CORALS UNDER STRESS

A STUDY OF THE CORAL INNATE IMMUNE SYSTEM

Thesis submitted by
Johannes A.J.M. van de Water, BSc, MSc

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ARC Centre of Excellence for Coral Reef Studies.
School of Marine and Tropical Biology,
James Cook University, Townsville, Australia
&
Australian Institute of Marine Science
Townsville, Australia

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Abstract of Thesis

Despite the absence of an adaptive immune system, scleractinian corals possess an array of innate immune mechanisms for defence against environmental and biological stressors. The rising prevalence of coral diseases worldwide, however, indicates that the corals' ability to respond to disturbances is being increasingly hampered, potentially because cumulative impacts of multiple stressors are overwhelming the immune system and threatening their health. A thorough understanding of how the coral immune system is affected by and responds to stressors is needed to understand the role that innate immunity can play in coral resilience in the face of increasing anthropogenic impacts and a changing climate. My primary objectives in this thesis were to investigate: 1) temporal patterns in immune parameters in healthy corals and how seasonal variation in environmental factors affect constitutive levels of these parameters, 2) impacts of anthropogenic stress associated with a tourist platform on the coral immune system and potential implications for reef degradation, 3) the coral immune response elicited by injury and how this response is affected by seawater temperature, and 4) impacts of elevated seawater temperature on the response of members of the coral holobiont to bacterial challenges, including responses of the coral host, its endosymbiont *Symbiodinium*, and coral-associated bacterial communities.

A study of temporal patterns in green fluorescent protein (GFP)-like protein expression and in activity levels of the pro-phenoloxidase (proPO)-activating system over a year in three common Indo-Pacific corals revealed that corals maintain differing constitutive levels of these immune parameters, and that the seasonality of peaks in temporal patterns varies among species (Chapter 2). Overall, high constitutive levels of PO activity in *Porites cylindrica* versus generally low constitutive levels in *Acropora millepora* are consistent with ecological characterisations of these species as stress-tolerant and stress-sensitive, respectively. Variation in temporal patterns of both parameters among species and in the manner that patterns correlate with environmental factors, including temperature, salinity and solar radiation, indicates that investment in immune mechanisms is a life history trait that varies among corals, and that species differ in how they prioritise energy allocation to immune parameters in resource investment trade-offs.

In Chapter 3, I analysed the effect of an anthropogenic disturbance on baseline levels of immune parameters in the disease-susceptible coral *A. millepora.* In summer,

anthropogenic stressors associated with tourist platforms caused disease in 30% of corals monitored near platforms, while corals at a control site unaffected by recreational activities, physical damage and platform shading remained healthy throughout the 7 month study. GeXP analyses revealed that both healthy and diseased corals adjacent to tourist platforms increased their expression of immune genes involved in Toll-like receptor (TLR) signalling cascades, such as *MAPK p38* and *MEKK-1*, compared to corals at the nearby control site. In addition, diseased corals exhibited a 2 to 3-fold increase in PO activity and fluorescence levels compared to control corals, as well as up to 2-fold increased expression of immune genes, including *cFos* and *Factor B*. Multiple stressors associated with platforms and warm temperatures appear to have overwhelmed the coral immune system in summer, resulting in higher coral disease prevalence at these locations. Once seasonal temperatures declined, disease subsided, suggesting that the immune response was able to cope with anthropogenic stressors in the absence of temperature stress.

Some corals near tourist platforms sustained significant levels of physical damage in summer, but did not develop disease, raising questions about the interactive effects of elevated seawater temperature and injury on the coral immune response. In a manipulative aquarium-based experiment at Heron Island, branches of *A. aspera* that were exposed to low level heat stress (32°C) and subsequently injured were found to exhibit a rapid immune response within 24 hours post-injury that was largely unaffected by heat stress (Chapter 4). For example, while immune genes Tx60 and apextrin were downregulated in uninjured corals at 32°C, their expression in injured heat stressed corals was similar to that in injured corals at ambient temperatures. The similarity in the responses of heat-stressed and control corals (maintained at ambient temperatures) to injury suggests that the immune response of *A. aspera* is robust to minor increases in temperature, and temperatures 1-2°C above typical summer maxima have a limited impact on its ability to recover from lesions.

The increasing likelihood of physical damage to reef corals from predator outbreaks, storms and anthropogenic disturbances, highlights the need for further detailed studies of how corals cope with injury. A longer-term field-based study of *A. aspera's* immune response to injury demonstrates that this species recovers from significant, artificially-induced lesions within ten days (Chapter 5). The immune response was dynamic, consisting of at least three distinct phases involving differing timing in the upregulation of components of the innate immune system, including the

proPO-activating system, GFP-like protein expression, Toll-like receptor signalling and the complement system. The initial response involved a 1.5-fold increase in expression of a TLR within 24 hours, which orchestrated the innate immune response that was most pronounced 48 and 96 hours post-injury. Overall, the immune response was sufficient to protect corals against bacterial infection, as bacteria did not infiltrate coral tissues and the coral-associated bacterial community did not change, enabling rapid recovery in the absence of additional stressors.

Patterns of higher disease prevalence in summer for many common coral diseases suggest that elevated seawater temperatures affect a coral's immune response to pathogens. In Chapter 6, I investigated the effects of elevated seawater temperatures (29.5°C and 32°C, compared to 27°C (ambient)) on the response of the coral *Montipora aequituberculata* to bacterial challenges over a 22 day period, concurrently with responses of *Symbiodinium* and coral-associated bacterial communities. Full transcriptome analysis using RNA Seq revealed that corals exhibit an immune response towards the pathogenic bacterium *Vibrio coralliilyticus,* regardless of seawater temperature, but not against a non-pathogenic bacterium (*Oceanospirillales* sp.). This ability to distinguish between pathogenic and non-pathogenic bacteria and respond only to pathogens, suggests that corals may actively regulate their associated bacterial communities. Unexpectedly, the number of disease cases did not differ between *V. coralliilyticus*-challenged corals and other treatments, regardless of seawater temperature, despite the occurrence of a white syndrome outbreak in the coral population from which *V. coralliilyticus* was isolated. Results suggest that, either corals have acquired higher disease resistance over time, or that virulence of the *V. coralliilyticus* strain used had been reduced. RNA Seq analysis of the coral response showed that under heat stress, a large suite of immune response mechanisms was upregulated, including genes involved in phagocytosis, TLR and pro-inflammatory cytokine signalling, and the complement system. These responses were potentially directed towards a temperature-induced shift in bacterial communities observed in heat stressed corals. Gene enrichment analysis revealed that *Symbiodinium* also exhibited an immune response toward this biotic stressor; concurrently, a significant photophysiological response was detected under elevated seawater temperatures, although no bleaching was observed. Taken together, this study shows that *M. aequituberculata* is relatively stress-resistant, possesses a complex suite of immune responses and is capable of distinguishing pathogenic bacteria.

Overall, the work presented in this thesis shows that corals are well equipped to cope with disturbances such as injury and bacterial challenges, even when exposed to mild temperature stress or single environmental disturbances. However, the immunocompetence and performance of the coral immune system is species-specific and cumulative environmental and anthropogenic pressures may overpower the coral's immune system, leading to disease. Identifying impacts of environmental and anthropogenic stressors on the coral immune system provides important information for coral reef managers faced with the challenge of prioritising resources to reduce anthropogenic pressures on increasingly threatened coral reef ecosystems.

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INTRODUCTION

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“The Innate Immune System of Marine Invertebrates”

1.1 - Corals and the threats to coral reefs

Coral reefs are one of the most diverse ecosystems in the world, harbouring one-third of all marine species, however, populations of framework-building corals that underpin this ecosystem are declining worldwide (Gardner et al., 2003; Wilkinson, 2008; De'ath et al., 2012). A wide range of microbial and macroscopic symbionts are integral to the health of the coral holobiont, a term for the collective consortium comprised of the coral host and its symbionts (Rohwer et al., 2002; Rosenberg et al., 2007). Among the microbial symbionts are protists, fungi, bacteria, archaea and viruses, as well as endosymbiotic dinoflagellates in the genus *Symbiodinium*, which are present in the coral's endodermal cell layer and provide the coral with nutrients formed through photosynthesis (reviewed by Davy et al., 2012). The highly complex and diverse bacterial communities harboured by corals are involved in nutrient cycling (Raina et al., 2009; Lema et al., 2012), as well as the production of antibiotic compounds that defend the coral against pathogen invasion (Ritchie, 2006; Teplitski and Ritchie, 2009; Shnit-Orland and Kushmaro, 2009; Alagely et al., 2011; Bourne and Webster, 2013). Disturbances associated with anthropogenic activities, including greenhouse gas emissions and pollution, can lead to changes in microbial community composition of the holobiont (Ritchie, 2006; Bourne et al., 2008; Mouchka et al., 2010; Littman et al., 2011; Witt et al., 2011), resulting in disease and demise of coral colonies and declines in coral populations (Osborne et al., 2011; De'ath et al., 2012).

To maintain healthy symbiotic microbial communities, defend themselves against pathogens and respond to injury, corals employ a suite of immune mechanisms. However, the effects of stressors, such as elevated seawater temperatures, on these processes may hamper their defence and recovery capability.

1.2 - Overview of the invertebrate innate immune system

In contrast to vertebrates, who have both an innate and adaptive immune system, invertebrates have only an innate immune system, which has long been considered to be non-specific and lack memory. However, evidence is accumulating that the invertebrate immune system has significant specificity and that a high degree of memory is present (Rowley and Powell, 2007). The innate immune system is comprised of cellular and non-cellular components that recognise, respond to and eliminate foreign or non-self particles in a generic, non-specific manner. However, the large array of pattern recognition receptors (PRR), which recognise microbe-associated molecular patterns (MAMP) or pathogen-associated molecular patterns (PAMP) that

are conserved among microbial taxa, provide some degree of specificity in and diversification of the invertebrate innate immune response. MAMPs or PAMPs are components of microbes (e.g. lipopolysaccharide (LPS) of Gram-negative bacteria, β-1,3-glucan of fungi, peptidoglycan (PGN) of Gram-positive bacteria) that can trigger a host response upon detection by PRRs. Together, these activated PRRs initiate signalling cascades that orchestrate the immune response involving one or multiple components of the invertebrate innate immune system. In the following section, I provide an overview of the most important innate immune mechanisms in marine invertebrates and corals. For a more detailed description of the invertebrate immune system and current knowledge about these mechanisms in invertebrate species, see Appendix A.

A highly conserved class of PRRs of the innate immune system is the Toll-like receptor (TLR) class (Fig 1-I). TLRs are transmembrane receptors that are activated following the binding of MAMPs to its extracellular domain. In turn, these receptor activates a range of signal transduction pathways, including the MAPK p38, JNK and NFκB pathways, although it can also induce caspase-mediated apoptosis under certain conditions (Kumar et al., 2009; Lester and Li, 2014). In addition to the TLR, the invertebrate innate immune system also contains NOD-like receptors (NLR), which detect intracellular MAMPs and activate the same pathways as TLR (Fig 1-I) (Franchi et al., 2009). Signal transduction via these pathways ultimately results in the activation of transcription factors that regulate the expression of genes involved in inflammation, immune regulation and microbe elimination, as well as cell survival and proliferation (Fig 1-II) (Newton and Dixit, 2012). Following translation, various immune proteins are exocytosed (Fig 1-III), however many are secreted in an inactive form to prevent aberrant immune responses. Cytokines are generally produced bound to the cell membrane and released following proteolytic cleavage by proteases such as immune system-activated metalloproteases (Fig 1-IV) (Van Lint and Libert, 2007). Cytokines (e.g. interferons (IFN), interleukins (IL), transforming growth factor (TGF) β) are present in high concentration at the site of release and the cytokine concentration reduces by distance, thereby forming a chemotactic gradient that is used to direct other immune cells to the site of infection (Luster, 2001). Cells detect these cytokines via cytokine-specific receptors (e.g. IFN receptors, IL receptors, TGFβ receptors) and, in turn, the activated receptors initiate various signal transduction pathways that lead to the transcription of other pro-inflammatory and immune effector molecules, as well as molecules involved in negative feedback loops that prevent aberrant immune activation

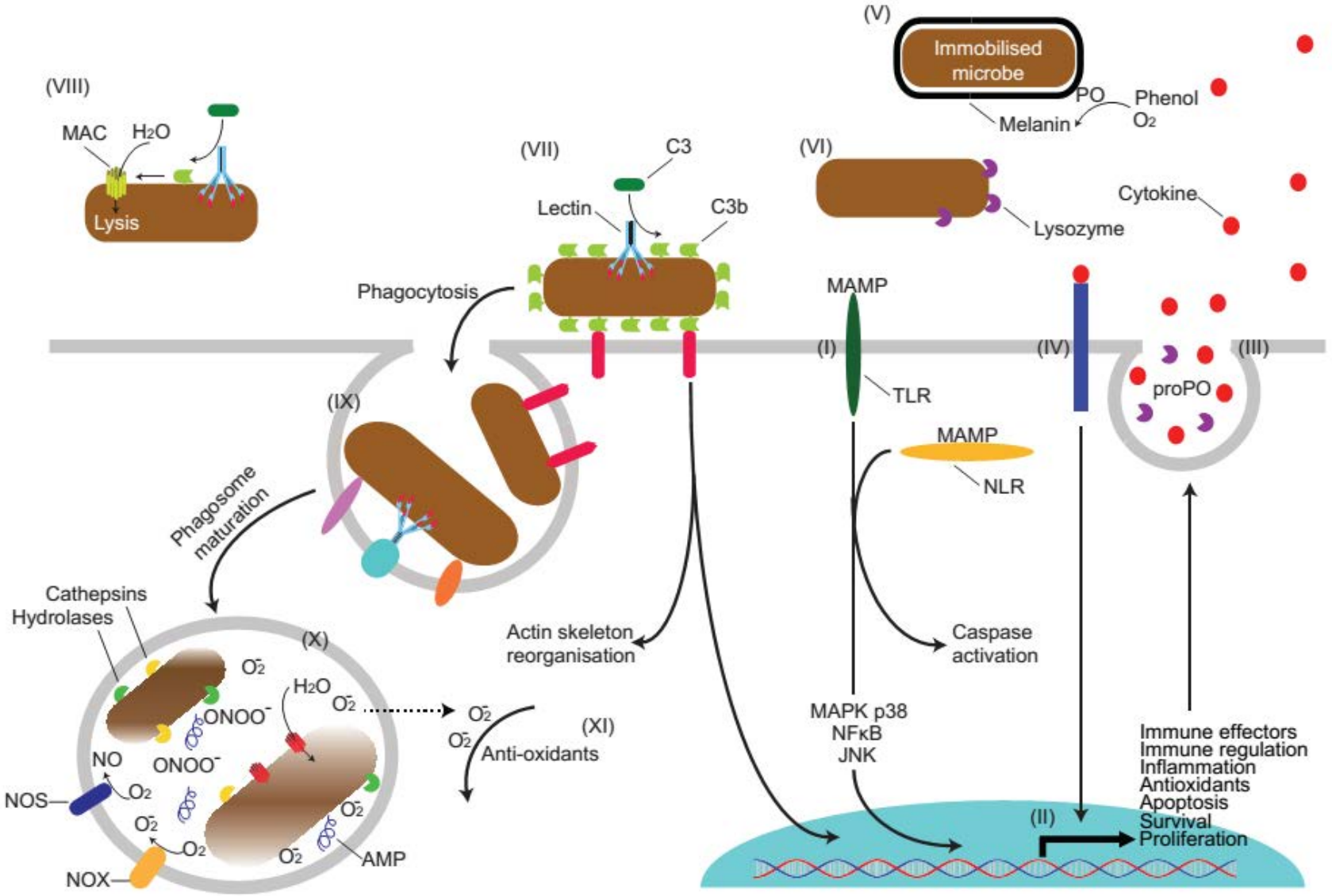

(V)
Immobilised microbe
PO
Phenol
O2
Melanin
(VIII)
MAC
H2O
Lysis
(VII)
C3
Lectin
C3b
(VI)
Lysozyme
Cytokine
Phagocytosis
MAMP
(I)
TLR
(IV)
(III)
proPO
(IX)
MAMP
NLR
Phagosome maturation
Cathepsins
Hydrolases
(X)
Actin skeleton reorganisation
Caspase activation
O2-
H2O
ONOO-
NO
NOS
O2
NOX
AMP
(XI)
Anti-oxidants
MAPK p38
NFκB
JNK
(II)
Immune effectors
Immune regulation
Inflammation
Antioxidants
Apoptosis
Survival
Proliferation

Figure 1: Schematic overview of the invertebrate innate immune system. (I) Toll and NOD-like receptor signalling pathways. MAMP-activated Toll-like and NOD-like receptors induce various signal transduction pathways, including NFκB, JNK and MAPK p38 pathways, resulting in transcription of immune genes (II). In addition, apoptosis may be induced via a caspase-8-mediated pathway. Products of transcribed immune genes may have intracellular functions or are exocytosed (III). Exocytosed cytokines have immunomodulatory functions, regulating immune gene expression and providing a chemotactic gradient for immune cell recruitment via cytokine receptor signalling (IV). In addition, proPO forms a microbe-immobilising barrier of melanin (V) and lysozyme digests the microbial cell wall causing lysis (VI). The lectin-complement system (VII) is initiated by binding of a lectin to MAMPs and results in the proteolytic cleavage of C3 into C3b, which is deposited onto the microbe. C3b can initiate the formation of a MAC (VIII) or via C3-specific receptors induce phagocytosis of the microbe (IX). Maturation of the phagosome leads to the formation of a microbicidal phagolysosome (X). Destructive reactive radicals are neutralised by antioxidants to prevent host damage (XI). Grey lines indicate surface cell layer of host; brown cylinders represent microbes. Abbreviations: MAMP, microbe-associated molecular pattern; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; NLR, nucleotide-binding oligomerisation domain (NOD-like) receptor; NFκB, nuclear factor kappa B; proPO, pro-phenoloxidase; C3, complement C3; MAC, membrane attack complex; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; AMP, anti-microbial peptide.

that may damage the host (Travis and Sheppard, 2014; Ivashkiv and Donlin, 2014; Schroder et al., 2004; Liew et al., 2005). The exact immune response exhibited by the host, however, depends on the combination of PRRs and cytokine receptors activated, which allows for a specific response towards the detected microbes. For example, IFN signalling is exclusive to an anti-viral response (Ivashkiv and Donlin, 2014; Schroder et al., 2004).

Several immune effector molecules are also exocytosed by host immune cells, including anti-microbial peptides (AMP), lysozyme and pro-phenoloxidase (proPO). The proPO-activating pathway (Fig 1-V) is highly conserved in invertebrates and is initiated upon the detection of MAMPs by MAMP-binding proteins (Cerenius et al., 2008). These proteins activate a serine protease cascade that leads to the cleavage of proPO into phenoloxidase (PO). PO subsequently oxidises phenolic compounds, which ultimately leads to melanisation of the invading microbe. Melanin forms a physical barrier, thereby immobilising the microbe, while during the PO catalysed reaction, reactive microbicidal molecules are formed. Lysozyme also has a strong antimicrobial function as it digests peptidoglycan resulting in the lysis of the microbe attacked (Fig 1-VI) (Callewaert and Michiels, 2010). AMPs can have a range of different anti-microbial functions, but most form pores in the microbial cell wall causing cell lysis or bind to intracellular molecules essential for the microbe's survival (Brogden, 2005).

The complement system has also been discovered in many invertebrates and is generally initiated through the binding of mannose-binding lectins (MBL) or ficolins to MAMPs on the microbial surface (Fig 1-VII) (Gadjeva, 2014). Binding initiates a proteolytic cascade where MBL-associated serine proteases (MASP) cleave complement proteins resulting in the cleavage of complement C3 or in the formation of

a C2b:C4b C3 convertase, which more efficiently cleaves C3 into C3a and C3b. While C3a has a chemotactic function and recruits immune cells, C3b is deposited on the microbe's surface. Here, C3b can be involved in two distinct processes. One of these processes is the formation of a membrane attack complex (MAC), which is a pore that is inserted into the microbe's cell wall and subsequently causes the lysis of the microbe (Fig 1-VIII). The other process involves the opsonin properties of C3b. Phagocytic immune cells express C3b-specific receptors that, when bound to C3b, initiate pro-inflammatory signal transduction cascades, as well as cascades involved in the reorganisation of the actin skeleton (Fig 1-VII). Actin reorganisation is crucial for phagocytosis, the process whereby cells ingest large particles (>0.5 µm). Via its opsonin properties, C3b significantly enhances the phagocytosis of the microbe (Fig 1-IX). Phagocytosis can, however, also be induced by other membrane bound receptors, including scavenger receptors or receptors that directly bind to microbe-bound lectins (Fig 1-IX). Following phagocytosis, a phagosome is formed in which the microbe is located (Flannagan et al., 2012). Through a multi-stage process of phagosome maturation, the phagosome becomes significantly more acidic. In the final maturation stage, lysosomes fuse with the phagosome forming a phagolysosome (Fig 1-X) (Flannagan et al., 2012). Here, the phagolysosome has turned into a true microbicidal structure with a pH of 4.5, containing NADPH oxidases (NOX) and nitric oxide synthases (NOS) that produce reactive oxygen and nitrogen intermediates, respectively, as well as digestive enzymes (cathepsins and hydrolases) and anti-microbial peptides. However, the highly reactive compounds produced indiscriminately target proteins, nucleotides and fatty acids and may also damage host structures. As a consequence, immune signalling also results in the upregulation of antioxidants, which neutralise these reactive molecules (Fig 1-XI).

1.3 - The innate immune system of corals

In corals, microbial communities on the surface of colonies and associated with different micro-habitats within polyps are important for immune functioning and health of the holobiont. Coral-associated microbial communities are highly diverse (Ainsworth et al., 2006; Dinsdale et al., 2008), but so far, most studies suggest host species-specificity and relative stability of bacterial communities on both geographically spatial and temporal scales (Rohwer et al., 2001; Rohwer et al., 2002; Littman et al., 2009b; Littman et al., 2009a; Kvennefors et al., 2010a). However, within a coral colony, the microbial community structure can vary substantially (Sweet et al., 2011), which may reflect the diverse physiological and symbiotic functions of these bacteria (Raina et al.,

2009; Lema et al., 2012). Corals regulate bacterial abundances by shedding bacteria from their surfaces (Garren and Azam, 2012), but whether and how they regulate the microbial community composition is not well understood. Antibiotics produced by the microbial community in the mucus may play a significant role in regulating its composition and preventing pathogenic species from establishing (Fusetani et al., 1996; Ritchie, 2006; Reshef et al., 2006; Gochfeld et al., 2006; Gochfeld and Aeby, 2008; Shnit-Orland and Kushmaro, 2009; Shnit-Orland et al., 2012; Kvennefors et al., 2012; Hunt et al., 2012), thereby showing the importance of a healthy microbial community (Krediet et al., 2013).

In other cnidarians, AMPs play an important role in shaping and maintaining healthy microbial communities (Fraune and Bosch, 2007; Fraune et al., 2010; Fraune et al., 2011; Franzenburg et al., 2013). The isolation of a constitutively expressed anti-bacterial and anti-fungal AMP from the coral *Pocillopora damicornis* (Vidal-Dupiol et al., 2011a) and the inducible expression of AMP genes in immune challenged gorgonians (Burge et al., 2013) suggests that AMPs play an important role in scleractinian innate immunity (Table 1.1). It is not fully understood how AMP expression is regulated in corals, but the presence of canonical Toll-like receptor homologues (Miller et al., 2007a; Shinzato et al., 2011), along with several TLR downstream signalling molecules (e.g. MyD88, IRAK4, ECSIT, TRAF-6, TAK-1, MEKK-1, MKK3/4, MAPK p38, JNK, TRAM, IκB, IκK, NFκB, ATF/CREB, cJun, cFos) (Miller et al., 2007a; Shinzato et al., 2011; Siboni et al., 2012) in corals suggests a functional TLR pathway is likely to be present (Table 1.1). In *Acropora cervicornis*, several TLR and associated genes are upregulated in response to white band disease (Libro et al., 2013). While corals can detect extracellular microbes using TLRs, intracellular pathogens may be detected by their repertoire of NOD-like receptors (Table 1.1) (Shinzato et al., 2011; Hamada et al., 2013). In addition, the apoptosis-inducing TNFR signalling pathway is present in the coral family *Acroporidae* along with various genes involved in the apoptosis processs (Shinzato et al., 2011; Grasso et al., 2011), and immune challenges appear to induce TNFR expression (Weiss et al., 2013).

The complement pathway, which can be activated via both the lectin and alternative pathways, is also present in corals (Table 1.1). A gene homologous to complement C3 was first isolated from the gorgonian coral *Swiftia exserta* (Dishaw et al., 2005). Analysis of the *Acropora digitifera* genome revealed the presence of two C3 homologues (Shinzato et al., 2011). In the scleractinian coral *Acropora millepora*, C3 expression is restricted to the endodermal cell layer (Miller et al., 2007a) and is increased following injury (Kvennefors et al., 2010b), as well as in response to a challenge by the bacterium *Alteromonas* sp. (Brown et al., 2013). The presence of a

Factor B (Bf) homologue in *A. millepora* suggests that complement C3 may be activated through the alternative pathway (Siboni et al., 2012). Generally, the lectin complement pathway is initiated following the binding of mannose-binding lectins (MBL) to foreign bodies (Table 1.1). The MBL millectin has been relatively well characterised, and although a function in the complement pathway has not yet been demonstrated, it is upregulated following exposure to LPS and PGN (Kvennefors et al., 2010b) and capable of agglutinating bacteria (Kvennefors et al., 2008). Millectin also recognises the coral dinoflagellate endosymbionts, *Symbiodinium*, suggesting a role in the maintenance, and potentially establishment, of this symbiosis (Kvennefors et al., 2008; Kvennefors et al., 2010b). Other C-type lectins in *A. millepora* have been suggested to be involved the early stages of coral development via tissue remodelling during metamorphosis (Grasso et al., 2011) and allorecognition during colony maturation (Puill-Stephan et al., 2012), but not in larval settlement (Siboni et al., 2012). Members of the tachylectin superfamily, which have agglutinating properties, may also be involved in coral innate immunity. Homologues have been found in several

Table 1.1 – Overview of current knowledge of the main coral immune mechanisms

Immune process		**Reference**
Receptors & signalling	NOD-like receptor	1, 2
	Toll-like and interleukin receptor	1, 3, 4
	TLR & NLR downstream signalling molecules	1, 3
	Lectins	5, 6, 7, 8
	Eicosanoid cytokines	4, 9
Effector mechanisms	Immune cells	10, 11
	Phagocytosis	4
	Oxidative burst	4, 12
	Complement pathway	3, 7, 13
	Membrane attack complex / perforin domain proteins	3
	Melanisation cascade	11, 14
	Coagulation pathway	14
	Anti-microbial peptides (AMP)	15, 16
Antioxidants	Antioxidant enzymes	17, 18
	Green Fluorescent Protein-like proteins	19, 20

[1](Shinzato et al., 2011); [2](Hamada et al., 2013); [3](Miller et al., 2007a); [4](Libro et al., 2013); [5](Grasso et al., 2008); [6](Grasso et al., 2011); [7](Kvennefors et al., 2008); [8](Vidal-Dupiol et al., 2011b); [9](Lõhelaid et al., 2014); [10](Palmer et al., 2011c); [11](Mydlarz et al., 2008); [12](Mydlarz and Jacobs, 2006); [13](Siboni et al., 2012); [14](Palmer et al., 2012a); [15](Vidal-Dupiol et al., 2011a); [16](Burge et al., 2013); [17](Shinzato et al., 2012); [18](Mydlarz and Harvell, 2007); [19](Palmer et al., 2009a); [20](Bou-Abdallah et al., 2006).

scleractinian coral genera (*Acropora*, *Montastraea*, *Oculina*) (Hayes et al., 2010), and in *Gorgonia ventalina*, tachylectins are known to be upregulated following a parasite infection (Burge et al., 2013).

The best characterised innate immune system component in corals is the proPO system. This system has been described for gorgonian and scleractinian corals, in both adults and larvae (Table 1.1). Two types of phenoloxidases are present in corals: tyrosinase and laccase-type PO (Mydlarz and Palmer, 2011; Palmer et al., 2012a), but the laccase-type PO is believed to have a non-immunological function (Palmer et al., 2012b). How PO activity is regulated in corals is currently unknown, however, melanin-containing granular amoebocytes are believed to store the proPO system components in vesicles, similar to crayfish (Johansson and Söderhäll, 1985). Upon wounding, these cells degranulate, resulting in the formation of a plug (Palmer et al., 2011c), which could be the result of a transglutaminase-mediated coagulation response (Table 1.1) (Palmer et al., 2012a) and hardened by melanin. Following plug formation, multiple other putative immune cell types (agranular and eosinophilic granular amoebocytes) are recruited to the lesion (Palmer et al., 2011c). The function of these cells is as of yet unknown, however, oesinophilic granular cells may be precursors of the melanin-containing granular cells (Palmer et al., 2011c). Immune cells (Table 1.1) are recruited in high numbers to lesions of *Aspergillus sydowii*-infections (Mydlarz et al., 2008) and allografts (Couch et al., 2013) in *Gorgonia ventalina*, where phenoloxidase activity is significantly higher than in non-affected tissues. Immune cells have been suggested to play a significant role in *Acropora cervicornis* in response to white band disease, showing significant upregulation of various genes involved in phagocytosis and the oxidative burst, including NADPH oxidases (Table 1.1) (Libro et al., 2013).

During the melanisation process, reactive oxygen species are formed and corals use antioxidants to protect themselves from ROS-induced damage. Anti-oxidant enzymes (SOD, catalase, peroxidase) are present in scleractinian corals (Table 1.1) (Shinzato et al., 2012), correlate with PO activity levels (Mydlarz and Palmer, 2011) and are upregulated in response to a fungal pathogen (Mydlarz and Harvell, 2007). Fluorescent proteins (FP) have also been reported to have antioxidative properties (Table 1.1) (Bou-Abdallah et al., 2006; Palmer et al., 2009a). Given the upregulation of FPs in the pigmentation response following trematode infection (Palmer et al., 2009b) and an increase in PO activity in pigmented coral tissues surrounding lesions (Palmer et al., 2008), a role of FPs in the antioxidant component of the innate immune system is plausible. However, FPs are also known to have a photoprotective role, in particular for

the algal endosymbiont and proliferating tissues (Salih et al., 2000; Dove et al., 2001; Dove et al., 2008; D'Angelo et al., 2012; Smith et al., 2013).

In addition to these relatively well characterised coral innate immune system components, other less well characterised components with putative immune functions are present. Homologues of the membrane attack complex / perforin (MAC/PF) domain-containing proteins Tx60 and apextrin, which may be complement effectors, are present in *A. millepora* (Table 1.1) (Miller et al., 2007a). Tx60 is a venom toxin from *Hydra*, but the function in *A. millepora* is currently unknown. Apextrin may have a role in coral larval settlement (Siboni et al., 2012). Similarly, peroxinectin, a key immune effector molecule with opsonic function in arthropods, was upregulated during *A.millepora* larval metamorphosis (Grasso et al., 2011). Heat shock proteins are also present (Tom et al., 1999; Shinzato et al., 2012), and a role in immunity has been suggested for Hsp70, because it is upregulated in response to *Vibrio coralliilyticus* (Brown et al., 2013). In contrast, lysozyme-like activity was upregulated in *Montastraea faveolata* following an infection with yellow band disease (Mydlarz et al., 2009). Eicosanoid cytokines may be involved in stimulating an immune response in white band disease-infected *A. cervicornis* (Table 1.1) (Libro et al., 2013), and in the inflammatory response signalling following wounding in the soft coral *Capnella imbricate* (Lõhelaid et al., 2014). A functional signalling pathway for the pro-inflammatory cytokine tumor necrosis factor (TNF) was also recently demonstrated (Quistad et al., 2014) and components of this pathway are know to be upregulated under stress (Barshis et al., 2013; Weiss et al., 2013). Integrins are important for cell-cell adhesion, and thus phagocytosis and immune cell mobility (Dupuy and Caron, 2008; Evans et al., 2009), but no immune function for coral integrins has been described to date (Brower et al., 1997). However, integrins have been shown to play a role in fertilisation and coral larval development (Iguchi et al., 2007; Knack et al., 2008). Other putative immune genes discovered in corals are the metal-binding protein ferritin (Shinzato et al., 2012), GiMAP/IAN (Weiss et al., 2013), which may play a role in phagocytosis, and several other immune-related domain-containing genes (Weiss et al., 2013).

1.4 - Immunocompetence and ecological immunology

Immunocompetence is the disease resistance capability of an organism and is defined as the magnitude and effectiveness of the immune response upon injury or infection (Adamo, 2004). The maintenance of a potent immune system is costly (Sandland and Minchella, 2003; Rolff and Siva-Jothy, 2003; Sadd and Schmid-Hempel,

2009) and is affected by the nutritional status of an individual (Siva-Jothy et al., 2005). Monitoring the immunocompetency of an organism can provide an understanding of its disease susceptibility, as well as the potentially immunosuppressive effects of environmental and chemical factors. In species other than corals, immunocompetence parameters are generally assayed using immunological tools (e.g. total immune cell count, phagocytosis, PO activity, antioxidant enzymes) (Adamo, 2004). Recently, basal levels of PO activity, melanin-containing granular cell size and FP concentration were also found to correlate with disease and bleaching susceptibility in ten families of Indo-Pacific corals (Palmer et al., 2010), potentially providing molecular tools to assess the ecological immune status of these organisms. However, the suitability of these parameters for monitoring purposes first needs to be addressed through ecological studies over seasonal timeframes.

1.5 - Coral disease and factors influencing immunocompetence

Despite the existence of many components of a robust immune system, corals have been affected by various diseases. At least 6.5% of the decline in coral cover on the Great Barrier Reef (GBR), Australia over the past 20 years can be attributed to coral diseases (Osborne et al., 2011). Disease is a multi-factorial phenomenon and may arise when environmental conditions change due to biological, environmental and / or anthropogenic disturbances (Adamo, 2012) that are detrimental to the host immune system, but beneficial to the pathogen. Preliminary data confirm that reduced immunocompetence plays a role in coral disease development (Mydlarz et al., 2006; Palmer et al., 2011a).

Coral disease has been correlated with localised anthropogenic impacts, including land-based anthropogenic impacts (Aeby et al., 2011; Guilherme Becker et al., 2013), nutrient enrichment (Kaczmarsky and Richardson, 2011), sewage run-off (Sutherland et al., 2011), tourism (Lamb and Willis, 2011), and the intensity of diving-related activities (Lamb et al., 2014). In addition, regular predation causes chronic stress with negative energetic consequences (Cole and Pratchett, 2011), and some corallivorous organisms are known vectors of disease (Nicolet et al., 2013). Immunocompetence is dependent on an organism's nutritional status (Siva-Jothy et al., 2005); hence, predation may result in immunocompromised corals.

Nutrient-enriched run-off from land-based practices and reduced seawater salinity in the tropical wet season are major concerns for coral reef health. Studies on abalone and clams have shown that reduced and increased salinity levels, as well as elevated ammonia levels negatively affect the number of immune cells present,

phagocytosis, and the proPO system (Reid et al., 2003; Cheng et al., 2004a; Cheng et al., 2004c; Ellis et al., 2011), while nitrite reduces phagocytosis and the number of immune cells, but increases proPO activation (Cheng et al., 2004b). Similar results were obtained for echinoderms (Reid et al., 2003) and shrimp (Liu and Chen, 2004; Tseng and Chen, 2004; Wang and Chen, 2005; Wang and Chen, 2006; Li et al., 2010b; Lin et al., 2012) under these stress conditions. Although some immune parameters were slightly increased, overall, abnormalities in seawater salinity and elevated nutrient levels adversely affected the immune system in all species tested, resulting in increased mortality as a consequence of disease (Reid et al., 2003; Cheng et al., 2004a; Cheng et al., 2004b; Cheng et al., 2004c; Liu and Chen, 2004; Tseng and Chen, 2004; Wang and Chen, 2005; Wang and Chen, 2006; Li et al., 2010b).

Various other factors are also known to negatively affect the immunocompetence of a range of marine invertebrates (Ellis et al., 2011). Phagocytic capabilities of immune cells are known to be affected by temperature, air exposure, seawater pH, hypoxia and anoxia, as well as a range of pollutants, including heavy metals and pesticides (Ellis et al., 2011). Anti-oxidant enzyme and lysozyme activities are strongly affected by season, but reports on the effects of temperature are contradictory (Ellis et al., 2011). However, both temperature and salinity do appear to reduce PO activity (Ellis et al., 2011). Similarly, AMP expression also has seasonal patterns and was upregulated at higher temperatures, whereas salinity affected only a few AMP types (Ellis et al., 2011). Also affected is the respiratory burst, when organisms are exposed to heavy metals (Coteur et al., 2005; Danis et al., 2006) and polychlorinated biphenyls (PCB) (Coteur et al., 2001; Coteur et al., 2005). In addition, metallothionein expression levels are reduced (Russo et al., 2003), while Hsp70 (Matranga et al., 2012) is strongly upregulated following exposure to cadmium. Hsp70 is also upregulated in acidic (Matranga et al., 2002), hypoxic (Holm et al., 2008), as well as extreme temperature (low and high) environments (Matranga et al., 2002), indicating significant stress under these conditions.

Several global scale impacts related to climate change may also play a role in promoting coral disease, including increasing seawater temperature, ocean acidification, altered rainfall and increased frequency of severe tropical storms (Sokolow, 2009; Ruiz-Moreno et al., 2012). Bleaching is the loss of *Symbiodinium* endosymbionts from the coral tissues, whereby the coral loses a major source of nutrients. Elevated pCO_2 levels (Anthony et al., 2008a) and high temperatures (Berkelmans and Oliver, 1999; Hoegh-Guldberg, 1999) can cause coral bleaching and likely have significant implications for the health of future reefs. Bleaching can also be caused by other stressors, including changes in seawater salinity (Hoegh-Guldberg

and Smith, 1989), unusually cold seawater temperatures (Muscatine et al., 1991; Gates et al., 1992; Hoegh-Guldberg et al., 2005) or pathogens (Kushmaro et al., 2001; Raymundo et al., 2005). Bleached and heat-stressed corals have been found to harbour a markedly altered microbial community (Ritchie, 2006; Bourne et al., 2008; Mouchka et al., 2010; Littman et al., 2011; Witt et al., 2011), exhibiting a shift towards a higher abundance of potential pathogens. A compounding effect is that the mucus of bleached corals lacks antimicrobial activity (Ritchie, 2006), which may be due to a shift towards a non-antibiotic-producing microbial community, reduced AMP production by the host or both. Recent studies demonstrate that bleached corals can be immunocompromised, although a considerable amount of variation in the response among coral species was observed. Bleached colonies of *Acropora millepora* exhibited signs of a compromised immune system, with lower peroxidase and PO activity and reduced melanin content (Palmer et al., 2011a), whereas bleached colonies of *Montastraea faveolata* showed upregulated PO activity (Mydlarz et al., 2009). Contrasting results were observed in another study, where heat-stressed, but unbleached, *M. faveolata* was found to have reduced melanin content and mucus bactericidal activity (Palmer et al., 2011b). Furthermore, *Pocillopora damicornis* was unable to exhibit an adequate immune and stress response against the coral pathogen *Vibrio coralliilyticus* at high temperatures when this bacterium is virulent (Vidal-Dupiol et al., 2011b), as evidenced by tissue lysis. In contrast, disease-resistant *Porites astreoides* appeared unaffected by heat stress, and exhibited an immune response under heat stress when challenged with PAMPs using the proPO-activating pathway (Palmer et al., 2011b), as did the gorgonian *Gorgonia ventalina* against *Aspergillus sydowii* using anti-fungal compounds (Ward et al., 2007). Overall, the effects of climate change on the immunocompetence and disease resistance of corals appears to be species-specific; some are likely to be severely affected, while others may be less affected under these conditions (Mydlarz et al., 2010).

1.6 - Thesis objectives and outline

Recent studies have shown that the innate immune system of scleractinian corals is more complex than previously thought based on their basal position in animal phylogenies. Although significant progress has been made, the field of coral immunology is still in its infancy, and understanding of the manner in which the coral innate immune system is modulated by multiple stressors, including global climate change (e.g. elevated sea water temperatures), anthropogenic influences (e.g. nutrient enrichment, tourism and fishing) and biological factors (e.g. pathogens and injury), is

generally poor. In light of the foundational role of corals as reef builders integral to the resilience of coral reef ecosystems and rising coral disease prevalence worldwide, there is an increasingly urgent need for a thorough understanding of how the coral immune system, and the holobiont as a whole, are affected by stressors and how corals respond to cope with these stressors.

The main objective of this work is to elucidate patterns in the coral innate immune response when exposed to biological, environmental and anthropogenic stressors. The following chapters build up an integrated picture of how the innate immune system of corals is affected by and responds to these stressors, both individually and in combination, and the implications of such stressors for the health of reef corals.

In Chapter 2, my aim was to determine temporal patterns in constitutive levels of immunocompetence parameters in corals and the suitability of these parameters as tools to monitor coral health. Determining constitutive levels of phenoloxidase activity and fluorescent protein concentrations *in situ* for three major reef-building corals representing the range from stress-sensitive to stress-tolerant species, throughout seasonal fluctuations over the course of one year, will provide insight into the different ecological immune strategies employed by corals and establish important baselines in key immunocompetence parameters. Establishing such baselines is critical for assessments of the effects of stressors on coral populations and will potentially form the basis for a tool for monitoring coral health. Outcomes can be used by reef managers to identify reefs that are most sensitive to disturbances and thereby assist with decisions on resource prioritisation for reef conservation.

In Chapter 3, my objective was to investigate the effects of multiple stressors, specifically anthropogenic stressors related to permanent tourist platforms combined with summer temperatures, on coral health. Comparing temporal patterns in phenoloxidase activity, GFP-like protein expression and immune gene expression over summer in corals at reef sites with and without tourist platforms will identify differences in immunocompetency parameters associated with healthy versus diseased corals. Determining the effects of tourist platforms on immunocompetence and coral disease development will provide insights into anthropogenic and environmental drivers of coral diseases. Identifying these drivers can help to inform management strategies focused on eliminating these stressors and improving coral health near tourist platforms.

In Chapters 4 and 5, my aims were to characterise the response of the coral immune system (immune gene expression analysis, phenoloxidase activity, GFP-like protein expression) and coral-associated bacterial communities (*in situ* hybridisation, 16S rRNA bacterial community profiling) to artificially inflicted injuries, both in the short term (24 hours) in the presence of mild heat stress (Chapter 4), and over a longer time course (10 days) in the absence of heat stress (Chapter 5). Given the increasing likelihood of damage to corals by severe storms and other anthropogenic factors, there is a need to establish a thorough understanding of the coral immune response following physical damage, the dynamics of the response, and processes associated with the regeneration of injuries. This will provide an understanding of the effect of injury on coral health and how these processes may be affected under future climate conditions.

In Chapter 6, my objective was to investigate the response of a moderately stress-tolerant coral and its microbial symbionts to pathogenic bacteria under elevated seawater temperatures. Determining the full transcriptome response of the coral and *Symbiodinium* using RNA Seq, coupled with impacts of these stressors on *Symbiodinium* photophysiology and bacterial community structure, will provide insights into how the coral holobiont may be impacted under future climate conditions. The outcomes of this study may aid in predictions of the impact of diseases on reef ecosystems.

In Chapter 7, I provide an overview of the main findings of this work, synthesise the results and place the outcomes into a broader perspective. Additionally, I suggest directions for future research to further our knowledge of coral immunology and the impacts of stressors on coral immunocompetency.

Chapter 2

Natural temporal variations in the prophenoloxidase-activating system and fluorescent protein expression are linked to environmental factors

This chapter has been submitted for publication to *Coral Reefs*

2.1 - Abstract

Extremes in seasonal environmental parameters can have significant impacts on the health of reef corals, thus there is need for sensitive biomarkers that could alert reef managers to impending stress. Even small increases in seawater temperature and sunlight intensity above summertime means, as well as increased terrestrial inputs and turbidity associated with high rainfall during wet seasons, can contribute to the development of disease and bleaching in corals. I analysed temporal patterns in parameters associated with innate immunity to establish baseline levels from which anomalies might be detected for representative species from three major reef-building coral families (Acroporidae, Faviidae, Poritidae). Patterns in phenoloxidase (PO) activity and in expression of green fluorescent protein (GFP)-like proteins on an inshore reef at nine time points over the course of one year show that both parameters are highly variable through time and that temporal patterns differ among species. Yearly averages of these parameters also differed among the three species. *Porites cylindrica* had 2.8-fold higher levels of PO activity than *Acropora millepora,* which had the lowest levels. In contrast, mean fluorescence was lowest in *P. cylindrica*, and highest in *Echinopora mammifomis*. Furthermore, the manner in which temporal patterns in these parameters correlated with seawater temperature, rainfall and salinity differed among the three species. For example, while PO activity positively correlated with seawater temperature in *A. millepora*, a negative correlation was found in both *E. mammiformis and P. cylindrica*. Differences in constitutive levels and temporal patterns in these parameters among species suggest that corals from these three families have evolved very different strategies for investing resources in innate immune parameters. Such differences highlight the need for baselines that are species-specific and for further long-term data before these ecological immunity parameters could be considered suitable for routine coral health monitoring.

2.2 - Introduction

Increasing environmental and anthropogenic pressures facing corals worldwide (Burke et al., 2011) highlight the urgent need for tools to evaluate and monitor coral health to manage and pre-empt activities that might cause further declines. Given the importance of immune system function for health, parameters associated with the innate immune system could make ideal stress biomarkers for coral health. A number of studies have identified activity of the prophenoloxidase (proPO) system, an important component of the innate immune system of invertebrates (Cerenius et al., 2010a), and expression of green fluorescent protein-like (GFP-like) proteins as potential coral stress biomarkers based on detectable responses to variations in environmental parameters (D'Angelo et al., 2008; Smith-Keune and Dove, 2008; Palmer et al., 2010; Roth et al., 2010; Palmer et al., 2011b; Roth and Deheyn, 2013). Both parameters respond to warm seawater temperature anomalies, which are known to significantly affect the health of the coral holobiont, causing shifts in coral-associated microbial communities (Littman et al., 2010), increases in the virulence of coral pathogens (Ben-Haim et al., 2003b; Vidal-Dupiol et al., 2011b), and disruptions to the coral-*Symbiodinium* endosymbiosis leading to bleaching (Douglas, 2003). Heavy monsoonal rainfall, which results in reduced salinity and increased terrestrial run-off leading to increased levels of turbidity and agricultural pollutants, is also a major stressor for corals. Warm seawater temperatures and changes in salinity and agricultural pollutants have all been shown to compromise the functioning of the innate immune systems of a range of marine invertebrates (Tseng and Chen, 2004; Ellis et al., 2011; Lin et al., 2012). In addition, both low salinity (Kerswell and Jones, 2003; Jones and Berkelmans, 2014) and cold sea surface temperatures (Saxby et al., 2003; Hoegh-Guldberg et al., 2005) have been implicated in coral bleaching. Extremes in seasonal environmental conditions can therefore significantly affect coral health, fitness and disease susceptibility. Although targeted, short-term studies are beginning to explore how environmental stressors affect the proPO-activating system and GFP-like protein expression, an understanding of what constitutes healthy baseline levels is needed to explore the potential of these two parameters as stress biomarkers.

The prophenoloxidase (proPO)-activating system is part of the melanin-synthesis pathway and forms an important, highly conserved component of the innate immune system of invertebrates (Mydlarz et al., 2006; Cerenius et al., 2010a), and has been described in both gorgonian and scleractinian corals (Mydlarz et al., 2008; Palmer et al., 2008). In corals, higher levels of immune parameters associated with this system, particularly phenoloxidase activity and melanin content, have been linked to

higher resistance to both bleaching and disease (Palmer et al., 2010). The proPO system is activated when components of microbial cell walls are detected, leading to activation of a protease cascade that cleaves proPO into PO (Cerenius et al., 2010a). In turn, PO oxidises phenolic compounds into cytotoxic quinone intermediates, which non-enzymatically form melanin. Whereas melanin forms a barrier and immobilises microbes, cytotoxic quinones and reactive oxygen species formed eliminate invading organisms. In corals, melanin and PO have been found to play a significant role in wound healing (Palmer et al., 2011c; D'Angelo et al., 2012) and in anti-microbial (Palmer et al., 2011a) and anti-parasite (Palmer et al., 2008) immune responses. Additionally, melanin deposits have been implicated in bleaching mitigation via photoprotection (Palmer et al., 2010). However, knowledge of baseline levels of PO activity characteristic of healthy corals and of the additional capacity for activation represented by the stored inactive proenzyme proPO is currently lacking. Studies of baseline levels of these parameters and how they vary seasonally are essential first steps in evaluating the efficacy of these immune parameters as stress biomarkers.

Expression of GFP-like proteins is thought to contribute significantly to cellular-based stress responses of corals, ameliorating bleaching by absorbing and dissipating solar radiation that could otherwise trigger photoinhibition (Baird et al., 2009). In contrast to their photoprotective role at high light intensities (Salih et al., 2000; Dove et al., 2001; Dove et al., 2008), fluorescent proteins (FPs) may also augment light for photosynthesis at low light intensities (Kawaguti, 1969; Schlichter et al., 1986). Similarly, there is strong evidence that non-fluorescent chromoprotein (CP) has a photoprotective role (Salih et al., 2000; Dove et al., 2001) for *Symbiodinium* photosystems (Smith et al., 2013). Increased expression of GFP-like proteins has been found in areas of high tissue proliferation, such as at branch tips and in regenerating tissues during wound healing (D'Angelo et al., 2012), potentially protecting both coral and *Symbiodinium* cells from light stress in areas with low levels of *Symbiodinium* pigmentation. GFP-like proteins may also exhibit anti-oxidant properties (Bou-Abdallah et al., 2006; Palmer et al., 2009a) by mitigating the effects of reactive oxygen species (ROS) produced by defective *Symbiodinium* photosystems (Lesser, 1996), and via the proPO-activating system and the oxidative burst as part of an innate immune response (Palmer et al., 2008; Palmer et al., 2009b; D'Angelo et al., 2012) .

Coral families differ in their susceptibilities to disease (Willis et al., 2004; Raymundo et al., 2005) and bleaching (Marshall and Baird, 2000; Loya et al., 2001), thus evidence that taxonomic (family level) trends in disease susceptibility are inversely correlated with constitutive levels of PO activity (Palmer et al., 2010) underscores the need to examine baseline levels that are at least family- or preferably species-specific.

On the Great Barrier Reef (GBR), coral species in the family Acroporidae are among the most vulnerable to coral diseases, whereas species in the family Poritidae are among the most resistant; species in the family Faviidae tend to be intermediate in their susceptibility to disease (Willis et al., 2004; Palmer et al., 2010). Similarly, there is a hierarchy in bleaching susceptibility among coral taxa, with massive taxa (e.g. Poritidae) being more resistant than fast-growing branching taxa (e.g. Acroporidae) (Marshall and Baird, 2000; Loya et al., 2001). However, within-species susceptibility can be significantly influenced by various other physiological and environmental factors, such as thermal history (Guest et al., 2012; Howells et al., 2013) and *Symbiodinium* endosymbiont composition (Howells et al., 2012) and density (Cunning and Baker, 2013). Such variations in bleaching and disease susceptibility highlight the need for universal coral biomarkers for stress. While potential biomarkers at colony, population and community levels have been identified (Cooper et al., 2009), data from longer term studies to test their efficacy are limited.

In this study, I investigated seasonal patterns in both activity of the proPO system and expression levels of GFP-like proteins to establish baseline levels for a common species in each of three major reef-building coral families. Species selected span the range from those typically resistant to those highly susceptible to bleaching and disease. Seasonal patterns in sea temperature, cloud cover, rainfall and salinity were assessed over the corresponding year-long period to identify potential links between these parameters and environmental factors. I hypothesised that corals upregulate GFP-like proteins and the proPO system during summer months to cope with increased environmental stress that can lead to bleaching and disease. To evaluate the applicability of these biomarkers as indicators for coral health monitoring, I tested if expression levels of these two biochemical parameters varied with seasonal patterns in environmental parameters.

2.3 - Material & Methods

2.3.1 - Field sampling design and sample collection

This study was conducted in Pioneer Bay (coordinates: 18° 36' 24.9114"S, 146° 29' 20.205"E), situated on the sheltered side of Orpheus Island within the Great Barrier Reef Marine Park. Samples of corals in the families Acroporidae (*Acropora millepora* – red colour morph), Faviidae (*Echinopora mammiformis)* and Poritidae (*Porites cylindrica)* were collected from similarly-sized tagged colonies over the course of 1 year, from October 2009 to September 2010 (Fig.. 2.1). Numbered plastic cattle tags and cable ties were used to tag ten colonies of *E. mammiformis* located at 2-4 m depth

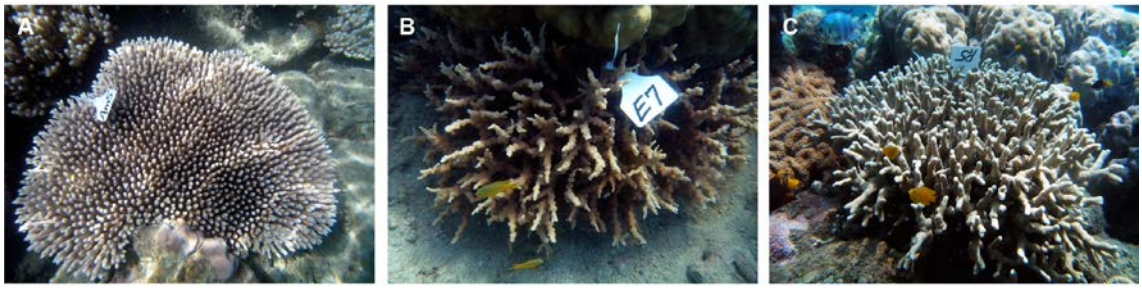


Figure 2.1 – Study species from three important reef-building coral families. A) *Acropora millepora* (Acroporidae), B) *Echinopora mammiformis* (Faviidae) and C) *Porites cylindrica* (Poritidae).

and eight colonies of each of *A. millepora* (2-3 m depth) and *P. cylindrica* (4-5 m depth). Colonies were sampled and photographed at nine time points: October 2009 (late austral spring); December 2009, and January, February and March of 2010 (austral summer); May, July and August of 2010 (austral winter); and September 2010 (early austral spring). At each time point, one branch (approximately 5 cm in length) was sampled from the middle of each tagged colony using surgical bone cutters, placed in a plastic bag underwater, transferred to a cryogenic tube and snap-frozen in liquid nitrogen, and stored at -80ºC until processed.

2.3.2 - Sample preparation

To prepare tissue lysates, frozen fragments were thawed on ice and approximately 4 cm^2 of coral tissue was removed from the fragment using an airbrush into 10 ml of ice-cold extraction buffer (50mM Tris-HCl, pH 7.8 with 50 mM dithiothreitol). Each tissue sample was homogenized for 45 seconds (IKA T10 Basic homogenizer, Malaysia). The resulting slurry was centrifuged at 3500 rpm for 5 minutes and the supernatant collected and stored at −30°C. Total protein content of each sample was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The endpoint absorbance was read at 750 nm using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA).

2.3.3 - Clarification of terminology

In previous studies of coral immune responses, the term phenoloxidase (PO) activity has been used to describe levels of the active enzyme detectable in a sample and pro-phenoloxidase (proPO or PPO) activity to describe the sum of active and potential enzyme activity. The latter activity is revealed by converting the inactive pro-phenoloxidase zymogen to the active PO form through trypsin-mediated experimental cleavage. Since proPO is a pro-enzyme or zymogen and thus lacks any form of activity, I argue that the term proPO activity is not an accurate reflection of the parameter that is being quantified by the assay. Here I use the term tpPO (total

potential phenoloxidase) activity to replace proPO activity. In my study, both PO and tpPO activity refer to tyrosinase-type phenoloxidase activity that is assayed by using dopamine as substrate.

2.3.4 - Phenoloxidase activity

Phenoloxidase activity was assayed according to procedures outlined in Palmer et al. (2011a), with some modifications. Both total potential (trypsin-activated) phenoloxidase (tpPO) activity and active phenoloxidase (PO) activity were measured to analyse the total capacity and the active fraction of the proPO system, respectively, in each sample. To analyse tpPO activity, 20 µl of coral tissue lysate was loaded in triplicate into wells of a 96-well plate, to which Tris buffered saline (50 mM, pH 7.8; 40 µl) and trypsin (25 µl 0.1 mg/ml) were added. Reaction mixtures were incubated for 20 minutes to allow for activation of pro-phenoloxidase by trypsin, and then 30 µl of 10 mM dopamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was added to each mixture. As a blank, 20 µl of extraction buffer was used. The same procedure was followed to analyse PO activity, except that 25 µl double distilled water was substituted for the trypsin solution. Absorbance was measured at 490 nm every 5 min for 45 min using the SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA). Data for each sample were independently obtained in triplicate. Phenoloxidase activity was calculated as the change in absorbance using the linear portion of the reaction curve over time and standardized to total protein content of each sample.

2.3.5 - Chromoprotein and fluorescent protein expression

Twenty µl of tissue lysate was added to each well of a black, clear bottom 384-well plate in triplicate for each sample. Expression of chromoprotein was analysed by measuring the absorbance at 588 nm using a SpectraMax M2. The fluorescence spectrum was analysed by measuring emission wavelengths between 400 and 700 nm, with a 5 nm resolution, emitted upon excitation of fluorescent proteins at 280 nm. All data were normalised to total protein content. Fluorescence spectra and fluorescent protein expression levels were calculated in R using the method described in Paley *et al.* (in review; procedure in Appendix B). In summary, the exponentially decaying background scatter was subtracted from each spectrum between 445 and 645 nm and multiple regression models based on purified FP spectra were fitted to the data to calculate the proportions of the individual fluorescent proteins (cyan, green and red fluorescent protein) present.

2.3.6 - Environmental parameters

Daily environmental data (seawater temperature, rainfall and salinity) were obtained from the Australian Institute of Marine Science Orpheus Island Platform and Sensor Float 1 (Integrated Marine Observing System (IMOS)) located in Pioneer Bay (see http://www.aims.gov.au). Daily cloud fraction data (Level 2, MOD06) were acquired from the Moderate Resolution Imaging Spectroradiometer (MODIS) on board the Terra satellite (downloaded via ladsweb.nascom.nasa.gov; algorithm description in King et al. (1997)). As cloud cover is variable on local scales and MODIS data result from a single snapshot (or, at most, two snapshots) around 10:30am local time, cloud fraction conditions across each daytime period were inferred by considering data from pixels in the vicinity of the study site. The radial extent of cloud influence considered was estimated using local wind information. Near-surface wind speeds in the IMOS data during the study period had a mean value of 19.8 km/hr, which for an eight-hour daytime period corresponds to a distance of 158.4 km. However, upper-level wind speeds are typically greater than those near the surface, plus the geometry of sun angle at the location and wind direction through each day confer additional variability affecting spatial values to be included in the daily cloud fraction estimate. Accordingly, the distance was reduced (by half) to 79.2 km, which is approximately 0.7 arc degrees, and cloud fraction values within a radius of 0.35 arc degrees of Pioneer Bay were averaged for each day. All environmental parameter data can be obtained from Supplementary Figures 2.1 and 2.2.

2.3.7 - Statistical analysis

Temporal data were analysed using a Linear Mixed Effects (LME) Model and comparisons were made between consecutive months. Pair-wise comparisons were made using LME models and the Bonferroni correction was applied to obtain the critical p-value based on $\alpha = 0.05$. Correlations between PO and tpPO activities, as well as between biological (PO and tpPO activities, total fluorescence and GFP-like protein expression) and environmental parameters (average of 10 days prior to sampling) were analysed using Pearson's r. All analyses were conducted using the statistical software package S-PLUS 8.0. Differences were considered significant when $p < 0.05$, or $p < 0.05/n$ in case of Bonferroni-adjusted p-values with n being the number of pair-wise comparisons.

2.4 - Results

2.4.1 - Phenoloxidase activity

On average, mean constitutive levels of the two parameters associated with the proPO-activating system differed among the three coral species over the one-year duration of the study. The yearly average of active phenoloxidase (PO) activity (measured as ΔOD 490 nm per mg protein per min) was more than two-fold greater in *Porites cylindrica* (10.7 ± 0.54) than in *Acropora millepora* (3.73 ± 0.48), while *Echinopora mammiformis* (5.62 ± 0.31) had intermediate levels of PO activity (dashed lines in Fig. 2.2A-C). A similar pattern was found for yearly averages of total potential phenoloxidase (tpPO) activity, which was more than two-fold greater in *P. cylindrica* (10.89 ± 0.55) than in *A. millepora* (4.65 ± 0.53), and again intermediate in *E. mammiformis* (6.88 ± 0.41) (Fig. 2.2D-F). A strong correlation between tpPO activity (measured as ΔOD 490 nm per mg protein per min) and PO activity was detected for

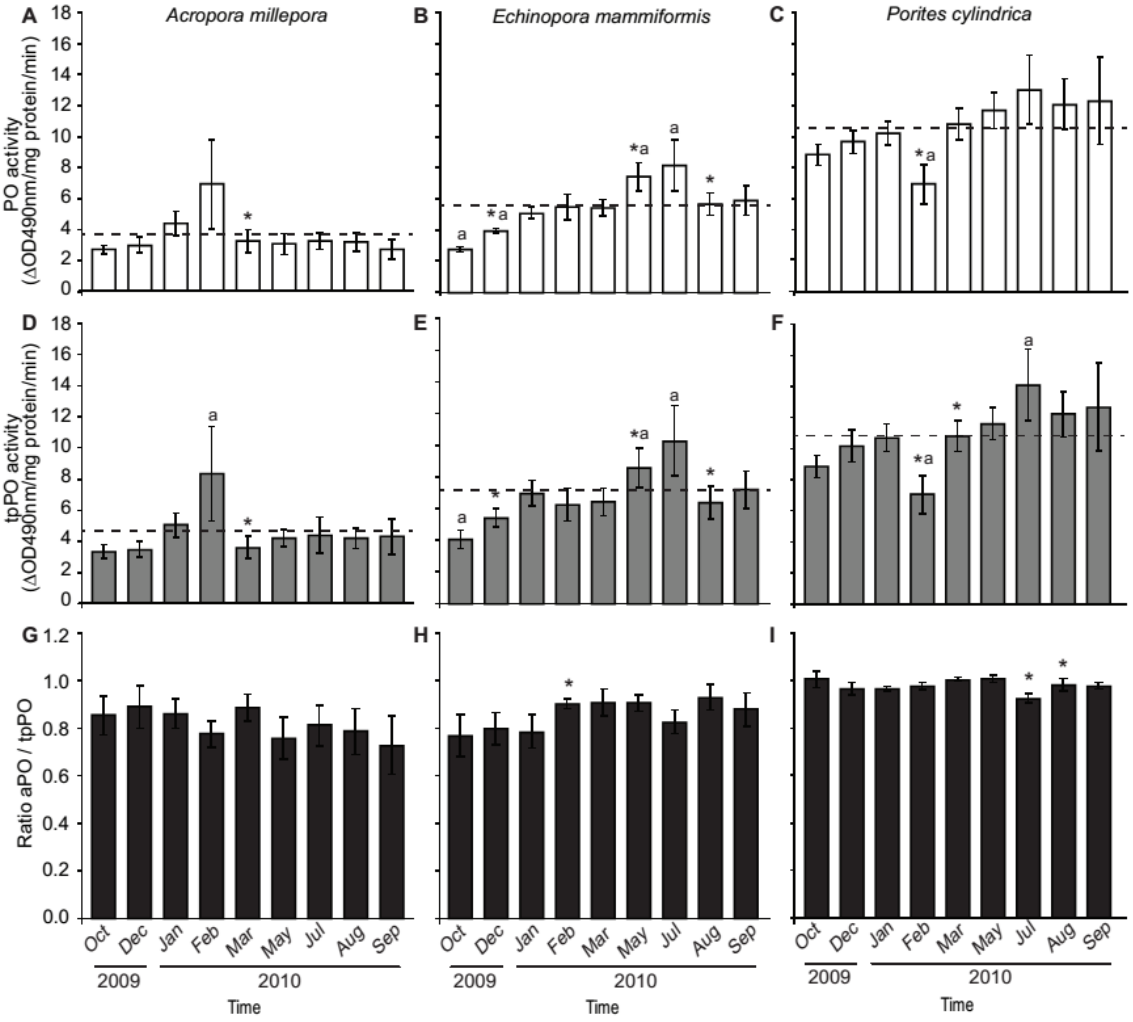


Figure 2.2 – Seasonal patterns in the proPO system. Temporal patterns in A-C) active phenoloxidase (PO) activity; D-F) total potential phenoloxidase (tpPO) activity; and G-H) ratio of PO activity and tpPO activity. Asterisk (*) indicates statistical significant difference with previous time point. Letter 'a' indicates statistical significant difference from the yearly average. Data were considered significant when $p < 0.05$.

all three species (*A. millepora*: r = 0.95; *P. cylindrica*: r = 0.99; *E. mammiformis*: r = 0.91). The average percentage of tpPO activity present in the active form (PO) ranged from 81.7±2.6% in *A. millepora,* to 86.0±1.9% in *E. mammiformis* and up to 98.0±0.7% in *P. cylindrica* (Fig. 2.2G-I), indicating that the majority of tpPO is present in its active state in all three species. Patterns in the ratios of PO to tpPO activities among species mean that *A. millepora* stores the greatest proportion of its total potential phenoloxidase activity in the inactive proPO zymogen form and *P. cylindrica* the least.

Seasonal patterns in phenoloxidase activity also tended to differ among the three species. In *A. millepora*, both PO and tpPO activity remained constant for most of the year, except for an increase between the summer months of December and February (levels 2.3-fold higher in February than in March; PO $p = 0.06$; tpPO $p = 0.04$), which resulted in 1.8-fold higher activity levels in February compared with the yearly average (PO $p = 0.06$; tpPO $p = 0.04$) (Fig. 2.2A, D). *P. cylindrica* also displayed a generally stable level of phenoloxidase activity throughout the year, but for this species both PO ($p = 0.01$) and tpPO activity ($p = 0.01$) were significantly reduced in February compared with January and March (Fig. 2.2C, F). Concomitantly, the highest levels occurred in winter for *P. cylindrica* (Fig. 2.2C, F). The most variable pattern of phenoloxidase activity was observed for *E. mammiformis* (Fig. 2.2B, E). Compared with the yearly average, PO activity was reduced in the late spring (October, $p < 0.01$) and early summer months (December, $p = 0.02$), and activity of tpPO was reduced in October ($p < 0.01$). Conversely, activity was significantly higher than the yearly average in the early winter months of May (PO $p = 0.01$; tpPO $p = 0.03$) and July (PO $p < 0.01$; tpPO $p < 0.01$).

2.4.2 - Green fluorescent protein-like proteins

Comparisons of mean fluorescence spectra between summer and winter revealed no major shifts in emission wavelength peaks in any of the three species (Fig. 2.3A-C). However, overall mean levels of fluorescence were higher in summer (December - March) than in winter (July - September) for *E. mammiformis* ($p = 0.001$). In contrast, overall mean levels of fluorescence did not differ significantly between summer and winter months for either *A. millepora* ($p = 0.878$) or *P. cylindrica* ($p = 0.405$) (Fig. 2.3A-C), although peaks in total fluorescence in both the cyan FP and green FP regions were higher in summer for both species. This was particularly the case for *P. cylindrica,* which had a three-fold higher peak in the cyan FP region (470 nm peak) in summer than in winter. More detailed analyses of temporal patterns in total fluorescence at the level of month also differed among species (Fig. 2.3D-F). Overall, mean total fluorescence throughout the year was nearly two-fold higher in *E.*

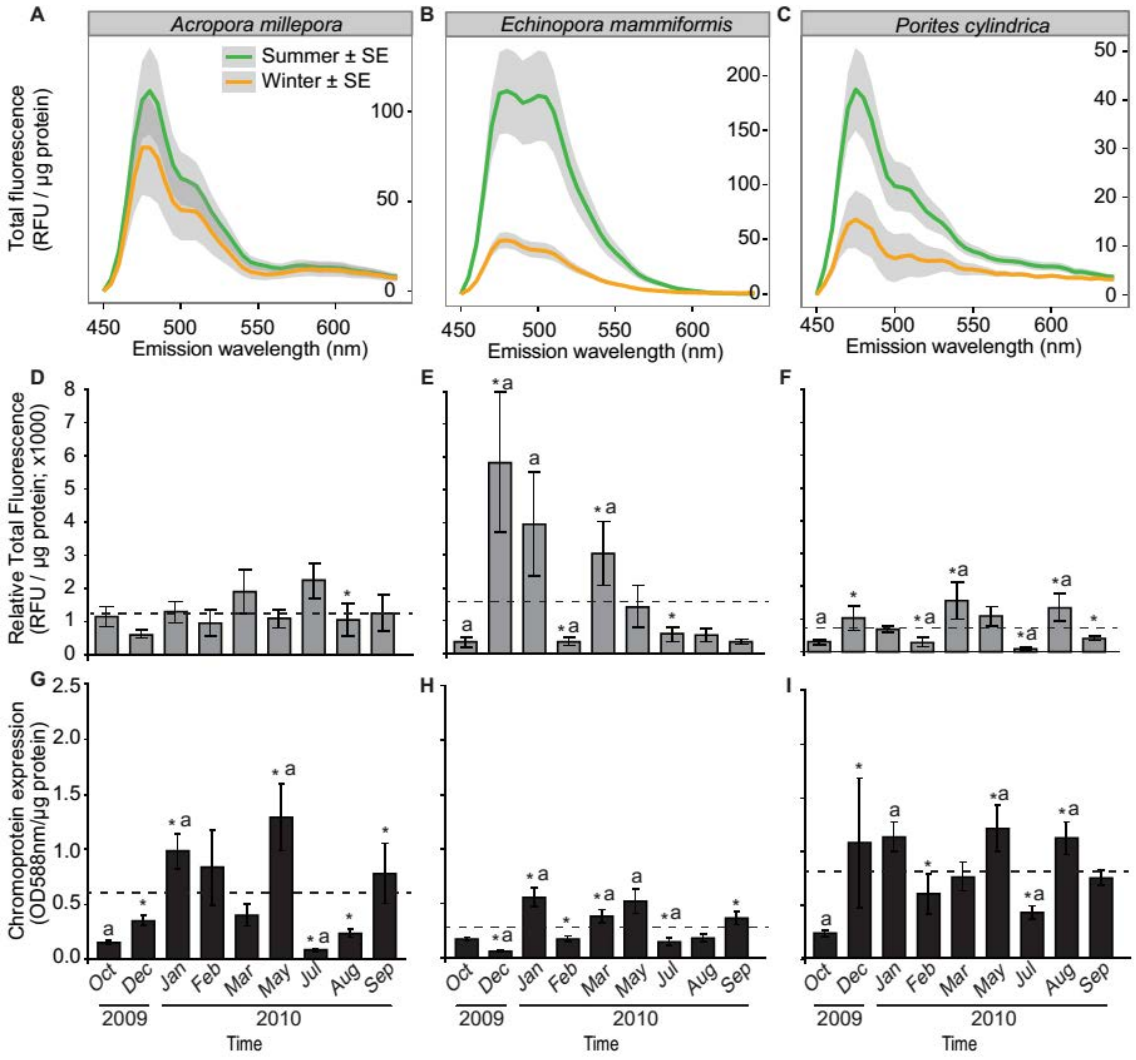


Figure 2.3 – Seasonal patterns in coral fluorescence. A-C) Fluorescence spectra in summer and winter, shaded area indicates the standard error of the mean (SEM); D-F) total fluorescence levels; and G-I) chromoprotein levels. Asterisk (*) indicates statistical significant difference with previous time point. Letter 'a' indicates statistical significant different from the yearly average. Data were considered significant when $p < 0.05$. RFU = relative fluorescence units; OD588nm = optical density at 588 nm wavelength.

mammiformis than in *P. cylindrica,* with mean levels intermediate in *A. millepora* (dashed lines in Fig. 2.3D-F). Total fluorescence levels in *A. millepora* were comparatively stable throughout the year (mean 1284 ± 153 RFU / µg protein) (Fig. 2.3D), whereas they fluctuated significantly in the other two species (*E. mammiformis*, 1690 ± 332 RFU / µg protein; *P. cylindrica*, 741 ± 71 RFU / µg protein). Fluctuations in total fluorescence were particularly pronounced in summer months in *E. mammiformis*, reflecting the 8.4 to 16.1-fold reduction in February compared with the other summer months, and in both summer and winter months in *P. cylindrica* (Fig. 2.3E-F).

