mitochondrial electron transport chain and the citric acid cycle (tricarboxylic acid cycle). Two transcripts encoding succinate dehydrogenase (ubiquinone) iron-sulfur subunit 2 (comp71033_c0 and comp47174_c0) and one transcript encoding succinate dehydrogenase (ubiquinone) flavoprotein (comp72308_c0) all significantly increased in cells exposed to elevated CO_2 concentrations. In addition, transcripts encoding cytochrome b5, fumarate reductase, cytochrome c oxidase subunit 3 and cytochrome bc_1 complex involved in oxidative phosphorylation (GO:0006119) were also detected and significantly increased.

Transcripts encoding gluconeogenic pathway proteins (GO:0006094) were also identified in the transcriptome. Nine transcripts encoding fructose-bisphosphate aldolase, an enzyme that is also involved in carbon utilization (GO:0015976) were significantly increased in cells exposed to elevated pCO_2 concentrations. In addition, one transcript encoding phosphoenolpyruvate carboxykinase (PEPCK) was detected with significant increase in transcript levels.

Transcription factors

The most expressed GO terms were genes involved in DNA binding (GO:0003677) and the cellular component ribosome (GO:0005840) involved in the cellular process

of translation (GO:0005840; Figure 5.3a). A total of 124 transcripts were identified for DNA binding. The majority of these transcripts encoded proteins containing cold shock-like and cold shock domains (CSDs), representing homologs of bacterial cold shock proteins (CSPs) CspA, B and D and the eukaryotic upstream of N-ras (UNR) involved in regulation of translation and RNA stability. Transcripts encoding CSD containing proteins were also identified and significantly increased in GO term regulation of transcription (GO:0006355). Transcripts encoding mitochondrial and chloroplastic proteins associated with ribosomal subunits (30S, 50S, 54S, and 60S) involved in protein synthesis were also identified and showed significant increases in cells exposed to elevated CO_2 conditions.

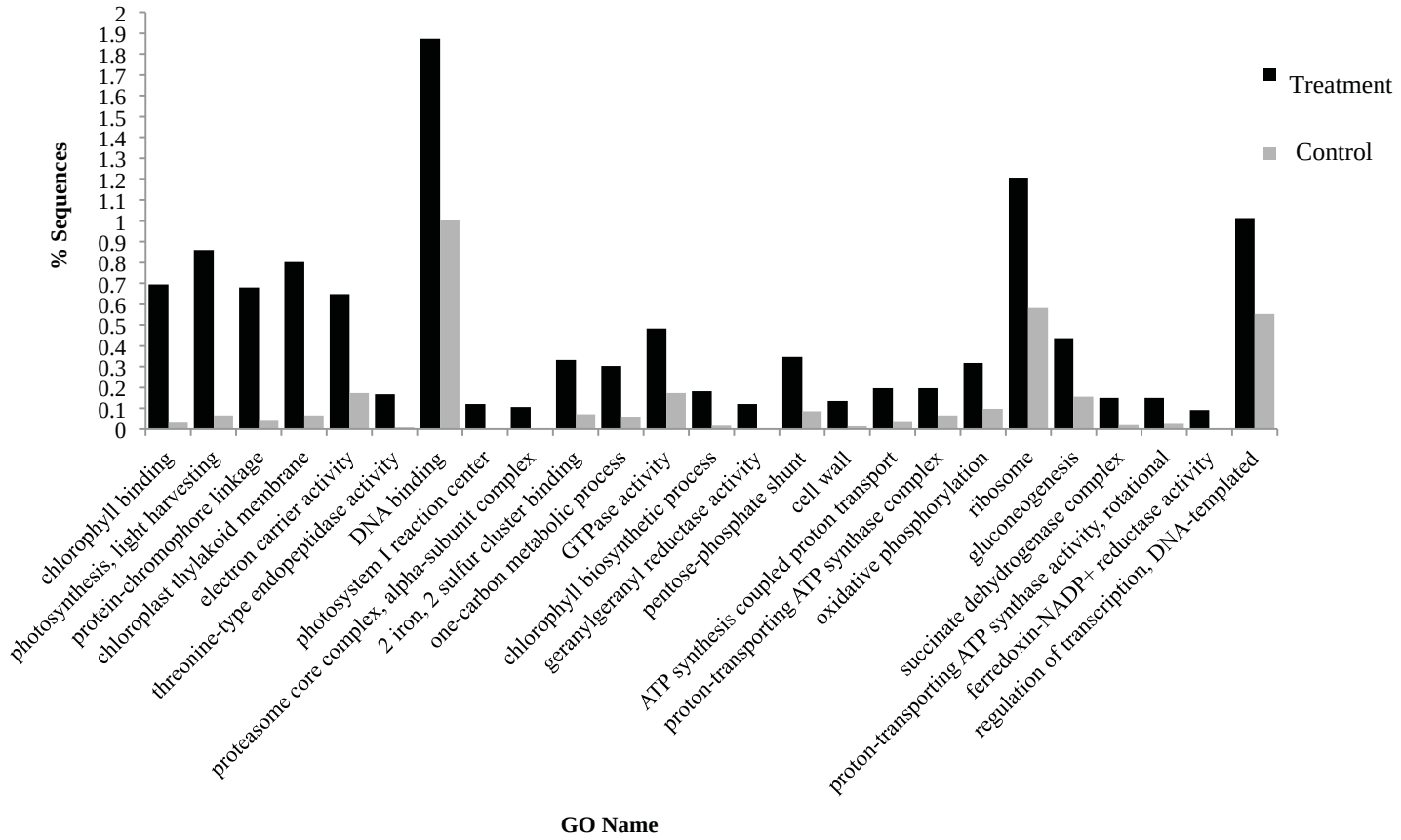


Figure 5.4. Gene enrichment analysis of most specific up-regulated GO categories in *Symbiodinium* clade F transcriptome. Enrichment analysis is expressed as significantly enriched GO terms (X-axis) and the relative percentage of sequences (%) within each GO term having a significance value of FDR < 0.05 (Y-axis). Black bars correspond to the treatment set (10 000 ppm pCO_2) and the grey bars represent control samples (450 ppm pCO_2).

Under-represented functions in *Symbiodinium* transcriptome

GO term enrichment analysis identified several under-represented functions in *Symbiodinium* cells exposed to elevated pCO_2 conditions (FDR <0.05; Figure 5.5). The most notable down-regulated molecular functions were protein binding (GO:0005515), ATP binding (GO:0005524), calcium ion binding (GO:0005509), and protein serine/threonine kinase activity (GO:0004674). Biological processes such as transmembrane transport (GO:0055085), protein phosphorylation (GO:0006468), and ammonium transport (GO:0015696) were also under-represented in *Symbiodinium* (Figure 5.5).

Transmembrane transport

A total of four hundred and fifty-one transcripts were significantly decreased for the GO term transmembrane transport (GO:0055085) in *Symbiodinium* cells exposed to elevated pCO_2 conditions (Figure 5.5). The most abundant transcripts within this GO category included channel proteins involved in ion transport such as, sodium channel proteins (type 2, 8, 9 and 11 subunit alpha A and type 4 subunit alpha B), potassium channel proteins (AKT2 and KAT1), and ATP-binding cassette (ABC) family transporters. ATP-binding cassette (ABC) family transporters are a large superfamily of membrane proteins that mediate the transport of a wide variety of molecules across cell membranes, including ions, amino acids, and peptides (Higgins 1992). ABC transporters convert the energy gained from ATP hydrolysis into trans-bilayer movement of substrates either into the cytoplasm (import) or out of the cytoplasm (export). Fifteen transcripts encoding various families (B, C, E, F, and G) of ABC transporters were identified and significantly decreased.

Transcripts encoding ammonium and nitrate transporter proteins were also identified and annotated. Ammonium transport is a key process in nitrogen metabolism. Sixteen transcripts encoding ammonium transporters (GO:0015696) belonging to the largest family of ammonium transporters (AMT1) were identified. Eight of the sixteen transcripts encoded different AMT1 genes (AtAMT1-1, AtAMT1-2 and AtAMT1-3) and were all significantly lowered in cells exposed to elevated pCO_2 conditions. Two transcripts encoding high affinity nitrate transporter 2.5 (AtNRT2.5), which is essential for nitrogen acquisition in *Arabidopsis* were also identified with significant decreases in transcript abundance.

Protein and ATP binding

Transcripts encoding molecular chaperones such as heat shock proteins (HSPs) were identified in the transcriptome. While HSPs were first identified as proteins whose synthesis was enhanced by stresses such as an increase in temperature, several HSPs have been shown to be involved in protein biogenesis. Three transcripts encoding HSP90 transcripts were identified and significantly decreased in *Symbiodinium* cells exposed to increased pCO_2 conditions. Twenty-one transcripts encoding Hsp40, also known as DnaJ, were identified and significantly decreased. The J domain of DnaJ interacts with Hsp70 and plays a role in regulating the ATPase of Hsp70. Two transcripts encoding HSP70 proteins were also identified and significantly lowered. In addition, two DNA damage repair RAD proteins (RAD23-1; comp45467 and RAD16 homolog; comp73817) were annotated and displayed significantly lower transcript levels.

