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Investigating the Driving Mechanisms Behind Differences in Bleaching and Disease Susceptibility Between Two Scleractinian Corals, Pseudodiploria Strigosa and Diploria Labyrinthiformis

Zoe A. Pratte
Florida International University, zpratte@fiu.edu

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

INVESTIGATING THE DRIVING MECHANISMS BEHIND DIFFERENCES IN BLEACHING AND DISEASE SUSCEPTIBILITY BETWEEN TWO SCLERACTINIAN CORALS, *PSEUDODIPLORIA STRIGOSA* AND *DIPLORIA LABYRINTHIFORMIS*

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To: Dean Michael R. Heithaus
College of Arts and Sciences

This dissertation, written by Zoe A. Pratte, and entitled Investigating the Driving Mechanisms Behind Differences in Bleaching and Disease Susceptibility Between Two Scleractinian Corals, *Pseudodiploria strigosa* and *Diploria labyrinthiformis*, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

_____
Christina Kellogg

_____
Krish Jayachandran

_____
Mauricio Rodriguez-Lanetty

_____
Eric Bishop von Wettberg

_____
Laurie Richardson, Major Professor

Date of Defense: June 15, 2015

The dissertation of Zoe A. Pratte is approved.

_____
Dean Michael R. Heithaus
College of Arts and Sciences

_____
Dean Lakshmi N. Reddi
University Graduate School

Florida International University, 2015

DEDICATION

To my mom, dad, and little brother, who answered every phone call.

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ABSTRACT OF THE DISSERTATION

INVESTIGATING THE DRIVING MECHANISMS BEHIND DIFFERENCES IN BLEACHING AND DISEASE SUSCEPTIBILITY BETWEEN TWO SCLERACTINIAN CORALS, *PSEUDODIPLORIA STRIGOSA* AND *DIPLORIA LABYRINTHIFORMIS*

by

Zoe A. Pratte

Florida International University, 2015

Miami, Florida

Professor Laurie Richardson, Major Professor

Disease and bleaching are two conditions which commonly lead to coral death. Among coral species, susceptibility to disease and bleaching is variable, and *Pseudodiploria strigosa* tends to be diseased more than *Diploria labyrinthiformis,* while *D. labyrinthiformis* bleaches more readily. The focus of this dissertation was to investigate and compare multiple components of these two coral species, and identify how they may relate to disease and bleaching resistance. Compenetnts examined included the surface mucopolysacharide layer (SML) thickness, gene expression, microbial associates, and a white plague aquarium study. The SML thickness decresased with increasing temperature regardless of coral species, indicating that SML thickness does not likely play a role in differences between susceptablities of these two coral species. However, *Diploria labyrinthiformis* had a lower mortality rate at 31°C, had fewer

differentially expressed genes assossiated with stress, and upregulated genes associated with innate immunity in the summer, all of which may contribute to its relative disease resistance. The bacterial associates of each coral species were also monitored. Differences between the two coral species were primarily caused by Clostridia, Gammaproteobacteria, and rare species which may contribute to the relatively higher disease susceptibility of *P. strigosa.* Lastly, an aquarium study suggested that a potential pathogen of the *Roseobacter* clade infects both *D. labyrinthiformis* and *P. strigosa*, and might be transmitted by the Cryptochiridae gall crab, indicating that potential disease vectors associated with these two coral species may also play a role in disease resistance and resilience.

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*Note: This paper was published before *Diploria strigosa* and *Diploria clivosa* were reassigned to the new genus *Pseudodiploria*.

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CHAPTER 1

INTRODUCTION

Climate change, warming, and anthropogenic impacts are among the primary causes of coral reef decline globally (Hoegh-Guldberg et al. 2007; Harvell et al. 2007). If coral reefs are to be protected and preserved for coming generations, mechanisms of coral resilience to such stressors must be understood (Hughes et al. 2010). Resilience and resistance to disease and bleaching are particularly important, as both conditions often lead to coral death (Harvell et al. 2001; Miller et al. 2006; Bruno et al. 2007). Corals exhibit a high variability in their resistance to bleaching and disease, both between different species and among colonies of the same species (Raymundo et al. 2003; Kaczmarsky et al. 2005; Pandolfi et al. 2011). Two closely related species, *Pseudodiploria strigosa* (formerly *Diploria strigosa*- Budd et al. 2012) and *Diploria labyrinthiformis* are prime examples of this variability. *Pseudodiploria strigosa* tends to be relatively susceptible to coral diseases, while *D. labyrinthiformis* bleaches readily (reviewed in depth in chapter two). Understanding the driving mechanisms that create these differences in disease and bleaching resistance are vital to of coral conservation and mitigation efforts.

When considering possible mechanisms of coral bleaching and disease resistance, all living and nonliving components must be considered at the microscopic and macroscopic scale. The surface mucopolysaccharide layer (SML) is an example of a nonliving component of corals that is absolutely imperative to coral health, as it serves as a coral's primary source of protection from environmental threats (Brown and Bythell, 2005). Chapter two begins with an introduction summarizing the critical roles the SML plays in coral health and disease, followed by an assessment of the effects warming and

acidification have on the SML thickness of *D. labyrinthiformis* and *P. strigosa* (as well as a third species, *P. clivosa*). The results of this chapter are critically analyzed with respect to changes in SML thickness, and how SML thinning may contribute to disease susceptibility.

A second aspect which may contribute to differential disease and bleaching susceptibilities is the genes that are actively being transcribed (Bellantuono et al. 2012; Barshis et al. 2013). Accordingly, chapter three evaluates the gene expression of *D. labyrinthiformis* and *P. strigosa,* monitored over 18 months, to identify seasonally differentially expressed genes. Laboratory experiments were also conducted to assess the gene expression of these two species in the presence of warming and acidification. The analysis and discussion of this chapter draws attention to differentially expressed genes between species, seasons, and treatments, and how they may be correlated with coral disease and bleaching resilience, with particular interest in genes associated with innate immunity and stress.

In addition to the coral animal itself, viruses, archaea, bacteria, fungi, and the endosymbiotic alga *Symbiodiumium* (zooxanthellae) are influential in maintining coral health. Collectively, these components are known as the coral holobiont (Rohwer et al. 2002; Reshef et al. 2006; Rosenberg et al. 2007). In particular, the bacterial members of the holobiont play crucial roles in maintaining coral health (Rosenberg et al. 2007). In parallel with chapter three, chapter four tracks changes in bacterial-associates seasonally for 18 months, and experimentally when exposed to warm temperatures and acidification. Analysis of data in chapter four focuses upon differences in the bacterial communities

between the two species, the manner in which they shift seasonally, and how this may affect disease resistance.

To facilitate disease resistance, the etiology of the disease itself must be understood. A particularly destructive coral disease, white plague, has been documented to affect both *D. labyrinthiformis* and *P. strigosa* (Croquer et al. 2003; Kaczmarsky et al. 2005). Chapter five examines a white plague outbreak (in aquaria) associated with these two coral species. Chapter five utilizes bacterial profiling and associations with a small cryptochiridae gall crab.

The focus of the present dissertation was to examine multiple aspects of the coral holobiont for contributing factors to disease and bleaching resistance and resilience, using *D. labyrinthiformis* and *P. strigosa* as model systems. These aspects include: 1) variations in SML thickness; 2) changes in the coral transcriptome in response to stressors; 3) changes in the bacterial associates in response to stressors; and 4) examination of white plague associated with both these coral species.