Yearly averages of chromoprotein (CP) expression showed the inverse of patterns found for total fluorescence, with mean levels more than two-fold higher in *P. cylindrica* (0.81 ± 0.08) than in *E. mammiformis* (0.28 ± 0.03), but with *A. millepora*

again being intermediate between these (0.58 ± 0.08) (Fig. 2.3G-I). Throughout the year, however, CP expression fluctuated significantly in all species. Temporal patterns in CP expression were roughly similar for all three species, with CP expression being upregulated by the greatest amount in January and May, and consistently downregulated in July (Fig. 2.3G-I).

Similar to patterns in total fluorescence, proportions of the individual fluorescent proteins, i.e. cyan, green and red fluorescent proteins (CFP, GFP and RFP, respectively), in relation to total fluorescence did not differ significantly from the yearly average in *A. millepora,* in any of the 9 months (Fig. 2.4A, D, G), although the proportions of CFP and GFP fluctuated among months (Fig. 2.4A, D). Proportional levels of each FP were also relatively stable in *P. cylindrica*, with the exception of levels recorded in March, when the contribution of CFP to total fluorescence was significantly increased, and concomitantly, levels of GFP and RFP were decreased (Fig. 2.4C, F,

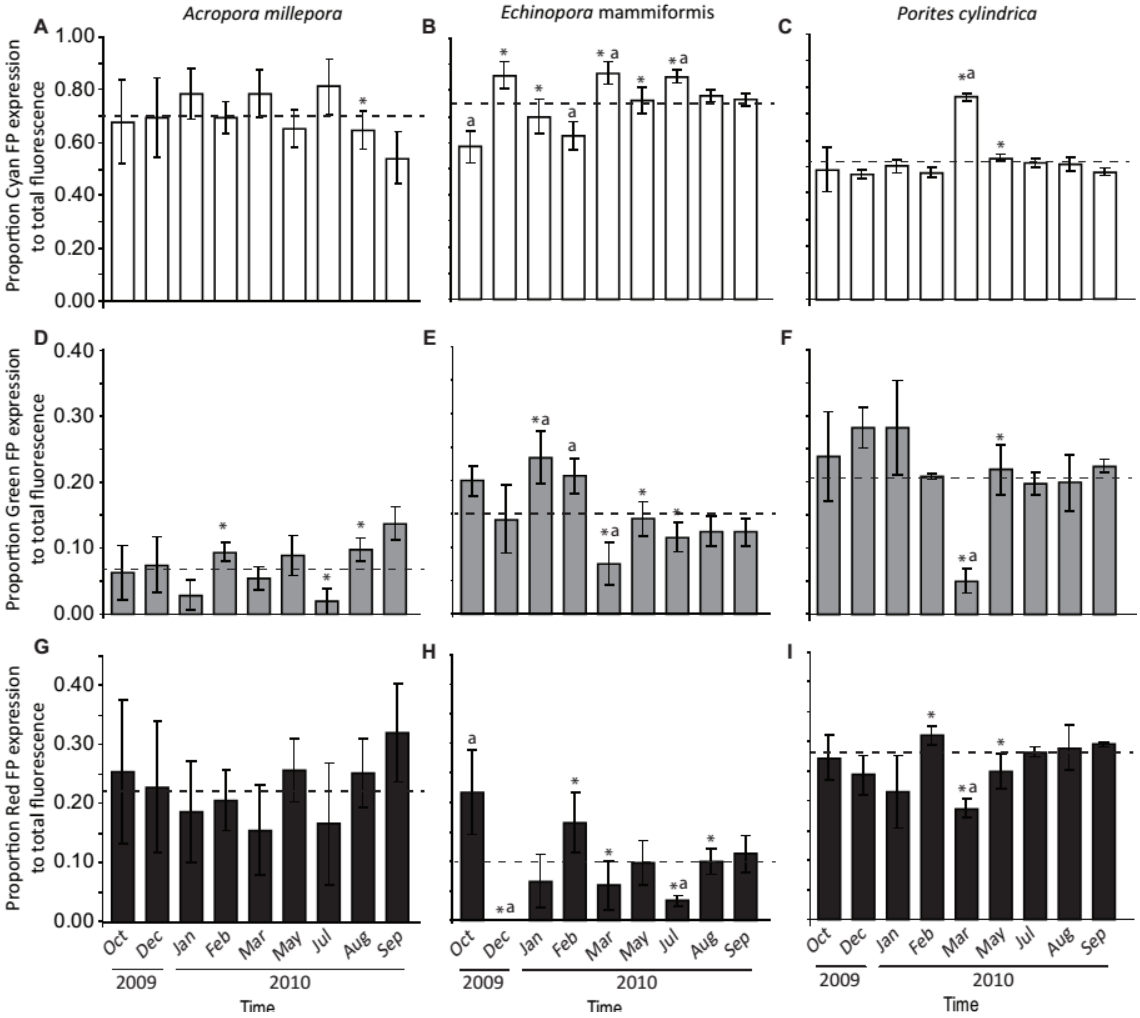


Figure 2.4 – Seasonal patterns in proportion of individual fluorescent proteins (FP) to total fluorescence. A-C) cyan fluorescent protein; D-F) green fluorescent protein; and G-I) red fluorescent protein. Asterisk (*) indicates statistical significant difference with previous time point. Letter 'a' indicates statistical significant different from the yearly average. Data were considered significant when $p < 0.05$.

I). In contrast, individual FP proportions in *E. mammiformis* fluctuated over time and around the yearly average, with no clear association with season (Fig. 2.4B, E, H).

2.4.3 - Correlations between biological and environmental parameters

Based on monthly average rainfall and seawater temperatures (Suppl. Fig. 2.2) from the five years prior to my study as a reference, no anomalies in these environmental parameters were observed during my sampling period. Between 2004 and 2009, the hottest monthly average temperature was 29.2 ± 0.2°C (mean ± SEM), while the highest temperature in my study was 29.4°C. Rainfall was also not considered anomalous, with a peak of 948 mm in January 2010, compared with peaks of 1068 and 1007 mm in October 2005 and January 2009, respectively (744 ± 134 mm (mean ± SEM) rainfall in the wettest month in previous years).

Levels of PO activity were significantly correlated with temperature across all three coral species (Table 2.1). However, whereas PO activity was positively correlated with temperature in *A. millepora*, it was negatively correlated with temperature in *E. mammiformis* and *P. cylindrica*. In *A. millepora*, PO and tpPO activity were also positively correlated with rainfall, but in contrast, in *P. cylindrica,* both parameters showed a negative correlation with rainfall (Table 2.1). No significant correlations between rainfall and any of the two biochemical parameters were observed in *E. mammiformis*.

Chromoprotein expression was correlated with one environmental parameter in one species, i.e. CP was positively correlated with temperature in *A. millepora* (Table 2.1). Interestingly, a strong correlation was observed between CP and both PO ($r = 0.72$, $p < 0.01$) and tpPO ($r = 0.67$, $p < 0.01$) in *A. millepora*, but not in the other two species tested. With the exception of the correlation with temperature in *E. mammiformis*, total fluorescence did not correlate with any of the environmental parameters in any of the species examined (Table 2.1). In addition, I did not observe any correlation between rainfall and GFP-like protein expression (Table 2.1). Proportions of GFP and CFP showed a positive and a negative correlation with temperature, respectively, in *E. mammiformis* (Table 2.1). In contrast, GFP was negatively correlated with cloud cover in *P. cylindrica*, whereas CFP was positively correlated with this parameter in this species (Table 2.1). My findings also show that there is a strong positive correlation between salinity and the proportion of GFP in *P. cylindrica,* and a strong negative correlation with the proportion of CFP in this species (Table 2.1). An overview of the environmental parameters assessed can be found in Supplementary Figure 2.1.

Table 2.1 – Correlations (Pearson's r) between immune parameters (PO activity and GFP-like proteins) and environmental factors (temperature, rainfall, salinity and cloud cover). Significant correlations ($p < 0.05$) are indicated with an asterisk (*) as well as **bold and underlined**.

	Temperature	Rainfall	Salinity	Cloud cover
PO activity				
Acropora millepora	**0.26** (p = 0.049) *	**0.34** (p=0.012) *	-0.18 (p=0.240)	0.24 (p=0.068)
Echinopora mammiformis	**-0.25** (p=0.022) *	-0.01 (p=0.949)	-0.01 (p=0.947)	0.15 (p=0.160)
Porites cylindrica	**-0.34** (p=0.005) *	**-0.34** (p=0.009) *	0.25 (p=0.106)	-0.13 (p=0.298)
total potential PO activity				
Acropora millepora	0.21 (p = 0.1065)	**0.35** (p=0.009) *	-0.19 (p=0.249)	0.24 (p=0.067)
Echinopora mammiformis	-0.20 (p=0.068)	-0.06 (p=0.639)	0.07 (p=0.614)	0.11 (p=0.315)
Porites cylindrica	**-0.36** (p=0.003) *	**-0.35** (p=0.006) *	0.29 (p=0.055)	-0.13 (p=0.314)
Chromoprotein expression				
Acropora millepora	**0.26** (p=0.046) *	0.22 (p=0.113)	-0.11 (p=0.130)	0.13 (p=0.308)
Echinopora mammiformis	0.10 (p=0.343)	-0.001 (p=0.989)	-0.12 (p=0.391)	0.08 (p=0.461)
Porites cylindrica	0.03 (p=0.815)	-0.12 (p=0.375)	0.22 (p=0.161)	-0.19 (p=0.112)
Total fluorescence				
Acropora millepora	0.05 (p=0.699)	-0.182 (p=0.248)	-0.18 (p=0.248)	0.21 (p=0.128)
Echinopora mammiformis	**0.28** (p=0.013) *	-0.09 (p=0.436)	0.14 (p=0.305)	-0.08 (p=0.505)
Porites cylindrica	-0.01 (p=0.939)	0.10 (p=0.472)	-0.07 (p=0.661)	-0.10 (p=0.405)
CFP proportion				
Acropora millepora	0.10 (p=0.450)	-0.03 (p=0.855)	-0.03 (p=0.855)	0.11 (p=0.429)
Echinopora mammiformis	**-0.25** (p=0.027) *	-0.204 (p=0.089)	0.13 (p=0.346)	-0.01 (p=0.999)
Porites cylindrica	0.06 (p=0.609)	0.10 (p=0.433)	**-0.53** (p=0.0002) *	**0.32** (p=0.008) *
GFP proportion				
Acropora millepora	-0.10 (p=0.457)	-0.12 (p=0.433)	-0.12 (p=0.433)	-0.05 (p=0.739)
Echinopora mammiformis	**0.31** (p=0.005) *	0.14 (p=0.241)	0.07 (p=0.605)	-0.03 (p=0.805)
Porites cylindrica	0.07 (p=0.589)	-0.16 (p=0.214)	**0.49** (p=0.0007) *	**-0.32** (p=0.009) *
RFP proportion				
Acropora millepora	-0.09 (p=0.491)	0.07 (p=0.638)	0.07 (p=0.638)	-0.12 (p=0.384)
Echinopora mammiformis	0.07 (p=0.518)	0.15 (p=0.209)	-0.25 (p=0.072)	0.02 (p=0.871)
Porites cylindrica	-0.18 (p=0.151)	0.08 (p=0.531)	0.01 (p=0.939)	-0.01 (p=0.912)

2.5 - Discussion

2.5.1 - Seasonal patterns in the pro-phenoloxidase system

In this study, I show that there is high variability in mean levels of both activated and total potential PO activity over a year, and this occurs both within and among corals from three common reef-building families. The typically more than two-fold

higher levels of PO activity found in *Porites cylindrica,* a disease- and bleaching-resistant species, compared with the more susceptible *Acropora millepora,* suggest a significantly higher level of resource investment in innate immunity by *P. cylindrica,* which likely contributes to its greater stress resistance. With one exception, year-long consistency in comparative levels of PO activity among the three species verifies and extends taxonomic patterns in innate immunity reported by Palmer et al. (2010), which were based on samples collected at one point in time. Contrasting patterns in February samples, of upregulated PO activity above typically low constitutive levels in *A. millepora* versus downregulated PO activity above typically high constitutive levels in *P. cylindrica,* highlight species-specific differences in investment in this immune parameter in response to summer environmental parameters.

In addition to significant temporal variation in both active and total potential PO activity, the seasonal timing of peaks in these parameters differed among the three coral species. Peaks in PO and tpPO activity in *A. millepora* occurred during summer months, and in combination with a positive correlation between PO activity and seawater temperature, this timing suggests that upregulation of these immune parameters minimises temperature stress that could lead to bleaching in this species. Increased PO activity in bleached colonies of *A. millepora* in an earlier study (Palmer et al., 2011a) corroborates this hypothesis. Such upregulation could counteract changes in coral-associated bacterial communities induced by heat stress or provide photoprotection for coral tissues and symbionts (Palmer et al., 2010). In contrast, peaks in baseline levels of PO activity were found in winter months in both *P. cylindrica* and *E. mammiformis*, showing a negative correlation between this parameter and seawater temperature. However, the higher constitutive PO levels throughout the summer in these two species compared to *A. millepora* may have been sufficient to cope with summer levels of environmental parameters in the absence of seasonal anomalies. Potential differences in resource allocation to other components of the innate immune system by each coral species may also explain the observed disparity among species. Variability in temporal patterns in PO and tpPO activity within and among coral species highlights the importance of long-term baselines and the need for multiple assays to assess coral health, as outcomes are dependent on the species sampled and the seasonal timing of the sampling.

2.5.2 - Seasonal patterns in GFP-like protein expression

Comparisons of temporal patterns in GFP-like protein expression among corals revealed that both total fluorescence and individual FP expression levels were also variable within and among coral species over time. In *A. millepora,* comparative

stability in FP expression through time and lack of correlation with normal seasonal variation for any of the four environmental parameters examined indicate that the typically high constitutive levels of FP expression may be sufficient to fulfil potential stress reduction function(s) in this coral species in the absence of seasonal anomalies. In contrast, 5.8-fold higher total fluorescence levels in summer compared with winter in *E. mammiformis* and a positive correlation with seawater temperatures highlight that corals species differ markedly in their strategies for investing in constitutive FP levels. Increasing investment in GFPs with increasing seawater temperature, in contrast to decreasing investment in CFP, suggests that GFP, and not CFP, may play a role in the heat stress response in *E. mammiformis*. The typically low but highly variable levels of total fluorescence in *P. cylindrica* over the year were not correlated with any of the four environmental parameters examined. Although total fluorescence was not correlated with salinity in *P. cylindrica*, increases in the proportion of CFP as salinity decreased and concomitant decreases in GFP raise the possibility that CFP expression may be upregulated under prolonged low salinity stress in *P. cylindrica* at the expense of GFP expression. However, such an interpretation requires confirmation through experimental studies with a higher sampling resolution and raises additional questions about the function of fluorescent proteins in corals.

Significant reductions in total fluorescence levels in both *P. cylindrica* and *E. mammiformis* in February compared with the other summer time points, despite seawater temperatures being the highest recorded around any of my sampling times, raise the possibility that fluorescence plays a role in mitigating stress induced by an environmental parameter other than temperature. Interestingly, the cloud cover signature at this time was distinctly different from all other time points, showing 13 consecutive days with more than 90% cloud cover in the study region, while cloud cover was below 90% at all other sampling times. Although solar radiation is increased in summer and heats up the seawater, clouds can reduce the amount of solar radiation that reaches corals. In accordance with a photoprotective function for fluorescent proteins, it may be that these two species increased their fluorescence levels in summer in response to higher levels of solar radiation and then down-regulated levels when clouds reduced solar radiation to non-stress levels. Taken together, total fluorescence levels appear to be upregulated in response to increased levels of solar radiation, providing corroborative evidence for a photoprotective function for fluorescent proteins in corals.

Non-fluorescent chromoprotein levels were highly fluctuating in all coral species, although a positive correlation with seawater temperature was found in *A. millepora*. This suggests a role for chromoprotein in the thermal stress response, which

is consistent with the observation of elevated chromoprotein expression levels in naturally bleached colonies of this species (Seneca et al., 2010). Chromoprotein exhibits anti-oxidant properties (Palmer et al., 2009a) and could therefore be upregulated in response to increased PO activity at higher temperatures to mitigate the adverse effects of reactive oxygen produced by the proPO system. The strong correlations found between CP expression and PO activities in this species, supports this interpretation. Surprisingly, in *E. mammiformis* and *P. cylindrica* there was no correlation between temperature and CP, or between CP and PO activities. Corals do, however, possess other more effective antioxidants, such as superoxide dismutase, catalase and peroxidase (Mydlarz and Palmer, 2011; Shinzato et al., 2012). Potentially, these enzymes are the primary antioxidants in *E. mammiformis* and *P. cylindrica*, while in *A. millepora* CP may play a more significant role in neutralising reactive oxygen species.

2.5.3 - Comparative ecological immunology

Comparative differences in immune and stress response parameters among coral species representing three coral families strongly suggest that corals differ in the allocation of resources to constitutive levels of immune and stress response systems. Higher levels of PO activity in the resistant coral *P. cylindrica* compared with the intermediately resistant *E. mammiformis* and the susceptible *A. millepora* are consistent with positive correlations between immune status and both disease and bleaching resistance, as found by Palmer et al. (2010). In addition, I also found that a significantly higher proportion of PO is in its active form in *P. cylindrica* than in *A. millepora*, which would further contribute to differences in disease and bleaching susceptibilities between these species. *P. cylindrica* appears to have constitutively high levels of PO activity and is thereby capable of preventing microbial infection and responding immediately to disturbances. Due to the short half-life of activated PO, maintaining such high PO activity requires significant resources. *A. millepora*, on the other hand, has lower constitutive levels of PO activity, but does have the capability of inducing additional activation of PO in response to infections when and where necessary. Although storing more PO in its inactive proPO-form may require fewer resources and thereby allows the allocation of more resources for other life history traits, such as colony growth, these relatively low constitutive PO activity levels may provide minimal protection against invading microbes, thus increasing the chance of infection and disease before a significant immune response can be orchestrated by the host. It should, however, be noted that none of the coral colonies followed in this study developed signs of disease or bleaching. Evaluating both PO and tpPO activity levels

in corals may significantly increase our understanding of the ecological immunology of corals.

Differences in constitutive levels of fluorescence among the coral species studied also correlate with life history differences. Fast growing *A. millepora* generally maintained relatively high fluorescence levels across seasons compared with the other two species, which exhibited typically lower and more variable total fluorescence levels and generally have slower growth rates. Fluorescent proteins are known to be more highly expressed in growing tissues (D'Angelo et al., 2012), possibly because of their photoprotective function, which may explain the relatively higher constitutive levels of fluorescence in the fast growing species. Maintaining fluorescence is considered costly due to relatively high FP expression levels, although studies of FP turnover suggest that due to their long half-life, maintaining high levels of FPs may be comparatively cheap (Leutenegger et al., 2007a). However, the apparent lack of induced FP expression in summer when solar irradiance and temperature, two important factors in coral bleaching, are highest was surprising. This could indicate that FP levels in *A. millepora* are not sufficiently adequate to mitigate the effects when these environmental factors reach stressful levels, or that summer temperatures and solar irradiance were not stressful. In contrast, *E. mammiformis* significantly upregulates FP levels, in particular GFP, in summer, which likely protects this coral from high irradiance and temperature and ameliorates bleaching (Baird et al., 2009). *P. cylindrica* may use its high constitutive levels of PO activity to produce photoprotective melanin, a function recently suggested for this compound (Palmer et al., 2010). Overall, these differences show variability in strategies for investing resources among life history traits by different coral species.

2.5.4 - Biochemical tools for coral reef management

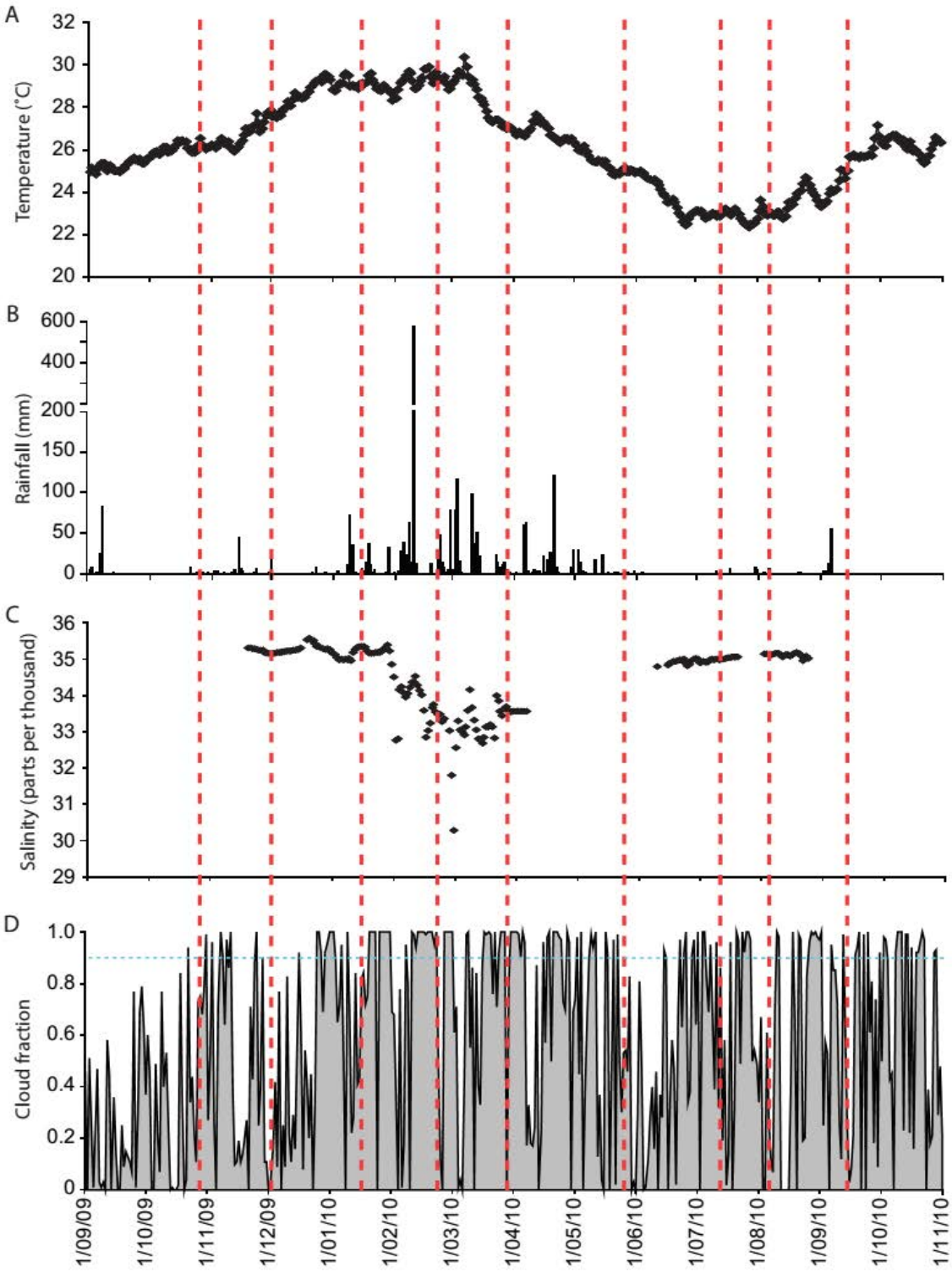
An urgent need for tools to evaluate and monitor coral health to manage and pre-empt activities that might cause further declines has arisen over recent years due to the increasing environmental and anthropogenic pressures facing corals worldwide (Burke et al., 2011). In this study, I assessed the suitability of PO activity and fluorescent protein expression as potential biomarkers for environmental stressors and the feasibility of providing an initial baseline reference for these parameters in three coral species. During the 2009-2010 sampling period, no anomalies in either seawater temperature or rainfall occurred, making this a suitable period for the establishment of baseline immune parameter levels. Recent studies have suggested that these immune parameters may be suitable tools for reef managers (Palmer et al., 2010), for example FP expression for coral health monitoring (Roth et al., 2010; D'Angelo et al., 2012).

Generally, biomarkers should: 1) be specific to a stressor and not other factors; 2) have a magnitude in change that reflects the magnitude of the stressor; and 3) have low background variability (Cooper et al., 2009). My results show, however, that there is significant variability over time in most parameters tested, although the degree of variability depended on the coral species. Temporal variability may be acceptable in cases where extensive baseline data are available. While changes in GFP-like protein expression do not appear to be specific to a single stressor (e.g. responses to high solar irradiation (D'Angelo et al., 2008), heat stress (Dove et al., 2006), bleaching (Leutenegger et al., 2007b) and coral translocation (Bay et al., 2009)), the range of stressors that induce changes in FP expression or in the ratio of individual FPs suggests that FP expression levels may function as a general stress biomarker for some corals. Although correlations are good indicators of relationships between immune parameters and environmental factors, experimental studies exploring how these potential biomarkers respond to stressors, as well as the magnitude of these responses need to be addressed under controlled conditions. In addition, several key common indicator species should be identified and, for each of these, extensive baseline data for each biomarker should be obtained on both temporal and spatial scales. Overall, PO activity and FP expression can potentially be used as biomarkers for coral health, but additional research on baseline data and stressors is required before their suitability and applicability can be addressed.

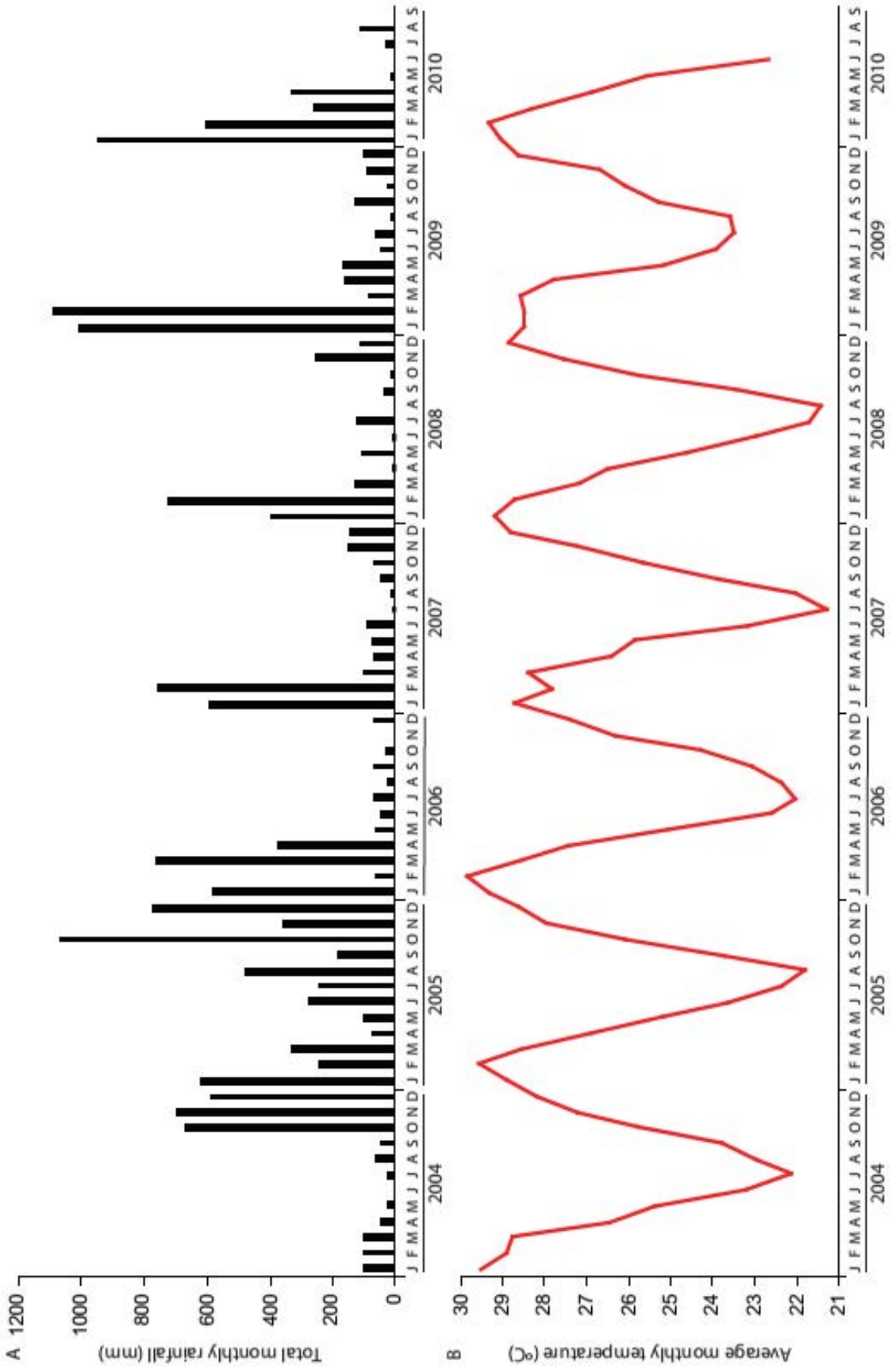
2.6 - Conclusion

Given the increasing environmental and anthropogenic pressures and stresses corals face worldwide, there is an urgent need for ecological coral health monitoring tools and techniques. In this study, initial baselines of multiple ecological immune parameters were established for three species from three major reef-building coral families. The significant differences observed in constitutive levels and temporal patterns in PO activity and GFP-like protein expression among these species suggest that they have evolved different ecological immune strategies. Such variations underscore the need to obtain species-specific baseline levels and further long-term data before considering the suitability of these ecological immune parameters for routine coral health monitoring.

2.7 - Supplementary Figures



Supplementary figure 2.1 – Temporal patterns of environmental parameters. A) seawater temperature; B) rainfall; C) seawater salinity; D) cloud fraction (fraction of area covered by clouds). Red dashed lines indicate sampling time points.



Supplementary figure 2.2 – Long term temporal patterns of environmental parameters. Monthly averages of A) rainfall and B) seawater temperature from January 2004 till September 2010.

Chapter 3

Comparative immune responses of corals to injury, disease and anthropogenic stressors associated with offshore tourist reef platforms

3.1 - Abstract

Unravelling anthropogenic and environmental factors suppressing the coral immune system is important for prioritising management actions at reefs exposed to high levels of recreational activities. Fifteen-fold higher prevalence of coral disease at reefs near permanent reef-based tourist platforms compared to reefs without platforms make these ideal study locations to tease apart the effects of anthropogenic and environmental stressors on coral health. Here, I monitored the health of the coral *Acropora millepora* adjacent to two reef platforms (one in use by tourists and the other unused) plus a control site without a platform over seven months spanning the Austral summer. Comparisons of temporal patterns in a range of biochemical and genetic immune parameters (Toll-like receptor (TLR) signalling pathway, lectin-complement system, prophenoloxidase-activating system, GFP-like proteins) among healthy, injured and diseased corals revealed that corals possess a diverse array of immune responses to environmental and anthropogenic stressors. Expression of genes involved in the TLR signalling pathway (*MAPK p38, MEKK1, cFos, cJun, ATF4/5, ERK-2*) and complement system (*C3* and *Bf*) was significantly modulated by seasonal environmental factors in summer months in corals at the control site. Corals at reef platform sites experienced additional stressors over the summer, as evidenced by increased expression of various immune genes, including *MAPK p38* and *MEKK-1*. Seasonal and anthropogenic factors may have interacted synergistically to overwhelm their immune systems, leading to disease in 31% of all tagged corals near the tourist platform in January. However, prior to disease onset, colonies that developed disease showed reduced expression of genes involved in the complement pathway (*HL-1* and *C3*), suggesting their immune systems may have been compromised. Responses to disease and physical damage primarily involved the melanisation cascade and fluorescent proteins, and appeared sufficient for recovery when summer heat stress subsided. Overall, I found that corals near reef platforms respond to platform-associated stressors, which in synergy with seasonal factors, potentially compromise the coral immune system, contributing to increased disease prevalence at these sites.

3.2 - Introduction

Increasing evidence that coral disease epizootics are causing significant declines in coral cover and degradation of coral reefs (Gardner et al., 2003; Osborne et al., 2011; Porter et al., 2001) suggests that coral immune systems are being overwhelmed by combinations of local and global environmental and anthropogenic disturbances. Teasing apart the roles of climate change-related environmental factors, like warming and acidifying oceans, in disease causation from anthropogenic disturbances, such as sedimentation, eutrophication caused by agricultural run-off, and sewage disposal, (Harvell et al., 2004; Harvell et al., 2007; Lesser et al., 2007; Bruno et al., 2007; Sutherland et al., 2011; Haapkylä et al., 2011; Ruiz-Moreno et al., 2012; Redding et al., 2013) is key to understanding current challenges facing coral immune systems. A report of 15-fold greater prevalence of coral disease near permanent tourist reef platforms compared to adjacent reefs without such platforms (Lamb and Willis, 2011) suggests that such sites are ideal microcosms for characterising responses of the coral innate immune system. Determining if differential coral immune responses can distinguish among anthropogenic and environmental drivers of reduced coral health would represent a significant step forward in the development of effective coral reef management and conservation strategies near permanent offshore platforms.

Corals have a large repertoire of innate immune defence mechanisms available to maintain fitness and defend against biotic and abiotic stressors. As discussed next, three of these have been relatively well documented: 1) the Toll-like receptor pathway, 2) the melanisation cascade, and 3) the complement system. However, the manner in which these immune mechanisms respond to different environmental and anthropogenic impacts is relatively unexplored. Toll-like receptors (TLR) are activated following the detection of microbial components (microbe-associated molecular patterns; MAMP) and subsequently activate various signal transduction pathways (e.g. JNK, MAPK p38, and NFκB pathways), which regulate the expression of target genes involved in immunity and cell survival thereby orchestrating the immune response (see section 1.2 and 1.3 for more detailed review). Recent molecular studies of the coral innate immune system have identified a large number of genes encoding TLRs and proteins involved in the downstream signalling pathways (Miller et al., 2007a; Shinzato et al., 2011; Hamada et al., 2013), however, functional studies of the TLR signalling pathways are limited.

The melanisation cascade, or proPO-activating system (Mydlarz et al., 2008; Palmer et al., 2008), is a rapidly-induced mechanism activated in response to MAMPs (Cerenius et al., 2010a). The capacity to activate this system within minutes, leading to

the production of a hostile cellular environment and ultimately, to the deposition of melanin that immobolizes microbes ((Cerenius et al., 2010a); see Section 1.3 for more detailed review), suggests that this immune mechanism may be directed primarily at events requiring a rapid response like pathogen invasion and injury. Significant correlations between PO activity levels and disease resistance in various invertebrates, including corals, plus evidence of the major role the proPO-activating system plays in the disease response and wound healing process (Palmer et al., 2010; Palmer et al., 2011a; Palmer et al., 2011c) corroborates this interpretation. Although the biological function of the melanisation cascade in corals has been studied extensively, the impacts of stressors other than elevated seawater temperatures are still unknown.

The complement system is another effector mechanism involved in the direct elimination of invading microbes, primarily via promoting phagocytosis and inducing the formation the membrane attach complex (MAC). Key components of the complement system, including complement C3, Factor B (Bf), lectins and mannose-binding lectin (MBL)-associated serine protease (MASP), are present in many invertebrates (Mydlarz et al., 2006; Cerenius et al., 2010a). In corals, lectins and C3 have been implicated in the anti-bacterial and wounding response (Kvennefors et al., 2008; Kvennefors et al., 2010b; Brown et al., 2013), and MAC/PF (membrane attack complex / perforin) domain-containing genes have been identified (Miller et al., 2007a). How this immune mechanism is affected by environmental and anthropogenic factors, however, remains to be elucidated in corals.

Elevated seawater temperatures are known to reduce immunocompetence, which is the ability of an organism to induce an immune response, in several coral species (Palmer et al., 2011a; Palmer et al., 2011b; Vidal-Dupiol et al., 2014). In other invertebrate systems, changes in salinity and elevated levels of nutrients or pollutants are also known to reduce immune function, resulting in increased disease-related mortality (Cheng et al., 2004a; Cheng et al., 2004b; Li et al., 2010b; Liu and Chen, 2004; Reid et al., 2003; Tseng and Chen, 2004; Ellis et al., 2011; Danis et al., 2006; Coteur et al., 2001). Higher prevalence of coral disease on reefs near permanent offshore tourist platforms than at reefs without such facilities (Lamb and Willis, 2011) suggest that platform-associated stressors also affect coral immunocompetence. Corals near platforms harboured distinct bacterial communities compared to corals at reefs without platforms, with significant shifts in bacterial communities during disease development (Pollock *et al.*; Appendix C). Moreover, differences in bacterial communities had been observed two months prior to the appearance of macroscopic signs of disease. As healthy bacterial communities are essential to the functioning of the coral holobiont, playing important roles in nutrient cycling (Raina et al., 2009; Lema

et al., 2012) and protection from pathogens (Ritchie, 2006; Shnit-Orland and Kushmaro, 2009; Teplitski and Ritchie, 2009; Alagely et al., 2011), changes in the structure of bacterial communities may signify that immune systems of corals living near tourist platforms are compromised.

In this study, I monitored colonies of the reef-building coral *Acropora millepora* near tourist platforms and control sites over a summer season and assessed their immunocompetence based on immune protein and gene expression. The occurrence of both disease and recreationally-related injury at platform sites, coupled with recovery of lesions and injuries as warm summer temperatures subsided, enabled comparisons of the functional responses of a range of immune parameters to both anthropogenic and environmental factors.

3.3 - Material & Methods

3.3.1 - Study site and sample collection

The study was conducted at Hardy Reef (19°44'33"S, 149°10'57"E) on the Great Barrier Reef of Australia between November 2010 and June 2011. Hardy Reef has two permanently moored offshore platforms: a 45 m x 12 m platform, which was in use at the time of the study as the primary docking pontoon and could accommodate up to 400 visitors and associated recreational activities per day (study site 1, tourist platform), and a second platform (24 m x 10 m), which had not been utilised by tourists the year prior to the study and was located 400 m south of the main tourist platform (study site 2, unused platform). Throughout the year, large numbers of seabirds live on both platforms, which are both located 5 m from the reef crest. In addition, a control site (study site 3) was established 800 m south of the unused platform in similar reef habitat. Additional details can be found in Pollock *et al*. (Appendix C).

Eight visually healthy and similarly-sized colonies of *Acropora millepora* were tagged using plastic cattle tags and cable ties at 2-3 m depth at each of the three study sites. Colonies were sampled at six time points: November (late austral spring), December (early austral summer), January (austral summer), February (austral summer), March (late austral summer) and June (early austral winter). At each sampling time, the health status of the tagged coral colonies was visually assessed and categorised as either healthy, damaged (branches recently broken with exposed skeleton) or diseased (signs of the coral disease white syndrome, as per Beeden et al. (2008)). One branch (~5 cm in length) was sampled from midway between the centre and the edge of each tagged colony, at each of the 6 time points during the study. For diseased and damaged colonies, an apparently healthy portion of each branch was

sampled approximately 1 cm from the lesion boundary or damaged area. In all cases, the disease lesion was radiating from the centre of the colony. A photograph of each tagged colony was taken before and after each sample was collected. Samples were collected in the same order for all time points and the sampling took approximately 2 hours to complete. Samples were immediately snap-frozen in liquid nitrogen and stored at -80ºC.

3.3.2 - Messenger RNA isolation

Frozen samples of *A. millepora* were crushed in a liquid nitrogen-chilled, stainless steel mortar and pestle using a hydraulic press. Messenger RNA (mRNA) was isolated from approximately 100 mg of crushed coral using the Dynabeads mRNA DIRECT kit (Invitrogen Dynal AS, Oslo, Norway) according to a modified protocol based on the manufacturer's recommendations. In short, crushed coral was added to 400 µl lysis buffer, incubated on a vortex at low speed for 7 min and centrifuged for 2 min at 12000 *g*. Supernatant was added to pre-washed oligo(dT)-Dynabeads and incubated on the vortex at medium speed for 8 min to allow mRNA annealing. Tubes were placed on a DynaMag-2 magnetic particle concentrator for 5 min and supernatant was removed. Using the DynaMag-2, oligo(dT) Dynabead/mRNA complexes were washed twice with 300 µl of Buffer A and subsequently twice with 400 µl of Buffer B. Complexes were resuspended in 27 µl ice-cold 10 mM Tris-HCl, incubated at 80°C for 2 min and rapidly cooled down on ice. Oligo(dT)-Dynabeads were concentrated on the DynaMag-2 and mRNA-containing supernatant was collected and stored at -80°C until use.

3.3.3 - Gene expression analysis

Expression levels of 17 immune system-related genes and 4 reference genes (Seneca et al., 2010; Siboni et al., 2012) were analysed using the GenomeLab GeXP Start Kit and the CEQ-8800 Genetic Analysis System (Beckman-Coulter, Brea, CA, United States of America) following the protocol described in Siboni et al. (2012) with some modifications. Description of all genes and primer sequences can be found in Appendix B Table 1. For each sample, cDNA was generated from 6.7 ng of mRNA. Forward primer concentrations were 200 nM. Reverse primer concentrations were optimised for the multiplex to ensure signals in the electropherogram were within the CEQ-8800 detection range: 2 µM for *CTL2*; 1 µM for *MEKK1*, *GAPDH*, *Bf*, *MAPK p38*, *ctg_1913*, *RPL9*, *CTL1*, *HL1* and *Apextrin*; 500 nM for *TRAF6*, *TIR-1*, *C3/A2M-2*, *ERK-2*, *Millectin*, *HL2* and *cJun*; 62.5 nM for *ATF4/5*; 25 nM for *cFos*; 12.5 nM for *NFκB*; 23 pM *RPS7*. PCR products were diluted 1:20 prior to loading on the CEQ-8800 Genetic

Analysis System. Data were filtered and analysed using the GeXP Analysis Software (Beckman-Coulter, Brea, CA, United States of America) (Siboni et al., 2012). Gene expression levels were normalised to an internal control (Kan^R) and to the geometric mean of the expression levels of three housekeeping genes (*RPS7*, *RPL9* and *ctg_1913*) selected using geNorm (Vandesompele et al 2002). Results were obtained for three independent technical replicates per sample.

3.3.4 - Protein and enzyme assays

To prepare samples for protein and enzyme assays, coral tissue lysates were prepared according to the methods described in Chapter 2, section 2.3.2. PO, and tpPO activity was analysed following the protocol described in Chapter 2, section 2.3.4 of this thesis. The procedure followed for the analysis of coral fluorescence and chromoprotein expression can be found in Chapter 2, section 2.3.5.

3.3.5 - Statistical analyses

Temporal patterns of individual gene and protein parameters in control site corals were analysed using a Linear Mixed Effect (LME) Model, with "Colony" as a random effect and with both immune parameter and time as fixed effects. Analysis of variance (ANOVA) was used to test for differences between corals at the control site and corals that 1) sustained damage, 2) developed disease, 3) were healthy near the tourist platform, and 4) were healthy near the unused platform, followed by a Fisher's least significant difference (LSD) test. Because of low levels of gene expression resulting in a value of 0 in a few cases, datasets with >20% zeros were tested for differences in the proportion of 0 values across sites and health conditions using logistic regression models, and differences in expression of samples with expression values > 0 between sites and health conditions using a LME model. All analyses were conducted using the statistical software package S-PLUS 8.0. Data were considered significant when $p < 0.05$ or when the 95% confidence interval excluded 0.

3.4 - Results

3.4.1 - Coral health assessment

At the control site, all eight tagged colonies remained visually healthy throughout the study period (November to June). Colonies at the platform sites (both the tourist platform and unused platform) were visually healthy at the start of the study in November and December. In January, five colonies near the platforms (1 at the tourist and 4 at the unused platform) developed macroscopic signs of the coral disease

white syndrome (WS) and sustained approximately 40-50% partial colony mortality. In addition, 3 of the tagged colonies at the tourist platform sustained severe physical damage, with several broken branches on each colony, likely due to reef-based snorkelling activities. In February, lesions associated with the damaged corals recovered and WS lesions ceased progressing in all but one colony (at the unused platform site). In March, all WS-affected colonies appeared healthy, except for areas of partial colony mortality; in June, all colonies were visually healthy. Additional information concerning health assessments of tagged corals and patterns in environmental factors can be found in Pollock *et al.* (see Appendix C).

3.4.2 - Temporal changes in the immune system of corals at the control site

Although corals at the control site all remained healthy, expression levels of most immune parameters fluctuated significantly over the seven months of the study. Four main patterns in the immune response of these healthy control corals were detected. First, mean PO and tpPO activity and total fluorescence levels all increased in March compared to levels in the previous summer months, although this increase was not significant for PO activity (Fig. 3.1A, B, C). Secondly, I observed increased expression in the complement system's *C3* and *Factor B* (*Bf*) genes in summer months (Suppl. Fig. 3.2B, Fig. 3.2B), as well as in genes associated with the TLR pathway, i.e., *ATF4/5, cJun,* and *ERK-2* (Fig. 3.2A; Suppl. Fig. 3.2C, F). In addition, the proportion of cyan fluorescent protein was relatively higher in summer (Suppl. Fig. 3.1A). The third pattern observed was that various genes involved in the TLR signalling pathway were expressed at lower levels in summer compared to other months, including *cFos, MEKK1, MAPK p38* and *TRAF6,* (Fig. 3.2C, E, F; Suppl. Fig. 3.2K). Finally, several parameters showed a pattern of reduced expression or activity in winter (June) compared to all other months. These parameters were PO activity and *HL-1*, *apextrin* and *NFκB* expression (Fig. 3.1A; Fig. 3.2D; Suppl. Fig. 3.2A, I). All other parameters showed some temporal fluctuations in expression, however, no clear patterns could be discerned. Details of the statistical significance of each comparison can be found in Supplementary table 3.1.

3.4.3 - The effect of anthropogenic disturbances associated with reef platforms on the immune system of corals

In general, temporal patterns in the immune parameters analysed for healthy corals near reef platforms followed profiles similar to those of healthy corals at the control site. In March, however, tpPO activity was reduced in corals at both platform sites in comparison to controls (Fig. 3.1B), while total fluorescence levels were reduced

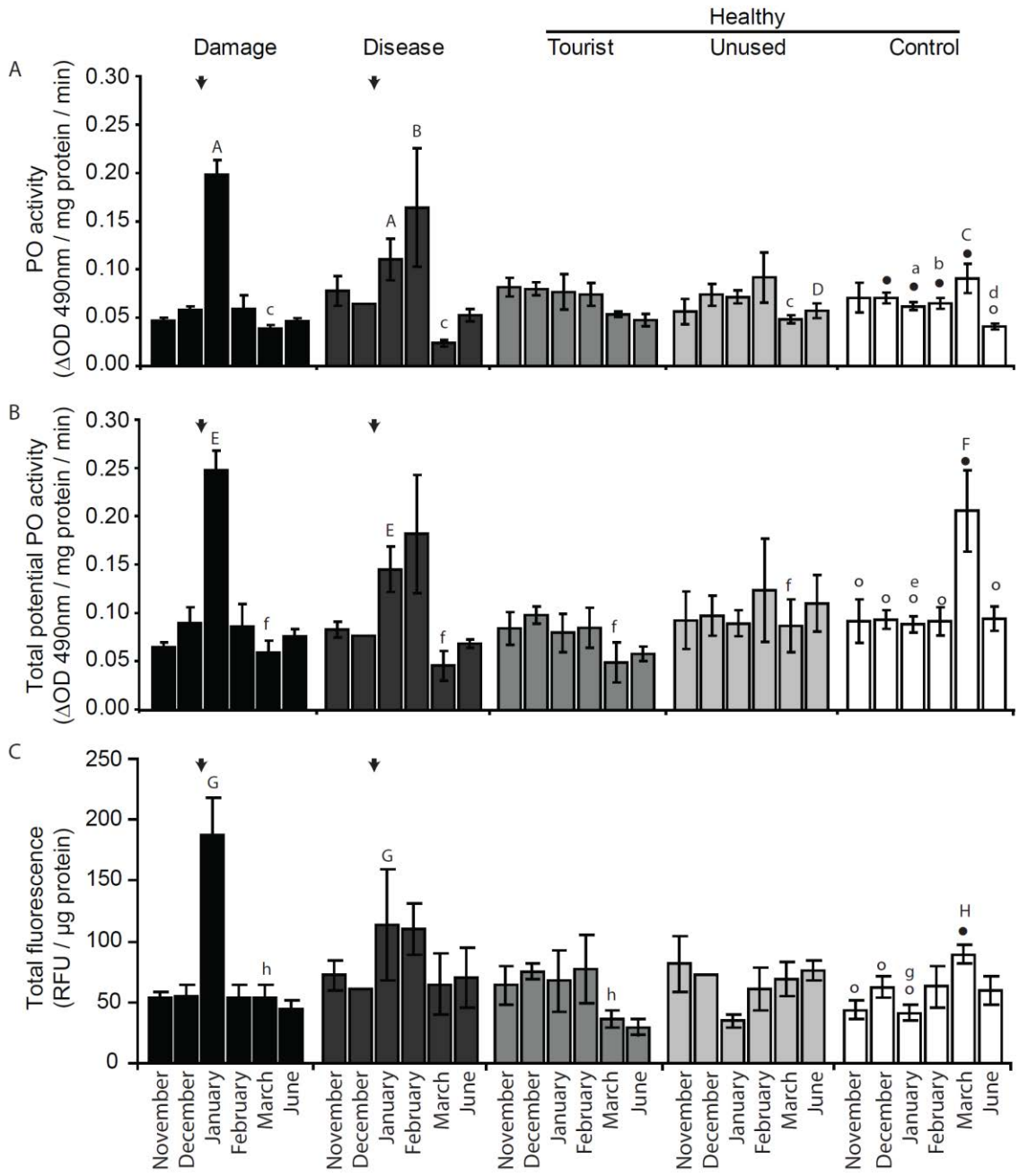


Figure 3.1 – Comparative temporal patterns in phenoloxidase activities and total fluorescence levels in *Acropora millepora* at Hardy Reef, central Great Barrier Reef. Patterns are compared among corals that were healthy at three study sites (tourist platform, unused platform and control site), and those that were damaged or diseased at platform sites, for: A) active phenoloxidase (aPO), B) the total potential phenoloxidase (tpPO) activity and C) total fluorescence levels. Data are grouped by health status, with healthy corals split up by study location. Arrows indicate when disease and damage occurred. Letters (a-h; A-H) indicate means that differ significantly from the corresponding mean at the control site, where upper case letters denote the significantly higher mean in the comparison, and lower case letters denote the significantly lower mean. For temporal patterns in control corals, symbols (• or ○) denote means that differ significantly from means with the other symbol. Results were considered significant when $p < 0.05$ or 95% confidence interval excluded 0.

only in corals at the tourist platform (Fig. 3.1C). In June, I observed an increase in PO activity at the unused platform (Fig. 3.1A), although the proportion of GFP was reduced (Suppl. Fig. 3.1B). Corals near platforms showed significant increases in the

expression of genes involved in the TLR signalling pathway in summer. *MEKK1* and *MAPK p38* were upregulated at both platform sites (Fig. 3.2E, F), as well as *Bf* at the unused platform in January (Fig. 3.2B). In addition, these genes and the transcription factors *cFos* and *cJun* were upregulated at the tourist platform in December (Fig. 3.2C; Suppl. Fig. 3.2C), while these corals had lower *ATF4/5* expression (Fig. 3.2A) and total fluorescence levels (Fig. 3.1C) in February and March, respectively.

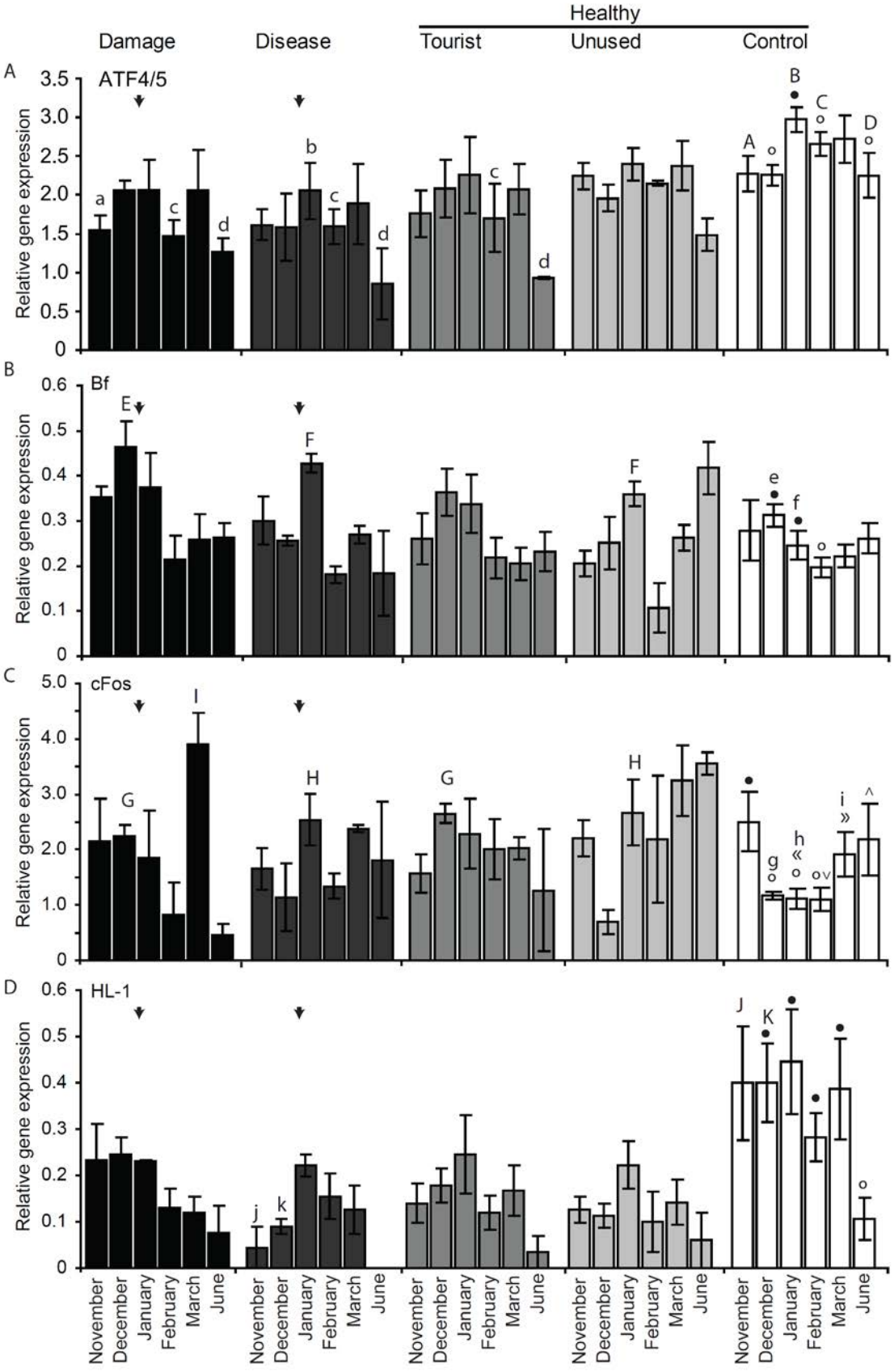
The putative Toll-like receptor *TIR-1* (Suppl. Fig. 3.2J) was present in a relatively small portion of the samples regardless of location and month of sampling. One exception occurred at the tourist platform, however, where all healthy corals expressed *TIR-1* at detectable levels during the summer months of January and February. In addition, I found a significant difference in the number of corals with detectable levels of the C-type lectin *CTL-2* between sites ($p = 0.03$) (Suppl. Fig. 3.2E). This effect was due to the presence of *CTL-2* in 67.5% and 58.2% of the coral samples collected at the control site and at the tourist platform, respectively, while only 35.3% of the samples collected at the unused platform expressed *CTL-2* at detectable levels.

3.4.4 - The immune response of injured corals

In damaged corals, several immune parameters were significantly higher in January, the month when corals sustained injury. Both PO and tpPO activity were increased in injured corals relative to corals at the control site (Fig. 3.1A, B), as well as total fluorescence (Fig. 3.1C). However, these parameters (Fig. 3.1A, B, C), along with *apextrin* (Suppl. Fig. 3.2A), were all reduced in March, while *TRAF6*, *MEKK1* and *cFos* were upregulated compared to controls (Suppl. Fig. 3.2K; Fig. 3.2C, E). The proportion of cyan fluorescent protein was significantly increased in March and June (Suppl. Fig. 3.1A), although the proportion of GFP was lower in June (Suppl. Fig. 3.1B). Signalling via the TLR pathway may also have been involved in the response, with upregulation of *MEKK1* and *MAPK p38* in January (Fig. 3.2 E, F). In addition, elevated expression of the lectin *CTL-1* was observed following physical damage in January ($p = 0.04$) (Suppl. Fig. 3.2D). Surprisingly, I also found increased expression of *Bf, cFos, and MEKK1* in December, prior to injury (Fig. 3.2B, C, E). *ATF4/5* expression was downregulated in both January and February (Fig. 3.2A).

3.4.5 - The immune response of white syndrome-affected corals

The immune system of corals that developed visual signs of disease showed significantly reduced expression of the lectin *HL-1* and complement gene *C3* prior to January, when symptoms became apparent (Fig. 3.2D; Suppl. Fig. 3.2B). In January, PO and tpPO activities, as well as total fluorescence levels, were increased relative to


Damage
Disease
Healthy
Tourist
Unused
Control
A
ATF4/5
B
Bf
C
cFos
D
HL-1
Relative gene expression
November
December
January
February
March
June

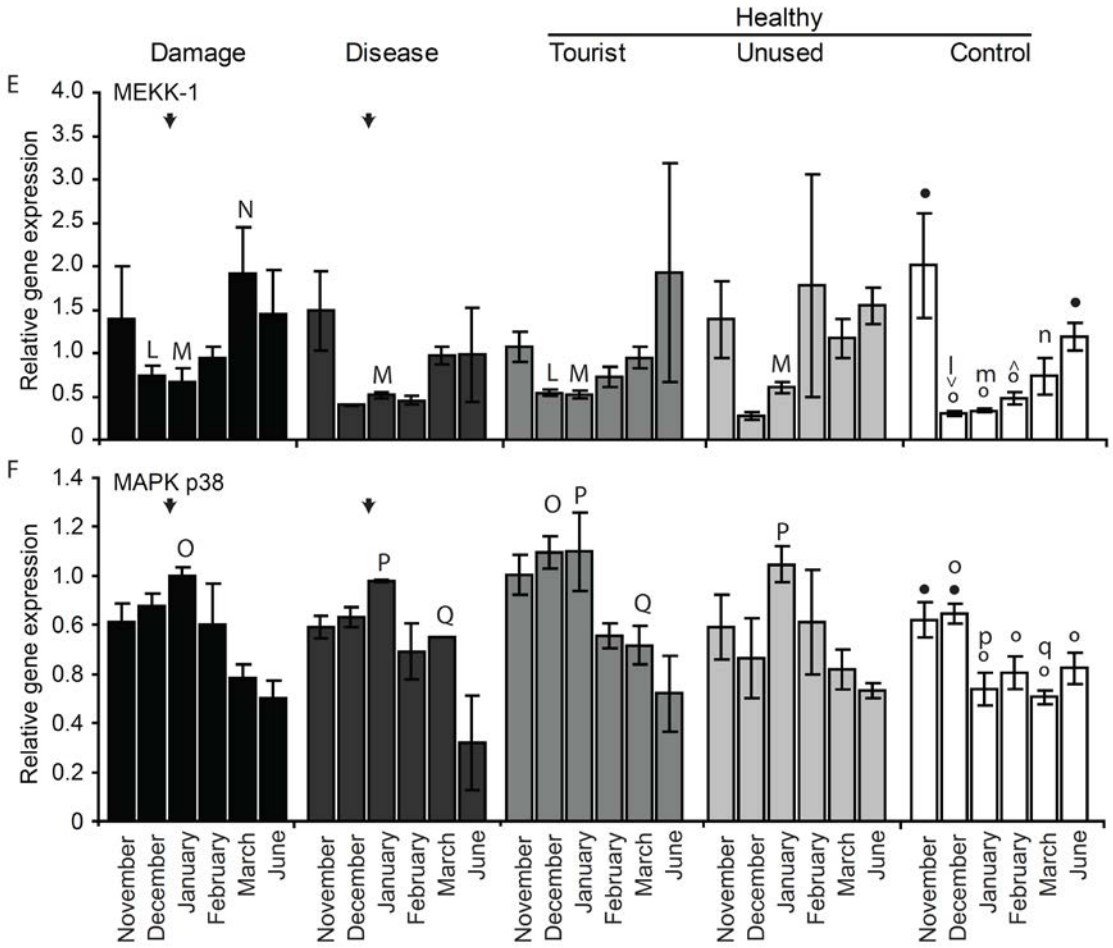


Figure 3.2 – Comparative temporal patterns in immune gene expression levels in *Acropora millepora* at Hardy Reef, central Great Barrier Reef. Patterns are compared among corals that were healthy at three study sites (tourist platform, unused platform and control site), and those that were damaged or diseased at platform sites, for: A) ATF4/5, B) Bf, C) cFos, D) HL-1, E) MEKK1, F) MAPK p38. Data are grouped by health status, with healthy corals split up by study location. Arrows indicate when disease and damage occurred. Letters (a-q; A-Q) indicate means that differ significantly from the corresponding mean at the control site, where upper case letters denote the significantly higher mean in the comparison, and lower case letters denote the significantly lower mean. For temporal patterns in control corals, symbols (• and ○) or (^ and ˅) denote means that differ significantly from means with the other symbol. Results were considered significant when $p < 0.05$ or 95% confidence interval excluded 0.

controls (Fig 3.1A, B, C), while expression levels of *Bf*, *cFos, MEKK1*, and *MAPK p38* were upregulated (Fig. 3.2B, C, E, F). However, only PO activity was elevated in February (Fig. 3.1A), while *ATF4/5* expression was downregulated in both January and February (Fig. 3.2A). In comparison with control site corals, various parameters were differentially expressed or activated in March, including reduced PO and tpPO activities (Fig. 3.1A, B), as well as *apextrin* levels and upregulation of *ERK-2* (Suppl. Fig. 3.2A, F).

Corals that showed macroscopic signs of disease had significantly higher levels of *TIR-1* expression ($p < 0.01$) (Suppl. Fig. 3.2J). However, it should be noted that, of all diseased corals analysed in this study (n = 4), two consistently expressed *TIR-1* at

high levels in all 6 months (average relative expression 5.6 ± 0.88), which was in contrast to the other two colonies, which had low*TIR-1* expression throughout the study (average relative expression 0.05 ± 0.035).

3.4.6 - The C-type lectin Millectin

Millectin, was found in the majority of samples, although there was a significant time effect ($p = 0.01$). This was likely to be explained by the absence of expression in 31.6% and 30% of the samples from January and February, respectively; although in contrast, 4.3%, 16.7% and 0% of corals lacked expression in November, December and March, respectively (Suppl. Fig. 3.2H).

Details of the statistical significance of comparisons between corals at the control site and 1) healthy corals near reef platforms, 2) corals that sustained injury in January and 3) corals that developed visual signs of white syndrome in January, can be found in Supplementary table 3.2.

3.5 - Discussion

The range of immune responses demonstrated in this study by the coral *Acropora millepora* highlights the complexity of the coral innate immune system. Overall, four general patterns in the expression and activity levels of known coral immune genes and proteins were found in healthy corals over a seven-month period spanning the austral summer. The presence of 15-fold higher levels of disease, as well as injury associated with recreational activities at sites near permanently moored offshore platforms (Lamb and Willis, 2011), provided an important opportunity to compare immune responses of healthy corals to those of corals exposed to a range of stressors. My results show that the immune systems of all corals near reef platforms, including healthy corals, responded to platform-associated stressors, and that corals further boosted their immune system in cases of disease or injury. Such studies help to advance research into the underlying mechanisms that are contributing to rising levels of coral disease globally (Ruiz-Moreno et al., 2012; Sokolow, 2009; Willis et al., 2004).

3.5.1 - Temporal patterns in the immune system of healthy corals

Variation in the expression of immune genes and proteins in healthy control colonies of *A. millepora* over seven months suggests that healthy immune systems respond to seasonal variation in environmental parameters, notably in summer. Although no visual signs of disease were detected at the control site, four distinct

temporal patterns in immune parameters were detected. First, significant upregulation of several genes in summer (*ERK-2, c-Jun, ATF4/5, C3, Bf*) suggests that apparently healthy colonies of *A. millepora* boost components of their immune system in response to summer-related stressors, potentially including increased seawater temperature or solar radiation, or reduced salinity associated with the summer wet season. ERK-2 is activated by a variety of stimuli, including TLR and pro-inflammatory cytokine signalling. This kinase subsequently activates AP-1 transcription factors, such as c-Jun and ATFs. Expression of complement C3 is known to be regulated via ERK-2 and its gene promoter contains binding sites for c-Jun and ATFs (Sugihara et al., 2010). In addition, C3 is activated by Bf in response to the detection of a microbe that requires elimination. Upregulation of several components of the complement system in an ERK-2-mediated manner in summer is consistent with the presence of a seasonally-related environmental stressor.

A second pattern detected in healthy control corals provides further support for this interpretation. Sudden increases of up to 2.2-fold in both PO and tpPO activities, as well as 1.7-fold increases in total fluorescence levels, in late summer (March), strongly suggest that corals experienced a stressor at this time. However, no anomalies were apparent in seawater temperature, light, rainfall or wind speed data at the sites in March (see Appendix C Fig. 1), and macroscopically, all corals remained visually healthy. The occurrence of peaks in PO activities at the end of summer may represent a response to 3 months of accumulated summer heat stress (see Appendix C Fig. 1C), but I can only speculate that this is the reason for the response. However, co-occurrence of peaks in PO and tpPO activity with the warmest summer month in my study of temporal patterns in the proPO-activating system at Orpheus Island (Chapter 2), lend support for the interpretation that warm summer temperatures are driving this response. Further studies of seasonal patterns in basal levels of immune parameters among populations on outer reefs, like Hardy Reef, and inshore reefs, such as in Pioneer Bay, Orpheus Island, are needed to test the generality of these patterns; they also highlight the need to further investigate the basal ecological immunological status of corals on larger temporal and spatial scales.

A third pattern detected in healthy control corals was the reduced expression of four genes involved in the Toll-like receptor signalling pathway (*TRAF6, MEKK1, MAPK p38, cFos*) in summer. This arm of the *TRAF6*-mediated TLR pathway is crucial in immune responses against microbes and may be involved in AMP-mediated regulation of healthy coral-associated microbial communities, such as in *Hydra* (Fraune and Bosch, 2007; Fraune et al., 2010; Franzenburg et al., 2012; Franzenburg et al., 2013). In addition, this signalling cascade is involved in the transcriptional regulation of

immune genes, such as immunostimulatory cytokines. I also detected reduced expression of the lectin Millectin in summer, suggesting a compromised lectin-complement pathway and possibly a breakdown of the coral-*Symbiodinium* symbiosis, given the role of Millectin in the maintenance of this symbiosis (Kvennefors et al., 2008). Reduced function of these immune mechanisms may lead to a less resilient coral. The reduced expression levels found in this study in summer may help explain reported increases in disease prevalence in summer (Willis et al., 2004; Harvell et al., 2007; Bruno et al., 2007).

Finally, significant reductions in various immune parameters, including PO activity and the expression of *HL-1, apextrin* and *NFκB* in winter (June) are consistent with a seasonally-reduced need for these immune parameters. Since these are primarily immune effector molecules, this may suggest that corals are exposed to significantly lower levels of microbial stress in winter. This would enable the coral to allocate fewer resources to the immune system and more to other life history traits, such as growth and reproduction.

3.5.2 - The effect of tourist reef platforms on coral immunocompetence

Significantly higher levels of TLR signalling pathway genes (*MAPK p38*, *MEKK1*) in all corals (healthy, diseased and injured) near both platforms in January, as well as at the tourist platform in December (MAPK p38, MEKK1, cFos, cJun), in comparison to control corals, indicates that offshore platforms have an impact on the coral immune system. While physical damage was likely to be the result of reef-based activities, such as snorkelling, consistent upregulation of this pathway suggests the presence of additional platform-associated stressors, which may have played a role in the development of white syndromes. Potential platform-associated stressors include nutrient influxes derived from guano from sea birds that frequent these reef platforms, as well as chemicals originating from human activities, such as cleaning products and sunscreen. While no cause can be pinpointed, there is a clear need to further investigate potential anthropogenic disturbances near offshore reef platforms to enable development of management actions that could mitigate the effects of these stressors and prevent localised coral reef degradation.

In a recent complementary study, it was found that bacterial communities associated with corals near the two offshore reef platforms were markedly less diverse (by ~50%) than those at the undisturbed control site, and differences were particularly striking in January (Pollock *et al.*; Appendix C). Since the TLR pathway may be involved in regulating the composition of coral-associated bacterial communities, the upregulation of various genes involved in the TLR pathway in corals near platforms in

summer could indicate that these corals were responding to changes within their bacterial communities and attempting to re-establish a more favourable community via TLR-induced AMP production. However, the fact that bacterial communities differed significantly between control and platform sites in November and December, prior to the significant TLR response in January, suggests that long-term anthropogenic impacts had already modified microbial communities associated with platform corals, and that an additional stressor, such as rising summer temperatures, potentially acting synergistically with platform-associated stressors, caused further changes in microbial communities and triggered the response.

3.5.3 - The coral response to injury

The primary response to injury detected in this study was up to 3.2-fold increases in the proPO-activating system and 4.5-fold increases in fluorescence levels in January, when broken branches were first detected. Despite potentially additional, unidentified stressors at platform sites (as discussed in 3.4.2), corals near the tourist platform exhibited a sufficient immune response following physical damage to enable recovery. While phenoloxidase activity may have an anti-microbial function or form a physical barrier to seal the lesion (Mydlarz et al., 2008; Palmer et al., 2011a; Palmer et al., 2011c), fluorescent proteins likely protect regenerating tissues from light stress by dissipating high energy wavelength light (Baird et al., 2009). Activation of the melanisation cascade and increased levels of fluorescent proteins in tissues at growth margins following physical damage found in an earlier study (D'Angelo et al., 2012) support this interpretation. FPs also have the capacity to scavenge reactive oxygen (Palmer et al., 2009a; Bou-Abdallah et al., 2006) and may neutralise radicals produced by the melanisation cascade; however, whether this is one of FPs primary functions is currently unclear. Furthermore, I found that the C-type lectin *CTL-1* was upregulated following damage, suggesting activation of the lectin-complement pathway, which is consistent with studies that show wounding-induced upregulation in the expression of various lectins ((Kvennefors et al., 2010b); Chapter 5). Another component of this pathway, *Bf*, was also upregulated, although this was observed a month prior to the significant damage event in January. While I have currently no functional explanation for the increased levels of *Bf* in corals that would sustain significant damage later on, it cannot be excluded that these corals were more prone to pressure from reef-based tourist activities, for example based on their proximity to the platform, and responded accordingly. Surprisingly, I did not observe a response via the TLR pathway following physical damage (as described in Chapter 5). However, the immune response following injury is very dynamic, and I may have missed the fully orchestrated response

involving the complement and TLR pathways, which are typically upregulated within days (see Chapter 5), because of low sampling resolution.