Protein phosphorylation

Serine/threonine-protein kinases are crucial components of various cellular processes, including regulation of cell proliferation, meiosis (Draetta 1990), and apoptosis (Cohen et al. 2005). Fifty-six transcripts representing various serine/threonine-protein kinases families were detected. One transcript encoding the Aurora-B class was identified along with one cyclin-dependent transcript and six never-in-mitosis A serine/threonine kinase (Nek). All transcripts were significantly lowered in cells exposed to elevated CO_2 concentrations. Glycogen synthase kinase (GSK) is a serine/threonine kinase with important roles in the regulation of glycogen synthesis, protein synthesis, gene transcription, and cell differentiation in various cell types. One transcript encoding GSK (GSK3B; comp75008) involved in protein phosphorylation (GO:0006468) was identified and significantly decreased under elevated pCO_2conditions ($p< 0.007$).

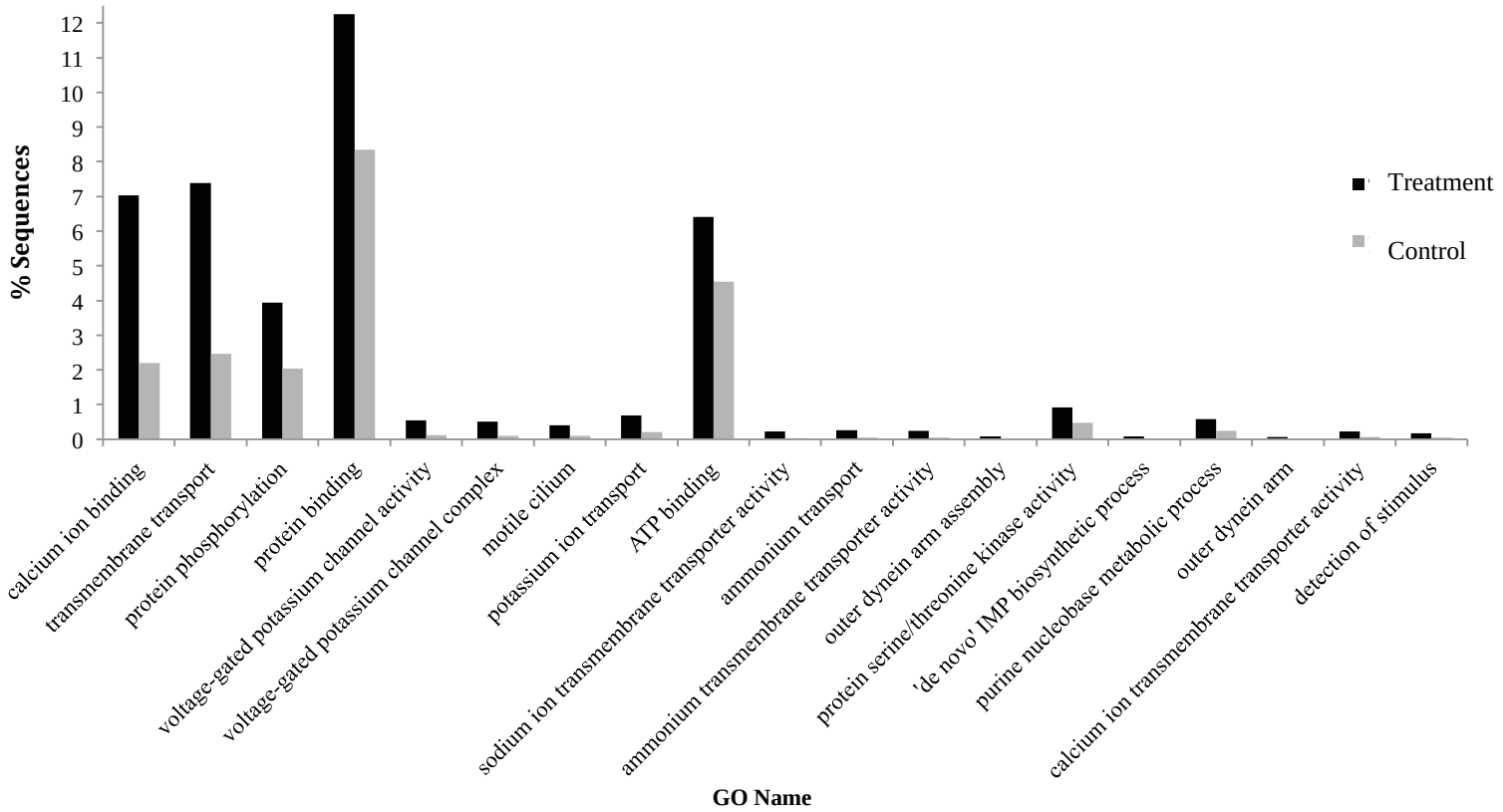


Figure 5.5. Gene enrichment analysis of most specific down-regulated GO categories in *Symbiodinium* clade F transcriptome (FDR < 0.05). Enrichment analysis is expressed as significantly enriched GO term (X-axis) and the proportion of genes that are down-regulated in the transcriptome with percentage of sequences represented within each GO term (Y-axis). Black bars correspond to the treatment set (10 000 ppm pCO_2) and the grey bars represent control samples (450 ppm pCO_2).

Validation of gene expression by qPCR

To validate the RNA-Seq results, qPCR was performed on six up-regulated GOI and six down-regulated GOI (Figure 5.6). The particular GOI were chosen to represent a wide range of expression levels and expression patterns under the pCO_2 conditions used in the experiment. Most of the selected genes showed a positive correlation between mRNA-Seq and qPCR (Figure 5.6).

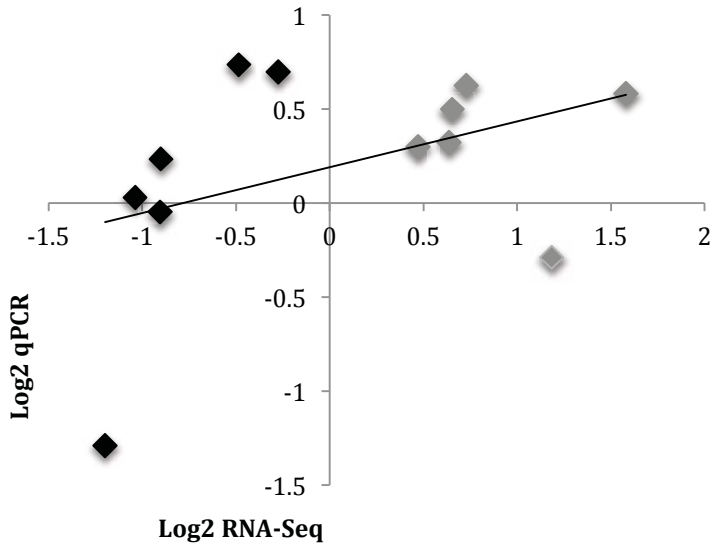


Figure 5.6. Validation of RNA-Seq approach using quantitative PCR. Gene expression levels of six up-regulated (grey diamonds) and six down-regulated (black diamonds) genes quantified by qPCR. Results were compared with those obtained using RNA-Seq method. The log2 change in expression of qPCR and RNA- Seq was positively correlated indicating the accuracy of the RNA-Seq approach for quantification.

Discussion

The present transcriptome study was designed employing a treatment condition of elevated pCO_2 (10 000 ppm pCO_2) and cells of *Symbiodinium* clade F. The primary objective was to gain a general insight into the transcriptome-wide changes that occur in response to elevated pCO_2 conditions and to further examine CA genes in *Symbiodinium*. The particular CO_2 concentration of 10 000 ppm, while extreme, was used to mirror the work carried out in Chapter 2. In addition, 5 % CO_2 is routinely used to study the effects of elevated CO_2 concentrations in many physiological studies (Spalding et al. 1983, Moroney et al. 1987, Fukuzawa et al. 1990, Marek and Spalding 1991, Bozzo and Colman 2000, Moroney and Ynalvez 2007).

Analysis of the *Symbiodinium* transcriptome revealed that approximately 20% of the transcriptome was differentially expressed under elevated pCO_2 conditions (FDR < 0.05). Of the 12,728 differentially expressed transcripts that were detected, 6620 transcripts significantly increased while 6108 transcripts were significantly lowered. This is similar to what has been observed in a transcriptome study of *C. reinhardtii* where shifts in gene expression caused by transition from high CO_2 to limiting CO_2 modifies the expression of around 14-38 % (about 2200 to 5880 genes) of the approximately 15 500 *chlamydomonas* genes (Brueggeman et al. 2012, Fang et al. 2012). In the present study, the majority of differentially expressed transcripts displayed ≤ 1-fold change in abundance, which correlates with work described previously in this thesis (Chapters 2, 3 and 4) and of targeted expression studies (Leggat et al. 2011a; Rosic et al. 2011; Ogawa et al. 2013; Gierz et al. 2016). Due to this it has been hypothesised that translation or post-translational regulation may be critical in *Symbiodinium* cellular responses. Overall, increasing CO_2 supply to *Symbiodinium* clade F cultures enriched functions such as, photosynthesis, light-

harvesting, chlorophyll biosynthetic processes, metabolic pathways and translation and transcription factors (Figure 5.3), while transcripts encoding proteins involved in protein and ATP-binding, transmembrane transporters, and ammonium transport were significantly lowered under elevated pCO_2 conditions.

