Genetic bases of immunity and disease resistance to White Band Disease in the Caribbean

Staghorn coral *Acropora cervicornis*

by

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ABSTRACT

Over the past thirty years, marine disease outbreaks have increased significantly, producing dramatic alterations in marine ecosystems worldwide. Reef-building corals have been particularly vulnerable to the increase in new epizootic diseases, and yet many aspects of the coral-pathogen interaction remain unresolved, including how corals respond to disease infections. One example is represented by White Band Disease (WBD), a coral disease that causes rapid tissue degradation in acroporid corals. Since the 1970’s, WBD outbreaks have caused catastrophic mass mortalities of two foundation species on Caribbean coral reefs, the staghorn coral *Acropora cervicornis* and the elkhorn coral *A. palmata*, which populations have collapsed by up to 98%. The etiology of WBD has not been fully elucidated yet, but presence of disease resistant genotypes and local recovery from WBD in natural populations of *A. cervicornis* suggest that staghorn corals have the ability to fight the disease and that genetic mechanisms may underlie resistance to WBD.

My thesis focuses on understanding the genetic basis of coral host immunity and resistance to WBD in the endangered staghorn coral *Acropora cervicornis* using next-generation sequencing (RNA-seq). To identify the key mediators of the immune response of staghorn corals to WBD disease, I conducted transcriptome analysis of healthy and WBD-infected staghorn corals from natural populations. My results show that WBD causes expression changes in 4% of the coral host transcriptome, and that the immune response to staghorn corals is characterized by pathogen recognition, production of reactive oxygen species, apoptosis and stress response genes, as well as eicosanoids, a class of lipid metabolites including leukotrienes and prostaglandins, that had not previously identified as immune mediators in scleractinian corals.

Using common garden experiments, I exposed resistant and susceptible corals to healthy and to WBD-infected grafts to identify expression changes associated to resistance during disease exposure (WBD grafts) and allore cognition (healthy grafts). I found that expression patterns associated to disease resistance are independent from exposure to WBD and do not include any of the immune pathways involved in the response against WBD corals. Rather, the gene expression signature of WBD resistance encompasses a very small number of genes that are constitutively up- or down-regulated in resistant corals. Mechanisms involved in WBD resistance
include RNA interference-mediated post-transcriptional regulation, antiviral defense and heat stress response, suggesting a link between thermal stress tolerance and disease resistance.

Expression changes associated to exposure revealed that allore cognition only induces expression changes for a small subset of genes involved in general stress response and nematoecyst discharge, while exposure to WBD has a large-scale effect on the transcriptome. Consistent with the results described above, disease exposure resulted in enhanced pathogen recognition, synthesis of ROS and production of eicosanoids. Hallmarks of early stage response to WBD included Toll-like receptors (TLR)-mediated NF-κB signaling, Prophenoloxidase activity and increased expression of Heath shock proteins and antivirals. These results also show that eicosanoids in staghorn corals are involved in the response to WBD but not to allogeneic tissue exposure and mechanical injury, indicating a pivotal role during pathogen infections rather than during wound repair and general stress response.

Taken together, these results indicate that *A. cervicornis* mounts a powerful response against disease infections involving several innate immune pathways. In addition, this response does not appear to be a general immune response, as indicated by the highly divergent expression profiles of corals exposed to WBD and healthy grafts, suggesting that *A. cervicornis* is able to discriminate between pathogen attack and allore cognition. By characterizing the genetic signature of resistance to WBD, this research represents a valuable contribution to understanding how corals respond to disease and can serve as a conservation tool to identify and farm WBD-resistant genotypes for large scale restoration of the endangered Caribbean *Acropora*. This knowledge is critical to the conservation of threatened reef corals and has direct conservation implications for the endangered Caribbean staghorn coral *A. cervicornis*. 
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CHAPTER 1
Innate immunity, Acropora cervicornis, and White Band Disease

Overview

This dissertation explores the genetic basis of immunity and resistance to disease in the endangered Caribbean staghorn coral Acropora cervicornis infected with White Band Disease (WBD). The choice of A. cervicornis and WBD as host-pathogen system for my research is driven not only by its importance for coral reef conservation but also for its characteristics that make this system well-suited for the study of coral diseases. Unlike other coral diseases, WBD is transmissible via direct contact (grafting), allowing experimental replication under controlled conditions. In addition, due to its branching morphology, A. cervicornis can be easily sampled by collecting small fragments with minimal damage to the parent colony.

Several factors can affect the ability of corals to recover from diseases, including the ability of the coral to identify and eliminate pathogens, the dialogue between the coral host and its symbionts, as well as environmental drivers (temperature, vectors ecology, pathogen dynamics) and shifts in the bacterial community living on and within the coral tissue. In this dissertation, I use RNA-sequencing techniques to characterize expression changes in the coral host transcriptome that are associated to disease infection, allore cognition and resistance to WBD. In Chapter 2, I identify differentially expressed genes between healthy and WBD-infected corals in the field to characterize the coral’s genetic response to disease infection. Chapter 3 and Chapter 4 focus on gene expression changes in resistant and susceptible corals that were experimentally exposed to healthy, WBD-infected grafts or left unexposed for three days. Specifically, in Chapter 3 I describe transcriptional changes associated to resistance in order to identify unique gene expression signatures of WBD-resistant corals. In Chapter 4 I focus on the expression changes induced by healthy and disease-infected tissue exposure to characterize differences and similarities between allore cognition (healthy grafts) and pathogen exposure (WBD grafts), and I identify the hallmarks of early and late response to WBD by comparing the immune response of staghorn corals exposed to WBD grafts (early response) and infected with WBD in the field described in Chapter 2 (late response).
The innate immune system

Traditionally, immunity is classified into two branches, the adaptive and innate immunity. Innate immunity is the first line of defense against infections and allows rapid detection of pathogens through germ line-encoded Pathogen Recognition Receptors (PRRs) that recognize conserved Pathogen-associated Molecular Patterns (PAMPs) on the surface of invading microorganisms. Adaptive immunity is a slower, more tailored response that relies on pathogen-specific antigen receptors generated by somatic rearrangements from a small number of genes. After infection, these adaptive receptors are maintained by a population of memory cells in order to produce an enhanced response upon subsequent exposure to the same antigen. Unlike vertebrates, which possess both adaptive and innate immunity, invertebrates lack an adaptive immune system and rely exclusively on innate immunity. A general invertebrate innate immune response starts with the activation of PRRs upon pathogen binding, followed by a downstream signaling cascade that alters the expression of target pro-inflammatory genes, promotes synthesis of antimicrobial peptides and eliminates pathogens via phagocytosis, encapsulation and melanization.

The most studied classes of PRRs include Toll-like receptors (TLRs), C-type lectins (CLRs), and (NOD)-like receptors (NLRs). TLRs are highly conserved transmembrane receptors composed of a TIR (Toll/interleukin-1 receptor) domain and leucine-rich repeats (LRR) that activate the transcription factors NF-κB and IFR3 to stimulate production of pro-inflammatory cytokines and antimicrobials [1].

CLRs are carbohydrate-binding proteins involved in multiple processes including cell-cell adhesion, apoptosis, self/non-self and pathogen recognition. CLRs are key mediators of both innate and adaptive immunity as they recognize and bind sugars on the surface of bacteria and viruses in a calcium-dependent manner [2]. In vertebrates, CLRs are classified into different subgroups based on the structure of their carbohydrate recognition-domains (CRDs). Type I and II transmembrane CLRs such as Macrophage Mannose Receptor (MMR) and selectins are expressed on the surface of immune cells including macrophages and dendritic cells [3], while soluble CLRs such as Collectins and Mannose Binding Lectins (MLBs) mediate humoral defense by activating the complement cascade, promoting opsonization and phagocytosis, and modulating inflammation [4,5]. Invertebrates possess an array of humoral CLRs and other functionally similar lectin-like proteins, however, with the only exception of the horseshoe crab -
whose lectins have a unique complex multidomain structure [6] - invertebrates CLRs are structurally simple and usually composed of a single CRD.

A third class of PRRs is represented by NLRs, that sense intracellular pathogens and stress by recognizing PAMPs and damage associated molecular patterns (DAMPs). NLRs can trigger inflammation and apoptosis in combination with TLRs via activation of NF-kB and type I interferons (IFNs). Their structure is similar to plant disease-resistance R proteins and is composed of a nucleotide-binding oligomerization domain (NOD), a c-terminal LRR domain similar to TLRs, and a variable N-terminal domain [7].

Downstream of PRRs, a variety of immune enzymatic cascades and effectors act to kill and eliminate pathogens, including the complement system, Prophenoloxidase cascade (PPO) and the chemical defensome. The complement system of vertebrates comprises three proteolitic cascades: canonical, alternative and lectin pathway, all resulting in the activation of a protease C3-convertase that cleaves and activates C3 into C3a and C3b. C3a is an anaphylatoxin that stimulates inflammation and generation of ROS, while C3b is an opsonin that tags pathogens for phagocytosis and initiates the assembly of the membrane attack complexes (MAC) on the membrane of pathogens, causing cell lysis [8]. Invertebrates lack the canonical complement cascade- activated by antigen-antibody binding- but several complement-related domains and components of the alternative and lectin pathways have been identified in multiple taxa [9].

The PPO cascade is an important immune pathway used by invertebrates to encapsulate pathogens and tissue lesions. Pathogen infection or mechanical injuries trigger the proteolytic activation of the enzyme phenoloxidase (PO) from its precursor PPO by a cascade of serine proteinases. Active PO catalyzes the oxidation of the aminoacid tyrosine to quinones, and their subsequent polymerization leading to deposition of melanin at the site of infection and generation of cytotoxic compounds [10].

The term chemical defensome was originally created to define a large set of stress – response genes in the genome of sea urchin Strongylocentrotus purpuratus [11]. Examples of these genes include proteins involved in the production of ROS and antimicrobial peptides, detoxification of potentially harmful exogenous compounds such as xenobiotic detoxicants and antioxidants, and stress-induced genes like heat shock proteins (HSPs) [12,13]. Production of ROS such as oxygen ions and peroxides is a major mechanism of defense and immune signaling in both adaptive and innate immunity. ROS are generated from oxygen by the enzymatic
complex NAPDH oxidase to kill pathogens during phagocytosis and as signaling molecules downstream the TLR pathway [14]. Increased ROS production, however, can lead to oxidative stress due to excessive intracellular accumulation of ROS and other radicals, causing cell and DNA damage. In order to overcome the detrimental effects of oxidative stress, organisms possess an array of antioxidant enzymes such as catalase, superoxide dismutase and peroxidases that catalyze the decomposition of ROS into less reactive products [15]. Detoxification of xenobiotics and other toxic compounds include their modification by the enzymes cytochrome P450 oxidases and glutathione-S transferases followed by their removal from the cells by transporters belonging to ATP Binding Cassette (ABC) and multidrug efflux transporters family that transport solutes against a concentration gradient across the membrane [16-18].

**Immunity in corals**

Corals are marine invertebrates, mostly colonial, belonging to the class Anthozoa -with the exception of few species belonging to the class Hydrozoa- in the phylum Cnidaria. Colonies are composed of several polyps with a simple body plan of two tissue layers, an inner gastrodermis and an outer epidermis separated by a cell-free mesoglea. Reef-building corals (order Scleractinia) live in symbiosis with photosynthetic microalgae from the genus Symbiodinium, called zooxanthellae, for which they depend on for their survival [19,20]. Zooxanthellae are acquired either from the coral’s parent colony [21] or from the water column via phagocytosis and reside within gastrodermis in vacuoles (symbiosomes) derived from phagosomes [22,23].

Until recently, coral immunity was largely unexplored and corals were believed to rely uniquely on their microbial symbionts to resist infections and tolerate stress [24]. As a consequence of the global rise in corals die-offs over the last two decades caused by increasing epizootic events and anthropogenic disturbances, the dynamics of coral-pathogen interactions have gained considerable attention, with a growing number of studies showing that corals are able to respond to environmental stress and fight disease infections [25-27].

With the recent introduction of Next Generation Sequencing (NGS), allowing large-scale genomic and expression studies previously possible only for model organisms, the number of studies focusing on coral immunity has increased exponentially. Transcriptome–wide analysis of gene expression in corals exposed to pathogens and environmental stressors have identified
several mediators of immunity in both the coral host and its algal symbionts [28-30], revealing not only that corals can mount an immune response, but also that they possess several components of innate immunity including mediators of TLR, complement and PPO pathways [27,31].

**Allorecognition in corals**

Allorecognition is the ability to discriminate between self and non-self and is based on the detection of genetic polymorphism between members of the same species by the host immune system. Allorecognition has been described in all Metazoan including protist [32], fungi [33], plants [34] and even bacteria [35]. In vertebrates, T cells recognize non-self proteins present on allogeneic tissue and trigger a cascade that culminates in allograft rejection. These allopeptides are antigens encoded by a highly polymorphic component of the adaptive immune system, the major histocompatibility complex (MHC) [36]. In invertebrates, the ability to distinguish their own tissues from those of their conspecifics is mediated by the innate immune system via recognition of allogeneic polymorphisms and can lead to histocompatibility reactions involving cytotoxicity and tissue necrosis [37]. The underlying molecular mechanisms of allorecognition are still unknown for most invertebrates but the high variability of their outcomes suggests that they have evolved independently in each phylum [38].

In sessile colonial marine organisms like ascidians and Cnidaria, allorecognition is a critical process during space competition and to avoid germline parasitism [39-43]. The tunicate *Botryllus schlosseri* possesses one of the best studied invertebrate allorecognition system. The colonies of *B. schlosseri* are composed of adult zooids connected by a tunic and a common vascular system. Colony growth by asexual budding often results in physical contact between neighboring colonies that can either fuse together into a chimera, or reject by developing an edge necrotic tissue along the contact interface [44,45]. The reaction is under the control of a single highly polymorphic Mendelian locus known as fusion/histocompatibility (*fuhc*) [46,47]. Fusion happens when the two colonies share at least one of the two alleles. According to the mechanism proposed, the product of *fuhc* is protein ligand that is recognized by two putative receptors, *fester* and *uncle fester* [48,49].

In Cnidaria, the ability to recognize self from non-self arises after metamorphosis [43,50-52]. Several studies have focused on the colonial hydroid *Hydractinia symbiolongicarpus* where
allorecognition is controlled by the allorecognition gene complex (ARC), represented by two loci -\textit{alr1} and \textit{alr2}- with multiple, co-dominantly expressed alleles [53-55]. Both \textit{alr} genes encode transmembrane receptors with a highly variable extracellular region similar to immunoglobulins that determines the allorecognition phenotype [56]. Contact between colonies of \textit{Hydractinia} that share alleles at both loci results in fusion with formation of a stable chimera, whereas colonies that do not share any allele undergo rejection with discharge of nematocysts and tissue necrosis [57]. When alleles are shared at only one locus, the two colonies undergo temporary fusion followed by separation with visible necrosis and autophagy along the contact zone [58]. Unlike \textit{Hydractinia}, in the solitary polyp \textit{Hydra} interspecific grafts do not lead to rejection suggesting that the selective pressure for self/non-self recognition is higher in sessile colonial organisms where the risk of germline parasitism is particularly elevated [59].