3.5.4 - Response of corals to white syndromes

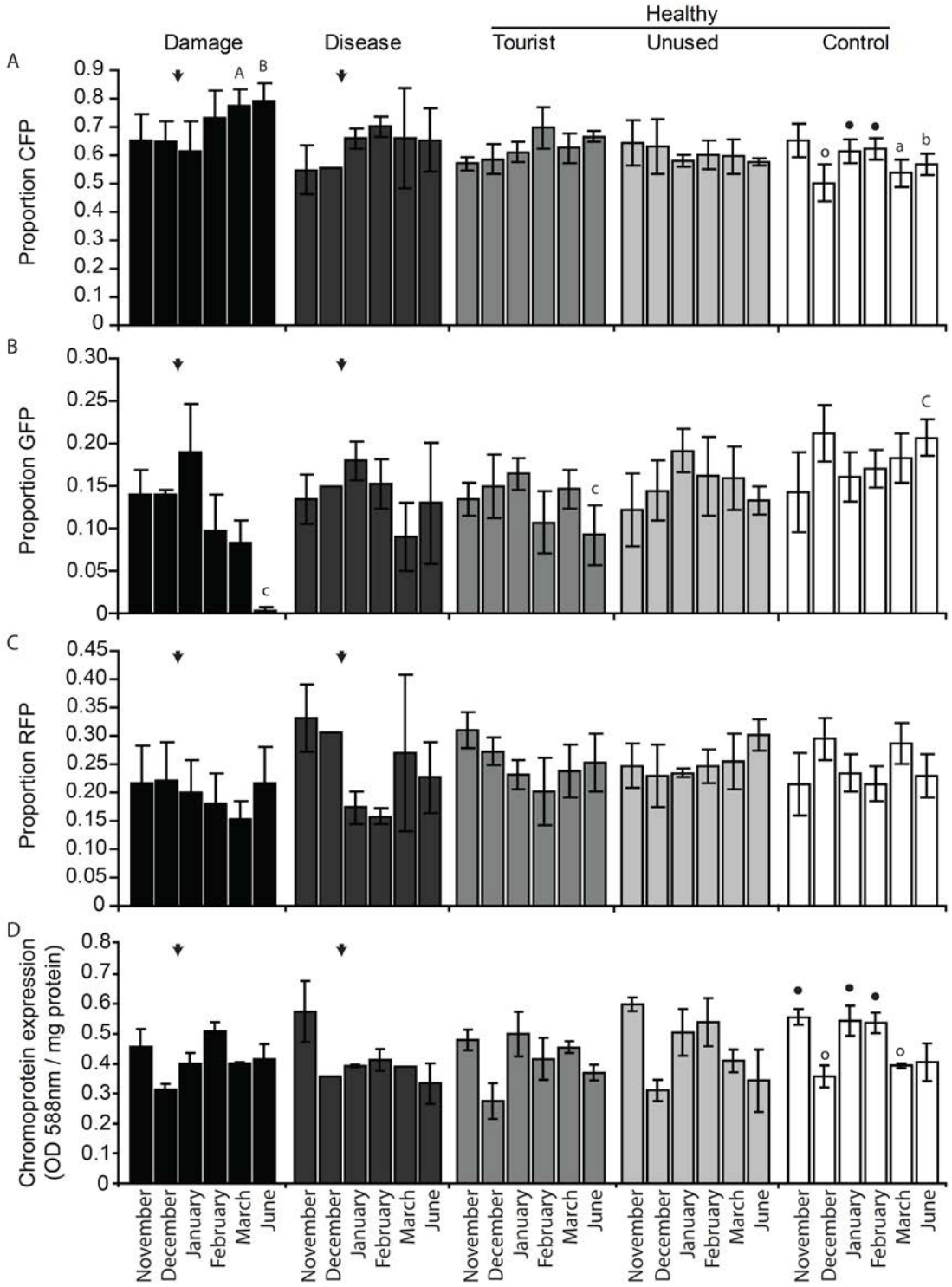
Only corals near reef platforms developed signs consistent with the group of coral diseases known as white syndromes. When combined with the observation that lesions appeared during the warm summer month of January, the results suggest that a seasonally-related stressor, such as temperature, solar radiation or rainfall, was acting to compound local stressors associated with platforms and cause disease. Interestingly, I found that prior to disease onset, these corals had significantly reduced levels of the lectin *HL-1* and complement *C3*, suggesting that they may have been immunocompromised, which would have made them more susceptible to disease. As progression of WS lesions had ceased in all diseased corals by March, I conclude that the immune response was sufficient to halt the disease. The 1.8 to 2.5-fold upregulation of PO activity in January and February, respectively, indicates that the immune response to disease involved activation of the melanisation cascade, as well as increased fluorescent protein expression, which was upregulated 2.7-fold compared to controls in January. In addition, I observed an increase in the expression of the putative TLR *TIR-1*. While this could indicate that these WS-affected corals regulated additional immune mechanisms in a TLR-dependent manner, it should be noted that expression was highly variable among colonies and these results should therefore be interpreted with caution.

In the complementary study of coral-associated microbial communities, it was found that corals developing WSs had markedly different microbial communities compared to corals that remained healthy, several months before visual signs of white syndrome became apparent (Pollock *et al*.; see Appendix C). Although the diversity of microbial communities associated with corals that would develop disease was significantly reduced, the lack of an early immune response suggests that the community did not negatively affect coral health prior to the onset of summer temperatures. However, the loss of microbial diversity is likely to have hindered maintenance of physiological processes essential to health of the coral holobiont, such as nutrient cycling (Lema et al., 2012; Raina et al., 2009) and anti-microbial functions (Fusetani et al., 1996; Ritchie, 2006; Reshef et al., 2006; Gochfeld et al., 2006; Gochfeld and Aeby, 2008; Shnit-Orland and Kushmaro, 2009; Shnit-Orland et al., 2012; Kvennefors et al., 2012; Hunt et al., 2012) reducing the corals' fitness, ultimately leading to additional shifts in microbial communities, and resulting in disease.

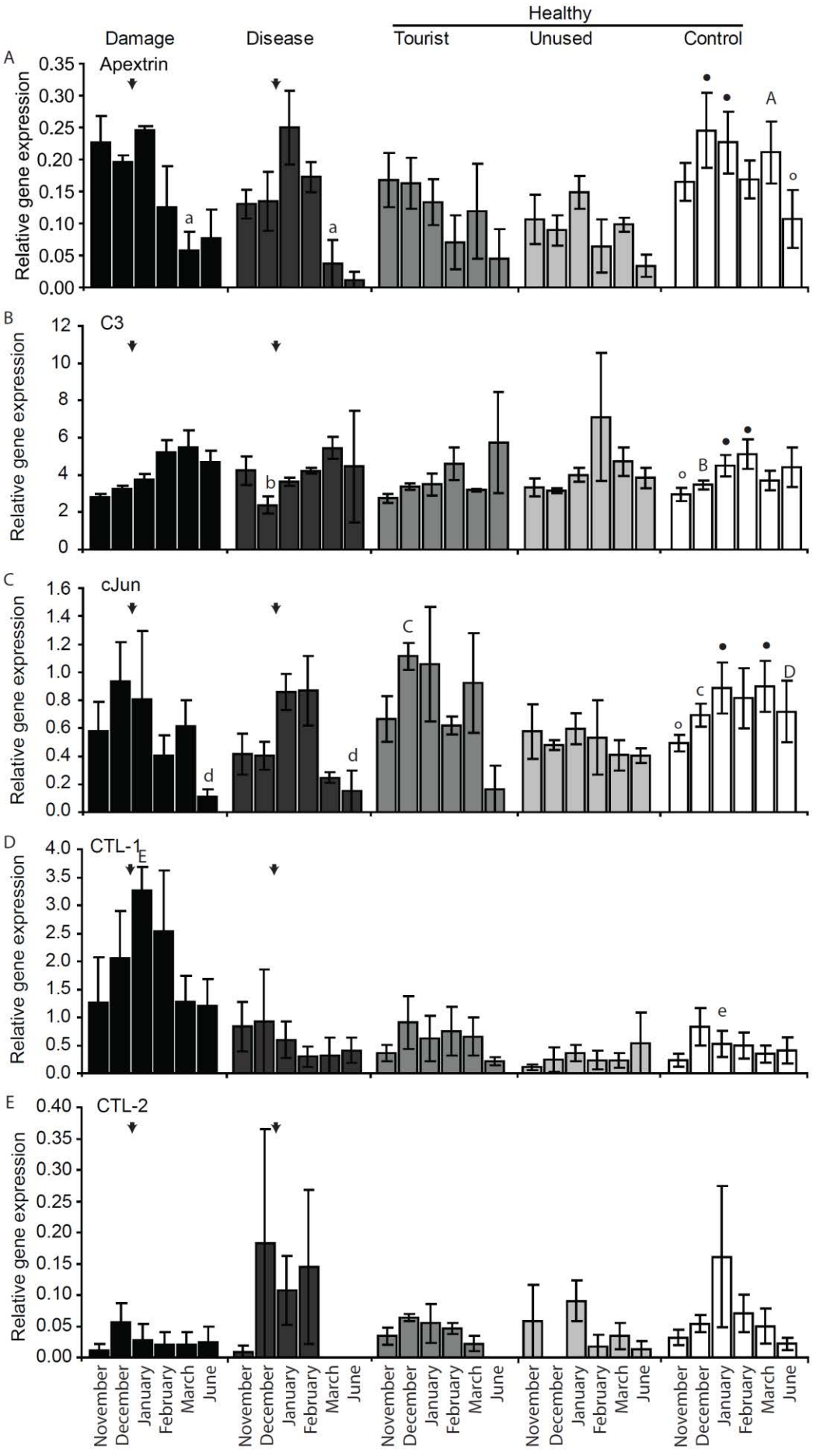
3.6 - Conclusion

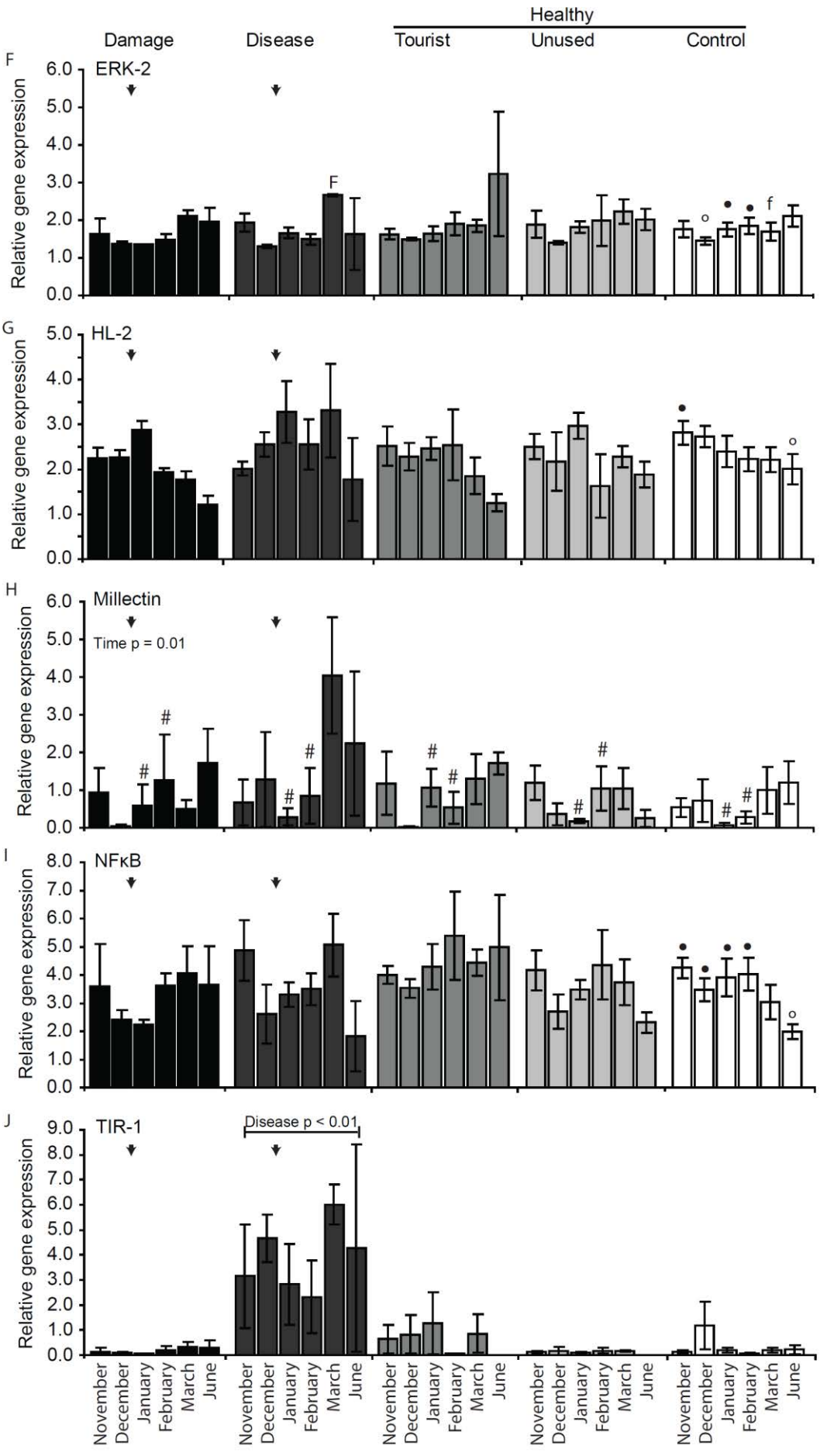
My study of the immune responses of the model coral *Acropora millepora* to a range of anthropogenic and environmental disturbances reveals that corals have a complex array of immune responses that are differentially regulated according to the type of disturbance. It is likely that full transcriptome or proteome analyses coupled with higher resolution sampling will reveal a greater diversity of coral immune responses. Overall, in corals at the undisturbed control location, expression of a range of immune genes was significantly modulated by seasonal environmental factors, in particular during the summer months. In addition, corals near tourist platforms showed upregulation of genes involved in the TLR signalling pathway in summer, suggesting increased pressure on the coral immune system from reef platform-associated factors. As a result, the coral immune system may have been overwhelmed by the combined or synergistic effects of these stressors, leading to disease in corals near platforms. However, corals that developed disease or sustained injury responded effectively, primarily using the melanisation cascade and enhanced fluorescent protein expression, and recovered when summer heat stress subsided. My study shows that corals are able to cope with normal seasonal stressors in summer, but that additional anthropogenic stressors compromise their immune system, contributing to increased disease prevalence and thereby localised reef degradation at these sites. Identifying the anthropogenic stressors responsible will enable the implementation of management actions to reduce stress on corals and will aid conservation efforts.

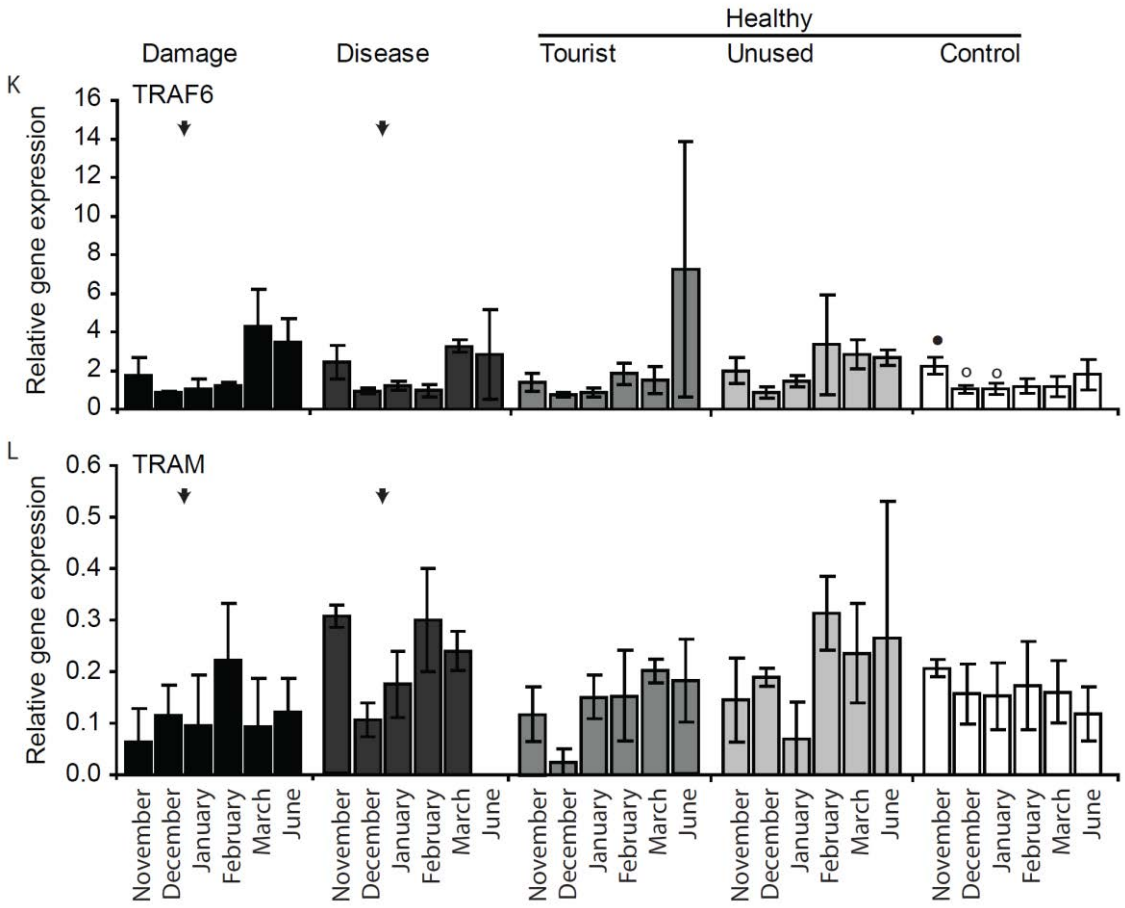
3.7 - Supplementary figures



Supplementary figure 3.1 - Comparative temporal patterns in GFP-like protein expression in *Acropora millepora* at Hardy Reef, central Great Barrier Reef. Patterns are compared among corals that were healthy at three study sites (tourist platform, unused platform and control site), and those that were damaged or diseased at platform sites, for: A) proportion of CFP, B) proportion of GFP, C) proportion of RFP and D) chromoprotein levels. Data are grouped by health status, with healthy corals split up by study location. Arrows indicate when disease and damage occurred. Letters (a-c; A-C) indicate means that differ significantly from the corresponding mean at the control site, where upper case letters denote the significantly higher mean in the comparison, and lower case letters denote the significantly lower mean. For temporal patterns in control corals, symbols (• or ○) denote means that differ significantly from means with the other symbol. Results were considered significant when $p < 0.05$ or 95% confidence interval excluded 0.


Damage
Disease
Healthy
Tourist
Unused
Control
A
Apextrin
B
C3
C
cJun
D
CTL-1
E
CTL-2
Relative gene expression
November
December
January
February
March
June


Damage
Disease
Healthy
Tourist
Unused
Control
F
ERK-2
G
HL-2
H
Millectin
Time p = 0.01
I
NFκB
J
TIR-1
Disease p < 0.01
Relative gene expression
November
December
January
February
March
June



Supplementary figure 3.2 - Comparative temporal patterns in immune gene expression levels in *Acropora millepora* at Hardy Reef, central Great Barrier Reef. Patterns are compared among corals that were healthy at three study sites (tourist platform, unused platform and control site), and those that were damaged or diseased at platform sites, for: A) *apextrin*, B) *C3*, C) *cJun*, D) *CTL-1*, E) *CTL-2*, F) *ERK-2*, G) *HL-2*, H) *Millectin*, I) *NFκB*, J) *TlR-1*, K) *TRAF6*, L) *TRAM*. Data are grouped by health status, with healthy corals split up by study location. Arrows indicate when disease and damage occurred. Letters (a-f; A-F) indicate means that differ significantly from the corresponding mean at the control site, where upper case letters denote the significantly higher mean in the comparison, and lower case letters denote the significantly lower mean. For temporal patterns in control corals, symbols (• or ○) denote means that differ significantly from means with the other symbol. Symbol # indicates months where proportion of corals expressing immune gene is significantly lower compared to other months. Results were considered significant when $p < 0.05$ or 95% confidence interval excluded 0.

3.8 - Supplementary tables

Supplementary table 3.1 – Overview of pairwise comparisons between means of immune parameters measured in corals at the control site. Only statistically significant comparisons are shown. Results were considered significant when $p < 0.05$.

	p-value
PO activity	
December – June	<0.0001
January – June	0.0005
February – June	0.0004
March – June	0.0103
tpPO activity	
November – March	0.0209
December – March	0.0111
January – March	0.0060
February – March	0.0130
March – June	0.0272
Total fluorescence	
November – March	<0.0001
December – March	0.0228
January – March	<0.0001
CFP	
December – January	0.0208
December – February	0.0291
Chromoprotein	
November – December	0.0013
November – March	0.0002
December – January	0.0040
December – February	0.0010
January – March	0.0064
February – March	0.0013

	p-value
Apextrin	
December – June	0.0489
January – June	0.0438
ATF4/5	
December – January	0.0022
January – February	0.0009
January – June	0.0332
Bf	
December – February	0.0006
January – February	<0.0001
C3	
November – January	0.0336
November – February	0.0207
cFos	
November – December	0.0184
November – January	0.0201
November – February	0.0202
January – March	0.0067
February – June	0.0452
cJun	
November – December	0.0144
November – March	0.0183
ERK2	
December – January	0.0130
December – February	0.0435
HL1	
December – June	0.0148
January – June	0.0020
February – June	0.0003
March – June	0.0344

	p-value
HL2	
November – June	0.0435
MAPK p38	
November – January	0.0069
November – February	0.0350
November – March	0.0003
November – June	0.0488
December – January	0.0003
December – February	0.0030
December – March	<0.0001
December – June	0.0060
MEKK1	
November – December	0.0085
November – January	0.0095
November – February	0.0176
December – February	0.0314
December – June	<0.0001
January – June	0.0001
February – June	0.0029
NFκB	
November – June	<0.0001
December – June	0.0064
January – June	0.0387
February – June	0.0092
TRAF6	
November – December	0.0126
November – January	0.0254

Supplementary table 3.2 – Overview of pairwise comparisons of immune parameters between corals at the control site and corals that either 1) remained healthy at platform sites (Tourist Healthy and Unused Healthy), 2) developed disease in January (Disease), or 3) sustained physical damage in January (Damage). Only statistically significant comparisons are shown. Results were considered significant when 95% confidence intervals excluded 0.

	Comparison	95% Confidence Interval	
PO activity			
January	Damage	0.0992	0.1730
	Disease	0.0148	0.0818
February	Disease	0.0256	0.1730
March	Damage	-0.0905	-0.0134
	Disease	-0.1120	-0.0222
	Unused	-0.0749	-0.0095
June	Unused	0.00140	0.0311
tpPO activity			
January	Damage	0.1120	0.2070
	Disease	0.0135	0.0996
March	Damage	-0.264	-0.0317
	Disease	-0.296	-0.0260
	Unused	-0.218	-0.0212
	Tourist	-0.274	-0.0414
Fluorescence			
January	Damage	79.4	210.0
	Disease	11.8	130.0
March	Damage	-69.8	-0.606
	Tourist	-87.2	-18.00
Cyan FP			
March	Damage	0.036	0.434
June	Damage	0.0426	0.397
Green FP			
June	Damage	-0.317	-0.0917
	Tourist	-0.217	-0.0133
Apextrin			
March	Damage	-0.299	-0.0047
	Disease	-0.344	-0.0019
ATF4/5			
November	Damage	-1.460	-0.0024
January	Disease	-1.69	-0.1570
February	Damage	-1.86	-0.537
	Disease	-1.67	-0.469
	Tourist	-1.62	-0.298
June	Damage	-1.89	-0.103
	Disease	-2.29	-0.505
	Tourist	-2.35	-0.302

	Comparison	95% Confidence Interval	
Bf			
December	Damage	0.0299	0.2730
January	Disease	0.07670	0.287
	Unused	0.01560	0.212
C3			
December	Disease	-1.920	-0.196
cFos			
December	Damage	0.560	1.620
	Tourist	1.010	1.970
January	Disease	0.148	2.71
	Unused	0.358	2.75
March	Damage	0.5140	3.46
cJun			
December	Tourist	0.0706	0.777
June	Damage	-1.130	-0.0737
	Disease	-1.090	-0.0367
ERK2			
March	Disease	0.053	1.930
HL1			
November	Disease	-0.515	-0.06160
December	Disease	-0.478	-0.011
MAPK p38			
December	Tourist	0.096	0.425
January	Damage	0.183	0.746
	Disease	0.225	0.666
	Tourist	0.324	0.808
	Unused	0.307	0.718
March	Disease	0.064	0.439
	Tourist	0.054	0.377
MEKK1			
December	Damage	0.295	0.585
	Tourist	0.106	0.369
January	Damage	0.143	0.515
	Disease	0.035	0.325
	Tourist	0.029	0.349
	Unused	0.135	0.407
February	Unused	0.071	2.52
March	Damage	0.366	1.99

Chapter 4

Elevated seawater temperatures have a limited impact on the coral immune response following physical damage

This chapter has been submitted for publication to *Hydrobiologia*

4.1 - Abstract

Recurrent disturbances on coral reefs, including predation, storm damage and elevated seawater temperatures, result in injury, which in turn, reduces coral fitness and immunocompetence. An effective immune response is essential for recovery from lesions to prevent disease and enhance coral survival. However, it is unknown how elevated seawater temperatures affect the coral immune response following injury. To address this knowledge gap, fragments of the reef flat coral *Acropora aspera* were exposed to ambient (27-29°C) or elevated (32-33.5°C) seawater temperatures for 8 days and subsequently experimentally injured. Expression patterns for 15 immune system-related genes involved in the Toll-like receptor (TLR) pathway and complement system 24 hours post-injury revealed that most genes of the TLR pathway were unaffected by elevated seawater temperatures. Exceptions to this pattern were *cFos* and *cJun,* which were upregulated and likely played a role in repair processes, and *TRAF-6* and *NFκB,* which were downregulated and may indicate reduced immune function. Components of the complement system were upregulated (*millectin*, *C3*) or downregulated (*Bf*, *Tx60*, *apextrin*) in corals exposed to high temperatures; however, corals that also sustained injury, showed normal *Tx60* and *apextrin* expression, suggesting that they play important roles in the wounding response. Overall, these results suggest that basal expression levels of immune genes are sufficient to mount a response to injury in the short term (24 hours), and that the immune response in *Acropora aspera* following injury is not significantly affected by the minor elevations in seawater temperatures experienced in this study.

4.2 - Introduction

Physical injury presents a major challenge to an organism's immune system, providing a point-of-entry for pathogens and requiring energy for tissue repair. On coral reefs, as many as 21-56% of coral colonies are commonly damaged at any given time (Lindsay, 2010). Predictions of increasing severity and/or frequency of impacts that cause loss of tissue integrity, including physical damage following severe tropical storms and dredging associated with escalating coastal developments, as well as more localised impacts (e.g., destructive fishing, anchoring, reef-based tourism activities and entangled fishing line), highlight the importance of understanding the capacity of the coral's immune system to respond to injury. In addition, demands on a coral's energy budget associated with climate change-related stressors, particularly ocean warming and acidification, are likely to increase in coming decades. Given the important role of the immune system in wound healing, understanding how the coral's immune response to injury is likely to be affected by additional stressors is becoming increasingly important.

Maintaining tissue integrity is of vital importance for the maintenance of fitness in any animal, including corals. To re-establish tissue integrity following injury, corals have a wound healing process similar to those in higher organisms (Palmer et al., 2011c). In this process, the immune system aids in wound sealing, elimination of microbes and tissue regeneration, by activating transglutaminase and phenoloxidase and recruiting immune cells to the lesion site (Palmer et al., 2011c; Palmer et al., 2012a). In addition, molecular studies have revealed that corals possess components of a range of innate immune pathways that are involved in eliminating potential harmful microbes and maintaining healthy microbial communities in other invertebrates (Fraune and Bosch, 2007; Franzenburg et al., 2012; Franzenburg et al., 2013). Various genes encoding the microbe-associated molecular patterns (MAMP)-detecting Toll-like receptors (TLR) and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR) have been reported to be present in corals, as well as components of their respective downstream signalling pathways, such as the JNK, MAPK p38, ERK-2 and NFκB pathways (Miller et al., 2007a; Shinzato et al., 2011; Hamada et al., 2013). Signalling via these pathways, results in the transcription of genes involved in the regulation of an immune response, as well as cell proliferation (see section 1.2 and 1.3 for more detailed review). The existence of genes involved in these pathways in corals suggests that they may play a significant role in their immune responses following wounding. Nevertheless, few studies have investigated the functions of various homologues and their roles in innate immunity, defence and disease in corals, and

therefore little is known about the molecular mechanisms that regulate the injury-induced immune response.

Discovery of various components of the complement pathway in corals, including complement C3, Factor B (Bf), lectins and mannose binding lectin (MBL)-associated serine protease (MASP) ((Miller et al., 2007a; Kvennefors et al., 2010b; Shinzato et al., 2011) see section 1.3 for more detailed review), suggest that corals also possess a functional complement system. This highly conserved immune effector mechanism promotes phagocytosis of microbes. Moreover, it induces the formation of a microbicidal membrane attack complex (MAC), for which several candidate MAC/PF (membrane attack complex / perforin) domain-containing genes have been identified in *Acropora millepora* (Miller et al., 2007a). Studies of two complement system proteins in corals have shown that the MBL millectin agglutinates bacteria and is upregulated upon challenge with immunostimulants (Kvennefors et al., 2008). In contrast, complement C3 was upregulated following injury (Kvennefors et al., 2010b). The presence of multiple components of the TLR pathway and complement system in corals suggests that they possess complex wound healing and immune systems, similar to those seen in higher organisms, although few studies have focused on functional responses of these pathways.

Various environmental and biological factors are known to significantly inhibit wound healing in corals, including elevated nutrient levels (Renegar, 2008, Denis et al., 2013), sedimentation (Mascarelli and Bunkley-Williams, 1999; Cróquer et al., 2002), light intensity (Titlyanov and Titlyanova, 2009), cyanobacteria (Titlyanov et al., 2005) algae (Mascarelli and Bunkley-Williams, 1999; Titlyanov et al., 2005), and the climate change-related variables pCO_2 and temperature. Increases in both pCO_2 levels seawater temperatures have been shown to reduce wound healing rates (Hess et al., 2004; Henry and Hart, 2005; Renegar, 2008; Edmunds and Lenihan, 2010; Lenihan and Edmunds, 2010; Denis et al., 2013). For example, corals that partially bleached following exposure to high temperatures healed at significantly lower rates than controls, and corals that fully bleached were unable to regenerate following injury (Meesters et al., 1997; Mascarelli and Bunkley-Williams, 1999; Fine et al., 2002). In contrast, regeneration capacity may also increase due to higher metabolic rates associated with a Q_{10} effect (Kramarsky-Winter and Loya, 2000). Increasing seawater temperatures predicted for the coming decades could have dire consequences for corals living near their upper thermal thresholds (Berkelmans and Willis, 1999), with large scale losses of coral reefs predicted (Hoegh-Guldberg et al., 2007). In addition, as seawater temperatures continue to rise, elevated seawater temperatures have also been linked to recent rises in coral disease (Willis et al., 2004; Sokolow, 2009; Ruiz-

Moreno et al., 2012). Shifts in coral-associated bacterial communities (Ritchie, 2006; Bourne et al., 2008; Mouchka et al., 2010; Littman et al., 2011; Witt et al., 2011), increases in coral pathogen virulence and a change in coral immune function (Mydlarz et al., 2008; Palmer et al., 2011a; Vidal-Dupiol et al., 2011b) have all been demonstrated to occur following exposure to elevated seawater temperatures. However, the combined effects of heat stress and injury on the various biochemical components and pathways involved in the coral's immune response are still largely unknown.

In this study, I used a manipulative aquarium-based experiment to assess the impacts of elevated seawater temperatures on the coral immune response exhibited in the first 24 hours following injury. In particular, I analysed the expression of several genes involved in the TLR pathway and complement system, to determine how the short-term wounding–induced immune response of corals is compromised by heat stress.

4.3 - Material & Methods

4.3.1 - Experimental design and sampling

Branches (approximately 8cm in length, n=56) of the scleractinian coral *Acropora aspera* were collected from colonies on the reef flat at Heron Island, Great Barrier Reef, Australia (23°26′31.20″S, 151°54′50.40″E) and transported in seawater to holding tanks at the Research Station. Branches were held upright in 4 racks (n=14 fragments per rack) and acclimated at ambient reef flat temperatures (27.1°C ± 1.3°C (mean ± SE)) for a period of 7 days in flow-through seawater. Racks were randomly assigned to 50 litre aquaria (n = 4) (1 rack per aquarium) supplied with seawater at ambient temperatures. Daytime seawater temperatures in two aquaria were gradually increased by 1°C per day over 4 days using thermostat-controlled heaters until the desired heat stress temperature of 32°C was reached. To simulate day-night temperature fluctuations on the reef flat, heaters were turned on at 07:00 and off at 19:00 hours each day. Therefore, temperatures in the elevated treatment tanks fluctuated between 27°C and 33.5°C (mid-day maximum because of solar heating) each day once the target temperature was achieved. Similarly, because of natural fluctuations in daily ambient temperatures of seawater supplied from the reef, corals in the control temperature treatment were exposed to daily temperatures fluctuations of 26°C - 28.5°C (the maximum daily water temperature on the reef flat at time of collection). After 8 days in each temperature treatment, half of the coral fragments in

each tank were injured (n=7 uninjured; n=7 injured per aquaria) by removing tissue using a high-pressure airgun. Lesions comprised a strip of exposed skeleton approximately 1 cm wide along half the length of each branch (~4 cm). Whole branches were collected 24 hours post-injury and snap-frozen in liquid nitrogen.

4.3.2 - Messenger RNA isolation

Frozen samples of each experimental branch of *A. aspera* were crushed in a stainless steel mortar and pestle, pre-chilled with liquid nitrogen, using a hydraulic press. Messenger RNA (mRNA) was isolated from the crushed coral using the Dynabeads mRNA DIRECT kit (Invitrogen Dynal AS, Oslo, Norway) according to a modified protocol based on the manufacturer's recommendations (as described in section 3.3.2). In summary, approximately 100 mg of crushed coral was added to 400 µl lysis buffer, incubated on a vortex at low speed for 7 min and centrifuged for 2 min at 12000 *g*. The supernatant was added to oligo(dT)-Dynabeads (pre-washed in lysis buffer) and incubated on the vortex at medium speed for 8 min to allow mRNA annealing. Tubes were placed on a DynaMag-2 magnetic particle concentrator for 5 min and supernatant was removed. Using the DynaMag-2, oligo(dT) Dynabead/mRNA complexes were washed twice with 300 µl of Buffer A and subsequently twice with 400 µl of Buffer B. Complexes were resuspended in 27 µl ice-cold 10 mM Tris-HCl, incubated at 80°C for 2 min and rapidly cooled down on ice. Oligo(dT)-Dynabeads were concentrated on the DynaMag-2 and mRNA-containing supernatant was collected and stored at -80°C until use.

4.3.3 - Quantification of gene expression

Expression levels of 20 immune system-related genes (Siboni et al., 2012) and 4 reference genes (Császár et al., 2009; Seneca et al., 2010) (Appendix B Table 1) were analysed using the GenomeLab GeXP Start Kit and the CEQ-8800 Genetic Analysis System (Beckman-Coulter, Brea, CA, United States of America) following the protocols described in Siboni et al. (2012) with minor modifications (as described in 3.3.3). In summary, cDNA was generated from 9 ng of mRNA. Reverse primer concentrations were optimised for the multiplex to ensure signals in the electropherogram were within the CEQ-8800 detection range: 500 nM for *TRAM*, *TIR-1*, *MEKK-1*, *TRAF6*, *Tx60*, *ERK2*, *Bf*, *MAPK p38, Millectin*, *ctg_1913*, *Apextrin*, *cJun*, *HL-1*, *HL-2*, *HL-3*, *CTL-1* and *CTL-2*; 250 nM for *GAPDH* and *RPL9*; 62.5 nM for *NFκB* and *C3/A2M-2*; 12.5 nM for *cFos* and *ATF4/5*; 3.75 nM for *RPS7*. Forward primer concentrations were 200 nM. All details for genes of interest and primer sequences can be found in Appendix B Table 1. Prior to loading on the CEQ-8800 Genetic

Analysis System, PCR products were pre-diluted 1:20. Data were filtered and analysed using the GeXP Analysis Software (Beckman-Coulter, Brea, CA, United States of America) (Siboni et al., 2012). Gene expression levels were normalised to the internal control (Kan^R) and to the geometric mean of the expression levels of three reference genes (*RPL9*, *RPS7* and *ctg_1913*) selected using geNorm (Vandesompele et al., 2002). Data were obtained for three technical replicates per sample.

4.3.4 - Statistical Analysis

For each gene, expression data were analysed for differences in expression using a two-factor analysis of variance (ANOVA), where heath status (healthy versus injured) and temperature (ambient vs elevated) were both treated as fixed factors. Differences between treatments were analysed using Tukey's HSD post-hoc tests. Differences were considered significant when $p<0.05$ (ANOVA) or when the value 0 did not fall within the 95% confidence interval (Tukey's HSD). All analyses were performed in the statistical software package S-PLUS 8.0.

4.4 - Results

Genes involved in the Toll-like receptor pathway were largely unaffected by injury or heat stress in the first 24 hours, with no significant differences in expression patterns found between health states or temperature treatments for *TIR-1*, *MAPK p38*, *MEKK-1* and *ERK-2* (Table 4.1; Fig. 4.1A, C-E). In contrast, injured corals had significantly lower *TRAF-6* expression compared to uninjured corals at ambient temperatures, although expression levels did not differ between health states under mild heat stress (Table 4.1; Fig. 4.1B). The transcriptomic response of *ATF4/5* to injury was dependent on seawater temperature, being downregulated at ambient temperatures, but upregulated in the elevated temperature treatment, as evidenced by a significant interaction effect between temperature and injury (Table 4.1; Fig. 4.1F). The transcription factors *cFos* and *cJun* were significantly upregulated in response to injury, by up to 1.6-fold for *cJun* (Table 4.1; Fig. 4.1G, H). Moreover, exposure to high temperatures caused further increases in expression of *cJun* in injured corals in comparison to controls (Table 4.1; Fig. 4.1H). *NFκB* was downregulated 24 hours post-injury, but this downregulation was only significant when corals were exposed to high seawater temperatures (Table 4.1; Fig. 4.1I).

Expression levels of genes involved in the complement system were affected by both elevated seawater temperatures and injury. Corals downregulated Factor B (*Bf*) by 1.2-fold, as well as the MAC/PF domain-containing genes *apextrin* and *Tx60* by 2.2

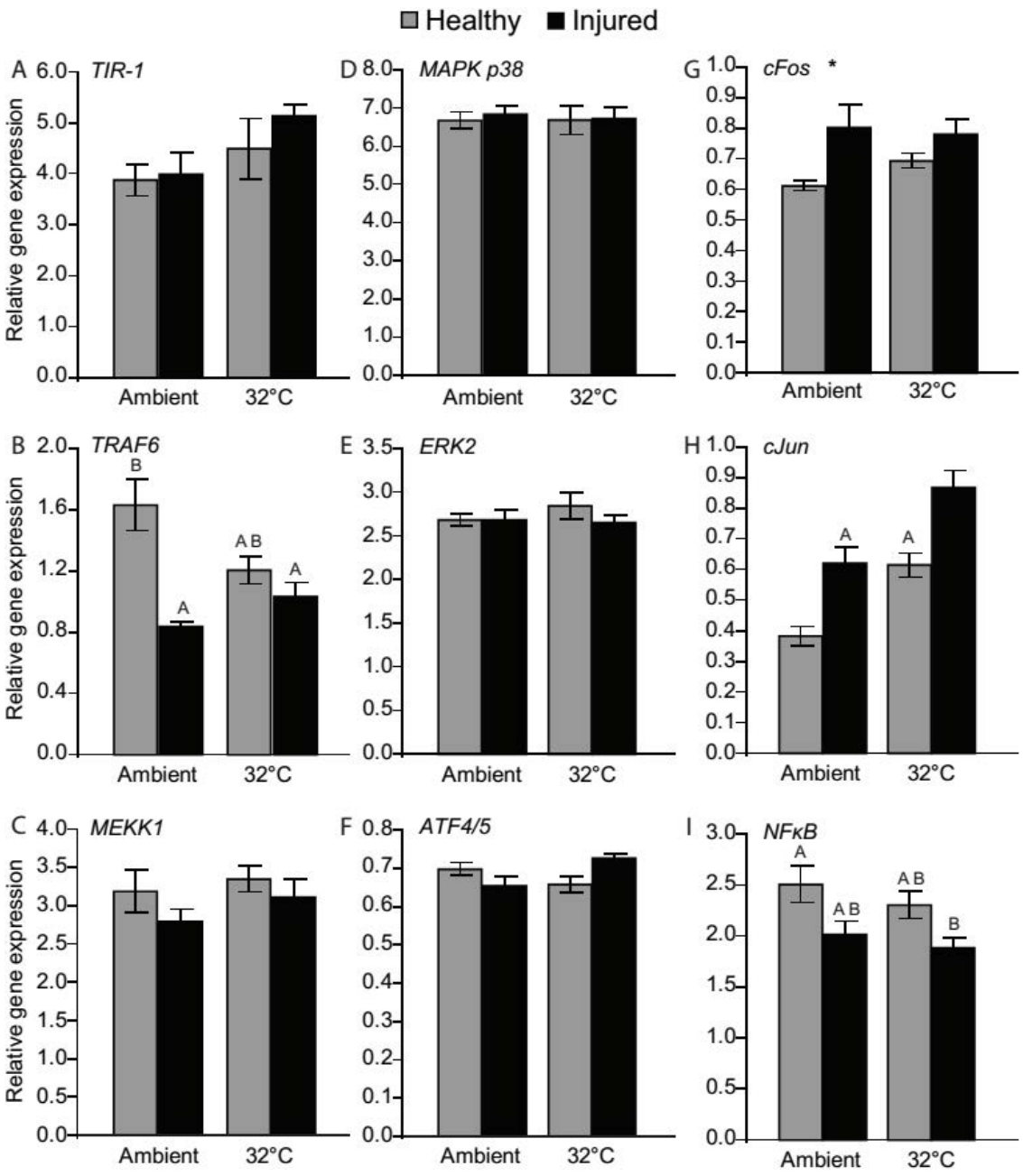


Figure 4.1 – Relative expression of genes involved in the Toll-like receptor pathway in injured and uninjured corals under ambient or elevated seawater temperatures. A) *TIR-1*, B) *TRAF-6*, C) *MEKK-1*, D) *MAPK p38*, E) *ERK2*, F) *ATF4/5*, G) *cFos*, H) *cJun* and I) *NFκB*. Capital letters (A, B, C) indicate experimental treatment groups that are statistically similar. For figures without letters, no significant differences were detected among the four treatment groups. Asterisk (*) identifies the gene for which expression levels differed between the two health states (but not between the two temperature treatments).

and 2.1-fold, respectively, when exposed to elevated temperatures (Table 4.1; Fig. 4.2C, E-F). Interestingly, no difference in a*pextrin* and *Tx60* expression was observed between corals injured under heat stress compared to injury at ambient temperatures (Fig. 4.2E-F). The lectins *HL-2 and millectin* were both significantly upregulated under heat stress, but expression was not altered by health state (Fig. 4.2A, B). Although, complement C3 was upregulated in response to heat stress for both healthy and

injured corals (Fig. 4.2D), expression levels were significantly lower in injured corals relative to those that were uninjured. Expression of *HL-1*, *HL-3*, *CTL-1*, *CTL-2* and *TRAM* was undetectable in all coral samples, potentially due to low expression levels. All results from statistical analyses can be found in Table 4.1.

4.5 - Discussion

Studies of the wounding response in invertebrates have addressed the cellular and molecular processes in tissue regeneration, but the effects of confounding environmental stressors on the immune response are largely unknown. Here, I demonstrate that components of the Toll-like receptor pathway and complement system involved in the wounding response are robust to mild increases in seawater temperatures in *Acropora aspera* 24 hours post-injury.

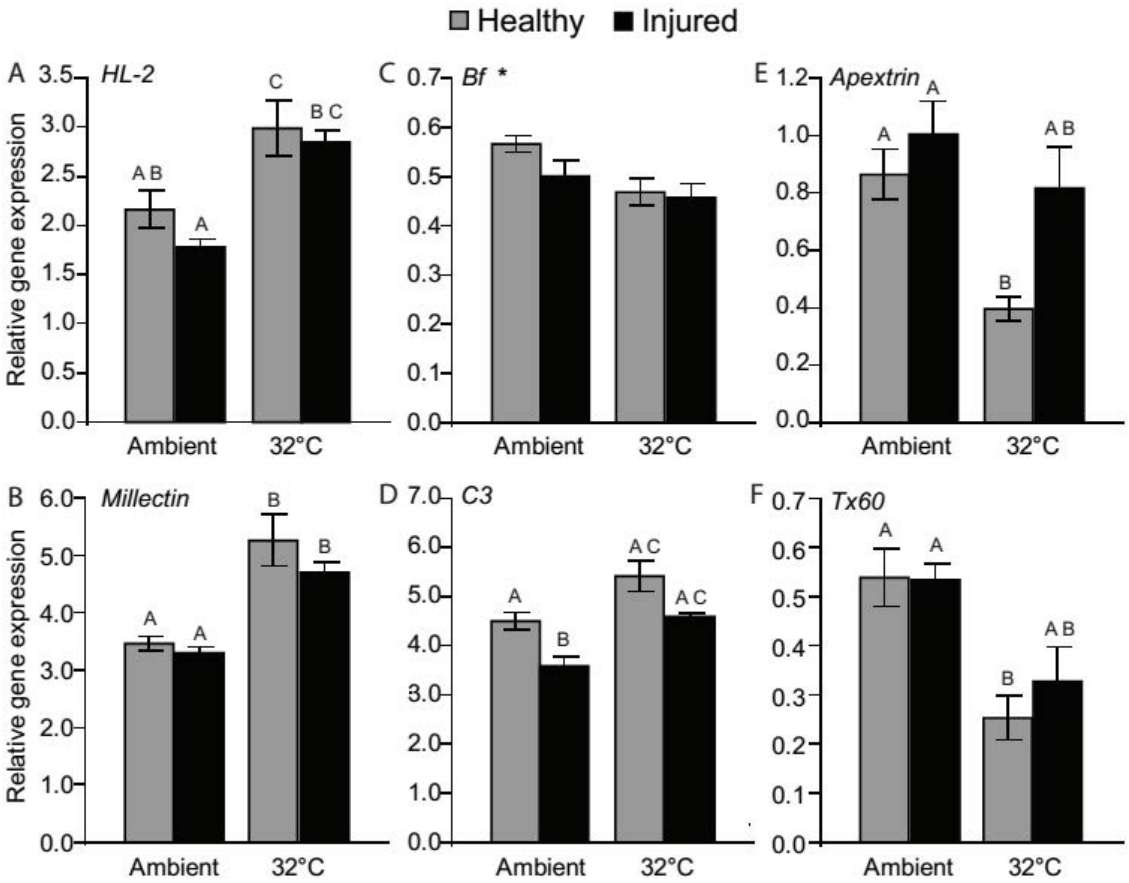


Figure 4.2 – Relative expression of genes involved in the complement system in injured and uninjured corals under ambient or elevated seawater temperatures. A) *HL-2*, B) *Millectin*, C) *Bf*, D) *complement C3*, E) *Apextrin* and F) *Tx60. NFκB*. Capital letters (A, B) indicate experimental treatment groups that are statistically similar. The asterisk (*) identifies the gene for which expression levels differed between the two temperature treatments (but not between the two health states).

Table 4.1 – Overview of statistical analysis. Statistically significant differences are indicated in ***bold and italics*** for the main ANOVA results. The 95% confidence interval is given for pair-wise comparisons where the difference was considered statistically different (i.e. it did not contain the value 0).

Gene	ANOVA	p-value	Tukey's HSD	95% Confidence Interval	
Apextrin	Temperature	***0.0074***	Ambient Healthy – 32℃ Healthy	0.0147	0.9240
	Injury	***0.0234***	Ambient Injured – 32℃ Healthy	0.1790	1.0400
	Temperature : Injury	0.2128			
ATF4/5	Temperature	0.3668			
	Injury	0.6372			
	Temperature : Injury	***0.0133***			
Bf	Temperature	***0.0323***			
	Injury	0.1765			
	Temperature : Injury	0.3414			
C3	Temperature	***0.0002***	Ambient Healthy – Ambient Injured	0.0971	1.7200
	Injury	***0.0007***	Ambient Healthy – 32℃ Healthy	-1.7700	-0.0593
	Temperature : Injury	0.8464	Ambient Injured – 32℃ Healthy	-2.6400	-1.0100
			Ambient Injured – 32℃ Injured	-1.8100	-0.1830
cFos	Temperature	0.7165			
	Injury	***0.0196***			
	Temperature : Injury	0.3523			
cJun	Temperature	***0.0003***	Ambient Healthy – Ambient Injured	-0.4250	-0.0520
	Injury	***0.0001***	Ambient Healthy – 32℃ Healthy	-0.4280	-0.0351
	Temperature : Injury	0.8737	Ambient Healthy – 32℃ Injured	-0.6810	-0.2880
			Ambient Injured – 32℃ Injured	-0.4330	-0.0601
			32℃ Healthy – 32℃ Injured	-0.4500	-0.0568
ERK-2	Temperature	0.5531			
	Injury	0.4315			
	Temperature : Injury	0.4086			
HL-2	Temperature	***0.0001***	Ambient Healthy – 32℃ Healthy	-1.5700	-0.0754
	Injury	0.1499	Ambient Injured – 32℃ Healthy	-1.9 200	-0.5010
	Temperature : Injury	0.4899	Ambient Injured – 32℃ Injured	-1.7900	-0.3640
MAPK p38	Temperature	0.8432			
	Injury	0.6897			
	Temperature : Injury	0.8290			
MEKK-1	Temperature	0.2473			
	Injury	0.1549			
	Temperature : Injury	0.7194			
Millectin	Temperature	***< 0.0001***	Ambient Healthy – 32℃ Healthy	-2.8000	-0.7920
	Injury	0.1680	Ambient Healthy – 32℃ Injured	-2.25 00	-0.2410
	Temperature : Injury	0.4201	Ambient Injured – 32℃ Healthy	-2.9100	-1.0000
			Ambient Injured – 32℃ Injured	-2.3600	-0.4510
NFκB	Temperature	0.3367	Ambient Healthy – 32℃ Injured	0.0266	1.2200
	Injury	***0.0060***			
	Temperature : Injury	0.7836			
TIR-1	Temperature	*0.0556*			
	Injury	0.3996			
	Temperature : Injury	0.5339			
TRAF6	Temperature	0.4919	Ambient Healthy – Ambient Injured	0.3880	1.2000
	Injury	***0.0003***	Ambient Healthy – 32℃ Injured	0.1700	1.0300
	Temperature : Injury	***0.0086***			
Tx60	Temperature	***0.0003***	Ambient Healthy – 32℃ Healthy	0.0673	0.5040
	Injury	0.5243	Ambient Injured – 32℃ Healthy	0.074 2	0.4890
	Temperature : Injury	0.4509			

4.5.1 – Toll-like receptor pathway

In the current study, I found that exposure of corals to mild heat stress for 8 days did not significantly affect the regulation of the TLR pathway, except for an increase in *cJun* expression. Injury, on the other hand, resulted in upregulation of both *cJun* and *cFos*, whereas *TRAF-6* was downregulated. Interestingly, the combination of injury and heat stress increased *cJun* expression in corals further, suggesting that it is involved both the thermal stress response and wound repair. The transcription factors *cJun* and *cFos* together form the AP-1 transcription factor complex (Hess et al., 2004). AP-1 is involved not only in the TLR pathway, but also plays a role in the regulation of

cell proliferation, differentiation and apoptosis. These processes are essential for sealing lesions and development of tissue layers and polyp structures, and are regulated via different signal transduction pathways (Hess et al., 2004). Since no other genes in the TLR pathway were upregulated following injury, my interpretation is that *cJun* and *cFos* are primarily involved in lesion regeneration rather than an immune response in the initial stages following injury.

Although no major changes in the expression of genes involved in the TLR pathway were detected under mild heat stress, injured heat stressed corals did have reduced levels of NFκB. Because the TLR signalling pathway regulates the expression of antimicrobial peptides (AMP) primarily via NFκB, these corals may have reduced AMP production and may thus be more susceptible to disease. Although no AMP has been identified from a species of *Acropora* yet, AMP-encoding genes are present in scleractinian corals and other cnidarians (Fraune and Bosch, 2007; Fraune et al., 2011; Vidal-Dupiol et al., 2011a; Burge et al., 2013; Vidal-Dupiol et al., 2014). Studies of the (septic) wounding response in a range of invertebrates (Fehlbaum et al., 1994; Rodriguez de la Vega et al., 2004; Pujol et al., 2008b; Xu and Faisal, 2010) have also shown upregulation of AMPs. The limited transcriptomic response of genes involved in TLR signalling under heat stress observed in my study could indicate that these corals were capable of maintaining stable bacterial communities under these conditions. While no evidence of active regulation of bacterial community composition by corals has been presented to date, AMPs have been shown to be crucial in maintaining healthy tissue-associated bacterial communities in the cnidarian *Hydra* (Fraune and Bosch, 2007; Franzenburg et al., 2012; Franzenburg et al., 2013).

In summary, I was unable to establish a link between the TLR pathway and the response to injury in *Acropora aspera*. As my study is preliminary, consisting of a single post-injury time point, potential earlier or later changes in gene expression levels may have been overlooked. Based on this study, the TLR pathway in *Acropora aspera* was uncompromised, suggesting that the coral has good potential to respond to injury under mild heat stress. However, understanding the impact of heat stress on the full dynamics of the wounding-induced immune response in corals requires study over longer time-frames and with greater sampling resolution.

4.5.2 - Complement system

The expression of genes involved in the complement system was affected by both elevated seawater temperatures and injury. Whereas heat stress caused downregulation of the MAC/PF domain containing *Tx60 and apextrin*, at ambient temperatures, expression of *Tx60* and *apextrin* was similar between healthy and

injured corals. This suggests that not only are *Tx60* and *apextrin* involved in the wounding response, but also that heat stressed corals are capable of inducing an immune response similar to those at ambient temperatures. My results are in line with a previous study on the bumble bee, where upregulation of genes encoding MAC proteins were observed following injury (Erler et al., 2011). The function of these genes in the recovery process in corals remains to be determined; however, the presence of a MAC/PF domain suggests a role in the anti-microbial response and could indicate infection of the lesion by microbes. The involvement of *Tx60* and *apextrin* in the wounding response, but the absence of upregulated expression at ambient temperatures in response to injury, indicates that the initial gene expression levels under normal conditions may be sufficient to prevent infection. This suggests that normal expression levels for other genes may also be sufficient, and that potentially, the immune protein expression at 24 hours post-injury is sufficient for an appropriate response. Alternatively, gene expression may be primarily regulated through increased mRNA stability (Wu and Brewer, 2012) rather than an increased transcriptomic response. This would enable the coral to direct its limited resources towards wound sealing and regeneration in the initial stages of the wounding response, although further studies are required to address this question.

The upregulation in expression of complement C3 and millectin observed in response to 8 days of exposure to elevated seawater temperatures may indicate that these heat stressed corals were directing resources to preserve the coral-*Symbiodinium* endosymbiosis. This is in accordance with previous studies that have implicated these proteins in the maintenance of the coral-*Symbiodinium* endosymbiosis in *Acropora millepora* (Kvennefors et al., 2008; Kvennefors et al., 2010b). The downregulation of *Bf* under heat stress suggests that this gene, which is a key player in the alternative complement pathway, may not be involved in the activation of C3 for the maintenance of symbiosis. This confirms a role for the lectin-complement pathway, with millectin as the MBL. In addition, my results for *millectin* are consistent with reports that injury does not change expression of this gene (Kvennefors et al., 2010b). However, I show reduced C3 expression 24 hours post-injury, which is in contrast to results from a recent study showing upregulation of C3 within 12 hours after corals were injured (Kvennefors et al., 2010b). Although this may appear inconsistent, very little is known about expression dynamics of immune genes in corals, and the 12-hour time difference between sampling in the two studies could explain differences in results. Investigations into expression levels over time will be required to gain further insights into these dynamics.

4.6 - Conclusion

In this study, I demonstrate that the immune response of *A. aspera* exhibited 24 hours post-injury, is not significantly affected by mild temperature stress. While heat stress did influence the corals' immune effector capacity, responses to injury under heat stress were as strong as those of non-heat stressed corals. Gene expression levels of both the TLR pathway components and other immune effectors were largely unaffected at 24 hours post-injury, while genes potentially involved in wound repair and tissue regeneration were upregulated. In addition, upregulation of components of the lectin-complement system, which is involved in the coral-*Symbiodinium* symbiosis, under heat stress suggests that corals were attempting to maintain the endosymbiosis. Normal expression levels of immune genes in the initial response following injury may be sufficient to ward off invading microbes, enabling the coral to invest more heavily in tissue repair. This study provides evidence that some corals are capable of withstanding the adverse effects of physical damage under elevated temperatures.

Chapter 5

The coral immune response facilitates protection against microbes during tissue regeneration following physical damage

This chapter has been submitted for publication to *Molecular Ecology*

5.1 - Abstract

The likelihood of increasing impacts of physical damage from predation, storms and anthropogenic disturbances on coral reefs highlights the need to understand the impact of injury on the coral immune system. In this study, I examined regulation of the coral immune response over 10 days in the field following physical trauma artificially inflicted on the coral *Acropora aspera*, simultaneously with bacterial colonisation of the lesions. Corals responded rapidly to injury by increasing the expression of immune system-related genes involved in the Toll-like and NOD-like receptor signalling pathways and the lectin-complement system in three distinct phases, corresponding to <48 hours, 96 hours and 10 days post-injury. Phenoloxidase activity was also significantly upregulated in two distinct phases (<72 hours and 10 days post-injury), while levels of non-fluorescent chromoprotein followed a similar temporal pattern but were significantly increased throughout the study. In addition, green fluorescent protein expression was upregulated in response to injury from four days post-injury; while cyan fluorescent protein expression was significantly reduced, suggesting a role for GFP, but not CFP in the wounding response. No shifts in the structure of coral-associated bacterial communities as a result of injury were evident based on a 16S rRNA gene amplicon pyrosequencing survey. Bacteria-specific fluorescent *in situ* hybridisation also showed no evidence of bacterial colonisation of the wound or regenerating tissues. Near complete regeneration of lesions within 10 days shows that corals exhibit immune responses that support rapid recovery following physical injury, maintain coral microbial homeostasis and prevent bacterial infestation that may compromise coral fitness.

5.2 - Introduction

Disturbances, such as severe tropical storms, sedimentation, fish bites and algal overgrowth, can result in wounding and compromised integrity of coral tissues. Other anthropogenic factors, including entangled fishing line, destructive fishing techniques, anchoring and reef-based tourism activities also contribute to significant physical damage to corals (Lamb and Willis, 2011; Lamb et al., 2014). Ecological surveys of coral reefs have reported that physical injury can be ubiquitous, with between 21 and 56% of scleractinian corals injured at any point in time (Lindsay, 2010). Given the recent rise in coral disease (Sokolow, 2009) and the likelihood of increasing levels of disturbances that create wounds and enable the entry of microbes, it is important to determine how corals respond to wounding and physical damage, and understand the capacity of corals to recover and regenerate tissues.

Wound healing is a vital process in maintaining tissue integrity and preventing infection and disease. In scleractinian corals, there are four phases in the healing process, similar to the wound healing process in higher organisms. The first step is a wound sealing-phase via coagulation, which results in the formation of a clot in the lesion and is characterised by the release of the granule contents of granular cells, possibly containing transglutaminase (Palmer et al., 2011c; Palmer et al., 2012a). During the second phase, immune cells (eosinophilic granular amoebocytes) that phagocytise microbes and cell debris, are recruited to the site of the lesion (Palmer et al., 2011c). Additional immune cells and fibroblasts infiltrate and proliferate at the lesion site in the third phase, termed the proliferation phase, resulting in the formation and reorganisation of an epidermal-like layer (Palmer et al., 2011c). Phase four encompasses wound maturation, which can occur within 48 hours (Palmer et al., 2011c), and is generally followed by remodelling of the epidermal cells into a new epithelium. Despite an understanding of the short term cellular mechanism of wound healing (Palmer et al., 2011c) and lesion regeneration (Bak and Steward-van Es, 1980; Bak, 1983; van Woesik, 1998; D'Angelo et al., 2012; Denis et al., 2013), the longer term response and underlying molecular immune mechanisms in corals remain to be elucidated.

Corals have a suite of innate immune defence mechanisms they can deploy to maintain a close symbiosis with associated bacterial communities and eliminate potential harmful microbes. Low but consistent background levels of anti-microbial peptide (AMP) expression result in the maintenance of a healthy microbial community in several invertebrates, including cnidarians (Fraune et al., 2011; Kounatidis and Ligoxygakis, 2012), and recently, an AMP was isolated from *Pocillopora damicornis*

(Vidal-Dupiol et al., 2011a). AMPs exhibit bactericidal properties, by forming pores in microbial cell walls or by binding to intracellular molecules essential for a microbe's survival (Brogden, 2005). During infection, AMP expression is generally upregulated upon detection of microbe-associated molecular patterns (MAMP) by pattern recognition receptors (PRR), such as Toll-like receptors (TLR). TLRs and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR) signal via the JNK, MAPK p38 and NFκB pathways, resulting in the transcription of target genes involved in immunity, cell survival and proliferation. A repertoire of genes encoding TLRs and NLRs, as well as components of their downstream signalling pathways, has recently been described in corals (Miller et al., 2007a; Shinzato et al., 2011; Hamada et al., 2013).

Invertebrates also employ several rapidly-inducible immune mechanisms to combat infections, such as transglutaminase. In arthropods, this enzyme polymerises haemolymph proteins into large aggregates, resulting in coagulation at the site of injury (Cerenius et al., 2010a). Recently, evidence of transglutaminase activity in corals was reported (Palmer et al., 2012a), which, combined with the identification of hemolectin homologues in *A. millepora* (Grasso et al., 2008; Grasso et al., 2011), suggests the presence of a coagulation system in corals. Another rapidly-inducible immune mechanism is the highly conserved melanisation cascade, or proPO system, which has recently been documented in both gorgonian and scleractinian corals (Mydlarz et al., 2008; Palmer et al., 2008). In this system, the inactive pro-enzyme pro-phenoloxidase (proPO) is cleaved into the active phenoloxidase (PO) following the detection of MAMPs (Mydlarz et al., 2006; Cerenius et al., 2010a). PO activity results in the deposition of melanin, which immobilizes microbes (Cerenius et al., 2010a), while at the same time, highly reactive and toxic quinone intermediates that attack the invading organisms are produced (Cerenius et al., 2010a). PO is also involved in hardening of the clot formed during the coagulation of haemolymph following wounding (Cerenius et al., 2010a; Kounatidis and Ligoxygakis, 2012). Melanin deposits have recently been found around the lesions of injured corals, confirming a role for PO in the coral wound healing process (Palmer et al., 2011c). In many invertebrates, components of the complement system, such as complement C3, Factor B (Bf), lectins and mannose-binding lectin (MBL)-associated serine protease (MASP), have been discovered (Mydlarz et al., 2006; Cerenius et al., 2010a). In this system, MBLs have been proposed to bind to the microbial cell wall, and activated MASPs cleave C3 and Bf. In association, these two proteins form a C3-convertase that efficiently cleaves C3 and deposits C3b on the microbe's surface. The opsonin-properties of C3b ensure enhanced phagocytosis and elimination of the microbe by specialised immune cells. In

addition, spontaneous hydrolysis of C3 and the subsequent binding of C3b to a microbial cell wall could lead to the formation of a membrane attack complex (MAC), which forms pores in microbial cell walls resulting in cell lysis. In corals, the MBL millectin was found to cause agglutination of bacteria *in vitro* and expression was increased upon challenges with immunostimulants (Kvennefors et al., 2008), while C3 was upregulated following trauma and injury (Kvennefors et al., 2010b). Millectin was also associated with *Symbiodinium*, suggesting that it may play a role in symbiosis (Kvennefors et al., 2008; Kvennefors et al., 2010b). In addition, genes encoding proteins containing a MAC/PF (membrane attack complex / perforin) domain have been identified in Acr*opora millepora* (Miller et al., 2007a).

Evidence of the presence and activation of several innate immune system pathways in corals suggests they possess complex wound healing and immune systems, encompassing coagulation, phagocytosis and antimicrobial properties, as seen in higher organisms. However, we still know very little about specific immune cells in cnidarians, their role in defence and the molecular mechanisms that govern wound healing and immune responses. In this study, I investigated the process of wound healing in scleractinian corals in the field over a 10-day period to determine the response of the innate immune system to tissue damage and its role in tissue repair. Specifically, I characterised the expression dynamics of immune system-related genes and proteins, as well as bacterial colonisation patterns and coral-associated bacterial community structures, during the wound healing and tissue regeneration process. My results show that tissue damage elicits a dynamic response in corals, involving various components of the coral immune system and tissue repair process, sufficient to prevent bacterial infection.

5.3 - Material & Methods

5.3.1 - Field experimental design and sample collection

Two large colonies of the scleractinian coral *Acropora aspera*, located within a 10 meter radius on the reef flat of Heron Island, Great Barrier Reef, Australia (23°26′31.20″S, 151°54′50.40″E), were tagged on 6 February 2011. To simulate physical damage, tissue was removed from 42 branches of each selected colony using a high-pressure airgun. Lesions were an approximately 1 cm wide strip along ¾ of the branch length (~7.5 cm). To account for within-colony differences in response, branches on three sides of each colony were damaged. At 16, 24, 48, 72, 96, 168 and 240 hours post-injury, 2 injured and 2 undamaged and healthy-looking nubbins were collected from each of the three sides per colony. Of these, one injured and 1 healthy-

looking nubbin were snap-frozen in liquid nitrogen, and the remaining nubbins were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) solution (see Supplementary Table 1 for an overview of samples used for each analysis).

5.3.2 - Fluorescence in situ *hybridisation*

Fixed coral nubbins were decalcified in 20% (w/v) Na-EDTA (pH 8.0) and subsequently cut into 3-5 pieces of equal length prior to histological processing. Samples were processed sequentially in 70%, 80%, 90%, 95%, and 100% (2 washes) ethanol, followed by three washes in xylene and three washes in Paraplast wax, each for 60 min, overnight in a Shandon Histo Centre 3 automated tissue processor, prior to embedding in Paraplast Wax. Paraffin blocks were sectioned at 7 µm and collected on Superfrost Plus slides (Menzel, Germany). Universal bacterial probe EUB338 (Amann et al., 1990), coupled to the fluorescent label ATTO647 (Thermo Fisher Scientific, USA), was used in a standardised FISH protocol (Ainsworth et al., 2006). Tissue sections were dewaxed in xylene (3x 10 min), dehydrated in absolute ethanol (3x 3 min) and air dried. Sections were covered with hybridisation buffer (900 mM NaCl, 20 mM Tris-HCl, 35% (v/v) formamide and 0.01% SDS in ultrapure water) and probes were added to a final concentration of 25 ng μl^{-1}, incubated at 46°C for 2.5 hours and washed in 50ml wash buffer (70 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA and 0.01% SDS in ultrapure water) at 48°C for 15 minutes. Sections were thoroughly rinsed with ice-cold ultrapure water to remove excess salts, air dried and embedded in Citifluor AF1 (Citifluor, UK). An LSM710 confocal laser scanning microscope (Carl Zeiss, Germany), combined with spectral emissions profiling, was used to visualise tissue-associated FISH-labelled bacterial communities as per (Ainsworth et al., 2006).

5.3.3 - Messenger RNA isolation

Frozen *A. aspera* coral nubbins were crushed in a pre-chilled (with liquid nitrogen) stainless steel mortar and pestle using a hydraulic press. Messenger RNA (mRNA) was isolated from the crushed coral using the Dynabeads mRNA DIRECT kit (Invitrogen Dynal, Norway) according to a modified protocol based on the manufacturer's recommendations. Approximately 100 mg of crushed coral was added to 400 µl lysis buffer, incubated on a vortex at low speed for 7 min and centrifuged for 2 min at 12000 *g*. Supernatant was added to oligo(dT)-Dynabeads (pre-washed in lysis buffer) and incubated on the vortex at medium speed for 8 min to allow mRNA annealing. Tubes were placed on a DynaMag-2 magnetic particle concentrator for 5 min and supernatant was removed. Using the DynaMag-2, oligo(dT) Dynabead/mRNA complexes were washed twice with 300 µl of Buffer A and subsequently twice with 400

µl of Buffer B. Complexes were resuspended in 27 µl ice-cold 10mM Tris-HCl, incubated at 80°C for 2 min and rapidly cooled down on ice. Oligo(dT)-Dynabeads were concentrated on the DynaMag-2 and mRNA-containing supernatant was collected and stored at -80°C.

5.3.4 - Gene expression analysis

Expression levels of 20 immune system-related genes (Siboni et al., 2012) and 4 reference genes (Seneca et al., 2010) were analysed using the GenomeLab GeXP Start Kit and the CEQ-8800 Genetic Analysis System (Beckman-Coulter, USA) following the previously described protocol Siboni et al. (2012) (Chapter 3, section 3.3.3). cDNA was generated from 9 ng of mRNA and forward primer concentrations were 200 nM. Reverse primer concentrations were optimised for the multiplex to ensure signals in the electropherogram were within the CEQ-8800 detection range: 500 nM for *TRAM*, *TIR-1*, *MEKK-1*, *TRAF6*, *Tx60*, *ERK2*, *Bf*, *MAPK p38*, *Millectin*, *ctg_1913*, *Apextrin*, *cJun*, *HL-1*, *HL-2*, *HL-3*, *CTL-1* and *CTL-2*; 250 nM for *GAPDH* and *RPL9*; 62.5 nM for *NFκB* and *C3/A2M-2*; 12.5 nM for *cFos* and *ATF4/5*; 3.75 nM for *RPS7* (Supplementary Table 2). PCR products were pre-diluted 1:20 prior to loading on the CEQ-8800. Data were filtered and analysed using the GeXP Analysis Software (Beckman-Coulter, USA) (Siboni et al., 2012). Gene expression levels were normalised to an internal control (Kan^R) and to the geometric mean of the expression levels of three housekeeping genes (*RPL9*, *GAPDH* and *ctg_1913*) selected using geNorm (Vandesompele et al., 2002). Results were obtained for three technical replicates per sample.

5.3.5 - Protein and enzyme assays

Approximately 120 mg of each frozen crushed coral sample were added to 500 µl phosphate buffered saline (50mM, pH7.8) (Sigma-Aldrich, Australia) and lysed using Lysing Matrix C and a FastPrep-24 (MP Biomedicals, USA) at 4.0MΩ/s for 1 minute. Samples were centrifuged for 45 minutes at 12000 g at 4ºC, and supernatants were collected and stored at -80ºC. Total protein content was determined for each sample using the DC Protein Assay (Bio-Rad) following the manufacturer's instructions. Absorbances were read at 750 nm using a SpectraMax M2 (Molecular Devices, USA). The assay to measure total potential phenoloxidase (tpPO) activity followed procedures outlined in (Palmer et al., 2011a), with some modifications. Twenty microlitres of coral tissue lysate was loaded in triplicate into wells of a 96-well plate, and phosphate buffered saline (50 mM, pH7.8; 40 µl) and 25 µl 0.1 mg/ml trypsin were added. For analysis of active phenoloxidase (PO) activity, trypsin solution was substituted by 25 µl

double distilled water. Reaction mixtures were incubated for 20 min to allow for activation of pro-phenoloxidase by trypsin and 30 µl of 10 mM dopamine hydrochloride (Sigma-Aldrich, Australia) were added. As a control, 20 µl of PBS was used. After a 15-min incubation, absorbance was measured at 490 nm every 5 min for a 45 min period using a SpectraMax M2. Data were independently obtained in triplicate. All data were normalised to total protein content. For chromoprotein and fluorescent protein expression, 20 µl of tissue lysate sample were added to each well of a black, clear bottom 384-well plate in triplicate. Expression of chromoprotein was analysed by measuring the absorbance at 588 nm using a SpectraMax M2. Fluorescence spectra were analysed by measuring the emission wavelengths between 400 and 700 nm, with a 5 nm resolution, upon excitation of fluorescent proteins at 280 nm. All data were normalised to total protein content and expression levels were calculated using the method described in Paley *et al.* (in review; Appendix B).

