Bleaching of the Cnidarian-Dinoflagellate Symbiosis: Aspects of Innate Immunity and The Role of Nitric Oxide

Thomas D. Hawkins

A thesis submitted to the Victoria University of Wellington in fulfilment of the requirements for the degree of Doctor of Philosophy in Science

Victoria University of Wellington
2013

Abstract

Driven by global warming and the increasing frequency of high temperature anomalies, the collapse of the cnidarian-dinoflagellate symbiosis (known as "bleaching" due to the whitening of host tissues) is contributing to worldwide coral reef decline. Much is known about the consequences of bleaching, but despite over 20 years of effort, we still know little about the physiological mechanisms involved. This is particularly true when explaining the differential susceptibility of coral hosts and their algal partners (genus *Symbiodinium*) to rising temperatures.

Work carried out over the past 10 years suggests that bleaching may represent an innate immune-like host response to dysfunctional symbionts. This response involves the synthesis of nitric oxide (NO), a signalling molecule widely dispersed throughout the tree of life and implicated in diverse cellular phenomena. However, the source(s) of NO in the cnidarian-dinoflagellate association have been the subject of debate, and almost nothing is known of the capacity for differential NO synthesis among different host species or symbiont types.

The aim of this study was to elucidate the role of NO in the temperature-induced breakdown of the cnidarian-dinoflagellate symbiosis and to assess differences in NO-mediated physiology at the level of both symbiont and host. The specific objectives were (i) to quantify NO synthesis in different types of symbiotic dinoflagellates, (ii) to determine a role for NO in the collapse of the cnidarian-dinoflagellate symbiosis, (iii) to confirm whether NO itself - as opposed to its more reactive derivatives - is capable of mediating cnidarian bleaching, and (iv) to measure the synthesis of NO and the regulation of associated pathways in different reef corals undergoing bleaching.

This thesis demonstrates that both partners of the symbiosis have a capacity for synthesising NO when stimulated by elevated temperature. However, their contributions to NO synthesis in the intact symbiosis may not be equal, as heightened symbiont NO production invariably occurred after that of the host, and at a time when bleaching had already commenced. Closer examination of host-derived NO in the model anemone *Aiptasia pulchella* revealed that the compound most likely mediates bleaching through

apoptotic-like cell death pathways, as either removing NO or inhibiting the activity of an important apoptosis-regulating enzyme could alleviate bleaching. NO's involvement in thermal bleaching also seems to be independent of its conversion to more toxic radicals such as peroxynitrite ($ONOO^-$), which, although present at elevated temperature, had little influence on symbiont loss in *A. pulchella*.

Different *Symbiodinium* types displayed significant variability in terms of their NO synthesis, tolerance and associated downstream pathways. For instance, a ten-fold increase in NO donor concentration (between types) was required to induce comparable declines in the photosynthetic yields of types A1 (a thermally robust type) and B1 (more sensitive). Synthesis of NO and activation of apoptotic pathways also differed significantly among differentially sensitive coral species. The thermally sensitive corals *Acropora millepora* and *Pocillopora damicornis* exhibited elevated NO production and host apoptosis during thermal bleaching, whereas *Montipora digitata* (a comparatively heat-tolerant species) produced little NO and appeared to lack the enzymes responsible for regulating apoptosis.

As is the case in a wide variety of animal-microbe interactions, NO appears to mediate the cnidarian-dinoflagellate symbiosis by influencing the activity of host apoptotic-like pathways. Interestingly, the activation of these host responses at elevated temperature may occur before the dinoflagellate becomes photosynthetically compromised. As such, the model of bleaching as simply a response to symbiont photoinhibition could require modification. Furthermore, the differential sensitivity of symbiont types to NO, coupled with the differential regulation of NO-synthetic and apoptotic pathways in the host, could contribute to corals' varying bleaching susceptibilities.

This thesis provides vital insights into the cell biology of the coral-dinoflagellate symbiosis and the events underpinning its breakdown during temperature stress. It also encourages a greater research emphasis on understanding physiological responses at the level of the coral host as well as during the early stages of a bleaching event.

Contributions and Publications

This thesis is written as a series of manuscripts either already published, submitted, or in preparation for submission in the near future. As such, there may be some repetition of themes between chapters (particularly in "Introduction" sections). All laboratory work, field-work, statistical analyses and writing were conducted by the Author, with assistance as described below

Chapter 2: This chapter is formatted as a stand-alone manuscript based on a published study. The article is attached to this thesis: Hawkins, T. D. and Davy, S. K. (2012) Nitric oxide production and tolerance differ among *Symbiodinium* types exposed to thermal stress. *Plant and Cell Physiology.* **53**: 1889-1898.
S. K. Davy advised on experimental design, interpretation, analyses and writing.

Chapter 3: This chapter is formatted as a stand-alone manuscript based on a published study. The article is attached to this thesis: Hawkins, T. D., Bradley, B. J., and Davy, S. K. (2013) Nitric oxide mediates coral bleaching through an apoptotic-like cell death pathway: Evidence from a model sea anemone-dinoflagellate symbiosis. *FASEB Journal.* **In Press.**
B. J. Bradley assisted with laboratory work (photosynthetic pigment analysis and cell counts). S. K. Davy advised on experimental design, interpretation, analyses and writing.

Chapter 4: This chapter is formatted as a stand-alone manuscript based on a published study. The article is attached to this thesis: Hawkins, T. D. and Davy, S. K. (2013) Nitric oxide and coral bleaching: is peroxynitrite required for symbiosis collapse? *Journal of Experimental Biology*. **216**: 3185-3188
S. K. Davy advised on experimental design, interpretation, analyses and writing.

Chapter 5: This chapter is formatted as a stand-alone manuscript and has been submitted for publication in the journal *Coral Reefs*: Hawkins, T. D., Krueger, T., Becker S., Fisher, P .L., and Davy, S. K. (submitted). Differential coral bleaching at a cellular level: Evidence of nitric oxide synthesis and host apoptosis in three reef corals.

S. Becker assisted with some of the laboratory work (photosynthetic pigment analysis and cell counts). T. Krueger, P. L. Fisher and S. K. Davy assisted with the field-work and experimental set up. P. L. Fisher and S. K. Davy advised on interpretation, analyses, and writing.

Acknowledgements

"*The drops of rain make a hole in the stone, not by force, but by oft falling*".
Titus Lucretius Carus, De Rerum Natura, 54 B.C.

Science works in the same way as the hollowing of Lucretius' stone, with small (often seemingly minor) discoveries built upon by successive generations. The quote has particular significance for an enthusiastic first-year Ph.D. student who might confess to delusions of grandiosity and the imminent publication, in a prestigious journal, of a seminal paper that explains much about their chosen field. Alas, the nature of research soon quells such misapprehensions.

In the completion of my Ph.D. thesis I must take this opportunity to acknowledge the support of innumerable colleagues, friends and family members. Without their constant encouragement I would almost certainly not be writing this. It is with regret, therefore, that I limit the following thanks and acknowledgements to those who have done most to help in this great adventure. For the original supply of the *Symbiodinium* cultures for my laboratory experiments, I would like to thank Scott Santos (Auburn University, Auburn, AL, USA), Mary-Alice Coffroth (University of Buffalo, Buffalo, NY, USA) and Gisele Muller-Parker (former Western Washington University, WA, USA). I also wish to thank the staff at Heron Island Research Station, University of Queensland, in particular Kyra Hay and Liz Perkins, for their assistance with fieldwork. Santiago Perez (Stanford University, CA, USA) also deserves my thanks for his encouragement during the earliest stages of my research. Of course, none of my work would have been possible without some financial support, which was provided by Commonwealth and JL Stewart Scholarships, Wellington Botanical Society, and Victoria University of Wellington Faculty of Science. The Royal Society of New Zealand Marsden Fund and Australian Coral Reef Society also deserve recognition for their provision of funds to allow me to travel and present my research overseas.

On a more personal note I must thank my parents, Jean and David, who, from my very earliest years, did everything they could to nurture and develop my curiosity. I also thank the staff at Newcastle University (UK), especially Peter Olive, John Bythell and

Sara Marsham, who helped me discover my passion for marine biology. Closer to home (New Zealand, that is), I must thank my wonderful and ever-supportive partner, Anita Eves. Enduring the ups and downs of a Ph.D. alongside me is something I wouldn't wish on anybody! Her constant encouragement and input, despite the relatively obscure nature of my research, has always been gladly received. Further thanks must go to Anita's family, who took in an itinerant postgrad student with the warmth and hospitality for which New Zealanders are famed, and also to all of the great friends I've made here. In particular, our man-of-a-thousand-skills *post*-doctoral researcher Paul Fisher and my fellow Ph.D. student Andrew Stringer must be congratulated on their efforts to keep me in touch with reality. My fellow Davy Lab researchers Emma Gibbin, Thomas Krueger, Susanne Becker, Stefanie Pontasch, Jennifer Howe, Anne Wietheger, Katie Hillyer, Ben Bradley, Dorota Starzak, Emily Neubauer, Dan Logan and Shaun Wilkinson (in particular for the endless supply of home-made pies) also deserve honourable mentions.

Last, but certainly not least, I wish to heartily thank my Ph.D. supervisor Simon Davy. I think he'd be the first to admit that taking on a new student just before a six-month sabbatical, followed by a further half-year of intermittent parental leave, wasn't an ideal situation. I have no complaints, however. Simon's passion for this subject meant that he was always available for a chat and to provide helpful advice. His guidance soon led me to realise that in the same way that stones are not eroded in one fell swoop, any discovery, however marginal and minor it may appear at the time, is still an important contribution. It is with this knowledge that I can now admit that although nitric oxide's role in coral bleaching may not initially have had me soaring with wonder, it remains an area with many more questions than answers. That is what makes it interesting, and it's why we're scientists.

List of Figures

List of Tables

Abbreviations:

Ac-DEVD-CHO: Acetyl-Asp-Glu-Val-Asp-aldehyde

Ac-DEVD-pNA: Acetyl-Asp-Glu-Val-Asp-p-nitroanilide

Ac-LEHD-CHO: Acetyl-Leu-Glu-His-Asp-aldehyde

Ac-LEHD-pNA: Acetyl-Leu-Glu-His-Asp-p-nitroanilide

AIMS: Australian Institute of Marine Science

ANOVA: Analysis Of Variance

APAF-1: Apoptotic Protease Activating Factor-1

APF: Aminophenyl fluorescein

APX: Ascorbate Peroxidase

ATP: Adenosine Triphosphate

AOX: Alternate Oxidase

AV-*fluor*: Annexin-V *Alexafluor* 488

Bcl-2: B-cell Lymphoma-2

BSA: Bovine Serum Albumin

CHAPS: 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate

Chl *a*: Chlorophyll *a*

CO_2: Carbon dioxide

cPTIO: 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide

Cyt *c*: Cytochrome *c*

DAF-FM-DA: 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate

DAN: 2,3-diaminonaphthalene

DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea

DIN: Dissolved Inorganic Nitrogen

DMF: N,N-dimethylformamide

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic Acid

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-Linked Immunosorbent Assay

FCM: Flow Cytometry

$\Delta F/F_m'$: Effective quantum yield of photosystem II

FP: Fluorescent Protein

FSW: Filtered Seawater

F_v/F_m: Maximum quantum yield of photosystem II

GBR: Great Barrier Reef

GSNO: S-nitrosoglutathione

H_2O_2: Hydrogen Peroxide

Hb: Haemoglobin

HB: Homogenisation Buffer

HCl: Hydrochloric Acid

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HTS: High Temperature Shock

HSP: Heat Shock Protein

IgE: Immunoglobulin E

IPCC: Intergovernmental Panel on Climate Change

ITS2: Internal Transcribed Spacer-2

LED: Light-emitting Diode

LMM: Linear Mixed Model

LPS: Lipopolysaccharide

LSD: Least Significant Difference

LSM: Laser Scanning Microscope

$\Delta\Psi_m$: Mitochondrial membrane potential

MAA: Mycosporine-like Amino Acid

MAMP: Microbe-Associated Molecular Pattern

MANOVA: Multivariate Analysis Of Variance

MFI: Mean Fluorescence Intensity

$MgCl_2$: Magnesium Chloride

MMP: Mitochondrial Membrane Permeabilisation

NA: Numerical Aperture

$NaNO_2$: Sodium nitrite

$NaNO_3$: Sodium nitrate

NF-κB: Nuclear Factor kappa-B

NO: Nitric Oxide

NO_2^-: Nitrite

NO_3^-: Nitrate

NO_x^-: Total oxidised nitric oxide (sum of nitrite and nitrate)

NOS: Nitric Oxide Synthase

NPQ: Non-photochemical Quenching

NR: Nitrate Reductase

$^1O_2$: Singlet Oxygen

O_2^-: Superoxide

OCl^-: Hypochlorite

OH^-: Hydroxyl

$ONOO^-$: Peroxynitrite

PAM: Pulse Amplitude Modulation

PCD: Programmed Cell Death

PI: Propidium Iodide

PS: Phosphatidylserine

PSII: Photosystem II

$Q_{A,\ B}$: Quinone A, B

RMANOVA: Repeated Measures Analysis Of Variance

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

Rubisco: Ribulose-1,5-bisphosphate carboxylase oxygenase

S.E.M.: Standard Error of the Mean

SIN-1: 3-morpholinosydnonimine

SNP: Sodium Nitroprusside

S-NONO: Spermine NONOate

SOD: Superoxide Dismutase

Table of Contents

Chapter 1

General Introduction

1.1. Endosymbiosis in the animal kingdom.

Symbiosis - the protracted association of individuals belonging to different species - is a ubiquitous phenomenon in the natural world. This is unsurprising, since in the "struggle for existence" (Darwin, 1859) it is almost inevitable that species should evolve to utilise each other and further their reproductive successes. To the layperson at least, alliances such as these - in which both partners benefit - are the very definition of symbiosis. However, these mutually beneficial relationships (known as mutualisms) represent only a fraction of possible symbioses (Douglas, 2010), as the term extends to parasitic (one partner existing at the expense of another) and commensal associations (where one partner receives neither benefit nor harm). Symbioses can also either be obligate or facultative depending on the partners' reliance on the association for their survival. For example, lichens are the product of an obligate association between certain fungi and photosynthetic microalgae in which neither partner can survive alone (Douglas, 2010). The association between tropical sea anemones and *Amphiprion* spp. anemonefish, however, is far more beneficial for the latter than for the anemone (Fautin, 1991). Adult anemonefish rarely survive in the wild without a host (and are therefore obligate partners), but vacant anemones are common on Indo-Pacific coral reefs. In this case, the anemone's cooperation is facultative.

Endosymbiotic relationships – in which one partner (the "symbiont") resides within the body of a larger "host" organism – are common in animals and particularly invertebrates. As well as spanning the range of symbioses described above, these associations can be divided further into two categories: intra- and extracellular. Intracellular symbionts exist within host cells whereas extracellular partners reside outside host cells, either in a specific organ or simply the body cavity. Probably the best-studied extracellular symbiosis in marine invertebrates is that of the bobtail squid (*Euprymna scolopes*) and its bioluminescent *Vibrio* bacteria. The squid acquires these bacteria during embryogenesis and concentrates its *Vibrio* population in a ventral organ

(Ruby, 1996) in order to provide a form of pelagic camouflage. In parallel with perhaps the most famous intracellular association - the cnidarian-microalgal symbiosis, described in detail below – *E. scolopes* shows remarkable selectivity regarding the species and strains of *Vibrio* with which it associates, and it can efficiently reject unsuitable, dysfunctional or potentially pathogenic bacteria (Visick *et al.*, 2000).

1.2. Cnidarian-microalgal symbiosis.

Cnidarians are among the simplest multicellular animals and one of the few metazoan phyla to lack a triploblastic (three tissue layer) body structure. They are also distinct from the majority of animals as they lack an alimentary canal - a cnidarian is essentially a blind sac with a single opening functioning both as a mouth and anus (Fig. 1.1). The presence of specialised stinging cells - nematocytes - is a diagnostic feature of Cnidaria, and these are distributed throughout the epidermal (outermost) tissue layer. By ensnaring small prey items such as zooplankters, cnidarians can feed heterotrophically. While all cnidarian species have this capacity for predation, a significant number gain much of their energy from symbiotic microalgae residing in their gastrodermal cells (Fig. 1.1). Early studies (e.g. Muscatine & Hand, 1958; Falkowski *et al.*, 1984; Steen & Muscatine, 1984) demonstrated the translocation of photosynthetically fixed carbon from the algal symbionts to the host and, furthermore, observed its incorporation into animal tissues (Muscatine & Hand, 1958; Trench, 1971; Muscatine *et al.*, 1984). Much of this work was carried out on the model organism *Hydra*, in which the symbionts are green algae of the genus *Chlorella* that are taken up alongside food particles before being selectively transported into the host's gastrodermis (see Muscatine *et al.*, 1975a; Muscatine *et al.*, 1975b; Trench, 1979; Davy *et al.*, 2012 for reviews).

The symbionts of Anthozoa (the class including corals and anemones) are acquired through pathways (see below) that are similar to those in *Hydra* (see Davy *et al.*, 2012 for review). Phylogenetically, however, the symbionts are quite different (Venn *et al.*, 2008). In the vast majority of symbiotic anthozoans (see Verde & McCloskey, 1996 for exceptions) the algal symbionts are photosynthetic dinoflagellates of the genus *Symbiodinium* (Fig. 1.2). Symbiotic anemones and corals benefit significantly from this arrangement and can acquire, given sufficient light, 100% or more of their energy requirements from algal photosynthesis (Falkowski *et al.*, 1984). In return, the algae

reside in an environment that, due to the metabolic activities of the host, is comparatively enriched with inorganic nutrients relative to the surrounding seawater (Muller-Parker & D'Elia, 1997).

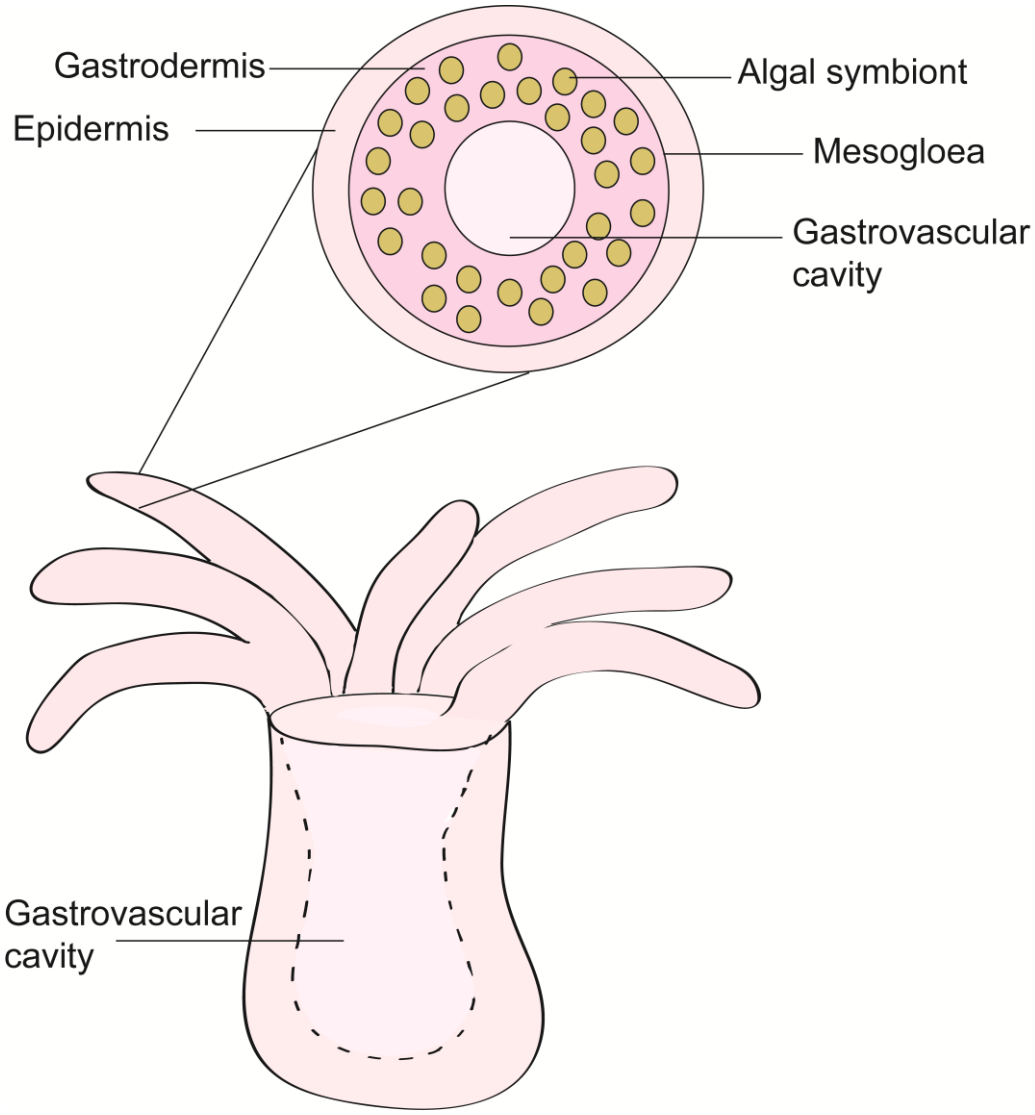

Figure 1.1. Simplified schematic diagram of a symbiotic anthozoan (phylum Cnidaria) polyp, showing diploblastic (two layer) tissue organisation, the blind gastrovascular cavity, and symbiotic microalgae in the gastrodermal cell layer.

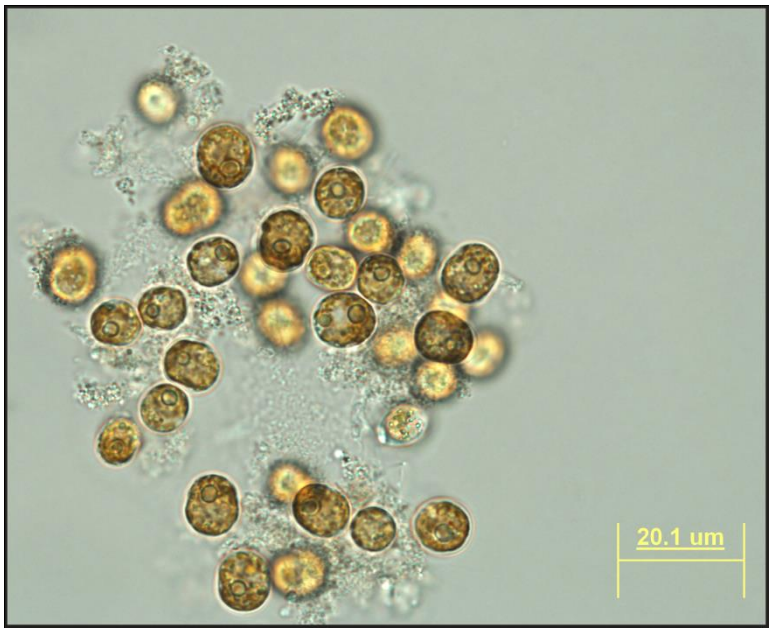

Figure 1.2. Bright field micrograph of *Symbiodinium* cells in culture (Culture ID A002), originally isolated from an *Acropora* sp. reef coral in Okinawa, Western Pacific.

The photosynthetic products thought to be released from *Symbiodinium* cells may also differ from those of *Chlorella*; glycerol was originally proposed as the dominant compound (Trench, 1971; 1979; Venn *et al.*, 2008) although this has been the subject of much debate and other photosynthetic products have since been suggested (Whitehead & Douglas, 2003; Burriesci *et al.*, 2012). In fact, and despite decades of research in this area, we still know very little about the identity of organic compounds provided to the host (Davy *et al.*, 2012). The regulation of their release is even less clear, with proteins, amino acids, and calcium/calmodulin antagonists all being suggested as possible mediators (see Venn *et al.*, 2008; Davy *et al.*, 2012 for reviews). In the other direction, the classic model of nutrient cycling in the cnidarian-dinoflagellate symbiosis proposes that ammonium produced by the host is assimilated by the alga during its photosynthetic activities and the nitrogen recycled back to the host as amino acids (Wilkerson & Muscatine, 1984; Trench, 1993). However, a study by Wang & Douglas (1998) found that ammonium release into the surrounding water declined when host organisms were incubated in the dark (to prevent symbiont photosynthesis) but supplemented with exogenous carbon. This suggests that, rather than the symbiont

simply acting as a sink for host-derived nitrogenous waste, its provision of photosynthetically-fixed carbon also allows for nitrogen conservation by the host (Wang & Douglas, 1998). We still know little about the physiology of inorganic nutrient cycling and carbon translocation in cnidarian-dinoflagellate associations and this remains a major area of current research (Davy *et al.*, 2012).

1.2.1. Establishment of anthozoan-dinoflagellate symbiosis.

Some anthozoan species transmit their symbionts vertically between generations and, perhaps unsurprisingly, this mechanism prevails among the minority of species that brood their larvae (Trench, 1993; Diekmann *et al.*, 2002; Baird *et al.*, 2009). The majority, however, spawn aposymbiotic larvae that must acquire their symbionts horizontally - from the external environment - prior to settlement and metamorphosis (Fadlallah, 1983; Baird *et al.*, 2009). The evolutionary advantages of this mode of transmission may seem at first unclear, but recent work has suggested that symbiotic larvae during planktonic life stages can incur significant physiological stresses associated with photosynthesis, and may be more susceptible to environmental stress (Yakovleva *et al.*, 2009). Moreover, horizontal transmission might encourage a greater symbiotic flexibility through an increased opportunity for host-symbiont recombination (Little *et al.*, 2004) and the establishment of novel "holobionts" (the symbiotic unit). This hypothesis appears to hold for most reef corals (Baird *et al.*, 2007; Baird *et al.*, 2009; Fabina *et al.*, 2012).

Horizontally transmitting species have to be able to select their symbionts from a background community of potentially numerous types. This selectivity is an emergent property of a number of cellular mechanisms acting both *pre*- and *post*-uptake. Symbiotic cnidarians possess a variety of pattern recognition receptors (PRRs) that selectively recognise microbe-associated molecular patterns (MAMPs) and thus could enable a host to discern "acceptable non-self" (a suitable *Symbiodinium* type for example) from "unacceptable non-self" (an unsuitable symbiont type). PRRs with similarity to complement-3 receptors, toll-like receptors, scavenger receptors, and lectins have all been observed in various symbiotic cnidarian species (see Davy *et al.*, 2012 for review). Symbiont markers such as cell-surface glycans (Logan *et al.*, 2010) and other glycoconjugates have also been characterised (reviewed by Davy *et al.*, 2012).

Little is understood about the specifics of PRR-MAMP interactions in symbiotic Cnidaria, but these mechanisms may represent the initial stages of a complex process of selective uptake and sorting that ensures a level of specificity in the symbiosis.

Following their successful phagocytic uptake, symbiont cells are selectively "packaged" into specialised vesicles ("symbiosomes" morphologically resembling the phagosomes of mammalian leukocytes) in gastrodermal cells within a matter of hours (Schwarz *et al.*, 1999). It appears that symbiont retention is restricted only to live, functional symbionts; dead and dysfunctional cells are quickly rejected (Dunn & Weis, 2009). This ability to prevent infection by the background microbial community implies the existence of an effective immune system. Indeed, but not without much debate (Rinkevich, 2011), cnidarians are believed to possess innate immune-like pathways (Kimbrell & Beutler, 2001; Altincicek, 2009; Dunn, 2009; Detournay *et al.*, 2012; Palmer *et al.*, 2012; Palmer & Traylor-Knowles, 2012) and these must presumably be modified in order for successful endosymbiosis to be achieved. *Symbiodinium* cells can successfully evade destruction through the manipulation of lysosome trafficking and binding (Schwarz & Weis, 2003; Chen *et al.*, 2005). Dead or photosynthetically compromised cells, however, have been seen to accumulate surface proteins that lead to lysosome targeting and eventual destruction (Chen *et al.*, 2003). Dunn & Weis (2009) also showed that larvae of the solitary coral *Fungia scutaria* employ apoptotic-like pathways (reviewed in detail below) to prevent colonisation by unsuitable symbionts, and that inhibition of these pathways results in persistent infection of the host by these previously incompatible *Symbiodinium* cells.

None of these phenomena are unique to mutualistic symbioses, however, as similar patterns of infection are seen in parasitisms including malaria and toxoplasmosis (Stevenson & Riley, 2004; Schwarz, 2008). The apicomplexan microbes responsible for these diseases (*Plasmodium* and *Toxoplasma* spp., respectively) are phylogenetically not far removed from dinoflagellates (Baldauf, 2003; Janouskovec *et al.*, 2010; Fig. 1.3). Moreover, with parasitism hypothesised to be an acquired characteristic in alveolates (Moore *et al.*, 2008), it seems probable that natural selection would have favoured the modification of existing cellular invasion strategies for a novel task - in this case parasitising the host. Our collective understanding of many parasitic infections is undoubtedly more advanced than our knowledge of the cnidarian-*Symbiodinium*

association, so it would be beneficial to view these models as potential mines of information regarding the strategies employed by *Symbiodinium* cells to avoid recognition/destruction (Schwarz, 2008; Sibley, 2011). It might also shed much-needed light on the innate immune-like mechanisms underpinning the breakdown of the symbiosis (Weis, 2008).

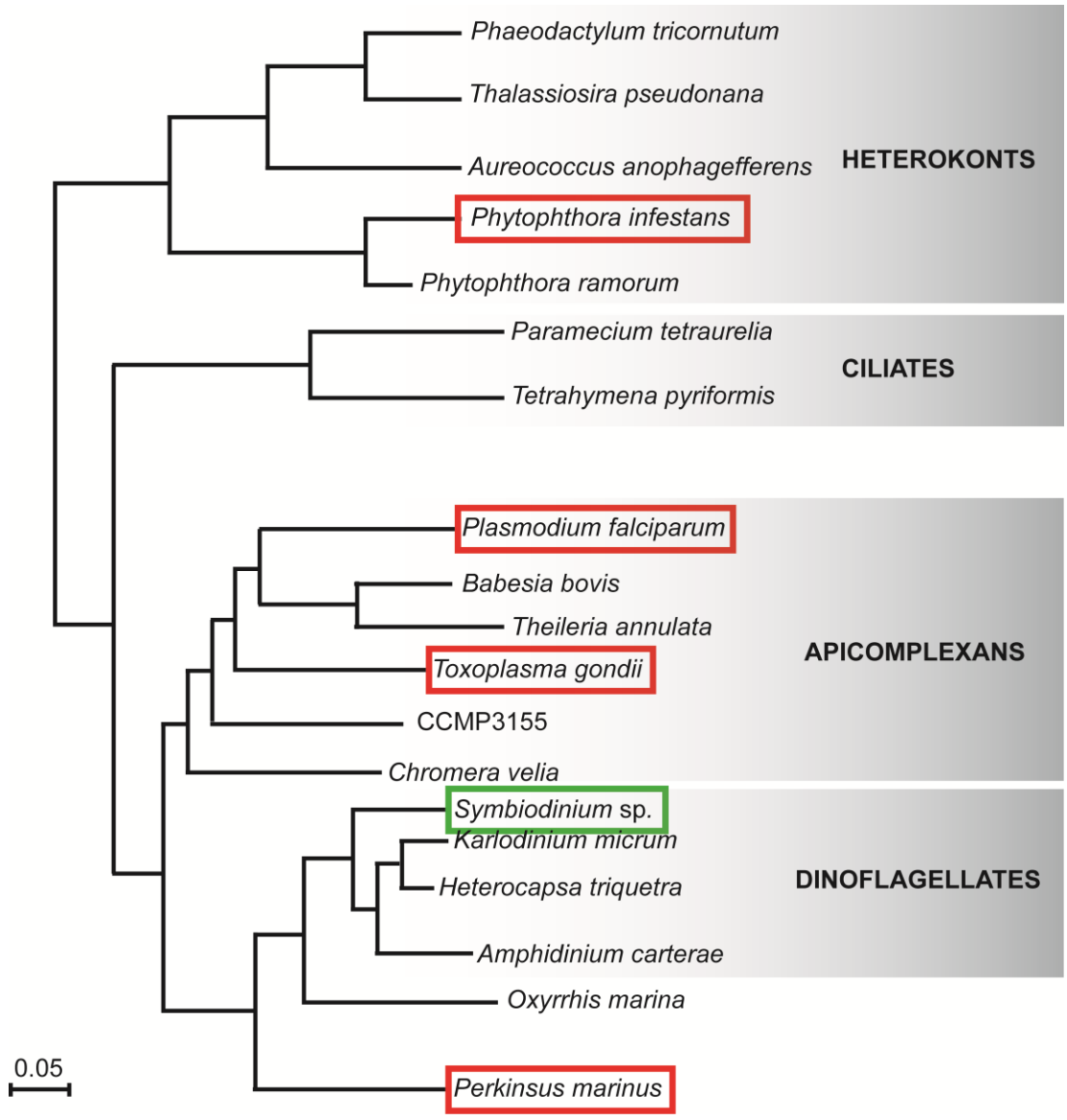

Figure 1.3. A phylogenetic examination of the origin of plastid structures in Apicomplexa and closely related taxa. Symbiotic dinoflagellates (green) group closely with some solely parasitic species (red) including *Phytophthora infestans, Toxoplasma gondii* and *Plasmodium falciparum*. The marine parasite *Perkinsus marinus* is itself descended from an early branch of the dinoflagellate lineage (Saldarriaga *et al.*, 2003). Figure adapted from Janouskovec *et al.* (2010).

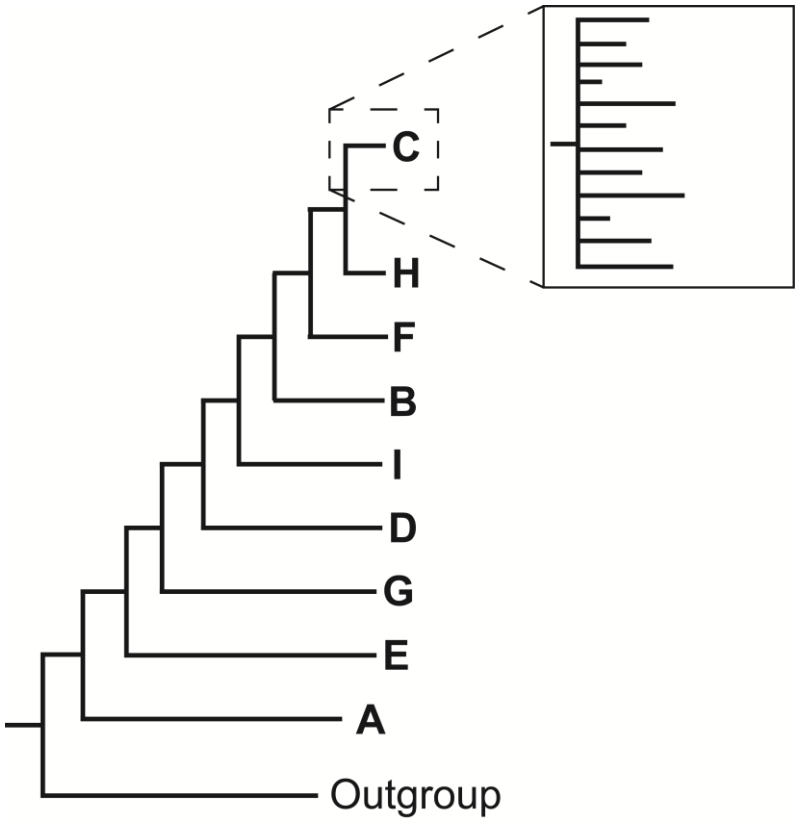

Figure 1.4. Diversity in the genus *Symbiodinium* [using the large ribosomal subunit to distinguish between clades (Rowan & Powers, 1991) and the internal transcribed spacer-2 - ITS2 - region as a within-clade "species" marker (LaJeunesse, 2002)]. Clade A appears to be descended directly from the ancestral group, with clades C and H the most recently derived groups. Adapted from Pochon *et al.* (2004) with additional information from Pochon & Gates (2010).

Evolutionary relationships between the different *Symbiodinium* clades (Fig. 1.4) are only recently becoming apparent (Pochon *et al.*, 2006; Stat *et al.*, 2006), but it appears that clade A may be the most primitive, forming a sister lineage to all other clades (Coffroth & Santos, 2005). It could also have been the first to enter symbiosis with invertebrates (Stat *et al.*, 2008). Clade C, on the other hand, represents the "crown of the *Symbiodinium* tree" (Pochon *et al.*, 2006); even given its relatively recent evolution (Stat *et al.*, 2006) it is the most diverse group by some distance (LaJeunesse, 2005). While diversity within clades A and B is less in comparison, they still dominate symbiont communities in the Caribbean Sea (LaJeunesse, 2002; Baker, 2003;

LaJeunesse *et al.*, 2003; Coffroth & Santos, 2005; LaJeunesse, 2005). On Indo-Pacific reefs including the Great Barrier Reef (GBR) of eastern Australia, clades A and B are displaced by clade C symbionts (LaJeunesse, 2002; Baker, 2003) and inter-cladal diversity is consequently depressed (LaJeunesse *et al.*, 2003; Goulet & Coffroth, 2004). Clade D is present at low levels in both regions (LaJeunesse, 2002; van Oppen *et al.*, 2005; Ulstrup *et al.*, 2006) and is often regarded as opportunistic and tolerant of a wide range of environmental conditions (Baker, 2003; Berkelmans & van Oppen, 2006; Ulstrup *et al.*, 2006; LaJeunesse *et al.*, 2008; Lien *et al.*, 2013). This is especially relevant in the context of oceanic warming and the breakdown of the coral-algal symbiosis, as described below.

Symbionts are released from their host even under non-stressful conditions (e.g. Fagoonee *et al.*, 1999), and this leads to a hypothetical "sink" of potentially infective symbionts in the surrounding seawater and sediment (Takabayashi *et al.*, 2012). Indeed, surveys at Kane'ohe Bay, Hawai'i, and the Caribbean coast of Mexico observed *Symbiodinium* cells in the water column (Manning & Gates, 2008), and recent work suggests that these cells can persist outside their host for long periods, supplementing their photosynthesis by feeding on bacteria (Jeong *et al.*, 2012). Interestingly, the water-borne *Symbiodinium* types identified by Manning & Gates (2008) (C3 in Hawaii and C21 in Mexico) were different from those dominating the *in hospite* communities at each location [C15 in Kane'ohe Bay (Apprill & Gates, 2007) and B1 in the Caribbean (LaJeunesse *et al.*, 2004)]. Together with laboratory observations of symbiont acquisition and sorting (described above), the distinctly non-random distribution of symbionts among host species (van Oppen *et al.*, 2001; LaJeunesse *et al.*, 2003; Coffroth & Santos, 2005; Sampayo *et al.*, 2007; LaJeunesse *et al.*, 2008; Macdonald *et al.*, 2008; Thornhill *et al.*, 2009) supports an impressive capacity for the host to selectively associate with particular symbiont types. The specificity of some coral species, for example *Fungia scutaria* (Weis *et al.*, 2001), for individual symbiont types appears highly stable over geographic and temporal scales, but a subject of much debate in the literature (Goulet, 2006; Baker & Romanski, 2007; Goulet, 2007) has been to extent to which this apparent specificity is simply an artefact of inadequate assessment methods (Silverstein *et al.*, 2012). Indeed, the number of host species associating with multiple *Symbiodinium* types does appear to be ever-growing (Baker, 2003; Fabricius *et al.*, 2004). For reasons that remain unclear, this is particularly so in

the Caribbean where many dominant reef corals host multiple *Symbiodinium* clades (Rowan & Knowlton, 1995; Rowan, 1998; Baker, 2003; Knowlton & Rohwer, 2003). These associations often exhibit bathymetric zonation, possibly linked to differences in the light environment at different depths (Rowan *et al.*, 1997). To a lesser extent, some Indo-Pacific communities also show depth-dependent changes in symbiont composition (albeit at a sub-cladal level) and Sampayo and co-workers (2007), studying the reef corals *Pocillopora damicornis* and *Stylophora pistillata* on the Great Barrier Reef, observed significant zonation of symbiont types from shallow (5-7 m) to deep (15-18 m) reef areas. These observations all imply a physiological diversity among different *Symbiodinium* types that may, in times of stress, have a significant impact on the stability of some cnidarian-*Symbiodinium* associations (van Oppen *et al.*, 2009).

1.3. Coral reefs: A pinnacle of marine symbiosis.

The cnidarian-dinoflagellate symbiosis underpins tropical coral reefs, the most biodiverse marine ecosystems on Earth. The efficient cycling of nutrients (see above) also neatly explains "Darwin's Paradox" - the once-intractable problem of how such high diversity and productivity could be maintained in the relatively nutrient-poor waters of the tropics (Muller-Parker & D'Elia, 1997; Sheppard *et al.*, 2009). Coral reefs sustain food webs and economically important fishing and tourist industries throughout the tropics. Furthermore, by assisting the incorporation of dissolved inorganic carbon into a physical framework (the coral's calcium carbonate skeleton), the coral-dinoflagellate association contributes significantly to geochemical and climate homeostasis. Recent climatic changes, however, are placing this mutualism under increasing strain (McClanahan *et al.*, 2009; Pandolfi *et al.*, 2011; De'ath *et al.*, 2012; Perry *et al.*, 2013) and the prognosis for reefs over the coming century is relatively bleak (van Hooidonk *et al.*, 2013).

1.4. Coral bleaching: The collapse of a symbiosis.

It has become clear over the past 30 years that coral reefs are becoming increasingly threatened (Glynn, 1983; 1984; Cortes & Risk, 1985; Liddell & Ohlhorst, 1986; Glynn & Dcroz, 1990; Done, 1992; Bythell *et al.*, 1993; Hughes, 1994; Anthony *et al.*, 2011; Perry *et al.*, 2013). This is a worldwide phenomenon and even well managed reef systems have suffered dramatic losses in terms of coral cover and diversity (De'ath *et al.*, 2012). Local and global stressors are implicated (Hughes, 1994; Hoegh-Guldberg *et al.*, 2007; Hughes *et al.*, 2010) and conservative projections have coral reefs, as we know them, not surviving far into the next century (Hoegh-Guldberg & Bruno, 2010; Hughes *et al.*, 2010; Pandolfi *et al.*, 2011; Perry *et al.*, 2013).

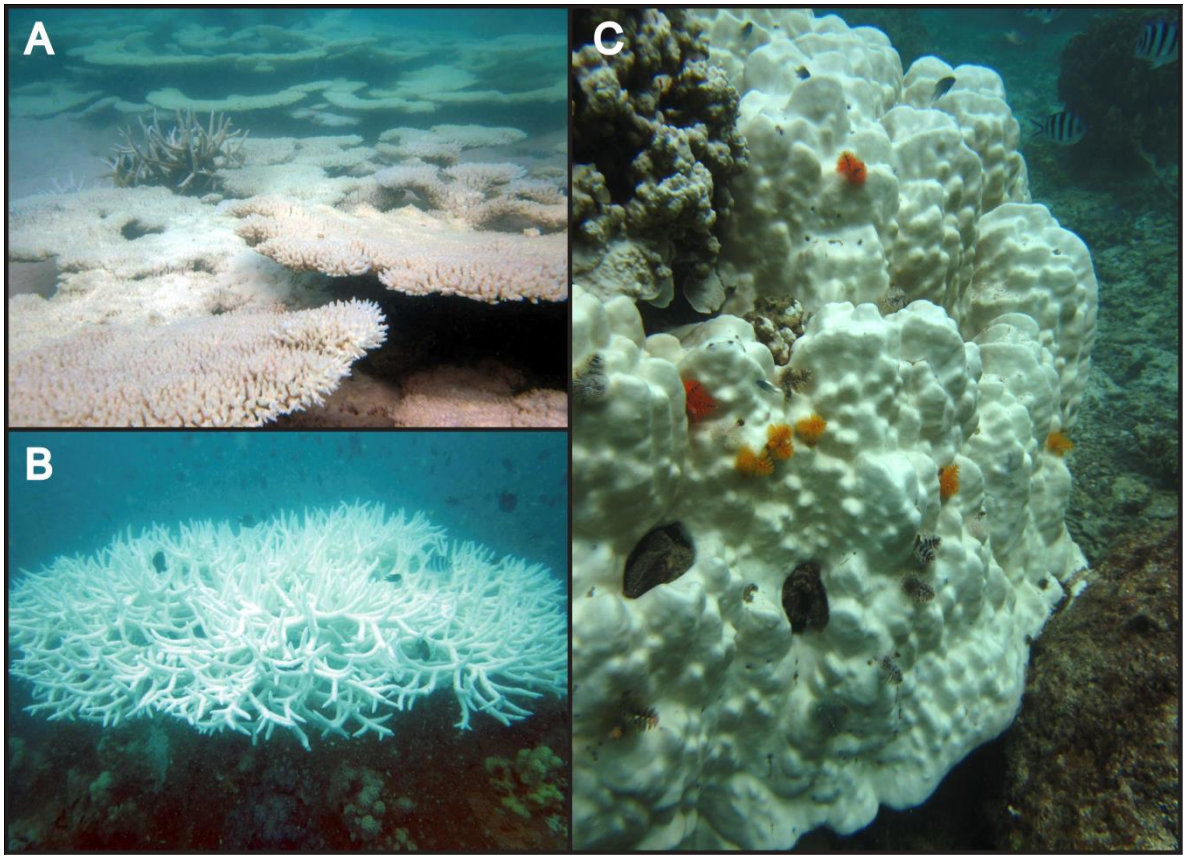

Figure 1.5. Thermal bleaching in reef corals in the Gulf of Thailand, 2010. **Panel A)** Bleached tabulate *Acropora* spp. corals; **B)** Fully bleached *Acropora formosa* colony; **C)** Partially bleached *Porites lobata* colony. Photos © Nathan Cook.

Out of the multitude of threats facing reefs, anthropogenic climate change is potentially the most pernicious. As the major driver of increasing sea surface temperatures, climate change is leading to more intense and damaging tropical storms (Webster *et al.*, 2005; Knutson *et al.*, 2010) as well as more frequent and intense episodes of coral "bleaching" (Hoegh-Guldberg, 1999; Fitt *et al.*, 2001; McClanahan, 2004; van Hooidonk *et al.*, 2013). This latter phenomenon is a process in which corals visibly pale (Fig. 1.5) due to declining numbers of symbiont cells and/or the degradation of symbionts' photosynthetic pigments. Bleached corals experience energetic and nutritional shortfalls (Goreau & MacFarlane, 1990) and often become more susceptible to pathogens (Harvell *et al.*, 1999; Mydlarz *et al.*, 2009, 2010). This can have pronounced and far-reaching impacts on the ecosystem as a whole and, although some species are able to compensate by increasing their heterotrophic activities (Grottoli *et al.*, 2006), others, including many of the dominant contributors to the reef structure itself, lack this ability. For these species, rapid symbiont population recovery (either through growth of residual *Symbiodinium* populations, heightened pigment biosynthesis, or uptake of new symbionts from the environment) is critical if they are to avoid the reduced growth rates (Goreau, 1990), reproduction (Szmant & Gassman, 1990) and mortality (McClanahan, 2004; Jones, 2008; McClanahan *et al.*, 2009) associated with coral bleaching. This symbiotic collapse can even affect the reef structure itself, which persists as a fine balance between mineral accretion (to which the calcifying activity of corals is a major contributor) and erosion (Done, 1992). Any long-term decline in calcification due to bleaching can result in structural as well as trophic ecosystem breakdown (Hughes *et al.*, 2007; Hoegh-Guldberg & Bruno, 2010; Perry *et al.*, 2013).

1.4.1. What causes coral bleaching?

Research over the past two decades (e.g. Iglesias-Prieto *et al.*, 1992; Warner *et al.*, 1996; Jones *et al.*, 1998; Warner *et al.*, 1999; Hennige *et al.*, 2009) has strongly implicated the dinoflagellate symbiont in the initial stages of thermal bleaching. Direct temperature-induced damage to the host almost certainly occurs (Dykens *et al.*, 1992), but coral bleaching appears to be a consequence of sustained damage to the symbiont's photosynthetic apparatus (reviewed by Smith *et al.*, 2005; Lesser, 2011) that associates with the overproduction of reactive oxygen species (ROS). The consequences of this are reviewed in detail below, but include oxidative stress (Dykens *et al.*, 1992; Lesser,

1996; 1997; Downs *et al.*, 2002; Lesser & Farrell, 2004; Lesser, 2006; Richier *et al.*, 2006) and the activation of host innate immune-like signalling pathways (Weis, 2008).

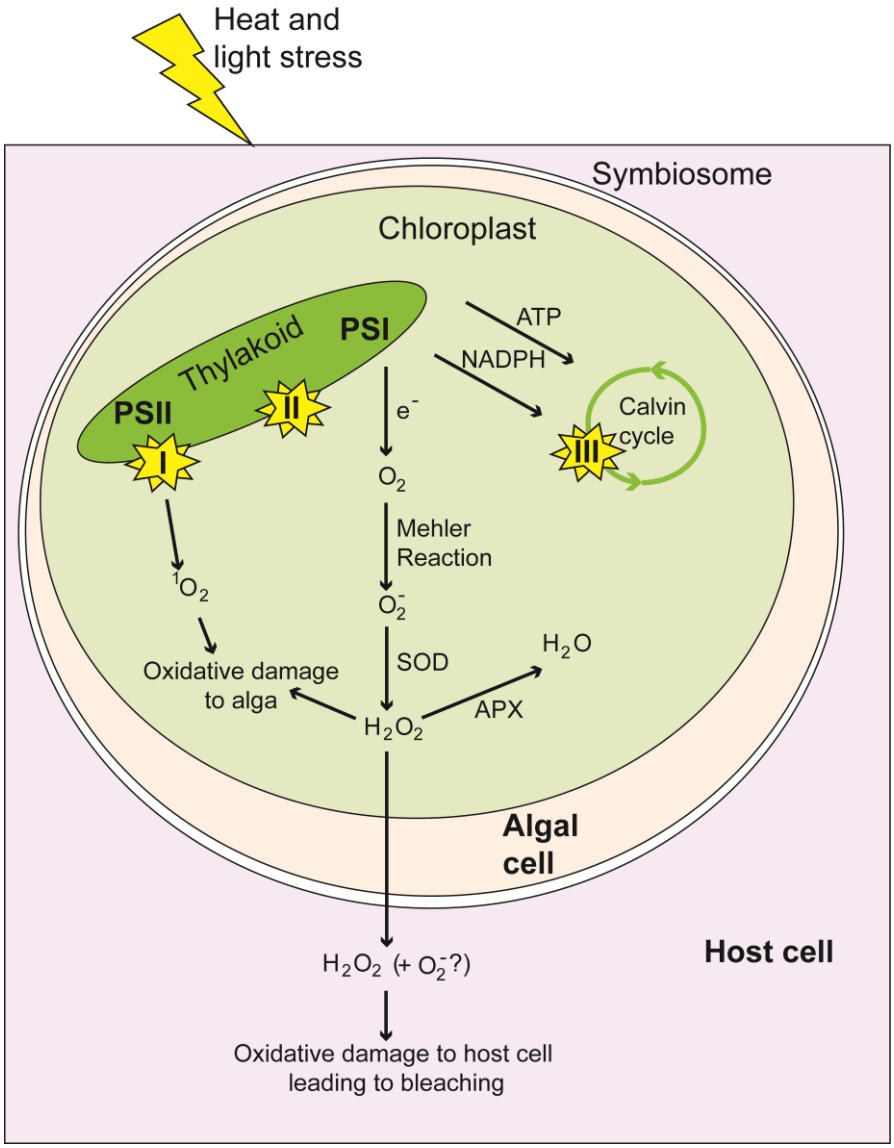

Figure 1.6. Simplified model of reactive oxygen species (ROS; including singlet oxygen: $^1O_2$; superoxide: O_2^-; and hydrogen peroxide: H_2O_2) generation in *Symbiodinium* cells exposed to heat and light stress. ROS generation occurs at photosystems (PS) I and II with subsequent damage to PSII (I), thylakoid membranes (II) and the components of carbon fixation (III). ROS may also leak into host cells. (SOD: superoxide dismutase; APX: ascorbate peroxidase). Adapted from Venn *et al.* (2008).

The precise points of injury within *Symbiodinium* cells are slowly becoming clear (Fig. 1.6) and include the D1 protein in photosystem II (PSII) (Warner *et al.*, 1999; Takahashi *et al.*, 2009; Ragni *et al.*, 2010) and the thylakoid membranes (Tchernov *et al.*, 2004). The Calvin cycle enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) has also been shown to be inhibited at elevated temperature (Jones *et al.*, 1998). The order in which these components become dysfunctional is less clear, however, as events at one location often have downstream effects on others. Despite this, the oxidative component of cnidarian bleaching, due to the build-up of ROS, is now well recognised (Lesser *et al.*, 1990; Dykens *et al.*, 1992; Lesser, 1997; Downs *et al.*, 2002; Lesser & Farrell, 2004; Lesser, 2006; Richier *et al.*, 2006; Weis, 2008). Several studies have observed elevated ROS generation in *Symbiodinium* cells during thermal stress (e.g. Tchernov *et al.*, 2004; Suggett *et al.*, 2008; Saragosti *et al.*, 2010; McGinty *et al.*, 2012) and one particular ROS, hydrogen peroxide (H_2O_2), has a high potential for diffusion out of the symbiont (Halliwell & Gutteridge, 2007). Co-evolution with photosynthetic endosymbionts has equipped symbiotic cnidarians well and they possess a wide variety of antioxidant mechanisms (Dykens & Shick, 1982; Furla *et al.*, 2005; Richier *et al.*, 2005; Bou-Abdallah *et al.*, 2006; Palmer *et al.*, 2009). Even so, influxes of H_2O_2 from stressed symbionts may be sufficient either to induce cell death directly or at least initiate signalling pathways within the host cell that eventually result in the loss of the symbionts (Martindale & Holbrook, 2002; Perez & Weis, 2006, 2008; Richier *et al.*, 2006; Dunn *et al.*, 2007; Perez & Weis, 2008; Weis, 2008).

1.4.2. Variation in bleaching susceptibility.

It has long been known that corals vary in their sensitivities to environmental stresses (Fig. 1.7), and while host-level physiological and anatomical differences (discussed below) have been identified (Baird *et al.*, 2008; Bromage *et al.*, 2009; Fitt *et al.*, 2009; Kramer *et al.*, 2013), their sensitivity is heavily influenced by the physiology of the resident *Symbiodinium* population (Rowan *et al.*, 1997; Jones *et al.*, 1998; Fabricius *et al.*, 2004; Rowan, 2004; Tchernov *et al.*, 2004; Berkelmans & van Oppen, 2006; Ulstrup *et al.*, 2006; Sampayo *et al.*, 2008; van Oppen *et al.*, 2009).

Figure 1.7. Differential bleaching in reef corals at Rarotonga, Cook Islands in 2011. **Panel A)** Partially bleached *Pocillopora eydouxi* colonies atop an unbleached massive *Porites lobata* colony; **B)** Bleached and unbleached encrusting *Favites* sp. colonies.

The role of the symbiont:

Many *Symbiodinium* ITS2 types such as A1 (e.g. from the jellyfish *Cassiopeia xamachana*), C15 (found in *Montipora digitata* and *Porites* spp.), and D1 (from numerous reef corals), are relatively robust in response to rising temperatures (Berkelmans & van Oppen, 2006; Robison & Warner, 2006; Suggett *et al.*, 2008; Ragni *et al.*, 2010; Fisher *et al.*, 2012). Others, however, such as B1 (e.g. from the anemone *Aiptasia*), A1.1 (e.g. from the anemone *Condylactis gigantea*) and C3 (from numerous reef corals) can, depending on the extent of their recent exposure to elevated temperatures, suffer pronounced physiological dysfunction (Robison & Warner, 2006; Ragni *et al.*, 2010; Fisher *et al.*, 2012). Tchernov & co-workers (2004) proposed that this variability in thermal tolerance stemmed from the symbionts' thylakoid membrane composition and, subsequently, the differential generation of ROS. For example, the temperature-sensitive B1-type symbionts from *Aiptasia* sp. and the reef coral *Stylophora pistillata* suffered greater membrane disruption and exhibited higher levels of ROS production than did more tolerant strains when exposed to the same temperatures (Tchernov *et al.*, 2004), a finding that has since been corroborated

(Suggett *et al.*, 2008). The ability of cells to repair the important D1 protein in PSII has also been found to vary between differentially temperature-sensitive *Symbiodinium* types (Ragni *et al.*, 2010). Furthermore, significant variation in accessory pigment concentration and the capacity for non-photochemical quenching (NPQ - the dissipation of excess light energy as heat) has recently been observed (Fisher *et al.*, 2012), even among closely related ITS2 types (Hennige *et al.*, 2009). Symbionts' capacities to detoxify ROS through its suite of antioxidants also vary taxonomically (McGinty *et al.*, 2012), and, together, these physiological characteristics all contribute to the physiological robustness of a holobiont and thus the likelihood of its collapse during a thermal stress event.

The role of the host:

Significant variability has also been observed among coral species hosting the same symbiont type. For example, the *Symbiodinium* ITS2 types C1 and C3 occur in temperature-sensitive *Acropora*, *Seriatopora* and *Pocillopora* spp. as well as in more thermally robust genera such as *Favia*, *Goniastrea* and *Platygyra* (LaJeunesse *et al.*, 2003). Kramer *et al*. (2013), in a recent study of *Pocillopora damicornis* and *Pavona decussata* reef corals, found that symbiont photosynthesis at elevated temperature differed significantly between the two species despite them both hosting type C1 *Symbiodinium* (Hill *et al.*, 2011). This suggests that the intracellular environment might differ between host species (Kramer *et al.*, 2013). There is also increasing evidence that changes occur within the cells of the coral host before the symbionts become compromised (Ainsworth *et al.*, 2008; Ainsworth *et al.*, 2011; Dunn *et al.*, 2012; Paxton *et al.*, 2013). A number of mechanisms, both anatomical and physiological, exist by which coral hosts can determine their own bleaching thresholds (Baird *et al.*, 2008). On an anatomical level, the morphology of a coral colony, together with the thickness of its tissues, strongly influences the internal light environment in which its symbionts reside and these characteristics can vary among differentially bleaching-susceptible taxa (Loya *et al.*, 2001; Kramer *et al.*, 2013). In terms of physiological mechanisms, fluorescent proteins (FPs), for example, are common in reef corals and are capable of absorbing much of the harmful excess radiation incident on shallow-water reef habitats (Salih *et al.*, 2000). Recent work suggests that they may also play an antioxidant role (Palmer *et al.*, 2009). Investment in FPs differs greatly among coral species and correlates with their bleaching susceptibilities (Salih *et al.*, 2000). Corals also contain mycosporine-like

amino acids (MAAs) that afford photoprotection by attenuating harmful UV radiation (Shick & Dunlap, 2002). In the context of heat stress, corals possess a range of heat shock proteins (HSP; molecular chaperones protecting vital enzymes and proteins from thermal degradation) that can be upregulated during exposure to high temperature (Brown *et al.*, 2002; Leggat *et al.*, 2011). Differential host expression of HSPs and the antioxidant enzyme superoxide dismutase (SOD) has been observed in corals from the genus *Stylophora* and *Porites* (Fitt *et al.*, 2009), two genera with divergent bleaching sensitivities. As is the case for the symbionts, a significant opportunity exists for host physiology to affect the overall thermal sensitivity of the holobiont (Baird *et al.*, 2008; Wicks *et al.*, 2012).