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***Note: This paper was published before *Diploria strigosa* and *Diploria clivosa* were reassigned to the new genus *Pseudodiploria*.**

CHAPTER 2

IMPACTS OF TEMPERATURE INCREASE AND ACIDIFICATION ON THICKNESS OF THE SURFACE MUCOPOLYSACCHARIDE LAYER OF THE CARIBBEAN CORAL *DIPLORIA* SPP.

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Abstract

Coral mechanisms of resilience and resistance to stressors such as increasing sea surface temperature and ocean acidification must first be understood in order to facilitate the survival of coral reefs as we know them. One such mechanism is production of the protective surface mucopolysaccharide layer (SML). In this study we investigated changes in the thickness of the SML in response to increasing temperature and acidification for the three Caribbean scleractinian coral species of the genus *Diploria* which have been shown to exhibit differential resilience to disease and bleaching. Among the three species, *D. strigosa* is known to have a higher susceptibility to disease, *D. labyrinthiformis* is known to bleach more quickly, and *D. clivosa* is relatively unstudied. When temperature was increased from 25°C to 31°C over a one week or six week period, the overall thickness of the SML decreased from 33% to 55% for all three species. Average SML thickness at 25°C for all three species ranged from 106 to 156 μm, while average thickness at 31°C ranged from 64 to 86 μm. SML thickness was significantly different among species at 25ºC, but not at 31°C. *D. labyrinthiformis* demonstrated lower fragment mortality due to thermal stress when compared to the other *Diploria* species. Acidification from pH 8.2 to 7.7 over five weeks had no effect on SML thickness for any species. The observed decrease in SML thickness in response to increased temperature might be attributed to a decrease in the production of mucus or an increase in the viscosity of the SML. These findings may help to explain the increased prevalence of coral disease during the warmer months, since increased temperature compromises an important aspect of coral innate immunity, as well as differences in disease and bleaching susceptibilities between *Diploria* species.

Introduction

The health of corals worldwide is currently in decline, in part due to increased bleaching and disease (Hughes et al. 2003; Rosenberg and Loya 2004). With the onset of global climate change, concern continues to rise for the preservation of coral reefs (Hoegh-Guldberg et al. 2007). It is known that anthropogenic impacts, such as a small 1-2ºC increase in sea surface temperature, can cause bleaching of corals (Mydlarz et al. 2009) and that bleaching increases susceptibility to disease (Bruno et al. 2007). Additionally, acidification can cause decreased calcification rates (Doney et al. 2009). Encouragingly, it is becoming evident that some corals demonstrate a degree of thermotolerance (Brown et al. 2002; Barshis et al. 2013; Mydlarz et al. 2009) and are capable of survival in acidified conditions (Fine and Tchernov 2007); thus corals may have innate resilience to these aspects of climate change.

Corals demonstrate variability in innate resilience to disease and bleaching, both among species as well as among individuals of the same species (Hughes et al. 2003; Sutherland et al. 2004; Grottoli et al. 2006; Pandolfi et al. 2011). Understanding the drivers behind this variability will be crucial to further our knowledge of potential coral resilience in the face of rising sea surface temperatures and acidification. This requires identifying aspects of the coral that may give rise to protection against these stressors. One such aspect is the surface mucopolysaccharide layer (SML), which protects all corals from stressors such as UV, sedimentation, desiccation and pathogen colonization, and harbors microbes beneficial to the coral (reviewed in Brown and Bythell 2005). The SML is known to be an important component of coral innate immunity, and may directly affect coral survival if its properties are compromised due to anthropogenic impacts.

There are relatively few studies of the dynamics, physical properties, and chemical composition of the SML. This is surprising since the SML is present on all corals (Bythell and Wild 2011). It is known that the SML is a polysaccharide protein lipid complex, secreted onto the surface of the coral tissue to form a mucus layer, that is primarily composed of protein and carbohydrate polymers (mucins) with a small fraction of lipids and amino acids (Brown and Bythell 2005). The chemical composition of coral mucus is highly variable among species, and shows no consistent baseline composition (Meikle et al. 1988). It is now understood that this variability may be in part due to different collection methods, as "milked" mucus has a different composition than that of mucus sampled directly from the coral surface (Jatkar et al. 2010 a). The mucins found in the SML are similar to those found in other vertebrates (Jatkar et al. 2010 a), while the lipid component of the SML is believed to be a byproduct of the symbiotic zooxanthellae that reside within the coral gastrodermis (Wooldridge 2009). Interestingly, it has been shown that the lipid content of the SML increases with rising temperature during the process of thermal bleaching (Wooldridge 2009).

Environmental stressors have been implicated in influencing the overall production of the SML. One study measured the SML thickness of four scleractinian coral species in varying environmental conditions (Jatkar et al. 2010 b). *Goniastrea aspera* colonies that had been recently exposed to air due to a dramatic low tide demonstrated an SML thickness significantly greater than those colonies which had remained submerged (Jatkar et al. 2010 b). Among colonies that experienced partial solar bleaching, *Platygyra daedalea* revealed a decrease in SML thickness, although not

significant, while *G. retiformis* SML thickness was highly variable. This pioneering study demonstrated that the SML is highly dynamic and responsive to the environment. It is possible that corals are capable of controlling SML production in response to environmental stressors by altering mechanisms such as the secretion of mucins, pH of the SML, transepithelial movement of electrolytes and water, as well as controlling the hydration of mucus (Brown and Bythell 2005). The extent of this control, as well as the effects that environmental parameters such as temperature and acidification have upon this control, remain unexplored.

The role of the SML in coral innate immunity includes both physical and biological functions. In addition to the protective physical properties, the SML also contains a microbial community that is crucial to the health of the coral (Bythell and Wild 2011). In the past ten years many studies have focused upon the composition and role of this microbial community (Rohwer et al. 2002; Kellogg 2004; Bourne 2005; Guppy and Bythell et al. 2006; Ritchie 2006; Sekar et al. 2008; Littman et al. 2009; Shnit-Orland and Kushmaro 2009; Vega Thurber et al. 2009; Meron et al. 2011; Sweet et al. 2011). This body of research has led to the development of the coral probiotic hypothesis, stating that coral-associated microbes may serve to protect their host based on their ability to rapidly evolve protection mechanisms (Reshef et al. 2006).

In spite of these recent studies, the ways in which the physical and chemical properties of the SML affect beneficial (or potentially pathogenic) microbes are relatively unknown. Factors such as genetic disposition of the coral host and fluctuating surroundings likely influence the composition and nature of the SML, which in turn may

alter the microbial communities found in the SML (Guppy and Bythell 2006). Coral mucus is a source of carbon and inorganic nutrients for heterotrophic marine bacteria (Nakajima et al. 2009), and varying amounts of dissolved organic carbon (DOC) released by corals may have a direct effect on the microbial communities (Tremblay et al. 2011). Environmental conditions may influence coral SML microbial communities as well. For example, increased temperature and acidification caused the SML-associated microbial functional gene community to change (Vega Thurber et al. 2009), and microbial communities shifted significantly at a lowered pH of 7.3 (Meron et al. 2011). It was not determined in these studies whether the observed microbial functional gene and community shifts occurred in response to the changes in environmental conditions, or due to altered properties of the SML under the influence of these factors, which secondarily influenced the microbial communities. To investigate these questions, it is first necessary to determine the response of the SML to conditions such as increased temperature and acidification.

