BIOLOGICAL MECHANISMS OF CORAL IMMUNITY

Thesis submitted by

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and

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To

Patricia Monica and Donald Charles Perch

&

Muriel Winifred and Francis James Palmer

whose love and wisdom have given me the courage to step forward

Thinking

If you think you are beaten, you are.
If you think you dare not, you don't.
If you'd like to win but you think you can't,
It's almost a cinch you won't.

If you think you'll lose, you're lost,
For out of the world we find
Success begins with a fellow's will -
It's all in the state of mind.

If you think you're outclassed, you are;
You've got to think high to rise;
You've got to be sure of yourself before
You can ever win a prize.

Life's battle doesn't always go
To stronger or faster men;
But sooner or later the man who wins,
Is the one who thinks he can.

Walter D. Wintle

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STATEMENT OF THE CONTRIBUTION OF OTHERS

This thesis was conducted as a conjoint agreement between Newcastle University, UK, and James Cook University, Australia and was co-supervised by Prof. John C Bythell and Prof. Bette L Willis, respectively. As my PhD supervisors, John Bythell and Bette Willis contributed to the design, analysis and review process of the various projects that comprise this thesis. Laura D Mydlarz, Ruth D Gates, Mikhail V Matz, Nikki G Traylor-Knowles, Robert Puschendorf, F G Palmer, Melissa S Roth and Chintan Modi also provided editorial assistance.

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ABSTRACT

Hard corals underpin the existence and biodiversity of tropical reefs, however they are declining globally at an alarming rate, largely because of increases in disease prevalence and thermal bleaching events. Despite extensive documentation of coral reef demise, no substantive investigations into the coral host's biological mechanisms for disease resistance, bleaching mitigation or wound healing have been conducted. Coral immunology may therefore provide important insights into understanding the capacity of corals to resist global coral declines. The principal objectives of this thesis were therefore, firstly, to investigate the presence of well-characterised invertebrate innate immunity effector responses, including the melanin-synthesis pathway, the coagulation pathway, immune cell activation and antioxidants, within a number of species from the anthozoan orders Scleractinia, Alcyonacea and Zoantharia. The second objective was to establish the extent to which coral immunity effector responses are activated following physical injury and infection. Finally, the potential influence of warmer seawater temperatures on coral immunity levels and activation were investigated. These objectives were addressed using anthozoans from the Great Barrier Reef Australia, the Caribbean and Hawai'i, using both colourimetric enzyme assays and histological techniques.

Principal findings include establishing the presence of each of the four invertebrate immunity effector responses investigated within corals. A coagulation enzyme, three types of enzymes involved in melanin synthesis and several immune cells were identified for the first time, indicating that anthozoan immune systems are as complex as phylogenetically higher invertebrates. Furthermore, an antioxidant property of coral fluorescent proteins (FP) was established and, in combination with the presence of FPs in coral tissues with increased immune activity, these findings suggest a further potential function of these colourful proteins. Comparative baseline levels of a suite of coral immunity components were found to explain among-family variation in both coral bleaching and disease susceptibility, suggesting immunity as a physiological link between, and mitigator of, these two threats. Furthermore, the ability of hard corals to activate an immune response was demonstrated by a discernable increase in all measured effector responses, both enzymatic and cellular, with both injury and infection. Similarly, the cells and phases of wound healing were characterised within a hard coral for the first time, demonstrating conservation of

mechanisms from the phylogenetically basal corals to humans. Additionally, within one coral species, elevated seawater temperature increased baseline levels of immunity but suppressed responses to injury, explaining links between warming sea surface temperatures and increasing disease prevalence that have been found for several coral diseases, but also suggesting the potential for corals to acclimatise to ocean warming.

Overall, this thesis presents novel findings in a number of areas of coral innate immunity, thereby laying the foundations for this new field of research for scleractinian corals. Coral immunology is an emerging field that is highly relevant to understanding the capacity of corals and coral reefs to persist in a warming world. Preliminary tools developed here will significantly advance the capacity to quantify coral health and, therefore, to better predict the future state of the world's coral reefs.

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Chapter 1 INTRODUCTION

1.1 IMMUNITY

Immunity refers to the ability of an organism to resist infection (Stedman 2000) and to maintain tissue integrity (Cooper and Koprowski 2002; Cerenius et al. 2008). Once the external protective barriers such as the exoskeleton, mucus or epidermal tissue layer have been breached, immunity can be divided into two broad categories: innate and acquired. Innate immunity is evolutionarily ancient (Rosenstiel et al. 2009) and therefore is responsible for the survival and persistence of multi-cellular organisms over eons (Rinkevich 1999) by providing defence against pathogens and parasites (Cooper 2002). Innate immunity provides the first-line of internal defence by initiating a non-specific and immediate response to invading organisms and tissue disruption for all Metazoa (Cooper 2002; Cooper and Koprowski 2002; Cooper 2008). Acquired immunity, which evolved with the vertebrates, thus is not present within invertebrates (Cooper 2002), is reliant on the activation of innate immunity (Fearon and Locksley 1996; Hoffmann et al. 1999; Rinkevich 2004) and is described as induced, adaptive, clonal and anticipatory (Cooper 2002; Hoffmann 2004).

Despite the division in immunity mechanisms between the invertebrates and vertebrates, the potential for invertebrates to possess elements of the more complex acquired immune system has been investigated. Immune memory, or specificity, which is demonstrated by an immune response being more efficient when reacting to previously encountered agents (Frank and Rinkevich 2001), has been documented in multiple invertebrates. These include crustaceans (Weinheimer et al. 1969; Kurtz and Franz 2003), gastropods (Ottaviani 1992), corals (Hildemann 1977) and sponges (Bigger et al. 1982), although within these organisms specificity is attributed to different mechanisms than those of vertebrates. Evidence of immune specificity within some invertebrates, in addition to multiple shared components of innate immunity with vertebrates, suggests that immune responses of even the most phylogenetically basal metazoans may be less simple than first assumed (Loker et al. 2004; Bosch 2008).

Corals (phylum Cnidaria; Class Anthozoa) are anatomically simple and occupy a basal position in metazoan phylogeny. Therefore coral immune systems were presumed to be basic (Loker et al. 2004; Bosch 2008). However, corals have

been in existence since the Palaeozoic era, approximately 475 million years ago, demonstrating their ability to persist through evolutionary time including six documented mass extinction events (Veron 2000). This demonstrated persistence raises the possibility that corals may have well-developed mechanisms for resisting infection and recovering from injury. However, to date, there have been a limited number of coral immunity investigations and consequently the field remains relatively unexplored.

The focus of this thesis is therefore on establishing the biological mechanisms of coral immunity and determining how these mechanisms respond to injury and infection.

1.2 CORAL IMMUNITY

Hard corals (Scleractinia) are the primary framework builders of modern tropical coral reefs. Therefore, a comprehensive understanding of the physiology and ecology of corals will provide a basis for investigating these diverse ecosystems. Investigations of corals are extensive particularly into life history traits of corals, including growth rates, morphologies (Barnes 1970; Highsmith 1979; Willis and Ayre 1985) and reproduction (Harrison et al. 1984; Willis and Ayre 1985; Harrison and Wallace 1990; Soong and Lang 1992; Hall and Hughes 1996). In addition, coral and coral reef general ecology has received attention (Hughes and Jackson 1985; Jackson and Hughes 1985) particularly with respect to declines and recoveries from disturbance events (Andres and Rodenhouse 1993; Hughes 1994; Hughes and Tanner 2000; Coles and Brown 2007). However, to date, immunological aspects of coral biology have only tentatively been explored.

The first studies of coral immunity were histocompatibility investigations with both gorgonians (Salter-Cid and Bigger 1991; Bigger and Olano 1993) and scleractinians (Hildemann et al. 1975; Potts 1976; Bak and Criens 1982; Willis and Ayre 1985; Chadwickfurman and Rinkevich 1994). These early experiments demonstrated the ability of anthozoans to discriminate between self and non-self (Grosberg 1988), a key aspect of innate immunity that enables the maintenance of tissue integrity (Cadavid 2005). As such, a diverse array of effector responses against allogenic tissue have been described including the extrusion of mesenterial filaments and the differentiation of sweeper tentacles (Buss et al. 1984). Early histocompatability studies provided the framework from which to explore cellular

components of tissue regeneration and wound healing, for example in gorgonian sea fans (Meszaros and Bigger 1999; Olano and Bigger 2000) and an anemone (Hutton and Smith 1996). However, until recently, the genes and molecules involved in non-self recognition had not been described (Cadavid 2005).

Fundamental studies of anthozoan immunity are so far lacking, particularly for hard corals, leaving the mechanisms of defence and disease resistance unexplored despite the key function of corals as coral reef framework builders. Currently, corals and the reefs that they comprise are declining globally (Wilkinson 2008). This loss demonstrates the urgent need to understand the biological mechanisms that enable corals to resist infection and recover from injury, two processes that are critical to the persistence of corals and coral reefs.

1.2.1 CORALS AND REEFS UNDER THREAT

Coral reefs represent the most bio-diverse marine ecosystems, supporting one-third of all marine species (Wilkinson 2008). This diversity is enabled, and contributed to, by scleractinian corals, the calcareous skeletons of which form the reef framework and enable benthic complexity, providing habitats for numerous invertebrate and fish species. Coral reefs provide a valuable food source and storm protection for coastal societies, particularly of developing nations, and significantly contribute to national economies *via* tourism and recreation. Coral reefs are thus an extremely valuable commodity for tropical countries. However, anthropogenic impacts including overfishing, poor fishing practices (e.g. with dynamite and cyanide) and pollution have historically led to the loss of coral cover in many locations. Subsequently, as of 2008, 19% of the world's reefs had been lost and a further 35% were considered threatened (Wilkinson 2008). With the rapidly changing climate compounding local human influences, one-third of hard corals are currently considered to be under threat of extinction (Carpenter et al. 2008).

Climatic changes induced by the excessive input of carbon dioxide and other greenhouse gases into the atmosphere since the industrial revolution, pose a threat to coral reefs worldwide. Of primary concern is the documented and predicted warming of sea-surface temperatures due to its direct link with coral bleaching and disease; two of the primary causes of coral decline (Hoegh-Guldberg 1999: Hoegh-Guldberg et al. 2009). Increased prevalence and epizootic outbreaks of some coral diseases are linked to elevated water temperatures (Willis et al. 2004; Bruno et al. 2007; Sato et al. 2009),

consistent with climate-change induced stress and increased disease in other phylogenetic groups (Harvell et al. 1999; Pounds 2001; Harvell et al. 2002; Pounds et al. 2006; Lafferty 2009). However, the mechanisms behind these temperature-associated patterns in coral declines remain to be determined. It has been hypothesised that temperature stress compromises the host immune defences or induces increases in the abundance and/or virulence of pathogens (Harvell et al. 1999; Harvell et al. 2002; Mydlarz et al. 2006; Harvell et al. 2007). Irrespective of the underlying mechanisms, given climate predictions of continuing ocean warming, coral disease epizootics, and therefore coral declines, are set to increase in frequency (Harvell et al. 2007).

Coral bleaching is the loss or dysfunction of symbiotic zooxanthellae, which reside within coral tissues (Gates et al. 1992; Brown 1997). Coral bleaching is a general stress response (Weis 2008) and can be induced by pathogens (Raymundo et al. 2005), salinity changes (Hoegh-Guldberg and Smith 1989), and cold water shock (Muscatine et al. 1991; Gates et al. 1992). However, mass bleaching events that cause widespread mortality, such as the 1998 event, are due predominantly to sustained elevated water temperatures coupled with high irradiance (Berkelmans and Oliver 1999; Hoegh-Guldberg 1999). As a result of warming sea-surface temperatures, the intensity and frequency of mass bleaching events are predicted to increase (Hoegh-Guldberg et al. 2007) and therefore significantly contribute to the loss of coral cover.

In addition to warming oceans, there are other climatic changes that are predicted to negatively influence coral biology. These include increasing ocean acidity and an increase in the intensity and frequency of tropical storms (Hoegh-Guldberg et al. 2007). The biological implications of increased ocean acidity for corals are just beginning to be discussed and investigated (Hoegh-Guldberg et al. 2007; Jokiel et al. 2008; Kurihara 2008; Sokolow 2009). Increasing ocean acidity poses a high threat to the persistence of hermatypic corals due to their necessity to secrete a calcareous exoskeleton, which may be readily dissolved in low pH environments. Concomitantly, studies suggest that coral growth rates will be compromised (De'ath et al. 2009) and both photosynthetic and immune pathways will be disrupted (Cheng et al. 2003).

Tropical storms cause direct mechanical damage to reef assemblages by fragmenting corals and creating lesions. Although the majority of corals are colonial, and therefore can recover from fragmentation, the survival of fragments will directly

depend on their ability to effectively seal wounds and prevent infection. Maintaining the ability to rapidly seal wounds under a changing climate is particularly pertinent as open lesions are more susceptible to infection and storm events are most frequent during the warmest months of the year when coral disease is also highest (Willis et al. 2004; Sato et al. 2009).

Coral reefs are under threat and declining globally, in part due to a reduction in coral cover because of thermal stress and disease-associated mortality (Wilkinson 2008). As immunity enables an organism to resist disease, fight infection and recover from injury (Cooper and Koprowski 2002), a thorough understanding of immune mechanisms is required in order to mitigate reef declines. Currently coral immunology is an emerging yet clearly vital field of investigation. In order to investigate coral immunity mechanisms a detailed understanding of immunity mechanisms utilised by other invertebrates is required. Invertebrate immunology is an established field that provides a solid start point from which to explore the previously unasked questions of coral immunity.

1.3 INVERTEBRATE INNATE IMMUNITY

As an evolutionarily ancient system, molecular components of innate immunity, such as cell-signalling pathways and transcriptional elements, are highly conserved among animal phyla (Beutler 2004; Rosenstiel et al. 2009), although defence strategies have diversified significantly (Loker et al. 2004). Since the origins of comparative immunology by Metchnikoff, and his predecessors (e.g. Metchnikoff 1892), invertebrate immunological studies have primarily focussed on model organisms in order to rapidly promote the understanding of mammalian innate immunity (Tzou et al. 2002; Loker et al. 2004). Model invertebrates include the fruit fly *Drosophila melanogaster* (Tzou et al. 2002) and the nematode worm *Caenorhabditis elegans* (Brown 2000), the investigations of which have recently been significantly aided by the complete genome sequencing of these organisms (Anon. 1998; Adams et al. 2000).

Phylogenetically basal invertebrates have not been intensively investigated from an immunological standpoint, until recently. Basal invertebrates, including cnidarians and poriferans, were largely discounted as model organisms due to their presumed genomic simplicity and to their phylogenetic distance from mammals. However, it is becoming apparent that the most basal metazoans, the Porifera, possess

immunity genes that are homologous to those of higher organisms (Muller 1997; Muller et al. 1997; Pancer et al. 1997; Perovic-Ottstadt et al. 2004; Wiens et al. 2007). This demonstrates that immunity genes are evolutionarily conserved from a common ancestor(s). Most significantly, however, is the recent discovery that anthozoans, of the phylum Cnidaria, are more similar to vertebrates in terms of genomic complexity than model invertebrates. This genomic complexity is indicated by the higher number of cnidarian gene sequences (Kuo et al. 2004; Technau et al. 2005; Miller et al. 2007; Sullivan et al. 2007; Schwarz et al. 2008; Sabourault et al. 2009), than that of model invertebrates, primarily because of extensive gene loss in these models including *D. melanogaster* (Kortschak et al. 2003). This discovery therefore suggests that cnidarians and their relatives may provide a better model system for studying mammalian immunity than *D. melanogaster* and *C. elegans*. Furthermore, as modular organisms that readily fragment, anthozoans present the unique opportunity to conduct controlled experiments on genetic replicates, thus reducing variation among replicates and enabling fine scale patterns to be detected.

The value of studying non-model species extends further than the potential insights they can provide to mammalian system functioning. It is increasingly evident that immunological defence systems are central to a species' resistance to and recovery from disease (Klein 1993). Therefore investigating immune mechanisms of threatened species is important in order to understand, and potentially mitigate, wildlife diseases (Klein 1993).

Innate immunity has three broad phases: recognition of pathogens, signalling cascades and effector response mechanisms (Hoffmann 2004). The non-specific nature of innate immunity requires the recognition of a broad array of potentially pathogenic microbes, as well as the disruption of tissue integrity (Rinkevich 1999; Tzou et al. 2002; Leulier and Lemaitre 2008) and the activation of signal transduction pathways that lead to the production of effector responses (Rinkevich 1999; Figure 1.1). Innate immunity effector responses include three key mechanisms (Hoffmann et al. 1999): antimicrobial peptides (Zasloff 2002), proteolytic cascades (e.g. phenoloxidase cascade) (Söderhäll 1982), and phagocytic cell activation (Metchnikoff 1892). These also represent cellular and humoral (within circulatory fluid) immunity, although distinctions between these categories are not always clear-cut (Iwanaga and Lee 2005).

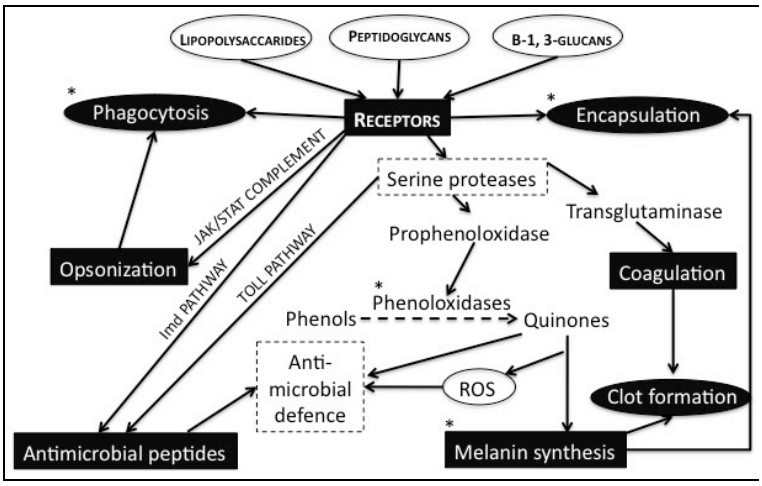


Figure 1.1: Schematic of the pathways and effector responses involved in innate immunity for invertebrates in general (based on Tzou et al. 2002). * effector responses currently identified for anthozoans.

1.3.1 RECOGNITION MOLECULES

Invertebrate **pattern recognition receptors (PRRs)** and soluble proteins recognise cellular disruption and pathogenic invasion (Hallman et al. 2001) and therefore underpin the ability of an organism to distinguish self from non-self (Medzhitov and Janeway 1997; Fujita et al. 2004). PRRs bind to the highly conserved **pathogen-associated molecular patterns (PAMPs)** (Khush and Lamaitre 2000; Cooper et al. 2006) that are composed of microbe cell wall components, such as the **lipopolysaccarides (LPS)** of gram-negative bacteria (Medzhitov and Janeway 1997; Yang et al. 1998; Hallman et al. 2001; Loker et al. 2004), β 1-3, glucans of fungi and peptidoglycans of gram-positive bacteria (Hallman et al. 2001; Medzhitov and Janeway 2002). Organisms generally possess a suite of receptors, each of which binds to one or more PAMP.

TOLL, inclusive of **TOLL-like receptors (TLRs),** are a family of highly conserved trans-membrane proteins (Belvin and Anderson 1996; Lemaitre et al. 1996; Medzhitov et al. 1997) that are key components of innate immunity due to their recognition of pathogens and activation of signalling pathways (Arancibia et al. 2007). TLRs have therefore been investigated and described for organisms across the metazoa (Iwanaga and Lee 2005). TOLL genes were first located within the model organism *Drosophila* (Lemaitre et al. 1996), some of which are vital in immuno-recognition whilst others appear to have a role in development (Royet 2004). For

anthozoans, five TOLL-like domains have been located within the anemone *Nematostella vectensis* (Miller et al. 2007; Sullivan et al. 2007). By contrast, only one TLR has been identified within the hard corals *Acropora millepora* and *Acropora palmata* (Miller et al. 2007). However, no functional studies have been conducted with anthozoan TLRs to demonstrate their role within coral immunity.

Distinctions between TLRs and the PAMPs that they bind to are determined by extracellular leucine-rich repeats (LRRs), which are flanked by cystein residues (Ferrandon et al. 2004). The *Drosophila* TOLL binds to gram-positive bacteria and fungi (Ferrandon et al. 2004; Ferrandon et al. 2007) and activates the TOLL signalling pathway (Figure 1.1) that produces antimicrobial peptides (e.g. Drosomycin) (Tzou et al. 2002; Iwanaga and Lee 2005; Ferrandon et al. 2007). Similarly, antibacterial peptides are produced in *Drosophila* upon binding of gram-negative bacteria to the trans-membrane receptor **Peptidoglycan recognition proteins (PGRP)** (Kim et al. 2000b) that activates the **immune deficiency (IMD)** pathway (Iwanaga and Lee 2005; Figure 1.1). The PGRP family was first identified in the silkworm *Bombyx mori* (Yoshida et al. 1996) and the mouse *Mus musculus* (Kiselev et al. 1998), demonstrating that these recognition proteins are evolutionarily conserved, although they are absent from the nematode worm *C. elegans* (Kang et al. 1998). Although the involvement of PGRPs (Michel et al. 2001) in both TOLL and IMD pathway activation has been demonstrated, the mechanisms behind this activation remain to be elucidated (Royet 2004). Furthermore, PGRPs from *B. mori* activate phenoloxidase pathways (Yoshida et al. 1996), indicating that they play a role in at least two effector response mechanisms (Table 1.1). Other innate immunity recognition molecules include receptors specific to certain PAMPs, for example the LPS-binding protein (LPS-BP) which detects bacteria and ß-1,3-binding protein which recognises the presence of gram-negative bacteria and fungi (Table 1.1).

Integrins are trans-membrane cell surface receptors (Brown 2000; Irving et al. 2005) involved in cell migration, cell-cell adhesion, cell-extracellular matrix adhesion and signal transduction (Takada et al. 2007). Each of these functions is essential to the encapsulation and phagocytosis responses of innate immunity (Johansson 1999; Irving et al. 2005). Some integrins bind to other trans-membrane proteins, however most are receptors for **extracellular matrix (ECM)** components (Brower et al. 1997). To date, within anthozoans, integrin heterodimers have been identified (Brower et al.

1997; Reitzel et al. 2008), although their roles within specific immunity pathways have not been investigated.

Integrins are activated by a diverse range of ligands that are either up-regulated or translocated to the ECM (Johansson 1999). Integrin-activating ligands include collagens, fibronectin, fibrogen, laminins and C3bi (Johansson 1999), all of which have been identified in invertebrates and are activated during tissue disruption or pathogen invasion. Additionally, the antioxidant enzymes peroxidase (peroxinectin; e.g. crayfish and shrimp) and superoxide dismutase are involved in cell-adhesion, in conjunction with integrins, and stimulate phagocytosis and encapsulation(Johansson et al. 1995; Johansson et al. 1997; Johansson et al. 1999).

Lectins (C-type) constitute a group of predominantly calcium-dependent (Takahashi and Ezekowitz 2005), carbohydrate-binding proteins (other than enzymes) that are dissolved within the ECM or are cell surface receptors (Matsushita and Fujita 2001; Endo et al. 2006). Lectins are involved in non-self and pathogen recognition (Endo et al. 2006) and phagocytic activities like cell adhesion (Hatakeyama et al. 2006) and opsonisation, whereby pathogens are flagged for destruction by host components, such as proteins (Muller 1997). Lectins have additionally been documented to activate the phenoloxidase cascade (Yu et al. 1999; Ling and Yu 2006) and induce the production of antimicrobial peptides (AMPs) (Olafsen et al. 1992). These effector responses are induced in the presence of numerous pathogens (Zhang et al. 2009) therefore lectins must play a vital role within innate immunity. Concomitantly, a Mannose-binding-like lectin was identified within *Acropora millepora* Expressed Sequence Tag (EST) databases, and demonstrated up-regulation with bacterial stimulus (Kvennefors et al. 2008). This documented up-regulation demonstrates a role of this coral lectin in an immune response, and suggests that corals possess similar innate immune mechanisms to higher invertebrates (Kvennefors et al. 2008).

Table 1.1: A summary of key innate immunity receptors explored in invertebrates

Receptor	Pathogen	Response	Phylum	Species	Author(s)
TOLL/ TLR (Toll-like receptor)	Gram-positive bacteria Fungi	PO AMP	Porifera	*S. domuncula*	Wiens et al. 2007
			Cnidaria	*A. millepora*	Miller et al. 2007
				H. magnipapillata	Zheng et al. 2005
				N. vectensis	Miller et al. 2007
			Mollusca	*C. farreri*	Qiu et al. 2007
			Nematoda	*C. elegans*	Pujol et al. 2001
			Echinodermata	*S. purpuratus*	Rast et al. 2006
			Arthropoda	*D. melanogaster*	Lemaitre et al. 1996
				B. mori	Wu et al. 2009
				T. tridentatus	Inamori et al. 2004
			Urochordata	*C. intestinali*	Azumi et al. 2003
				B. villosa	Azumi et al. 2003
PGRP (Peptido-glycan recognition proteins)	Gram-positive bacteria	AMP PO	Mollusca	*C. farreri*	(Su et al. 2007
				B. glabrata	Zhang et al. 2007
			Echinodermata	*S. purpuratus*	Rast and Messier-Solek 2008
			Arthropoda	*B. mori*	Yoshida et al. 1996
				D. melanogaster	Werner et al. 2000
				T. ni	Kang et al. 1998
LPS-binding protein (BP)	Bacteria	Opsonins Phagocyt.	Arthropoda	*P. americana*	Jomori and Natori 1992
				P. leniusculus	Lee et al. 2000
Beta glucan binding protein (BGBP)	Gram-negative bacteria Fungi	PO Opsonins Phagocyt.	Porifera	*S. domuncula*	Perovic-Ottstadt et al. 2004
			Mollusca	*P. viridis*	Jayaraj et al. 2008
			Arthropoda	*C. maenas*	Thornqvist et al. 1994
				P. leniusculus	Duvic and Soderhall 1990
				B. craniifer	Soderhall et al. 1988
				B. mori	Ochiai and Ashida 1988
				P. monodon	Sritunyalucksana et al. 2002
				M. sexta	Ma and Kanost 2000

A continuation of Table 1.1

Receptor	Pathogen	Response	Phylum	Species	Author(s)
Integrins		Phagocyt.	Porifera	*G. cydonium*	Pancer et al. 1997
				O. tenuis	Brower et al. 1997
			Cnidaria	*P. carnea*	Reber-Muller et al. 2001
				A. millepora	Brower et al. 1997
				N. vectensis	Reitzel et al. 2008
			Mollusca	*B. glabrata*	Davids et al. 1999
			Nematoda	*C. elegans*	Kramer 1994
			Echinodermata	*S. purpuratus*	Marsden and Burke 1997
					Susan et al. 2000
					Whittaker et al. 2006
			Arthropoda	*D.melanogaster*	Bunch et al. 1992
				P. leniusculus	Holmblad et al. 1997
			Urochordata	*H. roretzi*	Miyazawa et al. 2001
Lectins		PO Opsonins/ Phagocytosis	Porifera	*E. fluviatilis*	Funayama et al. 2005
				A. vastus	Gundacker et al. 2001
			Cnidaria	*A. millepora*	Kvennefors et al. 2008
				H.symbiolongicarpus	Schwarz et al. 2007
			Mollusca	*M. edulis*	Renwrantz and Stahmer 1983
				L. stagnalis	Horak and Deme 1998
			Nematoda	*C. elegans*	Drickamer and.Dodd 1999
			Echinodermata	*L. variegates*	Strumski and Kinsey 1983
			Arthropoda	*D.melanogaster*	Dodd and Drickamer 2001
			Urochordata	*H. pyriformis*	Form et al. 1979
				D. candidum	Vasta et al. 1982

1.3.2 SIGNALLING CASCADES

1.3.2.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) rapidly kill a broad spectrum of microbes and are produced by all multi-cellular organisms (Zasloff 2002). Most organisms have multiple AMPs that are produced by different signalling cascades, for example, the antifungal TOLL signalling cascade or the antibacterial IMD for *Drosophila* (Figure 1.1; Li and Karin 2000; Silverman and Maniatis 2001). Each cascade ultimately leads to the synthesis of AMPs and is regulated at the transcription level (Silverman and Maniatis 2001) primarily by a protein complex family known as nuclear factor kappa

B (NF-kB)/Rel (Li and Karin 2000). The NF-kB/Rel family of transcription factors regulates immune and inflammation responses, apoptosis, development and differentiation (Baldwin 1996). NF-kB pathways are therefore activated by multiple stimuli including pathogenic and environmental stress (Baldwin 1996).

Once the TOLL pathway of *Drosophila* (Figure 1.2a) is activated, the adapter proteins, called Tube and Pelle, are recruited to the intracellular portion of the TOLL trans-membrane receptor. The subsequent activation of Cactus kinase is thought to induce the degradation of the kinase NF-kB inhibitor, Cactus, from the Cactus-DIF complex, where DIF is a NF-kB homolog. This degradation of the NF-kB inhibitor (Inhibitory-kappa B; IkB) in the cytoplasm (Ghosh et al. 1995; Baldwin 1996) enables DIF to enter to the nucleus and induce the transcription of antifungal peptides (Silverman and Maniatis 2001). In contrast, the gram-negative bacteria-induced AMP production utilises the NF-kB homolog 'Relish' (Figure 1.2b) (Leulier et al. 2000; Silverman and Maniatis 2001). Relish is synthesised as a precursor within the cytoplasm and becomes active upon cleavage by a caspase (Dredd) (Ghosh et al. 1995; Leulier et al. 2000; Tzou et al. 2002).

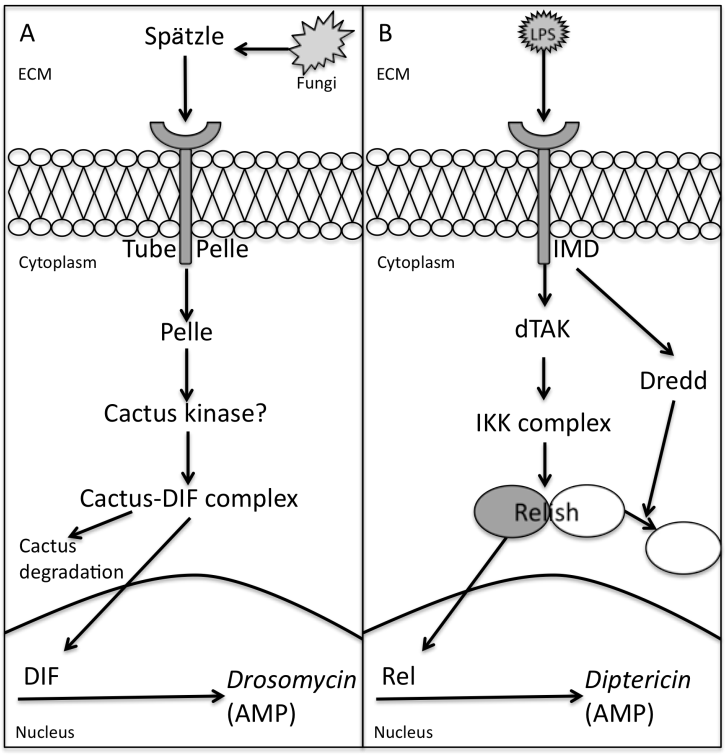


Figure 1.2: a) The fungal-induced TOLL pathway and b) the bacteria-induced Immune deficiency (IMD) pathway, leading to NF-kB (DIF/Rel)-transcriptopm of AMPs. (Adapted from Silverman and Maniatis 2001)

In addition to insects, NF-kB homologs and inhibitors have been found within ancestral metazoans, including the sponge *Amphimedon queenslandica* (Gauthier and Degnan 2008) and the cnidarians *Nematostella vectensis* (Sullivan et al. 2007) and *Acropora millepora* (Miller et al. 2007), demonstrating the presence of this pathway within non-bilaterian animals for the first time. This finding is contrary to previous suggestions that TLR pathways are unrelated to immune function in diploblastic animals, including cnidarians (Kanzok et al. 2004). However, in further support of TLR pathways in diploblastic animal immunity, additional components of the TLR signalling cascade have been identified within *N. vectensis,* including caspases that regulate apoptosis (Eckhart et al. 2007), AMP-associated domains and antioxidant enzymes (Reitzel et al. 2008). This documentation indicates that cnidarians possess the signalling pathway components to produce AMPs, although this specific function of identified components has not been investigated.

1.3.2.2 Proteolytic cascades: The melanin-synthesis pathway

The melanin-synthesis pathway, synonymous with the **prophenoloxidase (PPO)** cascade, is a fundamental component of invertebrate immune responses (Söderhäll and Smith 1986b; Rowley 1996; Asokan et al. 1997; Söderhäll and Cerenius 1998; Cerenius and Söderhäll 2004; Munoz et al. 2006; Butt and Raftos 2008) that are part of a non-self recognition system (Table 1.1; Söderhäll 1982; Perdomo-Morales et al. 2007; Bidla et al. 2008). Stimuli include certain microbial products e.g. PAMPs (Table 1.1; Cerenius et al. 2008), potentially enzymes released from pathogens (Galko and Krasnow 2004) or fatty acids and phospholipids generated during cell damage (Sugumaran and Kanost 1993; Cerenius et al. 2008). The melanin-synthesis pathway is therefore utilised during both physical injury and pathogen invasion in invertebrates (Söderhäll and Smith 1986b). The impermeable melanin product is utilised for encapsulation (Figure 1.1), barrier formation and/or structural integrity of damaged tissue (Sparks 1972; Rowley 1996; Nappi and Christensen 2005). Moreover, cytotoxic pathway intermediates (Figure 1.3) are utilised for antimicrobial defence (Nappi and Ottaviani 2000; Nappi and Christensen 2005). The potentially cytotoxic nature of these pathway intermediates dictates the necessity of tight temporal and spatial controls of pathway activation in order to prevent self-harm (Söderhäll and Cerenius 1998; Cerenius and Söderhäll 2004; Cerenius et al. 2008).

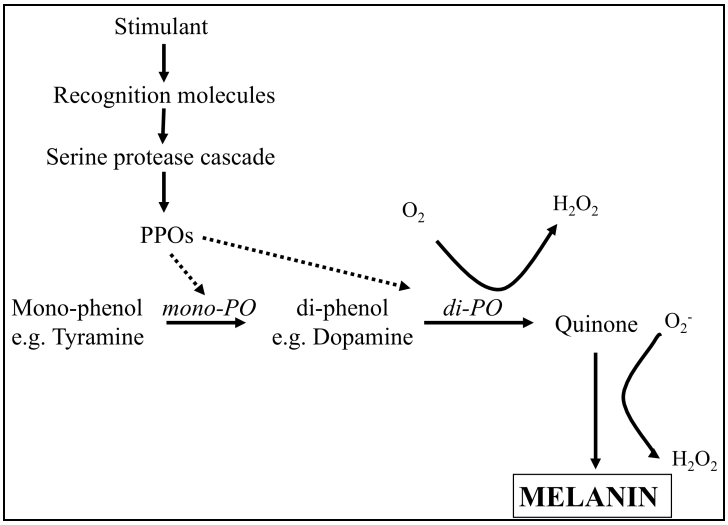


Figure 1.3: A schematic diagram of the melanin-synthesis pathway. Adapted from (Nappi and Christensen 2005).

Upon recognition of the presence of an invading organism or alternative stimulant, recognition molecules (Table 1.1) induce a serine protease cascade (Figure 1.3). PPOs are zymogens that are converted, *via* serine proteases (Söderhäll 1982; Söderhäll and Smith 1986b) and PPO-activating factors (PPAFs) (Piao et al. 2007; Cerenius et al. 2008), to the active form **phenoloxidase (PO)** (Cerenius and Söderhäll 2004; Nappi and Christensen 2005; Kanost and Gorman 2008). PO isoforms comprise a suite of multi-copper oxidases (Decker and Terwilliger 2000) that activate melanin synthesis (Figure 1.3) (Nappi and Christensen 2005). In arthropods, POs are involved in tyrosinase reactions by hydroxylating monophenols and oxidising *o*-diphenols to quinones (Figure 1.3). These reactions produce cytotoxic intermediates, including reactive oxygen species (ROS) such as hydrogen peroxide (Figure 1.3) and the quinones themselves (Nappi and Ottaviani 2000). PPAFs bind to the active PO (Piao et al. 2005) and ensure the enzyme stays in the locality of activation, thereby reducing excessive self-harm in synergy with specific melaninsation inhibitors (Cerenius et al. 2008).

Extensive enzymatic studies have confirmed the presence of the melanin-synthesis pathway within numerous invertebrates, for example; the moth *Manduca sexta* (Hall et al. 1995), the marine mussel *Perna viridis* (Asokan et al. 1997), the shrimp *Panaeus californiensis* (Gollas-Galvan et al. 1999) and *Litopenaeus schmitti* (Lamela et al. 2005) and the Pacific oyster *Crassostrea gigas* (Hellio et al. 2007). Furthermore, PO up-regulation has been documented during immune responses to

pathogenic infection as for the Sydney rock oyster, *Saccostrea glomerata* (Butt and Raftos 2008). Similarly, recent studies of the Caribbean sea fan *Gorgonia ventalina* (Mydlarz et al. 2008) and the hard coral *Montastraea faveolata* (Mydlarz et al. 2009) demonstrated an up-regulation of prophenoloxidase activity in diseased colonies. At the start of this project the presence of the melanin-synthesis pathway had not been investigated in reef-building scleractinian corals.

PPO activity is frequently documented within arthropod haemocytes and haemolymph indicating its role in both cellular and humoral immune responses (Sugumaran and Kanost 1993; Cerenius and Söderhäll 2004; Lee et al. 2004). As such, melanin-synthesis pathway activation demonstrates cross-talk between humoral and cellular immunity responses. However, significant cross-talk among multiple innate immunity pathways is likely to occur during an immune response because of the activation of different pathways by the same stimulants (Table 1.1) (Jiravanichpaisal et al. 2006; Cerenius et al. 2008). This cross-talk is often observed between the melanin-synthesis and coagulation pathways due to their synergistic roles in wound repair.

1.3.2.3 Proteolytic cascades: The coagulation pathway

Coagulation is a necessary component of innate immunity as it ensures the rapid re-establishment of tissue integrity after a wound has been created. This is particularly important for invertebrates with open circulatory systems to prevent the loss of fluids and pathogenic infection (Wang et al. 2001; Theopold et al. 2004). In invertebrates, clotting cascades were primarily described for the Horseshoe crab (*Limulus polyphemus*) (Bang 1956), with more recent investigations conducted on the Japanese Horseshoe crab (*Tachypleus tridentatus*). Within haemocytes LPS and ß-1,3-gluans initiate a proteolytic cascade (Figure 1.4) that induces the conversion of soluble coagulin to insoluble coagulin (Iwanaga 2002). This conversion is controlled by limulus intra-cellular coagulation inhibitors (Agarwara et al. 1996) and serine proteases zymogens: Factor B, Factor C, Factor G, and the proclotting enzyme (Bergner et al. 1997) found within the large haemocytes (Figure 1.4) (Iwanaga et al. 1992).

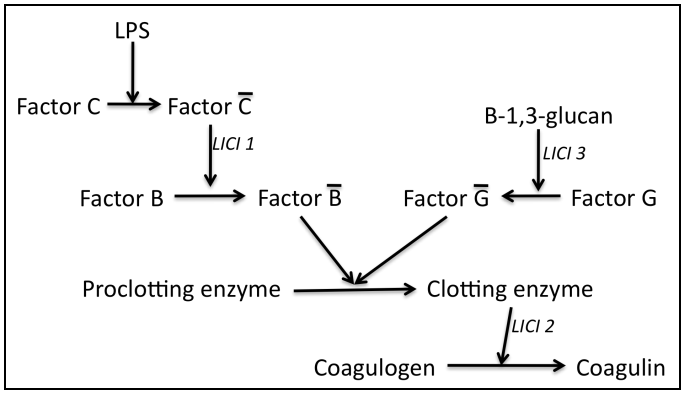


Figure 1.4: The coagulation pathway of the horseshoe crab. LICI = limulus coagulation inhibitor. Adapted from (Iwanaga et al. 1998; Theopold et al. 2004; Iwanaga and Lee 2005).

In the presence of LPS, Factor C is activated autocatalytically to Factor $\bar{}$C which in turn activates Factor B and converts the proclotting enzyme to the active form (Figure 1.4). The clotting enzyme then converts coagulogen to coagulin gel that is insoluble. Alternatively, ß-1,3-gluan activates Factor G, which also leads to the formation of coagulin (Figure 1.4) (Muta et al. 1995). Coagulin homopolymers are cross-linked to form a clot by cell-surface proteins in the presence of transgluatminase (Tokunaga and Iwanaga 1993). Additionally, cross-linking may also occur through factor B-activated hemocyanins and copper-containing oxygen carriers, which demonstrate PO activity (Theopold et al. 2004) and further indicate the cross-talk of immunity-related responses. Additionally, cell agglutination is a vital component of coagulation and wound healing.

Transglutaminases are a group of Ca^{2+}-dependent multi-functional enzymes (Kopacek et al. 1993; Lin et al. 2008) involved in both vertebrate and invertebrate clotting systems (Muta and Iwanaga 1996) and located within haemocytes in arthropods (Chung et al. 1977). Contrary to Horseshoe crab (chelicerate) coagulation (Tokunaga and Iwanaga 1993), transglutaminase is an essential component of crustacean coagulation (Wang et al. 2001) and has been investigated within lobsters (Fuller and Doolittle 1971), the crayfish *Pacifastacus leniusculus* (Kopacek et al. 1993; Hall et al. 1999) and the shrimp *Penaeus monodon* (Yeh et al. 1998). Once activated (in the presence of endogenous Ca^{2+}), transglutaminases catalyze clotting protein post-translational modifications (Cordella-Miele et al. 1990; Beninati and Piacentini 2004) to form stable clots utilising a proteinase inhibitor as a substrate (Wang et al. 2001). This proteinase substrate, a_2-macroglobin in the crayfish *P.*

leniusculus (Hall and Söderhäll 1994), can be cross-linked to the clotting-protein clot and enable effective resistance against pathogenic invasion (Wang et al. 2001). This stabilisation is due to inter- or intra-molecular isopeptide binding involved in cross-linking which increase tissue resistance to both physical and chemical disruption (Wang et al. 2001). Transglutaminases are also capable of influencing differentiation and proliferation (de Macedo et al. 2000), two processes that are vital to effective wound healing.

Transglutaminase primary structures demonstrate significant similarity across phyla, indicating evolutionary conservation of this catalytic clot-formation process (Greenberg et al. 1991). Transglutaminases have been identified within arthropods, including the grasshopper (Singer et al. 1992), the Horseshoe crab *Tachypleus tridentatus* (Tokunaga and Iwanaga 1993), the shrimp *Pandalus nipponensis* (Nozawa et al. 1997), *Penaeus vannamei* (Montano-Perez et al. 1999) and *Penaeus monodon* (Huang et al. 2004), the fruit fly *Drosophila* (Adams et al. 2000) and the freshwater crayfish *P. leniusculus* (Wang et al. 2001). They have also been identified from in molluscs including the scallop *Patinopecten yessoensis* (Nozawa et al. 1997; Nozawa and Seki 2001) and the squid *Todarodes pacificus* (Nozawa et al. 1997; Nozawa et al. 2001), and in the nematode *C. elegans* (Natsuka et al. 2001). However, to date there has been no investigation specifically of the components involved in the coagulation pathway, such as transglutaminase, within anthozoans.

1.3.2.4 Immune cells

Immune cells mediate immune responses (Smith et al. 1996b) and are responsible for phagocytosis (Stein and Cooper 1981), agglutination (Iwanaga and Lee 2005; Gowda et al. 2008), encapsulation (Valembois et al. 1992), release of enzymes, non-self recognition (Thornqvist et al. 1994) and coagulation (Figure 1.1). Immune cells communicate *via* chemical signalling within both their internal and external environments (Humphries and Yoshino 2003). Immune cells therefore comprise an integral component of innate immunity and are numerous and diverse, both across the Metazoa and within species groups (Humphries and Yoshino 2003). Sub-populations of immune cells, for example haemocytes, the circulating blood cells of invertebrates including arthropods and molluscs, coelomocytes of echinoderms and annelids and amoebocytes, are often described and classified by their morphology and physiology (Coteur et al. 2002). This division into sub-populations may additionally

indicate differential functional attributes of immune cells (Franceschi et al. 1991). Key effector cellular responses include the aggregation of immune cells at the site of infection or injury. This primarily occurs *via* the activation of the complement pathway.

The complement system mediates a chain of proteolysis and protein complex-forming reactions that form an integral part of an immune response, as well as providing the bridge between innate and adaptive immunity (Endo et al. 2006). A key output of the complement system is opsonisation, which enhances pathogen phagocytosis *via* host tagging of pathogens (Endo et al. 2006). The complement system can be activated by three distinct pathways: the classical, which is limited to vertebrates, lectin and alternative pathways (Fujita et al. 2004). Each of these pathways culminate in the activation of C3, a member of the a_2-macroglobin protein families (Armstrong et al. 1993) and the central component of the complement system (Endo et al. 2006). The lectin pathway is activated by the C-type recognition receptors like ficolin and the Mannose-binding lectin (MBL) (Matsushita and Fujita 2001,2002; Endo et al. 2006; Endo et al. 2010), which activate MBL-associated serine proteases (MASPs). These proteases subsequently cleave the components C4 and C2 to form C3 (Thiel et al. 2000). The alternative pathway is activated by the binding of small amounts of C3 to hydroxyl or amine groups on the cell surface of microorganisms; a processes which does not involve receptors (Thiel et al. 2000; Fujita et al. 2004). C3 forms a covalent bond with microbes using a intra-molecular thiloester bond (Law et al. 1980) that forms an opsonin tag on the pathogen and activates complement late components C5 – C9 and the membrane attack complex (MAC). Components of the complement system such as C3 homologs that contain a thiloester protein (TEP), and lectins, such as MBL and ficolin, have been located within a diverse range of invertebrates. These include the tunicates *Pyura stolonifera* (Raftos et al. 2003) and *Halocynthia roretzi* (Endo et al. 2006), the sea urchin *Strongylocentrotus purpuratus* (Smith et al. 1996b; Smith et al. 1999; Whittaker et al. 2006), and the anthozoans *Swiftia exserta* (Dishaw et al. 2005), *N. vectensis* (Reitzel et al. 2008) and the hard coral *Acropora millepora* (Miller et al. 2007; Kvennefors et al. 2008). The presence of complement components within anthozoans suggests that corals possess immune cells.

Integrins mediate interactions between cells and the ECM *via* signalling pathways and are involved in cell adhesion processes (Brown 2000) including

spreading, cell migration (Calderwood et al. 2000), growth, survival, differentiation, apoptosis, histocompatibility and formation of fibrillar matrices (Clark and Brugge 1995; Johansson 1999; Bokel and Brown 2002; Guo and Giancotti 2004; Plows et al. 2006; Johnson et al. 2009). Integrins are receptors on all nucleated cells, and unlike other receptors, integrin ligands span a diverse range of protein families, including ECM components (Clark and Brugge 1995) like fibronectin, collagens, peroxinectin of the crayfish *P. leniusculus* (Johansson et al. 1995), soluble ligands like fibrinogen, and intra-cellular adhesion molecules (Clark and Brugge 1995). Usually, ligands contain an adhesive motif, RGD, which is recognised by the integrin (reviewed by Johansson 1999). Upon ligand engagement, integrins may cluster and link to intra-cellular cytoskeleton complexes and actin filaments (Clark and Brugge 1995), leading to the mediation of cell adhesion, migration, re-shaping and spreading, which are key characteristics of immune cells.

Integrins signal both mechanically *via* cell anchorage and chemically by outside-in signalling into the cell (Miranti and Brugge 2002; Guo and Giancotti 2004). Overall integrin function therefore falls into two categories: 1) as mechanical links in cell adhesion and migration and 2) as signal transducers (Bokel and Brown 2002). The latter of the two potentially affects cytoskeleton arrangements and transcriptional activity (e.g. NF-kB) (Johnson et al. 2009) and enables responses to growth factor signalling (Miranti and Brugge 2002). Integrin-mediated signalling can occur directly where integrin clustering is the only extracellular stimulus, or collaboratively, whereby growth factors activate other receptors (Howe et al. 1998).

Integrin signalling induces tyrosinase phosphorylation and the activation of focal adhesion kinases (FAKs), a central component of cell migration and cell-cycling (Schwartz 2001). FAK develops physical associations with other signalling molecules, like the protein tyrosine kinase Src (Schwartz 2001; Plows et al. 2006). This association causes phosphorylation and signalling, which activate serine kinases, such as mitogen-activated protein kinase (MAPK), and lipid metabolism (Howe et al. 1998) and leads to a diverse array of effector responses (Schwartz 2001; Guo and Giancotti 2004). The presence of integrins and integrin signalling cascades has been determined in numerous invertebrates including the sponge *Ophlitaspongia tenuis* and coral *Acropora millepora* (Brower et al. 1997) insects (Metheniti et al. 2001) and the sea urchin *Lytechinus variegates* (Garcia et al. 2004). Despite the presence of

integrins (Brower et al. 1997), currently for anthozoans no functional studies exist that directly link integrin presence or activity to cellular defence.

1.3.3 Effector Responses

1.3.3.1 Antimicrobial peptides

Although there are a wealth of microbicidal substances, including for example secondary metabolites (Becerro and Paul 2004), antimicrobial peptides (AMPs) are strictly defined as polypeptide antimicrobial substances, encoded by genes and with fewer than 100 amino acid residues (Ganz 2003). AMPs can be categorised into three groups; linear peptides, peptides containing cystine residues, and peptides with one or two over-represented amino acids (Brogden 2005), of which it is common to have all three types within one organism (Lemaitre and Hoffmann 2007). Invertebrate AMPs are numerous, with seven types described for *Drosophila* alone (e.g. defensin; Lemaitre and Hoffmann 2007), and are primarily located within the granules of immune cells, (e.g. haemocytes) and in the haemolymph (Boman et al. 1991; Haug et al. 2002; Zhao et al. 2007), enabling both local and systemic defence (Lemaitre and Hoffmann 2007). AMPs have been identified and characterised in numerous invertebrates, e.g. the crabs *Carcinus maenas* (Schnapp et al. 1996) and *Callinectes sapidus* (Khoo et al. 1999), the shrimp *Penaeus vannamei* (Destoumieux et al. 1997), the mollusc *Mytilus edulis* (Charlet et al. 1996), the nematode *C. elegans* (Pujol et al. 2008) and the tunicate *Halocynthia aurantium* (Jang et al. 2002). AMPs demonstrate up-regulation in the presence of pathogens (Brey et al. 1993; Bachere 2000; Jang et al. 2002), highlighting their role in immune responses and the effectiveness of recognition and signalling pathways.

Bactericidal activity has been documented for soft corals (Slattery et al. 1995; Kelman et al. 2006), gorgonians (Kim 1994) and hard corals (Koh 1997; Kelman et al. 2006; Gochfeld and Aeby 2008; Geffen et al. 2009; Mydlarz et al. 2009). Antifungal activity of gorgonians has also been documented (Kim et al. 2000a). Antimicrobial activity of corals has varying origins, including small peptides and other antimicrobial compounds such as secondary metabolites (Slattery et al. 1995; Mydlarz and Jacobs 2006; Dunn 2009) and can be stimulated by mechanical disturbance and stress (Geffen et al. 2009). Additionally, the members of the bacterial community associated with the protective mucus layer of corals (Brown and

Bythell 2005) include antibiotic-producing microbes, which may defend corals against unfavourable pathogenic microorganisms (Ritchie 2006). However, the presence specifically of AMPs has yet to be elucidated for corals.

1.3.3.2 Proteolytic cascades: The melanin-synthesis pathway

The activation of the melanin-synthesis pathway, and therefore the presence of active PO, produces multiple highly reactive and cytotoxic intermediates (Figure 1.3) (Sugumaran 2002; Nappi and Christensen 2005). Cytotoxic compounds assist in antimicrobial defence by immobilising, aggregating and killing pathogenic microorganisms such as bacteria and fungi (Zhao et al. 2007; Cerenius et al. 2008). These compounds include reactive oxygen species (ROS), such as molecular oxygen and hydrogen peroxide, which demonstrate a wide range of toxic effects including lipid peroxidation and protein degradation (Bell and Smith 1994). Proteinases, oxidases and dopachrome conversion enzymes of the PO pathway are all contributors to the immobilisation and killing of pathogenic microbes (Zhao et al. 2007). Quinones are an integral component of the PO pathway (Figure 1.3), of which dopamine is non-enzymatically cyclised to form 5,6- dihydroxyindole (DHI). DHI and the resultant oxidation products are highly cytotoxic, as demonstrated by antifungal activity (Zhao et al. 2007). This melanin-synthesis pathway-induced cytotoxicity is therefore potentially advantageous during immune responses to microbial infection.

Melanins, although poorly characterised in lower invertebrates (Nosanchuk and Casadevall 2003), are pigments that are deposited as part of an immune response. Melanin deposits are used to encapsulate invading organisms, to create a physical barrier between healthy and compromised tissue and to provide structural support during tissue repair (Cerenius et al. 2008). The utilisation of melanin for encapsulating organisms or for melanisation in general, usually involves the degranulation of immune cells within which the melanin-synthesis pathway is active. These melanin-synthesis pathway-active cells are crystal cells within the fruit fly (Rizki et al. 1985) and haemocytes within crustaceans (Söderhäll and Smith 1986a). Encapsulation and melanisation have been documented in numerous invertebrates including in insects in response to both parasites (Nappi 1973; Collins et al. 1991; Beerntsen et al. 2000; Huang et al. 2005) and bacteria (Hillyer et al. 2003). These responses have also been described within worms (Porchet Hennere and Vernet 1992;

Field et al. 2004; Ling and Yu 2006), a tunicate (De Leo et al. 1997) and a gorgonian sea fan (Petes et al. 2003).

Melanins are also documented to have additional functions. These include desiccation resistance in plants (Laufer et al. 2006) and photoprotection *via* light absorption in both plants (Mayer and Staples 2002; Laufer et al. 2006) and higher metazoans (Meredith et al. 2006). Melanins also demonstrate free radical scavenging activity (Nappi and Christensen 2005), which may be beneficial for limiting self-harm during immune responses. Also, invertebrate clots become melanised and therefore stablised *via* melanin-mediated cross-linking of proteins. Melanin deposition therefore has multiple roles as an effector response within the invertebrates.

Melanin deposition has been documented histologically within the gorgonian sea fan, *Gorgonia ventalina*. *G. ventalina* deposited a layer of melanin to encapsulate fungal hyphae of the known pathogen *Aspergillus sydowii* (Petes et al. 2003; Ellner et al. 2007; Mydlarz et al. 2008) that causes a potentially fatal syndrome known as Aspergillosis (Smith et al. 1996a; Harvell et al. 2007). However, the presence of melanin deposits within in reef-building scleractinian corals has not been investigated.

1.3.3.3 Proteolytic cascades: The coagulation pathway

The effector response associated with the activation of the coagulation pathway is, primarily, the formation of a stable, insoluble clot of cross-linked proteins (Wang et al. 2001). The function of a clot, which usually consists of cross-linked fibrin, is to prevent excessive fluid loss immediately after physical injury and to minimise the invasion of pathogens into an open wound (Theopold et al. 2004; Haine et al. 2007). Clotting therefore rapidly re-establishes homeostasis (Haine et al. 2007). Depending on the location of the coagulation pathway, this usually involves the aggregation of immune cells and their interaction with humoral components (Smith et al. 2006).

Clot formation, as a result of coagulation pathway activation has been investigated within invertebrates including the tiger shrimp *Penaeus monodon* (Yeh et al. 1998), freshwater crayfish *Pacifastacus leniusculus* (Wang et al. 2001) and the fruit fly *Drosophila* (Karlsson et al. 2004). No investigations into the biochemical coagulation pathways responsible for anthozoan wound sealing have been conducted. However, physical injury is a major contributor to compromised coral health, because of fragmentation by tropical storms (Hughes 1994), fish predation (Rotjan and Lewis

2005, 2008) and boring organisms (Fang and Shen 1988). This highlights the need to investigate the mechanisms by which corals seal lesions and prevent infection.

The enzymatic nature of transglutaminases and the identification of the amine donor protein substrates (Greenberg et al. 1991) have enabled the biochemical investigation of the presence and activation of this vital coagulation component (de Macedo et al. 2000). This suggests the potential for the development of enzymatic assays that could indicate the presence of transglutaminase within a novel study species, such as corals. Conducting these enzyme assays would therefore establish the presence of coagulation pathway components within coral and potentially indicate an immunity mechanism.

1.3.3.4 Immune cells

Invertebrate immune cells are characteristically mobile, diverse, and functionally versatile and vary among phyla (Adamowicz 2005). Immune cells are activated or mobilised in response to foreign organisms and wounding and induce inflammatory responses (Zuk and Stoehr 2002). Inflammation is a key innate immune effector response and has two components: phagocytosis and encapsulation (Rowley 1996). Phagocytosis originally evolved as a feeding method, however it is utilised as an immune response to engulf and kill invading pathogens by host immune cells. Immune cells with phagocytic potential have been identified across the Metazoa, including within the inverterbates; anthozoans (Olano and Bigger 2000), molluscs (Foley and Cheng 1977) insects (Hillyer et al. 2003) and annelid worms (Stein and Cooper 1981). Immune cells are activated by, and associated with, each of the innate immune systems including antimicrobial peptide production, melanin formation, coagulation and the lectin complement network (Iwanaga and Lee 2005).

The intra-cellular killing of phagocytosed material occurs *via* the induction of a respiratory, or oxidative, burst. Oxidative bursts involve the generation of microbicidal reactive oxygen metabolites including superoxide, hydrogen peroxide and nitric oxide (Radomski et al. 1991; Pipe 1992; Hutton and Smith 1996). Additionally, hydrolytic enzymes, such as lysozyme (Cheng et al. 1975) are released from immune cell granules.

Encapsulation, as previously discussed in relation to the PO pathway, involves the aggregation and adhesion of immune cells, including granular cells, directly to an invading organism or to one another to form a capsule around the foreign object (Pech

and Strand 1996; Johansson 1999). Inflammatory factors such as lectins and melanin-pathway activators, in addition to antimicrobial cytotoxic material, are released during cell degranualtion (regulated exocytosis) (Foley and Cheng 1977; Perazzolo and Barracco 1997; Aladaileh et al. 2007; Vafopoulou 2009). Usually, degranulation is of granule-containing cells (Rowley 1996) such as granular and semi-granular cells of the earthworm *Dendrobaena veneta* (Adamowicz 2005) or the crystal cells of insects (Galko and Krasnow 2004; Bidla et al. 2008). Due to the release of intra-cellular components, the PO pathway is activated (Johansson and Söderhäll 1989) and triggers the aggregation of phagocytes, such as hyaline cells (Johansson and Söderhäll 1989) or amoebocytes (Adamowicz 2005), which form a sheath around the capsule (Rowley 1996). Therefore, invertebrate immune cells, although each with different functions, may act in concert to re-establish tissue integrity or to encapsulate a foreign organism. The mechanisms with which wounds are healed or foreign bodies encapsulated may therefore depend upon the type and function of immune cells.

Wound healing and tissue regeneration, two processes that occur immediately after clot formation, have been investigated within anthozoans including the gorgonian *Plexaurella fusifera* (Meszaros and Bigger 1999) and the hard corals *Montastrea annularis* (Meesters et al. 1994; Meesters et al. 1997a) and *Oculina patagonica* (Fine et al. 2002). Furthermore, the presence of immunity or putative immune cells, such as amoebocytes, have been investigated in an anemone (Hutton and Smith 1996), gorgonians (Meszaros and Bigger 1999; Olano and Bigger 2000; Mydlarz et al. 2008; Mydlarz et al. 2009) and scleractinain corals (Domart-Coulon et al. 2006; Vargas-Angel et al. 2007). Hutton and Smith (1996) found that only hyaline amoebocytes of the anemone *Actinia equina* were phagocytic, and that mixed cultures of amoebocytes, including granular cells, produced ROS and demonstrated bactericidal activity. This supports the activation of an oxidative burst to kill encytosed material during phagocytosis (Pipe 1992).

Meszaros and Bigger (1999), describe the cellular process of wound healing in the gorgonian *Swifter exserta*, whereby amoebocytes aggregate and are potentially involved in the extrusion of connective fibres. Olano and Bigger (2000) also investigated *S. exserta* and documented the presence of phagocytic granular cells. Mydlarz et al. (2008; 2009) went one step further and, using *Gorgonia ventalina*, confirmed that some granules of amoebocytic granular cells were melanin deposits

and that these cells aggregated around fungal infections (Mydlarz et al. 2008) and in response to thermal stress (Mydlarz et al. 2009). By comparison, the investigation of scleractinian immune cells lags behind. Domart-Coulon et al. (2005) documented the presence and infiltration of pigmented cells they thus termed chromophore cells, into growth anomalies, however no cellular function was investigated or attributed. Similarly, Vargas-Angel et al. (2007) observed amoebocytic cells in one *Montastraea cavernosa* sample and suggested that difficulty in the investigation and documentation of scleractinian amoebocytes is due, in part, to their small cell size. Again, no functional investigations were conducted on these scleractinain amoebocytes, therefore scleractinian immune cells remain poorly characterised and investigated.

1.3.4 The importance of Antioxidants

Reactive oxygen species (ROS) are toxic by-products of normal cell functions, including metabolism and signalling, and therefore represent a continuous threat for aerobic organisms (Thannickal and Fanburg 2000; Martindale and Holbrook 2002). ROS include oxygen metabolites that are more reactive than molecular oxygen, for example superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicals (Halliwell and Gutteridge 1985; Thannickal and Fanburg 2000). Although transient fluctuations in ROS levels serve a regulatory function, sustained high levels induce oxidative stress whereby ROS disrupt DNA, lipids and proteins (Thannickal and Fanburg 2000; Martindale and Holbrook 2002). Increased production of ROS and the potential induction of oxidative stress occur under environmental stress conditions and during immune responses (Halliwell and Gutteridge 1999; Fang 2004). As previously mentioned, ROS are synthesised as part of an innate immune response as by-products of the PO pathway (Nappi and Ottaviani 2000) and are produced as part of an oxidative burst during phagocytosis (Pipe 1992). Therefore a process needs to be in place to regulate levels of ROS and to prevent self-harm.

In order to mitigate and regulate ROS cytotoxicity, organisms possess antioxidants that scavenge and detoxify these radicals (Sugumaran and Kanost 1993; Halliwell and Gutteridge 1999). Antioxidants are primarily endogenous enzymes, for example superoxide dismutase which catalyzes the destruction of superoxide anions to water and H_2O_2 (Halliwell and Gutteridge 1999), catalase which catalyses the decomposition of H_2O_2 to water and oxygen (Gordon 1996), and peroxidases, which

are oxidant proteins that consume H_2O_2. Melanin also demonstrates redox potential (Nappi and Christensen 2005) and therefore represents a non-enzymatic antioxidant, whereas carotenoid pigments that may be incorporated into the tissue and skeleton from food sources or symbionts, represent exogenous antioxidants (Bendich 1989; Cornet et al. 2007). As such, these beneficial antioxidants, particularly the enzymatic ones, are usually up-regulated during an immune response to prevent self-harm (Bell and Smith 1994; Benzie 2000; Davies 2000; Company et al. 2007; Martinez-Dominguez et al. 2008).

Reef-building or hermatypic corals dwell in obligate symbioses with energy and nutrient-providing algal endosymbionts (Bythell 1988) commonly known as zooxanthellae (*Symbiodinium* sp.). Under normal conditions, these algal symbionts produce high quantities of ROS (Dykens et al. 1992; Lesser 1996; Mydlarz and Jacobs 2004; Lesser 2006), in addition to the ROS produced by the coral animal cells. ROS production by zooxanthellae is elevated during temperature and light stress (Lesser 1996,2006) and therefore poses a high threat of oxidative stress (Martindale and Holbrook 2002). The coral host also up-regulates ROS production during thermal stress (Weis 2008), injury (Meszaros and Bigger 1999; Mydlarz and Jacobs 2006) and infection (Mydlarz and Harvell 2007) therefore necessitating the presence of antioxidants.