1.4.3. Mechanisms of bleaching - linking thermal stress to holobiont collapse.

Despite the suite of protective measures acting at both symbiont and host levels, heat and light stresses can still destabilise the association. The transition to bleaching can proceed *via* a number of pathways (Fig. 1.8). Of particular interest in the context of this thesis are those pathways resulting in uncontrolled (necrotic) and programmed (including apoptotic) cell death of host cells, and the degradation of symbionts. Necrosis is often the result of the uncontrolled build-up of ROS, reactive nitrogen species (RNS), and harmful metabolites that eventually compromise a cell's physiological integrity (Cadenas, 1989; Lesser, 2006; Halliwell & Gutteridge, 2007). Programmed cell death [PCD; often referred to as apoptosis when cells display specific morphologies during death (Kerr *et al.*, 1972)], however, is a tightly regulated process of cell deletion followed by the assimilation/recycling of cellular material by neighbouring cells (Aravind *et al.*, 1999; Martindale & Holbrook, 2002; Bottger & David, 2003; Segovia, 2008). Bleaching has also been seen to involve autophagy-like pathways, whereby cells digest their own components through a complex procedure of organelle labelling, vesicle trafficking and lysosomal fusion (Gozuacik & Kimchi, 2004).

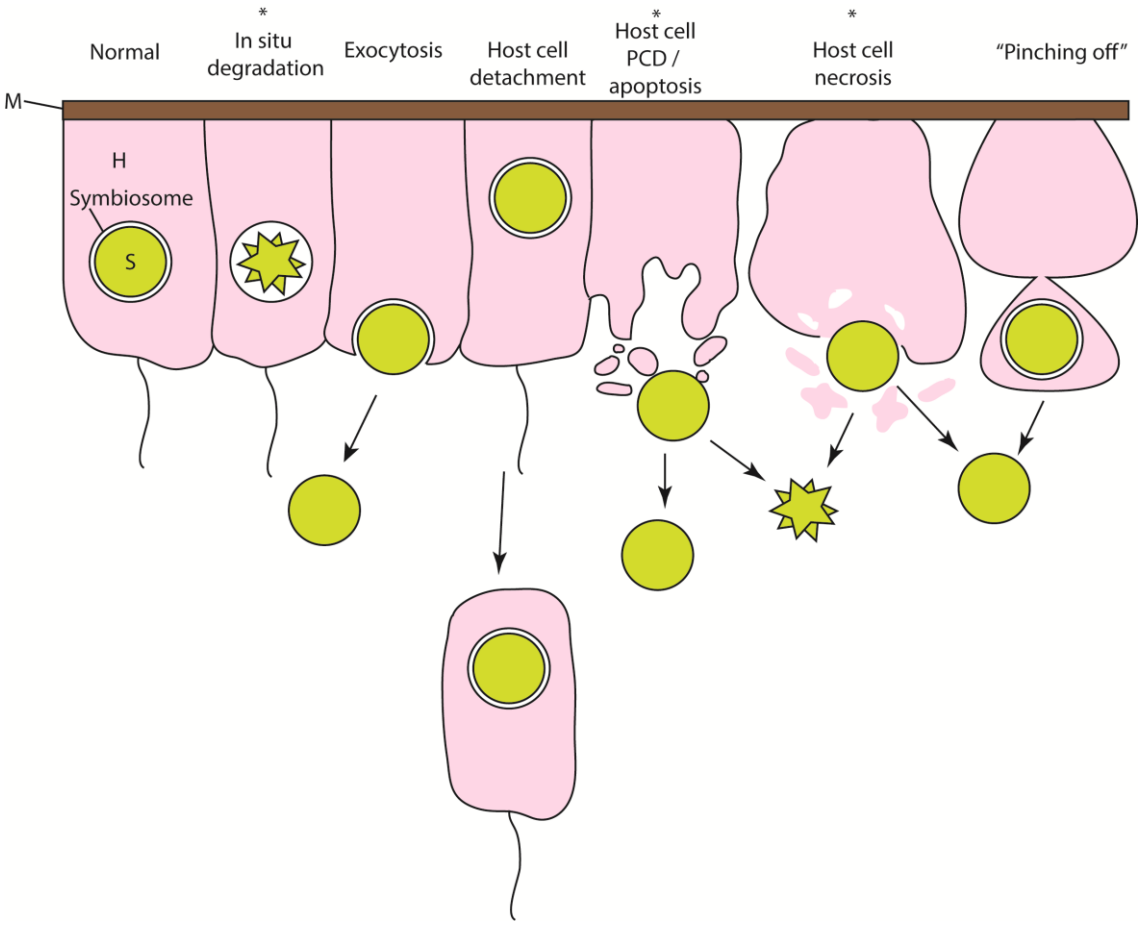

Figure 1.8. Symbiotic anthozoans have been known to bleach *via* several mechanisms. Symbionts (S) may be degraded within their host cells (H) or ejected from the host through a process of exocytosis or "pinching off". Host cells have also been observed undergoing programmed cell death (PCD) and necrosis, both of which lead to symbiont loss. Symbionts lost through PCD and necrosis can be either viable or degraded. Furthermore, entire host cells containing intact symbionts may be lost through detachment from the acellular mesogloea (M). Asterisks above pathways denote those most likely to be mediated by reactive oxygen species or nitric oxide and its derivatives. Re-drawn from Weis (2008) and Gates *et al.* 1992.

Characteristics of both PCD and necrosis have been observed in host and symbiont cells during thermal stress (Dunn *et al.*, 2002; Dunn *et al.*, 2004; Richier *et al.*, 2006; Dunn *et al.*, 2007; Bouchard & Yamasaki, 2009; Strychar & Sammarco, 2009), and enzymes with activities similar to those tasked with the execution of apoptosis in mammals (aspartate-specific cysteine proteases - "caspases") have been identified in symbiotic cnidarians (Cikala *et al.*, 1999; David *et al.*, 2005; Dunn *et al.*, 2006; Richier *et al.*, 2006; Kvitt *et al.*, 2011; Pernice *et al.*, 2011; Tchernov *et al.*, 2011) and their symbionts (Segovia, 2008; Bouchard & Yamasaki, 2009). While research into temperature-induced stress and cell death is proceeding apace, comparatively little has been achieved with respect to linking the two. The implication of RNS (specifically nitric oxide - NO) in the breakdown of the cnidarian-dinoflagellate symbiosis (Trapido-Rosenthal *et al.*, 2001; Trapido-Rosenthal *et al.*, 2005; Perez & Weis, 2006; Bouchard & Yamasaki, 2008) provides an attractive avenue of enquiry in this regard and forms the basis for this thesis.

1.5. Nitric oxide as a link in the bleaching cascade.

Nitric oxide is among the smallest signalling molecules in biology (Beckman & Koppenol, 1996). Originally discovered as the "endothelium-derived relaxing factor" promoting vasodilation in mammals (Ignarro *et al.*, 1987), and winning its discoverers a Nobel Prize in the process (Smith, 1998), NO is now recognised as ubiquitous in living organisms.

There are many pathways for biosynthesis of NO in eukaryotes and most are enzymatic in nature (Griffith & Stuehr, 1995; Moroz, 2001). In animals, NO is synthesised through the activities of nitric oxide synthase (NOS) enzymes, which convert arginine to citrulline with NO as a second product (Griffith & Stuehr, 1995; Colasanti & Suzuki, 2000). In mammals, three NOS isoforms are known: eNOS, responsible for endothelium-derived NO; nNOS, synthesising NO for neuronal signalling; and iNOS, responsible for synthesising NO in innate immune responses (Griffith & Stuehr, 1995). eNOS and nNOS are constitutive (expressed constantly at relatively low levels) and calcium-dependent, while iNOS is inducible, calcium-independent and capable of producing much greater quantities of NO than are the other two enzymes (Colasanti & Suzuki, 2000). Only one NOS isoform has been observed in any individual lower

invertebrate (Colasanti *et al.*, 2010) and it often bears a resemblance to both constitutive (calcium-dependent) and inducible (stimulated by heat or in immune-like response) mammalian NOS (Morrall *et al.*, 2000; Giovine *et al.*, 2001; Moroz *et al.*, 2004; Perez & Weis, 2006; Moroz & Kohn, 2007; Safavi-Hemami *et al.*, 2010). Plant and microbial synthesis of NO is significantly more complicated than it is in animals (Beligni & Lamattina, 2001) and multiple pathways capable of synthesising NO exist. These include NOS-like enzymes as well as the conversion of inorganic nitrogen *via* nitrate reductase (NR) enzymes (Ghigo *et al.*, 1995; Basu *et al.*, 1997; Yamasaki *et al.*, 1999; Yamasaki & Sakihama, 2000; Beligni & Lamattina, 2001; Sakihama *et al.*, 2002; Chandok *et al.*, 2003; Delledonne, 2005; Foresi *et al.*, 2010).

As expected for a molecule so widely distributed among taxa, NO plays a role in some diverse biological phenomena. These include neurotransmission (Colasanti *et al.*, 1995; Jacklet, 1997; Moroz, 2001; Torreilles, 2001; Moroz *et al.*, 2004; Cristino *et al.*, 2008), the alleviation of environmental stress (Beligni & Lamattina, 1999a; Gould *et al.*, 2003; Delledonne, 2005; Liu *et al.*, 2010) and the regulation of cell death (Wink & Mitchell, 1998; Brune *et al.*, 1999; Liu & Stamler, 1999; Borutaite *et al.*, 2000; Brookes *et al.*, 2000; Chung *et al.*, 2001; Brown & Borutaite, 2002; Almeida *et al.*, 2007; Snyder *et al.*, 2009). Importantly, given the context of this thesis, NO is also involved in a large number of symbiotic interactions (Wang & Ruby, 2011), described in more detail below.

1.5.1. Nitric oxide, innate immunity and microbial symbiosis.

As early as 1818 (Prout, 1818) it was observed that patients suffering acute fever displayed elevated levels of urinary nitrate (NO_3^-), a biomarker of elevated nitric oxide synthesis (Ignarro *et al.*, 1993). It is now widely recognised that NO is a critical component of innate immunity (Fang, 2004). For example, successful antimicrobial activity of mammalian macrophages is dependent on NOS enzymes (MacMicking *et al.*, 1997). These are upregulated in a wide variety of organisms during microbial infection (e.g. Eiserich *et al.*, 1998; Chan *et al.*, 2001; Villamil *et al.*, 2007; Carton *et al.*, 2009; Herrera-Ortiz *et al.*, 2011) and the resultant NO has been shown to inhibit bacterial respiration (Wink *et al.*, 2011), induce parasite cell death (Ali *et al.*, 2010), and generally reduce the pathogen load (MacMicking *et al.*, 1997; Leng *et al.*, 2009). NO

produced in concert with H_2O_2 plays an important role in the response of the mosquito *Anopheles* spp. to *Plasmodium berghei* infection (Herrera-Ortiz *et al.*, 2011), and heightened NOS activity in mice correlates with increased immunity to *Leishmania major* (Liew *et al.*, 1991). Nitric oxide is required for the effective killing of the same parasite in humans (Vouldoukis *et al.*, 1995). Conversely, inhibiting NOS has been shown to exacerbate diseases caused by *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Toxoplasma gondii*, *Plasmodium* and *Leishmania* spp., and *Salmonella* spp., as well as parasitic fungi and helminths (reviewed by MacMicking *et al.*, 1997; Nathan & Shiloh, 2000). Nitric oxide is also involved in the responses of plants to pathogenic microbes (Nürnberger *et al.*, 2004; Saito *et al.*, 2006) although less is known about its specific roles in plant immunity. It is also important to note that heightened NO synthesis in the innate immune responses of animals often occurs against a backdrop of upregulated programmed cell death pathways and the induction of apoptosis (Kimbrell & Beutler, 2001; Wilson *et al.*, 2009). Moreover, the modification of NO-synthetic pathways and apoptosis is an important strategy by intracellular parasites to avoid recognition and destruction by the host (Pannebakker *et al.*, 2007; Laliberte & Carruthers, 2008; Hippe *et al.*, 2009; Sibley, 2011).

A number of preventative mechanisms have been evolved by intracellular symbionts (both parasitic and mutualist) in order to bypass or deal with host-derived NO. These include the inhibition of NOS as well as the direct detoxification of NO. Both strategies are employed extensively by the bacterial symbionts of the squid *E. scolopes* (see above). A decade of intensive investigation has revealed host-derived NO to be critical during the establishment of this symbiosis (Davidson *et al.*, 2004), selectively "winnowing" (Nyholm & McFall-Ngai, 2004) the bacterial symbiont population by removing undesirable strains. Suitable *Vibrio* strains express a suite of attributes to either attenuate host NOS activity or detoxify NO (Wang & Ruby, 2011). These include specific MAMPs (Altura *et al.*, 2011), haem-NO/oxygen (H-NOX) binding protein (Wang *et al.*, 2010a), flavohaemoglobin (Wang *et al.*, 2010b), and alternative oxidase (AOX) enzymes (Dunn *et al.*, 2010). Haemoglobin (Hb)-like proteins also play a role in the plant-*Rhizobium* association (Shimoda *et al.*, 2005). Here, production of NO occurs in both symbiotic partners (Meilhoc *et al.*, 2011) - in the bacteria as a by-product of denitrification pathways (Zumft, 1997) and in the host as an immune response to symbiont or pathogen infection (Nagata *et al.*, 2011). Plant Hb is expressed partly in

order to detoxify excess NO (Shimoda *et al.*, 2005; Bustos-Sanmamed *et al.*, 2011; Nagata *et al.*, 2011) and the bacteria possess nitric oxide reductase (NOR) enzymes that perform a similar role (Sanchez *et al.*, 2011). NO also plays a significant role in the aphid-bacteria mutualism, where it is employed by the host as a defensive response to initial infection (Ganassi *et al.*, 2005). Interestingly, these authors proposed a role for NO similar to that in the squid-*Vibrio* association: mediating symbiotic specificity by selectively killing undesirable symbionts early in the onset of symbiosis (Ganassi *et al.*, 2005).

The cnidarian-dinoflagellate symbiosis bears a close similarity to many of the mutualist and parasitic associations described above (Schwarz, 2008) and this might even extend to NO signalling (Weis, 2008; Detournay *et al.*, 2012). A recent study observed an upregulation of NO synthesis in the anemone *Aiptasia pallida* in response to the MAMP lipopolysaccharide (LPS), a reaction that was compromised when specimens were infected with symbionts (Detournay *et al.*, 2012). However, a previous investigation showed that elevated host NO synthesis can be restored by exposing symbiotic *A. pallida* to elevated temperatures (Perez & Weis, 2006). Moreover, the same study found that removing NO at high temperature resulted in a lessening of bleaching intensity (Perez & Weis, 2006). Citing evidence from other metazoan systems (Chan *et al.*, 2001; Mendes *et al.*, 2003), the authors proposed NO as the "eviction notice", stimulated by symbiont-derived ROS (Fig. 1.9), that could lead to an apoptotic-like form of host cell death and eventual symbiont release (Perez & Weis, 2006; Weis, 2008). Complicating matters, however, are recent findings that the symbionts can also synthesise NO; Bouchard & Yamasaki (2008) provided strong evidence of NOS- and NR-derived NO in cultured *Symbiodinium* cells. Again, the authors suggested a role for NO as a mediator of PCD (Bouchard & Yamasaki, 2009).

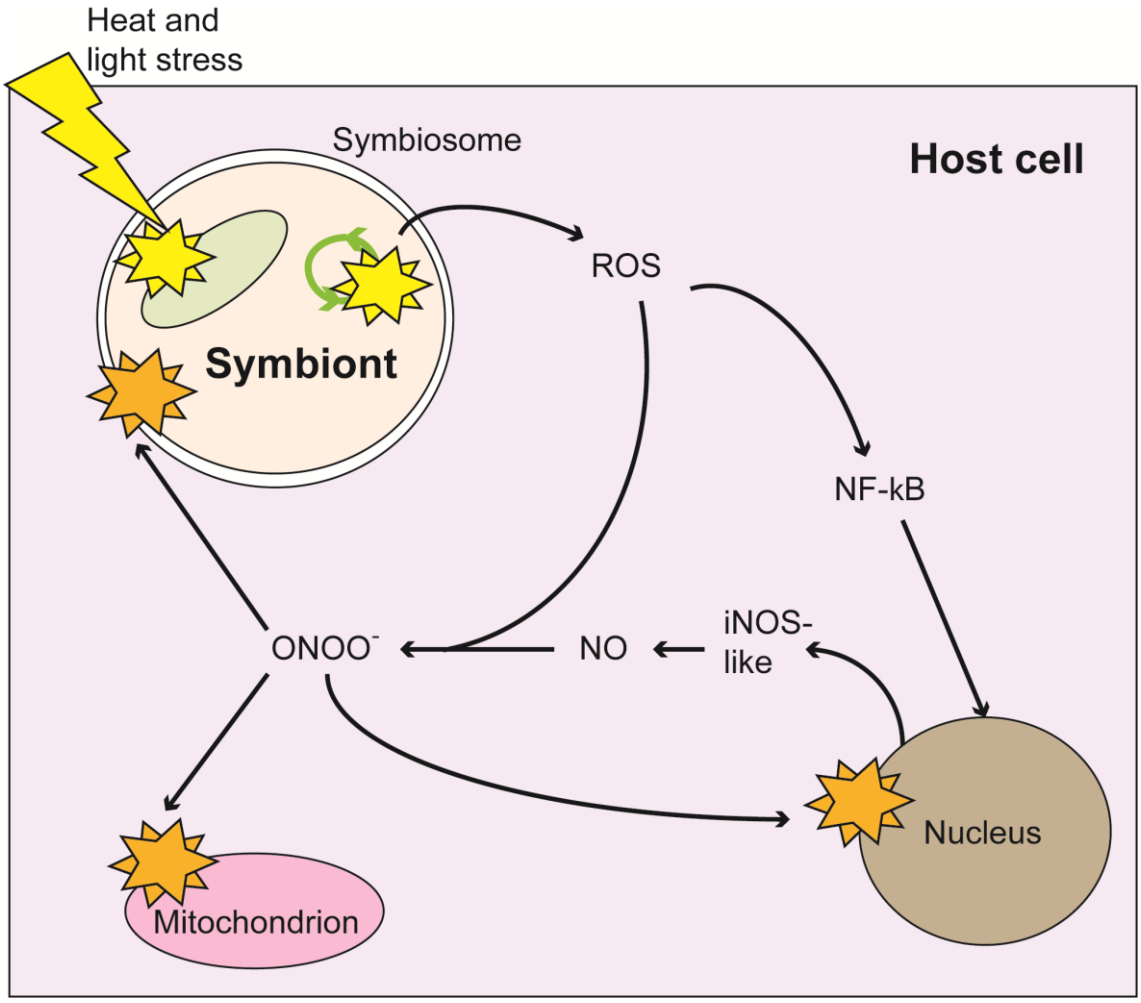

Figure 1.9. Hypothetical bleaching pathway proposed by Perez & Weis (2006). Excessive temperature/light stress-induced photoinhibition in the symbiont is associated with overproduction of ROS and the activation of host innate immune-like responses [e.g. nuclear factor-kappa B (NF-κB)-mediated pathways]. The upregulation of iNOS-like enzyme leads to NO accumulation that can, in the presence of certain ROS, result in the generation of toxic peroxynitrite ($ONOO^-$) and further damage to symbionts, host mitochondria, and DNA. This can lead to both apoptotic-like (programmed) and necrotic (uncontrolled) cell death. Adapted from Perez & Weis (2006) and Weis (2008).

Apoptotic-like PCD is a significant mechanism of symbiont loss during thermal stress (Dunn *et al.*, 2002; Dunn *et al.*, 2004; Richier *et al.*, 2006; Dunn *et al.*, 2007) and NO plays a major role in mediating this form of mortality (Brune *et al.*, 1999; Borutaite *et al.*, 2000; Chung *et al.*, 2001; Almeida *et al.*, 2007). The routes by which NO can regulate apoptosis are numerous, however, and include its direct action on mitochondria (Borutaite *et al.*, 2000; Brookes *et al.*, 2000; Shiva *et al.*, 2001; Brown & Borutaite, 2002), interactions with various pro- and anti-apoptotic molecules (Adams & Cory, 1998; Snyder *et al.*, 2009), and the diffusion-limited reaction of NO with O_2^- to produce peroxynitrite ($ONOO^-$) (Estevez & Jordan, 2002; Pacher *et al.*, 2007). $ONOO^-$ can cause serious damage to mitochondria (Gadelha *et al.*, 1997; Szabo *et al.*, 2007; Ahmad *et al.*, 2009; Bolanos & Heales, 2010), DNA (Szabo & Ohshima, 1997) and proteins (Beckman *et al.*, 1993; Ischiropoulos & Almehdi, 1995). Indeed it is possible that many of NO's effects during stress events are in fact due to its conversion to $ONOO^-$ (Radi *et al.*, 2001; Pacher *et al.*, 2007; Szabo *et al.*, 2007). Empirical confirmation of peroxynitrite's involvement in cell mortality is hampered, however, by the difficulties associated with detecting such a reactive and transient compound (Ischiropoulos *et al.*, 1995; Crow & Ischiropoulos, 1996; Herce-Pagliai *et al.*, 1998; Viera *et al.*, 1999; Ueno *et al.*, 2006; Chaki *et al.*, 2009; Ferrer-Sueta & Radi, 2009; Lesser, 2011).

1.6. Aim and scope of this study.

The primary aim of this study was to elucidate the role of nitric oxide in the temperature-induced breakdown of the cnidarian-dinoflagellate symbiosis. In achieving this aim, the project had a number of specific objectives:

1. Determine whether or not *Symbiodinium* cells (*in vitro* and *in hospite*) have the capacity to generate NO, and identify a role for the compound (if any) in temperature stress in these algae. Hypotheses: a) Biosynthesis of NO will be stimulated by exposure to high temperature; b) NO will adversely affect *Symbiodinium* cellular physiology.

2. Determine whether different types of *Symbiodinium* differentially produce NO during thermal stress, and whether they vary in their susceptibility to the compound. Hypotheses: a) Synthesis of NO during thermal stress will differ

significantly between *Symbiodinium* genotypes; b) Different types of *Symbiodinium* will vary in their sensitivity to exogenous NO.

3. Identify a specific role for NO in the breakdown of the *Aiptasia pulchella-Symbiodinium* association. Hypothesis: NO is involved in the activation of apoptotic-like pathways leading to thermal bleaching.

4. To determine whether NO and apoptosis might be critical events in the bleaching of *A. pulchella* treated with slow rather than rapid heating. Hypothesis: NO synthesis and apoptotic events in the host will correlate with algal photosynthetic dysfunction and symbiont loss during slow heating-induced bleaching.

5. Identify the generation of highly toxic peroxynitrite ($ONOO^-$) in thermally stressed cnidarians and confirm its importance for thermal bleaching. Hypotheses: a) Elevated temperature will induce $ONOO^-$ generation in *A. pulchella*; b) $ONOO^-$ is involved in the temperature-induced bleaching of symbiotic *A. pulchella*.

6. Determine whether different species of reef corals produce NO during thermal stress and whether production of the compound correlates with the induction of apoptosis and bleaching. Hypotheses: a) Differentially heat-sensitive corals will generate NO to varying degrees; b) This will correlate with host apoptotic events and will follow pronounced physiological dysfunction in the symbionts.

As a whole, this thesis sought to contribute to our knowledge of the cellular physiology of cnidarian bleaching by providing insights into the production and activity of reactive nitrogen species in various symbiotic cnidarians and their dinoflagellate symbionts. Drawing together previous cellular and biochemical research into an ecologically relevant framework will aid our understanding of the roles that cellular signalling might play in the breakdown of this important symbiotic association.

Chapter 2

Nitric oxide production and tolerance differ among *Symbiodinium* types exposed to heat stress.

2.1. Introduction

A symbiotic association between scleractinian corals and photosynthetic dinoflagellates of the genus *Symbiodinium* underpins the existence of coral reefs (Muller-Parker & D'Elia, 1997). This relationship is highly sensitive to changes in temperature, however, and thermal anomalies associated with global warming are now driving its collapse throughout tropical oceans (Hoegh-Guldberg & Bruno, 2010; van Hooidonk *et al.*, 2013). The loss of *Symbiodinium* cells from their cnidarian host (a major component of "coral bleaching") can result in colony mortality (Jones, 2008; McClanahan *et al.*, 2009) and the decline of reef systems (Graham *et al.*, 2008; Pratchett *et al.*, 2009). Despite its increasing frequency, little is known of bleaching's underlying physiological basis (Weis, 2008; Davy *et al.*, 2012). Given the timescales of projected reef decline (Pandolfi *et al.*, 2011), it is vital that this is addressed.

The genus *Symbiodinium* is highly diverse (Coffroth & Santos, 2005; Stat *et al.*, 2006), with nine distinct clades and numerous sub-clades (ITS2 "types") currently recognised (Pochon *et al.*, 2006; Pochon & Gates, 2010). This diversity is reflected in the sensitivities of different types to environmental stress (Robison & Warner, 2006; Suggett *et al.*, 2008; Takahashi *et al.*, 2009; Ragni *et al.*, 2010). Moreover, the presence of certain *Symbiodinium* types often correlates with the intensity of host bleaching responses (Berkelmans & van Oppen, 2006; Sampayo *et al.*, 2008; van Oppen *et al.*, 2009). One of the initial events in the bleaching process appears to be the chronic photoinhibition of the *Symbiodinium* cells (Warner *et al.*, 1999; Smith *et al.*, 2005), which is associated with the overproduction of ROS (Suggett *et al.*, 2008) and the onset of oxidative stress (Lesser, 1997; Lesser, 2006). If left unchecked, this can result in mortality (Dunn *et al.*, 2004; Pernice *et al.*, 2011) and/or the activation of immune-like responses that result in the symbionts' ejection (Perez & Weis, 2006; Weis, 2008).

PCD/apoptosis in particular has been proposed as a crucial step in coral bleaching (Dunn *et al.*, 2007) and this process has been reported in symbiotic dinoflagellates (Dunn *et al.*, 2004; Strychar *et al.*, 2004a; Sammarco & Strychar, 2013). Linking the oxidative component of coral bleaching with observed patterns of cellular mortality is now a major area of research, and it is here that NO could play a significant role (Brune *et al.*, 1999; Chung *et al.*, 2001; Almeida *et al.*, 2007).

NO is one of the smallest signalling molecules in biology (Smith, 1998) and its involvement in cell death (Brune *et al.*, 1999; Snyder *et al.*, 2009), ROS-based signalling (Crawford & Guo, 2005), innate immunity (Nürnberger *et al.*, 2004), and symbiosis (Trapido-Rosenthal *et al.*, 2001; Davidson *et al.*, 2004) has long been recognised. Perez & Weis (2006) proposed NO as an "eviction notice" in the bleaching of the sea anemone *Aiptasia pallida* during heat shock, and initial work suggested that symbiotic dinoflagellates possess their own nitric oxide synthase (Bhagooli *et al.*, 2001; Buxton *et al.*, 2002; Trapido-Rosenthal *et al.*, 2005) although this has since been contradicted (Safavi-Hemami *et al.*, 2010). Plants and green algae employ NOS-independent NO pathways (Yamasaki *et al.*, 1999; Sakihama *et al.*, 2002; Delledonne, 2005), however, and these appear to be present in symbiotic dinoflagellates isolated from the giant clam *Tridacna crocea* (Bouchard & Yamasaki, 2008). Furthermore, temperature-induced NO synthesis correlated with increasing caspase-like activity (enzymes involved in the execution of PCD), leading the authors to propose NO as a mediator of programmed cell death (Bouchard & Yamasaki, 2009). While NO has been shown to mediate mortality in some diatom species (Vardi *et al.*, 2006; Chung *et al.*, 2008), Bouchard & Yamasaki (2009) did not apply an NO scavenger to the cells and thus any link between NO and cell death in *Symbiodinium* remains correlative at best. As is the case in other photosynthetic organisms (Beligni & Lamattina, 1999b), heightened NO synthesis may be a protective strategy - through the compound's scavenging of ROS and consequent attenuation of oxidative stress (Delledonne, 2005) - that is simply overwhelmed during prolonged stress.

Whether protective or harmful, if temperature-induced synthesis of NO is widespread in the genus *Symbiodinium* then its production might vary with the differing thermal sensitivities of individual ITS2 types (Robison & Warner, 2006; Suggett *et al.*, 2008; Ragni *et al.*, 2010; Fisher *et al.*, 2012). As a small lipophilic molecule, NO could

potentially diffuse across symbiont membranes into the host cells. With the cnidarian-*Symbiodinium* association apparently sensitive to NO (Perez & Weis, 2006), *in hospite* leakage of the compound may have profound implications (Weis, 2008) and perhaps contribute to the differential bleaching of hosts containing different symbiont types (Perez *et al.*, 2001; Sampayo *et al.*, 2008; Weis, 2010).

This study sought to determine the role of NO in the stress responses of symbiotic dinoflagellates by examining its production in three different *Symbiodinium* ITS2 types exposed to elevated temperature, as well as its effects on the photophysiology and viability of these algae. Further elucidation of the mechanisms underpinning the declining health of thermally stressed *Symbiodinium* will greatly improve our understanding of the cellular basis of coral bleaching, and the role that symbiont diversity may play in this phenomenon.

2.2. Materials and Methods

2.2.1. Culture of Symbiodinium *dinoflagellates.*

The three *Symbiodinium* types used in this experiment were originally isolated from taxonomically and geographically distinct host populations (Table 2.1), and had been maintained in culture for at least 2 years. Their ITS2 types were identified by the methods of Logan *et al.* (2010). Prior to experimentation, *Symbiodinium* cell cultures were grown at 26°C under a 12 h light:12 h dark cycle (100-120 μmol photons m^{-2} s^{-1} provided by cool white fluorescent tubing - OSRAM DULUX L 36W 4000 K). Flasks containing Guillard's f/2 medium (- Si; AlgaBoost, AusAqua Pty Ltd, Wallaroo Austalia) were inoculated 20 days prior to experimental treatment and cells were maintained in exponential growth phase (50000-150000 cells mL^{-1}) by regular dilution with fresh f/2 medium. Cultures were diluted to a concentration of 30000 cells mL^{-1} 72 h prior to treatment and left undiluted throughout each experiment.

2.2.2. Experiments

To investigate the effects of temperature on photosystem II, NO production and cell viability, *Symbiodinium* cell suspensions (three independent replicate cultures per ITS2

type) were transferred to an illuminated water bath set at 26°C and an irradiance of 100-120 μmol photons m^{-2} s^{-1} (provided by a LED light bank - 20 RoHS 5W 6400 K - which provided illumination for all subsequent experiments), and allowed to acclimate for 48 h. Assessments of symbiont photosystem II were made during the acclimation period to ensure stability prior to treatment. The temperature was then either maintained at 26°C (control) or increased over a short period (< 1 hour) to 32°C or 34°C (Bouchard & Yamasaki, 2008; Bouchard & Yamasaki, 2009). Cells were exposed to the new conditions for 48 h.

Table 2.1. ITS2 type, original host species, and geographic origin of *Symbiodinium* cultures used in experiments.

Culture name	**Original host**	**Geographic origin**	**ITS2 type**
CCMP2467	*Stylophora pistillata*	Gulf of Aqaba, Egypt.	A1
Ap1	*Aiptasia pulchella*	Hawaii, USA.	B1
CCMP2466	*Discosoma sanctithomae*	Jamaica.	C1

The extent to which NO production is dependent on photoinhibition was examined by exposing cells to the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; Sigma Aldrich, Auckland, New Zealand) at 26°C (20 μM final) for 24 h in 100 mL f/2 medium at an irradiance of 100-120 μmol photons m^{-2} s^{-1} (see above).

The effects of NO on photosynthetic performance were examined in a further set of experiments, by exposing *Symbiodinium* cells to different nitric oxide donor and scavenger combinations, at either control (26°C) or elevated temperatures for 3-6 h under an irradiance of 100-120 μmol photons m^{-2} s^{-1}. The NO donors used were spermine NONOate (S-NONO; Life Technologies, Auckland, New Zealand) - known to release NO comparatively slowly and consistently (Miller *et al.*, 2004) - and S-

nitrosoglutathione (GSNO; Sigma-Aldrich, Auckland, New Zealand) - a donor specifically recommended for investigations into the influence of NO on photosynthetic pathways (Wodala *et al.*, 2008). The specific NO scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (c-PTIO; Life Technologies, Auckland, New Zealand) was used in order to examine the NO-dependency of a response. The NO donor sodium nitroprusside was also used in preliminary experiments, but its effects were mediated by compounds other than NO (Supplementary Fig. A1). Conditions for the elevated temperature + cPTIO treatment were determined by the responses of different *Symbiodinium* types to the 48-h high temperature treatments described above.

To assess the effects of NO on cell mortality, type A1 and B1 cells were exposed to control (26°C) and elevated temperatures for 24 h with and without 2 mM cPTIO.

All NO donor and scavenger treatments took place in 0.22-µm filtered seawater (FSW) in 1.5-ml tubes with cell suspensions (sourced from independent replicate cultures) at a concentration of *ca.* 1×10^6 cells mL^{-1}.

2.2.3. *Fluorometric assessment of photosynthetic competence.*

Fluorescence induction curves were conducted using a Water-PAM fluorometer (Walz, Effeltrich, Germany). Cells were dark-adapted for 30 min prior to measurement. Thus, values were obtained for maximum quantum yield of photosystem II (PSII) (F_v/F_m). During induction curves, cells were exposed to 250 µmol photons m^{-2} s^{-1} (irradiance increased to stimulate the cells' NPQ response) and the effective quantum yield of PSII ($\Delta F/F_m'$) was measured until steady state. Using these data, non-photochemical quenching (NPQ) was calculated as (F_m - F_m') / F_m', where F_m is maximum dark-adapted chlorophyll fluorescence and F_m' is maximum steady-state light-adapted chlorophyll fluorescence.

In order to ensure consistency between treatments, comparisons of PSII fluorescence yields and NPQ refer to values relative to pre-treatment (t = 0) conditions. In all cases, only algal cultures with initial dark-adapted yields (F_v/F_m) greater than 0.5 were used.

2.2.4. Flow cytometric (FCM) assessment of nitric oxide in Symbiodinium *cells*.

NO production was measured with the fluorescent nitric oxide indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA; Molecular Probes, Eugene, OR, USA) at a final concentration of 15 μM in 0.22-μm filtered seawater (FSW). DAF-FM-DA is more sensitive (*ca.* 3 nM detection limit), photostable, and cell-permeable than previously available fluorescent dyes and has been successfully used on a number of occasions to measure the low levels of NO present in microalgae (e.g. Kim *et al.*, 2008; Thompson *et al.*, 2008). After 90 min incubation in the dark (to prevent photobleaching of the DAF-FM fluorophore), cells were washed twice by repeated centrifugation (800 × g for 5 min) and resuspension in FSW. They were then kept in the dark for a further 30 min to allow cleavage of the DAF-FM-DA dye to its active DAF-FM form (Nagano & Yoshimura, 2002). The fluorescent product of DAF-FM's reaction with NO has an emission maximum at 515 nm when excited with blue light (Nagano & Yoshimura, 2002). This was measured using the FL1 (515-545 nm) channel on a FACScan 3-channel flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Excitation light was provided by a 488 nm argon laser and cells were gated on the basis of chlorophyll fluorescence (measured with a > 650 nm detector, see Fig. 2.1). Blank unlabelled samples were processed alongside probed cells to control for autofluorescence. Briefly, NO-dependent fluorescence was calculated by subtracting the mean fluorescence intensity (MFI) signal of *ca*. 50000 "blank" cells from the MFI value of the equivalent DAF-FM-incubated sample (Fig. 2.1). Positive controls, incubated for 30 min with 1 mM of the NO donor sodium nitroprusside (SNP) were prepared to ensure successful loading of the DAF-FM dye. NO-dependent fluorescence was standardised across treatments and genotypes by dividing the increase in MFI (DAF-FM MFI − blank MFI) by the mean cell volume of each sample. Volumetric measurements of individual cells (modelled as ellipsoids) were carried out using an eyepiece graticule and a ×100, 1.25 numerical aperture (NA) oil-immersion objective. At least 50 cells were measured per sample and final values for NO-dependent fluorescence were recorded as MFI μm^{-3}.

2.2.5. FCM assessment of cell death.

Propidium iodide (PI; Sigma Aldrich, Auckland, New Zealand) and annexin-V *Alexafluor* 488 (AV-*fluor;* Molecular Probes, Eugene, OR, USA) were used to identify different modes of mortality in *Symbiodinium* cells (Strychar *et al.*, 2004a; Sammarco & Strychar, 2013). PI cannot permeate live cells' plasma membranes, but diffuses through the dysfunctional membranes of non-viable cells wherein it binds strongly to nucleic acids and displays increased fluorescence. AV-*fluor* specifically binds phosphatidylserine (PS) residues. Translocation of PS to the outer plasma membrane surface is one of the earliest indicators of apoptosis and is highly conserved in eukaryotes (Li *et al.*, 2003). As such, live cells undergoing apoptotic-like cell death can be distinguished through the binding of AV-*fluor* only, while non-viable cells (whether due to necrosis or very late-stage apoptosis) are labelled with both PI and AV-*fluor*. Briefly, cells were centrifuged (800 × g for 5 min) and resuspended in 10 μg mL^{-1} PI and 50 μL mL^{-1} AV-*fluor* in calcium-enriched FSW (3 mM $CaCl_2$). Cells were incubated in 1.5-ml tubes (in the dark) for 30 min and additional calcium-enriched FSW was added prior to FCM processing. Upon excitation with a 488 nm argon laser and gating by chlorophyll fluorescence (Fig. 2.1), AV-*fluor* and PI fluorescence were detected at 515-545 nm and 560-605 nm, respectively. Gain, threshold, and compensation settings were kept constant throughout the experiment and blank unlabelled samples were processed alongside to control for autofluorescence (Fig. 2.1). Percentages of PI-positive ("non-viable") and AV-*fluor*-positive/PI-negative ("apoptotic-like") cells were calculated using graphical analysis of probed and blank samples (Weasel FCM analysis software, Walter & Eliza Hall Institute, Melbourne, Australia).

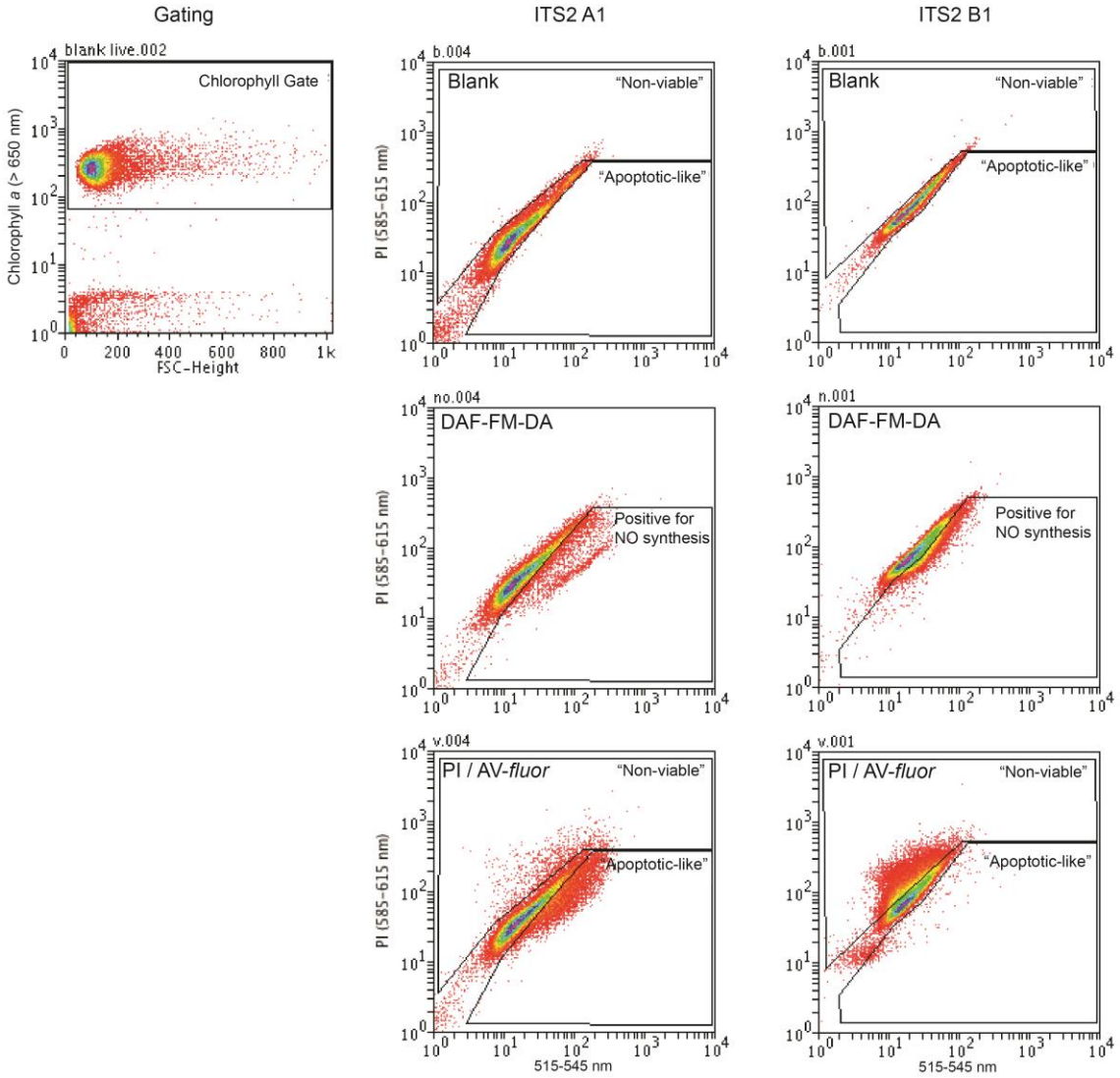

Figure 2.1. Flow cytometric (FCM) analysis of *Symbiodinium* cells of ITS2 type A1 (middle column) and B1 (right column). Cells were gated on the basis of their size and chlorophyll autofluorescence ("chlorophyll gate", left column) - thus, non-viable cells that were bleached or fragmented were not analysed. Samples were incubated in either FSW (top row - "blank"), 15 µM NO indicator DAF-FM-DA (middle row), or with a propidium iodide (PI) / Annexin-V *fluor* mix (bottom row). Each dot represents a single *Symbiodinium* cell, and dots are coloured according to their density (50000 cells per plot; red for individual cells, yellow-orange for > 100 cells, green-purple for > 500 cells). Shifts along the X-axis only (DAF-FM or AV-*fluor* signal) relative to blank samples represent NO synthesis or phosphatidylserine externalisation (apoptotic-like mortality), respectively. Upward shifts (Y-axis, PI fluorescence) indicate non-viability (either due to late-stage apoptotic-like mortality or necrosis).

2.2.6. Confocal microscopy of Symbiodinium *cells.*

To confirm cellular localisation of fluorescence, the dyes were visualised using an Olympus Fluoview FV-1000 inverted confocal laser scanning microscope (LSM; Olympus, Center Valley, PA, USA) and a ×100 1.45 NA oil immersion lens. Cells were aliquoted onto poly-l-lysine-coated glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) and left to settle for 15 min. The medium was then replaced with 1% w/v carboxymethylcellulose in FSW in order to immobilize the cells. DAF-FM and AV-*fluor* were excited with a 473 nm diode laser (which was also used to capture the confocal "phase contrast" TD1 image) and their fluorescence was detected at 485-545 nm. PI was excited by a 559 nm diode laser and fluorescence was detected at 595-645 nm. A 635 nm diode laser was used to visualise chlorophyll autofluorescence, which was detected at 655-755 nm (see Supplementary Figs. A2-A4 for separate confocal LSM filter images). Laser intensity, pinhole aperture (1 Airy unit), and all LSM detector settings were kept constant between blank and probed samples.

2.2.7. Statistical analyses.

Data analyses were carried out using a PASW Statistics 18.0 package (IBM, Armonk, NY, USA). Repeated measures analysis of variance (RMANOVA) was used to test null hypotheses regarding photosynthetic yields, NO production, and cell death for the three ITS2 types and treatments. One-way ANOVA was used to examine the effects of NO on cell physiology. *Post-hoc* analyses represent least significant difference (LSD) pair-wise comparisons of estimated marginal means between treatments and controls at a particular time point, unless otherwise indicated. Data were examined for normality and equal variance prior to any parametric analysis, and were transformed where necessary to fulfill these assumptions. In the case of sphericity (the variance parameter for RMANOVA), the Greenhouse-Geisser correction was used whenever Mauchly's Test returned a significant result.

2.3. Results

2.3.1. PSII fluorescence at elevated temperature.

Responses differed significantly between *Symbiodinium* types exposed to elevated temperatures (Table 2.2, Fig. 2.2). Types A1 and C1 were tolerant of moderate temperature stress (32°C) and A1 maintained dark-adapted yields at control levels even after 48 h at 34°C ($p = 0.606$). F_v/F_m of type C1 declined slightly at 34°C ($p < 0.001$), and type B1 exhibited significant and dramatic reductions in F_v/F_m, declining to near zero within 36 h at both 32°C and 34°C ($p < 0.001$).

Table 2.2. Statistical analysis of physiological parameters in three *Symbiodinium* ITS2 types exposed to temperature stress. Asterisks denote statistical significant ($p < 0.05$).

Parameter (treatment)	**Test (interaction)**	**Statistic**	**P-value**
F_v/F_m (heat stress)	RMANOVA (time × temperature × type)	$F_{5.475, 25.853} = 48.888$	< 0.001*
Nitric oxide (background)	One-way ANOVA (type)	$F_{2, 27} = 59.617$	< 0.001*
Nitric oxide (heat stress)	RMANOVA (time × temperature × type)	$F_{8, 42} = 12.022$	< 0.001*
Necrotic-like mortality (heat stress)	RMANOVA (time × temperature × type)	$F_{8, 36} = 5.073$	< 0.001*
Apoptotic-like mortality (heat stress)	RMANOVA (time × temperature × type)	$F_{5.742, 25.839} = 2.465$	0.053

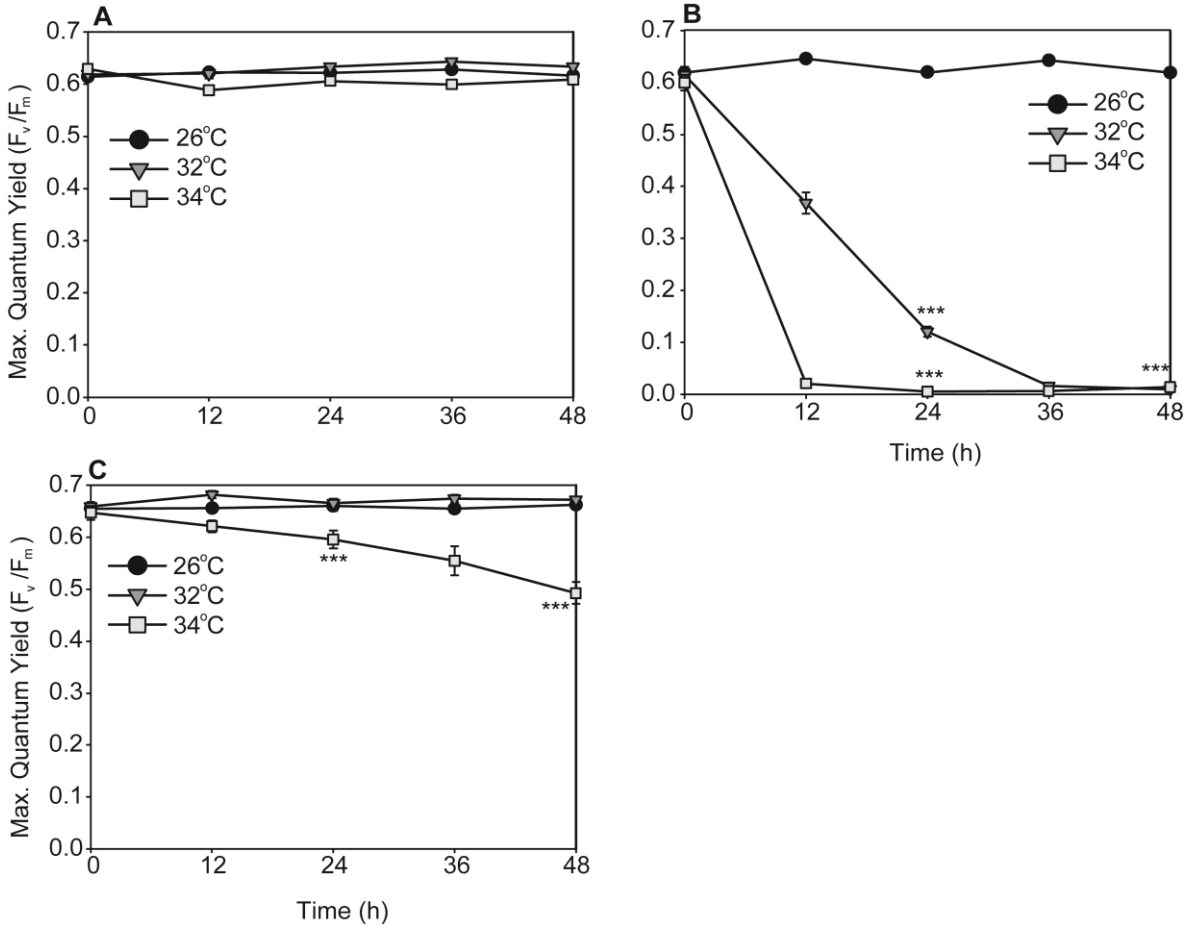

Figure 2.2. Effect of elevated temperature (under 100-120 μmol photons m^{-2} s^{-1} cool white LED light) on maximum quantum yield of PSII in *Symbiodinium* ITS2 types A1 (**Panel A**), B1 (**B**), and C1 (**C**). Values are means ± s.e.m. and asterisks indicate significant differences between each treatment and the control (26°C) group (RMANOVA with LSD pair-wise *post-hoc*, n = 3 independent cultures per time-point, *** $p < 0.001$).

2.3.2. Nitric oxide synthesis in Symbiodinium *cells.*

All cultures were capable of generating NO (Fig. 2.3), and pre-treatment levels differed significantly among types (Table 2.2). Responses to temperature stress also differed significantly (Table 2.2) with two distinct trends apparent. Types A1 and B1 up-regulated NO synthesis over 24 h at 32°C (A1: $p = 0.002$; B1: $p = 0.026$) and, while B1 cells sustained this increase over 48 h ($p < 0.001$), the response was transient in type A1 wherein NO levels declined to control levels after 48 h ($p = 0.458$). After 48 h at 34°C, however, both A1 and B1 cells showed elevated NO production (A1: $p = 0.021$; B1: $p < 0.001$). Type C1 cells behaved differently, with exposure to 32°C for 48 h or 34°C for 24 h resulting in a significant down-regulation of NO synthesis relative to controls (32°C: $p = 0.011$; 34°C: $p = 0.048$).

Exposure to the photosynthetic inhibitor DCMU (20 μM) at 26°C for 24 h resulted in significant increases in NO production in type A1 and B1 cells (t-tests on natural log-transformed data, A1: $t_7 = -5.012$, $p = 0.002$; B1: $t_{2.095} = -13.806$, $p = 0.004$), which occurred alongside dramatic declines in dark-adapted photosynthetic yield (Fig. 2.4). F_v/F_m in type C1 also declined during incubation with DCMU (Fig. 2.4), but NO production in these cells decreased relative to the control (t-test, $t_4 = 3.430$, $p = 0.027$).

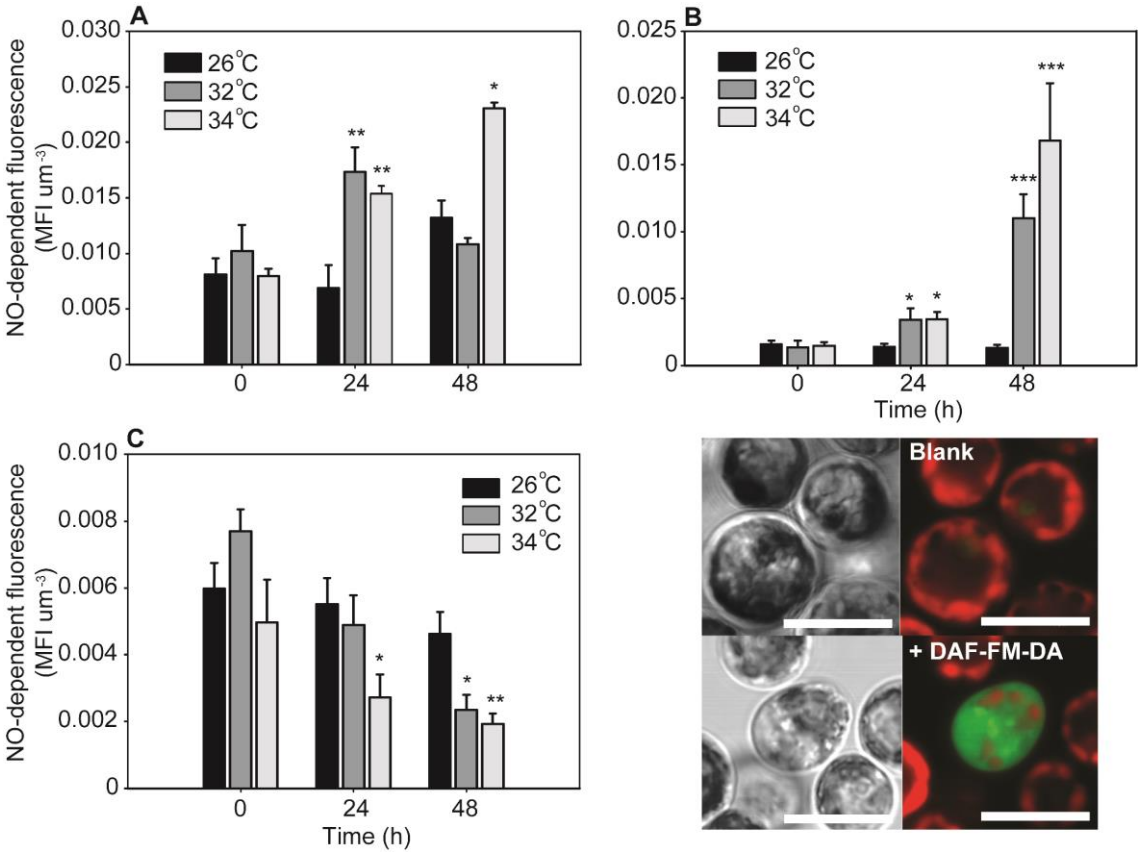

Figure 2.3. Effect of temperature (under 100-120 µmol photons m^{-2} s^{-1} cool white LED light) on the regulation of NO production in *Symbiodinium* ITS2 types A1 (**Panel A**), B1 (**B**), and C1 (**C**). Values are means ± s.e.m. and asterisks indicate significant differences between each treatment and the 26°C control group (RM-ANOVA with LSD pair-wise *post-hoc*, n = 3 independent cultures per time-point except for A1 at 26°C where n = 6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **Inset**: Phase contrast/TD1 and confocal LSM micrographs of *Symbiodinium* cells exposed to 34°C for 48 h and incubated with either FSW or 15 µM of the NO-sensitive probe DAF-FM-DA. NO-dependent fluorescence (485-545 nm) is labelled green and cells' chlorophyll autofluorescence (> 700 nm) is labelled red. Scale bar = 10 µm.

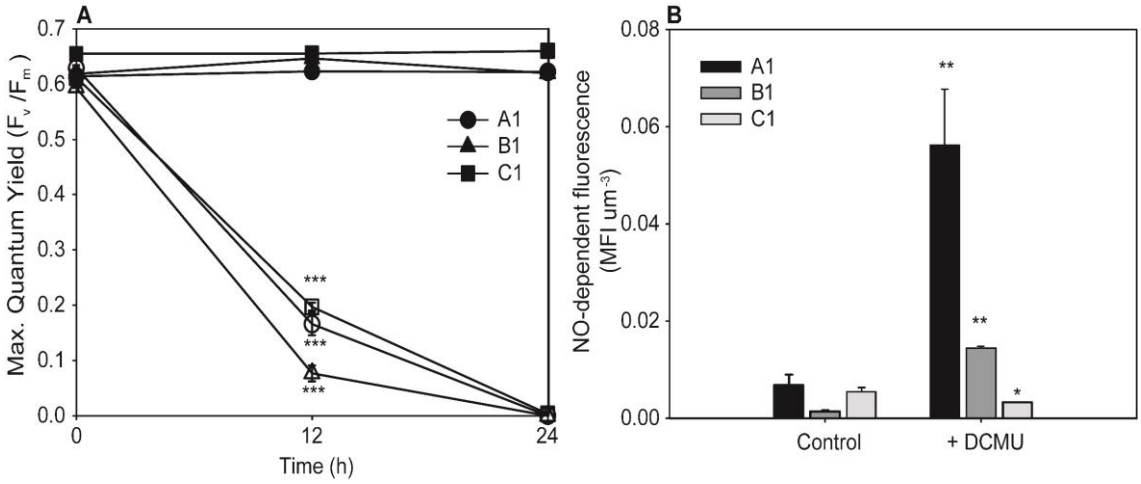

Figure 2.4. Effects of 24 h exposure to DCMU (20 µM) on maximum quantum yields of PSII (**A**) and NO production (**B**) in three *Symbiodinium* genotypes. Values are means ± s.e.m and significant differences between DCMU-treated (open symbols) and control samples (closed symbols) are indicated by asterisks (t-tests, n = 3 independent cultures except for A1 control where n = 6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

2.3.3. Effects of NO on chlorophyll a *fluorescence.*

Treatment with the NO donors S-NONO and GSNO adversely affected PSII fluorescence yields and revealed differences in the nitric oxide-sensitivity of different *Symbiodinium* ITS2 types. The addition of NO donors at 100 µM caused F_v/F_m to decline only in types B1 and C1; a higher concentration (1 mM) was required to induce significant declines in type A1 (Table 2.3, Fig. 2.5). Furthermore, GSNO appeared to be a much more potent photosynthetic inhibitor than S-NONO. The specific NO scavenger cPTIO was able to completely block the effects of 100 µM S-NONO and GSNO on F_v/F_m in B1 and C1 type cells, and of 1 mM S-NONO and GSNO in A1 cells (Fig. 2.5), confirming the NO-dependent nature of the effects. To determine whether a cell's own NO production might affect its PSII fluorescence, cultures were exposed to elevated temperature (36°C for A1 - increased from 34°C to ensure photoinhibition of PSII - and 34°C for B1) with and without 2 mM cPTIO. F_v/F_m declined in both types at the higher temperature and this was prevented to an extent by the addition of cPTIO (Fig. 2.5).