In sea anemones, agonistic behaviors between neighboring colonies are often associated with space competition [60]. Aggression involves discharge of specialized nematocyst-containing tentacles called acrorhagi, suggesting that allorecognition in these organisms is mediated by receptors located on the acrorhagial membrane [61]. In the genus \textit{Anthopleura}, both \textit{A. elegantissima} and \textit{A. xanthogrammica} can recognize and attack colonies from other species of the same genus as well as genetically different conspecific colonies, often resulting in physical separation between the colonies [60,62,63]. However, unlike \textit{A. elegantissima}, in \textit{A. xanthogrammica}, genetically dissimilar adults that have been in contact for extended time can develop a tolerance and coexist [64]. This difference in behavior may be explained by the different reproductive strategies adopted by the two species: while \textit{A. elegantissima} is mainly asexual, sexual reproduction in \textit{A. xanthogrammica} causes genetically distinct larvae to often settle in proximity, therefore increasing the chance of allogeneic encounters [64]. Similarly, mixed genotype aggregations and tolerance toward non-clonemate anemones has been observed in the plumose anemone \textit{Metridium senile} that alternates sexual and asexual reproduction. The frequency of agonistic behavior in this species appears to decrease with time [65] and it does not occur between clones of opposite sex (Kaplan 1983), supporting the hypothesis that aggressive behavior in anemones is associated to asexual reproduction. Interestingly, in the actinans \textit{Actinia equina} [66,67] and \textit{Phymactis clematis} [68] the extent of the aggression is also influenced by the size of the colonies, with bigger colonies being more aggressive than smaller ones.
In gorgonians, allorecognition has been documented in *Eunicella stricta* [69-71] and in *Swiftia exserta* [72]. In particular, in *S. exserta* the ability to reject allografts displays memory and specificity, the key features of an adaptive immune response. Colonies that are allografted for the first time develop signs of rejections including bleaching and necrosis along the contact interface after 7-9 days, while a second contact requires only 3-4 days to trigger a histocompatibility reaction [72]. This phenomenon can persists up to 8 weeks but is specific: faster secondary response only occurs if the two colonies have already been in contact, not when a “pre-sensitized” colony is grafted to a new allotype. Similar results showing alloimmune specificity and memory lasting up to 8 weeks after initial contact have also been observed in the encrusting coral *Montipora verrucosa*. Allografts between colonies show signs of rejection after few weeks [73,74] whereas secondary exposure results in shorter rejection time [73]. However, a similar a study on the congener species *M. dichotima* failed to demonstrate the same phenomenon [75], suggesting that if invertebrates possess immune memory, it cannot be generalized to all species.

In several species, histocompatibility reactions results in overgrowth of one colony over the other, like in the alectonacean soft coral *Parqthropodium fulvum, fulvum*, whose isografts consistently undergo fusions, while allografts can result in either retreat growth or overgrowth [76]. Similarly, in *Millepora dichotoma* contact between incompatible colonies results in overgrowth of one colony over the other but secondary contact often has a different outcome with reversal in overgrowth and necrosis [77].

Not all corals exhibit allogeneic discrimination: in certain species fusion can occur between both genetically identical and different colonies. Examples are the scleractinian leaf coral *Pavona cactus* [78], *Montipora* [79], *Porites* and *Seriatopora* [80]. In Pocilloporids, isogenic colonies always fuse but rejection between allogeneic tissues exhibits multiple degrees of incompatibility with intermediate reactions. These phenomena represent a less aggressive form of rejections and are called “apparent fusions” in *Pocillopora damicornis*, characterized by a white edge of tissue loss along the contact zone [52,81], and “non-fusion” in *Stylophora pystillata*, similar to fusion but characterized by physical separation between tissues, usually followed by overgrowth of one colony [82].

In the genus *Acropora*, fusion between isogeneic but not allogeneic colonies of *A. cervicornis* indicate high clonal discrimination in this species [83]. The study also shows that the
probability of fusion decreases when distance between colonies increase. However, this pattern seemed to differ between different geographic regions, possibly due to population structure. Colonies of *A. hampirichi* exposed to of allogeneic contact for few weeks manifested rejection as dark colored tissue along the interacting branches and unilateral overgrowth associated with necrosis [84]. Interestingly, the direction and rate of the overgrowth were specific to a given combination of colonies.

Based on the high degree of specificity displayed by several species, it is likely that self/non-self recognition is based on detection of genetic variability similarly to *Hydractinia alr* system. However, up to now, the molecular mechanisms of allore cognition in most cnidaria remain unknown. Lectins, in particular CELIII-type lectins, have been indicated as candidate mediators in *Acropora millepora* based on their expression profiles during coral development [85] and on the observation that in certain species allore cognition arises post settlement or after metamorphosis [51,52,86,87].

Interestingly, high expression of the heat shock protein hsp70 has been found in the tentacles of *A. elegantissima* and in the body and tentacles of *Corynactis californica* during aggressive behavior stimulated by contact with genetically different clones [88]. It has been proposed that up-regulation of hsp70 may be involved in tissues repair and recovery from the cytotoxic compounds produced during the rejection. Interestingly, expression of hsp70 was constantly high in *Corynactis californica*, while in *A. elegantissima* it depended on the competitor.

*Acropora cervicornis*

The Caribbean staghorn coral *A. cervicornis* is a scleractinian coral that inhabits tropical water at depths between 0 and 30 m. Together with its congener, the elkhorn coral *A. palmata*, *A. cervicornis* is a foundation species in the Caribbean reef systems, providing habitat for several marine organisms. The colonies of *A. cervicornis* are formed by polyps with 6-fold symmetry that secrete an aragonite calcium carbonate (CaCO$_3$) skeleton. Polyps feed on plankton during nighttime; during the day, they receive energy from the photosynthetic activity of zooxanthellae [19,20].

Fast branch growth rates (10-20 cm per year) and frequent asexual reproduction via fragmentation [89,90] have facilitated the high success of staghorn corals in colonizing and out-
competing neighboring species, making them one of the primary reef-building coral species in the Caribbean. Sexual reproduction via broadcast spawning typically occurs once a year, between August and September, when both female and male gametes are released simultaneously in the water column [91-93]. Planula larvae can live in the water column for approximately 4 days until settlement and metamorphosis in a new colony [92].

Since 1980, *Acropora* coral populations in the Caribbean have declined dramatically, mainly due to outbreaks of White Band Disease [94-96]. The disease, in conjunction to environmental stressors including overfishing, habitat destruction, and bleaching, has killed up to 98% of the living corals over the last thirty years [94,97,98]. Slow recovery rates and low post-settlement survival have raised serious concern about the survival of this species [99,100]. As a result, both *A. cervicornis* and *A. palmata* are listed as threatened under the U.S. Endangered Species Act [101] and critically endangered under the IUCN Red List [102,103]. In an attempt to preserve the existing staghorn coral populations, several conservation strategies are currently employed, including establishment of marine sanctuaries and protected areas, habitat restoration and coral nurseries [104].

**White Band Disease**

WBD is a host specific coral disease that infects the two Caribbean *Acropora* corals, the staghorn coral *A. palmata* and the elkhorn coral *A. cervicornis*. Since its first appearance in 1970s, WBD has been the leading cause of *Acropora* decline in the greater Caribbean [95,97]. The disease draws the name from its appearance in form of white bands that from the base of the colony and extend toward the branch tip at a rate of approximately five millimeters per day (personal observation). The white bands are caused by exposed skeleton resulting from tissue necrosis and are similar to the bands caused by White Syndrome (WS), a non-host specific disease that causes white tissue lesions in Indo-pacific Acroporids [105]. Two types of WBD have been described: type I occurs across the greater Caribbean, while type II has been described in the Bahamas. The two types are distinguished by the presence of a front of bleaching tissue in the lesions of corals infected with type II but not type I [106]; however, they could represent two morphological variants of the same disease.

The etiology of WBD is complex, and while shifts in the bacterial community have been observed in WBD-infected corals, no single causal agent has been identified. This is due to the
inability to fulfill Henle-Koch’s postulates for several bacteria that are associated with diseased but not healthy corals, including *Vibrio charachariae* [106], *Rickettsiales* [107] and Roseobacter-related bacteria [108]. As a result, it has been suggested that the disease may be caused by non-specific commensal microorganisms that become pathogenic when corals are immune compromised due to environmental stress [109]. However, multiple experiments demonstrate that the disease can be transmitted to healthy corals via direct grafting or tissue homogenates, implicating a pathogenic basis [110,111]. In one study, inhibition of WBD transmission by antibiotics (ampicillin: 0% transmission; tetracyclin: 20% transmission) and filtering (0.45 um: 80% transmission; 0.22 um: 10% transmission) indicates that WBD is caused by Gram negative bacteria and not by opportunistic pathogens [110]. Another recent study confirmed it and demonstrated that the selective antibiotic treatments ampicillin and paromomycin sulfate can stop disease infection [111]. This study also identified 16 candidate pathogens (14 bacteria, one archaea and one ciliate) and three primary pathogens, concluding that the disease is caused by a consortium of multiple bacteria. The three primary pathogens identified were bacterial, *V. charachariae* (KC737024), already implicated in WBD type II, the *Bacillus sp.* (KC737032) previously described in WBD in Indonesian *Acropora* [112], and *Lactobacillus suebicu*s (KC737026).

Interestingly, one of the secondary pathogens identified by this study, the ciliate *Philaster lucinda*, has been associated to WS in the Pacific and it has been observed feeding on corals tissue at the lesion interface [113], suggesting that the tissue lesions in WBD and WS may be caused histophagous activity of the ciliate.

Two modes of natural transmission of WBD have been described: via the corallivorous snail *Coralliophila abbreviata*, and via waterborne transmission in presence of tissue lesions. Transmission by animal vectors occurs independently whether the snail has previously fed on infected colonies, indicating that *C. abbreviata* can act as reservoir for WBD [114]. In 2008, a study published by Vollmer and Kline reported the presence of naturally resistant staghorn coral colonies in the region of Bocas Del Toro, Panama [115]. The study used in situ transmission experiments to survey disease transmission success in 49 unique staghorn coral genotypes. Over five transmission attempts, nine genotypes were highly susceptible to WBD (>70% transmission), 12 genotypes exhibited intermediate susceptibility (30–40% transmission) and five genotypes showed complete resistance to disease (no transmission). These data, combined
with further in situ transmission experiments and extensive field observations, allowed to identify and tag five additional resistant colonies (i.e. unique genotypes) exhibiting resistant phenotype in the region of Bocas Del Toro between 2008 and 2009 (Vollmer, unpublished data), suggesting that resistance to WBD in *A. cervicornis* has a genetic basis.
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CHAPTER 2

RNA-seq profiles of Immune Related Genes in the staghorn Coral *Acropora cervicornis* infected with White Band Disease


Abstract

Coral diseases are among the most serious threats to coral reefs worldwide, yet most coral diseases remain poorly understood. How the coral host responds to pathogen infection is an area where very little is known. Here we used next-generation RNA-sequencing (RNA-seq) to produce a transcriptome-wide profile of the immune response of the staghorn coral *Acropora cervicornis* to White Band Disease (WBD) by comparing infected versus healthy (asymptomatic) coral tissues. The transcriptome of *A. cervicornis* was assembled *de novo* from A-tail selected Illumina mRNA-seq data from whole coral tissues, and parsed bioinformatically into coral and non-coral transcripts using existing *Acropora* genomes in order to identify putative coral transcripts. Differentially expressed transcripts were identified in the coral and non-coral datasets to identify genes that were up- and down-regulated due to disease infection. RNA-seq analyses indicate that infected corals exhibited significant changes in gene expression across 4% (1,805 out of 47,748 transcripts) of the coral transcriptome. The primary response to infection included transcripts involved in macrophage-mediated pathogen recognition and ROS production, two hallmarks of phagocytosis, as well as key mediators of apoptosis and calcium homeostasis. The strong up-regulation of the enzyme allene oxide synthase-lipoxygenase suggests a key role of the allene oxide pathway in coral immunity. Interestingly, none of the three primary innate immune pathways - Toll-like receptors (TLRs), complement, and prophenoloxidase pathways, were strongly associated with the response of *A. cervicornis* to infection. Five-hundred and fifty differentially expressed non-coral transcripts were classified as metazoan (n = 84), algal or plant (n = 52), fungi (n = 24) and protozoans (n = 13). None of the 52 putative *Symbiodinium* or algal transcript had any clear immune functions indicating that the immune response is driven by the coral host, and not its algal symbionts.
Introduction

The global rise in disease epidemics linked to climate change has taken a heavy toll on tropical reef-building corals and the diverse ecosystems they support [1-4]. A prime example is White Band Disease (WBD), which beginning in the late 1970s [5], caused unprecedented Caribbean-wide die-offs of two species of Acropora corals, the staghorn coral A. cervicornis and the Elkhorn coral A. palmata [6-8]. As a result, both species are now listed as threatened on the US Endangered Species Act [9] and as critically endangered under the International Union for the Conservation of Nature (IUCN) Red List criteria [4]. Despite the devastating impacts of coral diseases on reefs world-wide, little is known about the basic etiology and ecology of most coral diseases [10-12] including basic information about how corals fight diseases [2,12,13], even though information about the coral immune response may be crucial to understanding the future resiliency of reef corals [2].

Genetic surveys indicate that corals and other cnidarians possess the genetic architecture underlying common innate immune pathways, including Toll-like receptors (TLR) as well as components of the complement and prophenoloxidase (PO) pathways [10,14,15]. PO activity and melanization responses have been elicited in corals exposed to pathogens [16-18] and components of the TLR pathway were differentially expressed in corals infected with non-host specific Symbiodinium types [19]. Elements of the complement pathway, such as mannose-binding lectins, appear to be involved in pathogen, symbiont, and self/nonself recognition in Acropora millepora [20]. Although cnidaria lack specialized immune cells, such as macrophages, cnidaria possess mobile amebocytes that are activated upon pathogen exposure or tissue damage [21-24]. Phagocytosis activity in cnidarians is commonly observed in flagellate gastrodermal cells during food uptake [25]. However, several studies have demonstrated that, upon immune stimulation, different populations of amebocytes can exhibit phagocytic activity directed toward wound healing and removal of necrotic tissue, as well as encapsulation of foreign particles [26, 27].