Enzymatic antioxidants documented for anthozoans include superoxide dismutase that is found in a suite of cnidarians (Dykens 1984) and histologically in the anemone *Anemonia viridis* (Hawkridge et al. 2000). Catalase activity has been found in sea anemones (Leutenegger et al. 2007a; Merle et al. 2007) and corals (Griffin et al. 2006), and peroxidase in a gorgonian (Mydlarz and Harvell 2007). Non-enzymatic antioxidants include melanins and carotenoids and have both been found in gorgonian sea fans (Petes et al. 2003; Leverette et al. 2008). Corresponding to a role in mitigating self-harm, up-regulation of anthozoan enzymatic antioxidants has been documented during thermal stress (Lesser 2006) and infection (Mydlarz and Harvell 2007). Also up-regulated during thermal stress events are coral fluorescent proteins (Smith-Keune and Dove 2008; Bay et al. 2009).

Coral fluorescent proteins (FPs) are largely responsible for the bright colours observed on coral reefs (Dove et al. 2001), yet their biological function remains debated. There are several hypotheses pertaining to the role of FPs including: 1) as photoprotectors of the symbiotic zooxanthellae (Salih et al. 2001), 2) light enhancers

to promote photosynthesis by the zooxanthellae (Dove et al. 2008), 3) as signals to reef fish (Matz et al. 2006) and 4) as scavengers of ROS (Bou-Abdallah et al. 2006). The latter of these hypotheses suggests an antioxidant function of FPs, however investigations were preliminary and conducted using FP isolated from a jellyfish. Further investigations are therefore required to determine whether coral FPs have an antioxidant function and whether it is utilised during immune responses.

1.4 CORAL IMMUNITY: A SUMMARY OF THE CURRENT KNOWLEDGE

The current knowledge of coral immunity mechanisms is limited but developing (Table 1.2). To date, aspects of each phase of immunity, as characterised by Hoffman (2004) have been investigated within corals (Table 1.2), although there are clear gaps in the knowledge. Gaps include, for example, the presence of receptors other than the TOLL-like receptor (TLR) such as peptidoglycan recognition proteins (PGBP), or LPS-binding proteins (LPS-BP). Furthermore, although various aspects of signalling pathways are present within anthozoans, as discussed by Reitzel et al. (2008), functional studies are extremely limited (Kvennefors et al. 2008).

Functional investigations of coral immunity parameters are more abundant for the effector responses. Documentation of coral effector responses include antimicrobial activity for both soft and hard corals (Koh 1997; Geffen et al. 2009; Mydlarz et al. 2009), although this activity has not been directly associated specifically to antimicrobial peptides (AMPs). The presence of the melanin-synthesis pathway has been established within gorgonian corals with documentation of both melanin deposit (Petes et al. 2003) and prophenoloxidase activity (Mydlarz et al. 2008) documented. At the beginning of the study in 2007, the presence of the melanin-synthesis pathway within scleractinian corals had not been investigated (Mydlarz et al. 2006), despite this effector response being a key component of invertebrate immunity (Söderhäll and Cerenius 1998). This represented a key gap in the knowledge of hard coral immunity. Similarly, the coagulation pathway has not been investigated within hard or soft corals, despite the life history strategy of corals to fragment and thus create open lesions, which must be sealed efficiently to prevent infection.

The majority of investigations relating to coral immunity have been applied to coral regeneration. Although most of these studies within hard corals addressed the rate of regeneration macroscopically (Lirman 2000a; Alvarado and Acosta 2009),

other studies, predominantly of gorgonians, included a histological examination of wound healing and/or documentation of immune-related cells (Meszaros and Bigger 1999; Olano and Bigger 2000; Mydlarz et al. 2008). Significantly lacking from scleractinian studies is a comprehensive documentation of the cellular processes involved in wound healing, including characterisation of immune cells. Similarly, antioxidants are a key aspect of an innate immune response but have only been documented within a few hard coral species (Hawkridge et al. 2000; Griffin et al. 2006). Coral immunology as a whole is currently also lacking a comprehensive, multi-species investigation of key immunity parameters.

Table 1.2: A summary of the current knowledge of coral immunity mechanisms

Phases of immunity	**Effector responses**	**Immunity parameter**	**Order**	**Reference**
Receptors and signalling		TLR	Scleractinia	Miller et al. 2007
		Integrin	Scleractinia	Brower et al. 1997
			Alcyonacea	
		MBL-like	Scleractinia	Kvennefors et al. 2008
Effector responses	Antimicrobial peptides	No. Bactericidal and antimicrobial activity	Scleractinia	Mydlarz et al. 2009
				Geffen et al. 2009
				Koh 1997
			Alcyonacea	Koh 1997
	Melanin-synthesis pathway	Prophenoloxidase activity	Alcyonacea	Mydlarz et al. 2008
		Melanin deposits	Alcyonacea	Petes et al. 2003
	Coagulation pathway	No.		
	Immune cells	Granular amoebocytes	Alcyonacea	Mydlarz et al. 2008
		Phagocytic cells	Alcyonacea	Olano and Bigger 2000
		Chromophore cells	Scleractinia	Domart-Coulon et al. 2006
		Amoebocyte	Scleractinia	Vargas-Angel et al. 2007
Antioxidants	Enzymatic antioxidants	Superoxide dismutase	Scleractinia	Hawkridge et al. 2000
		Catalase	Scleractinia	Griffin et al. 2006
		Peroxidase	Alcyonacea	Mydlarz and Harvell 2007

1.5 IMMUNOCOMPETENCE

Immunocompetence represents the magnitude and effectiveness of an organism's response to injury or infection (Adamo 2004b), and therefore can be indicative of disease resistance capabilities and applied in an ecological context. Obtaining quantifiable and representative measures of immunocompetence would enable, for example, inter-species comparisons to determine disease-susceptible species or the assessment of potentially immunosuppressive environmental effects, as utilised by ecological toxicologists. However, accurate measures of immunocompetence are difficult to obtain and are dependent on immunological assays that measure biologically relevant immunity parameters (Zuk and Stoehr 2002; Adamo 2004b). This necessitates that the quantified immunity parameters significantly correlate with ecological patterns of disease susceptibility, or the relevant environmental parameter(s) being examined.

Innate immunity parameters, predominantly effector responses, which have been quantified in numerous invertebrates, have also been correlated with disease resistance. For example, the melanic larvae of the moth *Spodoptera littoralis* were more resistant to fungal infection than their non-melanic counterparts (Wilson et al. 2001). Similarly, for the Sydney rock oyster, *Saccostrea glomerata,* phenoloxidase and phagocytosis activity correlated with disease resistance (Butt and Raftos 2008). Furthermore, for species of particular economical interest, immunity parameters are regularly used to monitor organism health, such as with oysters, mussels and crustaceans (Bachere et al. 2004). Monitoring the health of organisms within an experiment, aquaculture facilities or the environment, is potentially a highly valuable tool (Bachere 2000, 2003). However the assays used usually involve sacrificing individuals and therefore measuring a different genotype each time, giving rise to high background variation. Modular invertebrates, such as anthozoans, may offer better study systems in this respect, as the same genotype(s) can be sampled over time and without being removed from the system.

1.6 ECOLOGICAL IMMUNITY

All organisms possess immune systems, yet disease is a natural component of any ecosystem, indicating that the immune functioning sometimes gets overwhelmed. Ecological patterns of disease demonstrate varying susceptibility among individuals

and indicates that immune responses range in efficacy among both species and habitats (Sadd and Schmid-Hempel 2009). Immunity is an important life history trait that promotes organism fitness, although not without its costs (Stearns 1993; Rolff and Siva-Jothy 2003; Sandland and Minchella 2003; Sadd and Schmid-Hempel 2009). As such, relative investment in immunity is traded-off against investment in other life history traits such as growth and reproduction (Stearns 1993). Cost-induced trade-offs that have lead to the evolution of species-specific patterns of immunity (Langand et al. 1998; Koricheva 2002; Sadd and Schmid-Hempel 2009) and the costs involved in induction and maintenance of an immune response (Sandland and Minchella 2003; Sadd and Schmid-Hempel 2009), are largely responsible for the differences in immune responses observed at the ecological scale (Norris and Evans 2000; Sadd and Schmid-Hempel 2009).

Evolutionary costs related to increased investment into immunity have been experimentally demonstrated with *Drosophila,* whereby the offspring of genetic lines selected for increased encapsulation ability demonstrated reduced competitive ability (Kraaijeveld and Godfray 1997). Evolutionary costs are further demonstrated by the negative correlations that have been found between levels of immune function and other life-history traits, including growth (Stearns 1992, Schmid-Hempel 2003) and fecundity (Nordling et al. 1998, Simmons & Roberts 2005, McKean et al. 2008). The maintenance and induction of an immune response are largely dependent on nutritional status (Siva-Jothy & Thompson 2002). This dependency indicates a direct energetic cost and demonstrates the relationship between disease susceptibility, stress (Rolff and Siva-Jothy 2003) and seasonality (Nelson et al 1998). This suggests that investigating immunology in an ecological context will provide insights into inter-specific variations in susceptibilities to disease.

Understanding inter-specific variations in disease susceptibility may therefore provide a long-awaited explanation for the documented temporal (Willis et al. 2004; Page and Willis 2006), seasonal (Willis et al. 2004; Sato et al. 2009) and species-specific (Raymundo et al. 2005; Page and Willis 2008) patterns of coral disease. Gaining insight into the biological mechanisms that underpin these ecological patterns is therefore both timely and vital given the downward trajectory of coral cover based on observations (Wilkinson 2004; Bruno and Selig 2007; Wilkinson 2008) and future climate scenarios (Veron et al. 2009).

1.7 STUDY AIMS

As the field of coral biology currently lacks a firm understanding of immunology, the ability to accurately predict how corals and the resultant reef assemblages will respond to climatic changes is limited. The current knowledge of immune systems in scleractinian corals is lagging behind that of octocorals, particularly gorgonians. As scleractinians are the primary framework builders of tropical reefs, their persistence is integral to the sustained biodiversity of coral reefs and thus a lack of immunological understanding represents a gap in the current knowledge of coral biology. To directly address this shortfall, the general aim of this PhD is to investigate key immunity mechanisms within corals and to apply these mechanisms to the understanding of coral ecology and coral-associated climate change biology. To address this general aim, there were three primary aims, each of will be addressed by a series of objectives:

Aim 1: To determine the presence of innate immunity effector responses in scleractinian corals. *Determining the immunity mechanisms utilized by corals will provide the foundation of coral immunology, from which comparative and experimental studies can be conducted.*

Objective 1.1: To investigate the presence of the melanin-synthesis and coagulation pathways using enzymatic, biochemical assays on multiple scleractinian and alcyonacean corals.

Objective 1.2: To characterise coral immune cells using live tissue slurries and histological investigations for multiple coral species.

Objective 1.3: To develop a protocol to quantify coral fluorescent proteins (FPs) and to compare the type and concentration of FPs among multiple coral species.

Objective 1.4: To investigate antioxidant activities of coral FPs, and to establish the presence and activities of enzymatic antioxidants in multiple coral species.

Objective 1.5: To assess the relationship between coral immunity and bleaching and disease susceptibility, using data collected throughout aim 1 in conjunction with data obtained from the published literature.

Aim 2: To investigate the utilisation of characterised coral effector responses.
Establishing the utilisation of effector responses during periods of compromised tissue integrity or infection to help to determine the role of specific mechanisms during coral immune responses.

Objective 2.1: To compare melanin-synthesis pathway activity in healthy and compromised, non-normally pigmented tissue of two coral species, using biochemical and histological protocols.

Objective 2.2: To determine whether fluorescent proteins are responsible for the non-normal pigmentation of compromised coral tissues using spectral emissions analysis and cryo-histological techniques.

Objective 2.3: To compare immune responses of coral colour morphs to physical injury by measuring multiple coral effector responses characterised in aim 1.

Objective 2.4: To characterise the stages of wound healing in a scleractinian coral using histological techniques.

Aim 3: To investigate the potential effect of climate change on coral immunity.
Establishing the effect of adverse environmental factors on coral immunity to help to accurately determine the threat that corals and coral reefs are facing under a changing climate.

Objective 3.1: To determine the effect of elevated water temperature on the immunocompetence and constituent immunity of a coral species using controlled experimental conditions.

SECTION I. CORAL IMMUNE EFFECTOR RESPONSES

Establishing the presence of immunity effector responses and quantifying their activity, represent the first steps required for investigating immunocompetence (see Chapter 1, subsection 1.5) and are necessary for studies of comparative immunology among taxa (Adamo 2004b). However, at the beginning of this project, effector responses, such as the presence of antimicrobial peptides (AMPs), the melanin-synthesis and coagulation pathways, and cellular immunity components, were completely unexplored within the Scleractinia. Although the presence of effector responses has been extensively investigated in numerous invertebrates (e.g. Boman et al. 1972; Tzou et al. 2002; Lemaitre and Hoffmann 2007; Babcock et al. 2008; Irazoqui et al. 2010), there have been few studies of potential effector responses within anthozoans (Mydlarz et al. 2006). These published studies have primarily focussed on anemones (Young 1974; Hutton and Smith 1996) and gorgonians (Meszaros and Bigger 1999; Olano and Bigger 2000). Comparative studies of anthozoan effector responses, for example between healthy and diseased samples or among species, are even more limited (Mydlarz and Harvell 2007; Mydlarz et al. 2008; Mydlarz et al. 2009). Determining if various effector responses are present in hard corals will establish whether or not scleractinians possess a functional immune system that is potentially comparable to those of other invertebrates, as projected by preliminary genomic investigations of this group (Brower et al. 1997; Miller et al. 2005; Kvennefors et al. 2008).

Immunity underpins the ability of an organism to resist infection and to recover from injury (Cooper and Koprowski 2002), and in numerous invertebrates the direct relationship between levels of immunity parameters and disease resistance has been documented (e.g. Persson et al. 1987; Allam et al. 2001; Adamo 2004b; Newton et al. 2004; Schwarzenbach and Ward 2007; Butt and Raftos 2008). However, lack of knowledge of immunity in scleractinians has impeded the understanding of disease resistance mechanisms in this group, despite the clear urgency to acquire such knowledge given the current threatened state of coral reefs globally, because of the increasing frequency and severity of diseases (Harvell et al. 2007), bleaching events (Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2009) and other disturbances. The development of standardised assays to quantify coral effector responses is vital for

providing a relative measure of baseline levels of immunity and immunocompetence, and would enable comparative studies among corals (Adamo 2004b; Mydlarz et al. 2006). Furthermore, determining relative measures of coral immunity parameters will provide insights into the causes of variation in disease susceptibility among coral species.

Within this section, Chapters 2 to 4 (as listed below) explore evidence for three classes of effector responses that are typically found in invertebrate innate immune systems and one, fluorescence, that may be limited to anthozoans, within the anthozoan orders Scleractinia, Alcyonacea and Zoanthidea. Chapter 5 synthesises the information attained from Chapters 2 to 4 to assess the relevance of the identified immunity parameters to coral disease and bleaching.

Components of each of the chapters within this section have been published in:

Palmer CV, Modi CK, Mydlarz LD (2009b) Coral fluorescent proteins as antioxidants. PLoS ONE. 4: e7298 (Impact factor N/A)

Palmer CV, Bythell JC, Willis BL (2010) Levels of immunity parameters underpin bleaching and disease susceptibility of reef corals. The Federation of American Societies for Experimental Biology Journal, first published on February 2, 2010 as doi:10.1096/fj.09-152447 (Impact factor 7.049)

SAMPLING AND PROTEIN EXTRACTION METHODS

All Indo-Pacific anthozoans were sampled from within 1 to 5 m depth from Orpheus Island, Great Barrier Reef (GBR; 18°36'37.99"S and 146°29'15.80"E). Samples for all chapters, were collected one day before the mass-spawning event of November 2008. In Chapter 3, samples were additionally collected before the spawning of November 2007. Five samples of healthy tissue, approximately 4 cm^2, were collected from each of the anthozoan species list in Table I.I. All samples were immediately snap-frozen in liquid nitrogen and stored at -30 °C.

Three genetically distinct fragments of seven Caribbean hard coral species (Table I.II) were collected from the National Oceanic and Atmospheric Administration/Florida Keys National Marine Sanctuary (NOAA/FKNMS) Coral Nursery in Key West Fl in May of 2007. The fragments were obtained and transferred to Mote Tropical Marine Laboratory under the specifications of research permit number FKNMS-2007-050, snap-frozen in liquid nitrogen and shipped on dry ice to Dr LD Mydlarz' Laboratory at the University of Texas at Arlington where they were stored at -80 ºC.

Tissue was removed from each frozen sample using an airbrush with extraction buffer (50 $mmol.l^{-1}$ phosphate buffer, pH 7.8 with 0.05 $mmol.l^{-1}$ dithiothreitol) over ice. Tissue slurries were homogenised with a medium sawtooth (Fisher Scientific,

Power Gen 125) for 30 s and left on ice for 5 min. Samples were centrifuged at 4 ºC at 3,500 RPM for 5 min and the supernatants stored at -30 ºC for Indo-Pacific samples and at -80 ºC for Caribbean samples.

Table I.I: The Indo-Pacific anthozoan species used in each chapter.

Phylogeny			Chapter			
Order	**Family**	**Species**	**2**	**3**	**4**	**5**
Scleractinia	**Acroporidae**	*Acropora hyacinthus*	*	*	*	*
		Acropora tenuis	*	*	*	*
		Acropora millepora	*	*	*	*
		Montipora digitata	*	*	*	*
	Pocilloporidae	*Pocillopora damicornis*	*	*	*	*
		Seriatopora hystrix	*	*	*	*
	Merulinidae	*Merulina ampliata*	*	*	*	*
		Hydnophora	*	*	*	*
	Euphyllidae	*Physogyra* sp.	*	*	*	
	Faviidae	*Diploastrea heliopora*	*	*	*	*
		Platygyra sinensis	*	*	*	*
		Gonastrea aspera	*	*	*	*
	Mussidae	*Lobophyllia hemprichii*	*	*	*	*
		Symphyllia sp.			*	
	Fungiidae	*Fungia*	*	*	*	*
	Poritidae	*Porites massive* sp.	*	*	*	*
		Porites cylindrica	*	*	*	*
	Oculinidae	*Galaxea* sp.	*	*	*	
Alcyonacae	**Alcyoniidae**	*Lobophyton*	*	*	*	*
		Sarcophyton	*	*	*	*
		Sinularia			*	
Zoantharia	**Sphenopidae**	*Palythoa* sp.	*		*	

Table I.II: The Caribbean anthozoan species used in each chapter.

Phylogeny			Chapter			
Order	**Family**	**Species**	**2**	**3**	**4**	**5**
Scleractinia	**Faviidae**	*Montastraea annularis*			*	
		Montastraea faveolata			*	
		Montastraea cavernosa			*	
		Diploria strigosa			*	
	Poritidae	*Porites astreoides*			*	
		Porites cylindrica			*	
	Siderastreidae	*Sidastrea siderea*			*	
	Meandrinidae	*Dichocoenia stokseii*			*	

Chapter 2 PROTEOLYTIC CASCADES: THE MELANIN-SYNTHESIS AND COAGULATION PATHWAYS IN CORALS

2.1 ABSTRACT

The proteolytic cascades of melanin-synthesis and coagulation are important to multiple aspects of invertebrate immune responses, including wound sealing, antimicrobial defence and encapsulation. The presence of the melanin-synthesis and coagulation cascades was investigated enzymatically in a massive species of the hard coral *Porites*, by measuring the dose response activities of phenoloxidase (PO; *ortho*-diphenoloxidase) and transglutaminase, respectively. Additionally, 20 species, spanning 3 anthozoan orders and 11 families, were each investigated for three types of PO activity: mono-phenoloxidase, *o*-diphenoloxidase (tyrosinase-type) and *p*-diphenoloxidase (laccase-type) activities. Both the PO and transglutaminase dose response curves were consistent with a significant positive correlation between sample volume (protein concentration) and enzyme activity, and each enzyme was significantly inhibited by its inhibitor (sodium azide and iodoacetamide respectively). Furthermore, quantification of the activity of the three POs for each anthozoan revealed that activities varied among taxa, although *o*-diphenoloxidase generally had the highest activity. These findings document two immunity-related proteolytic cascades in hard and soft corals for the first time. The presence and activities of different POs suggest that different anthozoans preferentially use different melanin pathways and have differing levels of baseline immunity and therefore resistance to disease.

2.2 INTRODUCTION

The proteolytic cascades of melanin-synthesis and coagulation are key innate immune effector responses (Söderhäll and Smith 1986b; Rowley 1996; Söderhäll and Cerenius 1998), however prior to the commencement of this project, the presence of these effector response pathways had not been investigated within hard corals. Given the importance of the melanin-synthesis pathway in providing both cytotoxic defence and structural support to infected and damaged tissue in a range of invertebrates

(Söderhäll and Smith 1986b) reviewed in Chapter 1, investigating the presence and activity of enzymes key to this pathway provides an appropriate starting point to investigate effector responses of innate immunity in corals. Similarly, evidence that the coagulation pathway is important for sealing wounds and preventing infection in many invertebrates (Theopold et al. 2004), suggests that investigating the activity of enzymes associated with this pathway might also yield important information about innate immune function in corals.

2.2.1 THE MELANIN-SYNTHESIS PATHWAY

The presence of multiple different phenoloxidases (POs), the active form of their prophenoloxidase (PPO) zymogens, within some invertebrates (Sugumaran and Kanost 1993; Claus and Decker 2006), suggests that there may be a number of different melanin-synthesis pathway components and/or distinct pathways present within one organism. POs are a group of multicopper oxidases (Decker and Terwilliger 2000 46) and can be separated into two categories, the tyrosinase-type and laccase-type, and are classified by their ability to metabolise different phenolic substrates, including mono-phenols, *para*-diphenols and *ortho*-diphenols (Yatsu and Asano 2009). Tyrosinase-type POs have specificity to both mono-phenols (e.g. tyrosine) and *o*-diphenols (e.g. dihydroxyphenylalanine (DOPA)-based substrates; Sugumaran 2002; Nappi and Christensen 2005; Yatsu and Asano 2009). Conversely, laccase-type POs cannot utilise mono-phenols but can oxidise both *o*-diphenols and *p*-diphenols (Claus and Decker 2006; Yatsu and Asano 2009). It is worth noting however, that *o*-diphenoloxidases are sometimes referred to as a third PO-type called catecholoxidases (Cong et al. 2005; Pang et al. 2005). The presence of multiple melanin-synthesis pathway components and two separate pathways suggests that the different PO types may have different roles in invertebrate innate immunity (Sugumaran 2002).

For invertebrates, the utilisation of various POs during melanin synthesis is best described within insects (Figure 2.1; Sugumaran 2002; Nappi and Christensen 2005). Once the proteolytic cascade has been activated, melanin synthesis occurs *via* the hydroxylation of mono-phenol substrates (e.g. tyrosine or tyramine) to diphenol substrates (e.g. DOPA) by a tyrosinase-type PO demonstrating mono-phenoloxidase activity (Figure 2.1; Sugumaran 2002; Nappi and Christensen 2005). Diphenoloxidases oxidise *ortho*-diphenols, like dopamine or DOPA, to subsequent

chromatic compounds and quinones (*o*-diphenoloxidase or catecholase activity), which can then go on to produce melanin, usually non-enzymatically (Sugumaran 2002; Nappi and Christensen 2005; Claus and Decker 2006). Therefore, different tyrosinase-type POs represent different components of the same melanin-synthesis pathway (Nappi and Christensen 2005). Conversely, laccase-type PO(s) (referred to simply as laccase henceforth) may be involved in a separate melanin-synthesis pathway. Laccase is able to oxidise both *para*-diphenols and *o*-diphenols (Claus and Decker 2006), and may be involved in the oxidation of dopamine-derived *N*-acyldopamines to corresponding quinones (Figure 2.1(Sugumaran 2002)). Within arthropods, including the insect model, tyrosinase-type PO activity is a key component of innate immune responses and is involved in cytotoxic defence, encapsulation and phagocytosis (Söderhäll and Cerenius 1998; Iwanaga and Lee 2005). By contrast, laccase is documented to be involved in sclerotisation, also termed cuticle tanning, of arthropods (Barrett 1987; Cardenas and Dankert 2000; Arakane et al. 2005; Dittmer et al. 2009).

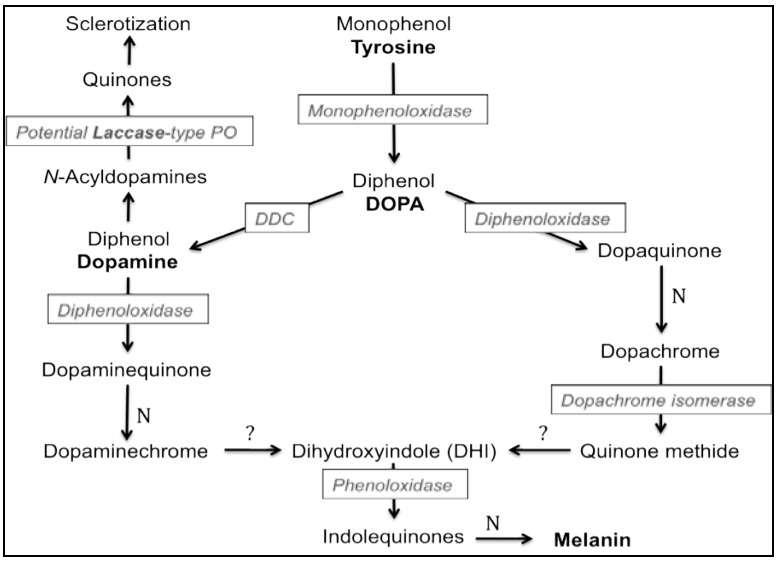


Figure 2.1: Melanin-synthesis pathway in insects. N = non-enzymatic conversion, ? = unknown mechanism, *DDC* = dopa decarboxylase. (Simplified and adapted from (Sugumaran 2002; Nappi and Christensen 2005)

In view of its integral role in innate immunity, it is not surprising that tyrosinse-type PO activity has been documented within multiple marine invertebrates, including the bivalves *Perna viridis* (Asokan et al. 1997), *Saccostrea glomerata* (Butt

and Raftos 2008) and *Crassostrea gigas* (Hellio et al. 2007), the ascidian *Ciona intestinalis* and the sea urchin *Diadema antillarum* (Smith and Soderhall 1991). However, documentation of the presence and role of laccase within non-arthropod and non-cuticle forming invertebrates is limited. So far, laccase activity has been found in the insect *Sarcophyaga bullata* (Barrett 1987), the crayfish *Procambarus clarkii* (Cardenas and Dankert 2000), the beetle *Tribolium castaneum* (Arakane et al. 2005), and the moth *Manduca sexta* (Dittmer et al. 2009). Laccase is also involved in immune responses within the insect *Drosophila* (Theopold et al. 2002) and has been documented within the haemolymph of the Pacific oyster *Crassostrea gigas* (Luna-Acosta et al. 2010). PO substrate-specificity investigations are important for determining effector response mechanisms, however only a few studies have been conducted, including within the freshwater crayfish *Pacifastacus leniusculus* (Aspan et al. 1995) the eastern oyster *Crassostera virginica* (Jordan and Deaton 2005) and the Pacific oyster *C. gigas* (Luna-Acosta et al. 2010), all of which demonstrated higher relative specificity for *o*-diphenol substrates.

Consistent with a higher specificity for *o*-diphenol substrates (Aspan et al. 1995; Jordan and Deaton 2005; Luna-Acosta et al. 2010), the default substrate for invertebrate PO studies has been L- 3,4-dihydroxyphenylalanine (L-DOPA). These studies include a suite of arthropods (Smith and Soderhall 1991; Cardenas and Dankert 1997; Perazzolo and Barracco 1997; Söderhäll and Cerenius 1998), the annelid *Eisenia fetida* (Prochazkova et al. 2006) and the cnidarian *Gorgonia ventalina* (Mydlarz et al. 2008). However, it has been documented that dopamine is a preferred substrate within arthropods (Sugumaran 2002), with L-DOPA a comparably poor substrate despite both being *o*-diphenols (Hall et al. 1995; Sugumaran 1998). Although inter-specific PO type and activity comparisons are limited (Barrett 1987; Smith and Soderhall 1991; Jordan and Deaton 2005), the species-specific differences found (Smith and Soderhall 1991), highlight the need for substrate specificity experiments to determine which substrate most accurately represents the PO type(s) and activity within the study organism. This is particularly important for studies comparing PO activities over time, between samples or among species. Determining whether hard coral species possess both the tyrosinase-type POs and laccase represents the first step in establishing quantifiable effector responses. Additionally, variations among taxa in substrate-specificity may indicate different uses of melanin pathways and may relate to disease susceptibility.

2.2.2 THE COAGULATION PATHWAY

The coagulation pathway (reviewed in Chapter 1) is vital for immediately sealing wounds through the formation of a clot to prevent opportunistic invasion of pathogens and fluid loss (e.g. Wang et al. 2001; Theopold et al. 2002; Theopold et al. 2004). Transglutaminase is the activating enzyme of the coagulation pathway in the majority of invertebrates investigated (Muta and Iwanaga 1996; Karlsson et al. 2004; Theopold et al. 2004) and catalyzes protein cross-linking (Cordella-Miele et al. 1990; Beninati and Piacentini 2004) during clot hardening (Theopold et al. 2004). Transglutaminases are a group of Ca^{2+}-dependent multi-functional enzymes (Lin et al. 2008) that have been located within arthropod haemocytes (Chung et al. 1977). Although wound-healing observations have been made for a variety of anthozoans, including an anemone (Young 1974) and gorgonians (Meszaros and Bigger 1999; Olano and Bigger 2000), there are no reports documenting clot formation *via* the activation of coagulation pathways. However, a recently released transcriptome database indicates the presence of transglutaminase genes within *Acropora millepora* (Meyer et al. 2009), which strongly suggests that the transglutaminase-associated coagulation pathway is active within hard corals.

This study aimed to determine if two innate immunity effector responses, the melanin-synthesis and coagulation pathways, are present within hard corals. The presence and relative activities of POs were investigated in 17 hard corals, 2 soft corals and a zoanthid using substrate specificity assays. In addition, an exploratory investigation into the presence and enzymatic activity of transglutaminase was conducted on one hard coral species.

2.3 METHODS

2.3.1 ENZYME ASSAYS

Indo-Pacific anthozoan samples were collected and protein extracted as described in Section I.I of the species listed in Table I.I. To determine the presence of phenoloxidase (PO) activity within a scleractinian coral, a dose response curve was created using pooled sample extracts from colonies (n = 5) of a massive poritid species, *Porites* sp.. Within 96-well microtitre plates, six wells each of 5, 10, 15, 20 and 25 µl of the pooled sample extract were set up and the corresponding 55 to 35 µl

of phosphate buffer (50 mmol.l^{-1} pH 7.8) added to make a total well volume of 60 µl. Next, 25 µl of double distilled water (ddH_2O) was added to each well and plates were left to incubate at room temperature for 20 min. Then 30 µl of 10 mmol.l^{-1} solutions of one of the following colourless substrates was added to two of the wells of each extract volume (0 to 20 µl): Tyramine (Fluka 93810) for mono-phenoloxidase activity, dopamine hydrochloride (Sigma-Aldrich H8502) for *o*-diphenoloxidase activity and hydroquinone (Sigma H9003) for *p*-diphenoloxidase (laccase) activity (Table 2.1; Jordan and Deaton 2005). The absorbance at 410 nm was recorded every 5 min for 45 min using a Spectramax M2 (Molecular Devices) spectrophotometer, and the change in absorbance for the linear portion of the reaction curve was plotted as PO activity.

For comparisons among anthozoans, activities of mono-phenoloxidase, *o*-diphenoloxidase and *p*-diphenoloxidase were determined, as described above, in triplicate for each species, i.e. with 20 µl of coral sample, 40 µl of phosphate buffer, 25 µl of ddH_2O and 30 µl of substrate, at 490 nm. Also, L-DOPA (3-(3, 4-Dihydroxyphenyl)-L-alanine; Fluka 37830) was used as an additional *o*-diphenol substrate and prophenoloxidase (PPO) activity was calculated for each coral and each substrate by repeating the assays with the substitution of the 25 µl of ddH_2O for 25 µl of trypsin (0.1 mg.ml^{-1} Sigma). PO and PPO activities were standardised to sample total protein as determined using the Bradford Quick-start assay (Bio-Rad).

Transglutaminase activity was determined using 5 to 25 µl of sample extracts pooled from 5 colonies of the massive coral *Porites* sp. and placed in triplicate wells of a microtitre 96-well plate. Then 45 to 25 µl of ddH_2O were added to each well to total 50 µl. Subsequently, 20 µl of z-Gln-Gly (Sigma C6154) and 70 µl of buffer (19.5% of 1 M Tris-acetate with 0.2 M EDTA, 4.5% 0.1 M $CaCl_2$ and 72.5% ddH_2O) with 2.8% 25 mmol.l^{-1} DMPDA (N, N – dimethyl-p-phenylenediamine; Sigma C7750) were added to each well. Transglutaminase activity was measured as the change in absorbance over 40 min at 278 nm, due to the oxidation of DMPDA (Table 2.1). Activity was inhibited by the addition of 10 mmol.l^{-1} iodoacetamide (Sigma I1149).

Table 2.1: A summary of the substrates used in enzyme assays

Substrates	λ max (nm)	Enzyme type
Tyramine	490	mono-phenoloxidase
Dopamine	490	*o*-diphenoloxidase
L-DOPA	490	*o*-diphenoloxidase
Hydroquinone	490	*p*-diphenoloxidsae
DMPDA	278	Transglutaminase

Data were tested for normality and homoscedasticity (Shapiro-Wilks and Levene's tests, respectively) prior to performing statistical analyses. Data sets that did not satisfy the assumptions were log transformed to meet the parametric criteria of normality and equal variances. To determine if enzyme activities were correlated with sample extract volumes (protein concentration), analyses of variance of regression (May and Bigelow 2005) were conducted using Sigma Plot (10.0). Enzyme activity in the presence and absence of inhibitors was compared using a t-test for transglutaminase and using a Mann-Whitney U test for PO, as parametric assumptions were not met in the latter case. PO activities and total PO were compared among coral families using one-way ANOVAs with Tukey's honestly significant difference (HSD) *post-hoc* tests or (where parametric assumptions were not met) Kruskal-Wallis tests, conducted in SPSS 17.0. For PPO activities, comparisons among coral families were made using a one-way multivariate ANOVA (MANOVA) in R.

2.4 RESULTS

2.4.1 THE MELANIN-SYNTHESIS PATHWAY

Dose-dependent responses were significant for all three types of phenoloxidase (PO) activity investigated (Figure 2.2). Activity was positively correlated with sample extract volume (protein concentration) for each PO, with the regression explaining 95% of activity variation in mono-phenoloxidase ($R^2 = 0.95$, $F_{(1, 4)} = 29.89$, $P = 0.01$), 89% in *o*-diphenoloxidase ($R^2 = 0.89$, $F_{(1, 4)} = 11.632$, $P = 0.04$) and 90% in *p*-diphenoloxidase ($R^2 = 0.90$, $F_{(1, 4)} = 13.108$, $P = 0.04$). At 20 µl, mono-phenoloxidase had approximately 10-fold lower activity than either *p*-diphenoloxidase or *o*-diphenoloxidase. The presence of PO activity within corals was further confirmed *via* the significant (~85%) inhibition of *o*-diphenoloxidase with sodium azide (Figure 2.3; n = 18; Mann-Whitney U = 3.0, $P < 0.001$).

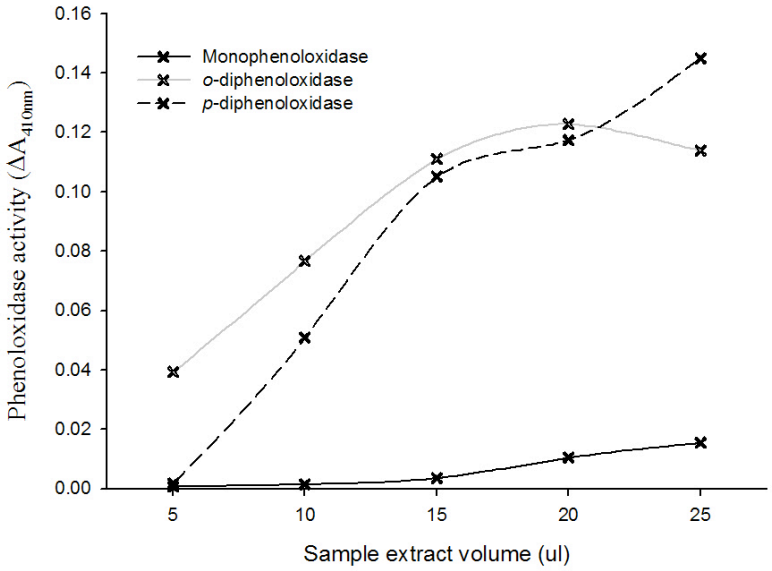


Figure 2.2: Dose-response curves of phenoloxidase (PO) activity of the hard coral *Porites cylindrica*. Each PO demonstrated an increase in activity with sample volume (mono-phenoloxidase: $R^2 = 0.95$, $F_{(1, 4)} = 29.89$, $P = 0.012$; *o*-diphenoloxidase: $R^2 = 0.89$, $F_{(1, 4)} = 11.632$, $P = 0.042$; and *p*-diphenoloxidase: $R^2 = 0.90$, $F_{(1, 4)} = 13.108$, $P = 0.036$).

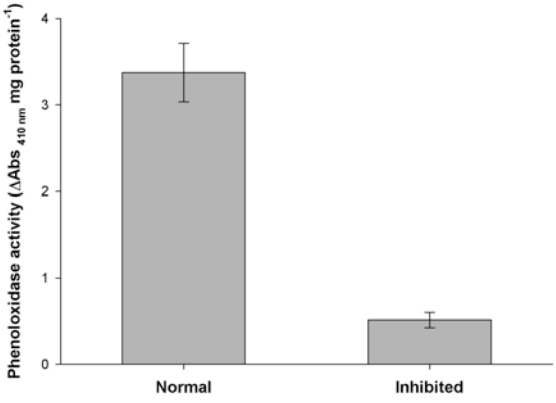


Figure 2.3: Comparison of mean *o*-diphenoloxidase activity (± SE) between assays with (inhibited) and without (normal) the addition of sodium azide (n = 18 samples; Mann-Whitney $U = 3.0$, $P < 0.001$).

2.4.2 THE MELANIN-SYNTHESIS PATHWAY IN MULTIPLE ANTHOZOANS

PO activity was demonstrated by each of the four substrates within all 11 anthozoan families tested (Figure 2.4). In general, dopamine PO activity (*o*-diphenoloxidase) represented the highest level of activity across families, regardless of variability among families in levels of activity ($F_{(10, 100)} = 16.77$, $P < 0.001$), which ranged from a 4-fold change in absorbance in the Euphyllidae to a greater than 60-fold change in the Oculinidae. L-DOPA PO activity also differed significantly among anthozoan families ($K\text{-}W_{(10)} = 67.74$, $P < 0.001$), with a 55-fold change in absorbance in the Oculinidae representing the highest activity. Activity was approximately 30-fold lower in the Pocilloporidae, which had the lowest activity overall. The third substrate type to demonstrate significant differences in PO activity among families was hydroquinone (*p*-diphenoloxidase), which also demonstrated greatest PO activity in the Oculinidae and least in the Pocilloporidae, although the Euphyllidae and Acroporidae had similarly low levels of activity ($F_{(10, 105)} = 9.137$, $P < 0.001$). PO activity using tyramine (i.e. mono-phenoloxidase activity) did not vary significantly among families, although mean activity values were greatest for the Poritidae and least for the Fungiidae. Overall, there were no clear family-level patterns in comparative activity among the four substrate types, for example, tyramine was the most active of the four substrate types in two families (the Pocilloporidae and Euphyllidae) but least active in five families (Figure 2.4).

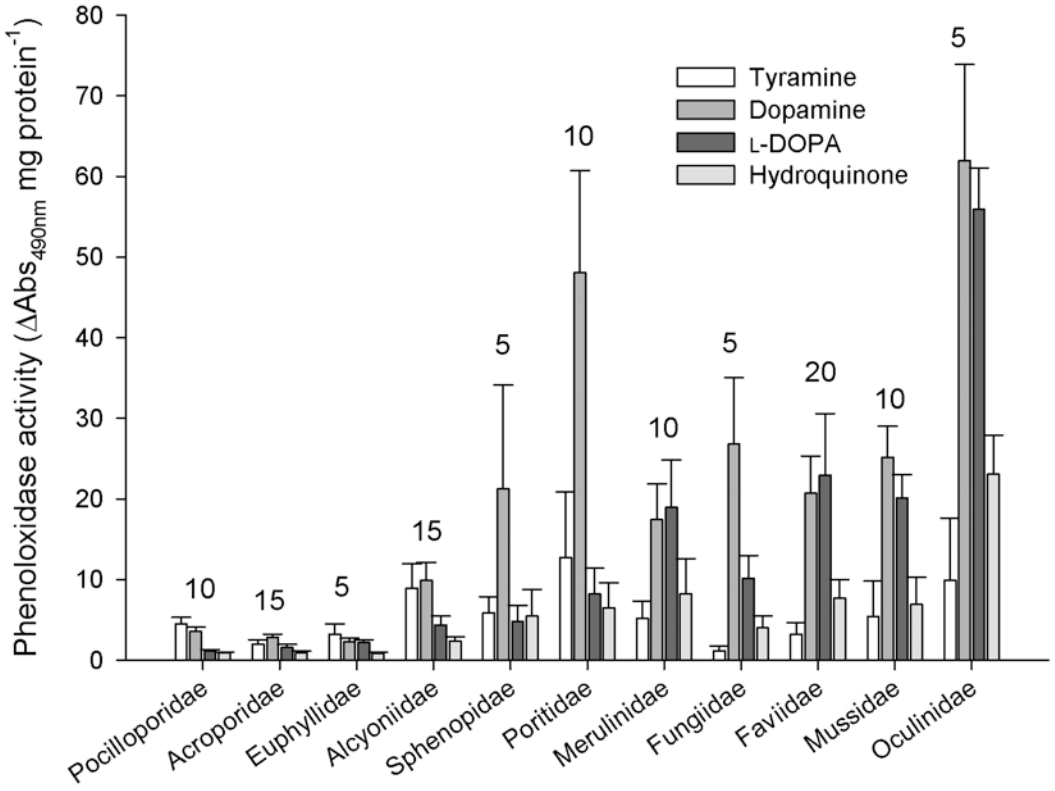


Figure 2.4: Mean phenoloxidase activities (± SE) for each of 9 scleractinian, 1 alcyonacean and 1 zoanthidean families. (Numbers above bars indicate sample size for each family).

Total phenoloxidase activity was found to vary significantly among coral families when activities were combined across all substrate types investigated (Figure 2.5; $F_{(10, 100)} = 13.33$, $P < 0.001$). The Pocilloporidae, Acroporidae and Euphyllidae had the lowest, and the Poritidae and Oculinidae the highest total PO activity (Tukey's HSD, Table 2.2). For each coral family, the relative contribution of dopamine to the total PO activity exceeded that of L-DOPA, with the exceptions of Merulinidae and Faviidae (Figure 2.5). This pattern of higher PO activity with dopamine as compared to L-DOPA was particularly noteworthy for the Poritidae where dopamine PO activity contributed nearly 6-fold greater than that of L-DOPA (11% and 64% of total PO activity respectively). By contrast, proportions of L-DOPA and dopamine PO activities were approximately equivalent for Euphyllidae, Faviidae and Mussidae.

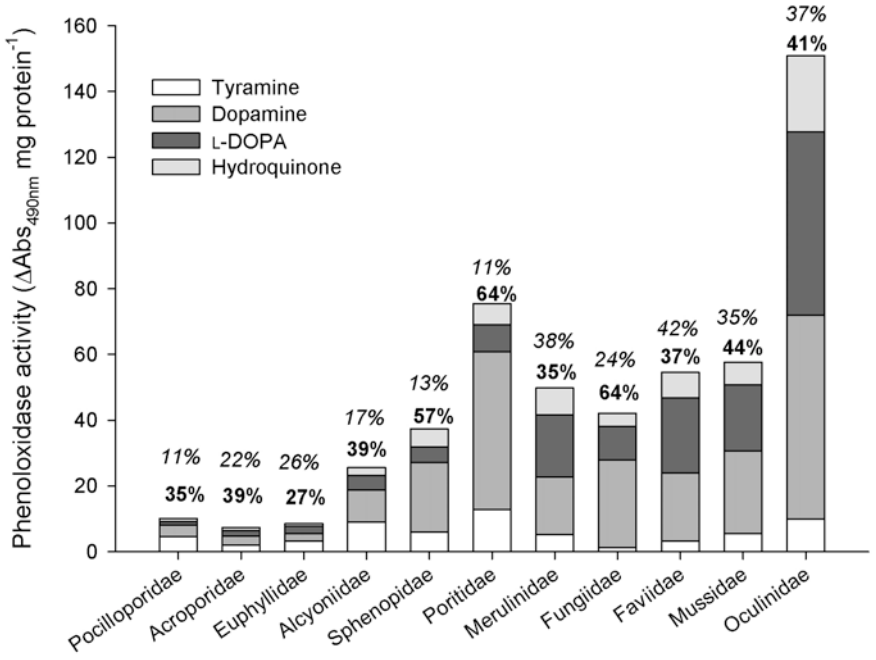


Figure 2.5: Comparison of total phenoloxidase (PO) activity and the mean proportions contributed by PO activity of each substrate type among eleven anthozoan families. Percentages above bars represent the contributions of L-DOPA *o*-diphenoloxidase (italics) and dopamine *o*-diphenoloxidase (bold) to total PO activity.

Table 2.2: Summary table of Tukey's HSD post-hoc comparison of total PO activity among coral families. Letters a-d denote families with statistically different levels of total PO activity ($p<0.05$).

Coral family	**Total PO activity**
Pocilloporidae	a
Acroporidae	a
Euphyllidae	ab
Alcyoniidae	abc
Sphenopidae	bc
Merulinidae	bc
Fungiidae	c
Faviidae	c
Mussidae	c
Poritidae	c
Oculinidae	d

Overall, there was a significant difference in prophenoloxidase (PPO) activities among anthozoan families (MANOVA, whole model Wilks lambda = 0.608, $F_{(10, 96)} = 15.47$, $P < 0.001$). However, univariate F-statistics of the MANOVA whole model indicated that PPO activity with the tyramine substrate did not vary significantly among families (Table 2.3), although activity levels were undetectable for Sphenopidae and Fungiidae, but comparably high (~20-fold change in absorbance) for Poritidae and Merulinidae (Figure 2.6). All other substrates; dopamine, L-DOPA and hydroquinone, varied significantly among coral families (Table 2.3). Dopamine *o*-diprophenoloxidase activity was approximately equivalent to L-DOPA *o*-diprophenoloxidase activity for the families Euphyllidae, Alcyoniidae, Fungiidae and Sphenopidae (Figure 2.6), although dopamine PPO activity was higher than L-DOPA PPO for the other coral families, most notably for the Poritidae and Oculinidae (Figure 2.6). Hydroquinone PPO (*p*-diprophenoloxidase) had the lowest activity of all substrates for all families and was, for example, 10-fold lower than dopamine PPO activity within the Oculinidae. However, in the Poritidae and Acroporidae, hydroquinone PPO was approximately equivalent to L-DOPA PPO, with approximately 10-fold and 3-fold changes in absorbance, respectively (Figure 2.6). Tukey's HSD grouped coral families similarly for each substrate (Table 2.4), with the Euphyllidae, Pocilloporidae and Acroporidae having approximately equivalent and lowest PPO activities and the Oculinidae the highest.

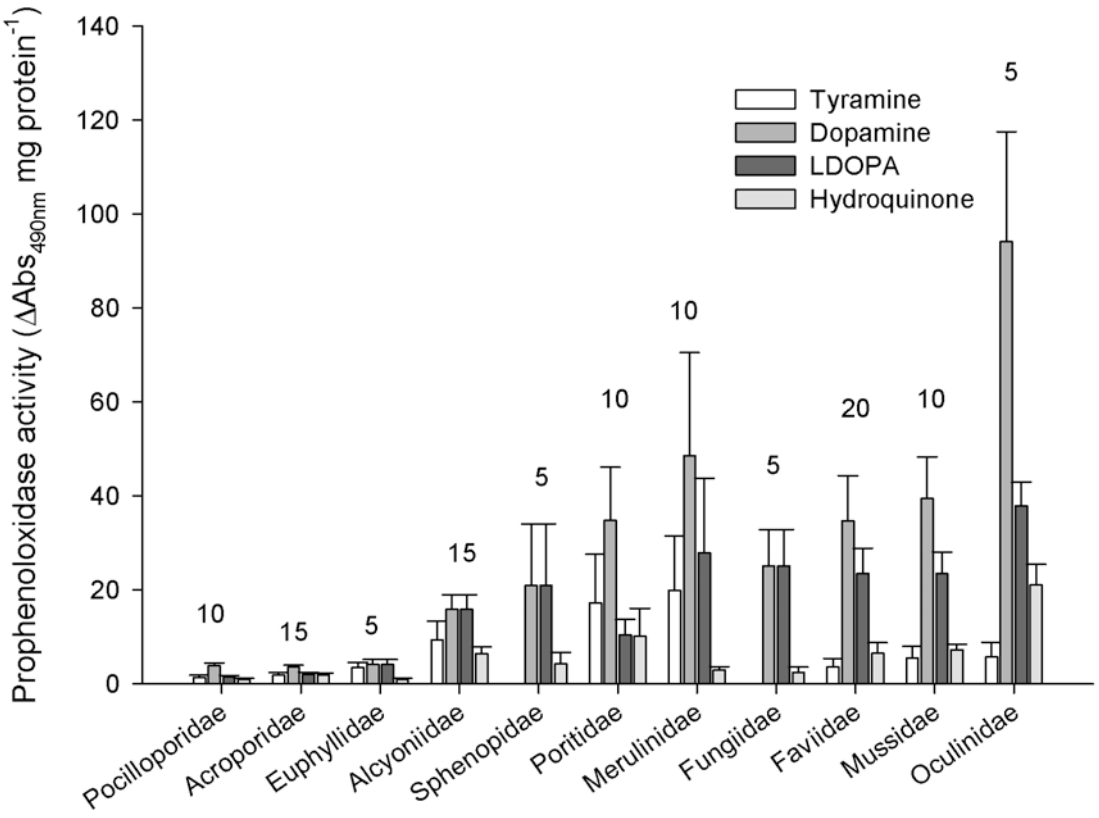


Figure 2.6: Comparison of mean prophenoloxidase (PPO) activities (± SE) among 9 scleractinian, 1 alcyonacean and 1 zoanthidean families. Numbers above bars indicate sample size for each family.

Table 2.3: Mulitvariate ANOVA (MANOVA) summary table of Univariate F-statistics for PPO.

Dependant variable	f-ratio	df	P
Tyramine	0.107	99	0.745
Dopamine	8.905	99	**<0.01**
L-DOPA	37.478	99	**<0.001**
Hydroquinone	6.446	99	**0.013**

Table 2.4: Summary table of Tukey's HSD post-hoc comparisons for PPO activities for each substrate. Each of the letters a-d denote families of similar PO activity; statistical differences in activity ($P < 0.05$) separate letters. Note that there was no significant difference among family groups for tyramine.

Coral family	**Dopamine**	**L-DOPA**	**Hydroquinone**
Pocilloporidae	a	a	a
Acroporidae	a	a	ab
Euphyllidae	a	ab	a
Alcyoniidae	ab	bcd	bc
Sphenopidae	ab	bcd	abc
Poritidae	b	bc	bc
Merulinidae	b	bcd	abc
Fungiidae	b	cd	abc
Faviidae	bc	cd	bc
Mussidae	bc	cd	c
Oculinidae	c	d	d

2.4.3 THE COAGULATION PATHWAY: TRANSGLUTAMINASE ACTIVITY

Transglutaminase activity was demonstrated within a massive species of the hard coral *Porites*. Transglutaminase activity increased significantly with increasing sample extract volume (protein concentration), demonstrating a dose-dependent response (Figure 2.7a; $R^2 = 0.97$, $F_{(2, 4)} = 36.05$, $P = 0.03$). Transglutaminase activity was significantly inhibited, by approximately 50%, with the addition of iodoacetamide (Figure 2.7b; $t_{(4)} = 2.85$, $P = 0.047$).

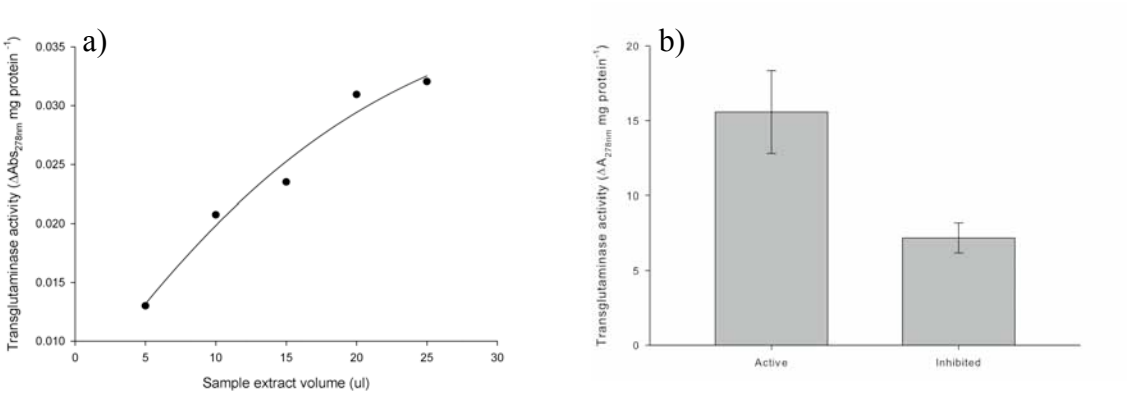


Figure 2.7: Transglutaminase activity of pooled samples of a *Porites* massive sp. showing: a) dose-dependent response ($R^2 = 0.97$, $F_{(2, 4)} = 36.05$, $P = 0.03$); and b) comparative activity with and without the inhibitor (± SE; $t_{(4)} = 2.846$, $P = 0.047$).

2.5 DISCUSSION

2.5.1 THE MELANIN-SYNTHESIS PATHWAY

Components of the melanin-synthesis pathway were documented within a suite of anthozoans for the first time, including within 17 hard corals (O: Scleractinia), 2 species of soft corals (O: Alcyonacea), and 1 species of zoanthid (O: Zoanthidea). Activity was detected for all three types of phenoloxidases (PO): mono-phenoloxidase, *o*-diphenoloxidase and *p*-diphenoloxidase, and their corresponding prophenoloxidases (PPOs). This documentation increases the number of anthozoan species for which a component of the melanin-synthesis pathway has been reported from one (*Gorgonia ventalina*; Mydlarz et al. 2008) to twenty-one. Moreover, this is the first documentation of mono-phenoloxidase and *p*-diphenoloxidase activities within anthozoans. These results demonstrate the presence of several melanin-synthesis pathway components and potentially two different pathways, including the tyrosinase-type and the laccase pathway (Sugumaran 2002; Jordan and Deaton 2005) in anthozoans. The presence of these two pathways (Sugumaran 2002) across diverse anthozoan taxa suggests that melanin synthesis is ubiquitous within the Anthozoa and demonstrates the presence of a key innate immune effector response within phylogenetically basal metazoans.

Different relative activities of each PO type demonstrate variability in baseline activity of components within the melanin-synthesis pathway among anthozoans. Generally, *o*-diphenoloxidase was greatest in the anthozoans tested, as indicated by

the general preference for the *o*-diphenol substrates dopamine and L-DOPA. This substrate preference for *o*-diphenols is consistent with previous reports from other invertebrates (Aspan et al. 1995; Jordan and Deaton 2005; Luna-Acosta et al. 2010), and the further specificity of anthozoans for dopamine is consistent with reports of similar substrate-specificity and preference associated with PO activity in arthropods (Hall et al. 1995; Sugumaran 2002). The presence of *o*-diphenoloxidase demonstrates baseline, or constituent, levels of the tyrosinase-type melanin-synthesis pathway, which is the main melanin-synthesis pathway used for encapsulation and phagocytosis during invertebrate immune responses (Söderhäll and Cerenius 1998; Iwanaga and Lee 2005; Bidla et al. 2008; Cerenius et al. 2008; Mydlarz et al. 2008; Cerenius et al. 2010), and which is typically correlated with disease resistance (Newton et al. 2004; Schwarzenbach and Ward 2007; Butt and Raftos 2008). However the presence of PO activity using hydroquinone as a substrate indicates laccase activity, which suggests that, in addition to constituent levels of the tyrosinase-type melanin-synthesis pathway, corals also utilise the laccase-type melanin-synthesis pathway (Sugumaran 2002).

The presence of laccase-like activity (*p*-diphenoloxidase), generally in lower concentrations than the *o*-diphenoloxidases, suggests that its presence is less crucial to the constant maintenance of tissue integrity and disease resistance than the tyrosinase-type melanin-synthesis pathway. Within other invertebrates, such as insects (Gorman et al. 2008) and crustaceans (Cardenas and Dankert 2000), laccase is utilised for tanning and hardening of the cuticle *via* the deposition of melanin (Arakane et al. 2005; Dittmer et al. 2009). As corals rely on efficient light penetration (Muscatine et al. 1984) to their obligate algal endosymbionts (Bythell 1988), melanin deposited as a hardened layer in anthozoans would be deleterious; correspondingly, it has not been generally observed. However, the presence of melanin deposits that absorb UV and visible light (Meredith et al. 2006) may be advantageous during times of temperature and light stress, such as during thermal bleaching events (Hoegh-Guldberg and Smith 1989; Hoegh-Guldberg 1999). It is probable that laccase activity in anthozoans may be more specific to clot stabilisation as part of the coagulation processes (Theopold et al. 2002) or another aspect of immunity, as hypothesised for laccase activity documented within the Pacific oyster, which, similarly to anthozoans, lacks an integument (Luna-Acosta et al. 2010). Mono-phenoloxidase activity (tyramine oxidation), although present in all anthozoan families, did not vary among them,

indicating that baseline levels of this enzyme are approximately constant among taxa. This is consistent with mono-phenoloxidase being required to activate subsequent stages of both the tyrosinase and laccase melanin-synthesis pathways (Figure 2.1; Sugumaran 2002). In combination, these results suggest that investigating total PO activity may be more informative for assessing relative immune capabilities among anthozoans, and therefore their potential baseline levels of disease resistance.

Total PO activity differed among anthozoan families, with the Oculinidae and Poritidae having significantly higher activity than, for example, the Pocilloporidae and Acroporidae. This pattern is broadly consistent with patterns in disease prevalence documented for families of corals on the Great Barrier Reef, where disease prevalence is typically greatest in the Pocilloporidae and Acroporidae and least in the Poritidae (Willis et al. 2004; Page and Willis 2006). Given the higher relative contribution of dopamine substrate than L-DOPA to total PO activity, the use of dopamine as an *o*-diphenoloxidase substrate is recommended as a better indicator of anthozoan total PO activity, and therefore should be used for future coral immunocompetence assays.

Quantifying PPO activity indicates the amount of phenoloxidase enzyme that is present in anthozoan tissue as an inactive pro-form. This therefore provides additional information about the extent of "immune reserves" and potential immunocompetence of a coral species by indicating the readiness to activate a response. Corals that have higher levels of active enzyme (PO) may be more effective at preventing infection and therefore resisting disease, as documented for other invertebrates (Newton et al. 2004). However, maintaining constant high levels of PO activity under healthy conditions may be energetically costly (Sadd and Schmid-Hempel 2009) and compromise a coral's ability to up-regulate an immune response once it is infected (immunocompetence). Conversely, corals that have a higher relative level of enzyme stored as the pro-form (PPO), may demonstrate a greater ability to up-regulate an immune response once infected or injured, although the likelihood of infection may be greater (Butt and Raftos 2008). Determining the relationship between PO and PPO activity within corals and among coral taxa needs further investigation, but may provide insights into inter-specific trade-offs in anthozoan life history traits and therefore contribute to the understanding of coral ecological immunity (Sadd and Schmid-Hempel 2009).

2.5.2 THE COAGULATION PATHWAY: TRANSGLUTAMINASE ACTIVITY

Detection of transglutaminase activity in a massive species of *Porites* demonstrates another aspect of immunity that is shown here for the first time in scleractinian corals. It is likely that, like the melanin synthesis pathway, this aspect of innate immunity is also conserved within the anthozoans, although only one species of coral was investigated in this study. This prediction is supported by the identification of transglutaminase family genes within a transcriptome database recently published for the hard coral *Acropora millepora* (Meyer et al. 2009).

2.5.3 CONCLUSION

In summary, this study significantly contributes to the investigation of hard coral immunity by establishing the presence of two quantifiable effector responses that are consistent with components of innate immune pathways in other invertebrates. Melanin-synthesis pathway components and transglutaminase activity of the coagulation pathway, which represent immunity effector responses, are shown for the first time within hard corals in this study. The quantification of these effector responses enables their direct comparison among coral species and provides a significant step towards investigating coral immunocompetence. Despite these significant findings, the potential presence of other coral immunity effector responses needs to be investigated, such as the presence of immune cells.

Chapter 3 CORAL IMMUNE CELLS

3.1 ABSTRACT

Immune cells are diverse among and within metazoan phyla and are vital for the encapsulation and phagocytosis of foreign organisms during infection, and also for tissue stabilisation during wound healing. Within anthozoans, granular amoebocytes have been identified in anemones and gorgonians, but there have been only two reports of putative amoebocytic cells in hard corals. In this study, live tissue slurries of the hard coral species *Acropora millepora, Porites cylindrica, Porites astreoides* and *Seriatopora hystrix* were investigated microscopically and the presence of amoebocytic cells identified in all four species. Moreover, granular amoebocytes from *A. millepora* demonstrated phagocytic activity, which represents the first documentation of phagocytosis for a hard coral cell. Phenoloxidase (PO) activity was located in the epidermis and calicodermis of *P. cylindrica,* as well as in unidentified smooth, oblong cells that are potentially developing nematocysts. Using a melanin-specific histological stain, chromophore cells, as previously described for *Porites* spp., were identified in all anthozoan species investigated and found to contain high densities of melanin. The mean volume fraction (Vf) of melanin in the free body epidermal layers varied among species, as did relative Vfs of melanin in the epidermis versus the gastrodermis and mean size of melanin-containing granular cells. This study demonstrates for the first time that scleractinian corals contain multiple cells that are morphologically similar to immune cells involved in phagocytosis, encapsulation and wound healing of other invertebrates. This therefore provides strong evidence that these immunity mechanisms are present within corals. The documentation of both PO activity and melanin within coral immune cells further reinforces the importance of the melanin-synthesis pathway within anthozoan immunity. Finally, the quantification of melanin-containing granular cells provides another effector response that can be potentially used as a measure of immunocompetence in corals.

3.2 INTRODUCTION

Immune cells are responsible for the cellular mechanisms of innate immunity (Smith et al. 1996b) and, as such, are involved in removing pathogens and repairing wounds, however immune cells within hard corals have not been thoroughly investigated. As the functions of immune cells in other invertebrates include encapsulation (Valembois et al. 1992) and phagocytosis (Stein and Cooper 1981) of foreign organisms, as well as the stabilisation of disrupted tissue after a physical injury (Franchini and Ottaviani 2000; Velnar et al. 2009), it is hypothesised that corals are likely to possess a suite of effective immune cells. Therefore, investigating the presence and characteristics of immune cells in corals would contribute significantly to the understanding of coral immunity effector responses. Despite the documented diversity in morphology and function of immune cells among phyla (Adamowicz 2005), morphologically similar cells have been described among multiple invertebrate groups and they may share common functions.