Table 2.3. Statistical analysis (one-way ANOVA, effect of treatment) of physiological parameters in three *Symbiodinium* ITS2 types exposed to combinations of NO donors, scavengers, and heat stress. Asterisks denote statistical significance ($p < 0.05$)

Treatment	**ITS2 type**	**Parameter**	**Statistic**	**P-value**
NO donors +/- cPTIO	A1, B1, C1	F_v/F_m	$F_{4, 30} = 245.983$	< 0.001*
		$\Delta F/F_m'$	$F_{4, 30} = 285.909$	< 0.001*
		NPQ	$F_{4, 30} = 25.832$	< 0.001*
NO donor concentrations	A1, B1, C1	F_v/F_m	$F_{4, 30} = 193.048$	< 0.001*
High temp. +/- cPTIO	A1, B1	F_v/F_m	$F_{2, 12} = 173.141$	< 0.001*
	A1, B1	Cell viability	$F_{2,12} = 112.885$	< 0.001*

The application of NO donors also affected light-adapted PSII fluorescence and significant declines in effective quantum yield ($\Delta F/F_m$) similar to those seen for F_v/F_m were observed (Table 2.3, Fig. 2.6). Non-photochemical quenching was inhibited by S-NONO in all three *Symbiodinium* types, although declines in type C1 were typically less dramatic than for A1 or B1 (Table 2.3, Fig. 2.6) and were absent in the GSNO treatment. Only in type A1 could the effects on NPQ of both NO donors be completely reversed by the addition of the NO scavenger cPTIO (Fig. 2.6).

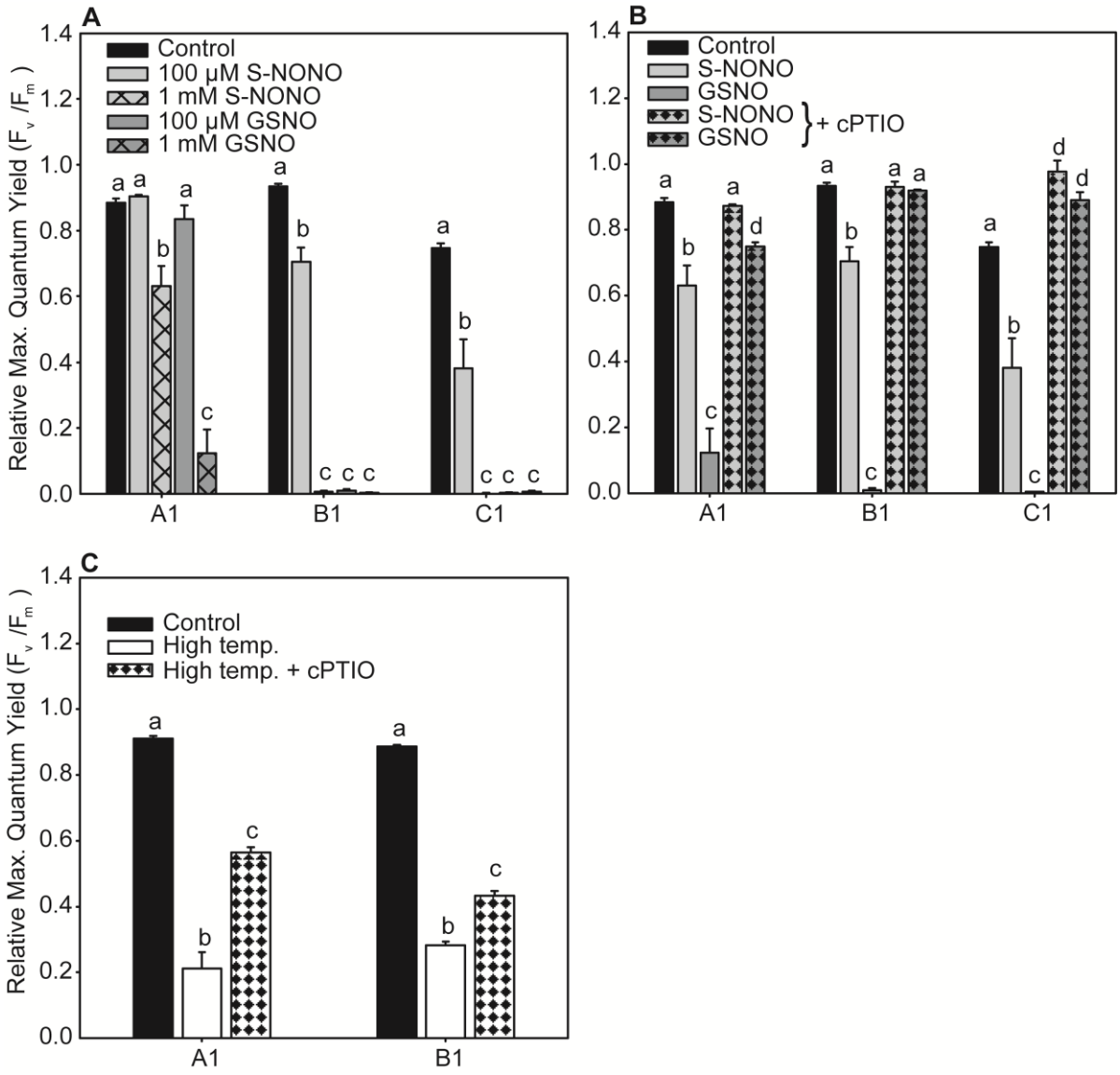

Figure 2.5. Effects of NO on the relative maximum quantum yields of PSII (F_v/F_m) in *Symbiodinium* cells under 100-120 µmol photons m^{-2} s^{-1} cool white LED light and **(Panel A)** treated with differing concentrations of S-NONO and GSNO for 3 h; (**B**) exposed to the nitric oxide donors S-NONO and GSNO (1 mM for ITS2 type A1, 100 µM for B1 and C1) for 3 h with and without the NO scavenger cPTIO (at equal concentrations to the donor); and (**C**) incubated at elevated temperature (36°C for A1 – increased from 34°C to ensure significant photoinhibition – and 34°C for B1) for 6 h with and without 2 mM cPTIO (scavenger concentration increased to raise the likelihood of scavenging all temperature-induced NO). Values are means ± s.e.m. and letters represent bars that are significantly different *within each type* (ANOVA with LSD pair-wise *post-hoc*, n = 3 independent cultures, $p < 0.01$ for all comparisons).

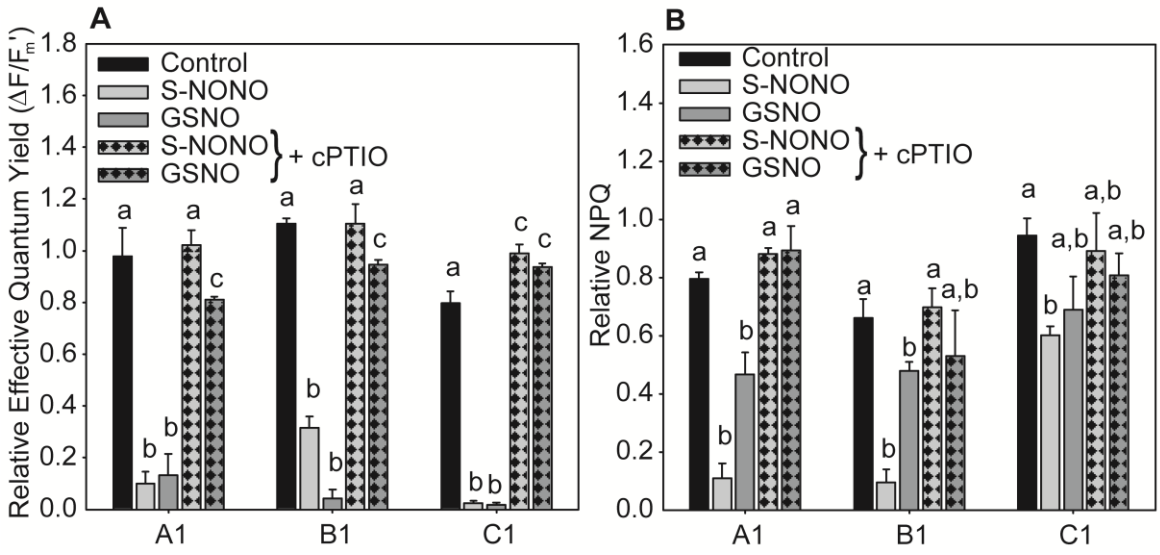

Figure 2.6. Effects of NO donors on **(Panel A)** the relative effective quantum yield of PSII ($\Delta F/F_m$) and (**B**) relative non-photochemical quenching (NPQ) in *Symbiodinium* cells exposed to the nitric oxide donors S-NONO and GSNO (1 mM for ITS2 type A1, 100 µM for B1 and C1) for 3 h under 100-120 µmol photons m^{-2} s^{-1} cool white LED light, with and without the NO scavenger cPTIO (at equal concentrations to the donor). Values are means ± s.e.m. and letters represent bars that are significantly different *within each type* (ANOVA with LSD pair-wise *post-hoc*, n = 3 independent cultures, $p < 0.01$ for all comparisons).

2.3.4. *Effects of temperature and NO on cell viability.*

Cell mortality differed among the three types exposed to elevated temperatures (Fig. 2.7, Table 2.2). At 32°C only B1 displayed increases relative to controls ($p < 0.001$), and this was more dramatic at 34ºC with up to 30% of cells being labelled as non-viable ($p < 0.001$). Increasing levels of cell death in other types were apparent only in type A1 exposed to 34°C for 48 h ($p = 0.012$), and even at this temperature mortality was relatively low (around 7% of treated cells). No significant changes were seen in the C1 population at either of the elevated temperatures (Fig. 2.7).

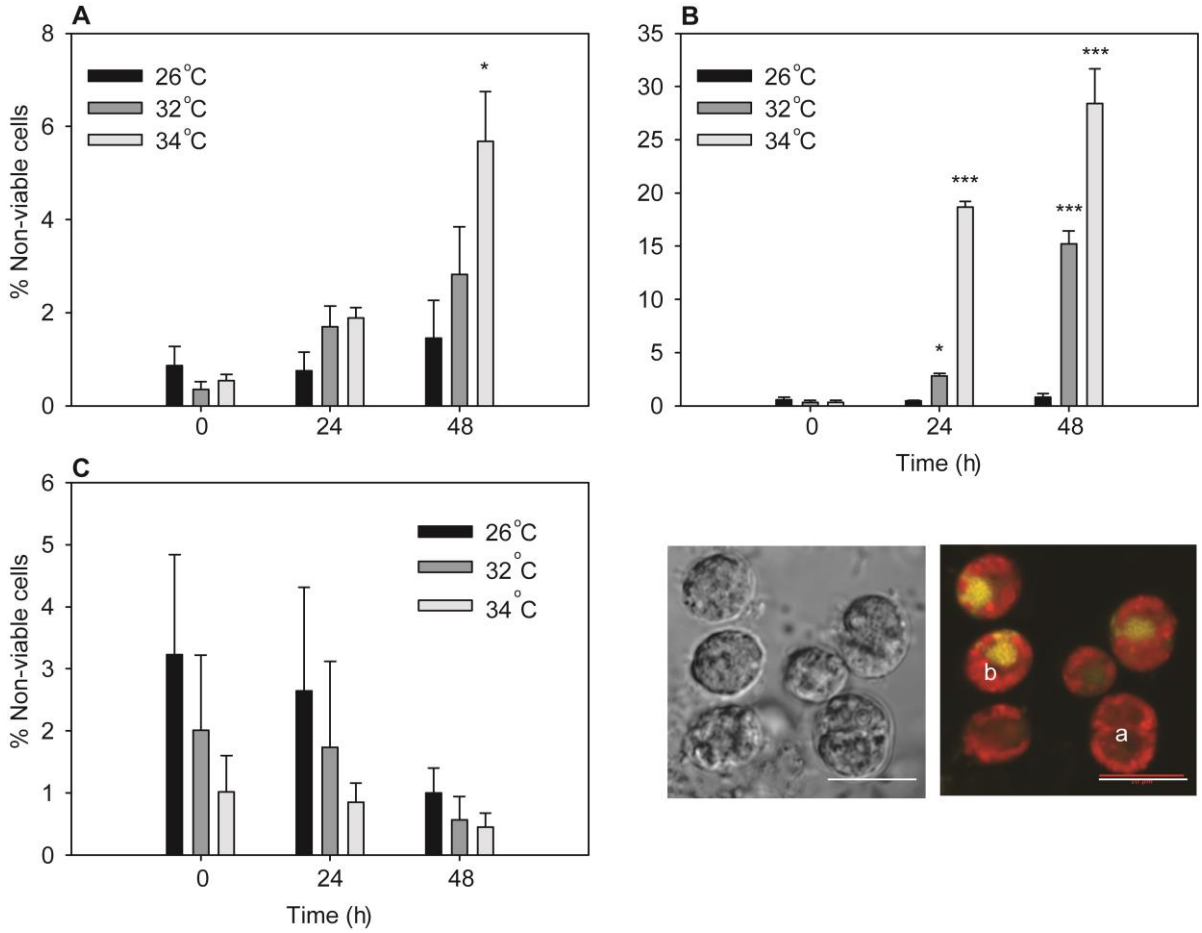

Figure 2.7. Effect of temperature (under 100-120 µmol photons m^{-2} s^{-1} cool white LED light) on the viability of *Symbiodinium* ITS2 types A1 (**Panel A**), B1 (**B**), and C1 (**C**) assessed using flow cytometric analysis of propidium iodide-labelled cells. Values are means ± s.e.m. Asterisks indicate significant differences between each treatment and the control (26°C) group (RM-ANOVA with LSD pair-wise *post-hoc*, n = 3 independent cultures, * $p < 0.05$, *** $p < 0.001$). **Inset:** Phase contrast/TD1 and confocal LSM micrographs of viable (a) and non-viable (b) *Symbiodinium* cells. Non-viable cells can be distinguished through the binding of propidium iodide (yellow, 560-605 nm) to nucleic acids. Chlorophyll autofluorescence (> 700 nm) is labelled red. Scale bar = 10 µm.

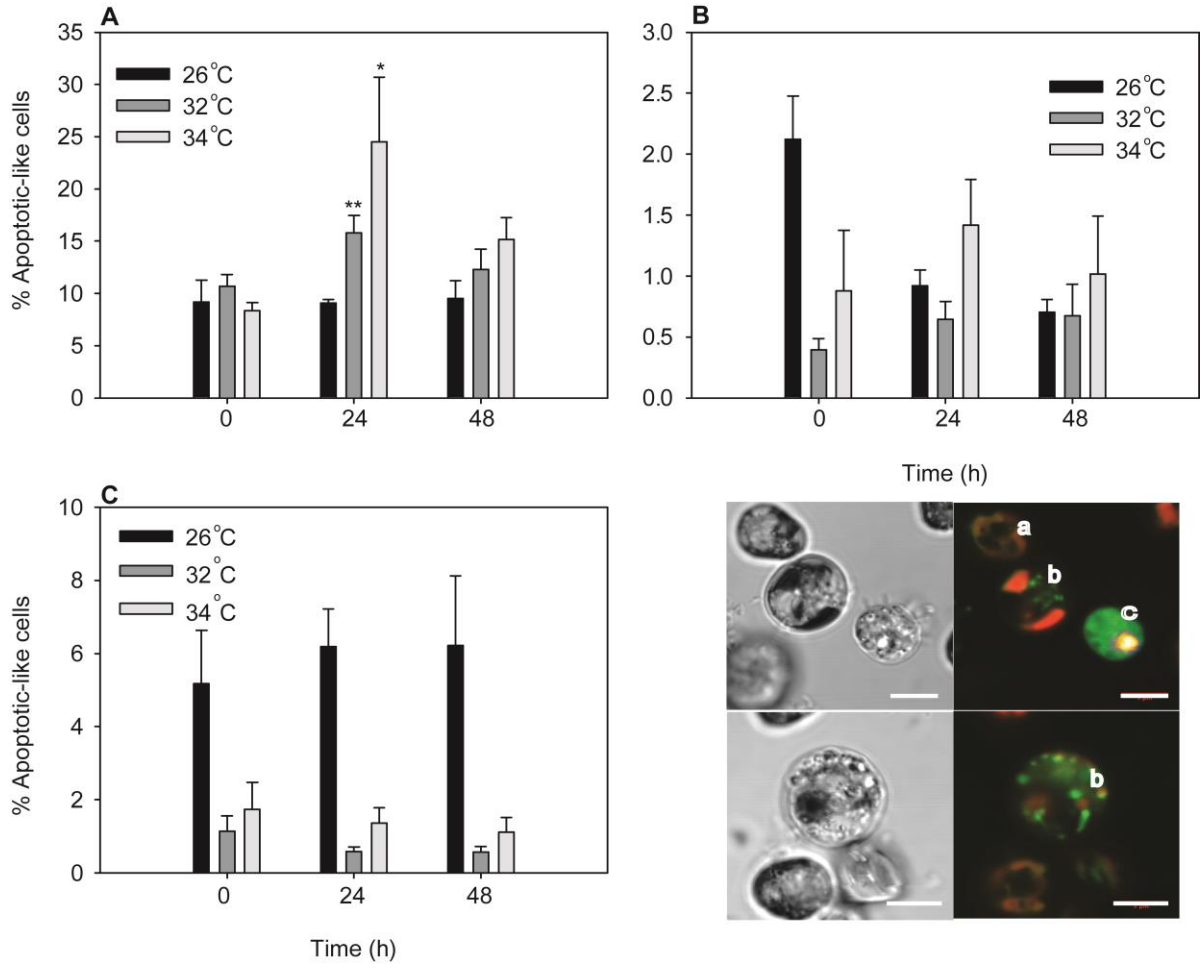

Figure 2.8. Effect of temperature (under 100-120 µmol photons m^{-2} s^{-1} cool white LED light) on apoptotic-like characteristics of *Symbiodinium* ITS2 types A1 (**Panel A**), B1 (**B**), and C1 (**C**) assessed using flow cytometric analysis of cells labelled with Annexin-V-*fluor* (488 nm), which selectively binds phosphatidylserine (PS) externalised during the earliest stages of apoptosis. Values are means ± s.e.m. Asterisks indicate significant differences between each treatment and the control (26°C) group (t-tests between "control" and treatments at each time point, n = 3 independent cultures, * $p < 0.05$, ** $p < 0.01$). **Inset:** Phase contrast/TD1 and confocal LSM micrographs of *Symbiodinium* cells identified as viable (**a**; not labelled), apoptotic-like (**b**; green AV-*fluor* only), and non-viable (**c**; orange-white propidium iodide and green AV-*fluor*). Chlorophyll autofluorescence (> 700 nm) is labelled red. Scale bar = 10 µm.

Baseline levels of AV-*fluor*-labelling were highly variable among genotypes (one-way ANOVA with Welch correction, $F_{2, 11.921} = 13.915$, $p < 0.001$), with type A1 displaying significantly higher percentages of cells labelled with AV-*fluor* (Tukey HSD *post-hoc*, $p < 0.01$). Analysis of AV-*fluor*-binding at elevated temperature revealed no significant time × temperature × type interaction, and pair-wise comparisons between treatments were therefore conducted with t-tests at each time point. The proportion of AV-*fluor*-labelled cells only increased significantly in type A1 after 24 h (t-tests, 32°C: $t_4 = -4.384$, $p = 0.008$; 34°C: $t_4 = -3.605$, $p = 0.023$). By 48 h, however, this had declined to control levels (Fig. 2.8). No significant changes were seen in the B1 or C1 populations (Fig. 2.8).

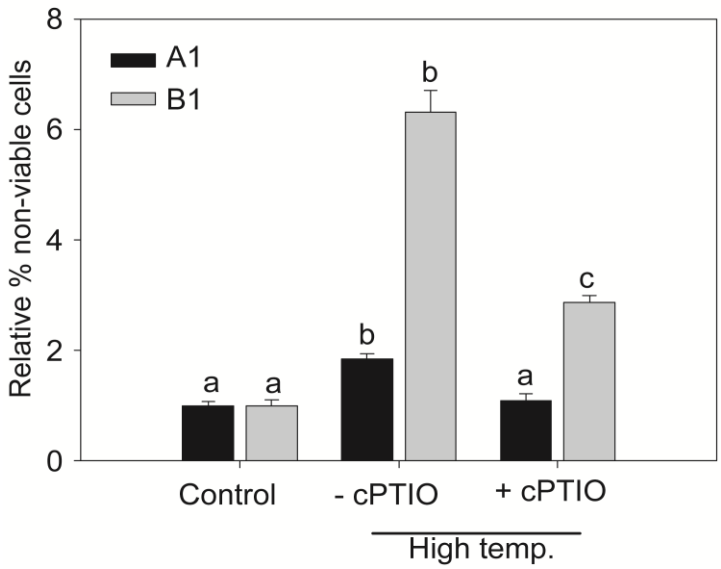

Figure 2.9. Effect of NO on the temperature-induced mortality of *Symbiodinium* types A1 and B1 under 100-120 µmol photons m^{-2} s^{-1} cool white LED light. Cells were exposed to control (26°C) and high temperature conditions (34°C for A1, 32°C for B1) for 48 h with and without 2 mM of the NO scavenger cPTIO. Non-viability was determined by incubating cells with the vital fluorescent dye propidium iodide, which selectively permeates through dysfunctional plasma membranes, and analysing samples using flow cytometry. Values are means ± s.e.m. and letters represent bars that are significantly different *within each type* (two-way ANOVA with LSD pair-wise *post-hoc*, n = 3 independent cultures, $p < 0.01$ for all comparisons).

Incubating cell suspensions at high temperature with the NO scavenger cPTIO confirmed NO as a causative agent of mortality in types A1 and B1 (Fig. 2.9, Table 2.3), where increases in the number of non-viable cells after 48 h at 32°C (B1) or 34°C (A1) were attenuated by cPTIO.

2.4. Discussion

Nitric oxide may be integral to the temperature-induced breakdown of the cnidarian-dinoflagellate symbiosis (Trapido-Rosenthal *et al.*, 2005; Perez & Weis, 2006; Weis, 2008; Bouchard & Yamasaki, 2009). This study confirms that symbiotic dinoflagellates produce NO and suggests that it could mediate their heat stress responses. It also highlights the physiological diversity of the genus *Symbiodinium*, even in the case of strongly conserved phenomena such as NO signalling.

2.4.1. Differential NO production in Symbiodinium *ITS2 types.*

Little is known about the activities of NO in microalgae, although its existence in these organisms has been recognised for some time (Sakihama *et al.*, 2002; Vardi *et al.*, 2006; Kim *et al.*, 2008; Thompson *et al.*, 2008). Heightened NO production in types A1 and B1, as seen in this study, supports the conclusions of recent investigations into NO synthesis by clade A *Symbiodinium* cells (Bouchard & Yamasaki, 2008; Bouchard & Yamasaki, 2009). Interestingly, the clade A cells used in the current work behaved differently to those examined in the study of Bouchard & Yamasaki (2009), in which exposure to 32°C induced chronic photoinhibition and persistent rather than transient increases in NO production. Bouchard & Yamasaki (2009) acknowledged that their cells were approaching stationary growth phase, and it is possible that the stresses associated with ageing algal cultures could account for their reduced thermal tolerance. Murik & Kaplan (2009) reported that the ability of *Peridinium* sp. dinoflagellates to withstand oxidative insults varied significantly with the population growth phase, and preliminary work carried out for this investigation suggests that *Symbiodinium* cells in stationary phase may produce higher levels of NO than do younger populations (see Supplementary Fig. A5). Of course, it is also possible that these discrepancies simply reflect differences in thermal tolerance at a sub-cladal level (Robison & Warner, 2006).

In spite of this, it seems likely that with both relatively ancestral (A) and derived (B and C) clades (Stat *et al.*, 2006) producing NO, the ability to generate the compound is widespread in symbiotic dinoflagellates. Inabilities to detect NO in *Symbiodinium* in other studies (e.g. Perez & Weis, 2006) might therefore reflect methodological differences. Genotypical differences in population-level expression of NO synthesis might have contributed to this. It appears from that the heightened DAF-FM signal from type A1 cells in this study was driven by a few cells fluorescing highly, while in type B1 there was a less obvious increase among a greater proportion of cells (Fig. 2.1). B1 would likely have been the genotype examined by these previous investigators – as it is a resident symbiont of *Aiptasia pallida* – and these relatively minor increases in individual cell DAF-FM fluorescence (Fig. 2.1) could have been missed in a confocal microscopy-only approach (Perez & Weis, 2006). In any case, additional work on a wider range of *Symbiodinium* types would enable further phylogenetic patterns of NO synthesis to be determined.

2.4.2. Effects of NO on Symbiodinium *cells.*

Research into NO synthesis in protists may be in its infancy, but even less is known about NO's physiological functions in these organisms. As is the case in plants, for example, NO might act as a potent antioxidant, preventing the overaccumulation of ROS and reducing the likelihood of the downstream generation of harmful agents such as the hydroxyl ($OH^{\cdot}$) radical (Caro & Puntarulo, 1998; Beligni & Lamattina, 1999a; b; Mittler, 2002; Besson-Bard *et al.*, 2008; Misra *et al.*, 2011; Yang *et al.*, 2011). While it cannot be discounted that, at low concentrations or under non-stressful conditions, similar phenomena could occur in *Symbiodinium* cells, this investigation proposes a cytotoxic capability for NO in some symbiotic dinoflagellate types. Exogenous NO affected both dark- and light-adapted photosynthetic parameters of *Symbiodinium*, and Takahashi & Yamasaki (2002) and Wodala & co-workers (2008) reported a similar pattern in plants, with the latter authors concluding that NO could inhibit electron transport between quinone A (Q_A) and Q_B. This is consistent with the data presented here, as a blockage of electron flow at Q_A would cause immediate reductions in effective quantum yield ($\Delta F/F_m'$). This inhibition of electron transport could stall the thylakoid membrane H^+ pump, leading to the collapse of NPQ (Genty *et al.*, 1989). NPQ is a major photoprotective mechanism in symbiotic dinoflagellates (Gorbunov *et*

al., 2001) and was affected by NO. Blockage of electron flow and the inhibition of NPQ could eventually lead to damage to the D1 protein in PSII (Hill *et al.*, 2011) and a declining maximum quantum yield (F_v/F_m). Furthermore, the alleviation of temperature-induced declines in maximum PSII fluorescence yields in the presence of an NO scavenger suggests that some symbiont types' own NO production at elevated temperatures may be playing a role in the photoinactivation of PSII. Further work is required, however, before any specific mechanistic conclusions can be drawn with confidence; in particular whether NO's apparent inhibition of *Symbiodinium* PSII is due to its direct actions on photosynthetic machinery or more generalised cellular damage. In addition, the situation appears different for type C1, as this displayed progressive photoinhibition at 34°C alongside decreasing NO synthesis. The observed inactivation of PSII in these cells may therefore have been purely temperature-driven, while in types A1 and B1 it could have resulted from increasing NO synthesis (as a general temperature response) exacerbating thermal photoinhibition. This is supported by the fact that F_v/F_m in these types could not be restored to control levels at high temperature even following the addition of an NO scavenger.

Bouchard & Yamasaki (2009) proposed NO as a cytotoxic molecule mediating apoptotic-like death in *Symbiodinium* cells and, although the results of this investigation support this hypothesis, it is clear that not all temperature-induced mortality in symbiotic dinoflagellates is apoptotic-like. While A1- and B1-type *Symbiodinium* cells produced similar levels of NO at elevated temperature, their modes of cell death differed markedly. B1 cells suffered acute uncontrolled necrotic-like mortality with little evidence of any apoptotic-like response. Conversely, type A1 displayed distinct apoptotic-like bursts at 32°C and 34°C coincident with heightened NO production. Use of an NO scavenger confirmed the NO-dependency of temperature-induced mortality in types A1 and B1. Given that these types were differentially susceptible to NO, it is unsurprising that the dominant form of mortality displayed by B1 – the least tolerant type – was necrosis. The more robust A1 cells, however, remained photosynthetically competent at 34°C despite significant increases in NO and this might have enabled a more regulated form of cell death (PCD/apoptosis) to be maintained.

2.4.3. Conclusions

NO appears harmful to *Symbiodinium* cells' photosynthetic function during heat stress and this may be important in the context of coral bleaching, as chronic photoinhibition can lead to oxidative stress (Liu *et al.*, 2004; Suggett *et al.*, 2008) that may, alongside symbiont-derived NO, trigger host responses resulting in symbiont ejection (Perez & Weis, 2006; Weis, 2008). Together with the varying NO production of different symbiont types, their differential NO tolerance may affect the levels of ROS leakage *in hospite*, cell mortality, and, potentially, the host's bleaching response.

This study is the first to examine NO production by different types of symbiotic dinoflagellate, and it is clear that the genetic diversity of these algae is reflected in their physiology even in the case of the strongly conserved phenomenon of NO signalling. Despite this, NO seems to be involved in the temperature-induced photoinhibition and mortality of some *Symbiodinium* types. As such, symbiont-derived NO may play a significant role in the coral bleaching phenomenon whether directly – inducing mortality in the symbionts (Strychar *et al.*, 2004a) – or indirectly, through leakage of itself or ROS into the host leading to an immune-like rejection response (Perez & Weis, 2006; Weis, 2008). The significance of NO in the longer-term and during the less acute temperature stresses that characterise most natural coral bleaching events, however, remains unclear. Further investigation of intact symbioses would yield vital information regarding the involvement of NO in the cnidarian bleaching phenomenon.

Chapter 3

Nitric oxide mediates coral bleaching through an apoptotic-like cell death pathway: Evidence from a model sea anemone-dinoflagellate symbiosis

3.1. Introduction

The collapse of a symbiosis can have devastating consequences not only for the organisms involved but also for the wider community. This is becoming increasingly apparent on tropical coral reefs, where the dominant ecosystem engineers, scleractinian corals, are undergoing a widespread decline. One of the most serious threats is global warming (Hoegh-Guldberg *et al.*, 2007; van Hooidonk *et al.*, 2013), as the associated increases in seawater temperatures are leading to a collapse of the coral-dinoflagellate symbiosis (Hoegh-Guldberg, 1999). While corals in shallow water are often exposed to temperature fluctuations of several degrees celsius over the course of a day, sustained increases (over several days / weeks) of only a few degrees can lead to "bleaching". Defined as the loss of symbiotic dinoflagellates (genus *Symbiodinium*) and/or their photosynthetic pigments from a coral host, bleaching can result in corals experiencing nutritional shortfalls (Goreau & MacFarlane, 1990) and mortality (McClanahan, 2004). Over longer timescales, it can also lead to the decline of whole reef systems (Graham *et al.*, 2006; Hoegh-Guldberg & Bruno, 2010; De'ath *et al.*, 2012). Despite the magnitude of the threat posed by coral bleaching, our knowledge of the physiological basis of this symbiotic breakdown remains limited (Weis, 2008).

The current model of bleaching proposes chronic photoinhibition arising in the symbiont alongside the overproduction of ROS (Suggett *et al.*, 2008). Depending on its severity, the resulting stress can not only harm the symbiont (Dunn *et al.*, 2004), but may also stimulate a host innate immune-like response leading to symbiont destruction or ejection (Weis, 2008). This response includes the upregulation of NO synthesis (Perez & Weis, 2006). NO is a ubiquitous signalling molecule that plays a vital role mediating microbial endosymbioses (Davidson *et al.*, 2004; Catala *et al.*, 2010; Wang & Ruby, 2011) and regulating intrinsic (mitochondria-mediated) apoptosis (Brune *et al.*,

1999; Tait & Green, 2010). This latter phenomenon is essentially a controlled "cellular suicide" mediated by cysteinyl proteases known as caspases. Recent work has identified a potential suite of caspase-like enzymes in various symbiotic cnidarians (Sunagawa *et al.*, 2009; Meyer & Weis, 2012), and apoptotic-like mortality is a major component of coral bleaching (Dunn *et al.*, 2004; Richier *et al.*, 2006; Dunn *et al.*, 2007; Dunn *et al.*, 2012). However, little is known about the specific molecular machinery of apoptotic-like death in cnidarians, and how extensive the similarities are to mammalian systems. The role of NO in the collapse of this symbiotic association is also currently unknown, although a plausible hypothesis involves its actions on cell death pathways in the presence of ROS. Furthermore, there is growing evidence that symbiotic dinoflagellates can produce NO during thermal stress (Trapido-Rosenthal *et al.*, 2005; Bouchard & Yamasaki, 2008; Bouchard & Yamasaki, 2009; Chapter 2), and the importance of this symbiont-derived NO during bleaching is also unclear. NO synthesis by endosymbionts is comparatively rare (Wang & Ruby, 2011), and with most research on *Symbiodinium* having been carried out on cultured (Bouchard & Yamasaki, 2008; Bouchard & Yamasaki, 2009; Chapter 2) or freshly isolated (Trapido-Rosenthal *et al.*, 2001) cells, it remains to be seen whether symbiont NO synthesis occurs *in hospite* (i.e. in the intact symbiosis).

This study sought to address these issues using the model symbiotic cnidarian *Aiptasia pulchella*. In particular, I examined the hypothesis that temperature stress-induced NO is involved in the induction of host apoptotic-like pathways (Weis, 2008) and is a consequence of symbiont photoinhibition (Perez & Weis, 2006). These questions were investigated using the rapid-heating methods similar to those employed by previous researchers (Dunn *et al.*, 2004, Perez & Weis, 2006; Detournay *et al.* 2011; Dunn *et al.*, 2012). However, of equal importance is the question of whether cellular events induced by these "heat shock" conditions accurately reflect those that occur during slower, more ecologically relevant rates of heating. To examine this question, I exposed symbiotic *A. pulchella* to a heating regime more similar to that experienced by bleaching corals in the field (Middlebrook & Anthony, 2010).

3.2. Materials and methods

Except where otherwise noted, all reagents were obtained from Sigma-Aldrich, Auckland, New Zealand.

3.2.1. Culture and treatment of Aiptasia pulchella *anemones*

Anemones (*Aiptasia pulchella*) were sampled from a laboratory-maintained, clonal population incubated under a 12 h light:12 h dark cycle (80-100 µmol photons m^{-2} s^{-1} provided by cool white fluorescent lamps - OSRAM DULUX L 36W 4000 K) at a temperature of 26°C, and fed twice weekly with freshly hatched *Artemia* sp. nauplii. Prior to rapid heating, anemones were starved for 48 h. Slow-heated anemones were fed twice weekly during acclimation and again immediately after the start of the experiment; they remained unfed for the remainder of the experiment.

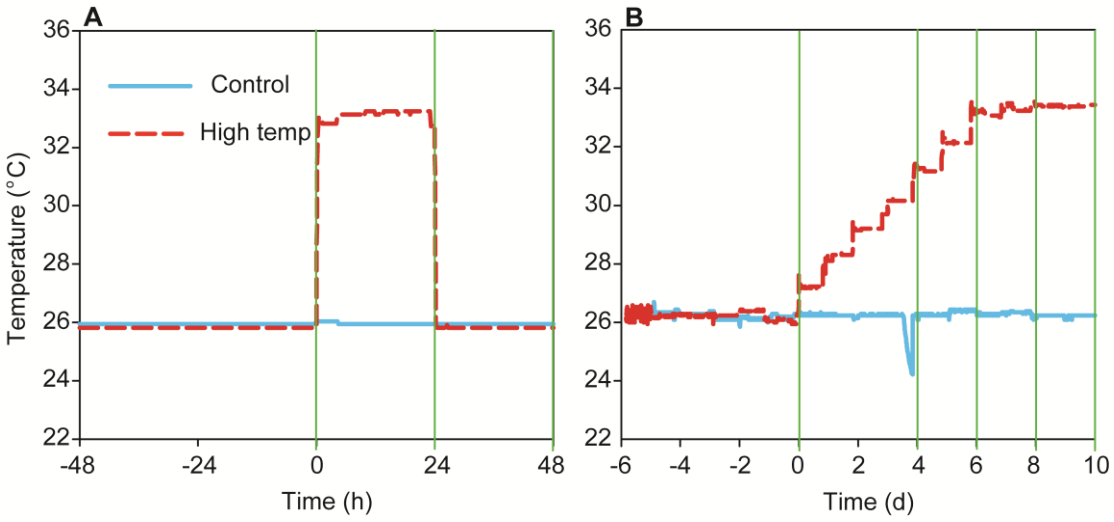

Figure 3.1. Temperature treatments of *Aiptasia pulchella*. **Panel A)** High temperature stress (HTS - 33°C) followed by a further 24 h at control (26°C) temperatures. The return to control temperatures for 24 h is required to induce symbiont release (Detournay & Weis, 2011); **B)** Slow heating (ramping of *ca.* 1°C day^{-1}). Green lines indicate sampling points.

The ability of *A. pulchella* to produce NO was confirmed by subjecting anemones to high temperature shock (HTS). Eight small (*ca.* 2 mm oral disc diameter) anemones were transferred to 25-mL glass beakers (one anemone per beaker) containing FSW, and maintained in a water bath at 26°C on a 12 h light:12 h dark cycle (70-90 µmol photons m^{-2} s^{-1}, LED light bank - 20 RoHS 5W 6400 K). The anemones were acclimated for 48 h and fluorometric assessments of PS II (see below) of the dinoflagellate symbionts were conducted every 12 h to confirm stability under the new photic regime. Temperature was then increased rapidly (over < 1 h) at midday to 33°C or kept constant at 26°C (control). The anemones were exposed to these conditions for 24 h.

To examine the interactions between high temperature shock, host NO synthesis, caspase-like enzyme activation and bleaching, a second batch of *A. pulchella* (5-7 mm oral disc diameter, n = 6 anemones for each of three time points) was acclimated in a water bath (see above). After removing one of the three anemones in each beaker (for initial symbiont density assays, n = 6, see below) the remainder were exposed for 24 h to control conditions (26°C), HTS, or HTS with one of the following reagents: a) 2 mM of the specific NO scavenger cPTIO (Life Technologies, Auckland, New Zealand); b) 100 µM of the initiator-caspase-9 inhibitor acetyl-Leu-Glu-His-Asp-aldehyde (Ac-LEHD-CHO); or c) 0.1% v/v DMSO (the solvent for Ac-LEHD-CHO). A third batch of anemones (n = 5 per time point) were treated for 24 h at 26°C with 1 mM of the NO donor GSNO in the presence and absence of 1 mM cPTIO or 100 µM Ac-LEHD-CHO. Assessments of host caspase-like enzyme activity were conducted on anemones (n = 6) sampled after 24-h exposure to the treatment or control conditions (see Dunn *et al.*, 2007; Detournay & Weis, 2011). As a positive control for caspase-like activity, anemones (n = 4) were incubated at 26°C for 24 h with 0.05% w/v colchicine (a microtubule inhibitor and apoptosis-inducer) in FSW (Pernice *et al.*, 2011). After both HTS and NO-donor treatments, remaining anemones were returned to control conditions for a further 24 h - to allow time for bleaching to occur (see Detournay & Weis, 2011) - and were then sampled for analysis of symbiont loss (see below).

To determine whether cellular responses in *A. pulchella* are affected by the rate of heating, eight 100-mL beakers each containing 10 anemones (one anemone for confocal assessments of NO and one for caspase enzyme and bleaching assays, for each of five

time points) were transferred to a water bath (see above) and acclimated for 7 days. Again, symbiont PSII performance was monitored to ensure stability prior to treatment. Temperature was then either kept constant at 26°C or increased by *ca.* 1°C per day (shortly after the start of the light cycle, with the new temperature reached within 1 h) to 33°C ± 0.3°C (SD) and maintained at this maximum for 4 days. Seawater was replaced daily to prevent a build-up of metabolic wastes.

3.2.2. Real-time visualisation of NO synthesis in Aiptasia pulchella*.*

Nitric oxide was detected *in situ* using the fluorescent dye DAF-FM-DA (Molecular Probes, Eugene, OR, USA). The seawater medium of anemones was replaced with 15 µM DAF-FM-DA in "relaxing solution" [50% FSW, 50% 0.37 M magnesium chloride ($MgCl_2$)]. Anemones were incubated in the dark for 90 min. After 60 min, Hoechst 33342 stain was added (10 µg mL^{-1} final) to label host cell nuclei and aid visualisation of gastrodermis tissue. Anemones were then washed twice to remove excess dye and left for a further 30 min to allow the DAF-FM-DA to be cleaved to its active DAF-FM form. Individual anemones were transferred to glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) and immobilised by adding a few drops of 1% (w/v) low-melting agarose in relaxing solution (boiled and cooled to *ca*. 28°C beforehand). Anemones were then visualised using an Olympus Fluoview FV-1000 inverted confocal LSM (Olympus, Center Valley, PA, USA) and ×40 0.9 NA water immersion lens. NO-dependent fluorescence was detected at 510-530 nm, with excitation provided by a 473 nm diode laser. Hoechst 33342 fluorescence was detected using a DAPI filter (430-460 nm) and 405 nm excitation, and symbiont chlorophyll autofluorescence was detected using a 635 nm laser and 655-755 nm emission filter. Fifteen images in the z-plane were acquired for each of three tentacles per replicate anemone (45 images in total per anemone) and NO-dependent fluorescence was quantified by measuring the 510-530 nm mean fluorescence intensity (MFI) of tentacle gastrodermis (positive for symbiont chlorophyll fluorescence, see Supplementary Fig. A6) using ImageJ software (National Institutes of Health, Bethesda, USA). Blank anemones (stained only with Hoechst 33342) were also visualized in order to control for host tissue autofluorescence. All instrument settings were kept constant and successful loading of DAF-FM-DA was confirmed by incubating non-fluorescing anemones in 1 mM SNP for 30 min.

3.2.3. Preparation of tissue homogenates and extraction of Symbiodinium *cells.*

Anemones were removed from experimental conditions at midday, rinsed with 0.37 M $MgCl_2$, immediately flash-frozen in liquid nitrogen and stored at -80°C.

Individual anemones were thawed on ice and homogenised in a 1.5-mL tube with a small tissue grinder and a known volume of homogenisation buffer (HB; 50 mM potassium phosphate, pH 7.8, 1 mM EDTA). A 50-µL aliquot was removed for symbiont counts (and to confirm that symbiont cells remained intact during the homogenisation process) and the remainder was centrifuged (1500 × g for 5 min) to pellet the algal cells. Aliqouts of supernatant (host fraction, to be used for analysis of caspase-like enzyme activity and NO_2^- content - see below) were then transferred to fresh 1.5-mL tubes, flash frozen and stored alongside the algal pellets at -80°C.

3.2.4. Symbiont density and *pigment analysis, and fluorometric assessment of PSII.*

Maximum quantum yields of PSII (F_v/F_m) were measured daily 30 min after lights-off using pulse amplitude modulated fluorometry (Diving-PAM, Walz, Effeltrich, Germany). Triplicate *in vivo* measurements of PSII yield were recorded for each replicate anemone at each time point.

Symbiont densities were quantified using Improved Neubauer haemocytometer counts (six replicate counts per sample; Boeco, Germany) normalised to host protein content, measured using the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard. For anemones used in the short-term experiment, the percentage of symbionts lost over the 48-h experimental period was calculated relative to mean initial values.

Symbiont chlorophyll *a* (Chl *a*) content was quantified by N, N-dimethylformamide (DMF) extraction carried out in darkness over 48 h at 4°C. Extracts were then centrifuged (16000 × g for 5 min) and triplicate 200-µL aliquots were measured in 96-well plates (UVStar, Greiner Bio-One GmbH, Germany) at 646.8 nm, 663.8 nm and 750 nm using a microplate reader (Enspire® 2300, Perkin-Elmer, Waltham, MA, USA). Chl *a* concentrations were determined after optical path length correction (0.555 cm) using the equations of Porra *et al.* (1989).

3.2.5. Assessments of nitric oxide (NO) production using 2,3-diaminonaphthalene (DAN).

Confocal LSM analysis of symbiont NO synthesis is hampered by the confounding effects of host fluorescence and the likelihood of inducing stress during the preparation steps (repeated washing and centrifugation) required to remove this host contamination. Moreover, the removal of symbionts from their host has been shown to be highly deleterious to the cells (Wang *et al.*, 2011). As such, analysis of NO production by slow-heated *Symbiodinium* cells *in hospite* was carried out on cells isolated from flash-frozen anemones.

Frozen algal pellets were thawed on ice and washed four times with chilled (4°C) HB by repeated centrifugation (1500 × g for 5 min), before resuspension in 250 µL HB. Cells were transferred to a 1.5-ml tube containing 50 mg glass beads (710-1180 µm diameter; Sigma-Aldrich, Auckland, New Zealand) and lysed in a Beadmill (Tissuelyser LT, Qiagen Inc., Hilden, Germany) for eight minutes at a frequency of 50 Hz. Disruption of cells was confirmed using visual haemocytometer counts of the lysate, which was then directly analysed (see below). Aliquots of anemone host fraction were analysed alongside symbiont cell lysates.

Nitric oxide has a short half-life in biological fluids and is quickly oxidised to nitrite (NO_2^-), which is comparatively stable and a reliable reflection of NO synthesis (Nussler *et al.*, 2006). *Symbiodinium* cell lysate and host homogenate NO_2^- content was quantified using the fluorescent indicator 2,3-diaminonaphthalene (DAN, Life Technologies, Auckland, New Zealand; Nussler *et al.*, 2006) according to the methods of Detournay & Weis (2011), with some modification. Briefly, after centrifugation (16000 × g for 20 min), proteins were removed by precipitation with 15 mg mL^{-1} zinc sulphate (Ghasemi *et al.*, 2007), vortexing for 20 s, and further centrifugation (16000 × g for 10 min). Aliquots (225 µL) of supernatant were then incubated with an equal volume of 158 mM DAN in 0.62 N HCl for 10 min at 28°C. NaOH (105 µL of a 2 N solution) was then added and samples were centrifuged again (16000 × g for 5 min). Triplicate aliquots (150 µL) of supernatant were then removed and NO_2^- content was calculated from DAN fluorescence (ex: 365 nm, em: 410 nm) measured using a plate

reader (see above) and a calibration curve of 10-10000 nM $NaNO_2$ in HB. As positive controls, cell lysates/homogenates (n = 8) were incubated with 1 mM NO donor sodium nitroprusside (SNP) for 4 h at 26°C.

3.2.6. Assessments of host caspase-like enzyme activity in Aiptasia pulchella.

Enzyme activities resembling those of mammalian caspase-3 (an "executioner" caspase; Nicholson, 1999; Lasi *et al.*, 2010) and caspase-9 (an "initiator" caspase in the intrinsic pathway; Lasi *et al.*, 2010, Tait *et al.*, 2012) were quantified using a colorimetric assay kit (Sigma-Aldrich, Auckland, New Zealand) with the substrates Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA) and Ac-Leu-Glu-His-Asp-pNA (Ac-LEHD-pNA), respectively. Caspase enzymes cleave the terminal aspartic acid residue in the tetrapeptide substrate with a specificity determined by the identity of the preceding three amino acid residues. Anemone homogenates were thawed on ice and centrifuged (16000 × g for 20 min). Aliquots of the supernatant were removed for protein quantification [Bradford assay with bovine serum albumin (BSA) as a standard (Bradford, 1976)] and the remainder was analysed in duplicate. Briefly, 10 µL caspase substrate (2 mM) was added to 40 µL "assay buffer" [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT)]. A 50-µl aliquot of centrifuged anemone homogenate was then added. After gentle shaking for 10 s, samples were incubated for 3 h at 37°C according to the manufacturer's instructions. Sample blanks were prepared alongside and, to control for non-specific substrate hydrolysis, some homogenates were pre-incubated for 30 min with 20 µM DEVD- or LEHD-aldehyde (-CHO), reversible inhibitors of caspases-3 and -9 respectively.

3.2.7. Statistical analyses

Data analysis was carried out using PASW Statistics 18.0 (IBM, Armonk, NY, USA). Where appropriate, data were analysed using repeated measures analysis of variance (RMANOVA) and transformed where necessary. The Greenhouse-Geisser correction was used whenever Mauchly's test returned a significant result. RMAMOVA outputs are for time × treatment interactions, and *post-hoc* reports represent pair-wise comparisons between treatments with Bonferroni correction. Other analyses were

carried out using uni- and multivariate ANOVA and t-tests. Where data did not meet assumptions of normality, Kruskall-Wallis non-parametric tests were conducted.

3.3. Results

3.3.1. NO synthesis, host caspase-like enzyme activation and bleaching during rapid high temperature stress in Aiptasia pulchella.

Exposure of symbiotic *A. pulchella* to HTS or the NO donor SNP for 24 h (Fig. 3.2A) resulted in significant increases in tentacle gastrodermis DAF-FM fluorescence (one-way ANOVA, $F_{2, 10} = 15.98$, $p = 0.002$) and tissue NO_2^- content (one-way ANOVA, $F_{2, 11} = 52.34$, $p < 0.001$). Treatment with 0.05% (w/v) colchicine in FSW caused heightened caspase-like enzyme activity (Kruskall-Wallis Tests, DEVDase: $p = 0.018$; LEHDase: $p = 0.02$) that was reversed in the presence of 20 µM caspase inhibitor (Fig. 3.2B).

Table 3.1. Statistical analysis of caspase-like enzyme activity and symbiont loss (MANOVA and ANOVA, respectively) in *Aiptasia pulchella* exposed to heating and the NO donor and scavenger GSNO and cPTIO, respectively.

Treatment	**Variable**	**F-value**	**P-value**
Nitric oxide donor	LEHDase	$F_{2, 11} = 22.841$	< 0.001
	DEVDase	$F_{2, 11} = 50.792$	< 0.001
	Symbiont loss	$F_{3, 17} = 6.848$	0.003
High temperature	LEHDase	$F_{2, 10} = 25.273$	< 0.001
	DEVDase	$F_{2, 10} = 21.833$	< 0.001
	Symbiont loss	$F_{4, 24} = 18.995$	< 0.001

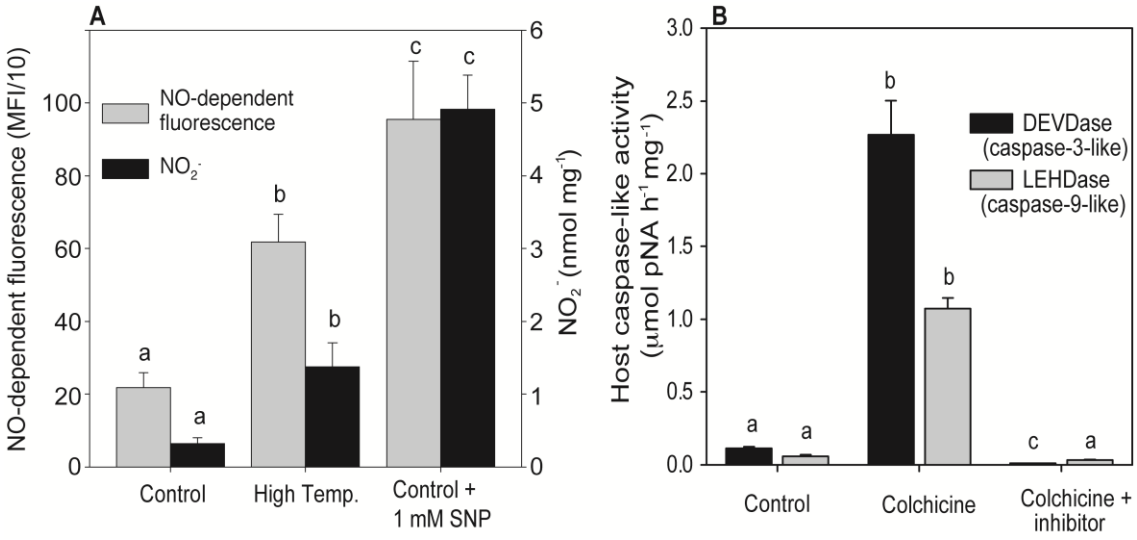

Figure 3.2. Detection of NO synthesis and caspase-like enzyme activity in *Aiptasia pulchella* (under 70-90 µmol photons m^{-2} s^{-1}). **Panel A**) NO synthesis (DAF-FM fluorescence intensity and tissue NO_2^- content) after HTS treatment (33°C for 24 h) or supplementation with an NO donor (1 mM SNP). **B**) Caspase-like enzyme activity of anemones treated with the microtubule inhibitor and apoptosis inducer colchicine (0.05% w/v for 24 h) in the presence and absence of caspase inhibitors. Values are means ± s.e.m. and letters indicate bars that are significantly different from each other within each parameter (**A**: n = 8 anemones, one-way ANOVA, Tukey HSD *post-hoc*; **B**: n = 4 anemones, Kruskall-Wallis test).

Addition of the NO donor GSNO (1 mM in FSW) also induced significant increases in caspase-like activity (Table 3.1), and these were reduced or absent in the presence of 1 mM cPTIO (Fig. 3.3A). Caspase-like activity increased at high temperature (Table 3.1, Fig. 3.3B) and in the case of LEHDase (caspase-9-like) this response was partially alleviated by the NO scavenger cPTIO (Fig. 3.3B). cPTIO had no significant effect on the activity of DEVDase (caspase-3-like) activity (Fig. 3.3B)

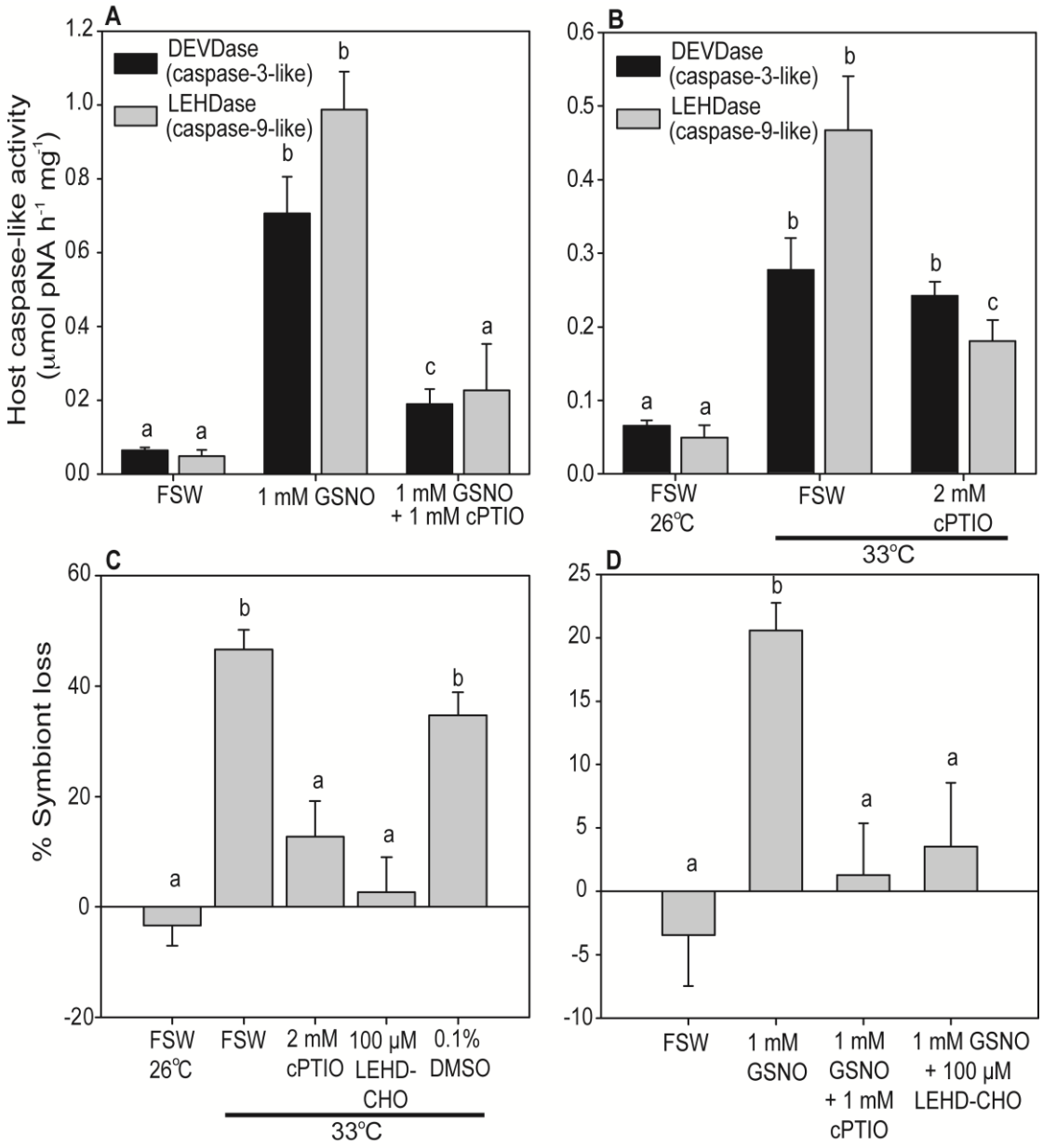

Figure 3.3. Cellular responses of symbiotic *Aiptasia pulchella* to short-term treatments. **A**) Caspase-like enzyme activity in anemones treated for 24 h with 1 mM of the NO donor GSNO in the presence or absence of 1 mM of the NO scavenger cPTIO. **B**) Caspase-like activity in heat-shocked anemones with and without 2 mM cPTIO. **C**) Bleaching (relative symbiont loss over 48 h) of heat-shocked anemones in the presence of 2 mM cPTIO, 20 µM of the caspase-9 inhibitor Ac-LEHD-CHO, or 0.1% (w/v) DMSO. **D**) Bleaching of GSNO-treated anemones with and without cPTIO and Ac-LEHD-CHO. Values are means +/- s.e.m. and letters indicate bars that are significantly different from each other (Tukey's HSD *post hoc*: A, B, D: $p < 0.01$; C: $p < 0.05$) within each parameter (A, B: one-way MANOVA, n = 5 anemones and n = 6, respectively; C, D: one-way ANOVA, n = 6 anemones and n = 5, respectively).

High temperature shock resulted in the loss of 47.0 ± 2.9% of symbiont cells from *A. pulchella*, and this was significantly reduced in the presence of the NO scavenger cPTIO (2 mM in FSW) or the LEHDase inhibitor Ac-LEHD-CHO (100 µM in FSW; Table 3.1, Fig. 3.3C). Interestingly, and as with the caspase-9-like activity described above, the alleviation of bleaching with cPTIO was not 100%. DMSO, the solvent for Ac-LEHD-CHO, had a negligible effect (Fig. 3.3C). The removal of NO with cPTIO also alleviated the temperature-induced declines in PSII fluorescence yield of *Symbiodinium* cells *in hospite* (RMANOVA, $F_{8, 56} = 19.465$, $p < 0.001$, Fig. 3.4). Furthermore, the addition of exogenous NO by GSNO (1 mM GSNO in FSW) at 26°C induced significantly heightened symbiont losses (20.5 ± 2.2%) (Fig. 3.3D) relative to controls maintained in FSW. This was not apparent when anemones were treated with 1 mM GSNO and either 1 mM cPTIO or 100 µM Ac-LEHD-CHO.

