EFFECTS OF ENVIRONMENTAL STRESSORS ON THE IMMUNE RESPONSE OF THE CARIBBEAN SEA FAN, *GORGONIA VENTALINA*

by

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Abstract

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Coral reef ecosystems are some of the most diverse on the planet and provide many important services such as fishery stock, tourism, and protection against storms. Despite this, coral reef ecosystems are on a steady rate of decline due largely to increases in disease prevalence. Coral diseases are associated with environmental stressors such as rising sea surface temperatures and acidified water conditions that have resulted from artificial increase atmospheric CO_2 levels by anthropogenic activities. It is hypothesized that the presence of environmental stressors is compromising coral immunity while also exacerbating pathogen virulence and contributing to the development of disease.

The Caribbean sea fan has been one of the many species greatly affected by disease outbreaks. Two of the most prevalent diseases include aspergillosis caused by fungal pathogen, *Aspergillus sydowii* and a yet, unnamed disease caused by an *Aplanochytrium* sp. protist pathogen (order: Labyrinthulomycetes). Using the sea fan and its associated pathogens as a coral disease model, this dissertation addresses several fundamental hypotheses regarding the effects of climate change on coral disease ecology. H_1 - Elevated temperatures can lower the sea fan immune responses, such as production of inhibitors of pathogen proteases while exacerbating putative pathogen virulence factors such as protease production. H_2 - Multiple stressors can have a synergistic adverse effect on the sea fan immune response versus the presence of only one stressor. H_3 - Responses of the sea fan can vary depending on the type of pathogen and can this can be additionally affected by environmental stress. Several key experiments were conducted using the sea fan to address these hypotheses.

In chapter 2, a new putative virulence factor (the production of proteases) in the sea fan fungal pathogen, *Aspergillus sydowii* and immune defense of the sea fan (protease inhibitor production) was

identified (Mann et al. 2014). Inhibitors of the sea fan were seen to directly interact with proteases extracted from *A. sydowii*. When grown at elevated temperatures (30°C), protease activity of *A. sydowii* was significantly higher than at ambient temperatures (25°C). Temperature stress did not induce a change in protease inhibitor activity, but in healthy *G. ventalina* colonies inhibitor activity against proteases were higher than in diseased individuals.

Chapter 3 utilized a multi-factorial experimental design to test the effects of multiple stressors (elevated temperature and acidification) and to exposure of the *Aplanochytrium* pathogen on the sea fan immune system. This experiment used a large suite of biochemical immunoassays to measure antimicrobial, anti-pathogenic, and antioxidant immune mechanisms. Together, the three factors (temperature, pH, and pathogen-exposure) had interactive and immuno-suppressive effect on the overall immune response. Although pH had effect on the sea fan immune response, this was only evident when combined with elevated temperature or pathogen-exposure. Additionally, an immune-suppressive effect on the sea fan's immune response was observed from the *Aplanochytrium* pathogen independent of the environmental stressors. The immuno-suppressive effect of a coral pathogen has yet to be fully documented.

Effects of thermal stress were further examined in chapter 4, where sea fans were exposed to a period of thermal stress prior to exposure to the two pathogens, *A. sydowii* and the *Aplanochytrium*. This study utilized a biochemical immunoassay suite as well as novel gene expression analysis to measure immune mechanisms such as immune signaling, antimicrobial, anti-pathogenic, and antioxidant activity. The exposure to preceding thermal stress adversely affected the overall capability of sea fans to respond to the pathogens and this was most evident in the immune signaling and antimicrobial defense mechanisms. Moreover, this chapter illustrates the variation in response to the two pathogens where response to *A. sydowii* was slightly greater than to the *Aplanochytrium* pathogen.

The combined works of this dissertation effectively demonstrate how compounding factors of climate change are adversely affecting coral immunity and leading to the development of diseases. Moreover, these studies incorporate several novel techniques and approaches towards the study of coral immunity that can be applicable to managers and conservationists to use towards better management of coral reef ecosystems around the Caribbean.

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Chapter 1

Introduction

Coral reef ecosystems

Coral reefs are among the most diverse ecosystems on the planet and provide several important ecosystem services. The creation of fisheries is perhaps one of the most important services provided by coral reefs which greatly influences marine and terrestrial biota, including humans (McClanahan et al. 2011). Coral reefs also serve as protection against hurricanes and storm damage to coastal areas, economical value through ecotourism, and an abundant source for novel pharmaceuticals (Barbier 2012). These ecosystems are found among the tropical regions of the globe and consist of large varieties of invertebrates, vertebrates, algae, and microbial communities (Done et al. 1996). The central reason for the high biodiversity of coral reef ecosystems is the existence of central ecosystem engineers: corals (Alper 1998). Corals are responsible for the large structures found on reefs which provide important habitat and breeding grounds for other reef organisms. In fact, regions that contain higher percentage of coral cover also have considerably greater overall diversity and healthy ecosystem states (Jones et al. 2004).

There are two major groups of corals belonging to the phylum Cnidaria: hard corals (scleractinians) and octocorals (gorgonians and true soft corals). Both groups live as colonial animals, comprised of many individual animals known as polyps. Primarily, scleractinian corals are responsible for the production of the reef structure. The polyps of scleractinians secrete large amounts of calcium carbonate to form a structural skeleton for the colonies. Octocorals also produce small amounts of calcium carbonate in the form of sclerites that are imbedded within dermal tissue and provide rigor to the colony; however, they do not create the large structures like scleractinian corals (Bayer 1973). In gorgonian corals, the colony structure is made up of both sclerites and hard, proteinaceous material known as gorgonin (Ehrlich 2010). Despite the creation of larger structures on reefs, octocorals make up a significant percentage of biomass on coral reefs and contribute immensely to the functionality of reef ecosystems by providing shelter, food sources, and nurseries for other reef inhabitants (Sánchez et al. 2003).

Much of the growth in corals is owed to an essential symbiotic relationship that exists between the coral host and an endosymbiotic, unicellular algae from the genus *Symbiodinium*. Corals are heterotrophic and are capable of filter feeding from the water column; however, *Symbiodinium* provide the majority of nutrients to the coral host through photosynthetic byproduct and aid in the secretion of calcium carbonate skeleton and the growth of the coral (Constantz 1986). The creation of reef ecosystems by corals would likely not exist without this important relationship.

Coral decline, disease, and climate change

In recent decades, coral reef ecosystems have been steadily declining. Globally, the amount of coral cover has decreased significantly and in some regions such as the Caribbean, cover has been reduced as much as 80% (Gardner et al. 2003; Alvarez-Filip et al. 2011). This decline of coral populations is due largely to increasing disease outbreaks (Altizer et al. 2013). As many as 22 diseases exist in the Caribbean and newer disease/syndromes are being identified each year (Harvell et al. 2007). The range of pathogens is large and includes several species of bacteria, fungi, and protists (Rosenberg et al. 2007). Several species originating from pollution and wastewater sources, such as *Serratia marcescens*, are discernibly infectious agents of disease (Patterson et al. 2002). However, many pathogens naturally existing in the water column can also cause infectious disease. Several *Vibrio* spp., common to marine environments, can be transmitted from one coral to another in the case of Yellow Band Disease (Cervino et al. 2008). Many microbes ubiquitous to the marine environment also exist as opportunistic pathogens where they can be found in and around healthy corals (Burge et al. 2013a). Increased environmental stressors are thought to be main drivers of coral diseases by altering or compromising the immune function of corals which lead the way for infectious and opportunistic pathogens to establish infection (Altizer et al. 2013).

Coral immunity

In invertebrates, innate immunity is the only line of defense against infection and operates in three broader processes: (1) recognition – often through the presence of toll-like receptors or lectins, (2) signaling pathways – proteolytic or phosphorylating cascades such as the melanin synthesis pathway or complementation, and (3) effector responses – including antimicrobial and opsonization proteins or other neutralizing enzymes such as antioxidants (Palmer and Traylor-Knowles 2012). In corals, several of these

response mechanisms have been confirmed and shown to play an important role in the response to both infection of pathogens and non-pathogenic, foreign invaders.

Few recognition processes, such as toll-like receptors, have been specifically identified in corals, but several exist in other members of the Cnidarian phylum (Dunn 2009). The melanin synthesis pathway, a signaling pathway and likely the result of an immunoreceptor, is one of the most studied pathways in coral immunology. The deposit of melanin in both corals as well as other invertebrates functions as physical-chemical barrier against foreign invaders/material, phagocytosis, and wound healing caused by mechanical damage (Cerenius et al. 2008). In the melanin synthesis pathway, the cascade is regulated by serine proteolytic enzymes (proteases) which are activated when specialized receptors along the host cellular membrane bind to pathogen-associated molecular patterns (PAMPs). These proteases cleave inactive pro-phenoloxidases into active phenoloxidases which then oxidize phenol substrates to create melanin and other by-products such as bursts of reactive oxygen species (ROS) (Cerenius and Soderhall 2004).

Oxidative bursts (of ROS), which can result from the melanin synthesis cascade or increased respiratory activity, are additionally important for invertebrates in protection against pathogen invasion (Philipp et al. 2012). Oxidative bursts aim to damage pathogen cellular membranes and slow or inhibit growth. However, these ROS also act against the host; therefore, it is essential for the host to minimize self damage through the use of antioxidant effectors. Antioxidants in corals have been identified for several coral species and contain an array of antioxidant types such as fluorescent proteins (Palmer et al. 2009), peroxidase (Mydlarz and Harvell 2007), and superoxide dismutase (Couch et al. 2008).

Other effector responses in corals include a variety of antimicrobial molecules. Corals, especially octocorals, have been well-known for being an abundant resource for antifungal and antibacterial compounds (Jensen et al. 1996; Kelman 2004). In general, this broad class of molecules aims to disrupt growth processes or virulence factors of the invading pathogen.

Sea fan disease ecology

In the Caribbean sea fan, *Gorgonia ventalina*, disease has been particularly widespread. Additionally, *G. ventalina* is vulnerable to multiple types of disease. Sea fan aspergillosis, caused by marine fungus *Aspergillus sydowii*, is one of the more recognized diseases of the sea fan (Geiser et al.

1998). Aspergillosis can be identified by the presence of dark purple lesions and necrotic tissue within the lesion. Histological analysis shows that when infected, the fungal pathogen commonly resides inside the tissue, along the gorgonin skeletal axis of the sea fan (Petes et al. 2003). Between 1997-2003, sea fan populations of the Florida Keys were nearly decimated by outbreaks of aspergillosis, where ~50% of the population was lost (Kim and Harvell 2004). Since this primary epidemic of aspergillosis, disease prevalence has recovered to about 2% in the Florida Keys. Although there are signs of recovery in some areas such as Florida, sea fan aspergillosis still persists at higher prevalence in several other areas of the Caribbean such as Puerto Rico (Flynn and Weil 2009a).

In addition to aspergillosis, outbreaks of a newly characterized unnamed disease, have become more prevalent among sea fan populations around the Caribbean within the past decade (Burge et al. 2012). This disease is identified histologically by having pathogen growth/presence along the gorgonin axis, destruction of the gorgonin axis, and polyp degradation. This disease can be caused by a protist from either the *Aplanochytrium* or *Thraustochytrid* genera of the Labyrinthulomycetes order depending on the region (Burge et al. 2012).

Many emerging diseases in marine ecosystems appear as opportunistic, particularly in corals (Harvell et al. 1999; Burge et al. 2013a). Both aforementioned sea fan pathogens (*A. sydowii* and the Labyrinthulomycetes), are known to be opportunists in other systems, both marine/aquatic and terrestrial. Aspergillosis is a common opportunistic infection among other disease systems include humans, birds, insects, and plants (Van Waeyenberghe et al. 2011). Likewise, opportunistic infections of Labyrinthulomycetes have been discovered and discussed throughout other marine disease systems including sea grasses, clams, and oysters (Ragan et al. 2000; Trevathan-Tackett et al. 2013). The presence of *A. sydowii* and the Labyrinthulomycetes are likely opportunistic pathogens to the sea fan. Several pieces of sea fan literature support this, confirming the presence of *Aspergillus* spp. and Labyrinthulomycetes can sometimes be present when there are no outwards signs of disease. In addition, both pathogens can exist together as a co-infection of the sea fan (Petes et al. 2003) and this may suggest that in the least, one pathogen may serve as an primary infecting pathogen while the other is an opportunist. However, the examination of the mechanisms for co-infections in the sea fan has not been thoroughly completed.

Like other corals of the Caribbean, diseases of the sea fan are thought to be associated with factors of climate change such as rising sea surface temperatures and ocean acidification. Changes to the environment can have significant impact on the dynamics and relationship between a host and its' associated microbes - which could well lead to opportunistic infections. With two distinct disease systems (aspergillosis and the Labyrinthulomycetes infection), readily available, isolates of both pathogens in culture, a large suite of biochemical immunoassays developed in corals, as well as several sequenced sea fan transcriptomes, the sea fan disease model provides exceptional opportunity for investigating how factors of climate change are impacting the dynamics between the corals and associate pathogens.

Dissertation aims

Overarching, this project aims to use the sea fan as a coral disease model to investigate several important questions regarding the relationship between coral disease and climate change. Specifically, I emphasize how elevated temperature and acidified water conditions affect the immunological response of the sea fan and the physiology of associated pathogens, *Aspergillus sydowii* and the *Aplanochytrium*.

Chapter 2 Aims

- Aim 1: To confirm the presence of proteases and protease inhibitors in *Aspergillus sydowii* and the sea fan (*Gorgonia ventalina*, respectively.
- Aim 2: To examine the effect of elevated temperature on the activity of protease and protease inhibitor activity of *A. sydowii* and the sea fan, respectively.

Chapter 3 Aims

- Aim 1: To investigate the synergistic or combinative effects of pH, temperature, and pathogen exposure on the immune response of the sea fan to an *Aplanochytrium* sp. pathogen.

Chapter 4 Aims

- Aim 1: To examine the immune response of the sea fan to pathogen-exposure after a period of preceding thermal stress.
- Aim 2: Determine differences in the immune response of the sea fan to each pathogen, *A. sydowii* and the *Aplanochytrium*

A shift in host-pathogen dynamics can often lead to development of diseases. Therefore, these aims collectively address several questions regarding host-pathogen dynamics in corals and further, how environmental stressors can shape this relationship.

Chapter 2

Interplay between proteases and protease inhibitors in the sea fan-*Aspergillus* pathosystem

Introduction

The number of infectious diseases and outbreaks has been increasing across marine and terrestrial ecosystems (Altizer et al. 2013). Host-pathogen interactions and disease development are key factors to understand the dynamics of epizootics (Harvell 2004). In order to prevent disease, a host must be able to mount a successful immune response and success in evading disease is determined by both the pathogen virulence and host immunocompetence.

Pathogen-produced proteases aid in the colonization of a host (Lee et al. 2002; Monod et al. 2002) by benefiting the pathogen in two ways: nutrient acquisition through host tissue breakdown and/or interfering or disrupting host immune function (Hoge et al. 2010). Therefore many hosts produce protease inhibitors as a response against pathogen proteases. In several plant and invertebrate species, protease inhibitors are key components of the innate immune system and can act directly against proteases produced by microbial pathogens, insects and other pests (Jongsma and Bolter 1997; Faisal et al. 1998; Donpudsa et al. 2009)

Fungal pathogens, particularly *Aspergillus* spp., are prevalent in many disease systems including plants, arthropods, birds, etc. (Kulshrestha and Pathak 1997; Asis et al. 2009; Rahim et al. 2013). Copious levels of proteases make pathogenic *Aspergillus* spp. especially virulent (Dunaevskii et al. 2006). In the past few decades, aspergillosis of the Caribbean sea fan coral, *Gorgonia ventalina* (Linnaeus: Geiser et al. (1998), has decimated sea fan populations throughout the Caribbean (Bruno et al. 2011). Aspergillosis infection can cause tissue necrosis that can spread through the entire coral colony leading to mortality. In some locations over 50% of the sea fan populations have been affected (Kim and Harvell 2004); and although there are signs of recovery in some areas, sea fan aspergillosis still persists in several Caribbean reefs (Flynn and Weil 2009a).

Increase of disease and consequent population decline has not only been observed in sea fans, but other corals worldwide (Ruiz-Morenol et al. 2012). In general, coral diseases and their outbreaks have been linked to changing environmental conditions such as elevated sea surface temperatures associated with global climate change (Hoegh-Guldberg and Bruno 2010). Chronic exposure to elevated

temperatures for extended periods of time has adverse effects on the immunity of corals likely increasing disease susceptibility (Mydlarz et al. 2010).

The sea fan-*Aspergillus* pathosystem serves as an excellent model for investigating yet unanswered questions on the effects of environment on pathogen virulence and host immunity. In this study we investigate how environmental stressors (e.g. elevated temperature) affect the relationship between the putative pathogen *A. sydowii* and the sea fan. Specifically, protease activity of *Aspergillus sydowii* was measured and the antagonistic interactions between protease inhibitor activity of the sea fan and *A. sydowii*-derived and commercial proteases.

Methods

Aspergillus sydowii culturing and maintenance

Five strains of *Aspergillus sydowii* from various sites and origins were used in this project: three strains were isolated from sea fans from reefs in the Florida Keys, Florida, San Salvador, Bahamas, and Saba, Netherland Antilles, one from a mangrove (NRRL250) and one from a human (NRRL254). Stock spore solutions of the sea fan *A. sydowii* strains were obtained from the Harvell lab at Cornell University while the other two spore stocks were acquired from the USDA Agricultural Research Service Culture Collection. Delineation between the sea fan-isolated strains of *A. sydowii* was first determined by metabolic profiles in Alker et al. (2001). Phylogeny was further established between strains of multiple origins (sea fan, mangrove, and human) in Rypien et al. (2008) and Rypien and Andras (2008).

Cultures of *A. sydowii* from the spore collections were grown on peptone-yeast-glucose agar (PYG: 1.25 g peptone, 1.25 g yeast, 3.0 g glucose, 30 g Instant Ocean salt mix L^{-1}). The human *A. sydowii* strain was grown with no added Instant Ocean salt mix. Spores were harvested from germinated cultures on PYG agar and strained through a 40 µm cell strainer (BD Falcon, San Jose, California, USA) and stored in quarter-strength PYG until use. Spore concentrations were quantified using a Bright-line hemocytometer before use in the assays described below (Sigma-Aldrich, St. Louis, Missouri, USA).

Aspergillus sydowii protease assays

Protease activity was quantified using two culture methods: growth on casein-enriched PYG agar plates (3% casein) and growth in PYG broth media (0.1% peptone, 0.1% yeast, 3% glucose, 3% Instant Ocean salt mix L^{-1}). In the first protease assay, the sea fan *A. sydowii* strains were inoculated on the

center of agar plates (3 per strain) from approximately 1 x 10^3 spores and incubated either at 25°C or 30°C for 48 hours. The radial colony growth and the protease activity (the zone of clearing, indicating cleavage of the casein protein) were quantified with ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Total protease activity was measured by calculating the ratio of radial colony growth to the radial zone of clearing around colony.

For the second protease assay, spores were grown from approximately 5 x 10^7 spores per 100 mL of broth media in vented Erlenmeyer flasks (VWR, Radnor, Pennsylvania, USA). Three flask replicates per strain (2 sea fan (San Salvador and Saba), mangrove, and human-isolated strainse) were incubated at either 25°C, 28°C, 30°C, or 32°C for 48 hours, and shaken at 100 rpm. After incubation, hyphal bodies were separated using 40 µm cell strainers (BD Biosciences, San Jose, California, USA) and centrifuged at 2880 x *g* using an Eppendorf 5810R (Eppendorf, Hauppauge, New York, USA) for 20 min at room temperature. Proteases are typically produced with high hyphal body mass and minimal sporulation (Krull et al. 2010). Lack of spore production in the cultures was confirmed with visual inspection. 1 mL of the supernatant was collected as extracellular proteases. Both the hyphal bodies and supernatant samples were flash frozen with liquid N_2 and stored at -80°C until processing for protein extractions.

Crude protein extracts from fungal hyphal bodies were prepared by grinding the samples in liquid N_2 with a mortar and pestle. Proteins including the proteases were extracted from the powder using 100 mM sodium phosphate buffer solution (PBS), pH 7.8 for approximately 45 min on ice. Protein extract was recovered after centrifugation at 2205 x *g* at 4°C for 10 min into 1.5 ml tubes. Total protein content was quantified using the Red660 protein assay (G Biosciences, St. Louis, Missouri, USA). Extracellular proteases were quantified from the media supernatant, while intracellular were quantified from protein extracts of the hyphal bodies.

Extracellular and intracellular fungal proteins were assayed for general serine protease activity in 96 well black microplates (Greiner Bio One, Monroe, North Carolina, USA), generally following the protocol of Twining (1984). 10 µL of sample extract was incubated with 20 µL 2.5% fluorescein isothiocyanate (FITC) casein substrate (Sigma Aldrich, St. Louis, MO, USA) dissolved in 20 mM PBS with 150 mM NaCl, pH 7.6 for 30 min at 37°C. The reaction was stopped with 10% trichloroacetic acid to

precipitate remaining proteins. Proteins were pelleted at 5590 x *g* (Baxter Scientific Products, Deerfield, Illinois, USA) and the fluorescence of the supernatant was observed with excitation at 485 nm and emission wavelength at 535 nm on a Synergy 2 Microplate Reader (Biotek Instruments, Winooski, Vermont, USA). Units (U) of protease in the intracellular and extracellular samples were quantified using a standard curve of 1000 U bovine trypsin (Sigma-Aldrich, St. Louis, MO, USA). Intracellular and extracellular protease activity was standardized to the total protein content of the extract to account for potential biomass differences between growth temperatures. PYG broth and extraction buffers were used as negative controls.

Gorgonia ventalina sample collections

Protease inhibitor activity against *Aspergillus sydowii* proteases in natural populations of *Gorgonia ventalina* was examined from ten healthy and ten diseased colonies collected from Looe Key Research Reef (24˚ 34.138N, 81˚ 22.905W), Florida Keys, USA using SCUBA equipment. The depth for these colonies ranged from 3 to 6 m (Florida Keys National Marine Sanctuary Collections Permit #2004-092). Samples consisted of approximately 15 cm^2 of tissue cut with scissors from each colony. Diseased colonies were sampled from the lesion site (as determined by purple coloration and lesion morphology – Mydlarz and Harvell 2007), and from a visually healthy area at least 10 cm away from the lesion site. All samples were immediately flash frozen in liquid N_2 and sent on dry ice to the University of Texas at Arlington (UTA).

To examine the effect of temperature on protease inhibitor activity in healthy sea fans (no sign of infection, necrosis, or other injury), we experimentally exposed twelve sea fan colonies to elevated temperatures. Using SCUBA, sea fans were collected from Media Luna reef (17˚ 56.091 N - 67˚ 02.577 W) in La Parguera, Puerto Rico. Samples (~20 cm^2) were brought back to the station and divided into 2 fragments (3x5 cm), one for temperature treatment and the other as control. Fragments for each treatment were evenly divided across 3 indoor tanks for a total of 6 tanks. All tanks were maintained as closed systems with 20% daily water changes and aerated using aquarium water pumps (TAAM Inc., USA). Artificial lighting with full-spectrum bulbs was set to a 12 hour day/night cycle and temperature was maintained with aquarium heaters (Hydor, Sacramento, California, USA). After a 2 day acclimation period, temperatures were increased over 2 hours to 30-32°C and held for fourteen days. Controls were held at

26-28°C. At the end of the experiment, no Aspergillosis lesions or otherwise were observed. Samples were immediately flash frozen in liquid N_2 and shipped on dry ice to UTA.

Crude protein extracts from sea fan fragments were prepared by grinding the entire sea fan sample (tissue and skeleton) in liquid N_2 with a mortar and pestle. Proteins including the proteases were extracted from the powder using 100 mM sodium phosphate buffer solution (PBS), pH 7.8 for approximately 45 min on ice. Protein extract was recovered after centrifugation at 2205 x *g* at 4°C for 10 min to remove cellular debris. Total protein content of the serum was quantified using the Red660 protein assay (G Biosciences, St. Louis, Missouri, USA).

Protease inhibitor assay

A protease inhibitor assay was developed to quantify activity of the sea fan protein extracts against commercial proteases and fungal-derived proteases from *A. sydowii*. The assay modifies the previously described protease activity assay by calculating decrease in proteolytic cleavage of a given protease. 10 µL of sea fan extract were incubated with either 30 µL of fungal extracellular protease extract (derived from the San Salvador sea fan strain, grown at 30°C, described above), 10 µL trypsin (0.1 mg mL^{-1}) (Sigma-Aldrich, St. Louis, MO, USA), or 10 µL α-chymotrypsin (1 mg mL^{-1}) (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature. The sea fan extract/protease mixture was then added to 40 µL 2.5% FITC-casein substrate (in 20 mM PBS, 150 mM NaCl, pH 7.6) and cleavage of fluorescently bound casein measured as described above. Protease inhibitor activity was calculated as a decrease in proteolytic cleavage by the coral extract-protease mixture compared to the protease alone. Activity was standardized to the total protein of the coral extract.

Controls for the assay included the protease, to determine the maximum protease activity in each assay, and sample blanks with coral protein extraction buffer alone and combined with PYG broth as control for the fungal protease. As reference, standard curves of commercial protease inhibitors (aprotinin and leupeptin, Sigma Aldrich, St. Louis, MO, USA) were analyzed to obtain a reference of activity for the coral protease inhibitors. A minimum of 0.6 mM aprotinin was required to inhibit trypsin and chymotrypsin 50-100% while trypsin was inhibited completely by 0.01 mM leupeptin. The extraction buffer, PYG broth and coral extracts did not affect or quench the fluorescence component of the FITC substrate by

themselves. The assay was also validated by testing heat-inactivated coral extracts to ensure the inhibitor activities were from a protein/enzyme source.

Data analysis

Homoscedasticity of all data sets was confirmed with the Brown-Forsythe test and normality was determined with the Shapiro-Wilk test. Any data sets that were non-normally distributed or of unequal variance were successfully transformed using the Box-Cox method. A two-way analysis of variance (ANOVA) of protease activity of *Aspergillus sydowii* was performed with temperature and strain as factors. A one-way ANOVA of sea fan protease inhibitor activity was performed for each type of protease inhibitor with either disease or temperature as an effect. In both cases, Tukey-Kramer post-hoc analyses were used to detect differences between factors/effects. Statistical analysis was performed using JMP 10.0 software (SAS, Cary, North Carolina).