5.3.6 - Statistical Analysis

Gene expression, protein expression and PO activity data were obtained from three technical replicates with a coefficient of variance of no more than 25%. Data were analysed for differences in expression using General Linear Models (GLM), followed by a Fisher's LSD test using the STATISTICA 10 statistical software package. The Fisher LSD post hoc test was used to determine the statistical significance of differences between treatments at different time points. Differences were considered significant when $p < 0.05$.

5.3.7 - 16S rRNA gene amplicon pyrosequencing

Samples of approximately 100 mg of crushed frozen coral were aseptically transferred to 1.5 ml Eppendorf tubes and total genomic DNA was extracted using the PowerPlant DNA Isolation Kit (MO BIO Laboratories, USA) following the manufacturer's instructions. Extracted DNA was quantified using a NanoDrop (ThermoScientific) and shipped to the Research and Testing Laboratory (RTL; Texas, USA). Bacterial 16S rDNA was amplified using the 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R primer (5'-GTNTTACNGCGGCKGCTG-3') set, followed by sequencing on a Roche GS FLX+ 454 pyrosequencer Research and Testing Laboratory. Sequence fasta and quality files were obtained from Research and Testing Laboratory and processed using the QIIME pipeline (Caporaso et al., 2010). The split_libraries.py script was used to remove poor quality (<25) sequences, reads of <200 bp or >550 bp in length, primers, and barcodes, and to add a sample identification to each sequence. Using UCHIME (Edgar et al., 2011), chimeric

sequences were identified against the chimera-free 16S rRNA gene sequence database (Green Genes database gg_13_5) and removed. Using the chimera-free fasta file, numbers of reads per sample were calculated and samples were normalised to 600 reads per sample. Sequences of 97% similarity were clustered using uclust (Edgar, 2010), with each cluster representing an operational taxonomic unit (OTU). The most abundant sequence per cluster was selected as representative sequence, and Green Genes taxonomy was assigned using BLAST (75% similarity). QIIME pipeline was used to generate OTU tables and tables were analysed for relative abundance. Alpha diversity metrics (total observed species (OTU), predicted species (chao1), Shannon-Weiner diversity and Fisher's alpha) were generated from OTU tables. Beta diversity was analysed using a two dimension principal coordinate analysis, based on Unifrac distances generated from weighted and unweighted Unifrac analysis using PRIMER 6 (PRIMER-E, UK). PERMANOVAs on square root-transformed OTU tables, as well as weighted and unweighted Unifrac distances, were performed to test for significant differences between coral-associated bacterial communities using PRIMER 6 (9999 permutations). The complete dataset was deposited in the NCBI Sequence Read Archive (SRA) database with accession number SRA##.

5.4 - Results

5.4.1 - Macroscopic and cellular assessment of coral recovery

Following artificially-inflicted physical damage, a pigmentation response was observed within 16 hours at lesion sites in *A. aspera* and was visible until the end of the monitoring period, 10 days post-injury (Fig. 5.1A). In the majority of injured coral fragments (11 out of 12), almost complete tissue regeneration occurred within 10 days, showing near-normal macroscopic tissue morphology and pigmentation (Fig. 5.1B-G). No identifiable signs of macroscopic microbial colonisation or overgrowth of tissues were associated with lesions.

5.4.2 - Molecular responses following physical damage

Total fluorescent protein (FP) expression did not change in injured corals throughout the 10 day experiment (Suppl. Fig. 5.1B), however expression of the typically dominant cyan fluorescent protein (CFP) was significantly reduced (by up to 0.84-fold) on Days 3, 7 and 10 post-injury ($p < 0.01$) and slightly reduced at Day 4 ($p = 0.06$) (Fig. 5.2A). Concomitantly, green fluorescent protein (GFP) expression was significantly upregulated (by up to 3.75-fold) at each time point that CFP was downregulated ($p < 0.01$), including Day 4 ($p = 0.03$) (Fig. 5.2B). Red fluorescent

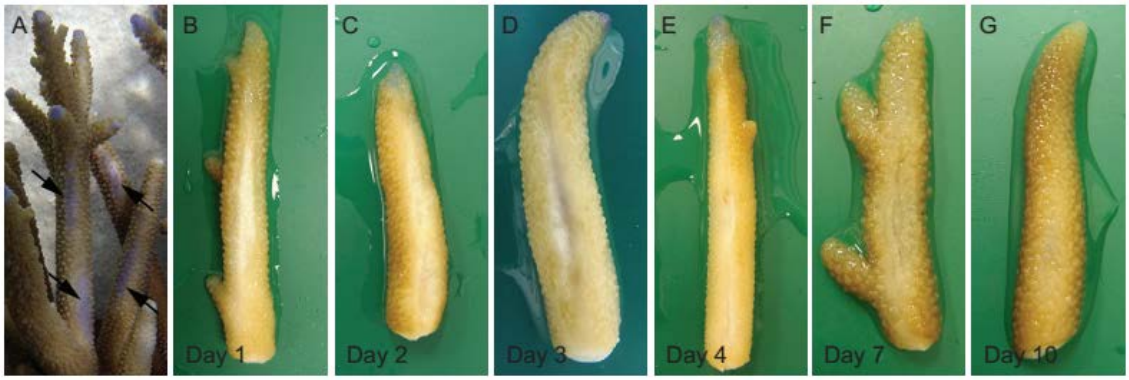


Figure 5.1 – Macroscopic analysis of injured coral. A) Pigmentation response (arrows) at lesion site 7 days post-injury, B-G) Wound healing in *Acropora aspera* during 10 day period following injury. Photos are representative images of decalcified nubbins of injured corals.

protein (RFP) was not differentially expressed in wounded tissues compared to controls (Suppl. Fig. 5.1A), remaining low (<17% of total fluorescence) throughout the study. The visual presence of blue-purple pigmented tissue in the vicinity of coral lesions suggested upregulation of a blue chromoprotein (CP) (Dove et al., 2001), and accordingly, expression levels for this protein were upregulated within 16 hours post-injury ($p = 0.04$) and remained upregulated compared to control coral nubbins throughout the 10-day study period ($p < 0.01$ at each time point) (Fig. 5.2C). The temporal expression pattern for CP showed two peaks: the first within 24 hours post-injury and a second lower peak on day 7 (Fig. 5.2C). Phenoloxidase (PO) activity

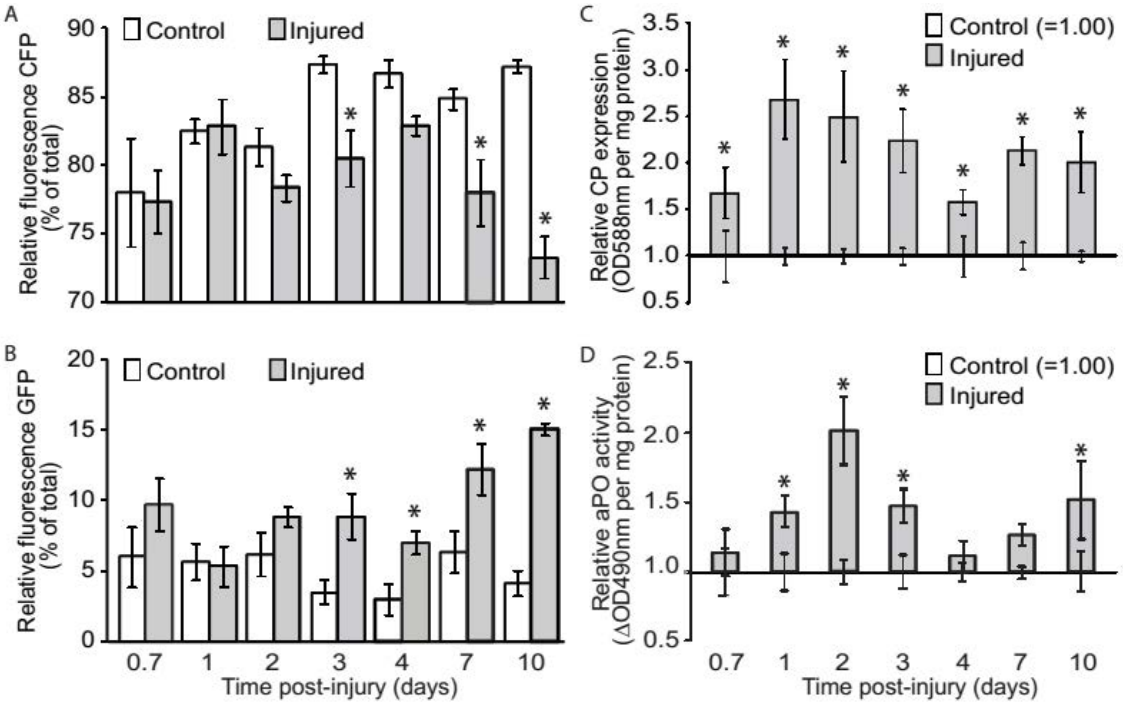


Figure 5.2 - Temporal GFP-like protein expression and phenoloxidase activity in response to injury. A-C) Temporal expression patterns of A) cyan fluorescent protein (CFP), B) green fluorescent protein (GFP), C) chromoprotein (CP) and D) phenoloxidase (PO) activity. CP and PO activity data are relative to control. Asterisk (*) indicates statistical significance of $p < 0.05$.

increased significantly between Days 1 and 3 post-injury ($p < 0.01$), with peak activity evident 2 days post-injury ($p < 0.01$), compared to control samples (Fig. 5.2D). Subsequently, activity decreased in injured corals, reaching expression levels similar to those of controls at days 4 and 7 ($p = 0.54$), although a second peak in PO activity was evident at 10 days post-injury ($p < 0.05$) (Fig. 5.2D). Total potential phenoloxidase activity mirrored this temporal pattern ($r = 0.99$) (Suppl. Fig. 5.1C).

At 16 hours post-injury, expression patterns of a suite of immune system-related genes demonstrated either non-significant trends for reduced gene expression or significant downregulation (*ATF4/5:* $p = 0.04$; *millectin:* $p < 0.01$) for all genes of interest, except *TIR-1* (Fig. 5.3, Suppl. Fig. 5.2). The putative Toll-like receptor homologue *TIR-1* was the first gene observed to be significantly upregulated (1.49-fold) within 24 hours post-injury ($p < 0.01$), and remained upregulated for a further 24 hours ($p = 0.03$). Following a return to control levels 3 days post-injury (Fig. 5.3A), a second upregulation in *TIR-1* expression (1.33-fold) was observed on day 4 ($p < 0.01$), but expression levels did not differ from those of controls by 7 days post-injury (Fig. 5.3A). Genes involved in signalling downstream of TLRs also showed variable temporal expression profiles, with upregulation in expression on day 2 (*MEKK1* 1.13-fold, $p = 0.03$; *ERK2* 1.33-fold, $p < 0.01$) and day 4 (*NFκB* 1.39-fold, $p < 0.01$; *TRAF6* 1.79-fold, $p < 0.01$; *cFos* 2.33-fold, $p = 0.02$; *MEKK1* 1.48-fold, $p < 0.01$; *ERK2* 2.16-fold, $p < 0.01$) (Fig. 5.3B-F). Some of these genes were also upregulated on day 10 (*TRAF6:* 1.36-fold, $p = 0.04$; *ERK2:* 1.80-fold, $p < 0.01$; and *cFos:* 2.14-fold, $p < 0.01$), whereas this was not observed for other genes involved in TLR signalling (Fig 5.3B-F). No changes in expression of *cJun* were observed, but *ATF4/5* (16h $p = 0.04$; day 3 $p = 0.02$; day 10 $p = 0.01$) and *MAPK p38* (day 4 $p = 0.04$; day 10 $p = 0.01$) were downregulated (up to 0.79 and 0.76-fold respectively) (Suppl. Fig. 5.2A-C).

Millectin was consistently downregulated in injured corals (up to 0.27-fold at 16 hours, and at Days 1, 3, 4 and 7; $p < 0.01$) throughout the 10-day sampling period (Suppl. Fig. 5.2D). Downregulation was also observed for the homologue of hemolectin *HL-2* at early time points (16 hours: $p < 0.01$; day 1: $p < 0.05$); however, this gene was significantly upregulated on day 10 (1.36-fold, $p < 0.05$) (Fig. 5.3G). Expression of the C-type lectin *CTL-2* was upregulated in response to physical damage compared to control corals, which had lectin levels that were either at very low levels or were undetected (Suppl. Fig. 5.3). The expression of *Bf* and complement *C3* were found to follow similar patterns to several of the TLR downstream signalling molecules, being significantly upregulated at day 4 (*Bf* 1.42-fold, $p < 0.05$; *C3* 1.41-fold, $p < 0.01$) (Fig. 5.3H-I). Ten days post-injury, expression levels of *Bf* were also significantly increased (2.56-fold, $p < 0.01$), which was in contrast to a downregulation in expression of *C3* at

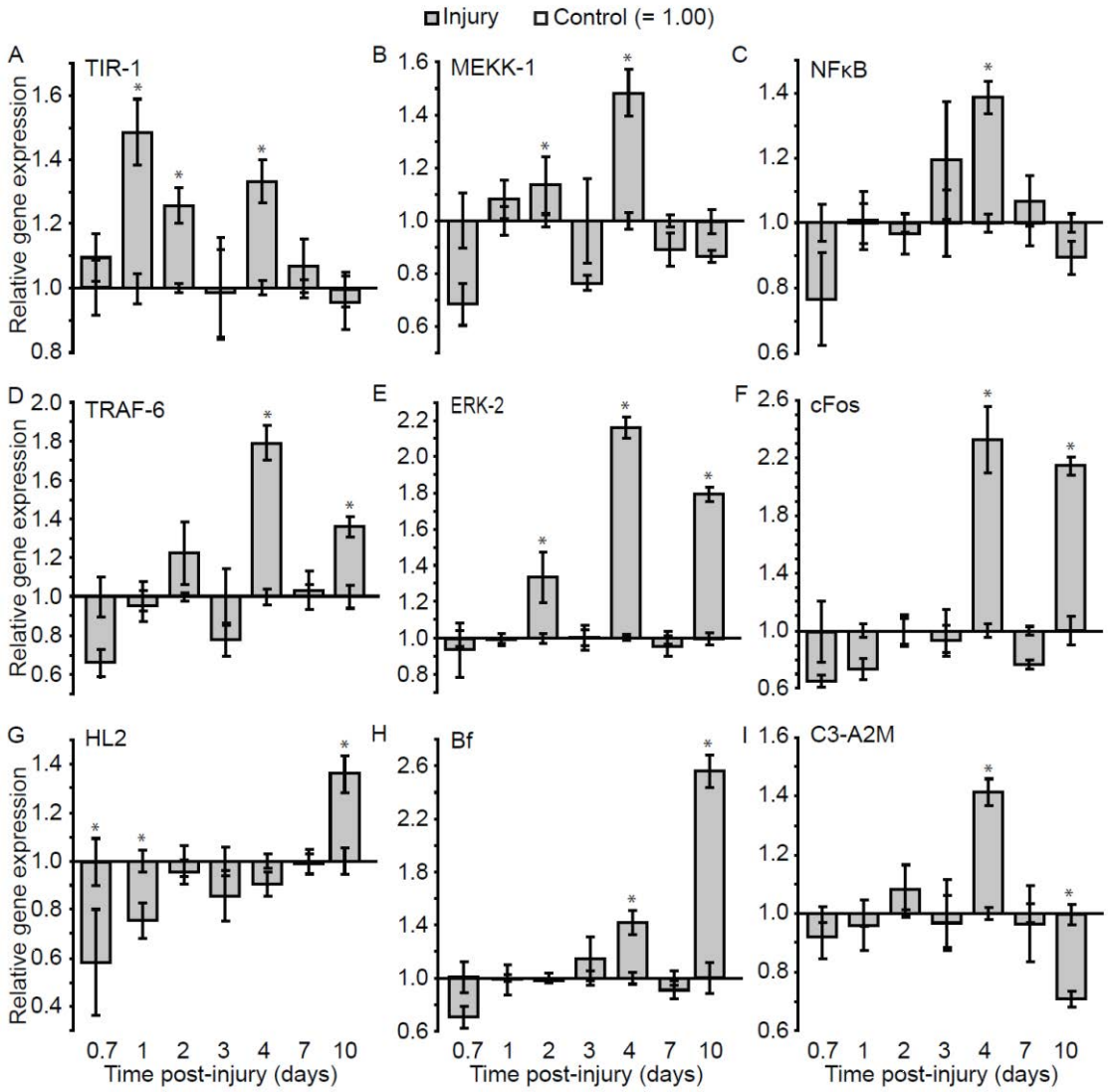


Figure 5.3 – Temporal expression patterns of immune system-related genes in response to injury. A-I) Temporal expression patterns of genes involved in PRR signalling A) Toll-like receptor homologue *TIR-1*, B) *MEKK-1*, C) *ERK2*, D) *NFκB*, E) *TRAF-6*, F) *cFos* and immune effectors G) *HL-2*, H) *Bf* and I) *C3*. All data are relative to control. Asterisk (*) indicates statistical significance of $p < 0.05$.

this time (0.71-fold, $p < 0.01$) (Fig. 5.3H-I). Genes that are potentially involved in the formation of the membrane attack complex were expressed either at similar levels to the control samples or downregulated (*apextrin* downregulated by up to 0.49-fold on Days 1 and 2 ($p < 0.05$) and Days 4 and 7 ($p < 0.01$); *Tx60* downregulated by 0.34-fold on Day 3 ($p < 0.01$); Suppl. Fig. 5.2F-G). Additional statistical details are presented in Supplementary Tables 5.2 and 5.3.

5.4.3 - Bacterial community response following physical damage

Examination of tissues using FISH with general bacterial-specific probes did not show signs of microbial invasion of tissues or exposed skeletal spaces following artificially-induced injury. Aggregates of bacteria were observed within the calicodermal and gastrodermal cell layers that comprise aboral tissues and within ectodermal and

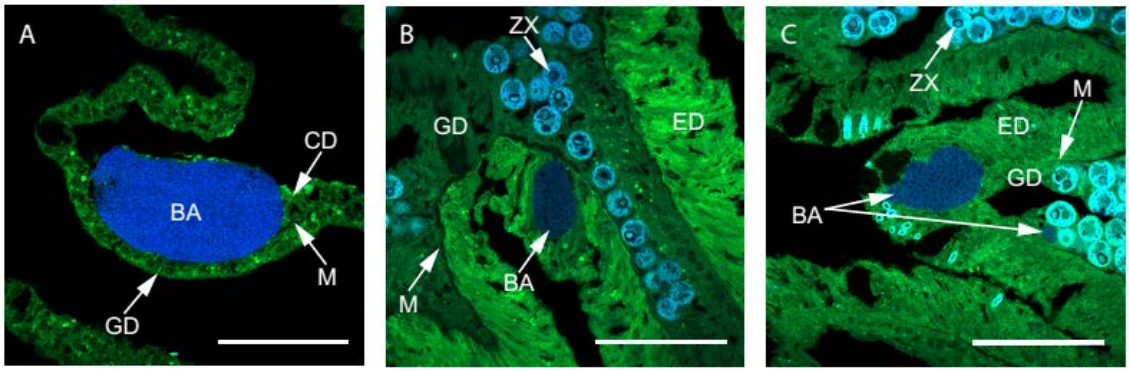


Figure 5.4 – Microscopic analysis of coral-associated bacteria in injured coral. A-C) Localisation of bacterial aggregates in coral tissues following injury: aggregates in A) calicodermis/gastrodermis tissue layers, B) ectodermis and C) both ectodermis and gastrodermis of the ectodermis/gastrodermis tissue layers. Bacteria were visualised using the bacteria-specific probe EUB338. Magnification: 63x. Scale bar: 50 micrometer. Abbreviations: BA: bacterial aggregate, ZX: zooxanthellae, E: ectodermis, G: gastrodermis, M: mesoglea.

gastrodermal cell layers that comprise oral tissues of both healthy and injured corals during FISH examination (Fig. 5.4A-C). Bacterial aggregates were not associated with lesions and were distributed throughout the coral tissues. There were no observable differences in number or size of bacterial aggregations in tissues from injured versus healthy corals.

Amplicon-based 16S rRNA gene amplicon pyrosequencing was used to profile the bacterial community of both healthy and injured corals. Although overall, injury had a significant negative effect on species richness (Chao1 $p < 0.05$) and alpha diversity (Shannon-index $p < 0.05$; Fisher's alpha $p < 0.05$) (both are reduced in injured corals at all time points, except on day 7; Table 5.1), Tukey's HSD post-hoc testing did not reveal any differences between experimental groups at any time point or among sampling time points. Similarly, principal coordinate analysis of the beta diversity of bacterial communities did not reveal any changes as a result of injury or over time (Fig. 5.5A, B). Results of a PERMANOVA confirmed that there were no changes in the coral-associated bacterial communities due to tissue damage ($p = 0.34$), sampling time ($p = 0.07$), or due to damage over time ($p = 0.83$). In addition, I did not observe

Table 5.1 – Overview of alpha diversity metrics of coral-associated bacterial communities.

		Total sequences	Rarefied sequences	OTU	chao1	Shannon-Index	Fisher's alpha
Day 2	Healthy	18982	5963	263	278.16	6.31	56.29
	Injured	17809	5963	174	182.63	5.89	33.55
Day 3	Healthy	13187	5963	171	181.06	6.09	32.84
	Injured	13091	5963	104	123.13	4.94	17.89
Day 4	Healthy	7143	5963	334	347.48	6.72	76.44
	Injured	10490	5963	84	90.50	4.56	13.84
Day 7	Healthy	8922	5963	86	89.21	4.56	14.24
	Injured	14454	5963	111	133.24	5.18	19.36
Day 10	Healthy	10015	5963	206	238.62	5.62	41.39
	Injured	5963	5963	77	83.50	4.84	12.48

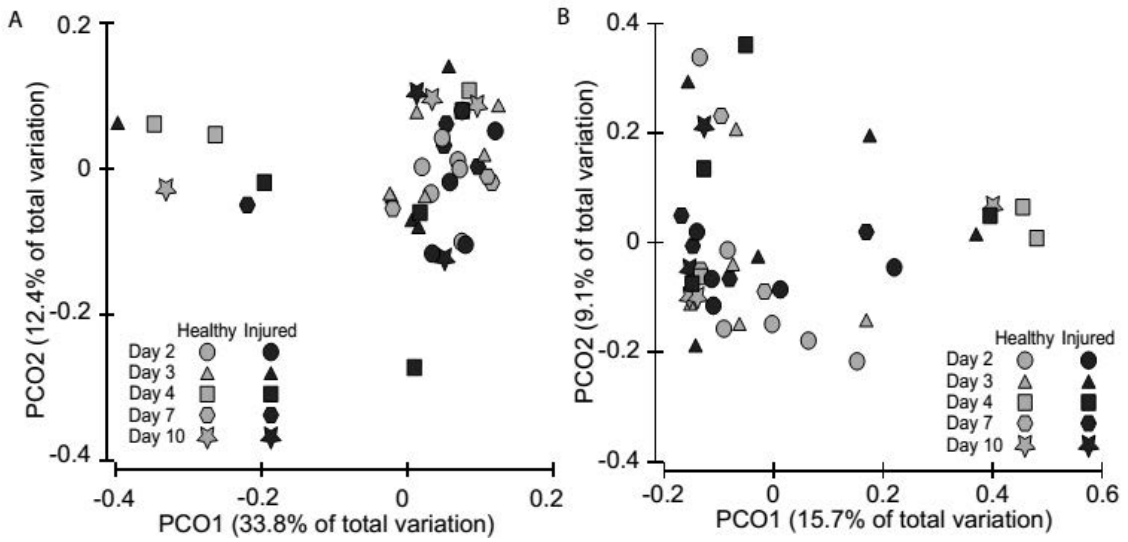


Figure 5.5 – Principal coordinate analysis representing bacterial community profiles of healthy and injured corals based on 16S rRNA amplicon sequencing. Analyses were based on A) weighted and B) unweighted UniFrac distances.

changes in abundance of the species present (damage: p = 0.29; sampling time: p = 0.14; interaction: p = 0.19), nor was a change in bacterial species associated with the corals observed (damage: p = 0.25; sampling time: p = 0.28; interaction: p = 0.76).

5.5 - Discussion

Investigations into wound healing in invertebrates have primarily focused on cellular and molecular wound repair and tissue regeneration processes, with limited attention to the immune responses induced by wounding. Here, I characterise the coral immune response to tissue damage and concurrent effects on coral-associated bacterial communities in the scleractinian coral *Acropora aspera* over a 10-day period.

5.5.1 - The coral immune response to physical damage

Near-complete wound healing and restored *Symbiodinium*-related pigmentation was observed macroscopically 10 days following artificially-induced injuries in *A. aspera*, which contrasts with the slower regeneration times of 80 days to up to 19 months previously reported for most scleractinian corals (Bak and Steward-van Es 1980; Bak, 1983; Meesters and Bak, 1994; van Woesik, 1998; Denis et al., 2013). Many intrinsic and extrinsic factors, e.g. colony morphology, genotype, wound characteristics and environmental factors, are known to affect the regeneration capacity of corals (Henry and Hart, 2005; Denis et al., 2013), providing a possible explanation for the much more rapid regeneration observed in this study.

Our study of the coral immune response to physical damage at the transcriptome level highlights the longer timeframe of the response than is typically characterised in studies of other invertebrates, which have tended to focus on the initial 48 hours post-injury. These studies report that many genes are upregulated in the first 2 days, including those involved in the stress response, metabolism and cell adhesion (Altincicek and Vilcinskas, 2008), as well as in the immune system, such as antimicrobial peptides (AMP) (Erler et al., 2011), membrane attack complex (MAC) proteins, and a NADPH oxidase (Altincicek and Vilcinskas, 2008). In contrast, the transcriptomic response of *A. aspera* occurred in three distinct phases over a 10-day period post-injury: 1) within 48 hours, 2) at 96 hours and 3) at 10 days.

The initial response following injury was primarily TLR pathway-dependent, as indicated by an upregulation of TIR-1 at 24 hours post-injury, followed by increases in expression of several of its downstream signalling proteins, including T*RAF6*, *MEKK-1*, and *ERK2* at 48 hours post-injury. Since AMP production is primarily regulated by the TLR signalling pathway, the observed upregulation of components of this pathway could indicate a significant production of AMPs in response to injury, which is consistent with previous short term (septic) wounding response studies in a range of invertebrates (Fehlbaum et al., 1994; Rodriguez de la Vega et al., 2004; Pujol et al., 2008a; Xu et al., 2010). Although scleractinian corals and other cnidarians are known to possess AMPs (Fraune and Bosch, 2007; Fraune et al., 2011; Vidal-Dupiol et al., 2011a), no AMP has been isolated from species of *Acropora* to date. Upregulation of components in the pathway leading to AMP production suggests the likelihood that *A. aspera* produces this class of molecules, which has an important role in the innate immune system of invertebrates.

I also found evidence of a possible positive feedback loop in the expression of TLR signalling pathway molecules in the first phase of the immune response. A positive feedback loop for TLRs has been described in mice, in which activation of TLR leads to the production of cytokines, which subsequently amplify TLR expression (Gomariz et al., 2005) and potentially its downstream signalling molecules. Upregulation of TLR signalling pathway genes in the early stages of wound healing and a positive feedback loop in expression of these genes enable the coral to respond more rapidly, thereby potentially enhancing the immune response when activated by injury and the detection of MAMPs.

The strongest regulation of genes was observed at 96 hours, and this marks the second phase, involving multiple genes of the TLR signalling pathway (*TIR-1*, *TRAF6*, *MEKK1*, *ERK2*, *cFos* and *NFκB*), as well as immune effector genes that are part of the complement system (*Bf* and *C3*). The third phase occurred 10 days post-injury, when

lesions had mostly healed, and was characterised by increases in expression levels of genes of the TLR pathway (*cFos*, *MEKK-1* and *ERK2*), complement system (*Bf*) and coagulation system (*HL2*). Together, these genes may have been involved in the maintenance of the healthy bacterial community structure, although a role in tissue regeneration and development for *cFos*, *MEKK-1* and *ERK2* is also likely based on their role in cell proliferation.

Expression of several coral genes displayed unexpected patterns in response to physical injury. For example, expression of *MAPK p38,* an important gene implicated in stress and innate immune responses, was downregulated in the second and third phases of the immune response on the transcriptome level. Such downregulation suggests that *MAPK p38* is unlikely to be involved in the wounding response in *A. aspera*. The low to undetectable levels of C-type lectin *CTL-2* in controls, combined with the up to 23.5-fold significantly higher levels of this lectin in injured corals compared to controls from 2 days post-injury onward, indicate that C-type lectin *CTL-2* may play a role (potentially in the complement system) in the wounding-induced immune response or during the establishment of endosymbiosis in regenerating tissues. *Millectin* was consistently downregulated following physical damage, which is in contrast to previous reports showing that injury does not change *millectin* gene expression (Kvennefors et al., 2010b). Consistent with a previous study reporting upregulation of C3 following injury (Kvennefors et al., 2010b), results from my study show a 1.41-fold increase in *C3* expression on Day 4. Factor B (*Bf*), part of the C3 convertase of the alternative complement pathway, which converts C3 into C3a and C3b, was also upregulated 4 days post-injury, confirming a role for this protein in the complement system. However, upregulation of *Bf* was also observed 10 days post-injury, in contrast to the downregulation of *C3*, suggesting an additional, currently unknown, role for *Bf*.

Gene expression patterns for the majority of the genes of interest were variable throughout my 10-day post-injury study period. Such variability has significant implications for the design of studies focused on detecting or characterising invertebrate immune responses, given that the time of sampling determines the extend of the transcriptomic response detected. Sampling at short intervals is required to provide sufficient resolution to gain an appropriate understanding of gene regulation in corals. A study by Schwanhausser *et al*. (2011) found that, on average, approximately 40% of protein expression levels in mammalian cells can be explained by mRNA expression, suggesting that the remaining 60% are regulated by post-transcriptional and post-translational processes. Accordingly, it is possible that immune response proteins in corals could be continuously upregulated following injury and that the

observed fluctuations in differential gene expression might be due to trade-offs among energy investment options, with corals switching transcription off when protein levels are sufficient and only investing in the expression of a gene when the encoded protein levels drop below a certain threshold level.

Upregulation of the proPO system in *A. aspera* in the first 3 days and at 10 days post-injury correlated with the first and third transcriptomic immune response phases, suggesting a positive feedback loop potentially involving components of the TLR or NLR pathway. As vesicles containing melanin and components of the proPO-activating system are rapidly released in response to wounding to seal the lesion by the formation and hardening of a clot (Palmer et al., 2011c), it is unlikely that the proPO system is involved in these processes at the later stage (10 days post-injury) when upregulation was observed. No increase in PO activity was observed at 4 days post-injury, when the most significant transcriptomic responses occurred, further indicating that the proPO system plays other roles in addition to wound sealing in the coral immune response. A recent study found increased levels of PO activity in proliferating tissues of two scleractinian corals (D'Angelo et al., 2012), suggesting that the upregulation in PO activity seen in my study might be correlated with tissue regeneration and wound healing. The second lower peak in PO activity at 10 days is consistent with a role of PO in proliferating tissues, given that wound healing was not complete. Lack up upregulation of PO activity on Days 4 and 7 post-injury suggests that the functioning of this system may be more complex than previously appreciated. For example, the coral may use a feed-back mechanism to regulate the amount of melanin in its proliferating tissues and thus regulate the activation status of the melanisation cascade. In the bumble bee *Bombus terrestris,* complex temporal PO activity patterns after injection with immunostimulants were observed until 14 days after injection (Korner and Schmid-Hempel, 2004). Patterns observed in *A. aspera* could therefore also be the result of normal temporal PO activity dynamics after wounding.

Temporal expression patterns of the non-fluorescent chromoprotein corresponded with those in PO activity. CP exhibits stronger anti-oxidant properties than FPs (Palmer et al., 2009a) and a possible explanation for the correlation between PO activity and CP expression is that *A. aspera* upregulates CP expression to mitigate the adverse effects of PO-produced ROS on its own tissues, possibly in addition to chromoprotein's potential photoprotective role. Together these results suggest a significant role for PO in the longer term immune response and during tissue regeneration following wounding, although the exact role remains to be elucidated.

A role for GFP, but not CFP or RFP, in the wound healing and tissue regeneration process is suggested based on my results. The relative expression of

GFP was upregulated >3 days post-injury, while the typically highly expressed CFP was downregulated >3 days post-injury, and RFP expression and total fluorescence levels remained constant throughout the 10 days following injury. This is in contrast with a recent study, in which CFP and RFP were implicated in coral tissue growth and wound repair in *Acropora pulchra* and *Porites lobata* (D'Angelo et al., 2012). The authors hypothesised that the types of GFP-like proteins in the growth zone could differ between species, which could account for my contrasting results. Upregulation of the non-fluorescent chromoprotein at the lesion site following physical damage potentially protects newly formed tissue and proliferating coral cells from high light conditions (Salih et al., 2000; Dove et al., 2001), and/or *Symbiodinium* cells from damage to their photosystems (Smith et al., 2013). Evidence strongly supports the photoprotective functions of some FPs in corals under high environmental light intensities (Salih et al., 2000; Dove et al., 2001). Contrasting results between my study and previous studies highlight the need for further research into the role of FPs in the wounding response of corals.

Elevated expression levels of *ERK-2* and *cFos* following wounding potentially link these genes with the tissue regeneration process. The regeneration process involves the lesion being sealed, followed by development of cell layers and polyp structures through cell proliferation and differentiation. ERK-2 is a protein kinase that is also activated via growth factor signalling and, in turn, activates the transcription factor complex AP-1, consisting of cFos and cJun, as well as ATF family members (Hess et al., 2004). In contrast to upregulation of *ERK-2* and *cFos*, *cJun* expression remained stable and *ATF4/5* was downregulated. As cFos and cJun form the AP-1 complex, it is unlikely that cJun is not involved in cell proliferation and differentiation during wound healing. Upregulation of cJun protein levels might still have been possible, for example as a result of an increased stability of mRNA transcripts allowing increased protein expression, in the absence of increased gene expression (Wu and Brewer, 2012). In addition, cJun and cFos only form the AP-1 complex whilst being in a phosphorylated state, and a consistent expression level, but increased phosphorylation status could result in an increased AP-1 activity during wound healing (Hess et al., 2004). Therefore a role for cJun in the regeneration process cannot be excluded and further proteomic approaches are required to address these expression patterns.

Coral tissue regeneration requires not only the differentiation and development of host tissues, but also uptake and establishment of symbiosis with endosymbiotic *Symbiodinium*. Previous studies suggest that the immune effectors of the lectin-complement pathway, C3 and millectin, are implicated in the maintenance of *Symbiodinium* endosymbiosis in *Acropora millepora*, in addition to playing a role in the

anti-microbial response (Kvennefors et al., 2008; Kvennefors et al., 2010b). Upregulation of components of the lectin-complement pathway, such as *C3* and *Bf* as well as the lectin *CTL-2,* observed in this study*,* indicate a role for this pathway in the tissue regeneration process that may be related to the establishment of *Symbiodinium* symbiosis in newly differentiated host cells. However, the *millectin* homologue was consistently downregulated in my study until 7 days post-injury. In combination with results of these previous studies, my results suggest that, whereas other components of the lectin-complement pathway may be involved in the establishment of symbiosis, millectin may be involved in the maintenance of the symbiosis. This interpretation is supported by the increases in expression to control levels observed 10 days post-injury, when lesions were almost completely healed and *Symbiodinium* density-based tissue pigmentation returned to normal.

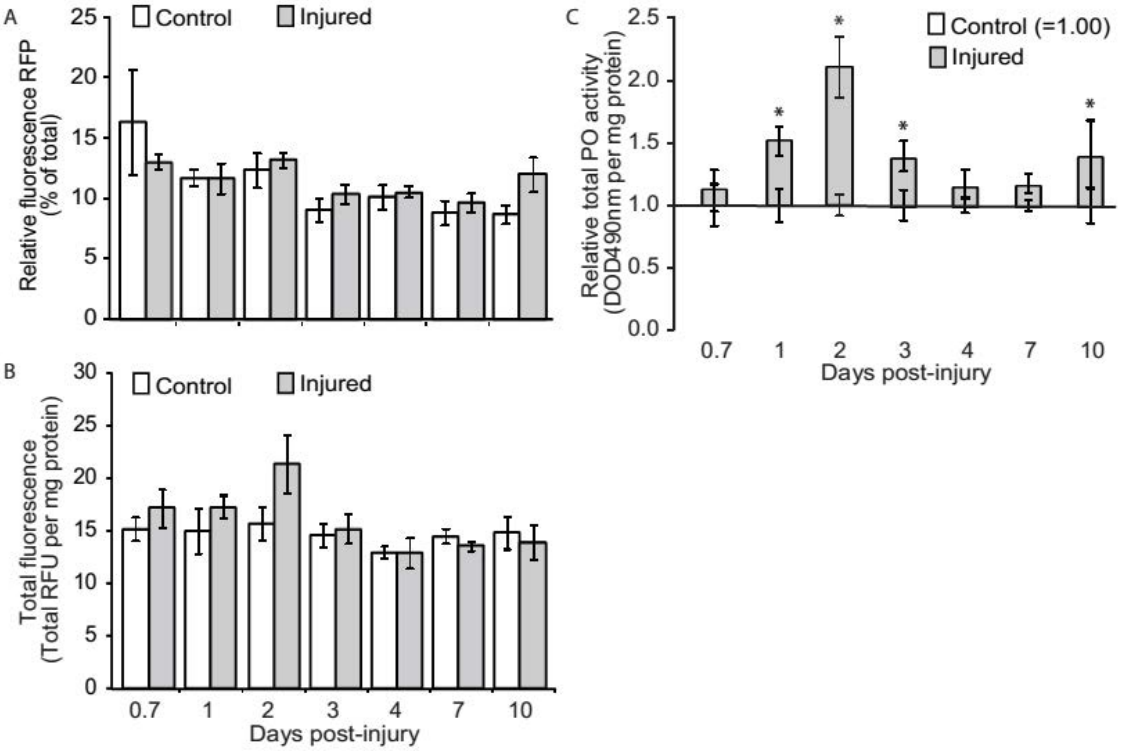
5.5.2 - Bacterial community responses following physical damage

Profiling of the coral-associated bacterial community demonstrated no difference in bacterial community structure associated with injured corals compared to healthy controls, and no community shifts throughout the 10-day experiment as lesions healed. FISH-based approaches also demonstrated no microbial infiltration of the lesion boundary for any tissues sampled throughout the experiment. Although bacterial aggregates were observed to be associated with coral tissues, these aggregates were consistent in number and appearance for tissues derived from both injured and healthy corals. Bacteria play a major role in the health of the coral holobiont and it is therefore highly advantageous for the coral to maintain a healthy beneficial microbial community. Several invertebrates, including cnidarians, have been shown to control the composition of their microbial community through the expression of anti-microbial peptides (AMP) (Fraune and Bosch, 2007; Kounatidis and Ligoxygakis, 2012). The recent isolation of an AMP from *Pocillopora damicornis* (Vidal-Dupiol et al., 2011a) suggests this may also hold true for corals. Further, Toll-like receptors fulfil an essential role in the detection and regulation of the bacterial community composition in the cnidarian *Hydra* (Franzenburg et al., 2012) and are the main regulators of AMP expression. As the potential Toll-like receptor TIR-1 was upregulated in *A. aspera* following injury, I speculate that this coral species is capable of maintaining a healthy bacterial community throughout the repair process of an artificially-inflicted wound via TLR-regulated AMP expression.

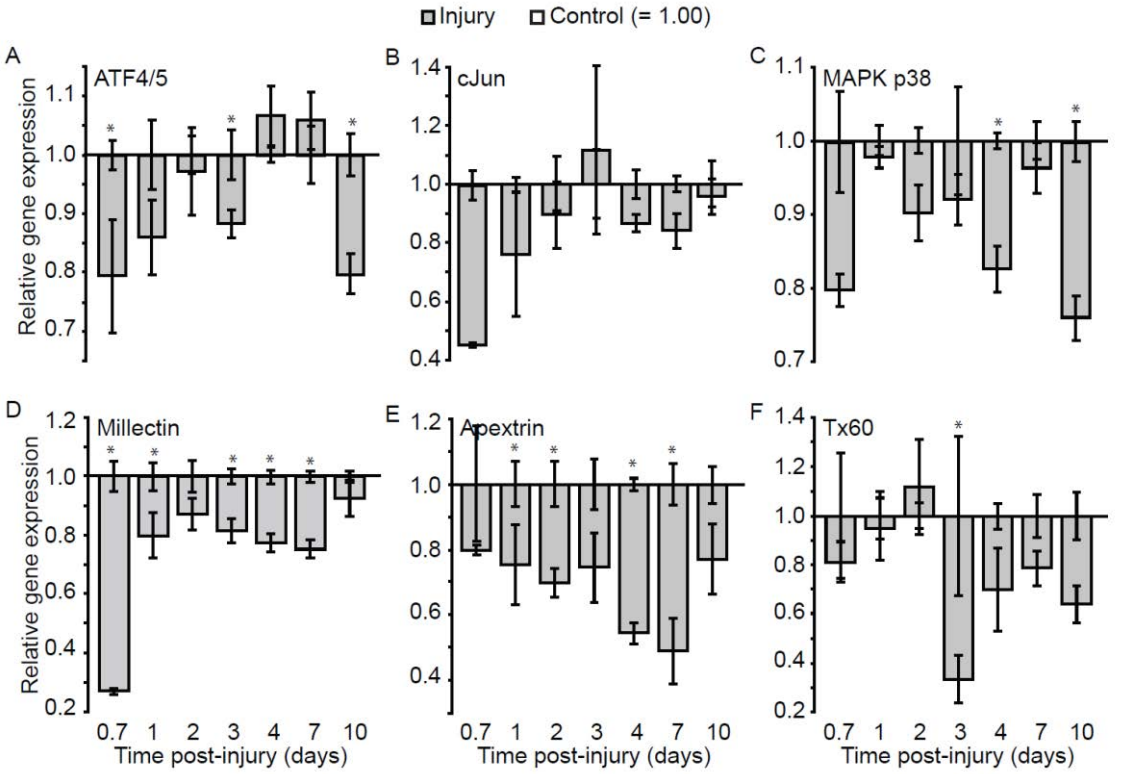
5.6 - Conclusion

In this study, I demonstrated that *A. aspera* exhibits a strong immune response following physical injury, likely preventing bacterial infection and enabling wound healing within 10 days. Physically damaged corals increased the expression of components of the TLR pathway, which potentially regulate the production of AMPs via a positive feedback loop. Elevated AMP production may allow the coral to maintain a healthy bacterial community that simultaneously produces anti-microbial compounds, thereby preventing pathogenic microbes from establishing. Simultaneously, wound repair and tissue regeneration processes commenced, including increased cell proliferation (*ERK2* and its downstream transcription factors) and the establishment and maintenance of symbiosis with *Symbiodinium* in newly formed tissues using the lectin-complement pathway. However, at the lesion front, these highly proliferating cells with their endosymbionts are exposed to high (UV) light radiation intensity and encounter microbes. Corals also increased the expression of photoprotective GFP-like proteins in these tissues, likely to protect the highly proliferating cells and *Symbiodinium* from high light intensity. Overall, the response following injury and during wound repair in *Acropora aspera* is dynamic, consisting of multiple phases, and is characterised by increased expression of a multitude of immune system-related genes, phenoloxidase, as well as several GFP-like proteins over time.

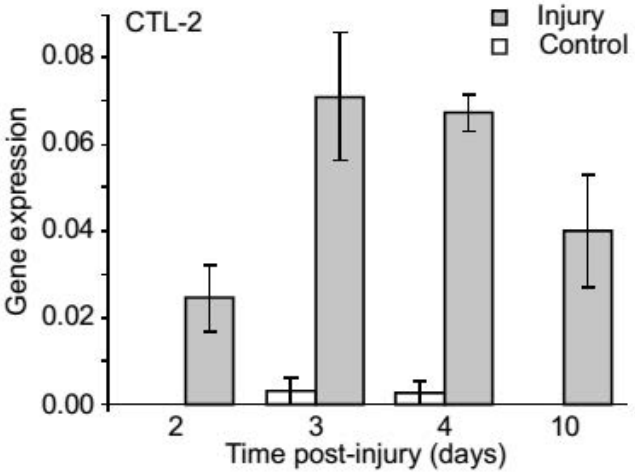
5.7 - Supplementary figures



Supplementary figure 5.1 - Temporal GFP-like protein expression and phenoloxidase activity in response to injury. A-C) Temporal expression patterns of A) red fluorescent protein (RFP), B) total fluorescence and C) total potential phenoloxidase (tpPO) activity. Data of tpPO activity are relative to control. Asterisk (*) indicates statistical significance of $p < 0.05$.



Supplementary Figure 5.2 – Temporal expression patterns of immune system-related genes in response to injury. A-C) Temporal expression patterns genes involved in PRR signalling A) *cJun*, B) *ATF4/5* and C) *MAPK p38* and immune effectors lectins D) *Millectin* and E) *CTL-2*, and the membrane attack complex-domain containing F) *apextrin* and G) *Tx60*. Data are relative to control, except for *CTL2*. Asterisk (*) indicates statistical significance of $p < 0.05$.



Supplementary Figure 5.3 – Temporal expression pattern of the lectin *CTL-2*.

5.8 - Supplementary tables

Supplementary table 5.1 – Overview of time points analysed using respective techniques and assays. Numbers listed represent the number of samples analysed.

Time point	Health status	Histology	Fluorescence *in situ* hybridisation	Gene expression	Phenoloxidase activity	GFP-like protein expression	16S rRNA amplicon
16 hrs	Healthy			6	6	6	
	Injured			6	6	6	
24 hrs	Healthy	6	6	6	6	6	
	Injured	6	6	6	6	6	
48 hrs	Healthy			6	6	6	6
	Injured			6	6	6	5
72 hrs	Healthy			6	6	6	6
	Injured			6	6	6	5
96 hrs	Healthy	6	6	6	6	6	3
	Injured	4	4	6	6	6	4
168 hrs	Healthy	6		6	6	6	3
	Injured	6		6	6	6	4
240 hrs	Healthy	3	3	6	6	6	3
	Injured	5	5	4	4	4	2

Supplementary Table 5.2 – Overview of statistical analysis of main effects. Statistically significant differences are indicated in ***bold*** for the main General Linear Model results.

	SS	df	MS	F	P	SS	df	MS	F	P
	Total potential phenoloxidase activity					*Phenoloxidase activity*				
Intercept	**228.5555**	**1**	**228.5555**	**3937.013**	**0.000000**	**233.2463**	**1**	**233.2463**	**3776.269**	**0.000000**
Damage	**1.8386**	**1**	**1.8386**	**31.672**	**0.000000**	**1.9626**	**1**	**1.9626**	**31.774**	**0.000000**
Time	**2.4339**	**6**	**0.4056**	**6.988**	**0.000010**	**2.5880**	**6**	**0.4313**	**6.983**	**0.000011**
Interaction	**0.8120**	**6**	**0.1353**	**2.331**	**0.042894**	0.6779	6	0.1130	1.829	0.107911
Error	3.5993	62	0.0581			3.8295	62	0.0618		
	Chromoprotein					*Cyan fluorescent protein*				
Intercept	**940.1668**	**1**	**940.1668**	**8431.133**	**0.000000**	**47.33421**	**1**	**47.33421**	**38661.38**	**0.000000**
Damage	**4.1612**	**6**	**0.6935**	**6.219**	**0.000039**	**0.04391**	**1**	**0.04391**	**35.87**	**0.000000**
Time	**9.6986**	**1**	**9.6986**	**86.974**	**0.000000**	**0.03421**	**6**	**0.00570**	**4.66**	**0.000573**
Interaction	0.3307	6	0.0551	0.494	0.810213	**0.03406**	**6**	**0.00568**	**4.64**	**0.000596**
Error	6.8022	61	0.1115			0.07591	62	0.00122		
	Green fluorescent protein					*Red fluorescent protein*				
Intercept	**0.375859**	**1**	**0.375859**	**390.7753**	**0.000000**	**0.900137**	**1**	**0.900137**	**1314.172**	**0.000000**
Damage	**0.037236**	**1**	**0.037236**	**38.7142**	**0.000000**	0.000404	1	0.000404	0.590	0.445379
Time	**0.021377**	**6**	**0.003563**	**3.7043**	**0.003281**	**0.019043**	**6**	**0.003174**	**4.634**	**0.000598**
Interaction	**0.018558**	**6**	**0.003093**	**3.2158**	**0.008167**	0.004422	6	0.000737	1.076	0.386561
Error	0.059633	62	0.000962			0.042467	62	0.000685		
	Apextrin					*ATF4/5*				
Intercept	**1.37268**	**1**	**1.372684**	**16.28036**	**0.000157**	**101.0955**	**1**	**101.0955**	**3394.316**	**0.000000**
Damage	**2.86215**	**1**	**2.862152**	**33.94580**	**0.000000**	**0.2767**	**1**	**0.2767**	**9.291**	**0.003422**
Time	**15.22769**	**6**	**2.537948**	**30.10066**	**0.000000**	**15.8002**	**6**	**2.6334**	**88.416**	**0.000000**
Interaction	0.68943	6	0.114905	1.36280	0.244348	0.3516	6	0.0586	1.967	0.084578
Error	5.05892	60	0.084315			1.7870	60	0.0298		
	Bf					*C3*				
Intercept	**3.791646**	**1**	**3.791646**	**1696.824**	**0.000000**	**40.41303**	**1**	**40.41303**	**1506.565**	**0.000000**
Damage	**0.012564**	**1**	**0.012564**	**5.623**	**0.021015**	0.00638	1	0.00638	0.238	0.627595
Time	**0.342539**	**6**	**0.057090**	**25.549**	**0.000000**	**4.72979**	**6**	**0.78830**	**29.387**	**0.000000**
Interaction	**0.091375**	**6**	**0.015229**	**6.815**	**0.000016**	**0.65452**	**6**	**0.10909**	**4.067**	**0.001739**
Error	0.131839	59	0.002235			1.60948	60	0.02682		
	cFos					*cJun*				
Intercept	**111.2192**	**1**	**111.2192**	**1492.499**	**0.000000**	0.66743	1	0.667427	3.85930	0.054353
Damage	0.0129	1	0.0129	0.173	0.678678	0.50094	1	0.500936	2.89659	0.094216
Time	**3.7961**	**6**	**0.6327**	**8.490**	**0.000001**	**16.08315**	**6**	**2.680524**	**15.49975**	**0.000000**
Interaction	**1.2804**	**6**	**0.2134**	**2.864**	**0.016086**	0.56079	6	0.093465	0.54045	0.775225
Error	4.4711	60	0.0745			9.85757	57	0.172940		

	SS	df	MS	F	P	SS	df	MS	F	P
	ERK-2					*HL2*				
Intercept	**36.35897**	**1**	**36.35897**	**2568.694**	**0.000000**	**47.16379**	**1**	**47.16379**	**1353.176**	**0.000000**
Damage	**0.76138**	**1**	**0.76138**	**53.790**	**0.000000**	**0.30612**	**1**	**0.30612**	**8.783**	**0.004355**
Time	**17.60303**	**6**	**2.93384**	**207.270**	**0.000000**	**26.44008**	**6**	**4.40668**	**126.432**	**0.000000**
Interaction	**1.83722**	**6**	**0.30620**	**21.633**	**0.000000**	**0.78042**	**6**	**0.13007**	**3.732**	**0.003211**
Error	0.84928	60	0.01415			2.09125	60	0.03485		
	MAPK p38					*MEKK-1*				
Intercept	**186.5574**	**1**	**186.5574**	**5427.857**	**0.000000**	**11.32840**	**1**	**11.32840**	**237.7840**	**0.000000**
Damage	**0.5136**	**1**	**0.5136**	**14.942**	**0.000275**	0.00212	1	0.00212	0.0446	0.833519
Time	**96.2009**	**6**	**16.0335**	**466.492**	**0.000000**	**11.33938**	**6**	**1.88990**	**39.6691**	**0.000000**
Interaction	0.1415	6	0.0236	0.686	0.661353	**1.09338**	**6**	**0.18223**	**3.8250**	**0.002705**
Error	2.0622	60	0.0344			2.85849	60	0.04764		
	Millectin					*NF-κB*				
Intercept	**0.33456**	**1**	**0.334557**	**19.1654**	**0.000049**	**19.55682**	**1**	**19.55682**	**673.0128**	**0.000000**
Damage	**2.13462**	**1**	**2.134617**	**122.2838**	**0.000000**	0.00520	1	0.00520	0.1789	0.673848
Time	**10.80196**	**6**	**1.800326**	**103.1336**	**0.000000**	**3.18862**	**6**	**0.53144**	**18.2884**	**0.000000**
Interaction	**1.45634**	**6**	**0.242724**	**13.9047**	**0.000000**	**0.46975**	**6**	**0.07829**	**2.6943**	**0.022068**
Error	1.04738	60	0.017456			1.74352	60	0.02906		
	TIR-1					*TRAF6*				
Intercept	**23.67410**	**1**	**23.67410**	**751.2469**	**0.000000**	**21.39026**	**1**	**21.39026**	**416.8912**	**0.000000**
Damage	**0.31185**	**1**	**0.31185**	**9.8958**	**0.002578**	0.05284	1	0.05284	1.0299	0.314262
Time	**11.26648**	**6**	**1.87775**	**59.5863**	**0.000000**	**49.61067**	**6**	**8.26844**	**161.1501**	**0.000000**
Interaction	**0.43098**	**6**	**0.07183**	**2.2794**	**0.047715**	**1.51729**	**6**	**0.25288**	**4.9286**	**0.000371**
Error	1.89078	60	0.03151			3.07854	60	0.05131		
	Tx60									
Intercept	**300.2254**	**1**	**300.2254**	**1426.784**	**0.000000**					
Damage	**1.7587**	**1**	**1.7587**	**8.358**	**0.005367**					
Time	**15.6034**	**6**	**2.6006**	**12.359**	**0.000000**					
Interaction	1.9742	6	0.3290	1.564	0.173853					
Error	12.4148	59	0.2104							

Supplementary table 5.3 – Overview of statistical analysis outcome of Least Significant Difference post-hoc tests. Statistically significant differences between control and injured corals at each day post-injury are given. NS indicates no significant differences were found.

	Day						
	0.7	1	2	3	4	7	10
Cyan FP	NS	NS	NS	>0.01	NS	>0.01	>0.01
Green FP	NS	NS	NS	>0.01	0.03	>0.01	>0.01
Red FP	NS	NS	NS	NS	NS	NS	NS
Chromoprotein	0.04	>0.01	>0.01	>0.01	>0.01	>0.01	>0.01
PO activity	NS	>0.01	>0.01	>0.01	NS	NS	>0.05
tpPO activity	NS	>0.01	>0.01	>0.01	NS	NS	>0.05
Apextrin	NS	>0.05	>0.05	NS	>0.01	>0.01	NS
ATF4/5	0.04	NS	NS	0.02	NS	NS	0.01
Bf	NS	NS	NS	NS	>0.05	NS	>0.01
C3	NS	NS	NS	NS	>0.01	NS	>0.01
cFos	NS	NS	NS	NS	0.02	NS	>0.01
cJun	NS	NS	NS	NS	NS	NS	NS
ERK-2	NS	NS	>0.01	NS	>0.01	NS	>0.01
HL2	>0.01	>0.05	NS	NS	NS	NS	>0.05
MAPK p38	NS	NS	NS	NS	0.04	NS	0.01
MEKK-1	NS	NS	0.03	NS	>0.01	NS	NS
Millectin	>0.01	>0.01	NS	>0.01	>0.01	>0.01	NS
NFκB	NS	NS	NS	NS	>0.01	NS	NS
TIR1	NS	>0.01	0.03	NS	>0.01	NS	NS
TRAF-6	NS	NS	NS	NS	>0.01	NS	0.04
Tx60	NS	NS	NS	>0.01	NS	NS	NS

Chapter 6

Immunological, physiological and microbial community responses of the holobiont of a moderately heat-tolerant coral to a pathogen under elevated seawater temperatures

6.1 - Abstract

Global increases in coral disease prevalence have been linked to ocean warming, which is causing shifts in coral-associated bacterial communities, increasing pathogen virulence and compromising the coral host immune system. To explore interactive effects of elevated seawater temperatures and bacteria on the coral holobiont, I challenged fragments of the coral *Montipora aequituberculata* with the coral pathogen *Vibrio coralliilyticus* and the commensal bacterium *Oceanospirillales* sp. under ambient (27°C) and elevated (29.5°C and 32°C) seawater temperatures for 22 days. Although no visual signs of bleaching were present in any of the treatments and there was an almost complete absence of disease development, significant responses were detected in three members of the coral holobiont. The photochemical efficiency of *Symbiodinium* was significantly reduced at 32°C, and further reduced in *V. coralliilyticus*-challenged corals. Activity of phenoloxidase, a component of the invertebrate host innate immune response, was significantly diminished in corals challenged by *V. coralliilyticus* at 32°C. In contrast, no differences in photochemical efficiency or phenoloxidase activity were detected between controls and corals challenged with *Oceanospirillales*. Analyses of the full transcriptomic response of both the coral host and *Symbiodinium* revealed a strong response under high temperature conditions. The coral upregulated genes in a range of cellular processes, in particular the innate immune system, including molecular chaperones, lectins and genes involved in Toll-like receptor signalling, complement system, phagocytosis, immune cell activation and function, cytokine production and availability, as well as regulatory negative feedback loops. Shifts in the coral-associated bacterial community detected at the end of the study were correlated with elevated seawater temperatures, whereas bacterial challenges had no effect. Overall, I show that the *M. aequituberculata* holobiont is moderately heat tolerant and exhibits a strong immune response involving a suite of immune mechanisms to combat potentially pathogenic bacteria under elevated seawater temperature stress, thereby preventing the development of disease.

6.2 - Introduction

The prevalence of coral disease epizootics is on the rise worldwide (Harvell et al., 2007; Sokolow, 2009), largely as a consequence of increasing anthropogenic disturbances, including global climate change-induced elevated seawater temperatures (Sokolow, 2009) and reduced water quality (Sutherland et al., 2011; Kaczmarsky and Richardson, 2011; Pollock et al., 2014). Understanding why interactions among coral hosts, pathogens and the environment are changing and how such changes are driving the increasing occurrence of disease is critical for managing the health and resilience of coral reefs. Intricate inter-kingdom symbioses within the coral holobiont (*sensu* Rohwer et al. (2002)) among the coral host and a range of microbial symbionts, including bacteria, archaea, viruses, fungi, and the endosymbiotic alga *Symbiodinium,* pose further challenges for understanding and managing coral health. This study aims to examine the immune response of the coral host, concurrently with changes in *Symbiodinium* and associated bacterial communities, in response to both a bacterial challenge and temperature stress to begin unravelling factors underpinning coral health.

Environmental disturbances like elevated seawater temperatures can have significant impacts on the dynamic microbial communities that govern coral holobiont health. Examples of ways in which coral-associated bacteria contribute to holobiont health include nitrogen fixation (Lema et al., 2012), sulphur-cycling (Raina et al., 2009), production of antimicrobial compounds (Nissimov et al., 2009; Shnit-Orland and Kushmaro, 2009; Shnit-Orland et al., 2012; Kvennefors et al., 2012), and the exclusion of harmful bacteria through occupation of available microbial niches (Rohwer et al., 2002). Elevated seawater temperatures have been shown to cause shifts in coral-associated bacterial communities towards potentially more pathogenic microbes (Ritchie, 2006; Bourne et al., 2008; Mouchka et al., 2010; Littman et al., 2011; Witt et al., 2011). Microbial community shifts in response to environmental disturbances may also depend on the type of *Symbiodinium* harboured by the coral host (Littman et al., 2010). When coupled with an increase in pathogen virulence at elevated temperatures (Sussman et al., 2008; Vidal-Dupiol et al., 2011b), microbial shifts may enhance the potential for disease. Indeed, the prevalence of many coral diseases is significantly higher in summer when seawater temperatures are above average or during warm temperature anomalies (Willis et al., 2004; Bruno et al., 2007), but whether this is caused by changes in the bacterial communities or their virulence has not been demonstrated for most coral diseases. In one group of coral diseases known as white

syndromes, the gram-negative bacterium *Vibrio coralliilyticus* has been implicated as a causative agent (Sussman et al., 2008; Ushijima et al., 2014). The virulence of this bacterium is highly temperature-dependent (Ben-Haim et al., 2003a; Ben-Haim et al., 2003b; Kimes et al., 2012) and its virulence factors attack both the coral and *Symbiodinium* cells (Sussman et al., 2009). Such interactions among host-microbe partners in the holobiont highlight that a thorough understanding of the impact of environmental factors on disease incidence in corals is unlikely unless the responses of individual members of the coral holobiont are assessed.

Corals possess a range of innate immune and stress response mechanisms for defence against biotic and abiotic disturbances. Genetic studies have discovered Toll-like receptors (TLR) and their downstream signalling molecules in various coral species, and functional studies have revealed that this pathway is involved in both the response to wounding (Chapter 4), bacteria and larval settlement and metamorphosis (Miller et al., 2007a; Shinzato et al., 2011; Siboni et al., 2012; Vidal-Dupiol et al., 2014). TLR signalling is crucial for the initiation of a pro-inflammatory response, as well as the regulation and maintenance of healthy associated bacterial communities via anti-microbial peptides (AMP) in the cnidarian *Hydra* (Fraune and Bosch, 2007; Fraune et al., 2010; Franzenburg et al., 2012; Franzenburg et al., 2013). Whether AMPs have a similar function in corals remains to be determined, but they have recently been implicated in the immune response of the coral *Pocillopora damicornis* to *V. coralliilyticus* (Vidal-Dupiol et al., 2011a).

Other immune mechanisms employed by corals include the lectin-complement system and the prophenoloxidase (proPO)-activating system. The lectin-complement system is involved in the immune response against bacteria (Brown et al., 2013), wounding response (chapter 4), allorecognition, and potentially in the maintenance of the coral-*Symbiodinium* symbiosis (Kvennefors et al., 2008; Kvennefors et al., 2010b). The proPO-activating system is induced in response to immune elicitors (Palmer et al., 2011b), pathogens (Mydlarz et al., 2008) and injury (Chapter 5), but has also been implicated in photoprotection (Palmer et al., 2010). Similarly, green fluorescent protein (GFP)-like proteins exhibit photoprotective functions (Salih et al., 2000; Dove et al., 2001; Dove et al., 2008; D'Angelo et al., 2012; Smith et al., 2013), as well as antioxidant properties (Bou-Abdallah et al., 2006; Palmer et al., 2009a). To prevent damage to coral tissues, corals use primarily antioxidant enzymes (Palmer et al., 2011b), and potentially GFP-like proteins, for the neutralisation of reactive oxygen and nitrogen species produced by various immune mechanisms, such as PO and the oxidative burst, to eliminate microbes. The oxidative burst is induced following the phagocytosis of microbes or cellular debris by immune cells, which are activated upon

pathogen exposure and physical damage, and play a role in the response to white band disease in *Acropora cervicornis* (Libro et al., 2013) (a detailed review of coral immune mechanisms can be found in Section 1.2 and 1.3 and Appendix A).

Elevated seawater temperatures are known to have severe effects on many physiological processes in corals (Desalvo et al., 2008; Rodriguez-Lanetty et al., 2009; Voolstra et al., 2009; DeSalvo et al., 2010; Leggat et al., 2011; Bellantuono et al., 2012; Roth and Deheyn, 2013; Kenkel et al., 2013), including several metabolic functions, calcification, fluorescence, apoptosis, antioxidant response, and the immune system; although the extent of the effect depends on the heat tolerance of the coral species, its acclimatisation potential and symbiont type (Bellantuono et al., 2011; Yuyama et al., 2012; Kenkel et al., 2013). In addition, corals respond to stress by increasing the expression of heat shock proteins (HSP) (Sharp et al., 1997; Fang et al., 1997; Tom et al., 1999; Downs et al., 2000; Wiens et al., 2000; Hashimoto et al., 2004; Chow et al., 2009; Rodriguez-Lanetty et al., 2009; Nakamura et al., 2011; Leggat et al., 2011; Meyer et al., 2011), which also play a role in the immune response to pathogens in corals (Brown et al., 2013) and other marine invertebrates (Sung et al., 2008; Baruah et al., 2011). Heat and light stress also cause reductions in phytopigments (Strychar and Sammarco, 2012) and damage to photosystems of *Symbiodinium,* resulting in the generation of cell-damaging reactive oxygen species (ROS). Prolonged heat stress can therefore cause coral bleaching, the loss of the endosymbiotic alga *Symbiodinium* from coral tissues, which may ultimately result in colony mortality (reviewed in Weis (2008)). Clearly, the health of corals depends on the efficient functioning of all partners within the coral holobiont and it is paramount to investigate how each of these partners is affected under environmental stress.

In this study, I examined the holobiont response (coral host, *Symbiodinium* and coral-associated bacterial communities) to a bacterial challenge by the known coral pathogen *V. coralliilyticus* under elevated seawater temperatures. To identify and characterise holobiont responses before irreversible damage occurs, I used the moderately heat-tolerant coral *Montipora aequituberculata* (Marshall and Baird, 2000). The study population had also been the subject of recurrent outbreaks of white syndromes and atramentous necrosis in spring-summer seasons since at least 2001 (Jones et al., 2004; Anthony et al., 2008b; Haapkylä et al., 2011), which were linked to *V. coralliilyticus* (Sussman et al., 2008). Here I assess the transcriptomic response of the coral and its endosymbiont *Symbiodinium*, the photosynthetic capacity of its endosymbiont, together with several well-characterised immune parameters and the structure of bacterial assemblages associated with the coral, to a bacterial challenge under heat stress.

6.3 - Material & Methods

6.3.1 - Collection of experimental corals

Large fragments of the scleractinian coral *Montipora aequituberculata* were collected from the reefs around Magnetic Island, Great Barrier Reef, Queensland, Australia (19.1333° S, 146.8333° E) in September 2012 and transferred to 1000-litre flow-through tanks at the Australian Institute of Marine Science (AIMS), Townsville, Queensland, Australia. Corals were allowed to recover and were monitored for signs of disease for 7 days, prior to fragmentation into 567 fragments of approximately 20 cm^2 using surgical bone cutters. Fragments were attached to large ceramic coral frag plugs (Oceans Wonders LLC, USA) using Quick Fix Supa Glue gel (Selley's Pty Ltd, Australia). Corals were kept in the flow-through system for 2 hours to wash off excess mucus and potential residual chemicals from the glue. Fragments were then rinsed in ultra-filtered seawater (0.04 micron) prior to placement in racks in experimental aquaria. Coral fragments were monitored daily for a 14 day acclimation period at 27°C, prior to commencement of heat stress treatments and bacterial challenges.

6.3.2 - Preparation, isolation and identification of bacteria for challenge treatments

Bacteria used in this experiment were: 1) non-pathogenic *Oceanospirillales* sp. strain S47 (control for bacterial addition), and 2) the coral pathogen *Vibrio coralliilyticus* strain P1 (LMG 23696) (Sussman et al., 2008). Bacteria were streaked onto Difco Marine Agar (BD, USA) and cultured in Difco Marine Broth medium (BD, USA) under aerobic conditions at 170 rpm at 27°C. *V. coralliilyticus* cultures were started from glycerol stocks stored at -80°C since September 2003, which had been derived from samples that had been collected from Magnetic Island.

To establish cultures of the non-pathogenic bacterium, two healthy colonies of the coral *Acropora millepora* were collected from Pelorus Island, Great Barrier Reef (18°33'S; 146°29'E) and maintained in aquaria at AIMS. Coral fragments (approximately 30 mm in length) were collected from each colony and washed in sterile artificial seawater (ASW) to remove loosely attached microbes. Tissue slurries were produced by airbrushing (80 lb/in^2) each coral fragment into 5 ml of ASW to remove coral tissues and associated microbes. The tissue slurries were homogenized to break down tissue clumps, and a dilution series was plated immediately on modified minimal marine agar (1% bacteriological agar, 25 g of NaCl, 0.7 g of KCl, 0.05 g of KH_2PO_4, 1 g of NH_4NO_3, 1 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $MgCl_2 \cdot H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.005 g of FeEDTA, 1 g of Tris, 5 g of sodium succinate, 1.35 g of glucose in 1 L of distilled

water). After 2 days of incubation at 28°C, single bacterial colonies were transferred into Difco Marine Broth (BD, USA) and grown overnight in a shaking incubator. Liquid cultures were re-plated on minimal marine agar; the procedure was repeated until pure isolates were obtained. The near complete 16S rRNA gene of strain S47 was PCR amplified with the bacterial specific primers 63F and 1387R (Marchesi et al., 1998). This strain of *Oceanospirillales* was selected based on its ubiquitous association with benthic invertebrates and more particularly reef-building corals (Bourne et al., 2013).

6.3.3 - Experimental set-up

Coral fragments were randomly assigned to one of 27 experimental aquaria (n = 21 fragments per aquarium). Each aquarium had a capacity of 34 litres and was filled with 32 litres of ultra-filtered seawater in an air-conditioned room held at 24°C at AIMS. Ultra-filtered seawater was provided by the National Sea Simulator at AIMS and generated using hollow fibre membranes with a nominal pore size of 0.04 µm and absolute pore size of 0.1µm. Seawater was aerated and water movement was maintained using an AquaClear 50 powerhead (Rolf C. Hagen Inc., Italy) at its full capacity of 1000 litres per hour. Aquaria were maintained as closed systems, with each aquarium placed in a fresh water-filled white 76-litre plastic tank and 20% of total seawater volume was manually changed every 3 days (starting on Day 2). Seawater salinity was monitored daily and adjusted to 35 parts per thousand (ppt) if required. Seawater temperatures were controlled using calibrated 100-watts aquarium heaters (JEBO, Guangdong Zhenhua Electric Appliance Co. Ltd., China) placed in the 76-litre fresh water-filled tank and heated fresh water was circulated at a 300 litres per hour flow rate using an AquaClear 30 powerhead (Rolf C. Hagen Inc, Italy) to ensure equal distribution. Each aquarium was illuminated with four 22-inch 55-watts compact ultra actinic 420 nm fluorescent lights (Catalina Aquarium, USA) yielding an irradiance of 100.7 ± 10.6 µmol photons m^{-2} s^{-1} set at a 12h light : 12h dark photoperiod. Irradiance was measured using a LI-1400 data logger (LI-COR Biosciences, USA) at the surface.