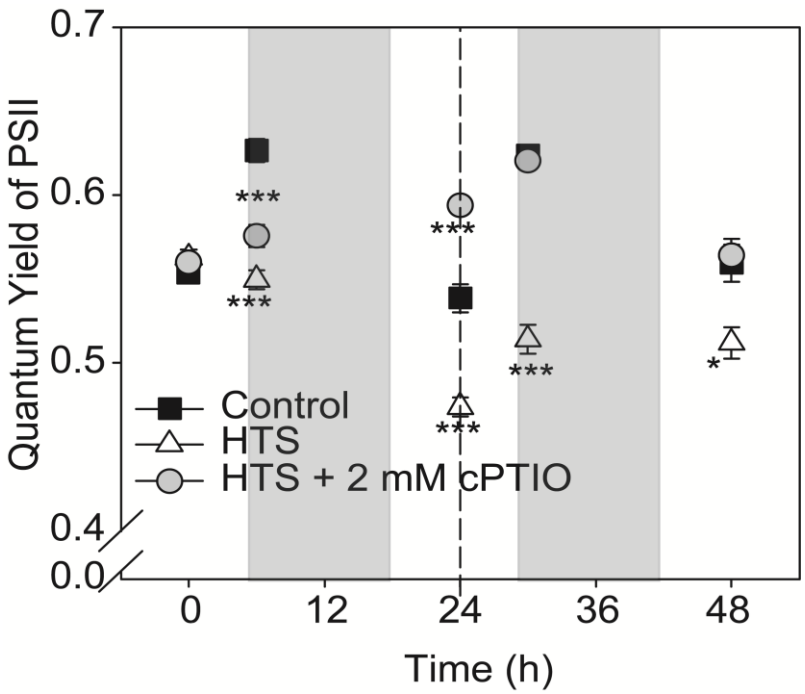

Figure 3.4. *In hospite* quantum yields of PSII in *Symbiodinium* cells in *Aiptasia pulchella* anemones exposed to HTS (under 70-90 µmol photons m^{-2} s^{-1}) with and without the NO scavenger cPTIO (2 mM final). Shaded areas indicate periods of darkness and the dotted line at 24 h indicates the time at which temperature was returned to 26°C (control conditions). Values are means ± s.e.m. and asterisks indicate significant differences relative to controls (RMANOVA, pair-wise *post-hoc* with Bonferroni correction, n = 6 anemones per time point, * $p < 0.05$, *** $p < 0.001$).

3.3.2. Photoinhibition and bleaching in slow-heated Aiptasia pulchella.

Slow heating resulted in significant reductions in the maximum quantum yield of symbiont PSII (RMANOVA, $F_{4.2, 55.5} = 63.85$, $p < 0.001$, Fig. 3.5A) from 0.60 ± 0.01 to 0.37 ± 0.01. This preceded significant declines in symbiont Chl *a* content (RMANOVA, $F_{2.5, 35.3} = 8.32$, $p < 0.001$, Fig 3.5B) - from 1.05 ± 0.06 pg per cell to 0.39 ± 0.02 pg per cell - and symbiont density within the host (RMANOVA, $F_{4, 36} = 4.38$, $p = 0.005$, Fig. 3.5C), which decreased from 6.13 ± 0.21 to 3.52 ± 0.29 million cells per milligram of soluble host protein.

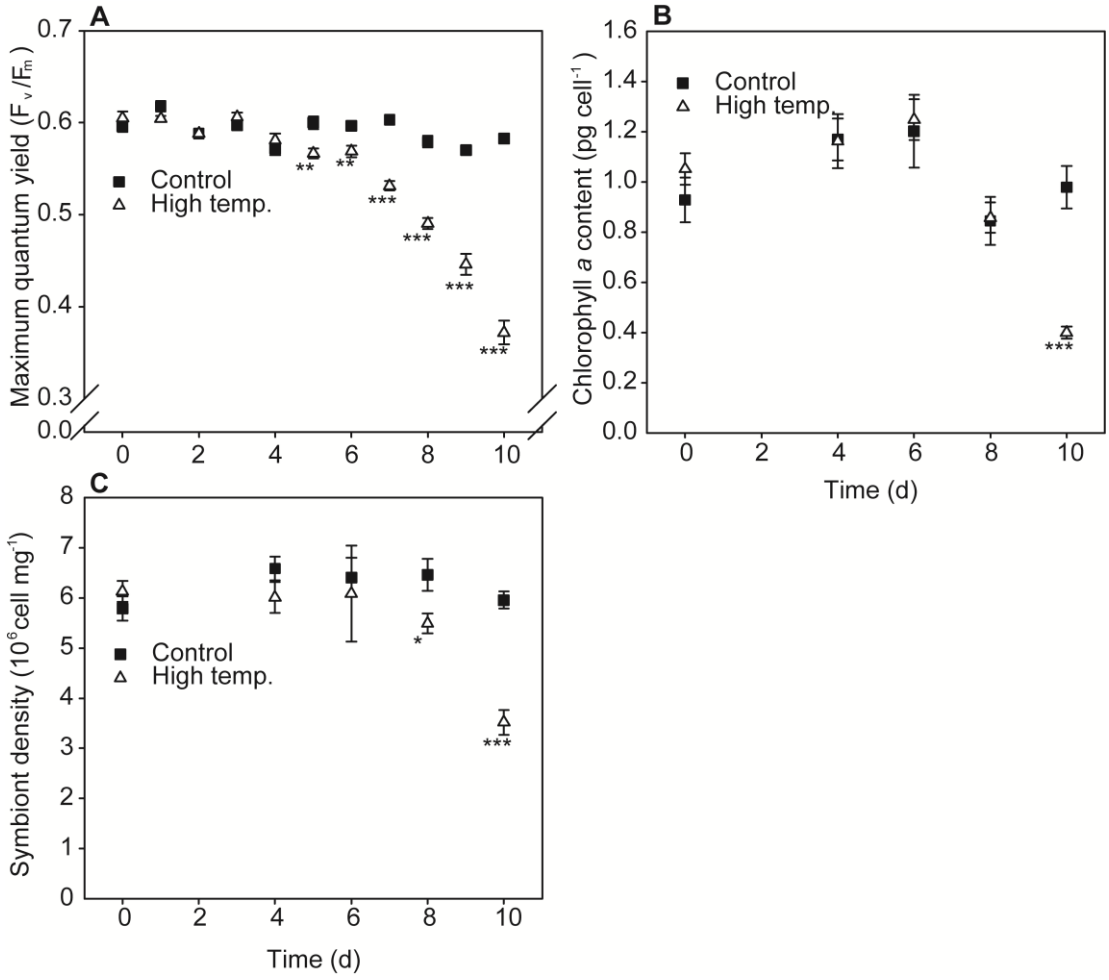

Figure 3.5. Photosynthetic dysfunction and bleaching of slow-heated *Aiptasia pulchella* (under 70-90 µmol photons m^{-2} s^{-1}). **Panel A)** Maximum quantum yield of PSII; **B)** Symbiont chlorophyll *a* content per cell; **C)** Symbiont density per mg soluble host protein. Values are means ± s.e.m. and asterisks indicate significant differences relative to controls at each time point (RMANOVA, pair-wise *post-hoc* with Bonferroni correction, n = 8 anemones per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3.3. NO synthesis and host caspase-like enzyme activity in slow-heated Aiptasia pulchella.

Confocal visualisation of NO in *A. pulchella* tentacle gastrodermis (Fig. 3.6) revealed that levels of NO increased significantly at elevated temperature (RMANOVA, $F_{4, 44} = 6.216$, $p < 0.001$), becoming 2.5-fold higher on Days 4 and 6 than in controls (Fig. 3.7A). NO synthesis at 33°C subsequently declined and, by Day 10, had returned to pre-treatment levels (Figs. 3.7A). A similar pattern was observed in the nitrite (NO_2^-) content of host anemone tissue (Fig. 3.7B), where a peak was observed on Day 6 and levels subsequently declined (RMANOVA, $F_{4, 40} = 3.658$, $p = 0.012$). These data were significantly more variable than those obtained through direct visualisation of NO, and the initial increase on Day 4 was missing in the NO_2^- analysis. Apparent NO synthesis in the algal symbionts (quantified as cellular NO_2^- accumulation) also responded to slow heating (RMANOVA, $F_{4, 36} = 4.377$, $p = 0.005$) although significant increases relative to pre-treatment values were only apparent after Day 10 (Fig. 3.7D).

Caspase-like enzyme activities were significantly affected by slow heating (RMANOVAs, DEVDase: $F_{4, 40} = 4.431$, $p = 0.005$; LEHDase: $F_{4, 44} = 12.263$, $p < 0.001$) and after 4 days at elevated temperature both enzymes' activities were at least two-fold greater than those of the control group (Fig. 3.7C). Further heating resulted in declining enzyme activity, although there was a secondary increase relative to the controls on Day 8 (Fig. 3.7C).

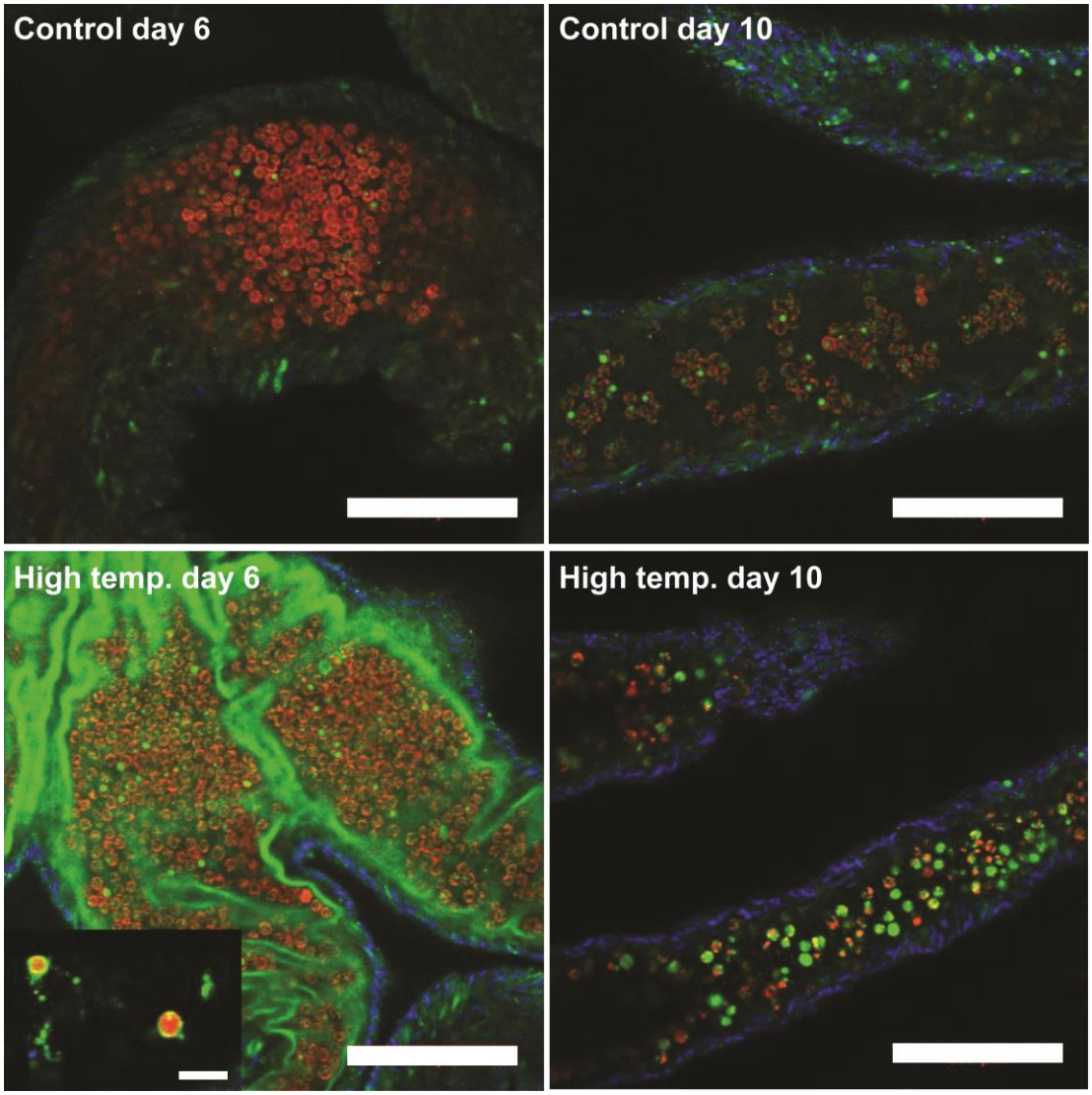

Figure 3.6. Confocal visualisation of nitric oxide (NO) production in *Aiptasia pulchella* anemones maintained for 10 days under either control or slow heating conditions (70-90 µmol photons m^{-2} s^{-1} irradiance) and loaded with 15 µM NO-sensitive dye DAF-FM-DA. NO-dependent fluorescence (510-530 nm, green) is superimposed on Hoechst 33342 (440-490 nm, blue) and symbiont chlorophyll (> 655-755 nm, red) fluorescence. **Inset:** *Symbiodinium* cells released from anemone tissue during slow heating, and displaying evidence of NO synthesis around their periphery (likely representative of host material). Scale bars for main images: 100 µm; for inset: 20 µm.

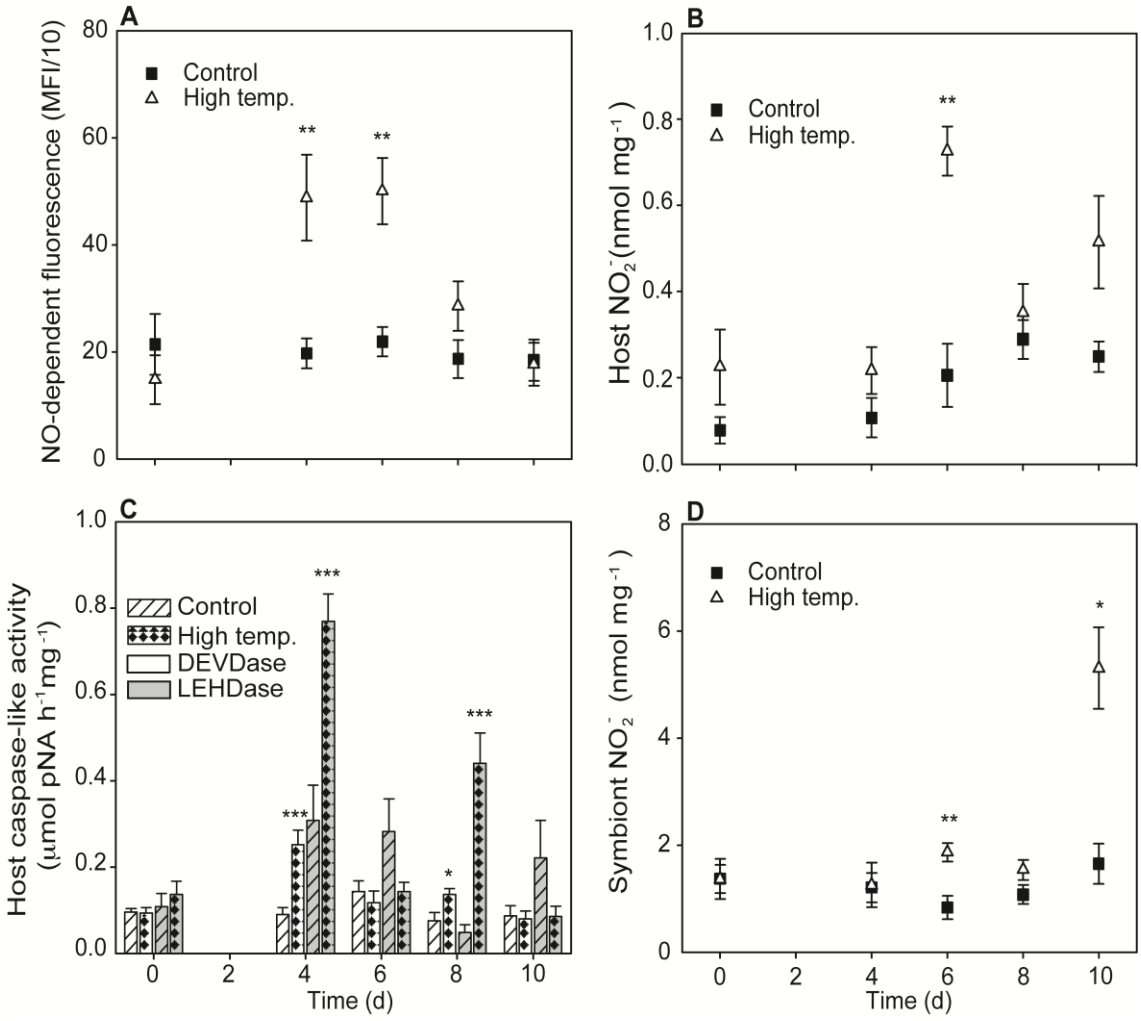

Figure 3.7. Cellular responses of *Aiptasia pulchella* to slow heating (under 70-90 µmol photons m^{-2} s^{-1}). **Panel A**) NO-dependent fluorescence of live anemone gastrodermis. **B**) nitrite (NO_2^-) content of host tissue . **C**) Activity of host caspase-like enzymes in anemone tissue. **D)** NO_2^- content of symbiont cells. Values are means ± s.e.m. and asterisks indicate significant differences relative to control values within each parameter (RMANOVA, pair-wise *post-hoc* with Bonferroni correction, n = 8 anemones per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4. Discussion

This study develops the role of the ubiquitous antimicrobial and signalling compound nitric oxide in the breakdown of an ecologically important endosymbiosis. While NO possesses significant antioxidant and anti-inflammatory properties in some biological systems (Wink *et al.*, 2011), its synthesis in heat stressed symbiotic cnidarians appears to be a deleterious phenomenon. Indeed, NO's involvement in the collapse of the cnidarian-dinoflagellate association arises through the activation of an enzyme closely linked with the initiation of apoptosis. This finding is significant in the context of intracellular symbioses in general, as the modification of host NO synthesis and cell death pathways is a common strategy employed by mutualistic and pathogenic microbes in order to persist within the host's internal environment (Carmen & Sinai, 2007; Pannebakker *et al.*, 2007; Wang & Ruby, 2011). To the authors' knowledge, this study also provides the first quantitative evidence of *in hospite* NO synthesis by an intracellular eukaryotic symbiont.

3.4.1. Temperature stress induces bleaching in Aiptasia pulchella *through a caspase-mediated, NO-dependent pathway.*

Exposing *A. pulchella* to elevated temperature led to elevated NO synthesis similar to that described previously (Perez & Weis, 2006). Through its interactions with important components of the intrinsic apoptotic pathway (Brookes *et al.*, 2000), NO has a capacity to regulate programmed cell death and thus form part of a host innate immune-like response (Perez & Weis, 2006; Weis, 2008).

The activity of caspase-like enzymes, often necessary for the initiation and execution of apoptotic cell death (Nicholson, 1999; Tait & Green, 2010), increased significantly in *A. pulchella* under heat stress conditions and when exposed to a nitric oxide donor. Although a range of potentially non-apoptotic functions exists for caspases (Kuranaga & Miura, 2007) and direct assessments of apoptosis were not carried out for this study, these findings are consistent with the hypothesised involvement of apoptosis in cnidarian bleaching (Dunn *et al.*, 2004; Richier *et al.*, 2006; Dunn *et al.*, 2007; Pernice *et al.*, 2011). The enzyme activities reported here are also likely to be those of two different proteases. Mammalian caspase-9 is an "initiator" caspase (Nicholson, 1999)

and its role lies in the induction of apoptosis following mitochondrial dysfunction (Tait & Green, 2010). Caspase-3, however, is an "executioner" caspase(Nicholson, 1999) activated downstream of caspase-9 or alternatively through the receptor-mediated (non-mitochondrial) pathway (Nicholson, 1999). While caution must be taken in inferring the existence of multiple caspase-like enzymes in *A. pulchella* from the observations of differential substrate cleavage, recent genomic analyses have revealed a number of caspase-like genes including those similar to mammalian caspases-3 and -9 (Meyer & Weis, 2012). Furthermore, sponges possess enzymes similar to caspases-3 and -9 (Wiens *et al.*, 2003), the anemone *Anemonia viridis* has a caspase-8-like enzyme (Richier *et al.*, 2006), and the cnidarian *Hydra* has a suite of caspase-like proteases (Lasi *et al.*, 2010b). It is likely, therefore, that more than one is present in *A. pulchella*. This hypothesis is supported by our observations that DEVDase and LEHDase activities responded to differing extents to colchicine, heat stress, and the experimental removal of NO. The differing influence of NO on apparent caspase-9- and caspase-3-like activity is surprising, as the activities of mammalian caspase-9 are thought to be exerted solely through its activation of caspase-3 (Kumar & Cakouros, 2003).

The differential effects of the NO scavenger cPTIO on temperature-induced caspase-9- and 3-like activity and bleaching raise another interesting question. The fact that removing NO alleviated thermal bleaching (and caspase-9-like activity) without affecting caspase-3-like activity implies an executioner-like role for *A. pulchella* "caspase-9-like" enzyme. Lower invertebrate caspases are known to possess characteristics of both initiator and executioner caspases (Bottger & David, 2003; Wiens *et al.*, 2003; Dunn *et al.*, 2006; Lasi *et al.*, 2010b) so this is a distinct possibility. It is also important to note that, while levels of caspase-like enzyme activity responded much more strongly to exogenous NO than to high temperature, this was not reflected in the relative intensities of bleaching. At this early stage, we do not know to what extent this might reflect the additional actions of NO (such as the S-nitrosylation of critical enzymes) - caused by the relatively high dosage of 1 mM GSNO - that might have affected the mechanisms of bleaching downstream of caspase-like enzyme activation. In any case, further work in this area would be greatly beneficial to our understanding of cell death regulation in symbiotic cnidarians. It would also be desirable to repeat some of these experiments using aposymbiotic *A. pulchella*. Treating the host with an NO donor in the absence of symbionts would allow us to determine

whether activation of host apoptosis by NO occurs directly (as outlined above) or whether the additional effects of NO on symbiont physiology (see Chapter 2) might play a role.

The findings here - that NO appears to be involved temperature-induced caspase-9-like enzyme activation, and that both NO and caspase-9-like enzyme are required for symbiont loss - are consistent with a developing paradigm of coral bleaching. This model proposes that temperature-induced ROS and NO generation could lead to intrinsic apoptotic-like cell death (Dunn *et al.*, 2004; Perez & Weis, 2006; Weis 2008), stimulated by the release from mitochondria of pro-apoptotic molecules such as cytochrome *c* (Dunn *et al.*, 2012). Our investigation measured caspase-like enzyme activity rather than apoptosis itself, but NO and its toxic breakdown product peroxynitrite [$ONOO^-$, produced through NO-ROS interactions (Radi *et al.*, 2001)] both have a well-known capacity to stimulate apoptosis by inducing the release of cytochrome *c* from mitochondria (Radi *et al.*, 2002a) or inhibiting anti-apoptotic Bcl-2 proteins (Snyder *et al.*, 2009). The subsequent activation of caspase-9 (Li *et al.*, 1997) might then initiate an apoptotic cascade. While this investigation did not extend to assessments of mitochondrial integrity, temperature-induced mitochondrial dysfunction and cell death have been observed in *A. pulchella* (Dunn *et al.*, 2012) and the reef coral *Pocillopora damicornis* (Downs *et al.*, 2010).

Our finding that activation of a caspase-like enzyme is critical for bleaching contrasts with that of Dunn *et al*. (2007), who observed, in the presence of a general caspase inhibitor, an upregulation of autophagy-like cell death that restored the bleaching response. Inhibition both of autophagic pathways and of caspase activity was required to alleviate bleaching. One possible explanation for this disparity may lie with the severity of bleaching in this earlier study, which was much higher [*ca*. 80% of symbionts lost (Dunn *et al.*, 2007)] than in the present investigation. This implies an increased intensity of thermal stress and thus a greater scope for the activation of additional cell death mechanisms. Furthermore, NO is known to be a potent inhibitor of autophagy (Sarkar *et al.*, 2011; Shen *et al.*, 2013) and the alleviation of bleaching when NO was scavenged implies a reduced role for this pathway in the responses of the organisms examined here.

3.4.2. Slow heating induces host caspase-like enzyme activation and both host- and symbiont NO synthesis.

The effects of slow heating reported here are consistent with those observed under rapid heat stress, both in this and other studies (Bhagooli *et al.*, 2001; Buxton *et al.*, 2002; Dunn *et al.*, 2004; Trapido-Rosenthal *et al.*, 2005; Perez & Weis, 2006; Richier *et al.*, 2006; Hawkins & Davy, 2012). Experimental heating resulted in heightened NO synthesis in both host and symbiont and led to increased expression of host apoptotic-like pathways. Interestingly, initial increases in NO synthesis preceded bleaching and appeared to be driven by host rather than symbiont physiology. For example, little change in symbiont F_v/F_m or NO_2^- content was apparent on Days 4 and 6 of the treatment, yet NO-dependent fluorescence of anemone gastrodermis increased markedly. This finding supports Perez & Weis' (2006) assertion that NO in bleaching *Aiptasia* sp. is primarily host-derived.

After 10 days of treatment, however, and at a time at which anemones had visibly bleached, there was evidence of elevated symbiont NO generation. While it cannot be discounted that this reflects contamination with host-derived material, the fact that algal pellets were washed repeatedly prior to cell lysing does reduce this possibility. Levels of symbiont NO_2^- on Day 10 were also an order of magnitude greater than those of the host and are thus unlikely to be driven by the latter. Moreover, there was little evidence of NO-dependent fluorescence in host gastrodermal tissue after 10 days, while algal cells did appear to display heightened green (510-530 nm) fluorescence (Fig. 3.6). Despite supporting the findings of previous investigations (e.g. Bouchard & Yamasaki, 2008), therefore, the timing of symbiont NO production in this study suggests that it might only exaggerate thermal bleaching. This could occur either through the further stimulation of already-active pathways in the host (Dunn *et al.*, 2007, Weis 2008) or by inducing further symbiont stress and mortality (Yamasaki *et al.*, 2000; Bouchard & Yamasaki, 2009; Chapter 2).

These observations are also interesting in a wider context. Many organisms produce NO in response to infection by parasitic or pathogenic microbes (MacMicking *et al.*, 1997; Fang, 2004; Wink *et al.*, 2011; Singh *et al.*, 2013). At the cellular level, at least, the cnidarian-dinoflagellate mutualism displays a remarkable similarity to many parasitic

associations (Schwarz, 2008). This study, together with other recent investigations (Perez & Weis, 2006; Detournay & Weis, 2011; Detournay *et al.*, 2012), suggests that this similarity extends to the phenomenon of nitric oxide signalling. An antimicrobial defensive compound such as NO might be evolutionarily valuable to a symbiotic cnidarian host such as *A. pulchella*, where there may be physiological costs associated with excessive growth of a symbiotic microbial population. In this context, our observations of stressed intracellular symbionts apparently producing NO *in hospite* is intriguing, as it implies that symbiont NO synthesis may, alongside host innate-immune like responses (Weis, 2008; Vidal-Dupiol *et al.*, 2009), contribute to the breakdown of this symbiosis.

3.4.3. Conclusions

NO seems to be necessary for the temperature-induced breakdown of the cnidarian-dinoflagellate symbiosis and appears to be involved in the activation of a host enzyme associated with apoptosis. However, the timing of NO production under slow heating suggests that a sizeable gap exists between initial NO synthesis and subsequent bleaching. Furthermore, the induction of NO and apoptotic pathways prior to algal photoinhibition implies that these may represent host responses to something other than ROS leakage from compromised symbionts. Perhaps they represent responses to metabolic dysfunction or mitochondrial disruption in the host itself (Dunn *et al.*, 2012)? The contribution of symbiont-derived NO is even less clear. While elevated NO production alongside photoinhibition and chlorophyll degradation is consistent with a general stress response for the B1-type symbionts present in *A. pulchella* (see Chapter 2), the fact that bleaching had already commenced suggests that the extent to which it can be influenced by symbiont-derived NO may be less significant than previously thought. Further examination of NO production in cnidarians and their symbionts, as well as the numerous pathways that NO can influence, would shed much-needed light on the communication dysfunction that leads to the collapse of this important symbiosis.

Chapter 4

Nitric oxide and coral bleaching: Is peroxynitrite generation required for symbiosis collapse?

4.1. Introduction

Microbial symbioses are ubiquitous in the natural world and sustain some of the most diverse ecosystems on Earth (Muller-Parker & D'Elia, 1997). One of the most ecologically important associations is between reef corals (Cnidaria; Scleractinia) and photosynthetic dinoflagellates. The dinoflagellate symbionts (genus *Symbiodinium*) provide fixed carbon to the host in exchange for inorganic nutrients that are typically absent from the surrounding seawater (Davy *et al.*, 2012). This association underpins the existence of coral reefs but is being placed under increasing strain by global warming (Hoegh-Guldberg & Bruno, 2010). It has long been known that excessive heating of corals can result in the loss of their symbiotic dinoflagellates, yet we still know little about the physiological events underpinning this symbiosis collapse (Weis, 2008; Lesser, 2011).

The collapse of the cnidarian-dinoflagellate association has been linked to the overproduction of ROS (Lesser, 1996; 1997; Lesser, 2006; Richier *et al.*, 2006). Often associated with chronic photosynthetic dysfunction in the symbiont (Tchernov *et al.*, 2004; Suggett *et al.*, 2008; McGinty *et al.*, 2012), ROS have a well-known capacity for cellular damage (Halliwell & Gutteridge, 2007), but at low concentrations may also act as important cellular signalling compounds (Martindale & Holbrook, 2002; Winterbourn, 2008; Wink *et al.*, 2011). It has been hypothesised that ROS leakage from dysfunctional symbionts could stimulate an innate immune-like signalling pathway resulting in host NO synthesis (Perez & Weis, 2006; Weis 2008), but very few data exist regarding ROS and cnidarian innate immunity. NO is a ubiquitous signalling compound (Moroz, 2001)) implicated in the regulation of numerous microbial endosymbioses (Wang & Ruby, 2011). At sufficiently high concentrations, however, NO can react with ROS (specifically superoxide) to generate peroxynitrite ($ONOO^-$),

which is much more toxic and has a capacity to irreversibly inhibit mitochondrial respiration as well as cause damage to enzymes, DNA, and lipid membranes (Beckman & Koppenol, 1996; Estevez & Jordan, 2002). In fact, the reaction between NO and O_2^- occurs faster than that of O_2^- with superoxide dismutase, thus the formation of $ONOO^-$ highly probable when NO and O_2^- are produced simultaneously (Radi *et al.*, 2001). The likelihood of its generation under thermal stress has therefore led to $ONOO^-$ being proposed as the effector of NO-mediated cnidarian bleaching (Perez & Weis, 2006; Weis 2008).

Using the model symbiotic cnidarian *Aiptasia pulchella*, this investigation sought to test the hypothesis that cnidarian bleaching is dependent on NO's conversion to $ONOO^-$ during thermal stress. Filling this gap in our knowledge is important if we are to better understand the cellular basis of coral bleaching and the breakdown of intracellular mutualisms in general.

4.2. Materials and Methods

Cultures of symbiotic *A. pulchella* were maintained as described in Chapter 3.

4.2.1. Experimental induction of $ONOO^-$ in Aiptasia pulchella *and its fluorometric assessment.*

Peroxynitrite was detected using the fluorescent probe aminophenyl fluorescein (APF; Molecular Probes, Eugene, OR, USA). APF is specific for highly reactive oxygen and nitrogen species and detects, alongside peroxynitrite, hydroxyl ($OH^\cdot$) and hypochlorite (OCl^-) radicals (Setsukinai *et al.*, 2003). By using scavengers of peroxynitrite and nitric oxide, however, one can determine the extent to which APF fluorescence is $ONOO^-$-dependent.

The suitability of APF was determined by preparing 100 µL solutions of 10 µM APF in 'anemone relaxing solution' (see Chapter 3) with and without 2 mM urate (Sigma-Aldrich, Auckland, New Zealand), a peroxynitrite scavenger that has been employed successfully in fluorescence-based assays (Saito *et al.*, 2006; Tewari *et al.*, 2013). The peroxynitrite donor 3-morpholinosydnonimine (SIN-1; Life Technologies, Auckland,

New Zealand), which generates $ONOO^-$ through the simultaneous release of O_2^- and NO, was then added (0 - 1 mM final SIN-1 concentration). APF fluorescence (ex: 490 nm, em: 515 nm) was monitored over 2 h using a fluorescent microplate reader (Enspire® 2300, Perkin-Elmer, Waltham, MA, USA). To determine whether the effects of urate on APF signal were truly due to scavenging of $ONOO^-$ (rather than quenching of APF fluorescence), the SIN-1/APF incubation was repeated with 2 mM urate added 30 min after SIN-1.

The ability of APF to detect peroxynitrite in the tissues of *A. pulchella* was assessed by incubating individual anemones for 60 min with 10 µM APF in a) relaxing solution only, b) 1 mM SIN-1, c) 1 mM SIN-1 with 2 mM urate, and d) 1 mM SIN-1 with 1 mM of the specific NO scavenger cPTIO (Life Technologies, Auckland, New Zealand).

Endogenous production of $ONOO^-$ by *A. pulchella* was investigated using the high temperature shock (HTS) method. Briefly, anemones (2-3 mm oral disc diameter) in FSW (n = 6 per treatment in individual glass beakers) were transferred to a 26°C water bath under 12-h light:12-h dark cycle, (light: 70-90 µmol photons m^{-2} s^{-1} provided by a LED light bank 20 RoHS 5 W 6400 K) and allowed to acclimate for 48 h (fluorometric assessments of symbiont photosynthesis - see below - were conducted to ensure stability prior to treatment). Temperature was then increased (over < 1 h) to 33°C or kept constant at 26°C (control) and anemones were exposed to these conditions for 24 h. Some additional anemones (n = 6 per treatment) were HTS-treated in the presence of 2 mM urate or 1 mM cPTIO.

To quantify $ONOO^-$ in live *A. pulchella*, the FSW of experimental anemones was replaced with 10 µM APF in relaxing solution and anemones were incubated in the dark for 60 min in order to prevent the photobleaching of the APF fluorophore. Individual anemones were prepared as described in Chapter 3 and visualised using an Olympus Fluoview FV-1000 inverted confocal LSM (Olympus, Center Valley, PA, USA) and ×40 0.9 NA water immersion lens. A 473 nm laser was used to excite APF, the fluorescence of which was measured at 510-530 nm. Symbiont chlorophyll autofluorescence was detected using 635 nm excitation and a 655-755 nm emission filter. Image acquisition and analysis followed the methods described in Chapter 3 and

any non-fluorescing anemones were incubated for 30 min with 1 mM SIN-1 to confirm successful loading of APF.

4.2.2. Examining the role of $ONOO^-$ in thermal bleaching of Aiptasia pulchella*.*

The role of $ONOO^-$ in temperature-induced bleaching was investigated by incubating *A. pulchella* (n = 5 in individual glass beakers) for 24 h at 26°C in FSW containing 1 mM SIN-1 with and without 2 mM urate. In addition, six replicate anemones were HTS-treated with and without 2 mM urate. After 24 h exposure to high temperature or SIN-1/urate, anemones were returned to control conditions for a further 24 h [to allow time for bleaching; Detournay & Weis (2011)] and then processed for symbiont density assays as described below.

4.2.3. Assessments of symbiont photosystem II fluorescence and host bleaching.

Fluorescence yields of photosystem II (PSII) were monitored regularly (midday and 30 min after lights-off) using pulse amplitude modulation fluorometry (Diving-PAM, Walz, Germany). Symbiont densities were determined as follows. Whole anemones were homogenised using a tissue grinder (Raylab NZ Ltd., Glendene, Auckland, New Zealand) in a 1.5-mL tube with a small volume of buffer (50 mM potassium phosphate, pH 7.8, 1 mM EDTA). An aliquot was removed for haemocytometer counts (at least 6 per sample, until coefficients of variation were ≤ 15%; Improved Neubauer, Boeco, Germany) and the remainder was centrifuged (16000 × g for 20 min) and analysed for soluble protein content (Bradford assay; Bradford, 1976) with bovine serum albumin as a standard. Changes in symbiont density relative to host soluble protein ("% symbiont loss") after 48 h were calculated relative to mean pre-treatment (t = 0) values.

4.2.4. Statistical analysis.

Data analysis was carried out using PASW Statistics 18.0 (IBM, Armonk, NY, USA). Where appropriate, data were analysed using RMANOVA and transformed where necessary. RMANOVA reports represent time × treatment interactions with pair-wise *post-hoc* comparisons (Bonferroni correction for multiple comparisons). Other analyses were carried out using one-way ANOVA.

4.3. Results

4.3.1. Detection of $ONOO^-$ in aqueous buffer using APF.

Aminophenyl fluorescein fluorescence (515 nm) successfully responded to SIN-1-derived peroxynitrite in a dose-dependent manner (Fig. 4.1A). Addition of the $ONOO^-$ scavenger urate (2 mM) prevented this increase, and adding urate after 30 min confirmed that this was due to $ONOO^-$ scavenging rather than quenching of fluorescence (Fig. 4.1B). Adding heat-treated (33°C for 24 h) urate had a similar inhibitory effect on APF fluorescence as freshly prepared urate (Fig. 4.1C), confirming that urate retained its $ONOO^-$-scavenging properties even under conditions used to induce bleaching in *A. pulchella*.

4.3.2. Confocal visualisation of $ONOO^-$ in live Aiptasia pulchella.

Incubation of APF-loaded *A. pulchella* with the peroxynitrite donor SIN-1 resulted in significant increases in tissue 510-530 nm fluorescence (one-way ANOVA, $F_{3, 20} = 59.753$, $p < 0.001$) that were absent in the presence of 2 mM urate or 1 mM cPTIO (Fig. 4.2A). HTS treatment of *A. pulchella* also induced increases (one-way ANOVA, $F_{3, 19} = 14.679$, $p < 0.001$), which were absent in the presence of urate or cPTIO (Fig. 4.2B). The decline in fluorescence intensity when anemones were treated with scavengers either of $ONOO^-$ itself (urate) or its precursor NO (cPTIO) confirmed that APF signal in *A. pulchella* was an accurate reflection of $ONOO^-$ generation rather than the dye's interactions with other highly reactive compounds.

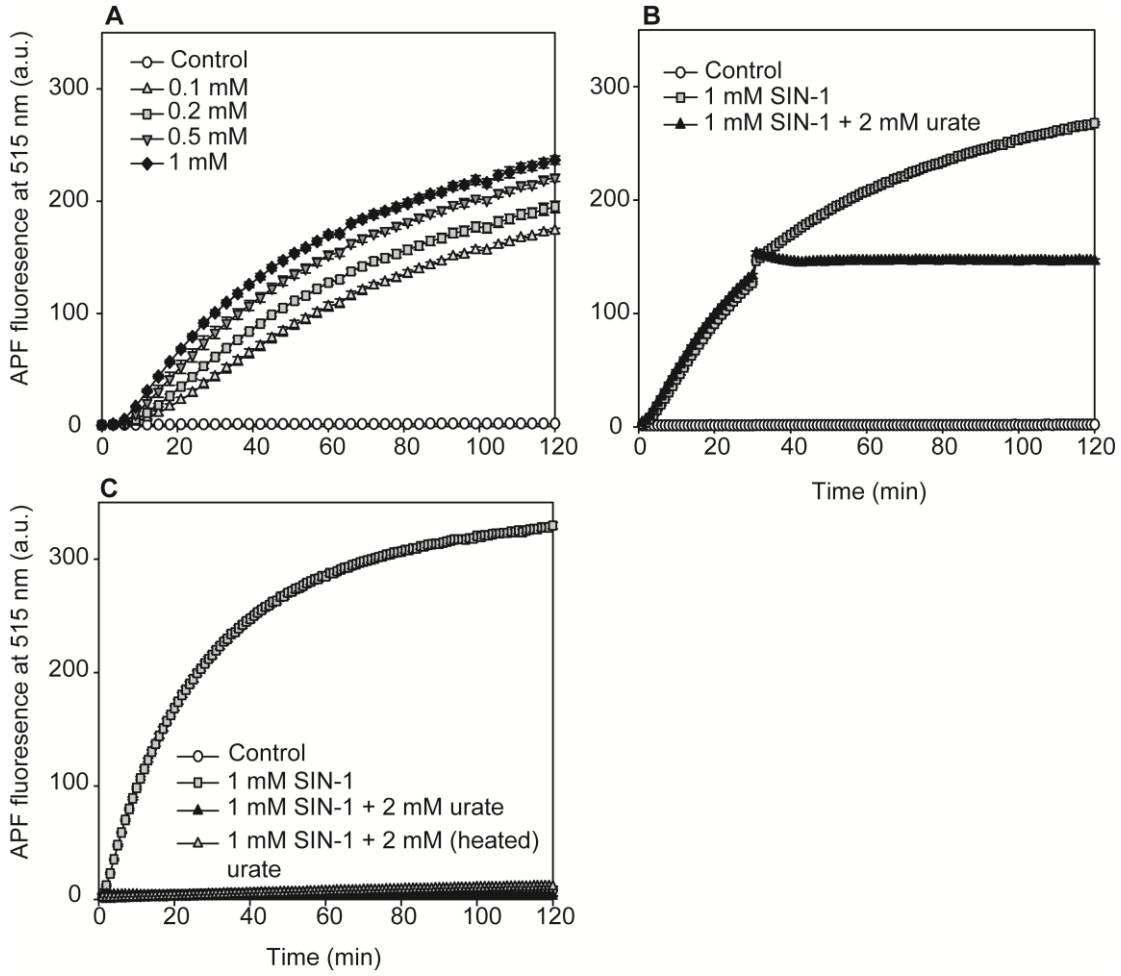

Figure 4.1. The suitability of aminophenyl fluorescein (APF) for detection of peroxynitrite ($ONOO^-$). **Panel A**) Fluorescence kinetics after addition of various concentrations of the $ONOO^-$ donor SIN-1 to APF (10 µM in relaxing solution - see text). **B**) Fluorescence kinetics of APF after addition of 1 mM SIN-1 in the presence or absence of 2 mM urate (a peroxynitrite scavenger), which was added 30 min after SIN-1. **C**) Fluorescence kinetics of APF after addition of 1 mM SIN-1 with either fresh or heat-treated urate (2 mM). Values are means ± s.e.m. (n = 4 independent experiments).

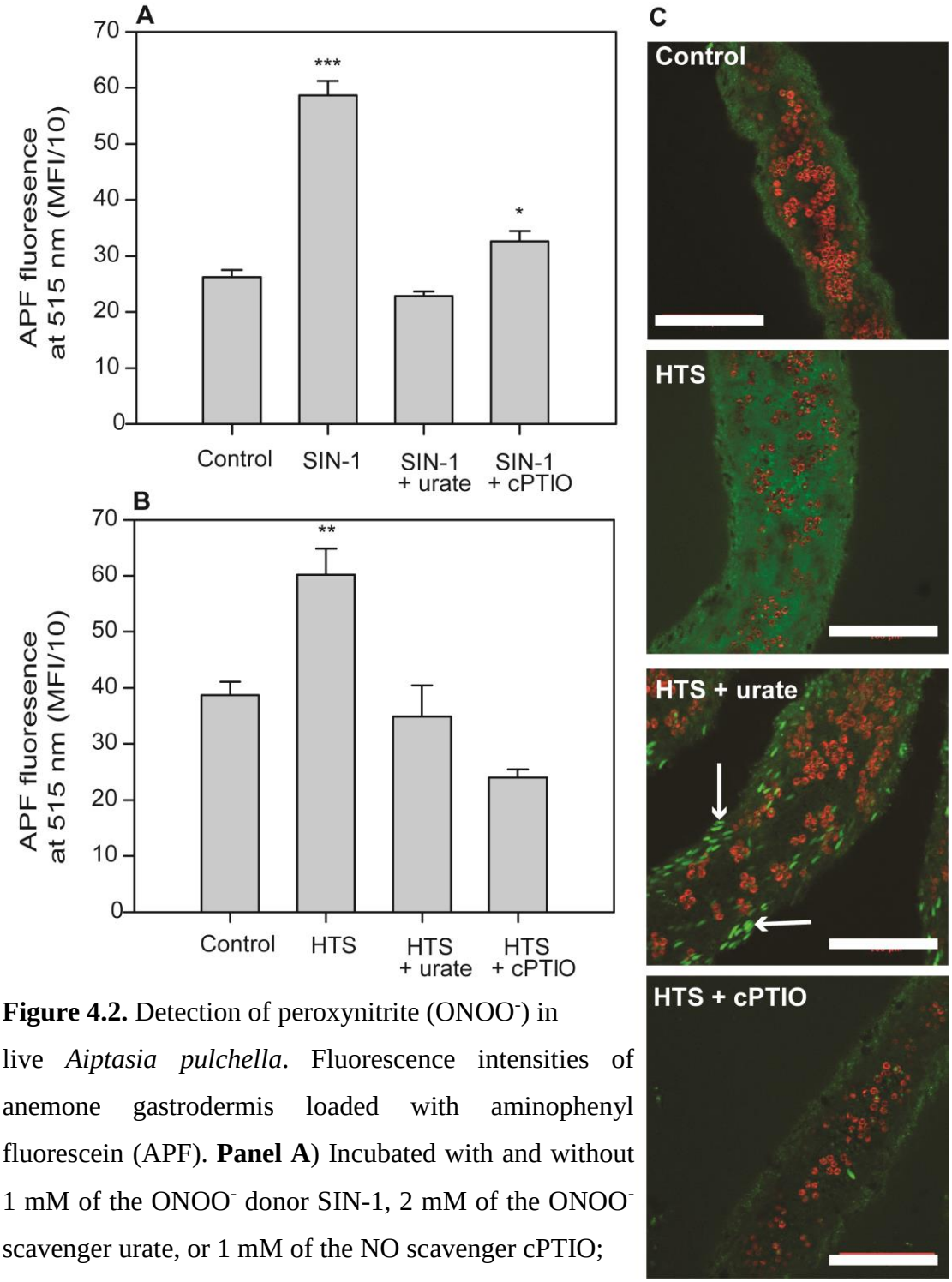

Figure 4.2. Detection of peroxynitrite ($ONOO^-$) in live *Aiptasia pulchella*. Fluorescence intensities of anemone gastrodermis loaded with aminophenyl fluorescein (APF). **Panel A**) Incubated with and without 1 mM of the $ONOO^-$ donor SIN-1, 2 mM of the $ONOO^-$ scavenger urate, or 1 mM of the NO scavenger cPTIO; **B**) Exposed to high temperature shock (HTS) with and without 2 mM urate or 1 mM cPTIO. Values are means ± s.e.m. and asterisks denote significant differences relative to the control group (one-way ANOVA with Tukey HSD *post-hoc*, n = 6 anemones, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **C**) Confocal LSM micrographs of anemone tentacles exposed to control conditions, HTS, HTS + 2 mM urate, and HTS + 1 mM cPTIO. Green indicates APF fluorescence, while symbiont chlorophyll autofluorescence is labelled red. Scale bar: 100 µm. Arrows indicate fluorescence of nematocytes.

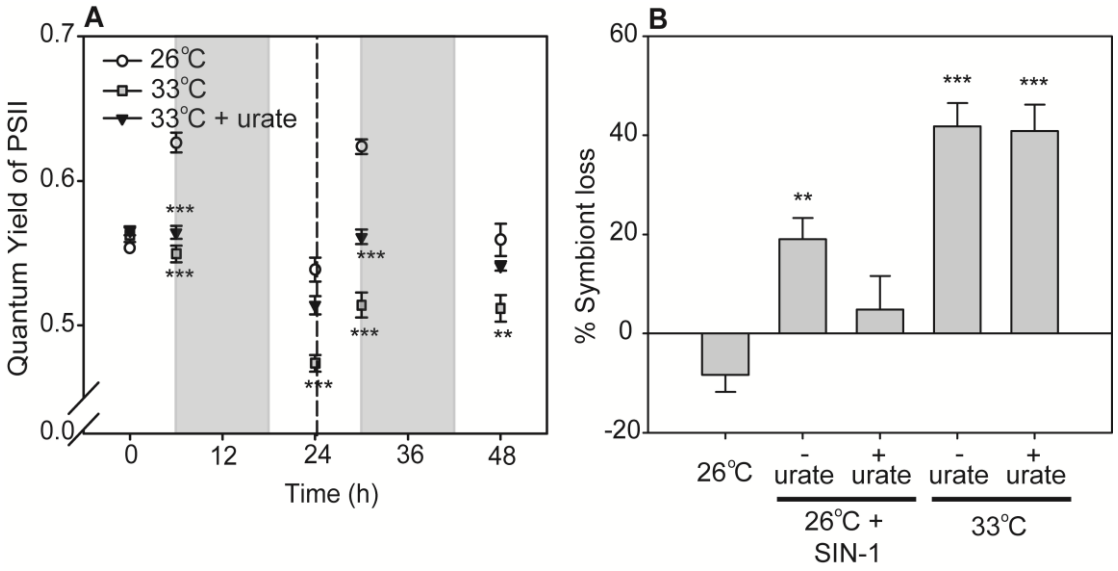

Figure 4.3. Effects of peroxynitrite ($ONOO^-$) on thermal photoinhibition and bleaching in *Aiptasia pulchella*. **Panel A**) Quantum yields of PSII over 48 h. Shaded areas represent periods of darkness and the broken line indicates the time at which anemones were returned to control (26°C) conditions from 33°C. **B**) Bleaching of anemones (% symbionts lost over 48 h) exposed to high temperature shock (HTS - 33°C) or 1 mM of the $ONOO^-$ donor SIN-1 at 26°C, both treatments with and without 2 mM urate. Treatments lasted 24 h before anemones were returned to control conditions. Values in both panels are means ± s.e.m. and asterisks denote significant differences relative to the control group (**A**: RMANOVA, pair-wise *post-hoc* with Bonferroni correction, n = 6 anemones per time point **B**: one-way ANOVA with Tukey HSD *post-hoc*, n = 6 for control and 33°C-treatments, n = 5 for SIN-1 incubations, ** $p < 0.01$, *** $p < 0.001$).

4.3.3. Effects of peroxynitrite on photoinhibition and bleaching.

The addition of the peroxynitrite scavenger urate (2 mM) significantly alleviated temperature-induced declines in PSII quantum yield (RMANOVA, $F_{8, 60} = 12.491$, $p < 0.001$, Fig. 4.3A) and, after 24 h at 33°C, quantum yield was significantly higher in the presence of urate ($p = 0.003$). Addition of the peroxynitrite donor SIN-1 (1 mM in FSW) induced significant bleaching of *A. pulchella* at control temperatures (one-way ANOVA, $F_{4, 27} = 20.94$, $p < 0.001$; Tukey HSD *post-hoc vs.* "control", $p = 0.006$; Fig. 4.3B) and the $ONOO^-$ scavenger urate restored levels of symbiont loss to those of the controls (Tukey HSD *post-hoc vs.* "control", $p = 0.365$). Symbiont losses were also

significantly higher after HTS treatment but urate had no effect on bleaching intensity (Tukey HSD *post-hoc vs.* "HTS", $p > 0.999$; Fig. 4.3B).

4.4. Discussion

The generation of peroxynitrite ($ONOO^-$) has been proposed as a significant step in the cellular cascade underpinning coral bleaching (Perez & Weis, 2006; Weis, 2008) and this study provides strong evidence that $ONOO^-$ generation occurs in thermally stressed cnidarians. To the authors' knowledge, it also represents the first observation of $ONOO^-$ in either a lower invertebrate (e.g. Porifera, Cnidaria, or Ctenophora) or an intracellular mutualism.

Peroxynitrite has the potential to cause cellular damage in numerous ways (Ischiropoulos & Almehdi, 1995; Szabo & Ohshima, 1997; Estevez & Jordan, 2002; Kim *et al.*, 2005; Pacher *et al.*, 2007; Szabo *et al.*, 2007; Ahmad *et al.*, 2009) but, in the light of recent investigations (e.g. Dunn *et al.*, 2012), it is the compound's interactions with mitochondria that may be most important. Due to differences in the diffusivity and reactivity of NO and O_2^-, peroxynitrite generation in animal cells occurs primarily at the sites of O_2^- production (Radi *et al.*, 2001), the most significant of which is the respiratory chain on the inner mitochondrial membrane (Halliwell & Gutteridge, 2007). $ONOO^-$ can irreversibly inhibit most mitochondrial complexes (Radi *et al.*, 2002a; Radi *et al.*, 2002b) and current research links mitochondrial dysfunction and the associated apoptotic pathways to bleaching in symbiotic cnidarians (DeSalvo *et al.*, 2010; Dunn *et al.*, 2012). Whether this can occur at physiologically relevant $ONOO^-$ concentrations has been the subject of debate (Fukuto & Ignarro, 1997), however, and we still know little about *in vivo* $ONOO^-$ generation outside of mammals.

It would appear from the data obtained using SIN-1 that addition of $ONOO^-$ to *A. pulchella* can stimulate bleaching at control temperatures. This isn't entirely surprising, as a bolus dose of an NO and O_2^- donor would be expected to induce significant physiological stress (Lesser, 2006; Perez & Weis, 2006; Weis, 2008). The absence of any alleviation of bleaching intensity when heat-treated anemones were incubated with the $ONOO^-$ scavenger urate, however, implies that $ONOO^-$ may not play such a significant role thermal bleaching. Taken together, these findings suggest that while

$ONOO^-$ has a bleaching-inducing capacity, either the levels of $ONOO^-$ generated *in vivo* are not sufficient to influence symbiont loss, or alternative pathways (perhaps involving NO or ROS directly) are more critical.

The removal of $ONOO^-$ did have a significant positive effect on the performance of symbiont PSII at elevated temperature. Given what we already know about NO synthesis by symbiotic dinoflagellates (Trapido-Rosenthal *et al.*, 2005; Bouchard & Yamasaki, 2008; Bouchard & Yamasaki, 2009; Chapter 2) their production of $ONOO^-$ and its apparent effects on photosystem II could represent an attractive avenue of inquiry into the thermal stress responses in these algae. Recent observations of naturally-occuring uric acid deposits in *Symbiodinium* cells (Kopp *et al.*, 2013) may have implications for such $ONOO^-$-mediated events, particularly regarding the localisation of $ONOO^-$ generation and scavenging. It should be noted, however, that urate is also a potent scavenger of superoxide radicals, and with ROS strongly implicated in the photosynthetic dysfunction of *Symbiodinium* cells, it remains to be seen how much of the urate-induced recovery in PSII yield is due to $ONOO^-$-scavenging rather than general antioxidant activity. Moreover, it would be greatly informative to conduct an experiment quantifying the bleaching of anemones exposed to SIN-1 alongside the NO scavenger cPTIO. This would enable us to discern the relative effects of NO and ROS more reliably.

In conclusion, it appears likely that any mediation of temperature-induced cnidarian bleaching by nitric oxide occurs independently of its conversion to peroxynitrite. NO has the capacity to directly influence the cell death pathways implicated in bleaching (Snyder *et al.*, 2009) so $ONOO^-$ generation may be unnecessary in this regard. The situation may of course be different in reef corals undergoing bleaching in the field, where light intensities greater than those employed in the present study could exaggerate NO / ROS synthesis in both host and symbiont. In any case, investigating where in the symbiosis $ONOO^-$ is produced, and how such a potent radical can have such modest effects during temperature stress are important subjects for future study.

Chapter 5

Differential coral bleaching at a cellular level: Evidence of nitric oxide synthesis and host apoptosis in three reef corals.

5.1. Introduction

Coral reefs as we know them may not survive the 21st century (Hoegh-Guldberg & Bruno, 2010; van Hooidonk *et al.,* 2013) as a number of factors, both local and global, are placing reefs under increasing stress (Perry *et al.*, 2013). Perhaps the most significant of these is global warming (van Hooidonk *et al.*, 2013). Reef-building corals live close to their upper thermal thresholds, and warming of only a few degrees can have significant consequences (Fitt *et al.*, 2001). This often becomes apparent as a "whitening" of the coral tissue resulting from the expulsion or degradation of the coral's intracellular symbionts and/or their photosynthetic pigments. It is these symbionts (dinoflagellates of the genus *Symbiodinium*) that, under normal circumstances, provide the host with photosynthetically-fixed carbon, and thus enable calcification and reef accretion by scleractinian corals (Davy *et al.*, 2012). Thermal bleaching can cause coral mortality directly, through the stresses associated with high temperature (Jones, 2008), or indirectly, as a result of depressed growth rates (Goreau & MacFarlane, 1990) or the ability to resist pathogens (Mydlarz *et al.*, 2010). While much is known of the ecological patterns and consequences of bleaching (e.g. Pratchett *et al.*, 2009), comparatively little is understood regarding its physiological and cellular basis (Weis, 2008; Lesser, 2011). This is especially true for differential bleaching, wherein corals of different species (or harbouring distinct types of *Symbiodinium*) display variation in their susceptibility to elevated temperatures (van Oppen *et al.*, 2009; Weis, 2010). It is vital, given the predictions for coral reefs over coming decades (van Hooidonk *et al.*, 2013), that we improve our understanding of corals' responses to warming oceans.

A number of mechanisms have been proposed as being vital to the bleaching process (Weis, 2008), but a critical factor is likely to be the development of oxidative stress (Lesser, 2006; Richier *et al.*, 2006; Lesser, 2011), a phenomenon caused by the overproduction of ROS. Often associated with thermal photoinhibition in the symbionts

(Tchernov *et al.*, 2004; Suggett *et al.*, 2008), ROS generation might lead to symbiont dysfunction and mortality (Dunn *et al.*, 2004; Sammarco & Strychar, 2013). It may also induce host innate immune-like responses (Perez & Weis, 2006; Dunn *et al.*, 2007; Weis, 2008) similar to those seen in organisms challenged by microbial pathogens (Nürnberger *et al.*, 2004; Rivero, 2006; Villamil *et al.*, 2007). However, few data exist regarding host signalling during temperature-induced ROS generation.

One host response involves the heightened synthesis of the signalling molecule nitric oxide (NO; Trapido-Rosenthal *et al.*, 2005; Perez & Weis, 2006; Detournay & Weis, 2011) and an upregulation of apoptotic-like programmed cell death pathways (Dunn *et al.*, 2004; Richier *et al.*, 2006; Dunn *et al.*, 2007; Paxton *et al.*, 2013). Ubiquitous in living organisms, NO is involved in a number of processes relevant to coral bleaching. These include cell death (Wink & Mitchell, 1998) - particularly apoptosis (Brune *et al.*, 1999; Almeida *et al.*, 2007) - innate immune responses to pathogenic infections (Liew *et al.*, 1991; MacMicking *et al.*, 1997; Fang, 2004; Nürnberger *et al.*, 2004; Leng *et al.*, 2009; Herrera-Ortiz *et al.*, 2011), ROS-based signalling and oxidative stress (Crawford & Guo, 2005; Catala *et al.*, 2010; Wink *et al.*, 2011), and the maintenance of microbial symbioses (reviewed by Wang & Ruby, 2011). The possible roles for NO in the breakdown of the coral-*Symbiodinium* association are various, but, given recent work on model symbiotic cnidarians (Perez & Weis, 2006; Detournay & Weis, 2011; Detournay *et al.*, 2012) its involvement in apoptosis is plausible. NO has a well-known capacity to induce apoptosis through the intrinsic (mitochondria-mediated) pathway (Brown & Borutaite, 2002; Pacher *et al.*, 2007), and recent investigations have observed evidence of mitochondrial dysfunction (Desalvo *et al.*, 2008; Downs *et al.*, 2010; Dunn *et al.*, 2012) as well as the modified expression of apoptosis-regulating genes (Bellantuono *et al.*, 2012a; Barshis *et al.*, 2013) in thermally-stressed cnidarians. The activity of enzymes similar to the aspartate-specific cysteine proteases (caspases), that are tasked with initiating and executing apoptosis has also been observed in corals (Dunn & Weis, 2009; Kvitt *et al.*, 2011; Pernice *et al.*, 2011; Tchernov *et al.*, 2011). Moreover, variation in their expression has been proposed as a possible cellular basis for the differential survival of bleached corals at elevated temperatures (Tchernov *et al.*, 2011). In the model symbiotic cnidarian *Aiptasia* sp., apoptotic activity has been hypothesised to result from temperature-induced NO synthesis (Perez & Weis, 2006; Chapter 3), and differential NO production has been observed in heat-stressed

anemones (*Aiptasia pallida*) hosting heat-tolerant and -sensitive *Symbiodinium* types (Perez, 2007). However, the hypothesis that the differential induction of NO synthesis/apoptosis could contribute to the varying bleaching susceptibilities of corals has yet to be examined in any reef-building species.