Specific functions enhanced under elevated CO_2

Analysis of chlorophyll pigments in *Symbiodinium* found significantly increased chlorophyll content in cells exposed to 10 000 ppm pCO_2 (Figure 5.1a). Chl *a* concentrations were significantly higher in *Symbiodinium* cells exposed to elevated pCO_2 conditions at the completion of the experiment (Figure 5.1a). Higher chlorophyll content in plants has been suggested to be an adaptation under elevated CO_2 to increase photosynthetic activity (Bhatt et al. 2010). Increases in *Symbiodinium* chl *a* have also been observed under numerous studies investigating thermal stress (Ogawa et al. 2013, Gierz et al. 2016, Gierz et al. 2017). Interestingly, comparison of growth rates and chlorophyll content in *Symbiodinium californium* found cultures exhibiting low growth rates, when incubated at different temperatures of 5, 10, and 30 °C, also showed an increase in chlorophyll *a* content (McBride et al. 2009). This pattern of increase chlorophyll *a* content and low growth rate has also been observed in the present study. In phytoplankton, variation of chl *a*-specific absorption has been attributed to packaging of chlorophylls within different pigment-protein complexes (Bissett et al. 1997). It may be possible that as with thermally stressed *Symbiodinium* cells, *Symbiodinium* exposed to elevated pCO_2 concentrations increase chl *a* content due to changes in specific pigment-protein complexes within the chloroplasts. Analysis of chl *c* content (Figure 5.1b) and the ratio of chl *c* to chl *a* (Figure 5.1c) found that there were no significant differences between controls and treatment cells.

In accordance with increases in chl *a* content, gene enrichment analysis of differentially expressed transcripts identified increases in transcripts encoding magnesium-chelatase subunits (*ChlH*), protochlorophylide reductase and geranylgeranyl diphosphate reductase involved in chlorophyll biosynthesis (GO:0015995). The synthesis of Chl *a* from glutamic acid occurs via a complex pathway consisting of at least fifteen reactions (Von Wettstein et al. 1995). Magnesium-chelatase subunits (*ChlI*, *ChlH* and *ChlD*) act as catalysts for the ATP dependant insertion of a magnesium ion into the chl *a* structure (Suzuki et al. 1997). The *ChlH* subunit is known to bind protoporphyrin and has also been suggested to be the catalytic subunit. Another important step in chlorophyll synthesis is the light-dependant conversion of pchlide into chlide, which is achieved by the enzyme protochlorophyllide reductase (POR). In contrast, a number of photosynthetic eukaryotes synthesize an enzyme that reduces protochlorophylide irrespective of light, thereby providing these organisms the ability to synthesize chlorophyll in the dark (Dring 1988). Transcripts encoding the third identified enzyme involved in chlorophyll biosynthesis, geranylgeranyl diphosphate reductase, were identified in the present study. This particular enzymes catalyses the reduction of geranylgeranyl pyrophosphate to phytyl pyrophosphate and provides phytol for chlorophyll synthesis.

Enhanced photosynthesis

Gene enrichment analysis identified increases in numerous transcripts encoding several genes involved in the primary stages of photosynthesis under elevated CO_2. This was typified by increases in transcript abundance of light harvesting complex (LHC) proteins, components of PSI reaction centre, PSII subunits, ATPsynthases and the final part of the photosynthetic electron transfer ferredoxin and ferredoxin-$NADP^+$

reductase activity (Figure 5.3). This pattern of up-regulation of photosynthetic genes under CO_2 enrichment is similar to that seen in other algae (Riebesell et al. 1993, Hein and Sand-Jensen 1997, Peng et al. 2016, Sun et al. 2016)

LHC proteins are peripheral components of PSI and PSII and are essential for capturing and transferring light energy to the photosystems as well as playing a protective role in dissipating excess energy as heat (Iglesias-Prieto et al. 1993, Takahashi et al. 2008). The LHC superfamily exhibits a high degree of sequence diversity and includes subfamilies such as the Chl *a* and *b* binding proteins (Cab), the Chl *a* and *c* binding proteins (Cac), the Chl *a* and phycobilin binding proteins (LhcaR, Lhc), and fucoxanthin-Chl *a* and *c* binding proteins (Fcp) and those associated with protective mechanisms and stress responses such as the LI818 and LI818-like proteins (Richard et al. 2000, Koziol et al. 2007). Of the fifty-seven transcripts identified in the present study all significantly increased, including transcripts encoding the LI818 and LI818-like proteins. The increase in chlorophyll from CO_2 enrichment observed in the present study may therefore be available for trapping more light energy for photosynthesis. Furthermore the increase in L1818 and L1818-like proteins may also indicate that CO_2 enrichment could improve the resistance to stress of *Symbiodinium*.

Photosynthetic ATP synthesis can be generated through at least two routes. Linear electron flow (LEF) is driven by photosystem II (PSII) and PSI, which involves electron transfer via ferredoxin (Fd) and ferredoxin-NADP+reductase activity (FNR) to reduce $NADP^+$, generating NADPH and ATP for the Calvin-Benson cycle (Hald et al. 2008). Transcripts encoding subunits of PSII that increased included *psb*J, which is involved in electron transfer within PSII, *psb*K which binds plastoquinone and also has a role in maintaining the dimeric structure of PSII and *psb*F which encodes the β-subunit of cytochrome b559. Cytochrome b559 is a ubiquitous component of PSII

located close to the D1 and D2 reaction centre subunits (Nanba and Satoh 1987). Previous studies have identified that cytochtome b559 plays a structural role during early stages of PSII assembly and may also be involved in a secondary electron transfer pathway that helps protect PSII from photodamage (re(Shinopoulos and Brudvig 2012, Hamilton et al. 2014). It is generally considered that the reactions of linear electron flow generate less ATP than is required for the metabolic reactions of photosynthesis (Osmond 1981, Kramer and Evans 2011, Foyer et al. 2012). In addition, the ATP demand varies depending on environmental conditions and on metabolic status. Cyclic electron flow is an alternate electron transfer route that involves only PSI. Cyclic electron flow generates extra proton gradient by recycling electrons around PSI, resulting in extra ATP in the absence of NADPH (Kramer and Evans 2011). The PSI complex acts as a light-driven enzyme that catalyses the transfer of electrons from plastocyanin in the thylakoid membrane to ferredoxin on the stromal side of the thylakoid membrane (Jensen et al. 2007). PSI is composed of 15 core subunits and associated with light-harvesting complex I (LCHI). Elevated pCO_2 enhanced several subunits PSI reaction centre (*psa*D, *psa*E, *psa*F and *psa*L). In particular, both *psa*D and *psa*E subunits are involved in the binding of ferredoxin (Jensen et al. 2007). Additionally, *psa*D is required for the assembly of *psa*E and *Psa*C into the PSI complex.

The last step of the primary phase of photosynthesis involves the production of NADPH via FNR. Previous studies on the microalga *Coccomyxa subellipsoidea* C-169 have demonstrated up-regulation of ferredoxin and FNR under elevated CO_2 and have implied that the increase in energy generated through photosynthesis might be converted and transferred through ferredoxin to sustain rapid growth and lipid accumulation (Peng et al. 2016). NADPH is also known to be a reducing agent

involved in protecting cells against reactive oxygen species (ROS) (Clement et al. 2017). In the present study, five transcripts encoding ferredoxin-NADP+reductase were identified with significant increases (Figure 5.3). The increased supply of NADPH, as a result of enhanced FNR activity, may therefore play a photoprotective role in *Symbiodinium* in the present study. However, further studies would need to be conducted to determine if this is the case in *Symbiodinium* clade F cells exposed to elevated pCO_2 concentrations.

Carbonic Anhydrase

CAs are proteins that form integral parts of carbon-concentrating mechanisms (CCMs), which play an important role in the acquisition of inorganic carbon for photosynthesis in many algae (Leggat et al. 1999; Giordano et al. 2005; Oakley et al. 2014). Genome studies on *Symbiodinium* have identified enrichment of CAs in the genomes of *S. microadriaticum*, *S. minutum*, and *S. kawagutii* in comparison to other eukaryotic genomes (Aranda et al. 2016). In the present study thirty-two transcripts encoding CAs were identified, one of which displayed homology to the SymBCA2 gene identified in Chapter 2 (comp 104242_c0). Of the thirty-two transcripts, nine (including comp 104242_c0 transcript) were differentially expressed with significant increases under elevated CO_2 conditions (Table 5.2). The increase in transcript levels of these CAs under elevated pCO_2 conditions is surprising given that previous chapters of this thesis have shown decreases of CA transcripts under elevated CO_2 conditions, which is also consistent with previous studies (Price and Badger 1989, Sültemeyer et al. 1989, Matsuda and Colman 1995, Giordano et al. 2005, Falkowski and Raven 2007). Of particular note is the increase in transcription of comp 104242_c0 transcript, which is in contrast to results obtained in Chapter 2 (Figure

2.7b). However it must be noted that the C1 phylotype was used in Chapter 2 as opposed to the F phylotype examined in the present study. While Chapter 3 examined cultures of *Symbiodinium* F grown under elevated pCO_2, the 10 000 ppm pCO_2 concentration was not used, therefore a comparison cannot be made between the two chapters. The contrasting results now raise the question as to whether the role of one particular CA in *Symbiodinium* can differ according to the phylotype examined. Further experiments would need to be conducted to investigate this hypothesis.

The majority of CA transcripts identified in the study, were represented by the β-class, and predicted to be chloroplast targeted. In addition, all nine transcripts that were differentially expressed represented the β-class and were also predicted to be chloroplast targeted. Given that the β-class of CAs are much more diverse in their quaternary structure, sequence, and distribution than any of the other CA families, the higher representation of members in the β-class CAs in the present study is not surprising. This is also evident in *C. reinhardtii* where six of the twelve known CAs belong to the β-class (Mitra et al. 2005, Ynalvez et al. 2008). In addition, chloroplastic CAs seem to be the most important CAs in the photosynthetic CO_2 flux of microalgal cells by facilitating the diffusion of CO_2 across the chloroplast envelope or by rapidly dehydrating bicarbonate to CO_2 (Price et al. 1994). Surprisingly, the results of this study predicted the transcript showing homology to SymBCA2 to also be chloroplast targeted. This is in contrast to chapter 2 that predicted SymBCA2 to be located in the cytosol. However, the prediction made in chapter 2 was due to the absence of any transit peptides, as the full-length sequence was not obtained.