6.3.4 - Experimental design

The 27 aquaria, each containing 21 coral fragments of *M. aequituberculata,* were randomly assigned to 9 treatments, comprising all combinations of 3 temperature treatments (27°C, 29.5°C, 32°C) and 3 bacterial treatments (no bacteria control, *Oceanospirallales* S47*, V. corallilyticus*) (Figure 6.1). Following the 14-day acclimation period at 27°C, seawater temperatures were maintained at 27°C (26.9 ± 0.063) (control) or gradually increased by 0.5°C every 24 hours, starting on day 0, until target seawater temperatures were reached: 29.5°C (29.5 ± 0.05) (medium heat stress; Day

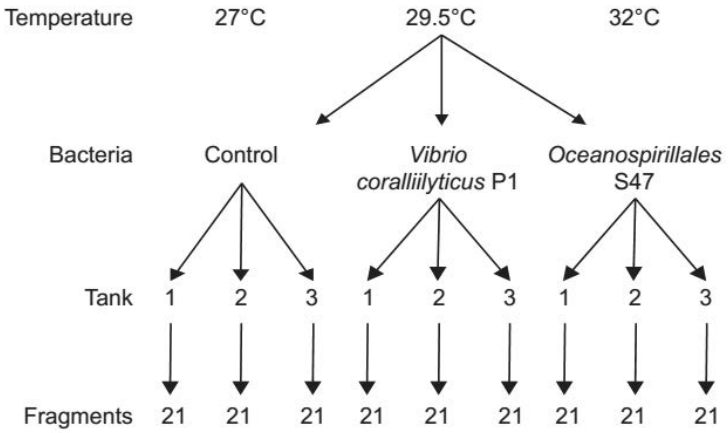


Figure 6.1 – Schematic overview of the experimental set-up. The set-up consisted of 27 aquaria (9 treatment combinations with 3 aquaria per treatment). Seawater temperatures remained constant (27°C) or were increased at a rate of 0.5°C per day until target temperatures were reached (29.5°C or 32°C) starting on Day 0. Every 3 days corals were challenged with bacteria (*Oceanospirillales* S47; *Vibrio coralliilyticus* P1) or no bacteria were added (control). Twenty-four hours after each bacterial challenge, 3 coral fragments were sampled per tank.

4) or 32°C (32 ± 0.12) (high heat stress; Day 9). Temperatures were maintained until the end of the experiment (Day 22) and monitored daily using a digital thermometer.

Corals were challenged with bacteria every 3 days, starting on Day 0. Bacteria were prepared, washed and resuspended in ultra-filtered seawater and spun at 2000 rpm in an Allegra X15R centrifuge (Beckman Coulter, USA) for 8 minutes. Resuspended active bacteria were counted using a Helber Bacteria Counting Chamber (ProSciTech, Australia) and added to aquaria. As a control for the bacterial addition, equal volumes of ultra-filtered seawater were added. Final bacterial concentrations were $1x10^5$ per ml of seawater on Days 0, 3, 6, 9, 12, and $1x10^6$ per ml of seawater on Days 15, 18 and 21. Powerheads were switched off for 4 hours after each addition to allow bacterial settlement.

Coral fragments (n = 3) were randomly sampled from each tank 24 hours following the addition of bacteria (Days 1, 4, 7, 10, 13, 16 and 22). Each sample was fragmented into three pieces, snap frozen in liquid nitrogen and stored at -80°C or stored in 4% para-formaldehyde-ultra-filtered seawater solution for histological analyses.

6.3.5 - Photography and microscopy

Coral fragments were visually monitored for signs of bleaching and disease daily. Every 3 days, photographs were taken of 6 coral fragments from each tank, which were also analysed for photochemical efficiency using a Panasonic TZ1 camera in a marine case (Panasonic Corporation, Japan). Photomicrographs of apparent signs

of disease were taken using a Leica DFC500 microscope camera (Leica Microsystems, Wetzlar, Germany).

6.3.6 - Photosynthetic efficiency

Maximum (F_v/F_m) and effective ($\Delta F/Fm$) quantum yields of photosystem II of the endosymbiotic *Symbiodinium* were measured using a pulse amplitude modulation (PAM) fluorometer (mini-PAM; Walz GmbH, Germany). Measurements were obtained at 8 mm from the coral tissue (controlled via a rubber spacer) by placing a 6 mm fibre-optic probe perpendicular to the surface of the coral. Maximum quantum yields were obtained at night following 2 hours of dark adaptation, whereas effective quantum yields were measured at noon.

6.3.7 - Protein and enzyme assays

Frozen coral fragments were thawed and tissue removed using an airbrush with 6 ml of autoclaved ultra-filtered seawater over ice. Tissue slurries were homogenised using a T10 basic homogenizer (IKA, Germany) for 30s and stored at -80°C. Protein concentrations were determined using the BIO-RAD DC protein assay (BIO-RAD, USA) according to the manufacturer's recommendations and absorbances were read at 750 nm. Phenoloxidase (PO) and total potential prophenoloxidase (tpPO) activities were analysed according to protocols described in Palmer et al. (2011a) (see Chapter 2 section 2.3.4), with some modifications. In short, to quantify PO activity, 20 µl of coral tissue lysate were loaded into wells of a 96-well microtitre plate and 40 µl of ultra-filtered seawater and 25 µl of Milli-Q water were added. To quantify tpPO activity, Milli-Q water was substituted with 0.1 mg/ml trypsin. Plates were left at room temperature to allow activation of prophenoloxidase (PPO) into PO. After a 20 min incubation, 30 µl of 10 mM dopamine hydrochloride (Sigma-Aldrich, USA) were added to each well. As a blank, 20 µl of sample was replaced by ultra-filtered seawater. The absorbance was read at 490 nm every five minutes for 45 min. Results were independently obtained in triplicate and standardised to total protein content. For chromoprotein and fluorescent protein expression, 20 µl of tissue lysate were added in triplicate to each well of a black, clear bottom 384-well plate (Greiner M1811, Sigma-Aldrich, USA). Fluorescence spectra were analysed by measuring the emission wavelengths between 400 and 700 nm, with a 5 nm resolution, upon excitation of fluorescent proteins at 280 nm. Expression of chromoprotein was analysed by measuring the absorbance at 588 nm. Fluorescence spectra and absorbances were measured using a Synergy H4 Hybrid Reader (BioTek Instruments Inc., USA). All data were normalised to total protein

content and expression levels were calculated using the method described in Paley *et al.* (in review; see Appendix B).

6.3.8 - Statistical analysis

All statistical analyses were conducted in R and the *lme4* package was used to develop linear mixed effects models. Maximum and effective quantum yields, total fluorescence and fluorescent protein contributions were analysed using linear mixed effects models (fixed effects: temperature, bacteria, time, and all possible interactions; random effects: aquaria). In addition, two nested random effects (tank: 9 unique treatment combinations of temperature and bacteria; fragment: 21 replicates per tank) were incorporated for maximum and effective quantum yield. Wald tests and t-statistics were used to construct models for each variable, and models were analysed for improvements using log-likelihood ratio test and Bayesian Information Criteria. Phenoloxidase activities (PO, tpPO) and chromoprotein content were analysed using linear fixed effects models, because no significant random effects were observed, and models were compared using analysis of variance (ANOVA).

6.3.9 - DNA isolation

DNA was extracted using a modified protocol of Wayne's DNA preparation method (Wilson et al., 2002). In short, for isolation of DNA from coral-associated bacteria and *Symbiodinium*, coral fragments were thawed on ice and airbrushed in 6 ml of ultra-filtered seawater. Tissue slurries were spun at 3500 rpm for 5 minutes, supernatants were discarded and tissue pellets were frozen at -80°C. Twenty micrograms of coral tissue were added to 750 ml of grinding buffer (100 mM Tris pH 9.0; 100 mM disodium ethylenediaminetetraacetic acid (EDTA); 100 mM sodium chloride (NaCl); 1% sodium dodecyl sulphate (SDS); 400 µg Proteinase K). Glass beads (3 mm diameter) were added and tissues were homogenised using a Mini-Beadbeater-96 (MBB-96) (BioSpec Products Inc., USA). Tissue lysates were incubated for 30 min at 65°C. Potassium acetate was added to a 1 M final concentration. After a 30 min incubation on ice, samples were centrifuged for 15 min at 18.000 x g and supernatants were transferred to clean tubes. Isopropanol (0.8x volume) was added. After a 15-min incubation at room temperature, DNA was precipitated by centrifugation for 15 min at 18.000 x g. Precipitated DNA was washed with 70% ethanol and spun for 5 min at 18.000 x g. DNA was air-dried and resuspended in buffer (10 mM Tris (pH 7.5), 1 mM EDTA). Extracted DNA was quantified using a NanoDrop ND-1000 (ThermoFisher Scientific, Australia).

6.3.10 - Symbiodinium genotyping

Identity of *Symbiodinium* endosymbionts was determined at the sub-cladal level via the nuclear ribosomal DNA internal transcribed spacer 1 (rDNA ITS1) in forty randomly chosen samples using a modified protocol by van Oppen et al. (2001) and compared to standard ITS1 profiles of *Symbiodinium* clades. In short, the ITS1 region was PCR-amplified from 50 ng of template DNA using 0.4 µM symITS1F forward primer, 0.4 µM symITS1R reverse primer (Appendix Table 2) and MyTaq DNA polymerase (BIOLINE Pty Ltd, Australia) according to the following profile: 3 minutes at 95°C, 35 cycles at 95°C for 30 seconds, at 58.5°C for 30 seconds and at 72°C for 30 seconds, followed by a final extension for 3 minutes at 72°C. PCR products were diluted 1:5 in loading buffer (80% formamide, 10 mM EDTA pH 8.0, 1 mg/mL xylene cyanol and 1 mg/ml bromophenol blue), denatured for 3 minutes at 95°C and size-separated using Single Strand Conformation Polymorphism (SSCP) on a 4% poly-acrylamide gel in 0.6x Tris / Borate / EDTA (TBE) buffer on a Gel-Scan 3000 electrophoresis system (Corbett Life Sciences Pty Ltd, Australia).

6.3.11 - Bacterial culture identification

To verify the identity of the *Oceanospirillales* sp. strain S47 and *Vibrio coralliilyticus* strain P1 at each time point, excess bacteria that were not used for the bacterial challenge, were spun down at 2500 rpm for 10 minutes and pellets were frozen at -80°C. To isolate DNA, a small volume (~3 µl) of bacteria was added to 300 µl of ultra-pure water. Tubes were placed in water and boiled in a microwave for 15 min. PCR amplification of 16S rRNA gene was confirmed using the 63F and 1387R primer set (Appendix Table 2) (Marchesi et al., 1998). DNA and 63F primer were sent to Macrogen (Seoul, South Korea) for Sanger sequencing. Bacterial identity was confirmed by analysing the sequencing results using BLAST.

6.3.12 - 16S rRNA gene amplicon pyrosequencing

DNA extracted via Wayne's method was tested for PCR amplification using the 63F and 1387R primer set (Marchesi et al., 1998). All samples showed amplification and were shipped to Molecular Research LP (MR DNA) (Shallowater, Texas, USA). Bacterial 16S rDNA was amplified using the 28F and 519R primer set (Appendix Table 2), followed by pyrosequencing on a Roche GS FLX+ 454 pyrosequencer. Sequence fasta and quality files were provided by Molecular Research LP (MR DNA) (Shallowater, Texas, USA) and processed using the QIIME pipeline (Caporaso et al., 2010) following the protocol described in Chapter 5 (see section 5.2.7). The split_libraries.py script was used to remove poor quality (<25) sequences, reads of

<200 bp or >550 bp in length, primers, and barcodes, and to assign each sequence to its respective treatment. Chimeric sequences were identified against the chimera-free 16S rRNA gene sequence database (Green Genes release May 2013) and removed using UCHIME (Edgar et al., 2011). The resulting chimera-free fasta file was used to calculate numbers of reads per sample and samples were normalised to 617 reads per sample. Using uclust (Edgar, 2010), sequences of 97% similarity were clustered, with each cluster representing an operational taxonomic unit (OTU) defined at the species level. The most abundant sequence per cluster was selected as a representative sequence, and Green Genes taxonomy was assigned using BLAST (at 97% similarity). OTU tables were generated using the QIIME pipeline and analysed for relative abundance. Alpha diversity metrics (total observed species (OTU), predicted species (chao1), Shannon-Weiner diversity and Fisher's alpha) were generated from OTU tables using the QIIME pipeline.

To visualise differences between coral-associated bacterial communities, principal coordinate analysis and CLUSTER analysis were performed on weighted and unweighted UniFrac dissimilarity matrices (square-root transformed) based on bacterial sequences at the OTU level. Permutational multivariate analysis of variance (PERMANOVA) was used to test for differences between bacterial communities associated with corals among different treatments. PERMANOVA and post-hoc pair-wise comparisons were performed on both weighted and unweighted UniFrac similarity matrices under Type III partial sums of squares and 9999 permutations of residuals under the reduced model. Similarity Percentage (SIMPER) analysis was used to elucidate the contribution of individual OTUs to the overall bacterial assemblages. All analyses were conducted using the statistical software package PRIMER 6 & PERMANOVA+ (PRIMER-E Ltd). The complete dataset was deposited in the NCBI Sequence Read Archive (SRA) database with accession number SRA##.

6.3.13 - RNA isolation

Frozen coral fragments were crushed in a stainless steel mortar and pestle, pre-chilled with liquid nitrogen, using a hydraulic press. RNA was isolated from a 50 µl volume of crushed coral for each sample using Ambion's RNAqueous Total RNA Isolation Kit (Life Technologies, USA) according to the manufacturer's recommendations. RNA was treated with DNAse I (Life Technologies, USA) according to the manufacturer's protocol to remove DNA contamination. RNA concentrations were measured using the NanoDrop ND-1000 (ThermoFisher Scientific, USA) and RNA quality was determined on 1.5% (w/v) agarose - Tris / acetic acid / EDTA (TAE) gel.

6.3.14 - Montipora aequituberculata transcriptome

The procedure to generate the cDNA library for transcriptome sequencing was based on the protocol by Meyer et al. (2009) and was adapted for sequencing on the Illumina platform. The transcriptome was obtained from a mix of equal amounts of DNAse I-treated RNA of 9 samples (one of each treatment), in order to provide a reference sequence for the analysis of the RNAseq data (see below). To synthesise first-strand cDNA, 5 µl of RNA (820 ng) were combined with 1 µl of 10 µM T-tr primer and incubated at 65°C for 3 min. The RNA-primer mixture was added to the reaction mixture (1 µl SMARTScribe Reverse Transcriptase (Clontech Laboratories Inc., USA) with 2 µl buffer, 0.5 µl 10 mM dNTP, 1µl 0.1 mM DTT and SW-tr primer) and the reaction mixture was incubated at 42°C for 1 hour. Reverse transcriptase was inactivated for 15 min at 65°C and the product was diluted 1:5 prior to storage at -20°C.

To amplify cDNA, 14 identical PCR reactions (30 µl) were set up as follows: 1 µl first-strand cDNA, 0.6 µl Advantage 2 polymerase mix (Clontech Laboratories Inc., USA) with 3 µl buffer, 0.3 µl 10 µM cDNA amplification primer 3ILL-tr, 0.75 µl 10 mM dNTP and 24 µl of water, and cDNA was PCR amplified using the following profile: 5 min at 94°C and 16 cycles of [40 sec at 94°C, 60 sec at 60°C and 5 min at 72°C]. The PCR product was purified using the GeneJet PCR Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. To concentrate cDNA, 5 µl 3 M sodium acetate pH 5.0 and 125 µl 100% ethanol was added to 50 µl eluate. The mixture was incubated for 20 min at -20°C and centrifuged at 18.000 x g for 20 min at 4°C. The pellet was subsequently rinsed with 80% ethanol, air dried and resuspended in water.

To remove over-abundant transcripts, 600 ng of cDNA was normalised using the Trimmer cDNA Normalisation Kit (Evrogen, Russia) following the manufacturer's protocol. Normalised cDNA was PCR amplified in 16 identical reactions (30 µl), with each reaction containing: 1 µl normalised cDNA, 0.6 µl Advantage 2 polymerase mix (Clontech Laboratories Inc., USA) with 3 µl buffer, 0.3 µl 10 µM cDNA amplification primer 3ILL-tr, 0.75 µl 10 mM dNTP and 24 µl of water. Following PCR (5 min at 94°C and 9 cycles of [40 sec at 94°C, 60 sec at 60°C and 5 min at 72°C]), the PCR product was purified using the GeneJet PCR Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations.

The normalised cDNA was then fragmented by sonication in 30 sec bursts for a total of 9 min using the ultrasonic liquid processor Sonicator 3000 (Misonix Inc., USA), with cup horn part number 431C. Fragmented cDNA (40 µl) was precipitated by mixing with 4 µl 3 M sodium acetate pH 5.0 and 100 µl 100% ethanol, incubating for 20 min at

-20°C and centrifugation at 18.000 x g for 20 min at 4°C. The pellet was rinsed with 80% ethanol, air dried and resuspended in buffer 2 (New England Biolabs, USA). Prior to adaptor ligation, 25 ng of cDNA fragments (3.5 µl) were blunt-ended in duplicate using a mixture (total 12.5 µl) of 0.6 µl Klenow fragment of DNA polymerase I (New England Biolabs, USA) with 1.25 µl buffer 2, 1.25 µl bovine serum albumin, 0.6 µl T4 DNA polymerase (New England Biolabs, USA), 0.6 µl 10 mM dNTP and 4.7 µl water, and a 1.5-hour incubation at room temperature, followed by enzyme inactivation for 15 min at 70°C.

For adaptor ligation, two adaptor mixes were prepared: 1) 5ILL-tr and anti-ILL, 2) 3ILL-tr and anti-ILL, with both primers at equimolar concentrations, with a final primer concentration of 5 µM. Adaptors were ligated overnight at 4°C in two separate reactions: mix 1) 12.5 µl blunt-ended cDNA, 1.25 µl T4 DNA ligase (New England Biolabs, USA) with 1.25 µl buffer, 2.5 µl 5ILL-tr/anti-ILL and 7.5 µl water and mix 2) 12.5 µl blunt-ended cDNA, 1.25 µl T4 DNA ligase (New England Biolabs, USA) with 1.25 µl buffer, 2.5 µl 3ILL-tr/anti-ILL, 2.5 µl 5ILL-tr/anti-ILL and 5 µl water. T4 DNA ligase was inactivated by incubating the mixture for 10 min at 65°C and DNA was purified using the GeneJet PCR Purification Kit (Thermo Fisher Scientific, USA).

Adaptor-cDNA fragments were PCR amplified in three reactions each with 8 replicates. For middle fragments: 1 µl mix 2, 3 µl ILL-BC34 primer (1 µM), 0.3 µl ILL-Mpx2n primer (10 µM), 0.6 µl Advantage 2 polymerase mix with 3 µl buffer, 0.75 µl dNTP and 21.4 µl water. For 5'-end fragments: 1 µl mix 1, 0.3 µl ILL-halfSW primer (10 µM), 0.3 µl ILL-Mpx2n primer (10 µM), 0.6 µl Advantage 2 polymerase mix with 3 µl buffer, 0.75 µl dNTP and 24 µl water. For 3'-end fragments: 1 µl mix 1, 0.3 µl ILL-halfT primer (10 µM), 0.3 µl ILL-Mpx2n primer (10 µM), 0.6 µl Advantage 2 polymerase mix with 3 µl buffer, 0.75 µl dNTP and 24 µl water. The PCR reaction was run according to the following profile: 5 min at 94°C, 14 cycles of 40 sec at 94°C, 60 sec at 65°C and 60 sec at 72°C. PCR products were purified using the GeneJet PCR Purification Kit (Thermo Fisher Scientific, USA) and loaded onto a 1.5% (w/v) agarose gel with SYBR Green I Nucleic Acid Gel Stain (Life Technologies Inc., USA). Fragments of 400-500 base pairs were excised and allowed to diffuse out of the gel into water by overnight incubation at 4°C. Middle, 3'-end and 5'-end cDNA fragments were combined and sent for sequencing on the Illumina MiSeq (Illumina Inc., USA) at the Genomic Sequencing and Analysis Facility of the University of Texas at Austin (Austin, Texas, USA). Primer sequences can be found in Appendix D Table 2. The complete dataset of raw trimmed reads was deposited in the NCBI Sequence Read Archive (SRA) database with accession number SRA##.

6.3.15 - Transcriptome assembly and annotation

The *de novo Montipora aequituberculata* transcriptome was assembled using a Trinity platform protocol described in Haas et al. (2013). Raw reads were trimmed of non-template sequences using Cutadapt (Martin, 2011). Quality filtering was done using Fastx Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). When filtering, non-paired reads were retained separately for later use in the assembly, which included both paired and unpaired reads.

After trimming and filtering, transcriptome assembly was performed using an input of 3,521,179 sets of paired reads and 1,592,295 unpaired reads. Average read length was 195.7 bp (standard deviation = 68.6). The assembly included 180,971 contigs greater than 200 bp, with a total size of 123.5 Mb. Average contig length was 682 bp and N50 was 1012 bp. BlastX of the assembly against the Uniprot database (e-value cut-off of 10^{-20}), returned 29,292 hits. Of these, 57% covered at least 40% of the length of hit sequence.

Assembled contigs were annotated based on BlastX (Altschul et al., 1997) hits against 1) the annotated proteome of *Nematostella vectensis* by the Joint Genome Institute (Putnam et al., 2007), and 2) the annotated *Acropora digitifera* proteome (Dunlap et al., 2013). The latter reference was pre-filtered to include only protein sequences of at least 60 amino acids in length and annotations assigned with an e-value ≤ 1e-20. GO, KEGG, KOG, and gene name annotations were transferred to *M. aequituberculata* contigs from matches in one or both the references using an e-value cutoff of 1e-4. Gene names derived from the *N. vectensis* proteome were KOG term names. When matches were provided from both the *N. vectensis* and *A. digitifera* proteome databases, names were concatenated in the final annotation. Several GO annotations were added manually based on gene names. The annotated transcriptome is freely available from http://www.bio.utexas.edu/research/matz_lab/matzlab/data.html.

6.3.16 - Tag-based RNA Seq library construction and data analysis

Tag-based RNA-seq (Meyer et al., 2011) is specifically designed to measure abundances of polyadenylated transcripts; it generates a single read per transcript, representing a random fragment near the 3'-end. RNA Seq cDNA libraries were prepared following a protocol described in Meyer et al. (2011) with optimisations in primer sequences and bioinformatics pipeline for sequencing on the Illumina HiSeq platform. In addition, the protocol was modified to remove PCR duplicates, resulting in the counts being unique observations of independent transcripts, called unique transcript counts (UTCs).

DNAse I-treated RNA (750 ng) of all 80 samples from Day 22 were fragmented in a 96-well plate in a thermocycler by heating for 15 min at 95°C. The primer 3ILL-30TV (1 µl of 10 µM) was added to each sample, incubated for 3 min at 65°C and cooled down on ice. To make cDNA, an RT mixture (1 µl S-ILL-swMW primer (10 µM), 1 µl SMARTScribe Reverse Transcriptase (Clontech Laboratories Inc., USA) with 4 µl buffer, 2 µl DTT (0.1 M), 1 µl dNTP (10 mM) and 1 µl water) were added and incubated for 1 hr at 42°C and 15 min at 65°C. To amplify the cDNA, a PCR mixture (1 µl 5ILL primer (10 µM), 1 µl 3ILL-30TV (10 µM), 1 µl of Titanium Taq polymerase (Clontech Laboratories Inc., USA) with 5 µl buffer, 5 µl dNTP (2.5 mM) and 32 µl water) was added, and PCR amplification was performed using the following PCR profile: 5 min at 95°C and 14 cycles of 40 sec 95°C, 60 sec at 63°C and 60 sec at 72°C. PCR product was purified using the GeneJet PCR Purification Kit (Thermo Fisher Scientific, USA). The 80 samples were divided into two sets of 40, and a different barcode was attached to each of the 40 amplified cDNA samples. To this end, 5 µl of cDNA (~75 ng) and 10 µl of 1 µM bar-coding primers (JLBC#, UTBC# or ILL-RAD#) were added to PCR reaction mixtures (1 µl ILL-Mpx2n primer (10 µM), Titanium Taq polymerase (Clontech Laboratories Inc., USA) with 5 µl buffer, 5 µl of dNTP (2.5 mM) and 23 µl of water) and run in a thermocycler using the following profile: 5 min at 95°C, 40 sec at 95°C, 60 sec at 63°C and 60 sec at 72°C. Bar-coded cDNA was size-separated on a 2% agarose gel with SYBR Green I Nucleic Acid Gel Stain (Life Technologies Inc., USA). Fragments with a size of 400-500 base pairs were excised and eluted into 15 µl of water by overnight incubation at 4°C. The quality of the cDNA was assessed by PCR using 1 µl of 1:10 diluted bar-coded cDNA in reaction mixture (0.2 µl IC1-P5 (10 µM), 0.2 µl IC2-P7 (10 µM), 0.2 µl Titanium Taq polymerase (Clontech Laboratories Inc., USA) with 1 µl buffer, 1 µl dNTP (2.5 mM) and 6.4 µl water) and the following PCR profile: 5 min at 95°C and 14 cycles of 40 sec at 95°C, 60 sec at 63°C and 60 sec at 72°C.

The concentration of the bar-coded cDNA from each sample was quantified by qPCR. For quantification, 1 µl of 1:250 diluted bar-coded cDNA was added to the reaction mixture (0.2 µl IC2-P7 primer (10 µM), 7.5 µl SYBR Green PCR Master Mix (Life Technologies Inc., USA) and 6.1 µl water) and reactions were run for 10 min at 95°C followed by 25 cycles of 15 sec at 95°C and 60 sec at 60°C. Based on Ct-values, concentrations were determined using the mix_illumina_qPCR.R script in R. Exact same quantities of cDNA from 40 differently bar-coded samples were multiplexed. The two multiplexed samples totalling 80 experimental samples were sent to Beijing Genomics Institute (BGI) (Shenzhen, China) for analysis on the Illumina HiSeq 2000. Samples were sequenced at a depth of 1.16 – 9.18 million (median: 2.85 million) 50 base pair single reads per sample. Primer sequences can be found in Appendix D

Table 2. The complete dataset of raw trimmed reads was deposited in the NCBI Sequence Read Archive (SRA) database with accession number SRA##.

6.3.17 - Differential gene expression analysis

The DESeq package was used to analyse the UTC data in R. The model-aware Cox-Reid adjusted maximum likelihood estimator (”pooled-CR” option) was used to estimate dispersions according to the formula:

“count ~ temperature+bacteria+temperature:bacteria”

Using a series of likelihood ratio tests between nested negative binomial models, the significance of each term was calculated for each gene. Using the genefilter package (Bourgon et al., 2010), transcripts in the bottom 30% for mean UTC across samples were excluded to maximise the number of differentially expressed genes (DEGs) following the false discovery rate correction. Heatmaps were generated using a custom R-script uniHeatmap.R.

6.3.18 - Gene ontology (GO) and EuKaryotic Orthologous Group (KOG) analysis

GO analysis was conducted according to a method developed by Voolstra et al. (2011). In short, this method involves 4 steps: 1) assembly of a GO database tailored to the set of annotated genes; 2) merging of GO categories that have significant overlap and determining the levels of gene sharing between GO categories; 3) Mann-Whitney U test to determine the significance of the mean rank change for each GO category based on continuous measures (in this study signed log-p-values); 4) creation of a dendrogram indicating the level of gene sharing, direction of change (up- or down-regulated; shown by different text colour) and level of significance (indicated by font size). In addition, the total number of genes assigned to each GO category within the dataset is listed.

For both GO and KOG analysis, “signed log-p-values” (negative logarithm of the gene’s p-value, multiplied by -1 if the gene was down-regulated) were used. Hereby, a larger value on the absolute scale was assigned to genes of higher significance (i.e. smaller p-value). This value was negative if the gene was downregulated or positive if the gene was upregulated relative to the control condition (ambient temperature, no bacteria). To determine if a GO category or KOG was up- or downregulated, a delta-rank value was computed based on the signed log-p-values as the difference between the mean rank of all genes within the GO category and the mean rank of all other genes. Using a two-sided Mann-Whitney U-test, the significance of the delta rank was calculated followed by the false discovery rate correction. Data were plotted in a dendrogram, as described above, for GO categories and in a heatmap for KOGs.

Both GO-MWU and KOG-MWU methodologies are available from Matz lab website (http://www.bio.utexas.edu/research/matz_lab/matzlab/Methods.html) as packages including scripts, documentation and example data files.

6.4 - Results

The majority of parameters analysed in this study did not show any interactive effect of temperature stress and bacterial treatment. From here on, when referring to: 1) the effect of a temperature stress, the statement is made for all treatment combinations at that temperature, regardless of bacterial treatment, and 2) the effect of a bacterial treatment, the statement is made for all treatment combinations that underwent that bacterial treatment, regardless of temperature. When both a bacterial and a temperature treatment are mentioned together, the result addresses that specific treatment combination.

6.4.1 - Macroscopic assessment of coral fragment health

Over the course of the experiment, the majority of coral fragments appeared healthy based on pigmentation and absence of tissue lesions. Only 7 fragments showed signs of disease, characterised by tissue loss and exposure of the underlying skeleton, signs that are consistent with a white syndrome, followed by development of a grey-black film covering the lesion, characteristic of atramentous necrosis ((Jones et al., 2004, Anthony et al., 2008b); Figure 6.2). Timing of disease development was variable, with disease found in one *V. coralliilyticus*-challenged fragment at 27°C on Day 7. All other cases occurred on Day 15 (two non-challenged at 32°C, two *V. coralliilyticus*-challenged at 29.5°C and 32°C, respectively, and on two *Oceanospirillales*-challenged corals at 29.5°C and 32°C, respectively). Progression was relatively slow and no fragment had sustained >25% mortality by the end of the experiment or sampling. No signs of bleaching or major changes in pigmentation from loss of *Symbiodinium* were observed for all other fragments (Fig. 6.3).

Figure 6.2 – Representative photograph of coral fragment showing tissue loss. exposed skeleton and signs of atramentous necrosis.

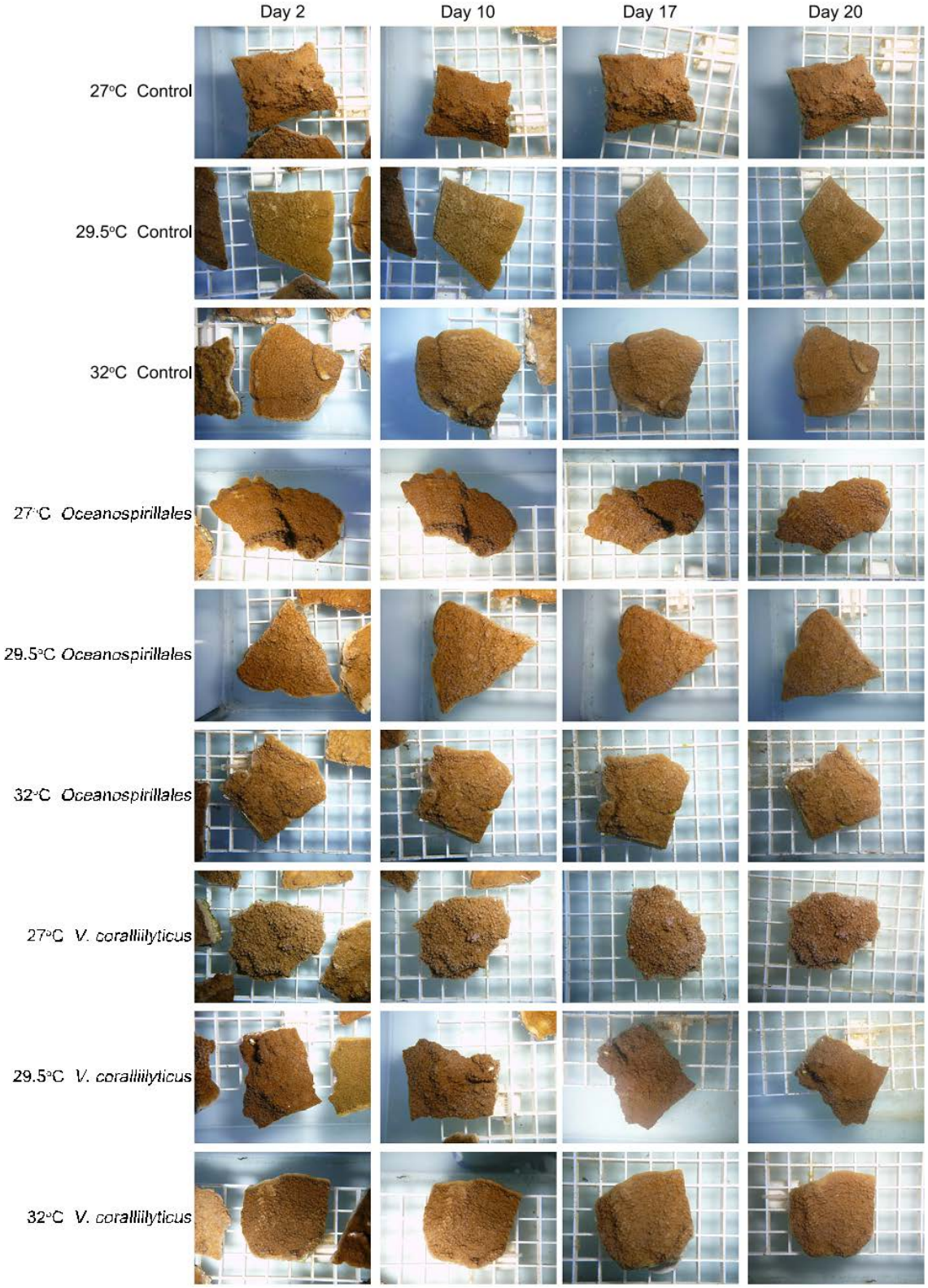


Figure 6.3 – Time series of representative coral fragments of *Montipora aequituberculata* for all 9 combinations of 3 temperature treatments and 3 bacterial treatments. Fragments followed photographically were randomly selected at the commencement of the experiment. The same light and camera settings and distance to the object were used in each series of photographs.

6.4.2 - Response of Symbiodinium to bacterial challenges and heat stress

Symbiodinium C• (van Oppen, 2004) was the only *Symbiodinium* type detected in all fragments, as determined by profiling of the nuclear ribosomal ITS1 region (Suppl. Fig. 6.1). Both effective (Y(II)) and maximum (Fv/Fm) quantum yield declined over time in all treatments (Fig. 6.4A, B; Suppl. Fig. 6.2A, B). Generally, a consistent pattern was observed, comprising an initial decrease in Y(II) and Fv/Fm during the heating stage (Days 0 – 9), followed by a slight recovery and then a subsequent second period of decline. The rate of decline differed significantly among treatments. For Fv/Fm, the slope of the models for all treatments at 32°C had a significantly stronger negative coefficient compared to all treatments at 27°C and 29.5°C; however no statistically significant difference was detected between the treatments at 27°C and 29.5°C. Similar results were obtained for Y(II), with the exception that yield for *Oceanospirillales*-challenged corals at 27°C was higher than for all treatments at 29.5°C. Notably, effective quantum yield of corals exposed to *V. coralliilyticus* at 32°C was significantly lower than the yields of *Oceanospirillales* S47-challenged or unchallenged corals at 32°C. The results of statistical analyses for both effective and maximum quantum yield can be found in Supplementary Table 6.1.

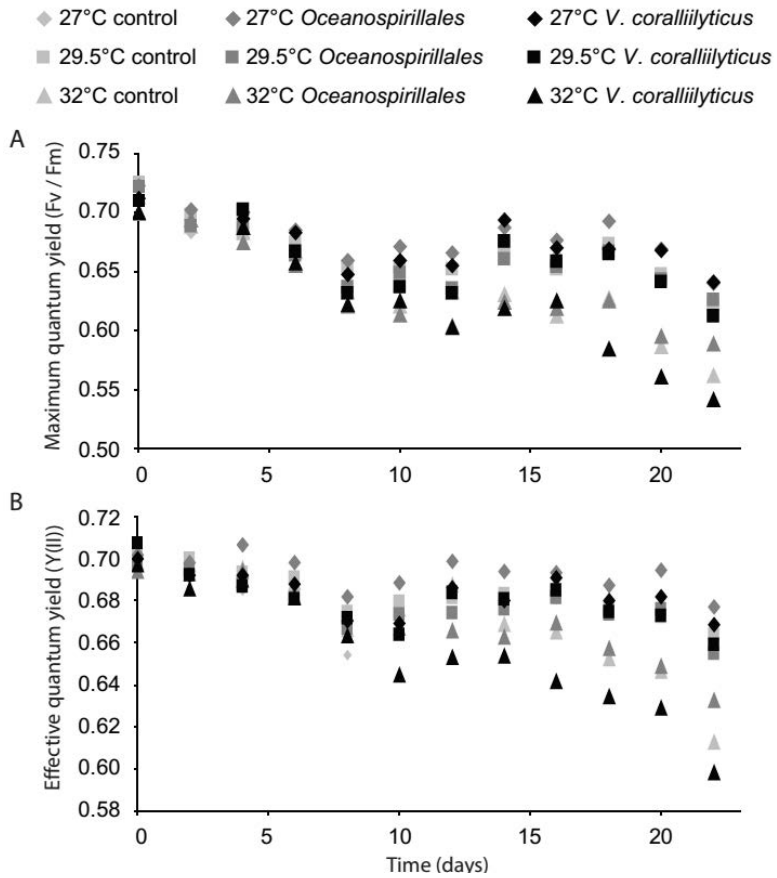


Figure 6.4 – Photosynthetic capacity of corals subjected to combinations of elevated seawater temperatures and bacterial challenges. Time series of: A) mean maximum quantum yield (Fv / Fm), and B) mean effective quantum yield (Y(II)) measured every 48 hours using pulse amplitude-modulated fluorometry.

Analyses of the *de novo* transcriptome of the *M. aequituberculata* holobiont identified 12,913 genes as *Symbiodinium* genes. Of these genes, 11,909 were also found in the RNA Seq dataset, and 200 were differentially expressed in response to temperature stress (Fig. 6.5; full overview, including gene names, found in Electronic Figure 6.5 supplied as a separate file). In the 32°C treatments, *Symbiodinium* upregulated 118 genes and downregulated 70 genes compared to the 27°C treatments. Gene enrichment analysis based on the Gene Ontology Biological Processes annotations showed that at 32°C, the cell cycle and various DNA processes of *Symbiodinium* were downregulated (Suppl. Fig. 6.3). However, *Symbiodinium* also showed a significant response to an unknown biotic stimulus, including immune responses, potentially involving a range of cell surface receptors (Suppl. Fig. 6.3). In addition, various metabolic processes and photosynthesis were downregulated, while genes involved in apoptosis showed a trend of increased expression (Suppl. Fig. 6.4).

The expression profile of *Symbiodinium* genes in corals at 29.5°C was intermediate, with expression characteristics of *Symbiodinium* at both 27°C and 32°C. Elevated expression was detected for 9 genes at 29.5°C compared to the other temperature treatments. Five of these genes were also expressed in a small subset of samples from the 32°C (n = 7) and 27°C (n = 7) treatments. At 29.5°C, however, *Symbiodinium* exhibited patterns opposite to those described for the 32°C treatment,

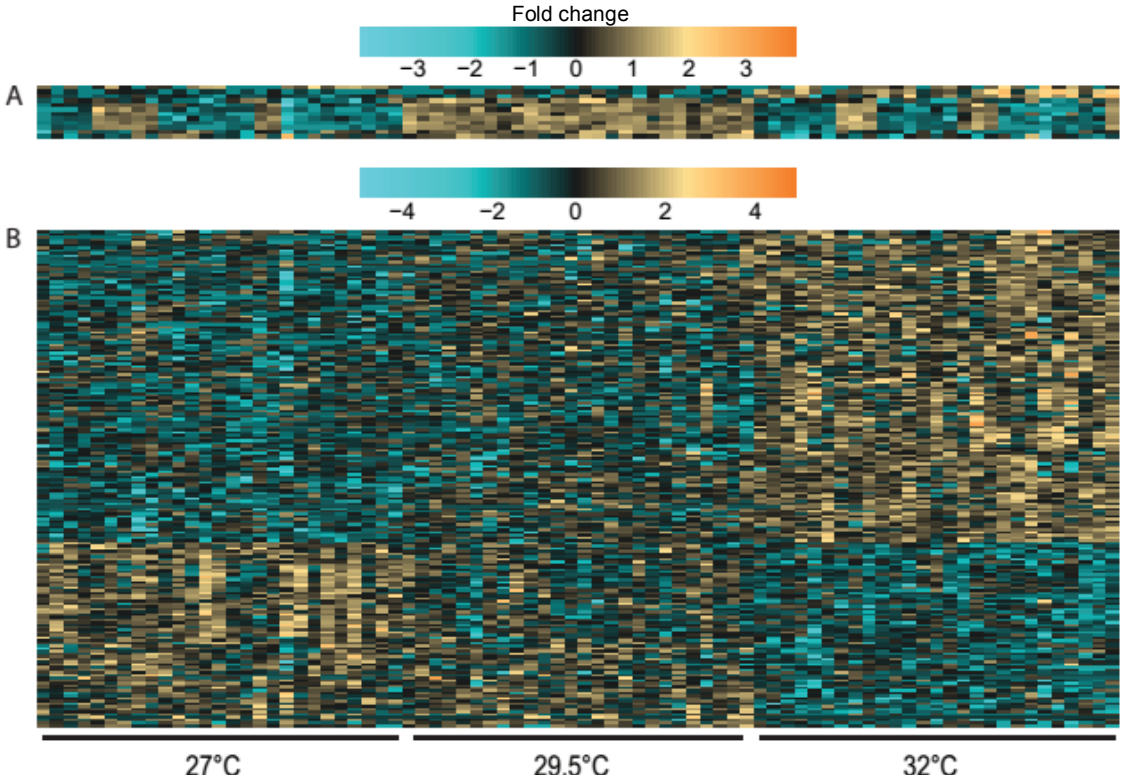


Figure 6.5 – Heatmap of the expression patterns of differentially expressed genes in *Symbiodinium* endosymbionts following exposure to elevated seawater temperatures. A) Profile of 12 differentially expressed genes in *Symbiodinium* at 29.5°C. B) Profile of 188 differentially expressed genes in *Symbiodinium* at 32°C.

with trends of increased metabolism and photosynthesis (Suppl. Fig. 6.5). No genes were differentially expressed by *Symbiodinium* in response to bacterial challenges.

6.4.3 - Gene expression response of the coral host under elevated temperatures

The *M. aequituberculata* holobiont transcriptome contained 54,196 sequences that were identified to be of coral host origin. Through the tag-based RNA Seq analysis, I obtained reads for 36,454 of these sequences. Analysis based on the EuKaryotic Orthologous Group (KOG; Fig. 6.6) annotation showed that, compared to corals at 27°C, those experiencing temperatures of 32°C had increases in expression of genes involved in defence mechanisms, vesicular traffic, RNA processing, as well as protein turnover and chaperones. In comparison, corals at 29.5°C had increased translation, cell membrane biogenesis and energy production processes (Fig. 6.6). In both elevated temperature treatments, I found a reduction in metabolic processes, transcription, as well as in cell growth/proliferation. Similar trends in differential regulation of processes based on GO categories were observed for 29.5°C (Suppl. Fig. 6.6A) and 32°C (Suppl. Fig. 6.6B). These differential expression patterns are consistent with a clear stress response and activation of the innate immune system at 32°C, but not at 29.5°C.

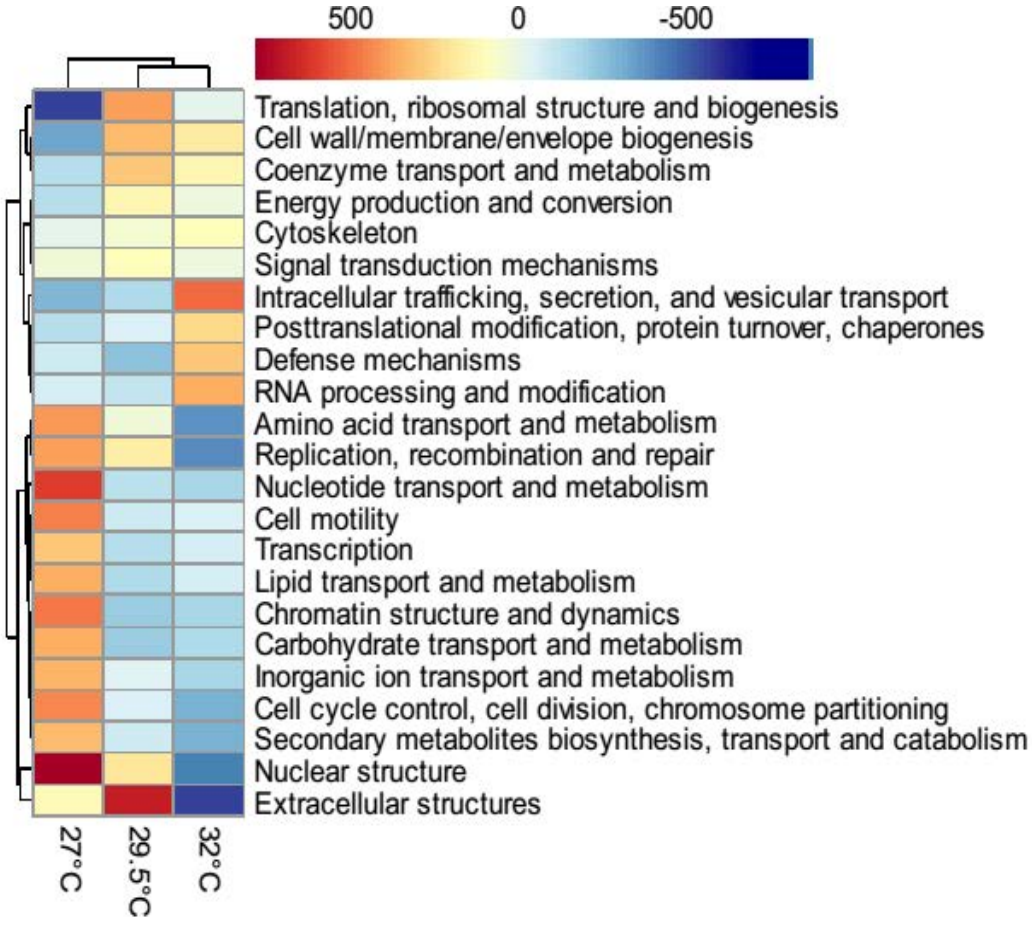


Figure 6.6 – Heatmap of cellular processes differentially affected by elevated seawater temperatures (29.5°C and 32°C). Analysis was based on EuKaryotic Orthologous Group (KOG) annotations.

In response to elevated seawater temperatures, the coral host differentially expressed 540 genes (10% False Discovery Rate), with expression profiles differing among the three temperature levels (Fig. 6.7A shows the differentially expressed gene profile at 29.5°C (n = 19 genes); Fig. 6.7B shows the profile at 32°C (n = 521 genes)). Here, I primarily focus on the stress and immune responses of the coral host towards bacteria under elevated seawater temperatures (a full overview, including gene names, can be found in Electronic Figure 6.7A and 6.7B, respectively, supplied as a separate file). At elevated temperatures, various molecular chaperones from the Hsp90, Hsp26/42 and DNAJ families were expressed at significantly higher levels than in corals at 27°C (Suppl. Fig. 6.7), with upregulation up to 2.9-fold greater at 32°C than at 29.5°C. Levels of transcription factors belonging to the Maf family, which are involved in the anti-oxidant response, were also elevated by 1.6 to 2.5-fold at 32°C (Suppl. Fig. 6.7). In contrast, the bicarbonate transporter SLC26 and a carbonic anhydrase were downregulated at elevated temperatures (Suppl. Fig. 6.7). In addition, 85 genes with a putative role in the coral immune response were differentially expressed; at 32°C, 61 genes were upregulated and 23 downregulated, while at 29.5°C, 48 were down-

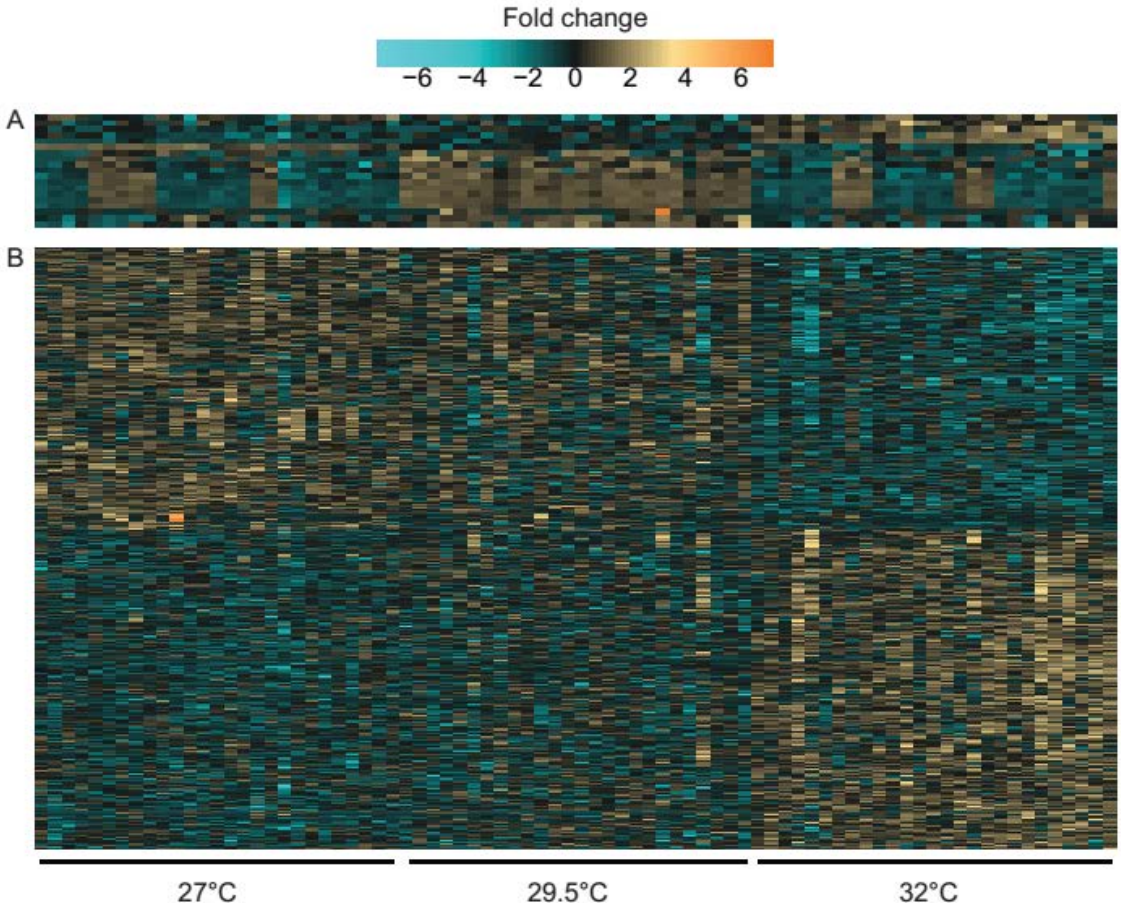


Figure 6.7 – Heatmap of the gene expression profiles of the coral host *Montipora aequituberculata* in response to elevated seawater temperatures on Day 22. A) profile of 19 differentially expressed genes following exposure to intermediate heat stress in seawater of 29.5°C; B) profile of 521 differentially expressed genes following exposure to high heat stress in seawater of 32°C.

regulated and only 9 upregulated (Suppl. Fig. 6.7). These genes were involved in various processes of the immune response including Toll-like receptor signalling, apoptosis, cytokine production, anti-viral responses, phagocytosis, complement system, immune cell activation, hypoxia-induced inflammation as well as in negative feedback loops that regulate the afore mentioned processes. Details of all differentially expressed stress and immune genes, including gene names, e-values of annotations and fold change in expression, can be found in Suppl. Fig. 6.7.

6.4.4 - Gene expression response of the coral host to bacterial challenges

The response of the coral host towards bacterial challenges appeared to be specific for the bacteria encountered. *Oceanospirillales*-challenged corals showed increased energy production, cell motility and translation, while signal transduction, cell membrane biogenesis and extracellular structures were downregulated (Fig. 6.8). This was confirmed by analysis of differential regulation of GO category-annotated processes, although this analysis revealed that various metabolic processes were also affected (Suppl. Fig. 6.8A). In contrast, the coral host appeared to recognise *V. coralliilyticus* as harmful, as shown by a trend for increased expression of the stress and innate immune responses, including coagulation, complement activation and apoptosis (Fig. 6.8, Suppl. Fig. 6.8B). In addition, increases in translational and energy production processes were observed in response to the *V. coralliilyticus* challenge (Fig. 6.8, Suppl. Fig. 6.8B).

The majority of genes involved in these GO category-annotated biological processes were not significantly differentially expressed. However, the expression profiles of 11 differentially expressed genes, of which only 7 were annotated, clearly differed among the three bacterial challenge treatments (Fig. 6.8B). Two of the annotated DE genes are involved in the circadian clock: 1) circadian locomotor output cycles kaput (*CLOCK*) gene, which had a 1.93-fold and 3.41-fold higher expression, and 2) cryptochrome (*CRY*), which was reduced in expression 1.31-fold and 2.40-fold in *Oceanospirillales* and *V. coralliilyticus*-challenged corals, respectively, compared to unchallenged corals. In addition, a subunit of protein phosphatase-1 (*PPP1R3C/D*) was downregulated 1.35-fold and 3.13-fold, while the hairy/ enhancer of split related with YRPW motif (*HEY*) gene was 1.54-fold and 1.64-fold upregulated in *Oceanospirillales* and *V. coralliilyticus*-challenged corals, respectively.

6.4.5 - Biochemical responses of the coral host

Overall, PO activity (Suppl. Fig. 6.9A) was significantly higher in *V. coralliilyticus*-challenged corals compared to controls ($p < 0.05$). In addition, PO activity

changed over time in some treatments ($p < 0.05$). In particular, PO activity was significantly higher in corals exposed to *Oceanospirillales* at 32°C on Day 10, compared to Days 0 and 22, and significantly lower on Day 22 compared to Days 0 and 10 in *V. coralliilyticus*-challenged corals at 29.5°C. Total potential PO activity (Suppl. Fig. 6.9B) changed over time depending on the bacterial challenge that corals were exposed to ($p < 0.05$). Corals that were challenged with *V. coralliilyticus* at 29.5°C or 32°C, or with *Oceanospirillales* at 32°C all showed higher tpPO activity levels on Day 10 compared to Day 22. *Oceanospirillales*-challenged corals at 29.5°C, however, showed significant increases over time in tpPO activity.

No changes in total fluorescence were observed under any treatment condition or over time (Suppl. Fig. 6.10A). The proportion of GFP to total fluorescence also remained constant throughout the study (Suppl. Fig. 6.10C); however, the proportion of

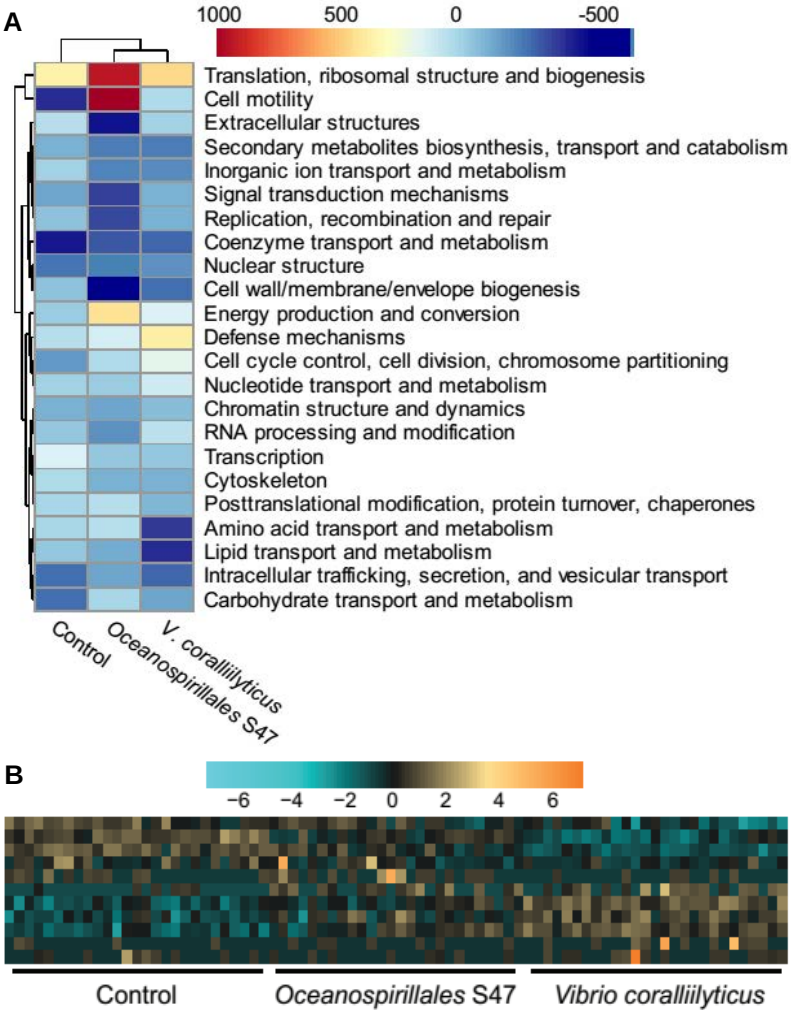


Figure 6.8 – Gene expression response of the coral host to bacterial challenges. Heatmaps of A) biological processes (EuKaryotic Orthologous Group annotation) differentially affected following bacterial challenges with *Oceanospirillales* S47 or *Vibrio coralliilyticus*. B) expression profile of 11 differentially expressed genes following bacterial challenges with *Oceanospirillales* S47 or *Vibrio coralliilyticus*.

CFP increased ($p < 0.05$) (Suppl. Fig. 6.10B), while RFP decreased over time ($p < 0.01$) (Suppl. Fig. 6.10D). The proportion of RFP was particularly affected when corals were challenged by bacteria ($p < 0.05$) (Suppl. Fig. 6.10D). Chromoprotein expression was only significantly affected in *Oceanospirillales*-challenged corals at 29.5°C with Day 22 being significantly higher than Day 1 ($p < 0.05$) and Day 10 ($p < 0.05$) (Suppl. Fig. 6.10E).

6.4.6 - Effect of bacterial challenge and heat stress on coral-associated bacterial communities

The alpha diversity of the coral-associated bacterial community was analysed based on four different indices: predicted species (chao1, Suppl. Fig. 6.11A), Shannon-Weiner index (SWI, Suppl. Fig. 6.11B), Fisher's alpha (FA, Suppl. Fig. 6.11C) and the number of unique OTUs (OTU, Suppl. Fig. 6.11D) (see summary in Supplementary Table 6.3). Overall, significant reductions were observed in the alpha diversity of bacterial assemblages associated with non-challenged (27°C: chao1, SWI, FA, OTU; 32°C: SWI) and *V. coralliilyticus*-challenged (27°C: SWI, FA, OTU; 32°C: chao1, FA) corals at 27°C and 32°C over time. However, no decreases in alpha diversity were detected in the bacterial communities of corals challenged with *Oceanospirillales*. Using principal coordinate analysis of both weighted and unweighted UniFrac distance matrices, I confirmed that there were significant changes within the bacterial communities over time. My results indicate that changes in bacterial community membership (unweighted; Fig. 6.9A), as well as in the abundance of species within the community (weighted; Fig. 6.9B) occurred. On Day 1, coral-associated bacterial communities were similar in the 27°C and 32°C treatments for all bacterial challenge treatments (Fig. 6.9A, B), as would be expected at the commencement of the experimental treatments. Bacterial communities had changed by Day 10, however, at which time corals had been exposed to the target heat stress for 1.5 days, but the pattern of change was similar across all treatments (Fig. 6.9A, B). The effect of temperature was only apparent at the end of the experiment (Day 22), when corals had been exposed to temperature stress for 13.5 days, at which time changes in coral-associated bacterial communities were best explained by temperature differences between the 27°C and 32°C treatments (Fig. 6.9C). In contrast, challenges by non-pathogenic or pathogenic bacteria had no effect (Fig. 6.9C). To assess the taxonomic affinity of the bacterial species driving the shifts in bacterial assemblages, I used a similarity percentage (SIMPER) analysis. Unexpectedly, the contribution of individual species or families to the change over time (Suppl. Table 6.4) or between temperature treatments on Day 22 (Suppl. Table 6.5) was very minimal, with a maximum

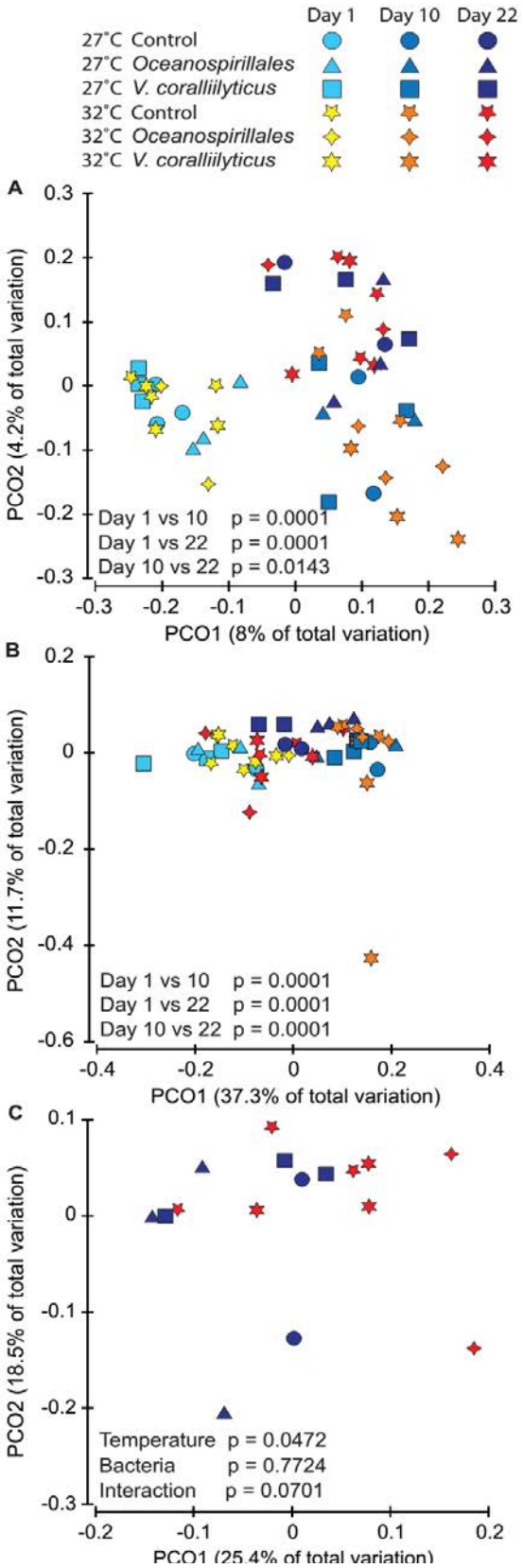


Figure 6.9 – Beta diversity of coral-associated bacterial communities. Principal coordinate analysis of beta diversity based on A) unweighted and B) weighted Unifrac distances. C) Principal coordinate analysis of coral-associated bacterial communities on Day 22 based on weighted Unifrac distances.

contribution of only 4.02% by the Rhodobacteraceae during the shift between Day 1 and 10. Overall, this indicates that the shifts were caused by restructuring of the complete bacterial assemblage and that it was not driven by single species or members of the *Oceanospirillales* or *Vibrionaceae*. All *Vibrionaceae* sequences found in my data were identified as *V. coralliilyticus* and were only present in corals challenged with this bacterium (Supplementary Table 6.4). *Oceanospirillales* sequences were nearly three times more abundant in *Oceanospirillales*-challenged corals compared with control or *V. coralliilyticus*-challenged corals (Supplementary Table 6.4).

6.5 - Discussion

In this study, I used a holistic approach to elucidate the responses of three components of the *Montipora aequituberculata* holobiont (coral host, *Symbiodinium* and the bacterial community) to a bacterial challenge under elevated sea water temperatures. The coral pathogen *V. coralliilyticus* was found to negatively impact photochemical yields of *Symbiodinium* when the holobiont was exposed to high temperatures; however, transcriptomic responses detected for *Symbiodinium* were to temperature stress rather than to

the pathogen challenge. In contrast, the coral host exhibited a differential immune response that distinguished between a pathogenic and non-pathogenic bacterium. The coral host also exhibited an immune response at high temperatures, potentially due to temperature-dependent shifts in the coral-associated bacterial community. Ultimately, these responses appeared to be sufficient to prevent any visual signs of coral disease development over the course of my 22-day study.

6.5.1 - Symbiodinium response to heat stress

The algal endosymbiont *Symbiodinium* C• differentially expressed a multitude of genes under elevated seawater temperatures, although functional analysis revealed that the majority of cases of differential expression did not involve genes that could be linked directly to a major heat stress response. Under experimental temperatures 2°C above summer means at the study site, the transcriptomic response involved upregulation of several heat shock proteins, combined with reduced expression of genes involved in metabolism and photosynthesis. Similarities with the transcriptomic response found for the *Pocillopora damicornis* holobiont in response to temperatures 7°C above its regular maintenance temperature (Vidal-Dupiol et al., 2014) suggest that such a limited heat stress response may be characteristic of *Symbiodinium*. Indeed, other studies have not detected any major transcriptomic responses in coral-associated *Symbiodinium* for types C3 and D under high (up to 34°C) temperatures (Leggat et al., 2011; Barshis et al., 2014). Differences between my study and these two latter studies suggest that the expression of heat stress tolerance genes differs among *Symbiodinium* types, and likely contributes to differences in bleaching susceptibility among corals harbouring different *Symbiodinium* types. In addition, I observed a response by *Symbiodinium* to a biotic stimulus, including an immune response, under heat stress, which was potentially due to a temperature-induced shift in the coral-associated microbial community in the elevated temperature treatments. Such a response indicates that *Symbiodinium* may also play a role in the disease resistance of corals.

Symbiodinium C• did not show a transcriptomic response to bacterial challenge, despite a significant impact of *V. coralliilyticus* on *Symbiodinium* photochemical efficiency at high temperatures. *V. coralliilyticus* becomes virulent at elevated seawater temperatures (Ben-Haim et al., 2003a; Ben-Haim et al., 2003b; Kimes et al., 2012) and secretes a zinc metalloprotease virulence factor that damages the photosystem II of the dinoflagellate (Sussman et al., 2009). In my study, the effect of *V. coralliilyticus* on the photosynthetic efficiency of the endosymbionts was apparent at 32°C, but not

under intermediate heat stress (29.5°C). This could indicate that this strain of *V. coralliilyticus* is only virulent towards the *M. aequituberculata* holobiont at temperatures above 29.5°C, when the photochemical efficiency of *Symbiodinium* is also reduced due to significant heat stress.

6.5.2 - Coral-associated bacterial community

Coral health is highly dependent on a healthy microbial community associated with its tissues. In this study, the coral-associated bacterial community of the *M. aequituberculata* holobiont underwent significant changes over time that were independent of the two bacterial treatments. By the end of the heat stress experiment (Day 22), significant differences in bacterial assemblages between corals in the 27°C and 32°C treatments were consistent with the important role that temperature has been shown to play in structuring coral-associated bacterial communities. Elevated seawater temperatures are known to cause shifts in bacterial assemblages towards more pathogenic species (Ritchie, 2006; Bourne et al., 2008; Mouchka et al., 2010; Littman et al., 2011; Witt et al., 2011), potentially resulting in increased disease prevalence. However, all shifts in my study were the result of an alteration in the bacterial community as a whole and not because of changes in the abundance of a small number of species or families. Hence, despite the repeated addition of large cell numbers of *Oceanospirillales* S47 and *V. coralliilyticus*, there were no major shifts in the bacterial assemblages towards these species. For example, *V. coralliilyticus* was detected only in *V. coralliilyticus*-challenged corals, but in low numbers. The ubiquitous coral-associated *Oceanospirillales* sp. were found in higher numbers in *Oceanospirillales*-challenged corals compared with control or *Vibrio coralliilyticus*-challenged corals, but while the contribution of Oceanospirillales to the community did change over time, it did not necessarily increase. Taken together, this suggests that the coral actively regulates the abundance of both bacterial species.

6.5.3 - Coral host transcriptome response to temperature

Overall, the coral immune response at 32°C was comprehensive, involving all major innate immune response mechanisms. The response was likely initiated by the upregulated TLR signalling pathway (Fig. 6.10(I)), which, in turn, was likely responsible for the production of pro-inflammatory cytokines. Immune cells are activated through cytokine receptor signalling and migrate towards the site of infection along a chemotactic gradient of cytokines (Fig. 6.10(II)). In addition, the lectin-complement system was induced to tag invading microbes for phagocytosis (Fig. 6.10(VII)). Using

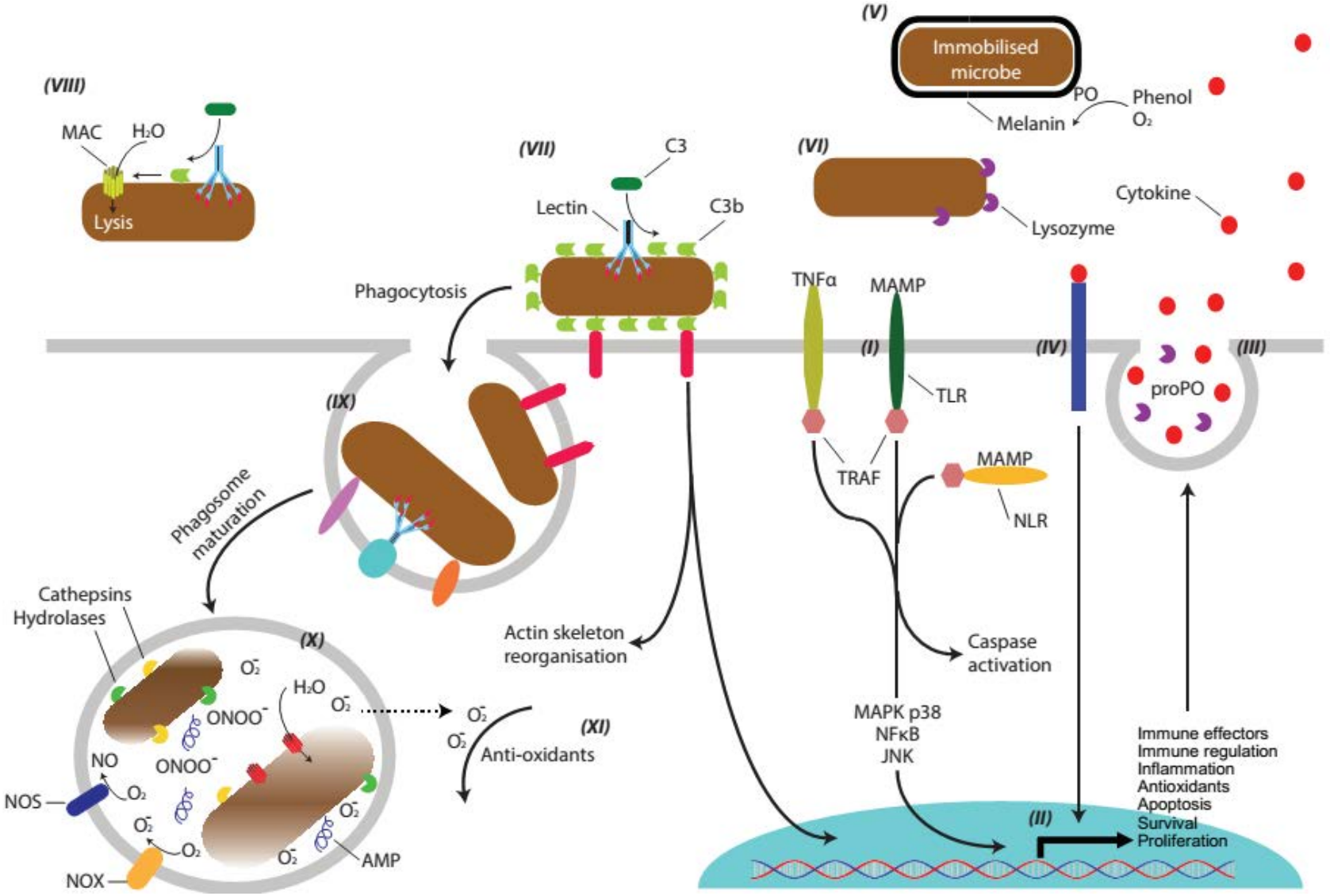

(V)
Immobilised microbe
PO
Phenol
O₂
Melanin
(VIII)
MAC
H₂O
Lysis
(VII)
C3
Lectin
C3b
(VI)
Cytokine
Lysozyme
Phagocytosis
TNFα
MAMP
(I)
(IV)
(III)
TLR
proPO
(IX)
MAMP
TRAF
NLR
Phagosome maturation
Cathepsins
Hydrolases
(X)
Actin skeleton reorganisation
Caspase activation
ONOO⁻
H₂O
NO
NOS
NOX
AMP
(XI)
Anti-oxidants
MAPK p38
NFκB
JNK
(II)
Immune effectors
Immune regulation
Inflammation
Antioxidants
Apoptosis
Survival
Proliferation

Figure 6.10 – Overview of the innate immune system, annotated to show genes upregulated by *Montipora aequituberculata* in response to a bacterial challenge under heat stress**.** (I) MAMP-activated Toll-like and NOD-like receptors as well as TNFα-activated tumor necrosis factor (TNF) receptors induce various signal transduction pathways via TRAFs, including NFκB, JNK and MAPK p38 pathways, resulting in transcription of immune genes (II). In addition, apoptosis may be induced via a caspase-mediated pathway. Products of transcribed immune genes may have intracellular functions or are exocytosed (III). Exocytosed cytokines have immunomodulatory functions, regulating immune gene expression and providing a chemotactic gradient for immune cell recruitment via cytokine receptor signalling (IV). In addition, proPO forms a microbe-immobilising barrier of melanin (V) and lysozyme digests the microbial cell wall causing lysis (VI). The lectin-complement system (VII) is initiated by binding of a lectin to MAMPs and results in the proteolytic cleavage of C3 into C3b, which is deposited onto the microbe. C3b can initiate the formation of a MAC (VIII) or via C3-specific receptors induce phagocytosis of the microbe (IX). Maturation of the phagosome leads to the formation of a microbicidal phagolysosome (X). Destructive reactive radicals are neutralised by antioxidants to prevent host damage (XI). Abbreviations: MAMP, microbe-associated molecular pattern; TLR, Toll-like receptor; NLR, nucleotide-binding oligomerisation domain (NOD-like) receptor; TNFR, tumor necrosis factor receptor; TRAF, TNF receptor-associated factor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; NFκB, nuclear factor kappa B; proPO, pro-phenoloxidase; C3, complement C3; MAC, membrane attack complex; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; AMP, anti-microbial peptide.

their ficolin/lectin receptors and scavenger receptors, immune cells phagocytose (Fig. 6.10(IX)) and subsequently eliminate the microbes (Fig. 6.10(X)). There were also indications of an anti-viral response by *M. aequituberculata* under heat stress. In addition, I found evidence of negative feedback mechanisms regulating the coral immune response. The exact causative agent eliciting the immune response in this coral at 32°C, however, remains to be identified. A likely cause is the shift observed in the coral-associated bacterial community, but other currently unknown challenges may also have played a role. However, as nearly 25% of all differentially expressed genes were immune genes that are involved in multiple immune defense pathways, including anti-microbial defenses, it is unlikely that the immune response at 32C is part of a broad stress response and a causative agent of microbial origin is therefore most probable.