Relatively few studies have studied the genetic response of corals infected with disease [28,29]. A microarray study of Pocillopora damicornis infected with Vibrio identified six candidate immune genes including three lectins and three putative antimicrobial proteins [28]. Exposure of A. millepora to bacterial and viral pathogen associated molecular patterns (PAMPs) resulted in up-regulation of few immune related genes including three GTPase of immunity
associated proteins (GiMAP) [29], a family of conserved small GTPases involved in the antibacterial response of plants and mammals [30].

White Band Disease represents a good system to investigate the immune response of a reef-building coral. It is one of the few coral diseases that is highly transmissible [31] and host-specific [5,11]. WBD is characterized by an interface of white dying tissue that advances rapidly along the coral colony (Figure 2.1). Current evidence suggests that the pathogen is bacterial [31-36], but Henle-Koch postulates have not been satisfied. To date, multiple bacteria have been associated with WBD infections, including *Vibrio harveyi* [33,37] as well as a marine *Rickettsia CAR1α* [34]. *In situ* transmission experiments have identified naturally resistant and susceptible genotypes of *A. cervicornis* [31], indicating that the immune response to WBD varies among individuals.

Here we used next-generation RNA-sequencing to produce a transcriptome-wide profile of the immune response of *A. cervicornis* to WBD by comparing infected versus healthy (asymptomatic) coral tissues. The transcriptome of *A. cervicornis* was assembled de novo from A-tail selected mRNA-seq data from whole coral tissues, and parsed bioinformatically into coral and non-coral transcripts using existing *Acropora* genomes in order to identify putative coral transcripts. Differentially expressed transcripts were identified in the coral and non-coral datasets to identify which genes were up- and down-regulated due to disease infection and characterize the immune response of the coral.

**Materials and Methods**

Total RNA was extracted from diseased and healthy *Acropora cervicornis* sampled from Crawl Cay reef in Bocas del Toro, Panama under Autoridad Nacional del Ambiente (ANAM) Collecting permit SE/A-71-08. For the diseased samples, corals with active mobile WBD interfaces were identified by monitoring the mobility of disease interfaces for two days, and then sampling a 2 cm region of tissue at and above the disease interface. A comparably sized and located tissue sample was taken from healthy (i.e. asymptomatic) corals. The coral tissues were flash frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted in TriReagent (Molecular Research Center, Inc.) following the manufacturer's protocol. Total RNA quality was assessed using the RNA Pico Chips on an Agilent Bioanalyzer 2100, and only extractions
showing distinctive 28S and 18S bands and RIN values of 6 or higher were prepped for RNA sequencing.

RNA sequencing was performed on five diseased and six healthy coral samples using a multiplexed Illumina mRNA-seq protocol [95] with the following modifications. Instead of fragmenting the mRNA prior to cDNA synthesis, we obtained much better success fragmenting the double stranded cDNA using DNA fragmentase (New England Biolabs) for 30 minutes at 37°C. RNA-seq libraries were then prepared using next-generation sequencing modules (New England Biolabs) and custom paired-end adapters with 4bp barcodes. Multiplexed samples were run (2-3 samples per lane) on the Illumina GAII platform (Illumina, Inc, San Diego, California, USA) at the FAS Center for System Biology at Harvard University. Barcoded samples were de-multiplexed and raw sequencing reads were quality trimmed to remove sequences and regions with a Phred score of less than 30 and a read length less than 15bp long using custom Perl Scripts in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).

A de novo transcriptome was assembled using Trinity [38] from 463.5 million single-end Illumina RNA-Seq reads from 39 A. cervicornis and 6 A. palmata samples, including the 11 A. cervicornis samples included in this paper. The assembled transcriptome produced 95,389 transcripts with a N50 of 363 and N75 of 696. RNA-seq data were produced using whole coral tissue, which putatively contains sequences from the coral host, its algal symbiont Symbiodinium, and other members of the coral holobiont (e.g. fungi, bacteria, and viruses).

In order to resolve the holobiont, and putatively classify the source of the transcripts that were assembled as either coral or non-coral, we utilized a multistep pipeline leveraging the existing genomes of two congener species – A. digitifera [39] and A. millepora [40]. RNA-seq reads were mapped against both Acropora reference genomes using Bowtie [96] to produce two exomes. Transcripts from our de novo assembly were aligned using BLAST [97] against each exome. Transcripts were assigned as putatively coral if they matched either exome with an e-value of less than \(10^{-10}\). Transcripts without significant coral hits were assigned as non-coral and could potentially include novel coral and/or algal symbiont Symbiodinium transcripts, as well as other associated eukaryotes, like endolithic fungi. Bacterial and viral transcripts are possible, but less likely given that A-tail selection to isolate eukaryotic mRNAs was performed prior to cDNA synthesis.
Putative gene identities for each transcript were identified by performing homology searches against the Swiss-Prot and TREMBLE protein databases [98], using tBLASTx. Matches with an e-value of less than $10^{-5}$ were considered homologous protein-coding genes. Subsequently, GenBank Flat Files corresponding to the hits’ Accession ID’s were downloaded and used to extract taxonomic data for each used as a second method to identify the putative source of the transcripts. GO terms and gene functions were obtained for the annotated transcripts on UniProt. The reference transcriptome sequences are available on Bioproject (accession number PRJNA222758).

Differences in gene expression between healthy and disease *A. cervicornis* specimens were estimated using the R package DESeq [38]. First, all contigs were separated into two datasets – i.e. coral and non-coral- based on their matches to the *Acropora* genomes. Size factor estimation and normalization were then performed separately on each dataset using the functions estimateSizeFactors and estimateDispersions, respectively. Differentially expressed contigs were detected by running a negative binomial test using the function nbinomTest. Only differentially expressed transcripts identified using p-values adjusted for multiple testing using Benjamini-Hochberg method (padj value < 0.05) that were also annotated (e-values < $10^{-5}$) were used for this study.

**Results**

A de novo assembly of the *A. cervicornis* transcriptome was assembled from 436.5 million Illumina RNA-sequencing reads from 45 coral samples of *A. cervicornis* and *A. palmata*. The total reads were de novo assembled using Trinity [38], resulting in 95,389 transcripts, with a N50 of 363 and N75 of 696. A total of 47,748 transcripts mapped against the existing *Acropora* genomes [39,40] and were classified as putative coral transcripts while the remaining 47,641 were classified as non-coral transcripts (Table 2.1).

For this study, five diseased (i.e. infected) and six healthy corals were used to profile the immune response of staghorn corals infected with WBD. The average number of putative coral reads (±SE) was 4,076,829 (± 898,542) in the diseased coral samples compared to 4,199,946 (±761,894) in the healthy samples. In total, 20,503 coral transcripts (43 %) and 14,253 (30%) non-coral transcripts had strong protein annotations (Blastx e-value < $10^{-5}$) (Table 2.1).

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Differentially Expressed Coral Transcripts

Statistical analysis in DEseq [41] identified 1,805 differentially expressed (DE) transcripts (adj p-value < 0.05) between healthy and WBD coral samples (Table 2.1); 559 of these DE transcripts had reliable protein annotations (Blastx e-values < 10\(^{-5}\)) that could be used to characterize the immune response of *A. cervicornis* infected with WBD (Figure 2.2a, Figure 2.3). Annotated transcripts were characterized by gene ontology (GO) and grouped into manually curated categories based on literature searches highlighting immune functions (Table 2.2). WBD-infected corals exhibited strong gene expression responses for genes related to immunity (n = 72), apoptosis (n = 18) and arachidonic acid metabolism (n = 5). Calcification (n = 14) and calcium homeostasis (n = 21) were also perturbed, as well as cell growth and remodeling (n = 134), cellular processes (n = 188) and general metabolism (n = 43).

Immune-related processes

Sixty-nine DE transcripts were associated with immunity. Three C-type lectins receptors, C-type mannose receptor 2 (MRC2), macrophage lectin 2 (CLEC10A) and collectin-12 (COLEC12) were up-regulated in infected corals. Two mediators of phagocytosis were up-regulated - the macrophage receptor multiple epidermal growth factor-like domains protein 10 (MEGF10) and actin-22 (act22), which is involved in the phagosome formation. All three subunits of NADPH oxidase (NOX) involved in reactive oxygen species (ROS) production were up-regulated, including cytochrome b-245 heavy chain (CYBB), NADPH oxidase 3 (NOX3) and neutrophil cytosol factor 2 (p67-phox). Other DE immune related genes included nine antioxidants participating in the detoxification of ROS such as peroxidasin (PXDN, n=3) and glutaredoxin (GLRX), and 12 transcripts associated with response to stress such as golgi-associated plant pathogenesis-related protein 1 (GAPR-1, n = 3) and universal stress protein A-like protein (UspA, n = 2).

Little or no differential expression was detected in the three primary innate immune pathways – Toll/TLR, complement and prophenoloxidase (PO) pathways. In the Toll/TLR pathway, two TLR2 homologs and the adaptor molecule TNF receptor-associated factor 3 (TRAF3) were up-regulated in WBD corals. In the complement pathway, two transcripts encoding macrophage-expressed gene protein 1 (MPEG1) were differentially expressed, but they
were down regulated in WBD corals. No differentially expressed transcripts were detected in the PO pathway.

**Arachidonic acid metabolism**

Six DE transcripts participating in the metabolism of arachidonic acid (AA) were up-regulated in diseased corals. Five matched coral allene oxide synthase-lipoxygenase (AOSL), a catalase related hemoprotein that catalyzes the biosynthesis of allene oxide, a precursor of marine eicosanoids. The sixth transcript matched the enzyme phospholipase A2 (PLA2), involved formation of AA from membrane phospholipids.

**Apoptosis**

Eighteen DE transcripts were associated with apoptosis, including both pro- and anti-apoptotic regulators such as the extracellular matrix protein thrombospondin 2 and fibroblast growth factor receptor 2 (n = 2), respectively. Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) and caspase 3 (CASP-3) were up-regulated while caspase 8 (CASP-8) was down-regulated in WBD corals.

**Calcification and Calcium homeostasis**

DE transcripts in this category included 14 proteins participating in carbon dioxide transport, biomineralization and skeletal growth. Two carbonic anhydrases were up-regulated (CA2 and CA3) and one was down-regulated (CA2) in WBD corals. Mediators of calcium homeostasis included 27 DE transcripts participating in calcium ion binding and transport such as calmodulin (CaM, n = 3), calumenin (CALU) and calsequestrin-2 (CASQ2) and were all up-regulated.

**Cell growth and remodeling**

Among the 138 DE transcripts related to cell growth and remodeling we identified 17 metallopeptidases (15 up, 2 down), 29 cytoskeletal proteins (all up-regulated) and 14 angiogenesis mediators (11 up, 3 down). A large group of DE transcripts were cell adhesion proteins (n = 29), including four up-regulated transcripts encoding sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 (polydom/SVEP1).
**Cell metabolism**

Forty-two DE transcripts were associated with cell metabolism. These included 14 mediators of lipid metabolism, in particular, five lipases involved in lipid and phospholipid catabolism (n = 5, all up), such as pancreatic triacylglycerol lipase (PL), pancreatic lipase-related protein 2 (PL-RP2) and phospholipase DDHD1 (DDHD1). Four transcripts participating in fatty acid biosynthesis, such as fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC) and acetyl-CoA carboxylase 1 (ACC1), were all down-regulated in WBD corals, and five transcripts involved in the breakdown of fatty acids such as long-chain-fatty-acid--CoA ligase 1 (LACS1) and 5 (LACS5) were up-regulated.

**Non-coral transcripts**

Out of the 47,641 putative non-coral transcripts in the dataset, 550 were differentially expressed in WBD infected corals (Table 2.1). Of these 550 DE transcripts, 251 were well-annotated and were all up-regulated (Figure 2.2b). About 33 % were metazoan, the remaining were putative zooxanthellae (23%), fungi (10%) and protozoa (5%). A small number of transcripts matched bacteria (4%) and viruses (0.1%), while the remaining 23 % were unknown.

Metazoan transcripts (n = 84) included mediators of cell growth and remodeling (n = 16), metabolism (n = 4), cellular processes (n = 61) and two uncharacterized transcripts. Only two immune-related transcripts were identified and were the antioxidant peroxiredoxin-2 (PRDX2) and the metallopeptidase aminopeptidase O (AP-O), which may be involved in leukotrienes synthesis from AA.

Fifty-nine transcripts had plant, algae or Alveolata protein IDs and are presumed or putative *Symbiodinium* transcripts. Based on GO terms, these *Symbiodinium* transcripts were associated with cell growth and remodeling (n = 8), cellular processes (n = 38) and metabolism (n = 10), while two were uncharacterized. One transcript matched cysteine proteinase RD21a (RD21), a peptidase involved in defense against fungi. Fungal transcripts (n = 24) belonged to cell processes (n = 20) and metabolism (n = 3) plus one uncharacterized protein. Out of the 14 transcripts matching protozoa, 13 were associated to cellular processes, two to metabolism and one to cell growth and remodeling.
Nine transcripts matched bacterial proteins, six of them were involved in cellular processes (n = 3), metabolism (n = 3) and three were uncharacterized. Two transcripts shared protein IDs annotating to virus proteins (glycoprotein gp2 and one uncharacterized), while the remaining 59 transcripts did not have functional annotations.

Discussion

Our study demonstrates that *Acropora cervicornis* mounts a vigorous immune response against White Band Disease (WBD) pathogen(s) involving dramatic changes in gene expression across 4% of the coral transcriptome. The identities of the differentially expressed (DE) coral transcripts indicate that the response of *A. cervicornis* to WBD infection is driven by phagocytosis of apoptotic cells (Figure 2.3, Table 2.2). Corals infected with WBD exhibited strong differential expression of transcripts involved in macrophage-mediated pathogen recognition and ROS production, two hallmarks of phagocytosis, as well as key mediators of apoptosis and calcium homeostasis. The strong up-regulation of transcripts involved in arachidonic acid (AA) metabolism and allene oxide synthesis suggests their key role in coral immunity.

The primary signature of phagocytosis activity in WBD infected corals was the up-regulation of four macrophage receptors that recognize and bind to conserved motifs on the surface of target cells. Three of these receptors, MRC2, CLEC10A and COLEC12 belong to the C-type lectin family of proteins that include several Pathogen Recognition Receptors (PRRs). MRC2 recognizes mannose and fucose on glycoproteins of bacteria, viruses and fungi [42] while CLEC10A recognizes galactose and N-acetyl-galactosamine residues [43]. COLEC12 is a scavenger receptor that shares structural similarity with macrophage scavenger receptor class A type I (SR-AI), a surface membrane receptor that mediates binding and phagocytosis of gram-positive, gram-negative bacteria and yeasts [44]. The fourth receptor, MEGF10, is membrane protein that promotes the clearance of apoptotic cells by causing macrophages to adhere and engulf them [45]. The stronger up-regulation of the three macrophage PRRs (2.2, 5.4 and 4.1 fold) compared to the one apoptotic cell recognizing receptor MEGF10 (2.17 fold) suggests the response is primarily driven by phagocytosis of microbes. A second signature of phagocytosis was the up-regulation of transcripts linked to ROS production, including three subunits of the
enzymatic complex NADPH oxidase (NOX). ROS production is a general and highly conserved response to invading pathogens and stress and the release of ROS from the mitochondria can induce apoptosis in metazoan and yeasts [46,47]. During phagocytosis, ROS are generated in mature phagosomes (i.e. specialized vacuoles in phagocytic cells) [48] to kill engulfed cells [49]. In cnidarians, ROS production has been observed in the hydroid *Hydra vulgaris* exposed to the immune stimulant lipopolysaccharide (LPS) [50] and in reef corals during thermal and UV-induced bleaching [51,52], possibly due to the breakdown of the mitochondrial and photosynthetic membranes [53,54].