Enhanced metabolism

While photophosphorylation in photosynthesis provides the ATP and NADPH for the

Calvin cycle, enhanced carbon fixation raises higher demand of ATP and NADPH, which can be generated from alternative pathways such as pentose-phosphate pathway and oxidative phosphorylation. In the present study, elevated CO_2 affected transcripts encoding proteins involved in metabolic process such as the pentose-phosphate pathway, oxidative phosphorylation and gluconeogenesis. These results suggest that under high CO_2 concentrations more fixed carbon is possibly driven into these pathways to produce more intermediates and metabolic energy for anabolism. In addition, the results of the present study indicate that energy production is matched to metabolic demand in *Symbiodinium* clade F exposed to elevated CO_2 conditions as both photosynthesis and metabolism are enhanced.

The oxidative pentose phosphate (OPP) pathway is a strategy to provide NADPH for biosynthetic processes. Transcripts encoding the critical enzymes for OPP pathway identified in the present study, glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), transketolase and fructose-bisphosphate aldolase were all significantly increased. These enzymes participate in several reactions of the pathway to generate NADPH and ribulose-5-phosphate (a precursor for ribose-5-phosphate) (Kruger and von Schaewen 2003). The increased production of NADPH observed in the present study may therefore be used to support carbon assimilation. Increased transcripts of both G6PD and 6PGD involved in the OPP pathway have also been observed in the diatom *Phaeodactylum tricornutum* under elevated CO_2 concentrations (Wu et al. 2015).

Genes encoding enzymes for the unique reactions in gluconeogenesis, PEP carboxykinase (PEPCK) and fructose-1,6-bisphosphatase (FBP) were also significantly increased in the present study, indicating that the gluconeogenic pathway was in use under elevated pCO_2 conditions. Enhanced gluconeogenic pathway

enzymes have been identified in *Symbiodinium* cells exposed to thermal stress and has been suggested as a mechanism to reduce free fatty acids in cells to carbohydrates (Gierz et al. 2017). PEPCK catalyzes the conversion of oxaloacetate into phosphoenolpyruvate (Pilkis and Granner 1992) and FBP is responsible for the conversion of fructose-1,6-biphosphate into fructose- 6-phosphate. Other than gluconeogenesis, FBP is also involved in chloroplast conversion of d-fructose 1,6-biphosphate into fructose-6-phosphate. Previous studies have demonstrated that over-expression of FBP in *C. reinhardtii* has a detrimental effect on growth and can led to decreases in biomass production (Dejtisakdi and Miller 2016). The results of the present study have also demonstrated this pattern of increased transcript abundance of FBP with decreases in transcripts encoding genes involved with cell regulation. These results therefore suggest that exposure of *Symbiodinium* to pro-longed periods of elevated pCO_2 concentrations has a detrimental effect on growth.

In eukaryotes, the electron transport chain found in the inner mitochondrial membrane is a composed of four multiprotein complexes (termed complex I-IV) and serves as the site of oxidative phosphorylation through the use of ATP synthase. Transcripts encoding genes involved in electron transport and oxidative phosphorylation were significantly increased in *Symbiodinium* exposed to elevated CO_2. These included transcripts encoding several subunits of Complex II (succinate dehydrogenase), Complex III (cytochrome bc1 complex), IV (cytochrome c oxidase) and ATP synthase. ATP synthase, also known as Complex V, is the final enzyme in the oxidative phosphorylation pathway and uses the energy stored in the proton gradient across the membrane to drive synthesis of ATP from ADP and phosphate. In the present study, four transcripts encoding ATP synthase coupled to mitochondrial electron flow were significantly increased. Previous transcriptome studies on

microalgae have demonstrated that enhanced oxidative phosphorylation is a way of supplying adequate ATP for rapid growth and biosynthetic processes under high CO_2 concentrations (Peng et al 2016).

Enhanced transcription and translation factors

Transcripts encoding DNA binding proteins were also significantly increased in *Symbiodinium* exposed to elevated pCO_2, the majority of which carried a cold shock domain (CSD). While the CSD is not common in other eukaryotes, previous studies on the dinoflagellate *Lingulodinium* identified that 68% of the identified DNA-binding domains fall into the class of CSD (Mihailovich et al. 2010), which has therefore been suggested to be a lineage-specific expansion in dinoflagellates (Bayer et al. 2012). Originally identified as a reaction to cold shock in *E. coli*, CSD containing proteins have now been found in all three domains of life and have been associated with various functions such as regulation of transcription (by binding DNA), splicing, translation (Chaikam and Karlson 2010), and can also function as RNA chaperones (Jiang et al. 1997, Phadtare 2004) Because these domains also bind mRNA, they are also known for their role in post-transcriptional regulation in eukaryotes (Mihailovich et al. 2010). However in the present study, the increased expression of DNA-binding proteins containing the CSD suggests that these particular proteins are involved in transcriptional regulation in *Symbiodinium* exposed to elevated pCO_2 as opposed to post-translational regulation. This fits with the observed increase in the GO category regulation of transcription (GO:0006355) also identified in this study. Proteins containing the CSD may therefore be responsible for much of the transcriptional regulation in dinoflagellates.

Specific functions down-regulated under elevated CO_2

Reduced transmembrane transport

Transmembrane transporters are involved in the translocation of diverse nutrients and ions into and out of cells that are all vital for cellular homeostasis. Recent *Symbiodinium* transcriptome studies have revealed that in comparison to other eukaryotes *Symbiodinium* display a large amount of domains involved in transmembrane transport (20 out of the identified 280 significantly enriched protein domains), which include transporter domains specific for bicarbonate, ammonium, amino acids as well as ABC and ion transporters (Aranda et al. 2016). The present study identified significant decreases in transcript abundance of various transcripts encoding transmembrane transporters in *Symbiodinium* exposed to elevated pCO_2, the majority of which were channel and voltage-gated proteins involved in the movement of sodium, potassium and calcium ions. Calcium ions are required for a number of processes that include the motility of eukaryotic cilia and flagella (Inaba 2015). In particular, in *C. reinhardtii* calcium ions have been shown to play a role in phototaxis, in the control of flagella waveform, in the maintenance of flagella length and in flagella surface motility (Goodenough et al. 1993, Wakabayashi et al. 2009). In addition, exposing cells of *C. reinhardtii* to lowered pH levels has resulted in the excises of flagella. Nutrient shortages have also been shown to freeze *Symbiodinium* in the G1 phase of the cell cycle (Muscatine et al. 1989, Muller-Parker et al. 1996, Smith and Muscatine 1999, Wang et al. 2008). In accordance with these previous studies, the results of the present study have also demonstrated lowered transcript abundance of proteins involved in transportation of ions and growth of *Symbiodinium* clade F under elevated pCO_2 conditions.

In addition to channel proteins, transcripts encoding proteins involved in ammonium transport were also significantly decreased in *Symbiodinium*. Ammonium is required for amino acid synthesis via the glutamine synthatase–glutamate synthase cycle (GS-GOGAT) (Mariscal et al. 2004). Ammonium is taken up directly via ammonium transporters (AMTs) and is the preferred source of nitrogen for many microorganisms due to its assimilation having a lower energetic cost than that of oxidised forms of nitrogen such as nitrate (Bloom et al. 2010). *C. reinhardtti* have the largest AMT1 family of ammonium transporters compared to other plants, consisting of eight members (González-Ballester et al. 2004) which are required for the regulation of intracellular flux. In addition, low- and high-affinity transport systems have been identified for both ammonium and nitrate (Crawford and Glass 1998). The low-affinity transporter system (LATS) is responsible for uptake when substrate is plentiful externally, whereas the high-affinity transporter system (HATS) is responsible for when substrate concentrations are low. In contrast to the present study, previous studies have shown that the AMT1 genes of *C. reinhardtii*, while strongly induced by N deficiency (González-Ballester et al. 2005), do not seem to be affected by carbon supply (González-Ballester et al. 2004, Fernandez and Galvan 2007).

The ATP-binding cassette (ABC) transporter family is one of the largest transporter families and is observed in all organisms from prokaryotes to eukaryotes (Dudler and Hertig 1992, Higgins 1992, Martinoia et al. 1993, Rees et al. 2009). Eukaryotic ABC transporters are classified into seven main families (ABCA to ABCG) (Anjard et al. 2002, Roth et al. 2003, Sheps et al. 2004). Despite being called transporters, two eukaryotic ABC transporter families (ABCE and ABCF) do not function as transporters but are involved in other cellular processes including

ribonuclease inhibition and translational control (Kerr 2004, Zhao et al. 2004). Translational control plays an essential role in maintaining homeostasis, and controlling cell proliferation, growth and development. In *Caenorhabditis elegans*, ABCE has been shown to be involved in regulation of growth and vulvae development (Zhao et al. 2004). In the present study, transcripts encoding ABC transporters were significantly decreased in elevated pCO_2, including transcripts encoding the ABCE and ABCF families. The decreases in ABC transcripts therefore suggest that the decrease transportation of nutrients and effective maintenance of cell homeostasis are inhibiting *Symbiodinium* cell growth at elevated pCO_2 conditions.