6.5.3.1 - Biological processes

Generally, I found that coral genes related to metabolism and cell proliferation were impacted at 32°C, showing that this temperature (not 29.5°C) elicits a stress response in the Magnetic Island population of *M. aequituberculata,* as manifested by an increased expression of genes related to protein turnover and chaperones. In addition, the coral also increased expression of genes involved in defense and intracellular trafficking, which may indicate an immune response to unknown temperature-dependent invading microbe(s), and a concomitant increase in phagocytosis. Below, I will discuss the importance and function of the genes involved in the observed stress and immune responses.

6.5.3.2 -Chaperones and antioxidants

The upregulation of a range of stress response proteins, including molecular chaperones of the DNAJ, Hsp90 and Hsp26/42 heat shock protein (HSP) families at 29.5°C, but significantly more at 32°C, is in agreement with earlier observations of the coral heat stress response (Rodriguez-Lanetty et al., 2009; Leggat et al., 2011; Maor - Landaw et al., 2014). These chaperones bind to proteins that have unfolded or are incorrectly folded as a consequence of stress, such as high temperatures, thereby preventing protein aggregation and facilitating their refolding (Tsan and Gao, 2009). HSP chaperone function is enhanced by stress-induced phosphoprotein 1 (STIP1), which was also upregulated at 32°C, via the formation of chaperone complexes (Odunuga et al., 2004). Since they bind to hydrophobic structures, these chaperones are also capable of binding MAMPs, and recent studies have indeed provided evidence that HSPs also play a significant role in the initiation of the immune response, likely mediated via Toll-like receptor signalling resulting in, amongst other processes, the production of pro-inflammatory cytokines (Fig. 6.10(I)-(II)) (Tsan and Gao, 2009). In addition, I observed the upregulation of several Maf genes. Although the biology of these transcription factors (MafF, MafK, MafG) is very complex because of their interactions with the six members of the Cap'n'collar (CNC) family, resulting in transcriptional repression or activation depending on the dimers formed, one of the major processes regulated by Maf transcription factors is the antioxidant response (Fig. 6.10(XI)) (Motohashi et al., 2002). These results indicate that the corals experienced stress under high temperatures (32°C), but to a lesser extent when exposed to intermediate heat stress (29.5°C), suggesting that the population of *M. aequituberculata* at Magnetic Island is moderately heat tolerant. Levels of reactive oxygen species (ROS) are known to increase as a result of defective photosystems of *Symbiodinium* at higher temperatures when corals experience heat stress (Weis, 2008). ROS can also be produced during the immune response, and the upregulation of Maf genes may be related to this process rather than *Symbiodinium*-derived ROS, or to both. The antioxidant enzyme peroxiredoxin 1 (Rhee et al., 2001) was also upregulated, suggesting that it is protecting cells from ROS produced during the immune response or by damaged *Symbiodinium* photosystems. Peroxiredoxin 1 is, however, also considered a danger-associated molecular pattern (DAMP), and when released from damaged cells, can elicit an inflammatory response via TLR2 and TLR4 signalling (Riddell et al., 2010; Shichita et al., 2012).

6.5.3.3 - Toll-like receptor and tumor necrosis factor receptor

The innate immune system is generally initiated through the detection of microbe-associated molecular patterns (MAMP) by Toll-like receptors (TLR) (Fig. 19(I)). These receptors activate various MAPK pathways, as well as NFκB via tumor necrosis factor receptor (TNFR)-associated factors (TRAFs), leading to the transcription of genes involved in cell survival and the immune response, including anti-microbial peptides (AMP), nitric oxide synthase (NOS) and pro-inflammatory cytokines (Fig. 19(II)) (Kumar et al., 2009). To prevent excessive signalling, MAPK activity can be negatively regulated by phosphatases (Arthur and Ley, 2013). The observed upregulation of TLR4 (a bacterial MAMP-specific TLR), TRAFs and a MAPK, combined with the downregulation of dual-specificity phosphatase, suggest that the TLR pathway is activated in *M. aequituberculata* under heat stress, potentially in response to the observed shift in bacterial community. I also detected elevated expression of TNFR family members under heat stress. TNFRs initiate similar pro-inflammatory signal transduction pathways to those by TLR via TRAFs, but are also capable of inducing apoptosis through the activation of caspases (Newton and Dixit, 2012).

6.5.3.4 – Apoptosis

The upregulation of PtdSerR (phosphatidylserine (PtdSer)-specific receptor) suggests that apoptosis may play a major role in the immune response observed. This receptor binds to PtdSer, a phospholipid that, under normal conditions, is present in the inner leaflet of the cell membrane, but flips to the outer layer of the cell membrane during apoptosis. Thereby, PtdSerR facilitates phagocytosis of apoptotic cells by immune cells, but without eliciting an inflammatory response (Fadok et al., 2001). Apoptosis has been suggested to be involved in coral bleaching (Dunn et al., 2007; Weis, 2008) as well as white syndromes (Ainsworth et al., 2007), and increased levels of TRAFs have recently been found in corals under heat stress conditions (Barshis et al., 2013).

6.5.3.5 - Cytokine signalling and regulation

TLR and TNFR signalling lead to the activation of various MAPK (JNK, p38, Erk1/2) pathways, as well as the NFκB pathway, which regulate the expression of effector immune mechanisms, cell survival and the production of pro-inflammatory cytokines (Fig. 19(II)) (Newton and Dixit, 2012). In turn, these cytokines (interleukins IL-1β, -6, -8, -12, -18 and TNFα) also have a range of immunostimulatory effects resulting in immune cell activation and the induction of other immune mechanisms, as well as a positive and negative feedback on cytokine expression (Fig. 19(III)). Most cytokines are

produced in a pro-form, however, and additional stimuli are required for release. For example, TLR, NLR and TNFR signalling induces transcription of pro-IL-1β (Netea et al., 2008) and the NLRP3 inflammosome (NACHT, LRR and PYD domains−containing protein 3) (Qiao et al., 2012), but only when NLRP3 detects MAMPs will it activate IL-1β (Gross et al., 2011). Similarly, the metalloprotease meprin A increases IL-1β (Herzog et al., 2009), although it inactivates IL-6 via hydrolysis (Keiffer and Bond, 2014).

To date, little is known about the role of cytokines in corals and only TNF (Barshis et al., 2013; Weiss et al., 2013; Quistad et al., 2014) and eicosanoid cytokine (Libro et al., 2013; Lohelaid et al., 2014) pathways have been shown to be present. In my study, NLRP3 was upregulated, while meprin A was downregulated under heat stress, showing the tight regulation of expression of genes with apparent overlapping functions. Cytokine signalling at elevated temperatures was also potentially mediated through histamine receptors, which showed increased expression and are known to enhance IL-1β-induced IL-6 production, but suppress IL-12 (O'Mahony et al., 2011). In addition, cytokine signalling was regulated via interleukin-binding proteoglycans, such as perlecans, that can limit interleukin availability (Miller et al., 2007b). Another example is TNF, which also plays a role in the initiation of the anti-viral interferon response by inducing the expression and activation of interferon regulatory factor (IRF) 1 resulting in the production of type I interferons (Yarilina et al., 2008). In addition, IL-12 and -18 regulate the expression of the type II interferon IFNγ (Puddu et al., 1997; Dinarello et al., 1998), which signals via its receptor and the JAK-STAT pathway and induces expression and activation of IRF1 and IRF8. The transcription factor IRF1 regulates a range of immune effectors, such as iNOS, type I interferons, stimulatory and inhibitory interleukins, cathepsins, caspases and IRF2 (Schroder et al., 2004). While IRF2 competitively inhibits IRF1 function (Harada et al., 1989), IRF1 and IRF8 act together to induce immune cell-mediated inflammation responses (Paun and Pitha, 2007). In this study, I observed increases in both IRF1 and IRF8 expression, and a decrease in IRF2, suggesting a significant function of the interferon pathways in the immune response in *M. aequituberculata* under heat stress that could indicate the involvement of viruses in an infection.

6.5.3.6 - Anti-viral responses

Interferons are the primary innate immune mechanism to combat virus infections (Randall and Goodbourn, 2008). However, viruses can also be eliminated through other mechanisms, such as the RNA interference machinery. The RNAi complex is capable of detecting and subsequently degrading double-stranded RNA

(dsRNA) viruses, as well as suppressing viral gene expression (Aliyari and Ding, 2009). I observed increased levels of Dicer, the dsRNA digesting enzyme, at high temperatures, suggesting there may be an anti-viral response, although it cannot be excluded that the elevated expression of Dicer is related to a microRNA-mediated regulation of the immune response (Lindsay, 2008) and/or other processes. An antiviral response is also suggested from the increased expression of ATRX, a DNA structure modifying enzyme involved in repression of the lytic cycle following viral infection (Lukashchuk and Everett, 2010), at medium heat stress, but downregulation at 32°C. This may indicate that *M. aequituberculata* is unable to respond adequately to viral infections at high temperatures. Supportive of an active anti-viral immune response at higher temperatures, however, is an increased level of expression of the cytochrome P450 family member CYP3A, which is capable of converting cholesterol into the antiviral factor 25-hydroxycholesterol (Croyle, 2009, Honda et al., 2011), as well as the upregulation of TLR4, which is known to be involved in the response to viral infections (Lester and Li, 2014).

6.5.3.7 - Complement system

The complement system is a highly conserved immune effector mechanism that can be initiated via three pathways: 1) the classical pathway, 2) the lectin pathway, and 3) the alternative pathway (Gadjeva, 2014). The classical and lectin pathways are activated following the binding of ficolins or lectins, respectively, to pathogens and result in the activation of a C3 convertase complex that cleaves complement C3 into C3a and C3b (Fig. 6.10(VII)). When deposited, C3b has opsonin properties and facilitates the phagocytosis of the C3b-marked microbe (Fig. 6.10(IX)). Binding of ficolins to the receptor calreticulin also induces phagocytosis (Lu et al., 2002). Phagocytosis is the process in which microbes are engulfed by immune cells leading to their lysosomal destruction. In this study, I found significant upregulation of several lectins and ficolins. as well as complement C3 and calreticulin. In addition, expression of the scavenger receptor deleted in malignant brain tumor 1 (DMBT1/gp340), which binds various microbes including bacteria and viruses and activates the lectin complement pathway (Madsen et al., 2010; Leito et al., 2011), was significantly elevated.

6.5.3.8 - Phagocytosis

Various genes potentially involved in phagocystosis (Fig. 6.10(IX)), including PIK3C2, Rho, TRIO, Rac1, WASP, profilin (actin reorganisation), Hrs, VAMP7 and CD63 (phagosome trafficking and maturation), as well as a nitrous oxide synthase

(NOS) (oxidative burst, microbe elimination) (Fig. 6.10(X)) were upregulated in this study (Bugyi and Carlier, 2010; Flannagan et al., 2012; Schmidt and Debant, 2014), although FGD5/6, a regulator of WASP-activating cdc42 (Kurogane et al., 2012; Steenblock et al., 2014), was downregulated. In addition, two bacteria-aggregating and phagocytosis-inducing scavenger receptors, macrophage receptor with collagenous structure (MARCO) (Kraal et al., 2000) and DMBT1 (Madsen et al., 2010), were upregulated. This shows that the anti-microbial defense in *M. aequituberculata* depends significantly on phagocytosis, with a potential role for the complement system.

6.5.3.9 - Immune cell activation

Phagocytosis requires the prior activation of immune cells. Besides detection of MAMPs, immune cells require other factors for full activation. In support of a cellular immune response, several genes potentially involved in immune cell activation and function, were found to be significantly upregulated under heat stress. These include the 5-hydroxy tryptamine (serotonin) receptor 4 (Baganz and Blakely, 2012), calcineurin/protein phosphatase 3 (Feske et al., 2003), agrin (Mazzon et al., 2012) and cytochrome P450 27A (Nagy et al., 2012). Since reorganisation of the actin skeleton is essential for cell migration, the observed upregulation of Rho, TRIO, Rac1, WASP and profilin could also indicate increased immune cell migration to sites of potential infection or lesion repair (Raftopoulou and Hall, 2004; Bugyi and Carlier, 2010; Schmidt and Debant, 2014), although FGD5/6 was downregulated (Kurogane et al., 2012; Steenblock et al., 2014). This indication is reinforced by the upregulation of Mrp4, which has been shown to be important for immune cell migration (van de Ven et al., 2008), and of ankyrin, which connects the spectrin skeleton with CD45 and thereby plays a role in cell adhesion and migration, as well as promotes Rac1 signalling (Bourguignon et al., 2000).

6.5.3.10 - Hypoxia

Phagocytes that move toward a site of infection experience an increasing hypoxic environment. Hypoxic conditions cause the activation of hypoxia-inducible factor (HIF) via the inhibition of HIF prolyl 4-hydroxylase (Zinkernagel et al., 2007). The transcription factor HIF subsequently induces the expression of pro-inflammatory cytokines, release of microbicidal molecules and enhances phagocytosis (Zinkernagel et al., 2007). Increased HIF activity is highly likely in my study, due to the observed downregulation of HIF prolyl 4-hydroxylase. However, a potential negative feedback loop may also be present, as Ras-like without CAAX 1 (ROC1), a negative regulator of HIF (Kamura et al., 2000), was upregulated.

6.5.3.11 - Coagulation and other immune effector processes

Other processes that were affected were the coagulation with upregulation of a coagulation factor V homologue, but downregulation of factor VIII, as well as the circadian cycle due to the downregulation of cryptochrome (see below for its role in immune function). Despite the significant immune responses observed and described above, I surprisingly found that various defense-related proteins of unknown function were downregulated at elevated temperatures. Similarly, histones H1 and H2 were downregulated, which could indicate a general increase in transcription due to loosening of the chromatin structure, but also reduced expression of antimicrobial peptides, as these histones have been found to be AMP precursors (Kawasaki and Iwamuro, 2008). In contrast, other immune effector enzymes, such as flavin-containing monooxygenase (FMO) and myeloperoxidase (MPO), involved in the production of reactive antimicrobial compounds, were highly upregulated. Whereas FMO detoxifies xenobiotics and simultaneously produces hydrogen peroxide (Siddens et al., 2014), myeloperoxidase (MPO) may be stored in granules of immune cells and released upon immune stimulation, subsequently oxidising chloride and other halide ions in the presence of hydrogen peroxide, thereby generating the highly microbicidal hypochlorous acid and other highly reactive compounds (Davies, 2011).

6.5.3.12 - Immune response modulation

To elicit an appropriate response to infections, the immune response requires modulation through negative and positive feedback loops. For example, TLR signalling also induces expression of Jagged, a ligand of Notch, which initially cooperates with TLR in pro-inflammatory cytokine production (IL-6, IL-12 and TNF) (Hu et al., 2008). At later phases, Notch signalling inhibits TLR-induced NFκB activation (Zhang et al., 2012b) and cytokine expression via its target genes Hes1 and Hey1. However, this can in turn be abrogated by interferon-γ (Hu et al., 2008), allowing tight regulation of the TLR-mediated immune response. This mechanism of TLR signalling may also be active in corals under heat stress, since I found overall increases in the expression of homologs of Jagged and Notch. Another important immunomodulatory mechanism is the transforming growth factor (TGF) β signalling pathway (Worthington et al., 2012). TGFβ has primarily been recognised as an anti-inflammatory cytokine, although some pro-inflammatory effects, depending on the context, have also been discovered. The upregulation of latent TGFβ-binding proteins and downregulation of TGFβ-activating metalloprotease function at 32°C suggests a reduced availability of the active form of this cytokine and thereby reduced anti-inflammatory modulation. Other indicators of

regulatory negative feedback loops were the downregulation of the transcription factor Elk-1, which is generally activated by MAPK signalling and directly or indirectly regulates the expression of various immune genes, including TLR9, as well as many cytokines and genes involved in cell migration (Takeshita et al., 2004a; Kasza, 2013), and the upregulation of some serine protease inhibitors (serpins) at high temperatures. Serpins regulate the activation of the serine protease cascades that lead to activation of the complement system (Jiang et al., 2001), melanisation cascade (De Gregorio et al., 2002) and coagulation process (Toubarro et al., 2013).

6.5.3.13 - Coral calcification

In line with a previous study on chronically heat stressed corals, I observed strong downregulation of SLC26 (solute carrier family 26) members, as well as a carbonic anhydrase (Kenkel et al., 2013). While carbonic anhydrases catalyse the formation of bicarbonate and are known to be involved in coral calcification (Moya et al., 2008), SLC26 proteins are putatively involved in coral calcification, given their functional role in bicarbonate (HCO_3^-) transport (Soleimani and Xu, 2006). Downregulation of these genes could indicate that the calcification rate in *M. aequituberculata* is severely reduced under heat stress.

6.5.4 - Transcriptomic response to bacterial challenge

Analysis of the differential regulation of biological processes in response to bacteria revealed that corals are highly effective at distinguishing potentially beneficial from harmful bacteria. While the commensal *Oceanospirillales* sp. improved metabolic processes, there was a trend for an immune response towards the potential pathogen *V. coralliilyticus*. This clear difference in responses suggests that corals can distinguish pathogenic bacteria from non-pathogenic bacteria. While the freshwater cnidarian *Hydra* is known to regulate its bacterial community using TLR-mediated AMP expression (Fraune and Bosch, 2007; Fraune et al., 2010; Franzenburg et al., 2012; Franzenburg et al., 2013), further experimental investigations are required to elucidate how corals potentially facilitate this process.

While there was a clear trend of an immune response towards *V. coralliilyticus*, the anti-bacterial response of the coral host still appeared relatively modest, with only 11 genes being significantly differentially expressed. Protein phosphatase-1 (*PP1*) was downregulated in response to bacterial challenges. This enzyme has various physiological functions (Ceulemans and Bollen, 2004) and reduced PP1 expression may potentially lead to increased transcription via eIF2α activation and increased immediate energy availability through reduced glycogen synthesis. In addition, PP1

inhibits the Erk2, MAPK p38 and NFκB pathways (Saxena et al., 1999; Nika et al., 2004; Jin et al., 2011), which play major roles in the immune response. Reduced PP1 expression may therefore result in higher activity of these pathways, leading to increased immune function, including enhanced phagocytosis and oxidative burst activation via Erk2 signalling and immune response regulation via NFκB-induced pro-inflammatory cytokine production, as well as cell proliferation and survival (Hayden and Ghosh, 2012). Interestingly, I show that two genes involved in the circadian cycle (cryptochrome and CLOCK) may also have an immune response function in corals, which has only recently been described for mice and humans (Curtis et al., 2014; Narasimamurthy et al., 2012). Cryptochrome, a repressor of expression of the *CLOCK* gene, was downregulated in response to bacterial challenges, resulting in the upregulation of *CLOCK*. Reduced levels of cryptochrome have previously been linked to increased expression of pro-inflammatory cytokines (IL-6, TNFα and CLCX-1) and inducible nitric oxide synthase (iNOS), which plays a role in the anti-microbial oxidative burst following phagocytosis or encapsulation (Curtis et al., 2014). In addition, CLOCK represses the anti-inflammatory function of the glucocorticoid receptor, enhances the activity of NFκB and positively regulates the expression of Toll-like receptors (e.g. TLR9) and their downstream transcription factors (FOS and JUN) (Scheiermann et al., 2013, Curtis et al., 2014). To orchestrate a proper immune response, TLR signalling is tightly regulated via the interferon-γ and Notch signalling pathways (Hu et al., 2008). Notch signalling is crucial in embryogenesis and the development of immune cells (Radtke et al., 2010; Yuan et al., 2010). My results showed an increased expression of the Notch target gene HEY, which selectively regulates expression of several interleukin cytokines (Hu et al., 2008), in response to bacterial challenge. I also found increased expression of a pathogenesis-related protein 1 that may exhibit antimicrobial properties (Gibbs et al., 2008). Overall, these results show that the transcriptomic response against *V. coralliilyticus* correlates with a broad immune response driven by TLR and NFκB signalling and also includes pro-inflammatory cytokine production, phagocytosis and the oxidative burst.

6.5.5 - The melanisation cascade

The melanisation cascade (Fig. 6.10(III)-(IV)) has been used as a proxy for coral immunity since its discovery in both gorgonian and scleractinian corals (Mydlarz et al., 2008; Palmer et al., 2008). PO activity is differentially regulated among coral species, however, depending on the stressors (Chapter 2; (Palmer et al., 2010)). For example, bleached colonies of *Montastrea faveolata* upregulate PO activity (Mydlarz et al., 2009), but have reduced melanin content when under heat stress (Palmer et al.,

2011b); while *Acropora millepora* has decreased PO activity when bleached (Palmer et al., 2011a). The disease-resistant coral *Porites astreoides* exhibits an immune response involving PO when exposed to PAMPs under heat stress (Palmer et al., 2011b). My results show that the capacity of tpPO activity in *M. aequituberculata* is not affected by increased temperatures, and that the amount of PO activated in response to bacteria depends on the seawater temperature. Both PO and tpPO activity decreased in corals challenged with *V. coralliilyticus* at 29.5°C and 32°C. This may indicate that *V. coralliilyticus* actively interferes with the melanisation cascade when virulent at high temperatures. Inhibition of this innate immune system component is a commonly employed mechanism by pathogens to evade the host immune response (Beck and Strand, 2007; Eleftherianos et al., 2007; Lu et al., 2008; Colinet et al., 2009). A member of the solute carrier family 16 (SLC16A10), which transports the PO substrate L-DOPA (Kim et al., 2002a), was found upregulated at 32°C and could indicate increased melanisation as part of the immune response, or with melanin potentially functioning as an antioxidant.

6.5.6 - GFP-like proteins

GFP-like proteins have also been implicated in various physiological processes. For example, they have photoprotective properties (Salih et al., 2000; Dove et al., 2001; Dove et al., 2008; D'Angelo et al., 2012; Smith et al., 2013) that can protect corals from bleaching or light damage to regenerating tissues (D'Angelo et al., 2012). However, this function has been debated (Mazel et al., 2003). GFP-like proteins also possess antioxidant capacity (Bou-Abdallah et al., 2006; Palmer et al., 2009a), which may play a role in mitigating the adverse effects of reactive oxygen species produced by the immune system (Mydlarz and Harvell, 2007; Palmer et al., 2009b), or heat stress-induced damage to the photosystems of the coral endosymbiont *Symbiodinium*. I therefore anticipated an increase in the expression of GFP-like proteins. In my study, however, I did not observe any significant changes in total fluorescence, GFP or chromoprotein expression, and only minor effects on the proportion of cyan and red FP, which were primarily time-dependent. Overall, this shows that GFP-like proteins are not involved in the response of *M. aequituberculata* to bacterial challenges or heat stress, suggesting that other coral antioxidant mechanisms (Shinzato et al., 2012) may be sufficient in neutralising ROS.

6.5.7 - The effect of the bacterial and temperature stressors on the coral holobiont

Overall, the *M. aequituberculata* holobiont was significantly affected by both elevated seawater temperatures and potentially pathogenic bacteria. Despite adverse

effects on its homeostasis, the coral exhibited a significant immune response towards the coral pathogen *V. coralliilyticus*, but also potentially in response to the heat stress-induced change in bacterial community. These responses were sufficient to prevent the development of disease over the course of this study, although a significant impact of the pathogen on *Symbiodinium* photosynthesis was evident. The immune response observed in *Symbiodinium* suggests that this endosymbiont may also play a role in the holobiont defence against infections and we may need to consider the "holobiont immune response" rather than just the coral immune response when assessing the disease resistance of corals.

The general lack of disease development in my study was surprising. The *V. coralliilyticus* P1 strain had been isolated from colonies of *M. aequituberculata* exhibiting white syndrome signs and used to successfully infect healthy colonies of *M. aequituberculata* with the isolated strain, thereby fulfilling Koch's postulates and identifying this bacterium as the causative agent of this disease (Sussman et al., 2008). To make sure that culturing would not affect properties of *V. coralliilyticus*, I conducted my study using the primary isolate of this strain and sourced my corals from the same population. Despite these precautions, I was unable to cause disease in healthy corals. I therefore hypothesise that this *M. aequituberculata* population has developed some degree of resistance to this coral pathogen, which could be the result of the selective elimination of white syndrome-sensitive corals from the population, adaptation or potentially immunological memory. However, I cannot exclude the possibility that this bacterium has reduced virulence, because of the long term in storage. It would be very promising for the future of coral reefs if coral populations are indeed able to develop resistance to diseases.

6.5.8 – Comparative immunology

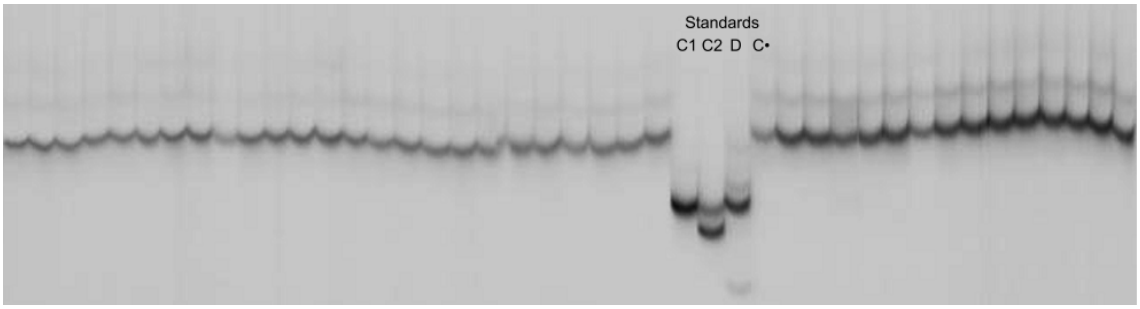
Montipora aequituberculata is a moderately stress-tolerant coral and showed a strong immune response when experiencing significant heat stress and the capacity to distinguish non-pathogenic *Oceanospirilales* sp. from the coral pathogen *V. coralliilyticus* regardless of temperature. My results are in stark contrast with recent observations by Vidal-Dupiol *et al*. (Vidal-Dupiol et al., 2014), where the disease and bleaching susceptible coral *Pocillopora damicornis* was found to exhibit an immune response to this pathogen under normal conditions (25°C), but this response was severely impaired under elevated seawater temperatures (32°C), resulting in disease and mortality (Vidal-Dupiol et al., 2011b). In addition, the immune response reported for *P. damicornis* encompassed the TLR signalling pathway, melanisation cascade, complement system, leukotriene cascade, antioxidants and several antibacterial

compounds, with the melanisation cascade appearing to play a primary role in the response to *V. coralliilyticus* at high temperatures (Vidal-Dupiol et al., 2014). In *M. aequituberculata*, the melanisation cascade was impaired in corals exposed to *V. coralliilyticus* under heat stress, but this coral was shown to possess a large suite of immune mechanisms that was not negatively impacted by temperature and was capable of preventing the development of disease. Taken together, this shows the comparative differences between these two species in: 1) the capacity of their immune systems, 2) the impact of temperature on the immune systems, and 3) disease susceptibility as a consequence of divergent stress tolerance levels.

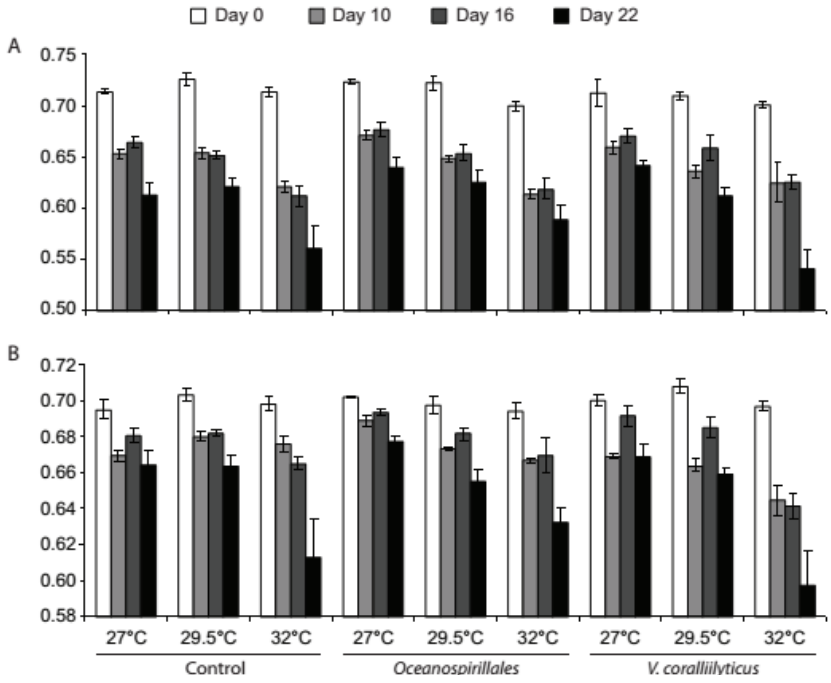
6.6 - Conclusion

Here, I assessed three components of the holobiont of the coral *Montipora aequituberculata* exposed to elevated seawater temperatures and pathogenic bacteria, and found that, despite being affected by heat stress, the coral was capable of orchestrating a strong immune response towards the pathogenic bacterium and a shift in the bacterial assemblages at high temperatures. These responses appeared to be sufficient to prevent the development of visual signs of disease and tissue loss. Overall, my results show that corals can effectively distinguish between bacterial species, suggesting active regulation of the microbial community by the coral host, and that the moderately heat tolerant coral *M. aequituberculata* is able to defend itself against potentially pathogenic bacteria under short-term heat stress.

6.7 - Supplementary figures

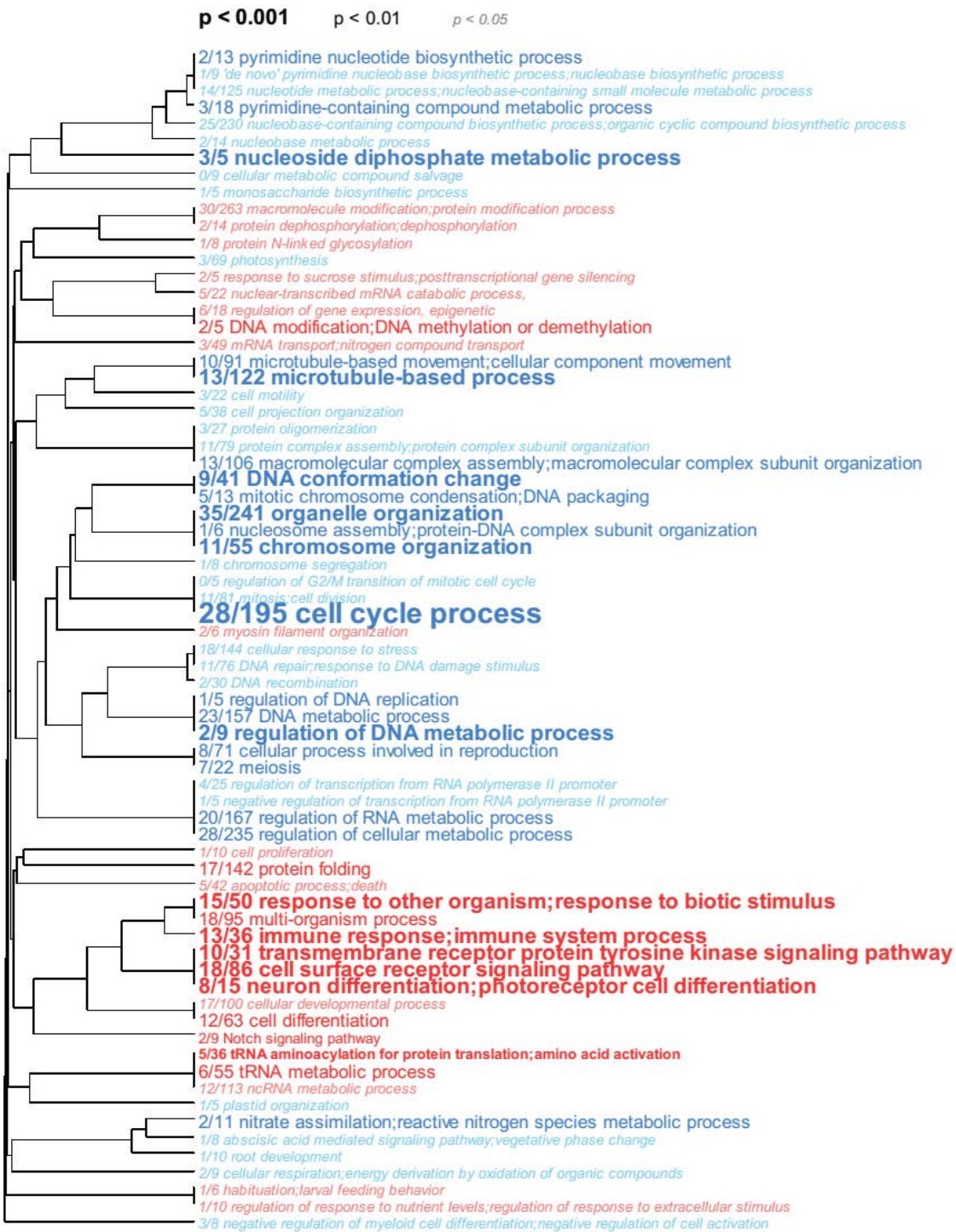


Supplementary figure 6.1 – *Symbiodinium* genotype. A subset of coral fragments (n = 39) was analysed for the genotype of *Symbiodinium* harboured within tissues of *Montipora aequituberculata,* along with known standards using Single Strand Conformation Polymorphism analysis of the nuclear ribosomal DNA internal transcribed spacer 1 (rDNA ITS1) region.

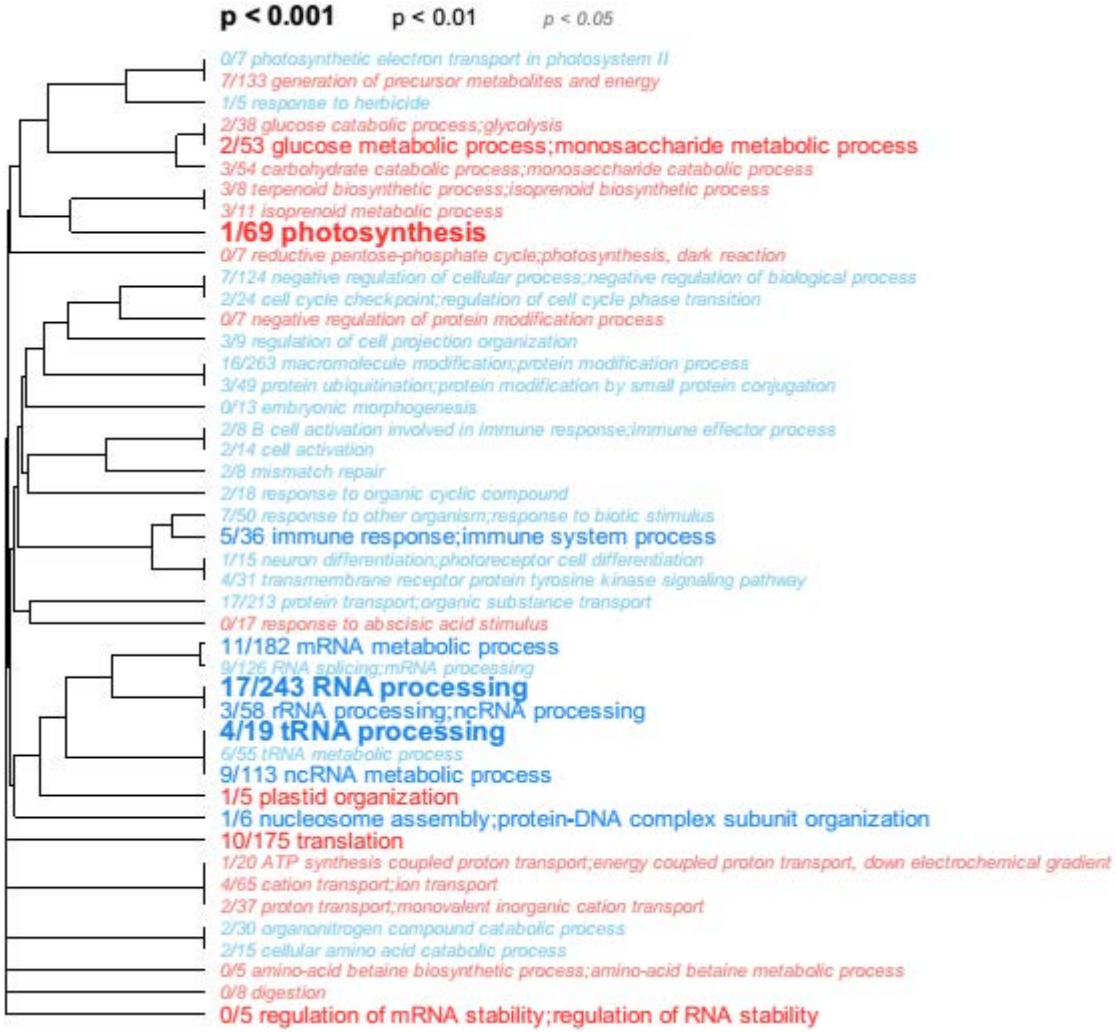


Supplementary figure 6.2 – Photochemical capacity of corals subjected to combinations of elevated seawater temperatures and bacterial challenges. Time series of selected time points, for: A) mean maximum quantum yield (Fv / Fm) and B) mean effective quantum yield (Y(II)) measured every 48 hours using pulse amplitude-modulated fluorometry.

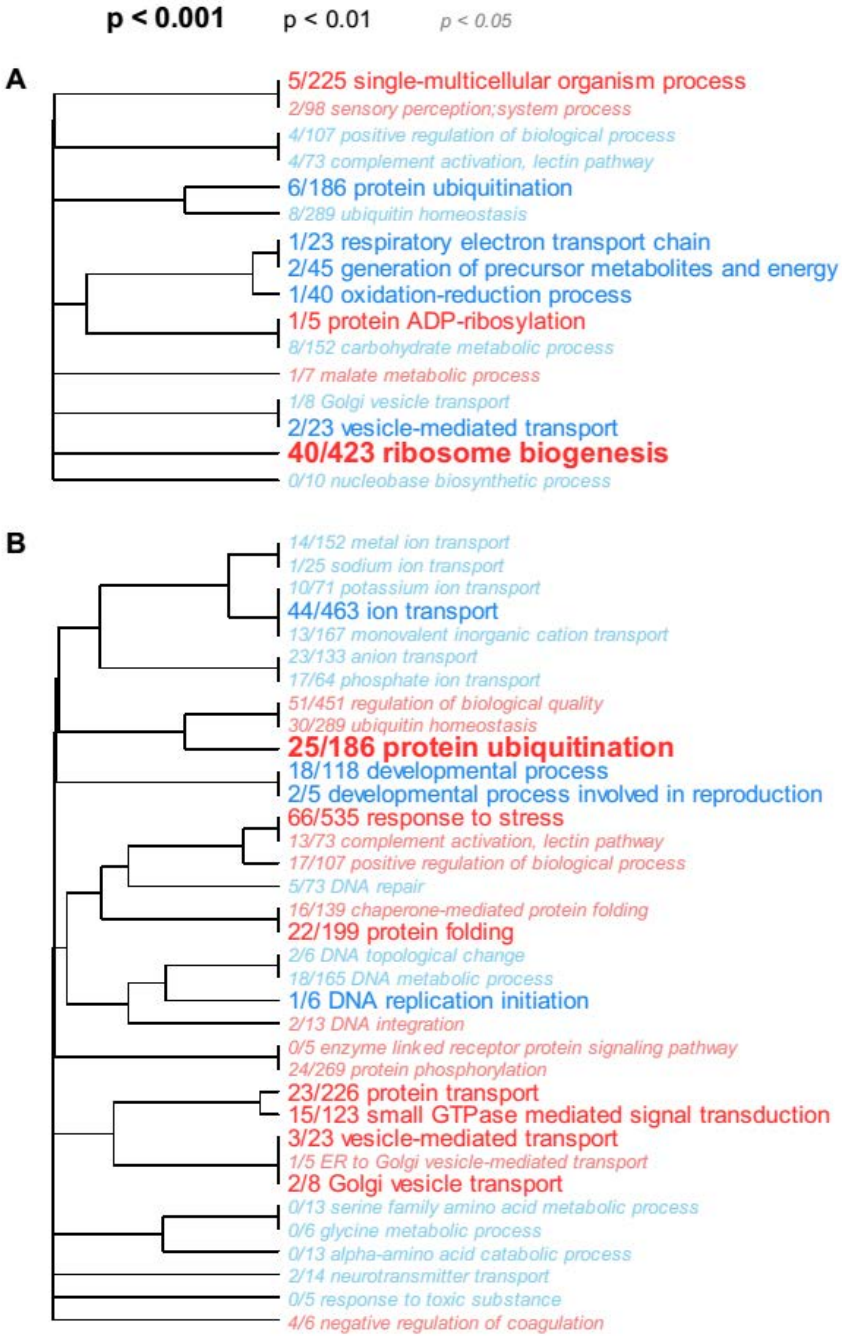
Supplementary figure 6.3 – Effect of temperature stress on biological processes of *Symbiodinium*. Overview of biological processes (Gene Ontology) that are differentially activated (red) or inhibited (blue) in response to elevated seawater temperatures at 32°C. Level of significance is $p < 0.05$.



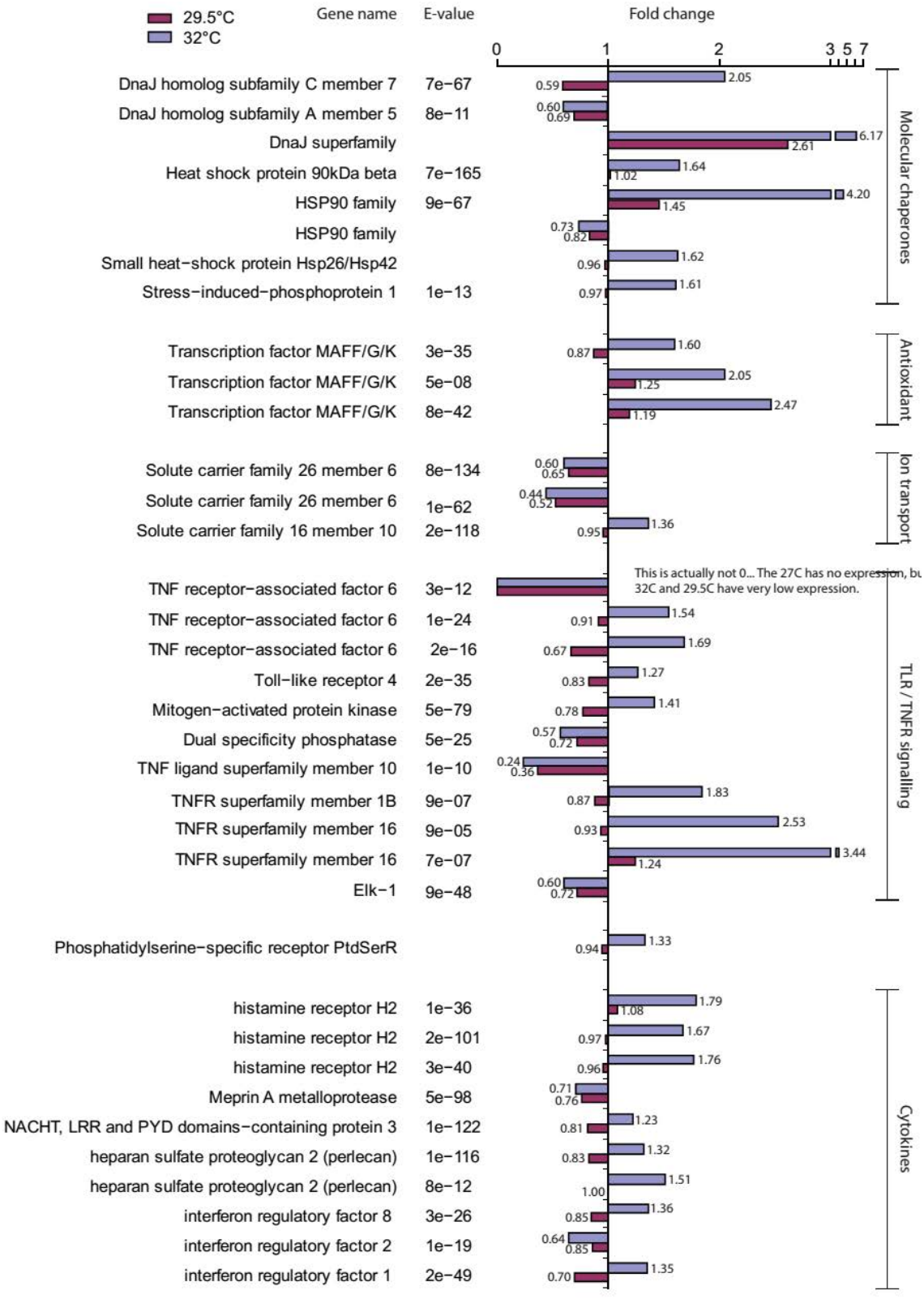
Supplementary figure 6.4 – Effect of temperature stress on biological processes of *Symbiodinium*. Overview of biological processes (Gene Ontology) that are differentially activated (red) or inhibited (blue) in response to elevated seawater temperatures at 32°C. Font size indicates level of significance. Analysis was conducted based on unadjusted p-values and results should therefore be considered trends.



Supplementary figure 6.5 – Effect of temperature stress on biological processes of *Symbiodinium*. Overview of biological processes (Gene Ontology) that are differentially activated (red) or inhibited (blue) in response to elevated seawater temperatures at 29.5°C. Font size indicates level of significance. Analysis was conducted based on unadjusted p-values and results should therefore be considered trends.



Supplementary figure 6.6 – Effect of temperature stress on biological processes of *Symbiodinium*. Overview of biological processes (Gene Ontology) that are differentially activated (red) or inhibited (blue) in response to elevated seawater temperatures A) at 29.5°C and B) at 32°C. Font size indicates level of significance. Analysis was conducted based on unadjusted p-values and results should therefore be considered trends.


29.5°C
32°C
Gene name
E-value
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0
1
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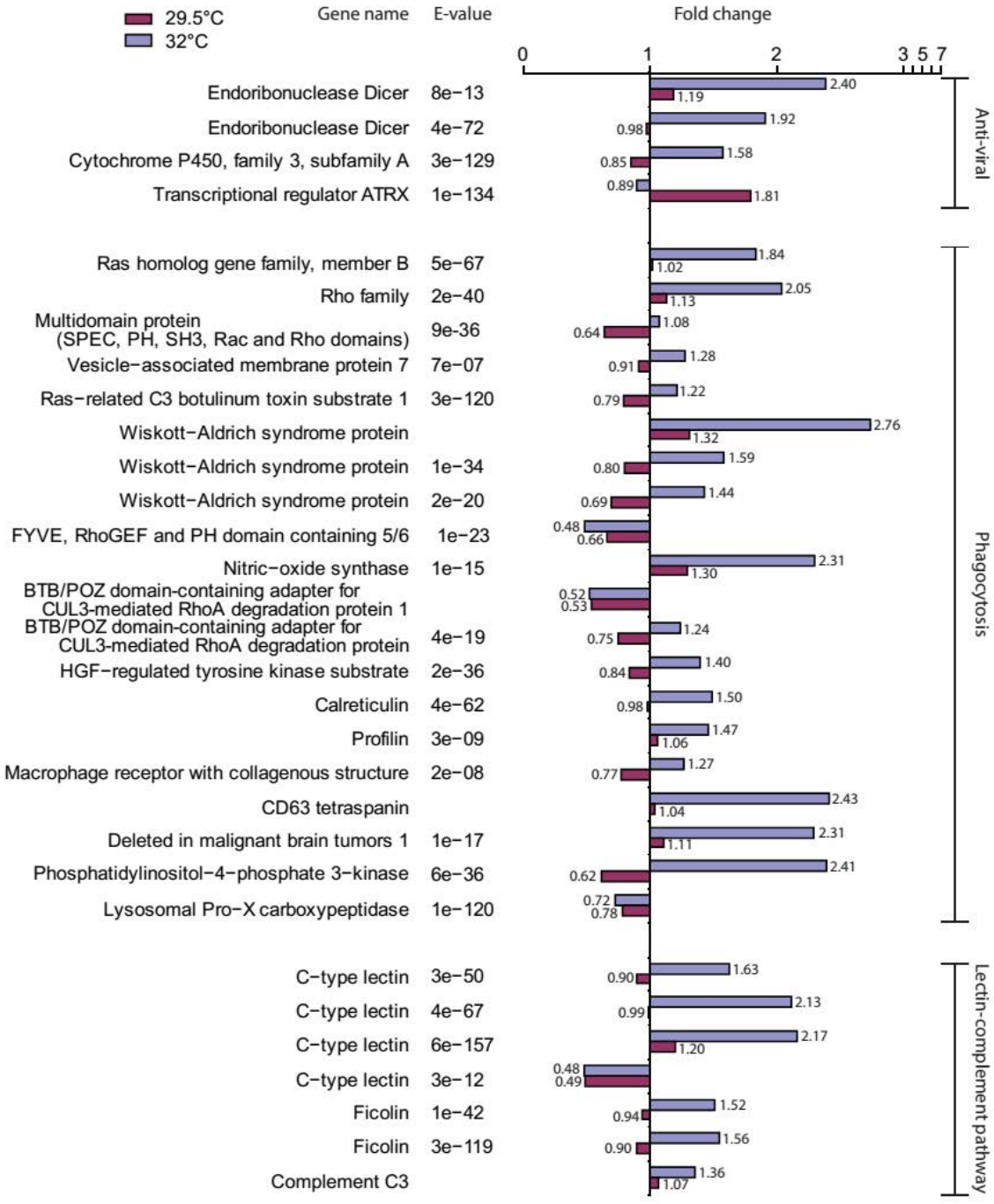
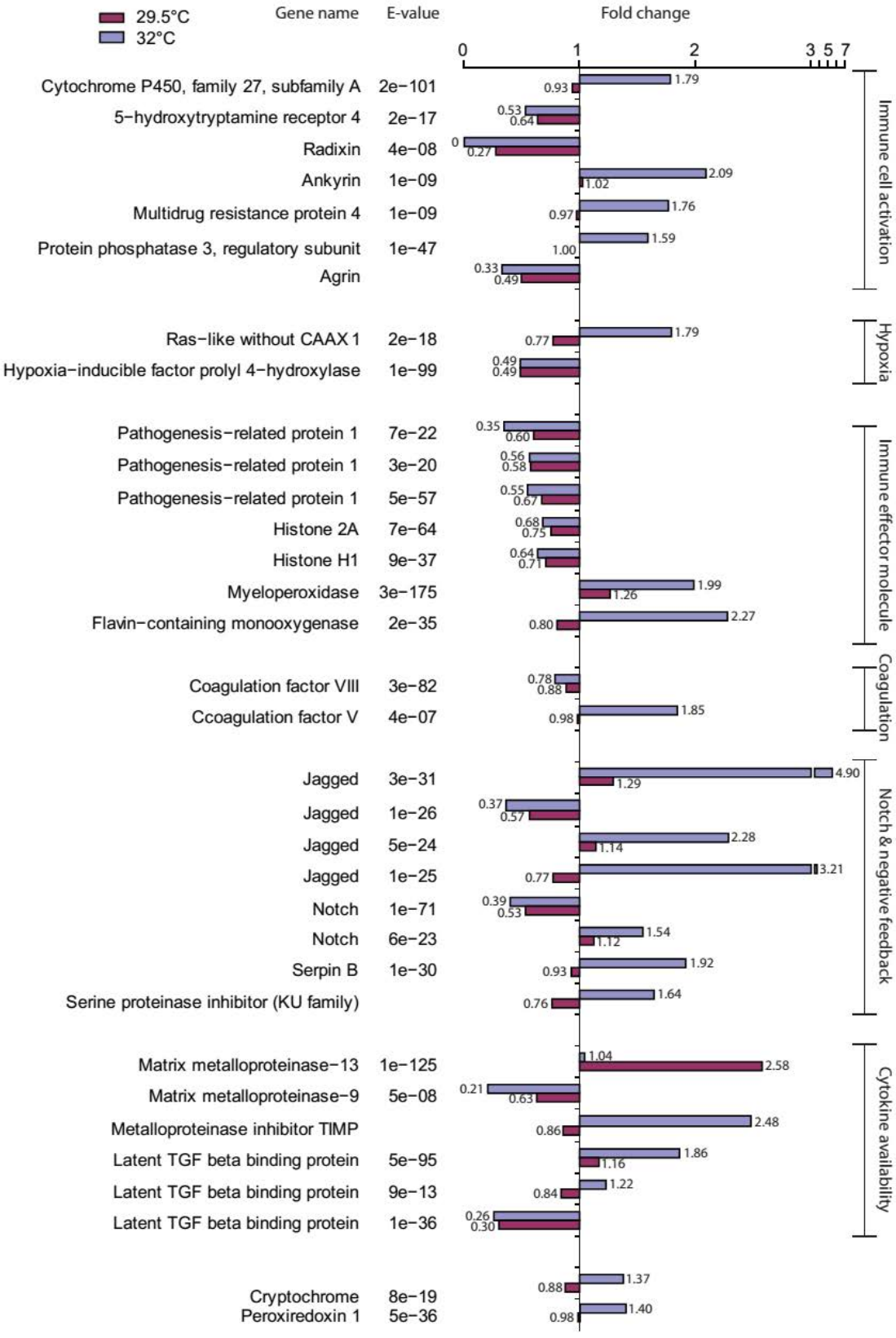
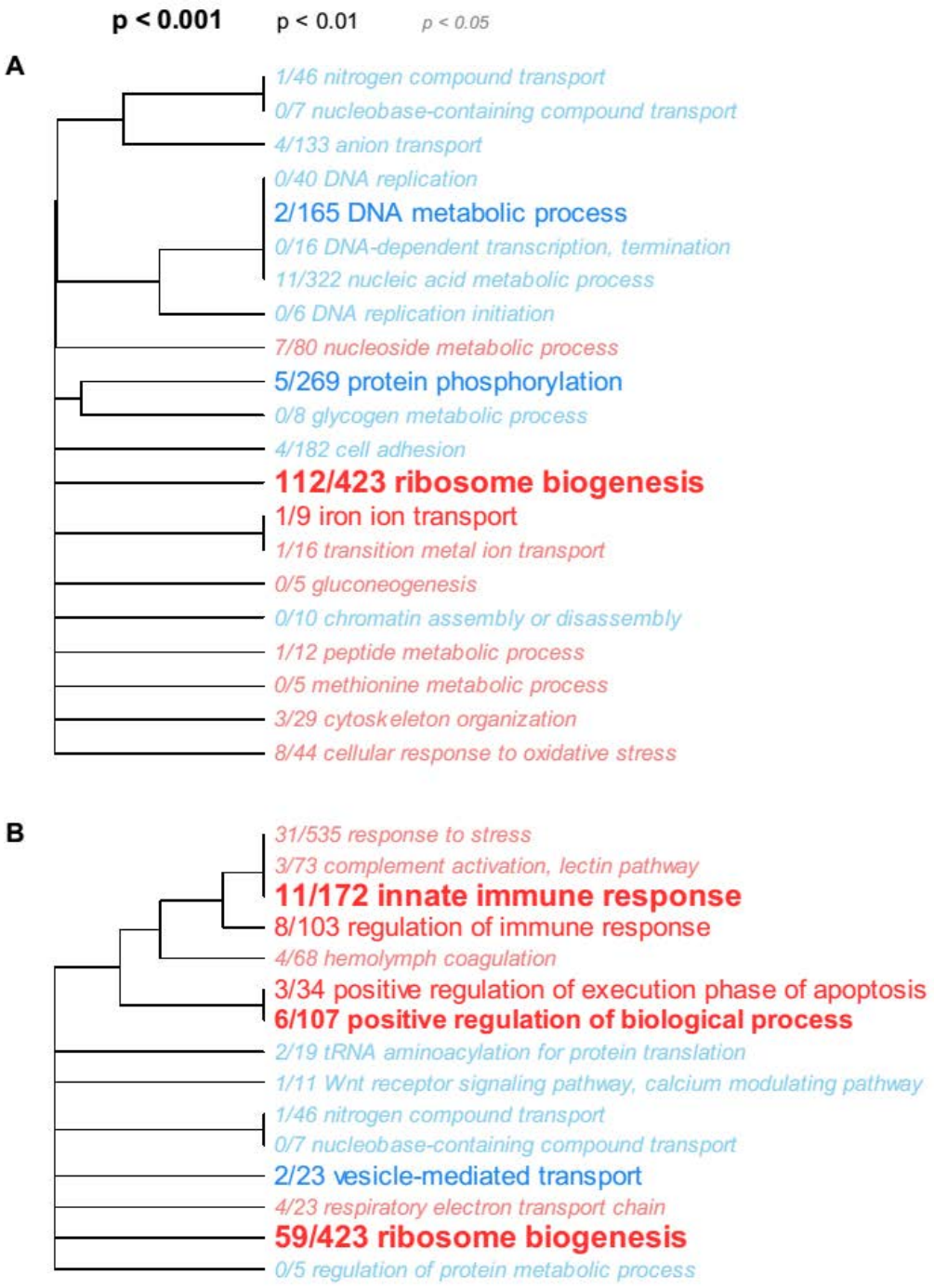
3 5 7
DnaJ homolog subfamily C member 7 7e−67
DnaJ homolog subfamily A member 5 8e−11
DnaJ superfamily
Heat shock protein 90kDa beta 7e−165
HSP90 family 9e−67
HSP90 family
Small heat−shock protein Hsp26/Hsp42
Stress−induced−phosphoprotein 1 1e−13
Transcription factor MAFF/G/K 3e−35
Transcription factor MAFF/G/K 5e−08
Transcription factor MAFF/G/K 8e−42
Solute carrier family 26 member 6 8e−134
Solute carrier family 26 member 6 1e−62
Solute carrier family 16 member 10 2e−118
TNF receptor−associated factor 6 3e−12
TNF receptor−associated factor 6 1e−24
TNF receptor−associated factor 6 2e−16
Toll−like receptor 4 2e−35
Mitogen−activated protein kinase 5e−79
Dual specificity phosphatase 5e−25
TNF ligand superfamily member 10 1e−10
TNFR superfamily member 1B 9e−07
TNFR superfamily member 16 9e−05
TNFR superfamily member 16 7e−07
Elk−1 9e−48
Phosphatidylserine−specific receptor PtdSerR
histamine receptor H2 1e−36
histamine receptor H2 2e−101
histamine receptor H2 3e−40
Meprin A metalloprotease 5e−98
NACHT, LRR and PYD domains−containing protein 3 1e−122
heparan sulfate proteoglycan 2 (perlecan) 1e−116
heparan sulfate proteoglycan 2 (perlecan) 8e−12
interferon regulatory factor 8 3e−26
interferon regulatory factor 2 1e−19
interferon regulatory factor 1 2e−49
0.59
2.05
0.60
0.69
6.17
2.61
1.64
1.02
4.20
1.45
0.73
0.82
1.62
0.96
1.61
0.97
1.60
0.87
2.05
1.25
2.47
1.19
0.60
0.65
0.44
0.52
1.36
0.95
This is actually not 0... The 27C has no expression, bu
32C and 29.5C have very low expression.
1.54
0.91
1.69
0.67
1.27
0.83
1.41
0.78
0.57
0.72
0.24
0.36
1.83
0.87
2.53
0.93
3.44
1.24
0.60
0.72
1.33
0.94
1.79
1.08
1.67
0.97
1.76
0.96
0.71
0.76
1.23
0.81
1.32
0.83
1.51
1.00
1.36
0.85
0.64
0.85
1.35
0.70
Molecular chaperones
Antioxidant
Ion transport
TLR / TNFR signalling
Cytokines

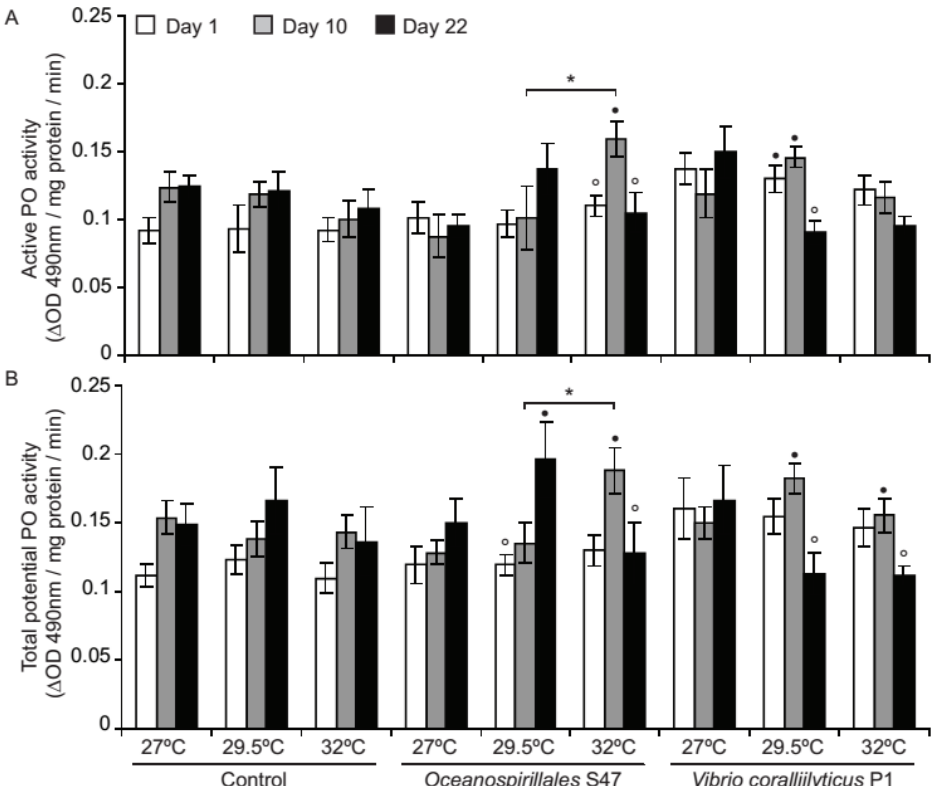

29.5°C
32°C
Gene name
E-value
Fold change
0
1
2
3 5 7
Endoribonuclease Dicer 8e−13 2.40 1.19
Endoribonuclease Dicer 4e−72 1.92 0.98
Cytochrome P450, family 3, subfamily A 3e−129 1.58 0.85
Transcriptional regulator ATRX 1e−134 0.89 1.81
Anti-viral
Ras homolog gene family, member B 5e−67 1.84 1.02
Rho family 2e−40 2.05 1.13
Multidomain protein (SPEC, PH, SH3, Rac and Rho domains) 9e-36 1.08 0.64
Vesicle−associated membrane protein 7 7e−07 1.28 0.91
Ras−related C3 botulinum toxin substrate 1 3e−120 1.22 0.79
Wiskott−Aldrich syndrome protein 2.76 1.32
Wiskott−Aldrich syndrome protein 1e−34 1.59 0.80
Wiskott−Aldrich syndrome protein 2e−20 1.44 0.69
FYVE, RhoGEF and PH domain containing 5/6 1e−23 0.48 0.66
Nitric−oxide synthase 1e−15 2.31 1.30
BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 1 0.52 0.53
BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 4e−19 1.24 0.75
HGF−regulated tyrosine kinase substrate 2e−36 1.40 0.84
Calreticulin 4e−62 1.50 0.98
Profilin 3e−09 1.47 1.06
Macrophage receptor with collagenous structure 2e−08 1.27 0.77
CD63 tetraspanin 2.43 1.04
Deleted in malignant brain tumors 1 1e−17 2.31 1.11
Phosphatidylinositol−4−phosphate 3−kinase 6e−36 2.41 0.62
Lysosomal Pro−X carboxypeptidase 1e−120 0.72 0.78
Phagocytosis
C−type lectin 3e−50 1.63 0.90
C−type lectin 4e−67 2.13 0.99
C−type lectin 6e−157 2.17 1.20
C−type lectin 3e−12 0.48 0.49
Ficolin 1e−42 1.52 0.94
Ficolin 3e−119 1.56 0.90
Complement C3 1.36 1.07
Lectin-complement pathway



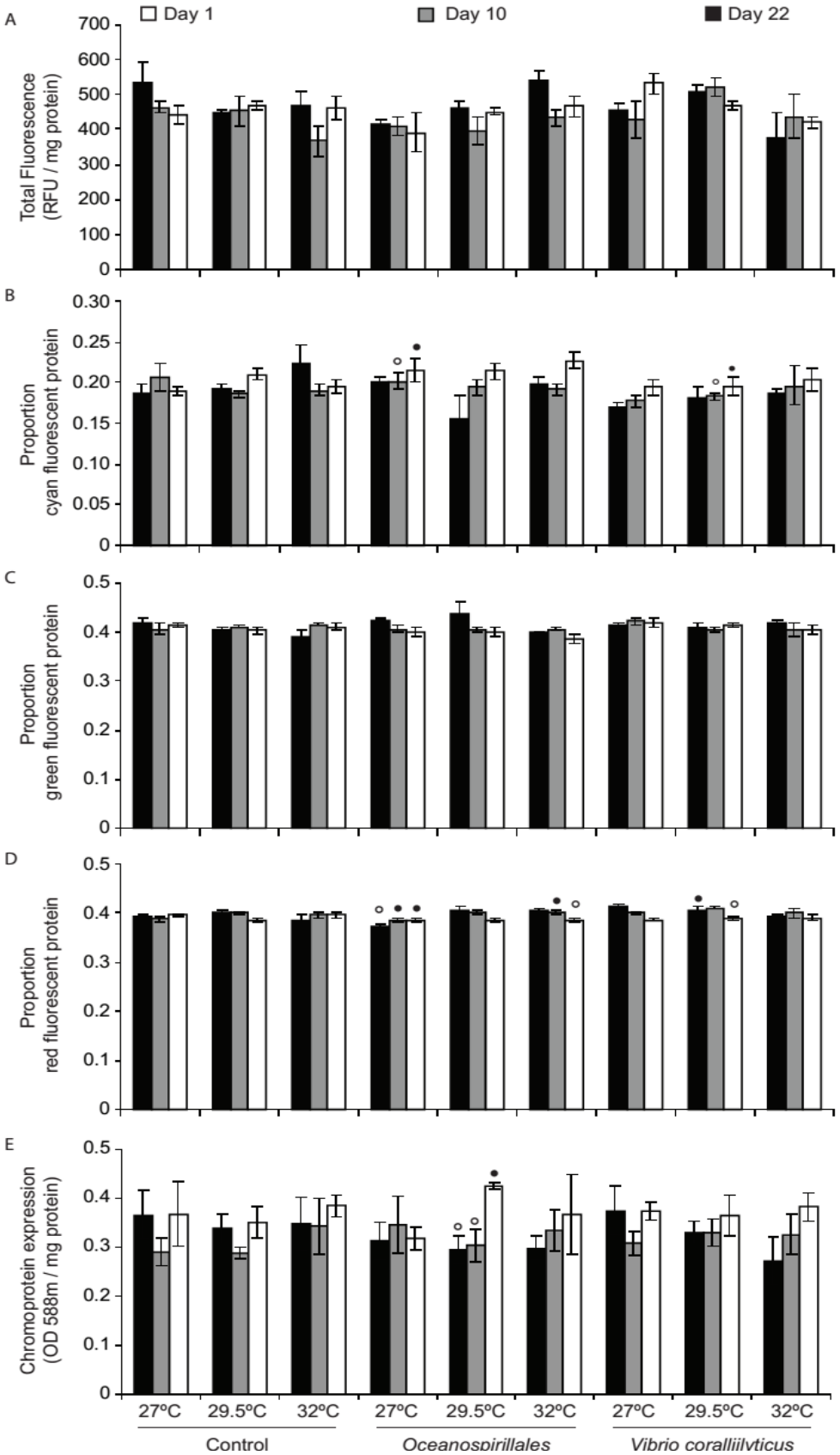
Supplementary figure 6.7 – Fold change in expression of differentially expressed stress and immune response genes following exposure of corals to elevated seawater temperatures. In addition, E-value of best BLAST match to indicate validity of gene name annotation is given.