Results

Aspergillus sydowii protease activity

All fungal strains showed measurable levels of protease activity as validated by independent culture methods (casein agar and broth), biochemical assays, or both. There were significant overall changes in protease activity for the three sea fan-isolated *A. sydowii* strains grown on casein agar (Two-way ANOVA, $F_{(2,5)}$=16.9230, P=0.003, Figure 2-1). There were also significant differences in protease activity between the three strains (Strain effect, ANOVA, $F_{(2,5)} = 230.2$, $P < 0.001$). Elevated temperature significantly increased extracellular protease activity for all three fungal strains (Temperature effect, ANOVA, $F_{(1,5)} = 221.8$, $P < 0.0001$). Sea fan fungal strains from San Salvador exhibited the highest overall protease activity at ambient (25°C) and elevated temperatures (30°C) while the Florida Keys strain had the lowest overall activity, with essentially no protease activity detectable at 25°C in this assay.

All *A. sydowii* strains had detectable amounts of intracellular and extracellular protease activity when grown in broth media. Both the strain identity and temperature had significant effect on intracellular protease activity (Two-way ANOVA, $F_{(9,15)} = 11.2725$ $P < 0.0001$, Figure 2-2). There were no significant differences in intracellular protease activity between the sea fan and mangrove-isolated strains but all were different from the human-isolated strain which had the lowest overall protease activity (Strain effect, ANOVA, $F_{(3,15)}$=53.80, P<.0001). Temperature effect was significant for intracellular protease activity in

the fungal strains from the two sea fan- isolated strains and the mangrove-isolated strains, but not in the human-isolated strain. Intracellular protease activity increased at 28-32°C and remained elevated at 30-32°C in the fungal strains from sea fans and mangrove, respectively (Temperature effect, ANOVA, $F_{(3,15)}$=67.50, P<.0001).

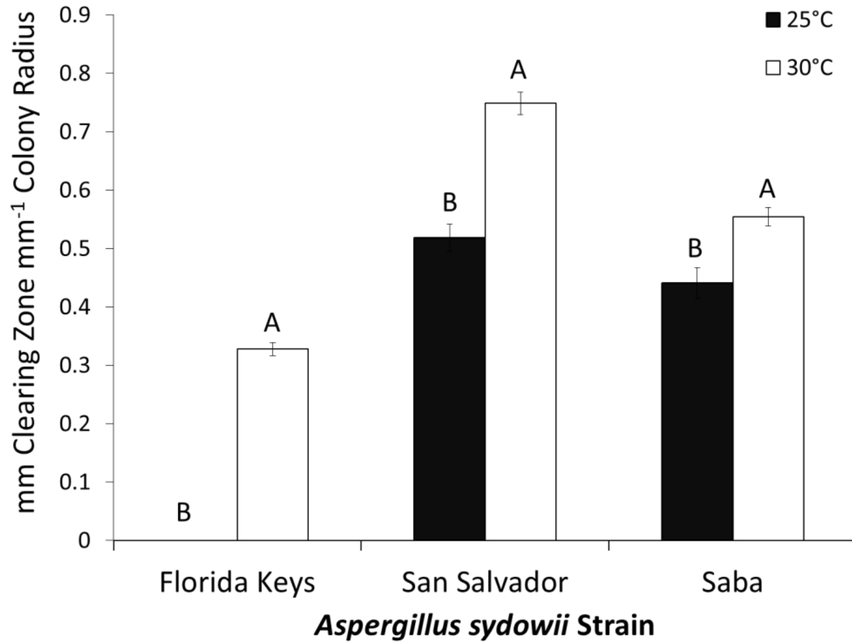

Figure 2-1 Protease activity of Aspergillus sydowii grown on casein agar

Mean (±SE) extracellular protease activity of Aspergillus sydowii sea fan strains grown on casein enriched agar (n=3). Letters indicate statistical differences under 25°C and 30°C at P<0.05 within strains.

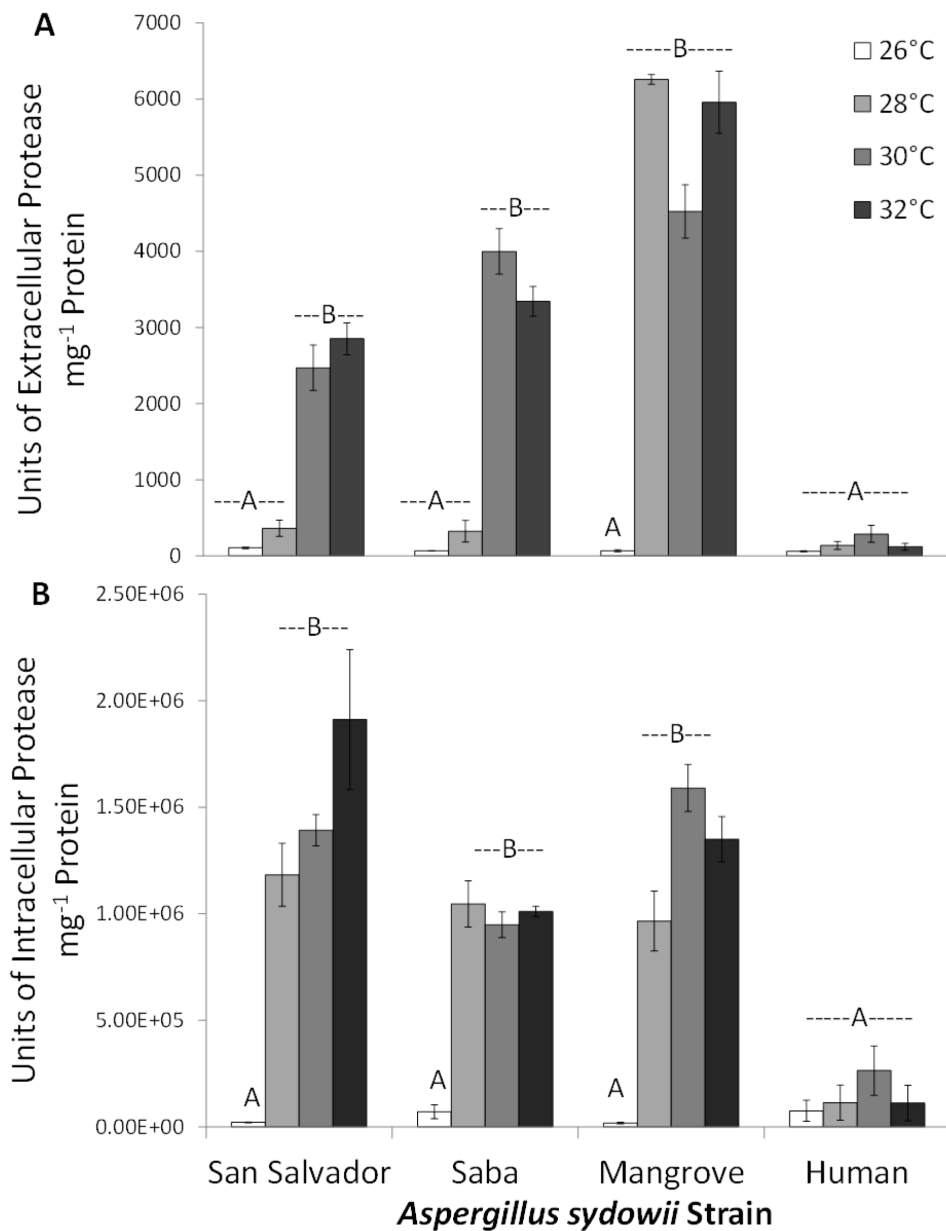

Figure 2-2 Protease activity of *Aspergillus sydowii* grown in PYG broth

Mean (±SE) intracellular protease activity mg^{-1} protein (A) and extracellular protease activity of *Aspergillus sydowii* strains from various sources (B). Letters indicate significant differences under different temperatures at $P<0.05$ within strains (n=3).

Similarly to the intracellular protease assay, the strain identity and temperature had significant effect on extracellular protease activity (Two-way ANOVA, $F_{(9,15)} = 9.37$, $P < 0.0001$, Figure 2-2). There was different protease activity among strains: the three sea fan-isolated strains had significantly higher protease activity than the human-isolated strain but not the mangrove-isolated strain (Strain effect, ANOVA, $F_{(3,15)}$=56.34, P<.0001). The effect of temperature was significant for extracellular protease activity in the *A. sydowii* strains isolated from sea fans and mangrove but not the human-isolated strain (Temperature effect, ANOVA, $F_{(3,15)}$=37.56, P<.0001). Specifically, extracellular protease activity was higher at 28-32°C for the mangrove-isolated strain and 30-32°C for the sea fan-isolated strains (San Salvador and Saba) compared to activity at 26°C.

Sea fan protease inhibitor activity

Sea fan extracts were able to inhibit the activity of all three proteases, fungal-derived, trypsin, and α-chymotrypsin. There was an overall effect of health condition on fungal-derived protease inhibition (ANOVA, $F_{(2)}$=4.0880, P = 0.0303), trypsin protease inhibition (ANOVA, $F_{(2)}$=5.1521, P=0.0130), and α-chymotrypsin protease inhibition (ANOVA, $F_{(2)}$=18.3048, P<0.001). Fungal protease inhibition was lower in lesion tissue of the diseased colony than both healthy tissue of diseased and healthy colonies (Figure 2-3A). Trypsin inhibition was systemically lower in diseased colonies (both lesion and healthy tissue) than the healthy control colonies (Figure 2-3B). In contrast, α-chymotrypsin inhibition was highest in the infected tissue and lower in both healthy tissues from the diseased and healthy colonies (Figure 2-3C).

Sea fans exposed to elevated temperatures for 14 days demonstrated no significant changes in protease inhibition activity against fungal-derived proteases (ANOVA, $F_{(11)}$=0, P=0.9977), trypsin (ANOVA, $F_{(11)}$=0.777, P = 0.3903), or α-chymotrypsin (ANOVA, $F_{(11)}$=0.0052, P=0.9434).

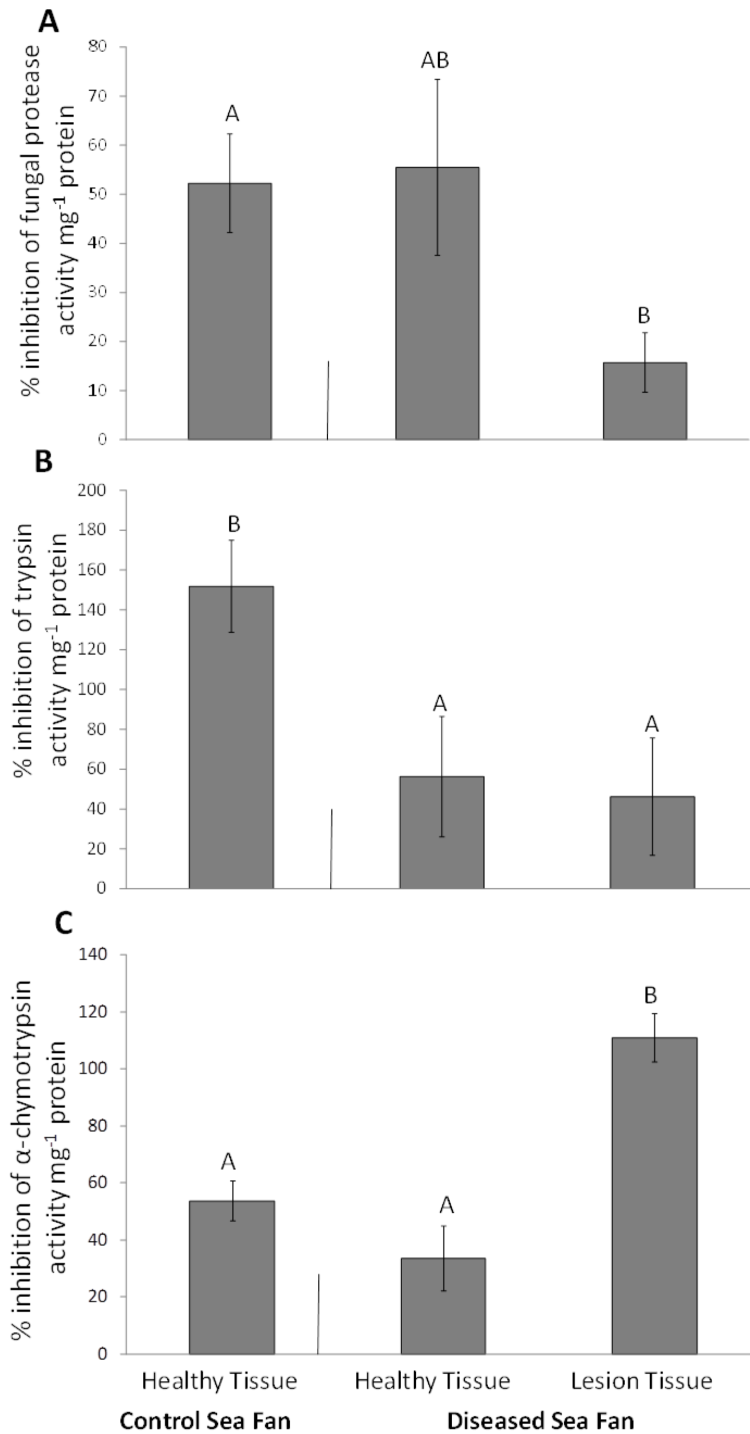

Figure 2-3 Protease inhibitor activity of the sea fan

Mean (±SE) protease inhibitor activity of A fungal-derived, B trypsin, and C α-chymotrypsin proteases (n=8). Letters indicate statistical differences at $P<0.05$.

Discussion

The interplay between host and pathogen can manifest itself through the specific virulence factors of a pathogen and countermeasures employed by the host (Rolff and Siva-Jothy 2003). Furthermore, host and pathogen dynamics can be shaped by changes to environmental conditions favoring a particular side of the relationship (i.e. pathogen virulence or host immunocompetence) (Altizer et al. 2013). Through the observation of pathogen protease and host protease inhibitor activity, this study demonstrates changing interplay of the sea fan – *Aspergillus sydowii* pathosystem under different temperatures and health conditions. The results highlight antagonistic mechanisms shaping host-pathogen interactions and further contribute to our understanding of how climate change may influence the future of coral disease dynamics.

Intracellular and extracellular proteases play varying roles in cellular metabolism but can also serve as potential virulence factors (Armstrong 2006). Intracellular proteases are commonly involved with cellular metabolic processes such as protein maturation and cascade initiation, (Bond and Butler 1987; McGillivray et al. 2012) while extracellular proteases are more involved in acquiring nutrients and/or evasion of host immune defenses (Hoge et al. 2010). Protease secretion is common in other species of *Aspergillus* (Hanzi et al. 1993) and this study confirms the presence and activity of both intracellular and extracellular proteases in *A. sydowii* strains isolated from sea fans, mangroves, and humans.

Increases of both extracellular and intracellular proteases can occur when there is metabolic demand for nutrients (Cohen 1973). In the sea fan and mangrove strains of *A. sydowii* used in this study, intracellular protease activity significantly increased at 28°C while extracellular activity increased at 30°C suggesting there may be an increase in metabolic activity and need for larger amounts of nutrients at these temperatures. It is likely that increasing metabolic demands translate into greater potential virulence and pathogenesis in sea fan-*Aspergillus* system. In the human *A. sydowii* strain, a clear out-group in this study, intracellular and extracellular proteases did not increase at elevated temperatures (28-32°C). In other *Aspergillus* species that cause human diseases, optimum temperatures for growth and virulence are closer to 37°C (Hedayati et al. 2007); therefore, the expected optimum temperature for protease activity in the human *A. sydowii* strain is beyond the ecologically relevant range examined here.

To maintain resistance to disease, the host must be able to conserve basic cellular function and produce a counteractive immune response to prevent pathogenesis (Little et al. 2005). As we show in this first part of this study, serine proteases are present in many fungal pathogens as virulence factors to help colonize host tissue (Dunaevskii et al. 2006). Sea fans have protease inhibitors that effectively work against trypsin, α-chymotrypsin, and fungus-derived proteases, suggesting a potential for resistance against *A. sydowii* infection and proliferation. Lower levels of trypsin and fungal protease inhibition were observed in infected sea fan tissue compared to healthy tissue from both disease and control colonies. Conversely, α-chymotrypsin inhibitory activity was higher or induced in the infected tissue compared to the healthy tissue of both diseased and control colonies. The varying patterns of protease inhibitor activity in diseased and healthy sea fans suggest differing functions of each inhibitor. Various types of protease inhibitors can have a distinct endogenous or exogenous function: either as intracellular regulatory factors for cellular processes (Van de Ven et al. 1993) or extracellular anti-pathogenic compounds (Xue et al. 2006).

The inhibitor activity against the fungal-derived proteases by the extracts from the infected tissue of the diseased sea fan colonies was lower in the lesion than the healthy tissues from both the diseased and control healthy colonies. As an anti-pathogenic defense, this is unexpected that inhibition of fungal-derived proteases is lower in the lesion than the rest of the colony. However, due to the high variability observed among the healthy tissue from the diseased colonies, it is possible that this defense is acute in nature. This may be an early onset defense that occurs when the fungus is invading host tissue, as possibly seen in some of the healthy tissue of the diseased colonies. Similar patterns of lower activity in the lesion than the healthy tissue have also been found in protein content and peroxidase activity of diseased colonies in another study (Mydlarz and Harvell 2007). The lesion represents the end point of an infection and so accordingly, melanin and other cellular defenses may be the remaining active defenses. Inducible anti-pathogenic responses could be occurring earlier in the infection process which many not be observed in the later infection as seen in the lesion. In sea fans experimentally exposed to pathogens (demonstrating this early stage of infection), induced protein and transcriptomic changes of putative anti-pathogenic processes have been observed, such as antifungal and peroxidase activity (Ward et al. 2007; Burge et al. 2013b). Therefore, the anti-fungal protease inhibitors examined here possibly serve as direct

pathogenic defenses that occur early in the infection process and the resources for these processes are spent by the time the lesion is visible or detectable for collection.

Trypsin-like proteases have shown to be involved in the melanin-synthesis and prophenoloxidase cascades of many invertebrates, including corals (Cerenius and Soderhall 2004; Mydlarz and Palmer 2011). Melanin-synthesis is a key mechanism in the encapsulation of pathogens and sea fans effectively use these pathways to fight *A. sydowii* (Petes et al. 2003; Mydlarz et al. 2008). In fact, early immune responses to immunogens show strong up-regulation of genes encoding a serine protease in hard corals (Weiss et al. 2013). Since the melanin-synthesis cascade including prophenoloxidase requires proteolytic cleavage to be activated, any inhibitors many prevent this. It is possible that the trypsin inhibitors in the sea fan are playing a role in the melanin-synthesis cascade rather than having direct anti-pathogen effects. The strong systemic decrease in trypsin inhibition in the entire diseased sea fan (both lesion and healthy tissue), may actually help prevent spread of infection throughout the colony by allowing activation of the melanin-synthesis cascades.

The pattern of α-chymotrypsin inhibition was opposite to that of trypsin and fungal-derived protease inhibition and showed a strong induction in infected tissue. Although chymotrypsin-like proteases have been suggested to be involved in the melanin-synthesis cascade in insects (Sugumaran et al. 1985), α-chymotrypsin inhibitors also have other functions (McManus et al. 1994). Chymotrypsin proteases are involved in digestion and growth, which need to be regulated by protease inhibitors (Novillo et al. 1997; Sunde et al. 2001). In heavily infected tissue of the sea fan, growth and digestion are not needed and may be suppressed by the α-chymotrypsin inhibitors.

Changes in environmental conditions can alter the dynamics and relationship between hosts and associated pathogens, compromising host immunity and/or exacerbating pathogen virulence (Harvell et al. 2002; Mydlarz et al. 2010). However, under experimental conditions, elevated temperatures did not affect the protease inhibitor activity in the sea fan tissue. It is possible the duration of the experiment was not long enough to elucidate any protease inhibitor response on its own, or exposure to a pathogen is needed to act in concert with elevated temperatures.

This study demonstrates how environmental change, such as elevated temperatures, can affect the dynamics of a host-pathogen relationship. Under current climate change conditions, increased

temperatures promote *A. sydowii* protease activity while having no immediate effect on sea fan inhibitor activity. The fact that sea fans still succumb to aspergillosis and many of the largest outbreaks have followed unseasonably warm air and sea temperatures (Flynn and Weil 2009a), indicates the sea fan is still losing the disease “arms race” against *A. sydowii*. In this case, perhaps the effects of temperature on pathogen growth (Ward et al. 2007) and virulence (i.e. extracellular protease activity) shift the power towards the fungus. Further studies are necessary to elucidate other specific mechanisms that may be changing within the host and/or pathogen under various climate change scenarios. Nonetheless, the contribution of this study to the understanding of host-pathogen relationships among corals is essential in determining causes behind epizootics and emergence of new diseases.

Chapter 3

Immunosuppression of the Caribbean sea fan (*Gorgonia ventalina*) resulting from interaction of environmental stressors and exposure to an *Aplanochytrium* sp. pathogen

Introduction

Although coral reefs cover less than 1% of the Earth's surface, these shallow benthic communities harbor the highest biodiversity in the ocean (Bellwood and Hughes 2001). Coral reefs are important for providing habitat for coastal fisheries, attracting tourism, and preventing coastal erosion (Worm et al. 2006). Despite the importance of coral reefs, degradation due to a variety of stressors, including climate change (increased sea surface temperatures and ocean acidification), eutrophication, sedimentation, and disease has occurred at an alarming rate (Fabricius 2005; Hoegh-Guldberg et al. 2007). The resulting decline of corals leads to lower coral cover among reefs which greatly affects the biodiversity and overall function of coral reef ecosystems (Altizer et al. 2013). Diseases outbreaks have been linked with elevated sea surface temperatures (Bruno et al. 2007), however, little is known current about the effects of ocean acidification on coral disease. These stressors are mostly consequence of anthropogenic-driven activities which have accelerated levels of atmospheric CO_2 (carbon dioxide). As of 2014, atmospheric CO_2 has reached 400 ppm and is projected to reach 750 ppm by the year 2100 (IPCC 2007; Tans and Keeling 2014). The continuing rise of atmospheric CO_2 is predicted to continue to elevate SST and acidify oceanic waters (Hönisch et al. 2012; Lima and Wethey 2012).

Both elevated temperature and ocean acidification have adverse impacts on corals. Thermal stress can affect many aspects of coral physiology and compromise vital functions, such as immunity, reproduction and survival rates of larvae (Baird and Marshall 2002; Mydlarz et al. 2010; Paxton et al. 2013; Ross et al. 2013). It can also cause disassociation of the endosymbionts (also known as bleaching). Likewise, acidified water conditions can decrease calcification rates, reproduction, and the ability to maintain cellular homeostasis (Kurihara 2008; Erez et al. 2011; Kaniewska et al. 2012). Ocean warming and acidification do not occur independently but rather act in concert and likely have combinative or synergistic effect on corals. The interaction between warming and acidification has been demonstrated in several other marine organisms, such as jellyfish, oysters, and fish and adversely affect reproduction, growth/development, and oxygen consumption capabilities (Munday et al. 2009; Parker et

al. 2009; Klein et al. 2014). However, data on how these environmental stressors interact to affect corals are lacking, particularly concerning coral disease and immunity (Ban et al. 2014).

Corals, like other invertebrates rely on the innate immune system to defend against against pathogens and foreign invaders. Broad components of coral immunity include antimicrobial defense, pathogen encapsulation, and phagocytosis (Palmer and Traylor-Knowles 2012). The general production of antimicrobial peptides or effectors is largely present in corals and shown to effectively stop or slow both bacteria and fungi growth (Kim et al. 2000; Gochfeld and Aeby 2008). Other anti-pathogenic defenses include the melanin-synthesis pathway and protease inhibitors. Melanin synthesis provides a physical-chemical barrier to block and encapsulate pathogens while protease inhibitors can be secreted to neutralize or deactivate pathogenic proteases (Mydlarz and Palmer 2011; Mann et al. 2014). The production of antioxidants (such as superoxide dismutase, peroxidase, and catalase) is also extremely important in not only stress response, but as an immunological response (Cornet et al. 2007). Antioxidants produced as immunological defense can protect against pathogen produced reactive oxygen species (ROS) as well as ROS resulting from melanin synthesis (Mydlarz et al. 2006). As ectothermic organisms, corals rely on a stable environment to maintain homeostasis and immunological responses can be severely disrupted when pathogens and environmental stressors are introduced (Pörtner 2008; Sokolova et al. 2012).

The Caribbean sea fan, *Gorgonia ventalina*, is an abundant gorgonian coral among Caribbean reefs. The sea fan is host to several diseases including aspergillosis, caused by fungal pathogen *Aspergillus sydowii* (Geiser et al. 1998) and a more recently characterized unnamed disease caused by an *Aplanochytrium* sp. protist pathogen (order: Labyrinthulomycetes; Burge *et al.* 2012). This disease can be identified histologically by the presence of *Aplanochytrium* aggregating primarily along the gorgonin axis or internal proteinaceaous skeleton and degradation of polyps.

Several species of Labyrinthulomycetes are known to be opportunistic pathogens to hosts such as clams and sea grasses (Burge et al. 2013a). In both of these systems, temperature has also been shown to significantly alter host-pathogen dynamics and disease development (Perrigault et al. 2011; Bishop 2013). Therefore, the introduction of environmental stressors (i.e. elevated temperature and/or

changes in pH) to the sea fan coral may also be leading to a compromised immune system and/or the introduction of more favorable conditions to the *Aplanochytrium* pathogen.

In this study we use the sea fan as a model to investigate how multiple stressors (e.g. elevated SST and ocean acidification) interact together to influence the coral immune response to a pathogen during short term and long term treatment lengths. We hypothesize that (1) the sea fan will display a response to the *Aplanochytrium* exposure through the production of antimicrobial, anti-pathogenic, and antioxidant effectors in the absence of environmental stressors (Figure 3-1). (2) Additionally, we hypothesize that environmental stressors synergistically lower immunocompetence of the sea fan by decreasing constitutive immunity levels (antimicrobial and anti-pathogenic responses). Because antioxidants are involved in the general stress response, we also hypothesize that antioxidant activity remains elevated to combat respiratory oxidative stress from host and the symbiont (Baird et al. 2009). (3) Lastly, we hypothesize that combination of both the environmental stressors and pathogen-exposure can have further synergistic effect on the sea fan immune system. Longer exposure to these stressors will have a greater effect than at a short term exposure.

Here for the first time, we illustrate how these factors can act in combination to adversely affect the immunity of corals. Short term treatment of the factors on the sea fan only revealed responses to the pathogen while over a the longer treatment period, exposure to both elevated temperature and acidified water conditions compromised the immunity of the sea fan and furthermore, was compounded with the exposure to an *Aplanochytrium* pathogen. These results show that multiple stressors significantly impact development of disease in corals and should encourage future research to encompass a broader range of stressors when studying coral ecology under climate change scenarios.