In WBD infected corals, it is possible that phagocytosis is aimed either at the removal of invading pathogens and/or used to clear damaged apoptotic cells [55]. The genetic signature of phagocytosis in WBD infected corals raises questions about the identity of these phagocytic immune cells in *A. cervicornis*. Cnidaria lack specialized immune cells, but do possess mobile amebocytes. Aggregations of amebocytes have been observed in the gorgonian coral *Gorgonia ventalina* infected with pathogenic fungi [24] and near wounded tissues in the soft coral *Plexaurella fusifera* [26]. Histological examination revealed that amebocytes exhibited phagocytic and PO activity [27] as well as antimicrobial activity against Gram-negative bacteria and ROS production [56]. Interestingly, certain populations of ameboid cells always show phagocytic activity, while others only acquire it upon immune activation [27]. These findings indicate that cnidaria, traditionally considered “simple” animals, are able to mount an innate immune response by employing the functional plasticity of amebocytes, which seem to represent the primary immune population of phagocytic cells.

Increased apoptosis in WBD infected corals was indicated by the differential expression of TNFRSF1A and CASP-3. During apoptosis, TNFRSF1A binds to tumor necrosis factor (TNF), which then recruits CASP-8 initiating the downstream activation of CASP-3, the main effector caspase of the apoptotic pathway [57,58]. While both TNFRSF1A and CASP-3 are upregulated, CASP-8 is down-regulated which may suggest that CASP-3 is activated by some alternative pathway. Active programmed cell death was also suggested by disruption of calcium homeostasis as indicated by the strong up-regulation of CaM and other calcium binding proteins. In both plants and animals [59,60], apoptosis can be triggered by LPS from gram-negative bacteria via alteration of TNFRSF1A expression [61]. Some bacterial pathogens are also able to induce or inhibit apoptosis in their host [60,62,63] via alteration of membrane permeability and
disruption of Ca2+ homeostasis [64], direct activation of TNF-α [65], TLR2 [66,67] or CASP-3 [68]. In corals, apoptosis occurs normally during metamorphosis [69] and the onset of symbiosis [70], but it has also been observed during bleaching as a possible mechanism to expel zooxanthellae in response to thermal stress [71-73]. Apoptosis has also been detected in the lesions of three Pacific species of Acropora infected by White Syndrome (WS), suggesting that it is a mechanism of tissue loss in WS [74].

Another key, yet unexpected, finding of this study is the potential role of the arachidonic acid (AA) pathway in the coral immune response. Genes involved in AA synthesis increased dramatically in WBD infected corals. The role of AA as an inflammation regulator is well-known in metazoans [75], but has not been described in Cnidaria or in association with any coral disease. In metazoans, AA is released by apoptotic cells as a chemotactic factor to promote clearance by phagocytes [76], but it can also induce apoptosis via rapid increase of calcium concentration and activation of CASP-3 in a CASP-8-independent way [77]. These findings are consistent with our data showing up-regulation of CASP-3, but not CASP-8, suggesting that AA may act similarly as immunomodulator in A. cervicornis. The five transcripts matching allene oxide synthase-lypoxigenases (AOSL) from the soft coral Plexaura homomalla, on the other hand, indicated that AA is converted into allene oxide, an intermediate compound of prostanoid synthesis in plants and soft corals [78-82].

Allene oxide has received considerable attention as a putative precursor of clavulones [83], a class of unique marine prostanoids known for their anti-viral and anti-cancer activity [84,85]. The link between the AOSL pathway and clavulones synthesis in corals, although still under debate, was suggested by the similarities with the biosynthetic pathway of jasmonic acid [83] a plant hormone that is produced via an allene oxide intermediate upon mechanical injury [86] and herbivore attack [87]. Although further study is needed to understand the role allene oxide in corals, our data represent the first evidence implicating AOSL in coral immunity and suggest that AOSL may be involved in controlling levels of free AA produced by apoptotic cells.

Several other immune related genes exhibited altered expression in infected corals. The majority were anti-oxidants including PXDN, peroxidasin-like proteins and GLRX - a glutathione-dependent enzyme. PXDN has been shown to be DE in some thermally stressed corals, but not in a consistent manner. For example, in Montastraea faveolata, PXDN was up-regulated in thermally-stressed larvae [88], but was down-regulated in thermally-bleached adult
colonies [89]. Active cell remodeling and cell matrix degradation was indicated by several DE cytoskeletal proteins, metalloproteases and cell adhesion proteins, probably associated with cellular and cytoskeletal rearrangements linked to phagocytosis and apoptosis. CASP-3 activation, in particular, initiates apoptosis by altering the expression of metalloproteases and hydrolytic enzymes such as cathepsins that degrade extracellular matrix components [90]. Interestingly, WBD infected corals up-regulated three transcripts encoding polydom, a cell adhesion protein belonging to the pentraxin family of lectins. Recent studies suggest an immune function for polydom based on its similarities in its protein domains to complement proteins and C-type lectins with antimicrobial activity [91]. In cnidarians, the potential immune role for polydom is bolstered by its up-regulation in the hydroid *Hydractinia symbiolongicarpus* after fungal and bacterial exposure [92].

Surprisingly, none of the three main innate immune pathways - TLR, complement and PO - played a prominent role in the immune signature of *A. cervicornis* infected with WBD, even though transcripts from these pathways are well-represented in our transcriptome. Only three transcripts in the TLR pathway were differentially expressed: two TLRs matching to human TLR2 and TRAF3. In the lectin complement pathway, the only two DE transcripts were two proteins matching MPEG1, a MAC/PF (membrane attack complex/perforin) containing protein that is involved in the response against Gram negative bacteria in sponges and is up-regulated upon LPS exposure [93]. None of the transcripts belonging to the PO pathway were differentially expressed during WBD infection, even though in other corals PO activity acts as an important defense against invading pathogens and tissue damage [16-18]. The absence of PO activity in WBD-infected staghorn corals may be due to the high energetic costs associated with the immune response, whereas the lack of TLR and complement pathways may indicate that the response against WBD involves different mechanisms or, alternatively, that these pathways are activated during the first stages of disease infection. In Chapter 4, I describe the differences between early and late-stage coral immune response to WBD.

*Non-coral transcripts*

The taxonomic distribution of non-coral transcripts highlighted the presence of several members of the coral holobiont, i.e. the coral host and associated symbiotic microorganisms, including zooxanthellae, fungi and protozoa. The majority of these non-coral transcripts matched
metazoan and putative zooxanthellae proteins, while the remaining transcripts matched fungi, protozoa and bacteria. GO term analysis revealed that most of these non-coral transcripts encoded mediators of cell homeostasis and general metabolism. Transcripts with metazoan identities were likely coral transcripts that did not have identities in the coral reference genomes and may thus represent transcripts unique to *A. cervicornis*. Putative zooxanthellae transcripts were identified as transcripts annotating to Viridiplantae, Heterokontophyta (i.e. algae), cyanobacteria and the superphylum Alveolata. Interestingly, no genetic signature of immune activity from the algal symbionts was evident in our transcriptome. Instead, our data suggest drastic changes in photosynthesis and cell metabolism of the zooxanthellae; this is consistent with a previous study showing that *Symbiodinum* undergo major alteration of carbon metabolism in response to stress [94].

**Conclusions**

Our data reveal that the coral host, but not its algal symbionts, undergoes dramatic alterations in gene expression during response to WBD infection. Transcriptional changes affected mediators of innate immunity, in particular receptors on the surface of phagocytic cells, enzymes involved in ROS production and modulators of apoptosis. Taken together, our data suggest that WBD infection in *A. cervicornis* is associated with apoptosis, and that WBD pathogen triggers a powerful immune response driven by phagocytic cells that encapsulate and degrade apoptotic cells. This study also indicates a key role for arachidonic acid and in particular the enzyme AOSL in *A. cervicornis* immunity.

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### Tables

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<th>All Transcripts</th>
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**Table 2.1: Summary of coral and non-coral transcripts.** Total number of transcripts (n), significantly differentially expressed transcripts (adj p-val<0.05) (DE), number of up-regulated (up) and down-regulated (down) transcripts among the entire dataset and annotated transcripts only (e-val<10^−5). First row refers to putative coral transcripts, second row to non-coral transcripts.
Table 2.2: Summary of the main pathways involved in *A. cervicornis* response to WBD. Functions are defined by GO terms and manually curated categories. Expression values are reported as log$_2$ fold change of WBD infected corals relative to healthy corals.
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<th>DE transcripts</th>
<th>Uniprot ID</th>
<th>Function</th>
<th>log2(FC)</th>
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Figures

Figure 2.1: White Band Disease on *Acropora cervicornis*. A colony of the staghorn coral *A. cervicornis* infected with White Band Disease showing the characteristic white band of dying and necrotic coral tissue.
Figure 2.2: Volcano plots displaying differential gene expression between healthy and disease staghorn corals. Figure a. plots gene expression values of the putative coral transcripts, figure b. plots putative non coral transcripts. Each point represents an individual gene transcript. Red points represent significantly differentially expressed transcripts (adj p-value < 0.05).
**Figure 2.3: Heatmap of immune-related differentially expressed coral transcripts.** Heatmap showing expression profiles of healthy (H) and WBD infected (D) *A. cervicorns*. Hierarchical clustering indicates similarity between the different samples (columns), with healthy and disease samples forming different clusters. Transcripts annotations are based on GO terms and manually curated categories. Relative expression levels are shown in red (up) and blue (down). The expression level for each transcript was calculated as log2 fold change.
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CHAPTER 3
Genetic signature of disease resistance in endangered Caribbean corals

Abstract

Coral reefs are declining worldwide due to a variety of factors including rising sea surface temperature, increasing ocean acidification, and disease outbreaks. Over the last 30 years, White Band Disease (WBD) alone has killed up to 95% of the Caribbean’s dominant shallow-water corals - the staghorn coral *Acropora cervicornis* and the elkhorn coral *A. palmata*. Both corals are now listed as endangered on the US Endangered Species Act. Recovery of the Caribbean *Acropora* has been slow, but recent transmission surveys indicate that more than 5% of staghorn corals are disease resistant. Here we used RNA sequencing (RNA-seq) to compare transcriptome-wide gene expression between resistant and susceptible staghorn corals exposed to WBD. We identified strong constitutive differences in gene expression underlying disease resistance in staghorn corals that are independent from the immune response associated with disease (pathogen) exposure. Genes involved in RNA interference-mediated gene silencing and antiviral defense, including Argonaute and NOD-like receptor 5 (NLRC5) were up-regulated in resistant corals, whereas heat shock proteins (HSPs) were down-regulated. Up-regulation of Argonaute proteins indicates that RNAi-mediated gene silencing plays a key, but previously unsuspected role in coral immunity and disease resistance. Down-regulation of HSPs has recently been linked to thermal resilience in other *Acropora* corals, suggesting that the down-regulation of HSPs in disease resistant staghorn corals may confer a dual benefit of thermal resilience.
Introduction

Coral reefs are the world’s most biodiverse marine ecosystems, hosting approximately 25% of all marine species. Reef-building corals are highly vulnerable to environmental stressors and anthropogenic impacts including the global rise in sea surface temperatures, ocean acidification and disease outbreaks associated with climate change. It has been estimated that one third of world’s reef-building corals are at risk of extinction [1]. Recent studies indicate that corals have the ability to recover from bleaching events and disease outbreaks [5, 6] highlighting the importance of understanding the mechanisms of resilience and resistance in coral reef restoration and conservation strategies but the mechanisms underlying coral resilience are not yet understood, especially the basis of disease resistance.

The Caribbean staghorn coral A. cervicornis declined dramatically due to WBD outbreaks starting in 1979 and is now considered at high extinction risk [7,8]. Koch-Henle’s postulates for WBD have not yet been fulfilled, but multiple studies indicate that the pathogen is bacterial [9,10] with *Vibrio charachariae* and *Rickettsia CAR1* both shown to be associated with the disease [11-13]. The disease can be transmitted by direct contact, via the water if the coral is injured, and by animal vectors like the corallivorous snail *Coralliophila abbreviata* [14]. *In situ* transmission experiments indicate that more than 5% of staghorn corals are resistant to WBD [15]. Recent RNA-seq analyses indicate that staghorn corals mount a vigorous immune response to WBD mediated by Pathogen Recognition Receptors (PRRs), apoptosis, production of Reactive Oxygen Species (ROS) and synthesis of eicosanoids [16], but the genetic differences between disease resistant and susceptible corals are still unknown.

In order to identify genetic signatures underlying disease resistance in *Acropora cervicornis*, we used RNA sequencing to obtain transcriptome profiles of resistant and susceptible staghorn corals from the archipelago of Bocas Del Toro, Panama, that were exposed to disease (WBD grafts), allogeneic tissue (healthy grafts) or unexposed (no-graft control) (Figure 3.1). Transcriptome-wide comparison of gene expression between resistant and susceptible corals revealed that none of the main immune pathways previously identified in WBD-infected staghorn corals was associated to resistance. Instead, resistant corals exhibited constitutive (i.e. independent from disease exposure) differential expression of few transcripts, particularly NOD-like receptors, Argonaute proteins and Heat Shock proteins.
Materials and Methods

In situ transmission experiment

The in situ transmission experiment was conducted in July 2009 in the region of Bocas Del Toro (Republic of Panama), in a common garden along the coasts of Isla Bastimentos. Four resistant and three susceptible genotypes of *A. cervicornis* that were previously identified by [15] were used for the experiment. Replicate fragments (30 cm tall) from each staghorn coral genotype were collected and placed in the common garden for 24 hours to acclimate. One fragment for each genotype was collected right after the acclimation periods as a control and the remaining replicate fragments were exposed to either WBD infected tissue (WBD grafts) and to allogeneic tissue (healthy grafts) (Figure 3.1). The grafted fragments were collected after 3 days of exposure, flash frozen in liquid nitrogen and stored at -80°C for RNA analysis.