By the end of this century, sea surface temperatures are expected to rise by at least 2ºC, and pH could decrease by as much as 0.4 units. Already, sea surface temperatures have increased by more than 0.5 ºC, and pH levels decreased by 0.1 units (reviewed in Hoegh-Guldberg et al. 2007). Acidification of the oceans is known to increase the dissolution rate of calcite, and to a greater degree, aragonite (Doney et al. 2009). However, apart from the reduction in calcification rates (Doney et al. 2009), very little is known regarding the effect of acidification on coral health. Rising temperatures have been associated with an increase in coral bleaching, with bleached corals being more

susceptible to disease (Miller et al. 2006; Bruno et al. 2007; Muller et al. 2008; Mydlarz et al. 2009; Sharp and Ritchie 2012). While many aspects of rising sea surface temperatures and acidification have been studied, the response of the SML to increasing temperatures and acidification is completely unknown.

The purpose of this study was to determine the effects of acidification and temperature increase on the SML thickness of three Caribbean corals, *Diploria clivosa*, *D. labyrinthiformis* and *D. strigosa*. *D. labyrinthiformis* and *D. strigosa* were selected because they are important reef-building corals that have well-documented differences in susceptibility to bleaching and disease. In the US Virgin Islands from 2002 to 2005, *D. strigosa* had a 0.7% prevalence value for yellow band disease (YBD), while *D. labyrinthiformis* showed no sign of disease (Calnan et al. 2008). From 2004 to 2008 on reefs of Bermuda, black band disease (BBD) infected 0.44% of *D. strigosa* colonies, much higher than the 0.01% of infected *D. labyrinthiformis* colonies (Jones et al. 2012). This trend has been observed throughout the wider Caribbean (Edmunds 1991), Belize, and Bermuda (Rützler and Santavy 1983). Despite its apparent relative resistance to disease, *D. labyrinthiformis* has been documented to bleach more quickly and intensely than *D. strigosa* on the reefs of the Caribbean (Villamizar et al. 2008) and Bermuda (Cook et al. 1990). In one study, *D. clivosa* was documented to have a substantially higher prevalence of BBD than both *D. strigosa* and *D. labyrinthiformis* (Kaczmarsky et al. 2005). The relative bleaching susceptibility of *D. clivosa* is unknown.

Materials and Methods

Coral collection

Small colonies of three coral species, *D. clivosa* (n = 7), *D. labyrinthiformis* (n = 8), and *D. strigosa* (n = 7), were collected from the Florida Keys National Marine Sanctuary Coral Nursery on February 28th 2011, July 1st 2011, and November 17th 2011, and immediately transported to Florida International University. Coral were allowed to recover for one week in a 340 liter flow-through holding tank maintained at 25^0C, 34 ppt salinity, a 12h light/dark cycle with metal halide and fluorescent lighting, and weekly partial water changes using artificial sea water (ASW). Light intensity (PAR) ranged from 8-15 x 10^{14} quanta/second/cm^2 depending on proximity to the light source, as measured using a Biospherical Instruments Model QSL100 averaging quantum meter. After recovery, colonies were fragmented by hand using a chisel and hammer into approximately 9 cm^2 pieces. Fragments were then placed in a mixture of plaster of Paris and quick dry cement to provide a base that allowed them to stand upright. Fragments were maintained in the holding tank until transfer to experimental tanks. The corals were not fed at any point, although zooplankton were observed in both the holding and experimental tanks. Fragments remained alive and healthy in all tanks prior to experimentation.

Experimental design

Experimental tanks contained 19 liters of ASW, live rock from the holding tank, and a recirculating filter. Microbial populations in each tank were allowed to establish for

two weeks prior to the addition of coral fragments. Each tank was exposed to an identical light source of 12:12 L:D with a light intensity of $1x10^{15}$ quanta/second/cm^2. For each experiment triplicate fragments of each species were utilized. Fragments were allowed to acclimate for an additional two weeks in the experimental tanks before temperature or pH manipulation. The temperature experiments were repeated three times (9 fragments of each species) and the pH experiment once (3 fragments of each species). Fragments were reused only if they demonstrated complete recovery for up to six months after experimentation.

Measurement of SML thickness

SML thickness was measured following the procedures outlined by Jatkar et al. (2010 b), in which the optimum number of measurements per fragment was determined, and the robustness of the method demonstrated. A coral fragment was carefully removed and placed in a 2 liter glass bowl, remaining submerged, and placed under a boom dissecting microscope (Figure 2.1). The SML is not easily visible due to its transparency, thus small activated carbon particles were allowed to settle onto the fragment, indicating the SML. The distance between the carbon particles (top of the SML) and the coral tissue was then measured by observing the tip of a thin pulled pipette attached to a micromanipulator (Narishigi, Japan) as it was carefully maneuvered from the carbon particles to the coral surface, which was easily visible. As the SML is a fluid substance, carbon particles removed by SML movement were reapplied for subsequent measurements. All measurements were made in the area between the polyp tentacles and the top of the ridge of the calices for uniformity. Because of the dynamic nature of the

coral surface, fifteen measurements were made and averaged per fragment, per time point, each at a spatial resolution of 10 μm. Care was taken to disturb the coral as minimally as possible during the measuring process. Measurements took place using the same light source as the experimental tanks, with no other significant source of light, in order to eliminate light as a confounding factor.

Temperature increase

To determine the response of SML thickness to increasing temperature, fragments were placed in the 19 liter experimental aquaria at 25^0C. Each species had a total of nine replicates, spread over three independent trials. As an internal control, two SML thickness measurements were taken while the temperature was maintained at 25^0C, the first at t = 0 and the second after one week, to ensure SML thickness stability. Temperature was raised rapidly at 1^0C per day for one week to the final temperature of 31^0C, after which the SML was measured again. Fragments were kept at 31^0C for an additional week, and the SML thickness was measured once more at the end of this period.

To determine whether the rate of temperature increase had an effect on SML thickness the experiment was repeated, slowing the rate of temperature increase. As before, tanks were initially maintained at 25^0C for one week with the SML thickness measured at t = 0 and after one week at this temperature, to ensure SML thickness stability (internal control). In these experiments temperature was increased 1^0C per week from 25^0C to 31^0C over a six week period. Subsequent measurements took place every

seven days for the duration of the temperature increase, and then after one week of maintaining the tanks at the final temperature of 31^{0}C.

Acidification

To determine the effect of acidification on SML thickness, three fragments of each species were placed in a 19 liter experimental tank maintained at 25^{0}C and acidified from pH 8.2 to 7.7. A CO_2 injection system (AZOO, Taiwan) was used to infuse CO_2 into experimental tanks to lower pH via the natural buffering system present in aquatic systems. This approach is based on well-described equilibrium reactions that include the following: $CO_{2\,(atm)} \rightleftharpoons CO_{2\,(aq)} + H_2O \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}$ (Doney et al. 2009), and has been widely used to manipulate pH in experimental setups (Leclercq et al. 2000, Renegar and Riegl 2005). Although the bubbling of CO_2 does affect carbonate/bicarbonate chemistry as well, only pH was measured in this experiment, using an EcoTester pH2 probe (Oakton). As above, the SML was measured at t = 0 and after one week at 25^{0}C and pH 8.2 (internal control). After this time point the pH was lowered at a rate of 0.1 units per week over a five week period, with SML measured at the end of each week. After the five week period the tank was held at pH 7.7 and 25^{0}C for an additional week, and the SML thickness was measured again. To investigate synergistic effects of acidification with temperature, the temperature was then increased from 25^{0}C to 31^{0}C over a one week period at a rate of 1^{0}C per day with pH maintained at 7.7. The SML thickness was then measured at the end of this period. Fragments were held for an additional week at pH 7.7 and 31^{0}C, followed by a final measurement of the SML. Through the entirety of the experiment pH was monitored six days a week at the same

time every day, to ensure pH stability. As the CO_2 injection system used an adjustable regulator, target pH levels were easily achieved.