Protein and ATP binding/Cell regulation

The molecular response to stress varies with many pathways in place to minimize damage and re-establish cellular homeostasis. Various cellular functions can be targeted during stress such as cell cycle control, molecular chaperoning, protein repair, protein degradation, and DNA repair. If cellular function cannot be regained, cell death (apoptosis) may occur (Kültz 2005). Heat shock proteins (HSPs) are molecular chaperones that are involved in maintaining regular cellular functions with crucial roles in protein folding, unfolding, aggregation, degradation, and transport (Sørensen et al. 2003). In addition, these proteins are involved in cell differentiation (Gunter and Degnan 2007), cell signaling and in the protection of cells against stress and apoptosis (Arya et al. 2007). Previous gene expression studies on *Symbiodinium* have identified HSPs (HSP70 and HSP90) and have quantified their expression mainly in response to thermal stress (Leggat et al. 2007, Leggat et al. 2011, Rosic et al. 2011, Barshis et al. 2014). Within *Symbiodinium* exposed to elevated CO_2 concentrations differentially expressed transcripts encoding HSPs, DnaJs, and RAD DNA repair proteins were

significantly decreased. The down-regulation of chaperones and DNA repair proteins may therefore indicate a higher rate of protein misfolding and as a consequence leads to higher endoplasmic reticulum (ER) stress. ER stress triggers cell death by several mechanisms, including activation of proteases, kinases, and transcription factors, which have been identified in the present study.

Protein phosphorylation is another pathway that plays a significant role in a wide range of cellular processes such as cell signaling, gene expression, differentiation, and apoptosis. In addition, protein phosphorylation is a crucial post-translational modification. Eukaryotic cell cycles are driven by a set of regulators (Morgan 2007). Among these are the serine/threonine protein kinases. For example aurora kinases regulate cell proliferation and are essential for mitotic progression (Nigg 2001) and the nimA-realated kinases (Neks) represent a family of serine/threonine kinases implicated in cell cycle control. In addition, cell cycle progression is controlled by the action of cyclin-dependent kinases (CDKs). The present study identified numerous transcripts encoding serine/threonine-protein kinases that were lowered in *Symbiodinium* exposed to elevated CO_2 conditions. These results suggest that elevated CO_2 hinders cell cycle in *Symbiodinium* clade F however physiological measurements of growth and development are needed in order to link transcript abundance profiles of these cell cycle regulatory proteins to physiological responses of *Symbiodinium*.

Conclusions

In the present study CO_2 enrichment (10 000 ppm pCO_2) increase transcripts encoding proteins involved in functions such as photosynthesis and metabolism in cultured *Symbiodinium* clade F while transcripts encoding proteins involved in transmembrane

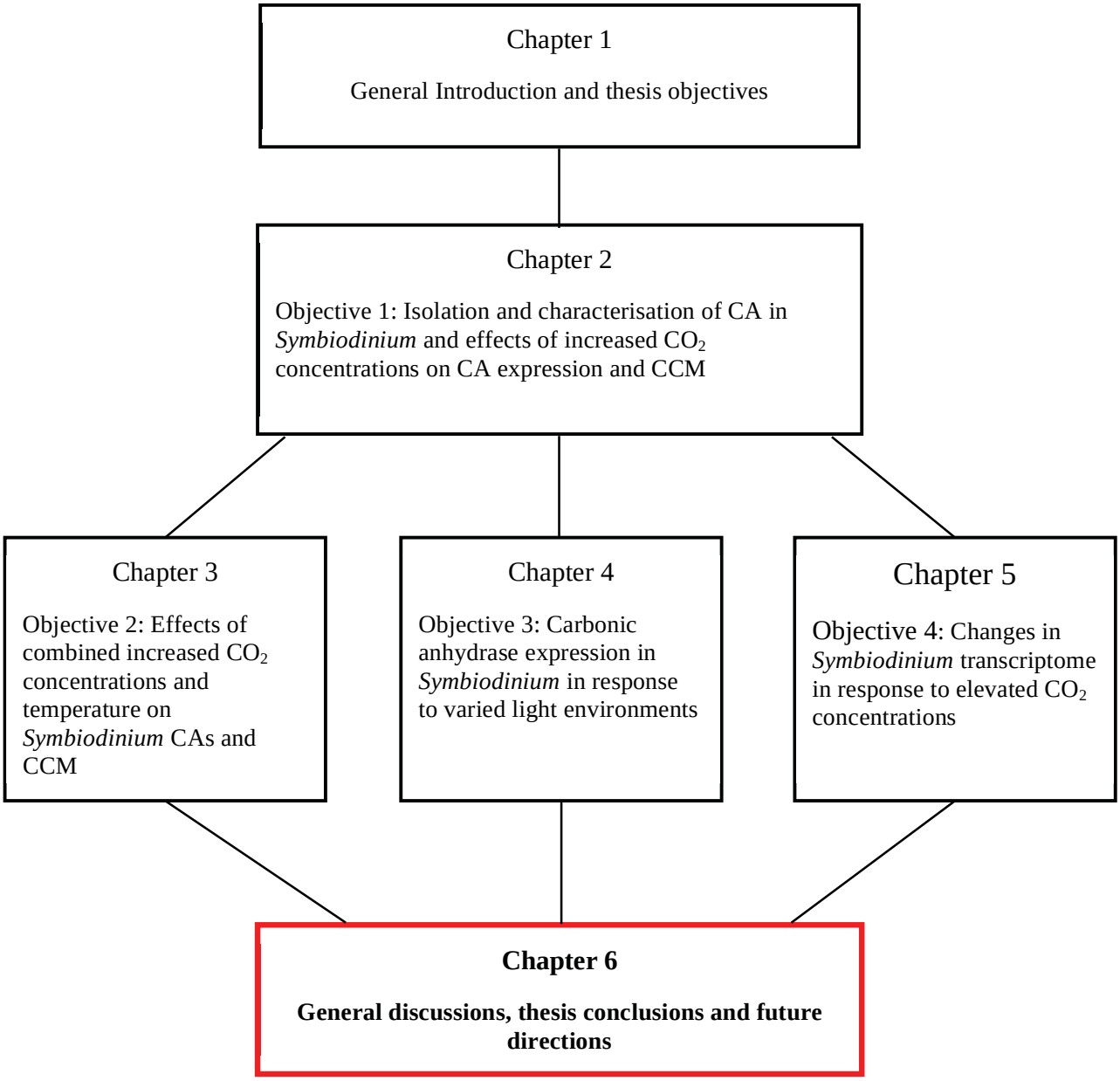
transport and cell regulation were decreased. While enhanced photosynthesis under elevated pCO_2 is consistent with previous studies, decreased transcript abundance of ammonium transporters and CAs are in contrast to previous studies. The increase in CA transcript abundance under 10 000 ppm pCO_2 is also in contrast to Chapter 2 results that demonstrated lowered *Symbiodinium* CA transcripts. These differences may be due to the different predicted cellular location of these transcripts. Interestingly, the one CA transcript identified as having homology to SymBCA2 (comp10424_c0) differed in both predicted location and transcript abundance. The results presented in this study have nevertheless provided significant insights into the acclimation and molecular response of *Symbiodinium* clade F to elevated pCO_2, which therefore provides important information for future climate change research.

Chapter 5 Summary points

- Elevated pCO_2 (10 000 ppm pCO_2) increased transcripts encoding proteins involved in:
    - Photosynthesis
    - Chlorophyll biosynthesis
    - Oxidative pentose phosphate pathway
    - Gluconeogenesis
    - Oxidative phosphorylation
    - Transcription and translation factors
- Elevated pCO_2 (10 000 ppm pCO_2) decreased transcripts encoding proteins involved in processes such as:
    - Transmembrane transport

    - Ammonium transporters
    - Cell regulation
- Thirty-two CAs were identified in the *Symbiodinium* transcriptome:
    - Six α-CAs
    - Twenty-two β-CAs
    - Two γ-CAs
- One transcript (comp 104242_c0) showed homology to SymBCA2 that was identified in Chapter 2
- Of the thirty-two CA transcripts identified, nine were DEG, all showing significant up-regulation under elevated CO_2 conditions. This included comp 104242_c0, which is in contrast to expression results obtained in Chapter 2.
- All nine differentially expressed CAs belong to the β-family and were predicted to be chloroplast targeted.
- The predicted location of comp 104242_c0 to the chloroplast is in contrast to the predicted location discussed in Chapter 2.

Schematic representation of thesis outline



Chapter 6: General Discussion

Coral reefs are among the most biologically diverse and productive ecosystems that not only support hundreds of thousands of plant and animal species, but also contribute significantly to the economic wealth of countries. The ecological success of coral reefs is largely due to the symbiotic partnership of the cnidarian host and their endosymbiotic dinoflagellates (Falkowski et al. 1993). Translocation of photosynthetic products generated by *Symbiodinium* delivers the majority of metabolic energy required by the host (Muscatine 1990). In return *Symbiodinium* are provided with inorganic nutrients from the host and protection from predation (Muscatine and Porter 1977). Dysfunction of this symbiosis under global climate change conditions, such as ocean acidification (OA) and warming, has led to worldwide decline of tropical coral reefs and a surge in scientific research on the causes, effects and implications of mass bleaching events. It is now indisputable that increases in anthropogenic CO_2 emissions are creating oceanic conditions that are both rapidly and increasingly becoming inhospitable to the worlds tropical coral reefs and symbiotic partnerships (Hughes et al. 2003, Hoegh-Guldberg et al. 2007, Anthony et al. 2008, Kleypas and Yates 2009). Understanding how coral reefs respond to stress events resulting from such anthropogenic influences therefore requires a better understanding of exactly how coral reefs function. This has always been challenging due to the complexity of such a biological structure that involves at least three different organisms; one an animal, one a plant, and the other a plethora of bacteria. The significance of the present research project is that it provides new information on the gene level for one of these organisms, the symbiotic dinoflagellate *Symbiodinim*.