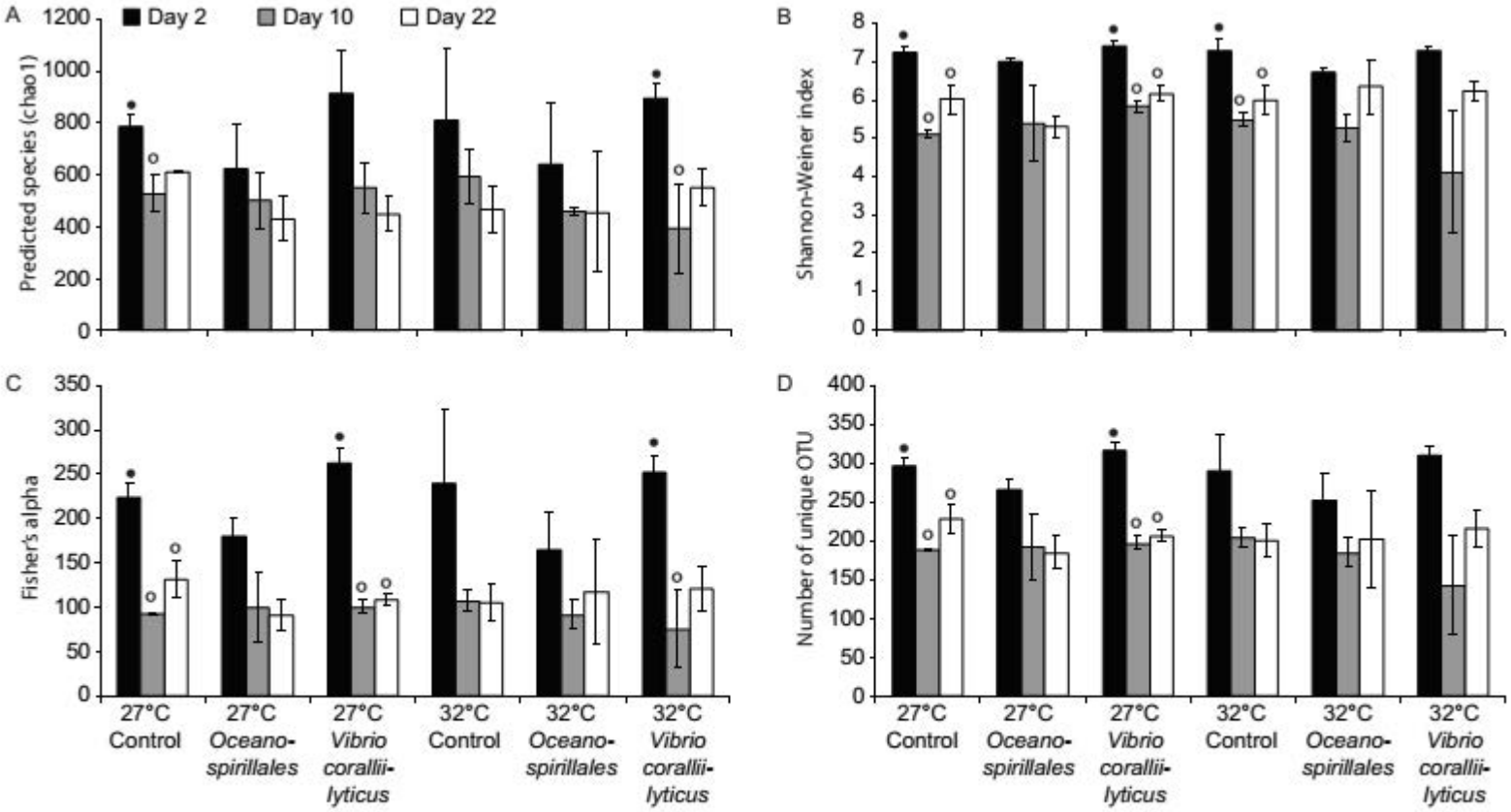
Supplementary figure 6.8 – Effect of bacterial challenges on biological processes. Overview of biological processes (Gene Ontology) that are differentially activated (red) or inhibited (blue) in response to bacterial challenges with A) *Oceanospirillales* S47 or B) *Vibrio coralliilyticus* P1. Font size indicates level of significance. Analysis was conducted based on unadjusted p-values and results should therefore be considered trends.



Supplementary figure 6.9 – Temporal patterns in phenoloxidase activity in response to bacterial challenges under elevated seawater temperatures. Temporal patterns of A) active phenoloxidase (aPO) activity and B) total potential phenoloxidase (tpPO) activity in corals exposed to bacteria (control, *Oceanospirillales* S47 or *Vibrio coralliilyticus* P1) under different seawater temperatures (27°C, 29.5°C or 32°C). Asterisk (*) indicates statistical significance ($p < 0.05$) between treatments at the same time point. Temporal differences between time points within the same treatment are indicated; black circles (•) indicate significantly higher levels than white circles (○).



Supplementary figure 6.10 – Temporal patterns of fluorescence and GFP-like protein following bacterial challenges under elevated seawater temperatures. Temporal patterns of A) total fluorescence, and proportions of B) cyan fluorescent protein, C) green fluorescent protein and D) red fluorescent protein, and E) chromoprotein expression. Black circle (•) indicates statistically significant ($p < 0.05$) higher levels than white circles (○).between time points within the same treatment



Supplementary figure 6.11 – Temporal patterns in alpha diversity of bacterial communities associated with corals exposed to different seawater temperatures (27C and 32C) and bacteria (control, Oceanospirillales S47 or Vibrio coralliilyticus). Alpha diversity metrics A) chao 1, B) Shannon-Weiner index, C) Fisher's alpha and D) number of unique operational taxonomic units are depicted. Temporal differences between time points within the same treatment are shown with black circles (•) indicating significantly higher levels than white circles (○).

6.8 - Supplementary tables

Supplementary Table 6.1 – Comparison of linear regression model coefficients of maximum quantum yield (Fv / Fm) and effective quantum yield (Y(II)).

Linear Mixed Effects Model		**Maximum Quantum Yield**	**Effective Quantum Yield**
		p-value	p-value
Temperature		<0.0001	0.9365
Bacteria		0.1065	0.2170
Time		<0.0001	<0.0001
Temperature:Bacteria		0.0433	0.0012
Temperature:Time		<0.0001	<0.0001
Bacteria:Time		0.8201	0.0385
Temperature:Bacteria:Time		0.3833	0.0030
Control 27°C	Control 29.5°C	0.4008	0.1582
	Control 32°C	<0.0001	<0.0001
	Oceanospirillales 27°C	0.8183	0.3302
	Oceanospirillales 29.5°C	0.3943	0.3376
	Oceanospirillales 32°C	0.0001	<0.0001
	Vibrio 27°C	0.3539	0.5131
	Vibrio 29.5°C	0.4625	0.3442
	Vibrio 32°C	<0.0001	<0.0001
Oceanospirillales 27°C	Control 29.5°C	0.3046	0.0144
	Control 32°C	<0.0001	<0.0001
	Oceanospirillales 29.5°C	0.2987	0.0475
	Oceanospirillales 32°C	0.0001	<0.0001
	Vibrio 27°C	0.5013	0.7416
	Vibrio 29.5°C	0.3567	0.0490
	Vibrio 32°C	<0.0001	<0.0001
Vibrio 27°C	Control 29.5°C	0.0938	0.0459
	Control 32°C	<0.0001	<0.0001
	Oceanospirillales 29.5°C	0.0902	0.1187
	Oceanospirillales 32°C	<0.0001	<0.0001
	Vibrio 29.5°C	0.1172	0.1221
	Vibrio 32°C	<0.0001	<0.0001
Control 29.5°C	Control 32°C	0.0006	0.0001
	Oceanospirillales 29.5°C	0.9749	0.6320
	Oceanospirillales 32°C	0.0033	0.0001
	Vibrio 29.5°C	0.9380	0.6209
	Vibrio 32°C	<0.0001	<0.0001
Oceanospirillales 29.5°C	Control 32°C	0.0006	<0.0001
	Oceanospirillales 32°C	0.0033	0.0001
	Vibrio 29.5°C	0.9117	0.9888
	Vibrio 32°C	<0.0001	<0.0001
Vibrio 29.5°C	Control 32°C	0.0003	0.0002
	Oceanospirillales 32°C	0.0016	0.0003
	Vibrio 32°C	<0.0001	<0.0001
Control 32°C	*Oceanospirillales* 32°C	0.7377	0.9190
	Vibrio 32°C	0.1205	0.0012
Oceanospirillales 32°C	*Vibrio 32*°C	0.0781	0.0017

Supplementary Table 6.2 – Alpha diversity of coral-associated bacterial communities in pooled samples.

		Total Sequences	Rarefied Sequences	OTU	chao1	Shannon-index	Fisher's alpha
Day 2	27°C Control	66396	8367	2015	3975.59	8.57	842.54
	27°C Oceanospirillales	11377	8367	1831	3245.57	8.58	723.44
	27°C V. coralliilyticus	10139	8367	2237	3975.99	9.11	999.86
	32°C Control	49670	8367	1646	3032.57	8.57	613.27
	32°C Oceanospirillales	8633	8367	1637	2691.79	8.26	608.14
	32°C V. coralliilyticus	11504	8367	2161	4074.46	9.00	944.25
Day 10	27°C Control	9086	8367	1177	2341.84	5.99	373.24
	27°C Oceanospirillales	10891	8367	1253	2288.84	6.90	408.52
	27°C V. coralliilyticus	8367	8367	1224	2075.12	6.79	394.90
	32°C Control	16023	8367	1378	2657.57	6.68	469.51
	32°C Oceanospirillales	12839	8367	1189	2361.34	6.01	378.72
	32°C V. coralliilyticus	18300	8367	1433	2956.39	6.65	497.55
Day 22	27°C Control	26957	8367	1227	2505.51	6.75	396.30
	27°C Oceanospirillales	17487	8367	1301	2506.00	6.58	431.50
	27°C V. coralliilyticus	26098	8367	1045	2183.01	6.95	315.14
	32°C Control	28386	8367	1339	2641.91	7.48	450.08
	32°C Oceanospirillales	21080	8367	880	1655.19	6.53	248.05
	32°C V. coralliilyticus	64605	8367	1233	2683.20	7.26	399.10

Supplementary Table 6.3 - SIMPER analysis based on a Bray-Curtis similarity matrix (square root-transformed) from coral-associated bacterial communities. Data is presented on the family level and indicates the contribution of OTUs to the change in bacterial assemblages between time points.

Class	Order	Family	Contribution (%)				
			Day 1 Day 10	Day 10 Day 22	Day 1	Day 10	Day 22
α-proteobacteria	Rhodobacterales	Rhodobacteraceae	4.02	3.65	18.88	38.28	28.01
α-proteobacteria	Rhizobiales	Hyphomicrobiaceae	1.30	1.52	5.63	6.71	6.14
Planctomycetia	Pirellulales	Pirellulaceae	1.97	1.57	5.04	4.91	4.54
α-proteobacteria	BD7-3		1.17	1.56	4.57	9.04	5.77
Flavobacteriia	Flavobacteriales	Flavobacteriaceae	1.89	2.29	4.27	3.55	6.05
γ-proteobacteria	Legionellales	Coxiellaceae	1.56	1.94	3.12	2.00	4.75
α-proteobacteria	Rhizobiales	Phyllobacteriaceae	1.33	1.04	3.03	2.05	2.06
Planctomycetia	Planctomycetales	Planctomycetaceae	1.34	1.77	2.79	1.65	3.92
α-proteobacteria			0.85	1.14	2.63	2.70	2.79
VHS-BH5-50			1.54	1.32	2.07	1.81	2.29
γ-proteobacteria	Marinicellales	Marinicellaceae	1.35	0.73	2.03	0.18	0.24
Saprospirae	Saprospirales	Saprospiraceae	1.19	1.22	2.02	1.06	1.74
α-proteobacteria	Sphingomonadales		1.24	2.10	1.96	4.13	0.70
γ-proteobacteria	Chromatiales		1.04	0.86	1.86	1.46	1.07
γ-proteobacteria	Alteromonadales	OM60	1.09	0.80	1.72	0.54	0.41
Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	1.30	0.82	1.71	0.47	0.71
EC214			1.36	0.93	1.66	0.98	0.84
γ-proteobacteria	Legionellales		0.94	1.05	1.62	1.53	0.98
δ-proteobacteria	Thiotrichales	Piscirickettsiaceae	1.18	0.80	1.45	0.34	0.5
Cytophagia	Cytophagales	Flammeovirgaceae	1.17	0.88	1.42	0.11	0.45
Acidimicrobiia	Acidimicrobiales	Wb1-P06	0.96	0.40	1.27	0.17	0.00
α-proteobacteria	Rhizobiales		0.83	0.87	1.09	0.00	1.04
Sva0725	Sva0725		1.30	0.86	1.08	0.24	0.69
γ-proteobacteria	Legionellales	Legionellacae	1.25	1.35	1.04	0.05	1.31
α-proteobacteria	Bdellovibrionales	Bacteriovoracaceae	0.89	0.67	1.03	0.24	0.24
Flavobacteriia	Flavobacteriales	Cryomorphaceae	0.90	0.62	1.00	0.00	0.15
α-proteobacteria	Kiloniellales		1.06	1.01	0.93	0.41	1.03
Acidobacteria-6	BPC015		0.89	0.45	0.88	0.04	0.09
γ-proteobacteria	Thiohalorhabdales		0.92	0.61	0.88	0.00	0.15
α-proteobacteria	Rhodobacterales	Hyphomonadaceae	0.52	0.84	0.86	1.27	1.04
δ-proteobacteria	Myxococcales		0.82	0.88	0.75	0.75	0.50
α-proteobacteria	Rickettsiales		1.19	1.39	0.72	0.59	0.96
SJA4			0.94	0.76	0.71	0.11	0.49
GN02 (phylum)			0.94	0.64	0.71	0.05	0.18
Acidimicrobiia	Acidimicrobiales		0.90	0.44	0.68	0.16	0.07
Cytophagia	Cytophagales	Amoebophilaceae	0.86	1.06	0.65	0.12	1.17
γ-proteobacteria	Alteromonadales		1.00	1.81	0.61	0.20	0.76
Phycisphaerae	Phycisphaerales		0.75	0.80	0.60	0.15	0.70
Opitutae	Cerasicoccales	Cerasicoccaceae	0.76	0.62	0.55	0.02	0.29
γ-proteobacteria			0.70	0.49	0.54	0.01	0.14
SR1 (phylum)			0.79	0.52	0.52	0.00	0.13
γ-proteobacteria	Oceanospirillales	Oceanospirillaceae	0.90	1.08	0.51	1.21	0.22
γ-proteobacteria	Alteromonadales	Alteromonadaceae	0.86	1.07	0.50	0.93	0.56
α-proteobacteria	Rickettsiales	Rickettsiaceae	0.71	1.15	0.44	0.38	1.38

α-proteobacteria	Rhodospirillales	Rhodospirilaceae	0.65	1.02	0.44	0.36	0.98
Clostridia	Clostridiales	Acidaminobacteraceae	1.22	2.31	0.37	1.19	1.71
γ-proteobacteria	Vibrionales	Vibrionaceae *	0.51	0.58	0.33	0.00	0.29
α-proteobacteria	Sphingomonadales	Sphingomondaceae	2.52	3.06	0.32	0.84	0.40
Clostridia	Clostridiales	Clostridiaceae	0.80	0.98	0.27	0.46	0.54
3-BR-5F			0.50	0.73	0.26	0.05	0.57
α-proteobacteria	Rhizobiales	Cohaesibacteraceae	0.31	1.15	0.13	1.24	0.25
Clostridia	Clostridiales	Lachnospiraceae	0.60	1.21	0.10	0.07	0.44
Bacilli	Bacillales	Staphyllococcaceae	0.51	0.95	0.02	0.07	0.69
GN07			0.13	0.91	0.01	0.00	0.91
Actinobacteria	Actinomycetales	Microbacteriaceae	0.00	1.00	0.00	0.00	0.09
Average Similarity			52.07	49.34	57.58	53.45	54.38

Supplementary Table 6.4 - SIMPER analysis based on a Bray-Curtis similarity matrix (square root-transformed) from coral-associated bacterial communities on samples taken on Day 22. Data is presented on the family level and indicates the contribution of OTUs to the total community under experimental conditions and comparison between 27°C and 32°C treatments.

Class	Order	Family	Contribution (%)					
			27°C 32°C	27°C	32°C	Control	*Oceano-spirillales*	*Vibrio coralliilyticus*
α-proteobacteria	Rhodobacterales	Rhodobacteraceae	2.25	28.32	27.61	28.80	28.39	27.34
α-proteobacteria	BD7-3		1.60	6.70	4.88	4.55	7.37	5.88
α-proteobacteria	Rhizobiales	Hyphomicrobiaceae	1.64	5.99	6.33	5.57	5.50	7.18
Flavobacteriia	Flavobacteriales	Flavobacteriaceae	1.61	5.76	6.45	5.98	6.85	5.30
Planctomycetia	Pirellulales	Pirellulaceae	1.61	5.08	4.23	5.07	3.31	4.90
γ-proteobacteria	Legionellales	Coxiellaceae	0.97	4.63	4.94	4.62	5.61	4.23
Planctomycetia	Planctomycetales	Planctomycetaceae	1.04	3.61	3.86	3.68	3.96	4.15
α-proteobacteria			1.13	2.63	2.96	2.73	2.58	2.81
α-proteobacteria	Rhizobiales	Phyllobacteriaceae	1.02	2.26	1.95	3.16	0.57	2.92
VHS-BH5-50			1.39	2.20	2.29	1.82	1.71	3.45
Cytophagia	Cytophagales	Amoebophilaceae	1.05	2.16	0.43	1.08	0.81	1.32
α-proteobacteria	Rickettsiales	Rickettsiaceae	1.08	1.69	1.03	1.32	1.18	1.44
Saprospirae	Saprospirales	Saprospiraceae	0.94	1.56	1.84	1.91	2.31	1.18
Clostridia	Clostridiales	Acidaminobacteraceae	2.89	1.46	1.71	0.16	1.65	3.32
α-proteobacteria	Rhodospirillales	Rhodospirilaceae	1.02	1.19	0.69	0.82	1.26	0.73
α-proteobacteria	Rickettsiales		1.03	1.08	0.74	0.56	0.65	1.33
α-proteobacteria	Sphingomonadales		1.48	1.04	0.36	0.17	0.63	1.00
γ-proteobacteria	Alteromonadales	Alteromonadaceae	1.02	0.99	0.19	1.13	0.49	0.16
γ-proteobacteria	Chromatiales		0.80	0.97	1.07	0.57	1.29	1.13
α-proteobacteria	Rhodobacterales	Hyphomonadaceae	0.92	0.94	1.07	2.02	0.16	1.15
α-proteobacteria	Rhizobiales		0.90	0.93	1.07	1.72	0.49	0.96
Clostridia	Clostridiales	Clostridiaceae	1.00	0.90	0.22	0.15	1.12	0.32
δ-proteobacteria	Desulfobacterales	Desulfobulbaceae	1.19	0.82	0.06	0.21	0.17	0.38
δ-proteobacteria	Myxococcales		0.89	0.73	0.22	1.40	0.16	0.11
BME43			0.85	0.72	0.07	0.16	0.33	0.35
Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	0.83	0.70	0.64	1.95	0.52	0.13
EC214			1.02	0.66	0.95	0.51	1.37	0.55
Cytophagia	Cytophagales	Flammeovirgaceae	1.02	0.65	0.20	0.44	0.30	0.35
Phycisphaerae	Phycisphaerales		0.75	0.63	0.71	0.55	0.66	0.79
γ-proteobacteria	Legionellales	Legionellacae	1.49	0.62	2.22	1.84	1.47	0.68
α-proteobacteria	Sphingomonadales	Sphingomondaceae	0.86	0.61	0.16	0.45	0.16	0.40
Bacilli	Bacillales	Staphyllococcaceae	0.97	0.60	0.68	0.94	0.50	0.49
γ-proteobacteria	Alteromonadales		2.45	0.51	0.90	1.81	0.52	0.10
γ-proteobacteria	Legionellales		1.10	0.49	1.52	1.40	0.66	0.77
δ-proteobacteria	Thiotrichales	Piscirickettsiaceae	0.85	0.43	0.48	0.20	0.59	0.54
3-BR-5F			0.74	0.43	0.64	0.16	2.15	0.11
α-proteobacteria	Kiloniellales		0.91	0.37	1.94	2.14	0.15	1.26
Acidimicrobiia	Acidimicrobiales	koll13	0.59	0.35	0.05	0.00	1.06	0.00
γ-proteobacteria	Oceanospirillales	Oceanospirillaceae	0.83	0.34	0.08	0.00	0.55	0.18
Bacilli	Lactobacillales	Streptococcaceae	0.70	0.34	0.08	0.15	0.55	0.00
GN07			1.68	0.26	1.82	0.30	0.82	1.44
Clostridia	Clostridiales	Lachnospiraceae	1.33	0.26	0.58	0.00	0.71	1.00
SR1 (phylum)			0.80	0.24	0.00	0.00	0.70	0.00
α-proteobacteria	Bdellovibrionales	Bacteriovoracaceae	0.67	0.20	0.20	0.00	0.58	0.43
γ-proteobacteria	Vibrionales	Vibrionaceae *	0.74	0.15	0.38	0.00	0.53	0.32
Bacteroida	Bacteroidales		0.84	0.11	0.34	0.00	1.05	0.11
Sva0725	Sva0725		1.03	0.17	1.48	1.01	0.21	0.71
Opitutae	Cerasicoccales	Cerasicoccaceae	0.83	0.10	0.54	0.46	0.15	0.10
Flavobactera	Flavobacteriales	Weeksellaceae	0.78	0.00	0.62	0.15	0.00	0.29
γ-proteobacteria	Alteromonadales	OM60	0.84	0.40	0.37	0.95	0.08	0.16
Leptospirae	Leptospirales	Leptospiraceae	0.58	0.17	0.07	0.58	0.00	0.00
SJA4			0.88	0.00	0.45	0.15	0.00	1.65
α-proteobacteria	Rhizobiales	Cohaesibacteraceae	1.91	0.05	0.51	0.48	0.00	0.15
Actinobacteria	Actinomycetales	Microbacteriaceae	1.82	0.19	0.00	0.00	0.19	0.00
Actinobacteria	Actinomycetales	Micrococcaceae	1.03	0.55	0.28	0.17	0.50	0.41
Average similarity			45.58	56.00	52.66	58.12	49.44	54.74

* Only *Vibrio* species identified was *Vibrio coralliilyticus* at 0.04% and 0.05% at 27°C and 32°C treatments respectively. In the respective bacterial treatment, this species contributed to the overall community by 0.00% (control), 0.00% (*Oceanospirillales*) and 0.08% (*V. coralliilyticus*).

Supplementary Table 6.5 – Overview of statistical analysis of differences in alpha diversity of coral associated bacterial communities.

Linear Mixed Effects Model	p-value	ANOVA	p-value	Tukey's HSD	95% Confidence Interval	
Chao1						
Time	0.0009	Time	0.0005	Day 1 – 10	83.8	468.0
Temperature	0.9725			Day 1 – 22	93.4	477.0
Bacteria	0.4152					
Time:Temperature	0.9004	Control 27°C	0.0352	Day 1 – 10	27.4	486.0
Time:Bacteria	0.5421	*Vibrio coralliilyticus* 32°C	0.0499	Day 1 – 10	11.3	991.0
Temperature : Bacteria	0.9994					
Time : Temperature : Bacteria	0.7521					
Fisher's alpha						
Time	<0.0001	Time	<0.001	Day 1 – 10	88.3	171.0
Temperature	0.9845			Day 1 – 22	71.4	154.0
Bacteria	0.2850					
Time:Temperature	0.9759	Control 27°C	0.0075	Day 1 – 10	55.9	209.0
Time:Bacteria	0.3454			Day 1 – 22	17.0	170.0
Temperature : Bacteria	0.9488	*Vibrio coralliilyticus* 27°C	<0.001	Day 1 – 10	113.0	212.0
Time : Temperature : Bacteria	0.8600			Day 1 – 22	104.0	203.0
		Vibrio coralliilyticus 32°C	0.0171	Day 1 – 10	41.4	312.0
Number of unique OTU						
Time	0.0001	Time	<0.001	Day 1 – 10	71.2	143.0
Temperature	0.6686			Day 1 – 22	50.3	122.0
Bacteria	0.4694					
Time:Temperature	0.8889	Control 27°C	0.0058	Day 1 – 10	50.9	165.0
Time:Bacteria	0.4206			Day 1 – 22	11.9	126.0
Temperature : Bacteria	0.8920	*Vibrio coralliilyticus* 27°C	<0.001	Day 1 – 10	85.1	155.0
Time : Temperature : Bacteria	0.6722			Day 1 – 22	75.8	146.0
Shannon-index						
Time	<0.0001	Time	<0.001	Day 1 – 10	1.3000	2.67
Temperature	0.7485			Day 1 – 22	0.5180	1.88
Bacteria	0.7321			Day 10 – 22	-1.4800	-0.0951
Time:Temperature	0.3563					
Time:Bacteria	0.7120	Control 27°C	0.0062	Day 1 – 10	0.9960	3.25
Temperature : Bacteria	0.3882			Day 1 – 22	0.0994	2.35
Time : Temperature : Bacteria	0.4603	*Vibrio coralliilyticus* 27°C	0.0016	Day 1 – 10	0.8170	2.32
				Day 1 – 22	0.4820	1.99
		Control 32°C	0.0118	Day 1 – 10	0.5410	3.07
				Day 1 – 22	0.0247	2.56

Chapter 7

GENERAL DISCUSSION

&

FUTURE DIRECTIONS

7.1 - General discussion

In this thesis, I explore the immune response of thermally tolerant (Chapters 2, 6) and sensitive (Chapters 3, 4, 5) species of reef-building corals, both in the field (Chapters 2, 3, 5) and in manipulative lab-based experiments (Chapters 4, 6), in the first 24 hours following injury (Chapter 4) and over a longer time period of 10 days (Chapter 5), and in response to a single stressor (injury: Chapter 5) and multiple stressors (injury and heat stress: Chapter 4; heat stress and pathogen challenge: Chapter 6). In combination, this suite of studies represents a significant contribution to the field of coral immunology and my understanding of the impact of anthropogenic stressors on the coral's ability to resist microbial infection. In this final chapter, I give an overview and synthesis of my main findings and place the outcomes into a broader perspective. Additionally, I suggest directions for future research to further our knowledge of coral immunology and the impacts of stressors on coral immunocompetency.

Ecological immunology is an emerging field of research that investigates immunity in the context of the natural environment (Sheldon and Verhulst, 1996) and aims to understand variation in disease resistance and immune responses among individuals, populations and species (Sadd and Schmid - Hempel, 2009). By considering the allocation of resources to immune mechanisms as a life history trait, the costs of maintaining an immune system and deploying an immune response can be explored from an ecological, as well as an evolutionary perspective (Sadd and Schmid - Hempel, 2009). The Scleractinia is a highly diverse order, comprised of both solitary and colonial corals with diverse reproductive strategies (broadcast spawning, brooding and asexual), morphologies and growth rates. The range of environments inhabited by corals, from the tropics to temporal regions, in shallow, high light habitats to mesophotic depths, and under conditions from low to high wave energy, highlights the need for different resource allocation strategies to various maintenance processes and life history traits, which collectively determine the optimal fitness of an organism (Sadd and Schmid - Hempel, 2009). Immunocompetency is a life history trait that competes for resources with other traits, such as reproduction and colony growth, hence it follows that immunocompetency is highly variable among species and populations (Sadd and Schmid - Hempel, 2009; Palmer et al., 2010). My study of year-long temporal patterns in baseline levels of biochemical immune parameters (expression of the proPO-activating system and fluorescent proteins) revealed clear

differences in the immunocompetency of three coral species. Generally higher constitutive levels of immune parameters in *Acropora millepora* than in *Porites cylindrica* accord with characterisations of these species as bleaching- and disease-susceptible versus resistant, respectively (Chapter 2).

The work presented in this thesis, in combination with several other recent studies (Hamada et al., 2013; Miller et al., 2007a; Shinzato et al., 2011), show that the immune system of corals is significantly more complex than previously appreciated. Here, I show that seasonal peaks in temporal patterns of these parameters differ among three coral species (Chapter 2). These patterns may be the result of evolutionarily fixed differences in the way these immune system components are impacted by, or used to respond to, environmental factors among coral species (Fig. 7.1A). Therefore, caution should be exercised when extrapolating results from one species to other coral species or families. In addition, immunocompetency may vary within a population in response to modulation of the coral immune response as a consequence of environmental or anthropogenic disturbances. Such within-population variability was shown for the susceptible coral *Acropora millepora* at sites that varied in their exposure to stressors associated with a tourist reef platform (Chapter 3). Although temperature-related stress in summer significantly affected the immune system, this did not result in higher coral disease prevalence in corals at a control site. However, in the presence of additional anthropogenic stressors, the immune systems of members of this population near tourist reef platforms were overwhelmed, resulting in the development of disease in a third of corals monitored at this site (Fig. 7.1B) (Chapter 3). Hence, corals within this population had the ability to prevent disease under seasonally elevated seawater temperatures, but not in the presence of multiple stressors.

Similarly, the immune response of corals following physical damage was not significantly affected by elevated seawater temperatures in a manipulative aquarium-based study (Chapter 4), corroborating my conclusion that, in absence of other anthropogenic stressors, corals can withstand and recover from environmental disturbances. Identifying the anthropogenic stressors that have the most significant impacts on coral immunocompetency and how cumulative effects of multiple stressors affect coral immune responses is of high importance for enabling reef managers to develop optimal coral reef management strategies. While certain stressors are more difficult to mitigate (e.g. ocean warming), others (e.g. mechanical damage from divers, pollution) can be addressed over relatively short time scales and on localised spatial scales.

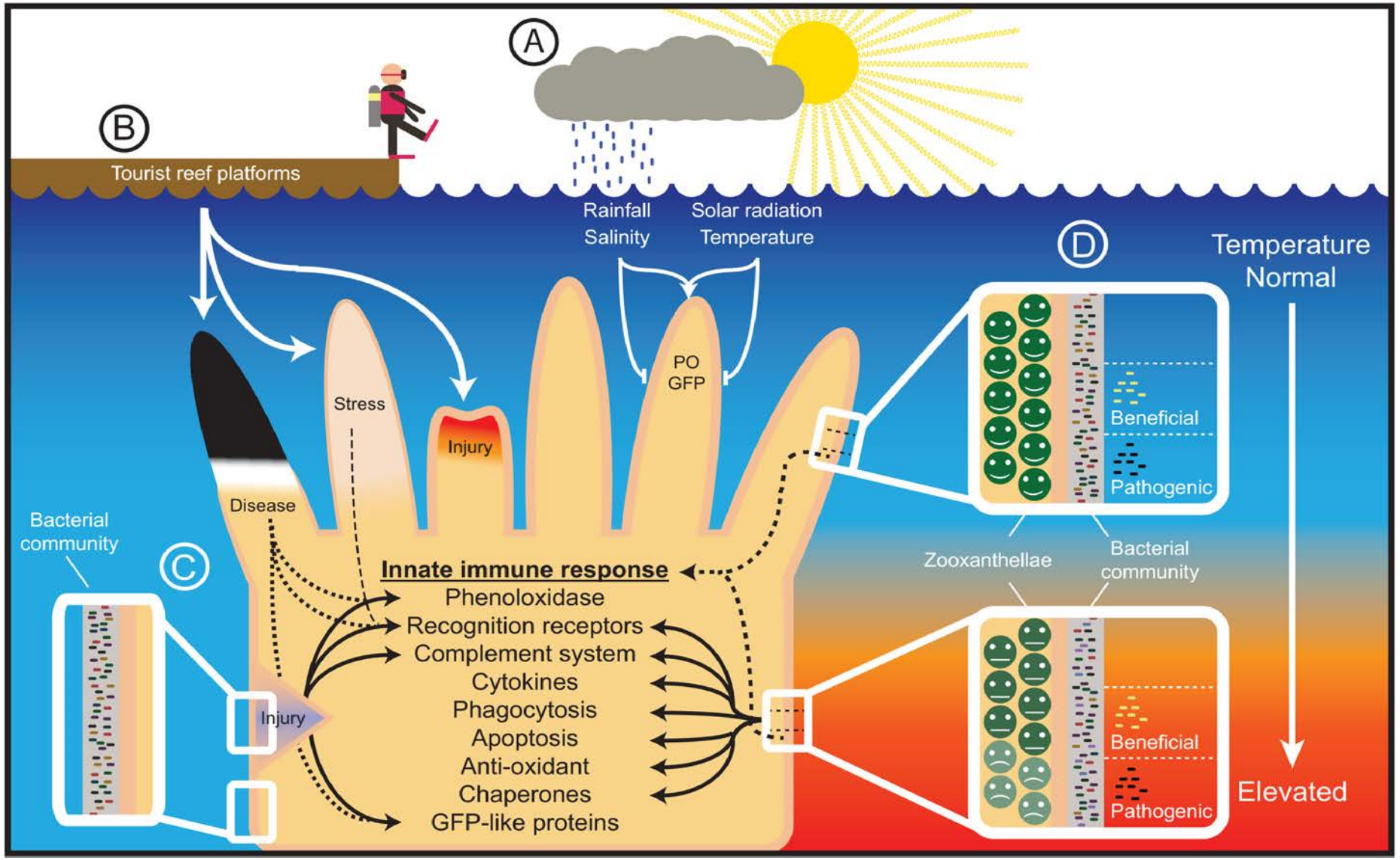

A
B
Tourist reef platforms
Rainfall
Salinity
Solar radiation
Temperature
PO
GFP
Stress
Injury
Disease
Bacterial
community
C
Injury
Innate immune response
Phenoloxidase
Recognition receptors
Complement system
Cytokines
Phagocytosis
Apoptosis
Anti-oxidant
Chaperones
GFP-like proteins
D
Temperature
Normal
Beneficial
Pathogenic
Zooxanthellae
Bacterial
community
Beneficial
Pathogenic
Elevated

Figure 7.1 - Overview of the main research findings presented in this thesis. A) Temporal patterns in the coral immune system components, pro-phenoloxidase-activating system and fluorescent protein expression, are influenced by environmental factors. B) Factors associated with permanent tourist reef platforms cause stress and elicit an immune response in corals. These additional anthropogenic stressors are responsible for increased prevalence of coral disease and physical injury. C) Corals elicit a dynamic immune response involving multiple immune system components following injury. The immune response is sufficient to maintain healthy coral-associated bacterial communities. D) Under elevated seawater temperatures, corals elicit a significant immune response involving a suite of immune mechanisms, likely in response to a temperature-induced shift in the bacterial community. The photophysiology of endosymbiotic zooxanthellae is affected by elevated temperatures, but harmful bacteria may cause significant additional damage. However, corals have the capability to distinguish between harmful and harmless bacteria and elicit an immune response only to harmful bacteria, regardless of temperature.

My finding that the immune response of *Acropora aspera* following physical damage is highly dynamic, consisting of several distinct phases involving multiple components of the innate immune system (Fig. 7.1C) (Chapter 5), provides further evidence that the immune system of corals is complex. Upregulation of a range of immune mechanisms following injury, including the Toll-like receptor pathway, complement system and proPO-activating system, provides evidence for the functional importance of these systems that hitherto, have primarily only been documented in genetic studies of corals. Overall, these immune pathway responses were sufficient to maintain a healthy coral-associated microbial community and provide protection against bacterial infection in this field-based study (Fig. 7.1C) (Chapter 5). Results of this study are corroborated by my findings in Chapter 3, where activation of the proPO-activating system was found to play a major role in the wounding response of *Acropora millepora* exposed to tourist-recreational activities. However, *A. millepora* also increased the expression of fluorescent proteins, whereas *A. aspera* did not, relying on the non-fluorescent chromoprotein. This disparity between outcomes of these two studies highlights differences in immune responses to stressors among species, and confirms results in Chapter 2 that immune responses are species-specific, varying even between species within the same genus. Surprisingly, the differential expression patterns in response to wounding of various immune genes in *A. aspera* showed inconsistencies at 24-hours post-injury in the aquarium-based study in Chapter 4 versus the field-based study in Chapter 5. For example, in the field-based study, TIR-1 was significantly upregulated 24 hours post-injury (Chapter 5), but did not show a change in expression in the aquarium-based study (Chapter 4), while the opposite pattern was found for *cJun* and *cFos*. The difference between a controlled, manipulative aquarium-based experiment versus a study conducted in the coral's natural habitat on corals that had been undisturbed prior to the experiment may explain the differential outcomes.

Because bacterial symbionts play a critically important role in coral health, I used a holobiont-focused approach in Chapter 6, which enabled me to unravel how the three major components of the holobiont of *Montipora aequituberculata* (coral host, endosymbiotic *Symbiodinium* and coral-associated bacterial communities) are affected by interactive effects of heat stress and a pathogen challenge. In a controlled aquarium-based experiment, evidence that corals respond differently to potentially pathogenic bacteria compared to non-pathogenic bacteria, regardless of temperature stress (Fig. 7.1D) (Chapter 6), suggests that the coral immune response recognises bacterial pathogens. Although the population from which the experimental corals were sampled had experienced recurrent white syndrome and atramentous necrosis outbreaks in several years prior to sampling (Jones et al., 2004; Anthony et al., 2008b; Haapkylä et al., 2011), these corals did not show visual signs of disease during experimental pathogen exposure in my study. It is possible that the study population may have acquired increased resistance to the pathogen *Vibrio corallilyticus*, as a consequence of natural selection of more disease resistant colonies or potentially through immunological memory (Chapter 6) (Rowley and Powell, 2007). Alternatively, the strain of *V. corallilyticus* used may have had reduced virulence after storage for 9 years. Nevertheless, a strong transcriptomic response by the coral host to *V. corallilyticus* challenge and heat stress, coupled with a transcriptomic response of the endosymbiont *Symbiodinium* to heat stress, enabled the holobiont to resist disease. In addition, I found that temperature-induced shifts in the bacterial community correlated with a change in expression of many coral immune system components (Fig. 1D) (Chapter 6).

In contrast to results found in Chapter 5, where bacterial communities associated with *Acropora aspera* remained stable over time, the *Montipora aequituberculata*-associated bacterial communities showed temporal changes. Several hypotheses can be posited to explain this difference. First, different coral species may interact differently with their bacterial communities, resulting in some that are temporally more stable than others, as has also been observed for the *Symbiodinium* communities of corals (Baker, 2003; Fautin and Buddemeier, 2004; Stat et al., 2009; McGinley et al., 2012). Secondly, the experiment on *A. aspera* was conducted *in situ*, while *M. aequituberculata* was collected from the field, fragmented and maintained in aquaria. Coral health and potentially the microbial community may be significantly impacted during each of these stages. Also, 43 days elapsed between collection of *M. aequituberculata* in the field and the conclusion of the experiment, suggesting that acclimation periods of > 40 days may be required to stabilise bacterial communities prior to experimentation. Thirdly, it is also known that the bacterial community structure

response under heat stress can be influenced by the *Symbiodinium* type harboured by the coral colony (Littman et al., 2010), which could suggest that *Symbiodinium* function may have differentially influenced the composition of the bacterial communities. Given that light intensities in the *M. aequituberculata* experiment were relatively low, this may have impacted *Symbiodinium* physiology and thereby the coral-associated bacterial community.

Contrary to findings of other recent studies (Leggat et al., 2011; Barshis et al., 2014), *Symbiodinium* associated with experimental fragments of *M. aequituberculata* exhibited a significant transcriptomic response *in hospite* during heat stress, which also significantly affected its photophysiology (Fig. 7.1D) (Chapter 6). Surprisingly, however, the transcriptomic response revealed that *Symbiodinium* had responded to an unknown biotic stimulus, most likely the shift in coral-associated bacterial communities. These results show that, in addition to the coral host, *Symbiodinium* also exhibits an immune response, which should be taken into account when considering the disease resistance of the coral holobiont.

Although the effect of short term heat stress on the overall health of the *M. aequituberculata* holobiont was relatively minor, prolonged heat stress could have significant detrimental effects on the holobiont health, resulting in bleaching or disease, even in the more stress-tolerant coral species. Given ongoing predictions of human-induced climate change, seawater temperatures are expected to rise, thereby exposing corals to longer term heat stress than under current climate conditions. Urgent action is needed to prevent or limit the rate of further climate change, improving the chances of coral survival and giving corals time to adapt to a changing climate.

7.2 - Future research directions

Significant advances have been made in the field of coral immunology since the first study (Mydlarz and Harvell, 2007) was published 7 years ago. Genome-wide studies have shown the complexity of the coral immune system and identified a suite of innate immune mechanisms (Hamada et al., 2013; Miller et al., 2007a; Shinzato et al., 2011), while a limited number of functional studies have addressed the role of some of these mechanisms in coral biology and ecology (Kvennefors et al., 2010b; Mydlarz et al., 2009; Palmer et al., 2011a; Vidal-Dupiol et al., 2014). Further functional, as well as full transcriptome studies, are needed to tease apart the workings of each immune system component, and to advance understanding of how corals use their immune system to cope with biotic and abiotic stressors, and how stressors affect coral immunocompetency.

Studies into the microbiome of the coral holobiont have proven the importance of a healthy microbial community in anti-microbial defence through the production of anti-microbial compounds (Fusetani et al., 1996; Ritchie, 2006; Reshef et al., 2006; Gochfeld et al., 2006; Gochfeld and Aeby, 2008; Shnit-Orland and Kushmaro, 2009; Kvennefors et al., 2012; Shnit-Orland et al., 2012; Hunt et al., 2012). Together with evidence that the composition of the bacterial community is dependent on the type of *Symbiodinium* associated with the coral host (Littman et al., 2010), this shows the intricacy of relationships among components of the coral holobiont and the importance of studying the holobiont, rather than only one of its components. The rapid development of molecular techniques provides researchers with increasing opportunities to use holistic approaches to address biological questions about corals. Using such a holistic approach, which addresses all components of the holobiont, will significantly enhance our knowledge of factors underpinning coral health.

Knowledge of constitutive levels of immune parameters can be used as a reference baseline for coral immunity. Characterising the immunocompetence of corals as part of coral health monitoring surveys may contribute to identifying reefs that are susceptible to disease and other stressors. However, temporal changes in constitutive levels of immune parameters and differences among species observed in my study highlight that additional long term data at higher sampling resolution are required before ecological immune parameters could be considered suitable for routine coral health monitoring. The need for further data is reinforced by differences in immune parameters found between populations of *A. millepora* at Orpheus Island (Chapter 2) and Hardy Reef (Chapter 3), which also suggest that there are spatial differences in these parameters, likely due to local conditions. In addition, experimental studies to confirm the links established between immune parameters and environmental factors are required and should include measurements of the effect of these environmental factors on coral health. A thorough understanding of natural fluctuations in coral immune parameters will enable the development of coral health monitoring tools.

Coral disease is a major contributor to the worldwide decline in coral cover and is linked to increasing levels of anthropogenic stress. Factors associated with global climate change, such as elevated seawater temperatures and ocean acidification, are known to affect the immune defence capability of marine invertebrates (Ellis et al., 2011), including corals (Mydlarz et al., 2010). However, coral species are not equally susceptible, with some being significantly more resilient than others (Chapter 6) (Vidal-Dupiol et al., 2011b). It is still largely unknown how other anthropogenic stressors that are known to cause disease in marine invertebrates, such as turbidity, tourism, pollution and eutrophication affect the coral immune system or the health of the coral

holobiont. Nevertheless, a combination of stressors is more likely to result in disease development in corals (Chapter 3). Studies addressing the effects of stressors and, in particular, combinations of stressors on the coral holobiont are warranted to identify stressors that have the most significant impact on coral health and recovery. Besides, identifying the anthropogenic stressors present at locations with high coral disease incidence, such as reef tourist platforms, that may be responsible for a compromised immune system and change in bacterial community leading to disease outbreaks is critical. Such studies are needed for informing management strategies aimed at coral disease mitigation.

The work presented in this thesis also provides leads to several other studies. For example, an anti-viral immune response was exhibited by the coral *Montipora aequituberculata* under elevated seawater temperatures (Chapter 6), suggesting a role for coral-targeting viruses in coral disease. Identifying the sequences corresponding to viral mRNA molecules may give insight into what viruses may be harmful to corals and ultimately lead to their isolation and subsequently experimental infection studies. Investigating viral communities associated with coral will be essential for a detailed understanding of coral disease development and should be included when analysing the microbial communities of diseased corals. The different types of coral disease that have been found so far are likely caused by different pathogens. I found that corals can distinguish beneficial from pathogenic bacteria (Chapter 6) and a comparative study among the immune responses elicited by different diseases may provide an indication on the specificity of the coral immune response to different pathogens. Active and specific regulation of the coral-associated microbial community was also suggested by the finding that the bacterial community associated with injured corals remained stable over time (Chapter 5). Although the work in this thesis has provided some evidence, the mechanism used by corals to distinguish bacteria and regulate the microbial community remains to be investigated. One striking observation made in this work was the apparent developed resistance of *M. aequituberculata* to the coral disease white syndrome, and suggests there may be some degree of immunological memory in corals or that the population has developed resistance as a result of natural selection. Investigating the biological factors that may result in disease resistance of coral populations should be a high priority and may help to future-proof coral reefs.

Increased understanding on the coral stress responses can also be obtained through improvements in research techniques used. While the currently used full transcriptome analysis is a very useful method and provides enormous amounts of data, it has some limitations. For example, mRNA levels can only explain an averaging 40% of protein expression levels (Schwanhausser et al., 2011). As proteins are the

functional response units, analysis of protein expression as well as post-translational protein modifications, that govern the activity and turn-over of proteins will provide more significantly more insight into the biology of corals. Developing a proteomics approach for coral should therefore be a priority. Overall, based on studies described in this thesis and proposed in this section, continued investigations into coral immunity will address key questions in the biology and ecology of corals and assist in the predictions of the fate of coral reefs.

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Appendix A

“Invertebrate innate immune system mechanisms”

A.1 Cellular responses

Most multicellular organisms have cells involved in the immune system. These immune cells may have very diverse functions ranging from the production of immune system components in intracellular granules and the secretion of these components following the detection of microbes, to phagocytosis where specialised cells engulf and subsequently destroy invading microbes.

A.1.1 Immune cells

Porifera, or sponges, are basal organisms comprising of cells that are totipotent and can change function depending on the circumstances. Sponges therefore do not have specialised immune cells, but archeocytes, which are large motile phagocytic cells that play an important role in immunity, but also in digestion and food transport (Van de Vyver, 1970). Like sponges, cnidarians also possess totipotent cells as well as specialised motile phagocytic cells, called amoebocytes, and different types have been described. In octocorals, these cells are involved in phagocytosis of bacteria (Olano and Bigger, 2000), melanisation of the lesions following a fungal infection (see A.3) (Mydlarz et al., 2008) and wound healing (Meszaros and Bigger, 1999). In sea anemones, additional functions have been ascribed to amoebocytes, including the production of anti-microbial compounds and reactive oxygen species (ROS) (Hutton and Smith, 1996). Similar functions are known for circulating haemocytes in a range of molluscs, where haemocytes are known to be involved in the phagocytosis(Cajaraville and Pal, 1995; Canesi et al., 2002; Garcia-Garcia et al., 2008) and the encapsulation (Laruelle et al., 2002; Batista et al., 2009) of microbes and larger parasites as well as the production of both ROS and nitric oxide (NO) (Bugge et al., 2007; Donaghy et al., 2009), lectins (Olafsen, 1995) and anti-microbials (Canesi et al., 2002). A large number of studies have focused on commercially important crustaceans and three main types of haemocytes have been found in these arthropods: hyaline cells, semi-granular cells and granular cells (Johansson et al., 2000). All these cells are important in the immobilisation and destruction of microbes. The semi-granular and granular cells are characterised by the number of intracellular granules. These granules, containing the components of the melanisation cascade, phagocytosis-promoting opsonins (e.g. peroxinectin) and anti-microbial peptides (Sritunyalucksana et al., 2001; Sricharoen et

al., 2005), are exocytosed following detection of microbe-associated molecular patterns (MAMPs), such as LPS, mannan, β-1,3-glucan or PGN, and ultimately results in the phagocytosis and elimination of the microbe (Jiravanichpaisal et al., 2006). Similar cellular responses have been described for various other non-aquatic invertebrates, including ticks (Eggenberger et al., 1990, Loosova et al., 2001; Pereira et al., 2001; Ceraul et al., 2002; Buresova et al., 2006), insect (Lavine and Strand, 2002; Hillyer et al., 2003; Castillo et al., 2006; Hillyer and Estevez-Lao, 2010) and annelids (Cameron, 1932; Valembois et al., 1992; Salzet et al., 2006; Lefebvre et al., 2008), showing the essentialness of this mechanism in invertebrate immunity. Echinoderms have a range of different immune cells with more specialised functions in their coelomic cavity, generally called coelomocytes (Gross et al., 1999; LC Smith, 2006). The coelomocytes can be subdivided into 1) phagocytes, which appear to exclusively target microbes via phagocytosis, 2) spherulocytes, which encapsulate and destroy larger foreign particles and seal wounds and 3) asteroids that are involved solely in coagulation.

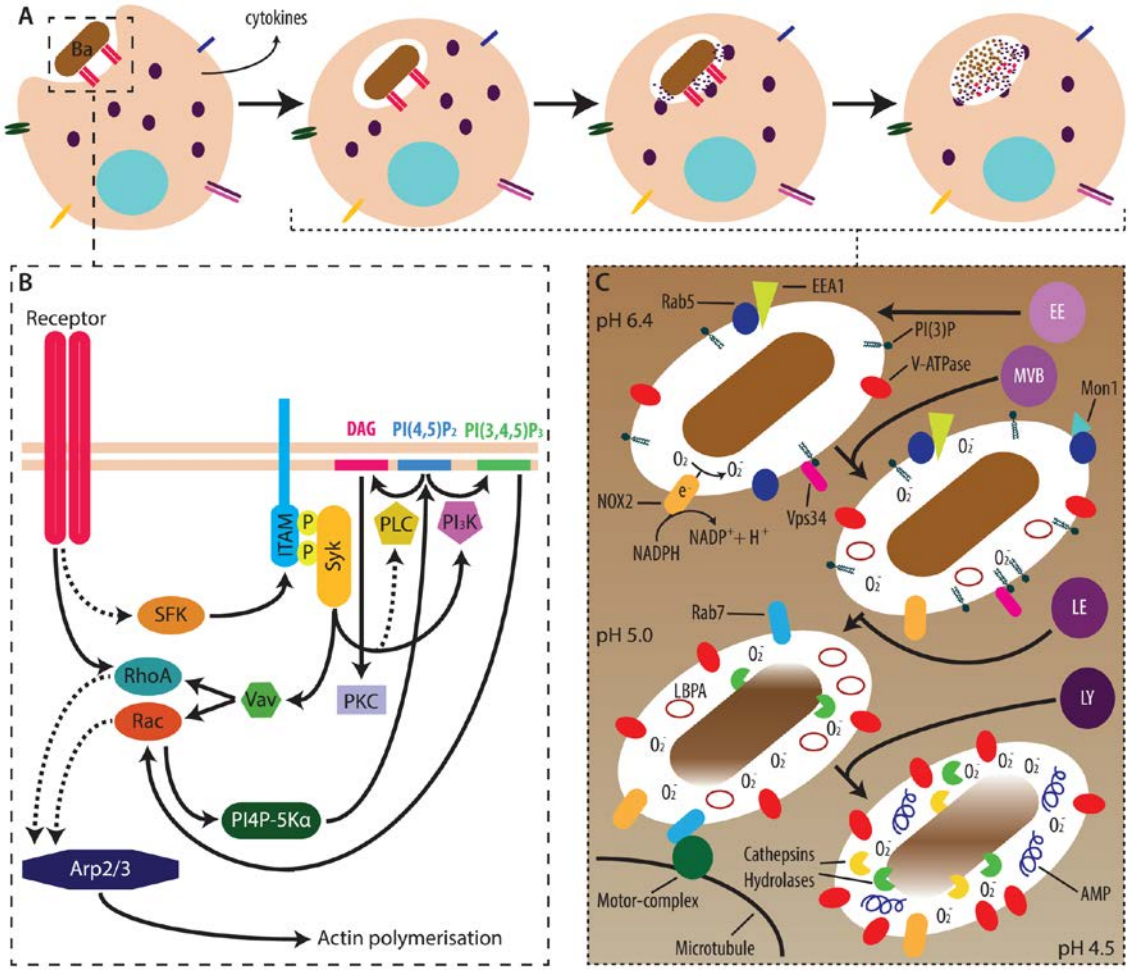
A.1.2 Phagocytosis

Phagocytosis, the process where cells ingest large particles (>0.5 µm), was first described by Metchnikoff over 100 years ago and is now considered one of the crucial components of the innate immune system, as well as the adaptive immune system of vertebrates (Appendix A Fig. 1A). The cell biology of phagocytosis was recently reviewed (Flannagan et al., 2012), and this will not be repeated here. In immunity, phagocytosis primarily depends on specialised immune cells. These cells express specific receptors on their outer membrane, which, upon detection of specific MAMPs (or indirectly via MAMP recognition/binding proteins) or opsonic immune factors, trigger a signal transduction cascade that results in cytoskeletal rearrangements and membrane remodelling allowing the engulfment and subsequent destruction of the detected foreign particle (Appendix A Fig. 1B) (Flannagan et al., 2012). Once the internalisation is complete, a membrane-bound vacuole is formed: the phagosome (Flannagan et al., 2012). The phagosome is cut from the membrane via scission and then matures rapidly via multiple stages to form a microbicidal organelle: 1) early, 2) intermediate, 3) late phagosome and 4) phagolysosome (Appendix A Fig. 1C). During the maturation, the pH in the phagosome is gradually decreased. The resulting phagolysosome has a pH of 4.5 and contains multiple antimicrobial proteins and peptides (Flannagan et al., 2012). While some of these peptides prevent the survival of phagocytised microbes by depriving them of essential nutrients (e.g. Fe^{3+}, Zn^{2+}, Mn^{2+}), others are cationic antimicrobial peptides (CAP), like defensins, that form pores in microbial cell walls, resulting in lysis. In addition, a cocktail of lysosomal hydrolases,

including lipases, nucleases, glycosidases, phosphatases and proteases (e.g. cathepsins) is present in the phagolysosome and is capable of the destruction of microbes through digestion (Flannagan et al., 2012). Further, reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) are rapidly produced by the NADPH oxidases and nitrous oxide synthases, also called inducible NOS (iNOS), respectively (Flannagan et al., 2012). This respiratory burst indiscriminately damages proteins, lipids and nucleic acids and thereby contributes to the destruction of the foreign body. iNOS expression is induced following the detection of MAMPs or pro-inflammatory cytokines and regulated through the p38 mitogen-activated protein kinase (MAPK), nuclear factor κB (NFκB), or the JAK-STAT-IRF (Janus-activated kinase - signal transducer and activator of transcription - interferon regulatory factor) signalling cascades (Gupta et al., 2009).

Phagocytosis, however, is not exclusive to immunity in the removal of foreign bodies (Flannagan et al., 2012). This process is also involved in the uptake and breakdown of apoptotic cells, where characteristic molecular markers of apoptotic cells, like phosphatidylserine, initiate phagocytosis by activating apoptotic corpse receptors. And whereas the phagocytosis of microbes and parasites can only be accommodated by immune cells, apoptotic cells can also be phagocytised by fibroblasts and epithelial cells.

Immune cells present in all invertebrates have phagocytic capabilities for the destruction of invading pathogens. However, the exact molecular pathways are currently poorly understood. Studies have revealed essential roles for PI3 kinase (Garcia-Garcia et al., 2008) and PKC (Plows et al., 2005; Lacchini et al., 2006; Wright et al., 2006; Garcia-Garcia et al., 2008) as well as extra cellular-regulated kinase (ERK) (Plows et al., 2005; Wright et al., 2006; Garcia-Garcia et al., 2008; Zahoor et al., 2008) in the phagocytosis process and respiratory burst in molluscs. In addition, NADPH oxidase and iNOS are crucial in the respiratory burst against bacteria in *Caenorhabditis elegans* (Chavez et al., 2009) and insects (Bergin et al., 2005; Hillyer and Estevez-Lao, 2010) and the Factor C-mediated NADPH oxidase activating p47phox was found to be upregulated following injury in *Hydra* (Laruelle et al., 2002). A gene encoding a Syk kinase homologue is present in sponges and *Hydra* (Steele et al., 1999; Muller and Muller, 2003) and phagocytes of echinoderms have been found to express the Arp2/3 complex and myosins (Henson et al., 1999; Henson et al., 2003). The cnidarian *Aiptasia pulchella* engages in symbiosis with *Symbiodinium* and the uptake of the symbionts occurs via phagocytosis, in which Rab proteins, including Rab5 and Rab7, play an essential role (Chen et al., 2003; Chen et al., 2004; Chen et al., 2005; Hong et al., 2009b; Hong et al., 2009a).



Appendix A Figure 1: Phagocytosis. ***A) Overview of the phagocytic process.*** Immune cells detect microbes via MAMP or opsonin receptors. Signal transduction cascades induce the production and release of cytokines and the internalisation of the microbe. Once internalised the microbe is destroyed in the phagosome by various anti-microbial molecules. ***B) Receptor signal transduction cascades leading to membrane remodelling.*** Receptors that detect MAMPs or opsonins activate downstream signal transduction cascades, in which the kinase Syk plays a central role. Syk activates the guanosine nucleotide exchange factor Vav and PI3 kinase. In turn, PI3 kinase converts membrane component phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$; produced from phospholipids by phosphatidylinositol 4-phosphate 5-kinase (PI4P-5K)) into phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$), thereby activating the small GTPases Rho and Rac. Together with Vav, these GTPases (in)directly activate the Arp2/3 nucleator complex. Syk also indirectly activates phospholipase Cγ (PLCγ), which hydrolyses $PI(4,5)P_2$ into diacylglycerol (DAG). DAG recruits protein kinase Cε (PKCε) that enhances phagocytosis independently of actin polymerisation. Nucleator complexes regulate actin polymerisation, directing the extrusion of the cell membrane thereby engulfing the foreign particle. ***C) Phagosome maturation.*** Following internalisation, the phagosome is formed and matures rapidly via four stages to form a microbicidal organelle: 1) early, 2) intermediate, 3) late phagosome and 4) phagolysosome. During the first stage, GTPase Rab5 recruits Vsp34, which produces phosphatidylinositol-3-phosphate (PI(3)P) from phosphatidylinositol. PI(3)P is a docking site for proteins such as early endosomal antigen 1 (EEA1), that facilitates the fusion of the phagosome with early endosomes (EE), and vacuole ATPase (V-ATPase). V-ATPase transports H^+ from the cytosol into the phagosomal lumen, thereby gradually reducing the pH (from pH 7.4 in the extracellular space to pH 4.5 in the phagolysosome). Further, the phagosome fuses with multivesicular bodies (MVBs) and late endosomes. At the late phagosome stage, Rab5-effector Mon1 indirectly activates Rab7, which is essential for the fusion of the late phagosome with lysomes. The acidic phagolysosome (pH 4.5) contains multiple antimicrobial proteins and peptides: 1) lysosomal hydrolases and cathepsins digest the microbe; 2) antimicrobial peptides (AMP) form pores in microbial cell walls resulting in lysis or deprive the internalised microbe from essential nutrient thereby preventing survival; and 3) oxygen radicals (e.g. superoxide anion, O_2^-), produced by the NADPH oxidase complex NOX2, damage lipids, proteins and nucleotides.

A.1.3 Encapsulation and nodule formation

When foreign bodies or an aggregate of agglutinated microbes are too large for phagocytosis by immune cells, multiple immune cells will encapsulate the foreign body. In some cases, melanisation of the capsule will occur and a nodule is formed. Encapsulated organisms are eliminated by various processes including the respiratory burst accommodated by NADPH oxidases and iNOS on the cell membrane of the encapsulating immune cells. Essential for encapsulation are Integrins (Wiegand et al., 2000; Lavine and Strand, 2003; Nardi et al., 2005; Levin et al., 2005; Zhuang et al., 2007; Zhuang et al., 2008), which are used by immune cells to attach to the foreign body and to keep the capsule intact via cell-cell adhesion. In addition, extracellular superoxide dismutase (SOD) and opsonins may cooperate in the respiratory burst against encapsulated parasites (Holmblad and Söderhäll, 1999). Encapsulation is further used in defence against a range of pathogens including viruses and fungi (Gillespie et al., 2000).

A.2 Proteolytic enzyme cascades

Several mechanisms of the innate immune system are under the regulatory control of proteolytic enzyme cascades. The high basal levels of proteins involved in these cascades allow for a rapid and immediate response upon initiation by MAMP-activated PRRs.

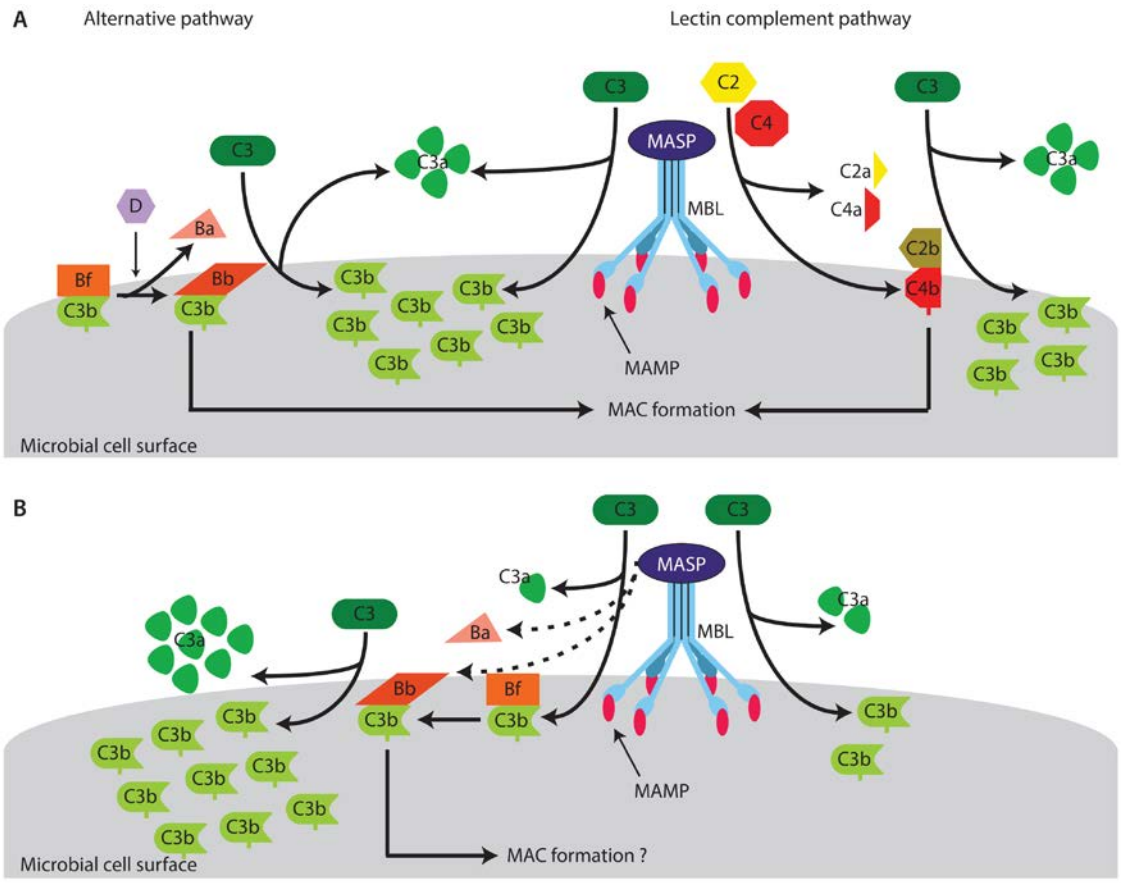
A.2.1 Coagulation

Activation of the coagulation system is triggered by the detection of microbial compounds and results in formation of a gel-like structure that traps microbes and seals lesions. The coagulation system has been investigated in detail only in crustaceans and horseshoe crabs and comparative analysis revealed that, although the function is identical, the two systems are significantly different from one another. In crustaceans, a large circulating 210 kDa homodimeric plasma clotting protein is rapidly polymerised into a gel by the enzyme transglutaminase, which is released from the animal's tissues (Hall et al., 1999; Maningas et al., 2013). Transglutaminase is also crucial for the coagulation processes in insects and horseshoe crabs (Matsuda et al., 2007; Dushay, 2009). The coagulation system of the horseshoe crab, however, is stored in its inactive form within vesicles harboured by granular haemocytes, a type of immune cell. Upon detection of LPS and β-1,3-glucan by the hemocyte membrane-bound pattern recognition protein (PRP) Factor C and Factor G, respectively, these serine protease zymogens are autocatalytically activated. Active Factor C initiates the inositol-1,4,5-triphosphate-signaling pathway mediated via a G protein-coupled

receptor (GPCR), thereby triggering the exocytosis and thus release of the vesicle contents (Ariki et al., 2004). In addition, Factor C and Factor G (in)directly activate the transglutaminase (Theopold et al., 2004), which produces a stable gel-like structure through the polymerisation of coagulin and cross-linking it with two other haemocyte-derived molecules (stablin and proxin) (Matsuda et al., 2007). In the vesicles, serine protease inhibitors (serpins) are also present and are believed, upon release, to prevent activation of the coagulation cascade in areas where coagulation is not needed (Miura et al., 1994; Miura et al., 1995; Agarwala et al., 1996). Interestingly, Factor C has also been found in complex with complement C3, suggesting a possible link between the coagulation and complement systems (Ariki et al., 2008). These two very distinct mechanisms that lead to coagulation, show that there are multiple ways to reach the same outcome. However, how the coagulation system works in other invertebrates, remains to be investigated. Transglutaminase and coagulation factors have been found in echinoderms (Canicatti and Rizzo, 1991; Ramirez-Gomez et al., 2008; Ramirez-Gomez et al., 2009), flies (Lindgren et al., 2008; Dushay, 2009) and ticks.

A.2.2 Complement and complement-like systems

The complement system has been well characterised in vertebrates (Appendix A Fig. 2A) and is comprised of proteins of the C3 protein family (C3, C4, C5) and proteases that belong to the Bf (complement factor B) family (e.g. Bf and C2) or the MASP (mannose-binding lectin-associated serine protease) family (e.g. MASP-1, MASP-2, MASP-3, C1r and C1s) (Daha, 2010). The main function of this system is promoting the opsonisation of foreign particles via phagocytosis. Binding of a mannose-binding lectin (MBL) to the mannose moieties on the surface of microbes, results in the activation of the MASPs, which subsequently proteolyse C4 and C2. In turn, C4b and C2b form a microbe surface-bound C3 convertase. This convertase continuously proteolyses C3 into C3a, a cytokine that initiates an inflammatory response, and C3b, that is covalently deposited on the microbe's surface thereby promoting phagocytosis of the microbe by specialised immune cells, which use the CR3 integrin receptor for C3 binding. However, there is also a lectin-independent pathway called the alternative complement pathway. Here, (spontaneously formed) C3b is stabilised by Bf when bound to the surface of a microbe. Together with Factor D-activated Bf (Bb), C3b forms a C3 convertase that proteolyses C3. These pathways are not mutually exclusive and can be active at the same time to amplify the effect of the complement system. In addition, the C3 convertases are also capable of proteolysing C5, which in turn initiates the terminal pathway via the formation of the cell-lysing membrane attack complex (MAC).



Appendix A Figure 2: The complement pathway. ***A) The complement system of vertebrates.*** In the lectin-complement pathway, MBL binds to microbial surfaces resulting in the activation of MASPs. MASP subsequently cleaves C2 and C4, which together form a C3 convertase. The C3 convertase cleaves C3 into C3a, a chemotactic cytokine, and C3b, which is deposited on the microbial surface and promotes phagocytosis. In addition, MASP is capable of cleaving C3. In the alternative pathway, Bf interacts with membrane-bound C3b, forming a C3 convertase once Bf is cleaved by D into Bb. This C3b:Bb convertase also initiates the formation of the microbial cell lysis mechanism MAC. ***B) Proposed model of the proto-complement system in invertebrates.*** Lectins bound to MAMPs activate MASPs. MASP cleaves C3 in C3a and C3b. In addition, MASP cleaves Bf into Bb and Ba, resulting in the formation of a C3b:Bb C3 convertase, which is significantly more efficient than MASP in converting C3. Abbreviations: MASP, MBL-associated serine protease; MBL, mannose-binding lectin; C2, complement C2; C4, Complement C4; C3, Complement C3; Bf, Factor B; D, Factor D; MAC, membrane attack complex.

Crucial in the complement system are members of the C3, Bf and MASP protein families. C3 and Bf appear to be present in all analysed invertebrates (except insects), while MASPs seem to be exlusive to deuterostomes. Cnidarians, such as sea anemones and corals, do possess members of the C3, Bf and MASP families (Kimura et al., 2009), in contrast to Porifera, Placozoa and Choanoflagellata. For the protostome complement system a hypothetical model has recently been developed, suggesting initiation by a microbe-bound lectin-MASP complex, which activates a Bf-containing C3 convertase via proteolysis (Appendix A Fig. 2B). A similar model may apply to the ascidian complement system, for which functional data is present and which contains not only C3 (Nonaka et al., 1999; Marino et al., 2002), Bf (Plows et al., 2005; Yoshizaki et al., 2005) and MASPs (Ji et al., 1997; Plows et al., 2005), but also a

mannose-binding lectin (MBL) (Bonura et al., 2009), glucose-binding lectin (GBL) (Sekine et al., 2001), ficolin (Kenjo et al., 2001) and integrins that could form the integrin-type complement receptor CR3 (Miyazawa et al., 2001; Miyazawa and Nonaka, 2004). Components of the ascidian system are involved in opsonisation and the induction of an inflammatory response. Interestingly, C3a was confirmed to have chemotactic activity (Pinto et al., 2003). In addition, a complement C6 gene is present in ascidians (Azumi et al., 2003) and other putative terminal pathway proteins as well as an MBL in echinoderms (Bulgakov et al., 2000; Multerer and Smith, 2004; Bulgakov et al., 2007). However, although it contains a membrane attack complex / perforin (MAC/PF) domain required for pore formation, a domain that interacts with other MAC forming proteins is absent and its function is therefore still unknown. In the complement system of the horseshoe crab, however, another alternative complement activation mechanism has been described in response to Gram-negative bacteria. In this process, a crucial role is played by Factor C, which upon binding to LPS, can activate C3 directly by proteolysis (Zhu et al., 2005; Ariki et al., 2008). The function of MASP appears therefore to be substituted by Factor C. However, Factor C does not directly activate C3 following infection by Gram-positive bacteria. The observation of an interaction between Bf and Factor C may indicate a functional role for Factor C in the activation of Bf and thus a role for a Bf-containing C3 convertase in response to Gram-positive bacteria (Zhu et al., 2005). In echinoderms, genes of C3 (Al-Sharif et al., 1998) and Bf (Smith et al., 1998) homologues have been characterised as well. Expression of C3 and Bf is significantly upregulated following challenges with LPS (Clow et al., 2000; Terwilliger et al., 2004) or bacteria (Shah et al., 2003) and results in phagocytosis of C3-coated microbes (Gross et al., 2000; Clow et al., 2004).

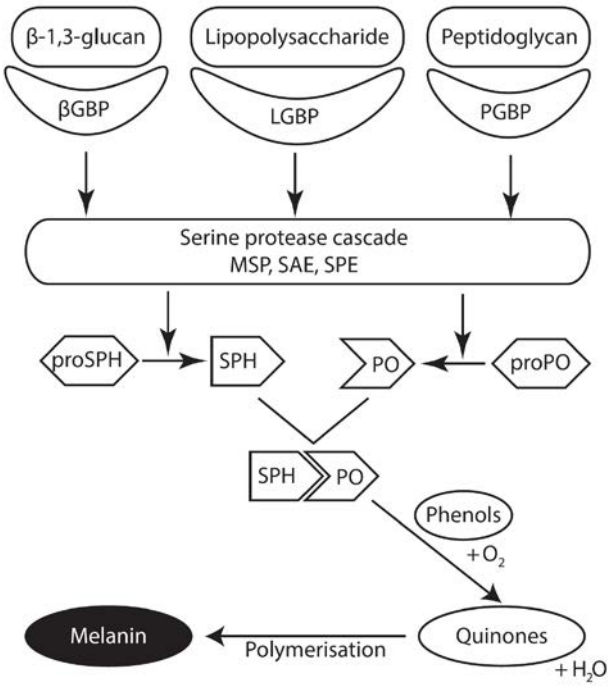
Although functionally poorly understood, homologues of components of the complement pathway have also been found in various invertebrate species, including C1q in *Biomphalaria glabrata* (Adema et al., 2010) and *Chlamys farreri* (Zhang et al., 2008a), C3 and Bf in *Ruditapes decussates* (Prado-Alvarez et al., 2009). In insects, however, no complement system has been found, albeit thioester-containing proteins (TEP) have structural similarities and biological functions to the complement proteins (Levashina et al., 2001; Moita et al., 2005; Dong et al., 2006a).