Statistical analysis

Analysis of variance (ANOVA) was used to compare SML thickness between all three species at 25^0C and 31^0C for both the rapidly and slowly increasing temperature experiment, as well as pH 8.2 at 25^0C, pH 7.7 at 25^0C and pH 7.7 at 31^0C. Significant results were further investigated using a Tukey's post-hoc test.

To ensure SML thickness was stable before temperature and pH manipulation, a t-Test (paired two sample for means) was performed between the first and second measurements (t = 0 and after one week) at 25^0C (or pH of 8.2) as well as after the final week when the temperature was held at 31^0C (or pH decreased to 7.7). A t-Test was also performed between the first and last measurements of all fragments of each experiment to ensure SML thickness was not affected by the measuring process. All analyses were done using IBM SPSS Statistics 19.

Results

Effect of rapidly increasing temperature

The thickness of the SML was highly variable among individual fragments while temperature was maintained at 25°C for one week. After the one week period, during the time in which temperature was raised from 25ºC to 31ºC, SML thickness decreased for each species (Figure 2.2). The SML remained relatively thinner when the temperature was held at 31 ºC for the final week. SML thickness decreased for *D. clivosa*, *D.*

labyrinthiformis, and *D. strigosa* an average of 37% (SD ± 11), 40% (SD ± 8), and 41% (SD ± 4) respectively. Average thicknesses were 126 µm (SD ± 24) at 25ºC and 78 µm (SD ± 11) at 31ºC for *D. clivosa*, 135 µm (SD ± 18) at 25ºC and 79 µm (SD ± 9) at 31ºC for *D. labyrinthiformis*, and 146 µm (SD ± 26) at 25ºC and 86 µm (SD ± 15) at 31ºC for *D. strigosa*. There was no significant difference between measurements at t = 0 and one week later at 25ºC, indicating a stable SML thickness in the initial two measurements. A slight significant difference was detected between measurements taken one week apart while the temperature was held at 31ºC for *D. clivosa* (t-test t(8), $p > 0.02$), however this may be due to a physiological response to the extremely rapid temperature change. No significant differences were detected between the first and last measurements of any experiment, indicating SML thickness was not affected by the measuring process (data not shown).

Effect of slowly increasing temperature

SML thickness was again highly variable at the individual level when the temperature was held at 25ºC for the one week internal control before experimental manipulation. SML thickness again decreased for all three species when temperature increased at a rate of 1ºC per week (Figure 2.3). SML thickness decreased for *D. clivosa, D. labyrinthiformis,* and *D. strigosa* an average of 43% (SD ± 10), 50% (SD ± 10), and 55% (SD ± 7) respectively. Average thicknesses were 121µm (SD ± 14) at 25ºC and 65µm (SD ± 12) at 31ºC for *D. clivosa*, 129µm (SD ± 15) at 25ºC and 64µm (SD ± 10) at 31ºC for *D. labyrinthiformis*, and 158µm (SD ± 42) at 25ºC and 66µm (SD ± 15) at 31ºC for *D. strigosa*. In these experiments 11 of the 27 fragments died, presumably due to heat

stress (Table 2.1). Four *D. clivosa* fragments died between 28-29ºC (week 5), one between 29-30ºC (week 6) and three while maintained at 31ºC (week 7). Three fragments of *D. strigosa* also died while maintained at 31ºC (week 8). All *D. labyrinthiformis* fragments survived. There was no significant difference between measurements taken during the week before the experiment (25°C) or the week after the experiment (31ºC). Statistical analysis was not possible for *D. clivosa* at 31ºC due to fragment mortality.

Effect of acidification and increasing temperature

Acidification of experimental aquarium water from pH 8.2 to 7.7 over a five week period had no detectable effect on SML thickness (Figure 2.4), although it was highly variable between individual fragments and across time. However, at the end of the pH manipulation when the temperature was raised to 31ºC over a one week period while maintaining the pH at 7.7, SML thickness decreased for *D. clivosa, D. labyrinthiformis,* and *D. strigosa* an average of 33% (SD ± 4), 40% (SD ± 3), and 36% (SD ± 4), respectively. Average thicknesses at pH 7.7 were 106 µm (SD ± 6) at 25ºC and 71µm (SD ± 5) at 31ºC for *D. clivosa*, 119 µm (SD ± 6) at 25ºC and 71µm (SD ± 5) at 31ºC for *D. labyrinthiformis,* and 109 µm (SD ± 8) at 25ºC and 69 µm (SD ± 5) at 31ºC for *D. strigosa*. There was a significant difference in the thickness of the SML between t = 0 and one week later at pH 8.2 and 25ºC for *D. clivosa* (t-test $t(8)$, $p < 0.01$). Although this indicates that SML thickness varied between the initial measurements, no significant difference was detected during acidification from pH 8.2 to pH 7.7. No significant difference was detected in the final week when temperature was increased to 31°C.

Comparison between species

When temperatures were rapidly increased from 25°C to 31°C over one week, there was a significant difference in SML thickness between species at 25°C (ANOVA $F(2,51)$, $p < 0.05$), but not at 31°C (Figure 2.5). A post-hoc Tukey test indicated that SML thickness was significantly thicker in *D. strigosa* compared to *D. clivosa* (M = 20.6, SE 3.76). SML thickness was also significantly different between species at 25°C in the slowly increasing temperature experiment (ANOVA $F(2,51)$, $p < 0.005$). A post-hoc Tukey test indicated that *D. strigosa* was significantly thicker than *D. clivosa* (M = 38.83, SE 9.98) and *D. labyrinthiformis* (M = 30.83, SE 9.98). As mentioned above, statistical analysis was not possible at 31°C due to fragment mortality. In the pH trial there was a significant difference between species at pH 7.7 and 25°C (ANOVA $F(2,15)$, $p < 0.02$). A post-hoc Tukey test indicated that *D. labyrinthiformis* SML thickness was significantly different from that of *D. strigosa* (M = 10.0, SE 3.76) and *D. clivosa* (M = 12.2, SE 3.76) There was no significant difference between species at pH 8.2 and 25°C or pH 7.7 at 31°C.

Discussion

The results of this study demonstrated that the SML thickness associated with all three species of the Caribbean scleractinian coral *Diploria* was highly sensitive to temperature increase. In all experiments and for all three species, average SML thickness decreased between 33-55% with increasing temperature (Figures 2.2, 2.3, 2.4), regardless of the rate of temperature change. SML thickness was highly variable among individual fragments, as well as week to week. Significant differences in SML thickness between

species occurred at 25°C, but not at 31°C (Figure 2.5). In all cases in which there were significant differences between species, *D. clivosa* had significantly thinner SML compared to *D. strigosa* and/or *D. labyrinthiformis.* In one experiment (slowly increasing temperature), *D. labyrinthiformis* had significantly thinner SML compared to *D. strigosa,* while the reverse was true in the acidification experiment (pH 7.7 at 25°C). It is possible that *D. labyrinthiformis* is more capable than the other *Diploria* species of withstanding stressors such as acidification without compromising SML production.