There are two main groups of morphologically similar invertebrate immune cells: granular cells, also known as granulocytes, and agranular cells, also known as hyaline cells or hyalinocytes (Ittoop et al. 2006). The majority of these characterised cells are amoebocytic and thus may be referred to as granular or agranular amoebocytes, but this varies within the literature (e.g. Ribeiro and Brehelin 2006). Although morphologically similar, invertebrate granular cells vary in size, biochemical content and sub-cellular structures both between and within species (Porchet Hennere and Vernet 1992) and are documented, for example, within insects (Liag and Iwashita 1996; Pech and Strand 1996; Li et al. 2002), bivalve molluscs (Pipe et al. 1997; Wootton and Pipe 2003; Aladaileh et al. 2007) and crustaceans (Johansson et al. 2000; Allam et al. 2001). A key distinguishing characteristic observed for granular cells is degranulation during phagocytosis (Foley and Cheng 1977; Porchet Hennere and Vernet 1992) and wound healing (Perazzolo and Barracco 1997; Aladaileh et al. 2007; Vafopoulou 2009), whereby controlled exocytosis expels the contents of the cell into the surrounding local environment, which may increase local cytotoxicitiy. *Via* this mechanism, granular cells play a key role in immune responses (Pech and Strand 1996; Johansson et al. 2000; Hillyer et al. 2003; Wootton and Pipe 2003; Aladaileh et al. 2007). Agranular cells, often termed hyaline cells because of their glassy appearance, are also documented within numerous

invertebrates including crustaceans (Bell and Smith 1994; Walton and Smith 1999; Johansson et al. 2000) and bivalve molluscs (Pipe et al. 1997; Wootton and Pipe 2003; Ittoop et al. 2006; Aladaileh et al. 2007). Hyaline cells in molluscs are documented to be smaller than granular cells and generally non-phagocytic (Wootton and Pipe 2003; Aladaileh et al. 2007), however they may be responsible for laying down an extracellular matrix during wound healing (Suzuki and Funakoshi 1992). These documented functional differences in immune cell types are indicated by their biochemical content (Wootton and Pipe 2003). Therefore, establishing the biochemical content of immune cells may provide a way to preliminarily determine cell function that can be applied to the investigation of potential immune cells within corals.

Investigations into the content of immune cells from multiple invertebrates have established that these cells usually contain biochemically active components of innate immunity effector response pathways. For example, the granular and hyaline cells of the bivalve *Scrobicularia plana* demonstrated phenoloxidase (PO) and lysosymal activity (Wootton and Pipe 2003), which respectively represent an active component of the melanin-synthesis pathway (Söderhäll and Smith 1986b) and a suite of bacteriolytic enzymes that aid phagocytosis (Cheng et al. 1975; Canesi et al. 2002). These cytoplasmic enzymatic activities were broadly consistent with the biochemical activities of the three types of immune cells isolated and described for other marine bivalves (Canesi et al. 2002), including the mussel *Mytilus edulis,* which additionally contained reactive oxygen species (ROS; Pipe et al. 1997). Furthermore, lysozymal activity has been documented within immune cells of a tunicate (Cheng et al. 1975) and antioxidant activities have been documented within haemocytes of the shore crab *Carcinus maenas* (Bell and Smith 1994). Also, the presence of melanin granules within both insect (Galko and Krasnow 2004; Bidla et al. 2008) and bivalve haemocytes (Aladaileh et al. 2007) supports the presence of melanin-synthesis pathway activity within multiple invertebrate immune cells (Iwama and Ashida 1986; Asokan et al. 1997; Gollas-Galvan et al. 1999; Jordan and Deaton 2005; Butt and Raftos 2008). Melanin deposits within immune cells demonstrate the direct involvement of proteolytic innate immunity effector responses in cellular components of immunity, including melanisation and encapsulation (Hillyer et al. 2003; Jiravanichpaisal et al. 2006; Cerenius et al. 2008).

In invertebrates with open vascular systems, such as crustaceans or worms, populations of circulating immune cells are ideally located to immediately respond to infection or injury (Persson et al. 1987; Porchet Hennere and Vernet 1992; Johansson et al. 2000; Babcock et al. 2008), however corals do not have a classic vascular system (Gateno et al. 1998). In the absence of circulating haemocytes, anthozoans have been reported to possess "wandering amoebocytes" that patrol the characteristically acellular mesogloea for foreign bodies (Bigger and Hildemann 1982). Amoebocytes are defined as mobile cells that move in an amoeboid fashion *via* the extension of pseudopodia, and thus they usually have an irregular shape (Hutton and Smith 1996; Coteur et al. 2002; Adamowicz 2005).

The accumulation of anthozoan amoebocytes at wound sites demonstrates their direct involvement in immune responses (Metchnikoff 1892). Granular cells have been documented to aggregate around wounds within the anemones *Calliactis parasitica* (Young 1974) and *Anthopleura elegantissima* (Patterson and Landolt 1979) and the gorgonians *Plexaurella fusifera* (Meszaros and Bigger 1999) and *Swifta exserta* (Olano and Bigger 2000). Granular amoebocytes have also been documented to aggregate in the presence of allografts within *S. exserta* (Bigger and Olano 1993) and were observed to aggregate at sites of infection and during temperature stress within *Gorgonia ventalina* (Mydlarz et al. 2008). Granular and hyaline cells, the latter of which contained small non-refractile granules unlike those of other invertebrates (Walton and Smith 1999), have been described from the mesenteries of the anemone *Actinia equina* (Hutton and Smith 1996). Within gorgonians, granular cells are arranged in cell chords within the mesogloea (Meszaros and Bigger 1999) and similarly, amoebocytes have been found in the mesogloea of a sediment-stressed sample of the hard coral *Montastraea cavernosa* (Vargas-Angel et al. 2007).

Putative amoebocytes have been described within the hard coral *Porites compressa* (Domart-Coulon et al. 2006) following observations of chromophore cells infiltrating the calicoblastic tissue layer of skeletal growth anomalies. The term "chromophore" was applied to these cells because they contain an unidentified yellow-brown pigment (Duerden 1903). Additionally, chromophore cells are presumed to be specific to the hard coral genus *Porites* (Duerden 1903; Domart-Coulon et al. 2006). Other than the documentation of amoebocytes (Vargas-Angel et al. 2007) and putative amoebocytes (Domart-Coulon et al. 2006) within two scleractinian species, immune cells of hard corals remain largely uncharacterised.

This may be due to their small size and apparently infrequent occurrence, as proposed by Patterson and Landolt (1979).

In this study, my aim was to determine if immune cells, similar in morphology and function to immune cells in other invertebrates, are present in a range of hard and soft coral species. If found, my specific objective was to use multiple histological techniques to determine the likely roles of immune cells in these species.

3.3 METHODS

3.3.1 MICROSCOPIC STUDIES OF AMOEBOCYTES AND PHAGOCYTOSIS

Live coral tissue of approximately 1 cm^2 was removed from the skeletons of three samples of each of *Acropora millepora, Porites cylindrica, Porites astreoides* and *Seriatopora hystrix,* using an airbrush with filtered seawater (0.25 µm). The Caribbean species *P. astreoides* was collected and processed at Mote Marine Laboratory, Summerland Key, Florida, whereas the other species were collected and processed at Orpheus Island Research Station, in the central Great Barrier Reef (GBR) region, Australia. Approximately 2 ml of tissue slurry were transferred into a 15 ml falcon tube and agitated with a disposable pipette for 1 min. The tissue slurry was centrifuged at room temperature (25 ºC) for 6 min at x1107*g*, and the supernatant removed and discarded. Next, 1 ml of 0.25 µm-filtered seawater was added and the tissue slurry was re-suspended by gentle agitation for 1 min and centrifuged for 3 min at x1107*g* to remove tissue debris. Note that non-enzymatic Cell Dissociation Solution (C5914 Sigma) was also trialled, but not deemed as effective as the described protocol.

One drop of the cell suspension was placed onto each of two cover-slips. One cover-slip was observed immediately for active amoebocyte spreading under a compound Olympus microscope with a camera head attachment, at 60 x magnification and 100 x magnification. The second cover-slip was incubated at room temperature (approximately 25 ºC) for 20 min, rinsed gently with 2 ml of filtered seawater, mounted onto a slide and observed immediately. Additionally, for *A. millepora,* one drop of cell suspension was added to a third cover-slip pre-treated with 10 µl of lipopolysaccharide (LPS)-coated microbeads and 20 µl seawater. For LPS-coated microbeads, 40 µl of 3.0 µm mean particle size latex microbeads (Sigma

LB30) were rinsed in filtered seawater. LPS from *Escherichia coli* (Sigma L2630) was then added (0.5 ml of 5 $\mu g.ml^{-1}$) and incubated at room temperature for 15 min, then stored at 4 ºC.

3.3.2 HISTOLOGICAL INVESTIGATIONS OF IMMUNE CELLS AND PHENOLOXIDASE ACTIVIY

Three samples (~ 1 cm^2) of the corals *P. cylindrica* and *A. millepora* were collected from within Pioneer Bay at Orpheus Island on the Great Barrier Reef (GBR) and fixed in 10% formalin-seawater solution and decalcified progressively in 3% - 10% formic acid. One *P. cylindrica* tissue sample was cut into two equivalent subsamples and one placed into 10 $mmol.l^{-1}$ dopamine hydrochloride to assay PO activity and the other into 10 $mmol.l^{-1}$ phosphate buffer. *P. cylindrica* samples were incubated for 90 min at 37 °C. Samples were then rinsed with distilled water and placed into 1 mol glacial acetic acid in 3% formalin for three hours, rinsed again with distilled water and stored in 70% ethanol. All histological samples were processed overnight in an automated tissue processor and embedded in paraffin wax. Wax blocks were sectioned at 5 µm and then stained with Haematoxylin and Eosin (H+E) stain with the exception of dopamine hydrochloride-treated sections of *P. cylindrica*, which were left unstained. Photographs were taken using an Olympus DP12 dedicated camera head mounted on an Olympus microscope.

3.3.3 INVESTIGATION OF CHROMOPHORE CELLS

Three samples of *P. cylindrica* were collected from Pioneer Bay, Orpheus Island (GBR), fixed in 4% formaldehyde-seawater solution, decalcified progressively in 3%-10% formic acid, rinsed with distilled water and stored in 70% ethanol. Samples were processed overnight in an automated tissue processor and embedded in paraffin wax. Wax blocks were sectioned at 5 µm and sections were stained with either Haematoxylin and Eosin (H+E) or Fontana-Masson melanin stain. In this melanin-specific stain, melanin granules reduce silver nitrate to metallic silver, which results in a histochemical reaction that precipitates black material wherever melanin is located (Porchet Hennere and Vernet 1992). Photographs were taken of the epidermal layer in the free body wall region in longitudinal sections at 20, 60 and 100 x magnification using an Olympus DP12 dedicated camera head mounted on an Olympus microscope.

3.3.4 HISTOLOGICAL QUANTIFICATION OF MELANIN DEPOSITS

Samples of healthy tissue from 15 scleractinian species and 2 alcyonacean corals, were collected from Pioneer Bay, Orpheus Island (GBR) as described in Section I and Table I.I. Samples were fixed in 10% formalin-seawater solution and processed histologically as described above, then stained with Fontana-Masson melanin stain.

Photographs were taken at 40 x magnification of the epidermal layer in the free body wall region of longitudinal sections of coral polyps and coenosarc for each sample using an Olympus DP12 dedicated camera head mounted on an Olympus microscope. Mean surface area of each melanin-containing granular cell was determined in Image J software using the "measure area" tool once the scale had been calibrated with a stage micrometer. Fifteen randomly selected Fontana-Mason stained cells were measured for each coral species.

The mean proportion of melanin in the tissue layers was determined as percentage surface area of melanin in the epidermis and gastrodermis and was calculated using Image J software by converting images to greyscale, and creating a histogram. The proportions of pixels within the selected area that represent melanin were recorded. Pixels that represented melanin were identified by their dark brown or black colouration and were determined separately for each photographed section because of differences in staining. Replicates of five measurements per tissue layer were used, from a minimum of three different photographs. Mean proportions of melanin found in epidermal and gastrodermal cell layers were compared for each species using paired t-tests, where assumptions of normality and homogeneity of variances were met. A Kruskal-Wallis non-parametric test was used to compare the mean total proportions of melanin among coral families.

3.4 RESULTS

3.4.1 MICROSCOPIC OBSERVATIONS OF AMOEBOCYTES AND PHAGOCYTOSIS

3.4.1.1 *Acropora millepora*

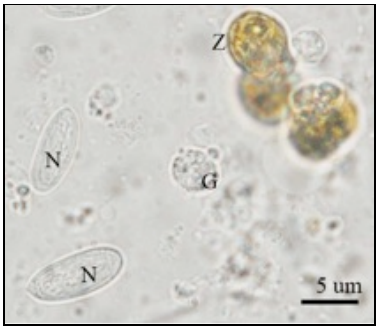


Figure 3.1: Zooxanthellae (Z), cnidae (N) and a round granular cell (G) isolated from *Acropora millepora*.

Cells that adhered to the cover-slip after 20-min tissue-slurry incubations included small round granular cells (Figure 3.1). These were numerous and difficult to distinguish from rounded-up epidermal cells, however, they were identified by the presence of higher densities of granules. Additionally, two types of amoebocytic cells were observed spreading with visibly extending pseudopodia (Figure 3.2a and b). These amoebocytic cells included small hyaline cells (Figure 3.2a), which were approximately 5 µm in diameter and contained non-refractile granules. The larger and more active amoebocytes contained granules of different sizes and colour densities (Figure 3.2b). After 20 min of incubation with LPS-coated microbeads, granular cells appeared to endocytose the beads (Figure 3.2c). This was observed twice and, although suggestive of phagocytosis, further experiments are required for confirmation and quantification of phagocytic potential. Easily identifiable and characteristic coral cells were intact and identifiable on cover-slips of each sample. These included zooxanthellae, which appeared as pigmented round cells approximately 5 to 8 µm in diameter, with a distinguishable cell wall (Figure 3.1). Although transparent because no histological stain was used, cnidae ranging in length from approximately 6 µm to 10 µm were also discernable and intact, although some had ejected their threads.

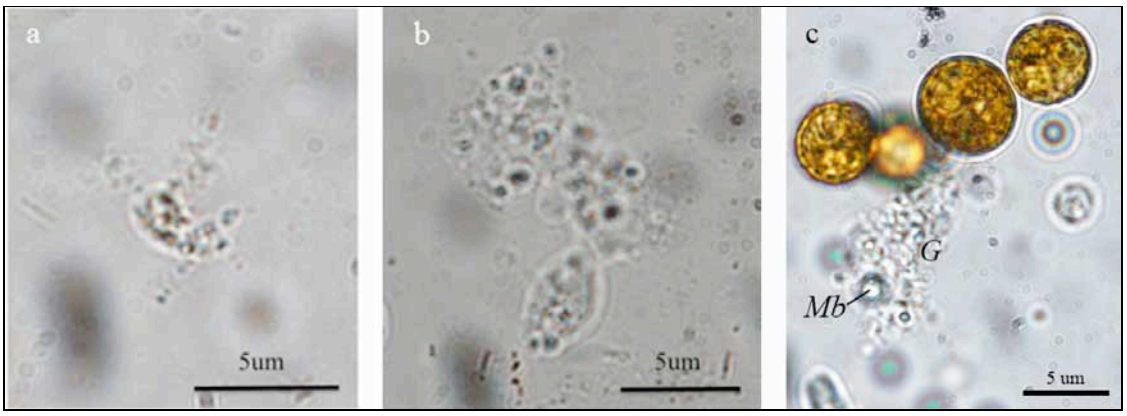


Figure 3.2: Immune cells isolated from *Acropora millepora,* including a) a hyaline cell with small granules, b) a granular amoebocyte, and c) a granular amoebocyte (*G*) phagocytosing an LPS-coated microbead (*Mb*). All cells had discernable pseudopodia and adhered to cover-slips.

3.4.1.2 *Porites cylindrica*

Granular cells (Figure 3.3a) and amoebocytic cells (Figure 3.3b) of *Porites cylindrica* adhered to cover-slips after 20-min tissue-slurry incubations. Granular cells were small (less than 2 µm in diameter), round and densely packed with small granules, some of which appeared to be pigmented. Conversely, amoebocytic cells (Figure 3.3b), identified because of their amoeboid characteristics, such as extending pseudopodia, were larger than the granular cells at approximately 4 to 6 µm in length. The amoebocytes contained larger granules than round granular cells, and they varied in size and colour density. Intact zooxanthellae were observed in each *P. cylindrica* tissue preparation.

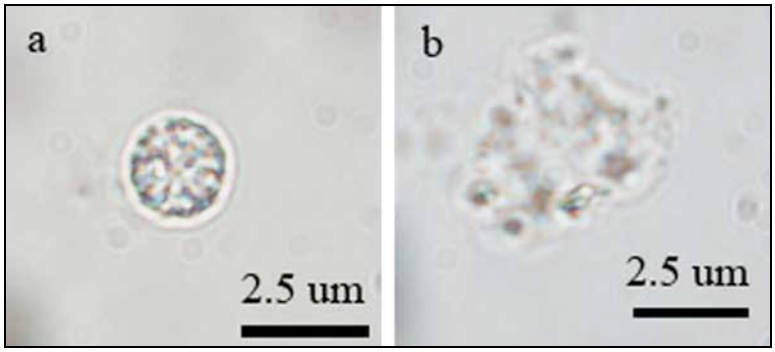


Figure 3.3: Two types of immune cells in *Porites cylindrica* identified from tissue slurry preparations*:* a) a round granular cell, and b) an amoebocyte.

3.4.1.3 *Porites astreoides*

Round chromophore cells of varying diameter (~ 3 µm to 7 µm; Figure 3.4a) were observed in live tissue slurries of *Porites astreoides*. Clear agranular (hyaline) amoebocytic cells adhered to and were observed spreading on the cover-slips (Figure 3.4b). Intact zooxanthellae were also identifiable on cover-slips with *P. astreoides* immune cells (Figure 3.4a).

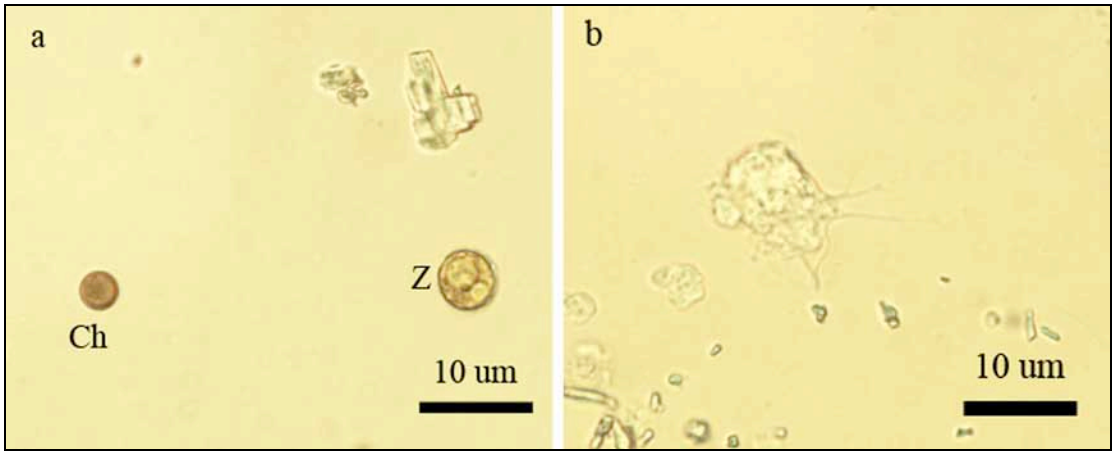


Figure 3.4: Cells found in tissue slurry preparations of *Porites astreoides:* a) an intact zooxanthella (Z) and a chromophore cell (Ch) containing a dense natural pigment, and b) a large amoebocyte with discernable pseudopodia.

3.4.1.4 *Seriatopora hystrix*

Within tissue slurries of *Seriatopora hystrix,* amoebocytic cells were identified by their ability to spread on cover-slips. Amoebocytic cells appeared to be agranular and to be approximately 7 µm in length (Figure 3.5a). Intact cnidae were also observed on cover-slip preparations (Figure 3.5b).

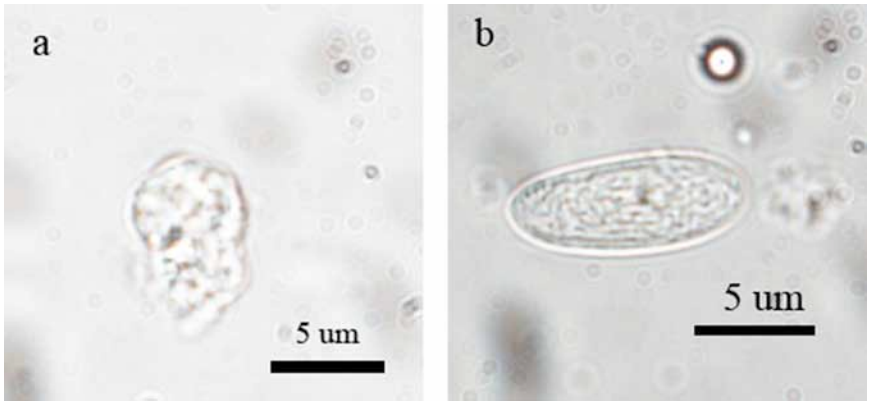


Figure 3.5: Cells isolated from tissue slurries of *Seriatopora hystrix:* a) a hyaline amoebocyte, and b) cnidae.

3.4.2 HISTOLOGICAL INVESTIGATION OF AMOEBOCYTES, GRANULAR CELLS AND PHENOLOXIDASE ACTIVITY

Histological sections of healthy *Acropora millepora* and *Porites cylindrica* tissues that had been stained with haematoxylin and eosin (H+E) demonstrated characteristic cell layers and sub-cellular organelles (Figure 3.6). For both species, the free body wall consisted of an epidermis comprised of columnar cells, an acellular mesogloea, which was characteristically thin, and a gastrodermis containing zooxanthellae that stained pink under H+E (Figure 3.6a and c). High densities of chromophore cells, distinguished by their brown colouration, occurred in the gastrodermal layer of the free body wall of *P. cylindrica* (Figure 3.6c), where cells appeared irregular in shape and packed around other cells such as zooxanthellae. Mesenterial filaments contained a diversity of cells, including putative amoebocytes, which contained blue staining nuclei (Figure 3.6b and d), and pigment cells, which contained large granules that stained bright pink under H+E. Chromophore cells were also apparent in both the mesenteries and mesenterial filaments of *P. cylindrica* (Figure 3.6d). Smooth, oblong, pink-staining structures were also observed, and did not appear to be located within a specific cell layer.

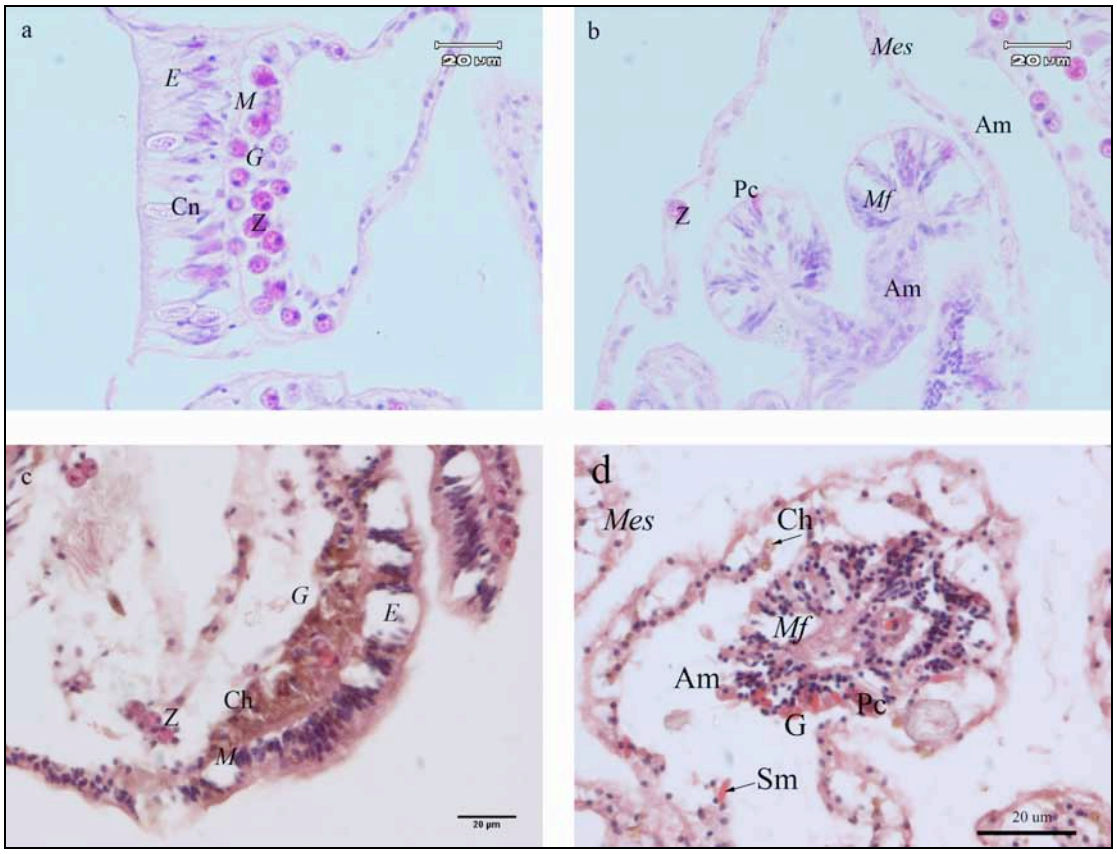


Figure 3.6: Histological sections of healthy tissues stained with haematoxylin and eosin showing: a) cell layers of the free body wall of *A. millepora*, b) mesenterial filaments of *A. millepora,* c) cell layers of the free body wall of *Porites cylindrica;* and d) mesenterial filament of *P. cylindrica*. *E* = epidermis, *M* = mesogloea, *G* = gastrodermis, *Mf* = mesenterial filament, *Mes* = mesentery and Z = zooxanthella, Cn = cnidae, Pc = pigment cell, Am = putative amoebocytes, Ch = chromophore cell and Sm = smooth cell.

3.4.3 HISTOLOGICAL LOCATION OF PHENOLOXIDASE ACTIVITY

The external edge of the free body wall epidermis of *Porites cylindrica* appeared dark brown to black in colouration in unstained tissue sections treated with dopamine hydrochloride (Figure 3.7a), demonstrating phenoloxidase (PO) activity. High densities of chromophore cells, observable as yellow masses due to their natural pigment, were observed in the gastrodermal layer (Figure 3.7a and b), but the lack of dark colouration suggests that they were not sites of PO activity. The calicodermis of the basal tissue layer also appeared brown to black in colouration (Figure 3.7b), as did the smooth oblong cells, which were dispersed throughout the mesenteries and tentacles (Figure 3.7b and c). H+E counter-stained sections of the tentacles (Figure 3.7c) and mesenteries (Figure 3.7d) displayed normal tissue characteristics, including

large cnidae, zooxanthellae and chromophores, as well as smooth oblong cells, which appeared black due to positive PO activity.

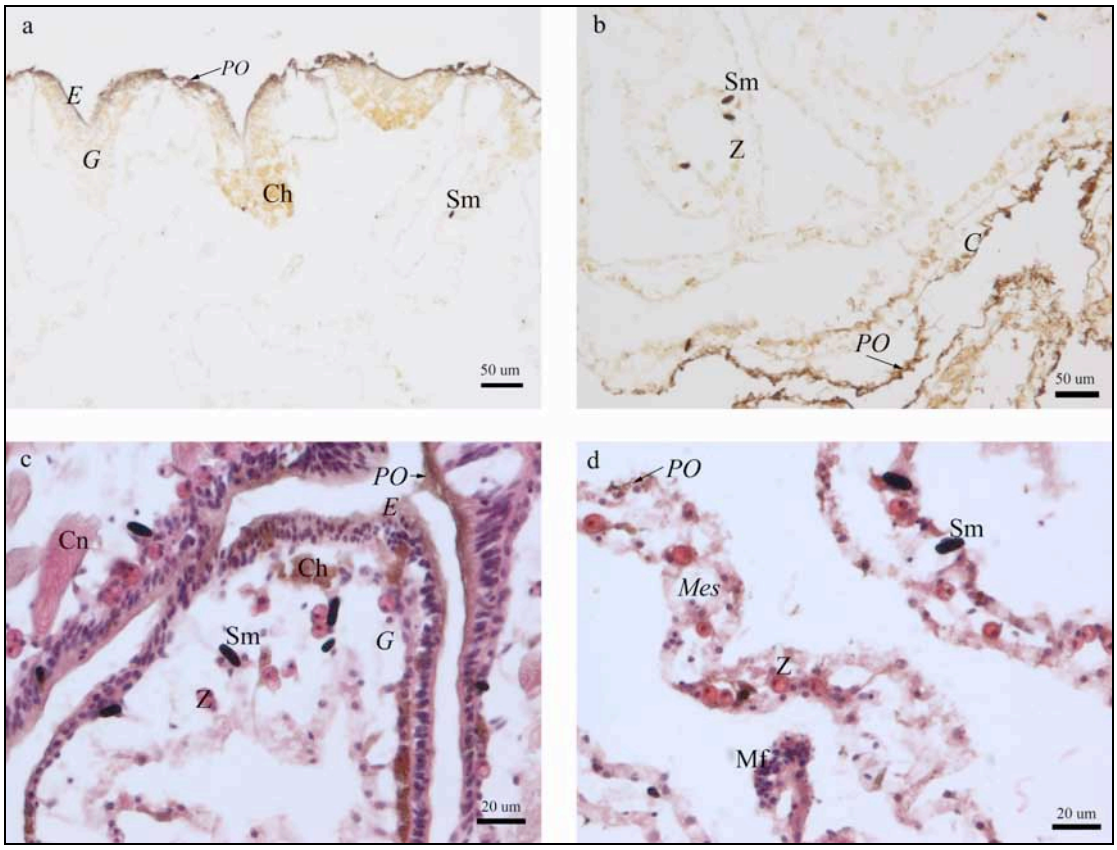


Figure 3.7: Histological sections of healthy *Porites cylindrica* tissues showing dark brown or black coloured structures due to phenoloxidase (PO) activity located in: a) the free body epidermis in unstained tissues; b) calicodermis in unstained tissues; c) smooth oblong cells in the free body epidermal layer in tissues stained with H + E; and d) the mesenteries in tissues stained with H + E. *E* = epidermis, *M* = mesogloea, *G* = gastrodermis, *C* = calicodermis, *Mf* = mesenterial filament, *Mes* = mesentery and Z = zooxanthella, Cn = cnidae, Pc = pigment cell, Am = putative amoebocytes, Ch = chromophore cell, Sm = smooth cell and *PO* = phenoloxidase activity.

3.4.4 INVESTIGATION OF CHROMOPHORE CELLS

Histological investigation of *Porites cylindrica* demonstrated that chromophore cells, identified as golden or brown cells when stained with H+E, were abundant in the free body wall epidermal layer of healthy tissue (Figure 3.8a). Chromophore cells were prevalent within both the epidermis and gastrodermis (Figure 3.8a). All chromophore cells stained black with the Fontana-Masson melanin stain (Figure 3.8b), indicating that they were packed with melanin.

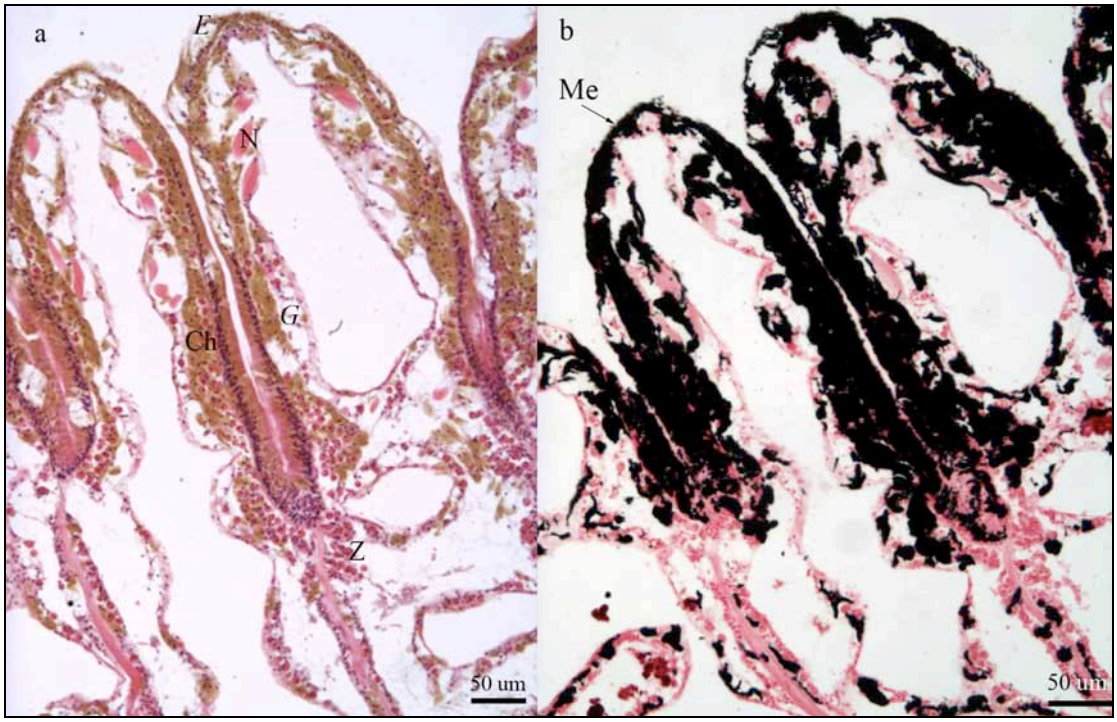


Figure 3.8: Longitudinal histological sections of healthy *Porites cylindrica* tissue showing the free body wall epidermal layer stained with: a) Haematoxylin and Eosin (H+E), and b) Fontana-Masson. *E* = epidermis, *G* = gastrodermis, N = cnidae, Ch = chromophore cell and *Me* = melanin.

3.4.5 HISTOLOGICAL QUANTIFICATION OF MELANIN DEPOSITS IN MULTIPLE CORAL SPECIES

Histological sections of healthy tissues of 15 coral species demonstrated that all routinely contained melanin-containing granular cells, identifiable by their dark brown to black colouration with the Fontana-Masson stain (Figure 3.9). Histological features of tissues in sections of all coral species examined were consistent with a normal healthy status, including normal conformation and appearance of the epidermal and gastrodermal cell layers and diagnostic cells of the free body wall tissues, i.e. nematocysts in the epidermis and zooxanthellae in the gastrodermis. Thicknesses of the two cellular layers and the connecting mesogloea varied between species, as did the distribution and density of melanin-containing granular cells. Melanin-containing granular cells were located predominantly in the epidermis in all species and varied in shape from oval and elongated in *Acropora* sp. (Figure 3.9a) to round in the soft coral family, Alcyoniidae. No melanin-containing granular cells were observed in the mesogloea of any of the scleractinian corals, although they were

present in the mesogloea of both of the Alcyoniidae species investigated (Figure 3.9c).

Mean volume fraction (Vf) of melanin in free body tissues differed significantly among coral families (K-W $_{(7)}$ = 83.56, P < 0.001; Figure 3.10). Mean Vf of melanin was lowest (less than 0.05) in the scleractinian families Pocilloporidae and Acroporidae, and more than seven-fold higher in the Faviidae, which had the highest (approximately 0.37) Vf of melanin. The mean Vf of melanin within both the epidermis and gastrodermis varied significantly between species (K-W $_{(11)}$ = 51.055, P < 0.001; and K-W $_{(11)}$ = 53.33, P < 0.001 respectively; Figure 3.11). For the majority of species, there was a significantly higher Vf of melanin in the epidermis as compared to the gastrodermis, for example in the pocilloporids *P. damicornis* and *S. hystrix* (t $_{(4)}$ = 3.61, P = 0.02 and t $_{(4)}$ = 5.70, P < 0.01), and three of the acroporids: *A. hyacinthus* (t $_{(4)}$ = 3.16, P = 0.03), *A. tenuis* (t $_{(4)}$ = 8.91, P < 0.01) and *M. digitata* (t $_{(4)}$ = 7.133, P < 0.01). Within the faviid *G. aspera,* there was almost two-fold higher Vf of melanin in the epidermis as compared to the gastrodermis, representing the highest proportion of melanin of all the species examined in the study (t $_{(4)}$ = 5.67, P < 0.01). Conversely, also within the Faviidae, *D. heliopora* had approximately two-fold lower Vf of melanin in the epidermis than the gastrodermis (t $_{(4)}$ = 6.51, P < 0.01). Both species examined within the Poritidae had equivalent proportions of melanin in the two cellular layers (t $_{(4)}$ = 1.79, P = 0.149 and t $_{(4)}$ = 1.12, P = 0.329). In addition to differences among species in the distribution of melanin between the two cell layers, the mean area of melanin-containing granular cells also differed significantly among species (K-W $_{(16)}$ = 164.01, P < 0.01). The Pocilloporidae and Acroporidae had the smallest cells (approximately 20 μm^2 and 40 μm^2 respectively), in contrast to the Poritidae and Fungiidae, which had the largest cells (approximately 140 μm^2; Figure 3.12).

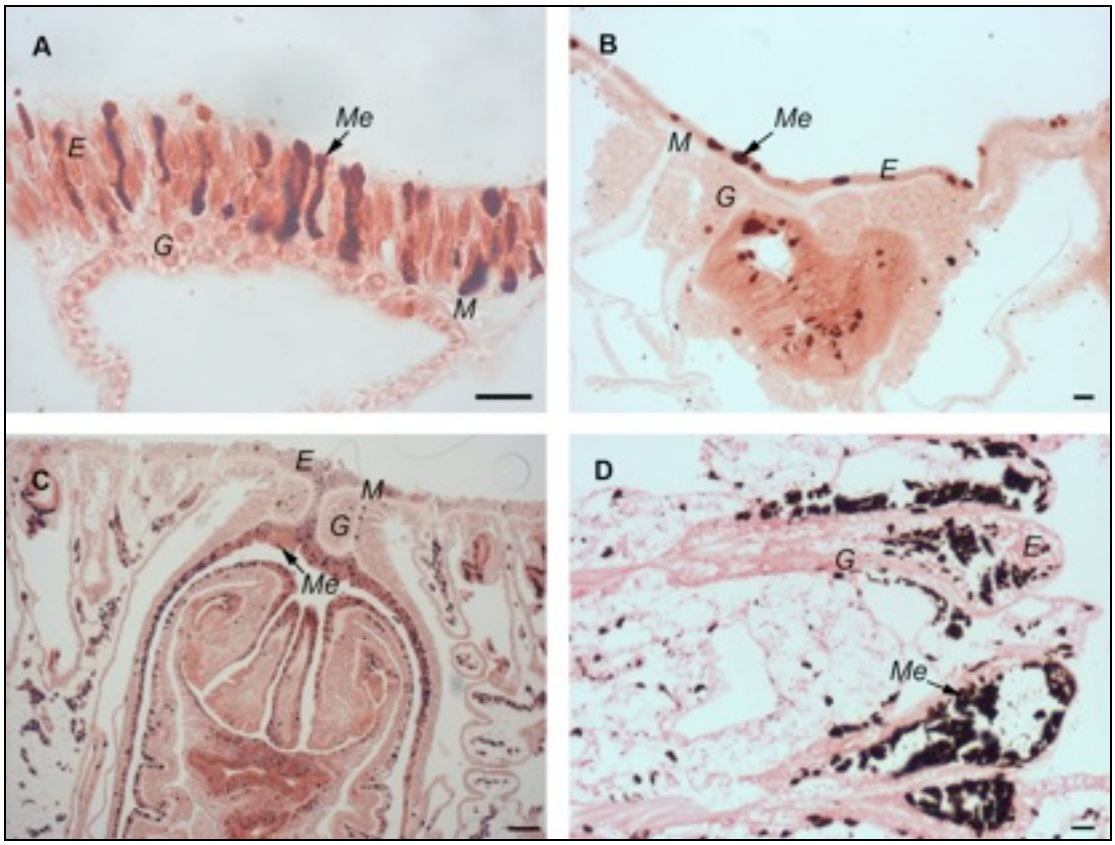


Figure 3.9: Histological sections of free body wall in: A) *A. hyacinthus,* showing extruding dark brown melanin-containing granular cells, which are limited to the epidermis (scale bar: 20 μm); B) *S. hystrix* showing melanin-containing granular cells predominantly in the epidermis (scale bar: 20 μm); C) *Sarcophyton* polyp with dark brown and black melanin-containing granular cells in the epidermis, gastrodermis and mesogloea (scale bar: 50 μm); and D) *Porites cylindrica* polyp with large melanin-containing granular cells in epidermal and gastrodermal cell layers. *E = epithelium, G = gastrodermis, M = mesogloea, Me = melanin.*

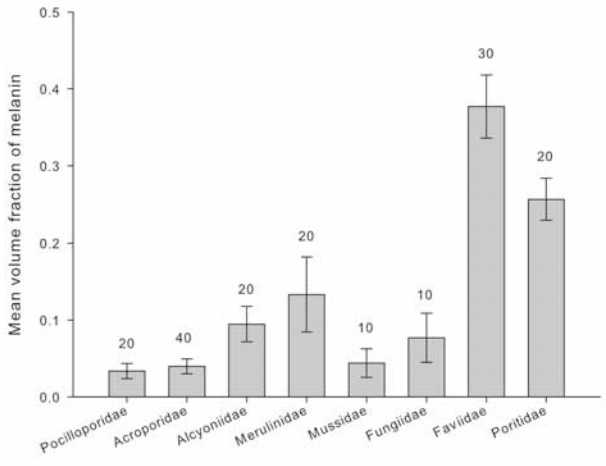


Figure 3.10: Comparison of mean (± SE) volume fraction (Vf) of melanin within the free body wall epidermal layers of a range of scleractinian and alyconacean families (sample sizes indicated above bars).

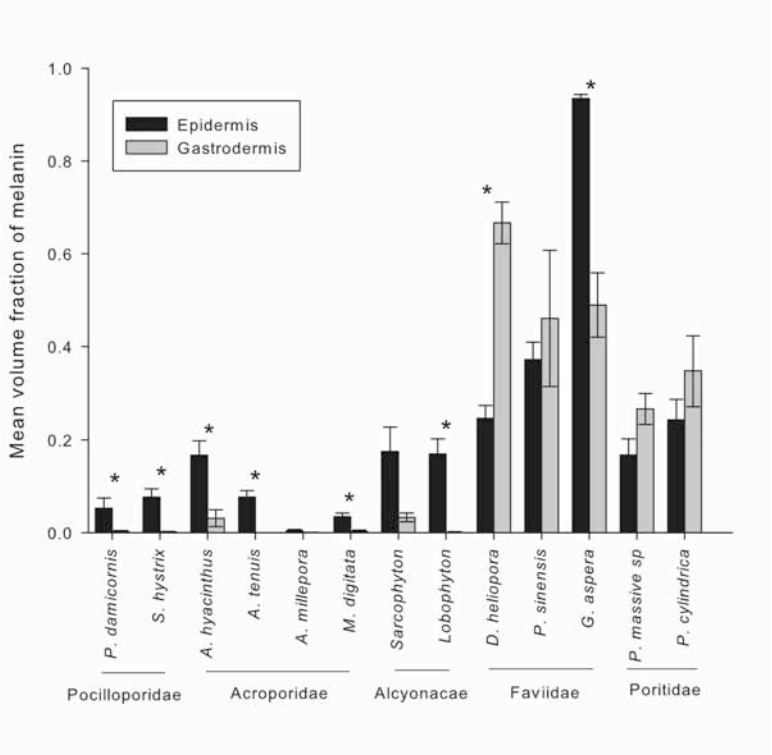


Figure 3.11: Comparison of mean Vf of melanin (± SE) in the epidermis and gastrodermis of representative scleractinian and alcyonacean species (n = 5 samples per species). Asterisks indicate that proportions differ significantly between the epidermis and gastrodermis.

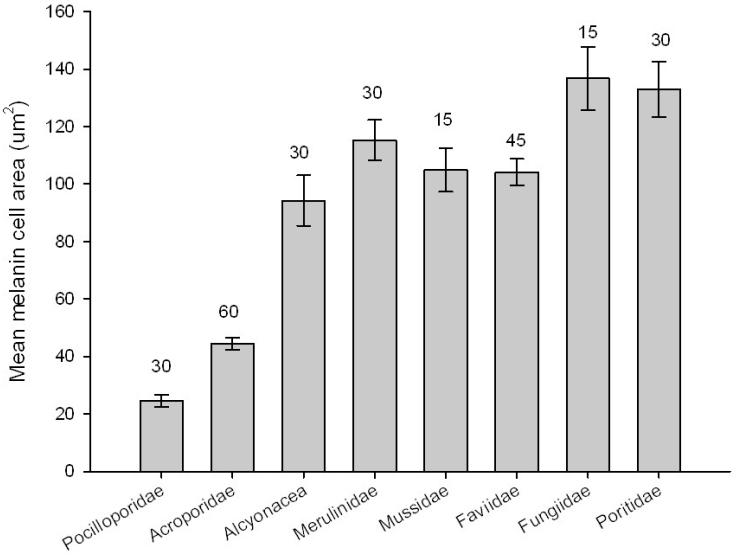


Figure 3.12: Comparison of mean size (cross-sectional surface area) of melanin-containing granular cells (± SE) among seven scleractinian and one alcyonacean families (sample sizes indicated above bars).

3.5 DISCUSSION

This study shows that scleractinian corals have several types of immune cells, which are broadly similar to those previously described for other anthozoans (Young 1974; Hutton and Smith 1996; Meszaros and Bigger 1999; Olano and Bigger 2000). Immune cell types identified here included round granular cells and both granular and hyaline amoebocytes. Phenoloxidase (PO) activity was located within coral tissue layers and in an unidentified cell type. Furthermore, the content of previously described chromophore cells was confirmed to be melanin and such cells were identified within multiple coral species. The presence of melanin and the ubiquity of these melanin-containing granular cells across all anthozoan species investigated strongly suggests that these cells are involved in immunity in these animals.

3.5.1 IDENTIFICATION OF AMOEBOCYTES AND PHAGOCYTOSIS IN CORALS

Hyaline and granule-containing cells with amoeboid characteristics were isolated from four hard coral species, *Acropora millepora, Porites cylindrica, Porites astreoides* and *Seriatopora hystrix*, establishing the presence of these immune cells in hard corals for the first time. These irregularly-shaped immune cells were observed to adhere to glass and to spread using pseudopodia, characteristics that confirm them to be amoebocytes (Hutton and Smith 1996; Coteur et al. 2002; Adamowicz 2005). Observations of granular amoebocytes in these species suggest that these cells are broadly similar to cells documented from tissue squashes of the gorgonian *Swifta exserta* (Olano and Bigger 2000).

Hard coral amoebocytes varied in size among species and their contents varied from being highly granular in *A. millepora* to agranular in *P. astreoides*. This is similar to the diversity of immune cells observed in other invertebrate phyla, such as molluscs (Wootton and Pipe 2003). Within *A. millepora*, the smaller spreading cells were termed hyaline cells because of similarities with those described for the anemone *A. equina* by Hutton and Smith (1996), which also contained numerous small non-refractile granules (Hutton and Smith 1996). However the larger agranular amoebocytes of *P. astreoides* have the more characteristic agranular cytoplasm of hyaline cells documented for other invertebrates, including molluscs (Wootton and Pipe 2003), although they are approximately twice the size of agranular cells documented within *S. plana* (Wootton and Pipe 2003).

Granular amoebocytes of *A. millepora* were observed to phagocytose LPS-coated latex microbeads. This is the first documentation of phagocytosis by a scleractinian coral cell, although further investigations are needed to quantify the phagocytic potential of these immune cells. Phagocytosis has also been documented in other anthozoans, for example by hyaline cells of the anemone *A. equina,* which also demonstrated antibacterial activity (Hutton and Smith 1996), and by colourless cells of *Swifta exserta* (Olano and Bigger 2000).

Round granular cells were isolated from *A. millepora* and *P. cylindrica* and, although smaller in size, were similar to those documented for the anemone *A. equina* (Hutton and Smith 1996). These round granular cells did not visibly extend pseudopodia and given their regular shape, are not characteristically amoebocytic and may not be mobile (Hutton and Smith 1996; Coteur et al. 2002; Adamowicz 2005). Consequently, these cells are considered to be distinct from the granular wound cells documented by Young (1974), which were irregular in shape and larger in size and therefore more similar to the granular amoebocytes observed in all hard coral species in this study. However, round pigment cells from the gorgonian *S. exserta,* although larger than the round granular cells observed within the hard corals, were observed to phagocytose particles (Olano and Bigger 2000). This demonstrates a potential role for non-characteristically amoebocytic cells within anthozoan immunity. However, further studies are needed to conclusively establish the role of round granular cells within scleractinian immune responses.

Tissue slurries used in the current study were composed of multiple, whole coral polyps, unlike the study by Hutton and Smith (1996), and therefore the origin of the cells could not be determined. It was therefore not established whether scleractinian immune cells are stored in the mesogloea of the mesenteries as previously documented for other anthozoans (Young 1974; Hutton and Smith 1996; Vargas-Angel et al. 2007). However, in this study, histological investigations of chromophore cells within multiple anthozoans suggest some fundamental differences in immune cell locations, for example between the epidermis and gastrodermis, within different members of the Anthozoa.

In healthy tissue sections of *A. millepora* and *P. cylindrica*, low numbers of putative amoebocytes were observed in mesenterial filaments, which is consistent with previous reports of anthozoan immune cell storage within these structures (Young 1974; Hutton and Smith 1996). No immune cells were observed within the

mesogloea of either species, which is typically thin in healthy samples of both *A. millepora* and *P. cylindrica,* in contrast to the mesogleoal layer of other anthozoans, and is inconsistent with suggestions that anthozoan amoebocytes patrol the mesogloea (Bigger and Hildemann 1982). Chromophore cells were observed in high abundance within the free body wall layers of *P. cylindrica* and were also observed in mesenterial filaments, although in lower densities and with a differing morphology. Smooth oblong eosinophilic cells were present in the mesenterial filaments and dispersed seemingly randomly throughout the mesenteries and occasionally in the free body wall layers. These cells bore similarities to developing cnidae (Buss et al. 1984; Kass-Simon and Scappaticci 2002), however no other transition phases of cnidae development were observed. The lack of easily discernable immune cells, such as amoebocytes, within histological sections of scleractinian corals is consistent with the small size of immune cells documented in this study and suggestions that their small size is responsible for problems with identification (Patterson and Landolt 1979).

Amoebocytic activity could not be confirmed for putative amoebocytes within mesenterial filaments as their potential migration or spreading could not be directly observed. However, putative amoebocytes bore similarities to the granular amoebocytes previously identified within live tissue slurries of *A. millepora* and *P. cylindrica*, in addition to granular cells of gorgonians (Olano and Bigger 2000; Mydlarz et al. 2008). Furthermore, the pink-stained cytoplasm of the putative amoebocytes under H+E was consistent with cytoplasm staining of granular amoebocytes from the mussel *Mytilus edulis* (Pipe et al. 1997). These molluscan amoebocytes were also demonstrated to have high phagocytic activity (Pipe et al. 1997) and demonstrated enzyme activity associated with immune functions, such as lysozyme and phenoloxidase (PO) activity (Wootton and Pipe 2003). To establish the presence and function of amoebocytic cells within scleractinian corals, investigations of tissues within which an immune response has been activated, such as injured coral tissue, may be more informative as immune cells would be expected to aggregate in high densities.

3.5.2 LOCATION OF PHENOLOXIDASE ACTIVITY IN CORAL TISSUES

Phenoloxidase (PO) activity was located within the free body wall epidermal layer and within the calicodermis of the basal disc within *P. cylindrica*. These two cell layers are the most exposed to external influence; the free body epidermis is

exposed to the water column and the basal body wall epidermis, the calicodermis, is exposed to endoliths, including potential pathogens, within the skeleton. Because of the increased chance of infection and injury in exposed tissue, maintaining levels of PO activity to prevent infection and to rapidly seal wounds would be beneficial (Nappi and Ottaviani 2000; Nappi and Christensen 2005). The location of PO activity and associated cytotoxicity (Nappi and Ottaviani 2000), in the free body wall epidermis may therefore provide an explanation for previously described antimicrobial activity observed in competing corals (Hildemann 1977). Concomitantly, PO activity in the calicodermis may explain why corals are not more frequently infected by organisms that reach the coral tissue through the skeleton.

In addition to PO activity within the exposed tissue layers, the smooth oblong cells that stained pink with H+E, demonstrated high PO activity. This suggests that either these cells are specific immune cells rather than developing cnidae, or that scleractinian cnidae utilise the melanin-synthesis pathway. The latter hypothesis could explain cytotoxicity associated with cnidae (Buss et al. 1984; Kass-Simon and Scappaticci 2002) and supports the presence of antioxidants within nematocysts (Hawkridge et al. 2000), although this needs further investigation. Use of the melanin-synthesis pathway in cnidae would be beneficial during aggressive direct competition, where cnidae are discharged towards the competing organism (Hidaka 1985; Rinkevich and Loya 1985).

3.5.3 CHROMOPHORE CELL CONTENTS IDENTIFIED IN CORALS

Histological investigations of the yellow-brown pigmented (Duerden 1903; Domart-Coulon et al. 2006) contents of chromophore cells of *Porites* spp. confirmed the presence of melanin in high density. This demonstrates for the first time that scleractinian cells are able to synthesise melanin. Therefore, it is likely that scleractinian corals are able to encapsulate foreign organisms, such as fungi, as observed within the gorgonian sea fan *Gorgonia ventalina* (Petes et al. 2003). The presence of melanin within the irregularly shaped granular chromophore cells strongly suggests that they are involved in scleractinian immunity, as described for melanin-containing cells of other invertebrates (e.g. Galko and Krasnow 2004). Granular cells of *G. ventalina* are also documented to contain melanin granules, although within in a heterogenous mix of granule types, including basophilic and acidopilic granules (Mydlarz et al. 2008). This mix of granules within gorgonian

granular cells is therefore distinct from *P. cylindrica* chromophore cells, which were densely packed with melanin granules. Chromophore cells are more consistent with granular cells of the annelid *Nereis diversicolor,* and are comparable to mammalian melanocytes both of which degranulate during an immune response (Porchet Hennere and Vernet 1992). Because of their dense melanin content, chromophore cells will be termed melanin-containing granular cells henceforth.

3.5.4 MELANIN DEPOSITS FOUND IN MULTIPLE CORAL SPECIES

Melanins play a key role in disease resistance in invertebrates (Söderhäll and Cerenius 1998; Cerenius et al. 2008), particularly for antimicrobial defence (Nappi and Ottaviani 2000) and free radical scavenging (Nappi and Christensen 2005), but in addition, they are known to function in desiccation resistance in plants (Laufer et al. 2006) and photoprotection via light absorption in both plants (Mayer and Staples 2002; Laufer et al. 2006) and higher metazoans (Meredith et al. 2006). The presence of melanin-containing granular cells in the free body wall tissues of all species investigated in this study suggests that they fulfil an important physiological role within scleractinian and alcyonacean corals.

The mean volume fraction (Vf) of melanin within free body wall tissues varied among families and, in broad terms, correlates inversely with documented patterns of disease prevalence among scleractinian families. For example, the Acroporidae and Pocilloporidae had the lowest Vf of melanin and correspondingly have been documented to have among the highest disease prevalence on the Great Barrier Reef (GBR; Willis et al. 2004; Page and Willis 2006). The Poritidae, documented as a more tolerant family to disease on the GBR, had the inverse pattern (Willis et al. 2004; Page and Willis 2006). This broad pattern of an inverse relationship between disease susceptibility and melanin content is consistent with other invertebrates, such as in the insects *Spodoptera exempta* (Wilson et al. 2001) and *Tenebrio molitor* (Barnes and Siva-Jothy 2000; Armitage and Siva-Jothy 2005), where quantities of melanin deposits positively correlate with immunocompetence. This correlation is due to the antibacterial properties of melanin deposits (Bull 1970) and the cytotoxicity of melanin pathway intermediates (Armitage and Siva-Jothy 2005; Nappi and Christensen 2005). For corals, it is advantageous to have antimicrobial-melanin within the potentially vulnerable epidermal cell layer for disease resistance. In addition, having UV and visible-light absorbing melanin

(Meredith et al. 2006; Brenner and Hearing 2008) in epidermal cell layers may be an important factor in determining the amount of light available to endosymbiotic algae that reside within the gastrodermis.

In addition to differences in the overall Vf of melanin in coral tissues, taxonomic differences in the distribution of melanin between the epidermal and gastrodermal cell layers highlight a further role for melanin in coral ecophysiology. The higher relative amount of UV and visible-light absorbing melanin deposits (Meredith et al. 2006; Brenner and Hearing 2008) found within the epidermis of shallow, high light-dwelling corals, such as the Acroporidae and Pocilloporidae, is potentially advantageous for limiting bleaching (Baird and Maynard 2008) and endosymbiont photoinhibition (Nishiyama et al. 2006). In further support of a role for melanin deposits as a photoprotector in coral, and therefore a host parameter that might contribute to mitigation of bleaching (Baird et al. 2008), the faviid *Goniastrea aspera*, a reef-flat dweller in the high intertidal zone, had the highest total proportion of melanin in its tissues. *G. aspera*'s more than two-fold greater investment in melanin deposits compared to all other corals in this study and the localisation of the majority of these deposits within the epidermis suggest a need for high investment in the production and maintenance of melanin in the most exposed cell layer. In contrast, *Diploastrea heliopora,* a faviid that thrives in deeper water with lower light levels, had more than a two-fold greater proportion of its melanin deposits located within the lower, gastrodermal layer. Such correspondence between the distribution of melanin within cell layers and the characteristic light environment experienced by the coral species provides further corroborative evidence that melanin functions as a photoprotector.

Amoebocytes, which characteristically aggregate in areas of injury or invasion (Metchnikoff 1892), may be phagocytic and are mobile, usually within the haemocoel of invertebrates (Wootton and Pipe 2003; Butt and Raftos 2008; Cooper 2008). The capacity of corals to control the location of melanin-containing granular cells would be highly advantageous for both bleaching and disease mitigation because of temporal variation in light conditions commonly experienced by corals and general unpredictability of entry points of invading pathogens. Additionally, a capacity to increase melanin-containing granular cells during exposures to high temperatures and light, such as reported for gorgonians during the 2005 bleaching event (Mydlarz et al. 2008), may limit bleaching and may also explain increased bleaching resistance after

prior exposure to high temperatures (Brown et al. 2002; Maynard et al. 2008; Thompson and van Woesik 2009). However, further investigations are required to fully characterise the amoebocytic properties of melanin-containing granular cells.

3.5.5 Conclusion

In summary, cytological investigations of multiple hard coral species indicate the presence of several, not previously described, immune cells that are broadly similar to those described for other invertebrates. The epidermal location of PO activity is consistent with its role in defence, although the PO within unidentified smooth cells needs further investigation. The presence of melanin-containing granular cells within all investigated species establishes the presence of melanin within hard corals for the first time and demonstrates the importance of the melanin-synthesis pathway within scleractinian cellular immunity. Furthermore, the quantification of melanin-containing granular cells enables the quantification of another scleractinian coral effector response, in addition to PO and transglutaminase activity (Chapter 2), which not only contributes to the fundamental knowledge of coral immunity mechanisms, but also to the development of effective measures of immunocompetence.

Chapter 4 VARIATION IN FLUORESCENT PROTEINS AMONG ANTHOZOAN TAXA AND THEIR POTENTIAL AS ANTIOXIDANTS

4.1 ABSTRACT

Reactive oxygen species (ROS) are typically produced as part of normal cell functioning and are readily produced during stress and immune responses, potentially causing self-harm. Consequently, antioxidants are an integral part of all organism defense systems and include proteins with either enzymatic or non-enzymatic modes of action. Enzymatic antioxidants have been investigated within a few coral species, but there have been no studies of non-enzymatic antioxidants, such as pigments, although fluorescent proteins (FPs) have previously been proposed to act as antioxidants. Therefore, the H_2O_2 scavenging rates of four pure scleractinian FPs, cyan (CFP), green (GFP), red (RFP) and chromoprotein (CP), and their mutant counterparts (without chromophores) were investigated. A method for quantifying FPs within the tissues was developed and used to compare emission spectra and total fluorescence among 22 Indo-Pacific and 7 Caribbean anthozoan species. Emission spectra demonstrated high diversity in FP types and concentrations among anthozoan species. There was a significant correlation between H_2O_2 scavenging and total FP concentration within the tissues for both Indo-Pacific and Caribbean species. *In vitro*, each FP type was able to scavenge H_2O_2, the most efficient type being CP, followed by CFP and RFP, which had equivalent activity. Hydrogen peroxide scavenging was significantly higher in the FP mutants, however, implying that scleractinian FPs have roles in addition to scavenging radicals, potentially involving their chromophores. This study documents quantitative variation in anthozoan FPs across a range of taxa for the first time and demonstrates H_2O_2 scavenging of scleractinian FPs *in vitro* and *in vivo*, thereby identifying a novel biochemical characteristic for these proteins. These data support a role for FPs in coral stress and immune responses and differing antioxidant efficiencies among coral species highlight potential multi-functionality of these conspicuous proteins.

4.2 INTRODUCTION

In nature, pigments and colouration patterns are both numerous and diverse, and have an equally diverse set of functions and roles (Protas and Patel 2008). In some organisms, such as snakes and birds, pigment functions include visual stimuli, for example as a warning to predators or to attract conspecifics (Protas and Patel 2008). In other organisms, pigments may have a direct function, such as protection from solar radiation by melanin in mammals (Meredith et al. 2006), as well as indirect functions relating to the by-products of pigment synthesis, as seen in immune resistance associated with melanin synthesis in numerous invertebrates (Roulin 2004; Nappi and Christensen 2005). However, the vivid colouration for which corals are renowned (Dove et al. 2001; Bandaranayake 2006) is caused primarily by fluorescent proteins (FPs) within the host animal tissue (Matz et al. 1999).

Anthozoan FPs fall into four basic colour types: cyan (CFP), green (GFP), red (RFP) and a blue/purple non-fluorescent chromoprotein (CP; Alieva et al. 2008). Analogous to other natural pigments, corals exhibit remarkable variation in the presence, and potentially concentration, of FP types, both inter- and intra-specifically, the latter of which may lead to conspecific colour morphs (Veron 2000; Dove et al. 2001; Matz et al. 2002; Kelmanson and Matz 2003). Additionally, coral FPs have been documented to vary over time within a coral colony (Dove et al. 2006a; Smith-Keune and Dove 2008; Bay et al. 2009) and to vary between locations for conspecifics (Cox et al. 2007; Kao et al. 2007). These studies suggest that anthozoan FPs vary in response to environmental conditions.

There are several hypotheses on the function of FPs within corals that may explain colour variation among coral colonies over space and time. One well-established hypothesis relates to the ability of FPs to convert shorter wavelengths of light into longer wavelengths. This spectral property has led to the suggestion that anthozoan FPs photoprotect zooxanthellae (Kawaguti 1944; Catala 1959; Kawaguti 1969; Salih et al. 1998; Salih et al. 2000). However, light-enhancing roles have also been proposed (Dove et al. 2001). Although the spectral properties of some FPs potentially support a light-enhancing role by providing remitted light at wavelengths that zooxanthllae can potentially use (Mazel et al. 2003; Salih et al. 2004; Cox and Salih 2006), this is not the case for all FPs, for example RFPs (Levy et al. 2003). Other proposed FP functions include their use as visual triggers for other organisms,

such as a warning to predators or to attract prey (Aeby 2002; Ward 2002; Matz et al. 2006). Additionally, oxygen radical quenching properties have been proposed for coral FPs after antioxidant activity was found for the GFP isolated from the jellyfish *Aequorea victoria* (Mazel et al. 2003; Bou-Abdallah et al. 2006).

The potential for anthozoan FPs to function as antioxidants needs further investigation; such a role would provide a direct link between the presence of FPs and the health status of corals. Exposure to reactive oxygen species (ROS) is continuous for aerobic organisms, whether as part of normal cell function, from exogenous sources (Martindale and Holbrook 2002) or during stress responses (Halliwell and Gutteridge 1999; Fang 2004; Lesser 2006; Richier et al. 2006; Weis 2008). Oxygen radicals are readily produced by a number of pathways and mechanisms (Mydlarz and Jacobs 2006), including by algal symbionts (Lesser 1996; Mydlarz and Jacobs 2004), therefore the ability of tissue to quench or decompose ROS is important for zooxanthellate scleractinian corals. The photosynthetic zooxanthellae generate high quantities of dissolved oxygen under normal conditions (Lesser 2006), but during thermal and light stress events, ROS levels are elevated (Lesser 1996; Jones et al. 1998; Lesser 2006), inducing oxidative stress (Martindale and Holbrook 2002) in both the symbiont and the coral host (Tchernov et al. 2004; Lesser 2006). In addition, coral responses to thermal stress (Weis 2008), injury (Meszaros and Bigger 1999; Mydlarz and Jacobs 2004) and infection (Mydlarz and Harvell 2007) also contribute to elevated ROS in host tissues. Of the different ROS, hydrogen peroxide (H_2O_2) particularly induces oxidative stress (Leutenegger et al. 2007a) because it is the most stable of the oxygen species and easily diffuses across biological membranes (Downs et al. 2002; Smith et al. 2005).