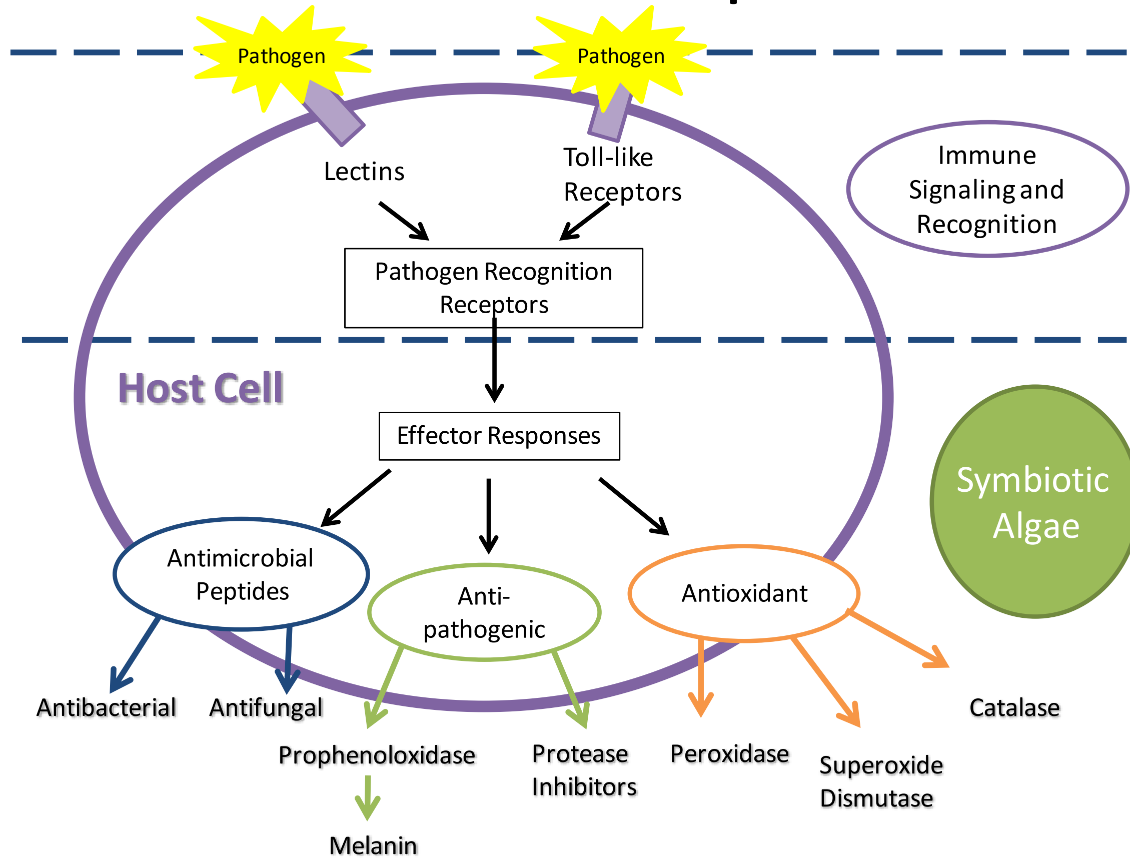

Figure 3-1 Oversimplified schematic of the coral immune response.

Methods

Sea fan collection

In summer of 2012, large fragments (72 x108 cm) from twelve colonies of sea fan corals (*Gorgonia ventalina*) were collected from Laurel patch reef in La Parguera, Puerto Rico (17° 56.608' N, 67° 03.208' W). Sixteen smaller clonal fragments (6x9 cm) were then cut from each colony and allowed to heal on the reef for two days. Afterwards, the fragments were brought back to the station (Isla Magueyes Marine Laboratories) and distributed into 24 plastic bin aquariums (8 fragments per bin; n=12; Figure **Error! Reference source not found.**). Each aquarium had individual water pumps that provided constant low of sand-filtered sea water at ambient temperatures (range of 26.0-29.0°C with a mean 27.90 ±.05°C) for two additional acclimation days. Artificial lighting with full-spectrum bulbs was set to a 12 hour day/night cycle.

Experimental setup

Water entering the facility went directly into 4 large drums, and then pumped free-flowing into the plastic aquariums with individual water pumps and air stones for aeration. After two days of acclimating to this system, the flow-through rates were slowed to 2 L/hr and heaters were turned on in half of the tanks and two of the large drums. Temperature was continuously maintained with individual aquarium heaters and elevated temperature was set to 30°C (mean: 30.43±.02) and ambient temperature at 26°C (mean: 26.65±.03). Once both drums and the tanks achieved similar temperatures (~3 hours), water flow was halted briefly and a predetermined CO_2/air concentration using mass flow-controllers (Dakota Instruments) was added to two of the drums. After achieving the desired CO_2 in the drums, free-flowing water was allowed into the aquariums until the desired pH was achieved (either 7.8 or 8.0). At this point, the flow-through was then returned to 2 L/hr. Flow-through was maintained at 2 L/hr throughout the experiment, except for tank maintenance.

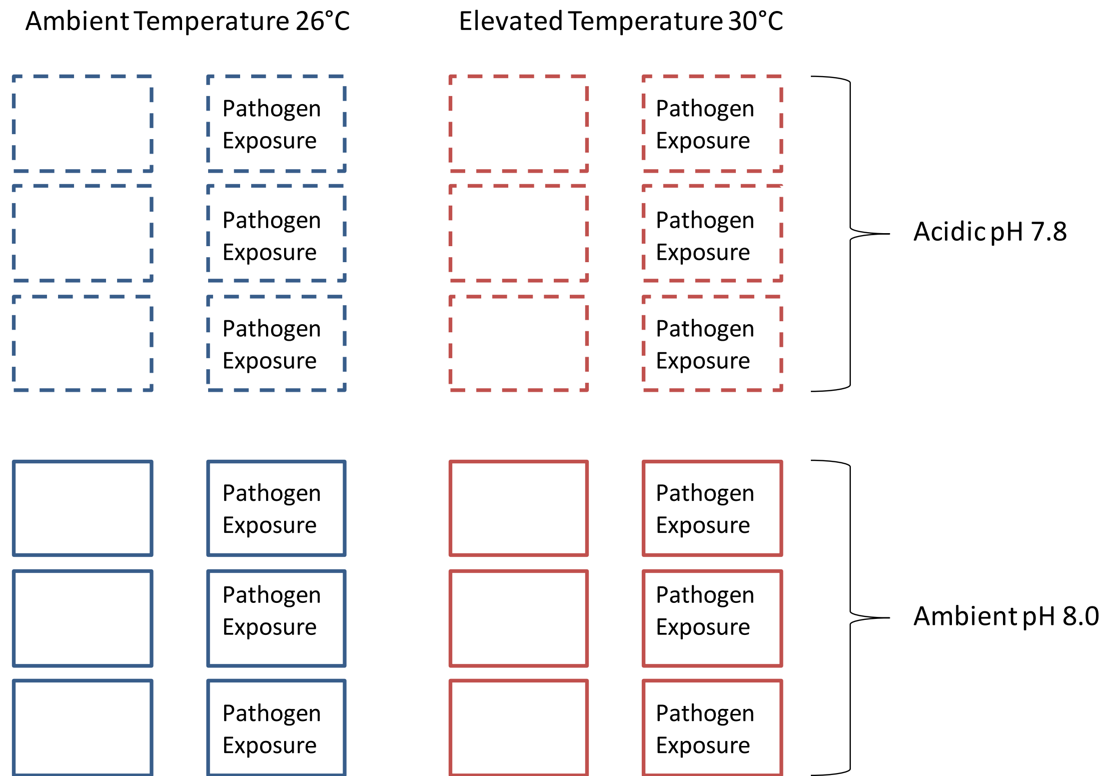

Figure 3-2 Experimental setup with all three treatments, pH, temperature, and pathogen-exposure

A set of two clonal fragments from 4 colonies were placed into each tank (8 fragments total). One set was collected after 18 hours of treatment and the other after 18 days of treatment (n=12).

Due to unequal flow into the facility, we were unable to maintain a constant flow of CO_2/air into the drums. Therefore, drums of sea water were prepared twice a day (with flow-through turned off) to ensure that the pH held steady in the tanks. The tank pH was measured before and after each change. pH values for each treatment were as follows: ~7.8 for acidified conditions and 7.99-8.05 for ambient conditions. pH was monitored with Ultrameter II TM 6P (Myron L® Company, Carlsbad, CA) which was checked daily against a certified reference material (CRM) for measuring oceanic carbon dioxide.

Once a week, water samples were taken from individual tanks for analysis for carbon chemistry parameters into 250 mL bottles with .05% $MgCl_2$ added following the protocol of SOP X of Dickson et al. (2007). Weekly samples for analysis of seawater pH and total alkalinity (A_T) processed as follows. An end point titration was used to measure A_T using a DL15 titrator (Mettler-Toledo, Columbus, OH,) with quality assurance following SOP 3b of Dickson et al. (2007). T_{CO2} was measured using a Licor LI-700 CO_2 detector following acidification as outlined in SOP2 Dickson et al. (2007). Spectrophotometic pH was measured on selected samples using SOP 6b of Dickson et al. (2007). The free-ware CO_2sys was used to calculate seawater pCO_2, pH, and saturation state (Ω) of aragonite (Ω_{ARAG}) and calcite (Ω_{CAL}) using the measured parameters of temperature, salinity, pH and A_T (Robbins et al. 2010). The following constants were used in the CO_{2CALC} program: CO_2 constants: Lueker et al. (2000); $KHSO_4$: Dickson (1990); pH scale: total scale (mol/kg SW); and air-sea flux: Wanninkhof (1992). Precision [(standard deviation / mean measured value) x 100] and accuracy ([(mean measured value - CRM) / CRM] x 100) value were calculated by repeated measurements of certified reference materials (CRMs) supplied by Andrew Dickson's laboratory (Scripps Institution of Oceanography, La Jolla, CA). The specifics of the carbon chemistry can be found in Table 3-1.

Table 3-1 Carbon chemistry results

Carbon chemistry and conditions of experimental setup including total alkalinity (A_T)

Treatment	Temp (C°)*	Salinity	pH	A_T	pCO_2	Calcium	Aragonite	$C0_3^{2-}$
26°C, Ambient CO_2	26.4 ± 0.87	35	7.98 ± 0.01	2292.43 ± 6.31	470.21 ± 8.76	4.78 ± 0.17	3.16 ± 0.12	198.04 ± 6.88
26°C, Elevated CO_2 (goal 750)	27.0 ± 0.45	35	7.81 ± 0.02	2292.43 ± 9.18	755.01 ± 32.27	3.52 ± 0.05	2.30 ± 0.03	145.45 ± 2.13
30°C, Ambient CO_2	30.6 ± 0.55	35	7.99 ± 0.01	2306.22 ± 2.62	463.94 ± 11.35	5.49 ± 0.06	3.68 ± 0.05	225.38 ± 2.30
30°C, Elevated CO_2 (goal 750)	30.2 ± 0.48	35	7.81 ± 0.01	2300.43 ± 6.31	763.55 ± 31.41	3.89 ± 0.04	2.61 ± 0.03	159.94 ± 1.96

*Mean continuous temp was 26.65± 0.03 °C & 30.43± 0.02, respectively

Experimental treatment conditions

The experiment was conducted with 3 treatments, temperature, pH, and pathogen- exposure, in a full-factorial design with clonal replication of the sea fan colonies (n=12). Sea fan fragments were exposed to one of eight treatments for a period of either 18 hours or 18 days. The treatment combinations included: (1) Elevated Temperature (2) Acidification, (3) Pathogen-Exposure (4) Elevated Temperature and Acidification, (5) Elevated Temperature and Pathogen-Exposure, (6) Acidification and Pathogen-Exposure, (7) Elevated Temperature, Acidification, and Pathogen-Exposure, and (8) Control treatment with no stressors.

Two hours following achievement of desired water quality parameters (pH and temperature), half of the sea fans were inoculated using sterile 1 cc syringes with ~5000 cells of an *Aplanochytrium* sp. (Burge et al. 2012) in 0.22 um filtered sea water and the remaining sea fans with 0.22 um filtered sea water as a control. After 18 hours and 18 days of treatment, the fragments were immediately flash frozen in liquid N_2 and shipped on dry ice to UTA for biochemical analysis.

Extract preparation. Coral fragments (skeleton, polyps and connective tissue) were ground to a powder in liquid nitrogen in a mortar. The frozen powder was immediately placed in approximately 2 ml of 100 mM sodium phosphate buffer (PBS), pH 7.8 and incubated on ice for 45 min. The mixture was centrifuged at 2205 x G at 4°C in an Eppendorf 5810R for 10 min and the supernatant was recovered into a 1.5 ml tube and kept frozen at -80°C between all assays. Total protein content was quantified with the Red660 protein assay using a standardized concentration curve of bovine serum albumin (G Biosciences, St. Louis, MO). All assays were standardized to protein concentration of the coral extracts. All assays were performed with a Synergy 2 spectrophotometer (Biotek, Winooski, VT) and ran in duplicates for each sample. The assays were grouped into three general immune mechanisms: direct antimicrobial defenses, anti-pathogenic defenses, and antioxidant defenses.

Biochemical assays

Direct antimicrobial defense

Antibacterial (AB) Assay. Antibacterial assay was conducted against a marine strain of *Vibrio alginolyticus* (courtesy from K. Ritchie, Mote Marine laboratory). *V. alginolyticus* was grown to exponential

phase in Luria-Bertani broth (VWR) with 3% Instant Ocean® salt. The culture was diluted to an optical density of 0.200, read at 600 nm wavelength. 10 µl of coral extract (diluted to 1 mg ml^{-1}) were then added to 140 µl of the *V. alginolyticus* culture in a sterile 96 well microplate (Greiner Bio-One). The plate was incubated at 30°C and was read at 600 nm every 10 min for 6 hrs using a spectrophotometer (BioTek). Optical density readings from the log phase growth period were used to calculate generation time of *V. alginolyticus*. From the data, higher generation times indicated higher antibacterial activity from the coral extract.

Antifungal (AF) Assay. Antifungal activity against a marine strain of *Aspergillus sydowii* (isolated from a sea fan in San Salvador) was performed. 140 µl of *A. sydowii* (100,000 spores ml^{-1}, quantified with a hemacytometer) was added to each well of a 96 well transparent sterile microplate (Greiner Bio-One) and incubated for 24 hrs at 30°C to allow for hyphae germination. 10 µl of coral extract (diluted to 1 mg ml^{-1}) was then added to the culture and the OD was read every 2 hrs for 48 hrs at 600 nm. OD readings from the log phase growth period were used to calculate growth rate of *A. sydowii*. A higher growth rate indicates lower antifungal activity of the sea fan extract (and vice versa).

Anti-pathogenic defense

Prophenoloxidase (PPO) Assay. 20 µl of coral extract was added to 30 µl of 100 mM PBS pH 7.8 and 10 µl of 0.01 mg ml^{-1} trypsin (Sigma Aldrich) in a 96-well microtiter transparent plate (Greiner Bio-One) and incubated at room temperature for 30 min. To initiate the reaction, 10 mM L-1,3-dihydroxphenylalanine (L-dopa, Sigma-Aldrich) was then added to each well and the OD was read at 490 nm every 30 s for 25 min. Rate of reaction was read by calculating slope during the linear phase of each reaction.

Protease Inhibitor (PI) Assay. The protease inhibitor assay was performed as per (Mann et al. 2014). 10 µL of sea fan extract (or 100 mM PBS, pH 7.8 as a control) were incubated with 10 µL trypsin (0.1 mg mL^{-1}) (Sigma-Aldrich) for 30 min at room temperature. The sea fan extract/protease mixture was then added to 40 µL 2.5% (w/v) FITC-casein substrate (Sigma-Aldrich) in 20 mM PBS, 150 mM NaCl, pH 7.6 and incubated for 30 min at 37°C. The reaction was stopped with 10% trichloroacetic acid to precipitate remaining proteins. Proteins were centrifuged down at 5590xg (Baxter Scientific Products,

Deerfield, Illinois, USA) and the fluorescence of the supernatant was observed with excitation at 485 nm and emission wavelength at 535 nm in a black 96 well plate (Greiner Bio-One). Percent inhibition of the protease activity was calculated against the control (PBS buffer only).

Antioxidant defense

Superoxide Dismutase (SOD) Assay. SOD activity was measured using the SOD Assay Kit (Sigma-Aldrich). 10 µl of coral extract was diluted with 10 µl sterile deionized water and added to Dojindo's highly water-soluble tetrazolium salt, (WST-1; 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) and xanthine oxidase in a transparent 96-well plate (Greiner Bio One) and incubated for 30 min at 37°C. After incubation, the OD was read at 450 nm. Activity was quantified by calculating percent reduction of superoxide ion present at the endpoint.

Peroxidase (POX) Assay. 10 µl of coral extract was added to 50 µl of 50 mM PBS pH 6 and 40 µl of 25 mM guaiacol (Sigma Aldrich, St. Louis, MO) in a transparent 96 well plate (Greiner Bio-One, Monroe, NC). The reaction was initiated by adding H_2O_2 to a final concentration of 10 mM and the optical density (OD) was read at 470 nm every 30 s for 15 min. Rate of reaction was determined by calculating slope during the linear phase of each reaction.

Catalase (CAT) Assay. 5 µl coral extract was added to 70 µl of 50 mM PBS pH 7 in a UV transparent 96-half well plate (Greiner Bio-One). The reaction was started immediately by adding 50 µl of 50 mM H_2O_2 and the OD was read every 30 s for 15 min at 240 nm. A standard curve was made from a two-fold serial dilution of 50 mM H_2O_2 and used to quantify the moles of H_2O_2 scavenged during the linear phase of the reaction. The change in H_2O_2 concentration was calculated by subtracting the final mM of H_2O_2 from the initial mM of H_2O_2.

Data analysis

Data were tested for normality and unequal variances using the Shapiro-Wilk and Brown-Forsythe tests, respectively. In the 18 hour treatment collection, CAT did not fit the criteria and was successfully transformed using Box-Cox best fit transformation. In the 18 day treatment collection, both PI and CAT data did not fit the criteria for parametric analysis and were transformed using Box-Cox best fit

transformation or multiplicative inverse function, respectively. Homoscedasticity and normality were achieved upon transformation.

Multivariate analysis of variance (MANOVA) was performed on each treatment length (18 hours and 18 days of treatment) separately to determine the effects of the three factors (temperature, pH, and pathogen-exposure) on the overall immune response (AB, AF, PPO, PI, SOD, CAT, and POX assays). To examine the effect and interactions between temperature, pH, and pathogen-exposure, three-factor univariate analysis of variance (ANOVA) was also run for each immune assay. Tukey's post-hoc test for multiple comparisons was used to determine differences between treatments in each immunoassay. Additionally, when there were two or more interactive factors in the analysis, the highest level interaction(s) with significance at $p \leq 0.05$ were chosen and interaction plots were created to examine specific interacting relationships between factors and the effect on the individual immune variable. Interaction plots that displayed parallel lines indicated an additive effect of the interacting factors on the immune variable, while diverging, crossed, or converging lines indicated a synergistic or combined effect (Dunne 2010).

Results

Overall immunity

At 18 hours of treatment, overall immunity was significantly affected (Wilks' Lambda, whole model, $F=4.1377$, $p<.0001$, Table 3-2). There was significant interaction between temperature and pH ($F=2.3264$, $p=0.0341$) as well as interaction between pH and pathogen-exposure ($F=2.2524$, $p=0.0398$). After 18 days of treatment, overall immunity was also significantly affected (Wilks' Lambda, whole model, $F=369.95$, $p<.0001$, Table 3-3). All three factors, temperature, pH, and pathogen exposure had interactive effect on immunity ($F=17.817$, $p<.0001$).

Table 3-2 Whole model MANOVA and univariate ANOVA analysis at 18 hrs of treatment
Whole model multivariate analysis of variance (MANOVA) and univariate analysis of variance (ANOVA) results for each immune parameter and each treatment effect after 18 hours of exposure. Significant p values (<0.05) are bolded.

		Whole Model			Temperature			pH			Temperature x pH		
		F	d.f.	p	F	d.f.	p	F	d.f.	p	F	d.f.	p
	MANOVA (Wilk's Lambda)	4.1377	49	**<.0001**	6.8494	7	**<.0001**	3.2555	7	**0.0048**	2.3264	7	**0.0341**
Immune Mechanism	Univariate ANOVA												
Antimicrobial	Antibacterial	15.7855	7	**<.0001**	2.4282	1	0.1233	0.3696	1	0.5450	2.3228	1	0.1316
	Antifungal	1.9499	7	0.0732	3.3721	1	0.0702	0.3387	1	0.5623	0.2620	1	0.6102
Anti-pathogenic	Prophenoloxidase	1.1195	7	0.3598	1.0597	1	0.3065	2.9379	1	0.0906	0.0133	1	0.9083
	Protease Inhibitor	1.3485	7	0.2397	1.9522	1	0.1664	1.1285	1	0.2915	0.2805	1	0.5979
Antioxidant	Superoxide Dismutase	1.8936	7	0.0822	1.4056	1	0.2395	3.4631	1	0.0666	0.9355	1	0.3365
	Peroxidase	1.3884	7	0.2226	1.8992	1	0.1722	3.6782	1	0.0589	0.3408	1	0.5611
	Catalase	9.9199	7	**<.0001**	21.894	1	**<.0001**	17.547	1	**<.0001**	5.0522	1	**0.0275**

		Pathogen-exposure			Temperature x Pathogen-exposure			pH x Pathogen-exposure			Temperature x pH x Pathogen-exposure		
		F	d.f.	p	F	d.f.	p	F	d.f.	p	F	d.f.	p
	MANOVA (Wilk's Lambda)	17.143	7	**<.0001**	1.9781	7	0.0703	2.2524	7	0.0398	2.0303	7	0.0632
Immune Mechanism	Univariate ANOVA												
Antimicrobial	Antibacterial	102.375	1	**<.0001**	1.9199	1	0.1699	0.0532	1	0.8182	0.0825	1	0.7747
	Antifungal	7.4400	1	**0.0079**	2.2824	1	0.1350	0.0667	1	0.7970	0.2134	1	0.6455
Anti-pathogenic	Prophenoloxidase	0.6067	1	0.4385	0.1273	1	0.7222	1.9816	1	0.1633	1.1306	1	0.2910
	Protease Inhibitor	4.1381	1	**0.0454**	0.0969	1	0.7564	0.0021	1	0.9633	1.6531	1	0.2024
Antioxidant	Superoxide Dismutase	4.5847	1	**0.0355**	0.5941	1	0.4432	0.1684	1	0.6827	1.7429	1	0.1907
	Peroxidase	1.1501	1	0.2869	0.0830	1	0.7741	1.7530	1	0.1895	0.2198	1	0.6406
	Catalase	0.0248	1	0.8752	5.2780	1	**0.0244**	10.547	1	**0.0071**	2.2731	1	0.1358

Table 3-3 Whole model MANOVA and univariate ANOVA analysis at 18 days of treatment

Whole model multivariate analysis of variance (MANOVA) and univariate analysis of variance (ANOVA) results for each immune parameter and each treatment effect after 18 days of exposure. Significant p values (<0.05) are bolded.

		Whole Model			Temperature			pH			Temperature x pH		
		F	d.f.	p	F	d.f.	p	F	d.f.	p	F	d.f.	p
	MANOVA (Wilk's Lambda)	369.95	49	**<.0001**	14.0836	7	**<.0001**	6.208	7	**<.0001**	11.449	7	**<.0001**
Immune Mechanism	Univariate ANOVA												
Antimicrobial	Antibacterial	134.8412	7	**<.0001**	72.2067	1	**<.0001**	27.625	1	**<.0001**	55.982	1	**<.0001**
	Antifungal	1.4367	7	0.2028	0.6380	1	0.4269	0.5577	1	0.4574	0.1324	1	0.7169
Anti-pathogenic	Prophenoloxidase	1.9660	7	0.0704	0.0053	1	0.9421	1.9707	1	0.1643	0.0450	1	0.8326
	Protease Inhibitor	2.1227	7	0.0507	1.4200	1	0.2370	0.7098	1	0.4021	2.9643	1	0.0891
Antioxidant	Superoxide Dismutase	5.0857	7	**<.0001**	7.6985	1	**0.0069**	3.4791	1	0.0659	4.1615	1	**0.0447**
	Peroxidase	2.4005	7	**0.0281**	0.2323	1	0.6312	1.6913	1	0.1973	1.3964	1	0.2409
	Catalase	9.9199	7	**<.0001**	21.8942	1	**<.0001**	17.547	1	**<.0001**	5.0522	1	**0.0275**

Table 3-3 Continued.

		Pathogen-exposure			Temperature x Pathogen-exposure			pH x Pathogen-exposure			Temperature x pH x Pathogen-exposure		
		F	d.f.	p	F	d.f.	p	F	d.f.	p	F	d.f.	p
	MANOVA (Wilk's Lambda)	92.3916	7	**<.0001**	10.5994	7	**<.0001**	15.9417	7	**<.0001**	17.817	7	**<.0001**
Immune Mechanism	Univariate ANOVA												
Antimicrobial	Antibacterial	537.6109	1	**<.0001**	43.3990	1	**<.0001**	83.5218	1	**<.0001**	88.9806	1	**<.0001**
	Antifungal	2.1645	1	0.1453	5.6519	1	**0.0199**	0.6120	1	0.4364	0.0683	1	0.7945
Anti-pathogenic	Prophenoloxidase	2.2303	1	0.1394	4.0693	1	**0.0471**	5.1629	1	**0.0258**	0.1062	1	0.7454
	Protease Inhibitor	3.8420	1	0.0536	0.0303	1	0.8623	2.0015	1	0.1611	2.4448	1	0.1220
Antioxidant	Superoxide Dismutase	6.5968	1	**0.0121**	0.0246	1	0.8758	4.3716	1	**0.0398**	5.5443	1	**0.0211**
	Peroxidase	0.5331	1	0.4675	8.3722	1	**0.0049**	3.8145	1	0.0544	1.0227	1	0.3150
	Catalase	0.0248	1	0.8752	5.2780	1	**0.0244**	10.5471	1	**0.0071**	2.2731	1	0.1358

Direct antimicrobial defense

After 18 hours of treatment, pathogen-exposure was the only factor to significantly affect AB activity (three-factor ANOVA, $F=102.3756$, $p<0.001$). Exposure to the *Aplanochytrium* pathogen greatly reduced the AB activity of the sea fan extracts (indicated by a higher growth rate of *V. alginolyticus*; Figure 3-3A). At 18 days of treatment, all three factors (temperature, pH, and pathogen-exposure) had significant interactive effect on AB activity (three-factor ANOVA, $F=88.9806$, $p<0.001$). Exposure to the *Aplanochytrium* also lowered AB activity of the sea fan extracts after 18 days of exposure (Tukey's HSD, $p<0.05$). In addition, the combination of elevated temperature and acidification (no pathogen-exposure) also lowered AB activity (Figure 3-3B). Interaction plots were created for the interactive effects occurring at 18 days of treatment. When sea fans were exposed to ambient temperature, low pH, and pathogen-exposure, the effect was only additive (Figure 3-4A); however, when temperature was also elevated, there was synergistic effect between the three factors (Figure 3-4B). The combination of temperature and acidification greatly decreased AB activity of the sea fan and was further compounded by exposure to the *Aplanochytrium* pathogen.