There were also differences in coral fragment mortality during the course of the experiments. Experimental fragments of both *D. strigosa* and *D. clivosa* exhibited mortality in the slowly increasing temperature experiment, while no *D. labyrinthiformis* fragments died (Table 2.1). Mortality rates of *D. clivosa* on the natural reef are undocumented. However, one study showed that *D. clivosa* was significantly more sensitive to temperature increase and high irradiance when compared to five other corals of a different genus (Fournie et al. 2012). In this study significant reductions in photochemical efficiency were observed, although no bleaching occurred. More studies are necessary to determine the exact cause of high mortality in *D. clivosa* and moderate mortality of *D. strigosa*, and whether these results are reflected in the field. The observed differences in survival rates in the slowly increasing temperature experiment and SML thickness in the acidification experiment correspond with the patterns of differences in disease prevalence rates observed between *D. labyrinthiformis* and *D. strigosa* (Rützler and Santavy 1983; Edmunds 1991, Calnan et al. 2008; Jones et al. 2012), and possibly contribute to the relative disease resistance of *D. labyrinthiformis.* Based upon the results

of this study, it is clear that *D. labyrinthiformis* displays higher tolerance to increased temperatures than the other *Diploria* species.

Average SML thickness ranged from 158 µm to 64 µm. In a comparable study, the SML thickness of four different species of scleractinian corals ranged from 145 µm to 700 µm (Jatkar et al. 2010 b). The difference in SML thickness ranges may be attributed to differences in the experimental set-ups and environmental conditions. For example, Jatkar et al. (2010 b) transported entire colonies from the reef and allowed only one hour of acclimation before SML measurements were taken. The stress of transport and environmental changes with only one hour of acclimation may have induced SML production resulting in overall thicker SML measurements. In the present study, fragments were acclimated for two weeks before experimentation, with prior acclimatization in the holding tank, and did not undergo transport. Jatkar et al. (2010 b) also studied different species (*Goniastrea aspera, G. retiformis, Favites abdita,* and *Platygyra daedalea*), possibly contributing to differences in SML thickness. However, there were similarities in the findings of these two studies. Mucus thickness was reduced by approximately half on solar-bleached tissues of *P. daedalea* (Jatkar et al. 2010 b), a similar proportion to the temperature-induced SML reduction detected in the current study. A 1.8 fold increase was also detected in the SML thickness of *G. aspera* after multiple exposures to air due to low tide. Although no increase in SML thickness was detected in the present study (and fragments were not exposed to air), both studies demonstrated that the SML is highly sensitive and responsive to the environment. Additionally, it was also found that the SML thickness of solar-bleached *G. retiformis*

was highly variable (Jatkar et al. 2010 b). High variability among coral fragments was also reflected in the present study. While each experiment isolated and manipulated specific environmental factors to determine the effect on SML, other experimental conditions may have contributed to the SML variation.

The injection of CO_2 and subsequent change in carbonate chemistry, as measured by acidification, had no detectable effect on SML thickness. The significant differences found in the first two measurements of this experiment at pH 8.2 and 25°C may be attributed to the highly dynamic nature of the SML. The absence of SML thickness change in the presence of acidification may be due to the fact that coral mucus is slightly acidic (Meikle et al. 1988; Wild et al. 2005), thus may have served as a natural buffer in the acidification experiment. However there is still potential for a negative effect. In our experiments corals were exposed to increasing acidification, for a final pH of 7.7, for only eight weeks. Other acidification studies used lower pH values of 7.3 (Meron et al. 2011) and 6.7 (Vega Thurber et al. 2009). In these studies, an increase in Alphaproteobacteria and decrease in Deltaproteobacteria and Bacteroidetes was detected within the SML at pH 7.3 (Meron et al. 2011), and a decrease in Alphaproteobacteria and increase in Beta-, Delta- and Epsilonproteobacteria was detected at pH 6.7 (Vega Thurber et al. 2009). Although the current acidification study did not involve microbial analysis, and did not detect changes in the SML thickness, the changes in microbial populations detected in other studies suggest that the SML is affected by acidification in other ways. For instance, it is known that a change in pH alters the selective permeability of eukaryotic mucus barriers, primarily when charged particles are involved (Lieleg and

Ribbeck 2011). The shifts in microbial composition detected in other coral acidification studies may occur due to the direct effects of acidification, or indirectly due to properties of the SML changing. These changes would not be detected in the present study.

Underlying reasons for the SML decrease

The decrease in SML thickness in the presence of rising temperature documented in this study may occur due to several reasons. For example, it could be caused by a decrease in overall SML production, or alternatively an increase in the viscosity of the SML. Brown and Bythell (2005) hypothesized that corals may be able to intentionally produce or secrete less mucus, controlling mucus production by mechanisms such as secretion of specific mucins, alteration of pH of the mucus, transepithelial movement of electrolytes and water, or mucus swelling. Upon the event of elevated temperatures, any number of these mechanisms could hypothetically be induced in order to conserve resources to increase survival chances or circumvent thermal bleaching and stress. However, it is unknown whether corals have the capacity to mediate their mucus production and if the reduction in SML thickness is intentional.

The reduction in SML thickness may be a consequence of increased viscosity in warmer temperatures. This could occur through a change in the physical properties of the mucus when temperature is increased. Up to 80% of mucus released by corals is dissolved into the water column (Wild et al. 2004). It is possible that warmer temperatures could increase the dissolution rate, thinning the amount of mucus remaining on the coral tissue. A change in the chemical composition could also be the reason for an increase in viscosity. It is known that the chemical composition of milked mucus is

different than that of the SML on the tissue surface, with milked mucus containing less mucins (Jatkar et al. 2010 a). These results suggest that corals are capable of producing different types of mucus with different properties (Jatkar et al. 2010 a). An increase in temperature may trigger the release of a different type of mucus by the coral.

It has been suggested that a rise in lipid content increases the viscosity of coral mucus (Wooldridge 2009; Meikle et al. 1988). This is intriguing, as it has been hypothesized that zooxanthellae are responsible for an increase in the lipid content of the SML during the process of thermal bleaching (Wooldridge 2009). All three *Diploria* species are known to harbor zooxanthellae clades B and C (summarized in Goulet and Coffroth 2004). Although zooxanthellae types were not determined in this study, different clades could potentially affect the lipids released into the SML, and thereby the viscosity of the SML, accounting for SML thickness differences between species and individuals. It is intriguing that no fragments bleached in this study, although a slight paling was observed in a few fragments. It is also interesting to note that while zooxanthellae density decreases in the presence of thermal stress, mucocyte density increases (Piggot et al. 2009). In the context of this study, increased mucocyte density may be a compensation mechanism for the decrease in SML thickness at higher temperatures. However, neither mucocyte nor zooxanthellae density were measured in the present study. These, and all other hypotheses regarding SML thinning in increased temperature, remains to be tested.

Potential consequences of warming sea surface temperatures on coral innate immunity

A thinning of the SML in response to increasing sea surface temperatures may pose a real threat to the health of all corals since elevated temperature has been shown to be directly associated with increases in both coral bleaching and disease (Rosenberg and Ben-Haim 2002; Miller et al. 2006; Bruno et al. 2007; Muller et al. 2008; Sharp and Ritchie 2012). Based on the results of this study, the association between increased temperature and bleaching/disease prevalence may be due in part to the thinning of the protective SML barrier. It has been shown that the SML loses its antimicrobial properties when a coral is thermally bleached (Ritchie 2006). The consequences of a thinning SML barrier in combination with loss of antimicrobial activity may synergistically contribute to the observed increase in disease during warmer months, and may potentially increase in severity as temperatures continue to rise.

In the same light, it has been shown that mucus supports the growth of diverse populations of coral-associated microbes (Kvennefors et al. 2012), many of which are believed to be beneficial to the health of coral (the coral probiotic hypothesis). Therefore, another potential consequence of SML thinning may be the reduction in the availability of DOC and nutrients in coral mucus. This may explain the population shift in the coral associated microbial community that was detected in *Porites compressa* fragments subjected to temperature stress (Vega Thurber et al. 2009). A reduction in the available nutrients for heterotrophic bacteria in the SML would limit the number and diversity of microbes within the SML, potentially affecting the symbiotic relationship between corals and their microbial associates.