The fundamental aim of this thesis was to establish a basis for understanding what affect elevated CO_2 conditions will have on the functioning of *Symbiodinium* photosynthesis, in particular, the enzyme carbonic anhydrase (CA). Research undertaken during this thesis therefore depended largely on first, the isolation and characterisation of CA enzymes in *Symbiodinium* (Chapter 2; aim 1) and second, assessing the expression of CA and other photosynthetic related genes under elevated CO_2 conditions (Chapter 2). In addition to elevated CO_2 conditions, the effects of other environmental stressors on *Symbiodinium*, such as increased temperature (Chapter 3) and varied light intensities (Chapter 4), were also investigated. This was achieved by employing the highly sensitive quantitative PCR (qPCR) technique that quantifies the expression of genes of interest by comparison to stably expressed internal control genes of *Symbiodinium* (Chapters 2, 3 and 4). This research also employed next-generation sequencing technique, (Illumina sequencing), to characterise the *Symbiodinium* transcriptome in response to prolonged elevated pCO_2 conditions (Chapter 5). In addition to transcript analysis, physiological parameters such as photosynthetic efficiency of PSII (measured as F_v/F_m of chlorophyll fluorescence; see Chapter 2 for details), cell density and chlorophyll pigment analysis were also investigated in an attempt to link any physiological changes with changes in transcript abundance (Chapter 2, 3 and 4).

The functional roles of CAs are diverse. CAs are fundamental to many biological processes such as respiration, CO_2 and ion transport, calcification and acid-base balance (Dodgson 1991). In addition, CAs play a major role in algal carbon-concentrating mechanisms (CCMs) as a key enzyme in the acquisition and concentration of inorganic carbon (Ci) for efficient photosynthetic activity (Badger and Price 2003, Moroney et al. 2011, Wang et al. 2011, Ludwig 2012). The extensive

literature on *Chlamydomonas* and cyanobacterial CCMs has provided a comprehensive platform on the functioning of CCMs and the involvement of multiple CA gene families. Many organisms express multiple CA gene families, which suggests that the specific function of one kind of CA gene may be different. Simultaneously, many organisms express multiple CA isoenzymes which can differ in oligomeric arrangement, molecular features, cellular localization, expression levels, kinetic properties, and distribution in organs and tissues (Alterio et al. 2012). For example, in *C. reinhardtii* there are at least 12 known genes that encode CAs of the α-, β-, and γ-class, which differ in subcellular location and function within the CCM (Moroney et al. 2011). Even though the evidence for a CCM in *Symbiodinium* was proposed over 20 years ago (Al-Moghrabi et al. 1996, Leggat et al. 1999), the role and extent of CAs in the *Symbiodinium* are still quite poorly studied and therefore promote studies on cultured *Symbiodinium* such as the characterisation study documented in Chapter 2. This is the first study to have isolated and characterised two β-class CAs (SymBCA1 and SymBCA2) and one δ-class CA (SymDCA1) from *Symbiodinium* (Chapter 2) and to examine the expression of these CAs under varied environmental stress conditions. The initial survey of the *Symbiodinium* β-class (through both expressed sequence tag (EST) analysis and PCR (Chapter 2)), identified that, as with *C. reinhardtii* there is a large diversity of CA sequences found within the one *Symbiodinium* strain (clade C3), indicating multiple copies within the genome. In addition, the majority of these proteins are encoded as polyproteins, which is consistent with other dinoflagellate chloroplastic genes that are present as multiple copies such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Rowan et al. 1996), peridinin-chlorophyll a-protein (PCP) (Norris and Miller 1994, Reichman et al. 2003) and peridinin chlorophyll a-c binding proteins (acPCPs) (Boldt et al. 2012).

In contrast to Rubisco, PCP and acPCPs however, both SymBCA1 and SymBCA2 were not chloroplast targeted, and instead, predicted to be localised to the cytosol. In addition, SymDCA1 was predicted to be localised to the plasma membrane (Chapter 2). A similar result has been identified in the genome of *S. kawagutii* in which the majority of the identified CAs were predicted to be cytoplasmic, suggesting that cytoplasmic CAs are critical for CO_2 acquisition, while two δ-CA were predicted to be localised to the plasma membrane (Lin et al. 2015). While the involvement of SymBCA1, SymBCA2 and SymDCA1 in the *Symbiodinium* CCM was not fully determined, the results of the present study did however illustrated additional CAs in *Symbiodinium* and identified changes in transcription under environmental stress.

Transcriptome analysis (Chapter 5) further identified the diversity of CAs within *Symbiodinium*. A total of 32 CA transcripts were identified in the transcriptome of *Symbiodinium* clade F, representing members from the α-, β-, γ-, and δ-CA gene family. In addition, one transcript showed homology to SymBCA2 identified in Chapter 2. Interestingly, more than half of the 32 CA transcripts were representatives of the β-class and were chloroplast targeted. Such high representation of the β-class in *Symbiodinium* is not surprising. This has also been observed in *C. reinhardtii* where six of the 12 known CA genes belong to the β-class (Ynalvez et al. 2008; Mitra et al. 2005). As a whole, the β-class of CAs are much more diverse in their quaternary structure, sequence and distribution than any of the other CA families. Such sequence diversity has therefore given rise to subsequent subgroupings within this class (Smith and Ferry 2000). The phylogenetic analysis of both SymBCA1 and SymBCA2 reported in this thesis has illustrated further this evolutionary diversity, and has also identified that the dinoflagellate CA sequences (including the sequences for both SymBCA1 and SymBCA2) are forming a novel subgroup within the β-CA class.

Of particular note, the characterisation study (Chapter 2) identified for the first time an amino acid substitution of the highly conserved arginine-aspartate dyad in the N-terminus of SymBCA2. While the significance of this mutation remains ambiguous and was not investigated, transcript abundance of SymBCA2 was still altered when *Symbiodinium* cells were exposed to elevated pCO_2 levels with a 9-fold decrease when exposed to 10 000 ppm pCO_2 conditions (Figure 2.6c, Chapter 2), an increase of 0.3-fold when exposed to 1500 ppm pCO_2 and 0.5-fold when exposed to 750 ppm pCO_2 combined with thermal stress (Figure 3.5b, Chapter 3) and decrease of 1.9-fold when transferred to high light conditions (Figure 4.3b, Chapter 4). Future work should therefore focus on obtaining full-length coverage of SymBCA2 and protein expression studies to assess the functionality of this particular CA in *Symbiodinium*.

One of the key findings of this research is that, while it has illustrated *Symbiodinium* CAs and other targeted genes are modulated by a variety of external environmental conditions, transcriptional regulation does not appear to play such a significant role in *Symbiodinium* stress response and that *Symbiodinium* is possibly reliant on post-transcriptional mechanisms for homeostatic regulation of its photosynthetic proteins. This is consistent with prior research that has demonstrated approximately 5-30% of genes are regulated at the level of transcription (Johnson et al. 2012), while most nuclear-encoded proteins are predominately regulated by post-transcriptional processes (Leggat et al. 2011). While transcriptional changes were observed throughout this thesis, these changes were predominantly small-scale. For example, the transcriptome study (Chapter 5) revealed that 20% of the transcriptome was regulated under elevated pCO_2 with the majority of differentially expressed transcripts being <1-fold change (Figure 5.3c and d). The largest fold change reported in this thesis however was a decrease in transcript abundance of phosphoglycolate

phosphatase (PGPase) by 25-fold under elevated CO_2 conditions (Figure2.6e). One possible explanation for such small-scale transcriptional changes may be due to the unique genomic organization and large size of *Symbiodinium* genomes (greater than 2Gb) (LaJeunesse et al. 2005). Such a unique genome organization may mean that *Symbiodinium* are not able to rapidly alter gene expression patterns (Leggat et al. 2011). Perhaps surprisingly, with the exception of SymDCA1 and Rubisco, only at extreme CO_2 concentrations (10 000 ppm pCO_2) was a change in gene expression observed for SymBCA1, SymBCA2, and PGPase, while at CO_2 concentrations of 550 ppm pCO_2 and 750 ppm pCO_2 no change in expression was observed (Chapter 2). These results imply that both SymBCA1 and SymBCA2 may be constitutively expressed in *Symbiodinium* and/or may have a role outside of the CCM. Alternatively, if both SymBCA1 and SymBCA2 are directly involved in the CCM then their expression may be needed for efficient functioning, as seen in the α-CA (*Cah3*) in *C. reinhardtii* (Karlsson et al. 1998). Future studies on *Symbiodinium* stress response may therefore benefit from proteomic or metabolomics approaches rather than transcriptomic (Gordon and Leggat 2010).