To mitigate and regulate ROS cytotoxicity, anthozoans possess a defensive suite of endogenous antioxidant enzymes (Sugumaran and Kanost 1993; Halliwell and Gutteridge 1999; Dunlap et al. 2003), such as superoxide dismutase (Dykens 1984; Hawkridge et al. 2000), which catalyzes the conversion of superoxide anions to water and H_2O_2 (Halliwell and Gutteridge 1999), catalase (Griffin et al. 2006; Leutenegger et al. 2007a; Merle et al. 2007), which catalyses the decomposition of H_2O_2 to water and oxygen (Gordon 1996), and peroxidases, which are oxidant proteins that consume H_2O_2 (Mydlarz and Harvell 2007). In addition, invertebrates, including octocorals, assimilate exogenous antioxidants, such as carotenoid pigments, into their tissue and skeleton from food sources or symbionts (Cvejic et al. 2007;

Leverette et al. 2008). However, these conserved antioxidant pathways can be overwhelmed during extreme temperature stress (Weis 2008), inducing the expulsion of symbionts (bleaching) (Lesser 1996) and self-damage during pathogen infections (Halliwell and Gutteridge 1999). Thus there may be a role for local proteins, potentially including FPs, as supplemental scavengers of ROS.

Variation in FP types has been documented among some coral phyla (Gruber et al. 2008) and also among different coral colour morphs (Dove et al. 2006b). However, in order to better understand the ecological relevance of observed FP variation, a comprehensive and quantitative study across multiple anthozoan species is required. If coral FPs are shown to be involved in mitigating bleaching and reducing self-harm during immune responses, quantitative investigations of coral FPs may provide insights into factors contributing to species-specific patterns of coral decline.

Current methods of investigating coral FPs are primarily observational, making accurate and quantifiable inter-specific comparisons of FP concentration difficult. These methods include observational assessment using UV lamps (Wiedenmann et al. 2004), photography (Gruber et al. 2008), a microspectrofluorometer with an epifluorescent microscope (Mazel 1995), and *in situ* spectrometry using an optical fiber and a blue light source, as described by Matz et al. (2006). The latter method makes comparisons among species difficult, as the skeletal structure, and therefore light emitted, varies considerably among phyla and may compromise the detection of variation in FPs among coral species. Additionally, quantification of FP gene expression has been used in comparative studies within species (Smith-Keune and Dove 2008; Bay et al. 2009), however the relationship between gene expression and FP production, activity and/or concentration has yet to be determined. Therefore an efficient, accurate, quantitative and comparable measure of coral FPs needs to be developed[1].

[1] The protocol developed as part of this chapter to enable coral fluorescent proteins to be standardised and quantitatively compared has recently been used in the following article: Bay LK, Ulstrup KE, Nielsen HB, Jarmer H, Goffard N, Willis BL, Miller DJ, Van Oppen MJH (2009) Microarray analysis reveals transcriptional plasticity in the reef building coral *Acropora millepora*. Molecular Ecology 18:3062-3075

In this study, my aims were to determine if pure coral FPs and coral tissue extracts demonstrate antioxidant properties and to develop a standardised method to quantitatively investigate FPs within coral tissues. If successful, my specific objective was to utilise this new protocol to compare the types and concentrations of FPs present among several Indo-Pacific anthozoans and Caribbean hard corals.

4.3 METHODS

4.3.1 SAMPLING AND TISSUE EXTRACTION PROTOCOLS

Samples from five colonies of each of 22 Indo-Pacific and three colonies of 7 Caribbean anthozoan species were collected and protein extracted, as described in Section I.I, of the species listed in Tables I.I and I.II. The samples from each of the geographical areas (Indo-Pacific and Caribbean) were collected from the same location, at the same time and from apparently healthy colonies, thus enabling inter-specific comparisons within each reef region (further details provided in Section I.I).

4.3.2 H_2O_2 SCAVENGING OF PURE FLUORESCENT PROTEINS *IN VITRO*

Four pure (wild-type) fluorescent proteins (FPs) were isolated and purified from *A. millepora,* and their corresponding mutants (without chromophores) were created by C. Modi and provided by M. V. Matz (University of Texas, Austin. U.S.A), who also supplied buffers used as procedural controls during these protocols. FPs were diluted to 2.5 $mg.ml^{-1}$ protein in phosphate buffer (pH 7.8, 50 $mmol.l^{-1}$) and 10 µl aliquots of each were added to triplicate wells of a 96-well UV-transparent microtitre plate. To each well, 40 µl of phosphate buffer (pH 7.0, 0.05 $mol.l^{-1}$) and 50 µl of 100 $mmol.l^{-1}$ H_2O_2 were added and the absorbance at 240 nm read immediately and every 31 s for ~1.5 min. Sample blanks randomly located throughout the microtitre plate were used to control for independent sample effects and samples were standardised to a H_2O_2 standard curve using a serial dilution from 50 $mmol.l^{-1}$ to 3.125 $mmol.l^{-1}$. In addition to pure FPs, their mutants and procedural controls, a range of common proteins including BSA, lysosyme and catalase were analysed to compare the efficiency of FP H_2O_2 scavenging. The significance of the relationship between H_2O_2 scavenging activity and protein concentration as well as differences between the wild-type FPs rate of H_2O_2 scavenging was compared among samples using a one-way ANCOVA, as assumptions of normality and variance were met.

4.3.3 STANDARDISATION OF FLUORESCENT PROTEINS

To determine whether fluorescence could be standardized to protein concentration, a dose response curve was created using pooled sample extracts from colonies (n = 5) of a massive coral species, *Porites* sp. From this pooled sample, 0 µl to 50 µl, in increments of 10 µl, were added to triplicate wells of a 384-black/clear microtitre plate. Phosphate buffer (50 mmol.l^{-1}, pH 7.8) was added to each well so that the total volume was 50 µl. Each well was excited at 280 nm and the relative fluorescence emission recorded. To determine if mean total fluorescence correlated with sample extract volumes, an analysis of variance of regression (May and Bigelow 2005) was conducted using Sigma Plot (10.0), as assumptions of normality and variance were met (Shapiro-Wilk's and Levene's tests, respectively).

As the proportion of buffer to sample extract varied among samples, a protocol was developed to remove this background fluorescence from the emission spectra of each species, to enable direct comparisons among samples. For each spectral emission, an exponential regression was used to fit the baseline fluorescence due to addition of buffer, and this was subtracted from the fluorescence emission of the sample.

4.3.4 CORAL SPECTRAL EMISSION

To obtain fluorescence emission spectra for the anthozoan tissues examined, aliquots of 30 µl of each sample were added to triplicate wells in a black/clear 384 well microtitre plate with parallel aliquots of extraction buffer to control for independent effects. Each well was excited at 280 nm, using a spectrophotometer (SpectraMax M2, Molecular Devices). This wavelength was used as wavelengths in the UV range are optimal for exciting FPs across the spectrum, whilst 280 nm is far enough away from the emission peak of the cyan FP to avoid cross over of the excitation and emission peaks (Shagin et al. 2004). Emission spectra were measured in 5 nm increments from 400 to 650 nm. The fluorescence of each sample was standardised to the sample's total protein concentration as determined by the Quick Start Bradford protein assay (Bio-Rad). The mean total fluorescence per mg protein for each sample was calculated by summing the standardised relative fluorescence units (RFUs) between 465 and 600 nm, which eliminated fluorescent emission from the zooxanthellae and further limited cross over with the emission peak, and a mean was then taken of total fluorescence for each species. The mean fluorescence of each

FP was calculated by summing across the relevant wavelengths, i.e. 465 to 500 nm for the cyan FP, 505 to 550 nm for the green FP and 555 to 600 nm for the red FP (Shagin et al. 2004; Alieva et al. 2008), and taking a mean for each sample (n = 5), for each species. Actual values of mean total fluorescence were plotted for Indo-Pacific species, but for clearness of graphical representation, emission spectra were plotted as a proportion of the highest fluorescence for Caribbean species (Sigma Plot 10.0). Caribbean species were compared statistically using log-transformed data, using a one-way ANOVA and Tukey's HSD *post hoc* tests, as assumptions of normality and homogeneity of variance were met for the log-transformed data. The mean total fluorescence was statistically compared among Indo-Pacific species using a Kruskal-Wallis non-parametric test, as assumptions of parametric analyses were not met and no suitable transformation could be found. To assess similarities among the coral species, Principal Components Analysis (PCA) was conducted on the mean of each FP (cyan, green and red) for both Caribbean and Indo-Pacific species, the latter of which was log-transformed beforehand. Statistical tests were conducted using SPSS 17.0 and PRIMER 6.1, and graphs were plotted using Sigma Plot 11.0.

4.3.5 ANTIOXIDANT ACTIVITY

Peroxidase activity of each of the samples collected for the 22 Indo-Pacific and 7 Caribbean species was measured in 96-well microtitre plates using 10 µl sample extract with 35 µl phosphate buffer (10 mmol.l^{-1}, pH 6.0) and 40 µl of guaiacol (Sigma G550225 mmol.l^{-1}), with the addition of 25 µl of hydrogen peroxide (H_2O_2; 20 mmol.l^{-1}) to activate the assay (Mydlarz and Harvell 2007). Absorbance at 470 nm was recorded for 45 min and the change in absorbance over time was calculated. A non-parametric Kruskal-Wallis test was used to statistically compare the mean peroxidase activity among coral families. To determine H_2O_2 scavenging activity of anthozoan tissue extracts, 20 µl aliquots of each sample extract were added to triplicate wells of a 96-well UV transparent microtitre plate (Costar). To each well, 30 µl of phosphate buffer (pH 7.0, 0.05 mol.l^{-1}) and 50 µl of 50 mmol.l^{-1} H_2O_2 were added and the absorbance at 240 nm read immediately and every 31 s for 8 min. Sample blanks were used to control for independent sample effects. For Indo-Pacific coral species, the change in absorbance over time was calculated, whereas for the Caribbean coral species, the mean mmol.l^{-1} H_2O_2 scavenged was calculated by subtracting the final absorbance from the initial absorbance and relating it back to the

mmol.l^{-1} of H_2O_2 using a standard curve (serial dilution from 50 mmol.l^{-1} to 3.125 mmol.l^{-1}) run on the same plate, to give an indication of the actual amount of H_2O_2 scavenged by hard corals. For all samples, scavenging activity was normalised to mg protein as determined from a Bradford Quick-start assay (Bio-Rad).

For the Indo-Pacific coral species, the correlation between mean scavenging activity and total fluorescence was analysed using an analysis of variance of regression in Sigma Plot (11.0). For Caribbean corals, the mean scavenging rate was compared among species using a one-way ANOVA and Tukey's HSD *post-hoc* tests. The correlation between the rate of H_2O_2 scavenging and the relative proportion of the summed standardised fluorescence for each colour was analysed using an analysis of variance of regression in Sigma Plot (11.0).

4.4 RESULTS

4.4.1 H_2O_2 SCAVENGING BY PURE FLUORESCENT PROTEINS *IN VITRO*

Overall, the pure (wild-type) FPs demonstrated significant dose-dependent H_2O_2 scavenging activity ($F_{(1, 11)} = 36.5$, $P < 0.001$; Figure 4.1a), which differed significantly among the FP types ($F_{(3, 11)} = 31.4$, $P < 0.001$), with CP scavenging the highest amount of H_2O_2 at each concentration, followed by CFP. At the highest FP concentration tested, GFP had the lowest scavenging activity. FP mutants scavenged H_2O_2 (Figure 4.1b) up to five-fold more than their wild type counterparts ($F_{(5)} = 34.4$, $P < 0.001$), with no significant difference among the three FP types ($P = 0.17$). Procedural controls of buffers did not demonstrate any scavenging activity. Of the common proteins investigated (Figure 4.2), catalase was by far the most efficient H_2O_2 scavenger, even when tested at a lower concentration than that used for the other proteins. GFP was the only other protein to demonstrate H_2O_2 scavenging. The amount of H_2O_2 within wells with BSA and lysozyme did not decrease over time and neither did the control of pure H_2O_2, indicating that there were no independent effects on H_2O_2.

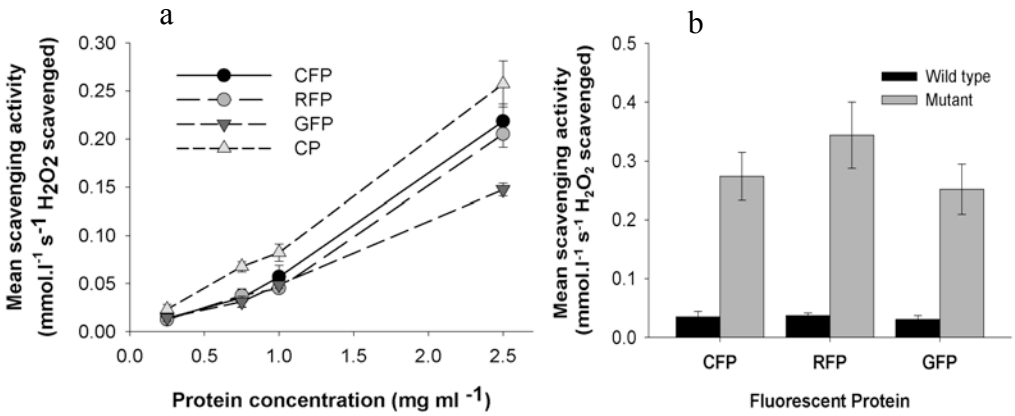


Figure 4.1: Mean hydrogen peroxide scavenging activity (± SE) for: a) each pure (wild-type) fluorescent protein (FP) extracted from *Acropora millepora* at a series of total protein concentrations (ANCOVA $F_{(3, 11)} = 31.4$, $P < 0.001$), and b) each FP wild-type and the corresponding mutant per mg of protein (wild-type compared to mutant $F_{(5)} = 34.4$, $P < 0.001$). n = 3 samples for each wild-type and mutant FP.

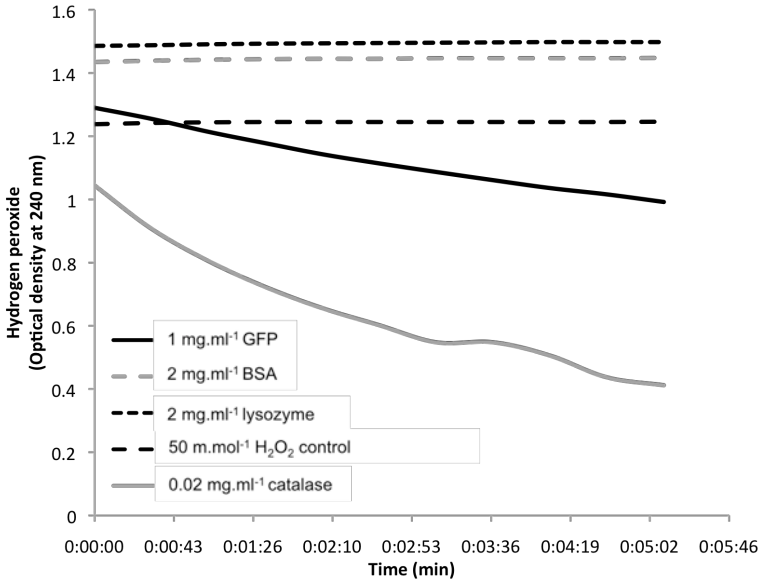


Figure 4.2: The hydrogen peroxide scavenging activity of several proteins, including pure GFP and pure catalase.

4.4.2 STANDARDISATION OF CORAL FLUORESCENT PROTEINS

For the pooled extracts of *Porites cylindrica,* there was a significant regression between sample volume (hence protein concentration) and relative fluorescence, with the relationship explaining 96% of the variation in fluoresence ($R^2 = 0.96$, $F_{(1, 5)} = 86.8$ $P < 0.001$; Figure 4.3). When 50 µl of phosphate buffer was tested without the addition of sample, a relative fluorescence of approximately 20% of the pure sample extract (50 µl) was observed.

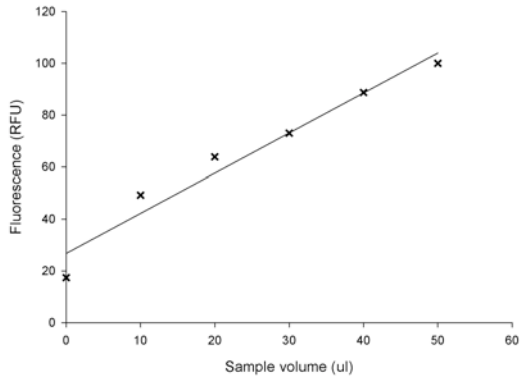


Figure 4.3: The relative fluorescence emission of pooled *Porites cylindrica* samples at different volumes ($R^2 = 0.956$, $F_{(1, 5)} = 86.82$ $P < 0.001$)

4.4.3 FLUORESCENCE OF INDO-PACIFIC ANTHOZOANS

Spectral emissions differed among the 22 Indo-Pacific species investigated (Figure 4.4), with inter-specific variation in both the presence and concentration of each FP colour type, as inferred from the emission spectra. Overall, species within the Pocilliporidae, Alcyonaciidae, Euphyllidae and three of the four acroporids had the lowest total spectral emission, the peaks of which were predominantly within the GFP range of wavelengths (Figure 4.4a). Indo-Pacific species of the families Sphenopidae, Oculinidae and Faviidae had fluorescence emissions of the greatest magnitude, with most spectra peaking primarily within the CFP range of wavelengths (Figure 4.4c).

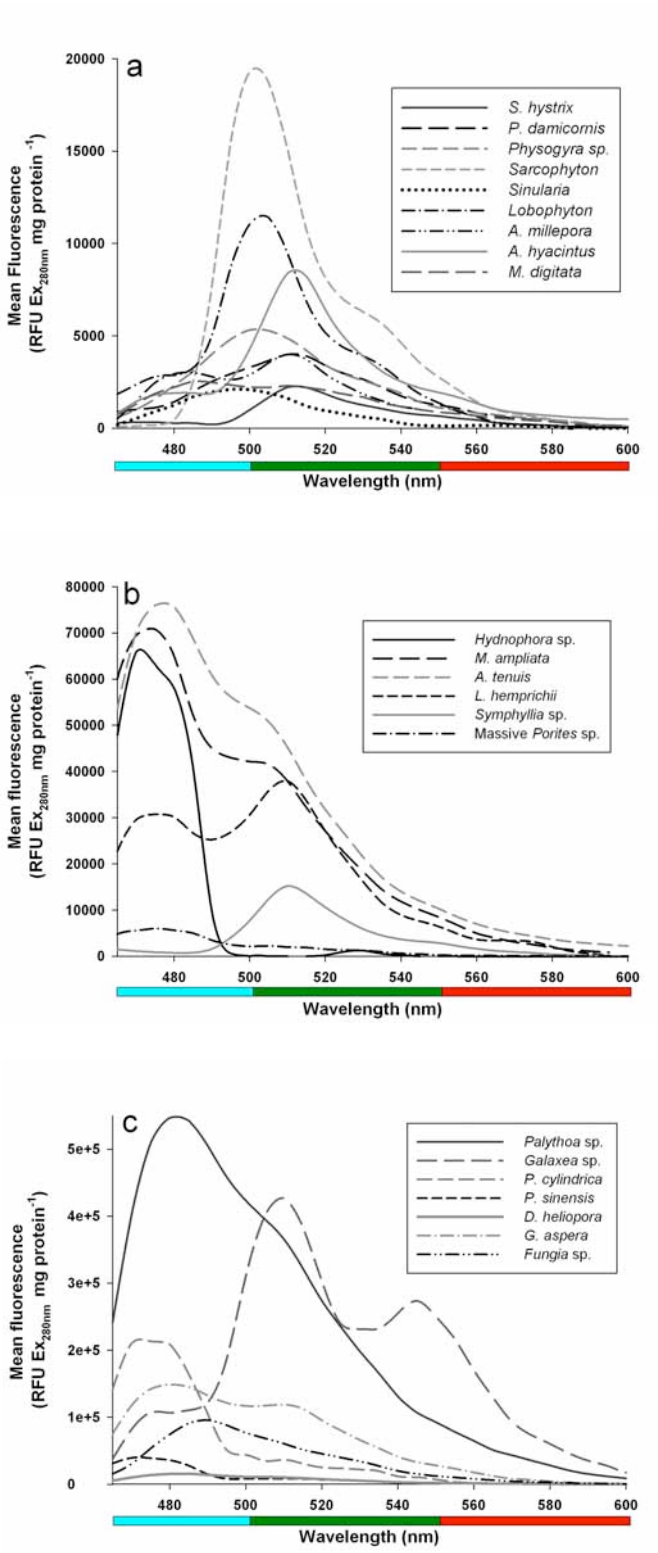


Figure 4.4: The mean fluorescence emission spectra (RFU Ex. $_{280\ nm}$ mg protein^{-1}) for 22 anthozoan species grouped by total emission spectra magnitude: a) up to 20,000 RFU including the families Pocilloporidae, Alcyonaciidae, some Acroporidae and Euphyllidae, b) up to 80,000 RFU including the families Merulinidae, Mussidae, a poritid and an acroporid, c) up to 500,000 RFU including the families Faviidae, Oculinidae, Fungiidae, Poritidae and Sphenopidae. (N = 5)

Mean total fluorescence varied significantly among the Indo-Pacific species tested (Table 4.1; $\chi^2_{(21)} = 75.7$, $P < 0.001$), with the zoanthid *Palythoa* sp. (F: Sphenopidae) demonstrating the highest total fluorescence, followed by the scleractinian corals *Galaxea* sp. (F: Oculinidae), *G. aspera* (F: Faviidae) and *P. cylindrica* (F: Poritidae). The pocilloporid coral *S. hystrix* had the lowest total fluorescence, with <2% of the fluorescence found within the zoanthid (Sphenopidae) and the oculinid coral *Galaxea* sp.. The euphyllid *Physogyra* sp. and the other pocilloporid investigated, *P. damicornis,* also demonstrated low total fluorescence (Table 4.1). Among the Faviidae, the total fluorescence of *G. aspera* was 11-fold higher than that of *D. heliopora*.

The Principal Components Analysis (PCA) plot of PC scores demonstrates similarities in FP signatures, comprised of comparative emissions across each of the three FP colour wavelengths, as opposed to total FP emission levels, among the Indo-Pacific species (Figure 4.5). As such, the FP signatures of *Palythoa* sp. and *Galaxea* sp. were distinct from those of the other Indo-Pacific species investigated (Figure 4.5). Similarly, a central cluster of species belonging to a heterogeneous group of families, including Mussidae, Acroporidae, Fungiidae, Faviidae and Merulinidae, demonstrated similar FP signatures. Overall there was a limited relationship between composite FPs and anthozoan family. PC1 appeared to represent combined FP concentrations of CFPs, GFPs and RFPs, whereas PC2 was a contrast between CFP and GFP/RFP (Figure 4.5). Therefore, *Palythoa* sp. had the overall highest FP concentration, whereas *S. hystrix* had the lowest (PC1; eigenvalue 86.5%). The FP signature of *Symphyllia* sp. was GFP/RFP dominated, whereas *Hydnophora* sp. was CFP dominated (PC2; eigenvalue 11.1%).

Table 4.1: Mean fluorescence (RFU $Ex._{280\ nm}$ mg $protein^{-1}$) of CFPs, GFPs and RFPs for each Indo-Pacific species, inferred from the emission spectra by summing across the 465 to 500, 505 to 550 and 555 to 600 nm wavelength emissions, respectively. Mean total fluorescence represents the emission spectra summed across 465 nm to 600 nm.

Species		Total	Cyan	Green	Red
S. hystrix	***Mean***	**19,543.8**	**3,108.6**	**1,671.2**	**2,840.9**
	Std. Err.	6,474.9	1,995.0	564.5	919.4
P. damicornis	***Mean***	**47,744.9**	**15,242.7**	**3,663.8**	**5,796.3**
	Std. Err.	7,965.8	3,905.7	376.8	1,304.7
P. cylindrica	***Mean***	**1,441,976.3**	**1,138,900.7**	**34,702.5**	**24,817.3**
	Std. Err.	420,021.7	371,728.7	7,030.8	8,835.1
***massive Porites* sp.**	***Mean***	**48,472.1**	**43,828.4**	**15,333.5**	**1,428.1**
	Std. Err.	30,614.3	30,932.6	8,718.5	1,428.1
A. tenuis	***Mean***	**856,202.8**	**492,719.1**	**298,615.2**	**64,868.5**
	Std. Err.	80,652.1	52,522.7	22,850.9	5,909.8
A. millepora	***Mean***	**58,751.7**	**3,641.8**	**13,209.6**	**5,295.0**
	Std. Err.	20,737.0	2,298.1	1,919.4	2,918.4
A. hyacinthus	***Mean***	**73,239.2**	**16,686.9**	**46,118.5**	**10,433.9**
	Std. Err.	24,686.6	3,446.9	20,195.6	3,614.4
M. digitata	***Mean***	**36,442.9**	**15,723.4**	**15,843.1**	**4,876.4**
	Std. Err.	3,454.5	1,429.2	1,834.5	546.2
***Hydnophora* sp.**	***Mean***	**289,790.9**	**286,044.8**	**3,303.4**	**442.6**
	Std. Err.	90,585.4	88,112.7	3,275.7	296.5
M. ampliata	***Mean***	**738,467.4**	**445,771.9**	**257,929.8**	**44,771.5**
	Std. Err.	514,584.9	298,700.4	195,874.5	34,311.9
L. hemprichii	***Mean***	**462,680.6**	**203,362.3**	**230,726.3**	**36,166.8**
	Std. Err.	92,795.3	52,446.0	53,365.7	6,818.4
Symphillia sp.	***Mean***	**126,556.9**	**10,530.2**	**142,833.9**	**19,000.8**
	Std. Err.	41,340.2	641.4	43,274.8	3,934.5
P. sinensis	***Mean***	**270,037.0**	**205,531.9**	**53,787.9**	**10,717.2**
	Std. Err.	102,335.5	40,497.6	52,922.6	10,717.2
D. heliopora	***Mean***	**168,938.6**	**98,839.1**	**60,252.7**	**9,846.9**
	Std. Err.	73,939.1	42,177.9	27,474.0	4,679.2
G. aspera	***Mean***	**1,827,268.0**	**536,291.7**	**517,206.0**	**78,158.4**
	Std. Err.	632,818.9	285,127.8	249,573.5	37,230.8
***Galaxea* sp.**	***Mean***	**2,363,158.2**	**1,966,269.1**	**3,238,670.3**	**1,099,455.2**
	Std. Err.	1,088,360.0	891,531.6	1,485,733.2	772,903.2
***Physogyra* sp.**	***Mean***	**57,131.7**	**27,447.3**	**24,652.9**	**5,031.5**
	Std. Err.	10,154.2	3,859.6	5,132.4	1,365.0

Species		Total	Cyan	Green	Red
***Fungia* sp.**	***Mean***	**943,896.3**	**526,227.0**	**363,828.0**	**53,841.3**
	Std. Err.	460,109.1	255,204.2	179,065.1	25,891.2
***Sarcophyton* sp.**	***Mean***	**135,617.9**	**55,433.4**	**97,864.4**	**12,611.1**
	Std. Err.	369,24.6	13,616.8	24,085.4	3,364.7
***Sinularia* sp.**	***Mean***	**24,501.3**	**13,408.4**	**9,964.1**	**2,257.6**
	Std. Err.	13,337.6	6,331.2	6,519.1	1,596.3
***Lobophyton* sp.**	***Mean***	**90,858.9**	**37,912.2**	**48,953.9**	**4,991.0**
	Std. Err.	20,444.5	9,337.5	10,006.4	2,346.5
***Palythoa* sp.**	***Mean***	**2,134,193.5**	**7,469,299.5**	**4,500,316.8**	**929,009.8**
	Std. Err.	312,229.0	3,657,019.0	2,182,832.4	455,366.0

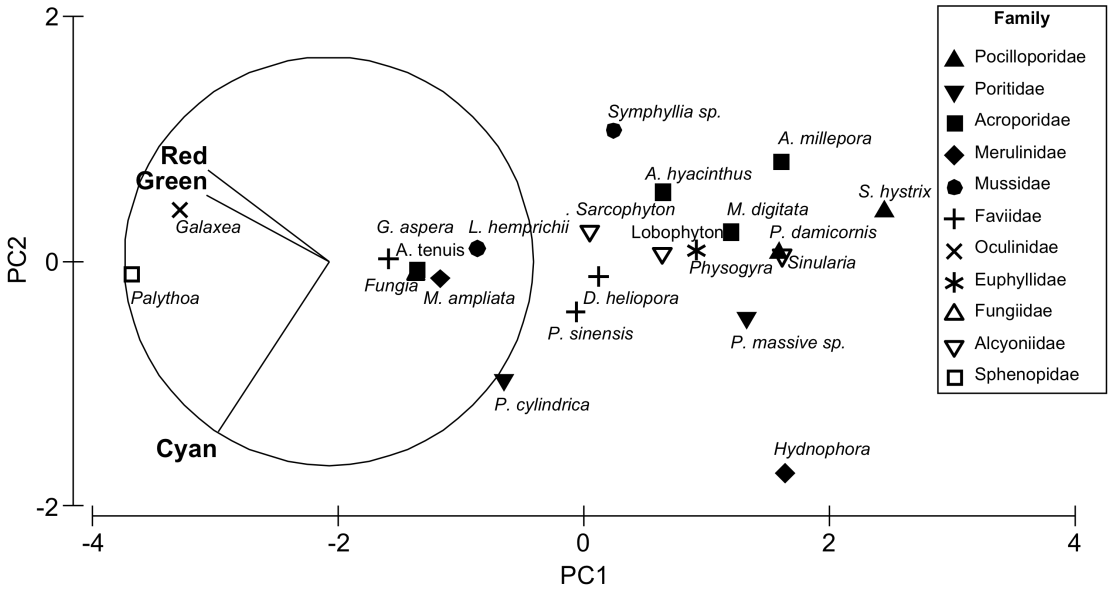


Figure 4.5: Principal Components Analysis of cyan, green and red FPs for each of the twenty-two Indo-Pacific anthozoan species.

4.4.4 ANTIOXIDANT ACTIVITY OF INDO-PACIFIC ANTHOZOANS

Each Indo-Pacific coral family demonstrated peroxidase activity, but the range of activity varied by more than 15-fold among them (Figure 4.6; K-W test $_{(9)}$ = 32.3, P <0.001). The lowest activity mg protein^{-1} was found for the hard coral families Acroporidae and Pocilloporidae and the soft coral family Alcyoniidae. The Merulinidae and Oculinidae had the highest mean activity, with between 7- and 17-fold greater peroxidase activity than the Acroporidae, but these families also had the highest variance. There was a positive correlation between hydrogen peroxide (H_2O_2) scavenging activity and mean total fluorescence for Indo-Pacific coral species,

whereby mean total fluorescence explained 55% of the variation in H_2O_2 scavenging activity (Figure 4.7).

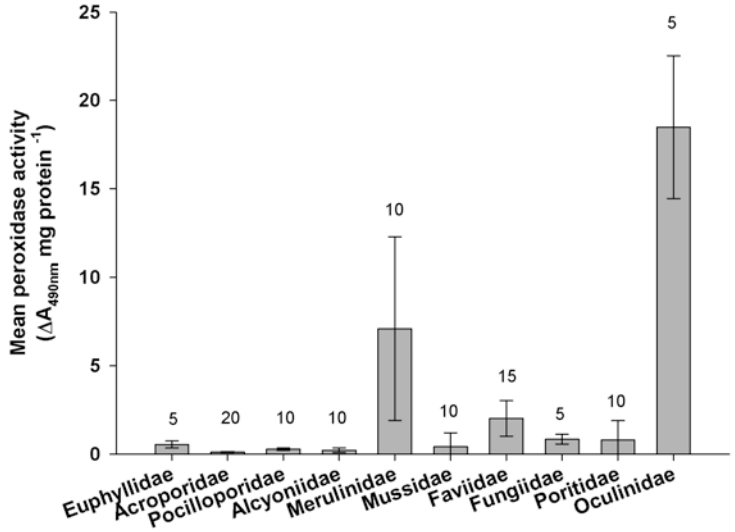


Figure 4.6: Comparison of mean peroxidase activity (± SE) among one alcyonacean and nine scleractinian families of Indo-Pacific corals.

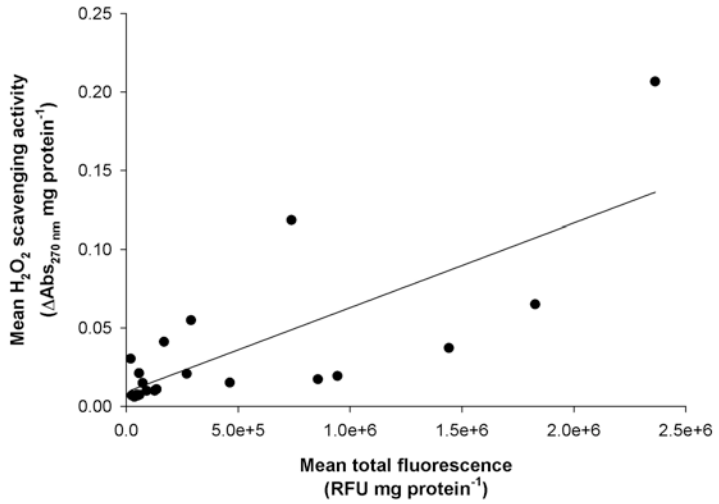


Figure 4.7: The correlation between the total fluorescence and the rate of H_2O_2 scavenged for 18 scleractinian and 1 alcyonacean Indo-Pacific coral species (R^2 = 0.55, F = 21.05, P < 0.001).

4.4.5 FLUORESCENCE OF CARIBBEAN CORALS

Spectral emissions also differed among the seven Caribbean coral species analysed (Figure 4.8), indicating inter-specific variation in the presence and concentration of each FP colour type. CFP was present only in the faviids *Montastraea annularis* and *Diploria strigosa* and the meandrinid *Dichocoenia stokseii*. Conversely, all species had fluorescence peaks within the green portion of the spectrum, indicative of the presence of GFP-like proteins, at wavelengths of either approximately 505 or 515 nm. Of these, *Siderastrea siderea* and *Montastraea cavernosa* had the highest peaks and *D. stokseii* the lowest. Four of the seven corals, *S. siderea, M. annularis, D. strigosa* and *Montastraea faveolata*, had broad peaks of relatively low magnitude between 575 and 590 nm, and slight peaks were detectable for *P. astreoides* and *D. stokseii,* indicating the presence of RFP. Additionally, *M. cavernosa* had a broad shoulder extending from the GFP peak at approximately 540 nm through the red spectrum. Although patterns in mean fluorescence emission peaks varied among species, when summed across the range of wavelengths, mean total fluorescence (RFU from 465 nm to 600 nm, Table 4.2) did not differ significantly among species ($F_{(6, 19)} = 0.468$, $P = 0.82$). However the three species with the highest fluorescent peaks, *M. cavernosa*, *S. siderea* and *M. annularis,* also had the highest mean total fluorescence, with the total fluorescence of *M. cavernosa* being 1.6-fold greater than that of *S. siderea*, although total fluorescence was highly variable for all three species. *D. stokseii* had the lowest mean total fluorescence, followed by *M. faveolata* and *P. astreoides*, and was 2.5-fold lower than *M. cavernosa*. Mean fluorescence values (RFU per mg protein^{-1}) for each of CFP, GFP and RFP did not differ among species ($F_{(6, 19)} = 0.489$, $P = 0.81$; K-W $\chi^2 = 1.84$, $P = 0.93$ and K-W $\chi^2 = 9.55$, $P = 0.15$ respectively, Table 4.2).

The PCA plot of PC scores demonstrated similarities in FP signatures among the Caribbean coral species (Figure 4.9). As such, the FPs of *S. siderea* were distinct from those of the other Caribbean species investigated, which fell along PC1. Overall, there appeared to be some relationship between composite FP signature and coral family, for example the Faviidae demonstrated similar FP signatures. Similar to the Indo-Pacfic PCA, PC1 appeared to represent a combined FP concentration of GFP and RFP, whereas PC2 was a contrast between CFP and GFP/RFP (Figure 4.9). Therefore, *M. cavernosa* and *S. siderea* had the overall highest FP concentration

whereas *D. stokseii* and *P. astreoides* had the lowest (PC1; eigenvalue 57.9%). The FP signature of *S. siderea* was distinct from the other corals and was CFP dominated, whereas the remaining families, but notably *M. cavernosa* and *M. annularis* were GFP/RFP dominated (PC2; eigenvalue 33.9%).

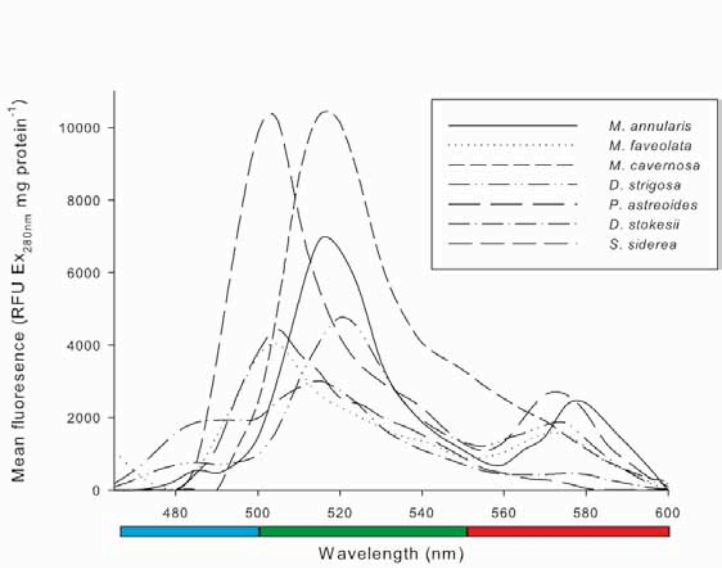


Figure 4.8: The mean relative fluorescence emission spectra (RFU $Ex._{280\ nm}$ mg $protein^{-1}$) for seven Caribbean coral species, plotted as relative proportions of the highest fluorescence.

Table 4.2: The mean fluorescence (RFU $Ex._{280\ nm}$ mg $protein^{-1}$) of CFPs, GFPs and RFPs for each Caribbean species inferred from the emissions spectra by summing across the 465 to 500, 505 to 550 and 555 to 600 nm wavelength emissions, respectively. Mean total fluorescence represents the emission spectra summed across 465 nm to 600 nm.

Species		Total	CFP	GFP	RFP
M. annularis	***Mean***	**53,900**	**3,548.3**	**37,780.7**	**11,910.6**
	Std. Err.	49,567.2	3,548.3	34,396	10,973.5
M. faveolata	***Mean***	**38,405.4**	**8,304.1**	**20,846.4**	**8,803.8**
	Std. Err.	21,757	7,968.2	13,664.3	1,240.9
M. cavernosa	***Mean***	**82,575.6**	**3,575.9**	**64,956.8**	**13,466.2**
	Std. Err.	60,383.6	3,321.6	46,514.2	9,977.3
D. strigosa	***Mean***	**44,328.9**	**4,779**	**28,692.8**	**10,646.5**
	Std. Err.	17,188.9	4,116.8	13,801.2	346.6
P. astreoides	***Mean***	**33,736.4**	**8,042.9**	**23,795.4**	**1,898**
	Std. Err.	9,965.5	3,798.1	6,903.1	800
D. stokesii	***Mean***	**33,482.4**	**11,052.2**	**19,174.6**	**3,166.4**
	Std. Err.	15,771.6	9,594.3	6,117	366.5
S. siderea	***Mean***	**77,430.8**	**21,224.4**	**41,853.5**	**13,886.4**
	Std. Err.	42,543.9	12,250.9	25,364.2	5,428.7

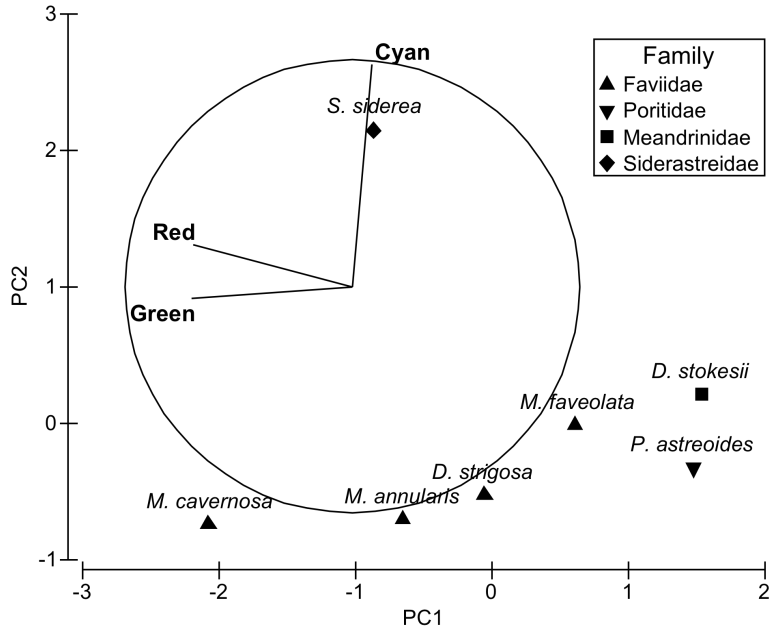


Figure 4.9: Principal Components Analysis (PCA) of CFP, GFP and RFP of the seven Caribbean coral species.

4.4.6 ANTIOXIDANT ACTIVITY OF CARIBBEAN CORALS

The mean rate of H_2O_2 scavenged per mg protein was not significantly different between the seven Caribbean coral species ($F_{(6, 19)} = 1.035$, $P = 0.45$; Table 4.3). There was a positive correlation ($R^2 = 0.25$, $F_{(1, 19)} = 6.05$, $P = 0.02$) between H_2O_2 scavenging rate and total fluorescence (Figure 4.10a). For the constituent fluorescent proteins, correlations between rate of H_2O_2 scavenging and relative fluorescence were strongest for the GFPs ($R^2 = 0.22$; $F_{(1, 19)} = 4.95$, $P = 0.04$; Figure 4.10c) and RFPs ($R^2 = 0.34$; $F_{(1, 19)} = 9.37$, $P < 0.01$; Figure 4.10d). In contrast, H_2O_2 scavenging rate was not significantly correlated with CFP relative fluorescence ($R^2 = 0.08$, $F_{(1, 19)} = 1.63$, $P = 0.22$, Figure 4.10b). The significant positive correlation between total fluorescence and rate of H_2O_2 scavenging demonstrates the viability of using this variable as a proxy for the contribution of FPs to overall levels of antioxidants.

Table 4.3: The mean H_2O_2 scavenging rate ($mmol.l^{-1}\ s^{-1}$ mg $protein^{-1}$) and standard errors for each coral species.

Species	Mean	Std. Err.
M. annularis	3.4	1.8
M. faveolata	1.7	0.3
M. cavernosa	0.9	0.2
D. strigosa	1.5	0.3
P. astreoides	1.3	0.3
D. stokesii	1.5	0.8
S. siderea	2.5	0.1

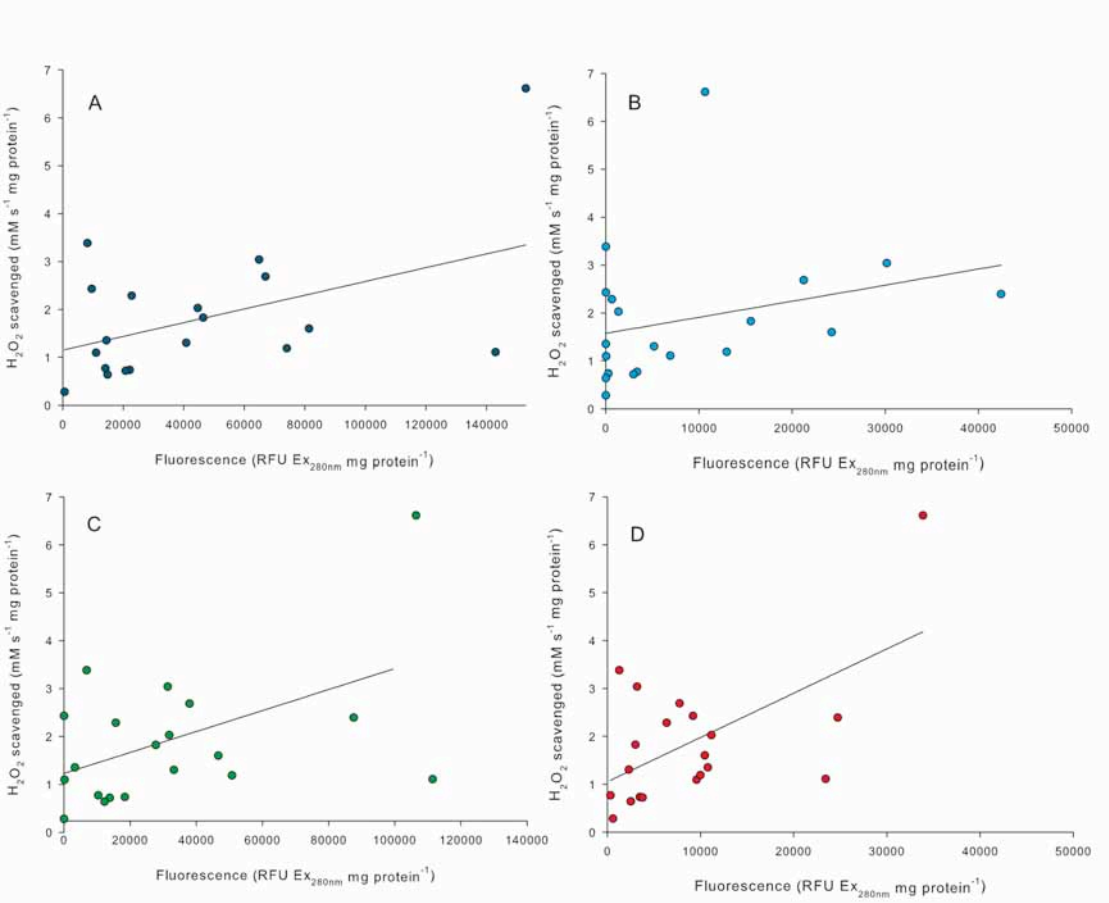


Figure 4.10: Regressions between mean fluorescence of seven Caribbean coral species and the rate of H_2O_2 scavenged for: a) total RFU from 465 nm to 600 nm ($R^2 = 0.25$, $F = 6.05$, $P = 0.024$), b) CFP from 465 nm to 500 nm ($R^2 = 0.08$, $F = 1.6295$, $P = 0.218$), c) GFP from 505 nm to 550 nm ($R^2 = 0.22$, $F = 4.95$, $P = 0.04$) and d) RFP from 555 nm to 600 nm ($R^2 = 0.34$, $F = 9.37$, $P < 0.01$).

4.5 Discussion

This study is the first to comprehensively quantify the fluorescence emission spectra and infer the concentration and type of fluorescent proteins (FPs) in a range of anthozoans, including 22 Indo-Pacific and 7 Caribbean species. This was enabled by the development of FP spectral emission standardisation and quantification methods. Additionally, coral FPs were found to scavenge H_2O_2 both *in vivo* and *in vitro*, demonstrating a novel biochemical role for these conspicuous proteins. Comparisons of anthozoan FP emission spectra highlighted inter-specific differences in type and concentration of FPs, however further work is needed to refine quantification methods and to determine whether these differences relate to differing life history strategies.

4.5.1 H_2O_2 SCAVENGING BY PURE FLUORESCENT PROTEINS

The four pure FPs: cyan (CFP), green (GFP), red (RFP) and the non-fluorescent chromoprotein (CP), all demonstrated dose-dependent H_2O_2 scavenging activity, providing evidence of antioxidant properties. The CP had the highest activity, followed by CFP and RFP, suggesting a potential advantage for corals possessing high CFP concentrations. GFP had the lowest activity, but still demonstrated greater scavenging than common proteins (BSA and lysozyme), therefore FPs may serve as supplemental antioxidants to catalase. Catalase was demonstrated to be a more efficient scavenger than the FPs in this study and has previously been noted to be the main H_2O_2 scavenging protein in corals (Merle et al. 2007). However, catalase activity can become limited during oxidative stress (Martindale and Holbrook 2002), which in corals can lead to bleaching (Lesser 1997). Therefore, corals that possess higher concentrations of FPs, particularly those with greater antioxidant activity, such as the CP and CFP, may be more effective at mitigating bleaching. However, further investigation into the molecular mechanisms of this proposed biochemical role for FPs is required to firmly establish their role in mitigating oxidative stress.

Despite the differential scavenging efficiency of the four wild-type FPs and the known role of pigments as antioxidants across the Metazoa (Bendich 1989; Vershinin 1999; Cornet et al. 2007), H_2O_2 scavenging rates were significantly higher for the non-fluorescent mutant counterparts. While this was unexpected, it suggests that the presence of the fluorophore inhibits scavenging. Therefore, whilst anthozoan FPs had demonstrable antioxidant capabilities, the evolutionary retention of the fluorophores suggests an alternative and colour-related primary role of these proteins. This supports the proposed role of coral FPs as visual triggers for other organisms (Ward 2002; Matz et al. 2006). Overall these findings support the suggestion that coral FPs serve multiple specific roles and functions that differ between the different types of FPs (Salih et al. 2000; Wiedenmann et al. 2002; Kelmanson and Matz 2003).

4.5.2 FLUORESCENT PROTEINS OF INDO-PACIFIC SPECIES

The fluorescence spectral emissions differed among Indo-Pacific species, indicating inter-specific diversity and variation in FP type and concentration, which, to a certain extent, reflects variation in host pigmentation observable directly on the reef (Matz et al. 1999; Veron 2000; Dove et al. 2001; Matz et al. 2002; Kelmanson and Matz 2003). As different anthozoan FPs demonstrate different H_2O_2 scavenging

efficiencies, variations in total fluorescence combined with diversity in the dominant FP type among species indicate that coral species vary in levels of antioxidants. As antioxidants have a role in mitigating bleaching and limiting self-harm during infection or injury, FP type and concentration are likely to contribute to variation in bleaching and disease susceptibility among coral species. Therefore, coral FP concentration may represent an ecologically important life-history trait, which needs further investigation. Furthermore, although total fluorescence is a good proxy for the relative contribution of coral FPs to hydrogen peroxide scavenging, as indicated by the positive relationship between these two variables, further refinement of this proxy, for example by incorporating the component FP colour types, may provide even better comparisons of antioxidant potential.

4.5.3 FLUORESCENT PROTEINS OF CARIBBEAN CORALS

As with the Indo-Pacific anthozoans, each Caribbean coral species had a different emission spectrum. However, unlike the Indo-Pacific coral families, statistically, the levels of total fluorescence did not differ among the Caribbean coral species. This is potentially attributable to high within-species variability in fluorescence, which might be reduced with higher sample sizes. Alternatively, a more refined proxy than total fluorescence might detect variation among Caribbean species. Members of the genus *Montastraea,* which represent the main framework builders on many Caribbean reefs (Hughes and Tanner 2000), demonstrated variation in FPs. Populations of several members of this genus are in decline (Edmunds and Elahi 2007), with *M. annularis* and *M. faveolata* currently listed as "endangered" on the IUCN Red List (Aronson et al. 2008b), partly due to their susceptibility to many of the characterised Caribbean diseases (Sutherland et al. 2004). In contrast, populations of *M. cavernosa* are not declining as rapidly and have been listed as a species of "least concern" (Aronson et al. 2008a). Concomitantly, *M. cavernosa* had a higher mean fluorescence than either *M. annularis* or *M. faveolata*, further suggesting that the presence of these antioxidant proteins in higher concentrations may be beneficial. All *Montastraea* species investigated showed a large degree of variability in fluorescence among genotypes, which may be due to their range of colour morphs, especially *M. cavernosa* (Kelmanson and Matz 2003). However, notwithstanding a propensity for intra-specific colour variation, all *M. cavernosa* colonies that were investigated by Kelmanson and Matz possessed genes for each of the four FP colours, demonstrating

that phenotypic plasticity in colony colour is linked to differential expression of FPs, potentially as a consequence of environmental variation (Kelmanson and Matz 2003). The difference between *M. cavernosa* and the other faviids, as indicated by the Principal Components Analysis, is in part due to the higher concentration of RFP, which had an emission peak of approximately 570 nm. This RFP peak is distinct from that of cyanobacteria that have been documented to occur within this species and demonstrate an orange-red fluorescence with a peak at 590 nm (Lesser et al. 2004). However, the cyanobacterial fluorescence may be contributing to the total red fluorecence exhibited by *M. cavernosa* in the current study.

Consistent with the Indo-Pacific poritids, *P. asteroides* is documented to be a very resistant coral, being tolerant to both disease and bleaching (Green et al. 2008; Croquer and Weil 2009). The presence of a GFP emission peak for *P. astreoides* in the current study is consistent with previous findings (Mazel 1995). However, the *P. astreoides* emission spectrum differed from that of the Indo-Pacific species, *P. cylindrica* and a massive *Porites* sp., which demonstrated higher CFP than GFP emission peaks. Overall, *D. stokesii* had the lowest FP concentration of all seven Caribbean species and *S. siderea* had an emission spectra similar in magnitude to that of *M. cavernosa*. Although there appear to be some broad ecological trends that support a relationship between concentration and type of coral FPs to coral health. This was discernable as corals were sampled at the same time and from the same locations, however this potential relationship needs to be quantitatively explored.

4.5.4 H_2O_2 SCAVENGING ACTIVITY *IN VIVO*

There was a positive correlation between the total fluorescence and H_2O_2 scavenging by both Indo-Pacific anthozoan species and Caribbean coral species, demonstrating *in vivo* antioxidant activity attributable to FPs. In contrast to scavenging activity *in vitro,* RFP and GFP accounted for the highest amount of H_2O_2 scavenging for Caribbean coral species *in vivo,* as compared to CFP, which did not show a significant relationship and does not conclusively account for any *in vivo* H_2O_2 scavenging. Even though the scavenging assays did not distinguish between catalase and FP scavenging in the coral extracts, the significant positive relationship between FP concentration and scavenging activity across a range of coral species provides supporting evidence for a novel role of anthozoan FPs as antioxidants.

The differing scavenging activities of the different FPs may partially explain the

differential allocation of FPs that has previously been described within coral tissue and among species (Cox et al. 2007; Gruber et al. 2008). GFP was found abundantly in the Indo-Pacific and Caribbean species investigated in this study, as well as in coral species previously investigated (Gruber et al. 2008), and is documented to occur consistently throughout coral cellular layers (Mazel et al. 2003; Matz et al. 2006). Therefore it is not surprising that GFP accounted for a significant amount of *in vivo* H_2O_2 scavenging in the Caribbean coral species. However, since pure GFP was the least efficient H_2O_2 scavenger in the *in vitro* assay, the *in vivo* GFP-scavenging correlation may be driven by the high within-tissue concentrations of GFPs in many of the species e.g. *M. cavernosa*. This relationship may be due to the need for corals to store higher levels of GFP in their tissues as a result of its less potent scavenging activity.

CFP did not have any detectable *in vivo* scavenging activity, although this result may be due to the relatively low presence of CFP within the seven Caribbean coral species used in this study. This result was not entirely unexpected since CFPs are documented to be limited in their prevalence (Gruber et al. 2008) and primarily located within a relatively small area of tissue on tentacle tips (Cox et al. 2007). However, investigations of Indo-Pacific species demonstrated high CFP activity, particularly for the zoanthid. Since pure CFP had high scavenging activity, this suggests that this anthozoan may significantly benefit from the high concentration of this FP. Alternatively, in other anthozoan species, due to the role of CFP as a more efficient antioxidant than the other FPs, it may be spatially and temporally regulated to maximise its efficacy.

The RFP was the most efficient *in vivo* scavenger and the purified RFP had potent *in vitro* activity, however, the pure CP was a superior H_2O_2 scavenger compared with its fluorescent counterparts. CP is observed to be predominantly limited to the extremities of colonies, such as branch tips and basal boundaries, however CPs have also been documented within healthy tissue of Pocilliporiidae (Dove et al. 1995), Acroporiidae and Poritiidae (Matz et al. 2002; Alieva et al. 2008). The correlation between total FP concentration and H_2O_2 scavenging supports an antioxidant role of FPs and also alludes to the biological significance of FPs as part of an anthozoan innate immune response.

Pigmentation responses, in areas of injury and infection, are common within the Anthozoa, and have been documented in the gorgonian sea fan (Mydlarz and Harvell

2007), due to carotenoids (Leverette et al. 2008), and in compromised tissue of multiple scleractinian species (Willis et al. 2004; Harvell et al. 2007). Although the cause of the pigmentation in scleractinian corals is currently unknown, it can be hypothesised that it is due to high concentrations of brightly pigmented, antioxidant FPs. Additionally, corals increase in fluorescence during temperature stress and bleaching (Dove et al. 2008), which supports their role as antioxidants. Overall, spectrally monitoring the dynamics of FPs potentially may provide a valuable and comparatively inexpensive tool for elucidating the relative health status and oxidative state of corals.

4.5.5 CONCLUSIONS

This study documents H_2O_2 scavenging activity of anthozoan FPs *in vitro* and *in vivo* demonstrating an additional role of anthozoan FPs as antioxidants. Furthermore, the diversity and concentration of FPs vary among anthozoans, a comparison that was enabled for the first time due to the development of novel standardisation protocols. This variation in FP type and concentration, in conjunction with differential antioxidant potentials, suggests that FP roles may differ between coral species or with changing environmental conditions. Overall, the findings highlight that anthozoan FP functions are potentially numerous, dynamic and not mutually exclusive. Further elucidation of FP functions, and their roles as antioxidants, could be gained through time series investigations of coral immune responses.

Chapter 5 LEVELS OF IMMUNITY PARAMETERS UNDERPIN BLEACHING AND DISEASE SUSCEPTIBILITY OF REEF CORALS

5.1 ABSTRACT

Immunity is a key life history trait that may explain hierarchies in the susceptibility of corals to disease and thermal bleaching, two of the greatest current threats to coral health and the persistence of tropical reefs. Despite their ongoing and rapid global decline, there have been few investigations into immunity mechanisms of reef-building corals. Coral immunity parameters quantified in preceding chapters were used in this study in conjunction with bleaching and disease susceptibility data from the published literature. These parameters included the presence of melanin, size of melanin-containing granular cells, and phenoloxidase (PO) activity, as well as concentrations of fluorescent proteins (FP), from hard (Scleractinia) and soft (Alcyonacea) corals spanning 10 families from the Great Barrier Reef (GBR). Overall levels of investment were inversely correlated to thermal bleaching and disease susceptibility. Correlations between taxonomic (family-level) variation in levels of these constituent immunity parameters and susceptibility to both thermal bleaching and disease suggest that baseline immunity underlies the vulnerability of corals to these two threats. This reinforces the necessity of a holistic approach to understanding bleaching and disease in order to accurately determine the resilience of coral reefs.

5.2 INTRODUCTION

The frequency and severity of coral bleaching events (Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2007) and coral disease epizootics (Harvell et al. 2002; Bruno et al. 2007; Harvell et al. 2007) are increasingly contributing to the decline of coral reefs globally (Hughes 1994; Aronson et al. 2003; Aronson et al. 2004; Bellwood et al. 2004). Recently, corals have been shown to possess a suite of innate immune mechanisms (Mydlarz et al. 2008; Chapters 2 to 4) that may contribute to their capacity to resist bleaching and disease, but our understanding of baseline levels of coral immunity is currently limited. Immunity is an important life history trait that promotes organism fitness (Stearns 1993; Sandland and Minchella 2003; Sadd and

Schmid-Hempel 2009), although there are physiological costs associated with the maintenance and use of immune functions (Rolff and Siva-Jothy 2003; Sadd and Schmid-Hempel 2009). Thus, species-specific investments in immune mechanisms develop throughout the evolution of a species' life history and represent trade-offs against investment in other life history traits, such as growth and reproduction (Stearns 1993; Rinkevich 1996). Differential allocation of energy to immunity traits may be indicative of the relative tolerance of species to disturbances (Rinkevich 1996; Sheldon and Verhulst 1996; Hosken 2001; Rolff and Siva-Jothy 2003). Consequently, determining differential investment in immune mechanisms among coral taxa may significantly enhance understanding of the mechanisms underlying their differential susceptibility to both bleaching and disease (Marshall and Baird 2000; Loya et al. 2001; Baird and Marshall 2002; McClanahan et al. 2004; Willis et al. 2004; Page and Willis 2006,2008).

Mass bleaching events that cause widespread coral mortality, such as the regional 1998 event in the Indian Ocean, are predicted to increase in frequency (Hoegh-Guldberg et al. 2007) and are due predominantly to sustained elevated water temperatures coupled with high irradiance (Berkelmans and Oliver 1999; Hoegh-Guldberg 1999). High UV and visible light radiation are major contributors to the breakdown of photosynthetic pathways *via* photodamage to photosystem II (Nishiyama et al. 2006), and consequently it is hypothesised that mechanisms which reduce UV or visible light flux to the zooxanthellae will limit bleaching (Jones et al. 1998; Baird et al. 2009). However, despite intensive investigation, the differential susceptibilities of coral taxa during and after bleaching events (Marshall and Baird 2000; Loya et al. 2001; McClanahan 2004; McClanahan et al. 2004) cannot be entirely explained by symbiont diversity or tolerance (Fabricius et al. 2005; Abrego et al. 2008; Fitt et al. 2009), thus affirming the importance of the coral host in determining bleaching susceptibility (Brown et al. 2000,2002; McClanahan et al. 2004; Baird et al. 2009). Host variables that potentially mitigate bleaching include mycosporine-like amino acids, which provide UV protection (Shick and Dunlap 2002; Fitt et al. 2009), tissue thickness (which relates to metabolic reserves; Loya et al. 2001; Fitt et al. 2009), antioxidant enzymes (Lesser 2006), heat shock proteins (Fitt et al. 2009) and fluorescent proteins (FPs; Salih et al. 2000; Dove 2004). FPs are responsible for the majority of the bright colours observed in reef corals (Dove et al. 2001) and are known to remit light at differing wavelengths, but despite a decade of

studies (Matz et al. 1999; Kelmanson and Matz 2003; Alieva et al. 2008), their biological roles within coral remain controversial (Tsien 1998; Dove et al. 2006b), particularly in relation to their photoprotective properties (Salih et al. 1998; Salih et al. 2000; Dove et al. 2006b). Recently, it has also been demonstrated that FPs exhibit reactive oxygen scavenging properties (Bou-Abdallah et al. 2006; Chapter 4), which would be beneficial during high oxidative stress conditions induced during both bleaching (Lesser 1996) and pathogen invasion (Costantini and Moller 2009), and may complement activities of antioxidant enzymes that are located within coral tissues (Hawkridge et al. 2000; Lesser 2006; Mydlarz and Harvell 2007).

Coral disease prevalence has increased over recent decades (Harvell et al. 2007), potentially in response to a combination of factors, including increasing pathogen abundance and virulence (Harvell et al. 1999), decreased host resistance (Mydlarz et al. 2006), ocean warming associated with climate change (Selig et al. 2006; Bruno et al. 2007), and environmental degradation (Bruno et al. 2003). Surveys documenting the presence of macroscopic signs of disease have been extensive (Willis et al. 2004; Raymundo et al. 2005; Page and Willis 2006,2008) and frequently document differential disease prevalence across family groups (Willis et al. 2004; Raymundo et al. 2005; Page and Willis 2006; Ward et al. 2006; Weil and Croquer 2009). Consistent differences in disease susceptibility across families suggest taxa-specific levels of disease resistance and investment in immunity mechanisms.

Immunity refers to the ability of an organism to resist infection (Stedman 2000), however coral innate immune mechanisms are only just beginning to be elucidated (reviewed in Chapter 1). Immunity parameters identified to date within corals, include the melanin-synthesis pathway, which provides cytotoxic defence (Chapters 2) and potentially photoprotection by melanin-containing granular cells (Chapter 3), as well as antioxidants, which protect the host tissue from oxidative stress (Chapter 4). Assuming that these parameters represent good measures of immunocompetence, the question that arises is whether differing levels of immunity parameters among coral families might explain inter-specific variations in disease prevalence and bleaching.

Disease resistance has historically been expressed in terms of immunocompetence, whereby the immune response to a given challenge is quantified (Adamo 2004b), however, the relevance of such measurements to the susceptibility of organisms to disease is complex, due to the limited number of immunity variables that

can be measured at one time (reviewed in Adamo 2004b). For a multitude of invertebrates, it has been shown that PO levels (Adamo 2004a; Newton et al. 2004; Schwarzenbach and Ward 2007; Butt and Raftos 2008) and melanin deposit abundance (Wilson et al. 2001; Armitage and Siva-Jothy 2005) correlate with disease resistance, suggesting that these parameters represent good candidates as potential indicators of disease resistance and relative investment in immunity for corals.