At 18 hours of treatment, pathogen-exposure was also the only factor to affect AF activity (three-factor ANOVA, $F=7.44$, $p=0.0079$). The exposure to the *Aplanochytrium* lowered AF activity when the sea fan was exposed to ambient temperature than elevated temperature regardless of acidification (Figure 3-5A). After 18 days of treatment however, there was interactive effect of temperature and pathogen-exposure on AF activity (three-factor ANOVA, $F=5.6519$, $p=0.0199$; Figure 3-6). Post-hoc comparisons did not reveal any significant differences among groups (Figure 3-5B).

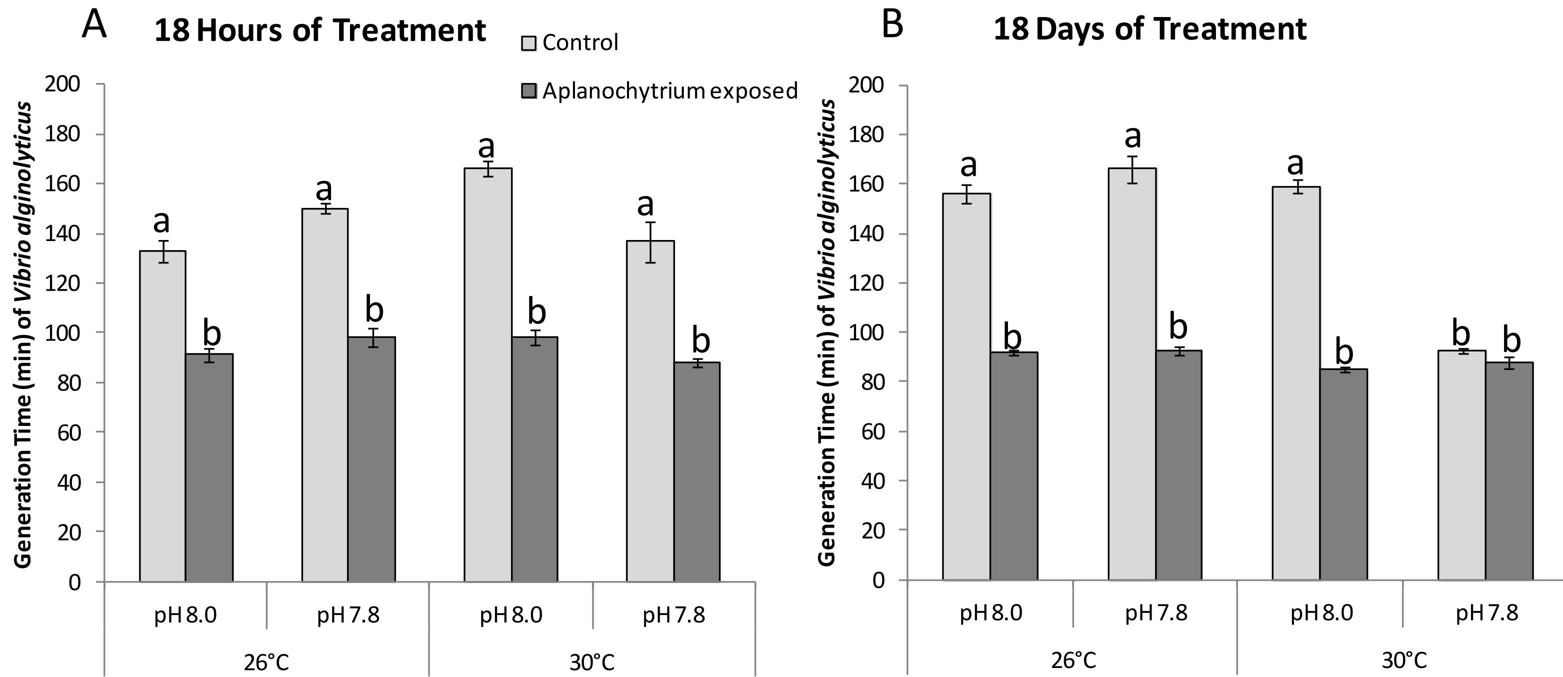

Figure 3-3 Antibacterial activity of the sea fan

Mean (±SE) generation time of *Vibrio alginolyticus* in the presence of the sea fan extracts exposed to elevated temperature, acidification, and *Aplanochytrium* pathogen treatments for a period of either 18 hours (A) or 18 days (B). Higher generation time indicates higher antibacterial activity. Letters indicate statistical significance at $p<0.05$ in each panel.

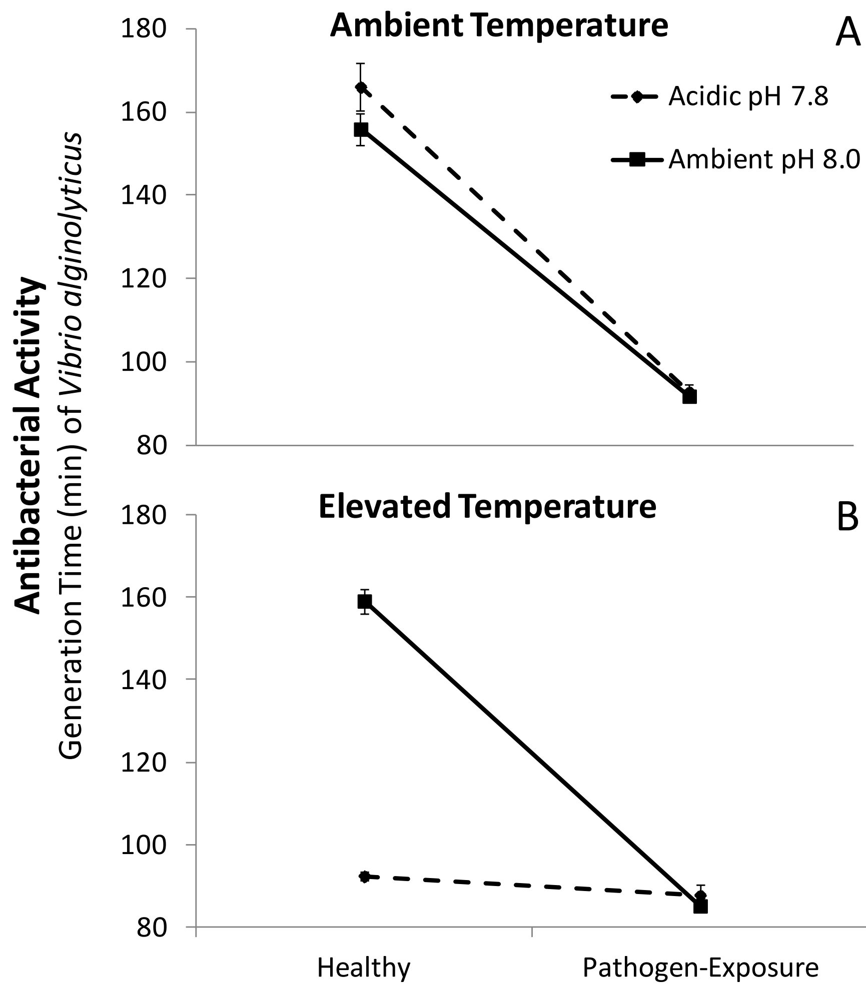

Figure 3-4 Antibacterial activity of the sea fan: interaction plot

Interactive plots of mean ± standard error growth rates of *Vibrio alginolyticus* in the presence of sea fan extracts from 18 days of treatment with elevated temperature, acidification and exposure to an *Aplanochytrium* pathogen. Higher growth rates of *V. alginolyticus* indicate low antibacterial activity of the sea fan and vice versa. Parallel lines indicate only an additive effect of the treatments (A) while non-parallel lines indicate a combinative/synergistic effect of the treatments (B).

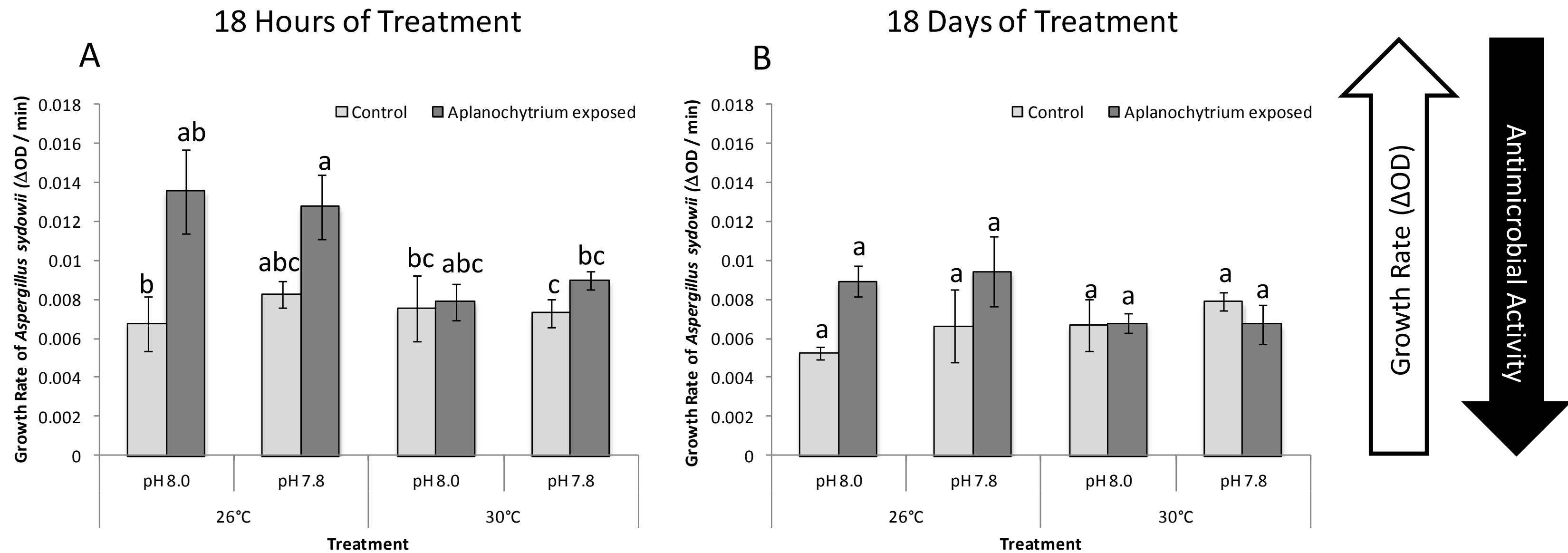

Figure 3-5 Antifungal activity of the sea fan

Mean (±SE) growth rate of *Aspergillus sydowii* in the presence of the sea fan extracts exposed to elevated temperature, acidification, and *Aplanochytrium* pathogen treatments for a period of either 18 hours (A) or 18 days (B). High growth rates of *A. sydowii* indicate low antifungal (AF) activity of the sea fan and vice versa. Letters indicate statistical significance at $p<0.05$ in each panel.

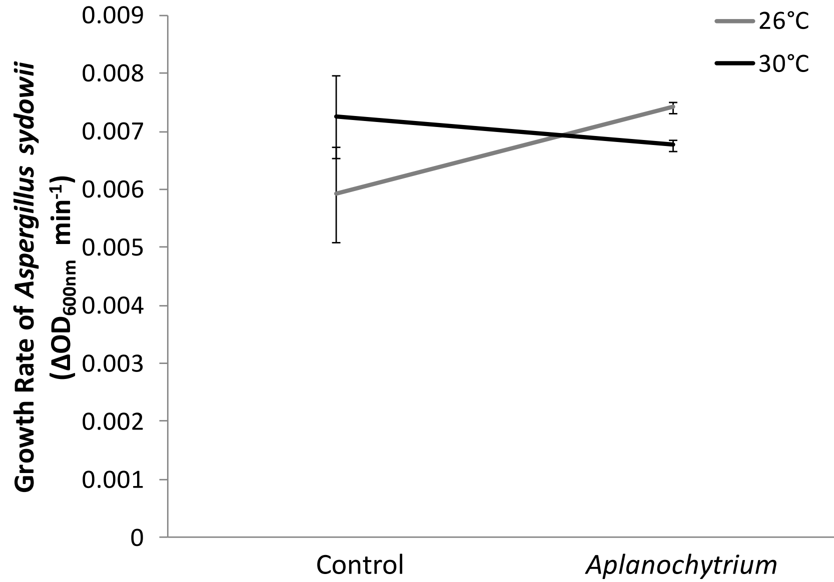

Figure 3-6 Antifungal activity of the sea fan: interaction plot

Interaction plot of mean (±SE) growth rates of *Aspergillus sydowii* in the presence of sea fan extracts with elevated temperature and pathogen-exposure factors at 18 days of treatment. High growth rates of *A. sydowii* indicate low antifungal (AF) activity of the sea fan. Exposure of the sea fan to both elevated temperature and the *Aplanochytrium* pathogen had significant interactive effect on antifungal activity of the sea fan. Non-parallel lines indicate synergistic effect of both factors.

Anti-pathogenic defense

At 18 hours of treatment, there were no interactive effects on PPO activity between treatments. There was difference in PPO activity under varying acidification treatment in sea fans treated exposed to ambient temperature and the *Aplanochytrium* pathogen (Tukey's HSD, $p<0.05$, Figure 3-7A). At the 18 days of treatment, there was significant interactive effect of pH and *Aplanochytrium* exposure (three-factor ANOVA, $F=5.1629$, $p=0.0258$; Figure 3-8A) as well as interactive effect of temperature and *Aplanochytrium* exposure on PPO activity (three-factor ANOVA, $F=.40693$, $p=0.0471$; Figure 3-8B). Both interactions indicate a synergistic effect of the treatments on PPO activity. Post-hoc comparisons did not reveal any significant differences among groups after 18 days of treatment (Figure 3-7B).

After 18 hours of treatment, exposure to the *Aplanochytrium* was the only factor to affect PI activity (three-factor ANOVA, $F=4.1381$, $p=0.0454$). Sea fans exposed to thermal stress and no pathogen had higher PI activity than sea fans exposed to the pathogen only (Tukey's HSD, $p<0.05$, Figure 3-9A). After 18 days of treatment, exposure to the *Aplanochytrium* had a slightly insignificant effect on PI activity (three-factor ANOVA, $F=3.842$, $p=0.0536$). There was even greater higher PI activity the sea fans exposed to thermal stress and no pathogen than sea fans exposed to the pathogen only (Tukey's HSD, $p<0.05$, Figure 3-9B).

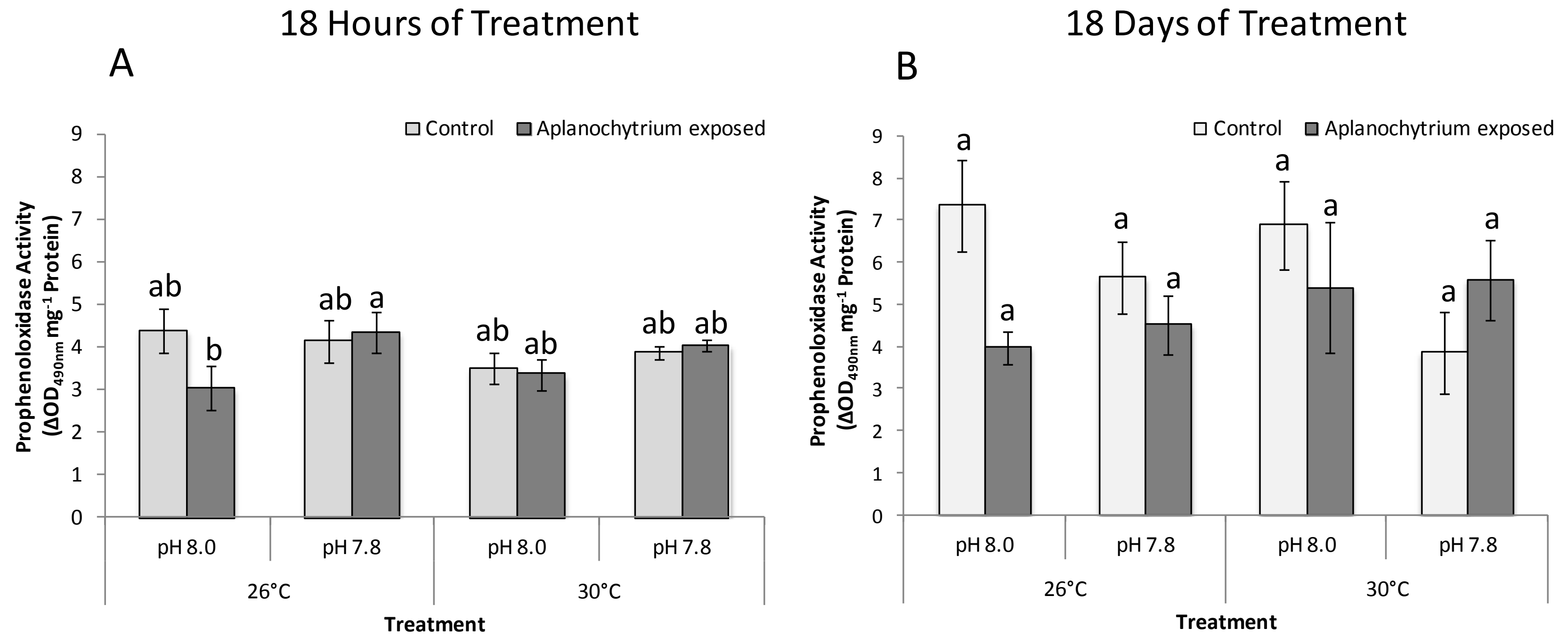

Figure 3-7 Prophenoloxidase activity of the sea fan

Mean (±SE) prophenoloxidase (PPO) activity of sea fan exposed to elevated temperature, acidification and an *Aplanochytrium* pathogen treatments for a period of 18 hours (A) or 18 days (B). Letters indicate statistical significance at $p<0.05$ in each panel.

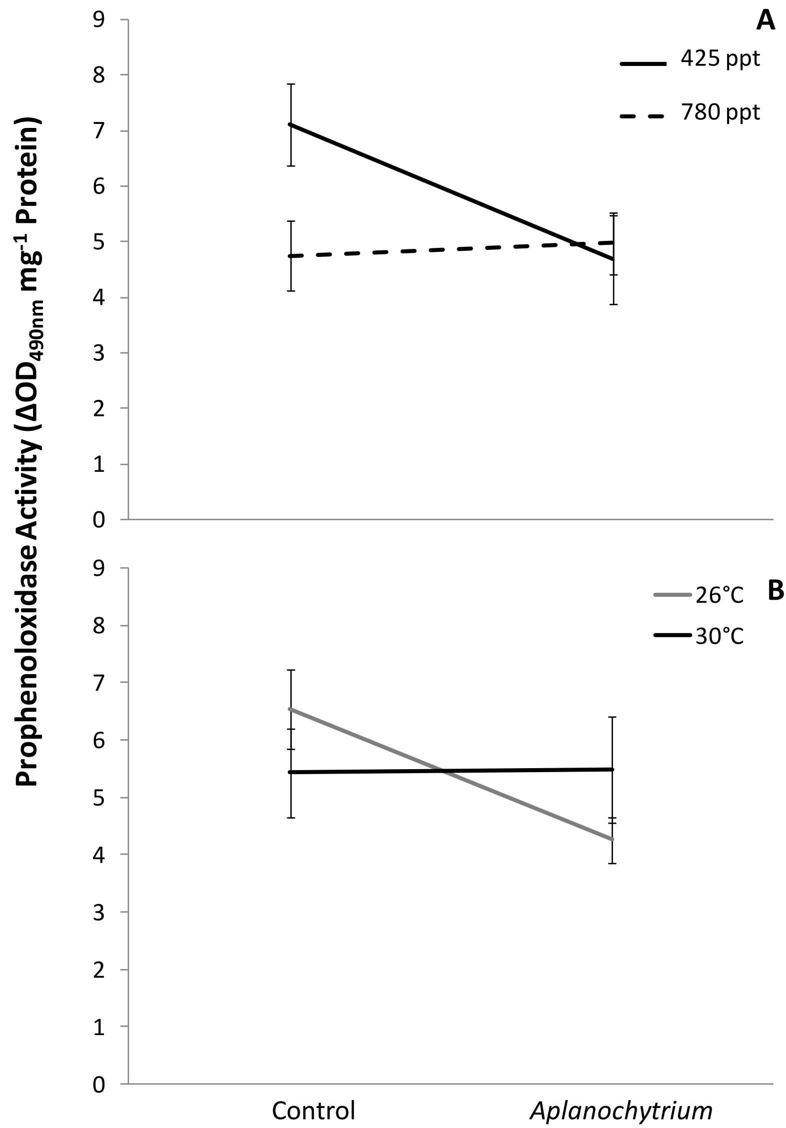

Figure 3-8 Prophenoloxidase activity of the sea fan: interaction plot

Interaction plots of mean (±SE) error prophenoloxidase (PPO) activity between factors (A) acidification and *Aplanochytrium* pathogen-exposure and (B) temperature and *Aplanochytrium* pathogen-exposure after 18 days of treatment. Non-parallel lines indicate synergistic effect of both factors.

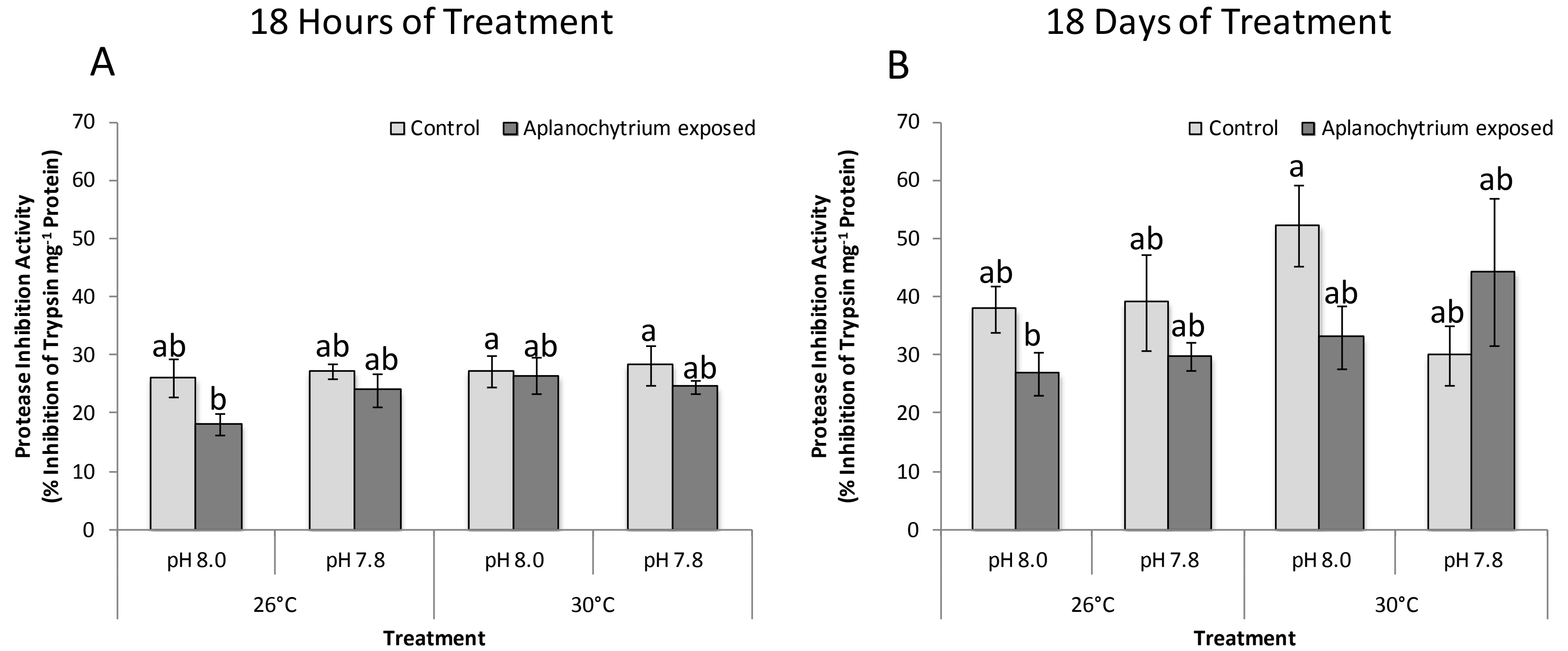

Figure 3-9 Protease inhibitor activity of the sea fan

Mean (±SE) protease inhibitor (PI) activity of sea fan exposed to elevated temperature, acidification and Aplanochytrium pathogen-exposure treatments for a period of 18 hours (A) or 18 days (B). Letters indicate statistical significance at $p<0.05$ in each panel.

Antioxidant defense

There was significant effect of pathogen-exposure on SOD activity after 18 hours of treatment (three-factor ANOVA, $F=4.5847$, $p=0.0355$). Independent of elevated stressors, SOD activity was lower in sea fans exposed to the *Aplanochytrium* (Figure 3-10A). At 18 days of exposure, there was significant interactive effect of all three factors on SOD activity (three-factor ANOVA, $F=5.5443$, $p=0.0211$; Figure 3-11). Sea fans that were exposed either to solely elevated temperatures or all three treatments had higher SOD activity than the remaining treatment groups (Tukey's HSD, $p<0.05$, Figure 3-10B).

After 18 hours of treatment, no significant interactions between treatments were observed in the POX assay. However, there were significant changes among groups where sea fans exposed to all three treatments had the highest POX activity (Figure 3-12A). After 18 days of treatment, there was synergistic interactive effect was observed between temperature and pathogen (three-factor ANOVA, $F=8.3722$, $p=0.0049$; Figure 3-13). However, post-hoc comparisons did not reveal any significant differences among groups after 18 days of treatment (Figure 3-12B).

After 18 hours of treatment, there was synergistic effect to pathogen-exposure and pH (three-factor ANOVA, $F=10.5471$, $p=0.0017$; Figure 3-15A) and pathogen-exposure and temperature (three-factor ANOVA, $F=5.278$, $p=0.0244$; Figure 3-15B) but no interactive effect was seen between all three factors together. CAT activity was highest in sea fans exposed to elevated temperature, low pH, and the *Aplanochytrium* and was lowest in sea fans exposed to only the *Aplanochytrium* (Figure 3-14A). After 18 days of exposure, there was significant interactive effect of all three factors on CAT activity (three-factor ANOVA, $F=8.2937$, $p=0.0051$). When there was elevated temperature, there was synergistic effect on CAT activity (Figure 3-16). Similar to 18 hours of exposure, sea fans exposed to elevated temperature, acidification, and the *Aplanochytrium* pathogen had the highest level of CAT activity (Figure 3-14).