The synergistic effects of OA and elevated SST on both transcript abundance of *Symbiodinium* β-CAs, Rubisco and PGPase and physiological parameters were also investigated in this thesis (Chapter 3; Aim 2). As these two environmental factors will be changing in tandem under future climate change scenarios, it is imperative to conduct studies to assess the impacts of these combined stressors on *Symbiodinium* photosynthesis. The results of this experiment demonstrated that the greatest changes in *Symbiodinium* cells exposed to combined elevated pCO_2 and temperature were on physiological parameters while transcriptomic analysis revealed few, small significant changes. Therefore, no clear link could be made between transcript abundance and

physiological response. In addition, minimal effects were observed in CO_2 alone treatments, while the main driver of these changes was thermal stress. This was evident with the significant decline of photosynthetic efficiency in *Symbiodinium* cells exposed to 31°C (Chapter 3; Figure 3.2), the decline in cell density from day 7 in cells exposed to thermal stress (Figure 3.3) and, the increase in both chl *a* and chl *c* in all thermal treatments (Figure 3.4). While the present study was conducted on cultured cells of *Symbiodinium*, many studies have pointed to *Symbiodinium* as the weaker partner in symbiosis during thermal stress. Impairment or damage to PSII, due to environmental stress, has been shown to result in the production of harmful ROS that may be a trigger for algal expulsion from the coral host (Lesser 1997, 2011). In a study exploring the interactive effects of near-term CO_2 increases (40 -90 ppm above current ambient) during a simulated bleaching event (34 °C for five days) of *Acropora aspera*, *Symbiodinium* photosynthetic efficiency was also significantly depressed while elevated pCO_2 only slightly mitigated the effects of increased temperature on F_v/F_m (Ogawa et al. 2013). In addition, no significant physiological changes were detected in *Symbiodinium* solely in response to increased CO_2 levels. Changes were however observed in response to temperature (Ogawa et al. 2013). These results, together with the results of this thesis demonstrate that thermal stress will most likely have a greater impact on *Symbiodinium* physiology than elevated pCO_2 in both free-living and symbiotic *Symbiodinium*.

A second key finding of this research is that both the physiological stress response and changes in transcript abundance displayed by *Symbiodinium* exposed to elevated pCO_2 seem to be phylotype specific. The results presented in this thesis therefore support previous studies on phylotype specific responses of *Symbiodinium* exposed to such conditions (Brading et al. 2011). Such phylotype specific responses

were evident in photosynthetic efficiency (as measured by F_v/F_m), between cells of *Symbiodinium* clade C1 and clade F exposed to elevated pCO_2 conditions. Cells of *Symbiodinium* clade C1 showed a significant decline in F_v/F_m when exposed to elevated CO_2 concentrations of 550 ppm pCO_2, 750 ppm pCO_2 and 10 000 ppm pCO_2 concentrations (Chapter 2; Figure 2.6), while F_v/F_m was not affected by elevated pCO_2 concentrations of 750 ppm pCO_2 and 1500 ppm pCO_2 in cells of *Symbiodinium* clade F (Chapter 3; Figure 3.2). Changes in transcript abundance also varied between the two clades examined in this thesis. Transcript levels of both SymBCA1 and SymBCA2 were significantly decreased in *Symbiodinium* clade C1 exposed to 10 000 ppm CO_2 conditions (Chapter 2; Figure 2.6b and c), while expression patterns were not altered at concentrations of 550 ppm and/or 750 ppm CO_2. Chapter 3 also reported that gene expression patterns were not altered when cells of *Symbiodinium* clade F were exposed to 750 ppm and/or 1500 ppm (Chapter 3; Figure 3.5 a and b) but were more affected by thermal stress. Furthermore, the differentially expressed CA transcripts identified in Chapter 5, including the transcript that showed homology to SymBCA2 (comp 104242_c0), increased under 10 000 ppm CO_2 conditions in *Symbiodinium* clade F, which is in contrast to Chapter 2. One possible explanation for the difference in CA transcription under elevated pCO_2 may relate to the location of these CAs. All differentially expressed CA transcripts identified in Chapter 5 were predicted to be located to the chloroplast, while the CAs identified in Chapter 2 were predicted to be located to the cytosol. It must be noted however that the full-length sequence was not obtained for SymBCA2 in Chapter 2. Therefore it is possible that SymBCA2 does contain a transit peptide and could be located to the chloroplast. It may also be possible that different phylotypes are more susceptible to elevated CO_2 conditions than other phylotypes (as has been displayed with thermal tolerance and

sensitivity of *Symbiodinium* (Rowan 2004, Berkelmans and Van Oppen 2006), and therefore a CO_2 tolerant linage may exists within *Symbiodinium.* This becomes extremely important when the environmental threshold of the coral-algal symbiosis relies upon the taxonomic composition of endosymbionts (Baker 2003, Berkelmans and Van Oppen 2006). Such diversity of stress response within and between *Symbiodinium* phylotypes has now led to attempts to facilitate genetic adaptation of existing *Symbiodinium* strains to mitigate coral bleaching (van Oppen et al. 2015). In this approach, strains of *Symbiodinium* can be subjected to a variety of environmental stressors in the laboratory with the end goal of eliciting an adaptive response through selection on random somatic mutations, which can be enhanced by exposing the cultures to a mutagen. The selected *Symbiodinium* strains may be able to establish symbiosis with the aposymbiotic early life stages of corals, resulting in corals with new phenotypes. This can ultimately lead to corals harbouring *Symbiodinium* strains with enhanced stress tolerance that can therefore be targeted for reef restoration (Van Oppen et al. 2015). Whether or not a CO_2 tolerant strain exists in *Symbiodinium* and if CA transcription abundance differ between the strains would need to be further investigated.

The effects of varied light intensities on *Symbiodinium* CA transcription were explored for the first time in this thesis (Chapter 4; aim 3). Light is recognized as a major regulator of photosynthetic proteins (Escoubas et al. 1995, Kobiyama et al. 2005, Murchie et al. 2005, Van Dolah et al. 2007), with CCM transcript levels of *C. reinhardtii* shown to vary at various times of light/dark cycles, even at air-level CO_2 concentrations (Tirumani et al. 2014). Both SymBCA1 and SymBCA2 transcript levels were not altered when cells were previously acclimated to HL conditions (250 – 350 μmol quanta m^{-2} s^{-1}) and transitioned to ML levels (80 - 100 μmol quanta m^{-2} s^{-1})

or LL levels (10 – 15 μmol quanta m^{-2} s^{-1}). Changes in transcript abundance however were noted when cells were acclimated to lower light intensities (ML; ~80 - 100 μmol quanta m^{-2} s^{-1}) intensities and changed to higher irradiance levels, with both SymBCA1 and SymBCA2 transcripts lowered in HL conditions (Chapter 4; Figure 4.3a and b). These results suggest that CO_2 may not be the sole factor in altering transcription of SymBCA1 and SymBCA2 and, that an interactive effect of both light and CO_2 may regulate these particular CAs as seen in *C. reinhardtii* (Tirumani et al. 2014). A key observation of this study was that while the physiological response of *Symbiodinium* moved to HL conditions is consistent with previous studies on *Symbiodinium,* and other photosynthetic algae, which report lower F_v/F_m values for HL conditions (Hennige et al. 2009; Robinson and Warner 2006), the acclimation process of cells acclimated to ML and moved to HL takes place mostly during the first day for PSII (Figure 4.1) while significant changes in transcript levels of SymBCA1, SymBCA2, acpPC_15 and acpPC_17 takes place much later on day 9 (Figure 4.3a and b). In addition, changes to transcript abundance of SymBCA1, SymBCA2 and acpPCs occurred much slower when compared to other microalgae that have shown to regulate expression within six hours of exposure to light (Tirumani et al. 2014). These results support the hypothesis that *Symbiodinium* may not able to rapidly alter gene expression patterns and that more changes are occurring post-transcriptionally. These results have also revealed that there is little understanding about the timing of gene expression in *Symbiodinium* and now raise the question as to whether SymBCA1 and SymBCA2 could be regulated by a circadian clock as seen in the periplasmic and mitochondrial CAs of *C. reinhardtii*. Future work should therefore investigate the effect of light/dark cycles on *Symbiodinium* CAs and also incorporate varying concentrations of pCO_2. In addition, since decreases in acpPCs transcripts were

observed, chlorophyll pigment content should also be investigated to link transcription changes to changes in chlorophyll content.

Advances in molecular and genomic approaches have enabled transcriptome analysis of *Symbiodinium* stress response under various environmental conditions. Whole-transcriptome analysis has already been used in studies on the impact of global climate change on many coastal organisms (Franssen et al. 2011, Meyer et al. 2011, Moya et al. 2012, Runcie et al. 2012). These studies have identified specific biological functions or phenotypic plasticity across populations facing different environmental conditions and, have also highlighted many unexpected pathways that may or may not be affected by such conditions. The major aim of the *Symbiodinium* transcriptome study (Chapter 5) was to initiate a whole-transcriptome analysis in *Symbiodinium* clade F to gain insight into cellular processes occurring in response to prolonged elevated pCO_2 conditions. While the CO_2 concentration used was not ecologically relevant, this concentration was used to mirror the work reported in Chapter 2. The results of Chapter 5 may therefore have a greater relevance to our understanding of the high CO_2 environment that surrounds *Symbiodinium* in the symbiosome when in symbiosis. Elevated pCO_2 conditions increased transcripts encoding for proteins involved in functions such as photosynthesis and carbon metabolism while transmembrane transport, protein phosphorylation, ATP and protein binding were all significantly decreased in *Symbiodinium* clade F. These results have indicated that *Symbiodinium* clade F cell cycle is affected by prolonged elevated pCO_2 conditions. The increased transcript abundance of photosynthetic proteins under CO_2 enrichment is similar to that seen in other algae (Riebesell et al. 1993, Hein and Sand-Jensen 1997, Peng et al. 2016, Sun et al. 2016). However, the decrease in transcript abundance of proteins involved in transmembrane transport, in particular transcripts

encoding ammonium transporters, is in contrast to previous transcriptome studies (González-Ballester et al. 2004, Fernandez and Galvan 2007). As noted earlier, the differentially expressed CA transcripts were all significantly increased in *Symbiodinium* clade F under elevated CO_2 conditions, which are in contrast to both previous studies that have identified decreases in CA transcripts when exposed to 1 or 5 % pCO_2 (Kaplan et al. 1980, Tsuzuki and Miyachi 1989, Raven 1991, Matsuda et al. 2001, Van de Waal et al. 2013) and, results obtained in Chapter 2. Therefore, it is essential that future studies examine the effects of elevated pCO_2 concentrations on both phylotypes of *Symbiodinium* to see if a similar response is observed.