The SML thicknesses detected in this study were very similar to those found in rat and human gastrointestinal tracts, measured using snap freezing and cryostat sectioning (Jordan et al. 1998), as well as the human colon (Pullan et al. 1994). A general thinning of mucosal layers has been detected in other vertebrates as well. In some cases this thinning has been linked to human diseases. For instance, ulcerative colitis has been associated with a significant thinning of the adherent mucus layer in the colon (McCormick et al. 1990; Pullan et al. 1994; Petersson et al. 2011). In induced colitis of mice, it was found that the mucosal barrier was crucial in protecting the colon from potential pathogens (Petersson et al. 2011). As with vertebrate diseases associated with reduced mucus, the link between mucus thinning and disease may be crucial to understanding the potential impacts of increasing temperatures and acidification on coral reefs.

Overall, this study found that the SML thickness of three Caribbean scleractinian coral species decreased with rising temperature, independent of the rate of temperature increase or with acidification. The consequences of this finding may compromise an important aspect of coral resilience and survival in the face of global climate change. The three species investigated have documented differences in susceptibilities to bleaching and disease, with a relatively resilient species demonstrating greater SML thickness in acidification and higher thermotolerance. Important next steps in this work are to determine the mechanisms involved in the observed SML thinning in response to increased temperature, and to assess the result of challenges by known coral pathogens to corals with temperature-induced thin SML.

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Figure 2.1 Experimental set-up. Submerged coral fragments were examined under a boom dissecting microscope with SML thickness measured using a pulled pipette attached to a micromanipulator at 10 μm resolution.

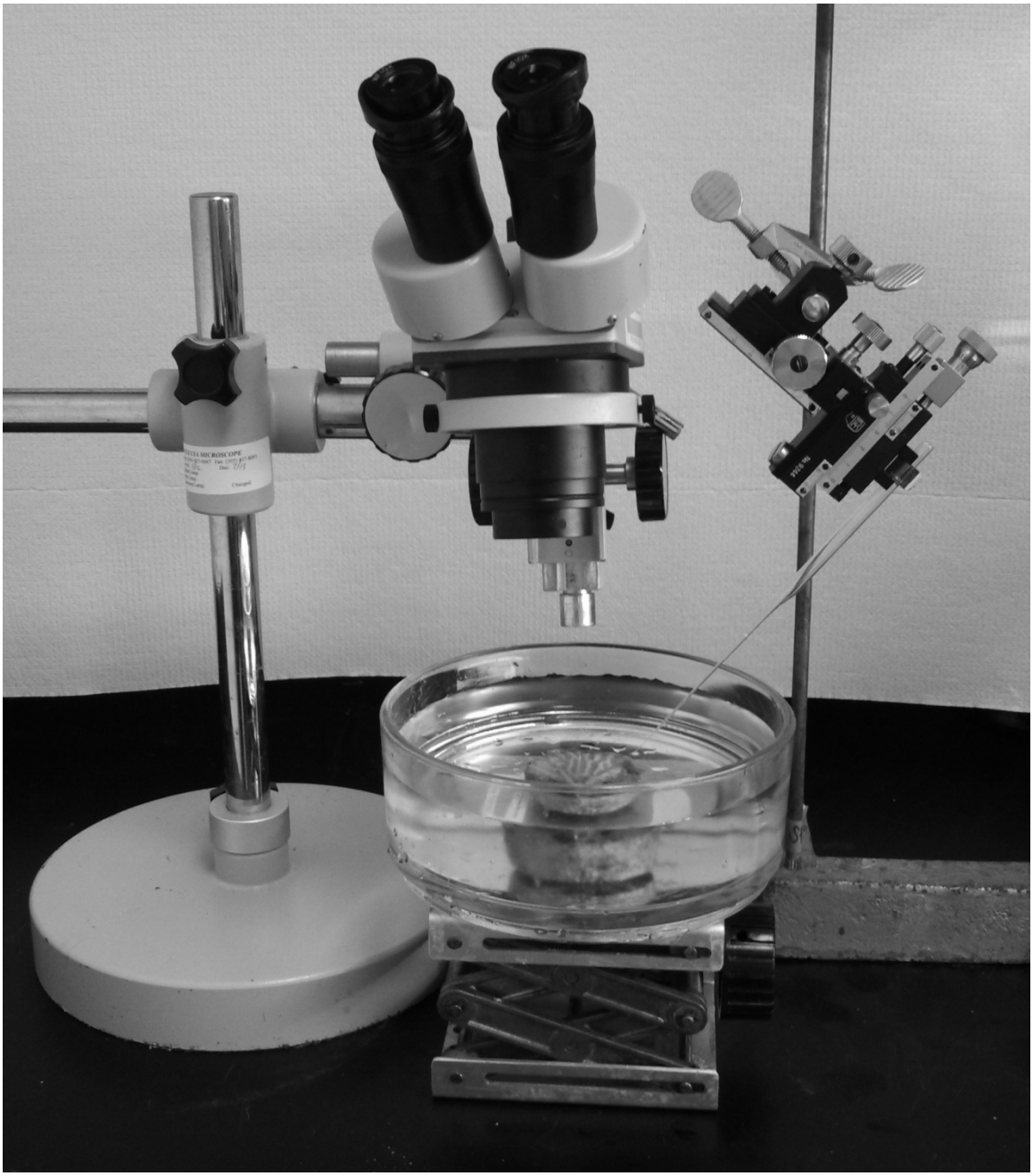

Figure 2.2 Normalized SML thickness (± SD) of three Caribbean coral species, *Diploria clivosa* (n = 9, light gray), *D. labyrinthiformis* (n = 9, medium gray), and *D. strigosa* (n = 9, dark gray), measured over a rapid temperature change from 25°C to 31°C. Measurements were made at t = 0 and one week later with temperature maintained at 25°C (internal control). Temperature (dotted black line) was subsequently raised from 25°C to 31°C at a rate of 1°C per day with a final measurement taken after one week at 31°C. Data were normalized for plotting relative to the initial SML thickness for each individual.

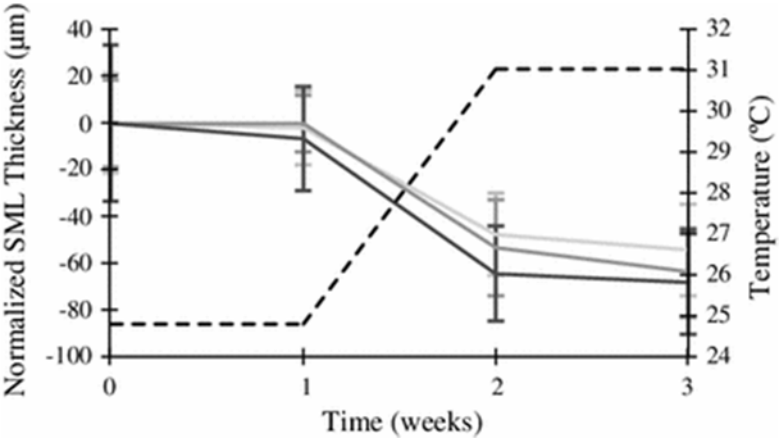

Figure 2.3 Normalized SML thickness (± SD) of *Diploria clivosa* (n = 9, light gray), *D. labyrinthiformis* (n = 9, medium gray), and *D. strigosa* (n = 9, dark gray), measured over a slow temperature change from 25°C to 31°C over a six week time period (weeks one to seven). Temperature (dotted black line) remained at 25°C from t = 0 to week one, and at 31°C from week seven to week eight (internal control). Data were normalized as in Figure 2.2. Measurements were discontinued in fragments that died presumably due to heat stress (8 *D. clivosa*, 0 *D. labyrinthiformis*, 6 *D. strigosa*).