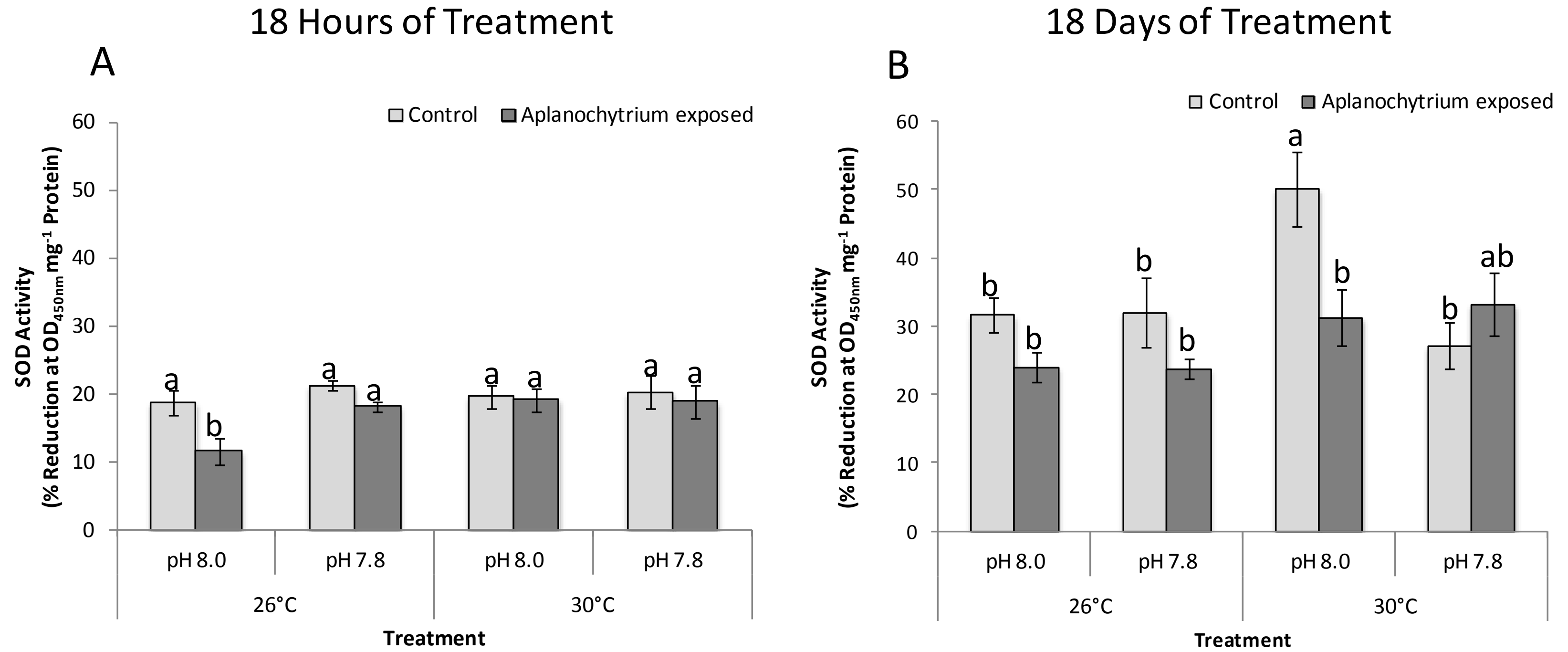

Figure 3-10 Superoxide dismutase activity of the sea fan

Mean (±SE) superoxide dismutase (SOD) activity of sea fan exposed to elevated temperature, acidification and an *Aplanochytrium* pathogen treatments for a period of 18 hours (A) or 18 days (B). Letters indicate statistical significance at p<0.05 in each panel.

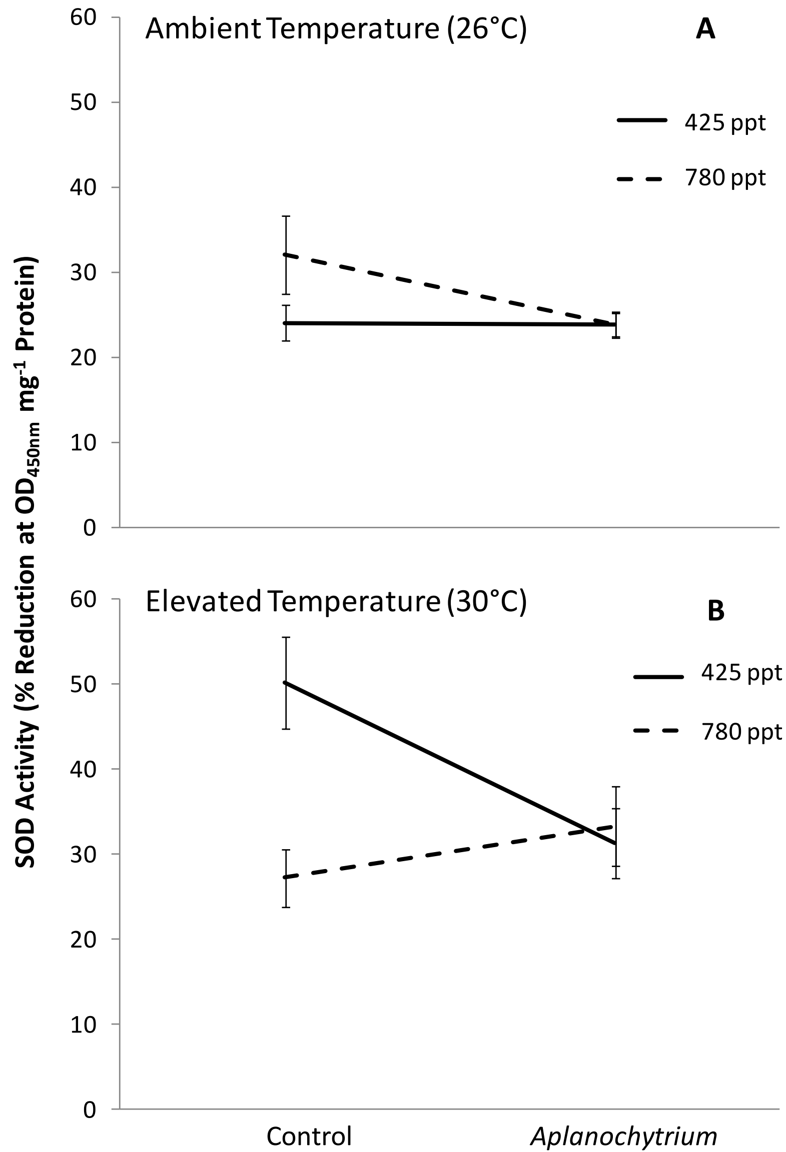

Figure 3-11 Superoxide dismutase activity of the sea fan: interaction plot

Interaction plots of mean (±SE) of superoxide dismutase (SOD) activity of sea fans treated with temperature stress, acidification, and *Aplanochytrium* pathogen-exposure for 18 days. Non-parallel lines indicate a synergistic interactive effect of the factors.

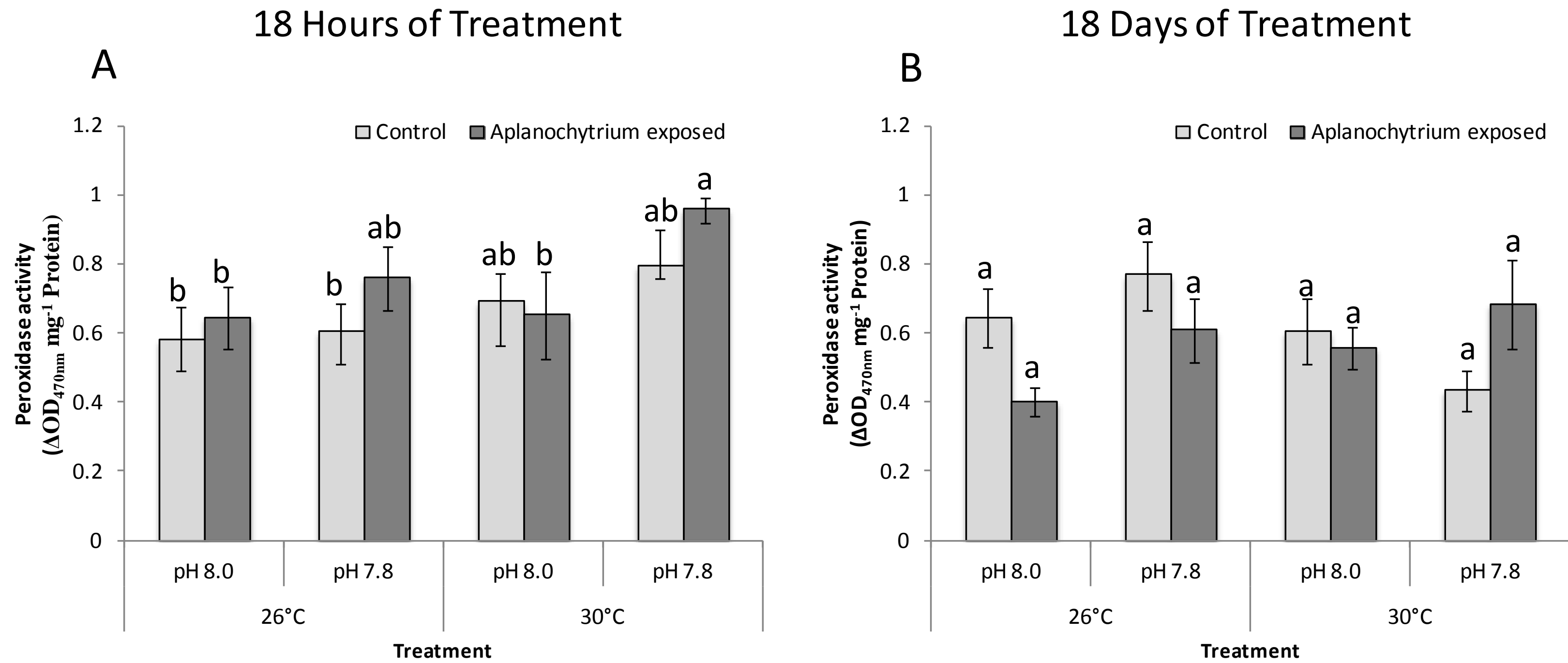

Figure 3-12 Peroxidase activity of the sea fan

Mean (±SE) peroxidase (POX) activity of sea fan exposed to elevated temperature, acidification, and an Aplanochytrium pathogen treatments for a period of 18 hours (A) or 18 days (B). Letters indicate statistical significance at $p<0.05$ in each panel.

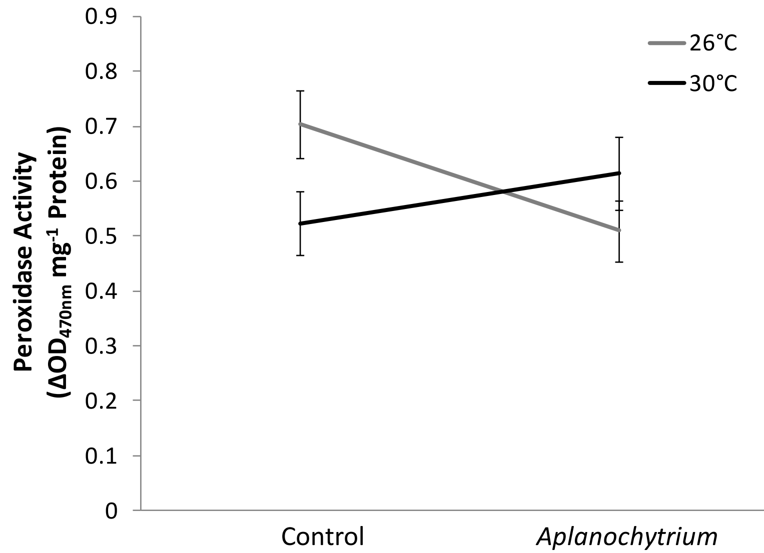

Figure 3-13 Peroxidase activity of the sea fan: interaction plot

Interaction plot of mean (±SE) peroxidase (POX) activity between temperature and *Aplanochytrium* pathogen-exposure factors after 18 days of treatment. Non-parallel lines indicate a synergistic interactive effect of the factors.

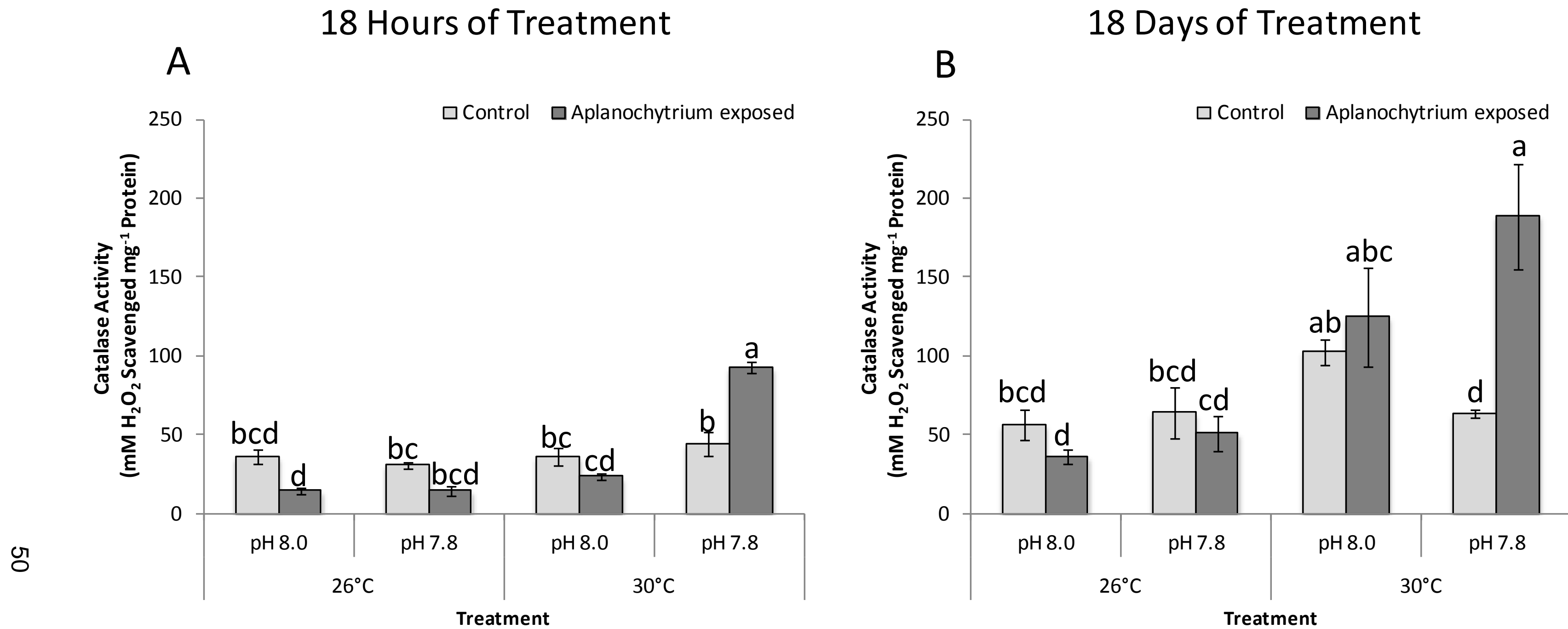

Figure 3-14 Catalase activity of the sea fan

Mean (±SE) catalase (CAT) activity of sea fan exposed to elevated temperature, acidification and an Aplanochytrium pathogen-exposure treatments for a period of 18 hours (A) or 18 days (B). Letters indicate statistical significance at $p<0.05$ in each panel.

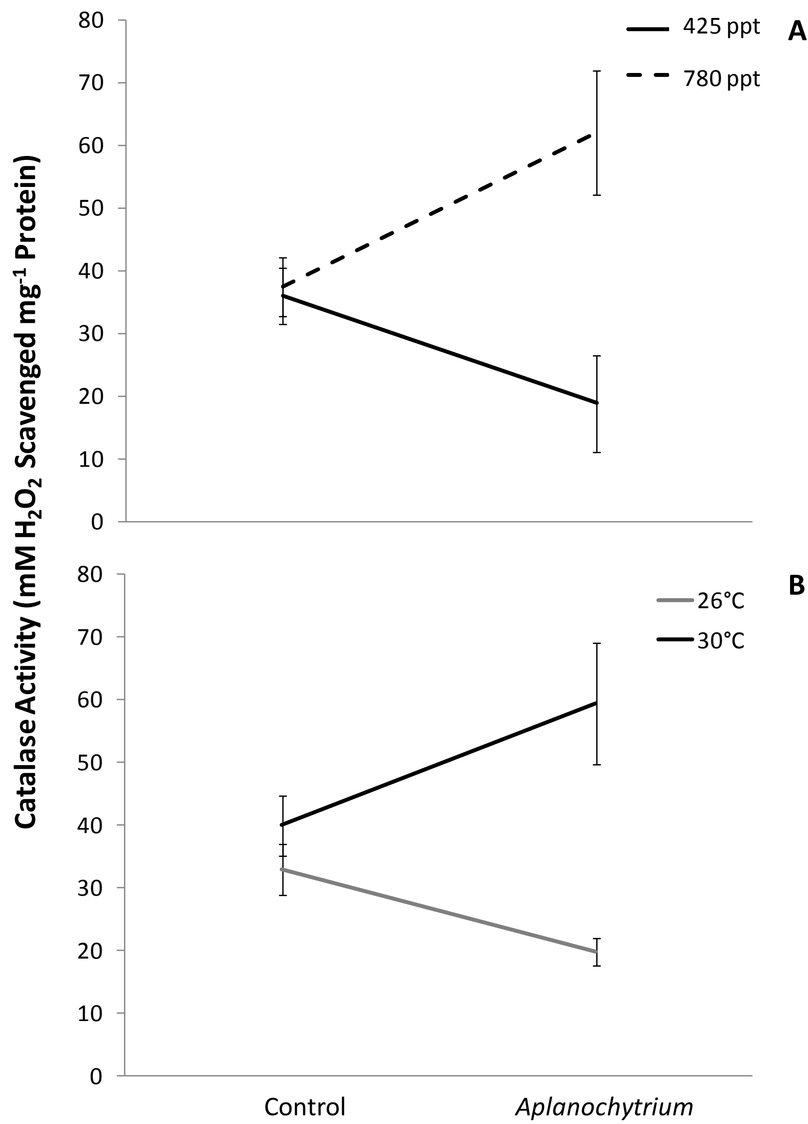

Figure 3-15 Catalase activity of the sea fan: interaction plot (18 hours of treatment)

Interaction plots of mean (±SE) catalase (CAT) activity between (A) acidification and Aplanochytrium pathogen-exposure and (B) temperature and Aplanochytrium pathogen-exposure after 18 hours of treatment. Non-parallel lines indicate a synergistic effect of the factors.

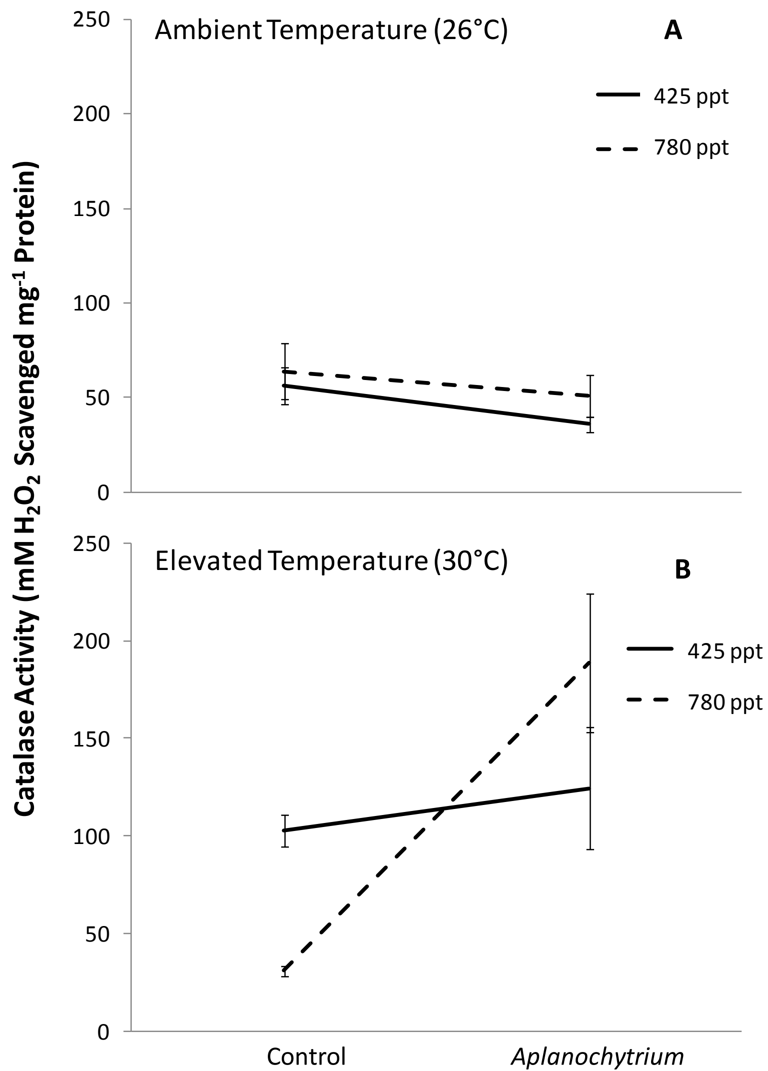

Figure 3-16 Catalase activity of the sea fan: interaction plot (18 days of treatment)

Interaction plots of mean (±SE) catalase (CAT) activity between all three factors temperature, acidification, and *Aplanochytrium* pathogen-exposure after 18 days of treatment. (A) Parallel lines indicate an additive effect between treatments while (B) non-parallel lines indicate a synergistic interactive effect.

Discussion

Changing environmental conditions as consequence of climate change are having significant impact on coral reefs (Wild et al. 2011). Environmental changes, such as increasing sea surface temperatures and ocean acidification, alter the physiology of corals and increase susceptibility towards bleaching and disease as well as compromising recruitment and growth (Mydlarz et al. 2010; Doropoulos et al. 2012). These factors in a natural setting interact and likely have compounding effects. The results of this experiment highlight the interacting effects of environmental factors (elevated temperature and acidity caused by high CO_2) and pathogen exposure (*Aplanochytrium* sp. pathogen) on the immune response of the Caribbean sea fan, *Gorgonia ventalina*.

Contrary to what was predicted, all three immune mechanisms (antimicrobial, anti-pathogenic, and antioxidant) were immediately affected by exposure to the *Aplanochytrium* pathogen at the short-term treatment length (18 hours). Interestingly, there appears to be a suppressive effect of the *Aplanochytrium* on many of the immune measures, particularly in the antimicrobial and anti-pathogenic mechanisms. The effects of environmental stress (elevated temperature and acidification) were not immediately observed with exception of the CAT immunoassay. Catalase enzymes have high stability and abundance in many organisms (Chelikani et al. 2004). As a result, the sea fan may have been exhibiting other changes in immune and/or stress responses to the environmental stressors but CAT activity was the only detectable measure from the immunoassays. Other early responses of the sea fan could have also been detected through gene expression analysis, as seen in other coral studies, but was not examined here (Desalvo et al. 2008).

After the long-term treatment (18 days), the full effect of the three factors (temperature, acidification, and pathogen-exposure) was apparent. The immunosuppressive effect of the *Aplanochytrium* pathogen seen after 18 hours remained evident in many of the immune mechanisms. This was particularly clear in the antimicrobial activity (AB and AF) which was largely hindered by the combination of the *Aplanochytrium* pathogen-exposure and one or both of the environmental stressors. Although several studies have shown decreases in antimicrobial activity of corals under environmental

stressors, (Mydlarz et al. 2009; Meron et al. 2010; Palmer et al. 2011) few have demonstrated a combined suppressive effect of both environmental stress and pathogen-exposure. The suppression of immunity was also seen in anti-pathogenic mechanisms (PPO activity). Conversely, there was overall increase in antioxidant activity in response to both the pathogen and environmental stressors. Although antioxidants can have an important role in the coral immune response (Mydlarz and Harvell 2007), the increases observed here could also be attributed to an overall stress response (Lesser 2006).

Synergistic effects of environmental stress and pathogen-exposure have recently been detailed in the *Pocillopora damicornis* - *Vibrio coralliilyticus* pathosystem where response to *V. coralliilyticus* was suppressed under elevated temperatures but not under ambient elevated temperatures (Vidal-Dupiol et al. 2014). Our results are consistent with Vidal-Dupiol et al. (2014); however, our results also show that throughout the entire experiment there was no clear, elicited immune response caused by the exposure solely to the *Aplanochytrium*. Without considering the environmental stressors, several immune measures (AB, AF, PPO, and SOD) showed a decrease in immune response to the *Aplanochytrium*. A comprehensive transcriptomics analysis of the sea fan exposed to *Aplanochytrium* (24 post infection) indicated both up- and down-regulation of potential immune genes, include down-regulation of a pathogen recognition receptor, neuronal pentraxin-2 and antimicrobial peptide, royalisin (Burge et al. 2013b). A lack of response to a Labyrinthulomycetes pathogen has also been observed in the subtropical sea grass, *Thalassia testudium* (Loucks et al. 2013). In *T. testudium* an immune response, through oxidative bursts, was observed in response to a lipopolysaccharide elicitor but not when exposed to the Labyrinthulomycetes. Considering the opportunistic behavior of many Labyrinthulomycetes spp. as discussed by Burge *et al*. (2013), lack of recognition may common among several marine hosts.

The combination of environmental stressors with a lower immunocompetence towards the *Aplanochytrium* is likely exacerbating this and other disease outbreaks, namely aspergillosis, that affect the sea fan. More recently, co-infections have also been identified in the sea fan with both the *Aplanochytrium* pathogen and *Aspergillus sydowii,* the causative agent of aspergillosis (Petes et al. 2003; Burge et al. 2012). The immunosuppression, particularly of the antifungal activity observed in this study

suggests that in the co-infection of the sea fan, there is likely an initial presence of the *Aplanochytrium* and subsequent infection with *A. sydowii.*

All organisms have an energy budget that is allocated to different components of physiology including immune response, stress response, growth and reproduction (Perrin and Sibly 1993). In ectothermic organisms such as corals, a significant amount of energy can be expended towards maintaining physiological homeostasis and mitigating effects of stressors such as elevated temperature or acidification. Under elevated temperatures, significant trade-off between growth or reproduction and stress response in hard corals has been observed (Baird and Marshall 2002). This is likely true for soft corals as well. Ocean acidification can affect the ability of corals to produce calcium carbonate which consequently can greatly impact skeletal production (Edmunds et al. 2013). Gabay et al. (2014) demonstrate that integrity of sclerites in Alcyonacean soft corals can also be significantly altered by exposure to low pH. These changes to the sclerite integrity significantly impact the overall structure of the soft coral colony. Although Gabay and colleagues show that thicker tissue layers in certain soft corals can buffer against effects of acidification, the sea fan tissue is significantly thinner than many other soft coral species. Therefore, acidified water conditions may be more likely to affect the sea fan than other thicker-tissued gorgonians. In combination, elevated temperature and acidification stress could be causing a shift of the sea fan energy budget from higher investment in stress response (the antioxidant response) to lower investment in costly immune response against the *Aplanochytrium* (antimicrobial or anti-pathogenic response).