This study used quantified bioindicator variables that have been shown (Chapters 2-4) to be key components of the immune responses of corals, including, PO activity, the density and size of melanin-containing granular cells and the concentration of fluorescent proteins, the combined measures of which will henceforth be referred to as constituent immunity. Using these variables, this study aimed to investigate whether there is a relationship between levels of constituent immunity and differential susceptibilities of corals to both thermal bleaching and disease on the GBR, in a phylogenetically diverse range of coral species. Results indicate that these immune variables are good proxies for coral bleaching and disease susceptibility, and raise the possibility that the same biological mechanisms underlie susceptibility to both of these impacts. Elucidating the vital components of coral immunity and determining differential utilisation of immune pathways among coral taxa will significantly enhance understanding of the mechanisms underpinning coral health and may enable more accurate predictions of future climate change impacts on reef corals.

5.3 METHODS

Immunity indicators quantified for samples described in Section I (Table I.I), including phenoloxidase activity (PO), cross-sectional area and volume fraction (Vf) of melanin-containing granular cells and fluorescent protein (FP) concentration (see Chapters 2, 3 and 4, respectively), were correlated to both bleaching and disease susceptibility data obtained from the published literature. In total, quantified immunity parameters and correlations were made for 15 scleractinian species and 2 alcyonacean corals (Section I, Table I.I). To establish a single measure of constituent immunity for each coral species and coral family, Principal Components Analyses (PCA) was conducted on the respective suite of immunity variables, i.e. including PO activity, FP concentration, cross-sectional area and Vf of melanin-containing granular cells.

Bleaching susceptibility indices were determined for each coral family using data from Marshall and Baird (2000), who documented the proportion of coral colonies (to genus) within the following four categories after the 1998 mass bleaching event on the Great Barrier Reef (including Orpheus Island, the sampling site of this study): unbleached, moderately bleached, severely bleached and dead. To attain comparable bleaching susceptibility data, a bleaching-mortality index (BMI) (McClanahan 2004) was calculated for each genus using the equation:

$$BMI = (0 \times c_1 + 1 \times c_2 + 2 \times c_3 + 3 \times c_4)/3,$$

where c_1 represents the proportion of unbleached colonies, c_2 represents the proportion of moderately bleached colonies, c_3 represents the proportion of severely bleached colonies and c_4 represents the proportion of dead colonies for each coral family. This provided an index where high mortality from bleaching equated to high BMI values. The mean BMI was then calculated per family group. As genera included in this study were limited to those documented by Marshall and Baird (2000), some scleractinian families including Euphyllidae, Oculinidae and the zoanthid family Sphenopidae, were not examined.

The prevalence of skeletal eroding band (SEB), a widespread coral disease found throughout the GBR, was obtained from Page and Willis (2008) for each coral family in this study and used as an indicator of disease susceptibility. Thus high prevalence equated to high disease susceptibility. Inclusion of a suite of described coral diseases was not possible because of the lack of published data at high taxonomic resolution.

Linear regression analyses were then used to determine the relationships between the PC1 scores of the PCA and either the BMI or the disease susceptibility rank for each coral family. At the species level (see Table 5.1), constituent immunity ranks were calculated as the PC1 score in a species-level PCA analysis. In addition, to predict bleaching and disease susceptibility for species with known levels of constituent immunity, predicted BMI and disease susceptibility ranks were calculated by using the family-level relationship between PC1 score and susceptibilities, and solving the respective regression equations to interpolate species level susceptibilities (the “y” value) for the known PC1 score of each species. A table of life history traits, including predominant growth form, linear extension rate, reproductive output and direct competitive ability, for each coral family was constructed using information from the published literature.

5.4 RESULTS

Principal Components Analysis (PCA) of immunity indicators for coral families demonstrated that these parameters were interdependent (Figure 5.1). The PCA indicated two main variables that contributed to the overall level of constituent immunity: PC1 (92%), which was primarily melanin-containing granular cell size and PC2 (5.7%), which was mainly PO activity. As such, the mussids, faviids and alcyoniids had similar melanin-containing granular cell size and the pocilloporiids the overall smallest. Poritidae had both the highest PC1 (cell size) and PC2 (PO activity).

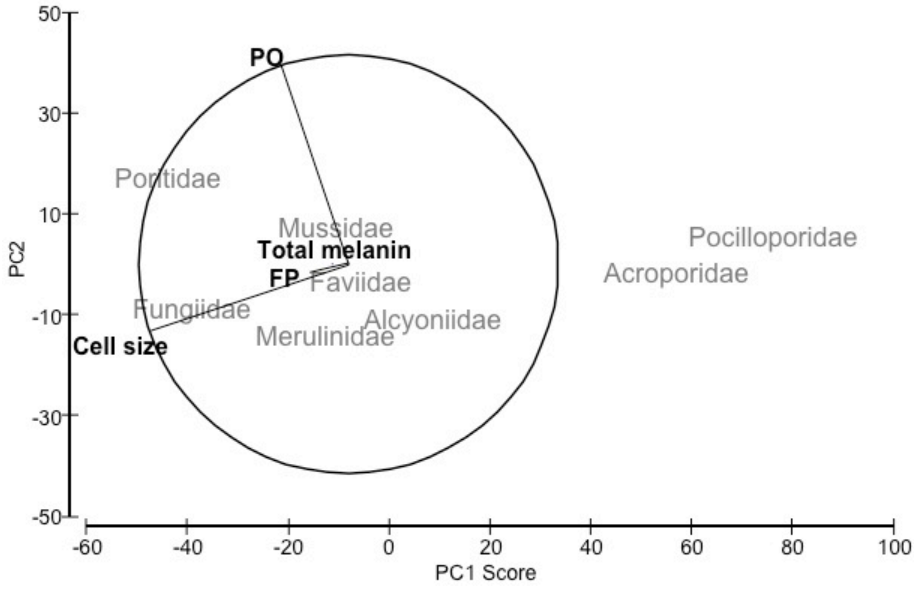


Figure 5.1: Principal Components Analyses (PCA) of immunity parameters for the 8 coral families.

There was a significant regression between the PC1 score of the PCA of immunity variables for each family, where a high score indicates low constituent immunity, and bleaching susceptibility, as measured by the bleaching-mortality index (BMI; Table 5.1) for 7 hard coral families and 1 soft coral family ($r^2 = 0.74$, $F_{(1, 7)} = 20.8$, $P = 0.004$; Figure 5.2). PC1 score and disease susceptibility rank (Table 5.1) were also strongly related for the 7 hard coral families ($r^2 = 0.83$, $F_{(1, 6)} = 25.2$, $P = 0.004$; Figure 5.3); the Alcyoniidae were excluded from this analysis because of a lack of disease prevalence data in the published literature.

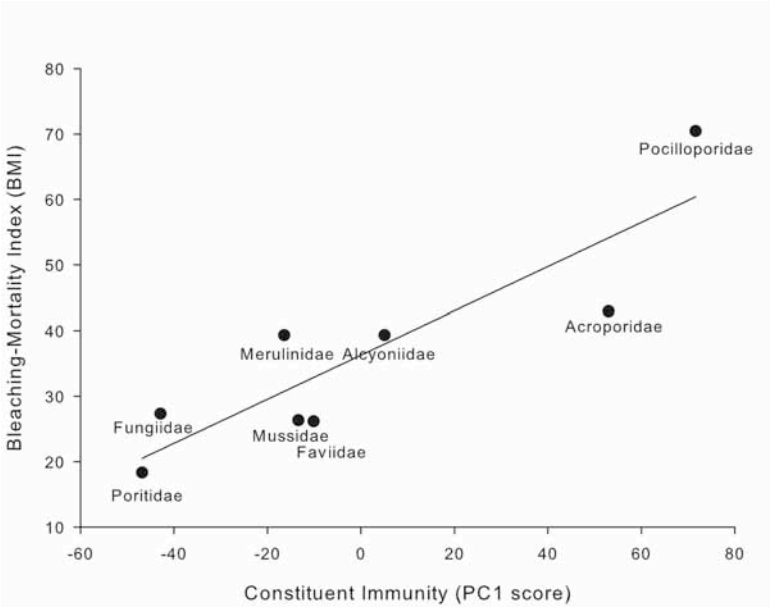


Figure 5.2: The relationship between PC1, the first principal components analysis score, which represents the overall suite of immunity indicators and where high investment in constituent immunity equates to a low PC1 score, and the bleaching mortality index (BMI) ($r^2 = 0.78$, $F_{(1, 7)} = 20.77$, $P = 0.004$) for each of seven hard coral families and one soft coral family.

Both the species-level BMI and disease susceptibility rank predictions, based on constituent immunity rank and calculated relative to the susceptibilities at the family level, indicate that *Pocillopora damicornis* is the most susceptible of the study species to both bleaching and disease; its BMI value (58.56) and disease susceptibility rank (4.86) were the highest of all the species investigated (Table 5.1). *Seriatopora hystrix*, the other species investigated in the family Pocilloporidae, had only slightly lower values than *P. damicornis.* The Acroporidae were the second most susceptible family. Of the four species in this family investigated, *Acropora tenuis,* was predicted to be the most susceptible and *Montipora digitata* the least. Of all 17 species, the poritid *P. cylindrica* was predicted to be the least susceptible to bleaching and disease (predicted BMI = 8.26 and disease susceptibility rank = -1.87), followed by the merulinid *Merulina ampliata* and then the fungiid *Fungia* sp.

Qualitative immunity ranks (low, intermediate or high levels of immunity parameters) for seven hard coral families and the soft coral family, Alcyoniidae, were the inverse of corresponding ranks for growth rate deduced from the literature for these taxa (Table 5.2). For example, the Acroporidae had low immunity, but high growth rates, whereas the Poritidae had high immunity, but low linear extension rates. Comparative patterns for immunity and reproductive ranks were less clear, but

followed a similar trend, with the Acroporidae having one of the highest ranks for reproductive output per cm^2 and the Poritidae the lowest. Additionally, families with predominantly massive growth forms had highest constituent immunity, whereas branching growth forms consistently demonstrated low constituent immunity. Patterns in direct competitive ability and immunity rank varied in a similar manner, with low competitive ability broadly correlated with low immunity rank, except that the Poritidae have apparently low competitive ability but high immunity rank.

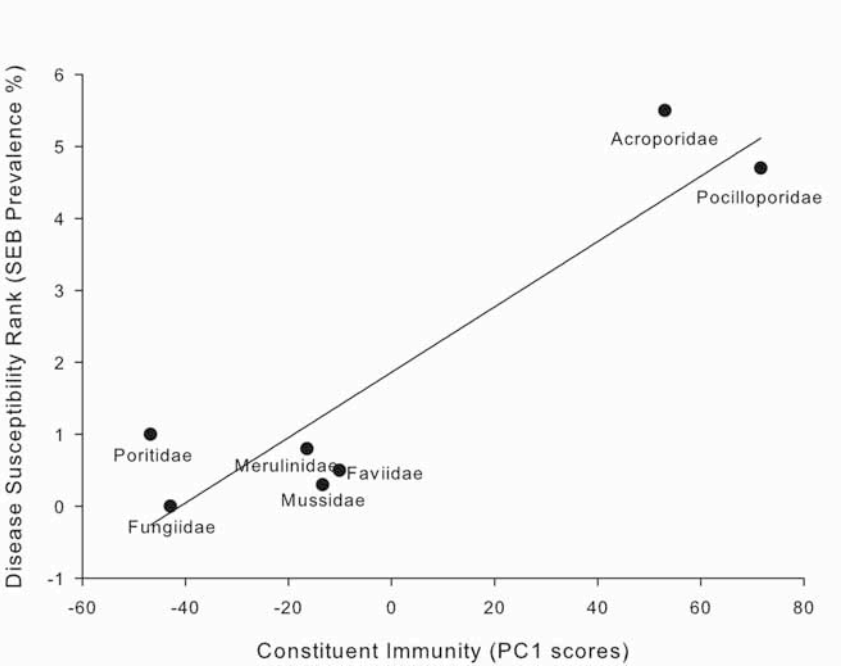


Figure 5.3: Relationship between the PC1 score from a principal components analysis of the overall suite of immunity indicators and disease susceptibility rank, as calculated by skeletal eroding band (SEB) prevalence, for seven scleractinian families ($r^2 = 0.83$, $F_{(1, 6)} = 25.22$, $P = 0.004$).

Table 5.1: Constituent immunity and predicted bleaching-mortality indices (BMI) and disease susceptibility ranks (as determined from equations calculated for regressions in Figures 6.1 and 6.2) for 15 species of scleractinian corals and two species of alcyonacean corals.

Family	Constituent immunity (PC1 score)	Species	Constituent immunity (PC1 score)	Predicted BMI	Predicted Disease susceptibility rank
Acroporidae	53.0	*A. hyacinthus*	45.29	51.53	3.92
		A. tenuis	48.31	52.55	4.06
		A. millepora	42.09	50.46	3.77
		M. digitata	40.28	49.85	3.69
Pocilloporidae	71.60	*P. damicornis*	66.15	58.56	4.86
		S. hystrix	59.49	56.31	4.56
Alcyoniidae	5.05	*Lobophyton*	26.30	45.14	3.06
		Sarcophyton	-35.61	24.29	0.25
Merulinidae	-16.42	*M. ampliata*	-54.40	17.96	-0.61
		Hydnophora	2.09	36.98	1.96
Faviidae	-10.11	*D. heliopora*	-4.38	34.80	1.66
		P. sinensis	-25.00	27.86	0.73
		G. aspera	-27.05	27.17	0.63
Mussidae	-13.41	*L. hemprichii*	-21.92	28.90	0.87
Fungiidae	-42.90	*Fungia*	-51.72	18.86	-0.49
Poritidae	-46.90	*P. massive sp.*	-27.80	26.92	0.60
		P. cylindrica	-82.12	8.62	-1.87

Table 5.2: Comparative immunity ranks and life-history traits for seven scleractinian coral families and the alcyonacean family, Alcyoniidae. ([1] Veron 2000, [2] Huston 1985; Crabbe 2009 [3] Harrison and Wallace 1990; Soong 1991; Leuzinger, 2003 #718, [4] Lang and Chornesky 1990)

Coral Family	Immunity rank	Predominant growth form[1]	Linear extension rate[2]	Reproductive output[3]	Direct competitive ability[4]
Acroporidae	Low	Branching	High	Intermediate/High	Low
Pocilloporidae	Low	Branching	High	Medium/High	Low
Alcyoniidae	Intermediate		High?	?	Intermediate/High
Merulinidae	Intermediate/High	Foliose	Intermediate/Low	?	Intermediate/High
Faviidae	Intermediate/High	Massive	Intermediate/Low	Intermediate/High	Intermediate/High
Mussidae	Intermediate/High	Massive	Low	Intermediate/High	Intermediate/High
Fungiidae	High	Solitary	?	High	Intermediate/Low
Poritidae	High	Massive	Low	Low	Low

5.5 DISCUSSION

This study demonstrates the importance of innate immunity variables in predicting both bleaching and disease susceptibilities of corals on the Great Barrier Reef (GBR). Constituent immunity, as measured by four immune function variables (size of melanin-containing granular cells, phenoloxidase activity (PO), fluorescent protein (FP) concentration, and volume fraction of melanin), was found to explain over 78% of inter-family variation in bleaching susceptibility, as calculated by the BMI index (McClanahan et al. 2004), confirming the significant contribution that the coral host makes to bleaching mitigation (Baird et al. 2009; Fitt et al. 2009). Additionally, 83% of inter-family variation in rank susceptibility to disease can be explained by the measured levels of constituent immunity, which is consistent with host immunity also playing a major role in pathogen resistance and disease prevalence (Harvell et al. 2007). Further investigations of these relationships would benefit from the inclusion of up-to-date and higher resolution susceptibility data spanning more coral species. Overall similarity in rank susceptibility of corals to bleaching and disease, both of which are contributing significantly to global reef decline and likely to intensify in the future (Harvell et al. 2007; Hoegh-Guldberg et al. 2007), suggest that a common underlying characteristic of the coral host explains these patterns. The positive correlations between PC1 scores and susceptibility ranks indicate this characteristic to be the level of investment in innate immune function, as high PC1 scores equate to low levels of constituent immunity. Therefore corals that invest more into innate immune functions show lower susceptibility to bleaching and disease across the GBR.

The accuracy of a parameter for quantifying immunocompetence is typically determined from the strength of its correlations with observed resistance to disease (Adamo 2004b; Siva-Jothy et al. 2005). Therefore, the strong positive relationships found between both bleaching and disease susceptibility and the four immunity parameters measured in this study suggest that these parameters (i.e. size of melanin-containing granular cells, PO activity, FP concentration, and melanin proportion) are accurate measures of coral constituent immunity and good indicators of immunocompetence (Adamo 2004b). As a consequence, these parameters may provide the basis for an effective tool for predicting levels of threat to coral communities. Such linkages highlight the vital role of the immune system in coral

physiology and ecology, which until recently has been overlooked (Mydlarz et al. 2006).

5.5.1 PHENOLOXIDASE ACTIVITY

The two main determinants of immunity rank and, by inference, bleaching and disease susceptibility were found to be PO activity and size of melanin-containing granular cells. The presence of PO activity in all coral species investigated (see Chapter 2) is consistent with the significant role that the melanin-synthesis pathway plays in the innate immunity of invertebrates (Söderhäll 1982; Rinkevich and Muller 1996; Söderhäll and Cerenius 1998; Nappi and Christensen 2005; Mydlarz et al. 2006). PO activity in healthy corals indicates a residual level of antimicrobial defence due to the production of cytotoxic melanin pathway intermediates such as quinones (Nappi and Ottaviani 2000; Nappi and Christensen 2005), and could explain biochemical cytotoxicity previously observed in directly competing corals (Hildemann 1977). Differences in residual PO activity among coral families indicate physiological differences that may have implications at an ecological scale in terms of disease resistance, with families having higher PO activity being more able to resist infection. This prediction is consistent with correlations found between PO activity and immunocompetence for numerous invertebrates, including oysters (Newton et al. 2004; Butt and Raftos 2008), yellow dung flies (Schwarzenbach and Ward 2007) and the water flea, *Daphnia magna*, (Mucklow et al. 2004). Based on constituent levels of PO activity, it can be predicted that the Pocilloporidae and Acroporidae are likely to be the most susceptible coral families to pathogenic invasion and the Poritidae the most resistant. These predictions are broadly consistent with previously documented taxonomic patterns in disease susceptibility on the GBR (Willis et al. 2004; Page and Willis 2008). The Pocilloporidae and Acroporidae are generally the most susceptible families to the majority of characterised syndromes, including skeletal eroding band (SEB; Willis et al. 2004; Page and Willis 2008), black band disease (BBD; Willis et al. 2004; Page and Willis 2006), brown band (BrB; Willis et al. 2004) and white syndromes (WS; Roff et al. 2006). Conversely, poritids are consistently one of the least susceptible families on the GBR (Willis et al. 2004; Page and Willis 2008).

5.5.2 THE PRESENCE AND LOCALISATION OF MELANIN

Melanins are known to play a key role in disease susceptibility in invertebrates (Söderhäll and Cerenius 1998; Cerenius et al. 2008), particularly for antimicrobial defence (Nappi and Ottaviani 2000), but in addition, they are known to function in desiccation resistance in plants (Laufer et al. 2006) and photoprotection *via* light absorption in both plants (Mayer and Staples 2002; Laufer et al. 2006) and higher metazoans (Meredith et al. 2006). The presence of melanin-containing granular cells in the free body wall tissues of all species investigated (Chapter 3) suggests that they fulfil an important physiological role within scleractinian and alcyonacean corals, potentially providing resistance to both bleaching and disease.

The variation in mean volume fraction of melanin within free body wall tissues that was found among families in Chapter 3 was negatively correlated with family rank order in disease susceptibility deduced from field surveys of disease prevalence. Thus the Acroporidae and Pocilloporidae had the lowest volume fraction of melanin but highest disease prevalence, whereas the Poritidae had the inverse pattern (Willis et al. 2004; Page and Willis 2006). Melanin-containing granular cell size demonstrated a similar pattern to melanin Vf and was a strong predictor of constituent immunity. This suggests that immune cells are a vital component of coral immunity and that possessing larger sized, potentially mobile, cells is advantageous. In other invertebrates, such as in the insects *Spodoptera exempta* (Wilson et al. 2001) and *Tenebrio molitor* (Barnes and Siva-Jothy 2000; Armitage and Siva-Jothy 2005), quantities of melanin deposits positively correlate with immunocompetence.

5.5.3 FLUORESCENCE

The third main determinant of susceptibility was found to be fluorescent protein (FP) concentration, which supports suggestions that FPs play a role within coral innate immunity (Chapter 4). Due to their light altering properties, investigations of FPs have focussed on the relationship of FPs to bleaching (Salih et al. 2001; Dove 2004; Leutenegger et al. 2007a; Dove et al. 2008; Smith-Keune and Dove 2008). Antioxidant properties of coral FPs (Chapter 4) are advantageous for mitigating self-harm and loss of symbionts during oxidative stress conditions, which occur during pathogen infection as part of an immune response and in response to thermal stress, which may lead to bleaching (Lesser 1996,2006; Weis 2008; Costantini and Moller 2009).

5.5.4 LIFE HISTORY PATTERNS OF INVESTMENT IN IMMUNITY IN RELATION TO BLEACHING AND DISEASE SUSCEPTIBILITY

Immunity and thus bleaching and disease resistance, vary among individuals largely because of species-specific differences in the allocation of energy to this life history trait (Sadd and Schmid-Hempel 2009). Given that growth, reproduction and immune function represent competing traits for the allocation of resources that are in excess of those required for core metabolic needs, investment trade-offs typically occur among these physiological functions (Sadd and Schmid-Hempel 2009). Life history theory predicts that species which prioritise investment in immunity would have lower resources available to invest in growth and/or reproduction, and the converse would be true for species that minimise investment in immune function (Stearns 1993; Sadd and Schmid-Hempel 2009). Thus, coral families that demonstrate low susceptibility to disease and bleaching are likely investing a high proportion of available resources into immunity, as alluded to in earlier studies of coral regeneration (Kramarsky-Winter and Loya 1996; Rinkevich 1996).

The diverse array of morphological, growth and reproductive strategies displayed by corals makes them an ideal group with which to explore life history trade-offs. When patterns of investment in constituent immunity are considered in light of investment in growth and reproduction, taxonomic patterns in resource allocation to innate immunity are in accord with the governing principle of trade-offs in life history evolution (Stearns 1993). For example, branching species of *Acropora* that invest comparatively heavily into rapid colony growth to access resources in the water column and to overtop competitors (Jackson and Hughes 1985), as well as into sexual reproduction (Harrison and Wallace 1990; Soong 1991; Soong and Lang 1992), would be predicted to have fewer resources available for investment in immune functions. Thus this finding, that the Acroporidae had the lowest immunity rank, which related to their high susceptibility to bleaching and disease (Berkelmans and Oliver 1999; Marshall and Baird 2000; Willis et al. 2004; Page and Willis 2006,2008), is consistent with the pivotal role that trade-offs play in life history evolution. Conversely, the Poritidae examined in this study are massive, gonochoric species with relatively slow rates of colony expansion and comparatively low reproductive output (Harrison and Wallace 1990). Correspondingly, the Poritidae had the highest immunity rank and have been found to be the least susceptible to

bleaching and disease on the GBR (Willis et al. 2004; Page and Willis 2006, 2008). It is therefore inferred that their comparatively low investment in colony expansion and reproductive output enables them to allocate comparatively greater resources to constituent immunity. This brief discussion of comparative coral life histories demonstrates likely trade-offs among patterns of energy investment into growth, reproduction and ecological immunity, and highlights the likelihood that further investigations into trade-offs among these three life history parameters at the level of genera, and preferably species, will provide a promising area of future research (Sadd and Schmid-Hempel 2009).

5.5.5 CONCLUSIONS

In summary, this study provides novel insights into constituent immunity variation across a broad range of Indo-Pacific corals, and demonstrates the importance of coral host immunity in determining susceptibility of the coral holobiont to bleaching and disease. It can be concluded that the variables measured in this study provide good indicators of coral immunity and underpin linkages between the susceptibility of corals to both bleaching and disease. Measuring constituent immunity may provide a useful tool for reef managers, particularly for species for which there is little ecological data on either bleaching or disease prevalence. Significantly, this study emphasises the importance of coral immunity in a species' predisposition to bleaching and disease and highlights the importance of understanding constituent immunity when predicting the future state of coral reefs, particularly in light of challenges posed by warming and acidifying oceans.

SECTION II. THE USE OF CORAL IMMUNITY PARAMETERS DURING IMMUNE RESPONSES

In Section I, it was demonstrated that anthozoans, including scleractinian, alcyonacean and zoantharian species, possess multiple effector responses that are known to be involved in innate immunity in other invertebrates, however, deployment of these immune cells, cytotoxic pathways and antioxidants for specific uses has not been explored previously in scleractinian corals. The presence of these components of innate immunity in corals is consistent with the hypothesis based on preliminary genomic investigations that coral immune mechanisms are as complex as those within other invertebrates (Brower et al. 1997; Miller et al. 2005; Kvennefors et al. 2008). Additionally, the correlation found between levels of anthozoan effector responses and both bleaching and disease susceptibility suggests that these immunity parameters are good measures of coral immunocompetence (Chapter 5), however the use of each parameter during an immune response and how these responses may vary under climate change conditions, such as elevated water temperature, remains poorly understood.

How and when the effector responses, such as those characterised in Section I, are employed during an immune response has been documented for numerous invertebrates (e.g. Ford et al. 1993; Asokan et al. 1997; De Leo et al. 1997; Field et al. 2004; Mucklow et al. 2004; Haine et al. 2008), including within gorgonians (Meszaros and Bigger 1999; Olano and Bigger 2000; Mydlarz and Harvell 2007; Mydlarz et al. 2008) and an anemone (Young 1974). These studies of anthozoan immune responses demonstrated the aggregation of immune cells in response both to injury (Young 1974; Meszaros and Bigger 1999; Olano and Bigger 2000) and infection (Mydlarz et al. 2008), and the up-regulation of the melanin-synthesis pathway and antioxidant enzymes in response to fungal infection (Mydlarz and Harvell 2007; Mydlarz et al. 2008). As anthozoans typically share common effector responses, including aggregating immune cells and cytotoxicity associated with the melanin-synthesis pathway (Section I), it can be hypothesised that similar challenges will activate these effector responses within hard corals. However, the potential use of fluorescent proteins as an immunity parameter during an anthozoan immune response,

as proposed in Chapter 4, has not previously been investigated. Similarly, as the presence of the coagulation pathway was documented within anthozoans for the first time in this project, the regulation of transglutaminase activity during an anthozoan immune response has not previously been investigated.

Investigating the activity of scleractinian effector responses, including phenoloxidase activity, melanin-containing granular cell density, fluorescent protein concentration, transglutaminase activity, hydrogen peroxide scavenging activity and enzymatic antioxidant activity, during an immune response will help to demonstrate the relevance and function of each parameter within anthozoan immunity. Understanding the use and regulation of immunity components during coral immune responses will provide insight into how corals may resist disease. Furthermore, inter- and intra-specific comparisons of how immunity parameters are used may highlight additional differences in life history strategies to those proposed in Chapter 5. Additionally, investigating the influence of elevated water temperature on coral immune responses will help to determine the likelihood of corals to persist under changing climatic conditions. This will enhance the study of anthozoan ecological immunology and add to the knowledge base for predicting the potential impacts of climate change.

Chapters within this section (see list below) collectively explore the use of coral immunity parameters that were identified in Section I, in relation to: 1) compromised coral tissue that appears inflamed, 2) parasite-infected tissue, 3) physical injury as a time series and 4) warmer water temperatures.

Components of each of the chapters within this section have been published in:

Palmer CV, Modi CK, Mydlarz LD (2009b) Coral fluorescent proteins as antioxidants. PLoS ONE. 4: e7298.

Palmer CV, Roth MS, Gates RD (2009a) Red fluorescent protein responsible for pigmentation in trematode-infected *Porites compressa* tissues. Biological Bulletin 215: 68-74.

Palmer CV, Mydlarz LD, Willis BL (2008) Evidence of an inflammatory-like response in non-normally pigmented tissues of two scleractinian corals. Proceedings of the Royal Society London B, Biological Sciences 275: 2687-2693.

Chapter 6 MELANIN-SYNTHESIS PATHWAY ACTIVITY IN NON-NORMALLY PIGMENTED TISSUES OF TWO SCLERACTINIAN CORALS

6.1 ABSTRACT

Increasing evidence of links between climate change, anthropogenic stress and coral disease, underscores the importance of understanding the mechanisms by which reef-building corals resist infection and recover from injury. Cellular inflammation and activation of the melanin-synthesis pathway are two mechanisms employed by invertebrates as part on an immune response to remove foreign organisms and have recently been documented to occur within hard corals (Section I). This study demonstrated the presence of the phenoloxidase (PO) activating melanin-synthesis pathway in two coral species, *Acropora millepora* and a massive species of *Porites*, which both develop local pigmentation in response to interactions with a variety of organisms. L-DOPA (3-(3, 4-dihydroxyphenyl)-L-alanine) substrate based enzyme activation assays demonstrated PO activity in healthy tissues of both species and up-regulation in pigmented tissues of *A. millepora.* Histological staining conclusively identified the presence of melanin in *Porites* tissues. These results demonstrate that the PO pathway is active in both coral species. Moreover, up-regulation of PO activity in areas of non-normal pigmentation in *A. millepora* and increased melanin production in pigmented *Porites* tissues suggest the presence of a generalised defence response to localised stress. Inter-specific differences in the utilisation of pathways involved in innate immunity may underlie the comparative success of massive *Porites* sp. as long-lived stress tolerators.

6.2 INTRODUCTION

Reports of coral disease have increased dramatically over the last decade; however, the biological mechanisms that corals utilise to limit infection and resist disease remain poorly understood. Coral disease identification is largely dependent on direct macroscopic observations (Harvell et al. 2007). As such, non-normally pigmented tissues have been used as the main signs identifying syndromes or diseases in several scleractinian species (Aeby 2003; Bongiorni and Rinkevich 2005;

Ravindran and Raghukumar 2006a,2006b). Discoloured tissues are typically pink, purple or blue and are observed in a variety of circumstances associated with stressful interactions (Kawaguti 1944; Rinkevich and Sakai 2001; Aeby 2003; Ravindran and Raghukumar 2006a,2006b). Because such observations are characteristically associated with agents that compromise coral tissue integrity, it has been suggested that these signs of non-normal pigmentation are associated with a generalised response of the coral to a physical or pathogenic challenge (Willis et al. 2004; Bongiorni and Rinkevich 2005; Ravindran and Raghukumar 2006b). The intriguing possibility therefore arises that such non-normal tissue pigmentation is associated with the activation of a general immune response.

Invertebrate effector responses have been thoroughly characterised (see Chapter 1), and include phagocytosis and the melanin-synthesis pathway. However, it is the up-regulation or activation of these effector responses that is indicative of the presence of an immune response and that may indicate its efficacy, or immunocompetence (Adamo 2004b). For example, increased phenoloxidase (PO) activity in response to physical injury in the fruit fly *Drosophila melangastor* and the wax moth *Galleria mellonella* (Bidla et al. 2008). Similarly, increased PO has been documented in response to infection in invertebrates including the crayfish *Procambarus clarkii* (Cardenas and Dankert 1997), the Sydney rock oyster *Saccostrea glomerata* (Butt and Raftos 2008), the pacific oyster *Crassostrea gigas* (Hellio et al. 2007) and the anthozoan *Gorgonia ventalina* (Mydlarz et al. 2008). Frequently linked with PO activity are the cellular effector responses including phagocytosis and encapsulation (Rowley 1996; Iwanaga and Lee 2005), which involve the aggregation of immune cells (see Chapter 1).

Many of the characterised invertebrate immune cells also demonstrate PO activity and/or contain melanin, for example those of the annelid *Neris diversicolor* (Porchet Hennere and Vernet 1992), crustacean *Panaeus californiensis* (Gollas-Galvan et al. 1999) and the sea fan *Gorgonia ventalina* (Mydlarz et al. 2008). Aggregation of immune cells at sites of infection or injury has been documented for multiple invertebrates including the insects *Pseudoplusia includens* (Pech and Strand 1996) and *Drosophila melangastor* (Galko and Krasnow 2004) ascidians (De Leo et al. 1997) and molluscs (Butt and Raftos 2008). Aggregation of anthozoan immune cells in response to injury or infection has also been documented for numerous species. These include *Anthopleura elegantissima* (Patterson and Landolt 1979)

Swifta exserta (Olano and Bigger 2000) *Plexaurella fusifera* (Meszaros and Bigger 1999) and *G. ventalina* (Mydlarz et al. 2008). However, the regulation of PO and cellular activity as part of an immune response has not been investigated within hard corals.

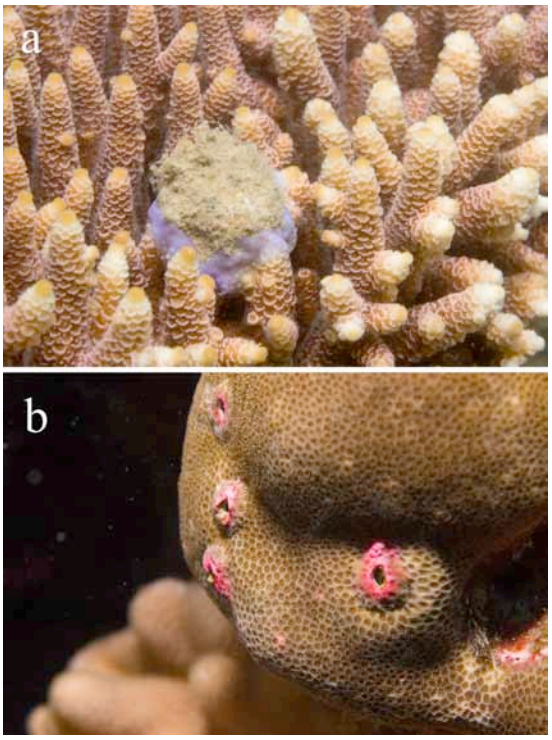


Figure 6.1: a) Blue pigmentation response of *A. millepora* and b) pink pigmentation response of *Porites* massive sp.

Despite an increasing focus on coral disease research to understand and reduce the rate of reef degradation worldwide, little attention has been paid to disease resistance mechanisms in scleractinian corals. This study investigated the presence of key invertebrate effector response mechanisms in areas of non-normally pigmented tissue in two species of scleractinian coral that have contrasting susceptibility to disease: *Acropora millepora,* from the family Acroporidae, which is one of the most susceptible families and a massive species of *Porites,* which has comparatively low susceptibility to disease on the Great Barrier Reef (Chapter 5). Comparisons of PO activity, cell infiltration and the localisation of melanin deposits between healthy and non-normally pigmented tissue (referred to simply as pigmented hereafter; Figure 6.1) provide novel insights into potential mechanisms of innate immunity in two scleractinian coral species.

6.3 METHODS

6.3.1 SAMPLE COLLECTION

Pigmented and healthy samples were collected from 28 colonies of *A. millepora* and 20 colonies of a massive species of *Porites* for comparisons of PO activity and histological microstructure (n = 5 samples per category from each species) from fringing reefs surrounding Orpheus Island, in the central sector of the Great Barrier Reef (GBR). Sampling pigmented and healthy tissues from each colony

controlled for potential genetic factors underlying differences in pigmentation. Healthy samples had colouration that was characteristic of the species, whereas pigmented samples displayed a distinct blue colouration in *A. millepora* and pink colouration in *Porites* sp. (Figure 6.1). Pigmented tissue sampled was generally associated with areas of wound healing. For protein and enzyme assays, samples were immediately snap-frozen in liquid nitrogen and stored at -20ºC. Samples for histology were fixed in 4% formaldehyde-seawater solution.

6.3.2 SAMPLE PROCESSING

For protein and enzyme assays, coral tissues were removed from the skeletons of all healthy and pigmented samples of *A. millepora* (n = 28 samples per category) and *Porites* sp. (n = 20 samples per category) using an airbrush (Panaache H2) and stored in 100 mmol.l^{-1} phosphate buffer with 5 mmol.l^{-1} 2-mercaptoethanol (Sigma-Aldrich M7522). Tissue adjacent to points of fragmentation caused by the sampling process was avoided. Tissue slurries were homogenised using a tissue homogeniser (Janke and Kunkel, IKA-Labortechnik) for one min, and freeze-dried to concentrate extracts and further disrupt cells. Samples were re-hydrated with 2 ml of molecular grade water (Sigma W4502) immediately before use.

6.3.3 BIOCHEMICAL ANALYSES

Ortho-diphenoloxidase (PO) activity was quantified by measuring the darkening of the colourless substrate L-DOPA to coloured dopachrome as a result of enzymatic oxidation as described in Chapter 2. For this assay, three 20 µl aliquots of each sample extract were placed in wells of a 96-well microtitre plate and left at room temperature for 20 min. Then 100 µl of molecular grade water (Sigma W4502) and 50 µl of 20 mmol.l^{-1} L-DOPA (3-(3, 4-dihydroxyphenyl)-L-alanine; Fluka 37830) in 100 mmol.l^{-1} phosphate buffer (pH 5.0) were added to each sample. The L-DOPA and extracts were tested for independent effects using controls. To quantify the oxidized product of (PO) activity, absorbance for each sample was read at 490 nm at time zero (immediately after the addition of L-DOPA) and after 30 min using a Multiskan Ascent plate reader (Labsystems, Finland). Data represent the linear phase of the reaction over 30 min and were normalised to mg protein for each sample. Protein concentration was determined using the Peterson's-Lowry total protein standard assay (Peterson 1977, Sigma-Aldrich TP0300) with BSA as a standard and performed

according to protocols provided by the manufacturer. Absorbance at 595 nm was measured for each sample using a Multiskan Ascent plate reader (Labsystems, Finland). PO activity between healthy and pigmented samples was compared using t-tests.

6.3.4 HISTOLOGICAL TISSUE COMPARISONS

Samples of healthy and pigmented tissue were collected from each of five *A. millepora* and five massive *Porites* colonies from Pioneer Bay, Orpheus Island. Samples were fixed in 4% formaldehyde-seawater solution, decalcified progressively in 3%-10% formic acid and stored in 70% ethanol. Histological samples were processed overnight in an automated tissue processor and embedded in paraffin wax. Wax blocks were sectioned at 5 μm and sections were stained with either Mayer's Haematoxylin and Young's Eosin-Erythrosin stain (H&E) or Fontana-Masson melanin stain, as described in Chapter 3.

Photographs were taken of the epidermal layer in the free body wall region in longitudinal sections of coral polyps for each *A. millepora* sample and in transverse sections of polyps for each *Porites* sample using an Olympus DP12 dedicated camera head mounted on an Olympus microscope. Counts of the symbiotic algae known as zooxanthellae, and melanin-containing granular cells per 100 μm^2 were conducted within fifteen randomly selected areas of gastrodermal tissue from both the healthy and pigmented tissue sections. The tissue area was calculated using imaging software (Image J). Mean numbers of zooxanthellae were compared statistically between healthy and pigmented samples of each genus and mean numbers of melanin-containing granular cells between healthy and pigmented samples of *Porites*. The non-parametric Mann Whitney U test was used in both cases, as assumptions of normality and homogeneity of variances were not met.

6.4 RESULTS

6.4.1 CHARACTERISATION OF PIGMENTED *ACROPORA MILLEPORA* TISSUE

PO activity was significantly greater in blue-pigmented tissues of *A. millepora* than in healthy tissues (Figure 6.2). This was indicated by a greater mean change in absorbance over time per mg protein (±SE; t = 2.212, P =0 .031). Conversely, the mean number of zooxanthellae per 100 μm^2 of gastrodermis (Figure 6.3) was three-fold greater in healthy tissues than in pigmented tissues of *A. millepora* (U = 7.0; P <0.01).

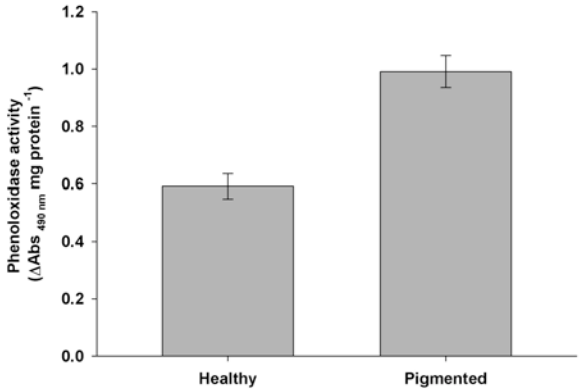


Figure 6.2: Comparison of mean (±SE) change in phenoloxidase activity, as measured by the oxidation of L-DOPA over time, between healthy and pigmented samples of *Acropora millepora* (n = 28; t = 2.212, P = 0.031).

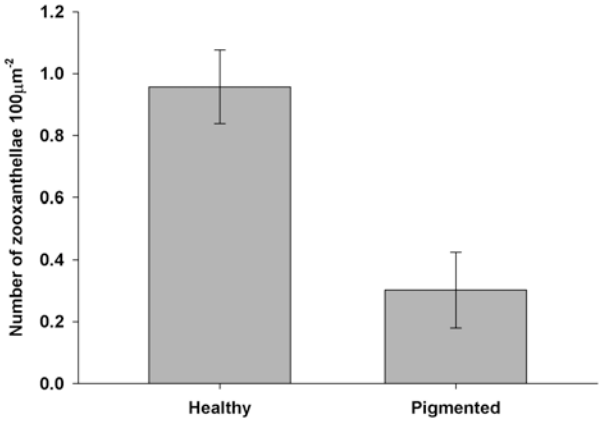


Figure 6.3: Comparison of mean (±SE) number of zooxanthellae per 100 μm^2 of gastrodermis between healthy and pigmented samples of *Acropora millepora* (n = 15; U = 7.0; P<0.01).

In healthy tissues of *A. millepora*, the two epithelial cell layers, the epidermis and gastrodermis, plus the connective layer, the mesogloea, were all present, and appeared normally structured and stained under H&E. Each contained intact diagnostic cells and structures, i.e. zooxanthellae in the gastrodermis and nematocysts in the free body wall epidermis (Figure 6.4a). All three layers were also discernible in the majority of pigmented tissue samples, however the mesogloea appeared reduced in thickness and tissue layers appeared highly granular (Figure 6.4b). Moreover, zooxanthellae densities were reduced and there were aggregations of unidentified oval cells in the epidermis. No melanin deposits were found to be located in the *A. millepora* tissue using the Fontana-Masson stain (Figure 6.4c).

6.4.2 CHARACTERISATION OF PIGMENTED *PORITES* SP. TISSUE

PO activity did not differ significantly between healthy and pigmented tissue samples of *Porites* (Figure 6.5), as demonstrated by a lack of significant difference in absorbance over time ($t = 0.479$, $P = 0.634$). Histological examination of *Porites* sp. tissues revealed the presence of granular cells in both healthy and pigmented tissues (Figure 6.4d and e). Both epithelial layers and the mesogloea were comparatively uniform in thickness and typical in appearance in healthy tissue sections (Figure 6.4d). In contrast, in pigmented sections (Figure 6.4e), the mesogloea was reduced in thickness, the epidermal cell layer was irregular and disrupted, and it contained an increased number of granular cells. Granules were stained black under the Fontana-Masson stain (Figure 6.4f), confirming that the granular cells contained melanin.

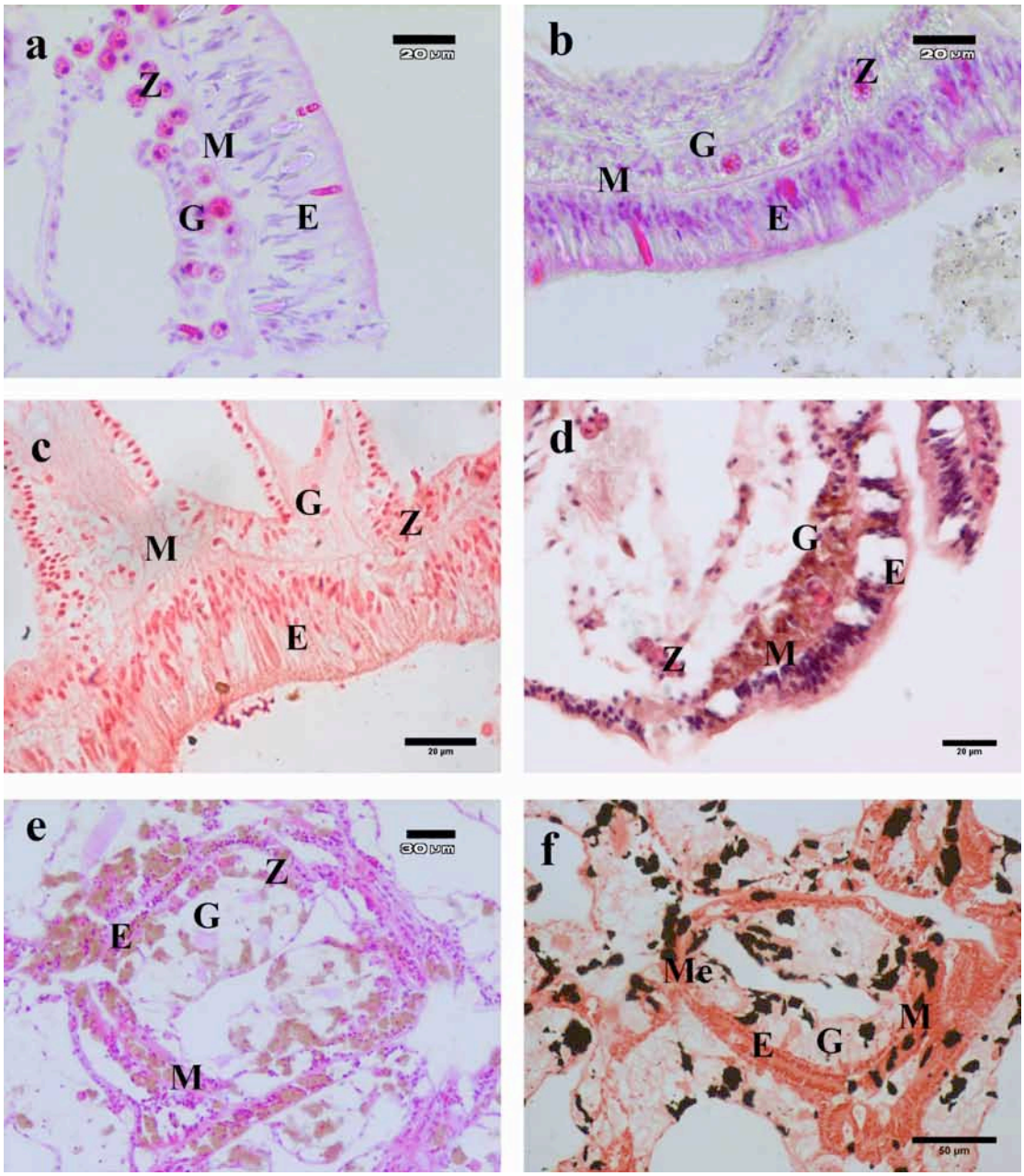


Figure 6.4: Histological sections showing (a) healthy free body wall epithelial layers of *Acropora millepora,* with abundant zooxanthellae in the gastrodermis (H&E); (b) pigmented free body wall epithelial layers of *A. millepora,* with granular cell layers and depleted numbers of zooxanthallae in the gastrodermis (H&E); (c) pigmented free body wall epithelial layers of *A. millepora* showing no melanin deposits (Fontanna Masson stain); (d) *Porites* free body wall epithelial layers showing pigment cells in the gastrodermis of a healthy sample; (e) *Porites* free body wall epithelial layers showing pigment cells in both the epidermis and gastrodermis of a pigmented sample; (f) *Porites* free body wall epithelial layers showing black stained melanin deposits (Fontana Masson stain) in the same pigmented sample as in e). *E, epithelium, G, gastrodermis, M, mesogloea, Z, zooxanthellae, Me, Melanin.*

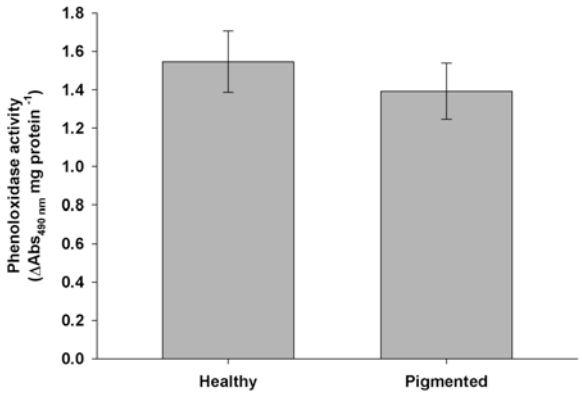


Figure 6.5: Comparison of mean (±SE) change in phenoloxidase activity, as measured by the oxidation of L-DOPA over time, between healthy and pigmented samples of *Porites* sp. (n = 20; t = 0.479, P = 0.634).

Comparisons of histological sections revealed that the mean number of zooxanthellae per 100 μm^2 of gastrodermis was more than two-fold greater in healthy (0.8 cells 100 μm^{-2} gastrodermis) than in pigmented (0.35 cells 100 μm^{-2} gastrodermis) samples (U = 669.0; P < 0.01; Figure 6.6). The mean number of granular cells per 100 μm^2 of tissue was more than four-fold greater in the epidermal layer of pigmented samples than in the epidermal layer of healthy samples (U = 52.5, P = 0.012), which corresponded to four times more melanin in pigmented tissues. In contrast, comparisons of gastrodermal tissue layers between healthy and pigmented samples of *Porites* sp. revealed no significant difference in the mean number of melanin-containing granular cells per 100 μm^2 of tissue (Figure 6.6), with the highest counts ranging between approximately 0.34 and 0.37 cells per 100 μm^2 (U = 73, P = 0.101). Overall, the lowest number of melanin-containing granular cells (<0.1 cells per 100 μm^2) was detected in the epidermal layer of healthy samples and the highest in the gastrodermal layer of healthy samples (0.38 cells per 100 μm^2). However, there was approximately 1.5 times the number of melanin-containing granular cells in the combined gastrodermal and epidermal layers of pigmented samples than in the combined epithelial layers of healthy samples (Figure 6.6).

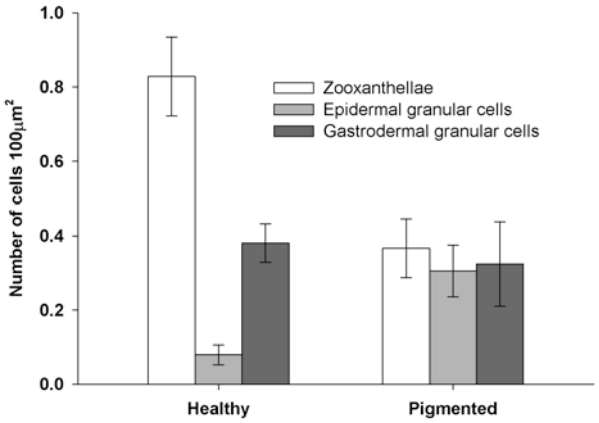


Figure 6.6: *Porites* healthy versus pigmented tissue comparisons for mean (±SE) densities of: zooxanthellae (n = 15; U = 669.0; P <0.01), epidermal granular cells (n = 15; U = 52.5, P = 0.012), and gastrodermal granular cells (n = 15; U = 73, P = 0.101).

6.5 DISCUSSION

Up-regulation of phenoloxidase (PO) activity in pigmented tissues of *Acropora millepora* and increased melanin-containing granular cells in pigmented tissues of *Porites* provide clear evidence that effector responses associated with innate immunity are up-regulated in corals with compromised health. The PO pathway is important in defence due to the production of cytotoxic intermediates (Nappi and Christensen 2005; Mydlarz and Harvell 2007) and can result in the formation of a physical barrier for encapsulation composed predominantly of melanin deposits (Rowley 1996; Petes et al. 2003). Although causes of pigmentation in tissues sampled in this study were unknown, associations with biochemical activities related to innate immunity suggest that pigmentation may be part of a generalised inflammatory-like response in these two species of corals.

6.5.1 CHARACTERISATION OF PIGMENTED *ACROPORA MILLEPORA* TISSUE

Symbiotic zooxanthellae are foreign organisms within coral tissues and therefore may be expelled locally as part of innate immune mechanisms activated in response to stress (Weis 2008). Low densities of zooxanthellae within the gastrodermal layer of pigmented tissues of *A. millepora* confirmed that their health was compromised (Weis 2008), or that zooxanthellae populations have not fully established (Dove et al. 2008). Increased PO activity, which has been shown to be

inducible in immunological studies of a variety of invertebrates as part of their resistance mechanisms (Cerenius and Söderhäll 2004; Munoz et al. 2006; Hellio et al. 2007; Butt and Raftos 2008), provided further evidence that pigmented tissues were immunologically challenged. Histologically, however, there was no evidence of cell infiltration indicative of an inflammation response, such as those that have been associated with phagocytic cellular responses in other anthozoans (Olano and Bigger 2000). Lack of cellular infiltration in immunologically challenged tissues of *A. millepora* provides support for speculations that cellular inflammation responses are not as well-developed in scleractinians as in gorgonians (Mullen et al. 2004; Vargas-Angel et al. 2007; Mydlarz et al. 2008). The lack of melanin-containing granular cells detected by the melanin-specific Fontana-Masson stain in *A. millepora* was unexpected, particularly as they have been previously observed within this species (Chapter 3). However, the acroporids had amongst the lowest number and size of these cells (Chapter 3), which, coupled with the relatively low sample size may be responsible for the lack of observation of melanin-containing granular cells in acroporids in this study.

6.5.2 CHARACTERISATION OF PIGMENTED *PORITES* SP. TISSUE

Similar levels of phenoloxidase activity in both the pigmented and healthy tissues of *Porites* sp. indicate a relatively high level of residual activity that could result in constant production and deposition of melanin in both tissue types. Histological investigations supported the comparatively constant PO activity found biochemically in *Porites* tissues, as melanin deposits were found in both the healthy and pigmented samples of *Porites* sp.. However, the 1.5-fold greater cell density of melanin-containing granular cells in pigmented tissues is consistent with these tissues being immunologically challenged. Since only activated PO was measured in this study, it is possible that the inactive form, prophenoloxidase, might be present in differing quantities and might provide a better indicator of melanin deposition in these two tissue types.

Differences in the distribution of melanin-containing granular cells in epidermal versus gastrodermal layers of *Porites* sp. between healthy and pigmented tissues are also consistent with their role in immunological defence. The four-fold greater density of melanin-containing granular cells in the epidermal layer of pigmented tissues compared to healthy tissues highlights their potential role in

defence, as the epidermal layer is the most exposed layer to an externally invading organism. In pigmented tissues, the high and approximately equal numbers of melanin-containing granular cells that were found in epidermal and gastrodermal layers suggests either their up-regulation or migration into the epidermal layer of compromised tissue, as would occur with amoebocytes accumulating in the external tissue layers as part of an inflammation response (Olano and Bigger 2000). It is hypothesised that melanin-containing granular cells in *Porites* sp. are involved in encapsulation, consistent with reports of the chromophore cells of *Porites compressa* (Domart-Coulon et al. 2006). These results support the role of melanisation and PO as part of a general inflammation-like response and highlight the potential for amoebocytes to be present within the tissue layers of scleractinian corals (see Chapter 3).

The significantly lower density of zooxanthellae in pigmented tissues than in healthy tissues is consistent with findings of Ravindran and Raghukumar (2006a, 2006b), and indicates that tissue health was potentially compromised in the pigmented samples of this study. Conversely, the presence of melanin-containing granular cells within the gastrodermis of healthy tissue may explain the relative tolerance of *Porites* sp. to thermal stress that results in bleaching in more sensitive coral genera (Brown 1997; Marshall and Baird 2000).

6.5.3 INTER-SPECIFIC COMPARISON

Although both *A. millepora* and *Porites* sp. demonstrate the presence and activation of PO associated pathways in tissues showing signs of compromised health (i.e. pigmentation combined with significantly reduced zooxanthellae densities), there are clear differences in the utilisation of the biochemical cascade between the two species. Differences in the characterisation of pigmented tissues between the two coral species suggest differing optimisation of the melanin pathway. *A. millepora* had more than two-fold lower constitutive levels of PO than *Porites* sp. and no observable melanin deposits or melanin-containing granular cells as putative amoebocytes (Domart-Coulon et al. 2006; Chapter 3). This is consistent with suggestions by Mullen et al. (2004) and Vargas-Ángel et al. (2007) that the use of cellular defences is variable among anthozoans. This not only supports a general lower resistance in *A. millepora,* as suggested in Chapter 5, but it can be hypothesised that *A. millepora* exploits the cytotoxic and non-cellular mechanisms of the PO cascade whereas

Porites sp. utilises a higher constitutive concentration of cellular PO and takes advantage of both the cytotoxic and barrier-forming potential of the PO cascade. The differences in the use of the PO cascade and cellular infiltration may relate to differences in constitutive disease and/or bleaching resistance, as in other marine invertebrates including oysters (Ford et al. 1993; Butt and Raftos 2008), clams (Allam et al. 2001) and crustaceans (Persson et al. 1987). Therefore the development of different immune mechanisms could be related to the differences in life history of these two corals (reviewed in Chapter 5).

The two genera *Porites* and *Acropora* have very disparate life history strategies, and undoubtedly partition energy differently among activities associated with growth, reproduction and maintenance (see Chapter 5). The continuous production of PO and melanin in *Porites* sp. requires a constant investment of energy with a corresponding reduction of resources available for growth and reproduction. Nevertheless, these PO levels would provide a continual level of resistance to infection, supporting observations of lower disease susceptibility for this genus on the Great Barrier Reef (Willis et al. 2004). In contrast, the more rapidly growing acroporids (Oliver 1985), which are one of the most susceptible families to both bleaching, (Brown 1997; Marshall and Baird 2000) and disease (Willis et al. 2004; Page and Willis 2006), see Chapter 5, have a lower level of residual PO and no melanin deposits. Such differences in immunity are particularly pertinent to the future biodiversity of coral reefs given the current rate of global climate change and the associated decline in coral health (Hoegh-Guldberg 1999; Hughes et al. 2003).

6.5.4 CONCLUSION

In summary, this study demonstrates the up-regulation of the melanin-synthesis pathway as part of an inflammatory-like immune response of scleractinian corals and demonstrates the involvement of melanin-containing granular cells within compromised tissue, thus supporting suggestions that they are amoebocytic (Chapter 3). This study also contributes to current understanding of how differential innate immune responses might contribute to ecological and life history differences among coral species.

Chapter 7 FLUORESCENT PROTEINS RESPONSIBLE FOR NON-NORMAL CORAL PIGMENTATION

7.1 ABSTRACT

Reports of coral disease have increased dramatically over the last decade; however, the biological mechanisms that corals utilise to limit infection and resist disease remain poorly understood. Compromised coral tissues often display non-normal pigmentation that potentially represents an inflammation-like response (Chapter 6), although these pigments remain uncharacterised. Using spectral emission analysis and cryo-histological and electrophoretic techniques, pink pigmentation associated with trematodiasis, an infection with larval stages of the trematode *Podocotyloides stenometre*, was investigated in *Porites compressa* from Hawai'i. Comparative studies of pigmented tissues of Great Barrier Reef corals further explored this inflammatory-like response. Relative abundance of non-fluorescent chromoprotein (considered a fluorescent protein because of its structure) within blue-pigmented tissues of *Acropora millepora* was compared to that of healthy samples. Similarly, the relative abundance of red fluorescent protein was compared between pink-pigmented and healthy tissues of massive *Porites* sp.. Spectral emission analysis revealed that pink-pigmented tissues of *P. compressa* fluoresce under blue light excitation (450 nm) and produce a broad emission peak at 590 nm (± 6). Electrophoretic separation of pigmented tissue protein extracts confirmed the red fluorescence to be a protein rather than a low-molecular-weight compound. Histological sections of *P. compressa* demonstrated green fluorescence in healthy tissue and red fluorescence in the trematodiasis-compromised tissue. The red fluorescent protein (FP) was limited to the epidermis, not associated with cells or granules, and appeared throughout the cell layer, potentially within interstitial spaces. Similarly, mean relative fluorescence of the red fluorescent protein was higher in pigmented than in healthy tissue of the massive *Porites* sp., and the non-fluorescent chromoprotein was higher in pigmented than healthy tissue of *A. millepora*, as determined by relative absorbtion at 588 nm. Consistently, pigmented tissue of both these GBR species demonstrated significantly greater hydrogen peroxide scavenging. These data collectively suggest that the red and the non-fluorescent chromoprotein FPs, which coincidently have the highest antioxidant capabilities, are produced and

localised in compromised coral tissue and therefore play a role within coral immune responses.

7.2 INTRODUCTION

Compromised tissues of anthozoans are frequently characterised by non-normal pigmentation (referred to as pigmentation henceforth; reviewed in Chapter 6) and descriptions of several anthozoan infections rely on the distinct macroscopic characteristics of this pigmented host response (Harvell et al. 2007). These distinct characteristics include pink swollen nodules of *Porites* spp., which are infected with larval trematodes of the species *Podocotyloides stenometre*, a condition called trematodiasis (Aeby 2003). Evidence that the melanin-synthesis pathway is associated with these pigmented areas of compromised tissue (Mydlarz and Harvell 2007) Chapter 7), combined with the frequency and diversity of non-normal pigmentation causes, suggest that this pigmentation in corals is part of a general immune response. Recently, the purple colouration of the sea fan was attributed to carotenoids that stain skeletal structures known as sclerites (Leverette et al. 2008), but the source of bright pigmentation associated with damaged or infected tissue of hard corals remains undetermined.

Fluorescent proteins (FPs) are largely responsible for the brightly coloured tissues found in many coral genera (Matz et al. 1999; Mazel et al. 2003), therefore it can be hypothesised that aggregations of specific FPs might cause the visible and distinct pigmentation commonly observed in coral tissues associated with a variety of competitive challenges or injuries. To date, four primary colour groups of FPs have been characterised from a variety of anthozoans (Matz et al. 1999; Ando et al. 2002; Sun et al. 2004; Leutenegger et al. 2007b; Alieva et al. 2008; reviewed in Chapter 4), and demonstrate differential antioxidant efficiencies (see studies of hydrogen peroxide (H_2O_2) scavenging described in Chapter 5). Antioxidant properties of FPs could serve an important function in corals, particularly in compromised tissues that may be under oxidative stress (Halliwell and Gutteridge 1999).

In this Chapter, I examine the potential involvement of FPs in the immune response of corals in three hard coral species for the first time. Pink tissue associated with the swollen nodules of trematodiasis infections (Aeby 2003) in *Porites compressa* presented a unique opportunity to investigate the characteristics of scleractinian coral tissue compromised by a known foreign organism, *Podocotyloides*

stenometre (Aeby 2003). Additionally, the presence of FPs and H_2O_2 scavenging activity was investigated in pink tissue associated with recovery from fish bites in a massive *Porites* sp. and blue tissue recovering from injury or an unknown interaction in *Acropora millepora.*

7.3 METHODS

7.3.1 SAMPLE COLLECTION

Six trematode-infected branches of *Porites compressa* (Dana, 1846; Figure 7.1) were collected from three colonies within Kāne'ohe Bay, O'ahu, Hawai'i, during June 2007. Branches were collected from similarly oriented areas of the colonies, and all were moderately infected with larval trematodes. Distinct infections were indicated by pink swellings surrounded by brown healthy tissue on individual polyps.

Figure 7.1: Pink pigmentation in response to larval trematode infections of *Porites compressa* from Kane'ohe Bay. (Photo credited to C. S. McKeon; used with permission).

During April 2009, three colonies of *Acropora millepora,* with distinct blue pigmentation as a result of physical breakage, and three *Porites* massive species with intense pink pigmentation were sampled in Pioneer Bay, Orpheus Island on the Great Barrier Reef (GBR). Equivalent areas of pigmented and healthy tissue were removed from each colony. GBR samples were snap-frozen in liquid nitrogen and stored at -30 ºC.

7.3.2 SPECTRAL EMISSION

For *P. compressa*, an airgun was used to blast areas of pigmented and healthy tissues from live branches (n = 3) into an extraction buffer containing 30 mmol.l^{-1} phosphate buffer and 5 mmol.l^{-1} 2-mercaptoethanol (Sigma-Aldrich M7522). Tissue slurries were homogenised and centrifuged for 7 min at 8050 x *g*. Samples were maintained at 4°C during processing. Three, 200 µl aliquots of the supernatant from each sample were placed in wells of a black (with transparent bottom) 96-well microtitre plate. Parallel 200 µl aliquots of extraction buffer were used to control for extract-independent effects. Each well was excited at 450 nm, using a

spectrophotometer (Spectramax M2, Molecular Devices), and the emission spectrum was measured in 6 nm increments. The data were normalised to the highest peak.