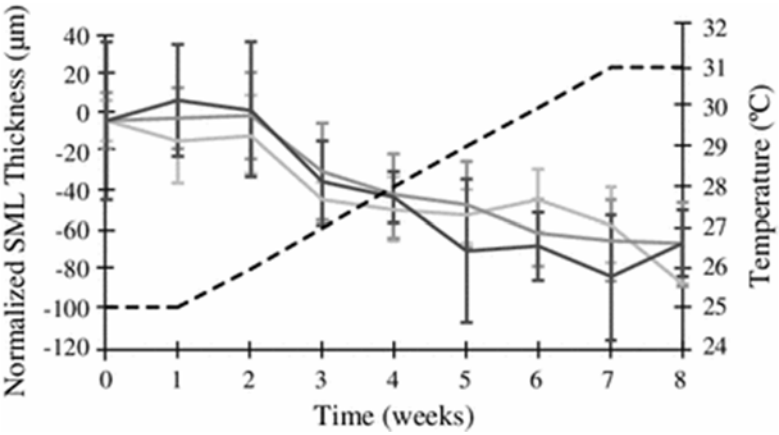

Figure 2.4 Normalized SML thickness (± SD) of *Diploria clivosa* (n = 3, light gray), *D. labyrinthiformis* (n = 3, medium gray), and *D. strigosa* (n = 3, dark gray), measured over a slow pH change (dotted black line) from 8.2 to 7.7 (at 25°C) at a rate of 0.1 units per week (weeks one to six). pH remained constant at 8.2 between t = 0 and week one (internal control). Temperature was increased from 25°C to 31°C (at pH 7.7) between weeks seven and eight at a rate of 1°C per day, and maintained at 31°C during weeks eight to nine (as indicated by *) Data were normalized as in Figures 2.2 and 2.3.

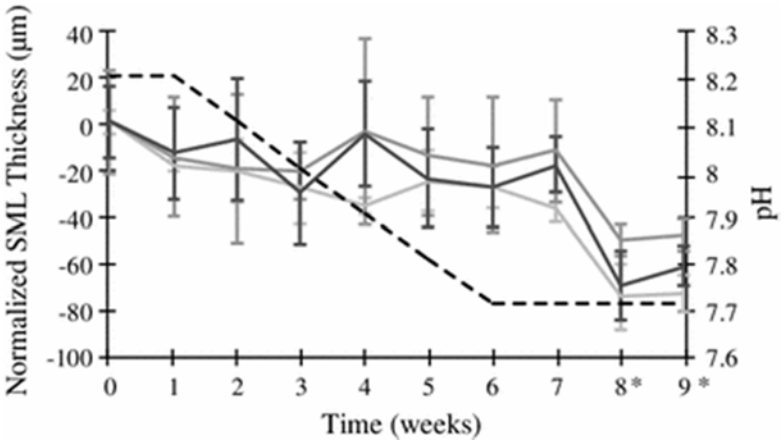

Figure 2.5 Average SML thickness (± SD) of *Diploria clivosa* (light gray), *D. labyrinthiformis* (medium gray), and *D. strigosa* (dark gray) in rapidly increasing temperature (1°C per day), slowly increasing temperature (1°C per week), and rapidly increasing temperature and acidification (pH 7.7). Statistical analysis was not possible at 31ºC in the slowly increasing temperature experiment due to fragment mortality.

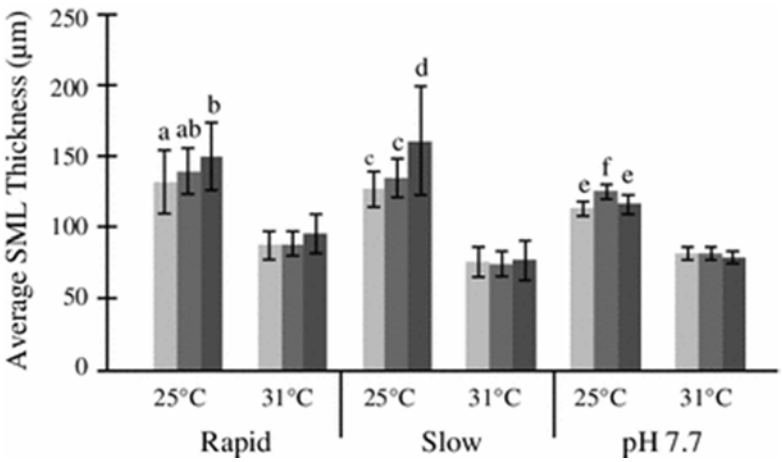

Table 2.1 Mortality of *Diploria clivosa*, *D. labyrinthiformis*, and *D. strigosa* fragments during the slow (six week) increase in temperature experiment from 25ºC to 31ºC (see Figure 2.3).

Species	Fragment Mortality			
	29ºC	**30ºC**	**31ºC**	**Total**
D. clivosa	4	1	3	8/9
D. labyrinthiformis	0	0	0	0/9
D. strigosa	0	0	3	3/9

CHAPTER 3

DIFFERENTIAL GENE EXPRESSION OF TWO SCLERACTINIAN CORAL SPECIES WITH DISSIMILAR DISEASE AND BLEACHING SUSCEPTABILITIES DURING ELEVATED TEMPERATURE AND LOWERED pH STRESS

Abstract

Pseudodiploria strigosa and *Diploria labyrinthiformis* are two closely related scleractinian coral species that are known to have different susceptibilities to both disease and bleaching. *Pseudodiploria strigosa* is relatively prone to disease, while *D. labyrinthiformis* bleaches readily. Both bleaching and disease have been shown to occur during warm summer months when reef temperatures are increased. In the present study we compared the gene expression of these two species *in situ* seasonally over 18 months (sampling every six months). Laboratory experiments were conducted to examine the effects of temperature increase and acidification on gene expression. In total, 359 unique transcripts were differentially expressed seasonally, and 261 in the laboratory experiments. In all conditions, *P. strigosa* displayed more differentially expressed genes (compared to controls) than *D. labyrinthiformis.* The higher number of differentially expressed genes, in particular those related to stress, may indicate that *P. strigosa* is more sensitive to environmental changes than *D. labyrinthiformis*. Conversely, *D. labyrinthiformis* may fail to respond to environmental changes, leaving it more prone to temperature-induced bleaching. *Diploria labyrinthiformis* tended to upregulate genes associated with innate immunity in the summer, perhaps explaining its relative disease resistance. The present study demonstrates that although closely related, these two species have different responses to warming and acidification, possibly contributing to their differences in disease and bleaching susceptibility and potentially affecting their capacity to survive global climate change.

Introduction

Coral cover is in decline worldwide (Pandolfi et al. 2011), and considerable efforts must be made to both reduce anthropogenic threats to corals and to improve their resilience (Carilli et al. 2009). Two imposing threats facing corals are increasing sea surface temperatures and ocean acidification (Hoegh-Guldberg et al. 2007; Anthony et al. 2008, 2011; Hughes et al. 2010). Increasing sea surface temperatures have been associated with coral bleaching, disease, and mortality (Hughes et al. 2003; Sutherland et al. 2004), while acidification is known to decrease the calcification process (Hoegh-Guldberg et al. 2007; Dove et al. 2013). There is a diversity of coral responses to these threats, ranging from high susceptibility to bleaching or disease to high resiliency to multiple stressors (Marshall and Baird 2000; Hughes et al. 2003). The range in responses can be between different species of coral (Grottoli et al. 2006) and even among different individuals of the same species (Sutherland et al. 2004).