Physiological characteristics of both symbiont and host contribute to the overall stability of the holobiont during stress (Baird *et al.*, 2008; Weis, 2010; Wicks *et al.*, 2012), but variability in coral bleaching has, more often than not, been attributed to differences in the *in hospite* symbiont population (Rowan *et al.*, 1997; Berkelmans & van Oppen, 2006; van Oppen *et al.*, 2009; Weis, 2010). The genus *Symbiodinium* is highly diverse (Stat *et al.*, 2006) and nine clades (A-I; identified using the ITS2 region of the nuclear ribosomal DNA) are currently recognised (LaJeunesse, 2002; Pochon & Gates, 2010). Moreover, it is clear that the physiology of different *Symbiodinium* ITS2 "types" can be highly variable (Tchernov *et al.*, 2004; Suggett *et al.*, 2008; Fisher *et al.*, 2012; McGinty *et al.*, 2012). This extends to the symbiont's own synthesis of NO (see Chapter 2), a phenomenon that has been the subject of some debate (Trapido-Rosenthal *et al.*, 2005; Perez & Weis, 2006; Bouchard & Yamasaki, 2008). However, little is known of the potential for NO synthesis by symbionts *in hospite*. Some early investigations (Bhagooli *et al.*, 2001; Buxton *et al.*, 2002) suggested that *Symbiodinium* cells could generate NO within their host. If this differs among coral species then it might contribute to their varying responses to thermal stress.

As outlined above, NO synthesis and apoptosis are common innate immune strategies in metazoans and NO appears to be involved in cnidarian apoptosis during heat stress. The aim of this investigation, therefore, was to quantify the expression of these innate immune-like responses in different reef corals exposed to high temperature stress. As such, there were two specific objectives to this study: firstly, to quantify host and *in hospite* symbiont NO synthesis, and secondly, to assess the expression of host apoptotic pathways. The species examined were *Acropora millepora*, *Montipora digitata* and *Pocillopora damicornis*. *M. digitata*, with its *Symbiodinium* C15-type symbiont (LaJeunesse, 2005), represents a physiologically robust Indo-Pacific coral species (Fisher *et al.*, 2012), while *Acropora* and *Pocillopora* are comparatively temperature-sensitive genera (Guest *et al.*, 2012). In addition *A. millepora* is a horizontally transmitting species and is comparatively flexible with respect to the types of symbionts

with which it associates (Tonk *et al.*, 2013). *M. digitata*, by contrast, is a vertically transmitting species which associates with only a few symbiont types (Baird *et al.*, 2009; Fabina *et al.*, 2012). *P. damicornis* uses vertical transmission, but is still somewhat flexible in its choice of symbionts (Tonk *et al.*, 2013) and indeed some evidence of horizontal uptake has been recorded for this species (Marlow & Martindale, 2007). By extending laboratory work carried out in this as well as previous investigations (Perez & Weis, 2006; Richier *et al.*, 2006) to reef corals in the field, we can gain physiological data of ecological significance, and further investigate the cellular basis for the varying responses of corals to climate change.

5.2. Materials and Methods

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich, Auckland, New Zealand, and were of the highest analytical grade available.

5.2.1. Collection and treatment of reef coral fragments.

Individual fragments (*ca.* 5 cm; n = 5 colonies per species) of *A. millepora* (cream colour morph), *P. damicornis* (pink colour morph), and *M. digitata* (green colour morph) were collected from Heron Island reef flat (23°26'43" S, 151°54'53" E) at low tide, mounted onto racks and transferred to experimental 30-L tanks under shade cloth (reducing incident midday photosynthetically active radiation (PAR) from *ca.* 2000 to 400-600 µmol photons m^{-2} s^{-1}). Treatment tanks contained four fragments (one per time point) from each of the five replicate colonies. A continuous flow system (*ca.* 1.5 L min^{-1}) ensured a constant turnover of seawater (from Heron Island reef flat), and fragments were acclimated for 6 days at 28.5°C (± 0.9°C; one standard deviation), conditions characteristic of summer daytime conditions on the Heron Island reef flat (AIMS; http://data.aims.gov.au/aimsrtds/station.xhtml?station=130). The temperature of two of the tanks was then increased by approximately 1°C per day and stabilised at either 31.5°C (± 0.6°C) or 33°C (± 0.8°C). Water temperatures were monitored using HOBO data loggers (Onset Corporation, Bourne, MA, USA), and treatment conditions were maintained for a maximum of 6 days for 31.5°C and 4 days for 33°C (Fig. 5.1).

Corals were sampled at midday immediately prior to heating, 5 days afterwards, and then every 48 h for a maximum of 4 days.

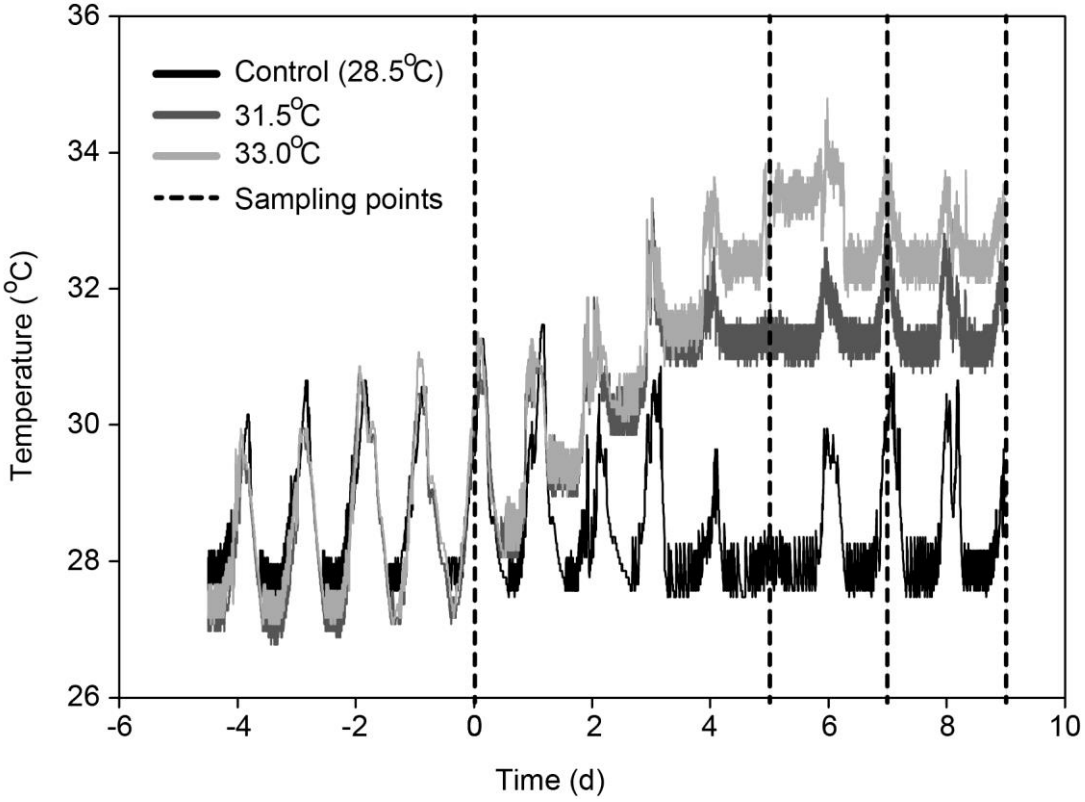

Figure 5.1. Acclimation and temperature treatments of coral fragments (ramping of *ca.* 1°C day^{-1}). Vertical green lines indicate sampling points.

5.2.2. Fluorometric assessment of in hospite *algal PSII.*

Quantum yields of algal PSII were measured using pulse amplitude modulation fluorometry (Diving-PAM, Walz, Effeltrich, Germany). Maximum (dark-adapted) quantum yields of PSII (F_v/F_m) were recorded 30 min after sunset, and presented values are means of three measurements of each coral fragment.

5.2.3. Coral fragment processing.

Coral fragments were removed from experimental tanks at midday, immediately flash-frozen in liquid nitrogen and stored at -80°C. All subsequent procedures were carried out at 4°C or on ice unless otherwise stated.

Tissue was removed from frozen coral fragments by airbrushing into approximately 7 mL of 50 mM potassium phosphate, pH 7.8, 1 mM EDTA homogenisation buffer (HB; prepared daily using 18.3 MΩ water). Tissue suspensions were homogenised with a sawtooth homogeniser (Labserv D-130, Thermo Fisher Scientific, Albany, New Zealand) for 10 s at 20000 rpm. Two 500-µl aliquots were removed for *Symbiodinium* cell counts (and to confirm the structural integrity of cells *post*-homogenisation) and photosynthetic pigment analysis (see below). Samples for cell counting were fixed with 10 µL Lugols solution and stored at -20°C. Pigment samples were centrifuged (16000 × g for 5 min), the supernatant discarded, and the pellet frozen and stored at -20°C. The remaining tissue homogenate was centrifuged (1500 × g for 5 min) to separate remaining *Symbiodinium* cells from host material. The supernatant was removed and stored in 1 mL aliquots at -80°C alongside the algal pellet.

5.2.4. Quantification of bleaching in reef coral fragments.

Symbiodinium cell densities were quantified using Improved Neubauer haemocytometer counts (Boeco, Germany), with at least six counts carried out per sample (until coefficients of variability were < 15%). Cell density was normalised to coral fragment surface area, measured with the parafin wax method (Stimson & Kinzie, 1991). Symbiont chlorophyll *a* (Chl *a*) content was quantified according to methods described in Chapter 3.

5.2.5. Symbiodinium *genotyping.*

Symbiodinium aliquots for genotyping were washed three times in HB (2000 × g for 5 min) and pellets were processed following the methods of Logan *et al.* (2010). PCR amplicons were obtained using the forward primer itsD (5'-GTGAATTGCAGAACTCCGTG-3') and reverse primer its2rev2 (5'-

CCTCCGCCTACTTATATGCTT-3'). All PCR amplicons were checked for correct size *via* gel electrophoresis (1.5% [w/v] agarose gel), purified (ExoSAP-IT; GE Healthcare, Life Sciences) and sequenced in both directions at Macrogen Korea (Seoul, South Korea). Sequences were identified using the NCBI and GeoSymbio databases (Franklin *et al.*, 2012).

5.2.6. Preparation of Symbiodinium *cell lysates.*

Algal pellets were processed and lysed according to methods described in Chapter 3.

5.2.7. Assessments of NO production.

With corals fragments having been flash-frozen immediately *post*-sampling, the live, real-time quantification of NO (with confocal LSM and fluorescent dyes, for example) was not possible. However, biogenic NO is quickly oxidised to nitrite (NO_2^-) and nitrate (NO_3^-). These stable products are reliable indicators of NO synthesis (Bryan & Grisham, 2007), and their relative contributions to total oxidised NO (NO_x^-; the total of NO_2^- and NO_3^-) depend on the abundance and proximity of *haem*-proteins in the tissues of interest (Bryan & Grisham, 2007). As such, overall assessments of NO synthesis benefit from measurements both of NO_2^- and total oxidised NO (NO_x^-).

NO_2^- and NO_x^- were quantified using the fluorescent indicator 2,3-diaminonaphthalene (DAN, Life Technologies, Auckland, New Zealand; Nussler *et al.*, 2006) according to the methods of Detournay & Weis (2011), described in Chapter 3. Samples were analysed as outlined below.

Nitrite (NO_2^-) assay:

NO_2^- content was calculated from DAN fluorescence (ex: 365 nm, em: 410 nm) measured using an ELISA microplate reader (see Chapter 3) alongside a calibration curve of 10-10000 nM $NaNO_2$ in HB (Detournay & Weis, 2011). Autofluorescence of host fractions was controlled for by preparing sample blanks. The autofluorescence of *Symbiodinium* cell lysates at 410 nm was negligible and 150 µL HB sufficed as a blank.

Total oxidised NO (NO_x^-) assay:

Quantification of NO_x^- with DAN requires the reduction of nitrate to nitrite, which was achieved by adding 0.1 M vanadium (III) chloride (VCl_3) solution in 1 N HCl (Miranda *et al.*, 2001) immediately prior to the DAN reagent. Host fractions were incubated as described previously (see Chapter 3) and DAN fluorescence was quantified using an ELISA microplate reader (see Chapter 3) and a calibration curve of 10-10000 nM $NaNO_3$ in HB. Addition of DAN-HCl solution to *Symbiodinium* cell lysates in the presence of VCl_3 promoted development of a persistent and interfering blue colour that could not be removed; these samples were therefore not included in the analysis.

As positive controls for the DAN assay, raw coral homogenates were incubated with 1 mM NO donor SNP at 25°C for 4 h.

5.2.8. Assessment of host caspase-like enzyme activity.

The activities of "executioner caspase-3"-like and "initiator caspase-9"-like enzymes (Nicholson, 1999) in the host were quantified according to the methods described in Chapter 3. Additionally, to confirm the absence of non-specific substrate hydrolysis, host fractions of homogenates (n = 3 untreated fragments per species) were pre-incubated for 30 min with 20 µM of the caspase-3 or -9 inhibitors DEVD- or LEHD-aldehyde (-CHO), respectively.

5.2.9. Statistical analyses.

Data analysis was carried out using a PASW 18.0 package (IBM, Armonk, NY, USA). Data regarding symbiont quantum yields, chl *a* content, cellular nitrite, and density within the host, and host tissue nitrite, nitrate, and caspase-like activity were analysed using RMANOVA with Greenhouse-Geisser correction used whenever Mauchly's test returned a significant result. RMANOVA *post-hoc* tests represent pair-wise comparisons between treatments at certain time points with Bonferroni correction. MANOVA was used to examine baseline caspase-like enzyme activity in *M. digitata*. Further analyses were carried out using one-way ANOVA and linear mixed models (LMM), with the latter employed to examine the correlation of coral fragments' host physiological parameters with bleaching in the context of a hierarchical experimental

design with repeated sampling. Each parameter was examined as a variable and the predictive power of putative upstream covariates was analysed individually. Data were transformed to fulfill assumptions of normality prior to any parametric analysis.

5.3. Results

5.3.1. Symbiodinium *genotypes.*

ITS2 sequences of *Symbiodinium* cells most closely aligned with the following genotypes: *Acropora millepora* - C3; *Montipora digitata* - C15; and *Pocillopora damicornis* - C42 (type 2). All five replicate colonies of each species hosted the same ITS2 symbiont type, and there was no evidence of individual colonies hosting more than one ITS2 type simultaneously.

Table 5.1. Statistical analysis **(**RMANOVA, time × temperature interaction) of symbiont physiological parameters in reef corals exposed to control (28.5°C) and elevated temperatures. Analysis of F_v/F_m values includes all three treatments (control, 31.5°C and 33°C). Chlorophyll *a* and nitrite (NO_2^-) analyses were carried out on control samples and either 31.5°C-treated (*Pocillopora damicornis*) or 33°C-treated (*Acropora millepora* and *Montipora digitata*) fragments. Asterisks denote statistical significance ($p < 0.05$).

Species (ITS2 type)	**Parameter**	**Statistic**	**P-value**
Acropora millepora (C3)	F_v/F_m	$F_{3.898, 19.489} = 97.189$	< 0.001*
	Chl *a* content	$F_{3, 21} = 1.834$	0.172
	NO_2^- content	$F_{3, 15} = 6.771$	0.043*
Montipora digitata (C15)	F_v/F_m	$F_{16, 96} = 4.684$	< 0.001*
	Chl *a* content	$F_{3, 24} = 4.18$	0.016*
	NO_2^- content	$F_{3, 15} = 0.407$	0.75
Pocillopora damicornis (C42)	F_v/F_m	$F_{1.71, 13.677} = 30.228$	< 0.001*
	Chl *a* content	$F_{3, 21} = 0.84$	0.487
	NO_2^- content	$F_{3, 18} = 1.137$	0.361

5.3.2. PSII fluorescence and bleaching of reef corals at elevated temperature.

Exposure to elevated temperature over 9 days led to significant declines in the dark-adapted PSII quantum yields (F_v/F_m) of all three species (Table 5.1), and these differed significantly between species (RMANOVA, time × species × temperature, $F_{7.615, 76.152} = 44.013$, $p < 0.001$). F_v/F_m in *A. millepora* and *P. damicornis* declined dramatically at the higher temperatures (Fig. 5.2A, C), while in *M. digitata* F_v/F_m was maintained at the control level until late in the experiment (Fig. 5.2B). Data are not available for *P. damicornis* after Day 6 at 33°C, when this species suffered 100% mortality (visible as tissue sloughing). Subsequent physiological analyses for *P. damicornis* were therefore carried out on fragments incubated at 31.5°C.

Baseline chl *a* content differed between species (one-way ANOVA, $F_{2, 28} = 23.667$, $p < 0.001$), with the C42-type symbionts of *P. damicornis* having the highest amount per cell (6.9 pg ± 0.1 [s.e.m.], Tukey HSD *post-hoc*, $p < 0.01$) and the C3-type symbionts of *A. millepora* the lowest (3.3 pg ± 0.2 [s.e.m.], Tukey HSD *post-hoc*, $p < 0.05$). C3 was also the only type to display any decline in chlorophyll content per cell at elevated temperature and, while there was no significant time × treatment interaction (Table 5.1), type C3 cells after 9 days at 33°C had a chl *a* content 58% lower than that the controls (Fig. 5.2D).

Symbiont densities per unit of coral surface area varied significantly between species prior to treatment (one-way ANOVA with Welch correction, $F_{2, 10.788} = 39.291$, $p < 0.001$); *P. damicornis* had at least 80% fewer *Symbiodinium* cells per cm^2 than did the two other species (Tukey HSD *post-hoc*, $p < 0.01$). When incubated at elevated temperature (31.5°C for *P. damicornis* or 33°C for *A. millepora* and *M. digitata*) for 9 days, symbiont densities declined significantly in *A. millepora* and *P. damicornis* (to *ca.* 10% and 50% of initial densities, respectively) but no significant changes were observed in *M. digitata* (Table 5.2, Fig. 5.2G-I).

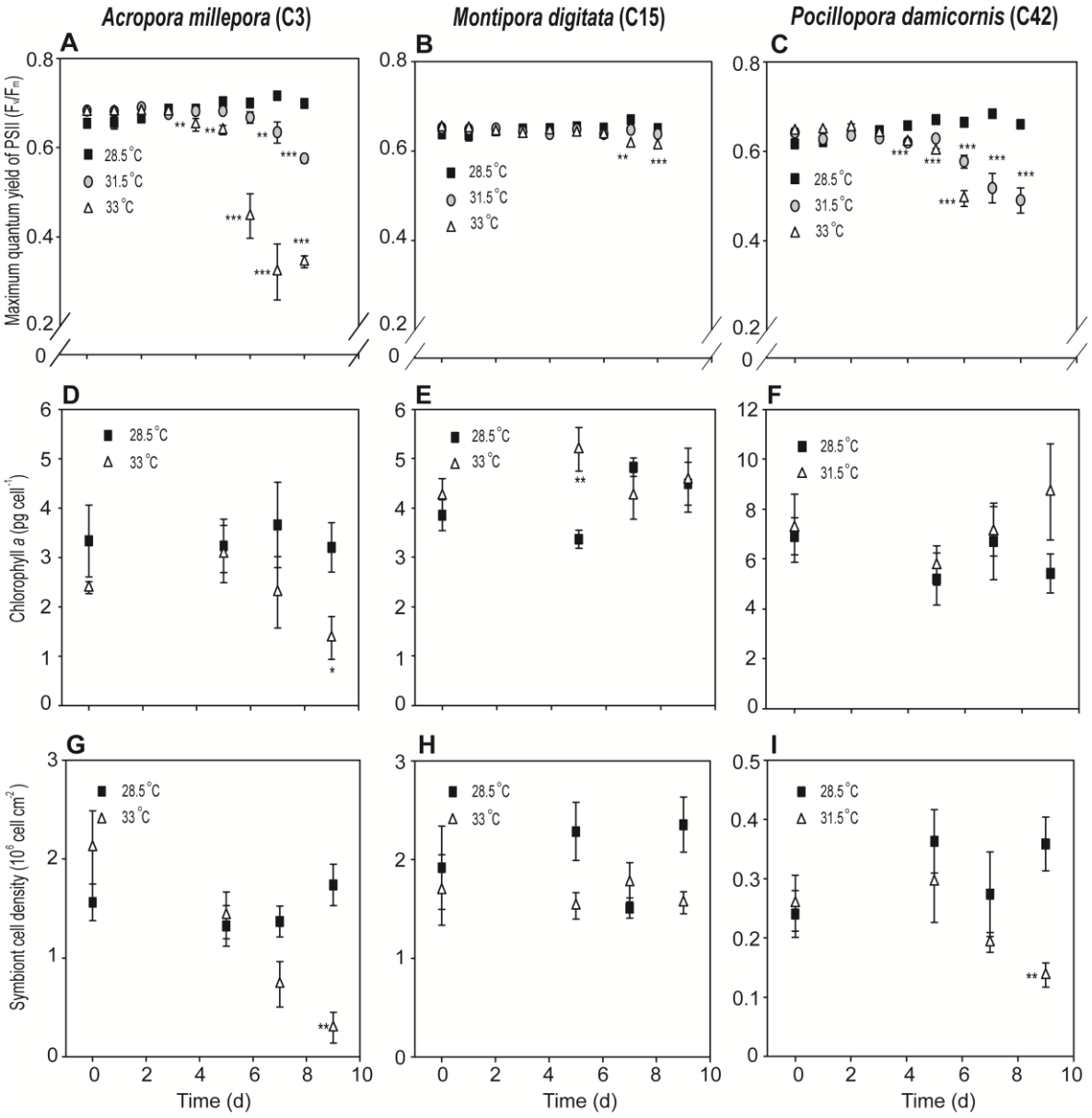

Figure 5.2. Quantum yields of photosystem II, symbiont chlorophyll *a* content and bleaching in *Acropora millepora*, *Montipora digitata*, and *Pocillopora damicornis.* **Panels A-C)** Maximum quantum yield of photosystem II (F_v/F_m); **D-F**) Chlorophyll *a* content per cell; **G-I**) Symbiont cell density normalised to coral fragment surface area. Values are means ± s.e.m. and asterisks denote significant differences relative to controls at each time point. (RMANOVA, pair-wise *post-hoc* with Bonferroni correction, n = 5 individual colonies per time point except *A. millepora* after 9 days at 33°C where n = 4, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Note the different y-axis scales in panels **D-F** and **G-I**.

5.3.3. NO synthesis in corals and their symbionts at elevated temperature.

Nitrite (NO_2^-) levels (nmol per unit soluble protein) in the type C3 *Symbiodinium* cells of *A. millepora* responded significantly to elevated temperature (Table 5.1, Fig. 5.3A) and this response was absent in the C15 symbionts of *Montipora digitata* (Fig. 5.3B). An increasing trend was apparent in the symbionts of *Pocillopora damicornis* but this was only significant relative to controls at Day 7 (Table 5.1, Fig. 5.3C).

Table 5.2. Statistical analysis **(**RMANOVA, time × temperature interaction) of host physiological parameters in reef corals exposed to control (28.5°C) and elevated temperatures (31.5°C for *Pocillopora damicornis;* 33°C for *Acropora millepora* and *Montipora digitata*). Asterisks denote statistical significance ($p < 0.05$).

Species	**Parameter**	**Statistic**	**P-value**
Acropora millepora	NO_2^- content	$F_{3, 18} = 0.89$	0.465
	NO_x^- content	$F_{3, 18} = 6.614$	0.031*
	LEHDase (caspase-9-like) activity	$F_{3, 21} = 3.82$	0.025*
	DEVDase (caspase 3-like) activity	$F_{3, 21} = 7.313$	0.002*
	Symbiont density	$F_{3, 18} = 6.572$	0.003*
Montipora digitata	NO_2^- content	$F_{3, 18} = 0.232$	0.873
	NO_x^- content	$F_{1.582, 9.459} = 1.228$	0.321
	LEHDase (caspase-9-like) activity	$F_{3, 24} = 0.085$	0.968
	DEVDase (caspase 3-like) activity	$F_{3, 24} = 0.464$	0.633
	Symbiont density	$F_{3, 18} = 1.426$	0.268
Pocillopora damicornis	NO_2^- content	$F_{3, 18} = 1.672$	0.209
	NO_x^- content	$F_{3, 12} = 3.920$	0.037*
	LEHDase (caspase-9-like) activity	$F_{3, 24} = 5.416$	0.005*
	DEVDase (caspase 3-like) activity	$F_{1.465, 11.722} = 4.714$	0.04*
	Symbiont density	$F_{3, 21} = 4.779$	0.011*

Host tissue NO_2^- (nmol per unit soluble protein) differed significantly between species at the start of the experiment (one-way ANOVA with Welch correction, $F_{2,\ 12.454} = 28.553$, $p < 0.001$), with *A. millepora* having significantly (approximately 2-fold) higher host tissue NO_2^- than the other two species (Tukey HSD *post-hoc*, $p < 0.01$). However, tissue NO_2^- content did not respond significantly to temperature in any of the coral species (Table 5.2, Fig. 5.3D-F).

Baseline total oxidised NO (NO_x^-) concentrations in the host also differed between species (one-way ANOVA with Welch correction, $F_{2,\ 12.686} = 4.012$, $p = 0.045$), but in this case *A. millepora* and *M. digitata* had similar NO_x^- levels while those of *P. damicornis* were lower (≤ 35%; Tukey HSD *post-hoc*, $p < 0.05$). *A. millepora* and *P. damicornis* showed a significant temperature × time interaction for host tissue NO_x^- (Table 5.2), with maximal NO_x^- observed on Day 5 of the experiment (Fig. 5.3G, I). No significant changes to NO_x^- content occurred in *M. digitata* (Table 5.2, Fig. 5.3H).

5.3.4. Responses of host caspase-like enzymes to elevated temperature.

Baseline host caspase-like enzyme activity was highly variable between the three coral species (Fig. 5.4); DEVDase (caspase-3-like) activity was at least 74% lower in *M. digitata* than in the other two species (one-way ANOVA, $F_{2,\ 29} = 42.577$, $p < 0.001$, Fig. 5.4B). Further examination revealed no significant difference between *M. digitata* homogenates treated with and without caspase-like enzyme inhibitors (MANOVA, Pillai's Trace, $F_{3,\ 12} = 0.899$, $p = 0.495$; Tukey HSD *post-hoc* DEVDase: $p = 0.867$, LEHDase: $p = 0.253$; Fig. 5.5).

Effects of heating on caspase-like enzyme activity differed depending on the species and the duration of treatment (Fig. 5.4). No changes in either DEVDase or LEHDase (caspase-9-like) activity were seen in *M. digitata* (Table 5.2, Fig. 5.4B). LEHDase activity in *A. millepora* and *P. damicornis* responded to increasing temperature (Table 5.2, Fig. 5.4A, C). After 5 days at 33°C in *A. millepora* and on Day 7 in 31.5°C-treated *P. damicornis* fragments, LEHDase activity was at least 2-fold higher than that in control samples. Patterns of DEVDase activity were similar to those of LEHDase, in that a 3-fold increase in activity was seen in *A. millepora* over the initial 5 days of

heating (to 33°C; Fig. 5.4A) and increases in *P. damicornis* DEVDase activity were only apparent on Day 7 at 31.5°C (Fig. 5.4C).

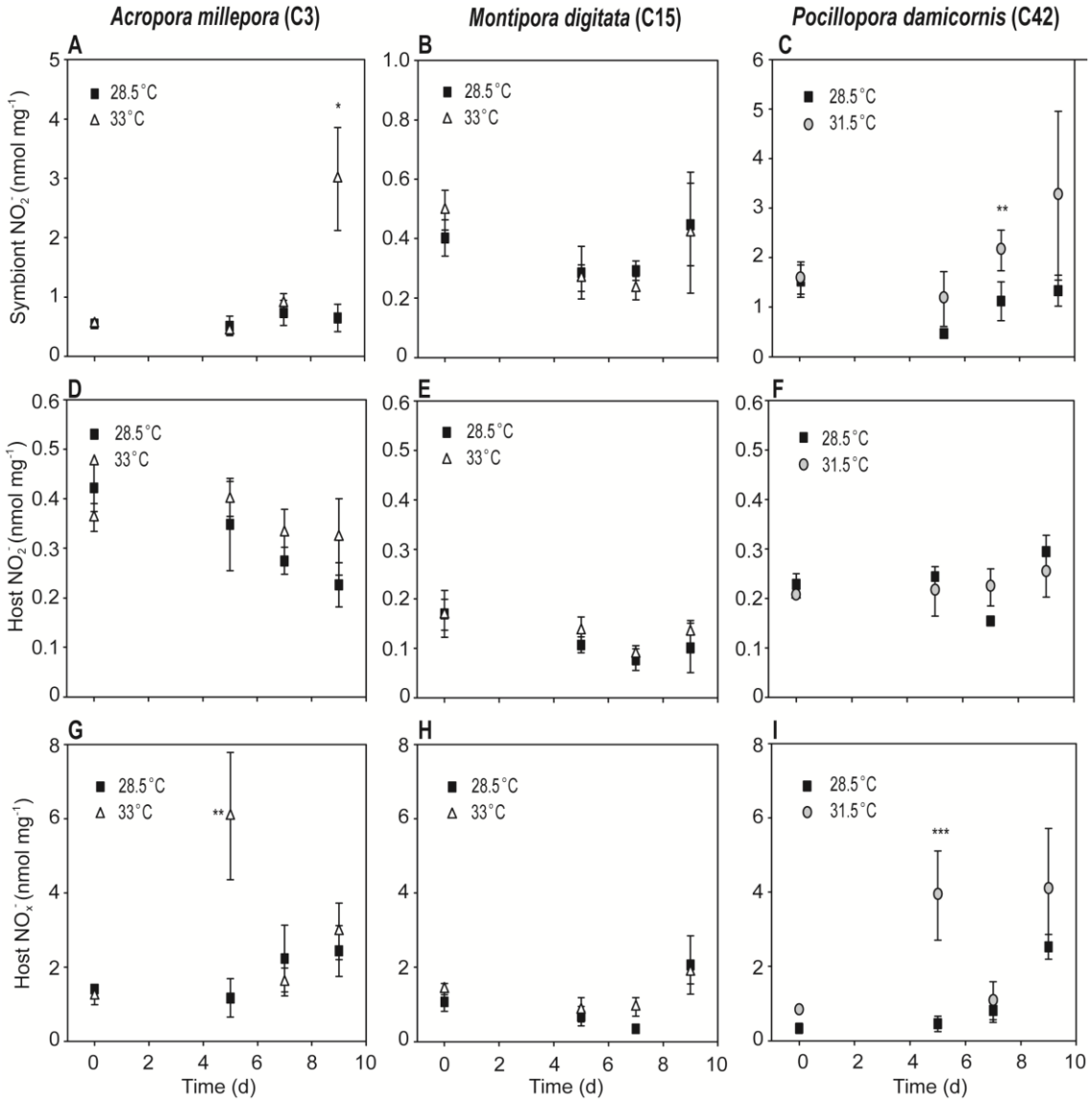

Figure 5.3. Nitric oxide synthesis in the reef corals *Acropora millepora*, *Montipora digitata*, and *Pocillopora damicornis*. **Panels A-C**) Symbiont nitrite (NO_2^-) content (nmol per unit soluble protein); **D-F**) Host nitrite (NO_2^-) content (nmol per unit soluble protein); **G-I**) Host total oxidised NO (NO_x^-) content (nmol per unit soluble protein). Values are means ± s.e.m. and asterisks denote significant differences relative to controls at each time point. (RMANOVA, pair-wise *post-hoc* with Bonferroni correction, n = 5 individual colonies per time point except *A. millepora* after 9 days at 33°C where n = 4, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Note the different y-axis scales in panels **A-C**.

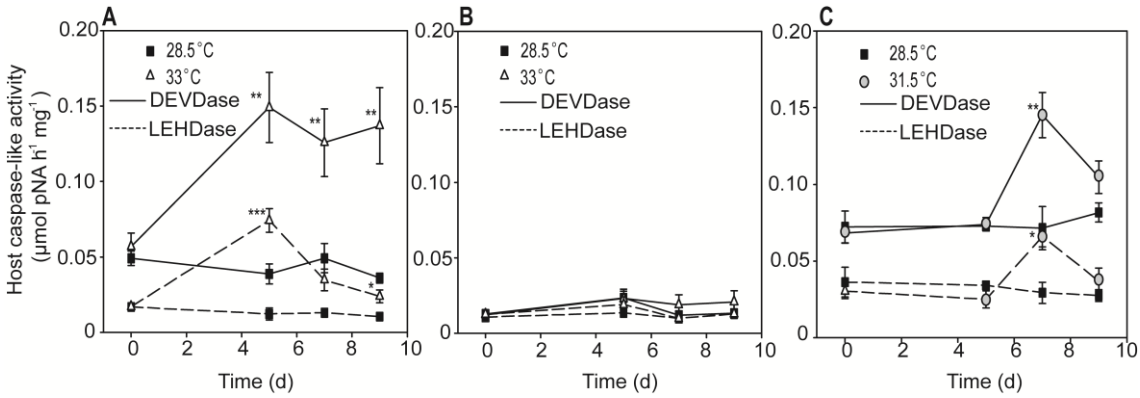

Figure 5.4. Host caspase-like enzyme activity in the reef corals *Acropora millepora* **(Panel A)**, *Montipora digitata* (**B**), and *Pocillopora damicornis* **(C)**. Values are means ± s.e.m. and asterisks denote significant differences relative to controls at each time point. (RMANOVA, pair-wise *post-hoc* with Bonferroni correction, n = 5 individual colonies per time point except *A. millepora* after 9 days at 33°C where n = 4, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

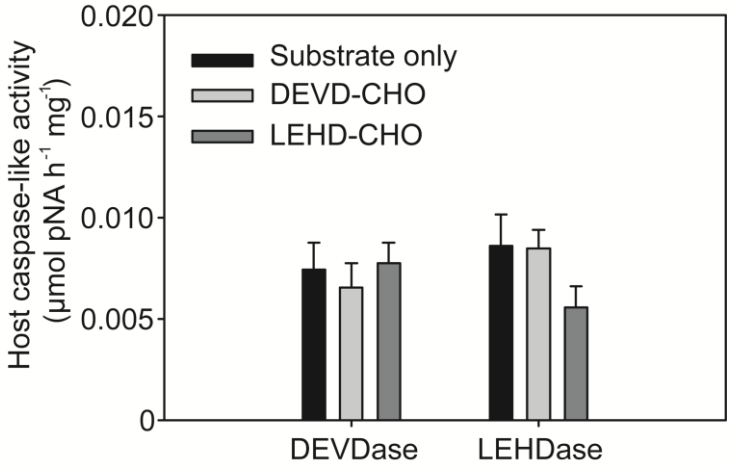

Figure 5.5. Baseline DEVDase (caspase-3-like) and LEHDase (caspase-9-like) enzyme activity in the reef coral *Montipora digitata*. Coral homogenates were incubated with various combinations of tetrapeptide-p-nitroanilide (-pNA) substrates and -aldehyde (-CHO) inhibitors. Values are means ± s.e.m. (n = 3 per treatment). Note the y-axis values relative to Fig. 5.4.

5.3.5. Relationships between NO synthesis, caspase activity and bleaching in Acropora millepora *and* Pocillopora damicornis.

LMM analysis of data from *A. millepora* (Table 5.3) revealed a number of significant findings regarding host tissue NO_x^- accumulation, caspase-like enzyme activity and bleaching. When treatment, colony and time effects were taken into consideration, NO_x^- was found to be a predictor only of DEVDase (caspase-3-like) activity. LEHDase activity was a significant predictor of DEVDase activity, but neither LEHDase nor DEVDase activity correlated significantly with symbiont density. In *P. damicornis*, LEHDase was a significant predictor of DEVDase activity (Table 5.4). *P. damicornis* host tissue NO_x^- content correlated significantly with LEHDase activity but, as in *A. millepora*, it was not a significant predictor of symbiont density.

Table 5.3. LMM analyses of log-transformed host physiological parameters in *Acropora millepora*. Each covariate (left column) was examined as a "predictor" of putative downstream "dependent" parameters. Asterisks denote statistical significance ($p < 0.05$) and the nature of any significant correlation is given in parentheses.

Dependent >	**LEHDase (caspase-9-like) activity**	**DEVDase (caspase-3-like) activity**	**Symbiont density**
Predictor V			
Tissue NO_x^- content	$F_{1, 9.754} = 2.753$ $p = 0.129$	$F_{1, 12.664} = 5.267$ $p = 0.040^*$ (positive)	$F_{1, 9.412,} = 0.078$ $p = 0.787$
LEHDase (caspase-9-like) activity	-	$F_{1, 10.107} = 23.400$ $p = 0.001^*$ (positive)	$F_{1, 5.447} = 0.058$ $p = 0.818$
DEVDase (caspase-3-like) activity	-	-	$F_{1, 9.550} = 1.749$ $p = 0.217$

Table 5.4. LMM analyses of log-transformed host physiological parameters in *Pocillopora damicornis*. Each covariate (left column) was examined as a "predictor" of putative downstream "dependent" parameters. Asterisks denote statistical significance ($p < 0.05$) and the nature of any significant correlation is given in parentheses.

Dependent >	**LEHDase (caspase-9-like) activity**	**DEVDase (caspase-3-like) activity**	**Symbiont density**
Predictor V			
Tissue NO_x^- content	$F_{1, 9.299} = 8.965$ $p = 0.015$* (positive)	$F_{1, 11.764} = 0.682$ $p = 0.425$	$F_{1, 13.799} = 2.275$ $p = 0.154$
LEHDase (caspase-9-like) activity	-	$F_{1, 5.657} = 12.958$ $p = 0.013$* (positive)	$F_{1, 7.162} = 4.042$ $p = 0.085$
DEVDase (caspase-3-like) activity	-	-	$F_{1, 16.789} = 0.364$ $p = 0.554$

5.4. Discussion

This investigation is the first to examine the production of the signalling molecule nitric oxide (NO) in different species of reef-building corals under experimental thermal stress. We report evidence of upregulated NO synthesis by reef corals and their symbiotic dinoflagellates during high temperature-induced bleaching. Secondly, these responses correlated with the activation of host apoptotic-like pathways. Furthermore, apoptotic events and NO synthesis in the host appeared to precede significant algal dysfunction and host bleaching. Perhaps most importantly, this study reports significant variability between differentially temperature-sensitive reef coral species in the apparent generation of NO and the constitutive and temperature-induced expression of apoptotic-

like pathways. These findings could contribute to a developing physiological framework for the varying sensitivities of corals to warming oceans.

5.4.1. Declining PSII functionality, symbiont NO synthesis, and bleaching in reef corals.

Exposure to heating of *ca.* 1°C per day (to 3-5°C above ambient) revealed significant differences among the symbionts of the reef corals *A. millepora*, *M. digitata* and *P. damicornis*. This differential algal performance was reflected in the intensity of bleaching in the three coral species. As observed previously (Fisher *et al.*, 2012), the *M. digitata*-C15 association was comparatively robust in response to heating while the *A. millepora*-C3 and *P. damicornis*-C42 (type 2) symbioses proved much more unstable.

Consistent with the apparent absence of stress in *M. digitata*, there was no evidence of upregulated NO synthesis in its C15-type symbionts. In contrast, the symbionts in *A. millepora* and *P. damicornis* displayed evidence of heightened NO production. The only previous investigation of NO in a clade C *Symbiodinium* (see Chapter 2) revealed a significant downregulation of NO synthesis during high temperature stress. A number of factors could explain this disparity. Firstly, the previous work examined *Symbiodinium* cells in culture, and they may well behave differently when inside their host. The nature of the treatments used by the previous study (rapid exposure to elevated temperature) *versus* the slow heating used here may also have contributed. In spite of these differences, the observation that heightened *in hospite* symbiont NO synthesis occurred well after pronounced declines in PSII function and the onset of bleaching support the hypothesis that NO produced by the symbiont may not be involved in the initiation of bleaching pathways.

5.4.2. Nitric oxide synthesis and apoptotic-like events in the coral host.

Heightened NO production by thermally stressed cnidarian hosts has been proposed as a critical component of a host innate immune-like response resulting in bleaching (Perez & Weis, 2006; Weis, 2008). The findings of this investigation support this hypothesis but suggest that the situation in reef corals may be different to that in laboratory model systems. For example, host tissue NO_2^- content - which responds to increasing temperature in the model cnidarian *Aiptasia pulchella* (Detournay & Weis, 2011) -

changed little in the coral species examined here. Only when total oxidised NO (NO_x^-; including both nitrite and nitrate) was quantified did any temperature-induced response become apparent. This was most obvious in *A. millepora* and less so in *P. damicornis. M. digitata*, the only species of the three not to bleach, showed very little evidence of temperature-induced NO synthesis. In all cases, NO synthesis appeared to be sporadice and transient, indicating significant temporal variability within coral tissues.

It could be argued that changes in host tissue NO_x^- content reflect varying dissolved inorganic nitrogen (DIN) cycling by coral-associated microbial communities, whose physiology and composition can change dramatically with temperature (Bourne *et al.*, 2008). However, the differing responses of NO_x^- among the three coral species and the strong positive effect of the NO donor SNP on tissue NO_x^- content (Supplementary Fig. A8) support the suitability of NO_x^- in assessing tissue NO synthesis. Detecting the biosynthesis of NO in biological systems is notoriously challenging (Bryan & Grisham, 2007). This is especially so in reef corals, where fluorescent proteins and the presence of an internal calcium carbonate skeleton preclude the use of the live-tissue imaging techniques that have been successfully employed with sea anemones (Perez & Weis, 2006). Future investigations could focus more closely on DIN cycling and accumulation in the tissues of heat-stressed corals, and this would certainly aid researchers in evaluating the suitability of NO_2^- and NO_x^- as markers of cnidarian NO synthesis.

Differences among the three coral species were also apparent in the regulation of host caspase-like enzyme activity. The activity of these proteases, well-known as the initiators and executioners of apoptotic-like cell death in metazoans (see Nicholson, 1999; Kumar & Cakouros, 2003 for reviews), has been observed previously in reef corals (Kvitt *et al.*, 2011; Pernice *et al.*, 2011; Tchernov *et al.*, 2011). Their differential regulation at high temperature has also been proposed as a mechanism underpinning the differing susceptibilities of corals to bleaching-induced mortality (Tchernov *et al.*, 2011). Concomitant with an absence of temperature-induced host NO synthesis, caspase-like activities in the thermally tolerant coral *M. digitata* showed no response to elevated temperature. Furthermore, closer examination revealed caspase-like enzyme activity in this species to be almost non-existent. Baseline caspase-like activity was detectable in the two bleaching-susceptible species and these enzymes responded strongly to increasing temperature. NO in mammals is implicated in caspase activation

and apoptosis (Brune *et al.*, 1999; Snyder *et al.*, 2009) and it may influence caspase-like activity in the anemone *A. pulchella* (see Chapter 3). While NO's involvement in coral apoptosis is supported by an apparent association between NO_x^- levels and caspase-like activities in *A. millepora* and *P. damicornis*, neither of these parameters were significant predictors of symbiont density. This might reflect a pre-bleaching induction of host NO synthesis/apoptosis, itself an interesting finding given the relative lack of attention that has been paid to host-level responses in bleaching corals (Baird *et al.*, 2008). Traditionally, the focus has been on symbiont sensitivity (van Oppen *et al.*, 2009) and the necessity of symbiont dysfunction and cell death (Warner *et al.*, 1999; Strychar *et al.*, 2004a; Smith *et al.*, 2005; Sammarco & Strychar, 2013) for the thermal bleaching of symbiotic cnidarians. This symbiont-centric view has led to our knowledge of host stress responses being comparatively slight. Recent work (e.g. Hill & Ralph, 2007; Ainsworth *et al.*, 2008; Vidal-Dupiol *et al.*, 2009; Ainsworth *et al.*, 2011; Dunn *et al.*, 2012; Paxton *et al.*, 2013), however, is challenging this paradigm, and together with earlier investigations of physiological stress in cnidarians (Dykens *et al.*, 1992; Nii & Muscatine, 1997), it is becoming clear that the host can mount significant cellular stress responses prior to algal dysfunction.

5.4.3. Conclusions

It is premature to conclude that innate immune-like responses such as elevated NO synthesis and apoptosis are essential for the bleaching of reef corals, but this investigation furthers our understanding in a number of areas. Firstly, our findings extend those of laboratory-based studies (Trapido-Rosenthal *et al.*, 2005; Perez & Weis, 2006; Richier *et al.*, 2006) to a more ecologically relevant setting. We observed, in two bleaching-susceptible coral species, evidence of a significant upregulation of host NO synthesis occurring alongside apoptotic pathway activation and preceding bleaching. These responses were conspicuously absent in a thermally tolerant species. Moreover, we report differences in the baseline expression of apoptotic pathways that correlated with thermal sensitivity and bleaching intensity among the three corals. This finding in particular could contribute to the developing model of bleaching as an out-of-control innate immune-like response varying in its intensity among differentially susceptible taxa (Perez, 2007; Weis, 2008; Tchernov *et al.*, 2011). Extending this work to a wider range of coral species would greatly improve our understanding of the mechanisms

underpinning differential coral bleaching. Given the changes predicted for the oceans over the coming decades, it is vital that this is achieved.

Chapter 6

General Discussion

6.1. Summary

Recent investigations have revealed nitric oxide (NO), a widespread gaseous signalling molecule, to be important in the regulation of the cnidarian-dinoflagellate symbiosis (Perez & Weis, 2008; Detournay & Weis, 2011; Detournay *et al.*, 2012), particularly during its stress-induced collapse (Perez & Weis, 2006). Much progress has been made uncovering the cellular physiology and signalling pathways that underpin this symbiosis (e.g. Weis, 2008; Dunn & Weis, 2009; DeSalvo *et al.*, 2010; Lesser, 2011; Venn *et al.*, 2011; Davy *et al.*, 2012), but the role played by NO has received comparatively little attention. The findings presented here further our understanding in a number of areas.

These include:
(1) The physiological responses of different *Symbiodinium* types to thermal stress and nitric oxide.
(2) The role of NO in the temperature-induced breakdown (bleaching) of the *Aiptasia pulchella-Symbiodinium* association.
(3) The relative importance of the toxic radical peroxynitrite ($ONOO^-$) in cnidarian bleaching.
(4) The contribution of NO synthesis and potentially NO-mediated cell death to the differential bleaching of reef corals.

In this chapter I will discuss more generally the findings of this thesis and formulate a framework describing the role of NO in coral bleaching.

6.2. Nitric oxide in symbiotic dinoflagellates.

6.2.1. Biosynthesis of NO.

As mentioned in Chapters 1 and 2, our knowledge of NO in photosynthetic microbes is extremely limited. A search of the literature may suggest that NO is common in microalgae, yet little is known of its functions or the pathways involved in its production. Yamasaki & Sakihama (2000) proposed that, in higher plants, stress-induced disruption of photosynthesis results in NO generation by nitrate reductase (NR). This has also been observed in the unicellular alga *Chlamydomonas reinhardtii* (Sakihama *et al.*, 2002), and by using a range of NR substrates and inhibitors Bouchard & Yamasaki (2008) provided strong evidence for a similar mechanism in symbiotic dinoflagellates. The NO-generating activity of NR might also have implications beyond pathways mediated by NO. Depletion of nitrite levels due to the enzymatic reduction of NO_2^- by NR could potentially affect the amount and nature of available inorganic nitrogen within a symbiont cell and, thus, its general metabolic activities during thermal stress.

The finding that type B1 *Symbiodinium* cells (both in culture and *in hospite*) appeared to increase their NO production alongside declining photosynthetic competence supports Bouchard & Yamasaki's (2008) model of nitrate reductase-derived NO. However, production of NO in a thermally tolerant *Symbiodinium* type (A1; Chapter 2) also increased with temperature despite an absence of photoinhibition. This suggests that NO synthesis in these algae may not always reflect photosynthetic dysfunction, but could be a component of a thermal response occurring prior to PSII damage. This may involve heat shock proteins, which are differentially regulated in *Symbiodinium* during temperature stress (Leggat *et al.*, 2011; Rosic *et al.*, 2011) and are known to influence the activity of nitric oxide synthase (NOS) (Yoshida & Xia, 2003; Zhang *et al.*, 2013). NOS-like genes and/or enzyme activity have been identified in unicellular algae (Kim *et al.*, 2006; Foresi *et al.*, 2010) and in a number of intracellular microbial parasites (Ghigo *et al.*, 1995) that are closely related to *Symbiodinium* (see Chapter 1). It would be surprising therefore, if the *Symbiodinium* genome did not encode a NOS-like protein. Although a recent attempt to characterise a NOS enzyme in symbiotic dinoflagellates was unsuccessful (Safavi-Hemami *et al.*, 2010), the NOS hypothesis receives strong

support from early studies (Buxton *et al.*, 2002; Trapido-Rosenthal *et al.*, 2005) as well as Bouchard & Yamasaki's (2008) work, in which addition of NOS substrates resulted in increased NO production. Certainly much more work is required, looking at individual components of NO-synthetic pathways in *Symbiodinium*, before we can draw any solid conclusions regarding the nature of NO synthesis in these microbes. Notwithstanding these limitations, temperature-induced NO generation in *Symbiodinium* does appear to represent a pathological response to environmental stress.

6.2.2. A working model for NO toxicity in symbiotic dinoflagellates.

The relatively low reactivity and highly diffusive nature of NO mean that few cellular components in the cnidarian-dinoflagellate symbiosis are beyond its reach (Fig. 6.1). In the symbiont, NO's apparent cytotoxicity (see Chapter 2) could occur *via* two routes. Firstly, it is clear that sufficiently high concentrations of NO can inhibit photosynthesis and, therefore, potentially exaggerate the effects of temperature (Takahashi & Yamasaki, 2002; Wodala *et al.*, 2008). Photosynthetic collapse would associate with increased ROS generation (Asada & Takahashi, 1987; Asada, 1999; Tchernov *et al.*, 2004; Halliwell, 2006; Lesser, 2006; Halliwell & Gutteridge, 2007) and potentially the further production of NO by nitrate reductase (Yamasaki, 2000; Yamasaki & Sakihama, 2000; Sakihama *et al.*, 2002; Bouchard & Yamasaki, 2008). As noted in Chapters 1 and 2, NO possesses significant cytoprotective properties (Beligni & Lamattina, 1999a; Delledonne, 2005); at low concentrations its scavenging of superoxide can prevent the generation of more toxic ROS such as $OH^{\cdot}$ and ameliorate O_2^{-}-mediated damage (Wink *et al.*, 1993; Beligni & Lamattina, 1999b; Beligni *et al.*, 2002; Wink *et al.*, 2011). Excessive ROS and NO synthesis, however, could overwhelm a symbiont's antioxidant defences and/or react to produce highly toxic peroxynitrite ($ONOO^{-}$) (Beckman & Koppenol, 1996). As noted in Chapter 4, $ONOO^{-}$ can irreversibly inhibit electron transport in chloroplasts and mitochondria (Ahmad *et al.*, 2009). It also permanently damages antioxidant enzymes such as SOD and catalase, can contribute to the formation of hydroxyl ($OH^{\cdot}$) radicals (the most toxic ROS; Ischiropoulos & Almehdi, 1995; Szabo & Ohshima, 1997; Radi *et al.*, 2001; Halliwell & Gutteridge, 2007; Ahmad *et al.*, 2009), and decay to the potent nitrosating agent N_2O_3 (Wink *et al.*, 2011). Production of $ONOO^{-}$ is yet to be observed in photosynthetic microbes but its existence

in *Symbiodinium* is suggested by the observations in Chapter 4 that removal of $ONOO^-$ alleviated temperature-induced declines in symbiont PSII quantum yield.

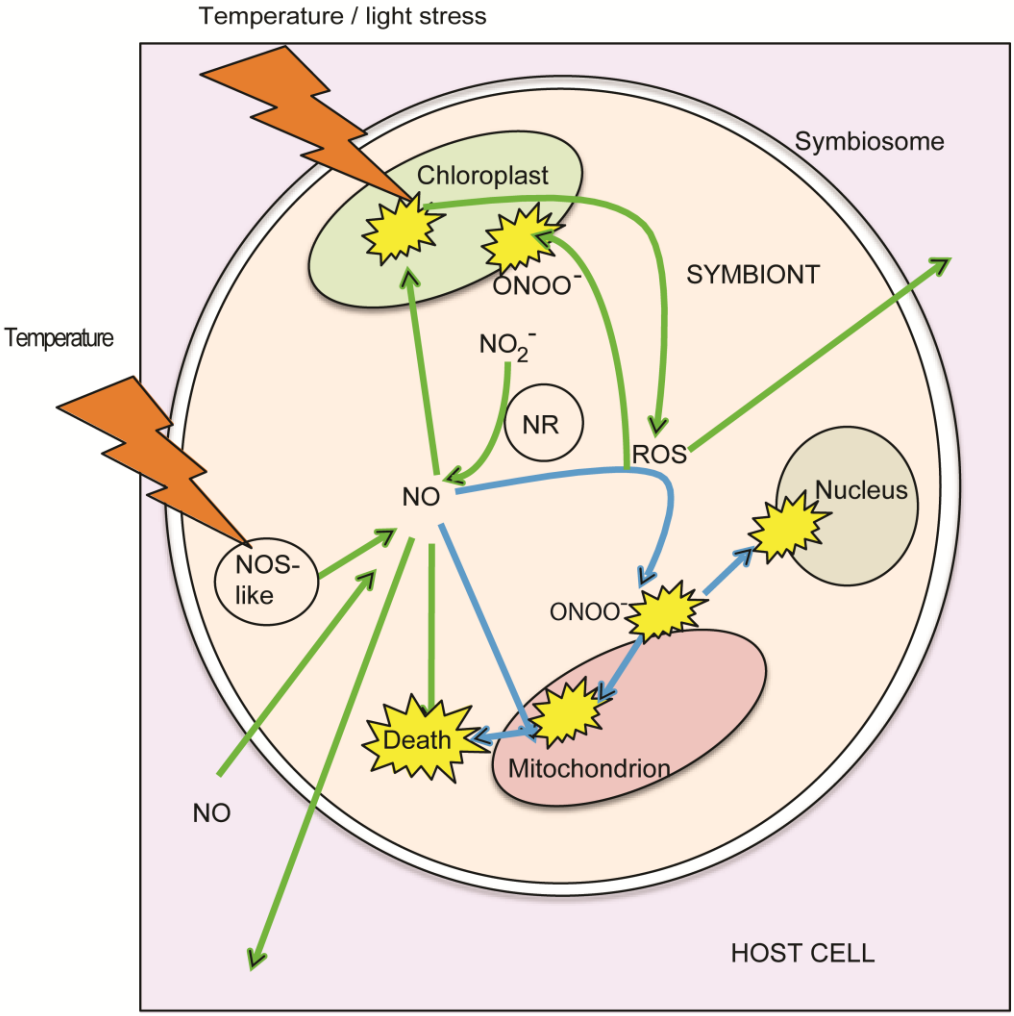

Figure 6.1. Cytotoxicity of nitric oxide (NO) in symbiotic dinoflagellates. Diffusion of host-derived NO across symbiont cell walls [inferred from studies that have detected NO in culture media (Bouchard & Yamasaki, 2008)], coupled with an upregulation of nitric oxide synthase (NOS)-like enzyme, could disrupt photosynthesis and modify cell death pathways. NO's effects could exacerbate those of high temperature and, in a sensitive genotype, lead to generation of reactive oxygen species (ROS) and further NO synthesis *via* nitrate reductase (NR). NO's presence alongside superoxide (O_2^-) may result in peroxynitrite ($ONOO^-$) formation. The high toxicity of $ONOO^-$ might cause widespread cellular dysfunction. Figure adapted from Bouchard & Yamasaki (2008). Green arrows indicate pathways supported by observations of *Symbiodinium* cells, blue arrows indicate events unknown in *Symbiodinium* cells but that have been observed in other photosynthetic organisms.

As outlined in Figure 6.1, $ONOO^-$-mediated damage to a symbiont's mitochondria (Brown & Borutaite, 2002) would likely amplify the consequences of PSII inhibition (by ROS and RNS) and could induce a widespread breakdown in cellular integrity. This might account for the uncontrolled (necrotic) mortality observed in type B1 cells in culture (see Chapter 2). Conversely, the more thermally robust nature of type A1 *Symbiodinium* could have resulted in comparatively depressed ROS production and thus a reduced likelihood of $ONOO^-$ generation. On its own, NO rarely has the capability of inducing necrotic cell death (Bonfoco *et al.*, 1995; Sandau *et al.*, 1997), but it can actively regulate the programmed cell death-like pathways (Almeida *et al.*, 2007) that appeared to be characteristic of temperature-induced mortality in type A1 cells (see Chapter 2).

6.2.3. Adaptive benefits of NO synthesis in Symbiodinium.

Exactly why a photosynthetic microbe should possess a capacity to synthesise a compound such as NO remains unclear. NO can be beneficial - through its antioxidant activities - and it is possible that, as is the case in mammalian systems (Michel & Feron, 1997; Wink & Mitchell, 1998), there exists a mechanism for a cell's constitutive NO synthesis (in order to scavenge ROS, for example) to be upregulated in an inducible response following a suitable stimulus (as observed here). The adaptive benefits of such a response, given NO's cytotoxic properties, are as yet unknown, but the compound's involvement in programmed cell death pathways could be a promising area for investigation. As outlined above, the regulation of PCD is one component of NO's activity in better-studied organisms under stress (Wink & Mitchell, 1998; Brune *et al.*, 1999; Brookes *et al.*, 2000; Chung *et al.*, 2001; Brown & Borutaite, 2002; Almeida *et al.*, 2007; Snyder *et al.*, 2009).