RNA-seq library preparation

Total RNA was extracted using TriReagent (Molecular Research Center, Inc.) following the manufacturer's protocol. Seventeen samples (n = 7 susceptible and n = 10 resistant) yielded high quality RNA (RIN = 6 or higher) and were processed for RNA-sequencing. Library preparation was performed with a modified Illumina mRNA-seq protocol as described in Libro et al [16]. Multiplexed paired-end libraries were sequenced on an Illumina GAII platform (Illumina, Inc, San Diego, California, USA) at the FAS Center for System Biology at Harvard University. De-multiplexing, read trimming and thinning were performed using custom Perl Scripts in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads were mapped to a reference transcriptome obtained from 39 *A. cervicornis* and six *A. palmata* whole coral tissue samples [1]. The reference transcriptome was composed of 95,389 transcripts, of which 47,748 were identified as coral (e-value < 10^-10) and 47,641 as non-coral based on alignment against two *Acropora* larval genomes [32,16]. Functional annotation was performed using tBLASTx with an e-value cutoff less than 10^-5 [3] against the Swiss-Prot and TReMBLE protein databases [34]. Gene functions for the annotated transcripts were retrieved using Gene ontology (GO) terms and manually curated categories, to highlight presence of immune related domains or putative immune role based on literature. The reference transcriptome sequences are available on Bioproject (accession number PRJNA222758)
**Differential gene expression analysis**

Two-factor, negative binomial GLM, implemented in the R package DESeq2 [35], was used to identify coral transcripts that differed significantly in their expression between the two disease resistance phenotypes (resistant vs. susceptible) and three exposure treatments [disease graft, allogeneic graft (i.e. healthy graft), or no graft control]. The fully factorial model allowed us to identify transcripts that differed due to resistance, exposure, or both factors as well as identify significant interactions between the factors.

**Results and Discussion**

We compared transcriptome-wide gene expression patterns between resistant and susceptible staghorn coral genotypes from Bocas Del Toro, Panama using next-generation Illumina mRNA sequencing (RNA-seq). To identify genes involved in disease resistance, we obtained RNA-seq profiles for four resistant and three susceptible staghorn coral genotypes exposed to disease (WBD grafts), allogeneic tissue (healthy grafts) or unexposed controls (no-graft control). Resistant and susceptible coral genotypes used in this experiment were previously identified in Vollmer and Kline [15]. The average number of high-quality reads per sample was 3,852,932 (±525,993) in resistant corals and 2,359,619 (±962,951) in susceptible corals. Two-factor GLM analyses of RNA-seq gene expression patterns comparing staghorn coral resistance phenotypes (WBD resistant vs. susceptible corals) and exposure treatments (disease graft vs. allogeneic graft vs. control) identified 87 (57 up- and 30 down-regulated) coral gene transcripts that were significantly differentially expressed (DE) due to the resistance phenotype, 3,721 (3,181 up- and 540 down-regulated) DE transcripts due to disease exposure, and 52 (37 up and 15 down-regulated) DE transcripts due to allogeneic grafting (Table 3.1). Out of the 87 DE disease resistance transcripts, 78 transcripts differed only due to resistance while the remaining nine transcripts (n = 5 annotated) differed due to disease exposure as well (resistance + exposure) (see Figure 3.1). No transcripts showed significant interactions between the two factors (resistance x exposure). This indicates that 90% (78 out of 87) of DE transcripts associated with resistance behave independently of disease exposure and thus represent constitutively expressed differences between resistant and susceptible staghorn coral genotypes. Thirty-five DE disease
resistance transcripts (24 up and 11 down-regulated), 2,692 DE disease exposure transcripts (2,390 up and 302 down-regulated) and 38 DE allogeneic graft transcripts (25 up and 13 down-regulated) had strong protein annotations (tBlastx e-value < 10^{-5}) and could be used to characterize the genetic response of the coral host. None of the DE transcripts associated with disease resistance have been previously identified as key mediators of staghorn coral immunity [1], indicating that the genetic basis of WBD resistance is distinct from the immune response elicited by disease (pathogen) exposure. In order to characterize the genetics of disease resistance, we focused on the 35 annotated coral transcripts that were DE between resistant and susceptible genotypes (Figure 3.1).

Resistant corals differed from susceptible corals in their higher expression of gene transcripts associated with RNA interference-mediated gene silencing (RNAi) and antiviral defense including three Argonaute proteins, AGO1 (3.6 fold up), AGO2 (3.5 fold up) and AGO3 (3.6 fold up), and two NOD-like receptor family CARD domain containing 5 (NLRC5) (3.0-2.1 fold up). Elevated Argonaute expression in resistant corals suggests there is link between RNAi-mediated gene silencing and disease resistance in staghorn corals, but it is not yet clear if the relationship is direct, as an antiviral defense via small interfering RNA (siRNA), or indirect, as part of host gene regulation via microRNA (miRNA). Argonaute proteins have been linked to pathogen resistance and immunity in animals and plants. For example, in Drosophila, AGO2 mutants are more susceptible to viral infection [17]. In plants, AGO2 expression is elicited by bacterial exposure and promotes the production of antimicrobials via miRNA-dependent gene regulation [18]. Data directly linking Argonaute expression to disease resistance is limited. Yet, in Arabidopsis, AGO4 regulates resistance to pathogens via RNA-directed DNA methylation [19], an epigenetic mechanism of transcriptional gene regulation.

NLRC5 is a Pathogen Recognition Receptor (PRR) in the NOD-like receptor (NLR) family that detect intracellular pathogens and regulate inflammatory cytokines production [20]. In vertebrates, NLRC5 can mediate antiviral defenses [21], regulate inflammation and immunity via NF-kB signalling in the cytoplasm [22], and even act as transcriptional regulator of major histocompatibility complex (MHC) class II genes [23]. While NLRs are broadly considered to be important innate immune regulators [24], relatively little is known about their roles in invertebrates, in part because they are absent in model organisms like Drosophila and Caenorhabditis elegans [25]. In plants, nucleotide-binding site–leucine-rich repeat (NBS–LRR)
proteins, which are structural similar to NLRs, detect pathogens and can confer disease resistance [26]. Interestingly, two genetic examples of host disease resistance (R) genes in plants involve endogenous siRNA-mediated gene silencing and miRNA-mediated regulation of auxin signalling [27]. Corals and other cnidarians possess an unusually high diversity of NLRs compared to other invertebrates [28], but no direct evidence for their immune function has been reported until now. Up-regulation of NLRs in resistant staghorn corals suggests they play an important role pathogen detection and immunity, possibly even allowing resistant corals to avoid pathogen infection.

One surprising feature of the gene expression patterns in the resistant staghorn corals was the down-regulation of 70 kDa heat shock proteins, specifically heat shock 70 kDa protein 12B (HSPA12b) (6.9 fold down) and heat shock 70 kDa protein 12A (HSPA12a) (n = 2; 8-5.8 fold down) (Figure 3.1). Heat shock proteins (HSPs) are ubiquitous molecular chaperones that regulate inflammation and immunity [29]. Members of this family, in particular Hsp70, are also induced during thermal stress to protect proteins and cell membranes from the damage caused by incorrect protein folding and aggregation [30]. In corals, increased HSPs expression has been documented in response to thermal stress during coral bleaching and due to increasing acidification [31], but not yet due to pathogen exposure [16]. However, thermally resilient A. hyacinthus corals living in high temperature environments have recently been shown to exhibit low HSPs expression [6]. Thus, it is possible that down-regulation of HSPs in disease resistant staghorn corals may confer increased thermal resilience as well.

Transcriptome-wide analysis of gene expression pattern underlying disease resistance in the staghorn coral A. cervicornis demonstrate that resistance to WBD infection is conferred by the constitutive expression of multiple gene pathways, including up-regulation of RNAi-mediated gene silencing and down-regulation of HSPs. How the up-regulation of Argonaute and NLRC5 confers disease resistance in staghorn corals is unknown. Recent research indicates that WBD infection can be blocked by antibiotic treatment, indicating that the pathogen is bacterial [9,10]. It is possible that WBD infection involves both bacterial and viral pathogens [9], and that the up-regulation of Argonaute in resistant corals is being used to respond to exogenous double-stranded RNA (dsRNA) via siRNA. However, NLRC5 and Argonaute were constitutively up-regulated and not up-regulated due to pathogen exposure. A second and more intriguing possibility is that the up-regulation of Argonaute transcripts in disease resistant corals is used for
miRNA-based post-transcriptional regulation. This suggests a novel role for miRNA-directed gene silencing in cnidarian immunity, which would be similar to the links between miRNA and pathogen resistance in plant immunity. Down-regulation of HSPs in disease resistant staghorn corals confirms the links of HSPs with coral immunity. This finding, coupled with recent evidence for lower HSPs expression in thermal resilient pacific Acropora corals [6], suggests the intriguing possibility that disease resistant corals may show increased thermal resilience as well. The ability to identify resistant staghorn corals in remnant populations across the Caribbean using these strong, constitutive differences in gene expression at Argonaute and HSP transcripts could also serve as a valuable conservation tool and aid nursery and out-planting efforts that are currently underway for this endangered species.

Acknowledgements

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Tables

Table 3.1: Differential expression analyses of RNA-seq data for staghorn coral resistance phenotypes and exposure treatments. Number of coral transcripts exhibiting significant differential expression (adj p-val<0.05) due to the resistance phenotype (resistant vs susceptible) and to exposure (allogeneic graft vs control; disease graft vs control). Only five transcripts were significant in both treatments (R+E). No significant interaction was found between resistance and exposure (R*E = 0).

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Figures

**Figure 3.1: Differentially expressed transcripts in resistant *A. cervicornis*.**
Gene expression analysis of resistant (R) and susceptible (S) corals exposed to (a) allogeneic tissue and (b) active White Band Disease (WBD). (c) Heatmap for gene expression of the 35 annotated differentially expressed genes that differ due to resistance. Hierarchical clustering indicates similarity between the different samples (columns), with resistant and susceptible samples forming different clusters. Red corresponds to up-regulated genes, blue indicates down-regulation. The expression level for each transcript was calculated as log2 fold change. Asterisks indicate transcripts that were DE in both factors (resistance + exposure).
References


CHAPTER 4
Immunity and allore cognition in the staghorn coral *Acropora cervicornis*

Abstract

Corals possess a complex innate immune system that enable them to fight disease infections, adapt to environmental stress, and recognize self from non-self. However, histocompatibility reactions in Cnidaria are highly variable among species, and it is not yet clear if the genetic bases of allore cognition and immunity overlap strongly. RNAseq-based gene expression profiles indicate that the staghorn coral *Acropora cervicornis* infected with White Band Disease (WBD) mount a vigorous immune response that includes up-regulation of genes involved in pathogen recognition, production of reactive oxygen species (ROS) and synthesis of eicosanoids via allene-oxide pathway.

Using RNA-seq, we identify the differences and similarities between coral immunity and allore cognition by comparing staghorn corals exposed to WBD and to allogeneic grafts. Our results indicate that the coral immune response is distinct from allore cognition. WBD-exposed corals display a strong immune response with changes in the expression of 2,692 gene transcripts. Expression changes associated with allore cognition were less pronounced, involving only 38 gene transcripts. By comparing the immune signature of disease-exposed corals (disease grafts) to WBD infected corals sampled from the field (Chapter 2), we are able to identify components of early and late-stage coral immune response. Shared gene pathways between early and late response include pathogen recognition, ROS production and synthesis of eicosanoids, while hallmarks of early response include up-regulation of downstream mediators of Toll-Like receptor (TLR) signaling pathway and NF-kB signaling, melanin synthesis, antiviral compounds and heat shock proteins. Increased eicosanoids production in disease corals, but not allogeneic grafts suggests that synthesis of eicosanoids in staghorn corals is induced by pathogen infection rather than by stress and mechanical injury.
Introduction

Coral disease epidemics have increased worldwide in frequency and severity over the last 30 years [1,2]. The combined effects of disease outbreaks and anthropogenic stress have caused mass mortalities of several reef-building coral species with coral cover declines up to 50% in the Indo-Pacific and to 80% in the Caribbean [3-5]. Recent studies indicate that corals actively fight disease infections (Chapter 2, [6]) and respond to environmental stress [7,8] with an array of innate immune pathways previously believed to be restricted to more complex organisms [9]. Known mediators of coral immunity include components of Toll-Like receptor (TLR) signaling pathway, complement, and prophenoloxidase cascade, as well as antimicrobial peptides and stress response proteins [10]. While corals possess the genetic components of the classic invertebrate innate immune repertoires, our RNA-seq profiles of WBD-infected staghorn corals suggest that recognition of pathogens via TLRs and C-type lectins, phagocytosis, apoptosis and production of eicosanoids via allene oxide represent the corals’ primary immune response (Chapter 2, [6]).

Corals are also capable of allore cognition, i.e. the ability of the host immune system to discriminate between self and non-self, which can trigger an allograft rejection [11]. In colonial and sessile marine organisms, allore cognition is important to avoid germline parasitism [12-15] and compete for space with neighboring colonies [16]. It has been suggested that cnidarians develop the ability to recognize self from non-self after metamorphosis [17-20], but the underlying mechanisms for the delayed allore cognition response is unclear.

In anthozoans, allore cognition responses have been documented in sea anemones [21-24], gorgonians [25,26], alcyonacean [27], and scleractinian corals [28-30]. Histocompatibility behaviors in scleractinian corals are highly variable across species, ranging from rejection of allogeneic colonies with discharge of nematocysts and cytotoxic reactions to less aggressive behaviors such as interspecific tolerance [28,31]. The best work on cnidarian allore cognition is from the hydrozoan Hydractinia symbiolongicarpus where the genetic basis of allore cognition has been characterized: fusion, rejection or temporary fusion between genetically distinct colonies is based on allelic similarity at two highly polymorphic allore cognition loci called alr1 and alr2 [32,33]. For scleractinian corals, the mechanisms that control fusion and rejection among conspecific colonies are still unknown and the link between immunity and allore cognition is largely unexplored [34].
Here we use the Caribbean staghorn coral *Acropora cervicornis* to investigate gene expression changes associated with pathogen exposure and with allore cognition. *A. cervicornis* and its congener, the elkorn coral *A. palmata*, have been heavily impacted by White Band Disease (WBD) that swept across the Caribbean in the 1980s and 1990s [35,36]. Both corals have not recovered from these unprecedented die-offs associated with WBD and are now listed as threatened under the US Endangered Species Act [37] and as critically endangered under the International Union for the Conservation of Nature (IUCN) Red List criteria [38,39]. While the WBD pathogen has not been fully characterized, it is known to be bacterial [40,41]. Multiple studies indicate that *Vibrio* [42,43] and *Rickettsiales* [44] bacteria are both associated with the disease. Transmission can occur via the water column in presence of tissue lesions, via direct contact or animal vectors, such as the snail *Coralliophila abbreviata* [45], and experimentally by grafting healthy corals with infected tissues [46].