Ultimately, the physiological performance of coral reefs is highly dependant upon the combined outcome of the coral host and its endosymbiotic algae *Symbiodinium*. Evidence that coral reefs can adapt at rates which are sufficient for them to keep up with rapid ocean warming and acidification is minimal, especially given that corals are long-lived and hence have slow rates of evolution. The present research however has improved the knowledge for one of these organisms, the symbiotic dinoflagellate *Symbiodinium* and has provided a basis for future climate change studies to further investigate the role and functioning of CA gene families in *Symbiodinium* photosynthesis under climate change conditions. Future research should therefore address the questions:

- Do certain CA gene families perform different roles in the CCM?
- Does the role of the same CA gene differ in different phylotypes of *Symbiodinium*?
- How many of the identified CAs are actively involved in the CCM?
- Are *Symbiodinium* CAs regulated by a circadian clock?

- Do *Symbiodinium* regulate transcription and how? Is this regulation the same for different phylotypes of *Symbiodinium*?
- Is there a regulatory gene controlling the induction of the *Symbiodinium* CCM as identified in *C. reinhardtii*?
- How will high levels of irradiance interact with elevated SST and OA to affect *Symbiodinium* photosynthesis?

In conclusion, this thesis has furthered the knowledge on CA enzymes in *Symbiodinium* and of the potential impacts of global climate change on the physiology and transcriptomic response of *Symbiodinium* photosynthesis. The data presented in this thesis have also illustrated and supported *Symbiodinium* stress response to vary according to the particular phylotype examined. Implicit in all the results obtained from this thesis is a strong foundation for understanding how a particular environmental driver such as ocean acidification and /or temperature will be transduced through *Symbiodinium* to possibly alter the tolerance and response of the coral-algal symbiosis to global climate change conditions. In addition, these results have raised additional questions that can be used to further investigate *Symbiodinium* stress response to climate change in order to protect such a biologically diverse and important ecosystem.

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Appendices

Method for DNA ligation

DNA ligations were performed using pGEM®-T Vector System (Promega, USA) with a ratio of insert to vector of 3:1 (10 µl final reaction volume). Purified DNA fragments were incubated with pGEM®-T vector (1 µl), 2X rapid ligation buffer (5 µL), T4 DNA ligase (3 Weiss units/ µL; 1 µL) and distilled water (10 µL final volume) overnight at 4 ^{0}C for the maximum number of transformants.

Method for preparation of competent cells

Competent cells of *E. coli*, strain: NM522) were prepared using a modified method of Cohen et al 1972 (Sambrook et al 1989). A single colony was picked from an LB agar plate that had previously been spread using a -80 º C glycerol stock and used to inoculate 5 ml of LB media (Appendix Table 2.5) and shaken overnight at 37 º C. The resulting culture was then used to inoculate 500 ml of LB media and again shaken overnight at 37 ºC for 3 – 4 hours or until the OD_{600} reading was between 0.5 – 0.6. The cultures were then cooled to 0 ºC on ice for 30 min and harvested in pre-cooled 50 ml Falcon tubes by centrifugation at 3000g for 20 min at 2 ºC. The resulting pellet was resuspended in 25 ml ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. After incubation on ice, cells were recovered by centrifugation at 3 000 x g for 20 min at 2 ºC and the cell pellet resuspended again in 2 ml of ice-cold 0.1 M $CaCl_2$. 200 µl of the cell suspension was then aliquoted into pre-chilled sterile 1.5 ml microcentrifuge tubes and snap-frozen in liquid nitrogen and stored at -80 ºC for later use.

Method for transformation of competent cells

An aliquot of frozen NM522 competent cells (200 µl) were thawed on ice before plasmid DNA (10 µl of ligation reaction) was added and incubated on ice for 20 min. Bacterial cells were then heat-shocked at 42 ºC for 90 sec in a water bath before returning to ice for a further

5 min. The transformation mixture was then plated onto pre-warmed LB/ampicillin plates that had also previously been spread with 50 µl of 20 mM IPTG/2% X-Gal (in N, N,-dimethylformamide) solution (Appendix Table 2.6-2.7) and incubated overnight at 37 ºC for blue/white selection. Sterile LB aliquots of 5 ml were inoculated with a single bacterial colony and grown overnight at 37 ^{0}C while shaking (260 rpm). Cells were collected by centrifugation (2 min and 10,000 x *g*) and plasmids were purified using the NucleoSpin® Plasmid kit (Macherey-Nagel, Germany). Multiple clones were then submitted to Macrogen Inc, Korea for sequencing.

Table 1. ASP-8A Medium used for *Symbiodinium* cell culture

Components	Molecular Weight (g / mol)	Stock Solution	1 L
NaCl	58.44	-	25 g
KCl	74.55	35 g / 500 ml	10 ml
$MgSO_4.7H_2O$	246.47	450 g / L	20 ml
$CaCl_2.2H_2O$	147.02	55 g/ 500 ml	10 ml
$NaNO_3$	84.99	2.5 g / 50 ml	1 ml
KH_2PO_4	136.09	0.5 g / 50 ml	1 ml
Nitrilotriacetic acid (NTA)	191.14	1.5 g / 50 ml	1 ml
Tris Base (pH 9.0)	121.14	100 g / L	10 ml
PII Metal Mix	-	(separate)	10 ml
NH_4NO_3	80.04	0.05 g / 50 ml	1 ml

8A Vitamin Mix (x2)	-	(separate)	0.25 ml
Vitamin B_{12}	1355.38	10 µg/ 1 ml	0.1 ml

Table 2. 8A Vitamin Mix for use in the preparation of ASP-8A Medium for culturing *Symbiodinium* cells

Components	Molecular Weight (g / mol)	Stock Solution (1 L)
p-Aminobenzoic acid, Na salt	159.12	10 mg
Biotin (d-)(Vitamin H)	244.32	0.5 mg
B_{12} (cyanocobalamin)	1355.38	0.5 mg
Choline di H_2 citrate	295.29	500 mg
Folic acid	441.40	2.5 mg
Folinic acid, Ca salt	511.51	0.2 mg
Inositol (myo-)	180.16	1000 mg
Nicotinic acid (niacin)	123.11	100 mg
Orotic acid, mono Na salt	178.08	20 mg
D-Pantothenic, hemi-Ca salt (Vitamin B5)	238.27	100 mg
Pyridoxamine, di HCl	241.11	20 mg
Pyridoxine, HCl (Vitamin B6)	205.64	40 mg
Putrescine, di HCl	161.07	40 mg

Riboflavin (Vitamin B2)	376.36	5 mg
Thiamine, HCl (Vitamin B1)	337.27	200 mg
Thymine	126.11	800 mg

Table 3. PII Trace Metal Mix for use in the preparation of ASP-8A Medium for culturing *Symbiodinium* cells

Components	Molecular Weight (g / mol)	Stock Solution (1 L)
$CoSO_4.7H_2O$	281.10	4.8 mg
EDTA.2Na	336.2	1000 mg
$FeCl_3.6H_2O$	270.29	49 mg
H_3BO_3	61.83	1140 mg
$MnSO_4.4H_2O$	223.06	164 mg
$ZnSO_4.7H_2O$	287.54	22 mg

Table 4. Standard components of DNase digestion reaction.

Components	**Volume (µl)**
RQ1 RNase-Free DNase 10X Reaction Buffer	0.9
RQ1 RNase-Free Dnase ($1U.\mu l^{-1}$)	1
mRNA in water or TE Buffer (100 ng)	1 – 7.1
DEPC water to total volume of 9 µl	X

Table 5. Standard components for Reverse transcription.

Components	**Volume (µl)**
2X RT Reaction Mix	10
RT Enzyme Mix (SuperScript™III Rand RNaseOUT™)	2
DNase-treated mRNA (100 ng)	10

Table 6. Overview of illumina sequencing data, assembly and annotation statistics of *Symbiodinium* clade F transcriptome.

	Control (n=5)	**Treatment (n=5)**
Raw read data		
Average reads per library size (pre-filter)	15767639	15534081
Average library size (post filter)	15667924	15435134
Assembly		
Number of contigs in assembled transcriptome	63908	
Average contig length	1023	
Annotation (number and percent)		
Number of annotated contigs with Blast2GO (FDR < 0.05)	20486 (32.05%)	
Number of contigs used for differential expression analysis	49538 (%)	
Number of differentially expressed genes (DEG)(p <0.05)	12728 (19.9%)	

Number of DEG Up-regulated (logFC > 0.0)	6620 (52% of DEGs)
Number of DEG Down-regulated (logFC < 0.0)	6108 (48% of DEGs)
Average fold change of DEG	0.32
Largest up-regulation (fold change log 2)	3.75
Largest down regulation (fold change log 2)	1.79 ($p < 0.01$)