It is also likely that both sides of the host-pathogen relationship are affected by environmental stressors. Unlike the host however, the *Aplanochytrium* pathogen may favor acidic pH conditions and elevated temperatures. Several Labyrinthulomycetes species have pH optima between 5-7 (Fan et al. 2002) and Burge et al. (2012) demonstrate in the sea fan that the *Aplanochytrium* sp. strain has optimal growth at 30°C. Exposure to a lower range of pH may be more conducive for growth and/or metabolic activity of the *Aplanochytrium*.

This potential increase in metabolic activity of the *Aplanochytrium* could play an additional role in the suppressed (or lacking) sea fan immune response. If the *Aplanochytrium* are more metabolically active, they may be able to produce immunosuppressive enzymes or proteins that allow for establishment of infection or colonization. For example, the overall decrease in antimicrobial activity (as seen in our study) may also include anti- *Aplanochytrium* defenses and may this may be moderated by the *Aplanochytrium*. Due to the growth patterns of the *Aplanochytrium* however, measure of antimicrobial activity against the *Aplanochytrium* was not possible for this study. Several fungal and fungal-like pathogens are capable of producing these type enzymes to surpass or hide from innate or adaptive host responses. For example, gliotoxin commonly produced by *Aspergillus fumigatus,* can induce apoptosis in mammalian macrophages (Kupfahl et al. 2006) and strongly reduces toll-like receptor activity (Hughes Jr et al. 1990; Hughes Jr et al. 1991). Considering the presence of analogous immune traits in corals, it is possible that the *Aplanochytrium* sp. (which although not true fungi, have some fungus-like characteristics; Tsui et al. (2009)) could also be producing types of immunosuppressive proteins, although further study is necessary.

The results of this study are one of the first to demonstrate how multiple environmental stressors and pathogen-exposure can interact to affect the coral immune response. The combination of environmental stress and pathogen-exposure was shown to suppress the coral immune response and this is likely contributing considerably to the development of disease in corals. Considering the large interactive effect of multiple stressors seen in this study, consideration of multiple stressor effects can provide deeper insight into the physiological responses of corals. Furthermore, with predictions of future climate change scenarios predicating the increase of oceanic temperatures and acidification, these results also implicate a critical need to improve strategies for mitigating the impacts of climate change to coral reef ecosystems.

Chapter 4

Preceding thermal stress causes immunocompromise in Caribbean sea fan, *Gorgonia ventalina*

Introduction

The number of diseases among marine species has been increasing considerably over the past few decades (Altizer et al. 2013). This rise in disease has been especially notable among corals. Coral reef ecosystems are some of the most diverse on the planet and loss of coral populations can have adverse effects to these ecosystems, such as loss of fishery stocks and protection against storms (Hoegh-Guldberg et al. 2007). The number of diseases in corals has also been increasing, with new diseases being identified every few years (Sutherland et al. 2004; Weil et al. 2006). This has made it more crucial than ever to increase our understanding of how corals are responding to pathogens and how subsequent disease develops.

Corals rely on an innate immune system as the first and only line of defense against pathogens. This response system begins with the recognition of a pathogen or foreign invader through special receptors such as toll-like receptors, lectins, and other specialized pathogen recognition receptors. When activated, these receptors initiate signaling cascades which lead to the production of effector responses. Main effector responses in corals include direct antimicrobial, anti-pathogenic, and antioxidant defenses. Corals have a wide range of antimicrobial activity that act as defense against bacterial and/or fungal pathogens (Kim et al. 2000; Gochfeld and Aeby 2008). Anti-pathogenic responses can include the synthesis of melanin and inhibition of pathogen proteases (Mydlarz and Palmer 2011; Mann et al. 2014). Antioxidants (such as peroxidase, catalase, and superoxide dismutase) are also important part of the immune response to neutralize reactive oxygen species resulting from pathogen oxidative bursts or products produced from immune processes such as melanin synthesis (Mydlarz and Jacobs 2006; Mydlarz and Harvell 2007).

Environmental stressors, particularly elevated sea surface temperatures, are assumed to be the largest driver of coral diseases, leading to an immuno-compromised host and/or the exacerbation of pathogen virulence (Mydlarz et al. 2006). Many studies demonstrate adverse effects that thermal stress

can have on host-pathogen dynamics in corals. Elevated water temperatures (>30°C) can affect many components of coral immunity including antimicrobial activity, phagocytosis, and anti-pathogenic or other effector responses (Ward et al. 2007; Mydlarz et al. 2008; Palmer et al. 2011). Higher temperatures also can increase several putative pathogen virulence factors including higher growth rates and increased protease and antioxidant production (Banin et al. 2003; Ward et al. 2007; Mann et al. 2014).

Despite the links made between elevated temperatures and coral disease/immunity thus far, many studies fail to simulate what may truly be driving the development of some diseases and infections. The link between coral disease and thermal stress has been explored largely by the simultaneous exposure to elevated temperature and a pathogen or pathogen-derived elicitor (Palmer et al. 2011; Vidal-Dupiol et al. 2011). However, it is likely that many infections occurring in corals follow a period of stress from elevated sea surface temperatures, water acidification, pollution, or other stressors. Furthermore, many studies have only examined responses in corals with one species of pathogen or particular pathogen elicitor (such as lipopolysaccharide). In truth, corals likely possess responses that are unique to the individual pathogen and additionally, responses to various pathogens may be differently affected by long-term environmental stressors such as elevated temperatures.

The Caribbean sea fan (*Gorgonia ventalina*) serves as an especially interesting model for investigating how thermal stress affects the immune response and how this response can vary between pathogens. In recent years, the two most common diseases in the sea fan have been: aspergillosis caused by the fungal pathogen *Aspergillus sydowii* (Flynn and Weil 2009b) and a newly described disease caused by a protist pathogen from the genus *Aplanochytrium* (Burge et al. 2012). Interestingly, co-infection between the two eukaryotic pathogens has been observed but not thoroughly examined (Petes et al. 2003). In this study, we examine how preceding thermal stress affects the immune responses of the sea fan and how this varies between the two pathogens.

As demonstrated in chapter 3, some immune responses of the sea fan are compromised by the *Aplanochytrium* pathogen. Nonetheless, transcriptomic analysis has shown there can be some response or recognition of the *Aplanochytrium* (Burge et al. 2013b). On the other hand, the sea fan has been

shown to present strong responses to *A. sydowii* (Kim et al. 2000; Mydlarz et al. 2008). Therefore, we hypothesize that under ambient temperatures the sea fan produces an immune response pathogens by increasing immune signaling and antimicrobial, anti-pathogenic, and antioxidant effectors but the degree of response can vary between *Aplanochytrium* and *A. sydowii*. (2) Further, we hypothesize that regardless of pathogen, environmental stressors can compromise the sea fan immune response and unable to fight the pathogen regardless of the type of pathogen.

Methods

Sea fan collection and experimental setup

A full factorial experimental design was utilized to examine the effects preceding and continuing thermal stress on the immune response of *Gorgonia ventalina* to an *Aplanochytrium* sp. (order: Labyrinthulomycetes) or *Aspergillus sydowii* pathogen. Healthy sea fans colonies (n=12) were collected from Media Luna reef (17˚ 56.091 N - 67˚ 02.577 W) in La Parguera, Puerto Rico during November of 2012. Absence of disease was confirmed with histological analysis. 120 cm^2 of tissue was cut from each sea fan colony and brought back to the station, divided into 12, 3x5 cm fragments (two for each collection time point), and held in tanks for a 2 day acclimation period with sand-filtered seawater pumped from two large drums that were filled daily. After acclimation, water was slowed to a rate of 2 L/hr. The remaining (12) fragments were evenly divided across a total of 18 plastic aquariums. Each aquarium had one water pump for aeration (TAAM Inc., USA). Lighting was provided with full-spectrum bulbs, set to a 12 hour day/night cycle.

2 fragments from each colony were assigned to one of the following treatments: (1) *Aplanochytrium* pathogen-exposure, (2) *A. sydowii* pathogen-exposure, (3) Thermal stress, (4) *Aplanochytrium* pathogen-exposure and thermal stress, (5) *A. sydowii* pathogen-exposure and thermal stress, and (6) Control with no pathogen-exposure or thermal stress. For a continuous period of 12 days, samples were exposed to elevated (30-31°C) or ambient temperature (26-27°C). To maintain ambient temperature, a chiller (Drs. Foster and Smith, Rhinelander, WI) was placed in one drum and the water was pumped from this drum into the ambient temperature treatment tanks. Water was pumped into the

elevated temperature treatment tanks from the second drum with no chiller. Elevated temperature was maintained in each individual tank with aquarium heaters (Hydor, Sacramento, California, USA). At day 6 of the experiment, water flow was briefly halted and all fragments were injected using sterile syringes with 500 µl of *A. sydowii* hyphae, ~5000 cells of *Aplanochytrium*, or sterile artificial sea water (as control). Prior to injection, pathogens were grown at 30°C in peptone-yeast-glucose media or QPX media for the *Aplanochytrim* sp. and *A. sydowii*, respectively (Burge et al. 2012; Mann et al. 2014). Injections were performed when the cultures had reached exponential phase of growth. Both pathogen strains were obtained courtesy of the Harvell lab at Cornell University. The strain of *A. sydowii* was isolated from a sea fan in San Salvador as described in Rypien et al. (2008). The *Aplanochytrium* sp. was isolated from a sea fan in Puerto Rico as described by Burge et al. (2012). At 1 d post-injection, half of the sample fragments from each treatment were collected. At the end of the experiment (6 days post-inoculation), the remainder of each of the samples was collected. During each collection point, samples were immediately flash frozen in liquid N_2 for biochemical and molecular analysis. Frozen samples were shipped back to UTA on dry ice.

Protein and RNA extractions

Coral fragments (skeleton, polyps and connective tissue) were ground to a powder in liquid nitrogen with a mortar and pestle. For the melanin assay (described below), a small portion of the frozen powder was placed in a pre-weighed 1.5 ml centrifuge tube and lyophilized for 48 hr. Afterwards, the weight of the lyophilized tissue was calculated and recorded. The remainder of the frozen powder was immediately placed in approximately 2 ml of 100 mM sodium phosphate buffer (PBS), pH 7.8 and incubated on ice for 45 min. The mixture was centrifuged at 2205xg at 4°C in an Eppendorf 5810R for 10 min and the supernatant was recovered into a 1.5 ml tube and kept frozen at -80°C between all assays. Total protein content was quantified with the Red660 protein assay using a standardized concentration curve of bovine serum albumin (G Biosciences, St. Louis, MO). All biochemical assays were standardized to protein concentration of the coral extracts. All assays were performed with a Synergy 2 spectrometer (Biotek, Winooski, VT) and ran in duplicates for each sample.

A subset of 6 sea fan colony fragments was utilized from the experiment for gene expression analysis. RNA was extracted using a RNeasy (Qiagen) and Trizol (Life Technologies) hybrid protocol as per Burge et al. (2013b). Genomic DNA was removed using DNAse I kit (Life Technologies). RNA quantification was performed on a Nanodrop spectrometer (Thermo Scientific) and samples were diluted to 12.5 ng/µl. Reverse transcription was performed on 50 ng RNA using a Smartscribe Kit (Clontech). Samples of cDNA were diluted to a final concentration of 1 ng/µl for qPCR amplification.

Immune signaling (gene expression)

Three immune-related genes of interest were used to measure immune signaling: Tachylectin – pathogen-associated molecular pattern receptor (Hayes et al. 2010), Peroxidasin – antioxidant (Shi et al. 2012), and Leucine Rich Kinase – common component for toll-like receptors (Roux et al. 2011). An Elongation Factor gene was used as a reference gene for relative quantification. All genes were obtained from transcriptomic data available from Burge et al. (2013b).

Quantitative PCR reactions (25 µl) were performed in duplicate in 96-well PCR plates in an ABI 7300 Real-Time PCR machine (Applied Biosystems, Life Technologies, Grand Island, NY). Each reaction contained 10 nM of each primer, 2 µl of cDNA template, and 7.5 µl of SYBR® GreenER qPCR SuperMix (Life Technologies). Negative controls (with no reverse transcription) were also run to confirm no amplification was from a genomic DNA source. The reaction conditions were: 95ºC for 20 s, followed by 40 cycles of 95ºC for 3 s and 65ºC for 3 s. After the reaction, a melting curve analysis was performed to confirm amplification of only one product. Relative expression (abundance) was standardized to the reference gene (Elongation Factor) and was calculated using the individual efficiencies of each gene as described by Pfaffl (2001).

Direct antimicrobial defense (biochemical immunoassays)

Antibacterial (AB) Assay. Antibacterial assay was conducted against a marine strain of *Vibrio alginolyticus* (courtesy from K. Ritchie, Mote Marine laboratory). 10 µl of coral extract (diluted to 1 mg ml^{-1}) were added to 140 µl of *V. alginolyticus* culture growing at exponential phase to a sterile 96 well transparent microplate (Greiner Bio-One, Monroe, NC). The plate was incubated at 30°C and the optical

density (OD) was read every 10 min for 6 hrs using a spectrophotometer (BioTek). Optical density readings from the log phase growth period were used to calculate generation time of *V. alginolyticus*. From the data, higher generation times indicated higher antibacterial activity from the coral extract.

Antifungal (AF) Assay. Antifungal activity against a marine strain of *Aspergillus sydowii* (isolated from a sea fan in San Salvador) was performed. 140 µl of *A. sydowii* (100,000 spores ml^{-1}, quantified with a hemacytometer) was added to each well of a 96 well transparent sterile microplate (Greiner Bio-One) and incubated for 24 hrs at 30°C to allow for hyphea germination. 10 µl of coral extract (diluted to 1 mg ml^{-1} protein) was then added to the culture and the OD was read every 2 hrs for 48 hrs at 600 nm using a spectrometer (BioTek). Growth curves were created from the OD readings and the growth rate was calculated from the exponential slope of the curves. A higher growth rate indicates lower antifungal activity of the sea fan extract (and vice versa).

Anti-pathogenic defense (biochemical immunoassays)

Prophenoloxidase (PPO) Assay. 20 µl of coral extract was added to 30 µl of 100 mM PBS pH 7.8 and 10 µl of 0.01 mg ml^{-1} trypsin (Sigma Aldrich) in a 96-well microtiter transparent plate (Greiner Bio-One) and incubated at room temperature for 30 min. To initiate the reaction, 10 mM L-1,3-dihydroxphenylalanine (L-dopa, Sigma-Aldrich) was then added to each well and the OD was read at 490 nm every 30 s for 25 min. Rate of reaction was read by calculating slope during the linear phase of each reaction.

Melanin Concentration (MEL) Assay. To extract melanin, 1 ml of 10 M NaOH was added to each tube of lyophilized sample and each sample tube was vortexed twice a day, for two days. The tubes were then centrifuged at 5590xg in a micro-centrifuge (Baxter Scientific Products, Deerfield, Illinois, USA) and the supernatant liquid was pipetted into a 96 well transparent plate and absorption was read at 490 nm. Melanin concentration was calculated using a standard curve made from 2 mg/ml of commercial melanin (Sigma-Aldrich). Samples were standardized to the total weight of the lyophilized tissue.

Protease Inhibitor (PI) Assay. The protease inhibitor assay was performed using a modified version of Twining (1984). 10 µL of sea fan extract (or 100 mM PBS, pH 7.8 as a control) were incubated

with 10 µL trypsin (0.1 mg mL^{-1}) (Sigma-Aldrich) for 30 min at room temperature. The sea fan extract/protease mixture was then added to 40 µL 2.5% (w/v) FITC-casein substrate (Sigma-Aldrich) in 20 mM PBS, 150 mM NaCl, pH 7.6 and incubated for 30 min at 37°C. The reaction was stopped with 10% trichloroacetic acid to precipitate remaining proteins. Proteins were centrifuged down at 5590xg (Baxter Scientific Products) and the fluorescence of the supernatant was observed with excitation at 485 nm and emission wavelength at 535 nm in a black 96 well plate (Greiner Bio-One). Percent inhibition of the protease activity was calculated against the control (PBS buffer only).

Antioxidant defense (biochemical immunoassays)

Peroxidase (POX) Assay. 10 µl of coral extract was added to 50 µl of 50 mM PBS pH 6 and 40 µl of 25 mM guaiacol (Sigma Aldrich, St. Louis, MO) in a transparent 96 well plate (Greiner Bio-One, Monroe, NC). The reaction was initiated by adding H_2O_2 to a final concentration of 10 mM and the optical density (OD) was read at 470 nm every 30 s for 15 min. Rate of reaction was determined by calculating slope during the linear phase of each reaction.

Catalase (CAT) Assay. 5 µl coral extract was added to 70 µl of 50 mM PBS pH 7 in a UV transparent 96-half well plate (Greiner Bio-One). The reaction was started immediately by adding 50 µl of 50 mM H_2O_2 and the OD was read every 30 s for 15 min at 240 nm. A standard curve was made from a two-fold serial dilution of 50 mM H_2O_2 and used to quantify the moles of H_2O_2 scavenged during the linear phase of the reaction. The change in H_2O_2 concentration was calculated by subtracting the final mM of H_2O_2 from the initial mM of H_2O_2.

Superoxide Dismutase (SOD) Assay. SOD activity was measured using the SOD Assay Kit (Sigma-Aldrich). 10 µl of coral extract was diluted with 10 µl sterile deionized water and added to Dojindo's highly water-soluble tetrazolium salt, (WST-1; 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) and xanthine oxidase in a transparent 96-well plate (Greiner Bio One) and incubated for 30 min at 37°C. After incubation, the OD was read at 450 nm. Activity was quantified by calculating percent reduction of superoxide ion present at the endpoint.

Data Analysis

All data sets were tested for normality and unequal variances using the Shapiro-Wilk and Brown-Forsythe tests, respectively. Gene expression data (immune signaling) were initially non-normally distributed and were transformed using Box-Cox power transformation method and normality was achieved upon transformation. Multivariate analysis of variance (MANOVA) for each treatment length (1 day post-inoculation and 6 days post-inoculation) was performed using the immunoassay or gene expression data to determine the effects of thermal stress and pathogen-exposure on the overall immune response and on each immune mechanism (immune signaling, direct antimicrobial, anti-pathogenic, and antioxidant). Two-factor univariate analysis of variance (ANOVA) was also performed to determine effects of these factors for each individual immunoassay or gene of interest. From the MANOVA and two-factor ANOVAs, the highest level of interaction with significance at $p<0.05$ was used to make inferences about the data. Additionally, post-hoc multiple comparisons were also used to determine differences among treatment groups (Tukey's HSD). All statistical analyses were performed using JMP Statistical Software Version 10.0 (SAS, Cary, NC).

Results

Overall Immunity

At 1 day post-inoculation (Table 1), both thermal stress and pathogen-exposure had interactive effect on overall immunity in the immunoassays (Wilks' Lambda, $F=4.8804$, $p<.0001$). However, there was no significant effect of the stressors on immune signaling alone. There was significant interactive effect of thermal stress and pathogen-exposure on direct antimicrobial defense (Wilks' Lambda, $F=2.9760$, $p<.0001$) and antioxidant defense (Wilks' Lambda, $F=3.7840$, $p=0.0017$; Table 1) but no effect on anti-pathogenic defense.

At 6 days post-inoculation (Table 2), there was also significant (but not interactive) effect of thermal stress and pathogen-exposure on overall immunity (Wilks' Lambda, thermal stress, $F=116.9028$, $p<.0001$, and Wilks' Lambda, pathogen-exposure, $F=2.1168$, $p=0.0122$). There was interactive effect between the two factors on both immune signaling (Wilks' Lambda, $F=4.1645$, $p=0.0018$) and

antimicrobial defense (Wilks' Lambda, $F=2.9760$, $p=0.0217$). Thermal stress was the only factor to significantly affect anti-pathogenic defense (Wilks' Lambda, $F=7.3426$, $p=0.0003$). Both thermal stress and pathogen-exposure had significant (but not interactive) effect on antioxidant activity (Wilks' Lambda, thermal stress, $F=46.1448$, $p<.0001$, Wilks' Lambda, pathogen-exposure, $F=2.7112$, $p=0.0165$).

Table 4-1 Whole model MANOVA and univariate ANOVA 1 day post-inoculation
Whole model multivariate analysis of variance (MANOVA) and univariate analysis of variance (ANOVA) results for each immune parameter and each treatment effect after 1 day of inoculation of *Aplanochytrium*.

		Whole Model			Temperature		
		F	d.f	p	F	d.f	p
	MANOVA (Wilk's Lambda)						
	Biochemical Immunoassays	3.8260	40	**<.0001**	18.1188	8	**<.0001**
	Gene Expression	1.0828	15	0.3926	0.2962	3	0.8277
Immune Mechanism	Univariate ANOVA						
Signaling	Tachylectin	0.5333	5	0.7488	0.9245	1	0.3486
(gene expression)	Peroxidasin	1.3032	5	0.2987	0.2411	1	0.6262
	Luecine Rich Kinase	1.8740	5	0.1400	0.0048	1	0.9452
Antimicrobial	Antibacterial	37.0984	5	**<.0001**	137.2613	1	**<.0001**
	Antifungal	23.2101	5	**<.0001**	95.0211	1	**<.0001**
Anti-pathogenic	Prophenoloxidase	3.2328	5	**0.0134**	6.7026	1	**0.0162**
	Melanin	0.7353	5	0.6005	1.4929	1	0.2276
	Protease Inhibitor	0.6097	5	0.6929	0.0139	1	0.9068
Antioxidant	Peroxidase	7.8879	5	**<.0001**	24.3260	1	**<.0001**
	Catalase	14.0387	5	**<.0001**	27.5515	1	**<.0001**
	Superoxide Dismutase	1.5516	5	0.1914	3.3378	1	0.0738

Table 4-1 Continued.

Immune Mechanism		Pathogen-exposure			Temperature x Pathogen-exposure		
		F	d.f	p	F	d.f	p
	MANOVA (Wilk's Lambda)						
	Biochemical Immunoassays	2.2813	16	**0.0084**	3.6758	16	**<.0001**
	Gene Expression	1.1187	6	0.3689	1.4593	6	0.2169
Immune Mechanism	Univariate ANOVA						
Signaling	Tachylectin	0.5019	2	0.6122	0.0868	2	0.9172
(gene expression)	Peroxidasin	1.0905	2	0.3535	2.4690	2	0.1078
	Luecine Rich Kinase	2.2074	2	0.1338	2.4403	2	0.1104
Antimicrobial	Antibacterial	8.4779	2	**0.0007**	12.4750	2	**<.0001**
	Antifungal	2.7603	2	0.0731	7.3281	2	**0.0016**
Anti-pathogenic	Prophenoloxidase	3.8645	2	**0.0276**	3.5746	2	**0.0356**
	Melanin	0.2594	2	0.7726	1.2071	2	0.3078
	Protease Inhibitor	1.3508	2	0.2685	0.2336	2	0.7925
Antioxidant	Peroxidase	2.0807	2	0.1357	5.4930	2	**0.0070**
	Catalase	4.1487	2	**0.0217**	11.9135	2	**<.0001**
	Superoxide Dismutase	2.0254	2	0.1428	1.0635	2	0.3531

Table 4-2 Whole model MANOVA and univariate ANOVA 6 days post-inoculation
Whole model multivariate analysis of variance (MANOVA) and univariate analysis of variance (ANOVA) results for each immune parameter and each treatment effect after 6 days of inoculation of the *Aplanochytrium* pathogen.

		Whole Model			Temperature		
		F	d.f.	p	F	d.f.	p
	MANOVA (Wilk's Lambda)						
	Biochemical Immunoassays	5.134	40	**<.0001**	70.9291	8	**<.0001**
	Gene Expression	5.6233	15	**<.0001**	2.9669	3	**<.0001**
Immune Mechanism	Univariate ANOVA						
Signaling	Tachylectin	5.0001	5	**0.0023**	5.5415	1	**0.0261**
(gene expression)	Peroxidasin	3.8676	5	**0.0090**	4.4965	1	**0.0433**
	Leucine Rich Kinase	14.0708	5	**<.0001**	0.0019	1	0.9652
Antimicrobial	Antibacterial	96.6919	5	**<.0001**	477.0181	1	**<.0001**
	Antifungal	30.9313	5	**<.0001**	153.4942	1	**<.0001**
Anti-pathogenic	Prophenoloxidase	0.2583	5	0.9341	0.0108	1	0.9175
	Melanin	4.6113	5	**0.0012**	18.0551	1	**<.0001**
	Protease Inhibitor	1.4995	5	0.2025	0.4496	1	0.5049
Antioxidant	Peroxidase	20.7685	5	**<.0001**	94.3726	1	**<.0001**
	Catalase	15.1700	5	**<.0001**	70.4462	1	**<.0001**
	Superoxide Dismutase	3.0258	5	**0.0163**	2.4213	1	0.1246

Table 4-2 Continued.

Immune Mechanism		Pathogen-exposure F	d.f.	p	Temperature x Pathogen-exposure F	d.f.	p
	MANOVA (Wilk's Lambda)						
	Biochemical Immunoassays	2.0368	16	**0.0307**	1.6689	16	0.0885
	Gene Expression	8.5957	6	**<.0001**	4.1645	6	**0.0018**
Immune Mechanism	Univariate ANOVA						
Signaling	Tachylectin	2.1573	2	0.1352	7.2870	2	**0.0029**
(gene expression)	Peroxidasin	3.8671	2	**0.0339**	3.2844	2	0.0529
	Leucine Rich Kinase	22.9585	2	**<.0001**	11.8382	2	**0.0002**
Antimicrobial	Antibacterial	0.7848	2	0.4605	1.3692	2	0.2617
	Antifungal	0.0597	2	0.9426	0.3364	2	0.7156
Anti-pathogenic	Prophenoloxidase	0.6161	2	0.5432	0.0275	2	0.9729
	Melanin	1.5131	2	0.2280	0.8936	2	0.4142
	Protease Inhibitor	3.4857	2	**0.0366**	0.1011	2	0.9040
Antioxidant	Peroxidase	3.8601	2	**0.0261**	0.1269	2	0.8811
	Catalase	2.8029	2	0.0681	0.4726	2	0.6255
	Superoxide Dismutase	8.5670	2	**0.0046**	0.6202	2	0.5411

Immune signaling

At 1 day post-inoculation neither thermal stress or pathogen-exposure had effect on the expression of tachylectin, peroxidasin, or leucine rich kinase. However, expression of all three genes was significantly affected by one or both treatments at 6 days post-inoculation.