Our recent RNA-seq analyses demonstrate that WBD-infected *A. cervicornis* in the field exhibit drastic gene expression changes across 4% of the coral transcriptome. The staghorn coral immune response is mediated by Pathogen Recognition Receptors (PRRs), including C-type lectins and TLRs, apoptosis, phagocytosis and synthesis of eicosanoids via allene oxide (Chapter 2, [6]). Previous research found that more than 5% of genotypes in natural populations of *A. cervicornis* in Bocas Del Toro, Panama, are fully resistant to WBD [46]. Recent transcriptome analysis reveals that WBD resistance staghorn corals have higher expression of intracellular PRRs like NOD-like receptors and of effectors of gene silencing via RNA interference, and lower baseline expression of heat shock proteins (Chapter 3).

Allore cognition in *A. cervicornis* has been studied by Tunnicliffe [47] and Neigel and Avise [48] using grafting techniques. Both studies demonstrate a clear and repeatable pattern of self- and non-self recognition among *A. cervicornis* genotypes, but the mechanisms used by staghorn corals to detect allogeneic tissue are not known. It is likely that detection of non-self determinants occurs during the first stages of exposure to allogeneic tissue, and may overlap with the detection of pathogens during the early stage of WBD infection.

In order to understand whether coral immunity and allore cognition are mediated by similar genetic pathways, we used RNA-seq to profile the genetic response of resistant and susceptible staghorn corals that were exposed to healthy allogeneic tissue, WBD-infected allogeneic tissue, and left unexposed (as a control). WBD grafts were used to characterize the
immune response of corals to WBD exposure and healthy allografts were used to control for allorecognition allowing us to separate gene expression changes associated with pathogen exposure versus allogeneic tissue exposure. By comparing the gene expression patterns between the experimentally transmitted WBD with disease grafts (three days of exposure) and the RNAseq data from WBD-infected corals from the field (Chapter 2), we identified hallmarks of early stages immune response against WBD infections.

Overall, our data reveal that the effects of allorecognition on the staghorn coral transcriptome are very different from the ones induced by pathogen exposure. The expression profiles of healthy and disease grafts showed little to no overlap, with only few shared mediators mainly involved in nematocysts activity and general stress response. Extensive overlap between early and late stage response, on the other hand, confirms that the immune response against WBD is mediated by PRRs, phagocytosis and production of chemical defenses including antimicrobials and eicosanoids. Hallmarks of early response included intense TLR-mediated NF-kB signaling, melanization as well as antiviral activity and heat shock proteins (HSPs). This study also represents the first evidence of a pivotal role for eicosanoids in coral immunity associated to pathogen infections rather than as general stress mediators.

Materials and Methods

Seventeen coral fragments were collected from three resistant and two susceptible colonies of *A. cervicornis*, previously identified as described by Vollmer and Kline [46] in the archipelago of Bocas Del Toro, Panama. The sampled coral fragments (30 cm tall) were acclimated in common gardens for 24 hours prior to grafting the experimental corals with disease or healthy (allogeneic) grafts. One fragment from each colony (n = 5) was collected immediately after acclimation (at 24 hours) as a control. The remaining coral fragments were left in the common garden, exposed to healthy (n = 5) and WBD infected allogeneic tissue (n = 7) via cable-tied coral grafts, and monitored daily (Figure 4.1). After three days, the grafted coral fragments were flash-frozen in liquid nitrogen, and stored at -80C for the downstream RNA extraction and analyses.

**RNAseq library preparation and sequencing**
Total RNA was extracted using Tri-Reagent (MRC, Cincinnati, OH, USA) following the manufacturer’s protocol with an additional ethanol wash. Total RNA quality and integrity were assessed with Agilent’s 2100 Bioanalyzer and high quality RNA extractions (RIN > 6) were used for Illumina RNA sequencing. RNA-seq libraries were prepared according to a modified multiplexed Illumina mRNA-seq protocol using custom paired-end adapters with 4bp barcodes (Chapter 2, [6]). Multiplexed samples were run on the Illumina GAII sequencer (Illumina, Inc, San Diego, California, USA) at the FAS Center for System Biology at Harvard University with 2-3 samples per sequencing lane to generate paired-end 30-bp reads. De-multiplexing, trimming and filtering of low quality reads (Phred < 30; read length > 15bp) were performed using custom Perl scripts in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).

Read counts for differential gene expression analysis were obtained by mapping reads against our Acropora reference transcriptome using Bowties. The reference transcriptome was assembled from 428 million Illumina RNA-sequencing reads obtained from 39 A. cervicornis and six A. palmata whole coral tissue samples (Chapter 2, [6]) and included 95,389 transcripts (N50 = 363 and N75 = 696), 47,748 of which aligned against Acropora exomes built from existing larval genomes [49] and were thus identified as coral (e-value < 10^{-10}). The remaining 47,641 transcripts did not align to the Acropora genomes and were classified as non-coral, possibly representing Symbiodinium, other associated eukaryotes as well as novel coral transcripts.

Protein annotations using a tBLASTx search [50] with an e-value cutoff less than 10^{-5} against the Swiss-Prot and TReMBLE protein databases [51] were obtained for 20,503 (43 \%) out of the 47,748 coral transcripts. Gene Ontology (GO) terms and gene functions were obtained for the annotated transcripts on UniProt using Blast2Go. The reference transcriptome sequences are available on Bioproject (accession number PRJNA222758)

Differential gene expression analysis was performed using a two-factor, negative binomial GLM, implemented in the R package DESeq2 [52] to identify differentially expressed (DE) coral transcripts between the two disease resistance phenotypes (resistant vs. susceptible) and three exposure treatments (disease graft, healthy graft, or no graft control). A fully factorial model was used to detect transcripts that differed due to resistance, exposure, or both factors as well as identify significant interactions between the factors.
Expression profiles associated to allorecognition were characterized by comparing expression changes between healthy grafts and unexposed controls, while DE transcripts between disease grafts and controls represented expression changes due to both allorecognition and disease exposure. Transcription profiles associated to resistance were characterized using transcripts that differed between resistant and susceptible corals, as described in Chapter 3.

To compare immune signatures of early-stage (disease grafts) to late-stage WBD infection (WBD-infected corals sampled from natural populations), we reanalyzed using DESeq2 the dataset generated from healthy and WBD-infected corals described in Chapter 2 (previously analyzed with DESeq) to create a matched statistical comparison between the datasets; this was necessary given the recent advances in DESeq2’s filtering algorithms. The functional identity of DE transcripts was determined by gene ontology (GO) terms and manually curated categories, with a particular interest in those with immune related domains or putative immune role based on literature.

Results

RNA-sequencing of 17 coral samples including seven disease grafts (four resistant and three susceptible), five healthy grafts (three resistant and two susceptible), five unexposed (three resistant and two susceptible) yielded an average of 3,702,307 reads (±681,591) per samples in disease grafts, 2,631,145 reads (±1,429,089) in healthy grafts and 3,194,956 reads (±854,355) in unexposed samples.

Two-factor differential expression analyses identified 87 DE coral transcripts associated with disease resistance and 3,772 DE transcripts associated to exposure (3,720 due to WBD exposure and 52 due to healthy graft exposure). Annotated (tBlastx e-value < 10-5) coral transcripts associated to exposure included 2,691 DE transcripts due to disease grafts (2,389 up and 302 down-regulated) and 38 DE transcripts due allogeneic grafts (26 up and 12 down-regulated). Transcripts associated to resistance included 35 DE transcripts (24 up and 11 down-regulated), of which five differed due to disease exposure as well (resistance + exposure). No significant interactions between the two factors (resistance x exposure) was detected, indicating that 90% (78 out of 87) of DE transcripts associated with resistance are expressed independently of disease exposure (See Table 3.1). The genetic basis of coral disease resistance is discussed in
Chapter 3. Here we focus on DE immune-related coral transcripts associated with disease exposure, represented by transcripts uniquely DE in disease grafts, and with allore cognition, represented by DE transcripts in both healthy and disease grafts.

Of the 3,720 (2,691 annotated) DE transcripts in disease grafts (WBD exposure + allore cognition), 49 (36 annotated) transcripts were also DE in healthy grafts (allore cognition), while the remaining 3,671 (2,655 annotated) represented disease infection-specific genes that were DE due to disease exposure but not to allore cognition.

After re-analysis, differential gene expression comparisons between healthy and WBD-infected environmental samples (late-stage response to WBD) yielded 2,817 (1,442 annotated) DE coral transcripts between healthy and diseased corals (Table 4.1). We merged the two datasets by contig ID and identified transcripts that had significant adjusted p-value in both experiments. Of the 49 (36 annotated) DE transcripts in both healthy and disease grafts (allore cognition), 11 transcripts (seven annotated) were DE in all comparisons (i.e. WBD-infected samples collected from the field, disease grafts and healthy grafts) indicating their role as general mediators of stress and immunity, while the remaining 37 (28 annotated) were DE only in corals exposed to healthy and WBD grafts and thus represented candidate mediators of allore cognition. A total of 984 transcripts (656 annotated) were DE in WBD infected samples, including diseased samples collected from the field and experimentally exposed disease grafts, but not healthy grafts, representing shared mediators between early and late response to WBD. Putative mediators of late and early response to WBD included respectively 1,822 (779 annotated) transcripts uniquely DE in WBD-infected environmental samples and 2,394 (1,904 annotated) transcripts uniquely DE in diseased grafts. Finally, three (two annotated) transcripts were exclusively DE in healthy grafts (Figure 4.2).

Out of all the 3,376 annotated DE transcripts associated with either allore cognition or disease infection, 460 transcripts had an immune-related function based on Blast identity, GO terms and manually curated annotations (Table 4.2) and were used in this study to identify mediators of allore cognition, disease exposure-specific genes and component of early response to WBD infection.

*Allorecognition*
Staghorn corals exposed to allogeneic healthy tissue exhibited expression changes in only 38 transcripts, seven of which had an immune related function. These included five transcripts belonging to the chemical defensome, including the toxin conodipine-M alpha chain, the antioxidants thioroedoxin reductase 1 (TXNRD1) and 3 (TXNRD3), universal stress protein A-like protein and the only down-regulated transcript, multidrug resistance-associated protein 1 (MRP-1), an efflux pump that removes xenobiotics from the cellular environment [53]. The remaining two transcripts included the receptor frizzled-5 belonging to the Wnt pathway - a signaling cascade already implicated in allorecognition and nematocysts production in the hydroid Hydractinia [54] - a transcript encoding hemagglutinin/amebocyte aggregation factor (18K-LAF), a protein that induces both aggregation of amebocytes and agglutination of erythrocytes in the horseshoe crab Limulus polyphemus [55].

All of these seven transcripts were also DE in corals exposed to disease grafts, but five were DE in WBD-infected corals in the field, possibly representing shared mediators of immunity and allorecognition. These included transcripts associated to nematocysts production and general response to stress, such as conodipine-M alpha chain, TXNRD1, universal stress protein A-like protein, MRP-1 and frizzled-5.

Candidate mediators of allorecognition, on the other hand, included two transcripts that were DE in corals exposed to healthy and disease grafts but not in WBD-infected corals in the field - TXNRD3 and hemagglutinin/amebocyte aggregation factor.

Response to experimental WBD-infection

Corals exposed to disease grafts exhibited DE in 2,691 annotated DE transcripts, of which 337 (13%) were immune-related. Of these, seven were also DE in corals exposed to healthy grafts (see above), representing shared mediators of immunity and allorecognition, while the remaining 330 transcripts represented disease exposure-specific genes.

Overall, expression profiles of staghorn corals experimentally exposed to WBD via disease grafts showed extensive overlap with the one of corals infected with WBD in the field. Of the 236 immune transcripts identified in corals infected with WBD in the field after reanalysis, 114 were shared with corals experimentally exposed to WBD (disease grafts) and included Pathogen Recognition Receptors (PPRs) such as TLRs and C-type lectins, mediators of complement and coagulation cascades, mediators of apoptosis, enzymes involved in the
production of ROS, lysosomal degradation and cytoskeletal rearrangements associated to
degradation associated to
phagocytosis, synthesis of antimicrobials and toxins as well as enzymes involved in the synthesis
of eicosanoids such as prostanoids (Table 4.2, Figure 4.3). The immune signature of early
response against WBD was characterized based on 211 transcripts that were only DE in disease
grafts and not in the WBD infected coral in the field. These early immune genes included
components of melanin biosynthesis pathway, downstream components of TLR and NF-KB
signaling, heat shock proteins and antivirals compounds (Table 4.2).

Among Pathogen Recognition Receptors (PRRs), two TLRs including TLR 2 and TLR 2
type-2 and three C-type lectins including collectin-12, C-type mannose receptor 2 (MRC2), and
ficolin-2, a serum opsonin that promotes activation of the lectin complement pathway, were DE
during both early and late response to WBD while TLR6 and a second transcript encoding
MRC2 were only DE in disease grafts.

Overall complement and coagulation cascades exhibited similar activity during early and
late response to WBD. In the complement cascade, C3 and PZP-like alpha-2-macroglobulin
domain-containing protein 8 (CPAMD8) was up-regulated, while two different transcripts-
possibly isoforms- encoding Macrophage-expressed gene 1 protein (MPEG1), a membrane
attack complex component/perforin MACPF domain containing protein, were down-regulated,
one during early and one during late response. One transcripts was only DE in corals exposed to
disease grafts, complement component 1 Q subcomponent-binding protein (C1QBP) - a receptor
for the complement component C1q. Coagulation seemed inhibited during WBD infection, as
indicated by the up-regulation of two anticoagulants - tissue Factor Pathway Inhibitor (TFPI) and
plasminogen, a coagulation protease that also acts as inhibitor of the complement cascade.
Coagulation factor VIII-a positive regulator of blood clotting, was down-regulated in disease
grafts but was up-regulated in fied samples.

Hallmarks of phagocytosis during both early and late response were active synthesis of
ROS associated to the oxidative burst, lysosomal degradation and cytoskeletal rearrangements
via CDC42, a member of rho family GTPases that controls the phagocytic cup formation [56,57].
These were represented by up-regulation of transcripts belonging to the NADPH oxidase
enzymatic complex involved in ROS production including NADPH oxidase 3, neutrophil cytosol
factor 2 and cytochrome b-245 heavy and light chain as well as of the lysosomal enzymes
cathepsin L, L1 and Z. While up-regulation of CDC42 was only significant in disease grafts, two
other mediators of CDC42-signaling were DE, Cdc42-interacting protein 4 homolog and Activated CDC42 kinase 1.