Tissue was removed from the frozen samples of *Porites* massive sp. and *A. millepora* using an airbrush (Paansche) and extraction buffer (50 mmol.l^{-1} phosphate buffer, pH 7.8 with 0.05 mmol.l^{-1} dithiothreitol) over ice. The tissue slurry was homogenised with a medium sawtooth (Fisher Scientific, Power Gen 125) for 20 s and left on ice for 5 min to extract the proteins. Samples were then vortexed with a spatula of glass beads for 20 s and left on ice for another 5 min. Tissue slurries were centrifuged at 4 ºC at x12300*g* for 5 min to remove the supernatant from the cellular debris and stored at -80 ºC until use.

Aliquots of 30 ml of each sample were added in triplicate to a black/clear 384-well microtitre plate with parallel aliquots of extraction buffer to control for independent effects. Samples of *Porites* massive sp. were excited at 540 nm and the emission between 570 nm and 590 nm recorded to measure relative red fluorescent protein (RFP) concentration. The endpoint absorbance at 588 nm was recorded for each well of *A. millepora* samples to measure the non-fluorescent chromoprotein (CP) concentration. Relative fluorescence and absorbance of each sample were standardised to total protein as detailed in Chapter 4. Mean total fluorescence in the red spectrum for samples of *Porites* massive sp. and mean CP absorbance at 588 nm for *A. millepora* (Alieva et al. 2008) were compared between healthy and pigmented samples using students t-tests, as assumptions of normality and homogeneity of variance were met.

7.3.3 HYDROGEN PEROXIDE SCAVENGING

Using the same tissue extract as described above for *Porites* massive sp. and *A. millepora*, 20 µl aliquots were added to triplicate wells of a 96-well UV transparent microtitre plate (Costar). To each well, 30 µl of phosphate buffer (pH 7.0, 0.05 M) and 50 µl of 50 mmol.l^{-1} H_2O_2 were added and the absorbance at 240 nm read immediately and every 31 s for 8 min. Sample blanks were used to control for independent sample effects. Mean scavenging rates of each species were compared using student t-tests.

7.3.4 CRYO-HISTOLOGY

Pigmented and healthy samples of *P. compressa* (n = 3 per category) were fixed in 4% paraformaldehyde phosphate buffer and decalcified in 20% disodium EDTA, pH 7.0, that was changed twice daily for 4 d. Samples were rinsed with deionized water and stored in 15% sucrose solution overnight at 4 °C, then transferred to optimum cutting temperature (OCT) compound. Samples were frozen onto histological chucks using OCT and isopropanol cooled with liquid nitrogen. Sections were cut at either 10 µm or 5 µm, using a cryo-stat at -30 °C. Sections were air dried on slides overnight and covered using aqueous cover-slip solution. Slides were observed and photographed using a Zeiss 510 laser scanning confocal microscope with excitations of 633 nm at 60%, 488 nm at 58%, and 543 nm at 100%. Filters used were LP 650, LP 505-550, BP 560-615.

7.3.5 GEL ELECTROPHORESIS

The supernatants of pigmented and healthy tissue extracts (obtained as described above) were analysed using polyacrylamide gel electrophoresis (10% resolving, 4% stacking gel). Auto-fluorescent protein bands were visualised and photographed on a Typhoon 8600 variable mode imaging system, at an excitation of 532 nm. An auto-fluorescent protein band was excised from the gel and excited at 450 nm in the spectrophotometer (Spectramax M2, Molecular Devices) to determine whether the band had the same spectral properties as the original sample extract.

7.4 RESULTS

7.4.1 SPECTRAL EMISSION OF *PORITES COMPRESSA*

The emission spectrum of pigmented tissues extracts of *Porites compressa* was distinct from that of the healthy tissue (Figure 7.2). The pigmented tissue extract had a broad emission peak at 590 ± 6 nm with a shoulder extending through 650 nm; this peak was absent from healthy tissue. Both healthy and pigmented tissue samples had a chlorophyll emission peak in the far-red (674 ± 6 nm).

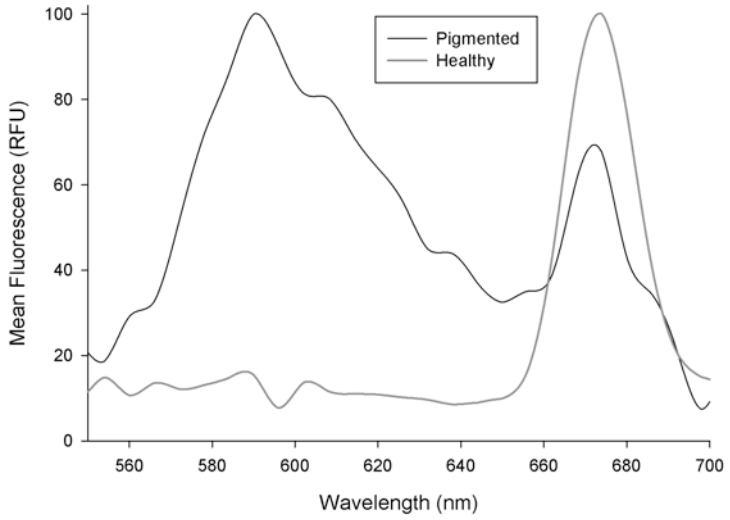


Figure 7.2: Representative emission spectra at an excitation of 450 nm for healthy and pigmented tissue extracts of *Porites compressa*

7.4.2 FLUORESCENCE AND H_2O_2 SCAVENGING OF *PORITES SP.* AND *ACROPORA MILLEPORA*

Mean relative fluorescence[2] of pigmented tissues of *Porites* massive sp. (Figure 7.3a) was significantly higher than that of healthy tissue ($T_{(2)} = 13.0$, $P = 0.048$). Additionally, the mean absorbance at 588 nm was significantly higher for pigmented tissue as compared to healthy tissue of *A. millepora* ($T_{(2)} = 4.0$, $P = 0.048$, Figure 7.3b). For H_2O_2 scavenging, there was a significantly higher activity for the pigmented tissues of both species (Figure 7. a and b) as compared to their respective healthy tissues ($t_{(4)} = 2.9$, $P = 0.047$ for *Porites* and $t_{(4)} = 3.6$, $P = 0.02$ for *A. millepora*). Additionally, the H_2O_2 scavenging activity of *Porites* pigmented tissue was 10-fold that of the pigmented tissue of *A. millepora*.

[2] Note the difference in the protocol for measuring fluorescence between Hawaiian corals and those from the Great Barrier Reef. This is because studies on Hawaiian corals were conducted prior to the development of standardisation protocols described in Chapter 4.

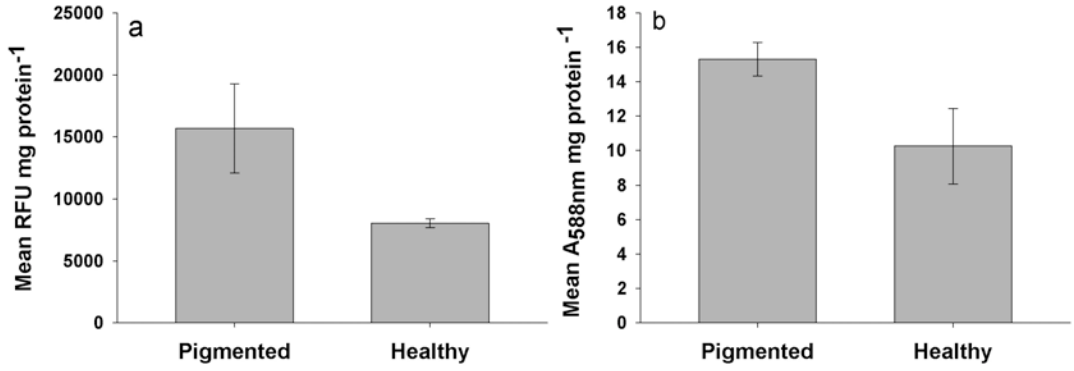


Figure 7.3: The mean relative FP concentration (± SE) in pigmented and healthy tissues for: a) red FP (Ex. 540 nm, Em. 570 nm to 590 nm) in a massive *Porites* sp. ($T_{(2)} = 13.0$, P = 0.048) and b) CP (absorbance at 588 nm) in *Acropora millepora* ($T_{(2)} = 4.0$, P = 0.048).

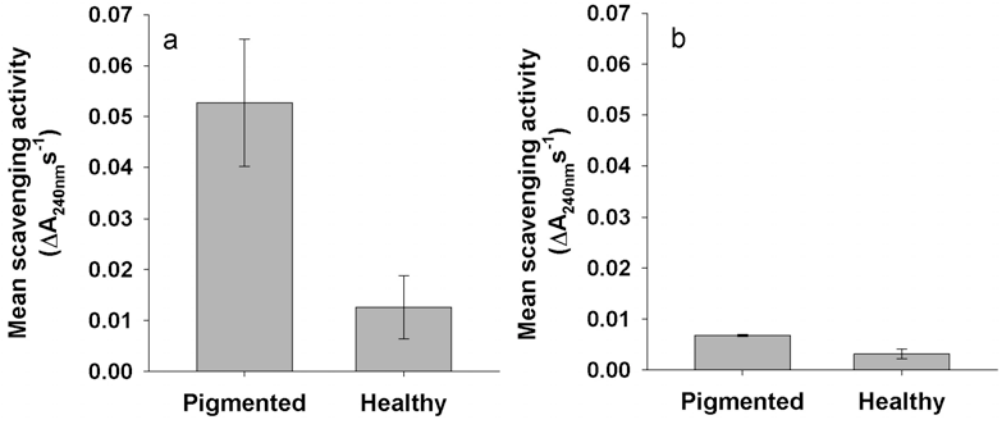


Figure 7.4: The mean H_2O_2 scavenging activity (± SE) of pigmented tissue and healthy tissue of: a) a massive *Porites* sp. ($T_{(4)} = 2.9$ P = 0.047), and b) *Acropora millepora* ($T_{(4)} = 3.6$, P = 0.02).

7.4.3 CRYO-HISTOLOGY

Confocal microscopy of pigmented and healthy tissue cryo-sections of *P. compressa* showed green fluorescence within the coral tissue. The pigmented tissue (Figure 7.5a) had a high density of melanin-containing granular cells in a relatively enlarged gastrodermal layer, which was devoid of zooxanthellae. Melanin-containing granular cells were also present in high densities within the epidermis of the pigmented tissue, although they were partially obscured by an unstructured red fluorescent substance. Both the epidermal and gastrodermal layers of healthy tissue (Figure 7.5b) also had melanin-containing granular cells, although they were in lower

abundance in healthy compared to pigmented tissues (data not shown). The gastrodermis of healthy tissues contained many zooxanthellae and did not display red fluorescence.

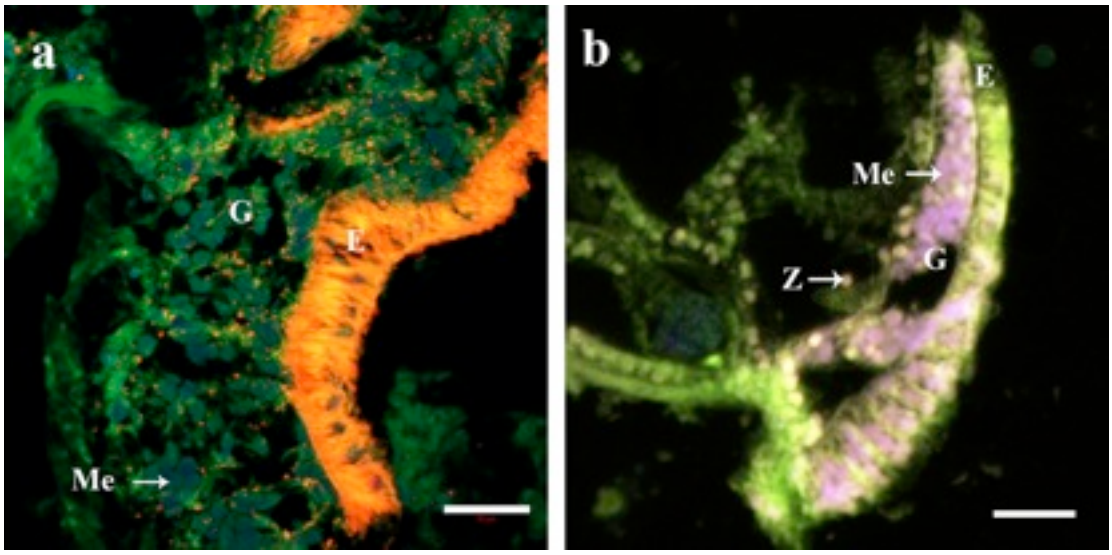


Figure 7.5: Autofluorescence of longitudinal sections of *Porites compressa* tissue. a) Pigmented tissue section (5 µm). b) Healthy tissue (10 µm). *E,* epidermis*, G,* gastrodermis, *Z*, zooxanthella*, Me,* melanin. Scale bars represent 50 µm.

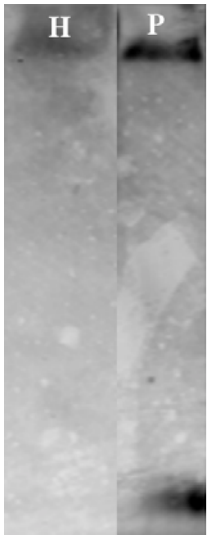


Figure 7.6: Auto-fluorescence image at 532 nm excitation of acrylamide gel with lanes of healthy (H) and pigmented (P) tissue extract.

7.4.4 GEL ELECTROPHORESIS

Electrophoretic separation of proteins resolved two bands in the pigmented tissue extracts and one band in the extracts of healthy tissue (Figure 7.6). The emission spectrum of the additional band from the pigmented tissue, although considerably less intense, was equivalent to that of the raw pink tissue extract, displaying the peak at 590 nm when excited with 450 nm (data not shown).

7.5 DISCUSSION

Red fluorescence (at 590 nm ± 6) of pigmented tissues associated with trematode infections in *Porites compressa* when excited with blue light (450 nm) indicates the presence of red fluorescent proteins (RFP) in compromised coral tissue. Similarly, pink pigmented tissues of *Porites* massive sp. had significantly higher relative fluorescence than healthy tissue. Blue pigmented tissues of *Acropora millepora* demonstrated greater absorbance, which corresponds to the non-fluorescent chromoprotein (CP; Alieva et al. 2008), than healthy tissues. This is the first demonstration that FPs are responsible for the distinct pigmentation of compromised coral tissue and supports their potential involvement in anthozoan immune responses (Chapters 4).

The highly localised RFP and CP in compromised, pigmented tissues (Willis et al. 2004), tentacle tips and in colony extremities (Dove et al. 1995; Shagin et al. 2004) of corals and anemones, supports the role of coral FPs as antioxidants (Chapter 4). Pigmented tissue of both *A. millepora* and the massive *Porites* sp. had comparatively higher concentrations of the FPs and higher H_2O_2 scavenging activity than the equivalent healthy tissue. This coincidence of high FP concentration and antioxidant activity supports the previous evidence that FPs have the ability to scavenge H_2O_2 *in vivo* (Chapter 4) and also alludes to the biological significance of FPs as part of innate immunity. Furthermore, these observations demonstrate that FPs with high H_2O_2 scavenging efficiency, CP and RFP, are preferentially up-regulated in tissue that is compromised or in frequent contact with foreign organisms and supports the suggestion that different FPs have different roles within the coral holobiont (Kelmanson and Matz 2003; Chapter 4).

In support of the hypothesis that different FPs have different functions (Kelmanson and Matz 2003; Shagin et al. 2004), the histological sections of *P. compressa* demonstrated different locations of green and red FPs. Healthy tissues showed heavy localisation of GFP in the gastrodermis and a complete lack of RFP, as confirmed by the absence of an emission peak within the red spectrum. In contrast, the compromised tissue had highly reduced GFP in the gastrodermis and high red fluorescence in the epidermis, again as confirmed in the emission spectrum. Furthermore, the highly reduced number of symbionts in the gastrodermal layer directly underlying the RFP suggests its ineffectiveness as a photoprotector, in

contrast to previously proposed hypotheses of anthozoan FP function (Salih et al. 1998; Salih et al. 2000). In addition, the location of the RFP in the epidermis and an emission wavelength that is not effective for photosynthesis (Levy et al. 2003) do not support a photo-enhancing, screen-scattering role (Salih et al. 2000) for the RFP. This further supports the role of FPs as antioxidants within compromised coral tissue.

The cryo-histology reveals that the RFP in *P. compressa* is coincident with an increase in melanin-containing granular cells, which is consistent with findings of Chapter 6. The presence of these cells in both the epidermal and gastrodermal layers of compromised tissue further suggests that the role of RFPs in these tissues is unlikely to be that of a photo-protector because melanin has highly effective light-absorbing properties (Meredith et al. 2006). Furthermore, the presence of melanin-containing granular cells, which have the potential to produce reactive oxygen species (Nappi and Ottaviani 2000), reinforces the necessity of increased antioxidant activity in these compromised areas of tissue (Halliwell and Gutteridge 1999).

7.5.1 CONCLUSION

Overall, these results demonstrate the direct relationship between coral immune responses and the localised up-regulation of efficient H_2O_2-scavenging FPs. This indicates a role of coral FPs within immune responses, potentially as antioxidants. However, investigations into the temporal regulation of FPs during a coral immune response would provide more information as to their specific role within anthozoan immunity.

Chapter 8 HETEROGENEOUS IMMUNE RESPONSES OF CORAL COLOUR MORPHS

8.1 ABSTRACT

Diversity of colour is commonly observed in reef corals, even among conspecifics within a single location, and is primarily due to the type and concentration of fluorescent proteins (FPs) present within coral tissues. The biological role of colour in the majority of corals remains undetermined, although differences in competitive ability and bleaching susceptibility suggest that colour relates to functional differences among colour morphs. In this chapter, I test whether colour morphs of the common reef coral *Porites cylindrica* have differing tissue concentrations of FPs and whether such differences relate to varying immune responses. Specifically, temporal regulation of coral immunity indicators, including tyrosinase-type phenoloxidase (PO), laccase-type PO, transglutaminase, peroxidase and H_2O_2 scavenging activity, in addition to FP concentration, were investigated within brown, yellow and green colour morphs of *P. cylindrica* in response to physical injury. Evidence that corals used these immunity pathways in wound healing confirmed that these basal invertebrates have mechanisms of innate immunity equivalent to those of other invertebrates. Colour morphs differentially regulated immunity parameters post-injury, potentially demonstrating variation in life history trade-offs within a species that may contribute to differences in the abundance of colour morphs found in the reef community. Overall, this study provides the first temporal comparison of a coral immune response to injury and confirms functional differences among conspecific colour morphs, posing intriguing questions as to how and why these differences evolved.

8.2 INTRODUCTION

Within mobile metazoan phyla, pigmentation and colour polymorphism is frequently attributed to sexual signalling and camouflage (Protas and Patel 2008), with temporal variations in colour associated with crypsis and social signalling (Protas and Patel 2008; Stuart-Fox and Moussalli 2008). Differential pigmentation has also been implicated in immunocompetence (Roulin 2004; Bonato et al. 2009), due to the generally positive relationship between physical condition and immune capability.

However, in sessile, spawning invertebrates that lack ocular faculties, such as corals, reasons for the presence of, and variation in, pigmentation are unclear, but presumably differ from those of higher organisms. It has been suggested that the diversity of coral colour and associated fluorescence has evolved in response to requirements of the obligate algal endosymbionts (Field et al. 2006), which is supported by the differing symbiont clades found within colour morphs of *Madracis pharensis* (Frade et al. 2008). Within conspecifics, it has also been proposed that colour polymorphism (Dove et al. 1995; Mazel et al. 2003) is due to phenotypic plasticity in response to a variety of environmental conditions (Kelmanson and Matz 2003). Furthermore, it has been proposed that fish detect and distinguish between coral tissue colours, which may enable the coral to influence predation (Ward 2002), for example to encourage removal of polyps infected with parasites (Aeby 2002).

Variation in the distribution and abundance of coral colour morphs suggests that there are physiological differences among them that are reflected in their general ecology. For example, pink morphs of *Pocillopora damicornis* were found to be more prevalent than their brown conspecifics in deeper water where light intensity is lower, and the pink morph had greater competitive ability in direct interactions (Takabayashi and Hoegh Guldberg 1995). Also, colour morphs of *Montipora monasteriata* are found within varying light environments (Dove et al. 2006a; Dove et al. 2008), which further suggest a relationship between colour and distribution across various habitats. The greater abundance of green morphs of *Porites astreoides* in shallow water compared to brown morphs is explained by the consistently greater tolerance of green morphs to UV-radiation (Gleason 1993). These light-related distribution patterns among colour morphs suggest physiological benefits related to the presence of FPs.

Coral FPs are up-regulated in compromised tissue (Chapter 7), which supports their role as antioxidants (Chapter 4) during coral immune responses. In particular, differing H_2O_2 scavenging efficiencies among FP colour types (Chapter 4) may affect the efficacy of a coral's immune response, as determined by various immunity parameters (Section I), therefore the relative abundance and distribution of coral colour morphs may be related to their immunocompetence. However, the regulation of immunity parameters following physical injury or infection has not been investigated within hard corals, or among colour morphs.

Hard corals frequently suffer physical injury and, moreover, fragmentation is an important component of the life histories of many species (Highsmith 1982; Willis

and Ayre 1985). In order to recover from an injury, whether caused by storm activity, predators or boring organisms (Rotjan and Lewis 2005), corals need to immediately activate an effective immune response. Inducing a physical injury on coral fragments (genetic replicates) not only provides a standardised method to induce an immune response, but also enables relative immunocompetence to be directly assessed as samples before (control) and after treatment can be taken (Adamo 2004b).

Coral colour morphs indicate the presence of different types and concentrations of FPs (Dove et al. 2001). As FPs are involved in coral immune responses, potentially as antioxidants for preventing self-harm, this may indicate trade-offs in the regulation of differing immunity parameters during wound healing. This study therefore aimed to investigate potential differences in the regulation of immunity parameters, including FPs, tyrosinase-type and laccase-type POs, transglutaminase, H_2O_2 scavenging and peroxidase activity, during wound healing among the brown, yellow and green colour morphs of the scleractinian coral *Porites cylindrica*.

8.3 METHODS

8.3.1 ABUNDANCE SURVEYS

Surveys of colony size and abundance of *Porites cylindrica* were conducted in Pioneer Bay, Orpheus Island, on the Great Barrier Reef (GBR) during April 2008. To determine the relative abundance of each of three distinct colour morphs, brown, yellow and green, within the Pioneer Bay population, the number of colonies of each colour morph was recorded within three 10 x 1 m belt transects. Transects were more than 5 m apart and were laid haphazardly on the upper reef slope, at a depth of between 3-5 m, parallel to depth contours. Colonies were defined as the set of attached branches that represented a physiological unit as a consequence of continuous tissue connections. Additionally, the number of branches per colony, to the nearest 5, was estimated as a measure of colony size. Size categories were as follows: small had 5 to 20 branches, medium had 25 to 75 branches and large had 80 or more branches. Relative abundance was calculated as the proportion of each colour morph out of the total number of *P. cylindrica* colonies per transect, to account for variation in the number of colonies among transects. The mean relative abundance of colour morphs was compared to expected values using a Chi-squared test. One-way

ANOVAs were used to compare the mean abundance and mean number of branches among colour morphs. A two-way ANOVA was used to compare the mean number of colonies per transect among different colour morphs and size classes. All assumptions of normality and homogeneity of variance were satisfied as determined using Shapiro-Wilk's and Levene's tests respectively.

8.3.2 SAMPLE COLLECTION AND AQUARIA SET-UP

Four colonies of each colour morph of *P. cylindrica* were haphazardly located and tagged, although all colonies were located at the same depth and were at least 5 m apart to increase the likelihood of sampling distinct genotypes. For macroscopic observations of wound healing, three branches were removed from one colony of each colour morph using bone-cutters. From the remaining three colonies of each colour morph, nine branches were removed and placed into individual zip-lock bags with ample seawater to be used in the immune response experiment. These branches were immediately transported to shore in shaded buckets of seawater and divided randomly among three shaded aquaria supplied with a constant flow of unfiltered seawater at Orpheus Island Research Station. Branches were held vertically using labelled clothes pegs cable-tied to small terracotta tiles.

8.3.3 MACROSCOPIC OBSERVATIONS OF WOUND HEALING

Three healthy branches from one colony of each colour morph were photographed under a dissecting microscope using a dedicated camera head (Olympus). Photographs were taken with both white and blue light (450 nm), the latter in conjunction with a blue light filter. Branches were placed in open-air aquaria with flow-through unfiltered seawater and a wound was created longitudinally along each branch using a scalpel blade. The wounds on each branch were photographed under the dissecting microscope at 1 h and 24 h post-injury.

8.3.4 WOUNDING EXPERIMENT

After the initial sample collection, branches from three colonies per colour morph were allowed to acclimatise for two days in the aquaria. On the third day, a branch from each of the three colonies per colour morph was snap-frozen in liquid nitrogen. A ring of injury, approximately halfway up each branch, was created using bone-cutters on the remaining branches. Three branches of each colour morph were

subsequently snap-frozen in liquid nitrogen at 1 h and 24 h post-injury. All samples were stored at -30 ºC.

8.3.5 BIOCHEMICAL ASSAYS

Tissue was removed from each branch using an air-blow gun kit (Spear and Jackson, Australia) with extraction buffer (50 mmol.l^{-1} phosphate buffer, pH 7.8, with 0.05 mmol.l^{-1} β-mercaptoethanol), on ice (Mydlarz et al. 2008). The resulting tissue slurry was vortexed with glass beads for 30 s and incubated on ice for 5 min before centrifugation at 3, 500 RPM at 4 ºC for 5 min. The supernatant (sample extract) was stored at –30 ºC. Phenoloxidase (PO) activity (tyrosinase-type and laccase-type) and transglutaminase activity were determined as described in Chapter 2. Peroxidase activity and hydrogen peroxide (H_2O_2) scavenging activity were determined as described in Chapter 4 and the fluorescence of each sample was determined and standardised as described in Chapter 4.

All biochemical assays were standardised to the total protein concentration (mg protein) of each sample, as determined using Peterson's Lowry total protein standard assay kit (Sigma-Aldrich TP0300). Enzymatic data represent the linear phase of reaction over 0 to 45 min. Mean activities were compared using repeated measures two-way MANOVAs, as assumptions of sphericity using the Mauchly criterion were met or corrected using the Geisser and Greenhouse correction (Schwertman 1978) using JMP Statistical Discovery Software version 8.0 (SAS Institute Inc., Cary, NC). H_2O_2 scavenging activity and transglutaminase activity data were log transformed to satisfy these assumptions. To assess similarities in the immune response among the colour morphs, a Principal Components Analysis (PCA) was conducted on the mean of each immunity variable for all three colour morphs using PRIMER 6.0. Graphs were plotted using Sigma Plot 11.0.

8.4 RESULTS

8.4.1 RELATIVE ABUNDANCE OF COLOUR MORPHS

Colour morphs of *Porites cylindrica* were unequally distributed at the survey site (X^2 = 6.49, P = 0.03) and they also differed significantly in their overall mean abundance ($F_{(2, 8)}$ = 5.95, P = 0.04; Figure 8.1a). In contrast, colony size, as indicated by the mean number of branches per colony, was similar among colour morphs ($F_{(2, 95)}$ = 1.15, P = 0.32) and this pattern was consistent across all size categories ($F_{(4, 18)}$ = 2.6, P = 0.07; Figure 8.1b). The smallest size category represented the highest proportion of colonies in all three colour morph populations ($F_{(2, 18)}$ = 22.4, P < 0.001). All colour morphs demonstrated normal population distributions (Shapiro-Wilk's statistic P > 0.05). The brown colour morph had the most symmetrical distribution with the lowest skewness value (2.19) and the yellow colour morph was the most asymmetrical with the highest skewness value (3.08).

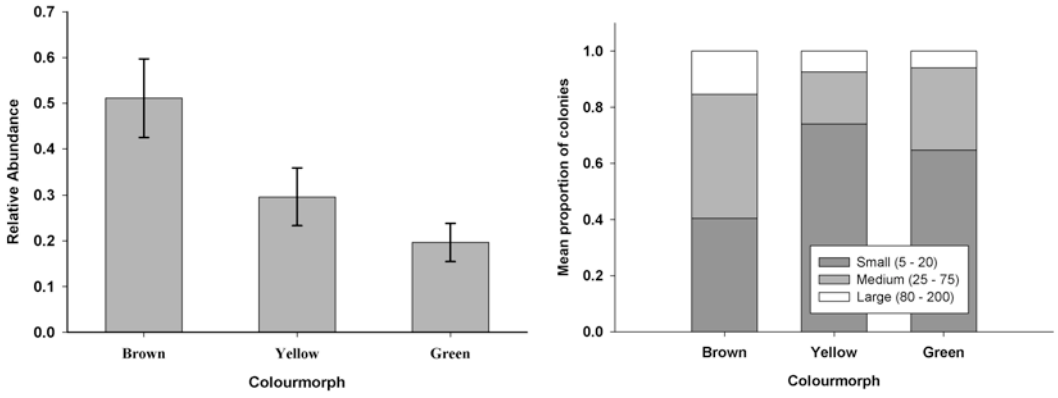


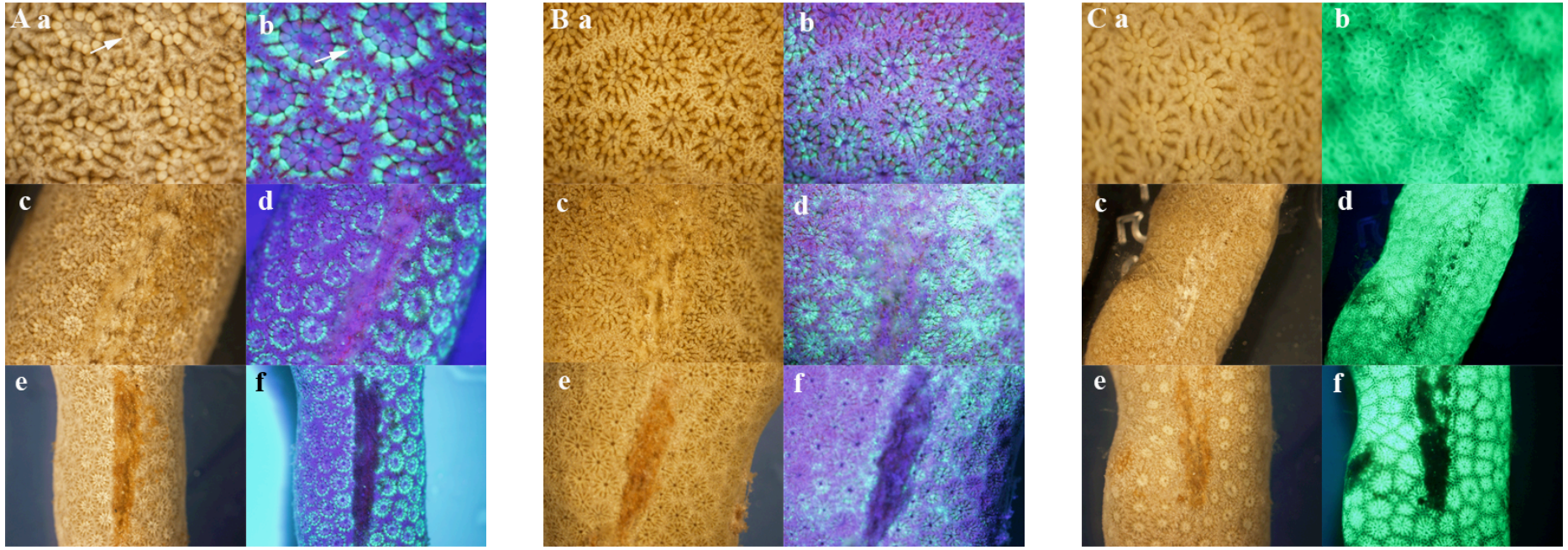
Figure 8.1: Comparisons among three colour morphs of *P. cylindrica* of: a) mean relative abundance (± SE), and b) relative abundance within small, medium and large size classes in Pioneer Bay, central Great Barrier Reef (n = 97 colonies).

8.4.2 MACROSCOPIC OBSERVATIONS OF WOUND HEALING

All healthy samples of *Porites cylindrica* appeared light brown under artificial white light, probably because of the low intensity and short wavelength spectrum of artificial light compared to daylight (Figure 8.2A, B and C). The retracted tentacles appeared lighter than the surrounding oral disc and coenosarc tissues, and apical areas of tissue covering the corallite walls as well as some distal surfaces of the polyps had

dark spots, which were also detectable with blue light (Figure 8.2Ab). Under blue light, the healthy tissue of the brown colour morph had distinct rings of green fluorescent tissue along the polyp walls, which extended into the tentacles in some areas. Red fluorescence was observable between the tissue folds, on the tentacles and around the polyp mouths. At 1 h post-injury, polyp tissues and upper layers of skeleton were visibly disrupted in the brown colour morph (Figure 8.2Ac) and the wound fluoresced red (Figure 8.2Ad). After 24 h, the wound area appeared dark brown under white light (Figure 8.2Ae), and this area absorbed blue light (Figure 8.2Af). The yellow colour morph (Figure 8.2B) demonstrated a similar healthy tissue appearance and response to injury as the brown colour morph. However, the green fluorescent rings around the polyps were more variable and there was a higher amount of red fluorescence throughout the tissue than in the brown colour morph.

Healthy tissue of the green colour morph appeared light brown under white light and was similar in appearance to the other colour morphs (Figure 8.2Ca). However, in contrast to the other morphs, the green morph demonstrated a high intensity of green fluorescence throughout its tissues (Figure 8.2Cb). The dark spots of on the apexes of the corallite walls appeared black and no red fluorescence was observable. At 1 h post-injury, the wound appeared pale under white light (Figure 8.2Cc), however the wound area had dark patches between the green fluorescence under blue light (Figure 8.2Cd). After 24 h, the wound site appeared brown under white light and strongly absorbed blue light, similar to the response observed for the brown and yellow colour morphs (Figure 8.2Ce and f).



Figures 8.2: Low-power microscope photos of *P. cylindrica* comparing tissue appearance among the brown (A), yellow (B) and green (C) colour morphs, when exposed to white light (left panels) and blue light, 450 nm, (right panels). Within each panel, tissue appearance is compared between: healthy branches (a and b), branches 1 h post-injury (c and d), and 24 h post-injury (e and f), White arrow heads indicate dark spots. To indicate the scale of each photograph, the mean corallite diameter was 1.2 mm.

8.4.3 FLUORESCENT PROTEINS OF COLOUR MORPHS

All colour morphs demonstrated a trend for increasing mean total fluorescence with time post-injury (Figure 8.3), however statistically this pattern was not consistent among colour morphs (Wilks' Lambda = 0.035, $F_{morph\ x\ time\ (4, 10)} = 10.9$, P = 0.001). The greatest increase was for the green colour morph, with total fluorescence increasing approximately 8-fold by 24 h post-injury compared to values prior to injury (0 h). Although mean total fluorescence increased 3-fold and 6-fold over this same time period in the brown and yellow colour morphs respectively, the significant interaction term indicates that the much smaller increases for these morphs were not statistically significant, potentially because of high variability among replicate samples. However, because trends in mean total fluorescence were consistent for all three colour morphs through time (Figure 8.3a), it is possible to interpret the main effect for colour morph in the analysis, despite this significant interaction effect, and conclude that there was a significant difference in mean total fluorescence among them ($F_{morph\ (2, 6)} = 55.7$, $P < 0.001$). Overall, the green colour morph had the highest mean total fluorescence at all time points compared to the brown and yellow colour morphs, which were approximately equivalent (Figure 8.3a). This difference was particularly notable at 24 h post-injury when the green colour morph demonstrated approximately 11-fold higher total fluorescence than either the brown or yellow colour morphs.

Of the constituent FPs (cyan, green and red), a similar trend of increasing fluorescence with time post-injury was observed (Figures 8.3b, c, and d respectively). Both the cyan and green fluorescence demonstrated a significant interaction between time and colour morph (Wilks' Lambda = 0.041, $F_{morph\ x\ time\ (4, 10)} = 9.90$, P = 0.002; Wilks Lambda = 0.02, $F_{morph\ x\ time\ (4, 10)} = 15.27$, $P < 0.001$, respectively), similar to the interaction described above for total fluorescence. Thus fluorescence of both FPs increased more than 6-fold through time in the green morph, but this was not statistically significant for the other two morphs. For the red FP, however, fluorescence increased significantly through time for all morphs consistently ($F_{time\ (2, 5)} = 7.99$, P = 0.03). This increase was particularly notable for the brown colour morph, which increased red fluorescence 15-fold by 24 h compared to initial levels, whereas the yellow and green colour morphs both increased by nearly 6-fold (Figure 8.3d).

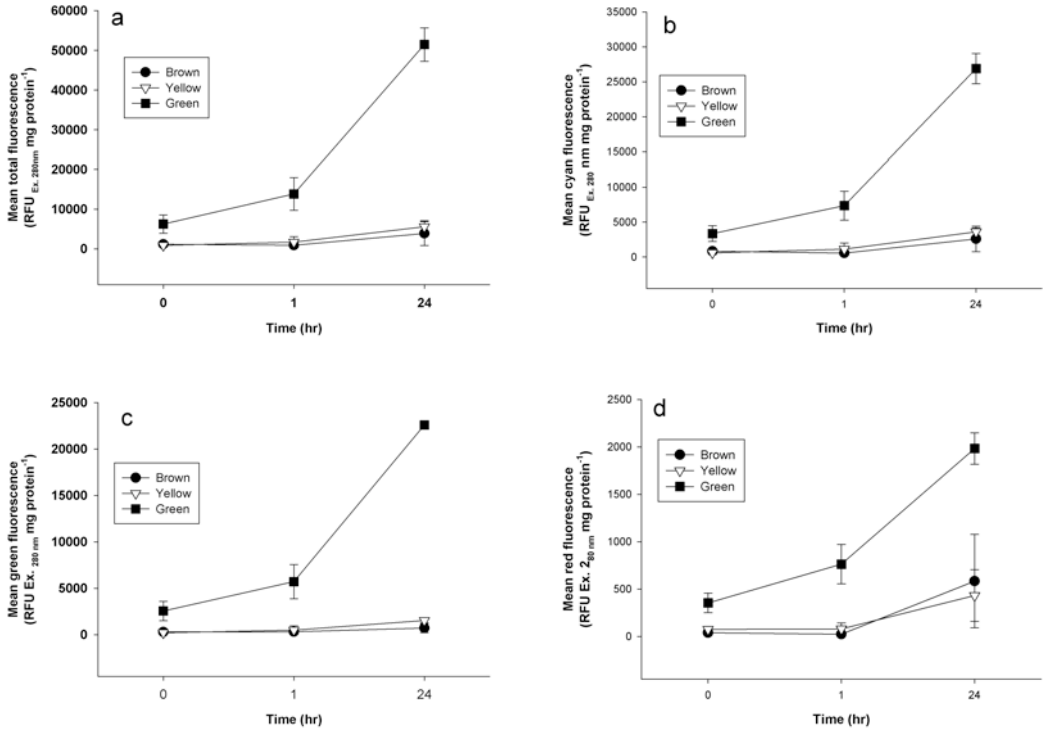


Figure 8.3: Mean fluorescence (± SE) of each colour morph at time = 0 h (control), 1 h post-injury and 24 h post-injury for: a) total fluorescence (465 nm to 600 nm), b) cyan, RFU summed across 465 nm to 500 nm, c) green, RFU summed across 505 nm to 545 nm, d) red, RFU summed across 550 to 600 nm.

8.4.4 IMMUNITY PATHWAY ACTIVITY OF COLOUR MORPHS

Patterns in tyrosinase-type PO activity over time, as measured by *o*-diphenoloxidase activity (Figure 8.4a), differed among the three colour morphs (Wilks' Lambda = 0.023, $F_{\text{morph x time (4, 10)}} = 13.84$, $P < 0.001$). Whereas PO activity increased in the green colour morph by approximately 6-fold between 0 h and 24 h, the brown and yellow colour morphs demonstrated decreasing activity with time (Figure 8.4a).

In contrast, patterns in laccase-type PO activity (Figure 8.4b), as measured by *p*-diphenoloxidase activity, were consistent through time for all three colour morphs (G-G Epsilon = 0.51, $F_{\text{morph x time (2, 6.1)}} = 3.4$, $P = 0.102$). In each case, laccase-type PO activity increased significantly through time (G-G Epsilon $F_{\text{time (1, 6)}} = 8.42$, $P = 0.03$), most notably for the yellow colour morph, which had a mean increase of approximately 34-fold between 0 h and 24 h. Increases in laccase-type PO activity for

the brown and green colour morphs between 0 h and 24 h were approximately equivalent, at between 3 and 4-fold the levels prior to injury.

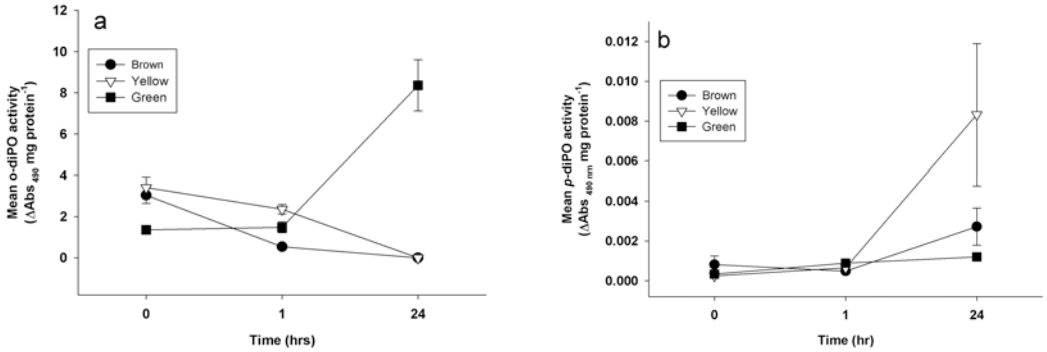


Figure 8.4: Mean phenoloxidase (PO) activity (± SE) at each time point for each colour morph: a) tyrosinase-type PO activity, and b) laccase-type PO activity.

Transglutaminase activity differed through time among the three colour morphs (Figure 8.5; Wilks' Lambda = 0.14, $F_{\text{morph x time } (4, 10)} = 4.23$, P = 0.03). Although overall the colour morphs demonstrated a general trend of increasing transglutaminase activity with time post-injury, the yellow and green morphs demonstrated an increase between 0 h and 1 h post-injury, whereas the brown morph decreased. However overall, between 0 h and 24 h, transglutaminase activity of the brown colour morph increased by nearly 7-fold, as compared to the yellow and green colour morphs which increased by over 5-fold and 4-fold respectively (Figure 8.5).

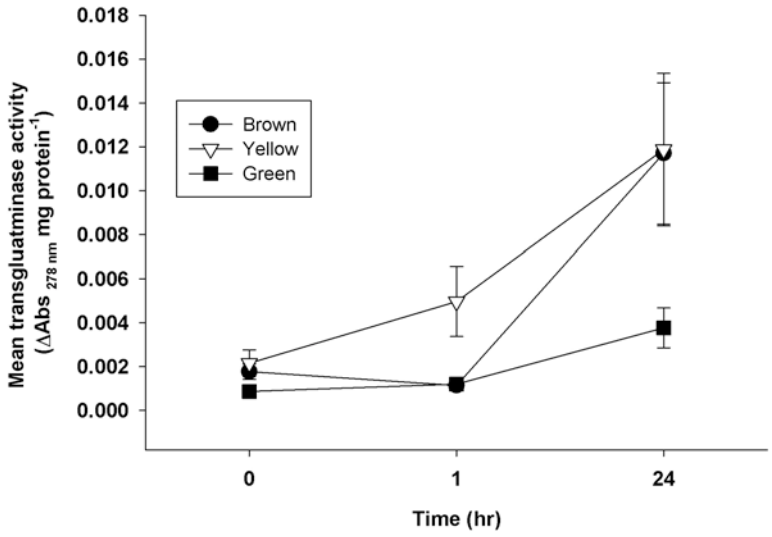


Figure 8.5: The mean transglutaminase activity (± SE) at each time point for each colour morph.

8.4.5 ANTIOXIDANT ACTIVITY OF COLOUR MORPHS

The mean peroxidase activity (Figure 8.6a) differed over time among the three colour morphs (Wilks' Lambda = 0.03, $F_{\text{morph x time (4, 10)}} = 11.3$, P = 0.001). The brown and green colour morphs showed an increase in peroxidase activity with time post-injury and the yellow colour morph demonstrated a slight decrease (Figure 8.6a). The peroxidase activity of the green colour morph at 24 h post-injury was 59-fold higher than the yellow morph and 7-fold higher than the brown morph at the same time point.

Hydrogen peroxide (H_2O_2) scavenging activity significantly increased over time post-injury for all colour morphs (Figure 8.6b; Wilks' Lambda = 0.68, $F_{\text{morph x time (4, 10)}} = 0.53$, P = 0.72; $F_{\text{time (2, 5)}} = 6.61$, P = 0.04). Between the controls at 0 h to 1 h post-injury there was an increase of between 1 and 2-fold for each colour morph. However, between 0 h and 24 h post-injury, the brown colour morph increased by over 60-fold, which was comparably more than that of the other colour morphs which increased by approximately 4-fold over the same time period.

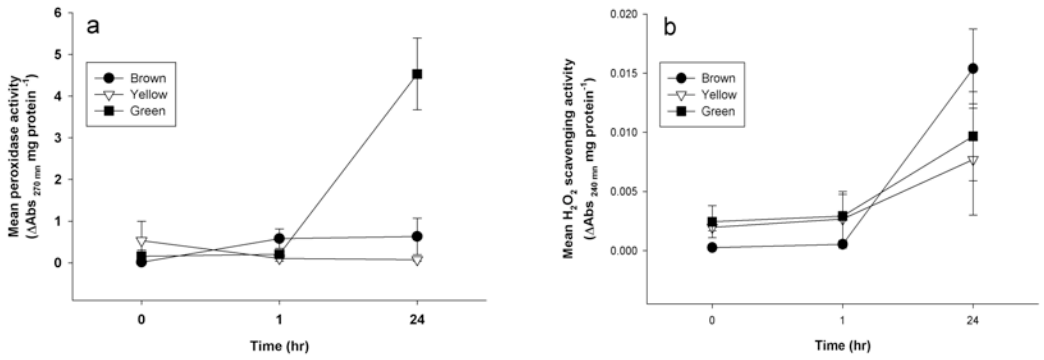


Figure 8.6: Mean antioxidant activity (± SE) at each time point for each colour morph: a) peroxidase activity and b) hydrogen peroxide (H_2O_2) scavenging activity.

8.4.6 COMPARISON OF IMMUNE RESPONSE AMONG COLOUR MORPHS

A Principal Components Analysis (PCA) plot for immunity parameters at both 1 h and 24 h post-injury, indicated that each colour morph had a distinct immune-response signature (Figure 8.7a and b, respectively). At 1 h post-injury, PC1 appeared to represent the regulation of FPs and of antioxidant activity (Table 8.1), with the brown morph having higher peroxidase and H_2O_2 scavenging activity and the green

and yellow morphs higher total, cyan and green FP as well as laccase-type PO. However, the overall antioxidant activity of the brown colour morph was lower relative to the overall activity of the green and yellow colour morph immunity parameters (PC1 eigenvalue; 80.4%). PC2 was a contrast between the activities of tyrosinase-type PO, which was highest for the green colour morph and lowest for the yellow, and transglutaminase activity, which demonstrated the opposite trend between the colour morphs (PC2 eigenvalue; 19.6%).

At 24 h post-injury, PC1 represented a combination mainly of transglutaminase, H_2O_2 scavenging activity and red FP concentration versus total fluorescent protein concentration (Figure 8.7b). The brown colour morph demonstrated high transglutaminase activity, as well as red FP and H_2O_2 scavenging. Conversely, the green colour morph had higher activity of the green and cyan FPs, which appeared to be driving the total FP. The yellow morph was also driven by these parameters, but to a lesser extent (PC1 eigenvalue; 72.7%). PC2 represents the contrasting regulation of laccase-type PO, tyrosinase-type PO and peroxidase (Table 8.2). As such, the yellow colour morph demonstrated the highest laccase-type PO activity and the green the highest tyrosinase-type PO and peroxidase (PC2 eigenvalue; 27.3%).

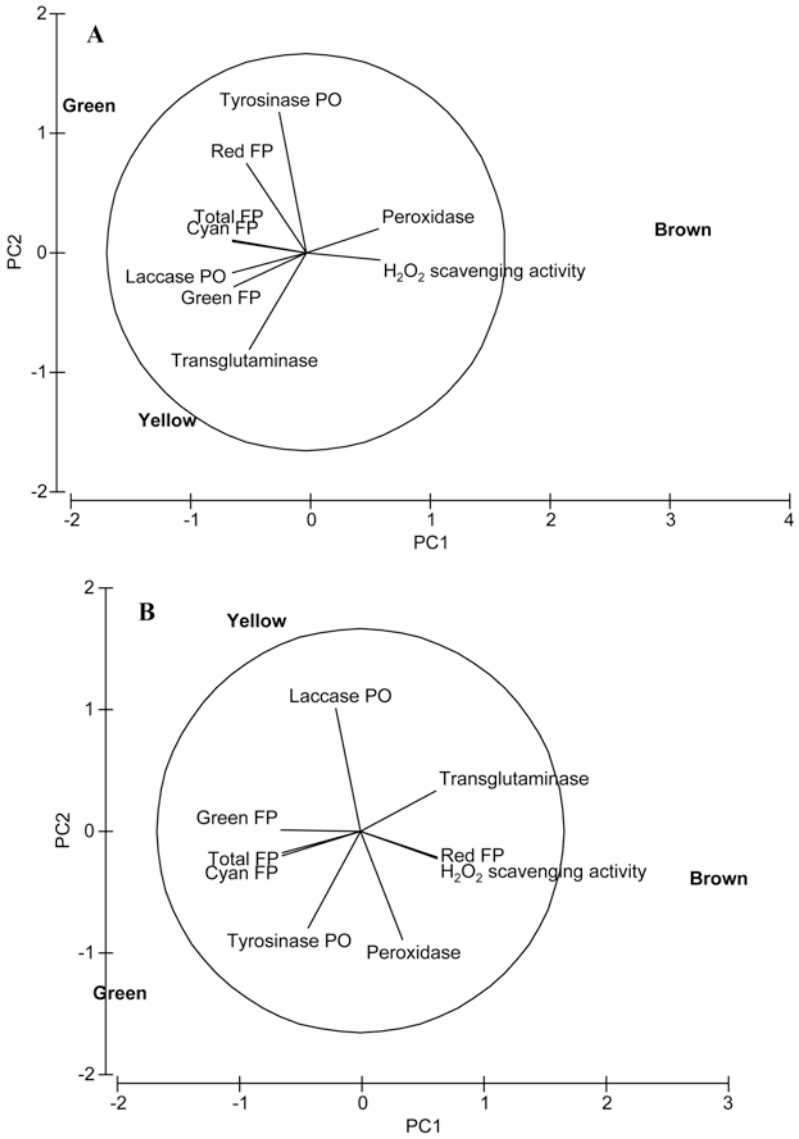


Figure 8.7: Principal Components Analysis of immune responses of each colour morph between: a) 0 h and 1 h post-injury, and b) 0 h and 24 h post-injury.

Table 8.1: Coefficients in the linear combinations of variables making up PC's (Eigenvectors) for the PCA of colour morphs within 1 h post-injury

Immunity parameter	PC1	PC2
Tyrosinase PO	-0.131	0.706
Laccase PO	-0.368	-0.101
H_2O_2 scavenging	0.371	-0.036
Transgluatminase	-0.284	-0.487
Peroxidase	0.367	0.119
Cyan FP	-0.370	0.061
Green FP	-0.362	-0.173
Red FP	-0.298	0.451
Total FP	-0.371	0.051

Table 8.2: Coefficients in the linear combinations of variables making up PC's (Eigenvectors) for the PCA of colour morphs within 24 h post-injury

Immunity parameter	PC1	PC2
Tyrosinase PO	-0.255	-0.483
Laccase PO	-0.3119	0.607
H_2O_2 scavenging	0.328	-0.140
Transgluatminase	0.372	0.196
Peroxidase	0.207	-0.541
Cyan FP	-0.383	-0.127
Green FP	-0.391	0.002
Red FP	0.383	-0.132
Total FP	-0.385	-0.114

8.5 DISCUSSION

8.5.1 RELATIVE ABUNDANCE OF COLOUR MORPHS

The brown colour morph was the most abundant of the *Porites cylindrica* colour morphs investigated in the Pioneer Bay population, and the green colour morph the least. Variation in colour morph abundance was consistent with patterns found for most other coral species (Gleason 1993; Takabayashi and Hoegh Guldberg 1995; Dove et al. 2006a). Interestingly however, the reverse pattern was documented for *Porites astreoides*; at 2 m depth, the green colour morph was more abundant than the brown and differences in UV-tolerance were attributed to mycosporine-like amino acids (Gleason 1993), rather than fluorescent proteins or their homologues, as proposed for *Montipora monasteriata* (Dove et al. 2006a). In addition to being more abundant, the brown colour morph had the most even size class distribution, as estimated by branch number. The greater abundance of the brown morph and potentially more stable population structure suggest that this morph may have a physiological fitness advantage (Stearns 1993; Sadd and Schmid-Hempel 2009) at this site. Overall, these results suggest that the presence of three colour morphs within this population of *P. cylindrica* is not due to phenotypic plasticity in response to environmental variation, as previously suggested as an explanation for the presence of colour morphs within coral populations (Kelmanson and Matz 2003), given that the three *P. cylindrica* morphs co-exist within the same reef zone. However, their differing abundances within this zone may reflect differing physiological advantages of each colour morph, although more extensive distribution and abundance surveys are needed to verify the general consistency of the patterns found in this study.

8.5.2 MACROSCOPIC OBSERVATIONS OF WOUND HEALING

Each colour morph demonstrated a macroscopically visible increase in brown colouration at wound sites, which is potentially due to melanin deposition within the affected tissues. This is likely because of its absorbance of light, which is consistent with melanin absorption (Meredith et al. 2006), but also the presence of melanin-synthesis pathway intermediates and melanin deposits previously located within coral tissues (Chapters 2, 3, 6 and 7). The deposition of melanin during wound healing is

well documented among other invertebrates (Fontaine and Lightner 1973; Galko and Krasnow 2004; Ancona Lunette 2005), and plays a role in clot stabilisation (Theopold et al. 2004). Therefore, if the brown substance observed in the wound site of all *P. cylindrica* colour morphs is melanin, it may be exuded by cells adjacent to the injury in order to form a rudimentary clot, similar to clot formation in other invertebrates (Galko and Krasnow 2004). This would immediately form a barrier between the environment and the disrupted tissue and potentially cleanse the area of microorganisms due to the cytotoxic by-products associated with melanin-synthesis (Nappi and Ottaviani 2000). However, the identity of the brown substance, as well as its origin needs to be confirmed, ideally with histological techniques. The increase in red fluorescence in the brown and yellow morphs following injury is coincident with increased local cytotoxicity as a result of melanin-synthesis. This adds to earlier evidence that fluorescent proteins (Chapter 4) and the melanin-synthesis pathway (Chapter 2) are present in healthy tissues of *Porites* sp. and are up regulated during an immune response (Chapters 6 and 7).

8.5.3 FLUORESCENT PROTEINS OF COLOUR MORPHS

The green morph was the most fluorescent of the three colour morphs investigated, however all colour morphs increased their relative fluorescence after injury. This suggests rapid up-regulation of fluorescent proteins (FPs) during an immune response, perhaps as supplemental antioxidants (Chapter 4). This interpretation is consistent with observations of pink colouration in tissues of *Porites* spp. that are associated with infection or injury (Chapters 7 and 8). Furthermore, this role during biological stress is consistent with the red FP, which was dominant within the green colour morph, being a more efficient antioxidant than the green FP (Chapter 4). Additionally, at 24 h post-injury, red FP was up regulated by an amount equivalent to the up-regulation of H_2O_2 scavenging activity. This further supports the general role of FPs as antioxidants (Chapter 4). Furthermore, evidence that catalase and other H_2O_2 scavengers can be overwhelmed during oxidative stress (Halliwell and Gutteridge 1999; Lesser 2006) suggests that the up-regulation of red FPs by 24 h post-injury may support these more efficient antioxidants (Chapter 4).

8.5.4 IMMUNITY PATHWAY ACTIVITY OF COLOUR MORPHS

Immunity pathways, including two melanin-synthesis pathways and the coagulation pathway, were present in all colour morphs and were differentially regulated post-injury. Consistent with wound healing in numerous insects, for example the mosquito *Armigeres subalbatus* (Lai et al. 2002), the fruit fly *Drosophila melanogaster* and the wax moth *Galleria mellonella* (Bidla et al. 2008), the green colour morph up-regulated the tyrosinase-type PO pathway within an hour after injury. The melanin-synthesis pathways serve two general immunological functions: structural support and antimicrobial defence *via* cytotoxicity of the melanin synthesis pathway intermediates (Nappi and Christensen 2005; Zhao et al. 2007). However, the tyrosinase-type PO pathway is documented to be significantly more cytotoxic than the laccase-type (Zhao et al. 2007). This may explain the down regulation of tyrosinase-type PO activity in the brown and yellow colour morphs during wound healing, which both had comparatively lower levels of hydrogen peroxide-scavenging FPs than the green morph. The brown and yellow morphs used the other, less toxic, enzymatic pathways, the laccase-type PO and transglutaminase, which may serve similar functional roles to the tyrosinase-type PO (Sugumaran 2002; Theopold et al. 2004; Nappi and Christensen 2005; Zhao et al. 2007).

The laccase-type melanin-synthesis pathway is involved in clotting, wound healing and sclerotisation in other invertebrates (de Marco and Roubelakis-Angelakis 1997; Mayer and Staples 2002; Laufer et al. 2006; Yatsu and Asano 2009 1511). The brown and yellow colour morphs up-regulated laccase PO to a greater extent than the green colour morph, which is consistent with the preferential use of a less cytotoxic melanin-synthesis pathway. This suggests that of the three colour morphs, the green morph is the least likely to suffer from infection post-injury, however, costs in terms of self-damage and high requirements for antioxidants may be incurred (Sheldon and Verhulst 1996; Sadd and Siva-Jothy 2006; Sadd and Schmid-Hempel 2009). Further studies to test the capacity of the green morph to resist infection following injury and costs associated with up-regulation of FPs would provide useful insights into ecological immunity of corals.

In addition to the melanin-synthesis pathways, transglutaminase activity associated with the coagulation pathway (Theopold et al. 2004) was up-regulated in all colour morphs upon injury. This is consistent with the documented role of

transglutaminase in the cross-linking of proteins during coagulation and clot formation in other invertebrates (Beninati and Piacentini 2004). However, the brown colour morph appeared to upregulate this coagulation pathway preferentially over either melanin-pathway, whereas these two proteolytic cascades have been shown to work in conjunction in several invertebrates (Theopold et al. 2004; Bidla et al. 2005). It is therefore possible that the transglutaminase pathway fulfils similar roles, to that of the melanin-synthesis pathways within anthozoan wound healing such as in clot formation. However, this study suggests a significant role for the coagulation pathway within anthozoan wound healing for the first time, which is broadly consistent with other invertebrates (Beninati and Piacentini 2004; Theopold et al. 2004).

8.5.5 ANTIOXIDANT ACTIVITY OF COLOUR MOTPHS

Oxidative stress conditions are induced during an immune response due to the disruption of cell integrity and the activation of cytotoxic defence pathways (Halliwell and Gutteridge 1999). This necessitates the up-regulation of endogenous antioxidants after physical injury in order to prevent self-harm (Halliwell and Gutteridge 1999; Fang 2004). Peroxidase activity was differentially regulated post-injury among the colour morphs. The brown and green colour morphs both up-regulated peroxidase activity, presumably due to increased oxidative stress induced during the immune response and with cell disruption (Halliwell and Gutteridge 1999). However, the yellow colour morph slightly down regulated the activity of this enzymatic antioxidant. The down-regulation of peroxidase activity in the yellow colour morph with injury suggests that, either oxidative stress conditions were not induced, or an alternative hydrogen peroxide (H_2O_2) scavenger was utilised, or perhaps that local tissues were maintained under conditions of oxidative stress, potentially to avoid infection. For all colour morphs, substrate-free H_2O_2 scavenging activity was approximately equivalent to control levels at 1 h post-injury, but was up regulated at 24 h post-injury. Thus oxidative stress conditions were induced in all colour morphs within a time frame that was similar to that of which cytotoxic immunity pathways were regulated.

8.5.6 COMPARISON OF IMMUNE RESPONSE AMONG COLOUR MORPHS

All colour morphs demonstrated activity of all six of the immunity parameters that were investigated, showing the use of a diverse suite of effector responses

(Section I). However, these immunity parameters were differentially regulated post-injury among the colour morphs, suggesting functional differences among them. At 1 h post-injury the brown colour morph had only up-regulated antioxidant activity, suggesting the lowest immunocompetence of the three colour morphs, based on regulation of the immunity parameters measured. However, the up-regulation of antioxidants indicates the induction of oxidative stress conditions, which is likely to be partly caused by the injury itself, but also as a result of an immune response (Halliwell and Gutteridge 1999).

Both of the PO types and transglutaminase are involved in the coagulation and clot formation phases of wound healing (Theopold et al. 2004; Bidla et al. 2005), however the brown morph did not up-regulate any of these pathways (tyrosinase-type PO, laccase-type PO or transglutaminase activity) to the same extent as the yellow and green morphs. It is possible that the brown morph uses different immunological mechanisms to other morphs, but further research is required to establish whether this is the case, and if so, what these alternative mechanisms are. As the brown morph was dominant within the reef surveys in this study, it might be expected to possess advantageous physiological characteristics over both the yellow and green morphs. In light of the apparently lower investment in immunity of the brown morph, it is hypothesised that this morph either allocates more resource to growth and reproduction than the other morphs, or invests in a more efficient suite of mechanisms (Sadd and Schmid-Hempel 2009).

Overall, the green colour morph appeared to be the most immunocompetent, with the greatest up-regulation of the most cytotoxic immunity pathway within the first hour post-injury. However, the low relative abundance of the green morph on the reef and the limited number of large colonies recorded within this study, suggests that this level of investment in immunity may be costly in terms of investment in growth and reproduction (Chapter 5; (Sadd and Schmid-Hempel 2009). Up-regulation of cytotoxic immunity pathways may therefore be advantageous for resisting infection during disease epizootics, but may not be advantageous over long time periods of repeated infections, due to the potentially high energetic costs involved.

A component of the *P. cylindrica* immune response to injury appears to be the accumulation of a brown substance, potentially melanin, at the wound site. Whilst this accumulation was observed in this study, it was not quantified and the substance was not confirmed to be melanin. However, the typical high abundance of melanin-

containing granular cells within the tissues of *Porites* species (Chapter 3) suggests that histochemical investigation of this substance is likely to be informative in discerning the origin of the brown substance. Additionally, the amount of this brown substance may be significant in distinguishing the immune response of the brown colour morph from that of the yellow and green morphs. In particular, the size and abundance of melanin-containing granular cells needs to be investigated and compared among colour morphs and may provide insights into the magnitude of the brown morph's immune response to injury, which might be more consistent with its relative dominance at the site.

8.5.7 CONCLUSION

Overall, this study provides the first controlled comparative investigation into the timing and regulation of immunity pathways within hard corals. Results confirm that all immunity parameters investigated are used during wound healing in corals and demonstrate that coral effector responses (Section I) have functions that are consistent with those documented for other invertebrates, with the exception of FPs. FPs appear to be uniquely employed as part of an immune response within cnidarians. Furthermore, comparisons among conspecific colour morphs demonstrate a heterogeneous use of immunity parameters that corresponds to functional differences among them. A valuable future direction would be to histologically investigate wound healing within *P. cylindrica*, which may explain the lack of melanin-synthesis pathway up-regulation within the brown colour morph and provide explanation for the brown substance at the wound sites.