Little is known about PCD in symbiotic dinoflagellates (Dunn *et al.*, 2002; Strychar *et al.*, 2004a; Strychar *et al.*, 2004b; Bouchard & Yamasaki, 2009; Sammarco & Strychar, 2013), and its widespread existence in microalgae (Moharikar *et al.*, 2006; Zuppini *et al.*, 2007; Deponte, 2008; Segovia, 2008; Bouchard & Purdie, 2011) has been something of an enigma (Nedelcu *et al.*, 2011). Explaining cellular suicide in a unicellular organism is obviously challenging, but adaptive mechanisms have been

proposed and some are particularly relevant to an endosymbiosis. For example, the host's internal environment exerts strong selective pressures (e.g. nutrient availability and physical space) on the symbiont, and excessive symbiont population growth might lead to nutritional shortfalls or the build-up of toxic metabolites (Deponte & Becker, 2004). Although host-level mechanisms such as the continuous ejection of symbionts (Hoegh-Guldberg *et al.*, 1987; Baghdasarian & Muscatine, 2000) undoubtedly play a role, an opportunity exists for PCD to act as a symbiont-level population regulator (Vardi *et al.*, 1999; Dunn *et al.*, 2004). Furthermore, with genetic relatedness within a host's symbiont population likely to be high, genes promoting pseudoaltruistic suicide would be strongly selected for. Up-regulating PCD pathways (maybe *via* heightened NO synthesis) during temperature stress could eliminate mutated or sensitive cells (Murik & Kaplan, 2009) and prevent the uncontrolled leakage of harmful compounds (such as H_2O_2) that might lead to a wholesale ejection of symbionts by the host (Weis, 2008). Interestingly, a similar model of sensitive cell PCD followed by replacement by more resilient cells has been proposed for cnidarian host cells during bleaching (Pernice *et al.*, 2011; Tchernov *et al.*, 2011).

The importance of symbiont NO synthesis and associated cell death pathways during high temperature stress greatly depends on the sensitivity of the host's own signalling pathways (described below). The data in Chapters 3 and 5 suggest that in many cases, an upregulation of NO synthesis/PCD by the symbionts may occur too late to influence their ejection from the host. Further work on a wider range of cnidarian-dinoflagellate symbioses may confirm whether or not this is a general phenomenon and whether symbiont NO synthesis *in hospite* is redundant in terms of affecting host bleaching.

6.2.4. A role for NO in the differential thermal sensitivity of Symbiodinium *types*.

On an ecological level, a symbiont's tolerance of NO is perhaps more important than its synthesis of the compound. While this thesis examined the effects of NO on only three ITS2 types of *Symbiodinium*, it nevertheless uncovered an interesting level of physiological diversity. In particular, not only was NO differentially generated by types A1, B1 and C1 but these types varied in their sensitivity to the compound. This was most obvious when assessing symbiont PSII efficiency, but, as noted above, was also evident in the contrasting patterns of mortality exhibited by types A1 and B1.

It is important to remember that NO is a common antimicrobial agent employed by organisms when challenged by microbial infection (mutualist or otherwise) (Liew *et al.*, 1991; MacMicking *et al.*, 1997; Nappi & Ottaviani, 2000; Torreilles, 2001; Davidson *et al.*, 2004; Nyholm & McFall-Ngai, 2004; Villamil *et al.*, 2007; Sorci & Faivre, 2009; Singh *et al.*, 2013). Together with the findings of Perez & Weis (2006) and Detournay & coworkers (Detournay & Weis, 2011; Detournay *et al.*, 2012), this thesis strongly supports the hypothesis that NO is produced by the host as part of an innate immune-like response (discussed below). The observation that symbiont types are differentially sensitive to exogenous NO has major implications, therefore, for the stability of different host-symbiont combinations. A more NO-tolerant type, as well as being potentially more physiologically robust, may be better placed to resist host-derived NO during the early stages of heating and thus delay its destruction or ejection.

We know almost nothing about how the varying tolerances observed in Chapter 2 may arise in symbiotic dinoflagellates, so this is obviously an important area for future work. In the squid-*Vibrio* symbiosis (see Chapter 1), the bacterial symbionts express an alternate oxidase (AOX) with a capacity to detoxify NO (Dunn *et al.*, 2010; Spiro, 2010), as well as MAMPs that attenuate the host's NO synthesis (Altura *et al.*, 2011). Other symbiotic microbes possess haemoglobin-like proteins that scavenge NO and NO-derived radicals (reviewed by Poole & Hughes, 2000). These are particularly important in the *Rhizobium*-legume association where they detoxify RNS (including NO) in the root nodules (Ott *et al.*, 2005; Shimoda *et al.*, 2005). Recent work suggests that symbiotic dinoflagellates have haemoglobin-like proteins (Weston *et al.*, 2012; Rosic *et al.*, 2013), potentially $ONOO^-$-scavenging urate deposits (Kopp *et al.*, 2013) and possibly an AOX protein (Suggett *et al.*, 2008). Differential expression of these mechanisms in different symbiont types might influence their tolerance of NO and thus their ability to withstand a host's innate immune-like response during thermal stress.

6.2.5. Nitric oxide in symbiotic dinoflagellates - conclusions and future work.

In conclusion, NO appears to be cytotoxic in thermally stressed symbiotic dinoflagellates, and this finding concurs with those of Bouchard & Yamasaki (2009). However, it raises an interesting area for further investigation: to what extent might

differential NO synthesis or -tolerance contribute to the mortality (or ejection from a host) of different *Symbiodinium* types during thermal stress? Physiological differences between types of symbiotic dinoflagellate have long been recognised, but these differences now appear to extend to highly conserved phenomena such as NO-signalling. Further investigation may reveal whether or not this is relevant during natural bleaching events and over a broader ecological scale.

6.3. Reactive nitrogen and innate immune-like events in the symbiotic cnidarian host.

6.3.1. The role of peroxynitrite in coral bleaching.

Trapido-Rosenthal & coworkers (2001; 2005) and Perez & Weis (2006) provided the first observations of NO biosynthesis in bleaching cnidarians and this thesis has built upon their findings. Under rapid heat-induced stress, the significant increases in host NO synthesis and $ONOO^-$ generation observed here are consistent with previous hypotheses (Perez & Weis, 2006; Weis, 2008). However, the evidence regarding the generation of $ONOO^-$ and its subsequent effects (or lack thereof) runs counter to expectation. While we still know little of the specific activities of $ONOO^-$ in biological systems, its existence in organisms under stress has been confirmed by numerous investigations (Yamasaki & Sakihama, 2000; Torreilles & Romestand, 2001; Saito *et al.*, 2006; Pacher *et al.*, 2007; Chaki *et al.*, 2009; Gaupels *et al.*, 2011).

$ONOO^-$ in mammals has been proposed as a significant mediator of NO-induced cytotoxicity (Beckman *et al.*, 1993; Estevez & Jordan, 2002; Pacher *et al.*, 2007; Szabo *et al.*, 2007) so it was therefore reasonable to suspect that this highly toxic radical plays a role in the temperature-induced breakdown of the cnidarian-*Symbiodinium* association. The data in Chapter 4 suggest that this may not be the case, however, and it is not altogether surprising when the biochemical characteristics of $ONOO^-$ are taken into account. As noted above, its extremely high reactivity and short half-life in biological systems restricts its diffusion (Pacher *et al.*, 2007; Ferrer-Sueta & Radi, 2009), and thus the number of its potential targets. Nevertheless, a role for $ONOO^-$ in the thermal stress response of cnidarians cannot be discounted. It remains a possibility that the experimental conditions used here, while sufficient to induce $ONOO^-$

generation and host bleaching, were not severe enough to result in widespread $ONOO^-$-mediated damage that could be alleviated with an $ONOO^-$ scavenger. This hypothesis is supported by the fact that a millimolar dose of an $ONOO^-$ donor induced bleaching at control temperatures (Chapter 4). Furthermore, heat-treated *A. pulchella* appeared to mount an apoptotic-like response (Chapter 3), which, like all forms of PCD, requires adenosine trisphosphate (ATP) as an energy source (Bernardi *et al.*, 2001; Elmore, 2007). ATP synthesis is severely inhibited by $ONOO^-$ (Brown & Borutaite, 2002; Radi *et al.*, 2002a) and apoptosis can become progressively more uncontrolled if ATP is depleted or the abiotic stimulus becomes too severe (Beltran *et al.*, 2000; Bernardi *et al.*, 2001; Dunn *et al.*, 2002; Dunn *et al.*, 2004; Nicotera & Melino, 2004; Elmore, 2007). This has been explicitly demonstrated for NO and O_2^- (Bonfoco *et al.*, 1995; Sandau *et al.*, 1997), the two "precursors" of $ONOO^-$. Maybe a more intense or prolonged heating treatment would have induced sufficient $ONOO^-$ to influence bleaching in this study. It could also be hypothesised that the generation of $ONOO^-$ in thermal stressed *A. pulchella* might an antioxidant strategy through NO's potent scavenging of superoxide radicals. The lack of any influence of $ONOO^-$ on thermal bleaching suggests that this pathway might actually be protective during thermal stress, ameliorating ROS buildup and the potential downstream oxidative stress. With very little known about peroxynitrite in invertebrates, this area is certainly one for future study.

6.3.2. Mediation of host apoptosis and bleaching by NO.

It appears to be the case, therefore, that elevated levels of NO can mediate thermal bleaching in *A. pulchella* independent of the compound's conversion to $ONOO^-$. How might this occur? In mammals, NO is implicated in the regulation of intrinsic apoptosis, which depends on mitochondrial (outer) membrane permeabilisation (MMP; Armstrong, 2006; Kroemer *et al.*, 2007; Tait & Green, 2010). Moreover, evidence of mitochondrial dysfunction in symbiotic cnidarians has been reported by recent investigations (Desalvo *et al.*, 2008; Downs *et al.*, 2010; Dunn *et al.*, 2012).

Nitric oxide has a capacity to mediate MMP *via* several mechanisms. Firstly, NO can inhibit mitochondrial respiration by blocking electron transport at complex IV (cytochrome oxidase) on the inner mitochondrial membrane (Cassina & Radi, 1996;

Brown, 1999; Beltran *et al.*, 2000; Borutaite *et al.*, 2000; Brown & Borutaite, 2002). Persistent inhibition of complex IV could lead to the termination of proton transport at complex V (ATP synthase) and the subsequent loss of mitochondrial membrane potential ($\Delta\Psi_m$) (Brown & Borutaite, 2002; Tait & Green, 2010). Rapid decreases in $\Delta\Psi_m$ can result in mitochondrial swelling (Johnson *et al.*, 1981; Brookes *et al.*, 2000; Kroemer *et al.*, 2007), outer membrane rupture and the subsequent release of cytochrome *c* (cyt *c*). The presence of cyt *c* in the cytosol stimulates the coupling of procaspase-9 with apoptotic protease activating factor (APAF)-1 and ATP (Fig. 6.2), and the activation of downstream executioner caspases (Brown & Borutaite, 2002). While the latter components of this pathway (downstream of cyt *c* release) are well-established (in mammalian cells, at least), the requirement of inner mitochondrial membrane disruption for MMP and cyt *c* release remains a subject of debate (Kroemer *et al.*, 2007). This would, however, represent a feasible strategy for investigating a model cnidarian system such as *A. pulchella.* Utilising cell isolation techniques (Gates & Muscatine, 1992; Paxton *et al.*, 2013) in concert with real-time measurements of $\Delta\Psi_m$ (Downs *et al.*, 2010; Tsai *et al.*, 2011), one could closely examine host mitochondrial membrane integrity under various conditions including high temperature and NO scavenger/donor combinations.

It is also known (again, predominantly from work on mammalian cells) that NO can induce MMP through more indirect routes. Damage to DNA by RNS can affect the expression of p53 protein (Messmer *et al.*, 1994), an cell-cycle regulator with an important role of determining cell survival or apoptosis (Brune *et al.*, 1999; Chung *et al.*, 2001). A change in p53 activity can shift the balance of Bcl-2 family proteins from anti- (Bcl-2, Bcl-xL) to pro- (Bak, Bax) apoptotic (Adams & Cory, 1998; Brune *et al.*, 1999; Chung *et al.*, 2001). The activation of Bax protein would ultimately induce MMP (Wolter *et al.*, 1997). There is evidence for the existence of p53 (Lesser & Farrell, 2004; Pankow & Bamberger, 2007) and an expanded Bcl-2 family in symbiotic cnidarians (Dunn *et al.*, 2006; Lasi *et al.*, 2010b; Pernice *et al.*, 2011). NO's relatively low reactivity, however, means that DNA damage and p53 activation are more likely to be caused by $ONOO^-$ (Szabo & Ohshima, 1997; Szabo *et al.*, 2007), which, as demonstrated in Chapter 4, may not be critical for cnidarian bleaching. A more likely scenario may involve NO's negative regulation of anti-apoptotic Bcl-2 family proteins (Fig. 6.2) without the need for $ONOO^-$ or DNA damage (Chae *et al.*, 2004; Snyder *et*

al., 2009). Much more work is required before we fully understand the involvement of NO-mitochondria interactions in cnidarian bleaching, but as noted above, recent technical advances such as the ability to visualise individual host mitochondria in live cnidarian cells (Downs *et al.*, 2010; Additional work Fig. B3, this thesis) will undoubtedly be of value.

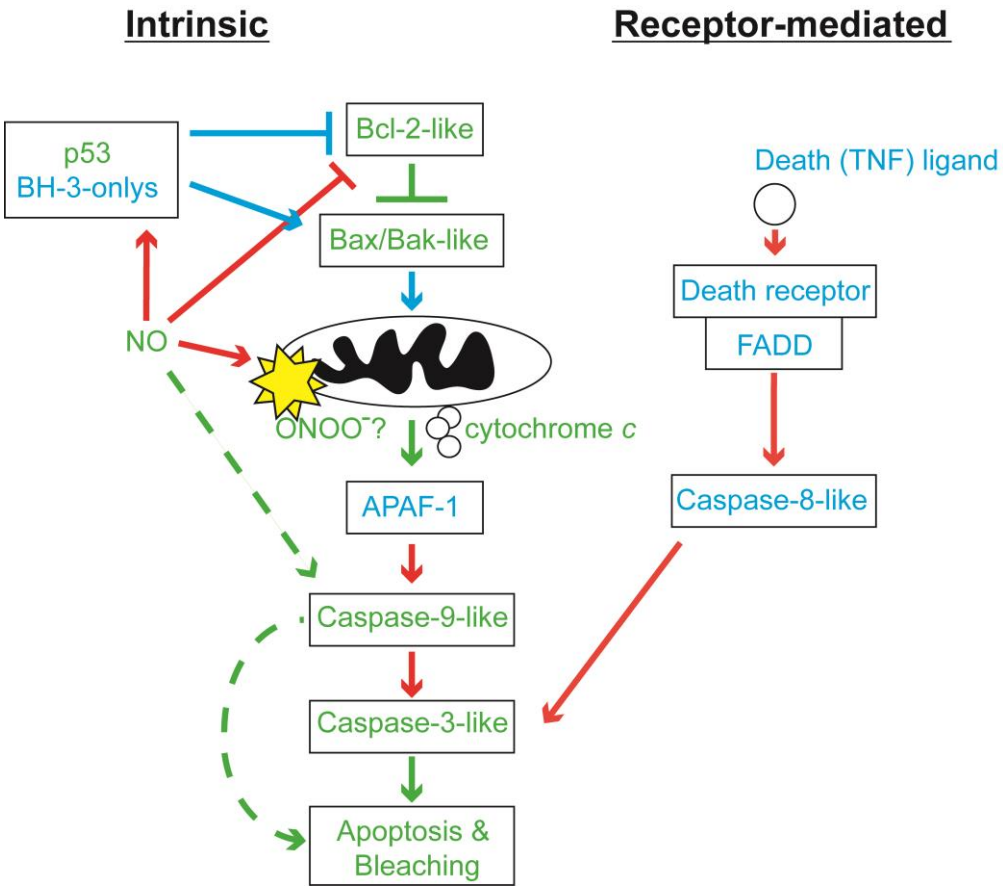

Figure 6.2. Putative apoptotic pathways in symbiotic cnidarians. Nitric oxide (NO) acts on the intrinsic pathway (see text for details). Pathways and components coloured green are supported by direct observations in symbiotic anthozoans, while blue indicates phenomena supported by evidence from other Cnidaria. Red indicates pathways yet to be observed in cnidarians. The green dashed lines represent the principle findings from Chapter 3 of this thesis. Adapted from Lasi *et al.* (2010b).

Whatever the mechanism of NO's involvement in MMP and cyt *c* release (and it remains to be seen whether or not NO is critical to cnidarian mitochondrial dysfunction), mitochondrial control of apoptosis is generally exerted through the recruitment of procaspase-9 to the apoptosome, and its subsequent activation (Kroemer & Reed, 2000; Kroemer *et al.*, 2007; Tait & Green, 2010). The pro-apoptotic actions of mammalian caspase-9 are thought to be expressed exclusively through the activation of procaspase-3 (Nicholson, 1999; Kumar & Cakouros, 2003). However, the differing effects of removing NO on caspase-3- and 9-like activity and bleaching (Chapter 3) suggest that this *A. pulchella* "caspase-9-like" enzyme may also play a role in the execution of apoptosis. Lower metazoan caspases bear a similarity to both initiator- and executioner caspases in mammals (Aravind *et al.*, 1999; Bottger & David, 2003; Wiens *et al.*, 2003; Dunn *et al.*, 2006; Lasi *et al.*, 2010a; Lasi *et al.*, 2010b; Podrabsky & Krumschnabel, 2010), and this may be the case for the enzyme activities observed here. As noted above, more research effort is needed before any mechanistic conclusions can be drawn regarding the suite of caspase-like enzymes present in symbiotic cnidarians.

It is also important to note that a number of studies on mammalian cells suggest that NO can inhibit, as well as stimulate, cell death (Kim *et al.*, 1997; Liu & Stamler, 1999; Zech *et al.*, 2003). As mentioned in Chapter 2, NO is known to possess cytoprotective properties (Beligni & Lamattina, 1999a; b; Delledonne, 2005) in the context of oxidative and abiotic stress, and the compound is actually capable of inhibiting critical apoptotic enzymes (Zech *et al.*, 2003). While the activation of host apoptotic-like cell death and bleaching pathways by NO remains the most parsimonious explanation for the results described in this thesis, a closer examination of the cell death machinery in symbiotic anthozoans will likely reveal a subtler situation in terms of NO-ROS-apoptosis regulation. More sensitive detection techniques may also allow us to examine the role of NO under normal growth conditions or at concentrations lower than those generated during stress.

6.3.3. Timing of host NO synthesis and apoptosis activation.

One of the most important findings of this thesis concerns the timing of host innate immune-like events (such as NO synthesis and apoptotic enzyme activation) during heating-induced symbiotic collapse. In the species that bleached at elevated

temperature, these events in the host (apparently necessary for bleaching; see Chapter 3) often preceded serious photosynthetic dysfunction in the symbionts. Whether this contradicts the current model of bleaching as a response to symbiont-derived ROS (Warner *et al.*, 1999; Smith *et al.*, 2005; Weis, 2008; Lesser, 2011) depends on how early in a stress event ROS leakage from a symbiont occurs.

Surprisingly, given the number of studies that invoke the oxidative theory of bleaching (see Lesser, 1996; Lesser, 2011 for reviews), relatively few investigators have looked at ROS leakage from *Symbiodinium* cells. Tchernov & coworkers (2004) observed evidence of heightened ROS generation/leakage occurring after symbiont maximum quantum yield of PSII (F_v/F_m) had declined to < 50% of pre-stress values. Saragosti *et al.* (2010) described elevated O_2^- generation in the coral *Stylophora pistillata* and Suggett & coworkers (2008) reported elevated H_2O_2 production by cultured *Symbiodinium* cells, but both studies employed light stress (≥ 1000 μmol photons m^{-2} s^{-1}) greater than anything experienced by the organisms examined here. Initial NO synthesis and apoptotic-like events in *A. pulchella* (Chapter 3) and *A. millepora* (Chapter 5) occurred with symbiont F_v/F_m > 90% of control values and under irradiances at least 50% lower than in these previous studies. The absence of elevated *in hospite* symbiont NO synthesis - a marker of physiological dysfunction (Chapter 2) - until late in the experiment also supports the assertion that, in this study at least, the activation of host pathways preceded catastrophic symbiont dysfunction.

Such early cellular changes in the host have been reported previously in reef corals (Ainsworth *et al.*, 2008; Ainsworth *et al.*, 2011) and a symbiotic anemone (Dunn *et al.*, 2012), and in earlier studies Dykens *et al*. (1992) and Nii & Muscatine (1997) observed primarily host-generated ROS in thermally stressed *Anthopleura elegantissima* and *A. pulchella*, respectively. Maybe these host-derived ROS are capable of inducing the NO-synthetic and apoptotic pathways invoked by Perez & Weis (2006) and Weis (2008). A recent study by Paxton *et al.* (2013) suggests that host cell death can precede symbiont mortality, and may therefore be a critical event in cnidarian bleaching. Furthermore, symbionts released from bleaching corals have also been found to be viable (Glynn *et al.*, 1985; Suharsono & Brown, 1992) and photosynthetically competent (Ralph *et al.*, 2001; Ralph *et al.*, 2005; Hill & Ralph, 2007). As a result of this previous work it is becoming increasingly clear that, despite being comparatively neglected in a field

dominated by algal-centric hypotheses, host physiology plays a significant role in determining bleaching sensitivity (Flores-Ramirez & Linan-Cabello, 2007; Baird *et al.*, 2008; Csaszar *et al.*, 2009; Fitt *et al.*, 2009; Wicks *et al.*, 2012).

So what might be stimulating these early host innate immune-like responses, if not the widespread influx of ROS from dysfunctional symbionts? As noted above, host-derived ROS may play a role, and it is possible that heightened NO synthesis at elevated temperature reflects the direct action of heating on host NOS-like enzymes (perhaps through the activity of heat shock proteins; Yoshida & Xia, 2003; Zhang *et al.*, 2013). However, if this was the case then one might expect NO synthesis to continue to increase with further heating rather than decline, as was the case in both *A. pulchella* (Chapter 3) and *A. millepora* (Chapter 5). It could be argued that declining NO generation after sustained heating reflects extensive metabolic dysfunction, but then energy-intensive pathways such as apoptosis should be similarly compromised. To the contrary, *A. millepora* caspase-like enzyme activity at 33°C was elevated throughout the 9-day treatment period, while that of *A. pulchella* and *P. damicornis* was maintained at least at the same level as in controls.

An attractive hypothesis involves processes invoked at the onset of symbiosis. Prior to the overproduction/leakage of ROS during thermal stress, perhaps the strategies employed by *Symbiodinium* to evade host innate immunity (Schwarz, 2008; Dunn, 2009; Detournay & Weis, 2011; Sibley, 2011; Detournay *et al.*, 2012) become compromised during the early stages of thermal stress, leaving symbiont cells vulnerable to recognition and rejection. Recent work on reef corals suggests that the expression of host lectins, involved in the onset of invertebrate endosymbioses (Bulgheresi *et al.*, 2006; Wood-Charlson *et al.*, 2006; Logan *et al.*, 2010), becomes modified well before any symbiont loss occurs (Desalvo *et al.*, 2008; Vidal-Dupiol *et al.*, 2009; Bellantuono *et al.*, 2012a; Barshis *et al.*, 2013). Furthermore, a lectin gene in *A. millepora* was upregulated at high temperature in colonies that had been preconditioned to thermal stress [and were less susceptible to bleaching (Bellantuono *et al.*, 2012b)], while the opposite (a lectin downregulation) was seen in non-preconditioned, bleaching-sensitive corals (Bellantuono *et al.*, 2012a). *A. millepora* lectin bears a close similarity to a mammalian CD23-like protein (Kijimoto-Ochiai, 2002; Kvennefors *et al.*, 2008), and the CD23-immunoglobulin E (IgE) pathway has a

capacity for regulating iNOS during innate immune-like responses to microbial infection (Dugas *et al.*, 1995; Vouldoukis *et al.*, 1995; MacMicking *et al.*, 1997; Padua Queiroz *et al.*, 2010). Could a dysfunction of lectin-glycan interactions alongside other symbiont "cloaking" mechanisms be sufficient to stimulate host an innate immune response prior to the inhibition of algal photosynthesis?

Much work is needed in this area, particularly examining the subtle changes in the physiology of both partners that occur prior to photosynthetic dysfunction or cellular mortality. With research into the molecular communication in the cnidarian-dinoflagellate symbiosis still in its infancy, the possible candidates for a host innate immune/iNOS elicitor are potentially numerous (see Dunn, 2009; Sunagawa *et al.*, 2009; Detournay *et al.*, 2012). It will also be interesting to uncover where exactly NO acts in the cnidarian-dinoflagellate association and whether it possesses a cytoprotective capacity in addition to its involvement in mortality. Technical advances in cell culture (Khalesi, 2008), microscopy (Downs *et al.*, 2010; Venn *et al.*, 2011; Dunn *et al.*, 2012), and reactive species detection (Ueno *et al.*, 2006; Yang *et al.*, 2006; Wardman, 2007; McQuade & Lippard, 2010) will no doubt provide unprecedented access to the inner workings of this symbiosis. Cell isolation techniques (Gates & Muscatine, 1992; Downs *et al.*, 2010; Paxton *et al.*, 2013) in concert with live cell imaging (Venn *et al.*, 2009; Downs *et al.*, 2010) will also enable us to examine, in real-time, the physiology of symbionts *in hospite* with no confounding influence of "fresh isolation" (Wang *et al.*, 2011). It would be particularly valuable to be able to directly assess the *in vivo* generation of RNS in corals (as opposed to relying on chemical proxies such as NO_x^-, for example). Recent improvements in the sensitive electrochemical detection of trace physiological gases (Bourne *et al.*, 2011; Glas *et al.*, 2012) might also allow such a non-destructive examination. The quantification of RNS in bleached and diseased corals in the field is an especially attractive area, as there appears to be convergence in the underlying cellular mechanisms of thermal stress and disease susceptibility in reef corals (Mydlarz *et al.*, 2009; Mydlarz *et al.*, 2010; Palmer *et al.*, 2010; Palmer *et al.*, 2011).

6.4. Incorporating host innate immune-like responses into a model of bleaching susceptibility in corals.

When developing a model of coral bleaching susceptibility that incorporates host innate immune-like responses, it is important that the numerous non-bleaching functions of such responses are acknowledged. This is particularly true for highly conserved phenomena such as ROS- and NO-signalling and apoptosis. As mentioned above, these are common host responses to infection by pathogens (MacMicking *et al.*, 1997; Nappi & Ottaviani, 2000; Fang, 2004; Saito *et al.*, 2006; Pannebakker *et al.*, 2007; Villamil *et al.*, 2007; Herrera-Ortiz *et al.*, 2011; Liu *et al.*, 2013; Singh *et al.*, 2013) and mutualists (Ruby, 1996; Shimoda *et al.*, 2005; Catala *et al.*, 2010; del Giudice *et al.*, 2011; Meilhoc *et al.*, 2011; Wang & Ruby, 2011).

Successful infection of *A. pallida* with homologous symbionts (those that occur naturally with that host) results in a downregulation of host NO production pathways (Detournay *et al.*, 2012), while infection of aposymbiotic *A. pulchella* with unsuitable or dysfunctional symbiont cells may lead to heightened host NO synthesis (see Supplementary Fig. A9). Furthermore, the selective removal of unsuitable symbionts ("winnowing" Nyholm & McFall-Ngai, 2004) in scleractinian coral larvae involves, among other mechanisms (Marlow & Martindale, 2007), an apoptotic-like pathway (Dunn & Weis, 2009). It seems that the more we learn about cnidarian-dinoflagellate symbiosis, the more we realise that the mechanisms underpinning both the onset and collapse of the association are homologous (Weis, 2008; Vidal-Dupiol *et al.*, 2009; Schnitzler, 2010; Davy *et al.*, 2012; Detournay *et al.*, 2012).

How might this be relevant to the phenomenon of differential coral bleaching? And could the pathways described above, which appear necessary for the selective uptake of suitable symbionts from the external environment, actually become a disadvantage during environmental stress? It is clear that many of the coral species most susceptible to bleaching, including *A. millepora* (Guest *et al.*, 2012), rely on the uptake of a wide range of symbiont types from the external environment (Baird *et al.*, 2009; Putnam *et al.*, 2012). These species could therefore be expected to possess apoptotic or NO-mediated "winnowing" mechanisms similar to those in the squid *Euprymna scolopes* (see Chapter 1), *Aiptasia* spp. and *F. scutaria*. As this thesis and other studies have

shown (Gloire *et al.*, 2006; Perez & Weis, 2006; Dunn *et al.*, 2007; Zuppini *et al.*, 2007; Segovia, 2008; Weis, 2008 for review), however, these pathways appear highly sensitive to environmental stress. Perhaps their heightened baseline expression might leave horizontally-transmitting corals such as *A. millepora* susceptible to NO/apoptosis-driven bleaching at high temperature.

Conversely, a species such as *M. digitata*, which utilises maternal (vertical) symbiont transmission (Baird *et al.*, 2009), and often has comparatively thick tissue (Loya *et al.*, 2001) and high symbiotic fidelity [associating with a specific symbiont type (Fabina *et al.*, 2012)] would less frequently encounter (on a cellular level, at least) multiple infective *Symbiodinium* types. This species would presumably receive little adaptive benefit from a "winnowing" mechanism involving the heightened expression of host apoptotic and NO-synthetic pathways. In concert with the increased robustness of a C15-type symbiont (Fisher *et al.*, 2012), *M. digitata* may therefore be more resistant to an overstimulation of these pathways that might eventually lead to bleaching. While this model remains hypothetical, it is supported by the fact that vertically transmitting coral species that associate with a few symbiont types are generally more resistant to increasing temperatures than are horizontally transmitting, more generalist species (Putnam *et al.*, 2012).

The hypothesis is complicated by the maternally transmitting coral *P. damicornis* (Baird *et al.*, 2009), which expressed similar baseline caspase-like enzyme activity to that of *A. millepora* and, in this study as well as others (McClanahan *et al.*, 2004; Guest *et al.*, 2012), was susceptible to thermal stress. However, pocilloporid corals are known to associate with a wide range of *Symbiodinium* types (Fabina *et al.*, 2012; Putnam *et al.*, 2012; Byler *et al.*, 2013) and, furthermore, horizontal uptake of symbionts has been observed in *Pocillopora* spp. during both adult (Glynn *et al.*, 2001) and embryonic (Marlow & Martindale, 2007) life stages. Such a coral might benefit from a "winnowing"-type mechanism as described above - a mechanism that may also be sensitive to environmental stressors.

This is speculative, of course, but could provide a valuable basis for future research. Assessments of baseline NOS/caspase activity and the expression of innate immune-like components across a range of differentially susceptible taxa - characterised by differing

life histories and resident *Symbiodinium* types - would be greatly beneficial in determining the plausibility of this model. It will be interesting more generally to see whether the cellular mechanisms underpinning bleaching are conserved across different coral functional groups and taxa.

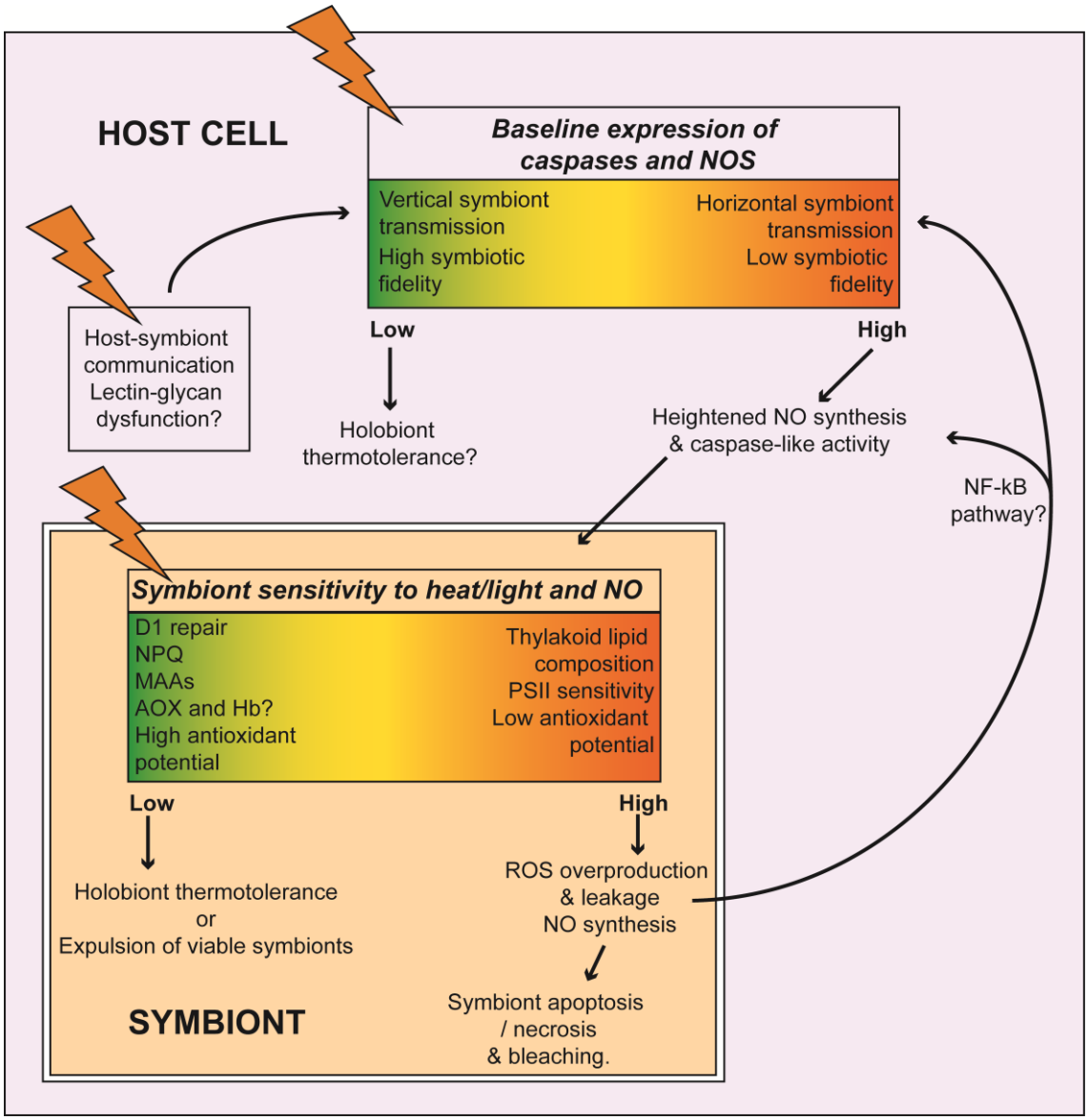

Figure 6.3. Simplified conceptual framework for the cellular basis of differential bleaching susceptibility in reef corals. Both the host's baseline expression of innate immune-like "winnowing" pathways (caspase-like enzymes and nitric oxide synthase - NOS; see text) and the symbiont's sensitivity to heat/light and host-derived NO contribute to the holobiont's stability during temperature disturbances.

6.5. The role of nitric oxide in cnidarian bleaching and the differential susceptibility of corals during thermal stress.

This investigation has examined the role of NO in cnidarian bleaching on a number of levels, ranging from the purely mechanistic to the more ecologically relevant. It reports a number of significant findings:

(1) NO is harmful to *Symbiodinium* cells *in vitro* and its effects (and their intensity) differ among *Symbiodinium* types.

(2) NO appears to mediate temperature-induced cnidarian bleaching through the activation of intrinsic apoptotic-like pathways.

(3) While toxic NO-derived radicals such as peroxynitrite ($ONOO^-$) have a capacity to induce cnidarian bleaching, it appears that NO alone is sufficient to mediate bleaching at elevated temperature.

(4) Host NO synthesis and apoptotic responses during thermal stress may occur prior to symbiont physiological dysfunction.

(5) Temperature-induced synthesis of NO and both baseline and temperature-induced expression of apoptotic-like pathways differ significantly among differentially temperature-sensitive reef coral species.

Drawing these findings together, this thesis proposes that prior to the development of cellular and physiological stresses in the symbiont (a phenomenon well-described in bleaching cnidarians; Smith *et al.*, 2005; Lesser, 2011), activation of host innate immune-like pathways - in which nitric oxide appears to play a major role - sets the symbiosis on the road to collapse (Fig. 6.3). The mechanisms of activation are as yet unknown but, as discussed above, they may involve pathways similar to those employed at the onset of symbiosis. Moreover, the road to bleaching is by no means one-way and may be significantly modified by the presence of a temperature-tolerant symbiont, or one capable of withstanding high concentrations of NO (Fig. 6.3). In this case, NO-driven apoptotic events in the host may result in sub-lethal bleaching, perhaps through the loss of host cells containing viable symbionts (Hill & Ralph, 2007) followed by re-growth of more temperature- or apoptosis-resistant host tissue (Tchernov *et al.*, 2011). Conversely, a sensitive symbiont might be unable to withstand the combined stresses of host-derived ROS, NO and heightened temperature, and would suffer significant oxidative stress and mortality (Lesser, 2006). Overproduction and leakage of symbiont-

derived ROS and/or NO into the host could then exaggerate host NOS activity through the mechanisms described by Perez & Weis (2006), increasing the likelihood of uncontrolled cell death in both symbiont and host and, consequently, catastrophic symbiont loss.

6.6. Cell biology of coral bleaching: physiological ecology predicting the reefs of the future?

We still have much to learn about the cell biology of cnidarians in the context of environmental change, but it seems that the cnidarian-dinoflagellate symbiosis is far more complicated (and flexible) than was previously thought (Baird & Maynard, 2008; Mieog *et al.*, 2009; Csaszar *et al.*, 2010; Jones & Berkelmans, 2010; Weis, 2010; Wicks *et al.*, 2010; Bellantuono *et al.*, 2012a; Bellantuono *et al.*, 2012b; Guest *et al.*, 2012; Silverstein *et al.*, 2012; Wicks *et al.*, 2012; Barshis *et al.*, 2013). The results of this thesis suggest, unsurprisingly, that multiple pathways exist for the determination of the thermal tolerance of corals and thus their scope for surviving the coming centuries. However, the physiological changes that are required for corals to remain as the dominant shallow marine ecosystem engineers in the tropics are substantial (Hoegh-Guldberg *et al.*, 2007; Hoegh-Guldberg & Bruno, 2010; Wild *et al.*, 2011). In the 150-200 years since the Industrial Revolution, atmospheric concentrations of CO_2 and other greenhouse gases have increased to levels greater than anything seen for at least the last million years (IPCC, 2007). To survive the proposed increases in seawater temperature (Hoegh-Guldberg, 1999), corals will have to increase their thermal tolerances by at least 0.2 - 1.0°C (Donner *et al.*, 2005). As Hoegh-Guldberg (2010) notes, nothing that has occurred over the 30 years since coral bleaching first became an issue (Glynn, 1983; 1984) suggests that this is achievable. Furthermore, the present rate of atmospheric CO_2 accumulation will soon see concentrations reaching 450 ppm, a level at which the catastrophic bleaching of coral reefs will become a regular occurrence (Hoegh-Guldberg *et al.*, 2007; Anthony *et al.*, 2011; van Hooidonk *et al.*, 2013). Coupled with ocean acidification (Orr *et al.*, 2005; Silverman *et al.*, 2009), it seems probable that only the hardiest species will exist as remnants of corals reefs' former biodiversity (Loya *et al.*, 2001; Fabricius *et al.*, 2011). Yet, the coral-algal association is evolutionarily ancient (Pandolfi *et al.*, 2011) and will likely persist in some form or another well beyond the effects of industrialisation (Knowlton, 2001; Jackson, 2008; 2010).

Identifying what the reefs of the future will look like, and where those reefs will be, may depend on our ability to understand the complicated physiology of the cnidarian-dinoflagellate symbiosis.

References

Adams J.M. & Cory S. (1998) The Bcl-2 protein family: Arbiters of cell survival. *Science*, **281**, 1322-1326.

Ahmad R., Rasheed Z. & Ahsan H. (2009) Biochemical and cellular toxicology of peroxynitrite: Implications in cell death and autoimmune phenomenon. *Immunopharmacology and Immunotoxicology*, **31**, 388-396.

Ainsworth T.D., Hoegh-Guldberg O., Heron S.F., Skirving W.J. & Leggat W. (2008) Early cellular changes are indicators of pre-bleaching thermal stress in the coral host. *Journal of Experimental Marine Biology and Ecology*, **364**, 63-71.

Ainsworth T.D., Wasmund K., Ukani L., Yellowlees D., Miller D. *et al.* (2011) Defining the tipping point. A complex cellular life/death balance in corals in response to stress. *Scientific Reports*, **1**, 160.

Ali M., Al-Olayan E.M., Lewis S., Matthews H. & Hurd H. (2010) Naturally occurring triggers that induce apoptosis-like programmed cell death in *Plasmodium berghei* ookinetes. *PLoS ONE*, **5**, 1-15.

Almeida B., Buttner S., Ohlmeier S., Silva A., Mesquita A. *et al.* (2007) NO-mediated apoptosis in yeast. *Journal of Cell Science*, **120**, 3279-3288.

Altincicek B. (2009) The innate immunity in the cnidarian *Hydra vulgaris*. *Invertebrate Survival Journal*, **6**, 106-113.

Altura M.A., Stabb E., Goldman W., Apicella M. & McFall-Ngai M.J. (2011) Attenuation of host NO production by MAMPs potentiates development of the host in the squid-*Vibrio* symbiosis. *Cellular Microbiology*, **13**, 527-537.

Anthony K.R., Maynard J.A., Diaz-Pulido G., Mumby P.J., Marshall P.A. *et al.* (2011) Ocean acidification and warming will lower coral reef resilience. *Global Change Biology*, **17**, 1798-1808.

Apprill A. & Gates R.D. (2007) Recognising diversity in coral symbiotic dinoflagellate communities. *Molecular Ecology*, **16**, 1127-1134.

Aravind L., Dixit V.M. & Koonin E.V. (1999) The domains of death: Evolution of the apoptosis machinery. *Trends in Biochemical Sciences*, **24**, 47-53.

Armstrong J.S. (2006) Mitochondrial membrane permeabilization: The *sine qua non* for cell death. *Bioessays*, **28**, 253-260.

Asada K. & Takahashi M. (1987) Production and scavenging of active oxygen in photosynthesis. In: *Photoinhibition* (eds D.J. Kyle, C.B. Osmond, & C.J. Arntzen), pp. 228-287. Elsevier, Amsterdam.

Asada K. (1999) The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. *Annual Reviews of Plant Physiology and Plant Molecular Biology*, **50**, 601-639.

Baghdasarian G. & Muscatine L. (2000) Preferential expulsion of dividing algal cells as a mechanism for regulating algal-cnidarian symbiosis. *Biological Bulletin*, **199**, 278-286.

Baird A. & Maynard J.A. (2008) Coral adaptation in the face of climate change. *Science*, **320**, 315-315.

Baird A.H., Cumbo V.R., Leggat W. & Rodriguez-Lanetty M. (2007) Fidelity and flexibility in coral symbioses. *Marine Ecology Progress Series*, **347**, 307-309.

Baird A.H., Bhagooli R., Ralph P.J. & Takahashi S. (2008) Coral bleaching: The role of the host. *Trends in Ecology and Evolution*, **24**, 16-20.

Baird A.H., Guest J.R. & Willis B.L. (2009) Systematic and biogeographical patterns in the reproductive biology of scleractinian corals. *Annual Reviews of Ecology, Evolution and Systematics*, **40**, 551-571.

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

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

Baldauf S.L. (2003) The deep roots of eukaryotes. *Science*, **300**, 1703-1706.

Barshis D.J., Ladner J.T., Oliver T.A., Seneca F.O., Tralyor-Knowles N. *et al.* (2013) Genomic basis for coral resilience to climate change. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 1387-1392.

Basu N.K., Kole L., Ghosh A. & Das P.K. (1997) Isolation of a nitric oxide synthase from the protozoan parasite, *Leishmania donovani*. *Fems Microbiology Letters*, **156**, 43-47.

Beckman J.S., Carson M., Smith C.D. & Koppenol W.H. (1993) ALS, SOD and peroxynitrite. *Nature*, **364**, 584-584.

Beckman J.S. & Koppenol W.H. (1996) Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *American Journal of Physiology-Cell Physiology*, **271**, C1424-C1437.

Beligni M.V. & Lamattina L. (1999a) Is nitric oxide toxic or protective? *Trends in Plant Science*, **4**, 299-300.

Beligni M.V. & Lamattina L. (1999b) Nitric oxide counteracts cytotoxic processes mediated by reactive oxygen species in plant tissues. *Planta*, **208**, 337-344.

Beligni M.V. & Lamattina L. (2001) Nitric oxide in plants: The history is just beginning. *Plant, Cell and Environment*, **24**, 267-278.

Beligni M.V., Fath A., Bethke P.C., Lamattina L. & Jones R.L. (2002) Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiology*, **129**, 1642-1650.

Bellantuono A.J., Granados-Cifuentes C., Miller D.J., Hoegh-Guldberg O. & Rodriguez-Lanetty M. (2012a) Coral thermal tolerance: Tuning gene expression to resist thermal stress. *PLoS ONE*, **7**, e50685.

Bellantuono A.J., Hoegh-Guldberg O. & Rodriguez-Lanetty M. (2012b) Resistance to thermal stress in corals without changes in symbiont composition. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 1100-1107.

Beltran B., Mathur A., Duchen M.R., Erusalimsky J.D. & Moncada S. (2000) The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 14602-14607.

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

Bernardi P., Petronilli V., Di Lisa F. & Forte M. (2001) A mitochondrial perspective on cell death. *Trends in Biochemical Sciences*, **26**, 112-117.

Besson-Bard A., Pugin A. & Wendehenne D. (2008) New insights into nitric oxide signaling in plants. *Annual Reviews of Plant Biology*, **59**, 21-39.

Bhagooli R., Archer J., Zielke S. & Trapido-Rosenthal H. (2001) Zooxanthellae released from the coral *Stephanocoenia intersepta* during heat-induced bleaching have elevated levels of nitric oxide synthase activity. *American Zoologist*, **41**, 1391-1392.

Bolanos J.P. & Heales S.J.R. (2010) Persistent mitochondrial damage by nitric oxide and its derivatives: Neuropathological implications. *Frontiers in Neuroenergetics*, **2**, 1.

Bonfoco E., Krainc D., Ankarcrona M., Nicotera P. & Lipton S.A. (1995) Apoptosis and necrosis - 2 distinct events induced, respectively, by mild and intense insults with n-methyl-d-aspartate or nitric-oxide & superoxide in cortical cell-cultures. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 7162-7166.

Borutaite V., Morkuniene R. & Brown G.C. (2000) Nitric oxide donors, nitrosothiols and mitochondrial respiration inhibitors induce caspase activation by different mechanisms. *FEBS Letters*, **467**, 155-159.

Bottger A. & David C.N. (2003) Evolution of cell death: Caspase-mediated mechanisms in early metazoans; noncaspase mechanisms in single-celled eukaryotes. In: *Genetics of Apoptosis* (ed S. Grimm), pp. 145-153. Garland Science, Abingdon, U.K.

Bou-Abdallah F., Chasteen N.D. & Lesser M.P. (2006) Quenching of superoxide radicals by green fluorescent protein. *Biochimica Et Biophysica Acta-General Subjects*, **1760**, 1690-1695.

Bouchard J.N. & Yamasaki H. (2008) Heat stress stimulates nitric oxide production in *Symbiodinium microadriaticum*: A possible linkage between nitric oxide and the coral bleaching bleaching phenomenon. *Plant and Cell Physiology*, **49**, 641-652.

Bouchard J.N. & Yamasaki H. (2009) Implication of nitric oxide in the heat-stress-induced cell death of the symbiotic alga *Symbiodinium microadriaticum*. *Marine Biology*, **156**, 2209-2220.

Bouchard J.N. & Purdie D. (2011) Temporal variation of caspase 3-like protein activity in cultures of the harmful dinoflagellates *Karenia brevis* and *Karenia mikimotoi*. *Journal of Plankton Research*, **33**, 961-972.

Bourne D., Iida Y., Uthicke S. & Smith-Keune C. (2008) Changes in coral-associated microbial communities during a bleaching event. *ISME Journal*, **2**, 350-363.

Bourne D.G., Muirhead A. & Sato Y. (2011) Changes in sulfate-reducing bacterial populations during the onset of black band disease. *ISME Journal*, **5**, 559-564.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye-binding. *Analytical Biochemistry*, **72**, 248-254.

Bromage E., Carpenter L., Kaattari S. & Patterson M. (2009) Quantification of coral heat shock proteins from individual coral polyps. *Marine Ecology Progress Series*, **376**, 123-132.

Brookes P.S., Salinas E.P., Darley-Usmar K., Eiserich J.P., Freeman B.A. *et al.* (2000) Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome *c* release. *Journal of Biological Chemistry*, **275**, 20474-20479.

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

Brown G.C. (1999) Nitric oxide and mitochondrial respiration. *Biochimica Et Biophysica Acta-Bioenergetics*, **1411**, 351-369.

Brown G.C. & Borutaite V. (2002) Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radical Biology and Medicine*, **33**, 1440-1450.

Brune B., von Knethen A. & Sandau K.B. (1999) Nitric oxide (NO): An effector of apoptosis. *Cell Death and Differentiation*, **6**, 969-975.

Bryan N.S. & Grisham M.B. (2007) Methods to detect nitric oxide and its metabolites in biological samples. *Free Radical Biology and Medicine*, **43**, 645-657.

Bulgheresi S., Schabussova I., Chen T., Mullin N.P., Maizels R.M. *et al.* (2006) A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates symbiont acquisition by a marine nematode. *Applied and Environmental Microbiology*, **72**, 2950-2956.

Burriesci M.S., Raab T.K. & Pringle J.R. (2012) Evidence that glucose is the major transferred metabolite in dinoflagellate-cnidarian symbiosis. *Journal of Experimental Biology*, **215**, 3467-3477.

Bustos-Sanmamed P., Tovar-Mendez A., Crespi M., Sato S., Tabata S. *et al.* (2011) Regulation of nonsymbiotic and truncated haemoglobin genes of *Lotus japonicus* in plant organs and in responses to nitric oxide and hormones. *New Phytologist*, **189**, 765-776.

Buxton L., Gutowska M., Sawney S., Owen R. & Trapido-Rosenthal H. (2002) Increases in the activity of zooxanthellae nitric oxide synthase are associated with the temperature-induced bleaching of the coral *Madracis mirabilis*. *Integrative and Comparative Biology*, **42**, 1205-1205.

Byler K.A., Carmi-Veal M., Fine M. & Goulet T.L. (2013) Multiple symbiont acquisition strategies as an adaptive mechanism in the coral *Stylophora pistillata*. *PLoS ONE*, **8**, e59596.

Bythell J.C., Gladfelter E.H. & Bythell M. (1993) Chronic and catastrophic natural mortality of three common Caribbean reef corals. *Coral Reefs*, **12**, 143-152.

Cadenas E. (1989) Biochemistry of oxygen toxicity. *Annual Reviews of Biochemistry*, **58**, 79-110.

Carmen J.C. & Sinai A.P. (2007) Suicide prevention: Disruption of apoptotic pathways by protozoan parasites. *Molecular Microbiology*, **64**, 904-916.

Caro A. & Puntarulo S. (1998) Nitric oxide decreases superoxide anion generation by microsomes from soybean embryonic axes. *Physiologia Plantarum*, **104**, 357-364.

Carton Y., Frey F. & Nappi A.J. (2009) Parasite-induced changes in nitric oxide levels in *Drosophila paramelanica*. *Journal of Parasitology*, **95**, 1134-1141.

Cassina A. & Radi R. (1996) Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Archives of Biochemistry and Biophysics*, **328**, 309-316.

Catala M., Gasulla F., Pradas Del Real A.E., Garcia-Breijo F., Reig-Armana J. *et al.* (2010) Fungal-associated NO is involved in the regulation of oxidative stress during rehydration in lichen symbiosis. *BMC Microbiology*, **10**, 297.

Chae I.H., Park K.W., Kim H.S. & Oh B.H. (2004) Nitric oxide-induced apoptosis is mediated by Bax/Bcl-2 gene expression, transition of cytochrome *c*, and activation of caspase-3 in rat vascular smooth muscle cells. *Clinica Chimica Acta*, **341**, 83-91.

Chaki M., Valderrama R., Fernandez-Ocana A.M., Carreras A., Lopez-Jaramillo J. *et al.* (2009) Protein targets of tyrosine nitration in sunflower (*Helianthus annuus* L.) hypocotyls. *Journal of Experimental Botany*, **60**, 4221-4234.

Chan E.D., Morris K.R., Belisle J.T., Hill P., Remigio L.K. *et al.* (2001) Induction of inducible nitric oxide synthase-NO by lipoarabinomannan of *Mycobacterium tuberculosis* is mediated by MEK1-ERK, MKK7-JNK, and NK-kappa B signaling pathways. *Infection and Immunity*, **69**, 2001-2010.

Chandok M.R., Ytterberg A.J., van Wijk K.J. & Klessig D.F. (2003) The pathogen-inducible nitric oxide synthase (iNOS) in plants is a variant of the P protein of the glycine decarboxylase complex. *Cell*, **113**, 469-482.

Chen M.C., Cheng Y.M., Sung P.J., Kuo C.E. & Fang L.S. (2003) Molecular identification of Rab7 (ApRab7) in *Aiptasia pulchella* and its exclusion from phagosomes harbouring zooxanthellae. *Biochemical and Biophysical Research Communications*, **308**, 586-595.

Chen M.C., Hong M.C., Huang Y.S., Liu M.C. & Cheng Y.M. (2005) ApRab11, a cnidarian homologue of the recycling regulatory protein Rab11, is involved in the establishment and maintenance of the *Aiptasia-Symbiodinium* endosymbiosis. *Biochemical and Biophysical Research Communications*, **338**, 1607-1616.

Chung C.C., Hwang S.P.L. & Chang J. (2008) Nitric oxide as a signaling factor to upregulate the death-specific protein in a marine diatom, *Skeletonema costatum*, during blockage of electron flow in photosynthesis. *Applied and Environmental Microbiology*, **74**, 6521-6527.

Chung H.T., Pae H.O., Choi B.M., Billiar T.R. & Kim Y.M. (2001) Nitric oxide as a bioregulator of apoptosis. *Biochemical and Biophysical Research Communications*, **282**, 1075-1079.

Cikala M., Wilm B., Hobmayer E., Bottger A. & David C.N. (1999) Identification of caspases and apoptosis in the simple metazoan *Hydra*. *Current Biology*, **9**, 959-962.

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

Colasanti M., Lauro G.M. & Venturini G. (1995) NO in hydra feeding response. *Nature*, **374**.

Colasanti M., Venturini G., Merante A., Musci G. & Lauro G.M. (1997) Nitric oxide involvement in *Hydra vulgaris* very primitive olfactory-like system. *Journal of Neuroscience*, **17**, 493-499.

Colasanti M. & Suzuki H. (2000) The dual personality of NO. *Trends in Pharmacological Sciences*, **21**, 249-252.

Colasanti M., Persichini T. & Venturini G. (2010) Nitric oxide pathway in lower metazoans. *Nitric Oxide-Biology and Chemistry*, **23**, 94-100.

Cortes J. & Risk M.J. (1985) A reef under siltation stress - Cahuita, Costa-Rica. *Bulletin of Marine Science*, **36**, 339-356.

Crawford N.M. & Guo F.Q. (2005) New insights into nitric oxide metabolism and regulatory functions. *Trends in Plant Science*, **10**, 195-200.

Cristino L., Guylielmotti V., Cotugno A., Musio C. & Santillo S. (2008) Nitric oxide signaling pathways at neural level in invertebrates: Functional implications in cnidarians. *Brain Research*, **1225**, 17-25.

Crow J.P. & Ischiropoulos H. (1996) Detection and quantitation of nitrotyrosine residues in proteins: *In vivo* marker of peroxynitrite. *Nitric Oxide, Part B*, **269**, 185-194.

Csaszar N.B.M., Seneca F.O. & van Oppen M.J.H. (2009) Variation in antioxidant gene expression in the scleractinian coral *Acropora millepora* under laboratory thermal stress. *Marine Ecology Progress Series*, **392**, 93-102.

Csaszar N.B.M., Ralph P.J., Frankham R., Berkelmans R. & van Oppen M.J.H. (2010) Estimating the potential for adaptation of corals to climate warming. *PLoS ONE*, **5**.

Darwin C. (1859) *The Origin of Species*. CRW Publishing Ltd., London.

David C.N., Schmidt N., Schade M., Pauly B., Alexandrova O. *et al.* (2005) *Hydra* and the evolution of apoptosis. *Integrative and Comparative Biology*, **45**, 631-638.

Davidson S.K., Koropatnick T.A., Kossmehl R., Sycuro L. & McFall-Ngai M.J. (2004) NO means 'yes' in the squid-*Vibrio* symbiosis: Nitric oxide (NO) during the initial stages of a beneficial association. *Cellular Microbiology*, **6**, 1139-1151.

Davy S.K., Allemand D. & Weis V.M. (2012) The cell biology of cnidarian-dinoflagellate symbiosis. *Microbiology and Molecular Biology Reviews*, **76**, 229-261.

De'ath G., Fabricius K.E., Sweatman H. & Puotinen M. (2012) The 27-year decline of coral cover on the Great Barrier Reef and its causes. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 17995-17999.

del Giudice J., Cam Y., Damiani I., Fung-Chat F., Meilhoc E. *et al.* (2011) Nitric oxide is required for an optimal establishment of the *Medicago truncatula-Sinorhizobium meliloti* symbiosis. *New Phytologist*, **191**, 405-417.

Delledonne M. (2005) NO news is good news for plants. *Current Opinion in Plant Biology*, **8**, 390-396.

Deponte M. & Becker K. (2004) *Plasmodium falciparum* - do killers commit suicide? *Trends in Parasitology*, **20**, 165-169.

Deponte M. (2008) Programmed cell death in protists. *Biochimica Et Biophysica Acta-Molecular Cell Research*, **1783**, 1396-1405.

Desalvo M.K., Rvoolstra C., Sunagawa S., Schwarz J.A., Stillman J.H. *et al.* (2008) Differential gene expression during thermal stress and bleaching in the Caribbean coral *Montastraea faveolata*. *Molecular Ecology*, **17**, 3952-3971.

DeSalvo M.K., Sunagawa S., Voolstra C.R. & Medina M. (2010) Transcriptomic responses to heat stress and bleaching in the elkhorn coral *Acropora palmata*. *Marine Ecology Progress Series*, **402**.

Detournay O. & Weis V.M. (2011) Role of the sphingosine rheostat in the regulation of cnidarian-dinoflagellate symbioses. *Biological Bulletin*, **221**, 261-269.

Detournay O., Schnitzler C.E., Poole A. & Weis V.M. (2012) Regulation of cnidarian-dinoflagellate mutualisms: Evidence that activation of a host TGFbeta innate immune pathway promotes tolerance of the symbiont. *Developmental and Comparative Immunology*, **38**, 525-537.

Diekmann O.E., Bak R.P.M., Tonk L., Stam W.T. & Olsen J.L. (2002) No habitat correlation of zooxanthellae in the coral genus *Madracis* on a Curacao reef. *Marine Ecology Progress Series*, **227**, 221-232.

Done T.J. (1992) Phase shifts in coral reef communities and their ecological significance. *Hydrobiologia*, **247**, 121-132.