A.2.3 Prophenoloxidase-activating (proPO) system

The melanisation process is present in both vertebrate and invertebrate animals and plants, although its function can differ considerably. In invertebrates, melanisation is the result of activity of a highly conserved immune mechanism: the proPO system or melanisation cascade (Appendix A Fig. 3) (Cerenius et al., 2008). This system is

activated following physical injury or the detection of microbes via their MAMPs, such as LPS, β-1,3-glucan or PGN. Recognition or binding proteins for these MAMPs (LGBP, βGBP and PGRP, respectively) trigger a cascade of serine proteinases, which are sequentially processed. The last serine proteinase in this cascade, the proPO-activating enzyme, interacts with a serine proteinase homologue (SPH) once processed. In turn, this complex cleaves prophenoloxidase (proPO) into active phenoloxidase (PO), thereby initiating the melanisation process, where PO oxidises monophenolic or diphenolic substances (including tyrosine) into quinones that subsequently non-enzymatically polymerise into melanin. This compound immobilises the microbe by the formation of a physical barrier around the organism and in addition hardens the gel-like structure formed during the coagulation process. During this melanisation process, the reactive and cytotoxic quinone intermediates potentially attack the microbe, while opsonic factors, that promote phagocytosis, are also formed.

Because of the production of cytotoxic compounds that could harm the animal, it is crucial that the proPO system is tightly regulated. Several proteins that regulate the melanisation cascade have been described in insects and crustaceans. For example, serpins have been found to inhibit the serine proteinases in the proPO-activating cascade, while melanisation inhibition proteins (MIP) also regulate the polymerisation of quinones into melanin (Zhao et al., 2005; Soderhall et al., 2009). However, not only the host organism itself actively modulates the activity of the proPO system. Several pathogenic viruses, bacteria and parasites have been shown to actively inhibit this immune mechanism, underlining its important role in invertebrate immunity (Beck and Strand, 2007; Eleftherianos et al., 2007; Lu et al., 2008; Colinet et al., 2009).



Appendix A Figure 3: Schematic overview of the pro-phenoloxidase (proPO)-activating pathway. Upon detection of MAMPs by MAMP-binding proteins (βGBP, LGBP, PGBP), a serine protease cascade is activated leading to the conversion of proSPH and proPO into SPH and PO, respectively, and the formation of a SPH:PO complex. PO oxidises phenolic compounds into quinones, which subsequently polymerise non-enzymatically into melanin, while SPH restricts spatially uncontrolled PO activity via binding to the microbial surface. Abbreviations: βGBP, β-1,3-glucan binding protein; LGBP, lipopolysaccharide and β-1,3-glucan binding protein; PGBP, peptidoglycan binding protein; MSP, modular serine protease; SAE, SPE-activating enzyme; SPE; Spätzle-processing enzyme; SPH, serine protease homologue; PO, phenoloxidase.

Despite the important role of the proPO system in invertebrate immunity generally (Fujimoto et al., 1995; Yasuhara et al., 1995; Jiang et al., 1997; Kim et al., 2002b; Hillyer and Christensen, 2002; Hillyer et al., 2003; Irving et al., 2005), gastropods appear to rely less on this system compared to other invertebrates. However, proPO-regulating serpins have been discovered in the snail *Biomphalaira glabrata* (Adema et al., 2010). In the horseshoe crab, no proPO is present and PO function is taken over by the oxygen carrier hemocyanin, which is activated by the same serine protease cascade involved in hemolymph coagulation (Nagai and Kawabata, 2000). In the melanisation cascade, the monophenolic amino acid tyrosine is the rate-limiting substrate that can be produced by an enzyme called phenylalanine hydroxylase (PAH). PAH was upregulated in granule-containing immune cells in response to an immune challenge in mosquitoes (Johnson et al., 2003), suggesting a role in the melanisation process and innate immune response. Insect proPO lacks secretion signals and are released following rupture of the granular immune cells (Matsumoto et al., 2003; Shrestha and Kim, 2008), while the simultaneously secreted serpins regulate the proPO cascade extracellularly (Liu et al., 1997; Zou et al., 2009).

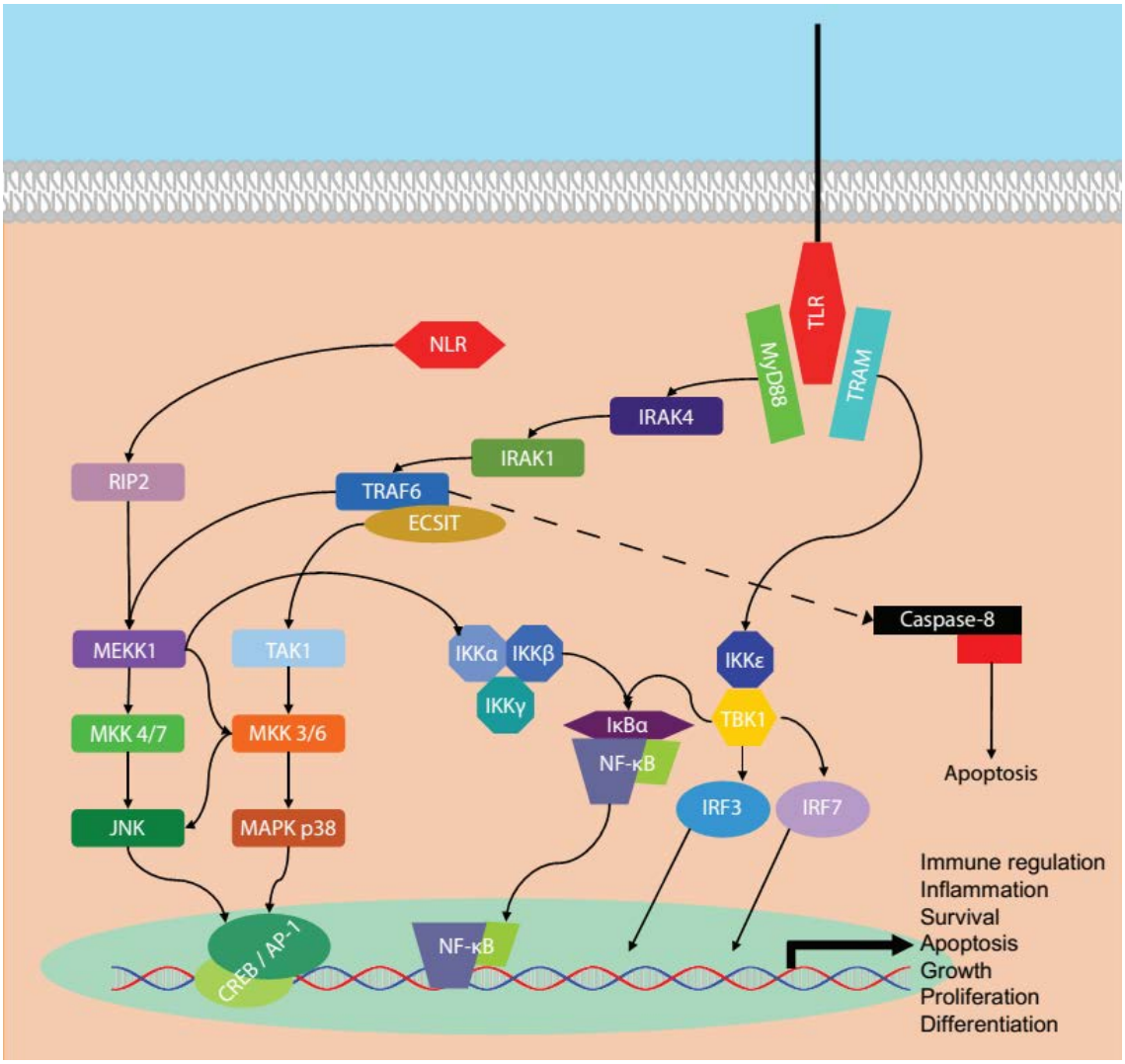
A.3 Immune receptor signalling

The detection of MAMPs by their respective receptors initiates signal transduction cascades that ultimately result in transcription of immune genes and potentially the activation of caspases. In addition to immune effector genes, the transcription of pro-inflammatory and immune system-regulatory cytokines is also induced. Signalling pathways involved appear to be conserved and can be initiated by multiple receptors of the innate immune system, each being activated by their own set of ligands (i.e. MAMPs). The combination of activated receptors and pathways will indicate the nature of the microbe and lead to an appropriate response. The ultimate immune response is further regulated via anti- and pro-inflammatory cytokine receptor signalling, thereby allowing additional fine tuning and boosting of the immune response.

A.3.1 Toll-like receptors

Toll-like receptors (TLR) are amongst the most conserved MAMP-detecting receptors in the animal kingdom. TLRs consist of an extracellular domain containing leucine-rich repeats (LRR) that are involved in the binding to MAMPs, and an intracellular domain containing a Toll-interleukin-like receptor (TIR) domain. Associated with the intracellular domain of TLRs are adaptor proteins including TRAM and MyD88. MAMP binding to these receptors generally leads to the activation of several signal transduction cascades (Appendix A Fig. 4) (Newton and Dixit, 2012). Signalling via TRAM results in

the activation of NFκB pathways as well interferon regulatory factors (IRF), while signalling via MyD88 and TRAF6 activates the cJun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) p38 and NFκB pathways. In addition, caspases can potentially be activated thereby inducing apoptosis (Appendix A Fig. 4).



Appendix A Figure 4: Toll and NOD-like receptor signalling pathways. Activation of the Toll-like receptor results in the activation of various signal transduction pathways: 1) signalling via TRAM leads indirectly to the activation of TBK1, which relays the signal through to IRFs and NFκB; 2) signalling via the MyD88-dependent pathway results in the activation of TRAF6 and ECSIT which activate the JNK and MAPK p38 pathways, respectively. Through activation of Tak1, TRAF6 also indirectly activates the NFκB pathway via the IKK complex. In addition, TRAF6 may induces apoptosis via a caspase-8-mediated pathway. NLR indirectly activates Tak1 thereby activating the JNK, MAPK p38 and NFκB pathways. Abbreviations: TLR, Toll-like receptor; MyD88, myeloid differentiation primary response; IRAK, interleukin-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; TNF, tumor necrosis factor; ECSIT, evolutionarily conserved signalling intermediate in Toll pathways; TAK1, transforming growth factor β-activated kinase-1; MEKK, MAPK/ERK kinase kinase; MKK, MAPK kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CREB, cAMP response element-binding protein; AP-1, activator protein-1; NLR, nucleotide-binding oligomerisation domain (NOD-like) receptor; RIP2, receptor-interacting serine/threonine-protein kinase-2; IKK, I-kappa-B kinase; NFκB, nuclear factor kappa B; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β; TIR, Toll/interleukin-1 receptor; TBK1, TANK-binding kinase; TANK, TRAF-family member-associated NFκB activator; IRF, interferon regulatory factor.

Interestingly, genomic analyses have revealed that the number of TLRs differ significantly between species. For example, whereas in tunicates only 2 TLR genes have been found (Azumi et al., 2003; Sasaki et al., 2009), amphioxus contains 72 (Huang et al., 2008) and the purple sea urchin a staggering 222 (Hibino et al., 2006). In the anthozoan *Nematostella vectensis* a gene of a prototypical TLR has been found as well as the typical signal transduction proteins downstream of TLR, MyD88 and NFκB (Miller et al., 2007a; Wolenski et al., 2011). In *Hydra*, however, regular TLRs are absent. Instead, two transmembrane proteins with LRR in their extracellular domain, but lacking an intracellular portion (HyLRR-1, HyLRR-2), and two transmembrane proteins with a TIR domain intracellularly, but no extracellular domain (HyTRR-1, HyTRR-2) were discovered (Miller et al., 2007a). Studies on HyTRR-1 and HyLRR-2 revealed that together these proteins form a complex with TLR function upon detection of the bacterial MAMPs flagellin or LPS (Bosch et al., 2009). In addition, it was shown that TLR function via MyD88 is critical for sensing bacteria (Franzenburg et al., 2012). Components of the TLR signalling pathways are also present in many other invertebrate. This includes TLR homologues in the gastropod *Lottia gigantean* and MyD88 in cephalopod *Euprymna scolopes* (Goodson et al., 2005). In bivalves, TLR homologues have been found as well (Tanguy et al., 2004; Song et al., 2006b; Qiu et al., 2007a; Perrigault et al., 2009), in addition to MyD88 (Gueguen et al., 2003; Qiu et al., 2007b; Wang et al., 2011), IRAK (Tanguy et al., 2004), TRAF3 (Roberts et al., 2009) and TRAF6 (Qiu et al., 2009; Wang et al., 2011). A role in immunity for TLR, MyD88 and TRAF6 in *Chlamys farreri* was suggested due to observed upregulation in response to PGN and LPS. In annelids, TLR function was induced following bacterial challenge, resulting in the upregulation of an inflammatory modulating cytokine with chemoattractant capabilities (Schikorski et al., 2009). Similar results were obtained for tunicates, where activation of TLRs by various MAMPs lead to downstream initiation of the NFκB pathway (Azumi et al., 2003; Sasaki et al., 2009), and in sponges, which upregulated a bactericidal protein in a MyD88-dependent manner following LPS detection (Wiens et al., 2005; Wiens et al., 2007). However, although present in horseshoe crabs, the TLR lacks MAMP-binding structures (Inamori et al., 2004) and its function is therefore unknown. In the nematode *Caenorhabditis elegans* TLR not only plays a role in resistance towards microbes (Tenor and Aballay, 2008), but is also involved in the behavioural avoidance of bacteria (Pujol et al., 2001; Pradel et al., 2007). TLR signalling has been studied in depth in *Drosophila* and is similar for all insects (Valanne et al., 2011). However, it differs from TLR signalling in other organisms in such that it requires MAMP-binding proteins to proteolytically release the ligand of Toll, Spätzle, which activates the Toll receptor, leading to initiation of the

NFκB pathway in a MyD88-dependent manner ultimately resulting in the production of anti-microbial peptides (AMP), which are essential for the defense against Gram-positive bacteria and fungi (Valanne et al., 2011). TLR signalling in crustaceans is similar to that in insects (Arts et al., 2007; Yang et al., 2008; Shi et al., 2009).

A.3.2 NOD-like receptors

Whereas TLRs detect microbes in the extracellular space, NOD-like receptors (NLR) detect MAMPs intracellularly. NLRs contain several distinct protein domains including a NACHT domain, a Pyrin domain (PYD), a DEATH or caspase recruitment domain (CARD) and a C-terminal LRR. MAMP binding to the LRR results in oligomerisation of NLRs and the subsequent activation of proinflammatory signal transduction cascades, e.g. NFκB pathway, autophagy or caspases (Appendix A Fig. 4) (Franchi et al., 2009; Newton and Dixit, 2012) and the formation of a protein complex called the inflammasome that induces cell death of infected cells via pyroptosis (Brodsky and Monack, 2009; Schroder et al., 2010). NLR homologues can be found throughout the animal and plant kingdoms, including in cnidarians (Lange et al., 2011). For example in *Hydra*, a DEATH and NACHT domain-containing NLR has been described that together with other DEATH domain-containing proteins may activate a caspase cascade resulting in apoptosis (Lange et al., 2011). In the sea urchin *Strongylocentrotus purpuratus* genome 222 NLR genes were discovered, although their function is still unknown (Brodsky and Monack, 2009).

A.3.3 TNF receptors

Signalling via the NFκB pathway, results in the transcription of multiple immune genes, including cytokines. One of these cytokines is tumor necrosis factor (TNF). TNF receptor signalling provides a positive feedback loop via the activation of the MAPK and NFκB pathways and could potentiate the induction of caspase-mediated apoptosis (Newton and Dixit, 2012).

Homologues of proteins involved in the TNF pathways have been found in many invertebrates, including TNFR in bivalves (Li et al., 2009b) and its ligand in abalone (De Zoysa et al., 2009a; De Zoysa et al., 2010), mussel (Hughes et al., 1990) and tunicates (Sasaki et al., 2009).

A.3.4 Scavenger receptors

Little is known about scavenger receptors (SR) in invertebrates, due to the isolation of only a few invertebrate SRs with immune function to date. SRs contain scavenger receptor cysteine-rich (SRCR) domains and can be found as membrane-bound or

secreted proteins. From *Drosophila*, dSR-C1 (Pearson et al., 1995), which binds double stranded RNA (Ulvila et al., 2006) and bacteria (Ramet et al., 2001), was isolated and putative SR genes are present in sponges (Blumbach et al., 1998). Another SR, CfSR, was isolated from the bivalve *Chlamys farreri* and was found to be expressed on the outer cell membrane of hemocytes and detect acetylated LDL, dextran sulphate and various PAMPs, playing a role in opsonisation. In *C. elegans*, SRs play a major role in the immunity against fungus (Means et al., 2009) as well as in reponse to toxins released by *Bacillus thuringiensis* that elicits an unfolded protein response in a MAPK p38 dependent-manner (Huffman et al., 2004, Bischof et al., 2008; Haskins et al., 2008). Although functionally uncharacterised, the purple sea urchin contains over 1000 different SRCR domains, indicating their importance in immunity in this species (Hibino et al., 2006). Interestingly, SR SCARF1 is, in vertebrates, also involved in the phagocytosis of apoptotic cells by specialised immune cells in a complement C1q-dependent manner (Ramirez-Ortiz et al., 2013).

A.3.5 Signal transduction pathways

The NFκB pathway (Appendix A Fig. 4) is a highly conserved pathway crucial in the immune response. The transcription factor NFκB is normally associated with the inhibitor of κB (IκB), which is destined for degradation by serine kinase I kinase (IKK) upon activation of IKK by TLR, TNFR or NLR. NFκB translocates to the nucleus where it regulates the expression of immune genes (Hayden and Ghosh, 2012). The importance of this pathway is demonstrated by the presence of NFκB homologues in many invertebrates, including insects (Valanne et al., 2011), gastropods (Jiang and Wu, 2007; Adema et al., 2010), bivalves (Wu et al., 2007; Wang et al., 2011), crustaceans (Wang et al., 2006; Li et al., 2009a; Li et al., 2010c). In bivalves, homologues of the NFκB regulators IκB (Song et al., 2006b; Montagnani et al., 2008; Wang et al., 2009b; Zhang et al., 2009a) and IKK (Xiong et al., 2008) have also been characterised and were shown to form an active NFκB pathway in pearl oysters. Similarly, in the horse shoe crab, IκB was rapidly degraded and NFκB translocated to the nucleus in response to a Gram-negative bacterial infection, resulting in the upregulation of immune-related genes, including iNOS, which is responsible for the respiratory burst, and Factor C, which is involved in the coagulation and complement cascades (Wang et al., 2006). In addition, NFκB signalling is crucial for mounting an AMP-based defense (Valanne et al., 2011).

Activation of innate immune system receptors also lead to the activation of several MAPK pathways via MyD88 and TRAF6, including the JNK, p38 and ERK pathways (Appendix A Fig. 4) (Arthur and Ley, 2013). MAPK pathways are

characterised by at least 3 kinases that activate one another via phosphorylation: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK. While the JNK and p38 pathways are activated by the MAP3K TAK-1, the ERK pathways depend on IKK signalling. Ultimately signalling results in the activation of transcription factors (e.g. the AP-1 complex (Hess et al., 2004)) that regulate the production of pro- and anti-inflammatory cytokines (TNF, interleukins, prostaglandins, interferons) and the expression of iNOS, activates NADPH oxidases as well as regulates phagocytosis and stimulates cell survival and proliferation (Arthur and Ley, 2013). Components of MAPK pathways have been found in bivalves (Canesi et al., 2003; Kang et al., 2006; Zhang et al., 2012a) and are essential for the defense against bacteria and fungi in *C. elegans* (Aballay et al., 2003; Huffman et al., 2004; Nicholas and Hodgkin, 2004; Begun et al., 2007; Pujol et al., 2008a). In addition, MAPK p38 appears to play a crucial role in the spreading of snail hemocytes and the respiratory burst.

Anther important pathway is the JAK-STAT pathway, which has been shown to be activated by interferon-γ in oysters (Canesi et al., 2003), and is important for the anti-viral response in flies (Dostert et al., 2005). In addition, interferon regulatory factors have been found in bivalve (Huang et al., 2013).

A.4 Immune effector molecules

A.4.1 Anti-microbial peptides

Signalling initiated by PRRs via the NFκB pathway is also essential for the production of anti-microbial peptides (AMP). In general, AMPs are produced as proAMPs and cleavage results in a functional microbicidal AMP. The modes of action of AMPs are diverse, but the majority forms pores in the microbial cell wall or bind to intracellular molecules essential for a microbe's survival. A wide range of AMPs has been described in invertebrates, with some being exclusively taxon-specific, having no orthologues in other organisms. One of those taxon-specific bactericidal AMPs is Periculin-1, which was isolated from *Hydra* (Canicatti and Rizzo, 1991). Also from *Hydra* is the AMP Arminin 1a, which is very potent against bacteria, including methicillin and vancomycin-resistant human pathogens (Augustin et al., 2009a), whereas Aurelin from the jellyfish *Aurelia aurita* effectively targets both Gram-negative and Gram-positive bacteria (Ovchinnikova et al., 2006). Another AMP extracted from *Hydra* with a different bactericidal action is Hydramycin-1 (Jung et al., 2009). This AMP induces aggregation of bacteria, potentially by interfering with the bacteria quorum sensing, and which is subsequently followed by apoptosis of the bacteria. Functional studies on the importance of AMP expression in *Hydra* have shown that the composition of bacterial communities associated with this cnidarian are strictly

regulated by AMPs in both larvae (Fraune et al., 2010; Fraune et al., 2011) and adults (Fraune and Bosch, 2007; Franzenburg et al., 2013).

AMPs with activity against Gram-positive and Gram-negative bacteria and fungi have also been found in gastropods, such as aplysianins of *Aplysia* (Iijima et al., 1995; Kamiya et al., 1986) and Abhisin in the abalone (De Zoysa et al., 2009c). Interestingly, the precursor of Abhisin is histone H2A, one of the main histones in the chromatin structure and therefore ubiquitously expressed. Anti-bacterial, anti-fungal, anti-viral and anti-protist activity has been documented for the many AMPs in bivalves (Mitta et al., 2000; Gonzalez et al., 2007; Zhao et al., 2007; Xu et al., 2010; Xu and Faisal, 2010; Balseiro et al., 2011), insects (Okada and Natori, 1983; Iijima et al., 1993; Lowenberger et al., 1995; Ekengren and Hultmark, 1999; Lamberty et al., 1999; Schuhmann et al., 2003; Bartholomay et al., 2004), nematodes (Banyai and Patthy, 1998; Pujol et al., 2008b; Zugasti and Ewbank, 2009; Roeder et al., 2010), echinoderms (Beauregard et al., 2001; Chludil et al., 2002a; Chludil et al., 2002b; Lee et al., 2007), arachnids (Nakajima et al., 2003a; Nakajima et al., 2003b; Fogaca et al., 2006; Machado et al., 2007) and crustaceans (Smith et al., 2008; Cuthbertson et al., 2008), while for annelids currently only Gram-positive bacteria-targeting AMPs are known (Tasiemski et al., 2000; Tasiemski et al., 2004). Interestingly, the expression of some annelid AMPs is not induced upon microbial stimulation (Cho et al., 1998; Wang et al., 2003), suggesting a role in the constitutive defense. AMPs in horse shoe crabs are primarily stored in the dense granules of hemocytes (Iwanaga et al., 1998) and have bactericidal and fungicidal activity, in addition to chitin recognition (Saito et al., 1995b; Kawabata et al., 1996; Kawabata et al., 1997; Osaki et al., 1999).

A.4.2 Serine protease inhibitors

In addition to AMPs, *Hydra* also expresses kazal-type serine protease inhibitors (SPI), such as Kazal-2, which exhibits strong anti-bacterial properties through the inhibition of essential bacterial-specific serine proteases (Augustin et al., 2009b). Other Kazal-type SPIs have been found in scallop (Wang et al., 2008a) and crustaceans (Cerenius et al., 2010b).

A.4.3 Anti-microbial proteins

Anti-microbial proteins are also found in invertebrates. Perforin is a potent cytolytic protein that forms pores in cell membranes, for which the membrane attack complex (MAC) / perforin (PF) domain is essential. In vertebrates this protein is present in the granules of cytotoxic T lymphocytes and Natural Killer cells and exerts its action following degranulation. Although the exact function is unknown, perforin-like

molecules have been discovered in invertebrates, including abalone (Mah et al., 2004; Wang et al., 2008b), *Hydra* (Altincicek and Vilcinskas, 2008) and sponges (Wiens et al., 2005) as well as apextrins in sea urchins (Haag et al., 1999).

A.4.4 Lectins

Lectins are a class of PRRs that specifically recognise terminal sugar moieties of glycoproteins and glycolipids. C-type lectins contain one or multiple carbohydrate-recognition domains (CRD) and can be structurally diverse (Zelensky and Gready, 2005). Depending on their specificity they bind to sugar moieties of microbial cell wall components, such as mannose, galactose, n-acetylglucosamine (GlcNAc) or n-acetylgalactosamine (GalNAc) as well as sialic acid. However, lectins which recognise the same sugar residue do not necessarily bind the same microbes, showing diversity and specificity of lectins in targeting microbes (Hardison and Brown, 2012; Hoving et al., 2013). Functionally, most C-type lectins play a role in agglutinating microbes, thereby immobilising them, and promote phagocytosis or encapsulation (via Syk-kinase signalling through C-type lectin receptors (CLR) (Drummond and Brown, 2013)), while others are part of the lectin-complement pathway and are associated with MASPs, and initiate this effective immune mechanism upon binding (Lis and Sharon, 1998; Vasta et al., 2007; Kerrigan and Brown, 2009). Such lectins have been found in most invertebrates, including ascidians (Suzuki et al., 1990; Vasta et al., 2001), bivalves (Fisher and DiNuzzo, 1991; Olafsen et al., 1992; Minamikawa et al., 2004; Bulgakov et al., 2004; Gourdine and Smith-Ravin, 2007; Kim et al., 2008a; Takahashi et al., 2008; Yamaura et al., 2008; Jayaraj et al., 2008; Adhya et al., 2009; Itoh and Takahashi, 2009; Wang et al., 2009b; Adhya et al., 2010), horseshoe crabs (Saito et al., 1995a; Gokudan et al., 1999; Inamori et al., 1999; Iwaki et al., 1999; Nagai et al., 1999; Beisel et al., 1999; Chiou et al., 2000; Kairies et al., 2001; Chen et al., 2001; Harrington et al., 2009), nematodes (O'Rourke et al., 2006; Schulenburg et al., 2008; Wong et al., 2007), gastropods (Hertel et al., 1994; Wang et al., 2008d; Hathaway et al., 2010), arachnids (Rego et al., 2005; Rego et al., 2006), crustaceans (Gross et al., 2001; Ma et al., 2008; Sun et al., 2008; Wang et al., 2009d; Zhao et al., 2009), echinoderms (Giga et al., 1987; Hatakeyama et al., 1994; Kakiuchi et al., 2002; Hibino et al., 2006; Bulgakov et al., 2007; Bulgakov et al., 2013), insects (Jomori and Natori, 1992; Shin et al., 2000; Yu and Kanost, 2003; Ling and Yu, 2006; Schnitger et al., 2009; Takase et al., 2009), sponges (Pfeifer et al., 1993; Kawagishi et al., 1994; Schroder et al., 2003; Dresch et al., 2012; Carneiro et al., 2013) and the cnidarian *Nematostella vectensis* (Wood-Charlson and Weis, 2009). Another class of C-type lectins is involved in the coagulation process: hemolectins. These lectins are important in immune function by

forming a secondary barrier, immobilising infiltrating microbes and promoting their elimination (Goto et al., 2001; Goto et al., 2003; Scherfer et al., 2006; Lesch et al., 2007; Lindgren et al., 2008; Bajzek et al., 2012; Chang et al., 2012).

Galactins are lectins that specifically bind β-galactoside and may be the most conserved lectins. Several galactins have been isolated from bivalves (Tasumi and Vasta, 2007; Yamaura et al., 2008; Kim et al., 2008b; Song et al., 2010) and function in agglutination of bacteria or promoting phagocytosis of various organisms, such as protists, bacteria and unicellular algae. For arachnids, however, no functional role for galectins have been documented (Huang et al., 2007). Interstingly, in *C. elegans* galectins also competitively bind to glycolipids on the host with bacterial toxins, thereby inhibiting toxicity (Ideo et al., 2009).

A.4.5 Fibrinogen-related proteins

Fibrinogen-related proteins (FREP) also have agglutinating functions, in addition to anti-microbial activity (Hanington and Zhang, 2011). Functional analyses have revealed that FREPs are responsive to bacteria, fungi and parasites and exert anti-microbial activity in different ways, including melanisation and bacteriolysis. The importance and complexity of FREP function in invertebrate immunity is shown by the large diversity of FREPs, their complementary and synergistic effects through intra- and inter-molecular associations and their widespread use among invertebrates (Wang et al., 2004; Waterhouse et al., 2007; Zhang et al., 2008b; Dong and Dimopoulos, 2009; Zhang et al., 2009b; Adema et al., 2010; Hanington and Zhang, 2011).

A.4.6 Lysozyme

Lysozyme is regarded as one of the crucial innate immune effector molecules against bacteria. This enzyme is ubiquitously present in many animals and hydrolyses the β-1,4-glycosidic linkage between N-acetylmuramic acid and N-acetylglucosimne of peptidoglycan, thereby causing lysis of bacteria cells (Callewaert and Michiels, 2010). Interestingly, lysozyme appears to synergistically increase the effect of AMPs (Haug et al., 2004).

Lysozyme proteins have been found in many bivalves (Nilsen et al., 1999; Takeshita et al., 2004b; Xue et al., 2004), annelids (Quaglino et al., 1996; Joskova et al., 2009), nematodes (Mallo et al., 2002; O'Rourke et al., 2006), insects (Yu et al., 2002; Dziarski and Gupta, 2006; Lemaitre and Hoffmann, 2007; Chapelle et al., 2009; Wang et al., 2009c), arachnids (Kopacek et al., 1999; Grunclova et al., 2003; Simser et al., 2004; Tanaka et al., 2010), echinoderms (Canicatti and Roch, 1989; Shimizu et al.,

1999) and sponges (Thakur et al., 2005), exhibiting bactericidal activity against Gram-positive and Gram-negative bacteria.

A.4.7 Cytokines

Cytokines are small signalling proteins and their expression is largely regulated by MAPK pathways. Tumor necrosis factor (TNF), interleukins, prostaglandins, interferons are examples of cytokines, which have a range of pro- and anti-inflammatory functions, including chemotaxis, phagocytosis, cell motility and cytotoxicity.

Besides TNF signalling (see A.3.3 and A.3.5), other cytokines have also been discovered in invertebrates. Currently two interleukins have been described in bivalves: IL-1 (Hughes et al., 1990) and IL17 (Roberts et al., 2008), of which IL-17 expression has been shown to be elevated following bacterial challenge. In the abalone, expression of a homologue of the apoptosis-inducing Fas ligand was found to be significantly upregulated in response to bacteria, viruses and LPS and induce the production of ROS (De Zoysa et al., 2009b). Another signalling molecule, transforming growth factor β (TGFβ), is upregulated in response to infection by *Serratia marcescens* in *C. elegans* and induces the expression of AMPs, lectins and lysozyme via MAPK p38 signalling (Mallo et al., 2002; Zugasti and Ewbank, 2009). In contrast, TGFβ appears to suppress an immune response in the cnidarian *Aiptasia pallida* allowing the successful establishment of symbiosis with dinoflagellates (Detournay et al., 2012). In addition, cytokines are known to direct the migration of immune cells via chemotaxis, aid in clotting at wound sites (Nakatogawa et al., 2009) and promote adhesion of immune cells (Clark et al., 1997; Wang et al., 1999), which is important for encapsulation of pathogens (Eleftherianos et al., 2009), while others stimulate proliferation of immune cells and induce the release of granule contents (Matsumoto et al., 2003). Other eicosanoid cytokines are produced by cyclooxygenase (COX) or lipoxygenase (LOX), and capable of inducing encapsulation and the expression of immune-related genes, such as proPO, lysozyme, serpins and AMPs (Shrestha and Kim, 2009), while only prostaglandins stimulated the release of proPO upon microbial challenge (Shrestha and Kim, 2008; Shrestha and Kim, 2009).

A.4.8 Anti-oxidant enzymes

Reactive oxygen species (ROS) play a major role in normal cell function, including cell signalling. ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•), is also produced in response to microbial infection during the destruction of encapsulated or phagocytised pathogens by NADPH oxidases and iNOS. However, ROS indiscriminately damages proteins, lipids and nucleotides and

therefore may also target the host, leading to oxidative stress and possibly to apoptosis or necrosis. Anti-oxidant enzymes neutralise ROS into harmless molecules during an immune response as well as stress due to contamination in aquatic invertebrates. The anti-oxidant enzymes super oxide dismutase (SOD) converts O_2^- into H_2O_2 and oxygen, while catalase and glutathione peroxidase (GPx) reduce H_2O_2 into water and oxygen. In addition, GPx reduces lipid peroxides into their respective alcohols. Another crucial enzyme is glutathione S-transferase (GST), a phase II detoxification enzyme that conjugates glutathione to hydrophobic compounds. As a substrate for anti-oxidant enzymes it is involved in the elimination of ROS.

In the cnidarian *Anemonia viridis*, a large number of superoxide dismutase isoforms have been found, including two copper- and zinc-containing SODs. These SODs are likely involved in the protection of the animal against reactive oxygen species produced during photosynthesis by the endosymbiotic zooxanthellae, resulting in hyperoxic states (Plantivaux et al., 2004). In gastropods, SOD has been found in eggs of *Biomphalaria*, showing parental investment in the protection of their brood (Hathaway et al., 2010), whereas snails resistant to trematode infection expressed different SOD compared to infected snails (Bayne, 2009) and sponges upregulate GPx in the allorecognition response (Kruse et al., 1999). Involvement of SOD (Estrada et al., 2007; Xing et al., 2008; Bao et al., 2008), catalase (Li et al., 2008) and GPx (Box et al., 2009) in the immune response has been documented in bivalves. In addition, GST appears to be exclusive to the response to exposure to xenobiotic compounds or other environmental stresses (Hoarau et al., 2001; Hoarau et al., 2002; Le Pennec and Le Pennec, 2003; Hoarau et al., 2004; Moreira and Guilhermino, 2005; Umasuthan et al., 2012), although in combination with SOD (Bebianno et al., 2004; Vlahogianni et al., 2007; Monari et al., 2007; Monari et al., 2009), catalase (Bebianno et al., 2004; Damiens et al., 2004; Vlahogianni et al., 2007; Contardo-Jara et al., 2009; Letendre et al., 2009) and GPx (Bebianno et al., 2004; Vlahogianni et al., 2007).

A.5 Other innate immune system components

A.5.1 Epithelial barriers

The internal milieu of organisms is separated from the environment by epithelial cell layers and can therefore be considered the first line of defense against microbes (Davis and Engstrom, 2012). Epithelial cells are connected via tight junctions, forming a barrier that physically prevents microbes from entering the host. Mucosal cells in the epithelia produce mucus, which also has protective functions, such as preventing microbes from adhering to the tissues. In addition, epithelial cells produce and secrete AMPs that have been shown to be crucial in the maintenance of healthy tissue-

associated microbial communities in various organisms, including *Hydra* (Fraune and Bosch, 2007; Fraune et al., 2010; Fraune et al., 2011; Franzenburg et al., 2013). Other immunological functions of epithelial cells of the freshwater polyp *Hydra* are phagocytisis and AMP secretion (Bosch et al., 2009), while gland cells produce anti-microbial serine protease inhibitors (Augustin et al., 2009b).

A.5.2 Apoptosis and r*egeneration*

In addition, the cell layers in cnidarians have a strong regenerative capacity and thereby rapidly replace cells following damage or infection. In combination with the apoptosis-inducing capabilities of TLR and NLR signalling, the rapid regeneration of affected tissues could be considered to be part of the innate immune system in cnidarians as well. Apoptosis has been shown to play a crucial role in the antiviral response in lepidopterans (Clem, 2007), while in crustaceans such a function is debatable and may depend on the pathogen (Wang et al., 2008c; Rijiravanich et al., 2008).

A.5.3 Danger Associated Molecular Patterns (DAMP)

An innate immune response is also induced following the detection of danger-associated molecular patterns (DAMP) (Kono and Rock, 2008). Under normal conditions, DAMPs are harboured within cells. However, when damaged, cells may release DAMPs and thereby notify neighbouring cells of danger and eliciting an immune response. In *Hydra*, extracellular nucleotides and monosodium urate cause an immune response (Bosch et al., 2009), while DAMPs in insects may activate the proPO system (Bidla et al., 2009).

A.5.4 Anti-viral proteins

Viruses are also highly prevalent in the marine environment and an anti-viral innate immune system component is therefore also expected in invertebrates. Indeed, in abalones homologues of a Mx protein and a cytidine deaminase (Wu et al., 2009), which potentially interfere with viral reproduction (Haller et al., 2007; Wu et al., 2009) and mark virus-infected cells for elimination (Bekerman et al., 2013) respectively, have been discovered. Protection against viruses could also be mediated through the RNA silencing mechanism, Dicer and Argonaute (Unajak et al., 2006; Robalino et al., 2007; Yao et al., 2010).

A.5.5 MAMP-binding proteins

There are several classes of MAMP-binding proteins such as the Gram-negative binding proteins (GNBP), which binds LPS and β-1,3-glucan, and peptidoglycan recognition proteins (PGRP). Although the exact mechanisms of most of these proteins are still poorly understood, they play a major role in diverse innate immune responses such as melanisation, bacterial agglutination, lysozyme activation, TLR pathway initiation and phagocytosis. PGRPs and GNBPs have been found in insects (Ochiai and Ashida, 1988; Lee et al., 1996; Dimopoulos et al., 1997; Ochiai and Ashida, 1999; Werner et al., 2000; Ma and Kanost, 2000; Michel et al., 2001; Zhang et al., 2003; Lee et al., 2004; Ju et al., 2006), gastropods (Zhang et al., 2007; Hathaway et al., 2010), crustaceans (Duvic and Soderhall, 1990; Cerenius et al., 1994; Lee et al., 2000) and bivalves (Christophides et al., 2002; Ni et al., 2007; Su et al., 2007; Wang et al., 2009b).

A.5.6 Alpha-2-macroglobulin

Proteases play a major role in disease as virulence factors from pathogens. One of the mechanisms by which organisms defend themselves against these proteases is by producing protease inhibitors such as α2-macroglobulin (A2M or α2M), which enfold the protease and marks it for opsonisation and destruction (Rehman et al., 2013). This opsonin function is not exclusive to free proteases and A2M is known to play a significant role in microbe phagocytosis. This complement-related protein has been described for the horseshoe crab (Iwaki et al., 1996), arachnids (Saravanan et al., 2003; Buresova et al., 2009) and crustaceans (Ma et al., 2010; Perazzolo et al., 2011).

A.5.7 Acute Phase Proteins

Acute Phase Proteins (APP) are involved in the protection of organisms against xenobiotic compounds and microbes. In the immune response they have functions in infection prevention, clearing microbes from the host, initiate inflammatory responses and aid in healing processes. Heat shock proteins (HSP) are highly conserved stress proteins (Hickman-Miller and Hildebrand, 2004) that prevent host damage following various stressors, including high temperatures, salinity and chemicals. HSPs have also been suggested to activate the innate immune system (Srivastava, 2002; Tsan and Gao, 2004), for example in response to *Vibrio* bacteria (Song et al., 2006a; Gao et al., 2008; Li et al., 2010a; Zhang et al., 2010) and wound healing and regeneration (Tsan and Gao, 2004; Pinsino et al., 2007). Another class of APPs are metallothioneins (MT), which generally have a function against oxidative stress and xenobiotic compounds (Bigot et al., 2009). However, expression of MT was also significantly upregulated

following a bacterial challenge in scallops (Wang et al., 2009a), suggesting a role in immunity. In echinoderms, the APP ferritin is also upregulated following an immune challenge resulting in the storage of iron (Smith et al., 1996; Beck et al., 2002; Nair et al., 2005; Ramirez-Gomez et al., 2008), thereby depriving microbes from this essential element and inhibiting their growth.

A.5.8 Down Syndrome Cell Adhesion Molecules

Down syndrome cell adhesion molecules (Dscam) are best characterised in insects and play roles in neural development (Shi and Lee, 2012) and innate immunity, e.g. by promoting phagocytosis (Watson et al., 2005). These proteins are part of the immunoglobulin (Ig) superfamily and contain multiple Ig domains. Gene duplications and alternative splicing have resulted in a huge array of Dscam extracellular domains, e.g. 19000 in flies (Schmucker et al., 2000) and up to 31000 in mosquitoes (Dong et al., 2006b). This extreme diversity may relate to the recognition of different antigens in the immune response with evidence that TLR signalling via the NFκB pathway results in the activation of the splicing factors that determine the production of pathogen-specific Dscam (Dong et al., 2012). However, Dscam is suggested to have originated after the split between cnidarians and bilaterians (Armitage et al., 2012). In addition, other Ig superfamily genes have also been shown to play roles in controlling and eliminating bacteria (Garver et al., 2008).

A.5.9 Microbial community

Epithelial cell layers are associated with a highly diverse microbiota, of which the structure can be highly regulated by the host, such as is described for *Hydra* (Fraune and Bosch, 2007; Fraune et al., 2010; Fraune et al., 2011; Franzenburg et al., 2013). The microbiota consists of commensal or symbiotic microbes that compete with pathogenic microbes for space and nutrients. In addition, some microbes produce antimicrobial compounds (e.g. bacteriocins) and thereby influence the composition of the microbiota. In addition, microbes can provide the host with essential nutrients, or play a role in homeostasis maintenance or development. This underlines the importance the epithelia-associated microbial community in host fitness (Fraune and Bosch, 2010; Nyholm and Graf, 2012).

Appendix B

"Relative quantification of fluorescent proteins"

(Methods adapted from Paley *et al*., in review PLoS ONE)

Relative contributions of individual FPs (cyan, green, and red; CFP, GFP and RFP, respectively) to total fluorescence were determined using multiple regression analysis (implemented in R) of the fluorescence spectra of coral protein extracts excited at 280 nm. The analysis was based on the emission spectra of purified A. millepora FPs expressed in bacteria (Alieva et al., 2008). The exponentially decaying background was calculated using the formula B = F450 / exp(k*(w-450)), where F450 is the fluorescence reading at 450 nm (where no FP emission is observed), w is wavelength in nanometers, and k is the empirically determined coefficient resulting in a good fit of the multiple regression model. The parameter k was kept the same (k = 0.1) for all samples analysed. The relative proportions of FPs were calculated based on the area of the background-subtracted fluorescence spectrum attributable to each FP component, multiplied by the coefficient reflecting the relative excitability of purified A. millepora FPs at 280 nm (CFP: 0.85, GFP: 0.92, RFP: 1). The relative amounts of each FP thus determined were then multiplied by the background-subtracted total fluorescence and standardized to total protein concentration determined. The resulting values are directly proportional to the concentration of each FP in the sample.

Appendix C

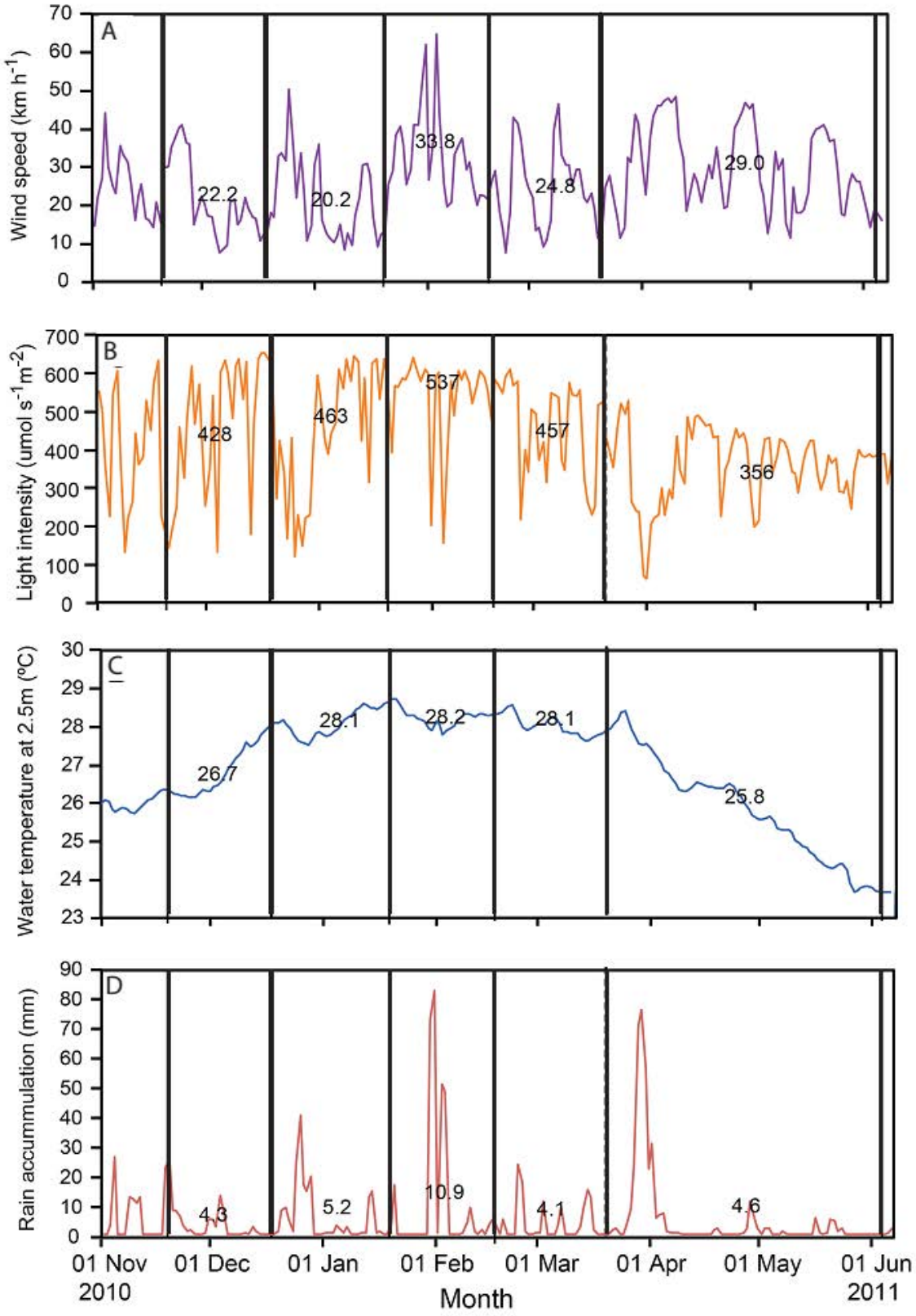
"Reduced coral-associated bacterial diversity coincide with increased white syndrome coral disease levels adjacent to reef platforms"

F. Joseph Pollock, Joleah B. Lamb, **Jeroen A.J.M. van de Water**, Britta Schaffelke, Bette L. Willis and David G. Bourne (manuscript in preparation)

Select sections from the Results of the draft manuscript by Pollock *et al*. adapted in support of statements made in Chapter 3.

C.1 - Physical environment characteristics and colony condition

All tagged colonies monitored at the control site (n=8) remained visually healthy throughout the duration of the study. All tagged colonies located adjacent to the platforms (n=16) were visually healthy in November and December, at the beginning of the study. In January, five colonies located at the platform sites (31% of platform colonies) displayed exposed skeleton lesion radiating from the center of the colony with severe tissue necrosis characteristic of WS (i.e. diffuse, acute-to-sub-acute areas of tissue loss revealing white, intact skeleton with no evidence of predation). WS-induced tissue loss ranged from 40% to 50% partial-colony mortality per affected coral. Between December and January, the reef experienced the greatest change in mean daily water temperature (+1.4 ºC; Appendix C Figure 1A) observed during the 8-month study and seven consecutive days with rain accumulation greater than 1 standard deviation above the mean accumulation recorded over the entire study period (mean±SD=5.4 mm day^{-1}±13.1 for the study, 20.4±9.6 mm day^{-1} over this 7 day period). In February, no new colonies developed disease signs and apart from 1 colony at the platform site with progressing WS, all disease lesions had ceased progression and no characteristic WS bands were evident. Between January and February, the reef experienced the highest mean daily wind speed, light intensity, water temperature and rain accumulation (Appendix C Figure 1B-F). During this period, a severe tropical cyclone passed 270 km north of the Whitsunday region and the reef experienced four consecutive days with rain accumulation 3 standard deviations above the study mean



Appendix C Figure 1 - Daily means of the environmental variables: (B) wind speed (km h^{-1}), (C) light intensity (photosynthetically active radiation, µmol s^{-1} m^{-2}), (D) water temperature (2.5 m depth, ºC) and (E) rainfall accumulation (mm). Bold vertical lines indicate sampling time points Numbers between vertical lines represent mean daily values between time points. Environmental data obtained from the Australian Institute of Marine Science (AIMS) monitoring station located on the main tourist platform at Hardy Reef.

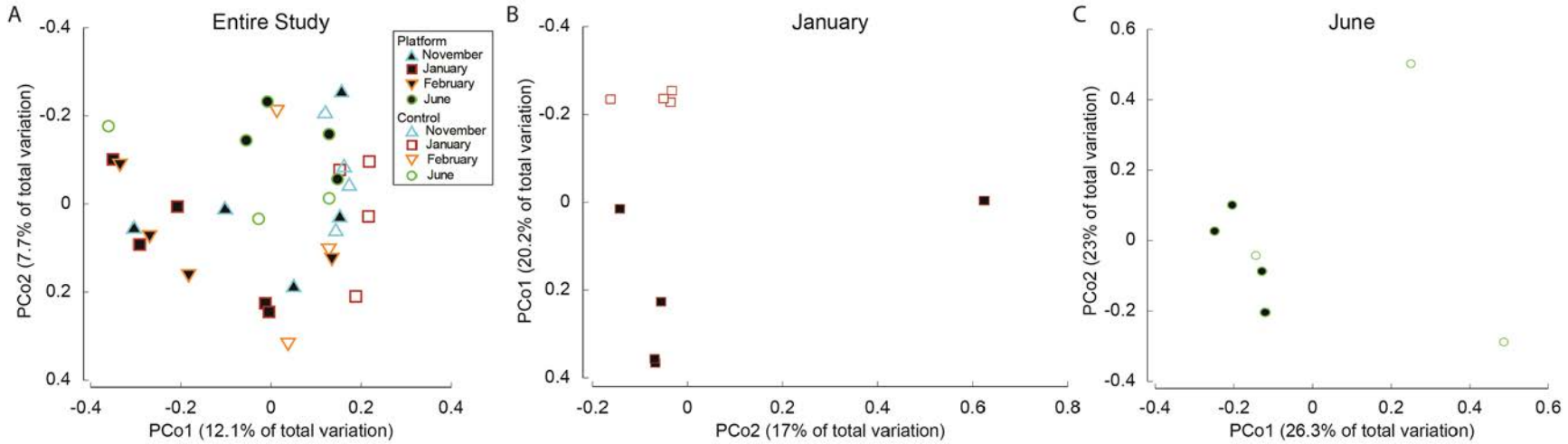
(3 day mean±SD=63.2 mm day^{-1}±16.6). No further disease development or lesion progression was observed in March, and all colonies previously recorded with WS at the platform site were visually healthy apart from healed, partial-colony mortality. In June, all colonies were again visually healthy. Mean daily water temperature ranged from 23.7ºC (June) to 28.8ºC (February) during the study period (Appendix C Figure 1D).

C.2 - Reduced coral-associated bacterial diversity on corals near reef platforms

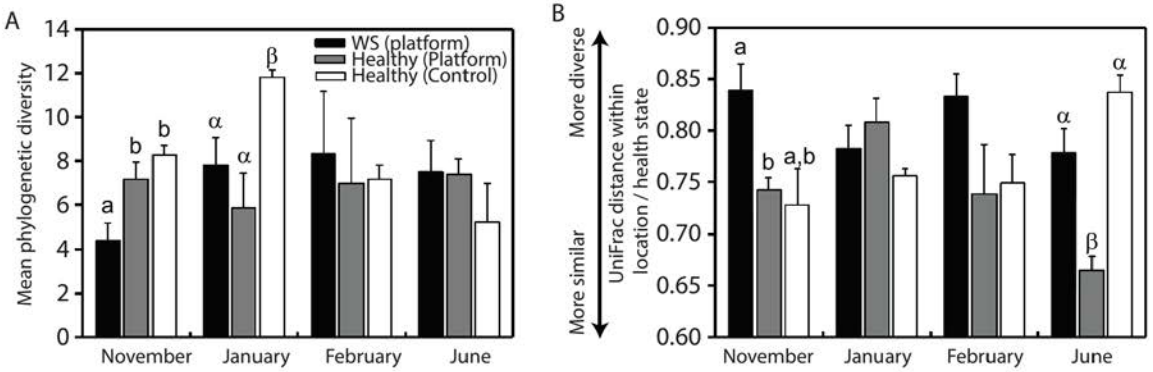
Bacterial communities associated with healthy colonies at each location (i.e. healthy colonies at platform sites v. healthy colonies at control site) fell into consistent phylogenetic clusters over the 8-month sampling period (df=1, pseudo-F=1.43, *P*=0.03). Clustering within location, visualized by a partitioning of the samples in the PCO ordination (Appendix C Figure 2A), indicates that healthy samples from platform sites were more similar to each other in bacterial phylogenetic structure than they were to healthy control site samples (df=1, pseudo-F=1.43, *P*=0.03). While bacterial communities did not differ significantly between sampling time points (df=3, pseudo-F=1.04, *P*=0.34), differences between locations were most pronounced in January (df=1, pseudo-F=1.58, *P*=0.005, Appendix C Figure 2B) and June (df=1, pseudo-F=1.32, *P*=0.03, Appendix C Figure 2C).

In January, bacterial diversity on platform site corals (mean phylogenetic diversity: 6.2±1.4, mean ± standard error) was nearly 50% lower than at the control site (11.8±0.3) (HSD, *P*=0.008, Appendix C Figure 3A). Bacterial community heterogeneity, which can be roughly visualized as the amount of within-location dispersion (i.e. variability) in the PCO ordination (Appendix C Figure 2B), did not differ significantly between platform (mean within-location Unifrac distance: 0.81±0.02) and control sites (0.76±0.01) (HSD, *P*>0.05, Appendix C Figure 3B), suggesting that differences in bacterial community diversity rather than heterogeneity underpins the detected dissimilarity between locations.

In June, disparities in bacterial community heterogeneity appeared to drive the observed separation between locations. Bacterial community structure was far more consistent (i.e. homogeneous) among platform site corals (mean UniFrac distance: 0.66±0.01) than control site corals (mean within-location UniFrac distance: 0.84±0.02) (HSD, *P*=0.002, Appendix C Figure 3B). These differences in bacterial community heterogeneity can be visualized as the large dispersion of control site samples relative



Appendix C Figure 2 - Two-dimensional principal coordinate ordination plots visualizing dissimilarity between bacterial communities (unweighted Unifrac distance) associated with A. millepora coral colonies remaining visually healthy throughout the study at control (white symbols) and platform sites (black symbols) (a) throughout the 8-month study, (b) in January only and (c) in June only. November = blue triangles, January = red squares, February = orange triangles and June = green circles.



Appendix C Figure 3 - A) Mean phylogenetic diversity and B) phylogenetic variation (measured as average within-category UniFrac distance) of bacterial communities associated with healthy A. millepora coral colonies at control (white, n = 4 colonies) and platform sites (grey, n = 5 colonies) and WS-affected colonies at platform sites (black, n = 5 colonies) at four time points (November, January, February and June). Error bars represent the standared error of the mean. Roman and greek characters indicated significant within-month differences (phylogenetic diversity by Tukey's HSD and unifrac distance by Student's t-test with 999 Monte Carlo permutations).

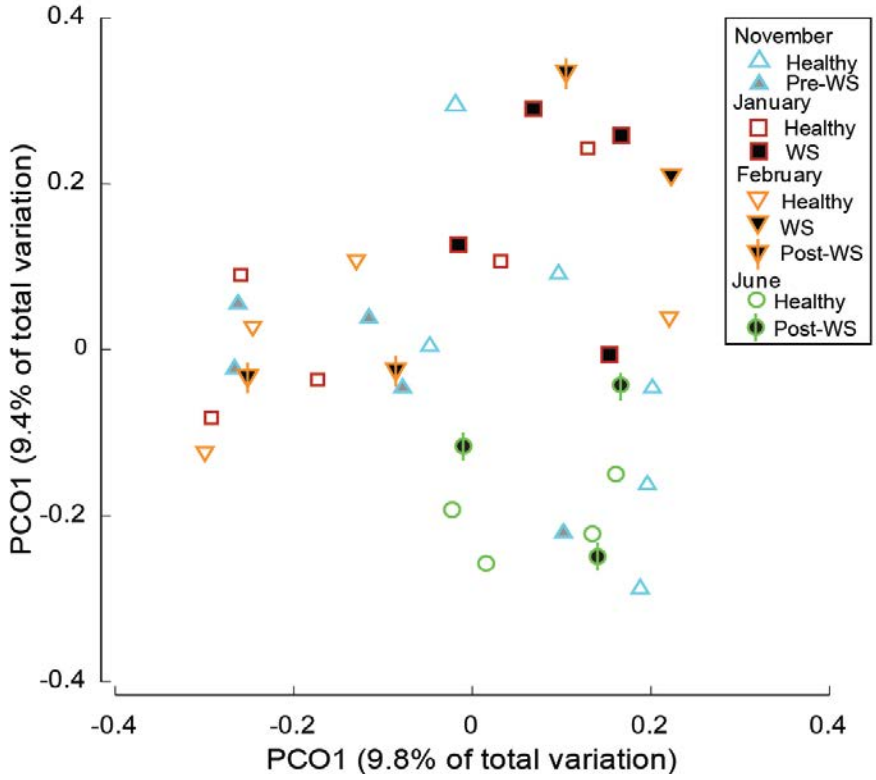
to the more tightly clustered platform samples in the PCO ordination (Appendix C Figure 2C). Unlike in January, loss of specific bacterial taxa and reduced overall diversity did not appear to drive community differences. Nearly 30% (10 out of 37) of OTUs explaining ≥ 1% of the separation between locations were more abundant at control sites (Supplementary Table S3) and overall bacterial diversity did not differ significantly between locations (HSD, $P>0.05$, Appendix C Figure 3A).

C.3 - Influence of WS on coral-associated bacterial communities

Bacterial communities on platform site corals that developed WS in January did not differ significantly from those remaining healthy over the course of the study (i.e. healthy colonies at platform sites v. WS-affected colonies at platform sites) (df=1, pseudo-F=1.16, $P=0.20$) and no significant differences were detected between sampling time points (df=3, pseudo-F=1.13, $P=0.14$). The lack of consistent clustering between health states, visualized as the absence of partitioning of the samples in the PCO ordination (Appendix C Figure 4), indicates that bacterial communities on corals developing WS were no more similar to each other than they were to communities on corals remaining healthy throughout the study.

Although consistent shifts in bacterial community membership were not detected between healthy and WS-affected corals, two key changes in bacterial community structure were observed in apparently healthy corals that would develop WS disease signs. In November, two months prior to the first recorded visual signs of disease, the diversity of bacteria on apparently healthy colonies that would subsequently develop WS (4.4±0.8) was nearly 40% lower than those remaining healthy (7.2±0.8) (HSD, $P=0.03$, Appendix C Figure 3A). Additionally, bacterial community heterogeneity was significantly higher (i.e. variation between samples was greater) on pre-WS colonies (mean UniFrac distance: 0.84±0.03) relative to corals remaining healthy throughout the study (mean UniFrac distance: 0.74±0.01) (HSD, $P=0.001$, Appendix C Figure 3B). These results indicate that bacterial communities on pre-WS corals were less diverse and more variable than on corals remaining healthy, even before the first visible appearance of macroscopic disease signs.

In June, following the cessation of disease progression, bacterial community heterogeneity was significantly higher on post-WS colonies (mean UniFrac distance: 0.78±0.02) compared to those remaining healthy throughout the study (mean UniFrac distance: 0.66±0.01) (HSD, $P=0.002$, Figure 3B) while bacterial diversity did not differ significantly between groups (HSD, $P>0.05$, Appendix C Figure 3A).



Appendix C Figure 4 - Two-dimensional principal coordinates plot visualizing separation between bacterial communities (unweighted UniFrac distance) associated with *A. millepora* coral colonies located at platform sites remaining visually healthy throughout the study (white symbols), colonies prior to visual signs of white syndrome (pre-WS, grey symbols), colonies displaying signs of WS in January and February (black symbols) and colonies without WS lesion progression (post-WS, black hash symbols). November = blue triangles, January = red squares; February = orange triangles; June = green circles.

Appendix D

"Tables"

Appendix D Table 1 – Overview of reference and immune genes included in the GeXP gene expression analysis assay

Gene name	Acronym	Accession number	PCR product length	Sequence of primer coding region	References
Reference gene					
Glyceraldehyde 3 phosphate dehydrogenase	*GAPDH*	EZ026309	187	F: AGGTGGAGCCAAGAAAGTCA R: TTAGCTAGAGGAGCCAGGCA	(Császár et al., 2009, Seneca et al., 2010)
Ribosomal protein S7	*RPS7*	EZ031290	197	F: CAGGCATGCTTACAACCAAA R: TCAACCTCCTTTGCTCCAGT	
Ribosomal protein L9	*RPL9*	EZ026324	292	F: CGTGTAAACGTGTGGTTTGC R: TTTGACACCTGAATTCGCAC	
Unknown transcript	*Ctg_1913*	EZ040581	280	F: GATTTAACCACCGGCAGTGT R: ATGGTAGGGAGGAGGCTGTT	
Toll-like receptor pathway					
Toll interleukin receptor	*TIR-1*	EF090256	137	F: AAAGCCGCAGTCATCAGTTT R: GAAATTGGCGTTGAATTCGT	(Miller et al., 2007a)
TNF receptor associated factor 6	*TRAF-6*	DY583189	127	F: TGATGAATGTCCTTTCGCAG R: ACATGCTTTGCAAGCTGATG	
Mitogen-activated protein kinase/ERK kinase kinase-1	*MEKK-1*	DY581208 DY581138 DY582675	117	F: CTGCGGATATTTGGTCCTGT R: TTTCTTTGTCGGTTGATCCC	
Extracellular signal regulated kinase	*ERK-2*	EZ025389	217	F: CCAAAGGTTACAGCAAGGCT R: TGCGTGCCTTTTCATTCATA	
Mitogen-activated protein kinase p38	*MAPK p38*	EZ031759	237	F: AAAATCAGCAGTGAATCCGC R: TCGGGGTCTGAATACGTAGC	
Component of AP-1 transcription factor	*cFos*	EZ016042	177	F: CTGGAAAGAGAATTGCTGGC R: GACGATTGCACTTCGGACTT	
Component of AP-1 transcription factor	*cJun*	EZ020860	366	F: TCGATCGAAGGGACAGTTCT R: GTGCTAGTTGCGGTGTTCAA	
Activating transcription factor 4	*ATF4/5*	DY577805	247	F: GGCCAGAACGTATCACCAAT R: TCTTCGAAATCAAACCCCTG	

Nuclear factor kappa B	*NF-κB*	G0000491 G0002043 DY582971 DY580118	152	R: CTCATATGCAGGTTGGTGGA F: GATGTTGCAGGCTCAGTTCA	
TRIF-related adaptor molecule	*TRAM*	EZ047194	157	F: AAGCTAACGGCTCACCAAGA R: TGTGCCATGCACAAGAAAAT	
Alternative complement pathway					
Complement factor B	*Bf*	GO001635	227	F: TTATCCATCCCGACGCTAAC R: AGGATCATCTTTTCCTGCGA	(Kimura et al., 2009)
Complement C3	*C3*	EF090257	167	F: CCGCTACACGCTAGACAACA R: CCGCAGAGTCGATGTACAAA	
Lectins					
Mannose-binding lectin	*millectin*	EU717895	257	F: AGCGAGTATCCACAACACCC R: GGCTTTTTCGATGTTTTCCA	(Kvennefors et al., 2008)
C type lectin-1	*CTL-1*	GO001638	312	F: GGGTTGTGTACAACGGCTTT R: CTTTCCATTCGGTTCTCCTG	(Grasso et al., 2008, Grasso et al., 2011)
C type lectin-2	*CTL-2*	GS01UH10	267	F: CAGGTCTGGATCGGACTCAT R: CATGTCCAGTGGTTGTACGC	
Hemolytic lectin-1	*HL-1*	EU863776 EU863777	335	F: TTCGCTCCAGAGGGAAACTA R: GCAGAAATGCCTTTGGTTGT	(Grasso et al., 2008, Grasso et al., 2011)
Hemolytic lectin-2	*HL-2*	EU863776	302	F: AACAGTTGAGATAACCGCCG R: TTGATTCCTGGTGCATTTGA	
Hemolytic lectin-3	*HL-3*	EU863777	379	F: TTCTGGAGATTGGGTAACGC R: TCGTTCTCAGCGTGTTGTTC	
Membrane attack complex / perforin					
Apical extracellular protein	*Apextrin*	EF091848	352	F: GGATTCGTACCAAAAAGGCA R: GAGGGGTCTGATATGGGGTT	(Miller et al., 2007a)
60 kDa proteinaceous toxin	*Tx60-A*	DY579588	207	F: TACTGCCCTTGAGGTTTGCT R: CTGAAAATCCCGCTGACTGT	

At the 5'-end, all primers contained universal fluorescent tags. Forward primer: AGGTGACACTATAGAATA . Reverse primer: GTACGACTCACTATAGGGA

Appendix D Table 2 – Sequences of primers used for 1) *Symbiodinium* genotyping, 2) coral-associated bacterial community profiling using 16S rRNA 454 pyrosequencing and the construction of cDNA libraries for 3) development of a *Montipora aequituberculata* transcriptome and 4) full transcriptome sequencing using a modified version of the 3'-end RNA Seq method(Meyer et al., 2011).

	Primer name	Primer sequence
Symbiodinium	symITS1F	5'-CTCAGCTCTGGACGTTGYGTTGG-3'
	symITS1R	5'-TATCGCRCTTCRCTGCGCCCT-3'
Bacteria 16S	28F	5'-GAGTTTGATCNTGGCTCAG-3'
	519R	5'-GTNTTACNGCGGCKGCTG-3'
	63F	5'-CAGGCCTAACACATGCAAGTC-3'
	1387R	5'-GGGCGGWGTGTACAAGGC-3'
Transcriptome	T-tr	5'-TCAGACGTGTGCTCTTCCGATCTAACGGACTTTTTTTTTTTTTV– 3'
	SW-tr	5'-ACCCCAUGGGGUCAGACGUGUGCUCUUCCGAUCUAACGCUA UGGG-3'
	3ILL-tr	5'-AGTTCAGACGTGTGCTCTTCCGATCT-3'
	5ILL-tr	5'-TTCCCTACACGACGCTCTTCCGATCT-3'
	anti-ILL	5'-AGATCGGAAGAGC/3InvdT-3'
	ILL-Mpx2n	5'-AATGATACGGCGACCACCGAAAAATACACTCTTTCCCTACACGACGCTCTTCCGAT-3'
	ILL-halfSW	5'-CAAGCAGAAGACGGCATACGAGATGCCATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CCGATCTAACGCTATGG-3'
	ILL-halfT	5'-CAAGCAGAAGACGGCATACGAGATGCCATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT CCGATCTAACGGACTTT-3'
	ILL-BC34	5'-CAAGCAGAAGACGGCATACGAGATGCCATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT-3'

RNA Seq	3ILL-30TV	5’-ACGTGTGCTCTTCCGATCTAATTTTTTTTTTTTTTTTTTTTTTTTTTTTV-3’
	S-ILL-swMW	5’-ACCCCATGGGGCTACACGACGCTCTTCCGATCTNNMWGGG-3’
	5ILL	5’-CTA CAC GAC GCT CTT CCG ATC T-3’
	IC1-P5	5’-AATGATACGGCGACCACCGA-3’
	IC2-P7	5’-CAAGCAGAAGACGGCATACGA-3’
	JLBC51	5’-CAAGCAGAAGACGGCATACGAGATCCTTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC53	5’-CAAGCAGAAGACGGCATACGAGATGACGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC55	5’-CAAGCAGAAGACGGCATACGAGATCACACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	UTBC56	5’-CAAGCAGAAGACGGCATACGAGATTTCTTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC57	5’-CAAGCAGAAGACGGCATACGAGATCTCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC58	5’-CAAGCAGAAGACGGCATACGAGATTAACCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC59	5’-CAAGCAGAAGACGGCATACGAGATAAAGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC60	5’-CAAGCAGAAGACGGCATACGAGATAGACCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC61	5’-CAAGCAGAAGACGGCATACGAGATGGGATAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC62	5’-CAAGCAGAAGACGGCATACGAGATACGACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC63	5’-CAAGCAGAAGACGGCATACGAGATGTGGGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	UTBC64	5’-CAAGCAGAAGACGGCATACGAGATTCGTATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC65	5’-CAAGCAGAAGACGGCATACGAGATCAAGGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC66	5’-CAAGCAGAAGACGGCATACGAGATGCCGGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC67	5’-CAAGCAGAAGACGGCATACGAGATCAGTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC69	5’-CAAGCAGAAGACGGCATACGAGATAATAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC70	5’-CAAGCAGAAGACGGCATACGAGATACTTTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’

	JLBC71	5’-CAAGCAGAAGACGGCATACGAGATTCCCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC72	5’-CAAGCAGAAGACGGCATACGAGATATACTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC73	5’-CAAGCAGAAGACGGCATACGAGATAGATGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC74	5’-CAAGCAGAAGACGGCATACGAGATAATCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC75	5’-CAAGCAGAAGACGGCATACGAGATCGGCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC77	5’-CAAGCAGAAGACGGCATACGAGATGATTCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	JLBC78	5’-CAAGCAGAAGACGGCATACGAGATCCCAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD79	5’-CAAGCAGAAGACGGCATACGAGATACGCGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD80	5’-CAAGCAGAAGACGGCATACGAGATAGGGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD81	5’-CAAGCAGAAGACGGCATACGAGATCTGCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD82	5’-CAAGCAGAAGACGGCATACGAGATAACTTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD83	5’-CAAGCAGAAGACGGCATACGAGATGGGTGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD84	5’-CAAGCAGAAGACGGCATACGAGATTCCTGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD85	5’-CAAGCAGAAGACGGCATACGAGATCGCGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD86	5’-CAAGCAGAAGACGGCATACGAGATACCGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD87	5’-CAAGCAGAAGACGGCATACGAGATTAATACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD88	5’-CAAGCAGAAGACGGCATACGAGATCACGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD89	5’-CAAGCAGAAGACGGCATACGAGATATGTGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD90	5’-CAAGCAGAAGACGGCATACGAGATTATAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD91	5’-CAAGCAGAAGACGGCATACGAGATTTTGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD92	5’-CAAGCAGAAGACGGCATACGAGATGTGCCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD93	5’-CAAGCAGAAGACGGCATACGAGATCTAACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’
	ILL-RAD94	5’-CAAGCAGAAGACGGCATACGAGATATAGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’