The expression pattern of 10 transcripts involved in the biosynthesis of eicosanoids indicated that these compounds play a key role in both early and late response against WBD infection. The enzyme phospholipase A2 (PLA2), involved in the synthesis of arachidonic acid from phospholipids during the first steps of the pathway [58], was up-regulated during both early and late response. Downstream conversion of AA into leukotrienes and prostaglandins was indicated by up-regulation of arachidonate 5-lipoxygenase (one DE during both early and late response, and one only during early response) that catalyzes oxidation of AA into leukotrienes and of hematopoietic prostaglandin D synthase, a key enzyme in the synthesis of prostaglandins [59]. Four transcripts in the pathway appeared to be DE only during early response including phospholipase A2 activating protein, that stimulates eicosanoids production by increasing PLA2 activity [60] and another prostaglandin synthetic enzyme, Prostaglandin E synthase 2 [61], both up-regulated. Allene oxide synthase-lipoxygenase (AOSL) (up) and 15-hydroxyprostaglandin dehydrogenase [NAD(+)]) (down) involved respectively in the synthesis of allene oxide [62] and catabolism of prostanoids [63], were only DE in during late response.

Mediators of apoptosis and angiogenesis included 51 transcripts encompassing pro and anti-apoptotic genes. In addition to caspase 3 and thrombospondin-2, previously identified in Chapter 2 [6], mediators of apoptosis involved in both early and late response to WBD included the anti-apoptotic protein lifeguard 2 and 4 [64] and apoptosis regulator R1, a homologous of the anti-apoptotic Bcl-2 [65]. Transcripts associated to early response to WBD included pro-apoptotic genes such as death-associated protein kinase 1 (DAPK1) involved in cell death in response to oxidative stress [66], tumor necrosis factor receptor superfamily member 16 (TNFRS16) and two programmed cell death proteins -PDCD4 and PDCD6- that promote caspase-3 activity via Ca^{2+}-dependent signaling [67].

Enhanced angiogenesis and wound healing activity were indicated by increased fibroblast growth factor signaling activity, as indicated by up-regulation of multiple isoforms of fibroblast growth factor 1, 2and 22, and of fibroblast growth factor receptor 2 and 3.

Both early and late response to WBD were associated with sustained alteration of calcium homeostasis, possibly associated to apoptosis and increased ROS production as indicated by up-
regulation of six transcripts encoding calmodulin (CaM), two of which were DE in both early and late response.

Among inflammation mediators, two transcripts with pro-inflammatory activity were up-regulated in both early and late stage WBD infection: C-X-C chemokine receptor type 7 (CXCR-7) and a homolog of lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF). CXCR-7 was the most up-regulated gene in corals exposed to disease grafts, and is regulated during cell growth and survival [68], chemotaxis [69] and macrophage mediated phagocytosis [70] by NF-kB and by hypoxia-inducible factor 1α (HIF-1α), -also up-regulated in early but not during late stage response to WBD. The other transcript, LITAF is involved in the regulation of pro-inflammatory cytokines production in response to LPS [71].

Signature RNA interference (RNAi)-mediated gene silencing during both early and late stage response to WBD was indicated by down-regulation of dicer-like protein 1 and up-regulation of staphylococcal nuclease domain-containing protein 1 (SND1). Dicer-like protein 1 is a ribonuclease that cuts exogenous double-stranded RNA (dsRNA) or genomically encoded pre-microRNA (pre-miRNA) into small interfering RNA (siRNA) or microRNA (miRNA) during the first steps of the pathway, while SND1 is, a component of the RNA-induced silencing complex (RISC), that induces post transcriptional gene silencing by recognizing and degrading target mRNA sequences complementary to siRNA or miRNA generated by dicer [72].

We identified several involved in the detoxification of xenobiotics and in the defense against chemicals and exogenous toxicants, collectively known as “chemical defensome” [73]. Shared mediators of early and late immune response included delta-latroinsectotoxin-Lt1a, a pore-forming toxin from spiders [74], and golgi-associated plant pathogenesis-related protein 1, a protein that shares structure similar to NEP-16, nematocyst toxin from Nematostella [75], both up-regulated. Antibacterial compounds included a L-amino-acid oxidase from the sea hare Aplysia californica [76], a cysteine-rich with EGF-like domain protein 2 (CRELD1), and multiple transcripts encoding the antimicrobial sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 (SVEP1/polydom). Genes involved in response to stress, possibly induced in response to the oxidative stress generated by increased ROS production, included universal stress protein A-like protein, hypoxia up-regulated protein 1 and antioxidant such as catalase, glutaredoxin and thioredoxin reductase 2.
Early immune genes, i.e. transcripts that were only DE in disease grafts, included the enzyme tyrosinase involved in synthesis of melanin, a key component of coral immunity. In the tyrosinase-type pathway, tyrosinase catalizes conversion of L-tyrosine into L-DOPA, and then into the antimicrobials O-quinone radicals that polymerize into melanin, encapsulating pathogens and damaged tissues [77].

Corals exposed to diseased grafts exhibited DE of several downstream mediators of TLR and NF-KB signaling. These included NLRC3, an intracellular nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) that inhibits inflammation via TLR-dependent inhibition of the transcription factor NF-kb [78]. TIR1, a TIR-domain containing protein previously identified in Acropora millepora, and myeloid differentiation primary response protein MyD88, a key adaptor in the transduction of TLR signaling [9]. Inhibitors of TLR-mediated NF-Kb activation included the TNF receptor-associated factor 4 (TRAF4) and TNFAIP3-interacting protein 1 (TNIP1), while activators included TGF-beta-activated kinase 1 and MAP3K7-binding protein 1 (TAB1), akirin-2 and calcium-calmodulin–dependent kinase IV (CaMKIV).

Transcripts with putative antiviral activity and heat shock proteins (HSPs) were DE almost exclusively during early stage response. Candidate antivirals were identified based on literature search and included components of the retinoic acid-inducible gene 1 (RIG-I) signaling that promote interferon-mediated antiviral response: DNA-directed RNA polymerase III subunit RPC4 [79] was up-regulated, while probable ATP-dependent RNA helicase DDX60 (DDX60) [80] and DDX60-like were down-regulated.

One of the clearest signatures of early response to WBD was represented by 11 DE Heat shock proteins. The expression pattern exhibited by HSPs was highly variable. Hsp10, hsp60, hspa14, hsp23, hsp11 and hspa2 were up-regulated, while hsp70, hsp68, hsp82 and hsp90 were down-regulated. The transcription factor hypoxia-inducible factor 1-alpha was also up-regulated as part of the stress response.

Discussion

Allorecognition response
Overall, gene expression profiles of staghorn corals exposed to allogeneic grafts were distinct from the genetic response of corals exposed to disease grafts. The lack of the main mediators of coral immunity in the DE transcripts in healthy grafts indicates that allorecognition is not mediated by the same immune pathways involved in the defense against pathogen infection. While the genetic response of the coral to allografts was far more modest than its response to the WBD pathogen, we were able to identify seven immune genes with distinct roles in allorecognition.

Exposure to allogeneic tissue triggered the production of toxins, possibly associated to nematocysts discharge along the contact between allogeneic tissues as indicated by up-regulation of conodipine-M alpha chain, a phospholipase-type toxin from a marine snail [81], and of the horseshoe crab hemagglutinin/amebocyte aggregation factor, a protein related to cytotoxin-1 from the fire coral _Millepora dichotoma_ [55]. Increased nematocyst activity associated to allorecognition was also indicated by up-regulation of the Wnt receptor frizzled-5. In _Hydractinia_, frizzled proteins and active Wnt/beta catenin signaling can induce production of nematocysts in pluripotent stem cells, facilitating their migration into the gonads of neighboring colonies during germ-line parasitism [82]. The Wnt signaling pathway also plays a direct role in allorecognition, as suggested by the up-regulation of a Wnt-responsive gene, the transcription factor chicken ovalbumin upstream promoter transcription factor (COUP-TF), during histocompatibility reactions in _Hydractinia_ [54].

Increased expression of antioxidants such as universal stress protein A-like protein and thioredoxin reductase 3 may indicate response to oxidative stress associated to ROS production during cytotoxic reactions. However, unlike in disease grafts, there was no evidence of up-regulation of ROS-generating enzymes, suggesting that antioxidant enzymes could be involved in general stress response.

It is noteworthy that the majority of the immune genes that were DE in corals exposed to allogeneic grafts (five out of seven), including conodipine-M alpha chain universal stress protein A-like protein, thioredoxin reductase 1, Frizzled-5 and multidrug resistance-associated protein 1, were not uniquely associated to allorecgnition and were also DE in corals infected with WBD in the field (no grafts), indicating that they could instead represent general mediators. The only exceptions were thioredoxin reductase 3 and hemagglutinin/amebocyte aggregation factor, for which a different isoform was DE in non-grafted WBD infected corals, but was down-regulated.
Interestingly, we did not observe the DE of lectins or HSPs associated with allorecognition. Lectins have been suggested as putative mediators of coral allorecognition, based on their expression profiles during coral development. In juvenile *A. millepora*, a CELIII type lectin was strongly up-regulated during the metamorphosis [83] suggesting a role in allorecognition maturation. High expression of HSPs proteins during aggressive behavior stimulated by contact with genetically different clones have been found in the tentacles of the sea anemone *Anthopleura elegantissima* and in the body and tentacles of *Corynactis californica*, possibly to promote tissues repair and recovery from the cytotoxic compounds produced during the rejection [84]. However, while expression of Hsp70 was constantly high in *C. californica*, in *A. elegantissima* was variable [84]. This is not surprising, as reactions to allogeneic tissue exposure are known to be highly variable from taxa to taxa. For example, in the colonial hydroid *Hydractinia*, allorecognition is controlled by a single locus with multiple, co-dominantly expressed alleles [85,86] and is not followed by elimination of non-self cells [87], while in *Hydra*, heterografts between *H. vulgaris* and *H. oligactis* stimulate aggregations of epithelial cells within the contact zone [88].

Histological examination of allografts and additional research is necessary to confirm our findings, however, the small number of genes DE in the allografted samples indicates that, unlike the immune response against WBD pathogen, allorecognition in *A. cervicornis* does not rely on inducible defenses. Alternatively, it may be that the allogeneic-induced changes in expression changes appear only at later stages of allogeneic tissue exposure (i.e. after 3 days). This hypothesis is supported by the observation that visible signs of histocompatibility reactions appear after weeks from initial allogeneic contact in *Acropora hemprichii* [30] and after 7-9 days to in the gorgonian *Swiftia exerta* [26]. Intriguingly, the study on *S. exerta* also found that secondary contact elicited a significantly faster immune reaction, and that the cytotoxic reaction varied greatly across different treatments, suggesting that cnidarians are able to use their innate immune repertoire to achieve specificity and memory, two characteristics previously thought to be limited to adaptive immunity [26,34].

*Pathogen exposure*
Direct exposure to WBD-infected corals via grafting resulted in dramatic gene expression changes in the transcriptome of *A. cervicornis* (Table 4.1). The reanalysis of the dataset obtained from healthy and disease samples described in Chapter 2 [6] allowed us to compare the differences between early (i.e. transmitted) and late (field sampled) response to the disease. Comparisons between the two datasets show a strong overlap in the immune signature of WBD previously described in Chapter 2 [6] and confirm that the hallmarks of immune response against WBD include pathogen recognition via C-type lectins and TLRs, activation of complement cascade, as well as ROS production and phagocytosis, eicosanoids synthesis and production of chemical defenses.

One of the most interesting hallmarks of the coral immune response was the increased production of eicosanoids associated with WBD infection. Transcripts matching arachidonate 5-lipoxygenase, PLA2, and hematopoietic prostaglandin D synthase were up-regulated in staghorn corals infected with WBD, but not exposed to allogeneic tissue. This indicates that eicosanoids biosynthesis in staghorn corals is elicited by pathogen exposure and not by allore cognition and mechanical injury. The different expression of prostaglandin E synthase 2, AOSL, and 15-hydroxyprostaglandin dehydrogenase [NAD(+)]) during late and early response suggests that different parts of the pathway may be up-regulated at different times or used in different ways. In vertebrates, membrane phospholipids are converted into arachidonic acid by the enzyme PLA2. The pathway splits into two branches, both accompanied by release of ROS as byproduct of lipid oxidation, but catalyzed by different enzymes. One branch leads to synthesis of leukotrienes via conversion of AA into 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by the enzyme lipoxigenase (LOX), while in the cyclooxygenase (COX) pathway, the enzyme PGH$_2$ Synthase produce prostaglandins from AA [89].

In soft corals, evidence of COX, LOX, and AOSL enzymatic activities have been detected, suggesting the existence of multiple pathways [90-92].

The enzyme AOSL is a fusion protein with catalase–like allene oxide synthase (AOS) and an 8R-lipoxygenase (LOX) and catalyzes the formation of allene oxides from fatty acid hydroperoxides [62,93]. The mechanism is similar to the way plants convert fatty acid hydroperoxides into allene oxide during the synthesis of the stress hormone jasmonic acid [94].

AOSL has been suggested to be a general stress response mediator in the soft corals due to its increased gene expression in response to mechanical injury, and to temperature stress
In scleractinian corals, the first evidence of eicosanoids synthetic pathway have been described in *Acropora cervicornis* infected with WBD (Chapter 2 [6]), where three enzymes, were differential expressed. PLA2, arachidonate 5-lipoxygenase, and AOSL were strongly up-regulated in response to WBD infection, indicating AOSL may play a pivotal role as mediators of immunity. However, unlike soft corals, our results indicate that AOSL is not elicited during mechanical injury upon tissue grafts.

The contrasting expression patterns exhibited by apoptosis mediators, including up-regulation of caspase 3 and other pro-apoptotics but down-regulation of caspase 8 (only significant during late stage response), and up-regulation of transcripts with pro-angiogenic and anti-apoptotic function, indicate a complex role of apoptosis and angiogenesis during WBD infection. It is possible that, like in mammals [97-100], the increase in ROS synthesis and cytosolic calcium, indicated by up-regulation of calmodulin, acts as fine tune modulators of tissue remodeling and programmed cell death in WBD-infected corals.

Among the immune signaling pathways, we detected the signature of RNA interference (RNAi)-mediated gene silencing. RNAi is a RNA-dependent mechanism of antiviral defense and post-transcriptional regulation. In the RNAi pathway, exogenous dsRNA deriving from viral infection or pre-miRNAs expressed from RNA-coding genes are first processed into short interfering RNA (siRNA) or microRNA (miRNA) by the enzyme dicer, and then loaded into RISC where they denature and hybridize with a the target mRNA, inhibiting its translation or promoting its degradation by the enzyme argonaute [101]. While the up-regulation of SND1 suggests increased RNAi activity in diseased corals, the down-regulation of the key component of the pathway, dicer, is less clear and may be a consequence of the up-regulation of stress response genes in particular HIF1A, whose expression is increased by ROS [102] and it is known to have has suppressive effects on miRNA pathway [103]. In cnidarians, multiple components of the RNAi machinery and a wide miRNA repertoire have been characterized in several species including *Nematostella* [104], *Hydra* [105] and the coral *Stylophora pistillata* [106]. Interestingly, WBD-resistant corals exhibited constitutive higher levels of another component of RISC, the enzyme argonaute (Chapter 3), indicating that RNA-based mechanism of immune regulation play a crucial role in the ability of staghorn corals to fight WBD infection.