Donner S.D., Skirving W.J., Little C.M., Oppenheimer M. & Hoegh-Guldberg O. (2005) Global assessment of coral bleaching and required rates of adaptation under climate change. *Global Change Biology*, **11**, 2251-2265.

Douglas A.E. (2010) *The Symbiotic Habit*. Princeton University Press, Princeton.

Downs C.A., Fauth J.E., Halas J.C., Dustan P., Bemiss J. *et al.* (2002) Oxidative stress and seasonal coral bleaching. *Free Radical Biology and Medicine*, **33**, 533-543.

Downs C.A., Fauth J.E., Downs V.D. & Ostrander G.K. (2010) *In vitro* cell-toxicity screening as an alternative animal model for coral toxicology: Effects of heat stress, sulfide, rotenone, cyanide, and cuprous oxide on cell viability and mitochondrial function. *Ecotoxicology*, **19**, 171-184.

Dugas B., Mossalayi M.D., Damais C. & Kolb J.-P. (1995) Nitric oxide production by human monocytes: Evidence for a role of CD23. *Immunology Today*, **16**, 574-580.

Dunn A.K., Karr E.A., Wang Y.L., Batton A.R., Ruby E.G. *et al.* (2010) The alternative oxidase (AOX) gene in *Vibrio fischeri* is controlled by NsrR and upregulated in response to nitric oxide. *Molecular Microbiology*, **77**, 44-55.

Dunn S.R., Bythell J.C., Le Tissier M.D.A., Burnett W.J. & Thomason J.C. (2002) Programmed cell death and cell necrosis activity during hyperthermic stress-induced bleaching of the symbiotic sea anemone *Aiptasia* sp. *Journal of Experimental Marine Biology and Ecology*, **272**, 29-53.

Dunn S.R., Thomason J.C., Le Tissier M.D.A. & Bythell J.C. (2004) Heat stress induces different forms of cell death in sea anemones and their endosymbiotic algae depending on temperature and duration. *Cell Death and Differentiation*, **11**, 1213-1222.

Dunn S.R., Phillips W.S., Spatafora J.W., Green D.R. & Weis V.M. (2006) Highly conserved caspase and Bcl-2 homologues from the sea anemone *Aiptasia pallida*: Lower metazoans as models for the study of apoptosis evolution. *Journal of Molecular Evolution*, **63**, 95-107.

Dunn S.R., Schnitzler C.E. & Weis V.M. (2007) Apoptosis and autophagy as mechanisms of dinoflagellate symbiont release during cnidarian bleaching: Every which way you lose. *Proceedings of the Royal Society B-Biological Sciences*, **274**, 3079-3085.

Dunn S.R. (2009) Immunorecognition and immunoreceptors in the Cnidaria. *Invertebrate Survival Journal*, **6**, 7-14.

Dunn S.R. & Weis V.M. (2009) Apoptosis as a post-phagocytic winnowing mechanism in a coral-dinoflagellate mutualism. *Environmental Microbiology*, **11**, 268-276.

Dunn S.R., Pernice M., Green K., Hoegh-Guldberg O. & Dove S.G. (2012) Thermal stress promotes host mitochondrial degradation in symbiotic cnidarians: Are the batteries of the reef going to run out? *PLoS ONE*, **7**, e39024.

Dykens J.A. & Shick J.M. (1982) Oxygen production by endosymbiotic algae controls superoxide dismutase activity in their animal host. *Nature*, **297**, 579-580.

Dykens J.A., Shick J.M., Benoit C., Buettner G.R. & Winston G.W. (1992) Oxygen radical production in the sea-anemone *Anthopleura elegantissima* and its endosymbiotic algae. *Journal of Experimental Biology*, **168**, 219-241.

Eiserich J.P., Hristova M., Cross C.E., Jones A.D., Freeman B.A. *et al.* (1998) Formation of nitric oxide derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature*, **391**, 393-397.

Elmore S. (2007) Apoptosis: A review of programmed cell death. *Toxicological Pathology*, **35**, 495-516.

Estevez A.G. & Jordan J. (2002) Nitric oxide and superoxide, a deadly cocktail. *Nitric Oxide: Novel Actions, Deleterious Effects and Clinical Potential*, **962**, 207-211.

Fabina N.S., Putnam H.M., Franklin E.C., Stat M. & Gates R.D. (2012) Transmission mode predicts specificity and interaction patterns in coral-*Symbiodinium* networks. *PLoS ONE*, **7**, e44970.

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

Fabricius K.E., Langdon C., Uthicke S., Humphrey C., Noonan S. *et al.* (2011) Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations. *Nature Climate Change*, **1**, 165-169.

Fadlallah T.H. (1983) Sexual reproduction, development and larval biology in scleractinian corals, a review. *Coral Reefs*, **2**, 129-150.

Fagoonee I., Wilson H.B., Hassell M.P. & Turner J.R. (1999) The dynamics of zooxanthellae populations: A long-term study in the field. *Science*, **283**, 843-845.

Falkowski P.G., Dubinsky Z., Muscatine L. & Porter J.W. (1984) Light and the bioenergetics of a symbiotic coral. *Bioscience*, **34**, 705-709.

Fang F.C. (2004) Antimicrobial reactive oxygen and nitrogen species: Concepts and controversies. *Nature Reviews Microbiology*, **2**, 820-832.

Fautin D.G. (1991) The anemonefish symbiosis: What is known and what is not. *Symbiosis*, **10**, 23-46.

Ferrer-Sueta G. & Radi R. (2009) Chemical biology of peroxynitrite: Kinetics, diffusion, and radicals. *Acs Chemical Biology*, **4**, 161-177.

Fisher P.L., Malme M.K. & Dove S. (2012) The effect of temperature stress on coral-*Symbiodinium* associations containing distinct symbiont types. *Coral Reefs*, **31**, 473-485.

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

Fitt W.K., Gates R.D., Hoegh-Guldberg O., Bythell J.C., Jatkar A. *et al.* (2009) Response of two species of Indo-Pacific corals, *Porites cylindrica* and *Stylophora pistillata*, to short-term thermal stress: The host does matter in determining the tolerance of corals to bleaching. *Journal of Experimental Marine Biology and Ecology*, **373**, 102-110.

Flores-Ramirez L.A. & Linan-Cabello M.A. (2007) Relationships among thermal stress, bleaching and oxidative damage in the hermatypic coral, *Pocillopora capitata*. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology*, **146**, 194-202.

Foresi N., Correa-Aragunde N., Parisi G., Calo G., Salerno G. *et al.* (2010) Characterisation of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance- and growth phase-dependent. *The Plant Cell*, **22**, 3816-3830.

Franklin E.C., Stat M., Pochon X., Putnam H.M. & Gates R.D. (2012) GeoSymbio: A hybrid, cloud-based web application of global geospatial bioinformatics

and ecoinformatics for *Symbiodinium*-host symbioses. *Molecular Ecology Resources*, **12**, 369-373.

Fukuto J.M. & Ignarro L.J. (1997) *In vivo* aspects of nitric oxide (NO) chemistry: Does peroxynitrite (-OONO) play a major role in cytotoxicity? *Accounts of Chemical Research*, **30**, 149-152.

Furla P., Allemand D., Shick J.M., Ferrier-Pages C. & Richier S. (2005) The symbiotic anthozoan: A physiological chimera between alga and animal. *Integrative and Comparative Biology*, **45**, 595-604.

Gadelha F.R., Thomson L., Fagian M.M., Costa A.D.T., Radi R. *et al.* (1997) Ca2+-independent permeabilization of the inner mitochondrial membrane by peroxynitrite is mediated by membrane protein thiol cross-linking and lipid peroxidation. *Archives of Biochemistry and Biophysics*, **345**, 243-250.

Ganassi S., Tagliazucchi D. & Mola L. (2005) Occurrence of nitric oxide synthase in *Megoura viciae* Buckton (Homoptera, Aphididae): A histochemical and immunohistochemical localisation. *European Journal of Histochemistry*, **49**, 385-393.

Gates R.D., Baghdasarian G. & Muscatine L. (1992) Temperature stress causes host cell detachment in symbiotic cnidarians: Implications for coral bleaching. *Biological Bulletin*, **182**, 324-332.

Gates R.D. & Muscatine L. (1992) Three methods for isolating viable anthozoan endoderm cells with their intracellular symbiotic dinoflagellates. *Coral Reefs*, **11**, 143-145.

Gaupels F., Spiazzi-Vandelle E., Yang D. & Delledonne M. (2011) Detection of peroxynitrite accumulation in *Arabidopsis thaliana* during the hypersensitive defense response. *Nitric Oxide-Biology and Chemistry*, **25**, 222-228.

Genty B., Briantais J.M. & Baker N.R. (1989) The relationship between the quantum yield of photosynthetic electron-transport and quenching of chlorophyll fluorescence. *Biochimica Et Biophysica Acta*, **990**, 87-92.

Ghasemi A., Hedayati M. & Biabani H. (2007) Protein precipitation methods evaluated for determination of serum nitric oxide end products by the Griess reaction. *Journal of Medical Sciences Research*, **2**, 29-32.

Ghigo D., Todde R., Ginsburg H., Costamagna C., Gautret P. *et al.* (1995) Erythrocyte stages of *Plasmodium falciparum* exhibit a high nitric-oxide synthase (NOS) activity and release an NOS-inducing soluble factor. *Journal of Experimental Medicine*, **182**, 677-688.

Giovine M., Pozzolini M., Favre A., Bavestrello G., Cerrano C. *et al.* (2001) Heat stress-activated, calcium-dependent nitric oxide synthase in sponges. *Nitric Oxide-Biology and Chemistry*, **5**, 427-431.

Glas M.S., Sato Y., Ulstrup K.E. & Bourne D.G. (2012) Biogeochemical conditions determine virulence of black band disease in corals. *ISME Journal*, **6**, 1526-1534.

Gloire G., Legrand-Poels S. & Piette J. (2006) NF-kappa B activation by reactive oxygen species: Fifteen years later. *Biochemical Pharmacology*, **72**.

Glynn P.W. (1983) Extensive bleaching and death of reef corals on the Pacific coast of Panama. *Environmental Conservation*, **10**, 149-154.

Glynn P.W. (1984) Widespread coral mortality and the 1982-83 El-Nino warming event. *Environmental Conservation*, **11**, 133-146.

Glynn P.W., Peters E.C. & Muscatine L. (1985) Coral tissue microstructure and necrosis: Relation to catastrophic coral mortality in Panama. *Diseases of Aquatic Organisms*, **1**, 29-38.

Glynn P.W. & Dcroz L. (1990) Experimental-evidence for high-temperature stress as the cause of El-Nino-coincident coral mortality. *Coral Reefs*, **8**, 181-191.

Glynn P.W., Mate J.L., baker A.C. & Calderon M.O. (2001) Coral bleaching and mortality in Panama and Ecuador during the 1997-1998 El Nino-Southern oscillation event: Spatial/temporal patterns and comparisons with the 1982-1983 event. *Bulletin of Marine Science*, **69**, 79-109.

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

Goreau T.F. (1990) Coral bleaching in Jamaica. *Nature*, **343**, 417.

Goreau T.F. & MacFarlane A.H. (1990) Reduced growth in *Montastrea annularis* during the 1997-1998 coral bleaching event. *Coral Reefs*, **8**, 211-215.

Gould K.S., Lamotte O., Klinguer A., Pugin A. & Wendehenne D. (2003) Nitric oxide production in tobacco leaf cells: A generalized stress response? *Plant, Cell and Environment*, **26**, 1851-1862.

Goulet T.L. & Coffroth M.A. (2004) The genetic identity of dinoflagellate symbionts in Caribbean octocorals. *Coral Reefs*, **23**, 465-472.

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

Goulet T.L. (2007) Most scleractinian corals and octocorals host a single symbiotic zooxanthella clade. *Marine Ecology Progress Series*, **335**, 243-248.

Gozuacik D. & Kimchi A. (2004) Autophagy as a cell death and tumor suppressor mechanism. *Oncogene*, **23**, 2891-2906.

Graham N.A.J., Wilson S.K., Jennings S., Polunin N.V.C., Bijoux J.P. *et al.* (2006) Dynamic fragility of oceanic coral reef ecosystems. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 8425-8429.

Graham N.A.J., McClanahan T.R., MacNeil M.A., Wilson S.K., Polunin N.V.C. *et al.* (2008) Climate warming, marine protected areas and the ocean-scale integrity of coral reef ecosystems. *PLoS ONE*, **3**, e3039.

Griffith O.W. & Stuehr D.J. (1995) Nitric oxide synthases: Properties and catalytic mechanisms. *Annual Reviews of Physiology*, **57**, 707-736.

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

Guest J.R., Baird A.H., Maynard J.A., Muttaqin E., Edwards A.J. *et al.* (2012) Contrasting patterns of coral bleaching susceptibility in 2010 suggest an adaptive response to thermal stress. *PLoS ONE*, **7**, e33353.

Halliwell B. (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiology*, **141**, 312-322.

Halliwell B. & Gutteridge J.M.C. (2007) *Free radicals in biology and medicine, 4th Edition*. Oxford University Press, Oxford.

Harvell C.D., Kim K., Burkholder J.M., Colwell R.R., Epstein P.R. *et al.* (1999) Emerging marine diseases: Climate links and anthropogenic factors. *Science*, **285**, 1505-1510.

Hawkins T.D. & Davy S.K. (2012) Nitric oxide production and tolerance differ among *Symbiodinium* types exposed to heat stress. *Plant and Cell Physiology*, **53**, 1889-1898.

Hennige S.J., Suggett D.J., Warner M.E., McDougall K.E. & Smith D.J. (2009) Photobiology of *Symbiodinium* revisited: Bio-physical and bio-optical signatures. *Coral Reefs*, **28**, 179-195.

Herce-Pagliai C., Kotecha S. & Shuker D.E.G. (1998) Analytical methods for 3-nitrotyrosine as a marker of exposure to reactive nitrogen species: A review. *Nitric Oxide-Biology and Chemistry*, **2**, 324-336.

Herrera-Ortiz A., Martinez-Barnetche J., Smit N., Rodriguez M.H. & Lanz-Mendoza H. (2011) The effect of nitric oxide and hydrogen peroxide in the

activation of the systemic immune response of *Anopheles albimanus* infected with *Plasmodium berghei*. *Developmental and Comparative Immunology*, **35**, 44-50.

Hill R. & Ralph P.J. (2007) Post-bleaching viability of expelled zooxanthellae from the scleractinian coral *Pocillopora damicornis*. *Marine Ecology Progress Series*, **352**, 137-144.

Hill R., Brown C.M., DeZeeuw K., Campbell D.A. & Ralpha P.J. (2011) Increased rate of D1 repair in coral symbionts during bleaching is insufficient to counter accelerated photo-inactivation. *Limnology and Oceanography*, **56**, 139-146.

Hippe D., Gais A., Gross U. & Lueder C.G.K. (2009) Modulation of caspase activation by *Toxoplasma gondii*. *Methods in Molecular Biology*, 275-288.

Hoegh-Guldberg O., McCloskey L.R. & Muscatine L. (1987) Expulsion of zooxanthellae by symbiotic cnidarians from the Red Sea. *Coral Reefs*, **5**, 201-204.

Hoegh-Guldberg O. (1999) Climate change, coral bleaching and the future of the world's coral reefs. *Marine and Freshwater Research*, **50**, 839-866.

Hoegh-Guldberg O., Mumby P.J., Hooten A.J., Steneck R.S., Greenfield P. *et al.* (2007) Coral reefs under rapid climate change and ocean acidification. *Science*, **318**, 1737-1742.

Hoegh-Guldberg O. (2010) The impact of climate change on coral reef ecosystems. In: *Coral Reefs: An Ecosystem in Transition* (eds Z. Dubinsky & N. Sambler), pp. 391-403. Springer, New York.

Hoegh-Guldberg O. & Bruno J.F. (2010) The impact of climate change on the world's marine ecosystems. *Science*, **328**, 1523-1528.

Hughes T.P. (1994) Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral reef. *Science*, **265**, 1547-1551.

Hughes T.P., Rodrigues M.J., Bellwood D.R., Caccarelli D., Hoegh-Guldberg O. *et al.* (2007) Phase shifts, herbivory, and the resilience of coral reefs to climate change. *Current Biology*, **17**, 360-365.

Hughes T.P., Graham N.A.J., Jackson J.B.C., Mumby P.J. & Steneck R.S. (2010) Rising to the challenge of sustaining coral reef resilience. *Trends in Ecology & Evolution*, **25**, 633-642.

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

Ignarro L.J., Buga G.M., Woods K.S., Byrns R.E. & Chaudhuri G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 9265-9269.

Ignarro L.J., Fukuto J.M., Griscavage J.M., Rogers N.E. & Byrns R.E. (1993) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 8103-8107.

IPCC (2007) *Synthesis report. Contribution of Working Groups I, II, and II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change.*

Ischiropoulos H. & Almehdi A.B. (1995) Peroxynitrite-mediated oxidative protein modifications. *FEBS Letters*, **364**, 279-282.

Ischiropoulos H., Beckman J.S., Crow J.P., Ye Y.Z., Royall J.A. *et al.* (1995) Detection of peroxynitrite. *Methods in Enzymology*, **7**, 109-115.

Jacklet J.W. (1997) Nitric oxide signaling in invertebrates. *Invertebrate Neuroscience*, **3**, 1-14.

Jackson J.B.C. (2008) Ecological extinction and evolution in the brave new ocean. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11458-11465.

Jackson J.B.C. (2010) The future of the oceans past. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **365**, 3765-3778.

Janouskovec J., Horak A., Obornik M., Lukes J. & Keeling P.J. (2010) A common red algal origin of the apicomplexan, dinoflagellate, and heterokont plastids. *Proceedings of the National Academy of Sciences of the United States of America*.

Jeong H.J., Yoo Y.D., Kang N.S., Lim A.S., Seong K.A. *et al.* (2012) Heterotrophic feeding is a newly identified survival strategy of the dinoflagellate *Symbiodinium*. *Proceedings of the National Academy of Sciences of the United States of America*.

Johnson L.V., Walsh M.L., Bockus B.J. & Chen L.B. (1981) Monitoring of relative mitochondrial-membrane potential in living cells by fluorescence microscopy. *Journal of Cell Biology*, **88**, 526-535.

Jones A. & Berkelmans R. (2010) Potential costs of acclimatization to a warmer climate: Growth of a reef coral with heat tolerant *vs.* sensitive symbiont types. *PLoS ONE*, **5**, e10437.

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

Jones R.J. (2008) Coral bleaching, bleaching-induced mortality, and the adaptive significance of the bleaching response. *Marine Biology*, **154**, 65-80.

Kerr J.F.R., Whyllie A.H. & Currie A.R. (1972) Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. *British Journal of Cancer*, **26**, 239-257.

Khalesi M. (2008) Cell cultures from the symbiotic soft coral *Sinularia flexibilis*. *In Vitro Cellular & Developmental Biology - Animal*, **44**, 330-338.

Kijimoto-Ochiai S. (2002) CD23 (the low-affinity IgE receptor) as a C-type lectin: A multidomain and multifunctional molecule. *Cell and Molecular Life Science*, **59**, 648-664.

Kim D., Yamaguchi K. & Oda T. (2006) Nitric oxide synthase-like enzyme mediated nitric oxide generation by harmful red tide phytoplankton, *Chattonella marina*. *Journal of Plankton Research*, **28**, 613-620.

Kim D., Kang Y.S., Lee Y., Yamaguchi K., Matsuoka K. *et al.* (2008) Detection of nitric oxide (NO) in marine phytoplankters. *Journal of Bioscience and Bioengineering*, **105**, 414-417.

Kim M.Y., Dong M., Dedon P.C. & Wogan G.N. (2005) Effects of peroxynitrite dose and dose rate on DNA damage and mutation in the supF shuttle vector. *Chemical Research in Toxicology*, **18**, 76-86.

Kim Y.M., Talanian R.V. & Billiar T.R. (1997) Nitric oxide inhibits apoptosis by preventing increases in caspase-3 like activity *via* two distinct mechanisms. *Journal of Biological Chemistry*, **272**, 31138-31148.

Kimbrell D.A. & Beutler B. (2001) The evolution and genetics of innate immunity. *Nature Reviews Genetics*, **2**, 256-267.

Knowlton N. (2001) The future of coral reefs. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 5419-5425.

Knowlton N. & Rohwer F. (2003) Multispecies microbial mutualisms on coral reefs: The host as a habitat. *American Naturalist*, **162**, S52-S62.

Knutson T.R., McBride J.L., Chan J., Emanuel K., Holland G. *et al.* (2010) Tropical cyclones and climate change. *Nature Geosciences*, **3**, 157-163.

Kopp C., Pernice M., Domart-Coulon I., Djediat C., Spangenberg J.E. *et al.* (2013) Highly dynamic cellular-level response of symbiotic coral to a sudden increase in environmental nitrogen. *mBio*, **4**, e00052-00013.

Kramer W.E., Schrameyer V., Hill R., Ralph P.J. & Bischof K. (2013) PSII activity and pigment dynamics of *Symbiodinium* in two Indo-Pacific corals exposed to short-term high-light stress. *Marine Biology*, **160**, 563-577.

Kroemer G. & Reed J.C. (2000) Mitochondrial control of cell death. *Nature Medicine*, **6**, 513-519.

Kroemer G., Galluzzi L. & Brenner C. (2007) Mitochondrial membrane permeabilization in cell death. *Physiological Reviews*, **87**, 99-163.

Kumar S. & Cakouros D. (2003) The role of caspases in apoptosis. In: *Genetics of Apoptosis* (ed S. Grimm), pp. 31-45. Garland Science, Abingdon, U.K.

Kuranaga E. & Miura M. (2007) Nonapoptotic functions of caspases: Caspases as regulatory molecules for immunity and cell-fate determination. *Trends in Cell Biology*, **17**, 135-144.

Kvennefors E.C.E., Leggat W., Hoegh-Guldberg O., Degnan B.M. & Barnes A.C. (2008) An ancient and variable mannose-binding lectin from the coral *Acropora millepora* binds both pathogens and symbionts. *Developmental and Comparative Immunology*, **32**, 1582-1592.

Kvitt H., Rosenfeld H., Zandbank K. & Tchernov D. (2011) Regulation of apoptotic pathways by *Stylophora pistillata* (Anthozoa, Pocilloporidae) to survive thermal stress and bleaching. *PLoS ONE*, **6**, e28665.

LaJeunesse T.C. (2002) Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs. *Marine Biology*, **141**, 387-400.

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

LaJeunesse T.C., Bhagooli R., Hidaka M., DeVantier L., Done T. *et al.* (2004) Closely related *Symbiodinium* spp. differ in relative dominance in coral reef host communities across environmental, latitudinal and biogeographic gradients. *Marine Ecology Progress Series*, **284**, 147-161.

LaJeunesse T.C. (2005) "Species" radiations of symbiotic dinoflagellates in the Atlantic and Indo-Pacific since the Miocene-Pliocene transition. *Molecular Biology and Evolution*, **22**, 1158-1158.

LaJeunesse T.C., Bonilla H.R., Warner M.E., Wills M., Schmidt G.W. *et al.* (2008) Specificity and stability in high latitude eastern Pacific coral-algal symbioses. *Limnology and Oceanography*, **53**, 719-727.

Laliberte J. & Carruthers V.B. (2008) Host cell manipulation by the human pathogen *Toxoplasma gondii*. *Cellular and Molecular Life Sciences*, **65**, 1900-1915.

Lasi M., David C.N. & Bottger A. (2010a) Apoptosis in pre-Bilaterians: *Hydra* as a model. *Apoptosis*, **15**, 269-278.

Lasi M., Pauly B., Schmidt N., Cikala M., Stiening B. *et al.* (2010b) The molecular cell death machinery in the simply cnidarian *Hydra* includes an expanded caspase family and pro- and anti-apoptotic Bcl-2 proteins. *Cell Research*, **1**, 1-14.

Leggat W., Seneca F., Wasmund K., Ukani L., Yellowlees D. *et al.* (2011) Differential responses of the coral host and their algal symbiont to thermal stress. *PLoS ONE*, **6**, e26687.

Leng J., Butcher B.A. & Denkers E.Y. (2009) Dysregulation of macrophage signal transduction by *Toxoplasma gondii*: Past progress and recent advances. *Parasite Immunology*, **31**, 717-728.

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

Lesser M.P. (1996) Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. *Limnology and Oceanography*, **41**, 271-283.

Lesser M.P. (1997) Oxidative stress causes coral bleaching during exposure to elevated temperatures. *Coral Reefs*, **16**, 187-192.

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

Lesser M.P. (2006) Oxidative stress in marine environments. *Annual Reviews of Physiology*, **68**, 253-278.

Lesser M.P. (2011) Coral bleaching: Causes and mechanisms. In: *Coral Reefs: An Ecosystem in Transition* (eds T.J. Dubinsky & J.S. Stamler), pp. 405-419. Springer, Berlin.

Li M.O., Sarkisian M.R., Mehal W.Z., Rakic P. & Flavell R.A. (2003) Phosphatidylserine receptor is required for clearance of apoptotic cells. *Science*, **302**, 1560-1563.

Li P., Nijhawan D., Budihardjo I., Srinivasula S.M., Ahmad M. *et al.* (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**.

Liddell W.D. & Ohlhorst S.L. (1986) Changes in benthic community composition following the mass mortality of *Diadema* at Jamaica. *Journal of Experimental Marine Biology and Ecology*, **95**, 271-278.

Lien Y.T., Keshavmurthy S., Nakano Y., Plathong S., Huang H. *et al.* (2013) Host genetics and *Symbiodinium* D diversity in a stress-tolerant scleractinian coral, *Oulastrea crispata*, in the West Pacific. *Marine Ecology Progress Series*, **473**, 163-177.

Liew F.Y., Li Y., Moss D., Parkinson C., Rogers M.V. *et al.* (1991) Resistence to *Leishmania major* infection correlates with the induction of nitric oxide synthase in murine macrophages. *European Journal of Immunology*, **21**, 3009-3014.

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

Liu B., Behura S.K., Clem R.J., Schneemann A., Becnel J. *et al.* (2013) p53-mediated rapid induction of apoptosis conveys resistance to viral infection in *Drosophila melanogaster*. *PLoS Pathogens*, **9**, e1003137.

Liu K., Sun J., Song Y., Liu B. & Xu Y. (2004) Superoxide, hydrogen peroxide, and hydroxyl radical in D1/D2/cytochrome b-559 photosystem II reaction centre complex. *Photosynthesis Research*, **81**, 41-47.

Liu L. & Stamler J.S. (1999) NO: An inhibitor of cell death. *Cell Death and Differentiation*, **6**.

Liu Y.J., Jiang H.F., Zhao Z.G. & An L.Z. (2010) Nitric oxide synthase like activity-dependent nitric oxide production protects against chilling-induced oxidative damage in *Chorispora bungeana* suspension cultured cells. *Plant Physiology and Biochemistry*, **48**, 936-944.

Logan D.D.K., LaFlamme A.C., Weis V.M. & Davy S.K. (2010) Flow-cytometric characterization of the cell-surface glycans of symbiotic dinoflagellates (*Symbiodinium* spp.). *Journal of Phycology*, **46**, 525-533.

Loya Y., Sakai K., Yamazato K., Nakano Y., Sambali H. *et al.* (2001) Coral bleaching: The winners and the losers. *Ecology Letters*, **4**, 122-131.

Macdonald A.H.H., Sampayo E.M., Ridgway T. & Schleyer M.H. (2008) Latitudinal symbiont zonation in *Stylophora pistillata* from southeast Africa. *Marine Biology*, **154**, 209-217.

MacMicking J., Xie Q.W. & Nathan C. (1997) Nitric oxide and macrophage function. *Annual Reviews of Immunology*, **15**, 323-350.

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

Marlow H.Q. & Martindale M.Q. (2007) Embryonic development in two species of scleractinian coral embryos: *Symbiodinium* localisation and mode of gastrulation. *Evolution and Development*, **9**, 355-367.

Martindale J.L. & Holbrook N.J. (2002) Cellular response to oxidative stress: Signaling for suicide and survival. *Journal of Cellular Physiology*, **192**, 1-15.

McClanahan T.R. (2004) The relationship between bleaching and mortality of common corals. *Marine Biology*, **144**, 1239-1245.

McClanahan T.R., Baird A.H., Marshall P.A. & Toscano M.A. (2004) Comparing bleaching and mortality responses of hard corals between southern Kenya and the Great Barrier Reef, Australia. *Marine Pollution Bulletin*, **48**, 327-335.

McClanahan T.R., Weil E., Cortes J., baird A.H. & Ateweberhan M. (2009) Consequences of coral bleaching for sessile reef organisms. In: *Coral Bleaching: Patterns, Processes, Causes and Consequences* (eds M.J. van Oppen & J.M. Lough), pp. 121-134. Springer-Verlag, Heidelberg, Germany.

McGinty E.S., Pieczonka J. & Mydlarz L.D. (2012) Variations in reactive oxygen release and antioxidant activity in multiple *Symbiodinium* types in response to elevated temperature. *Microbial Ecology*, **64**, 1000-1007.

McQuade L.E. & Lippard S.J. (2010) Fluorescent probes to investigate nitric oxide and other reactive nitrogen species in biology (truncated form: Fluorescent probes of reactive nitrogen species). *Current Opinion in Chemical Biology*, **14**, 43-49.

Meilhoc E., Boscari A., Bruand C., Puppo A. & Brouquisse R. (2011) Nitric oxide in legume-*Rhizobium* symbiosis. *Plant Science*, **181**, 573-581.

Mendes A.F., Caramona M.M., Carvalho A.P. & Lopes M.C. (2003) Differential roles of hydrogen peroxide and superoxide in mediating IL-1-induced NF-kappa B activation and iNOS expression in bovine articular chondrocytes. *Journal of Cellular Biochemistry*, **88**, 783-793.

Messmer U.K., Ankarcrona M., Nicotera P. & Brune B. (1994) p53 expression in nitric oxide-induced apoptosis. *FEBS Letters*, **355**, 23-26.

Meyer E. & Weis V.M. (2012) Study of cnidarian dinoflagellate symbiosis in the "omics" age. *Biological Bulletin*, **223**, 44-65.

Michel T. & Feron O. (1997) Nitric oxide synthases: Which, where, how, and why? *Journal of Clinical Investigation*, **100**, 2146-2152.

Middlebrook R. & Anthony K.R. (2010) Heating rate and symbiont productivity are key factors determining thermal stress in the reef-building coral *Acropora formosa*. *Journal of Experimental Biology*, **213**, 1026-1034.

Mieog J.C., Olsen J.L., Berkelmans R., Bleuler-Martinez S.A., Willis B.L. *et al.* (2009) The roles and interactions of symbiont, host and environment in defining coral fitness. *PLoS ONE*, **4**.

Miller M.R., Okubo K., Roseberry M.J., Webb D.J. & Megson I.L. (2004) Extracellular nitric oxide release mediates soluble guanylate cyclase-independent vasodilator action of spermine NONOate: comparison with other nitric oxide donors in isolated rat femoral arteries. *Journal of Cardiovascular Pharmacology*, **43**, 440-451.

Miranda K.M., Espey M.G. & Wink D.A. (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*, **5**, 62-71.

Misra A.N., Misra M. & Singh R. (2011) Nitric oxide ameliorates stress responses in plants. *Plant Soil and Environment*, **57**, 95-100.

Mittler R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, **7**, 405-410.

Moharikar S., D'Souza J.S., Kulkarni A.B. & Rao B.J. (2006) Apoptotic-like cell death pathway is induced in unicellular chlorophyte *Chlamydomonas reinhardtii* (Chlorophyceae) cells following UV irradiation: Detection and functional analyses. *Journal of Phycology*, **42**, 423-433.

Moore R.B., Obornik M., Janouskovec J., Chrudimsky T., Vancova M. *et al.* (2008) A photosynthetic alveolate closely related to apicomplexan parasites. *Nature*, **451**, 959-963.

Moroz L.L. (2001) Gaseous transmission across time and species. *American Zoologist*, **41**, 304-320.

Moroz L.L., Meech R.W., Sweedler J.V. & Mackie G.O. (2004) Nitric oxide regulates swimming in the jellyfish *Aglantha digitale*. *Journal of Comparative Neurology*, **471**, 26-36.

Moroz L.L. & Kohn A.B. (2007) On the comparative biology of Nitric Oxide (NO) synthetic pathways: Parallel evolution of NO-mediated signaling. In: *Advances in Experimental Biology* (eds T. Professor Bruno & T. Professor Barry), pp. 1-44. Elsevier, Amsterdam.

Morrall C.E., Galloway T.S., Trapido-Rosenthal H.G. & Depledge M.H. (2000) Characterisation of nitric oxide synthase activity in the tropical sea anemone *Aiptasia pallida*. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, **125**, 483-491.

Muller-Parker G. & D'Elia C.F. (1997) Interactions between corals and their symbiotic algae. In: *Life and death of coral reefs* (ed C. Birkeland), pp. 96-114. Chapmand & Hall, New York.

Murik O. & Kaplan A. (2009) Paradoxically, prior acquisition of antioxidant activity enhances oxidative stress-induced cell death. *Environmental Microbiology*, **11**, 2301-2309.

Muscatine L. & Hand C. (1958) Direct evidence for the transfer of materials from symbiotic algae to the tissues of a coelenterate. *Proceedings of the National Academy of Sciences of the United States of America*, **44**, 1259-1263.

Muscatine L., Cook C.B., Pardy R.L. & Pool R.R. (1975a) Uptake, recognition, and maintenance of symbiotic *Chlorella* by *Hydra viridis*. *Symposia of the Society of Experimental Biology*, **29**, 175-203.

Muscatine L., Pool R.R. & Trench R.K. (1975b) Symbiosis of algae and invertebrates - aspects of symbiont surface and host-symbiont interface. *Transactions of the American Microscopical Society*, **94**, 450-469.

Muscatine L., Falkowski P.G., Porter J.W. & Dubinsky Z. (1984) Fate of photosynthetically fixed carbon in light-adapted and shade-adapted colonies of the symbiotic coral *Stylophora pistillata*. *Proceedings of the Royal Society B-Biological Sciences*, **222**, 181-202.

Mydlarz L.D., Couch C.S., Weil E., Smith G. & Harvell C.D. (2009) Immune defenses of healthy, bleached and diseased *Montastraea faveolata* during a natural bleaching event. *Diseases of Aquatic Organisms*, **87**, 67-78.

Mydlarz L.D., McGinty E.S. & Harvell C.D. (2010) What are the physiological and immunological responses of coral to climate warming and disease? *Journal of Experimental Biology*, **213**, 934-945.

Nagano T. & Yoshimura T. (2002) Bioimaging of nitric oxide. *Chemistry Reviews*, **102**, 1235-1270.

Nagata M., Murakami E., Shimoda Y., Shimoda-Sasakura F., Kucho K. *et al.* (2011) Regulation of a class 1 haemoglobin gene and production of nitric oxide in response to symbiotic and pathogenic bacteria in *Lotus japonicus. Molecular Plant-Microbe Interactions*, **21**, 1175-1183.

Nappi A.J. & Ottaviani E. (2000) Cytotoxicity and cytotoxic molecules in invertebrates. *Bioessays*, **22**, 469-480.

Nathan C. & Shiloh M.U. (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 8841-8848.

Nedelcu A.M., Driscoll W.W., Durand P.M., Herron M.D. & Rashidi A. (2011) On the paradigm of altruistic suicide in the unicellular world. *Evolution*, **65**, 3-20.

Nicholson D.W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death and Differentiation*, **6**, 1028-1042.

Nicotera P. & Melino G. (2004) Regulation of the apoptosis-necrosis switch. *Oncogene*, **23**, 2757-2765.

Nii C.M. & Muscatine L. (1997) Oxidative stress in the symbiotic sea anemone *Aiptasia pulchella* (Carlgren, 1943): Contribution of the animal to superoxide ion production at elevated temperature. *Biological Bulletin*, **192**, 444-456.

Nürnberger T., Brunner F., Kemmerling B. & Piater L. (2004) Innate immunity in plants and animals: Striking similarities and obvious differences. *Immunological Reviews*, **198**, 249-266.

Nussler A.K., Glanemann M., Schirmeier A., Liu L. & Nuessler N.C. (2006) Fluorometric measurement of nitrite/nitrate by 2,3-diaminonaphthalene. *Nature Protocols*, **1**, 2223-2226.

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

Orr J.C., Fabry V.J., Aumont O., Bopp L., Doney S.C. *et al.* (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, **437**, 681-686.

Ott T., van Dongen J.T., Gunther C., Krusell L., Desbrosses G. *et al.* (2005) Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology*, **15**, 531-535.

Pacher P., Beckman J.S. & Liaudet L. (2007) Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews*, **87**, 315-424.

Padua Queiroz L., Jr., Mattos M.E., Jr., Silva M.F. & Silva C.L. (2010) TGF-B and CD23 are involved in nitric oxide production by pulmonary macrophages activated by B-glucan from *Paracoccidioides brasiliensis*. *Medical Microbiology and Immunology*, **199**, 61-69.

Palmer C.V., Modi C.K. & Mydlarz L.D. (2009) Coral fluorescent proteins as antioxidants. *PLoS ONE*, **4**, Article No.: e7298.

Palmer C.V., Bythell J.C. & Willis B.L. (2010) Levels of immunity parameters underpin bleaching and disease susceptibility of reef corals. *FASEB Journal*, **24**, 1935-1946.

Palmer C.V., McGinty E.S., Cummings D.J., Smith S.M., Bartels E. *et al.* (2011) Patterns of coral ecological immunology: Variation in the responses of Caribbean corals to elevated temperature and a pathogen elicitor. *Journal of Experimental Biology*, **214**, 4240-4249.

Palmer C.V., Bythell J.C. & Willis B.L. (2012) Enzyme activity demonstrates multiple pathways of innate immunity in Indo-Pacific anthozoans. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 3879-3887.

Palmer C.V. & Traylor-Knowles N. (2012) Towards an integrated network of coral immune mechanisms. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 4106-4114.

Pandolfi J.M., Connolly S.R., Marshall D.J. & Cohen A.L. (2011) Projecting coral reef futures under global warming and ocean acidification. *Science*, **333**, 418-422.

Pankow S. & Bamberger C. (2007) The p53 tumor suppressor-like protein nvp63 mediates selective germ cell death in the sea anemone *Nematostella vectensis*. *PLoS ONE*, **2**.

Pannebakker B.A., Loppin B., Elemans C.P.H., Humblot L. & Vavre F. (2007) Parasitic inhibition of cell death facilitates symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 213-215.

Paxton C.W., Davy S.K. & Weis V.M. (2013) Stress and death of host cells play a role in cnidarian bleaching. *Journal of Experimental Biology*.

Perez S. & Weis V.M. (2006) Nitric oxide and cnidarian bleaching: An eviction notice mediates breakdown of a symbiosis. *Journal of Experimental Biology*, **209**, 2804-2810.

Perez S. & Weis V. (2008) Cyclophilin and the regulation of symbiosis in *Aiptasia pallida*. *Biological Bulletin*, **215**, 63-72.

Perez S.F., Cook C.B. & Brooks W.R. (2001) The role of symbiotic dinoflagellates in the temperature-induced bleaching response of the subtropical sea anemone Aiptasia pallida. *Journal of Experimental Marine Biology and Ecology*, **256**, 1-14.

Perez S.F. (2007) *Exploring the cellular mechanisms of cnidarian bleaching in the sea anemone Aiptasia pallida*. PhD Thesis, Oregon State University.

Pernice M., Dunn S.R., Miard T., Dufour S., Dove S. *et al.* (2011) Regulation of apoptotic mediators reveals dynamic responses to thermal stress in the reef building coral *Acropora millepora*. *PLoS ONE*, **6**, e16095.

Perry C.T., Murphy G.N., Kench P.S., Smithers S.G., Edinger E.N. *et al.* (2013) Caribbean-wide decline in carbonate production threatens coral reef growth. *Nature Communications*, **4**, 1402.

Pochon X., Montoya-Burgos J.I., Stadelmann B. & Pawlowski J. (2006) Molecular phylogeny, evolutionary rates, and divergence timing of the symbiotic dinoflagellate genus *Symbiodinium*. *Molecular Phylogenetics and Evolution*, **38**, 20-30.

Pochon X. & Gates R.D. (2010) A new *Symbiodinium* clade (Dinophyceae) from soritid foraminifera in Hawai'i. *Molecular Phylogenetics and Evolution*, **56**, 492-497.

Podrabsky J. & Krumschnabel G. (2010) Cell death beyond worms, flies and humans: Unusual model systems for cell death research. *Apoptosis*, **15**, 243-248.

Poole R.K. & Hughes M.N. (2000) New functions for the ancient globin family: Bacterial responses to nitric oxide and nitrosative stress. *Molecular Microbiology*, **36**, 775-783.

Porra R., Thompson W. & Kriedemann P. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica Et Biophysica Acta - Bioenergetics*, **975**, 384-394.

Pratchett M.S., Wilson S.K., Graham N.A., Munday P.L., Jones G.P. *et al.* (2009) Coral bleaching and consequences for motile reef organisms: Past, present and uncertain future effects. In: *Coral Bleaching: Patterns, Processes, Causes and Consequences* (eds M.J. van Oppen & J.M. Lough), pp. 139-152. Springer-Verlag, Heidelberg, Germany.

Prout W. (1818) Further observations on the proximate principles of the urine. *London Medico-Chirugical Transactions*, **9**, 472-484.

Putnam H.M., Stat M., Pochon X. & Gates R.D. (2012) Endosymbiotic flexibility associates with environmental sensitivity in scleractinian corals. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 4352-4361.

Radi R., Peluffo G., Alvarez M.N., Naviliat M. & Cayota A. (2001) Unraveling peroxynitrite formation in biological systems. *Free Radical Biology and Medicine*, **30**, 463-488.

Radi R., Cassina A. & Hodara R. (2002a) Nitric oxide and peroxynitrite interactions with mitochondria. *Biological Chemistry*, **383**, 401-409.

Radi R., Cassina A., Hodara R., Quijano C. & Castro L. (2002b) Peroxynitrite reactions and formation in mitochondria. *Free Radical Biology and Medicine*, **33**, 1451-1464.

Ragni M., Airs R.L., Hennige S.J., Suggett D.J., Warner M.E. *et al.* (2010) PSII photoinhibition and photorepair in *Symbiodinium* (Pyrrhophyta) differs between thermally tolerant and sensitive phylotypes. *Marine Ecology Progress Series*, **406**, 57-70.

Ralph P., Larkum A. & Kuhl M. (2005) Temporal patterns in effective quantum yield of individual zooxanthellae expelled during bleaching. *Journal of Experimental Marine Biology and Ecology*, **316**, 17-28.

Ralph P.J., Gademann R. & Larkum A.W.D. (2001) Zooxanthellae expelled from bleached corals at 33oC are photosynthetically competent. *Marine Ecology Progress Series*, **220**.

Richier S., Furla P., Plantivaux A., Merle P.L. & Allemand D. (2005) Symbiosis-induced adaptation to oxidative stress. *Journal of Experimental Biology*, **208**, 277-285.

Richier S., Sabourault C., Courtiade J., Zucchini N., Allemand D. *et al.* (2006) Oxidative stress and apoptotic events during thermal stress in the symbiotic sea anemone, *Anemonia viridis*. *FEBS Journal*, **273**, 4186-4198.

Rinkevich B. (2011) The 'immunology trap' of anthozoans. *Invertebrate Survival Journal*, **8**, 153-161.

Rivero A. (2006) Nitric oxide: An antiparasitic molecule of invertebrates. *Trends in Parasitology*, **22**, 219-225.

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

Rosic N.N., Pernice M., Dove S., Dunn S. & Hoegh-Guldberg O. (2011) Gene expression profiles of cytosolic heat shock proteins Hsp70 and Hsp90 from symbiotic dinoflagellates in response to thermal stress: Possible implications for coral bleaching. *Cell Stress & Chaperones*, **16**, 69-80.

Rosic N.N., Leggat W., Kaniewska P., Dove S. & Hoegh-Guldberg O. (2013) New-old hemoglobin-like proteins of symbiotic dinoflagellates. *Ecology and Evolution*, **3**, 822-834.

Rowan R. & Powers D.A. (1991) A molecular genetic classification of zooxanthellae and the evolution of animal-algal symbiosis. *Science*, **251**, 1348-1351.

Rowan R. & Knowlton N. (1995) Intraspecific diversity and ecological zonation in coral-algal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 2850-2853.

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

Rowan R. (1998) Diversity and ecology of zooxanthellae on coral reefs. *Journal of Phycology*, **34**, 407-417.

Rowan R. (2004) Coral bleaching - Thermal adaptation in reef coral symbionts. *Nature*, **430**, 742-742.

Ruby E.G. (1996) Lessons from a cooperative, bacterial-animal association: The *Vibrio fischer-Euprymna scolopes* light organ symbiosis. *Annual Reviews of Microbiology*, **50**, 591-624.

Safavi-Hemami H., Young N.D., Doyle J., Llewellyn L. & Klueter A. (2010) Characterisation of nitric oxide synthase in three cnidarian-dinoflagellate symbioses. *PLoS ONE*, **5**, e10379.

Saito S., Yamamoto-Katou A., Yoshioka H., Doke N. & Kawakita K. (2006) Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells. *Plant and Cell Physiology*, **47**, 689-697.

Sakihama Y., Nakamura S. & Yamasaki H. (2002) Nitric oxide production mediated by nitrate reductase in the green alga *Chlamydomonas reinhardtii*: An alternative NO production pathway in photosynthetic organisms. *Plant and Cell Physiology*, **43**, 290-297.

Saldarriaga J.F., McEwan M.L., Fast N.M., Taylor F.J. & Keeling P.J. (2003) Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. *International Journal of Systematics and Evolutionary Microbiology*, **53**, 355-365.

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

Sammarco P.W. & Strychar K.B. (2013) Responses to high seawater temperatures in zooxanthellate octocorals. *PLoS ONE*, **8**, e54989.

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

Sampayo E.M., Ridgway T., Bongaerts P. & Hoegh-Guldberg O. (2008) Bleaching susceptibility and mortality of corals are determined by fine-scale

differences in symbiont type. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 10444-10449.

Sanchez C., Cabrera J.J., Gates A.J., Bedmar E.J., RIchardson D.J. *et al.* (2011) Nitric oxide detoxification in the rhizobia-legume symbiosis. *Biochemical Society Transactions*, **39**, 184-188.

Sandau K., Pfeilschifter J. & Brune B. (1997) The balance between nitric oxide and superoxide determines apoptotic and necrotic death of rat mesangial cells. *Journal of Immunology*, **158**, 4938-4946.

Saragosti E., Tchernov D., Katsir A. & Shaked Y. (2010) Extracellular production and degradation of superoxide in the coral *Stylophora pistillata* and cultured *Symbiodinium*. *PLoS ONE*, **5**, e12508.

Sarkar S., Korolchuk V.I., Renna M., Imarisio S., Fleming A. *et al.* (2011) Complex inhibitory effects of nitric oxide on autophagy. *Molecular cell*, **43**, 19-32.

Schnitzler C.E. (2010) *Temperature Stress, Gene Expression, and Innate Immunity at the Onset of Cnidarian-dinoflagellate Symbiosis*. PhD Thesis, Oregon State University.

Schwarz J.A., Krupp D.A. & Weis V.M. (1999) Late larval development and onset of symbiosis in the scleractinian coral *Fungia scutaria. Biological Bulletin*, **196**, 70-79.

Schwarz J.A. & Weis V.M. (2003) Localisation of a symbiosis-related protein, sym32, in the *Anthopleura elegantissima-Symbiodinium muscatinei* association. *Biological Bulletin*, **205**, 339-350.

Schwarz J.A. (2008) Understanding the intracellular niche in cnidarian-symbiodinium symbioses: Parasites lead the way. *Vie Et Milieu-Life and Environment*, **58**, 141-151.

Segovia M. (2008) Programmed cell death in dinoflagellates. In: *Programmed cell death in Protozoa* (ed J.M.P. Martin), pp. 126-142. Landes Bioscience & Springer, Berlin.

Setsukinai K., Urano Y., Kakinuma K., Majima H.J. & Nagano T. (2003) Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *Journal of Biological Chemistry*, **278**, 3170-3175.

Shen C., Chen X.-D. & Yan J. (2013) Nitric oxide inhibits autophagy in human chondrocytes: Comment on the article by Sasaki *et al*. *Arthritis & Rheumatism*, **65**, 287-288.

Sheppard C.R.C., Davy S.K. & Pilling G.M. (2009) *The Biology of Coral Reefs*. Oxford University Press, New York.

Shick J.M. & Dunlap W.C. (2002) Mycosporine-like amino acids and related gadusols: Biosynthesis, accumulation, and UV-protective functions in aquatic organisms. *Annual Reviews of Physiology*, **64**, 223-262.

Shimoda Y., Nagata M., Suzuki A., Abe M., Sato S. *et al.* (2005) Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in *Lotus japonicus*. *Plant and Cell Physiology*, **46**, 99-107.

Shiva S., Brookes P.S., Patel R.P., Anderson P.G. & Darley-Usmar V.M. (2001) Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome *c* oxidase. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 7212-7217.

Sibley L.D. (2011) Invasion and intracellular survival by protozoan parasites. *Immunological Reviews*, **240**, 72-91.

Silverman J., Lazar B., Cao L., Caldeira K. & Erez J. (2009) Coral reefs may start dissolving when atmospheric CO2 doubles. *Geophysical Research Letters*, **36**, L05606.

Silverstein R.N., Correa A.M.S. & Baker A.C. (2012) Specificity is rarely absolute in coral-algal symbiosis: Implications for coral response to climate change. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 2609-2618.

Singh R., Kone B.C., Gounni A.S. & Uzonna J.E. (2013) Molecular regulation of *Trypanosoma congolense*-induced nitric oxide production in macrophages. *PLoS ONE*, **8**, e59631.

Smith D.J., Suggett D.J. & Baker N.R. (2005) Is photoinhibition of zooxanthellae photosynthesis the primary cause of thermal bleaching in corals? *Global Change Biology*, **11**, 1-11.

Smith O. (1998) Nobel prize for NO research. *Nature Medicine*, **4**, 1215.

Snyder C.M., Shroff E.H., Liu J. & Chandel N.S. (2009) Nitric oxide induces cell death by regulating anti-apoptotic Bcl-2 family members. *PLoS ONE*, **4**, e7059.

Sorci G. & Faivre B. (2009) Inflammation and oxidative stress in vertebrate host-parasite systems. *Philosophical Transactions of the Royal Society B-Biological Sciences*, **364**, 71-83.

Spiro S. (2010) An alternative route to nitric oxide resistance. *Molecular Microbiology*, **77**, 6-10.

Stat M., Carter D. & Hoegh-Guldberg O. (2006) The evolutionary history of *Symbiodinium* and scleractinian hosts - Symbiosis, diversity, and the effect of climate change. *Perspectives in Plant Ecology Evolution and Systematics*, **8**, 23-43.

Stat M., Morris E. & Gates R.D. (2008) Functional diversity in coral-dinoflagellate symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 9256-9261.

Steen R.G. & Muscatine L. (1984) Daily budgets of photosynthetically fixed carbon in symbiotic zoanthids. *Biological Bulletin*, **167**, 477-487.

Stevenson M.M. & Riley E.M. (2004) Innate immunity to malaria. *Nature Reviews Immunology*, **4**, 169-180.

Stimson J. & Kinzie R.A. (1991) The temporal pattern and rate of release of zooxanthellae from the reef coral *Pocillopora damicornis* (Linneaus) under nitrogen-enrichment and control conditions. *Journal of Experimental Marine Biology and Ecology*, **153**, 63-74.

Strychar K.B., Coates M., Sammarco P.W. & Piva T.J. (2004a) Bleaching as a pathogenic response in scleractinian corals, evidenced by high concentrations of apoptotic and necrotic zooxanthellae. *Journal of Experimental Marine Biology and Ecology*, **304**, 99-121.

Strychar K.B., Sammarco P.W. & Piva T.J. (2004b) Apoptotic and necrotic stages of *Symbiodinium* (Dinophyceae) cell death activity: Bleaching of soft and scleractinian corals. *Phycologia*, **43**, 768-777.

Strychar K.B. & Sammarco P.W. (2009) Exaptation in corals to high seawater temperatures: Low concentrations of apoptotic and necrotic cells in host coral tissue under bleaching conditions. *Journal of Experimental Marine Biology and Ecology*, **369**, 31-42.

Suggett D.J., Warner M.E., Smith D.J., Davey P., Hennige S. *et al.* (2008) Photosynthesis and production of hydrogen peroxide by *Symbiodinium* (Pyrrhophyta) phylotypes with different thermal tolerances. *Journal of Phycology*, **44**, 948-956.

Suharsono S. & Brown B.E. (1992) Comparative measurements of mitotic index in zooxanthellae from a symbiotic cnidarian subject to temperature increase. *Journal of Experimental Marine Biology and Ecology*, **158**, 179-188.

Sunagawa S., Wilson E.C., Thaler M., Smith M.L., Caruso C. *et al.* (2009) Generation and analysis of transcriptomic resources for a model system on the rise: The sea anemone *Aiptasia pallida* and its dinoflagellate endosymbiont. *BMC Genomics*, **10**.

Szabo C. & Ohshima H. (1997) DNA damage induced by peroxynitrite: Subsequent biological effects. *Nitric Oxide-Biology and Chemistry*, **1**, 373-385.

Szabo C., Ischiropoulos H. & Radi R. (2007) Peroxynitrite: Biochemistry, pathophysiology and development of therapeutics. *Nature Reviews Drug Discovery*, **6**, 662-680.

Szmant A.M. & Gassman N.J. (1990) The effects of prolonged bleaching on the tissue biomass and reproduction of the reef coral *Montastrea annularis*. *Coral Reefs*, **8**, 217-224.

Tait S.W.G. & Green D.R. (2010) Mitochondria and cell death: Outer membrane permeabilization and beyond. *Nature Reviews Molecular Cell Biology*, **11**, 621-632.

Takabayashi M., Adams L.M., Pochon X. & Gates R.D. (2012) Genetic diversity of free-living *Symbiodinium* in surface water and sediment of Hawai'i and Florida. *Coral Reefs*, **31**, 157-167.

Takahashi S. & Yamasaki H. (2002) Reversible inhibition of photophosphorylation in chloroplasts by nitric oxide. *FEBS Letters*, **512**, 145-148.

Takahashi S., Whitney S.M. & Badger M.R. (2009) Different thermal sensitivity of the repair of photodamaged photosynthetic machinery in cultured *Symbiodinium* species. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 3237-3242.

Tchernov D., Gorbunov M.Y., de Vargas C., Yadav S.N., Milligan A.J. *et al.* (2004) Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 13531-13535.

Tchernov D., Kvitt H., Haramaty L., Bibby T.S., Gorbunov M.Y. *et al.* (2011) Apoptosis and the selective survival of host animals following thermal bleaching in zooxanthellate corals. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 9905-9909.

Tewari R., Prommer J. & Watanabe M. (2013) Endogenous nitric oxide generation in protoplast chloroplasts. *Plant Cell Reports*, **32**, 31-44.

Thompson S.E.M., Taylor A.R., Brownlee C., Callow M.E. & Callow J.A. (2008) The role of nitric oxide in diatom adhesion in relation to substratum properties. *Journal of Phycology*, **44**, 967-976.

Thornhill D.J., Xiang Y., Fitt W.K. & Santos S.R. (2009) Reef endemism, host specificity and temporal stability in populations of symbiotic dinoflagellates from two ecologically dominant Caribbean corals. *PLoS ONE*, **4**.

Tonk L., Bongaerts P., Sampayo E. & Hoegh-Guldberg O. (2013) SymbioGBR: a web-based database of *Symbiodinium* associated with cnidarian hosts on the Great Barrier Reef. *BMC Ecology*, **13**.

Torreilles J. (2001) Nitric oxide: One of the more conserved and widespread signaling molecules. *Frontiers in Bioscience*, **6**, D1161-D1172.

Torreilles J. & Romestand B. (2001) *In vitro* production of peroxynitrite by haemocytes from marine bivalves: C-ELISA determination of 3-nitrotyrosine level in plasma proteins from *Mytilus galloprovincialis* and *Crassostrea gigas. BMC Immunology*, **2**, 1.

Trapido-Rosenthal H., Zielke S., Owen R., Buxton L., Boeing B. *et al.* (2005) Increased zooxanthellae nitric oxide synthase activity is associated with coral bleaching. *Biological Bulletin*, **208**, 3-6.

Trapido-Rosenthal H.G., Sharp K.H., Galloway T.S. & Morrall C.E. (2001) Nitric oxide and cnidarian-dinoflagellate symbioses: Pieces of a puzzle. *American Zoologist*, **41**, 247-257.

Trench R.K. (1971) Physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. 1. Assimilation of photosynthetic products of zooxanthellae by two marine coelenterates. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **177**, 225-&.

Trench R.K. (1979) Cell biology of plant-animal symbiosis. *Annual Reviews of Plant Physiology and Plant Molecular Biology*, **30**, 485-531.

Trench R.K. (1993) Microalgal-invertebrate symbioses, a review. *Endocytobiosis and Cell Research*, **9**, 135-175.

Tsai S., Spikings E., Huang I.C. & Lin C. (2011) Study on the mitochondrial activity and membrane potential after exposing later stage oocytes of two gorgonian corals (*Junceella juncea* and *Junceella fragilis*) to cryoprotectants. *Cryoletters*, **32**, 1-12.

Ueno T., Urano Y., Kojima H. & Nagano T. (2006) Mechanism-based molecular design of highly selective fluorescence probes for nitrative stress. *Journal of the American Chemical Society*, **128**, 10640-10641.

Ulstrup K.E., Berkelmans R., Ralph P.J. & van Oppen M.J.H. (2006) Variation in bleaching sensitivity of two coral species across a latitudinal gradient on the Great Barrier Reef: The role of zooxanthellae. *Marine Ecology Progress Series*, **314**, 135-148.

van Hooidonk R., Maynard J.A. & Planes S. (2013) Temporary refugia for coral reefs in a warming world. *Nature Climate Change*, **3**.

van Oppen M.J., Palstra F.P., Piquet A.M.T. & Miller D.J. (2001) Patterns of coral-dinoflagellate associations in *Acropora*: Significance of local availability and physiology of *Symbiodinium* strains and host-symbiont selectivity. *Proceedings of the Royal Society B-Biological Sciences*, **268**, 1759-1767.

van Oppen M.J., Baker A.C., Coffroth M.A. & Willis B.L. (2009) Bleaching resistance and the role of algal endosymbionts. In: *Coral Bleaching: Patterns, Processes, Causes and Consequences* (eds M.J. van Oppen & J.M. Lough), pp. 83-96. Springer-Verlag, Heidelberg, Germany.

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

Vardi A., Berman-Frank I., Rozenberg T., Hadas O., Kaplan A. *et al.* (1999) Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO2 limitation and oxidative stress. *Current Biology*, **9**, 1061-1064.

Vardi A., Formiggini F., Casotti R., De Martino A., Ribalet F.ß. *et al.* (2006) A stress surveillance system based on calcium and nitric oxide in marine diatoms. *PLoS Biology*, **4**, e60.