Chapter 9 HISTOLOGICAL CHARACTERISATION OF WOUND HEALING IN A SCLERACTINIAN CORAL

9.1 ABSTRACT

Corals frequently suffer from physical injury, for example by storm-induced fragmentation, predation and boring organisms, which necessitates effective wound healing mechanisms. Although biochemical aspects of scleractinian innate immune responses have recently been investigated during wound healing (Chapter 8), the possible roles of immune cells (described in Chapter 3) in sealing wounds have not. Accordingly, a histological investigation into wound healing of *Porites cylindrica* was conducted on tissues at 0 h, 6 h, 24 h and 48 h after injury and cellular components compared to those of healthy tissue. This study therefore represents the first comprehensive histological investigation of wound healing in a scleractinian coral. *Symbiodinium* cell counts and melanin volume fraction analysis revealed a rapid decrease in both zooxanthellae and tissue cross-sectional area occupied by melanin-containing granular cells after injury. Four phases of wound healing were identified, which are similar to the phases described for both vertebrates and invertebrates. The four phases included plug formation *via* melanin-containing granular cell degranulation, immune cell infiltration (inflamation), granular tissue formation (proliferation) and maturation. These results demonstrate the conservation of wound healing processes from the phylogenetically basal anthozoans to humans. The brown-pigmented substance documented to cover the wound site after injury in Chapter 8 was confirmed to be melanin exudate originating from melanin-containing granular cells. The role of melanin exudates in wound healing in corals was established by histological observations of degranulation, as occurs in other invertebrates to seal wounds. This study documents the processes involved in scleractinian wound healing for the first time and further elucidates the roles of previously described immune cells (Chapter 3) in addition to characterising novel coral cells such as fibroblast-like cells.

9.2 INTRODUCTION

A wound is defined as damage to or disruption of normal anatomical structure and function (Robson et al. 2001), and therefore includes epithelial breaks as well as extensive tissue damage and/or skeletal damage (Velnar et al. 2009). Corals are frequently subjected to wounding, for example by fish bites (Rotjan and Lewis 2008), algal abrasion and/or direct overgrowth (Jompa and McCook 2003), storm damage (Edmunds and Witman 1991; Bythell et al. 1993; Bythell et al. 2000) and boring organisms (Fang and Shen 1988). However, histological studies of wound healing mechanisms in anthozoans are surprisingly few (Young 1974; Meszaros and Bigger 1999); most studies of wound healing have focused on observations of regeneration rates under varying conditions in scleractinian corals (e.g. Meesters et al. 1994; Meesters et al. 1997a; Lirman 2000a; Fine et al. 2002; Alvarado and Acosta 2009). The histological aspects of wound healing in hard corals have therefore not been investigated to date, yet are likely to represent an important contribution to the understanding of coral immune responses.

Wound healing is a vital process, during which specialised immune cells invade the wound site in a specific sequence (Grotendorst et al. 1985) and seal the lesion to prevent the loss of fluids, infection by foreign organisms (Wang et al. 2001; Theopold et al. 2002) and to aid in regeneration of the tissue (Meesters et al. 1994; Biressi et al. 2010). In mammals, wound healing follows four phases, which are broadly sequential, although overlapping (Kirsner and Eaglstein 1993; Martin and Leibovich 2005), and which have also been identified during wound healing within invertebrates (Fontaine and Lightner 1973; Burke 1974; Franchini and Ottaviani 2000; Ancona Lunette 2005; Biressi et al. 2010).

The first of the four wound healing phases, coagulation and clot formation, is similar among both invertebrates and vertebrates. Coagulation, whereby fluids such as blood or haemolymph become semisolid (soft clot) to seal the wound, begins immediately upon physical injury, in both vertebrates like humans (Velnar et al. 2009) and invertebrates such as arthropods (Wang et al. 2001; Theopold et al. 2002). Coagulation leads to the formation of a stable (hard) clot, where platelets aggregate, change shape and degranulate to form a plug (Martin and Leibovich 2005), which is stabilised by fibrin molecules in mammals (Velnar et al. 2009). In insects, clot formation occurs by the degranulation of immune cells and the incorporation of

cellular debris and extracellular matrix components into an extracellular aggregate (Theopold et al. 2004), which may also be referred to as a "plug" (Galko and Krasnow 2004). After clot, or plug, formation, the second phase of wound healing is the infiltration of immune cells into tissues in the wound area, which is also referred to as the inflammation phase in vertebrates (Velnar et al. 2009).

Infiltration of immune cells from tissue surrounding the wound site is triggered by immunity factors released by the cells involved in clot formation, thereby initiating an inflammatory response (Rowley 1996) and the second phase of wound healing (Velnar et al. 2009). During the inflammatory phase in both vertebrates (Velnar et al. 2009) and invertebrates (Menton and Eisen 1973; Franchini and Ottaviani 2000), the recruited immune cells phagocytose microorganisms and cellular debris (Martin and Leibovich 2005). In the sea cucumber, *Holothuria polii*, for example, the second wound-healing phase has been identified by the infiltration of amoebocytes and pigment cells, and the presence of fibroblast-like cells noted (Menton and Eisen 1973). The presence of fibroblasts in mammals signals the beginning of the third phase of wound healing, the proliferation phase (Martin and Leibovich 2005).

The proliferation phase of wound healing, so named because of the rapidly reproducing immune cells, primarily fibroblasts, includes the development of granulation tissue and the process of re-epithelialisation in mammals (Kirsner and Eaglstein 1993). Fibroblast-like cells are characterised by discernable extended pseudopodia and an ability to control extra-cellular matrix production and collagen release (Tettamanti et al. 2004; Velnar et al. 2009). Proliferating fibroblast-like cells are key to the perfusion of granulation tissue *via* the deposition of collagen, which is stimulated by various growth factors (Grotendorst et al. 1985; Franchini and Ottaviani 2000). Granulation tissue consists of multiple cell types and a basic extracellular matrix, which enables epithelial cells to migrate across it as part of the re-epithelialisation process (Robson et al. 2001; Ancona Lunette 2005; Velnar et al. 2009). During re-epithelialisation, epithelial cells proliferate and migrate from the wound edges and join, this occurs within a few hours in humans (Velnar et al. 2009), after which tissue reorganisation begins underneath the newly formed epidermis (Burke 1974). In the insect *Drosophila*, proliferation of the epidermal cells does not occur during re-epithelialisation, rather, epidermal cells surrounding the lesion orientate towards the wound and extend cytoskeleton actin projections known as

lamellipodia (Galko and Krasnow 2004). Similarly, orientation of epidermal cells at the lesion edge pointing towards the centre of the lesion has been documented within the crayfish (Vafopoulou 2009). Additionally, Meszaros and Bigger (1999) noted an extension of epithelial tissue over the wound towards the exposed axial skeleton of the gorgonian sea fan *Plexurella fusifera* within one day, although the mechanism was not described.

The fourth phase of wound healing is the maturation or remodelling phase, which is responsible for epithelium development and scar tissue formation (Kirsner and Eaglstein 1993; Velnar et al. 2009). In mammals, this involves a reduction in fibroblast density *via* apoptosis (Bolitho et al. 2010; Greenhalgh 1998), which also serves to contract the wound, as well as extensive deposition of collagen (Velnar et al. 2009). For invertebrates, apoptosis of excess cells has been recorded (Tettamanti et al. 2008) and remodeling involving collagen deposition, as part of regeneration, has been documented in the sea cucumber *H. glaberrima* (Miguel-Ruiz and Garcia-Arraras 2007) and leeches (Tettamanti et al. 2005). For anthozoans, reorganisation of new epithelium has been documented (Meszaros and Bigger 1999), and collagen production noted within lesions (Young 1974), however the potential presence of the aforementioned four phases of wound healing have not been investigated within hard corals. This study therefore aimed to histologically investigate the cellular mechanisms of wound healing in the scleractinian coral *Porites cylindrica.*

9.3 METHODS

9.3.1 SAMPLE COLLECTION

Three large (> 50 cm diameter) and visually healthy colonies of *Porites cylindrica* were located and tagged within Pioneer Bay at Orpheus Island, Great Barrier Reef (GBR), during May 2008. Injuries were created on four branches per colony between 1 and 2 cm from the branch tip. To create the injury, bone-cutters were used to form tissue and skeletal damage approximately 2 mm deep around the circumference of each branch. Injuries were created on branches that were on equivalent areas of each colony. Branches were sampled for histology using bone-cutters to remove the top 3 to 4 cm of each branch, inclusive of the injury. Samples were taken of a healthy branch as a control, then at 0 h, 6 h, 24 h and 48 h after injury from each colony.

9.3.2 HISTOLOGY

Porites cylindrica samples were fixed in 4% paraformaldehyde-phosphate buffer solution, decalcified progressively in 3% - 10% formic acid and stored in 70% ethanol. Samples were processed overnight in an automated tissue processor and embedded in paraffin wax. Sub-samples of branches were sectioned longitudinally at 5 μm to give cross-sections of polyps, including polyps in the areas of injury. Sections were alternately stained with Mayer's Haematoxylin and Young's Eosin-Erythrosin stain (H&E) and Fontana-Masson melanin stain. Using an Olympus DP12 dedicated camera head mounted on an Olympus high power microscope, six photographs were taken of the injured area, which manifested as U-shaped indentations into the sides of longitudinal sections of branches, as *P. cylindrica* tissue extended deeper within the skeleton than the lesion. This enabled two photos of each side and the base of the U-shaped lesion (n = 6 photos) to be taken of each sample (n = 3 colonies), for each stain at both 20 x and 60 x magnification. Similarly, six photographs were taken haphazardly of the free body epidermal layer of healthy tissue sections for each colony. Cells of interest were photographed at 100 x magnification.

The number of *Symbiodinium* cells (zooxanthellae) per unit cross-sectional area of gastrodermis was counted within three randomly selected areas of gastrodermal tissue from the two sides and base of each U-shaped lesion (n = 9 areas per lesion), for one sample from each of the three colonies (n = 27 cell counts), from each time point (0 h, 6 h, 24 h and 48 h after injury). Similarly, zooxanthellae were counted within nine haphazardly selected areas of gastrodermis from each healthy sample (n = 27 cell counts). The tissue area was calculated using imaging software (Image J) and the mean density of zooxanthellae was compared statistically between healthy and injured samples over time using Kruskal-Wallis non-parametric tests, as assumptions of normality were not met.

The mean proportion of melanin in the tissue layers (volume fraction; Vf) was determined from Fontana-Masson stained sections, as a percentage surface area of melanin in the epidermis and gastrodermis of healthy tissue, and in disrupted tissue of injured samples, which resembled gastrodermis. Vf was calculated using Image J software by converting images to greyscale, and creating a histogram. The proportion of pixels within the selected area that were dark brown or black, thus indicating melanin, was recorded. Similar to the zooxanthellae counts, measurements of melanin

Vf were made from three randomly selected areas of each side and the base of each lesion (n = 9 per lesion) for each colony (Tn = 27 measurements). Equivalent measurements were taken of the melanin Vf within the epidermis and gastrodermis of the healthy samples. Mean Vf of melanin was compared among time points using a Kruskal-Wallis non-parametric test.

9.4 RESULTS

9.4.1 ZOOXANTHELLAE DENSITY AND MELANIN VOLUME FRACTION

The mean zooxanthellae density was highest in the healthy samples and varied significantly among healthy and injured samples (Figure 9.1; K-W $x^2_{(4)} = 88.4$, $P < 0.001$). Zooxanthellae density was lowest in the freshly injured tissues (0 h), at 12.5% of that of the healthy tissue, suggesting a rapid release or expulsion of the symbionts after injury. After 1 h post-injury, there was an increase in zooxanthellae density over time, with a 4-fold increase between 1 h and 6 h post-injury and a 1.5-fold increase between 6 h and 48 h post-injury. The zooxanthellae density at 48 h post-injury was at approximately 75% of the healthy tissue density.

The mean melanin volume fraction (Vf) differed significantly among gastrodermal measurements of healthy and injured samples over time post-injury (Figure 9.2; K-W $x^2_{(4)} = 35.9$, $P < 0.001$). Melanin Vf was the highest in the epidermis of healthy samples, followed by the gastrodermis of healthy samples where the Vf was approximately half that of the epidermis. Immediately after injury (0 h), melanin Vf decreased to approximately half that of the healthy gastrodermis Vf, and was approximately the same at 6 h post-injury. This suggests a rapid release of melanin from the tissues after injury. By 24 h, the melanin Vf had increased to approximately 80% of the Vf of the healthy gastrodermis and remained approximately the same at 48 h post-injury. This increase demonstrated a gradual replenishment of melanin to near that of healthy levels.

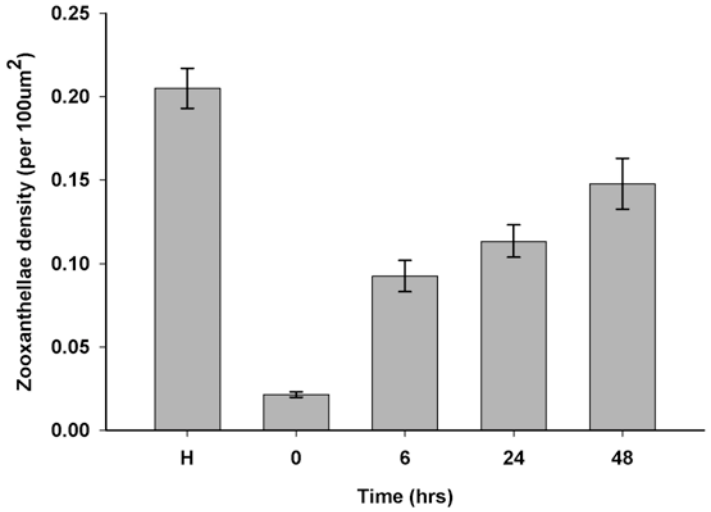


Figure 9.1: Mean zooxanthellae density (± SE) over time post-injury. H = healthy tissue, n = 27 (K-W $x^2_{(4)} = 88.35$, $P < 0.001$).

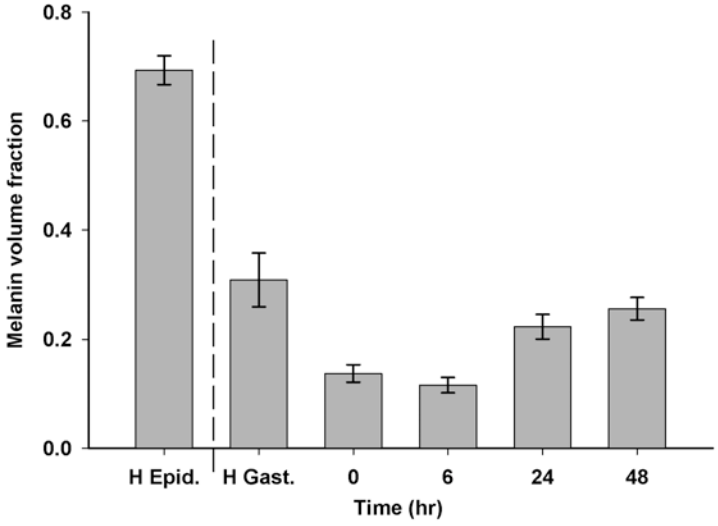


Figure 9.2: Mean melanin volume fraction (Vf; ± SE) over time post-injury (n = 27). H Epid = healthy coral epidermis. Melanin Vf compared among H. Gast. (= healthy gastrodermis) and gastrodermal layers within injured tissue (K-W $x^2_{(4)} = 35.94$, $P < 0.001$).

9.4.2 WOUND HEALING

9.4.2.1 Healthy tissue

Histological sections of healthy tissues revealed intact cell layers that contained cells characteristic for each layer, for example, zooxanthellae within the gastrodermis (Figure 9.3). The epidermis was densely packed with melanin visible within melanin-containing granular cells, although individual cells were difficult to discern due to their density. Melanin-containing granular cells were a characteristic deep golden brown colour in Haematoxylin and Young's Eosin-Erythrosin (H&E)-stained sections (Figure 9.3a) and black with the Fontana-Masson stain (Figure 9.3b). *Porites cylindrica* cnidae were large and dispersed primarily throughout the gastrodermis, although were also observed occassionally within the mesenteries and mesenterial filaments.

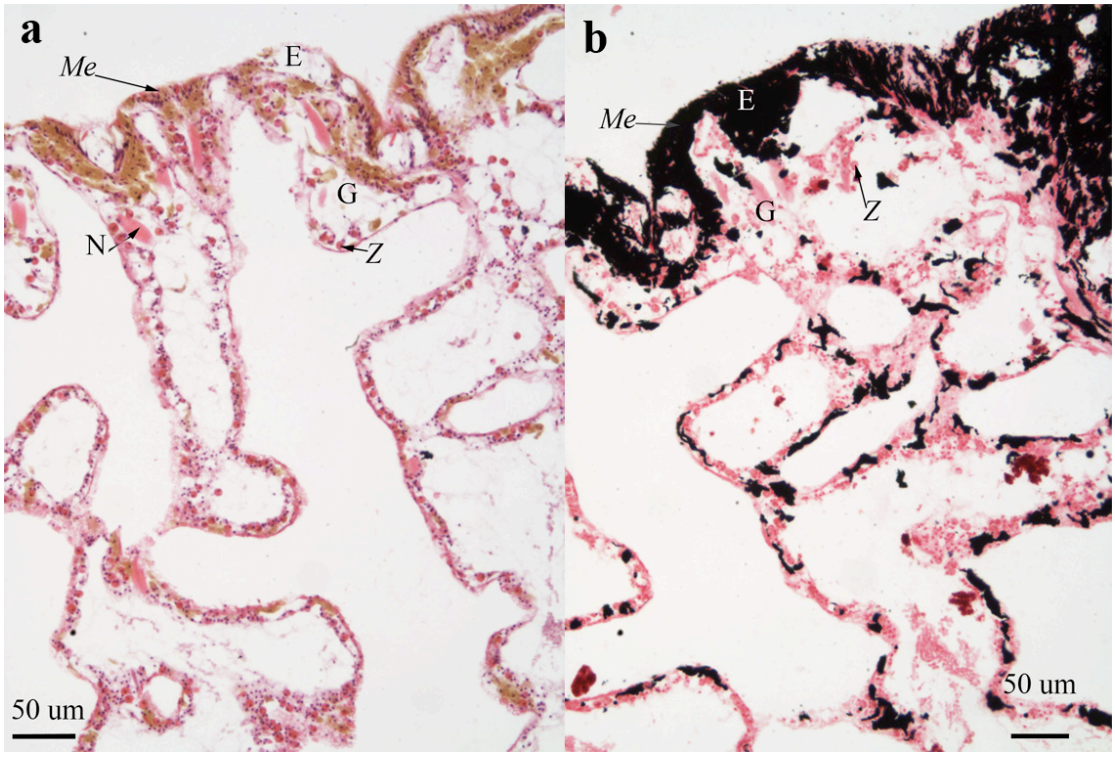


Figure 9.3: Healthy *Porites cylindrica* tissue sections stained with a) Haematoxylin and Young's Eosin-Erythrosin stain (H&E) and b) Fontana-Masson stain. E = epidermis, G = gastrodermis, *Me* = melanin and/or melanin-containing granular cell, Mes = mesentery, *Z* = zooxanthellae and N = cnidae.

9.4.2.2 Histopathology immediately post-injury (0 h)

Immediately post-injury, free-body wall epidermal tissue was disrupted and cell layers could not be clearly differentiated. Moreover, there was a distinct lack of

the characteristic golden-brown melanin-containing granular cells. Characteristic cell types present immediately post-injury included cnidae, which were relatively abundant, and zooxanthellae, which were in low density (Figure 9.4). Melanin granules were visible in H&E-stained sections (Figure 9.5a), and were associated with enlarged, round nuclei, discernable by the purple to blue colouration under H&E. Light pink stained (eosinophilic) cells with a granular appearance and dark oval nuclei were observed as putative amoebocytes. Smooth pink-stained cells were also present as well as mucus cells and unidentified large cells, which appeared empty although with a slight brown colouration. The Fontana-Masson stained sections immediately post-injury indicated that melanin was associated with the empty unidentified cells, suggesting that they were degranulated melanin-containing granular cells (Figure 9.5b). Nuclei were stained red with the Fontana-Masson stain due to counter-staining with Nuclear-fast red.

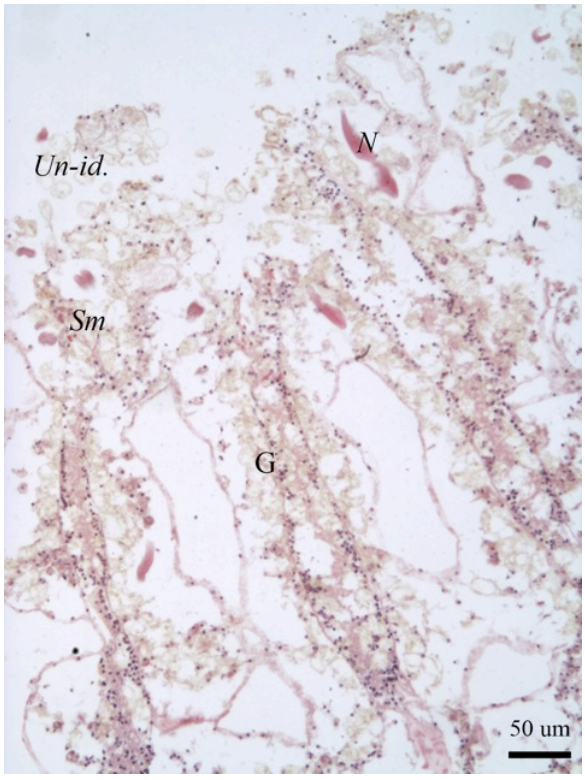


Figure 9.4: H&E stained free-body wall area of *Porites cylindrica* 0 h post-injury with free-body wall epidermis having been removed by the injury. G = gastrodermis, *Un-id.* = unidentified cells, *Sm* = smooth cell, *N* = cnidae.

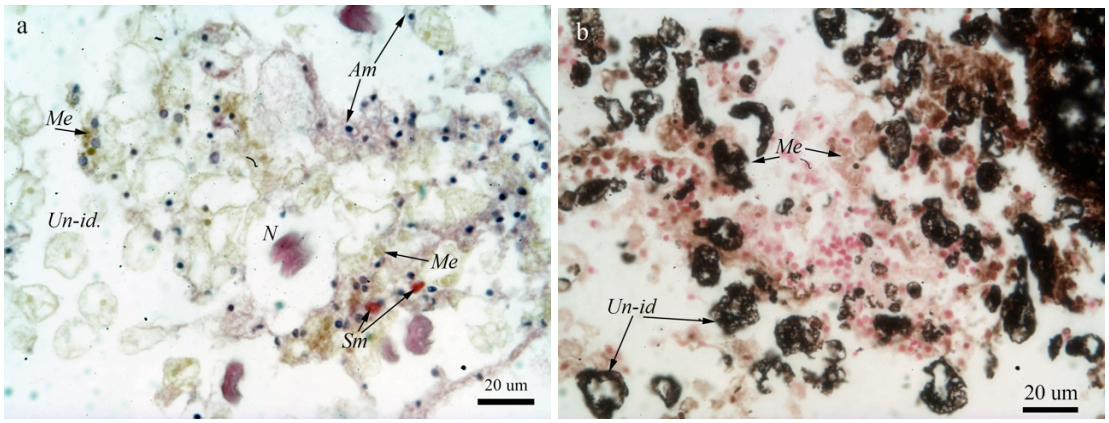


Figure 9.5: Disorganised *Porites cylindrica* tissue immediately post-injury (0 h) stained with a) H&E and b) Fontana-Masson stain. *Un-id.* = unidentified cells, *Am* = granular amoebocytes, *Me* = melanin-containing granular cells, *Sm* = smooth cell, *N* = cnidae.

9.4.2.3 Histopathology 6 hours post-injury

Dark pink-stained (under H&E) highly granular cells observed at 6 h post-injury (Figure 9.6) were morphologically identical to the pale pink stained eosinophilic amoebocytes that were observed at 0 h post-injury (Figure 9.5). This suggests a potential change in the protein concentration of eosinophilic granular amoebocytes with time post-injury. Eosinophilic granular amoebocytes were in dense aggregations and established an epithelial front at the wound edge. These granular amoebocytes formed a boundary between the disrupted but live tissue and the foreign organisms such as ciliates, as well as the dead and expelled components that formed cellular-debris plug (Figure 9.6).

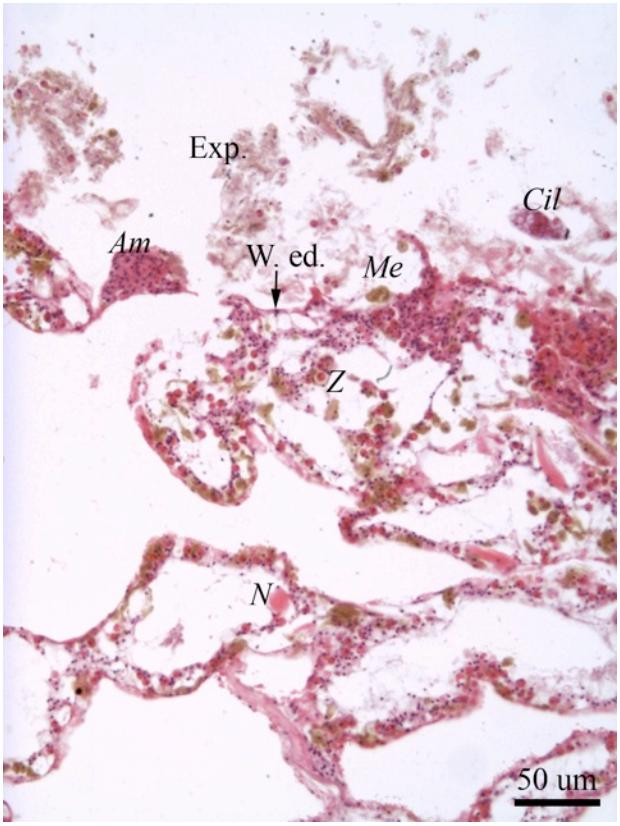


Figure 9.6: The lesion edge of *Porites cylindrica* 6 hours post-injury demonstrating amoebocyte aggregations, disrupted tissue and expelled, dead or dying tissue with microorganisms. Exp. = expelled, dead and dying tissue, W. ed. = wound edge, *Am* = granular amoebocytes, *Me* = melanin-containing granular amoebocytes, *Cil*= ciliate, *N* = cnidae.

Expelled components that contributed to the plug included zooxanthellae, which were identifiable by their deep pink colouration and spherical shape, and melanin, potentially from the degranulated melanin-containing granular cells (Figure 9.7a). Golden brown melanin-containing granular cells were densely aggregated with eosinophilic granular amoebocytes at the lesion edges (Figure 9.7b), where cell layers remained indistinguishable, although zooxanthellae, smooth cells and cnidae were all present. A thin epithelial layer of eosinophilic granular amoebocytes formed along the lesion edge (Figure 9.7c), with melanin-containing granular cells and pigment cells forming a front directly interior to this layer. Also interior to the newly formed epithelial layer, agranular fibroblast-like cells had extensive pseudopodia, which connected the disorganised tissue components. The lack of staining with H&E indicated that the cytoplasmic content of the fibroblast-like cells had a low protein

concentration, potentially due to high collagen levels (Figure 9.7d). Coincidently, dense aggregations of fibroblast-like cells appeared to be laying down a collagen-like substance as indicated by its striated appearance and light pink colouration under H&E. Agranular amoebocytes were also present, but in low densities and sporadically throughout the wound site (Figure 9.8).

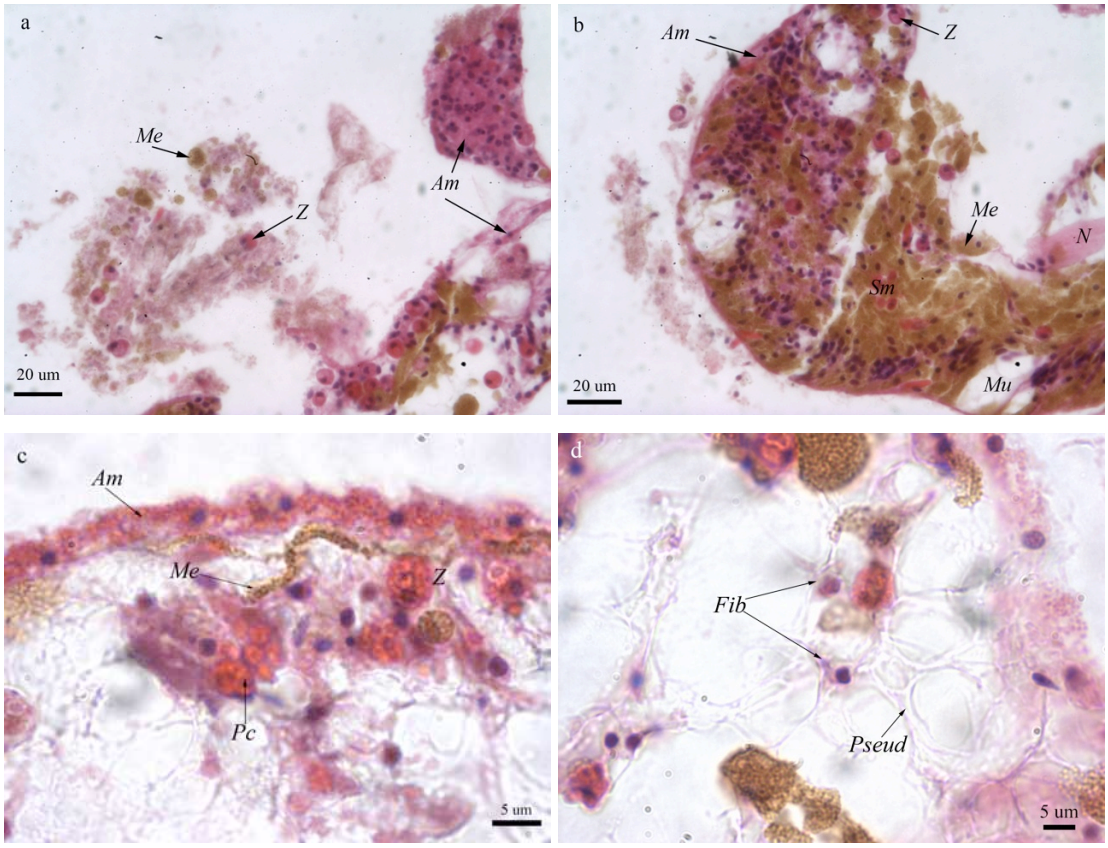


Figure 9.7: *Porites cylindrica* 6 hours post-injury with a) aggregating amoebocytes and expelled and/or disrupted tissue components, b) amoebocytes aggregating with melanin-containing-granular cells, c) granular amoebocytes forming a front with pigment cells, melanin and zooxanthellae forming a second front immediately interior to the first one, and d) fibroblasts displaying characteristic pseudopodia secreting connective collagen-like fibres. *Me* = melanin and/or melanin-containing granular cell, *N* = cnidae, *Mu* = mucus cell, *Pc* = pigment cell, *Am* = granular amoebocyte, *fib* = fibroblast and *Pseud* = pseudopodia.

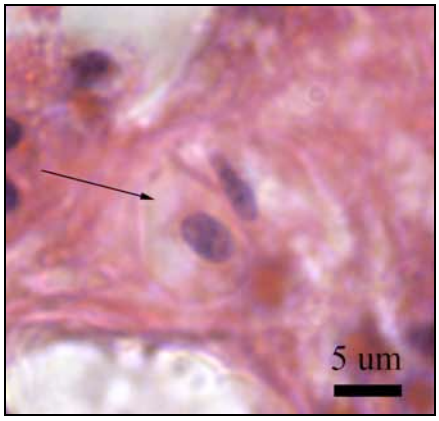


Figure 9.8: Agranular amoebocytes indicated with an arrow, present at 6 hours post-injury. Stained with H&E.

9.4.2.4 Histopathology 24 hours post-injury

At 24 h post-injury, granular amoebocytes were aggregated and aligned perpendicular to the established epithelial front (Figure 9.9a). Cell layers, although not apparently separated by mesogloea, had begun to form with zooxanthellae furthest away from the newly-forming epidermis (Figure 9.9a). Melanin-containing granular cells aggregated interior to the pink stained (under H&E) granular amoebocytes (Figure 9.9b).

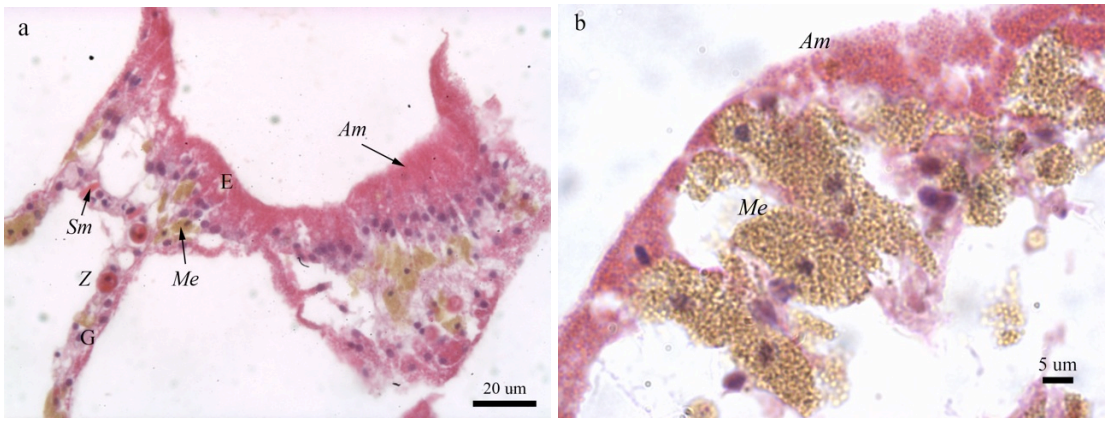


Figure 9.9: *Porites cylindrica* tissue 24 hours post-injury demonstrating a) granular amoebocytes aligning at the newly-fornimg epidermal layer, at the edges of the lesion b) granular amoebocytes being reinforced by melanin-containing granular cells. E = epidermis, G = gastrodermis, *Me* = melanin and/or melanin-containing granular cell, *Sm* = smooth cell, *Am* = granular amoebocyte.

9.4.2.5 Histopathology 48 hours post-injury

At 48 h post-injury, melanin-containing granular cells were in dense aggregations and the eosinophilic granular amoebocytes were in lower densities. The epithelial front was better developed than at 24 h post-injury, with cell layers distinguishable particularly at the lesion edges, and separated by a thin and developing mesogloea (Figure 9.10a). In less well-recovered areas, melanin-containing granular cells were observed behind the eosinophilic granular amoebocytes as observed at 6 h and 24 h post-injury, although the distinction between the cell types was less clear (Figure 9.10b). Eosinophilic granular amoebocytes, previously observed as pink in colouration contained the golden-brown granules consistent with those of the melanin-containing granular cells (Figure 9.10b *Am*). This suggests that the eosinophilic granular amoebocytes may represent a pre-stage of the melanin-containing granular cell. Additionally, some of the nuclei of both the eosinophilic granular amoebocytes and the melanin-containing granular cells appeared enlarged (Figure 9.10b) whereas others appeared granular or clumping with distinct dark blue (basophilic) spots that may be indicative of apoptosis (Figure 9.11).

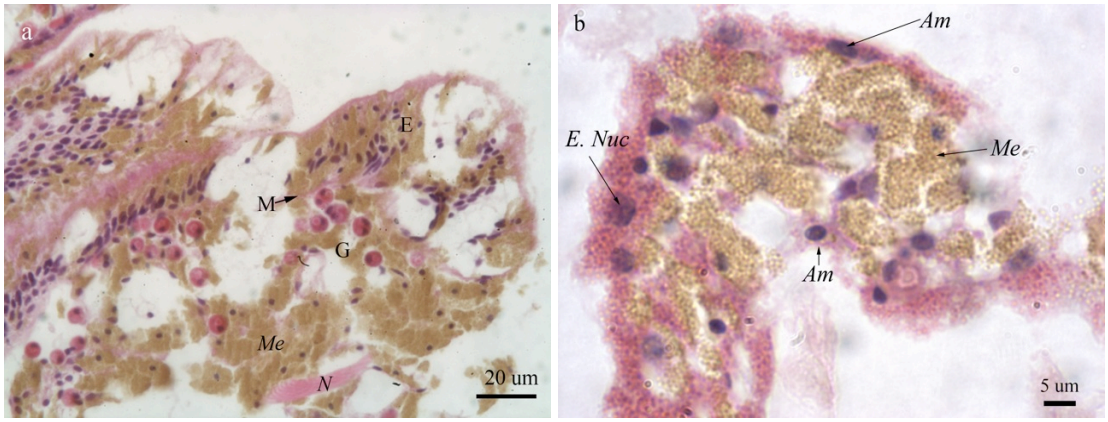


Figure 9.10: *Porites cylindrica* tissue 48 hours post-injury demonstrating a) a developing epithelial front and b) merging granular amoebocytes and melanin-containing granular cells. E = epidermis, M = mesogloea, G = gastrodermis, *Me* = melanin and/or melanin-containing granular cells, *N* = cnidae, *E. nuc* = enlarged nucleus, *Am* = granular amoebocyte.

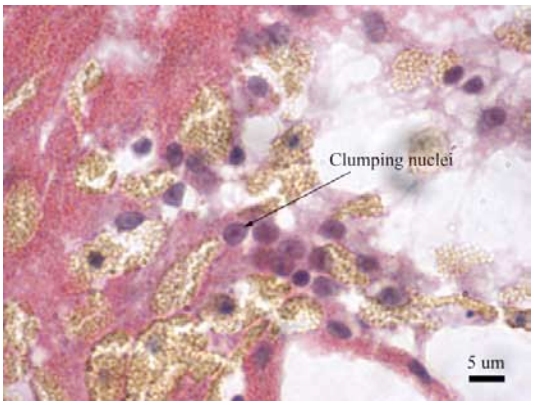


Figure 9.11: Apoptotic cells indicated by their clumping nuclei.

9.4.2.6 Interpretation of the wound-healing process

A series of schematic diagrams (Figure 9.12), based on the histological observations of *P. cylindrica* wound-healing, demonstrate the various stages that were observed. There were four discernable stages that relate to the established phases of wound healing identified in other animals (Figure 9.12, 2 to 5), from plug formation within the first hour post-injury, to wound maturation at 48 h post-injury.

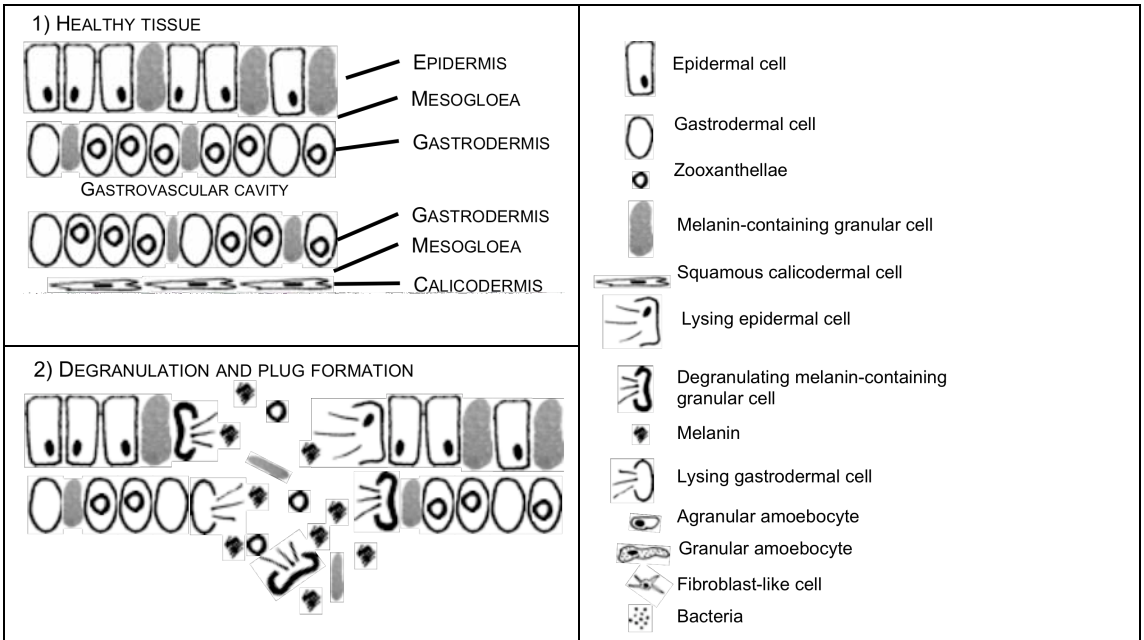


Figure 9.12: A series of schematic diagrams representing the wound healing process between 0 and 48 h in *P. cylindrica*. 1) Healthy coral tissue (1) is composed of a free-body wall layer including the epidermalis, a thin mesogloea and a gasterodermal layers. The cells layers are then mirrored on the other side of the gastrovascular cavity, to form the basal body wall with a squamous epithelium forming the calicoblastic layer. 2) Upon injury of the free-body wall epidermal layer (2), cell lysis and degranulation of melanin-containing granular cells starts to forms a rudimentary clot, or plug. (3). Infiltration of granular amoebocytes, some potentially *via* the mesogloea, occurs at 6 h to form a cell barrier behind which melanin-containing granular cells aggregate, along with agranular amoebocytes and some fibroblast-like cells (3). 4) At 24 h, infiltrated cells have begun to proliferate and the granular amoebocytes are reorientated and elongated to form an undifferentiated epidermis. 5) By 48 h, (5) differentiation begins to occur and the cell layers begin to form from the lesion edges. The high abundance of amoebocytes and fibroblast-like cells, due to proliferation, isare reduced by apoptosis.

9.5 DISCUSSION

Hard corals are subjected to physical damage from a variety of sources (Fang and Shen 1988; Edmunds and Witman 1991; Jompa and McCook 2003; Rotjan and Lewis 2008), which injure tissues and cause open wounds. Injury is a frequent occurrence for some coral species, for example those that are heavily preyed upon such as *Porites* spp. (Rotjan and Lewis 2008). However, whole colony mortality is rarely a consequence of physical injury (Bythell et al. 1993; Bythell et al. 2000). Although the ability of coral colonies to regenerate lost tissue has been documented (Meesters et al. 1994; Meesters et al. 1997a; Lirman 2000a; Fine et al. 2002), this study is the first to investigate and characterise the cellular components and processes that enable effective wound healing in hard corals. Wound healing in *Porites cylindrica*, from injury to 48 h, occurred in four main phases: 1) Degranulation and plug formation, 2) infiltration of immune cells, 3) cell proliferation and finally 4) wound maturation *via* tissue layer differentiation and potentially apoptosis.

9.5.1 DEGRANULATION AND PLUG FORMATION: 0 HOURS POST-INJURY

After a physical injury, the first phase of wound healing is clot formation (Theopold et al. 2004). In invertebrates such as butterflies and *Drosophila*, a soft clot or "plug" forms to seal the wound and establish haemostasis (Galko and Krasnow 2004; Theopold et al. 2004), which is similar to clot formation in vertebrates (Velnar et al. 2009). This plug is formed of extracellular aggregates *via* degranulation of granular cells and the aggregation of cell debris (Theopold et al. 2004). Immediately after injury, there was a distinct lack of the characteristic golden-brown melanin-containing granular cells in *Porites cylindrica*, which coupled with the presence of extracellular melanin, demonstrates the degranulation of melanin-containing granular cells. This wound-healing process left both empty melanin-stained cells and expelled, extracellular melanin as observed histologically, which contributed to the formation of a rudimentary clot or plug. These histopathological observations of free melanin may also explain the brown colouration observed at wound sites of *P. cylindrica* colour morphs in Chapter 8.

The melanin-containing granular cells observed in *P. cylindrica* wound healing, appear similar to cells involved in wound healing of other organisms, for example, melanocytes described for mammals (Mackintosh 2001; Park et al. 2009)

and melanophores described for other vertebrate species such as frogs (Herrick 1932) and invertebrates such as the fiddler crab (Vacca and Fingerman 1983). Invertebrate granular cells, which can also be termed granulocytes, are extensively documented in immunity literature (Porchet Hennere and Vernet 1992; Pech and Strand 1996; Pipe et al. 1997; Ancona Lunette 2005; Aladaileh et al. 2007), where they are reported to contain many active products involved in immunity, including phenoloxidase, melanin, peroxidase and lysozyme (Smith and Soderhall 1991; Wang et al. 2001; Aladaileh et al. 2007). Degranulation of granular cells during wound healing (Foley and Cheng 1977; Perazzolo and Barracco 1997; Aladaileh et al. 2007; Vafopoulou 2009) releases antimicrobial and cytotoxic material, including melanin, which can kill foreign organisms. This mechanism is also documented for mammalian mast cells and granulocytes (Mackintosh 2001) and for crystal cells of insects (Galko and Krasnow 2004; Bidla et al. 2008). The degranulation of melanin-containing granular cells in *P. cylindrica* is consistent with soft clot or plug formation described for other invertebrates (Fontaine and Lightner 1973; Galko and Krasnow 2004).

Melanin-containing granular cell degranulation explains the immediate decrease in both zooxanthellae density and melanin volume fraction (Vf) of surrounding tissue. The local and immediate cytotoxicity induced as a result of melanin-containing granular cell degranulation is potentially responsible for the loss of zooxanthellae. The measured decrease in zooxanthellae during wound healing in this study is contrary to documentations of gorgonian wound healing (Meszaros and Bigger 1999), where symbiont concentrations were observed to increase. Furthermore, although wound-healing observations of *Plexurella fusifera* began immediately after injury, there is no documentation of plug formation, only of disorganised tissue (Meszaros and Bigger 1999). Similarly, plug formation or degranulation is not explicitly described for the anemone *Calliactis parasitica*, potentially because observations begin at 1 h post-injury (Young 1974). However, Young (1974) mentions the presence of a layer of cell debris and mesogloeal fibres covering the wound, which is similar to that observed for *P. cylindrica*.

9.5.2 INFILTRATION OF IMMUNE CELLS AND THE ONSET OF PROLIFERATION: 6 HOURS POST-INJURY

After a soft clot has formed during wound healing, immune cells infiltrate the area of injury as part of an inflammation response to resist infection (Franchini and

Ottaviani 2000; Velnar et al. 2009). In *P. cylindrica,* there were dense aggregations of eosinophilic (acidophilic) granular amoebocytes, indicated by their deep pink staining with H&E by 6 hours post-injury. These eosinophillic granular amoebocytes are similar to those documented within the mollusc *Mytilus edulis*, where they are described to have phagocytic activity, demonstrate strong superoxide radical production and phenoloxidase activity (Pipe et al. 1997). Due to these immunity-related activities, eosinophillic amoebocytes are reportedly superior pathogen killers to other immune cells, and have the ability to proliferate (Pipe et al. 1997; Aladaileh et al. 2007). Although not conclusively established in this study, proliferation of eosinophilic granular amoebocytes would explain their high density in injured tissue sections. This is particularly the case as histological searches for these cells within healthy tissues were inconclusive, it is therefore hypothesised that they are not stored in high abundance (Chapter 3). This difference in cell density between healthy and injured tissues therefore suggests a high rate of proliferation of these coral amoebocytes.

Eosinophilic granular amoebocytes observed in this study appear similar to the amoebocytes described during wound healing of *P. fusifera* (Meszaros and Bigger 1999) and to those observed during fungal infections of *Gorgonia ventalina* (Mydlarz et al. 2008). Meszaros and Bigger (1999) pointed out the similarities of amoebocyte nuclei morphology to those of epidermal cells, suggesting that they may play an important role in re-epithelialisation. Similar to these observations by Meszaros and Bigger (1999), eosinophilic granular amoebocytes rapidly formed an epidermal layer by joining and flattening (spreading) along the lesion edge (Franchini and Ottaviani 2000). This cell layer appeared to be reinforced by melanin-containing granular cells and appeared to represent the first stages of re-epithelialisation (Velnar et al. 2009). Re-epithelialisation observed in this study 6 hours post-injury in *P. cylindrica* is consistent with results reported for other invertebrates, such as *Drosophila,* for which the epidermis migrated across the lesion within 8 hours (Galko and Krasnow 2004), however, re-epithelialisation reportedly took 48 hours in the earthworm *Eisenia foetida* (Burke 1974).

In addition to granular cells, hyaline cells, or hyalinocytes are another key group of immune cells and are described for molluscs (Pipe et al. 1997; Wootton and Pipe 2003; Aladaileh et al. 2007), crustaceans (Söderhäll and Smith 1986a) and corals (Chapter 3). Hyaline cells are identified by their agranular cytoplasm and are round

when unactivated or before spreading (Aladaileh et al. 2007), consistent with the agranular cells observed at 6 hours post-injury in *P. cylindrica*. These round agranular anthozoan cells are comparable in morphology to mollusc haemocytes that stained positively for fibronectin and demonstrated fibroblast activity (Franchini and Ottaviani 2000). *Porites cylindrica* round agranular cells were in low density and were similar in size and in nuclei morphology to both the eosinophilic granular amoebocytes, melanin-containing granular cells and putative fibroblast-like cells. These consistencies among different cell types suggest that they originate from a common stem cell type that is triggered to differentiate during the various phases of wound-healing, as described within other invertebrates including the crayfish (Soderhall et al. 2003).

Fibroblasts are characterised as collagen and lipid-containing cells with the ability to fold membranes into discernable pseudopodia (Tettamanti et al. 2004; Velnar et al. 2009). Fibroblasts-like cells observed in *P. cylindrica* were agranular and had extensive pseudopodia that connected the disorganised tissue components. The presence of fibroblast-like cells is consistent with wound healing in vertebrates, such as humans (Velnar et al. 2009) and rats (Gabbiani et al. 1971) as well as in invertebrates including the leech (Tettamanti et al. 2004). The role of fibroblast cells appears consistent across the metazoa, including being instrumental in the reformation of the extracellular matrix *via* the secretion of collagen. Fibroblast cells are observed to infiltrate wound sites soon after injury and to indicate the onset of the proliferation phase (Tettamanti et al. 2004; Velnar et al. 2009). Although collagen staining was not conducted in this study, the lack of fibroblast-like cell staining with H&E indicates a low protein content of the cytoplasm, potentially due to high collagen. However, further investigations are required to fully determine their role within coral wound healing, although this study represents the first documentation of fibroblast-like cells in anthozoans.

9.5.3 PROLIFERATION AND EPIDERMIS THICKENING AT 24 HOURS POST-INJURY

At 24 hours post-injury the melanin volume fraction (Vf) was higher than at 6 hours post-injury, indicating an increase in melanin, potentially to provide structural support (Sparks 1972). The newly formed epidermal layer of eosinophilic granular cells was thickened by this time point, which was consistent with observations of *P.*

fusifera (Meszaros and Bigger 1999). This observed thickening is partly due to the reorientation of cells and reorganisation of their morphology from flattened cells aligned parallel to the lesion edge and joined end to end at 6 hours post-injury, to cells aligned perpendicular to the lesion edge and joined side-by-side at 24 hours post-injury. The latter of these cell organisation descriptions is characteristic of epidermal columnar cells of healthy tissue. The disorganised cell layer located interior to the new epithelium in *P. cylindrica*, may represent maturing granulation tissue (Velnar et al. 2009), which has been documented to develop in the mollusc *Limax maximus* at 24 hours post-injury (Franchini and Ottaviani 2000). However, circulatory components that are usually characteristic of granulation tissue (Franchini and Ottaviani 2000; Velnar et al. 2009), were absent from *P. cylindrica* as they are not a normal part of anthozoan anatomy.

9.5.4 WOUND MATURATION AND APOPTOSIS AT 48 HOURS POST-INJURY

Consistent with recovering tissue, zooxanthellae density was higher at 48 h than at all earlier time points following injury and granulation tissue had started to differentiate into discernable cell layers. However, eosinophylic granular amoebocytes also contained melanin granules and were in various stages of melanisation, suggesting that these cells were morphing into a new cell type, as observed in the Sydney rock oyster (Aladaileh et al. 2007) and crayfish (Soderhall et al. 2003). Melanisation of eosinophilic granular amoebocytes suggests that their highly proteinaceous content may be prophenoloxidase, the precursor to melanin-synthesis, thus facilitating the conversion into melanin-containing granular cells. Following a similar logic, the agranular cells may be inactive forms of the fibroblast-like cells, as the cytoplasm and nuclei appeared similar (Soderhall et al. 2003).

The nuclei of a number of the eosinophilic granular amoebocytes at 48 h post-injury contained clumped material, which appeared as dark blue dots under H&E stain, potentially indicating apoptosis (Vafopoulou 2009). Apoptosis, or programmed cell death, is a component of wound healing, which eliminates excessive cells, that potentially have harmful content, in a non-damaging process (Greenhalgh 1998). In the crayfish, apoptosis of immune cells occurred four days after injury and was demonstrated by clumped nuclear material including chromatin (Vafopoulou 2009), consistent with observations in the current study.

9.5.5 CONCLUSION

This study demonstrates the cellular process of wound healing in a hard coral for the first time and identifies four overlapping phases that are broadly consistent with those of other invertebrates as well as vertebrates. This suggests that wound-healing processes are conserved across the metazoans, including Anthozoa. Furthermore, immune cells of *P. cylindrica* were characterised and their functions identified, or proposed, for the first time. These cells include melanin-containing granular cells, eosinophilic granular cells, agranular cells and fibroblast-like cells. Common characteristics between some of these cells suggest that they may originate from a common stem cell. Further studies into longer-term mechanisms involved in wound healing and comparative inter-specific studies would help to explain the differences in regeneration rates among species.

Chapter 10 WARMER WATER SUPPRESSES THE IMMUNE RESPONSE OF A HARD CORAL

10.1 ABSTRACT

Increasing numbers of coral bleaching events and disease epizootics in recent years have been correlated with increasing sea surface temperatures but, although it has been shown that constituent levels of immunity are linked with susceptibility to both disease and bleaching (Chapter 5), the immediate effects of warming seawater on coral immune responses are unknown. Accordingly, the effects of elevated seawater temperature (32 ºC) on activity levels of immunity parameters were quantified and compared between injured and uninjured (control) branches of the brown colour morph of *Porites cylindrica*. Activities of immunity parameters were measured at 0 (control), 1, 6, 24, 48 and 168 h post-injury, including for the proteins mono-phenoloxidase, *ortho*-diphenoloxidase and *para*-diphenloxidase, and the H_2O_2 scavengers peroxidase and red fluorescent protein (RFP). At ambient seawater temperatures (27 ºC), the activities of all four enzymes were significantly greater in injured compared to uninjured branches at 1 h post-injury, however quantities of RFPs did not become significantly greater in injured samples until after 24 h post-injury under ambient conditions. At elevated temperatures, activity levels of the three phenoloxidases (POs) increased in control branches over 7 days, whereas RFP fluorescence and peroxidise activity remained the same for uninjured branches through time. Increased PO activities at 32 ºC through time for uninjured branches suggest that corals are either acclimating to warmer water, or responding to sub-lethal cellular damage induced by the elevated temperature. Overall, activity levels did not differ significantly between injured and uninjured branches in the elevated temperature treatment. Moreover, in general, activity levels of injured branches were not as great in the elevated temperature treatment as they were in the ambient temperature treatment or were delayed, suggesting an overall suppression of immunity responses in *P. cylindrica* following injury in warmer water. As the elevated seawater treatment was within the non-bleaching summer range, and no other signs of stress were observed in control branches, this study suggests that corals become immuno-compromised before visible signs of stress are detected. The effects of up-regulated baseline levels of immunity that were measured in uninjured corals in

response to elevated temperatures, on energy resources, over both short and long time frames, warrant further investigation. Overall, the results of this study are consistent with observations of increased coral disease prevalence during summer months and highlight the threat that warming sea surface conditions pose to coral health. However, further studies including higher replication and multiple coral species would help to confirm the consistency of these patterns.

10.2 INTRODUCTION

Currently, one-third of hard corals are believed to be under threat of extinction (Carpenter et al. 2008), which, coupled with ongoing declines in coral cover, will severely affect biodiversity, societies and national economies (Hoegh-Guldberg 1999; Hughes et al. 2003; Hoegh-Guldberg et al. 2009). In particular, accelerating changes in environmental conditions induced by anthropogenic impacts, especially climate change, are jeopardizing the future of coral reefs (Hoegh-Guldberg et al. 2007). Thermally induced coral bleaching and coral disease epizootics are amongst the foremost contributors to coral reef declines. It is widely accepted that both coral bleaching and disease have increased and will continue to increase in frequency and severity with climate-related environmental shifts, particularly elevated sea surface temperatures (Hoegh-Guldberg 1999; Harvell et al. 2002; Harvell et al. 2007; Hoegh-Guldberg et al. 2007; Sokolow 2009). However the manner in which these environmental threats affect underlying response mechanisms of corals remains to be determined.

Extreme or unfavourable environmental conditions have been shown to compromise resistance mechanisms in numerous organisms; for example, increased water temperature reduced innate immunity of the Taiwan abalone *Haliotis diversicolor supertexta* and its resistance to pathogenic infection (Cheng et al. 2004). Pathogen resistance of the giant freshwater prawn *Macrobrachium rosenbergii* was also compromised in unfavourable temperature, pH, salinity and ammonia treatments (Cheng et al. 2003). Similarly, compromised immunity under simulated unfavourable environmental conditions was reported for the freshwater crayfishes *Pacifastacus leniusculus* and *Astacus astacus* (Jiravanichpaisal et al. 2004) and the yellowleg shrimp *Penaeus californiensis* (Vargas-Albores et al. 1998). In contrast, some organisms demonstrate increased immunity activity with elevated temperature, including insects (Thomas and Blanford 2003), crabs (Truscott and White 1990) and

the gorgonian sea fan *Gorgonia ventalina* (Mydlarz et al. 2008). These studies demonstrate a direct influence of environmental conditions on immunity responses and therefore disease resistance, but highlight variation in immunity responses to increased temperature among invertebrates.

Climate-induced physiological stressors, such as elevated sea surface temperatures, are hypothesised to negatively affect the ability of a coral host to resist infection and recover from injury (Harvell et al. 1999; Mydlarz et al. 2009). Anthozoan responses to thermal stress have been investigated extensively (e.g. Lesser 1996; Brown 1997; Anthony et al. 2007; Smith-Keune and Dove 2008; Fitt et al. 2009) and, as for other organisms such as plants (Fujita et al. 2006), may be interlinked with immune responses (Mydlarz et al. 2008; Weis 2008). However, obligate symbiosis with photosynthetic zooxanthellae (Muscatine et al. 1984; Bythell 1988) introduces further complexity into the responses of corals to biotic and abiotic stressors. In particular, reductions in symbiont density, such as during a thermal bleaching event (Gates et al. 1992), may limit resource availability and compromise the effective up-regulation of an immune response (Sadd and Schmid-Hempel 2009).

Reduced resource availability as a consequence of thermal stress undoubtedly contributes to increases in coral disease prevalence post-bleaching (Harvell et al. 2001; Miller et al. 2006; Bruno et al. 2007) and during summer months when water temperatures are highest (Mullen et al. 2004; Willis et al. 2004; Sato et al. 2009). However, some immune activity parameters, such as prophenoloxidase, were found to increase in bleached colonies of *Montastraea faveolata* in comparison to both healthy and diseased colonies (Mydlarz et al. 2009). Other immunity parameters, such as antibacterial activity, showed differing trends (Mydlarz et al. 2009), emphasising the complexity of coral stress and immune responses (Mydlarz et al. 2008; Mydlarz et al. 2009). One such complexity is the distinction between immunity and immunocompetence. An increase in baseline levels of immunity under adverse environmental conditions may occur in response to sub-lethal cellular damage induced by these conditions. However, controlled experiments are required to determine the relationship between baseline levels of immunity and immunocompetence, which may become compromised due to resource limitation.

This study investigated the regulation of coral immunity parameters, including activity levels associated with three types of phenoloxidase (PO) enzymes, as well as H_2O_2 scavenging activity of peroxidise and fluorescence of the red fluorescent protein

(RFP), within *Porites cylindrica* in response to injury under two temperature conditions. This experiment is both timely and vital for understanding how changing climatic conditions, particularly sea surface warming, may affect coral immune responses and ultimately, coral persistence.

10.3 METHODS

10.3.1 SAMPLES

Three large (> 50cm diameter) healthy colonies of the brown colour morph (see Chapter 8) of *Porites cylindrica,* were sampled in May 2008. All colonies were located on the upper reef slope in Pioneer Bay at Orpheus Island, Great Barrier Reef (GBR), and were greater than 5 m apart to increase the likelihood of sampling distinct genotypes.

10.3.2 EXPERIMENTAL DESIGN FOR AQUARIUM-BASED STUDY

From each of the three colonies of *P. cylindrica*, 63 branches were removed and transferred to a container of 1 μm filtered seawater. Each branch was randomly allocated to a temperature treatment, either ambient (27 ºC) or elevated (32 ºC), placed randomly in one of three aquaria per temperature treatment, and held upright using plastic clothes pegs labelled with the colony number (1 to 3). The aquaria were within temperature-controlled rooms at Orpheus Island Research Station, supplied with flow-through filtered seawater (1 μm), and provided with 12 h light/12 h dark light regimes using metal halide lights. All six aquaria (three per temperature treatment) were supplied with ambient seawater temperature (~27 ºC) at the start of the experiment, when branches were placed in aquaria, and for the three subsequent days to allow branches to recover from sampling and to acclimate to aquaria conditions. Water temperatures in the three aquaria allocated to the elevated temperature treatment were then increased by 1 to 1.5 ºC per day for three days and stabilised at 32 ºC. After two days with the elevated water temperature stable at 32 ºC, three branches from each colony (n = 9, Time = 0 h) were sampled randomly from both temperature treatments. Then half of the remaining branches were injured, using bone-cutters to score a ring of damage approximately 2 mm deep located 1 cm below the branch tip. Three branches were then sampled from each colony at 1 h, 6 h, 24 h, 48 h and 168 h (7 d) post-injury (n = 15 branches from each colony), from each of the

four experimental treatments; uninjured control samples at ambient water temperature, uninjured control samples at elevated water temperature, injured samples at ambient water temperature and injured samples at elevated water temperature (total n = 60 per colony). All samples were immediately snap-frozen in liquid nitrogen and stored at -30 ºC. No mortality occurred during the experiment and all coral branches appeared healthy with no paling (visible loss of zooxanthellae) or infection.

10.3.3 SAMPLE PROCESSING

A band of tissue, approximately 6 polyps wide, was removed from injured areas and from equivalent areas on control branches, using an airgun with 100 mmol.l^{-1} phosphate buffer with 5 mmol.l^{-1} 2-mercaptoethanol (Sigma-Aldrich M7522). Tissues from branch tips and bases were avoided. Resulting tissue slurries were frozen in liquid nitrogen and stored at -30 ºC. Thawed samples were then homogenised using a vortex with glass beads for 1 min. Tissue slurries were centrifuged for 5 min to remove tissue debris, glass beads and the majority of the zooxanthellae. Supernatants were carefully removed and aliquots stored at -30 ºC.

10.3.4 BIOCHEMICAL ANALYSES

Phenoloxidase (PO) activities were quantified by measuring the conversion of colourless substrates to coloured chromatic compounds as a result of enzymatic oxidation, as determined by changes in absorbance over time at 410 nm (cf. Chapter 2) for the substrates: tyramine (Fluka 93810) for mono-phenoloxidase activity, dopamine hydrochloride (Sigma-Aldrich H8502) for *o*-diphenoloxidase activity and hydroquinone (Sigma H9003) for *p*-diphenoloxidase activity. For each of these PO assays, three 10 µl aliquots of each sample extract were placed in wells of clear 96-well microtitre plates. To each well, 50 µl of 50 mmol.l^{-1} phosphate buffer (pH 7.5) and 25 µl of deionised water (DI) were added. The plates were then covered and incubated at either 27 ºC or 32 ºC, depending on the experimental treatment regime of each sample, for 15 min in order to record the activity of enzymes under experimental treatment conditions. The appropriate substrate (30 µl of 50 mmol.l^{-}) was then added and the change in absorbance for each well, including blank controls, was measured at 410 nm for 45 min. Peroxidase activity was measured by adding 40 µl of guaiacol (25 mmol.l^{-1}) to 10 µl of sample and 35 µl of 50 mmol.l^{-1} phosphate buffer (pH 6.0). The reaction was activated by the addition of 25 µl of H_2O_2 (20 mmol.l^{-1}) and measured at

470 nm for 45 min. The change in absorbance for each sample was standardised to its protein concentration as determined by the Quick Start Bradford assay (Bio-Rad). Fluorescence was measured and standardised to protein content as described in Chapter 4, using an excitation of 550 nm to specifically demonstrate red fluorescent emission, which was measured across the wavelength spectrum from 570 nm to 650 nm. The sum of the standardised relative fluorescent units (RFU) between 580 nm and 590 nm was used to obtain fluorescence values specifically for the *Porites* red fluorescent protein (Alieva et al. 2008). All assays were conducted using a spectrophotometer (SpectraMax M2, Molecular Devices).