Thermal stress and pathogen-exposure had interactive effect on tachylectin (two-factor ANOVA, $F=7.2870$, $p=0.0029$). Although insignificant from the Tukey's HSD test, expression of tachylectin at 6 days post-inoculation was higher in sea fans exposed to the *Aplanochytrium* than sea fans exposed to *A. sydowii* or the control under ambient temperature (Figure 4-1A). Further, sea fans had the lowest expression of tachylectin when both pathogen and thermal stress were combined.

Both factors also had significant (but not interactive effect) on peroxidasin activity (thermal stress, two-factor ANOVA, $F=4.4965$, $p=0.0433$; pathogen-exposure, two-factor ANOVA, $F=3.8471$, $p=0.0339$). The combination of thermal stress and pathogen-exposure appears to synergistically lower expression of peroxidasin, particularly when the sea fan was exposed to the *Aplanochytrium* (Tukey's HSD, $p<0.05$, Figure 4-2). Although slightly insignificant, there is interactive effect of thermal stress and pathogen-exposure on peroxidasin (two-factor ANOVA, $F=3.2844$, $p=0.0529$).

Thermal stress and pathogen-exposure had interactive effect on expression of leucine rich kinase (two-factor ANOVA, $F=4.3540$, $p=0.0180$). Expression of leucine rich kinase was the highest in sea fans exposed to thermal stress and no pathogen. In addition, similar patterns of expression were also seen in leucine rich kinase as seen in the expression of tachylectin: sea fans had higher expression of leucine rich kinase when exposed to the *Aplanochytrium* under ambient temperature but exposure to either pathogen under thermal stress hindered expression (Tukey's HSD, $p<0.05$, Figure 4-3).

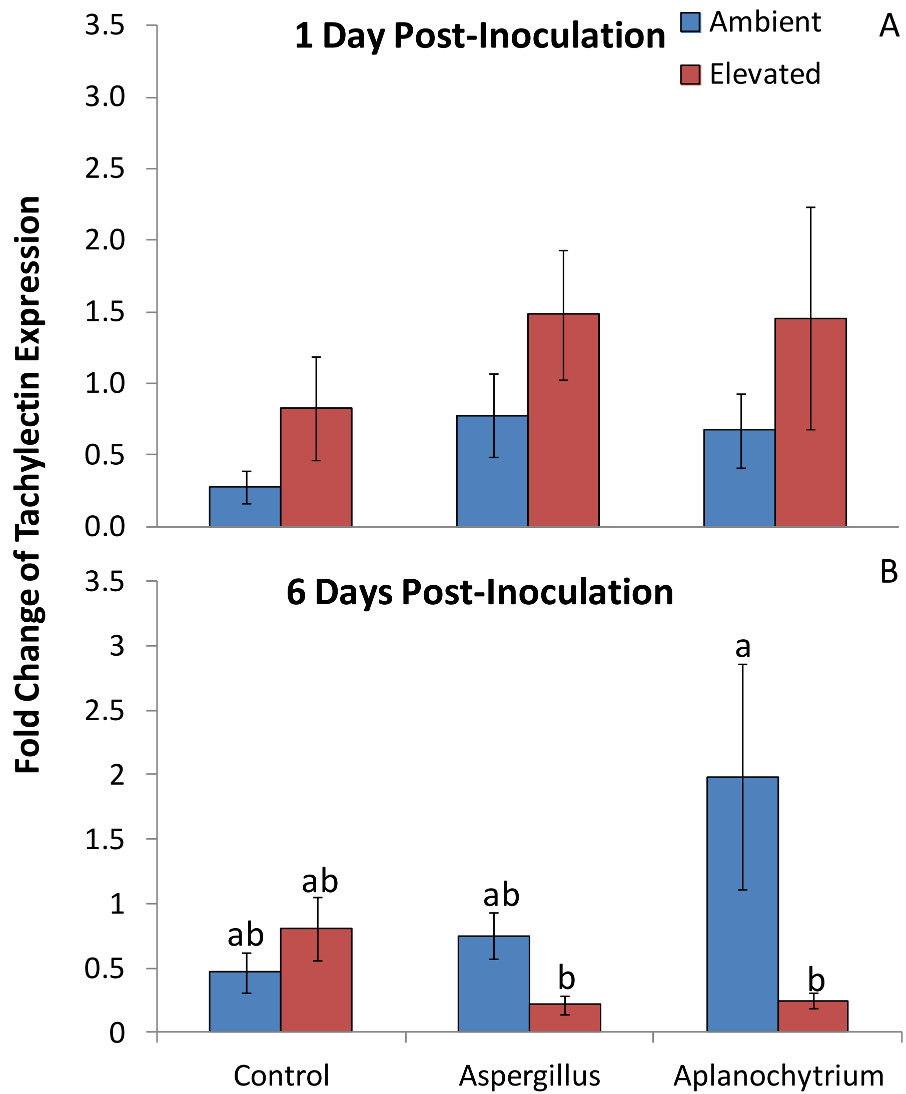

Figure 4-1 Expression of tachylectin in the sea fan

Mean (±SE) fold changes in expression of tachylectin of sea fans exposed to either of two pathogens (an *Aplanochytrium* sp. or *Aspergillus sydowii*) following a period of thermal stress. Thermal stress also continued after inoculation. Fragments were either sampled at 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD). Panels without letters have no significance.

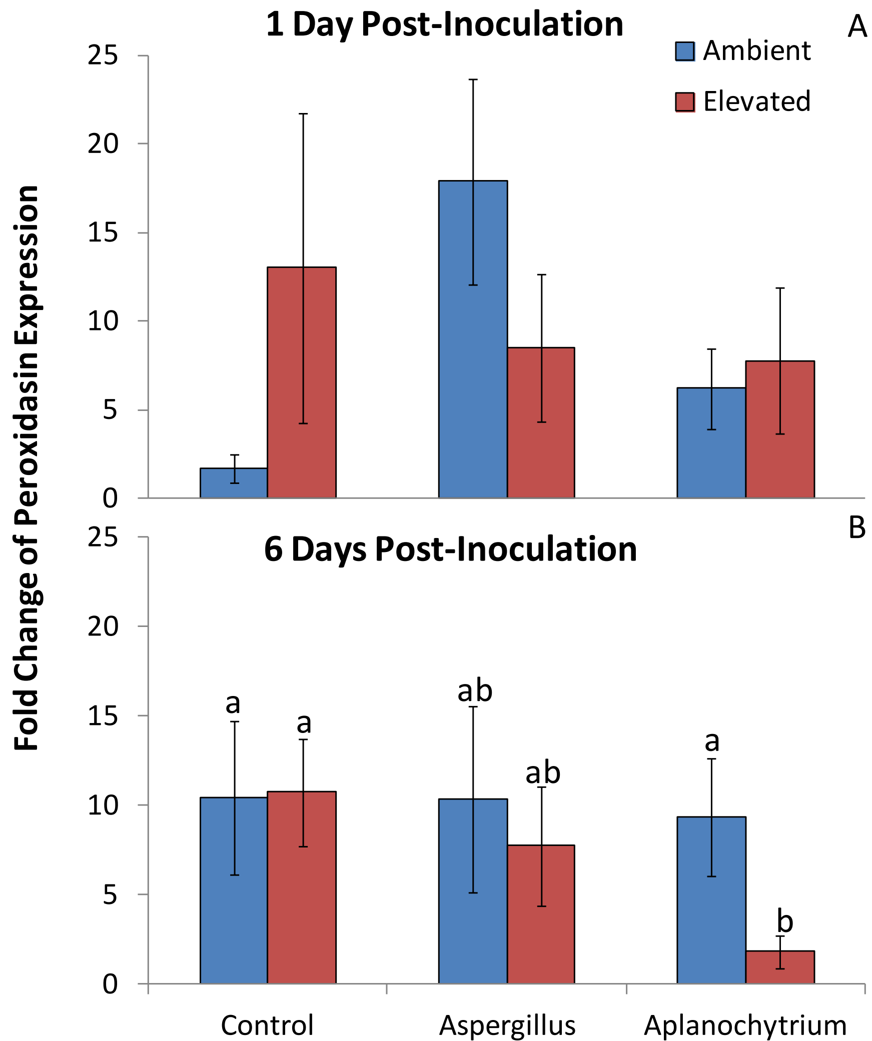

Figure 4-2 Expression of peroxidasin in the sea fan

Mean (±SE) fold changes in expression of peroxidasin of sea fans exposed to either of two pathogens (an Aplanochytrium sp. or Aspergillus sydowii) following a period of thermal stress. Thermal stress also continued after inoculation. Fragments were either sampled at 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD). Panels without letters have no significance.

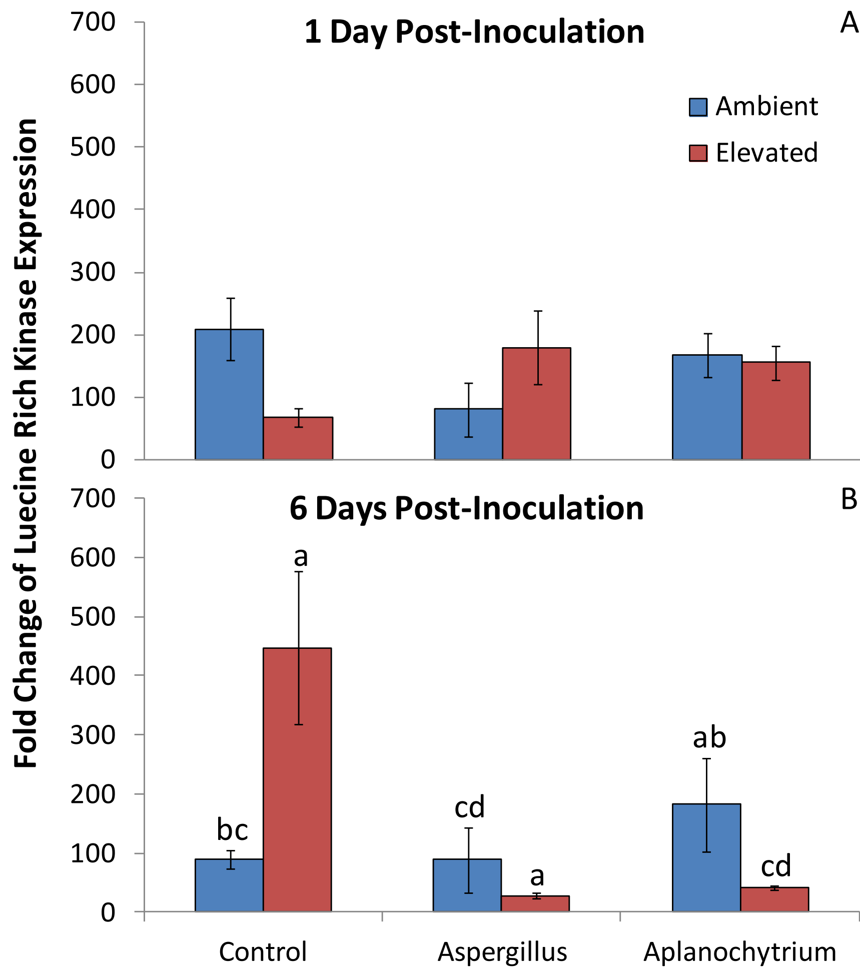

Figure 4-3 Expression of leucine rich kinase in the sea fan

Mean (±SE) fold changes in expression of leucine rich kinase of sea fans exposed to either of two pathogens (an *Aplanochytrium* sp. or *Aspergillus sydowii*) following a period of thermal stress. Thermal stress also continued after inoculation. Fragments were either sampled at 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD). Panels without letters have no significance.

Direct antimicrobial defense

There was significant interactive effect between thermal stress and pathogen-exposure on AB activity at 1 day post-inoculation (two-factor ANOVA, F=12.4750, p<.0001). Thermal stress decreased AB activity in the control sea fans and sea fans exposed to the *Aplanochytrium* but not *A. sydowii* (Tukey's HSD, p<0.05, Figure 4-5A). There was also significant interaction between thermal stress and pathogen-exposure on AF at 1 day post-inoculation (two-factor ANOVA, F=7.3281, p=0.0016). Under thermal stress, AF activity was overall lower than under ambient temperature. However, under thermal stress AF activity was higher in sea fans exposed to *Aspergillus* than the *Aplanochytrium* or the control (Tukey's HSD, p<0.05, Figure 4-5B).

At 6 days post-inoculation thermal stress was the only factor to strongly affect AB or AF activity (AB – ANOVA, F=477.0181, p<0.001; AF – ANOVA, F=153.4942, p<0.001). Overall, thermal stress decreased AB activity regardless of pathogen-exposure (Tukey's HSD, p<0.05, Figure 4-4B) and had similar effect to AF activity (Tukey's HSD, p<0.05, Figure 4-5B).

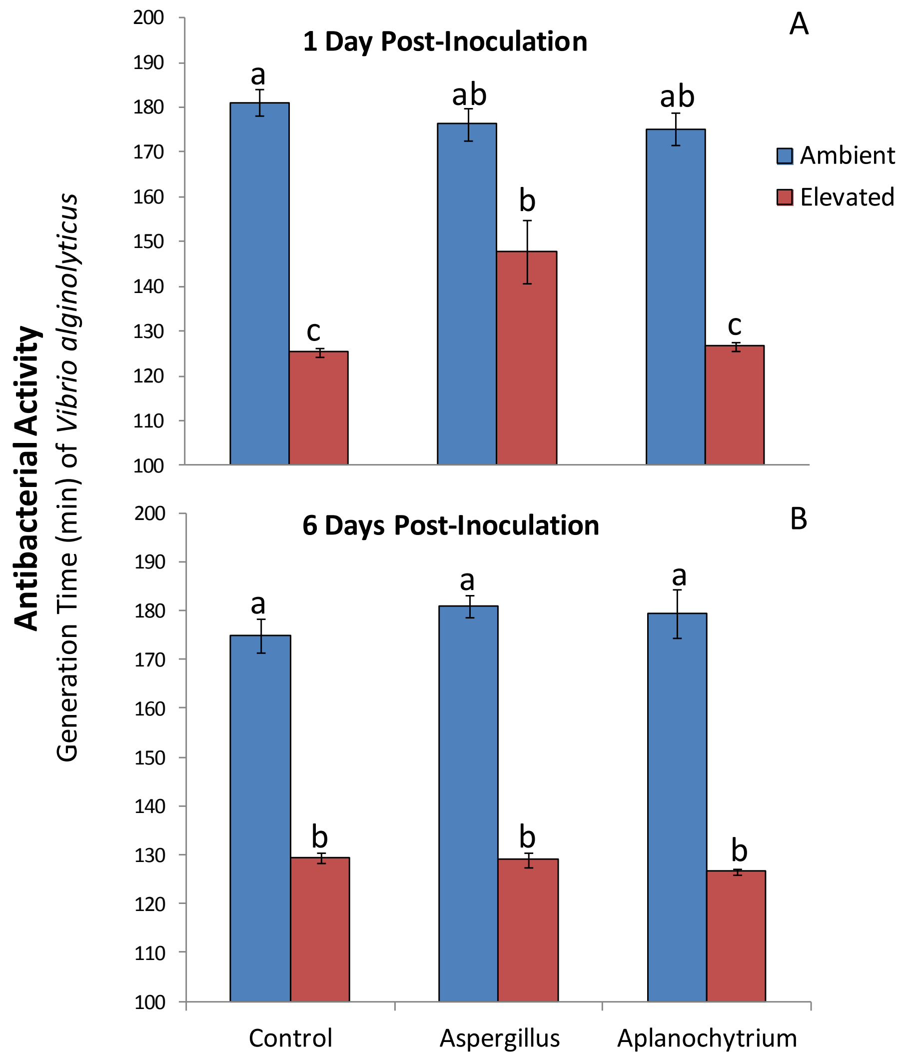

Figure 4-4 Antibacterial activity of the sea fan

Mean (±SE) antibacterial of sea fans exposed to either of two pathogens (an *Aplanochytrium* sp. or *Aspergillus sydowii*) following a period of thermal stress. Thermal stress also continued after inoculation. Fragments were either sampled at 1 day post-inoculation (A) or 6 days post-inoculation (B). Slower generation time indicates higher antibacterial activity. Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD).

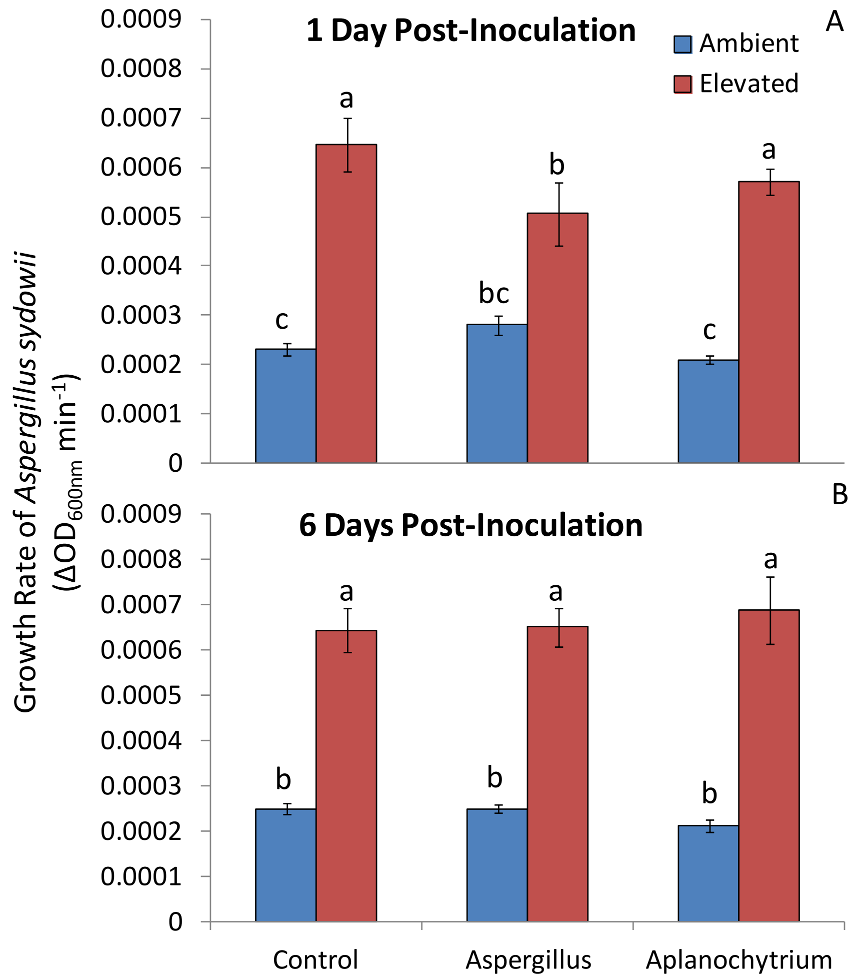

Figure 4-5 Antifungal activity of the sea fan

Mean (±SE) growth rate of *Aspergillus sydowii* in the presence extracts from sea fans exposed to either of two pathogens (an *Aplanochytrium* sp. or *Aspergillus sydowii*) following a period of thermal stress. Thermal stress also continued after inoculation. Fragments were either sampled at 1 day post-inoculation (A) or 6 days post-inoculation (B). Higher values indicate high growth rates but lower antifungal activity. Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD).

Anti-pathogenic defense

At 1 day post-inoculation, PPO activity was the only immune measure affected. There was significant interactive effect between thermal stress and pathogen-exposure on PPO activity (two-factor ANOVA, F=3.5746, p=0.0356). Thermal stress increased PPO activity but only in the control sea fans with no exposure to either pathogen (Tukey's HSD, p<0.05, Figure 2-1A).

PPO activity did not significantly change at 6 days post-inoculation but did appear overall higher than at 1 day post-inoculation. MEL activity was significantly increased under thermal stress at 6 days post-inoculation (ANOVA, F=18.0551, p<0.0001). Although not significant in the post-hoc analysis, there is higher MEL activity in the thermally stressed sea fans exposed to *A. sydowii* than the *Aplanochytrium* or control (Figure 4-7). Pathogen-exposure had significant effect on PI activity at 6 days post-inoculation (two-factor ANOVA, F=3.4857, p=0.0366). PI activity was higher in sea fans exposed to either pathogen than the control and was the highest when exposed to the *Aplanochytrium* (Tukey's HSD, p<0.05, Figure 4-8B).

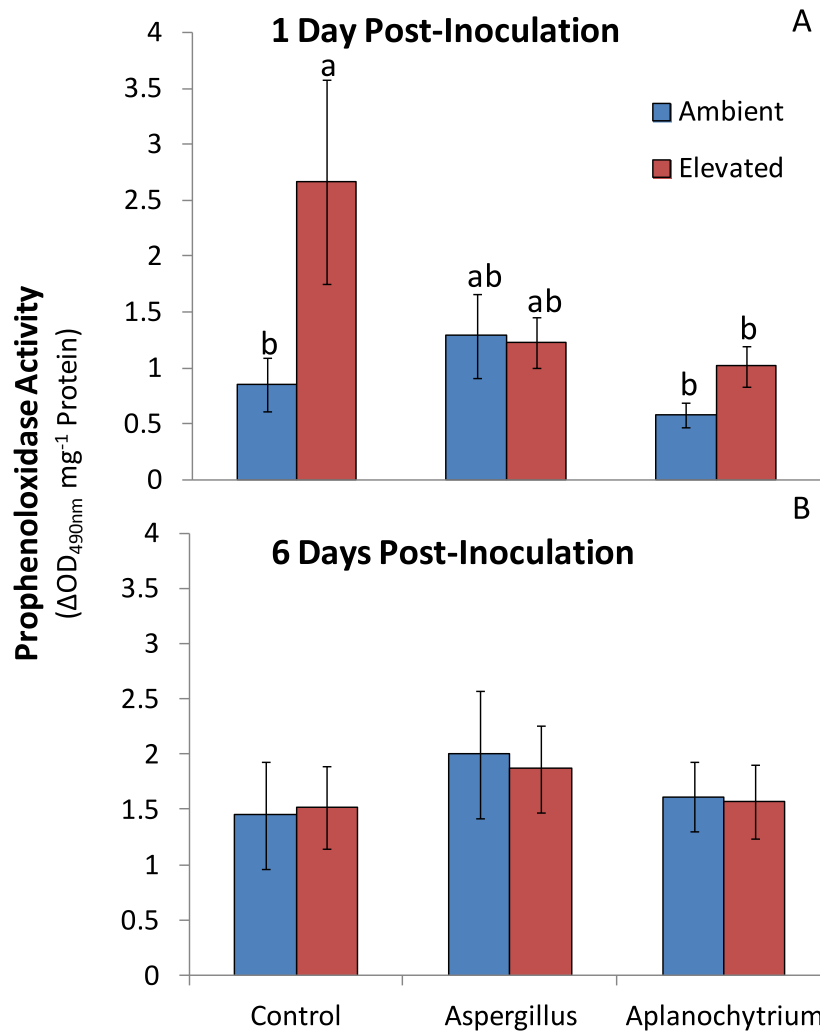

Figure 4-6 Prophenoloxidase activity of the sea fan

Mean (±SE) prophenoloxidase activity of sea fans exposed to either of two pathogens (*Aspergillus sydowii* or an *Aplanochytrium* sp.) after a period of thermal stress. Thermal stress also continued after inoculation. Fragments were sampled at either 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD). Panels without letters have no significance.

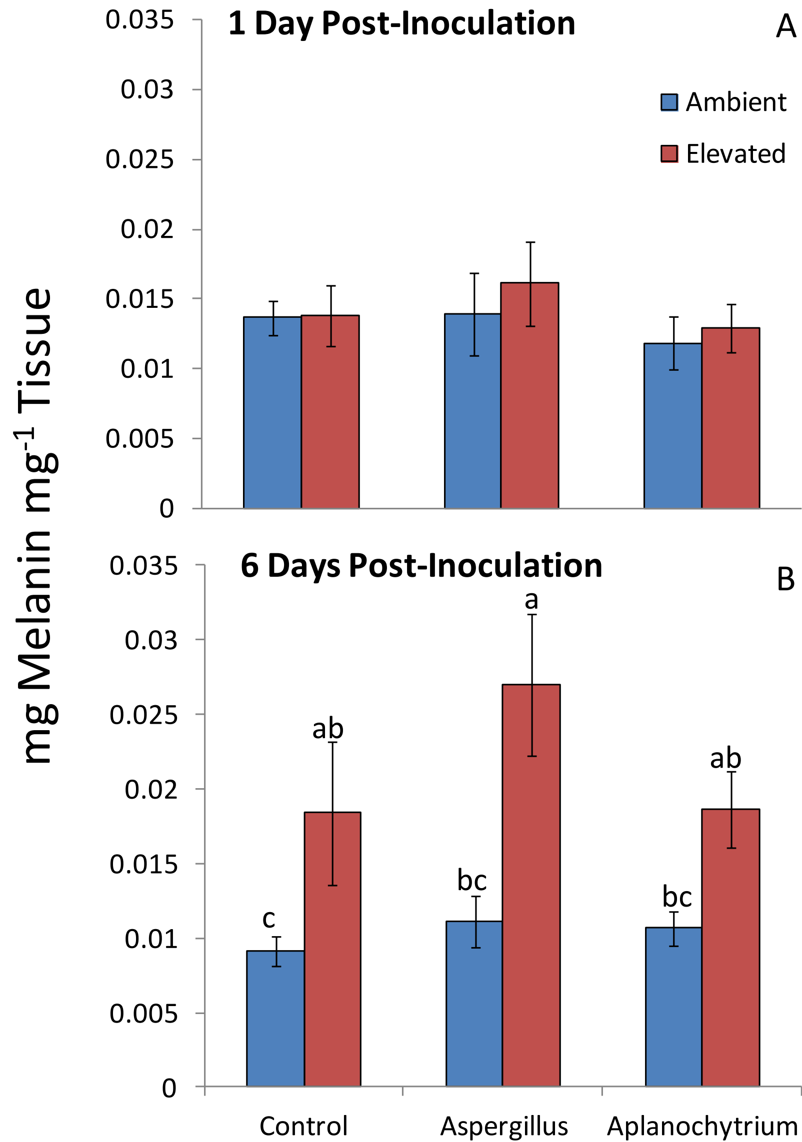

Figure 4-7 Melanin concentration of the sea fan

Mean (±SE) melanin concentration in sea fans exposed to either of two pathogens (*Aspergillus sydowii* or an *Aplanochytrium* sp.) after a period of thermal stress. Thermal stress also continued after inoculation. Fragments were sampled at either 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD). Panels without letters have no significance.