Venn A., Tambutte E., Holcomb M., Allemand D. & Tambutte S. (2011) Live tissue imaging shows reef corals elevate pH under their calcifying tissue relative to seawater. *PLoS ONE*, **6**, 1-9.

Venn A.A., Loram J.E. & Douglas A.E. (2008) Photosynthetic symbioses in animals. *Journal of Experimental Botany*, **59**, 1069-1080.

Venn A.A., Tambutte E., Lotto S., Zoccola D., Allemand D. *et al.* (2009) Imaging intracellular pH in a reef coral and symbiotic anemone. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 16574-16579.

Verde E.A. & McCloskey L.R. (1996) Photosynthesis and respiration of two species of algal symbionts in the Anemone *Anthopleura elegantissima* (Brandt) (Cnidaria; Anthozoa). *Journal of Experimental Marine Biology and Ecology*, **195**, 187-202.

Vidal-Dupiol J., Adjeroud M., Roger E., Foure L., Duval D. *et al.* (2009) Coral bleaching under thermal stress: Putative involvement of host/symbiont recognition mechanisms. *BMC Physiology*, **9**, 14.

Viera L., Ye Y.Z., Estevez A.G. & Beckman J.S. (1999) Immunohistochemical methods to detect nitrotyrosine. *Nitric Oxide, Pt C*, **301**, 373-381.

Villamil L., Gomez-Leon J. & Gomez-Chiarri M. (2007) Role of nitric oxide in the defenses of *Crassostrea virginica* to experimental infection with the protozoan parasite *Perkinsus marinus*. *Developmental and Comparative Immunology*, **31**, 968-977.

Visick K.G., Foster J., Doino J., McFall-Ngai M.J. & Ruby E.G. (2000) *Vibrio fischeri* lux genes play an important role in colonisation and development of the host light organ. *Journal of Bacteriology*, **192**, 4578-4586.

Vouldoukis I., Riveros-Moreno V., Dugas B., Ouaaz F., Becherel P. *et al.* (1995) The killing of *Leishmania major* by human macrophages is mediated by nitric oxide induced after ligation of the Fc epsilon RII/CD23 surface antigen. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 7804-7808.

Wang J.T. & Douglas A.E. (1998) Nitrogen recycling or nitrogen conservation in an alga-invertebrate symbiosis? *Journal of Experimental Biology*, **201**, 2445-2453.

Wang J.T., Meng P.J., Sampayo E., Tang S.L. & Chen C.A. (2011) Photosystem II breakdown induced by reactive oxygen species in freshly-isolated *Symbiodinium* from *Montipora* (Scleractinia; Acroporidae). *Marine Ecology Progress Series*, **422**, 51-62.

Wang Y., Dufour Y.S., Carlson H.K., Donohue T.J., Marletta M.A. *et al.* (2010a) H-NOX-mediated nitric oxide sensing modulates symbiotic colonization by *Vibrio fisheri*. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 8375-8380.

Wang Y., Dunn A.K., Wilneff J., McFall-Ngai M.J., Spiro S. *et al.* (2010b) *Vibrio fischeri* flavohaemoglobin protects against nitric oxide during initiation of the squid-*Vibrio* symbiosis. *Molecular Microbiology*, **78**, 903-915.

Wang Y.L. & Ruby E.G. (2011) The roles of NO in microbial symbioses. *Cellular Microbiology*, **13**, 518-526.

Wardman P. (2007) Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls, and prospects. *Free Radical Biology and Medicine*, **43**, 995-1022.

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

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

Webster P.J., Holland G.J., Curry J.A. & Chang H.-R. (2005) Changes in tropical cyclone number, duration, and intensity in a warming environment. *Science*, **309**, 1844-1846.

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

Weis V.M. (2008) Cellular mechanisms of cnidarian bleaching: Stress causes the collapse of symbiosis. *Journal of Experimental Biology*, **211**, 3059-3066.

Weis V.M. (2010) The susceptibility and resilience of corals to thermal stress: Adaptation, acclimatization or both? *Molecular Ecology*, **19**, 1515-1517.

Weston A.J., Dunlap W.C., Shick J.M., Klueter A., Iglic K. *et al.* (2012) A profile of an endosymbiont-enriched fraction of the coral *Stylophora pistillata* reveals proteins relevant to microbial-host interactions. *Molecular and Cellular Proteomics*, **11**, M111.015487.

Whitehead L.F. & Douglas A.E. (2003) Metabolite comparisons and the identity of nutrients translocated from symbiotic algae to an animal host. *Journal of Experimental Biology*, **206**, 3149-3157.

Wicks L.C., Sampayo E., Gardner J.P.A. & Davy S.K. (2010) Local endemicity and high diversity characterise high-latitude coral-*Symbiodinium* partnerships. *Coral Reefs*, **29**, 989-1003.

Wicks L.C., Gardner J.P.A. & Davy S.K. (2012) Host tolerance, not symbiont tolerance, determines the distribution of coral species in relation to their environment at a Central Pacific atoll. *Coral Reefs*, **31**, 389-398.

Wiens M., Krasko A., Perovic S. & Muller W.E.G. (2003) Caspase-mediated apoptosis in sponges: Cloning and function of the phylogenetic oldest apoptotic proteases from metazoa. *Biochimica Et Biophysica Acta-Molecular Cell Research*, **1593**, 179-189.

Wild C., Hoegh-Guldberg O., Naumann M.S., Colombo-Pallotta M.F., Ateweberhan M. *et al.* (2011) Climate change impedes scleractinian corals as primary reef ecosystem engineers. *Marine and Freshwater Research*, **62**, 205-215.

Wilkerson F.P. & Muscatine L. (1984) Uptake and assimilation of dissolved inorganic nitrogen by a symbiotic sea anemone. *Proceedings of the Royal Society B-Biological Sciences*, **221**, 71-86.

Wilson N.S., Dixit V. & Ashkenazi A. (2009) Death receptor signal transducers: Nodes of coordination in immune signaling networks. *Nature Immunology*, **10**, 348-355.

Wink D.A., Hanbauer I., Krishna M.C., Degraff W., Gamson J. *et al.* (1993) Nitric-oxide protects against cellular-damage and cytotoxicity from reactive oxygen species. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 9813-9817.

Wink D.A. & Mitchell J.B. (1998) Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radical Biology and Medicine*, **25**, 434-456.

Wink D.A., Hind H.B., Cheng R.Y.S., Switzer C.H., Flores-Santana W. *et al.* (2011) Nitric oxide and redox mechanisms in the immune response. *Journal of Leukocyte Biology*, **89**, 873-891.

Winterbourn C.C. (2008) Reconciling the chemistry and biology of reactive oxygen species. *Nature Chemical Biology*, **4**, 278-286.

Wodala B., Deak Z., Vass I., Erdei L., Altorjay I. *et al.* (2008) *In vivo* target sites of nitric oxide in photosynthetic electron transport as studied by chlorophyll fluorescence in pea leaves. *Plant Physiology*, **146**.

Wolter K.G., Hsu Y.T., Smith C.L., Nechustan A., Xi X., G. *et al.* (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *Journal of Cell Biology*, **139**, 1281-1292.

Wood-Charlson E.M., Hollingsworth L.L., Krupp D.A. & Weis V.M. (2006) Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis. *Cellular Microbiology*, **8**, 1985-1993.

Yakovleva I.M., Baird A.H., Yamamoto H.H., Bhagooli R., Nonaka M. *et al.* (2009) Algal symbionts increase oxidative damage and death in coral larvae at high temperatures. *Marine Ecology Progress Series*, **378**, 105-112.

Yamasaki H., Sakihama Y. & Takahashi S. (1999) An alternative pathway for nitric oxide production in plants: New features of an old enzyme. *Trends in Plant Science*, **4**, 128-129.

Yamasaki H. (2000) Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition *in vivo*. *Philosophical Transactions of the Royal Society B-Biological Sciences*, **355**, 1477-1488.

Yamasaki H. & Sakihama Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: *In vitro* evidence for the NR-dependent formation of active nitrogen species. *FEBS Letters*, **468**, 89-92.

Yamasaki H., Shimoji H., Ohshiro Y. & Sakihama Y. (2000) Inhibitory effects of nitric oxide on oxidative phosphorylation in plant mitochondria. *Nitric Oxide*, **5**, 261-270.

Yang D., Wang H.L., Sun Z.N., Chung N.W. & Shen J.G. (2006) A highly selective fluorescent probe for the detection and imaging of peroxynitrite in living cells. *Journal of the American Chemical Society*, **128**, 6004-6005.

Yang W., Sun Y., Chen S., Jiang J., Chen F. *et al.* (2011) The effect of exogenously applied nitric oxide on photosynthesis and antioxidant activity in heat stressed chrysanthemum. *Biologia Plantarum*, **55**, 737-740.

Yoshida M. & Xia Y. (2003) Heat shock protein 90 as an endogenous protein enhancer of inducible nitric-oxide synthase. *Journal of Biological Chemistry*, **278**, 36953-36958.

Zech B., Kohl R., von Knethen A. & Brune B. (2003) Nitric oxide donors inhibit formation of the Apaf-1/caspase-9 apoptosome and activation of caspases. *Biochemical Journal*, **371**, 1055-1064.

Zhang L., Liu Q., Yuan X., Wang T., Luo S. *et al.* (2013) Requirement of heat shock protein 70 for inducible nitric oxide synthase induction. *Cellular Signalling*.

Zumft W.G. (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews*, **61**, 533-616.

Zuppini A., Andreoli C. & Baldan B. (2007) Heat stress: An inducer of programmed cell death in *Chlorella saccharophila*. *Plant and Cell Physiology*, **48**, 1000-1009.

A: Supplementary Information

Chapter 2

A-2.1. Genotyping Symbiodinium *cultures*

Sequences obtained for the ITS2 region were as follows:

Culture CCMP2467:

GGACCATATGGACGTGATTGGGCACTTGCCAATGCCTGAGAGCATGTCTGC
TTCAGTGCTTCTACTTTCATTTTCTGCTGCTCTTGTTATCAGGAGCAGTGTAT
GCTGCATGCTTCTGCCAGCGAGACTGACATGCTATATATCAAGTTTTGCTTG
CTGTTGTGACTGATCGACATCTCACGTCGGATCAGT.

Culture Ap1:

GCCGATACAACATTTATTACTGCAGAGCTTTAGCATATAAGTAGCGGAGGA
AGCTTCAATGCTTAGCATTATCTACCTGTGCTTGCAAGCAGCATGTATGTCT
GCATTGCTGCTTCGCTTTCCAACAAGTCATCGATCGCTTTTGTGTTCGTAAAT
GGCTTGTTTGCTGCCTGGCCCATGCGCCAAGCTTGAGCGTACTGTTGTTCCA
AGCTTTGCTTGCATCGTGCAGCTCAAGCGCGCAGCTGTCGGGATGCTGATGC
ATGCCCTTAGCATGAAGTCAGACAAGAGAACCCGCTGAATTTAAGCATATA
AGGTAAGCGGAGGAGCGCCCCCCCTCAAATAACAACCAGGCCCCCCGTGTT
TTTTTAGGGGGTCCTCCTCCCCCGGGTGTGTTAAAAAAAAAAAAAAGGTT

Culture CCMP2466:

GGGATACTTTAATTTACTGCAGAGTTAAGCATATAAGTAAGCGGAGGAAGC
TTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATAGGTTTCTGCCTTGC
GTTCTTATGAGCTATTGCCCTCTGAGCCAATGGCTTGTTAATTGCTTGGTTCT
TGCAAAATGCTTTGCGCGCTGTTATTCAGGTTTCTACCTTCGTGGTTTTACTT
GAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGCCTCTA
GCATGAAGTCAGACAAGTGAACCCGCTGAATTTAAGCATATAAGTAAAGCG
GAGGAACGCAGTTCTGTCAAATCACACGCCGCC

A-2.2. Effects of the NO donor SNP on Symbiodinium PSII quantum yields.

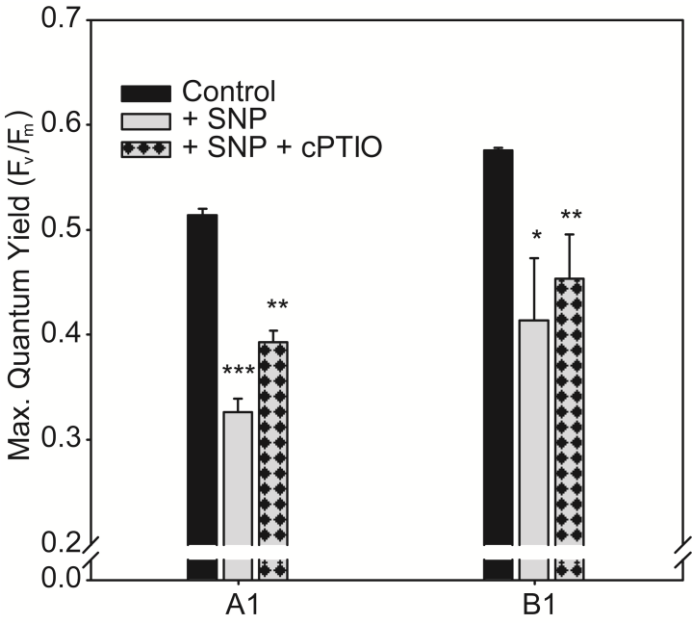

Figure A1. Effect of the NO donor sodium nitroprusside (SNP; 1 mM for A1, 100 µM for B1) on maximum quantum yield (F_v/F_m) in two ITS types of *Symbiodinium*. The incomplete restoration of F_v/F_m upon application of the NO scavenger cPTIO suggests that SNP's effects are mediated only partly by NO. Values are means ± s.e.m. and asterisks indicate significant differences relative to the control group *within each type* (ANOVA with LSD pair-wise *post-hoc*, n = 3 independent cultures, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

A-2.3. Confocal LSM filter images of Symbiodinium cells loaded with fluorescent dyes to detect NO synthesis and cell death.

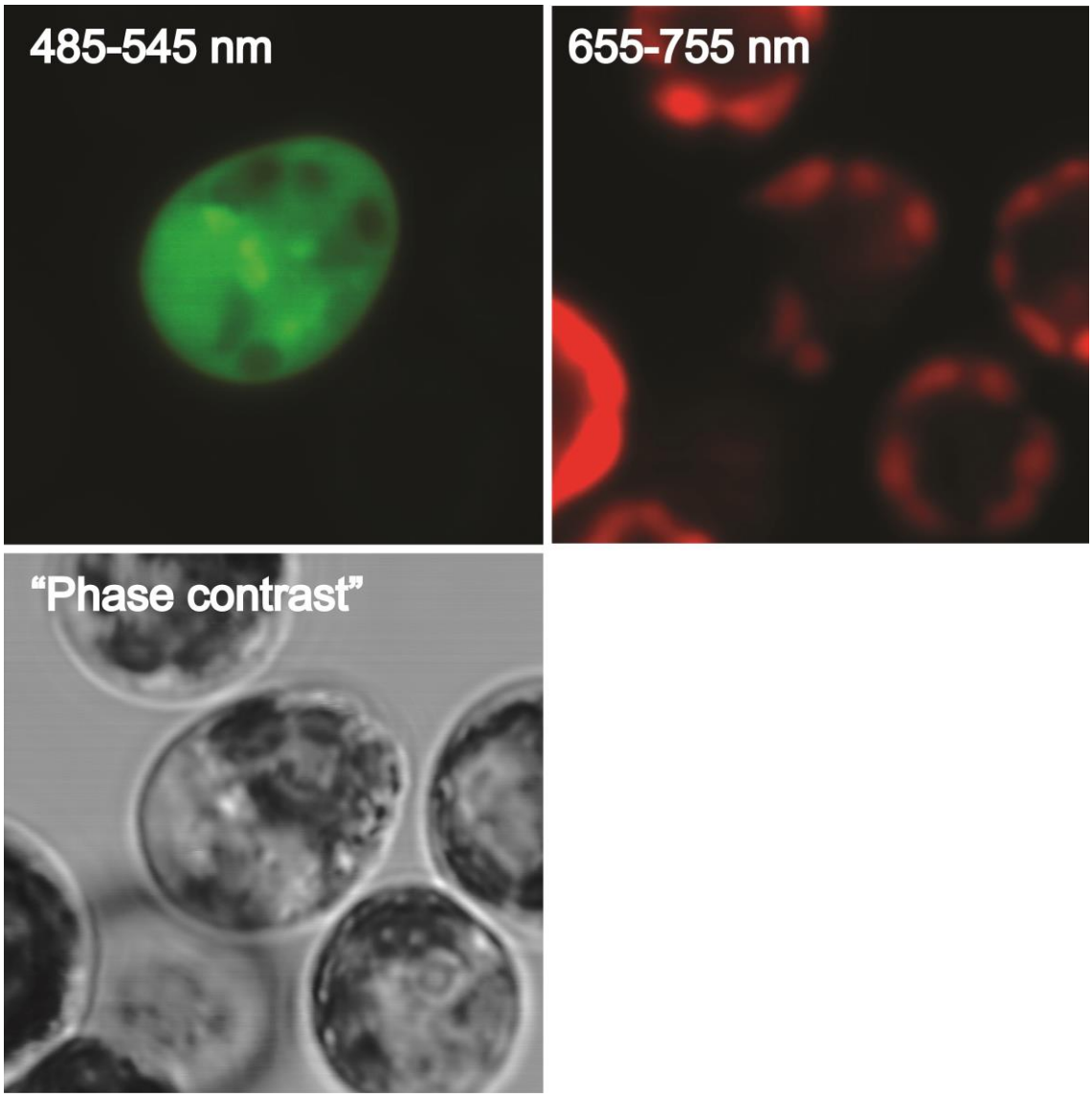

Figure A2. Confocal visualisation of DAF-FM in *Symbiodinium* type B1. In the production of Figure 2.3, NO-dependent DAF-FM fluorescence (green, top-left) is superimposed on chlorophyll autofluorescence (red, top-right).

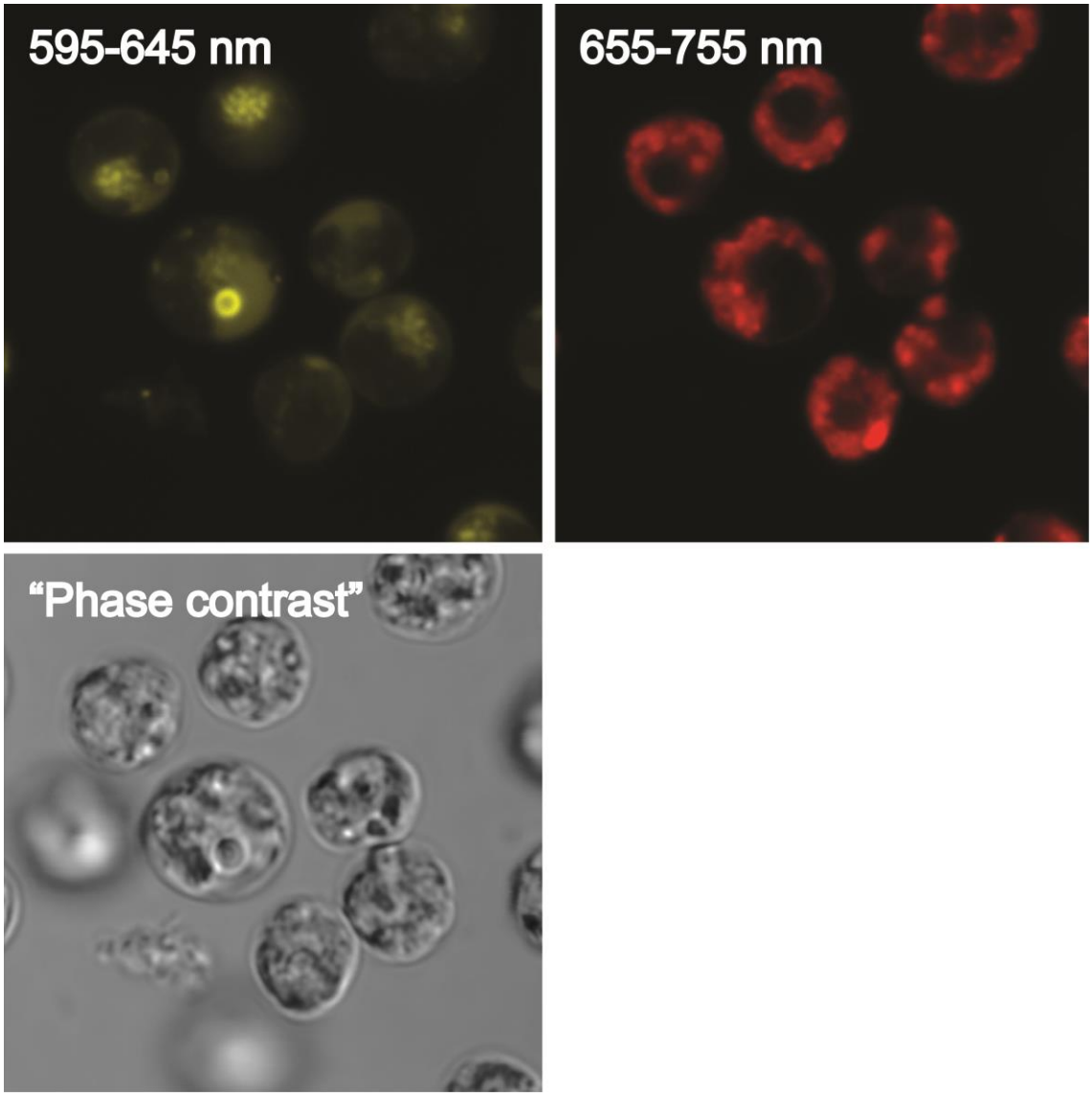

Figure A3. Confocal visualisation of propidium iodide in heat-killed (75°C for 4 h) cells of *Symbiodinium* type B1. In the production of Figure 2.7, propidium iodide fluorescence (yellow, top-left) is superimposed on chlorophyll autofluorescence (red, top-right).

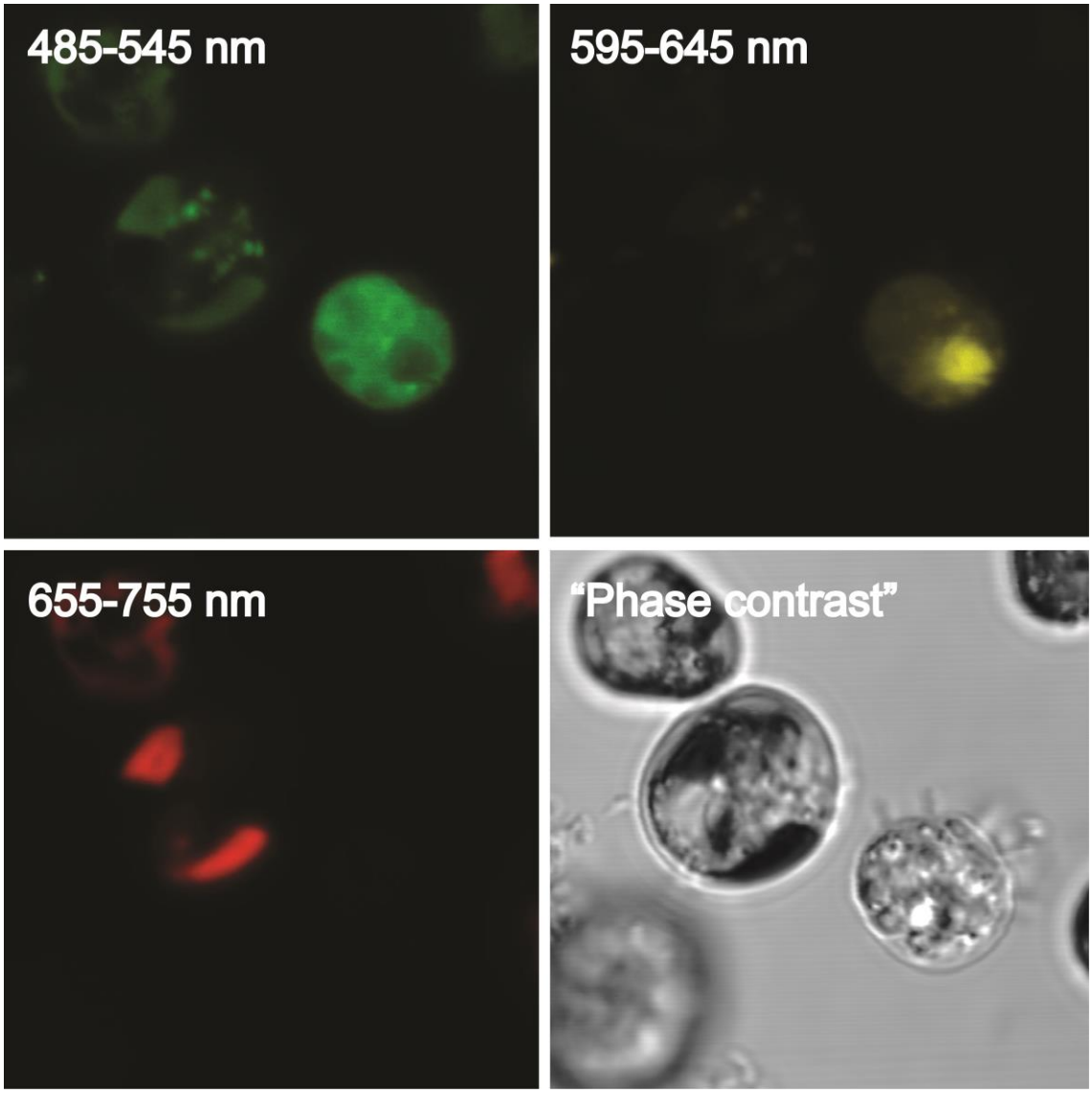

Figure A4. Confocal visualisation of the fluorescent dyes Annexin-V *fluor* 488 and propidium iodide in *Symbiodinium* type A1. In the production of Figure 2.8, AV-*fluor* fluorescence (green, top-left) is superimposed on propidium iodide fluorescence (yellow, top-right) and chlorophyll autofluorescence (red, bottom-left).

A-2.4. NO synthesis in ageing *Symbiodinium* cultures

Materials and methods

Cultures of Ap1 (ITS2-B1) were grown in 350 mL f/2 media in conical flasks (n = 4) with an initial inoculation concentration of 10000 cells mL^{-1}. Incubation conditions were as follows: 26°C constant temperature, 100-120 µmol photons m^{-2} s^{-1} (OSRAM DULUX L 36W 4000K) 12 h light:12 h dark cycle. Cells were removed for analysis on Days 4, 7, 10 and 14, and then weekly up to Day 42. At each sampling point, two 1-mL aliquots were removed for haemocytometer counts (Improved Neubauer, Boeco, Germany) and assessments of photosystem II fluorescence. After 30 min dark adaptation, the maximum quantum yield of PSII (F_v/F_m) was measured using a Water PAM (Walz, Effeltrich, Germany).

An additional aliquot (10-50 mL depending on cell concentration) was removed, centrifuged twice (1500 × g for 5 min) and resuspended in 100 µL 15 µM DAF-FM-DA NO-sensitive fluorescent dye (Molecular Probes, Eugene, OR, USA) in FSW. Cells were incubated for 90 min in the dark, after which they were washed by repeated centrifugation (twice, 1500 × g for 5 min) and resuspension in 500 µL FSW. An aliquot was removed for cell counts, and after 30 min (to allow cleavage of the DAF-FM-DA to its active DAF-FM form) 150000 cells were transferred to wells in a black-sided 96-well plate (CoStar®, Corning Inc. Life Sciences, Tewksbury, MA, USA). NO-dependent fluorescence was quantified using a fluorescent plate reader (Enspire® 2300, Perkin-Elmer, Waltham, MA, USA) with excitation wavelength 495 nm and detection at 515 nm.

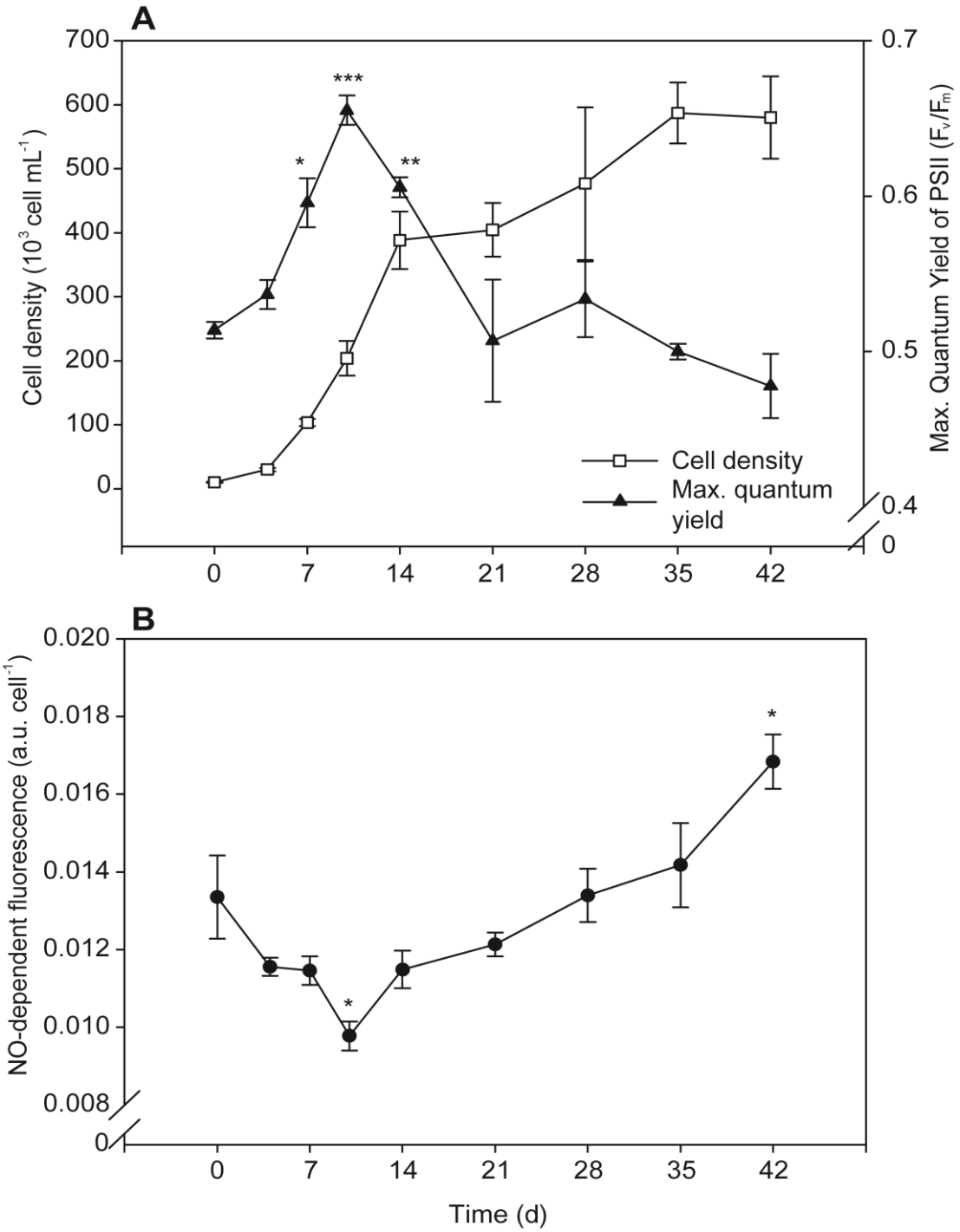

Figure A5. Growth, PSII fluorescence and NO synthesis in *Symbiodinium* culture Ap1 (ITS2-type B1) over 6 weeks. **Panel A)** Cell density per mL and maximum quantum yield of PSII (F_v/F_m). **B**) NO-dependent fluorescence of cells incubated with the NO-sensitive dye DAF-FM-DA. Values are means ± s.e.m. and asterisks indicate significant differences relative to Day 0 *within each parameter* (F_v/F_m and NO-dependent fluorescence only; RMANOVA with Simple contrasts of each time point *versus* t = 0, n = 4 independent cultures, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Symbiodinium cells maintained logarithmic growth for the initial fourteen days, after which growth rates slowed considerably (Fig. A5A). Maximum quantum yield of PSII changed significantly over time (RMANOVA, time, $F_{1.706,\ 5.118} = 9.007$, $p = 0.023$), increasing to Day 10 and then subsequently declining (Fig. A5A). F_v/F_m at the end of the experiment (Day 42) was not significantly different to the initial (Day 0) value (Simple contrast, $F_{1,\ 3} = 2.309$, $p = 0.226$).

NO-dependent fluorescence changed significantly over time (RMANOVA, time, $F_{1.647,\ 4.4} = 13.269$, $p = 0.016$), declining over the initial ten days (Simple contrast, $F_{1,\ 3} = 16.311$, $p = 0.027$) but then subsequently increasing (Fig. A5B). After forty-two days, levels of NO-dependent fluorescence were significantly higher than initial values (Simple contrast, $F_{1,\ 3} = 24.7$, $p = 0.016$).

Chapter 3

A-3.1. Quantifying NO-dependent fluorescence in symbiotic Aiptasia pulchella using confocal LSM.

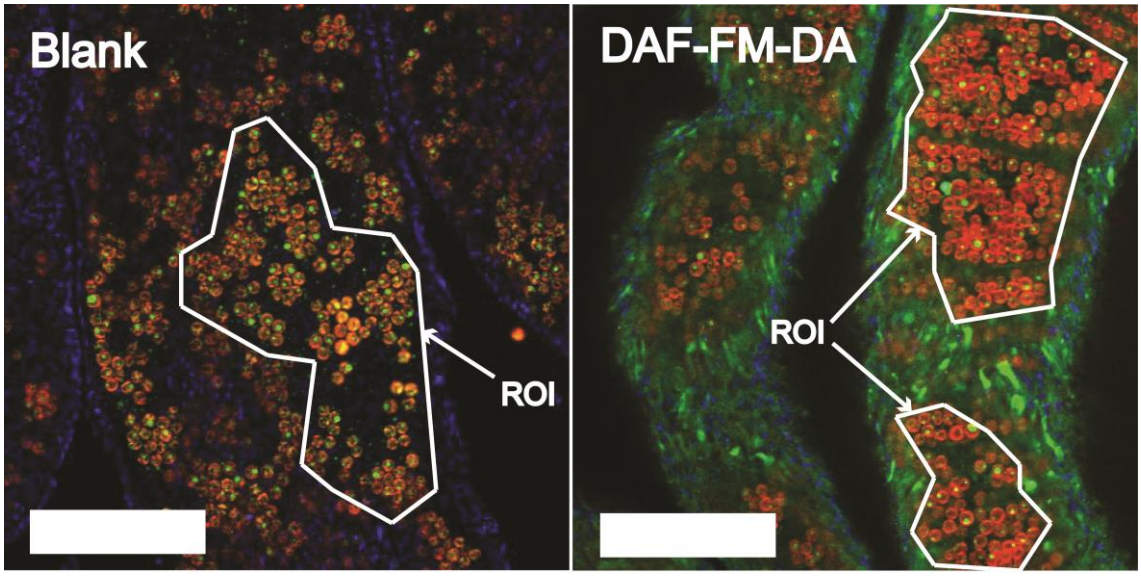

Figure A6. Quantification of NO-dependent fluorescence in tentacles of *A. pulchella* using DAF-FM-DA and ImageJ software. Regions of in-focus gastrodermis (positive for the presence of symbiotic dinoflagellates and negative for Hoechst 33342 fluorescence (blue) were selected as regions of interest (ROI). Mean 510-530 nm fluorescence intensity (MFI) of the ROI was then calculated for respective "blank" and "+DAF-FM-DA" images, with the former subtracted from the latter to give "NO-dependent fluorescence". Scale bar: 100 µm.

Chapter 5

A-5.1. ITS2 sequences of Symbiodinium *cells isolated from reef corals*

Acropora millepora

Colony 1 - C3

AACCAATGGCCTCCTGAATGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCGTATGAGTTATTGCCCTCTGAGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 2 - C3

AACCAATGGCCTCCTGAATGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCGTATGAGTTATTGCCCTCTGAGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 3 - C3

AACCAATGGCCTCCTGAATGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCGTATGAGTTATTGCCCTCTGAGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 4 - C3

AACCAATGGCCTCCTGAATGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCGTATGAGTTATTGCCCTCTGAGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 5 - C3

AACCAATGGCCTCCTGAATGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCGTATGAGTTATTGCCCTCTGAGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCA----------

Montipora digitata

Colony 1 - C15

AACCAATGGCCTCCTGAACGTGCGTTGCACCCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCTTCTGCGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGCGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 2 - C15

AACCAATGGCCTCCTGAACGTGCGTTGCACCCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCTTCTGCGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGCGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 3 - C15

AACCAATGGCCTCCTGAACGTGCGTTGCACCCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCTTCTGCGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGCGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 4 - C15

AACCAATGGCCTCCTGAACGTGCGTTGCACCCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCTTCTGCGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGCGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 5 - C15

AACCAATGGCCTCCTGAACGTGCGTTGCACCCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCTTCTGCGCCAATGGCTTGTTAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAAGTTTCTACCTTCGCGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Pocillopora damicornis

Colony 1 - C42 (type 2)

AATCAATGGCCTCCTGAACGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCCTCTGAGCCAATGGCTTGTGAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAGGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 2 -C42 (type 2)

AATCAATGGCCTCCTGAACGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCCTCTGAGCCAATGGCTTGTGAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAGGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 3 - C42 (type 2)

AATCAATGGCCTCCTGAACGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCCTCTGAGCCAATGGCTTGTGAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAGGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 4 - C42 (type 2)

AATCAATGGCCTCCTGAACGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCCTCTGAGCCAATGGCTTGTGAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAGGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

Colony 5 - C42 (type 2)

AATCAATGGCCTCCTGAACGTGCGTTGCACTCTTGGGATTTCCTGAGAGTAT
GTCTGCTTCAGTGCTTAACTTGCCCCAACTTTGCAAGCAGGATGTGTTTCTG
CCTTGCGTTCTTATGAGCTATTGCCCTCTGAGCCAATGGCTTGTGAATTGCTT
GGTTCTTGCAAAATGCTTTGCGCGCTGTTATTCAGGTTTCTACCTTCGTGGTT
TTACTTGAGTGACGCTGCTCATGCTTGCAACCGCTGGGATGCAGGTGCATGC
CTCTAGCATGAAGTCAGACAA

A-5.2. Assessments of bleaching in reef corals.

In addition to the quantification of bleaching as the density of *Symbiodinium* cells relative to coral fragment surface area, host soluble protein was also presented relative to coral fragment surface area, thus providing a proxy measurement for host tissue thickness. No significant changes in tissue thickness (time × temperature) were observed in any of the three species (Fig. A4).

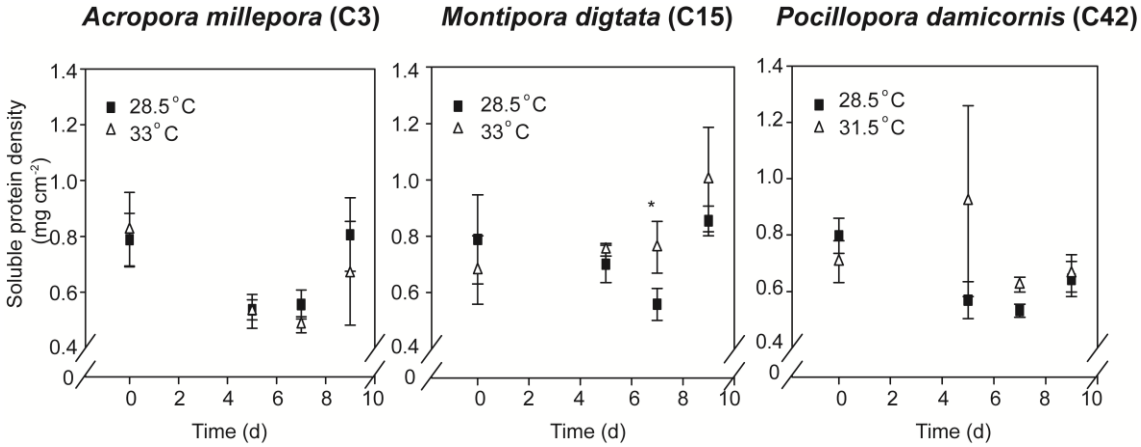

Figure A7. Changes in host tissue thickness (milligrams of protein per unit of fragment surface area) at elevated temperature in the reef corals *Acropora millepora, Montipora damicornis,* and *Pocillopora damicornis*. Parentheses indicate ITS2 types of *Symbiodinium* hosted by each species. Asterisks indicate significant differences relative to the controls (RMANOVA, pair-wise *post-hoc* with Bonferroni correction, n = 5 except for *A. millepora* after nine days at 33°C where n = 4, * $p < 0.05$).

A-5.3. Quantifying NO in coral host tissues with 2,3-diaminonaphthalene.

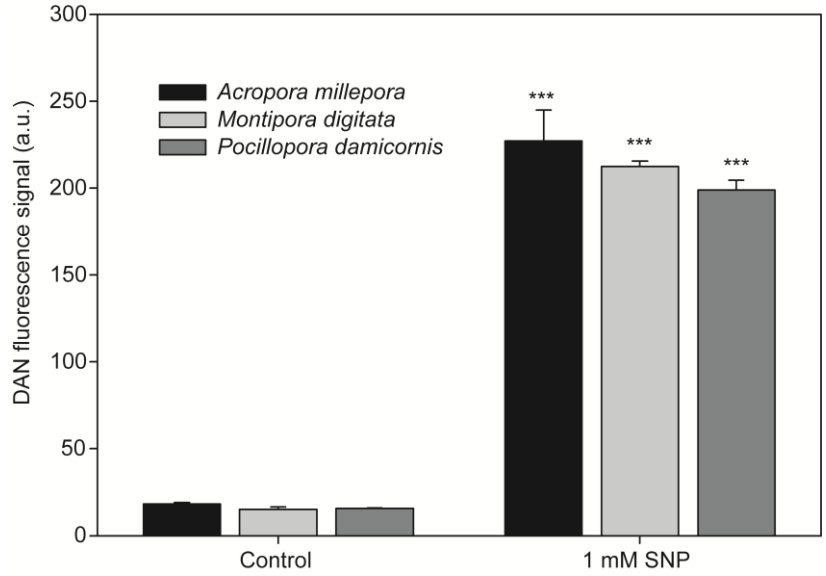

Figure A8. Detection of NO with 2,3-diaminonaphthalene (NO_x^- assay - see Chapter 5). *Acropora millepora*, *Montipora digitata* and *Pocillopora damicornis* homogenates (host fractions) incubated for 4 h with the NO donor SNP. Values are means ± s.e.m. and asterisks indicate significant differences within each species (t-tests, n = 3, *** $p < 0.001$).

Chapter 6

A-6.1. Host nitric oxide synthesis in response to infection

Aposymbiotic *A. pulchella* anemones were obtained as described above. Individual anemones (n = 3) were then injected (see above) with a suspension either of cultured homologous (ITS2 type B1) *Symbiodinium*, homologous *Symbiodinium* cells that had been heat-killed (80°C for 4 h), or cultured heterologous *Symbiodinium* cells (culture CCMP421, a "free-living" *Symbiodinium* type originally isolated from Wellington Harbour, New Zealand). Four hours *post*-injection, anemone synthesis of NO was visualised as described in Chapter 3. Unfortunately due to mortality of some *A. pulchella* specimens *post*-infection, data could only be obtained from one of the anemones infected with heat-killed algae and from two of the heterologous infections. Still, there appeared to be a trend in the data for elevated NO synthesis upon exposure to heterologous or non-viable symbiont cells (Fig. A7).

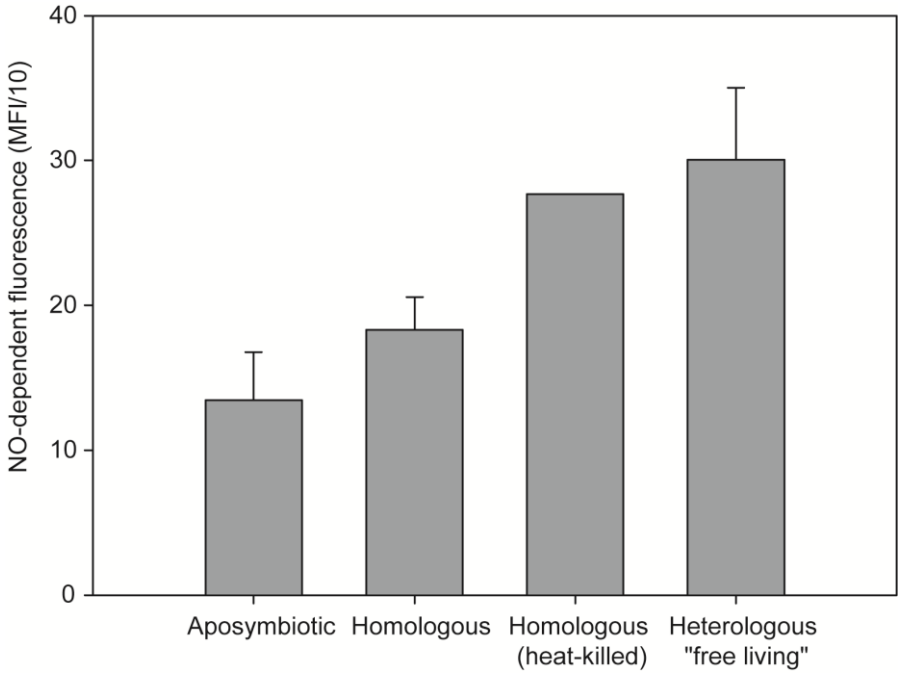

Figure A9. Production of NO in *Aiptasia pulchella* 4 h after infection by homologous, heat-killed, or heterologous *Symbiodinium* cells. Values are means ± s.e.m. (n = 3 for "aposymbiotic" and "homologous", n = 2 for "heterologous free-living", n = 1 for "homologous heat-killed".

B: Additional work

B-1. Reinfection of *Aiptasia pulchella* with different *Symbiodinium* types

The original proposal for Chapter 3 was to experimentally obtain aposymbiotic *A. pulchella* and experimentally reinfect them with various types of *Symbiodinium*. The induction of NO synthesis upon exposure to high temperature shock was then to be compared between these different holobionts.

Anemones were bleached following a standard cold-shock protocol alongside dark-incubation and starvation. Briefly, *A. pulchella* specimens were exposed to pre-chilled (4°C) sterile (autoclaved) FSW for 6 h before being returned to 26°C for 72 h. Anemones were maintained in the dark throughout. This procedure was repeated twice weekly for at least 6 months. Invididual *A. pulchella* were transferred to wells on a 24-well plate and placed under fluorescent lighting (100 µmol photons m^{-2} s^{-1}) at 26°C in sterile FSW. Any anemones that failed to recover their *Symbiodinium* populations after 4 weeks (visible as a developing colouration of tentacles) were then returned to dark-incubation). Anemones that recovered their symbiont populations invariably did so with 14 days and were discarded.

Reinfection of anemones was achieved by concentrating *Symbiodinium* cultures (by centrifugation [800 × g for 5 min] of *ca.* 50 mL algal culture and resuspension in 100 µL FSW) and then injecting a small volume into the anemones' mouths using a sterile Pasteur pipette drawn out to a fine tip. Uptake of *Symbiodinium* was assisted by providing a small quantity of *Artemia* nauplii alongside the algal cells. Anemones infected with differing symbiont types were maintained in separate bowls in sterile FSW. After at least 6 months, a subset of anemones was removed from each bowl and their resident *Symbiodinium* cells were extracted as described in Chapter 3 and processed for ITS2 genotyping (Logan *et al*., 2010).

Single strand conformation polymorphism (SSCP) analyses (Fig. B1) suggested that all *Aiptasia pulchella* cultures other than those reinfected with homologous (ITS2 type B1) *Symbiodinium* contained mixed *Symbiodinium* populations. This was confirmed by

sequencing; only the B1-infected population had an ITS2 sequence that could reliably be compared to GenBank sequences.

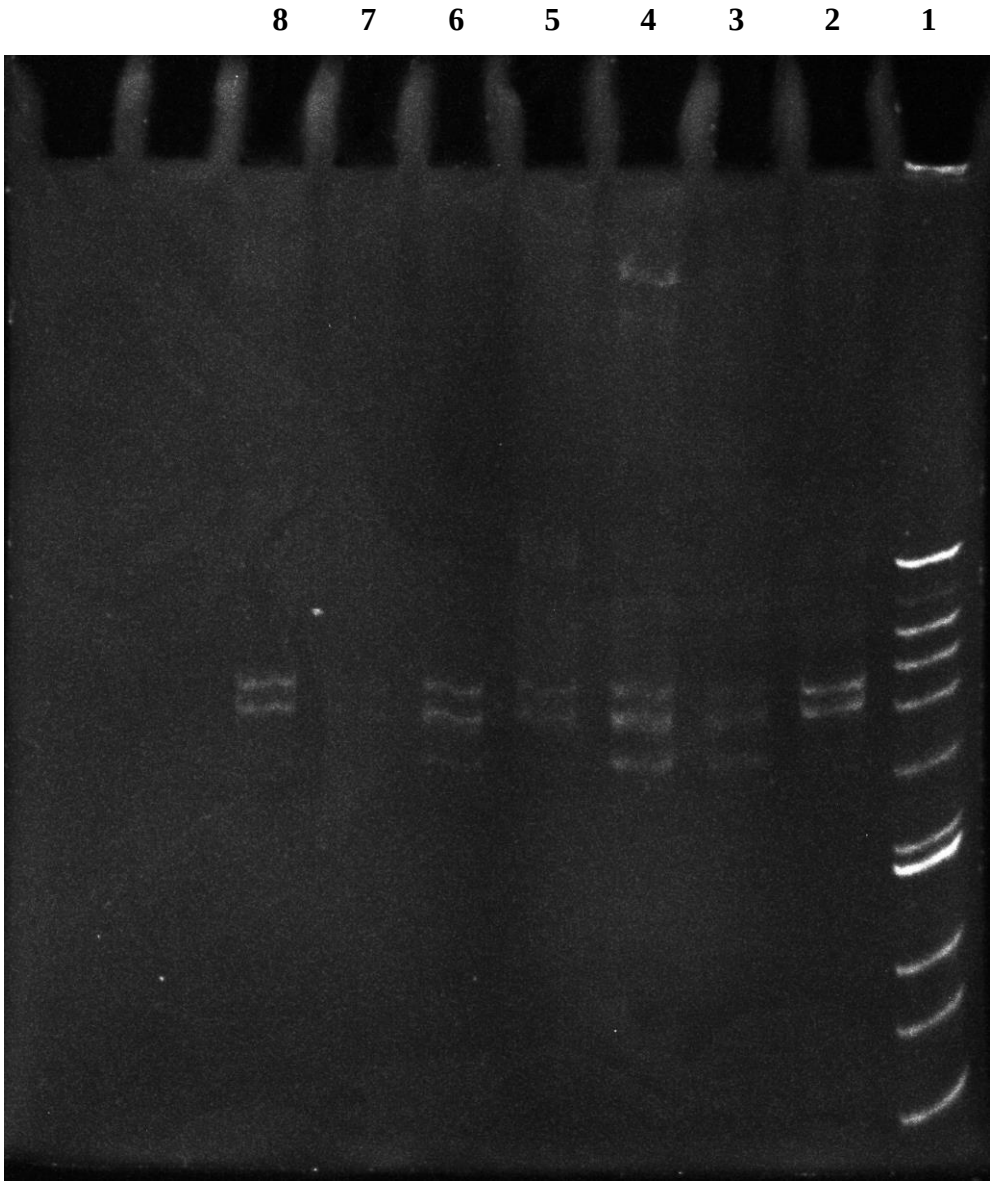

Figure B1. SSCP analysis of *Symbiodinium* ITS2 region extracted and amplified from cells isolated from reinfected *Aiptasia pulchella* populations. More than 2 bands on a lane indicates multiple *Symbiodinium* ITS2 genotypes. Location of bands can also distinguish different ITS2 types.

Lane 1: DNA Hyperladder®

Lane 2: Ap1 (ITS2 B1) reinfection

Lane 3: CCMP2466 (C1) reinfection

Lane 5: CCMP2467 (A1) reinfection

It appeared, therefore, that anemones were either hosting only B1-type *Symbiodinium*, or contained these B1-type algae alongside the introduced population. The cause of this contamination seemed to be a residual population of *Symbiodinium* that remained in the host over a period of at least 18 months in darkness, during cold-shock treatment, and that had not recovered over the 4-week period under lights (Fig. B2). Unfortunately, this meant that experiments comparing host NO synthesis at elevated temperature in different host-*Symbiodinium* combinations could not be carried out.

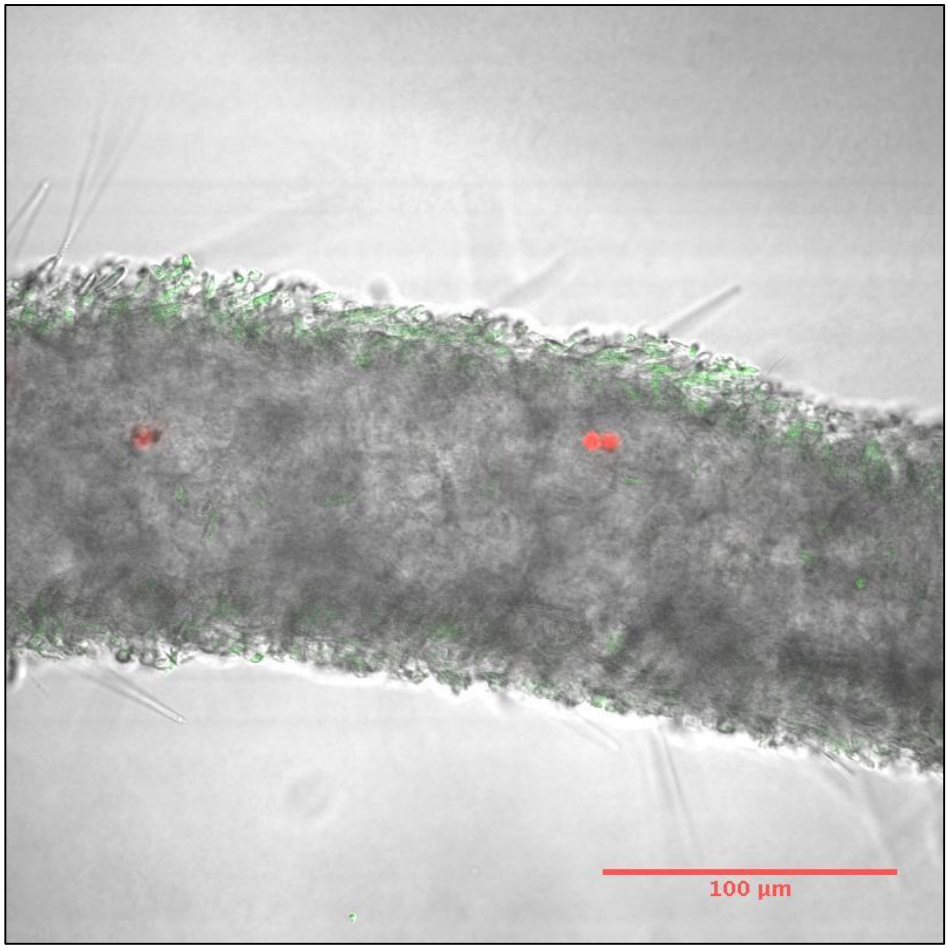

Figure B2. Confocal LSM micrograph of an "aposymbiotic" *Aiptasia pulchella* tentacle incubated in the dark (with regular cold-shock treatment) for 18 months, and still containing residual *Symbiodinium* cells (red chlorophyll fluorescence). Green areas indicate fluorescence of the NO-sensitive probe DAF-FM-DA.

B-2. Visualisation of host mitochondria in isolated *Aiptasia pulchella* cells.

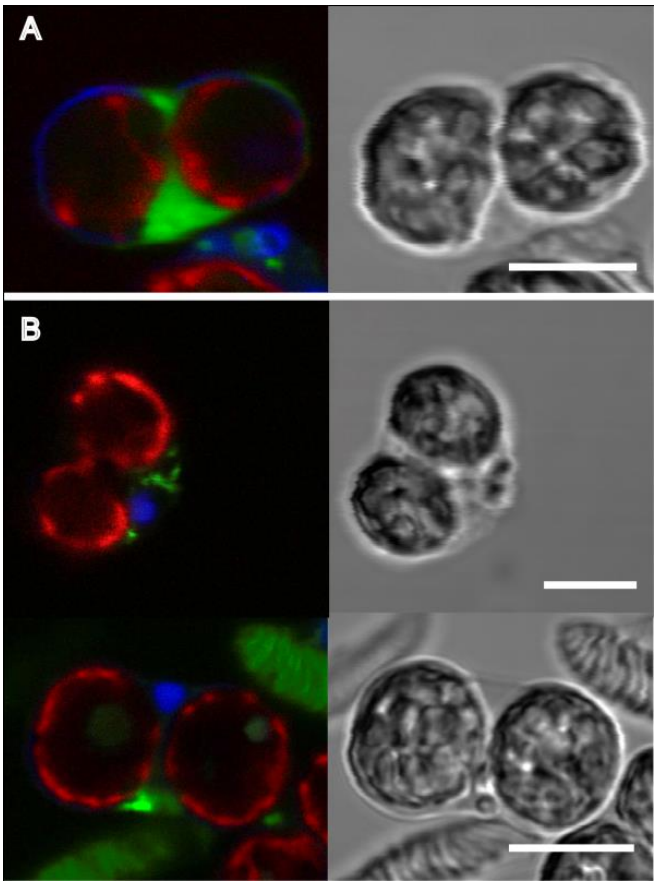

Figure B3. Confocal LSM micrographs of isolated *Aiptasia pulchella* cells. **Panel A**) Cells incubated with fluorescein diacetate (green - stains the cytosol in live cells) **B)** Cells incubated with Hoechst 33342 and rhodamine 123 (green - stains functional mitochondria). Scale bar in each set of images is 10 µm.

Intact live host cells of *Aiptasia pulchella* were obtained through tissue maceration (Gates & Muscatine, 1992). Briefly, anemones were incubated in calcium-free artificial seawater (Ca-FSW) for 1 h. A short length of tentacle (*ca.* 5 mm) was then removed and cut into 1 mm sections. Tissue sections were then subjected to tryptic maceration (0.1% w/v porcine trypsin in Ca-FSW) on an agitation platform for 45 min. The resulting tissue slurry was then transferred to poly-l-lysine-coated glass-bottom dishes and cells were allowed to settle for 10 min. Fluorescent dyes in Ca-FSW were then added, and cells were visualised on a confocal LSM (see Chapter 2). The ability of cells to survive

extraction and isolation was confirmed by incubating them with 5 µM fluorescein diacetate (FDA), which is cleaved to fluorescein (Fig. B3a; ex: 473 nm, em 510-530 nm) by cytosolic esterases in live cells. Host mitochondria were then visualised (Fig. B3b) using 5 µM of the fluorescent dye rhodamine 123 (R123; ex; 473 nm, em: 510-550 nm). Host nuclear material was stained with Hoechst 33342 (ex: 405 nm, em 440-490 nm) at a concentration of 5 µg / mL. All fluorescent dyes were purchased from Life Technologies, Auckland, New Zealand.