*Early stage response to WBD*
The main differences between early and late response were represented by increased melanin synthesis, NF-kB signaling downstream TLR pathway, antiviral activity and expression of HSPs in the disease grafts. Increased melanin synthesis activity, one of the best-characterized coral immune pathways, was indicated by up-regulation of the enzyme tyrosinase during early response to WBD. In hard and soft corals, melanization is induced upon fungal infection [107], tissue damage [108] and pathogen exposure [109]. Three melanin synthesis pathways have been detected in corals - the mono-phenoloxidase, tyrosinase-type and laccase-type pathways [110]. In Caribbean corals, the tyrosinase pathway exhibits the highest activity during bleaching and response to disease infections, suggesting it is involved in the production of cytotoxic compounds, melanization and phagocytosis like in insects [110]. One possible explanation for the increased melanin synthesis during the early, but not the late stages of the response to WBD is energy allocation. Immune responses are costly and therefore a sustained infection may result in resource depletion affecting the immune ability of the colony. Additionally, melanin-synthesis activity is known to be highly variable among coral taxa, with Acroporidae exhibiting the lowest activity [111].

Early response to WBD was characterized by DE of several TLR and NF-kB signaling mediators in corals exposed to disease grafts. Multiple mediators of TLR pathways in corals have been identified [9], however, very limited data are available on their direct role in coral immunity. Transcriptional changes in both activator and inhibitors of LPS-mediated activation of TLR pathway and NF-KB signaling described here suggest that TLR pathway in corals may have an immune function similar to other invertebrates. In particular, the up-regulation of TIR-domain containing protein TIR1 indicates a direct role for TIR proteins in defense against pathogen infection. Interestingly, differential expression of TIR1 upon immune challenge has been previously described in A. millepora, but in that study TIR1 was down-regulated in corals exposed to the bacterial PAMP muramyl dipeptide (MDP) [8]. Activators of NF-kB signaling during early response to WBD included the nuclear factor akirin-2 that acts synergistically with NF-kB upon bacterial challenge to induce production of pro-inflammatory cytokines [112], TAB1, that activates NF-kB by interacting with the adaptor protein TRAF6 downstream myd88, and CaMKIV that acts in the anti-apoptotic branch of the LPS-mediated TLR4 signaling pathway by regulating the temporal expression of pro-apoptotic and anti-apoptotic members of the Bcl-2 family [113]. Inhibitors were represented by TRAF4 and TNIP1, which inhibit TLR
signaling via a variety of mechanisms. TRAF4 acts via direct association with the adaptor proteins TRAF6 and Toll-IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF), by interaction with p47 (phox), a component of the ROS-producing enzyme NADPH oxidase, and also with NLR1 and 2 to inhibit NF-kB [114]. TNIP1 inhibits NF-kB activation induced by TLR4 and IFN upon LPS challenge and act downstream TRAF6 [115].

Early response differed from late response also for the up-regulation of the NOD-like receptor NLRC3. NLRs are PRRs activated upon viral infection and stimulate production of type-I interferons and pro-inflammatory cytokines. Unlike the other NLRs, NLRC3 is a negative regulator of interferonmediate immune response and its expression has been shown to decrease after viral infection [116]. In macrophages, NLRC3 is a negative regulator of the response to LPS by inhibiting MyD88-mediated activation of NF-kB and by interacting with TRAF6, possibly in order to protect cells from the cytotoxic effects produced by uncontrolled inflammatory response caused by LPS exposure [78].

Another hallmark of early stage response to WBD was the DE of several stress-induced proteins including HSPs. Their expression during early but not late stage response to WBD was not surprising, as HSPs are rapidly induced in response to high temperature and other environmental stressors, representing ubiquitous mediators of an organism’s response to rapid changes [117]. In corals, the expression of HSPs is known to increase during heat stress [118], however, down-regulation has been associated to increased thermal tolerance in A. hiacynthus [7] and to resistance to WBD in A. cervicornis (Chapter 3). The increased expression of four out of eight HSPs in is consistent with the up-regulation of other stress-response genes, including hypoxia inducible factor 1 alpha, while the down-regulation of the other four transcripts may be related to their complex role as regulators of a variety of cellular processes including stress response, apoptosis, and immunity during extreme conditions.

Conclusions

Our RNAseq analyses indicate that allore cognition does not produce dramatic changes in gene expression in staghorn corals and is characterized by increased expression of genes involved in production of toxins, nematocysts formation and general stress response. In contrast, WBD-infected staghorn corals display a strong immune response. Expression profiles associated to short-term experimental exposure to disease confirm that immune response against WBD in
staghorn corals includes pathogen recognition, ROS production and synthesis of eicosanoids. In addition, increased production of eicosanoids by corals infected with WBD, but not by corals exposed to allogeneic tissue grafts contradicts recent studies that suggest a role for prostanoids as mediators of mechanical injury and general stress, and indicates instead their key role to fight disease infections. The primary mediators of early response to WBD infection suggests that the first line of defense against the WBD pathogen involves melanin synthesis via tyrosinase-type pathway, TLR –mediated NF-kB signaling and heat shock proteins.
Tables

Table 4.1: Differences in gene expression associated to allore cognition and to WBD. Number of total differentially expressed (DE) transcripts (adj p < 0.05) and DE annotated transcripts (e-val < 10^{-5}) in healthy grafts (allore cognition), in disease grafts (early immune response) and in environmental WBD-infected samples (late response). * re-analysis of the dataset generated from Libro et al, 2013 (Chapter 2).

<table>
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<tr>
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<th>%</th>
<th>Annotated</th>
<th>Total</th>
<th>Up</th>
<th>%</th>
<th>Down</th>
<th>%</th>
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<td>14</td>
<td>27%</td>
<td>38</td>
<td>26</td>
<td>68%</td>
<td>12</td>
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<tr>
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<td>289</td>
<td>10%</td>
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<td>1,266</td>
<td>88%</td>
<td>176</td>
<td>12%</td>
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Table 4.2: DE immune related transcripts involved in allore cognition, early and late response to WBD. Differential expression analysis results for selected immune-related transcripts exhibiting differences in expression due to WBD infection in the field (reanalysis of the dataset from Chapter 2) or to exposure treatment (healthy and disease grafts). Expression levels (given as log2-transformed fold differences) and adj p-values are reported. General response mediators are transcripts that are DE in all three comparisons. Mediators of allore cognition are DE transcripts in graft-exposed corals. Response to WBD includes transcripts that are DE in both disease grafts and in WBD field samples, early response include DE transcripts in disease grafts only.

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<th>Disease graft vs Control</th>
<th>Disease vs Healthy (field)</th>
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<td>adj p-val</td>
<td>log2(FC)</td>
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### Table 4.3: Abbreviations. List of abbreviations used in this chapter.

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<tr>
<td>TXNRD1</td>
<td>Thioroedoxin reductase</td>
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<tr>
<td>TLR</td>
<td>Toll-Like receptor</td>
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<tr>
<td>MRC1</td>
<td>Macrophage mannose receptor 1</td>
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<td>MRC2</td>
<td>C-type mannose receptor 2</td>
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<td>CPAMD8</td>
<td>C3 and PZP-like alpha-2-macroglobulin domain-containing protein 8</td>
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<td>C1QB</td>
<td>Complement component 1 Q subcomponent-binding protein</td>
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<td>MPEG1</td>
<td>Macrophage-expressed gene 1 protein</td>
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<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<tr>
<td>CXCR-7</td>
<td>C-X-C chemokine receptor type 7</td>
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<td>LITAF</td>
<td>LPS-induced tumor necrosis factor-alpha factor homolog</td>
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<td>Myeloid differentiation primary response protein</td>
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<td>UspA</td>
<td>Universal stress protein A-like protein</td>
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<td>Heat shock protein</td>
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<td>Heat shock 70 kDa protein</td>
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<td>Fibroblast growth factor</td>
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<td>Fibroblast growth factor receptor</td>
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<td>Phospholipase A</td>
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<td>Prostaglandin E synthase 2</td>
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<tr>
<td>H-PGDS</td>
<td>Hematopoietic prostaglandin D synthase</td>
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<td>AOSL</td>
<td>Allene oxide synthase-lipoxygenase</td>
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<tr>
<td>15-PGDH</td>
<td>15-hydroxyprostaglandin dehydrogenase [NAD(+)]</td>
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Figures

Figure 4.1: *In situ transmission experiment*. Replicate fragments of resistant and susceptible corals were exposed to allogeneic tissue and to active WBD using healthy grafts (a) and WBD grafts (b) and placed in common gardens for three days (c).
Figure 4.2: Venn diagram representing the overlap between allore cognition, early, and late response to WBD. Circle A represents the number of annotated DE transcripts in WBD grafts (disease + allore cognition, n = 2,691); circle B represents DE transcripts in WBD-infected corals in the field (n = 1,442) and circle C represents DE transcripts in healthy grafts (allore cognition, n = 38). The overlap between the three circles represents transcripts (n = 7) that were DE in all comparisons (i.e. WBD-infected samples collected from the field, disease grafts and healthy grafts). The overlap between A and B (but not C) represents the 656 (19% of all annotated DE transcripts) shared mediators between early and late response to WBD that were DE in WBD-infected environmental samples and WBD grafts. Mediators of allore cognition are represented by 28 DE transcripts in healthy and diseased grafts (1%), i.e. the overlap between A and C. Mediators of late response to WBD were represented by 779 DE transcripts in B (23%) while early response mediators were represented by 1,904 DE transcripts (56%) in A.
Figure 4.3: Summary of the immune response to WBD in *A. cervicornis*. Transcripts highlighted in red represent up regulated transcripts, transcripts highlighted in blue represent down-regulated transcripts. Underlined text indicates DE only during early response.* indicate DE only during late response. Dotted lines indicate unknown pathways.
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Species. TIRLoT, editor.

TIRLoT, editor.


potential pathogens of white band disease in the endangered Caribbean coral Acropora


CHAPTER 5
General discussion and future research

General discussion

In this dissertation, I show that the staghorn coral *A. cervicornis* is able to fight WBD infections by mounting a strong immune response with evidence of pathogen recognition, apoptosis, phagocytosis as well as synthesis of eicosanoids (Chapter 2 and 4). I also identify the genetic basis of the differential ability of staghorn corals to respond to disease infection and demonstrate that 1) there are genetic differences between resistant and susceptible corals that are independent on pathogen exposure and 2) disease resistance does not rely on the innate immune pathways involved in the response against WBD infection (Chapter 3). Moreover, I show that the genetic underpinnings of allore cognition and pathogen detection in staghorn corals differ substantially, revealing that the response to WBD relies on complex disease-induced immune defenses rather than on general mechanisms of stress response (Chapter 4).

By experimentally exposing resistant and susceptible staghorn corals to healthy and WBD-infected grafts I was able to characterize expression changes associated to resistance. Intriguingly, I found that the transcriptional differences between resistant and susceptible corals are not influenced by immune stimulation and are indicative of a constitutive basis for WBD-resistance. My results show that resistant staghorn corals avoids disease infection not by mounting a stronger immune response against WBD, but rather via different baseline expression levels in a very small gene repertoire compared to susceptible corals. In particular, resistant corals exhibited higher expression levels of mediators of RNA interference-mediated post-transcriptional regulation and antiviral defense- indicating a novel immune role for RNAi in corals- and down-regulation of heat shock proteins.

Until recently, the only known mechanism for acquiring thermal tolerance in corals was the ability to replace *Symbiodinium* clade C with the thermally resistant clade D [1,2]. The discovery of low expression levels of HSPs in thermally resilient corals in the pacific *Acropora* corals [3] indicates that adaptations to stress and environmental disturbances can occur in the coral host as well. The data presented here support this hypothesis and further expand it, pointing
toward a link between constitutive higher thermal stress tolerance and disease resistance. Constitutive high levels of heat stress proteins could confer thermal tolerance to corals, making them less prone to disease infections.

Overall, these results indicate that the immune system of the coral host plays a fundamental role in detecting and eliminating pathogens, and provide new evidence for an immune-related function for several genes, contributing to expand the existing knowledge on the immune repertoire of cnidarians. In particular, the fundamental role of eicosanoids during both early and late immunity but not during allore cognition in staghorn corals diverges from recent findings that describe eicosanoids synthesis via allene oxide synthase- lipoxygenase (AOS-LOX) as a mediator of stress in soft corals [4,5]. Similarly, prophenoloxidase activity represents the main immune defense during pathogen recognition and mechanical injury in several soft and scleractinian corals [6-8] but appears to have a marginal role during the response to WBD. Taken together, these findings remark the high intra and interspecific variation of coral immune responses [9] and emphasize the importance of comparative and functional studies [10].

Due to its role as foundation species, A. cerviconis represents a proxy for predicting the effects of environmental change on Caribbean reef-coral. Therefore, understanding the mechanisms underlying staghorn coral’s immunity and resistance is critical for protecting and enhancing the survival of existing coral populations and thus provide valuable information to the development of conservation strategies.

Future research directions

Disease resistance and thermal tolerance
In order to elucidate the mechanisms of resistance and whether they are linked to increased thermal tolerance, the thermal resilience of resistant and susceptible corals needs to be assessed. Additionally, transcriptome surveys combined with transmission experiments can determine whether low baseline HSPs expression is as a general proxy to identify resistant staghorn coral colonies in natural populations across the Caribbean.

DNA-based component of resistance: variations at the DNA level may also underlie the differential ability of staghorn corals to avoid WBD infection. Genome-wide association studies
could be conducted to assess presence of genetic polymorphisms linked to disease resistance and susceptibility. A further hypothesis to test is whether resistance is associated to introgression of *A. palmata* loci into *A. cervicornis*. Asymmetrical introgression between *A. palmata* and *A. cervicornis* occurs through backcrossing of *A. cervicornis* with hybrid *A. prolifera* and high frequencies of introgressed genes have been found in staghorn coral populations in Panama.

*Role of eicosanoids in staghorn corals*

Functional genomic studies need to characterize molecular mechanisms behind eicosanoids biosynthesis and immune activity in staghorn corals. Several studies indicate that soft corals possess multiple eicosanoid biosynthesis pathways [11-15] but, besides this dissertation, there is no data available on eicosanoids activity in scleractinian corals.

*Recognition specificity*

The data presented here indicate differences between pathogen and non-self recognition as well as between early and late response to WBD in staghorn corals. Exposure of corals to different pathogen elicitors (i.e. commercial pathogen-associated molecular patterns such as lipopolysaccharide, peptidoglycan and double strand RNA) can be useful to assess the specificity of pathogen recognition in staghorn corals and to identify transcriptional changes associated to pathogen recognition without the confounding effects of disease infection and tissue degradation.
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