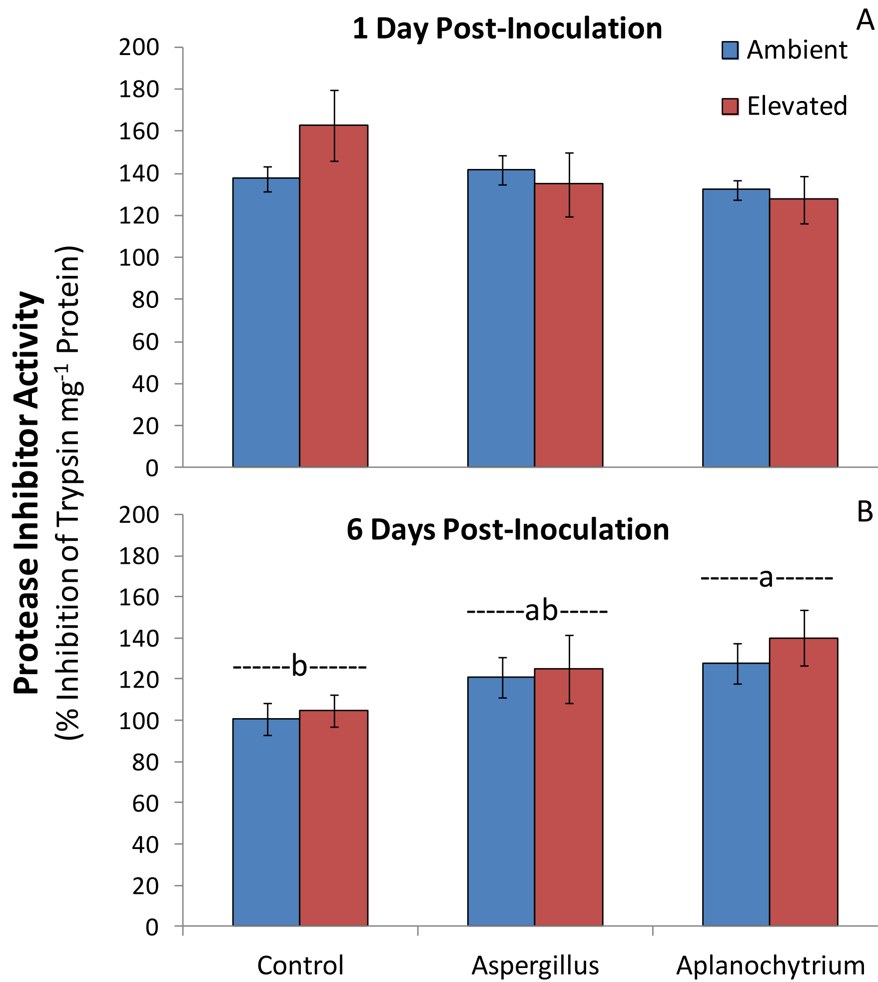

Figure 4-8 Protease inhibitor activity of the sea fan

Mean (±SE) protease inhibitor activity of sea fans exposed to either of two pathogens (*Aspergillus sydowii* or an *Aplanochytrium* sp.) after a period of thermal stress. Thermal stress also continued after inoculation. Fragments were sampled at either 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD). Panels without letters have no significance.

Antioxidant defense

At 1 day post-inoculation, there was significant interactive effect of thermal stress and pathogen-exposure on both POX and CAT activity (POX – two factor ANOVA, F=5.4930, p=0.0070; CAT – two factor ANOVA, F=11.9135, p<0.0001). Thermal stress increased activity in both POX and CAT assays. POX activity was highest when sea fans were thermally stress and exposed to the *Aplanochytrium* or no pathogen (Tukey's HSD, p<0.05, Figure 4-9A). In the CAT assay, sea fans that were thermally stress and not exposed to either pathogen had the highest activity (Tukey's HSD, p<0.05, Figure 4-10A).

At 6 days post-inoculation, thermal stress increased overall antioxidant activity (all assays: POX, CAT, and SOD). POX and SOD were also significant affected by pathogen-exposure (POX – ANOVA, F=3.8601, p=0.0261; SOD – ANOVA, F=8.5670, p=0.0046). POX activity was lower when the sea fan was exposed to either pathogen than the control, regardless of thermal stress (Figure 4-9B). Conversely, SOD activity was increased in sea fan exposed to either pathogen and was highest in sea fan exposed to the *Aplanochytrium* (Figure 4-11B). Similar was seen in the CAT assay but was slightly insignificant (ANOVA, F=2.8029, p=0.0681; Figure 4-10B).

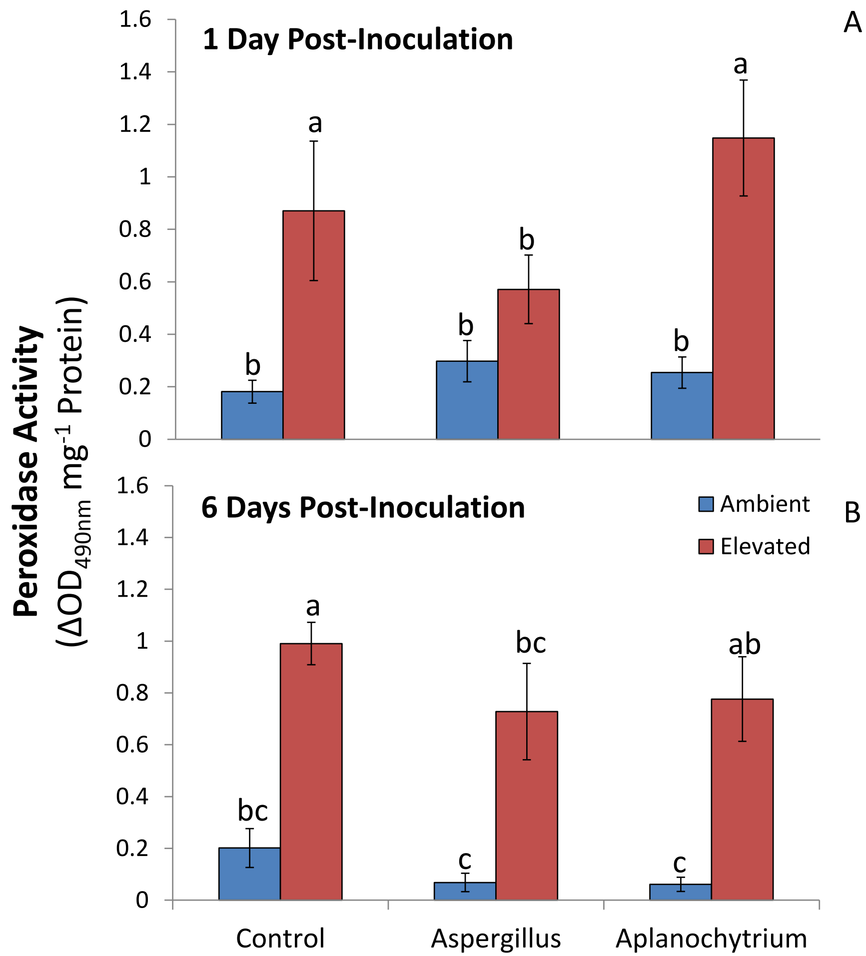

Figure 4-9 Peroxidase activity of the sea fan

Mean (±SE) peroxidase activity of sea fans exposed to either of two pathogens (*Aspergillus sydowii* and an *Aplanochytrium* sp.) after a period of thermal stress. Thermal stress also continued after inoculation. Fragments were sampled at either 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD). Panels without letters have no significance.

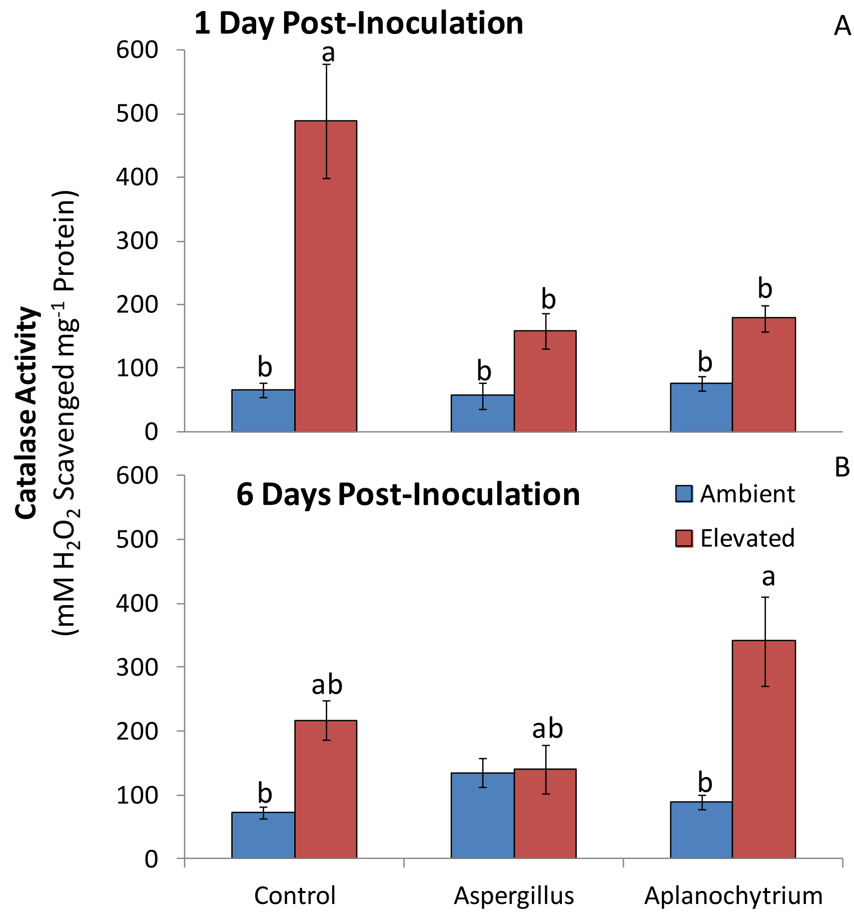

Figure 4-10 Catalase activity of the sea fan

Mean (±SE) catalase activity of sea fans exposed to either of two pathogens (*Aspergillus sydowii* and an *Aplanochytrium* sp.) after a period of thermal stress. Thermal stress also continued after inoculation. Fragments were sampled at either 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD).

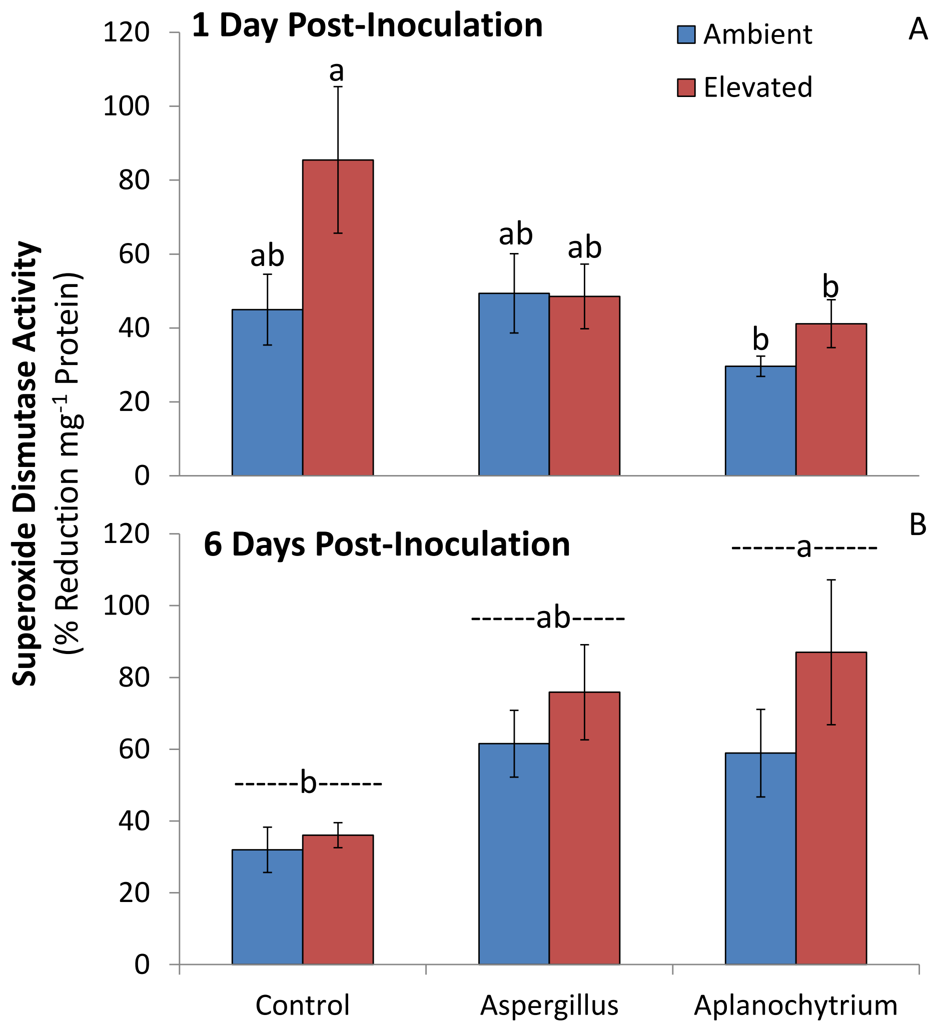

Figure 4-11 Superoxide dismutase activity of the sea fan

Mean (±SE) superoxide dismutase activity of sea fans exposed to either of two pathogens (Aspergillus sydowii and an Aplanochytrium sp.) after a period of thermal stress. Thermal stress also continued after inoculation. Fragments were sampled at either 1 day post-inoculation (A) or 6 days post-inoculation (B). Letters indicate statistical significance at $p<0.05$ in each panel (Tukey's HSD). Panels without letters have no significance.

Discussion

Increasing coral epizootics and disease outbreaks have lead to drastic changes in the function and biodiversity of coral reef ecosystems (Hughes et al. 2010). These outbreaks of disease are largely associated with environmental stressors, particularly rising sea surface temperatures. It is hypothesized that environmental stressors are compromising coral immune systems and/or exacerbating virulence of pathogens (Mydlarz et al. 2006). In the Caribbean sea fan (*Gorgonia ventalina*), both elevated temperature and acidification stressors have been shown to have compounding adverse effects on sea fan immunity (Chapter 2, p. 21). Furthermore, elevated temperatures also likely increase putative virulence in the sea fan protist pathogen *Aplanochytrium* sp. and the fungal pathogen *Aspergillus sydowii* (Burge et al. 2012; Mann et al. 2014).

Our results build upon these findings by demonstrating how preceding thermal stress can differentially affect and compromise the overall as well as individual mechanistic immune responses of the sea fan to two different pathogens (*Aspergillus sydowii* and an *Aplanochytrium* sp.). Moreover, we utilize novel methods such as gene expression analysis and a larger biochemical immunoassay suite to understand how under environmental stressors the immune response changes in the sea fan. Our results show that preceding thermal stress can increase some immune responses initially but decreases the overall response over an extended period of time. Furthermore, some immune responses varied between the two pathogens, *A. sydowii* and the *Aplanochytrium*, while others had no noticeable difference between the two pathogens.

Overall, expression levels observed at both 1 day post-inoculation and 6 days post-inoculation were largely consistent with Burge et al. (2013b) where expression was higher in *Aplanochytrium* exposed sea fans versus the control (independent of thermal stress exposure). Although statistically insignificant, there appears to be higher levels of expression of tachylectin and leucine rich kinase when sea fans were exposed to thermal stress (regardless of pathogen) at 1 day post-inoculation versus at 6 days post-inoculation. This has also been observed in the hard coral, *Acropora millepora*, where overall

gene expression in the short term was higher than longer term exposure to thermal stress (Meyer et al. 2011).

Effect of both factors, thermal stress and pathogen-exposure, had significant effect on immune signaling at 6 days post-inoculation. The effect of pathogen-exposure was more notable at 6 days post-inoculation where there was much higher expression of both receptor components (tachylectin and leucine rich kinase) in sea fans exposed to the *Aplanochytrium* (with no thermal stress). However, when the sea fan was exposed to thermal stress and either *A. sydowii* or the *Aplanochytrium* expression was lower compared to control sea fans (with no pathogen). This is the first study to clearly demonstrate the effects of both thermal stress and pathogen-exposure on the gene expression of the sea fan and suggests that there is indeed lowered immunocompetence under the combined stressors. Nonetheless, the lowered response seen only represents a small component of the molecular response and it is still possible that expression of other immune related genes would not be lowered in response to both stressors but a larger study of gene expression is required.

Direct antimicrobial defense was also affected by the combination of thermal stress and pathogen-exposure. Compared to the other immune mechanisms measured in this study, differences between responses to the pathogens were most prominent in the these assays, particularly at 1 day post-inoculation. At 1 day post-inoculation, both antibacterial and antifungal activity was highest in sea fans exposed to both thermal stress and exposure to *A. sydowii*. Sea fans exposed thermal stress and the *Aplanochytrium* were more similar to the control sea fans under thermal stress.

The production of antimicrobial compounds is an important component of immunity and can originate from different sources including the coral host and/or its natural microbial flora (Gil-Turnes et al. 1989; Ritchie 2006). Therefore, the responses observed from the immunoassays could originate from either source but the observed production of tachylectin (a common receptor for pathogen-associated molecular patterns) indicates a response from the sea fan host. While a greater antimicrobial response was observed in sea fans exposed to *A. sydowii* than the *Aplanochytrium*, antimicrobials against *Aplanochytrium* could still be present. Due to the nature of growth in the *Aplanochytrium* pathogen, no

“anti- *Aplanochytrium*” assay was performed to detect this. These changes in antimicrobial production play an important role in determining if the sea fan is able to fight off infection from *Aplanochytrium* or *A. sydowii* under environmental stressors.

Anti-pathogenic defenses (PPO, MEL, and PI) of the sea fan were also affected by the exposure to thermal stress and/or pathogen-exposure. Unlike the antimicrobial activities observed, there appears to be a general overall increase in anti-pathogenic response when sea fans are exposed to thermal stress. In a previous study, increase in other anti-pathogenic responses has also been observed such as ameobocyte activities, where densities of ameobocytes significantly increased under elevated temperatures (Mydlarz et al. 2008). Furthermore, Mydlarz et al. (2008) demonstrate the presence of granulosomes in the ameobocytes which are suggested to be facilitators for the production of MEL. This corroborates our current findings where MEL activity significantly increased in response to thermal stress (regardless of pathogen exposure). Although PPO activity had increased initially (at 1 day post-inoculation) in sea fans exposed to thermal stress only, there was no other significant increase in PPO overall. PPO is one of the precursor molecules in the melanin synthesis cascade and it would be expected to see a spike in PPO activity followed by MEL although this was not clearly seen in our study. Nonetheless, it still remains possible that there was an increase in PPO for sea fans but may have been missed in the time points analyzed.

No effect of the treatments was seen on PI activity at 1 day post-inoculation. However, at 6 days post-inoculation, there was a strong overall increase in PI activity in response to pathogen-exposure (regardless of thermal stress). In the study of Mann et al. (Chapter 1; 2014) as well as Mann et al. (Chapter 2), no effect of thermal stress on PI activity was also observed at a similar or longer periods of thermal stress treatment. Mann et al. (2014) also demonstrated lower PI activity in sea fans infected with aspergillosis compared to whole, healthy sea fans. However; our results show that there is an increase in PI activity in response to either pathogen at 6 days post-inoculation and this may represent an early response to infection. Mann et al. (2014) demonstrate higher PI activity in healthy tissue of diseased sea

fans and suggest that it is likely that PI activity may be an early response to pathogens rather than a later response as may be seen in the lesioned tissue.

Thermal stress had a large effect on the antioxidant mechanisms and appeared overall higher at both 1 day post-inoculation and 6 days post-inoculation in sea fans exposed to thermal stress. At 6 days post-inoculation, effect of pathogen-exposure was observed across the assays. In the CAT and SOD assays, antioxidant activity increased with exposure to either pathogen (and was highest in sea fans exposed to the *Aplanochtyrium*). However, in the POX assay activity decreased in sea fans exposed to either pathogen by the end of the experiment. The combined effect of both thermal stress and the pathogen-exposure suggests that the sea fans were indeed under stress from either or both factors the entire experiment.

The preceding thermal stress appears to overall have considerable impact on how the sea fan may respond to subsequent pathogen exposure. Several of the assays show that sea fans exposed to the preceding period of thermal stress only, had an initially elevated level of constitutive immunity which declined considerably by the end of the experiment. This decline suggests that the sea fan is capable of providing a short term immune response under thermal stress but diminishes when the thermal stress becomes more chronic. Furthermore, our results show that effects of chronic thermal stress can begin as soon 12 days (or possibly sooner). This provides important implications in understanding the resilience of corals to thermal stress. In the field, thermal stress events (with +1°C anomalies) can last much longer than the period observed in this study (Mydlarz et al. 2009; Guest et al. 2012). It is exceedingly likely that events of chronic thermal stress (which are becoming more and more common; Donner, 2009) can be quick to leave corals susceptible towards pathogens.

This study has additional importance towards understanding the co-infection of the sea fan with both pathogens, *A. sydowii* and the *Aplanochytrium* (Petes et al. 2003). The dynamics occurring between host and these two pathogens has not been thoroughly examined and this study provides better insight into mechanisms of how the co-infection may be established. This study shows how there can be variation in responses to each pathogen. In several of the immune measures a larger response to *A.*

sydowii than the *Aplanochytrium* was observed. In Chapter 2 as well as the current study, there is also evidence of suppression of the sea fan immune response by the *Aplanochytrium* pathogen. If suppression occurs more frequently from exposure to the *Aplanochytrium* than *A. sydowii*, it is possible that in the co-infection, the primary infection may be the *Aplanochytrium* while *A. sydowii* is the later infecting pathogen.

By and large, a preceding exposure to thermal stress can greatly affect the immunity of corals and leave them susceptible towards diseases. Moreover, immunity can be greatly affected by the exposure to different pathogens leaving some pathogens potentially more virulent than others. Together these results suggest that coral diseases have a wide range of uniqueness. Therefore, this should be considered when implementing conservation and mitigation strategies for coral reef ecosystems.

Chapter 5

Concluding Remarks

Global climate change has been a critical issue of particular concern among marine biologists and conservationists. Environmental stressors, such as elevated sea surface temperatures and acidification, have been increasing as a result of artificial increased levels of atmospheric CO2. This increase in stressors has made it increasingly challenging for the survival of marine organisms, especially corals. Moreover, outbreaks of coral disease have often been associated with the introduction of these stressors. Therefore, it is has been essential to expand upon our understanding of how corals are responding to these environmental stressors. Using the Caribbean sea fan as a coral disease model system, this dissertation addresses pertinent questions regarding how stressors (such as elevated temperature and acidification) affect both the virulence of coral pathogens and the immune responses of corals to these pathogens.

In chapter 2, I identified a putative virulence factor (the production of proteases) in the sea fan fungal pathogen, *Aspergillus sydowii* and a direct immune defense (inhibition against the fungus protease) of the Caribbean sea fan, *Gorgonia ventalina*. While elevated temperatures were seen to considerably increase the protease activity of *A. sydowii* no changes were seen in the sea fan; however, protease inhibitor activity was significantly lower in diseased tissue versus healthy tissue in the sea fan. This suggests that at some point in disease progression, this defense is compromised and no longer active against *A. sydowii* proteases. In subsequent chapters, the protease inhibitor assay was incorporated into a larger suite of immune measures which I used to further explicate how environmental stressors are affecting the overall immunity of the sea fan.

I also examined how multiple stressors (elevated temperatures and acidification) can interact to affect the immune system of the sea fan. In Chapter 3, I used a full-factorial experiment to demonstrate that elevated temperatures and acidified water conditions interact synergistically and adversely affect the several immune mechanisms (i.e. antimicrobial and antioxidant activity) of the sea fan in response to an *Aplanochytrium* sp. pathogen. Not only did this experiment show that immuno-compromise can result

from environmental stressors but also shows for the first time, the immunosuppression capability of a coral pathogen.

Although temperature did interact synergistically with acidification, the effects of thermal stress appeared to have the largest overall impact on the sea fan immune system. Chapter 4 follows up on this and examined how preceding and continued thermal stress can compromise the sea fan immune system and subsequent response to two different pathogens (*A.sydowii* and the *Aplanochytrium*). Similar to what was observed in Chapter 3, antimicrobial and antioxidant mechanisms as well as immune signaling (through gene expression analysis) were all affected by the thermal stress. Additionally, this chapter demonstrated variation in response to two different sea fan pathogens. In many of the immune mechanisms, responses to *A. sydowii* were often greater than that of the *Aplanochytrium* pathogen. This is consistent with what was observed in Chapter 3 where the *Aplanochytrium* had immuno-suppressive effect on the sea fan.

The results of this dissertation discuss several important conclusions regarding disease ecology of corals under global climate change which were not yet fully explicated. Environmental stressors (such as elevated temperature or acidification) can have considerable impact on the dynamics of host-pathogen relationships in corals. Environmental stressors can compromise host immunity while also increasing putative virulence in pathogens. Moreover, the presence of more than one stressor is likely to have compounding effects on coral immunity and while not observed in this dissertation, could potentially have similar effects to pathogen virulence. In general, there seems to be great consensus on the adverse impacts of environmental stress on coral immunity; however, as demonstrated by results in Chapter 4, it is also important to consider the individualistic response that may occur to different pathogen types.

Future Directions

The outcomes of this dissertation are significant in the context of future research for coral immunity and disease. Future directions should include an emphasis on poly-microbial diseases such as that seen in the co-infection of the sea fan. Furthermore, this research should have consideration of all environmental impacts to the coral (i.e. pollution, thermal stress, acidification, physical damage, etc.). My

research suggests that there are certain trade-offs that may be occurring within the coral host during periods of chronic stress and this should be further explored. The existing data from this work can also be used by coral reef managers and conservationists to better understand the host-pathogen dynamics in corals and help explain future disease outbreaks. Predictions can also potentially be made through the use of biological modeling. These predictions could consequently be used by reef managers to make recommendations to policy makers for implementing better management strategies of these precious ecosystems.

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Biographical Information

Whitney Mann was born in Dallas, Texas in 1986 to Sheryl Ann Whitmer and John Joseph Mann, Jr. After beginning her undergraduate degree at Texas State University in 2005, she transferred in 2006 to the University of Texas at Arlington where she graduated with her bachelor's degree in Biology in 2009. While preparing to attend veterinary school, she began working at a local pet store and was responsible for maintaining the aquarium habitats. As a result of an acquired interest in aquatic and marine environments, she began working as an undergraduate researcher under supervision of a Ph.D. student (Liz McGinty) and principal investigator, Dr. Laura Mydlarz. In January 2010, she started as a graduate student and continued researching in the Mydlarz lab. Throughout her graduate career she has performed many field experiments with corals in variety of places including Panama, the Florida Keys, and Puerto Rico. She has presented her work at local, regional, and international meetings and is co-author in several publications in the journals *Marine Biology* and *Journal of Experimental Marine Biology* (in review). Her future plans after graduation include obtaining a post-doctoral fellowship and continuing to study the field coral ecology and invertebrate immunity.