10.3.5 STATISTICS

Activity levels of the immunity proteins; mono-phenoloxidase, *o*-diphenoloxidase, *p*-diphenoloxidase, peroxidase and the fluorescence of red fluorescent proteins (RFP), were compared between injury treatments (uninjured or injured), temperature treatments (27 ºC or 32 ºC) and over time using a three-way block design MANOVA, with colony as the block level (branch as the replicate level; n = 9 per colony per treatment). Tukey's Honestly Significant Difference *post-hoc* tests were conducted on univariate two-way ANOVAs with significant outcomes. Analyses were executed in SPSS using log-transformed data, which satisfied assumptions of normality and homogeneity of variance according to Shapiro-Wilk's and Levene's tests, respectively.

10.4 RESULTS

Overall, mono-phenoloxidase activity of the brown morph of *Porites cylindrica* at the different temperatures (ambient and elevated; Figure 10.1) was dependent upon the time post-injury (Temperature x Time; Table 10.1). This was due to a trend of increasing activity at elevated water temperature (Figure 10.1a), which was absent at ambient water temperature (Figure 10.1b). This validates the overall significant effect of time on mono-phenoloxidase activity (Time; Table 10.1). Additionally, different *P. cylindrica* colonies demonstrated significantly different magnitudes of mono-phenoloxidase responses to the treatments (Colony; Table 10.1), however overall trends in activity were consistent (Figure 10.1). At ambient water temperature, mono-phenoloxidase activity was significantly higher, approximately 3-fold, for injured samples than uninjured samples at 1 h post-injury (Tukey P = 0.025).

This up-regulation suggests that mono-phenoloxidase activity was used rapidly as an immune response. Consistent with the significant effect of time, at elevated water temperature (Figure 10.1b) mean mono-phenoloxidase activity of uninjured branches significantly increased by approximately 3-fold between 1 and 168 h post-injury (Tukey P = 0.015). Although injured and uninjured control samples were not statistically different from the control treatment at any time point at elevated temperature (Tukey P < 0.05), injured branches demonstrated a peak of activity at 24 h post-injury (Figure 10.1b).

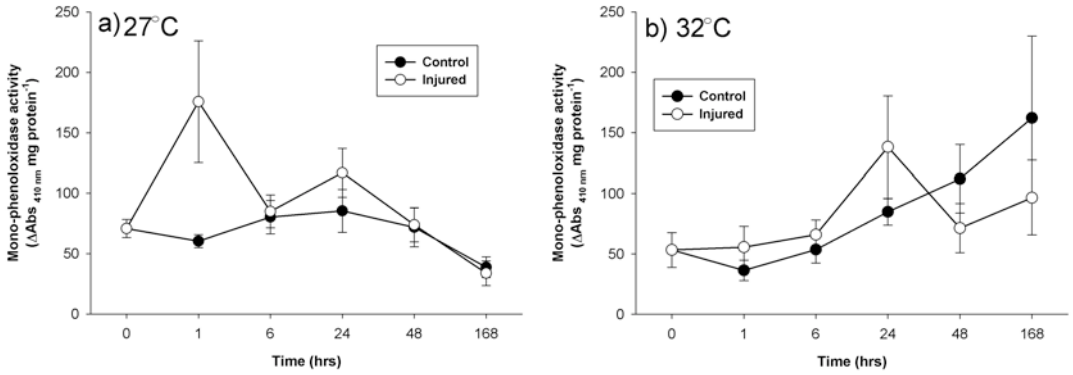


Figure 10.1: Mean mono-phenoloxidase activity (± SE) for uninjured (control) and injured branches of *P. cylindrica* at: a) ambient (27 ºC), and b) elevated (32 ºC) water temperature over time post-injury.

The variation in mean *o*-diphenoloxidase activity (Figure 10.2) in response to both temperature and injury was dependent on time (Temperature x time and Injury x Time, Table 10.1). Similar to mono-phenoloxidase activity, the variation in *o*-diphenoloxidase activity with temperature over time can be explained by the increase in uninjured control levels at elevated water temperature (Time, Table 10.1; Figure 10.2). The variation in *o*-diphenoloxidase activity with injury treatment and time is consistent with the increase in activity immediately post-injury at ambient water temperature, but the delay in up-regulation at elevated water temperature. At ambient water temperature, the injured treatment had more than 2-fold higher *o*-diphenoloxidase activity than the uninjured control treatment at both 1 h and 24 h post-injury (Tukey P < 0.05). This indicates a rapid response of *o*-diphenoloxidase activity to injury, which was sustained for 24 h. At elevated water temperature (Figure 10.2b), there was no difference in *o*-diphenoloxidase activity between injured and uninjured branches at 1 or 6 h post-injury, indicating no immediate response to injury.

However, at 24 h activity was significantly up-regulated (Tukey $P < 0.001$; Figure 10.2b). This increased activity suggests that the response of *o*-diphenoloxidase activity to injury may be delayed at higher temperatures. The approximate 3.5-fold increase in control levels of *o*-diphenoloxidase activity was significant over time by at elevated water temperature (Tukey $P < 0.05$; Tables 10.1). This increase indicates a significant response of *o*-diphenoloxidase activity to warmer water.

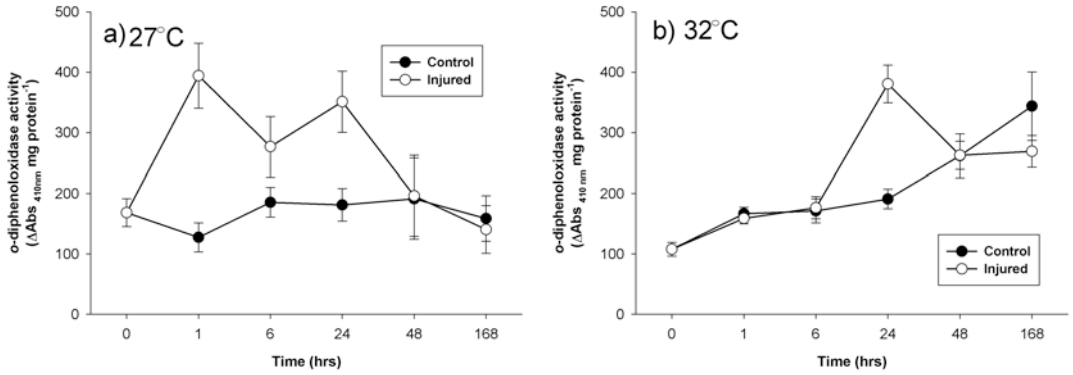


Figure 10.2: Mean *o*-diphenoloxidase activity (± SE) for uninjured (control) and injured *P. cylindrica* at: a) ambient (27 ºC) and, b) elevated (32 ºC) water temperature over time post-injury.

The mean level of *p*-diphenoloxidase activity (Figure 10.3) varied significantly with both the time post-injury and water temperature (Table 10.1). Furthermore, activity in response to injury was dependent upon the water temperature as well as time post-injury (Table 10.1). This is consistent with the significant up-regulation of *p*-diphenoloxidase activity in injured samples by approximately 2.5-fold (Tukey $P = 0.002$) under ambient temperature at 1 h post-injury as compared to uninjured controls (Figure 10.3a). This up-regulation in activity suggests a direct response of *p*-diphenoloxidase, indicative of laccase-type PO activity, to injury. At elevated water temperature (Figure 10.3b), there was a slight increase in activity at 1 and 24 h post-injury, although this up-regulation was not significant (Table 10.1). However, overall, *p*-diphenoloxidase activity of uninjured controls was higher at elevated water temperature than at ambient water temperature (Tukey $P < 0.05$), indicating a response of laccase-type PO to warmer water temperature.

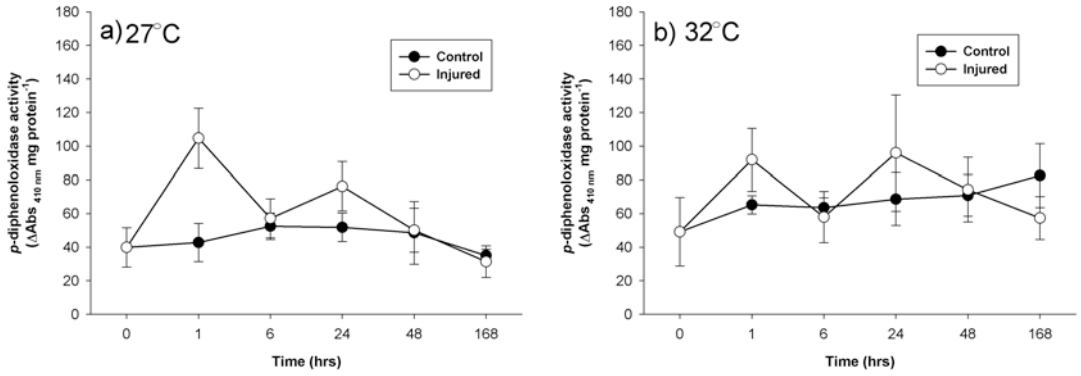


Figure 10.3: Mean *p*-diphenoloxidase activity (± SE) for uninjured (control) and injured *P. cylindrica* at: a) ambient (27 ºC) and, b) elevated (32 ºC) water temperature over time post-injury.

Overall peroxidase activity varied with injury, however this response to injury was dependent upon the water temperature (Temperature x Time, Table 10.1). At ambient water temperature (Figure 10.4a), there was a 10-fold increase in peroxidase activity with injury at 1 h post-injury (Tukey $P < 0.001$), which remained up regulated compared to uninjured controls for 48 h. This increased activity demonstrates a direct and sustained response of this antioxidant to injury. At elevated water temperature (Figure 10.4b), there was no difference in peroxidase activity between uninjured controls and injured samples, although overall (for both temperatures) activity varied between the injured treatments over time (Table 10.1). Overall, these results demonstrate an increase in peroxidase activity in response to injury.

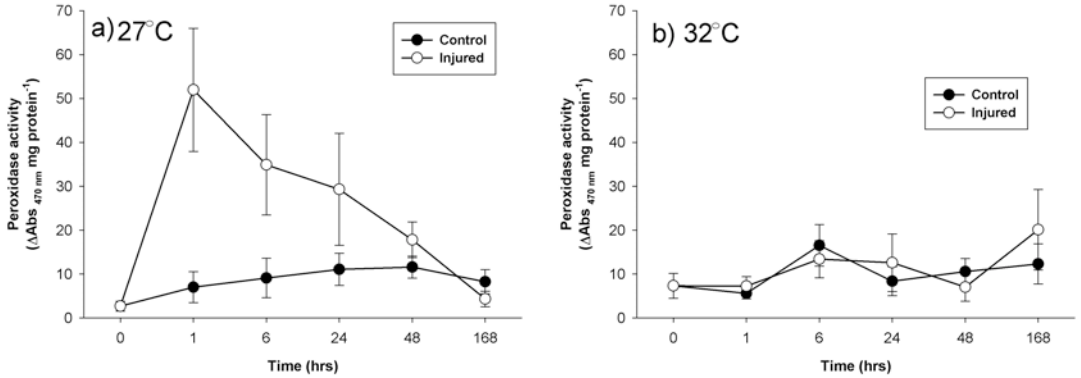


Figure 10.4: Mean peroxidase activity (± SE) for control and injured *P. cylindrica* at a) ambient (27 ºC) and b) elevated (32 ºC) water temperature over time post-injury.

Overall, red fluorescence (Figure 10.5) was significantly higher with injury as compared to the uninjured controls (Injury, Table 10.1), which was primarily driven

by the 2-fold higher red fluorescence activity in injured samples at 168 h post-injury within ambient water temperature (Tukey P = 0.005; Figure 10.5a). Red fluorescence also varied over time (Time, Table 10.1). At elevated water temperatures, although red fluorescence was higher in injured samples as compared to uninjured controls from 6 h post-injury, these differences were not significant (Figure 10.5b). Similar to peroxidase activity, these results demonstrate the up-regulation of red fluorescence in response to injury.

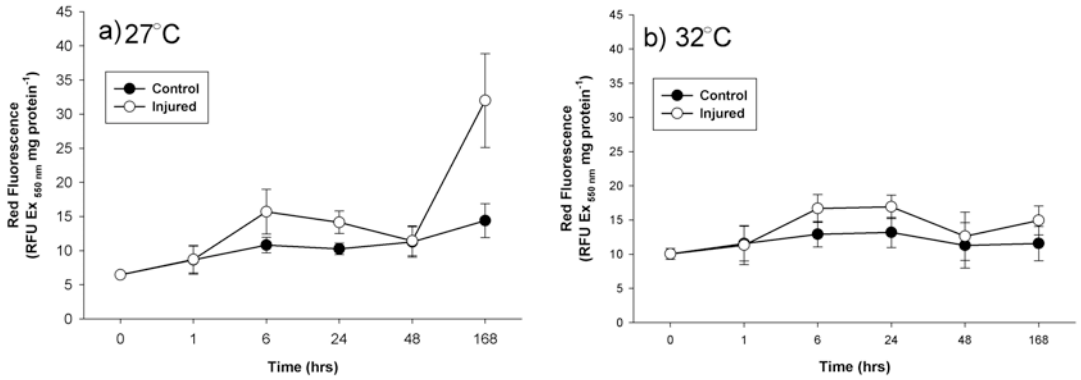


Figure 10.5: Mean red fluorescence (± SE) for control and injured *P. cylindrica* at a) ambient (27 ºC) and b) elevated (32 ºC) water temperature over time.

Table 10.1: A summary of results of the block design MANOVA with whole model and univariate F and P values examining the effects of colony, temperature (27 ºC and 32 ºC), injury (uninjured controls vs injured samples), time post-injury (1, 6, 24, 48 and 168 h), and their interactions on immunity protein levels. Mono-PO = mono-phenoloxidase, *o*-PO = *o*-diphenoloxidae, *p*-PO = *p*-diphenoloxidase and RFP = red fluorescence.

Immunity protein		Whole model (Wilks' Lambda)	Mono-PO	*o*-PO	*p*-PO	Peroxidase	RFP
Colony	df	10	2.00	2.00	2.00	2.00	2.00
	F	1.9	6.37	0.14	0.80	0.56	1.60
	P	**0.04**	**0.002**	0.869	0.449	0.571	0.205
Temperature	df	5	1.00	1.00	1.00	1.00	1.00
	F	4.4	0.13	11.08	11.48	1.30	0.09
	P	**0.001**	0.717	**0.001**	**0.001**	0.256	0.769
Injury treatment	df	5	1.00	1.00	1.00	1.00	1.00
	F	2.9	0.36	8.62	1.50	2.85	6.59
	P	**0.015**	0.55	**0.00**	0.22	0.09	**0.01**
Time	df	20	4.00	4.00	4.00	4.00	4.00
	F	2.7	2.63	2.44	3.25	1.07	4.66
	P	**<0.001**	**0.037**	**0.049**	**0.014**	0.373	**<0.001**
Temp x injury	df	5	1.00	1.00	1.00	1.00	1.00
	F	2.4	1.38	3.31	3.76	7.91	0.11
	P	**0.042**	0.24	0.07	**0.05**	**0.01**	0.74
Temp x time	df	20	4.00	4.00	4.00	4.00	4.00
	F	4.07	9.14	8.14	1.00	2.69	2.04
	P	**<0.001**	**<0.001**	**<0.001**	0.411	**0.033**	0.091
Injury x time	df	20	4.00	4.00	4.00	4.00	4.00
	F	2.12	1.85	2.37	3.58	2.41	1.24
	P	**0.003**	0.122	**0.055**	**0.008**	**0.051**	0.298
Temp x Injury x Time	df	20	4.00	4.00	4.00	4.00	4.00
	F	0.82	0.45	1.39	0.36	2.30	0.25
	P	0.69	0.776	0.238	0.838	0.061	0.909

10.5 DISCUSSION

At ambient water temperature, all measured coral immunity proteins increased upon injury, but at elevated water temperature they did not increase with injury even though the water was within the normal summer temperature range for the location. Therefore, immunocompetence, defined as the up-regulation of an immune response to a specific stimulus (Adamo 2004b), was suppressed under warmer water conditions. However, control samples (uninjured) at elevated water temperature increased their phenoloxidase (PO) activity over time, suggesting that temperature stress may increase constituent levels of anthozoan immunity, either as a protection mechanism or as a response to unobserved cellular damage. These results represent a preliminary step towards unravelling the intertwined complexities of anthozoan immune responses and their responses to thermal stress (Mydlarz et al. 2008; Weis 2008; Mydlarz et al. 2009).

The increase in activities of all three PO, plus peroxidase and the red fluorescence (RFP) upon physical injury at ambient water temperature, further supports the role of these parameters in coral immune responses (Section I and Chapter 8). The differences in the regulation of the POs post-injury may be indicative of their various roles within a coral immune response. Consistent with the predominantly reported role of the tyrosinase-like POs (mono-phenoloxidase and *o*-diphenoloxidase) in invertebrate immune responses (Söderhäll and Cerenius 1998; Iwanaga and Lee 2005), these enzymes increased in activity within one hour after injury under ambient water temperature. Although mono-phenoloxidase activity varied among coral colony, this did not affect the overall trends in activity. It is therefore likely that an increased sample size would eliminate this effect. The different regulation of mono-phenoloxidase and *o*-diphenoloxidase activity post-injury potentially highlights the timing of the use of the various stages of the melanin-synthesis pathway. This is because mono-phenoloxidases convert mono-phenols to diphenol substrates, which can then be used by *o*-diphenoloxidases (Sugumaran 2002; Nappi and Christensen 2005) and may explain why mono-phenoloxidase is only up-regulated at 1 h post-injury. Similarly, the consistent regulation of *p*-diphenoloxidase activity with mono-phenoloxidase activity with injury at ambient water temperature, implies its dependency on mono-phenoloxidase as a pathway precursor (Sugumaran

2002). Additionally, the up-regulation of *p*-diphenoloxidase activity further supports its role in wound healing, potentially during clot formation (Chapter 8).

In addition to the POs, activity of the antioxidant enzyme peroxidase increased immediately with injury at ambient water temperature. This increase is consistent with the use of an oxidative burst to prevent pathogenic infection (Pipe et al. 1997; Mydlarz and Jacobs 2006) and with the up-regulation of the cytotoxic melanin-synthesis pathways (Nappi and Ottaviani 2000). Furthermore, as RFP activity in injured samples was highest when peroxidase activity had returned to control levels (at 48 h), RFP activity levels in this study support the proposed role of the RFP as a supplemental antioxidant during an immune response (Chapters 4 and 7).

The PO responses in this experiment are different from those previously documented for the brown colour morph of *Porites cylindrica* with injury (Chapter 8). In the previous study, the brown colour morph demonstrated a decrease in tyrosinase-type PO (*o*-diphenoloxidase) with injury and showed an increase in laccase-type PO activity (*p*-diphenoloxidase) only after 24 h (Chapter 8), whereas under ambient conditions in this experiment, all POs increased within 1 h post-injury. The different response to injury detected in the this experiment compared to the previous injury experiments with three colour morphs may potentially be due to the differing light environments used. Artificial light was used for the duration of this experiment, whereas the three colour morphs were injured and held under natural light conditions.

The additional cytotoxicity of the immune response in the this study, as indicated by the three PO and peroxidase activities, compared to previous investigations (Chapter 8), may have been enabled by the use of artificial light. Although the metal halide lights used provide adequate light for photosynthesis (Abrego et al. 2008), they are not equivalent to natural light and specifically do not provide significant UV outputs. As natural sunlight may subject the corals and symbionts to low levels of UV-stress, reduced cytotoxicity in studies using natural light may reflect plasticity in *P. cylindrica* immunity functions, in response to altered environmental conditions, to avoid high incidence of self-harm. For example, when corals are at higher risk of oxidative stress, such as with high irradiance, the benefits of up-regulating a highly cytotoxic immune response may be outweighed by the potential for additional oxidative damage to host tissues and therefore this response may be limited. The potential regulation of coral immune responses based on light

environments has possible implications regarding the comparative immunocompetence of conspecifics living in high light versus shaded reef habitats.

The lack of immediate up-regulation of innate immunity variables at elevated water temperature indicates that temperature directly inhibits coral immunocompetence. This inhibition is consistent with reports from other invertebrates (Vargas-Albores et al. 1998; Cheng et al. 2003; Cheng et al. 2004; Jiravanichpaisal et al. 2004), and suggests that the ability of *P. cylindrica* to heal wounds may be compromised under warmer water temperatures. However, contrary to previous studies of the effects of thermal stress on coral immune responses (Mydlarz et al. 2008; Mydlarz et al. 2009), the elevated temperature treatment in this experiment was within the range of normal (non-bleaching) summer temperatures for the reef slope environment from which the samples were collected. Furthermore, no mortality occurred throughout the experiment and coral branches showed healthy pigmentation for the duration. This implies that corals become immuno-compromised before signs of stress are visible. This finding may enable the development of a new tool to contribute to early warning systems for coral bleaching and disease that are currently being developed by the Great Barrier Reef Marine Park Authority (GBRMPA); specifically it suggests that measurements of coral immunity may be used as bioindicators of coral health.

The increased baseline level of *o*-diphenoloxidase activity at elevated water temperature and the general increasing trend of the other POs over time suggests that *P. cylindrica* utilises melanin-synthesis pathways (tyrosinase-type and laccase-type) during thermal stress, potentially in response to sub-lethal cellular damage (Lesser 1997; Weis 2008). The use of the melanin-synthesis pathway during thermal stress is consistent with increased PPO activity (Pro-*o*-diphenoloxidase) documented in bleached *Montastraea faveolata* (Mydlarz et al. 2009) and with the involvement of immunity parameters in bleaching susceptibility (Chapter 5). As water temperatures were maintained at a constant 32 ºC for the duration of the experiment, and as assays were run at this temperature, it is unlikely that the observed increase over time was a result of temperature effects on enzyme activity (Mydlarz et al. 2009) and was more likely to be a demonstration of an increased presence of active POs. Mydlarz et al. (2009) proposed two hypotheses for the increase in melanin-synthesis pathway activity with temperature stress: calcium stimulation of the biochemical cascade and a release from immuno-suppression by the zooxanthellae when symbionts are lost

during bleaching. From the current study, neither of these hypotheses can be discounted as calcium stimulation may be cumulative over time and, although no paling was observed, zooxanthellae densities were not quantified. However, alternative hypotheses include, firstly, that during periods of elevated water temperature, corals respond to cellular damage with immunity variables. However, in the current study as elevated water temperatures were within a normal summer range and no signs of bleaching or compromised health were observed, this is unlikely. The second hypothesis is that elevated water temperatures cue corals to up-regulate baseline levels of immunity.

Seasonal fluctuations in immune responses and/or disease susceptibility are documented for numerous organisms, including the European abalone *Haliotis tuberculata* (Travers et al. 2008), deer mice *Peromyscus maniculatus* (Demas and Nelson 1998) and cyprinid fish *Rutilus rutilus* (Kortet et al. 2003). Seasonality of immune responses is a result of varying environmental conditions, such as temperature (Bowden et al. 2007; Bowden 2008; Brockton and Smith 2008), but also may be tightly linked with reproductive cycles. For example, the Pacific oyster *Crassostrea gigas* suffers high mortality during summer months due to a synergistic effect of elevated water temperature and the redirection of resources to spawning (Li et al. 2007). However, in fish, unfavourable environmental conditions and potentially high pathogen loads result in an up-regulation of immunity (Bowden et al. 2007), potentially because of the redirection of energy resources from growth and reproduction to immunity, as documented for vertebrates including *P. maniculatus* (Demas and Nelson 1998). Resource redirection, such as during reproduction, usually occurs as a result of an environmental cue, such as a change in photoperiod or ambient temperature, as documented for both vertebrates (Demas and Nelson 1998) and invertebrates (Kemp 2000). For *P. cylindrica,* elevated water temperature may be an environmental cue to up-regulate constituent immunity levels for the summer months.

Elevated water temperature on coral reefs is usually coupled with high irradiance and therefore a higher threat of coral bleaching (Hoegh-Guldberg 1999). Therefore, water temperature would make a useful environmental cue for corals to up-regulate baseline levels of immunity, particularly the melanin-synthesis pathway for its photoprotective (Meredith et al. 2006) and radical scavenging properties (Nappi and Ottaviani 2000). With these properties, melanin-synthesis may both mitigate

bleaching (Chapter 5) and prevent infection (Söderhäll and Cerenius 1998; Nappi and Ottaviani 2000; Nappi and Christensen 2005) during a season when pathogen virulence and load may be highest (Harvell et al. 1999; Harvell et al. 2002; Mydlarz et al. 2006). Temperature-induced seasonality in coral immunity would broadly complement reproductive patterns of Indo-Pacific corals. The majority of coral species on the Great Barrier Reef take part in an annual mass-spawning event during October or November (Harrison et al. 1984; Willis et al. 1985; Baird et al. 2009), which is cued by lunar periodicity (Willis et al. 1985; Babcock et al. 1986; Johnson 1992). As mass spawning takes place only a few months prior to the hottest time of year, coral resources may be directed away from reproduction towards immunity after this event, and thus enable both photo- and antimicrobial protection during the summer months. Field samples of corals, preferably of several species, taken frequently throughout the year and comparisons of both biochemical immunity assays and histological investigations of the stages and timing of gametogenesis would test the efficacy of this hypothesis. The inclusion of molecular techniques to assess the relative expression of relevant immunity and reproductive genes has the potential to greatly enhance this type of study.

Porites cylindrica is among one of the most tolerant corals to both bleaching and disease on the Great Barrier Reef (Willis et al. 2004; Page and Willis 2006, 2008), perhaps largely owing to the relatively high allocation of energy towards immunity, which enables the species to maintain a comparatively high baseline level of immunity (Chapter 5). However, the ability of a coral to sustain elevated baseline levels of immunity, such as under thermal stress, will largely depend upon energy reserves, particularly if corals are bleached and therefore nutrient-deprived (Bythell 1988). This suggests that corals with lower energy reserves, indicated by tissue thickness or biomass (Fitt et al. 2000), may not be able to sustain increased activity levels and will therefore be more susceptible to disease during thermal stress events. This hypothesis is supported by studies of the tissue biomass of four Caribbean corals, which was highest during the winter/spring and lowest in late summer/autumn (Fitt et al. 2000). Furthermore, tissue biomass was lower for corals from the family Acroporidae than those from Faviidae (Fitt et al. 2000), which is consistent with bleaching and disease susceptibilities previously investigated (Chapter 5).

10.5.1 CONCLUSION

This study suggests that warmer water temperatures suppress *P. cylindrica* immunocompetence to physical injury and therefore are likely to inhibit wound healing and reduce resistance to pathogenic infection. These results are of particular concern in light of the coincidence of increasing storm frequencies and warming sea surface temperatures (Hoegh-Guldberg et al. 2007), which suggest increasing likelihood of physical disturbance and tissue damage. Additionally, the results of this study take us a step closer to understanding increases in coral disease prevalence during summer months (Willis et al. 2004; Sato et al. 2009), by demonstrating suppression of immunocompetence with warmer water in one of the most disease-resistant corals. However, this suggests a lowered resistance to disease and has potentially vast implications for less resistant coral species if they demonstrate the same patterns of compromised immunity with elevated water temperatures. The suppression of coral immunocompetence with warmer water further implies a direct role of coral immunity in underpinning the temporal fluctuations of coral disease prevalence, as previously proposed (Harvell et al. 1999; Harvell et al. 2002; Mydlarz et al. 2006). Additionally, the results show a gradual increase in constituent levels of PO at elevated water temperature, which may enable corals to survive warming water conditions. This conclusion can be made in light of visible evidence that corals remained healthy and thus increased activity represents up-regulation of baseline immunity rather than a response to temperature-induced sub-lethal damage. However, the potential for coral to survive warming water conditions is likely to be directly linked to their energetic resources. Increases in baseline immunity with warming seawater have implications for temperature-cued seasonal fluctuations in constituent levels of immunity and further highlights the complex trade-offs in coral life history traits. However, field collections and controlled experiments with multiple simulations of climate-change scenarios, with higher sample sizes and multiple coral species, would help to further establish the trends identified within this study and may help to determine the potential for corals to cope with climatic changes.

Chapter 11 GENERAL DISCUSSION AND FUTURE DIRECTIONS

Investigations conducted as part of this thesis demonstrate that corals not only possess and use innate immunity components similar to those of higher invertebrates, but they also use immune functions that may be unique to cnidarians. These results refute the assertion that coral immune systems are basic, an argument that has been put forward because of their basal position in the phylogeny of animals and their simplistic body plan (Loker et al. 2004; Bosch 2008). On the contrary, my studies suggest that corals have well-developed mechanisms for resisting infection and recovering from injury (Table 11.1), which partly explain their persistence through evolutionary time (Veron 2000) and may provide optimism for their continued persistence under a rapidly changing climate.

11.1 CORAL IMMUNITY IN THE CONTEXT OF INVERTEBRATE IMMUNITY

Invertebrates comprise the majority of metazoan phyla and demonstrate a heterogeneous array of morphologies, habitat preferences and life histories, partly because of their multiple independent evolutionary lineages (Loker et al. 2004). Thus it is likely that terrestrial, short-lived, unitary invertebrates, such as insects, may have evolved disparate defence mechanisms to those of marine, long-lived, obligately symbiotic, modular organisms that compete for benthic space, such as corals (Loker et al. 2004). Conversely, similarities in innate immunity mechanisms are also present among organisms many from plants and fungi to invertebrates (Loker et al. 2004; Schulenburg et al. 2004). These similarities suggest a common origin of immunity mechanisms and their subsequent conservation through evolutionary time. Thus model organisms are commonly used to understand invertebrate immune systems (Schulenburg et al. 2004). Whilst comparative studies of the conservation of immunity mechanisms between model invertebrates (e.g. *Drosophila melanogaster* (Tzou et al. 2002) and *Caenorhabditis elegans* (Brown 2000)), and vertebrates, are extensive (e.g. Medzhitov et al. 1997; Hoffmann et al. 1999; Azumi et al. 2003; Fujita et al. 2004; Schulenburg et al. 2004), the origins of metazoan immunity are less well

explored (Hemmrich et al. 2007). The presence of gene homologues in basal and higher metazoans has been noted, and although functions of these genes in lower organisms are typically presumed to be consistent with their higher counterparts, molecular immunity mechanisms in basal metazoans remain largely unexplored (e.g. Brower et al. 1997; Muller 1997; Muller et al. 1997; Pancer et al. 1997; Perovic-Ottstadt et al. 2004; Miller et al. 2007; Wiens et al. 2007). In light of the absence of functional studies, establishing whether metazoans across the phylogenetic spectra utilise the same effector responses provides an alterative approach for addressing the question of whether immunity mechanisms are evolutionarily conserved.

Of the three innate immunity effector response mechanisms, i.e. antimicrobial peptides (AMPs), proteolytic cascades (phenoloxidase and coagulation cascades) and cell activation (Hoffmann et al. 1999), the latter two were investigated in anthozoans within this thesis (Table 11.1). Cnidaria, including the class Anthozoa, are amongst the most phylogenetically basal animals, preceded only by the Porifera and Ctenophora, which are therefore considered to be more basal (Dunn et al. 2008). My studies addressed the current lack of knowledge on mechanisms of innate immunity in corals (Loker et al. 2004; Mydlarz et al. 2006) and they significantly advance the current understanding of coral immunity components (see Table 11.1, which highlights knowledge of coral immunity prior to and after the completion of this thesis). My results provide an important base from which to further examine the phylogenetic context for the evolution of coral immune mechanisms.

Table 11.1: Overview of knowledge on coral immunity, highlighting findings from this thesis **(bolded)**

Phases of immunity	Effector responses	Immunity parameter	Order	Reference
Receptors and signalling		TLR	Scleractinia	Miller et al. 2007
		Integrin	Scleractinia	Brower et al. 1997
		MBL-like	Scleractinia	Kvennefors et al. 2008
Effector responses	Antimicrobial peptides	No. Bactericidal and antimicrobial activity	Scleractinia	Mydlarz et al. 2009
				Geffen et al. 2009
				Koh 1997
			Alcyonacea	Koh 1997
	Melanin-synthesis pathways	**(Pro)phenoloxidase activity**	Alcyonacea	Mydlarz et al. 2008
			Scleractinia	**Chp. 2, 5, Sec. II**
			Alcyonacea	**Chp. 2 & 5**
			Zoantharia	**Chp. 2**
		Melanin deposits	Alcyonacea	Petes et al. 2003
			Scleractinia	**Chp. 3, 5, Sec. II**
			Alcyonacea	**Chp. 3 & 5**
	Coagulation pathway	**Transglutaminase activity**	**Scleractinia**	**Chp. 2 and 8**
	Immune cells	**Granular amoebocytes**	Alcyonacea	Mydlarz et al. 2008
			Scleractinia	**Chp. 3 & 9**
		Phagocytic cells	Alcyonacea	Olano and Bigger 2000
			Scleractinia	**Chp. 3**
		Chromophore cells	Scleractinia	Domart-Coulon et al. 2006
		Melanin-containing granular cells	**Scleractinia**	**Chp. 3 & 9**
			Alcyonacea	
		Amoebocyte	Scleractinia	Vargas-Angel et al. 2007
		Agranular amoebocyte	**Scleractinia**	**Chp. 3 & 9**
		Fibroblast-like cell	**Scleractinia**	**Chp. 9**
Antioxidants	**Enzymatic antioxidants**	Superoxide dismutase	Scleractinia	Hawkridge et al. 2000
		Catalase(-like)	Scleractinia	Griffin et al. 2006
			Scleractinia	**Chp. 4, 5 & 7**
			Alcyonacea	**Chp. 4 & 5**
		Peroxidase	Alcyonacea	Mydlarz & Harvell 2007
			Scleractinia	**Chp. 4, 5 & 7**
	Non-enzymatic antioxidants	**Fluorescent proteins**	**Scleractinia**	**Chp. 4, 5 & 7**
			Alcyonacea	**Chp. 4, 5 & 7**
			Zoantharia	**Chp. 4**

The most basal invertebrates for which a melanin-synthesis pathway has been documented are anthozoans, including 20 Indo-Pacific species that span the orders Scleractinia, Alcyonacea and Zoantharia (Chapter 2), and one Caribbean species within each of the Gorgonacea (Mydlarz et al. 2008) and Scleractinia (Mydlarz et al. 2009). In addition, within the three Indo-Pacific orders of anthozoans, both tyrosinase-type phenoloxidase (PO) and laccase-type PO were found, demonstrating the presence of multiple activating enzymes for the melanin-synthesis pathways (Chapter 2). Whilst tyrosinase-type PO has been documented within invertebrates, including echinoderms (Smith and Soderhall 1991), molluscs (Asokan et al. 1997), arthropods (e.g. (Söderhäll 1982) and ascidians (Smith and Soderhall 1991), laccase-type PO has only previously been found within arthropods (Barrett 1987; Theopold et al. 2002) and a mollusc (Luna-Acosta et al. 2010). Biochemical evidence for laccase-type PO in anthozoans (Chapters 2, 8 and 10) indicates that this melanin synthesis pathway is present in phylogenetically basal metazoans, and has potentially been conserved from fungi (Claus 2003; Baldrian 2006). Furthermore, demonstrations of functional roles for these melanin-synthesis pathways in coral immunity (Section II) suggest that not only innate immunity components, but also their functions are conserved throughout the invertebrates (Loker et al. 2004; Schulenburg et al. 2004; Miller et al. 2007; Sullivan et al. 2007). However, melanin-synthesis pathway components appear to be absent from *C. elegans* (Brown 2000) and have not been investigated within the most basal metazoans, the sponges and ctenophores (Mydlarz et al. 2006).

The presence and demonstration of transglutaminase function in coral wound healing (Chapters 2 and 8) suggest that this Ca^{2+}-dependent enzyme is an important component of coral immunity, particularly given the potential frequency of coral physical injury (Bythell et al. 1993; Bythell et al. 2000; Rotjan and Lewis 2008). This is also the first documentation of transglutaminase activity in such a phylogenetically basal animal and further implies that anthozoans are complex organisms with immune components that are equivalent to those of higher organisms (Brower et al. 1997; Muller 1997; Miller et al. 2007).

The roles of immune cells as part of an innate immune response have been extensively documented for organisms across the Metazoa, including within basal invertebrates such as the Anthozoa (Olano and Bigger 2000), as well as vertebrates (Velnar et al. 2009). My investigations of anthozoan immunity identified novel

immunity-related cells, demonstrated phagocytosis-like behaviour of these cells (Chapter 3) and characterised wound healing (Chapter 9) within the Scleractinia for the first time. The cells involved in scleractinian wound healing, as well as the phases, were broadly consistent with those documented for higher invertebrates, such as insects (Galko and Krasnow 2004), as well as vertebrates, including humans (Velnar et al. 2009). Overall, this demonstrates that both cell types, such as fibroblasts, and processes involved in animal wound healing responses have been evolutionarily conserved. These studies also pave the way for understanding processes that may lead to different rates of coral regeneration among species and under different environmental conditions (Meesters and Bak 1993; Meesters et al. 1994; Fine et al. 2002; Alvarado and Acosta 2009).

11.1.1 CORALS AS MODEL ORGANISMS

Model organisms are extensively investigated, non-human species, whose study provides insights into processes and mechanisms relating to other organisms (Fields and Johnston 2005). The conservation of the investigated coral immunity parameters throughout invertebrates suggests that corals may be viable as model organisms for the study of invertebrate immunity, and potentially mammalian immunity with respect to wound healing (Chapter 10). There are several advantages of corals as model organisms for immunity studies that surpass those of current models, such as *D. melanogaster* (Tzou et al. 2002) and *C. elegans* (Brown 2000). The modular nature of corals provides unique opportunities for the production of multiple genetic replicates for use in experimental studies. Such clonal replicates reduce variation in results due to the use of different genotypes, which may mask fine-scale responses. They also enable temporal studies, whereby control and treatment levels of immunity parameters are measured for the same genotype(s) (e.g. Chapters 8, 9 and 10). This directly addresses problems arising from measuring the magnitude of immune responses, and thereby quantifying immunocompetence, in different genotypes (Adamo 2004b). Using corals as model organisms for invertebrate immunity would, for example, enable investigations of temporal effects of infection or adverse environmental conditions on levels of immunity and, additionally, provide an indication of the key response times of various innate immunity mechanisms.

11.2 CORAL IMMUNITY IN THE CONTEXT OF CORAL BIOLOGY

11.2.1 FLUORESCENT PROTEINS

The presence of fluorescent proteins is perhaps most conspicuous within cnidarians, however genes homologous to the jellyfish Green Fluorescent Protein (GFP) are present within some Bilateria, including copepods (phylum Arthropoda) and lancelets (subphylum Cepalochordata) (Shagin et al. 2004). Fluorescent proteins (FPs) were quantified in anthozoans for the first time in this thesis, using newly developed protocols (Chapter 4). Moreover, their antioxidant properties (Chapter 4) and up-regulation upon infection and injury (Section II) were demonstrated. Although mechanisms giving rise to antioxidant properties need to be determined, these results suggest important roles of coral FPs during an immune response, potentially as supplemental antioxidants (Chapter 4). However, the greater hydrogen peroxide (H_2O_2) scavenging activity of FP mutants, which lack chromophores, suggests that their primary role may not be as antioxidants. Given their reduced H_2O_2 scavenging activity when associated with chromophores, the accumulation of high concentrations of FPs in areas of compromised coral tissue (Chapters 6, 7, 8 and 10) suggests another immune or recovery-related key function, potentially related to colour.

The presence of FPs within symbiotic cnidarians supports hypotheses that they function as photoprotectors (Kawaguti 1944; Salih et al. 2000) and enhancers of photosynthesis by zooxanthellae within coral tissues. However, photoprotection mechanisms intrinsic within *Symbiodinium* sp. are more effective than those of FPs (Mazel et al. 2003; Wiedenmann et al. 2004) and thus far, studies into FPs as photosynthesis enhancers have not provided support for this hypothesis (Gilmore et al. 2003). Similarly, the presence of GFPs within some non-symbiotic Bilateria and both GFPs and GFP-homologues within the Cnidaria (Shagin et al. 2004) suggest that FPs may have an alternative general function. The up-regulation of anthozoan FPs in areas of injury (Chapters 6, 7, 8 and 10) may provide insights into such an alternative common role for FPs. However, although the GFP is the common ancestor, colour diversification from GFPs occurred within the Cnidaria, although along different lineages (Shagin et al. 2004). The evolution of the same FP colour types cyan and red and a purple/blue chromoprotein, along different lineages indicates convergent evolution (Shagin et al. 2004). Thus each FP colour may fulfil a specific role and, as a group, FPs may be multi-functional.

Coral FPs all demonstrated H_2O_2 scavenging activity, an antioxidant function that is key for mitigating thermal stress (Lesser 1997) and also critical during immune responses to injury and infection (Halliwell and Gutteridge 1999). Coincident with GFPs possessing the simplest chromophore and being the "default" state (Shagin et al. 2004; Alieva et al. 2008), the coral GFP was the least efficient at scavenging H_2O_2. The chromoprotein was the most efficient scavenger (Chapter 4), evolved within three distinct cnidarian lineages and possesses a more complex chromophore (Shagin et al. 2004; Alieva et al. 2008). Antioxidant properties associated with each FP colour type indicate a general role among cnidarians, however, the greater antioxidant efficiency of FPs in the absence of their chromophores, as well as convergent evolution of all FP types across lineages, suggests that a primary function of these coral proteins relates to their colour.

The accumulation of FPs with greater antioxidant efficiency in compromised coral tissue (Chapter 6) supports their role within anthozoan immunity and thus their likely multi-functionality. Similarly, the heterogeneous responses of coral colour morphs to injury implies an influence of colour on immune responses, however the up-regulation of FPs within all morphs further indicates their role in immunity (Chapter 8). Additional immunity-related roles of coral FPs could include extracellular matrix formation, which is the primary role documented for GFP homologues of the same superfamily within bilaterians (Willem et al. 2002), but has not yet been investigated within anthozoans. Also, although azooxanthellate anthozoans possess FPs (Wiedenmann et al. 2004), the relationship between the type and abundance of coral FPs and zooxanthellae needs to be better established. Assessing the possible connection between these biotic factors using data accumulated throughout this thesis indicates an inverse relationship between zooxanthellae density and FP concentration. My results demonstrate that during an immune response, zooxanthellae density is reduced (Chapters 6 and 9) but FP concentration is increased (Chapter 6, 7, 8 and 10). The timings and mechanisms behind this inverse relationship need to be closely examined in order to determine, for example, whether FPs induce the loss of zooxanthellae or whether they are up-regulated in order to re-establish a favourable environment for zooxanthellae re-colonisation. Although the inverse correlation might be coincidental, given the antioxidant properties of coral FPs (Chapter 4) and their presence in healthy coral tissue, the latter suggestion of creating a favourable environment seems more

plausible. However, although colour is likely to be involved in the primary role of FPs, few studies have conclusively or beneficially linked FP emission spectra with the requirements of zooxanthellae (Mazel et al. 2003; Salih et al. 2004; Cox and Salih 2006). Immunological functions of coral FPs that pertain to colour also remain to be established. A colour-based function of coral FPs represents a conundrum, given that corals are sessile, free-spawning invertebrates that are not affected by vision-driven sexual selection.

11.2.2 SYMBIOSIS

The obligate intracellular symbiosis of photosynthetic zooxanthellae within coral hosts (Bythell 1988) has the potential to influence, and be influenced by, immune responses within each of the two partners (Weis and Allemand 2009). The very establishment of such a symbiosis brings into question the efficacies of host and symbiont immune defences that allow the coexistence of foreign organisms (Muscatine et al. 1975). Furthermore, to enable the establishment and maintenance of a successful symbiosis, continuous cross-talk between the symbiotic partners is required (Weis 2008).

The onset of symbiosis between corals and zooxanthellae has been proposed to occur *via* pattern recognition receptors (PRRs) and associated symbiont ligands, such as lectin/glycan interactions (Wood-Charlson et al. 2006; Weis 2008). This method of recognition underpins the distinction between self and non-self and is therefore consistent with host-pathogen interactions and the initiation of an immune response (Medzhitov and Janeway 1997; Fujita et al. 2004). For example, zooxanthellae are documented to enter coral host cells *via* phagocytosis and remain within the phagosome, thus protected from host-derived antimicrobial defenses (Schwarz et al. 2008). Notably, this mechanism is similar to ones involved in obligate parasitism of eukaryotic cells, for example by the intracellular bacterium *Coxiela burnetii* (Hackstadt and Williams 1981), indicating that zooxanthellae may use parasitic mechanisms to enter coral host cells. Evasion of host immune responses by parasites enables them to persist within the host without detection (Sadd and Schmid-Hempel 2009). Avoidance and manipulation mechanisms employed by parasites include anatomical seclusion, such as within the phagosome, as well as active suppression of the host's immune responses (Damian 1997; Sadd and Schmid-Hempel 2009). The recent demonstration that competent zooxanthellae establish an

effective symbiosis within host tissues with only minor alterations to the host transcriptome, primarily involving the up-regulation of immunity-related genes (Schwarz et al. 2008; Voolstra et al. 2009), suggests that zooxanthellae infection may primarily depend on evading host immune detection systems. Thus, once phagocytosed into the host cell and stabilised within the phagosome, zooxanthellae may evade coral immune responses rather than suppress them, although the latter has been proposed in light of differences in immune activity within bleached and unbleached hard coral (Mydlarz et al. 2009).

The manner in which the relationship between coral hosts and their zooxanthellae endosymbionts is established and maintained is important for determining the interactions that lead to coral bleaching (Weis 2008; Weis and Allemand 2009). Coral bleaching, which is a general sign of stress, typically involves the loss of symbionts *via* one of several different mechanisms and may be in response to multiple causes (Gates et al. 1992). The best documented and most devastating cause of coral bleaching is thermal stress (e.g. Brown 1997; Hoegh-Guldberg 1999; Marshall and Baird 2000), during which elevated oxidative stress is largely responsible for the disruption of photosynthesis and the break-down of symbiosis (Lesser 1996,1997; Jones et al. 1998; Weis 2008). Components of innate immune signalling pathways are up-regulated by both organisms during thermal bleaching and include reactive oxygen species (ROS) and nitric oxide (Perez and Weis 2006; Weis 2008). If oxidative stress conditions originating within the zooxanthellae are detected as a threat by the host, host immune responses are likely to be activated in order remove the source of the threat, i.e. to expel dysfunctional zooxanthellae. Increased prophenoloxidase (PPO) activity detected in bleached, as compared to healthy, hard corals (Mydlarz et al. 2009) supports this interpretation. However, susceptibility to thermal bleaching was found to be higher in corals with lower baseline levels of immunity (Chapter 5), which suggests that the host immune system plays a significant role in preventing bleaching and therefore maintaining the symbiosis. Coral responses to environmental stress, inclusive of interactions with endosymbionts, are likely to be complex, thus some aspects of the coral immune response may potentially enable and protect the symbiosis, whilst others may initiate its breakdown.

Production of melanin as part of coral innate immune function may play a significant role in protecting photosynthetic zooxanthellae and thus in maintaining the symbiosis. Melanin is an effective absorber of light, including UV wavelengths (Riley

1997; Burkhart and Burkhart 2005; Meredith et al. 2006; Brenner and Hearing 2008), which are particularly damaging to the photosynthetic mechanisms of zooxanthellae (Nishiyama et al. 2006). The ability of corals to regulate the production of melanin (Chapter 2 and Section II) and the presence of melanin within mobile cells (Chapters 3 and 9), has large implications for mitigating bleaching *via* the up-regulation and movement of melanin to photoprotect the threatened zooxanthellae, as well as scavenge ROS (Riley 1997; Nappi and Ottaviani 2000). The presence of such an effective photoprotector within corals suggests that FPs may be redundant in this role and argues against the hypothesis that photoprotection is the primary role of FPs (Salih et al. 2000). Furthermore, if melanin plays a major role in photoprotection of corals (Chapter 5), this would explain the increased prophenoloxidase activity detected in bleached hard corals (Mydlarz et al. 2009). Additionally, such a role for melanin would explain increased phenoloxidase activity detected in experimental elevated temperature treatments, as increases in temperature may represent a cue for increased immune and photoprotective activity due to higher bleaching and infection risks associated with warm summer temperatures (Chapter 10).

Unusual coral bleaching patterns are used as a field sign of compromised health that are unrelated to thermal bleaching (Willis et al. 2004). Similarly, zooxanthellae density was significantly reduced in coral tissues that demonstrated immune activity associated with physical injury (Chapters 6 and 9). These decreases in symbiont density could either be due to 1) expulsion by the host, for example, if activation of immune responses triggers the coral to recognise the zooxanthellae as foreign organisms, 2) exocytosis of viable zooxanthellae triggered by the symbionts in response to unfavourable, oxidative stress environments, or 3) disruption of the symbiosis as collateral damage from cytotoxic immune pathways. The normal shape and staining characteristics of zooxanthellae observed within expelled cellular debris in response to injury, suggest that they remained viable and thus were not affected by the potentially cytotoxic local environment (Chapter 9), arguing against the third of these possible explanations. The loss of zooxanthellae, seemingly as a by-product of initiating an immune response to injury, appears counter-productive because of their function as nutrient providers (Muscatine et al. 1984; Bythell 1988). However, the apparent localisation of immune responses may ensure that recolonisation by zooxanthellae can occur rapidly from neighbouring tissue. The rapid increase in zooxanthellae density at wound sites within 48 h post-injury strongly supports this

hypothesis (Chapter 9). Similarly, the active expulsion of zooxanthellae by the host may also serve as an additional defense mechanism in order to prevent further damage caused by an immune response of the zooxanthellae, or as a more general response to eliminate microorganisms, the primary role of immune defense systems (Weis 2008).

Establishing the mechanisms and activation of zooxanthellae immunity were not addressed within this thesis, although future research on this topic would provide significant insights into holobiont physiology and the sensitivity of this symbiosis. As my measurements of immunity were either biochemical or histological, accidental measurements of zooxanthellae immunity instead of coral immunity are extremely unlikely. This is because precautions were taken, for example, to include a substantial centrifugation step within the sample extraction protocol for biochemical assays and thus ensure the removal of the majority of zooxanthellae.

11.2.3 THE IMMUNOLOGICAL LINK BETWEEN CORAL DISEASE AND BLEACHING

Organisms frequently respond to adverse environmental conditions with components of their immune systems that are also used during infection and injury. Thus changes in immunity variables in response to abiotic stress within invertebrates, particularly molluscs and plants, are commonly used as bioindicators of environmental degradation (Yu et al. 2000; Malanga et al. 2004). Induction of oxidative stress conditions is the predominate mechanism involved in cross-talk among biotic and abiotic stress responses (Yu et al. 2000; Malanga et al. 2004; Hansen et al. 2006; Weis 2008). Within corals, complexity in responses to stress is taken to a new level because of the obligate symbiosis with zooxanthellae, as previously discussed. Whilst not fully established, the immunological link between coral diseases and bleaching is likely to be on multiple levels and involve both partners of the symbiosis (Chapter 5).

The observed link between levels of immunity and coral disease (Chapter 5) is expected because of the primary role of immunity in resisting infection (Stedman 2000; Cooper and Koprowski 2002; Cerenius et al. 2008). However, as thermal coral bleaching is primarily a response to environmental stress (e.g. Jones et al. 1998; Hoegh-Guldberg 1999), the relationship between levels of coral immunity and bleaching and/or bleaching-associated mortality (Chapter 5) are perhaps less understandable. Loss of zooxanthellae associated with bleaching, and therefore of the

source of the majority of a coral's energetic requirements (Muscatine et al. 1984; Bythell 1988), will lead to "food shortage" (Fitt et al. 1993). If this induced energetic deficit (Porter et al. 1989) is not addressed by, for example, re-establishment of symbiont populations or by increased heterotrophy (Grottoli et al. 2006), then mortality rates will increase (Feder et al. 1997; Moret and Schmid-Hempel 2000; Seppala et al. 2008). The rate at which corals reach critical points leading to mortality is likely to be largely determined by their energetic reserves, which may be quantifiable as tissue biomass (Fitt et al. 2000).

Coral mortality post-bleaching may directly reflect levels of immunity, because if energy reserves are low, corals may become immuno-suppressed and thus more susceptible to infection (Feder et al. 1997; Moret and Schmid-Hempel 2000; Seppala et al. 2008). This would explain the high disease prevalence often documented after bleaching events (e.g. Harvell et al. 2001; Miller et al. 2006; Miller et al. 2009). However, elevated immunity levels in bleached corals (Mydlarz et al. 2009) and in corals exposed to warmer water (Chapter 10) indicate that an understanding of the timing of these processes is key for determining the potential success of a coral during and after bleaching. For example, corals that allocate energy into elevating baseline levels of immunity with warming seawater are likely to be more successful than those that do not have the resources to do so. This is primarily because an up-regulation of innate immunity responses, including up-regulating quantities of photoprotective and oxygen radical scavenging melanin, provides a number of benefits that mitigate bleaching. However, the ability of a coral to sustain this up-regulation of immunity if bleaching occurs is directly dependent upon its energy stores, which in turn are dependent on the duration and intensity of bleaching.

11.2.4 ECOLOGICAL IMMUNITY OF CORALS

The field of ecological immunity strives to understand immunity in the context of the natural environment (Sheldon and Verhulst 1996) and to explain variations in levels of immunity among individuals and species, which are primarily manifested as differential susceptibilities to disease (Sadd and Schmid-Hempel 2009). Ecological immunity therefore involves understanding the relative allocation of resources to various life history traits, including immunity (Sadd and Schmid-Hempel 2009). With rapid developments in knowledge relating to coral immunity (e.g. Mydlarz et al. 2006; Miller et al. 2007; Kvennefors et al. 2008; Mydlarz et al. 2008; Mydlarz et al.

2009) including chapters within this thesis, the inter-specific and seasonal patterns of coral disease and bleaching are beginning to be better understood (Chapters 5 and 10).

Anthozoans are a heterogenous class and include both solitary and colonial species, brooding and broadcast spawners and numerous morphologies. Furthermore, different species within the Anthozoa exploit a diverse array of habitats, for example, from habitats that are shallow with high light to those that are deep and/or cryptic with low light, or from habitats that have high wave energy to those with minimal water movement. Given the correspondingly distinct life histories, variation in baseline levels of immunity among different coral species is understandable, because different patterns in the allocation of resources will determine optimal fitness (Sadd and Schmid-Hempel 2009; Chapter 5).

Coral disease epizootics and thermal bleaching events are serious threats to the persistence and biodiversity of coral reefs. Variations in host immunity levels, both within and among species, have been proposed to contribute to patterns in mortality observed on reefs following these events, particularly in relation to disease epizootics (Harvell et al. 2004; Mydlarz et al. 2006; Harvell et al. 2007; Mydlarz et al. 2009). The relationship between coral immunity level and disease was better established within this thesis (Chapter 5). Phenotypic plasticity in coral immune responses is suggested by the comparatively higher amount of melanin in shallow-dwelling versus deeper-dwelling faviids (Chapter 5), although a series of transplant experiments would be needed to confirm that these observations represent phenotypic plasticity rather than genetic variation. Additionally, the role of environmental phenotypic plasticity in explaining differences in immune responses among three *Porites cylindrica* colour morphs is not clear-cut as they were all located within the same shallow reef environment. The location of each colour morph within the same environmental conditions suggests an alternative, probably colour-related determinant of immunity. This link between coral colour morph and immunity supports the suggestion that a primary role of coral FPs is to do with colour and additionally that FPs are involved in coral immunity, as previously discussed. However, it also remains unclear whether genetics or phenotypic plasticity determine the colour of coral colour morphs. Overall, increasing numbers of emerging diseases coupled with climate change (Harvell et al. 1999; Harvell et al. 2004) suggest that the success of corals will depend on a very fine balancing act of resource allocation, particularly as warmer

water appears to compromise the ability of corals to up-regulate an immune response (Chapter 10).

11.3 CORAL IMMUNITY AND WARMER WATER

Climatic changes and concomitant increases in bleaching and disease have contributed to the loss of an estimated 19% of the world's reefs (Wilkinson 2008). Declines in coral cover are set to increase with rising levels of atmospheric CO_2 (Veron et al. 2009) and subsequent elevated seawater temperatures, which to date have received the most scientific investigation. Additionally however, increasing ocean acidity is predicted to have severe detrimental effects on reef assemblages (Veron et al. 2009). The impairment of coral immune responses under warmer seawater temperatures is particularly concerning given predicted increases in ocean temperatures with climate change (Hoegh-Guldberg et al. 2007). The implications of this immunosuppression are potentially far reaching, given the relationships between immune activity and both bleaching and disease susceptibility (Chapter 5). It is highly likely that with warmer conditions, corals will be more susceptible to mortality associated with pathogenic infections and are less able to recover from physical injury.

Despite the suppression of *Porites cylindrica* immune responses at elevated seawater temperatures, baseline levels of phenoloxidase activity gradually increased over time (Chapter 10). This suggests that corals may be able to acclimate to warmer ocean temperatures such as those observed during the summer months. This also indicates that under these conditions, corals may better resist pathogen infection. Although corals may be better able to resist infection as temperatures raise, their inability to up-regulate a response to, for example, physical injury (Chapter 10) such as induced by storm events, may increase the likelihood of infection and limit wound healing. This is particularly pertinent as storm events occur primarily during the summer months when ocean temperatures are warmest. Moreover, severe storm events have increased in frequency and severity in the past decade, particularly within the Caribbean, and are predicted to do so further with climate change (Bythell et al. 1993; Hughes 1994; Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2009). However, it is unclear whether corals are able to regain the ability to up-regulate an immune response after extended time under new conditions, which would indicate the potential for acclimation.

It is costly to up-regulate constituent levels of immunity (Sandland and Minchella 2003; Sadd and Schmid-Hempel 2009) during times of warmer water, but potentially beneficial because anomolously warm seawater temperatures are typically correlated with bleaching events (Hoegh-Guldberg 1999) and disease epizootics (Willis et al. 2004; Sato et al. 2009). Thus increasing seawater temperature may act as a proximate cue (Bowden et al. 2007) to up-regulate immunity because of links to elevated threats of bleaching and disease. Similarly, shaping of immune responses by environmental parameters has been shown for *Drosophila* (Corby-Harris and Promislow 2008). Further investigations into how environmental cues relate to constituent levels of immunity may contribute to the explanation for observed differences in coral responses to bleaching after a prior bleaching event (Brown et al. 2002).

Energetic costs associated with inducing and maintaining an elevated state of immunity (Sandland and Minchella 2003; Sadd and Schmid-Hempel 2009) may limit its duration. It is likely that the ability of a coral to survive a bleaching event and/or resist disease during summer months is directly related to energy reserves and/or the ability of the coral to maintain them. It can therefore be hypothesised that corals with high biomass will be less susceptible to bleaching and to post-bleaching mortality (Loya et al. 2001) because of their comparatively elevated capacity to allocate energy into preventative immunity measures (Sadd and Schmid-Hempel 2009). Evidence that tissue biomass is lower in a more susceptible coral family (Acroporidae) than a more resistant one (Faviidae) supports this notion, as does the seasonal variation in tissue biomass with the lowest levels recorded after the summer (Fitt et al. 2000). Furthermore, corals that activate or up-regulate alternative feeding methods when autotrophic nutritional sources are depleted (Yamashiro et al. 2005) are more likely to survive bleaching events (Grottoli et al. 2006; Rodrigues and Grottoli 2007). Thus corals that are able to maintain energy reserves are more likely to maintain elevated baseline levels of immunity and may have higher survival rates, all of which may relate to colony morphology (Loya et al. 2001). Investigating comparative energy reserves among multiple coral species, in addition to their ability to maintain reserves during stress events, may provide a novel way forward in establishing the most susceptible corals to thermal climatic changes.

11.4 FUTURE DIRECTIONS

Coral immunology is a cutting-edge field of research that has the potential to advance the understanding of fundamental aspects of coral biology that are particularly pertinent under rapid climate change. Declines in coral cover and species richness are influencing economies, societies and biodiversity on a global scale (Hoegh-Guldberg et al. 2007; Hoegh-Guldberg et al. 2009), yet the underlying mechanisms need further elucidation. Further studies of coral immunology may lead to the development of novel tools that could provide early warning of compromised coral health and thereby mitigate reef degradation and help maintain coral biodiversity.

Of the three key immunity effector response mechanisms, i.e. antimicrobial peptides (AMPs), proteolytic cascades and cellular responses (Hoffmann et al. 1999), AMPs (Zasloff 2002) are largely unexplored within corals and warrant further study. To date, antimicrobial activities of corals and their tissue extracts have been documented in soft corals (Slattery et al. 1995; Kelman et al. 2006), gorgonians (Kim 1994) and hard corals (Koh 1997; Kelman et al. 2006; Gochfeld and Aeby 2008; Geffen et al. 2009; Mydlarz et al. 2009). These have included antimicrobial activity from small peptides and secondary metabolites (Slattery et al. 1995; Mydlarz and Jacobs 2006; Dunn 2009). However, the specific presence of AMPs has yet to be conclusively established, although they are reportedly present in all organisms (Zasloff 2002). Establishing the presence, type, abundance and use of anthozoan AMPs would provide further insight into coral immunity mechanisms and potentially provide another quantifiable parameter with which to assess and compare relative immune capabilities among species. Such improved comparisons would provide a more solid foundation from which to understand and study coral ecological immunity.

The investigation of constituent immunity in multiple coral species from the Great Barrier Reef (Section I) is a significant contribution to the emerging field of coral ecological immunity, and has begun to provide a quantifiable explanation for long-described patterns in inter-specific bleaching and disease susceptibility. However, in order to progress the understanding of coral ecological immunity, further investigations of temporal and spatial fluctuations in normal baseline levels of immunity are needed for several coral species. Further studies of the effects of bleaching events and disease on immunity are also needed, including the use of

known coral pathogens in controlled experiments. These studies will help to elucidate interactions between coral immunity and the environment, establish the potential for immunity-related phenotypic plasticity and thus acclimatisation, and also further determine why some species may be more susceptible to disturbance events than others. All of these outcomes would significantly contribute to the understanding of larger scale temporal and spatial patterns in the distribution and abundance of coral species.

A thorough understanding of coral immunity mechanisms and natural fluctuations in immunity levels will potentially enable the development of a novel "health-check" protocol for corals. A diagnostic tool of coral health has the potential to provide forewarning of the likely impact of disturbance events before signs are directly observed. For example, although coral samples appeared healthy at elevated water temperatures described in Chapter 10, differences in immunity levels in comparison to those at the ambient water temperatures, demonstrated health changes that could not otherwise be observed. Determining the relative health of a coral assemblage therefore represents a new approach to enable the development of more realistic models of future reefs scenarios.

Simulation models of future reef states are based on various climatic change scenarios, combined with known detrimental effects of predicted environmental changes on corals and coral reefs (e.g. Tanner et al. 1996; Hoegh-Guldberg 1999; Hughes et al. 2003; Anthony et al. 2007). To provide realistic inputs into simulation models, it is imperative to conduct controlled experiments that investigate the effect of predicted climate change scenarios on coral health. The temperature treatment experiment described in Chapter 10 provides the first pieces of this puzzle, however more extreme thermal stress experiments with multiple coral species that span the range of bleaching and disease susceptibilities are needed. Furthermore, in addition to rising sea surface temperatures, increasing ocean acidity is becoming a serious threat to coral persistence (Hoegh-Guldberg et al. 2007; Veron et al. 2009), but currently there are no studies that directly explore the effect of pH on coral immunity. As variations in pH affect the efficacy of immune responses in other invertebrates (e.g. Cheng et al. 2003), increased ocean acidity is likely to influence coral immunity (Mydlarz et al. 2006).

As an emerging field, coral immunology has numerous avenues that need extensive exploration. In particular, these avenues include further establishing the

components and mechanisms of anthozoan immune systems and applying the knowledge to urgent ecological immunity questions that pertain to coral persistence under a changing climate. To strengthen future studies, it would be advantageous to include molecular approaches to the investigation of coral innate immunity mechanisms and to merge these with the biochemical and histological methodologies described within this thesis. Overall, from the base of knowledge provided by the research described in this thesis, the continued study of anthozoan immunity is likely to provide answers to fundamental questions in coral biology that will help progress the understanding of coral ecology and help to accurately predict the future state of the world's coral reefs.

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