Understanding the differences between resilient corals and those that are sensitive to environmental change is key to coral management and conservation efforts (Hughes et al. 2010). In an "omics" era, many tools have recently become available to compare resilient and susceptible corals, to elucidate possible mechanisms of disease and bleaching resistance. In particular, transcriptomic analysis has become a commonly used technique to evaluate a large number (in the case of microarrays) or all (in the case of RNA-seq) mRNA genes currently being expressed, and can serve to identify genes that are significantly upregulated or downregulated between genotypes, populations, or conditions (Wang et al. 2009). Currently, the majority of coral transcriptomic research

has been conducted on a few select corals: *Acropora millepora, A. palmata, Orbicella* (formerly *Montastraea*) *faveolata,* and *Porites asteroids* (Miller et al. 2011). The technique has been used to evaluate thermally stressed larvae of *Acropora* (Rodriguez-Lanetty et al. 2009; Portune et al. 2010; Meyer et al. 2011) and *Orbicella* (formerly *Montastraea*) *faveolata* (Polato et al. 2010), thermally stressed *O. faveolata* (DeSalvo et al. 2008) and *Stylophora pistillata* adults (Maor-Landaw et al. 2014), and dark-bleached *O. faveolata* (DeSalvo et al. 2012). Transcriptomic analysis has also been used to identify differentially expressed genes (DEGs) between *Acropora* larvae in response to acidification (Moya et al. 2012; Kaniewska et al. 2012), and applied to investigate coral host response to white band disease (Barshis et al. 2013) and yellow band disease (Closek et al. 2014).

While these studies have provided invaluable information regarding coral transcriptomic response to rapid temperature increase, acidification, and disease, there is a distinct gap in this newly growing field. There are no studies (to the authors' knowledge) examining the seasonal nature of transcriptomic responses to temperature changes *in situ*, although it has been shown that coral transcriptomes are highly variable among individuals in the same environmental conditions, independent of coral genotype (Granados-Cifuentes et al. 2013). Another missing component in coral transcriptomics is direct comparison between species or genera. Comparisons using RNA-seq between closely related species of other taxa are beginning to emerge, lending clues to how closely related species react differently to identical conditions (Liu et al. 2011; Guan et

al. 2013). Comparing similar coral species with known variation in disease and bleaching susceptibility may potentially elucidate molecular resistance mechanisms.

In the present study we targeted the corals *Diploria labyrinthiformis* and *Pseudodiploria* (formerly *Diploria*) *strigosa* (Budd et al. 2012), two closely related species that are known to have different susceptibilities to bleaching and disease. *Diploria labyrinthiformis* is known to be resistant to diseases such as black band disease, yet more susceptible to bleaching, while *P. strigosa* is more susceptible to disease and resistant to bleaching (summarized in Pratte and Richardson 2014). In the present study RNA-Seq was performed to identify DEGs between *D. labyrinthiformis* and *P. strigosa* both seasonally on the reef and in controlled laboratory warming and acidification experiments, in order to elucidate underlying mechanisms of disease and bleaching susceptibility and resistance.

Materials and Methods

Field sample collection

Six pairs (each pair consisting of one *D. labyrinthiformis* colony and one *P. strigosa* colony), were identified in the Florida Keys National Marine Sanctuary, three at Horseshoe Reef (N 25' 08.362 W 80' 17.641) and three at Algae Reef (N 25' 08.799 W 80' 17.579). Each pair separated by less than 3 meters, to limit varying environmental conditions between the two species, thus ensuring that DEGs would be as a result of species differences, rather than environmental differences. Tissue, SML, and skeleton samples were taken from each coral colony at three time points: August 2013 and 2014,

to investigate the effects of warmer temperatures, and February 2014 to examine gene expression at cooler temperatures. These time points are hereafter referred to as Summer 2013, Summer 2014 and Winter, respectively. Temperature upon the time of collection was 30°C in both Summers and 25 ° C in Winter. Using a hammer and chisel small (1 cm^2) tissue/skeleton samples were removed, placed in Whirlpak bags, and promptly brought to the surface where they were immediately placed into 1 ml NucleoSpin RNA Plant kit (Macherey-Nagel) lysis buffer. Samples were then placed on dry ice for transport to the laboratory and frozen at -80°C until processing.

Experimental set-up and collection

Laboratory experiments were conducted to validate field (temperature variation) results, as well as to compare coral microbial associates and gene expression in the presence of acidification (pH 7.7) and warming (31°C) in synergy. *Psuedodiploria strigosa* (n = 3) and *D. labyrinthiformis* (n = 3) colonies were collected from the Florida Keys National Marine Sanctuary Coral Nursery between November 2012 and October 2013. Colonies were fragmented into approximately 3 cm^2 pieces, set onto small cement pedestals, and allowed to recover a minimum of 3 weeks before experimentation. Experimental aquaria were set up according to Pratte and Richardson (2014) and chapter 2. The temperature treatment group was subject to increasing temperature from 25-31°C over a period of six weeks (1°C per week and collected upon reaching 31°C), hereafter referred to as the Temperature Group. The second treatment group underwent increasing temperature as described above, as well as acidification from pH 8.2-7.7 at a rate of 0.1 pH units per week using a CO_2 injection system (AZOO, Taiwan), hereafter referred to as

the Temperature + pH Group. The Control Group was maintained at 25°C and a pH of 8.2. All aquaria were set up in duplicate, with 2-5 fragments of each species in each aquarium, depending upon availability. A total of 5 *D. labyrinthiformis* fragments and 8 *P. strigosa* fragments were used for each treatment. At the termination of the experiment, skeleton, tissue, and SML samples were collected as described in the field sample collection, and stored at -80°C until further processing at Florida International University.

RNA extraction and processing

The Nucleospin® RNA Plant Kit (Machery-Nagel) was used for all RNA extractions. The SML, tissue and skeleton samples frozen at -80°C in 1 ml of lysis buffer were allowed to partially thaw in a sterile Petri dish, with the addition of 10 µl β-Mercaptoethanol (Fisher Scientific). Tissue and semi-frozen lysis buffer were macerated using sterile razor blades, and 700 µl of the resulting slurry was used for further processing according to the manufacturer's protocol, with the exception of an equal volume (700 µl) of 70% ethanol when adjusting the RNA binding conditions. Samples were centrifuged at 13,000 x g and the resultant eluted RNA was stored at -80°C until further processing.

Total RNA was quantified using the Qubit® 2.0 Fluorometer (Life Technologies) and RNA integrity number (RIN) was determined using the Agilent RNA 6000 Pico Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies) following the manufacturer's protocol. All samples achieved an RIN 7.0 or higher. The RNA samples were pooled according to species for each treatment or time point collected, and submitted to the Sequencing Facility at Florida International University. Isolation of mRNA from total

RNA pooled samples was completed using Dynabeads mRNA Direct Micro Kit following the manufacturer's recommendations (Life Technologies). Construction of the transcriptome cDNA library was accomplished using the Ion Total RNA-Seq Kit v2 (Life Technologies). Each species and/or treatment was given a separate barcode using the Ion Xpress RNA-Seq Barcode 01-16 Kit (Life Technologies). Prior to template preparation, cDNA libraries were assessed for DNA concentration and fragment size distribution using the Agilent High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Template preparation was performed on the Ion OneTouch 2 instrument (Life Technologies) using the Ion PGM Template OT2 200 Kit (Life Technologies). Samples were then enriched using the Ion OneTouch ES instrument (Life Technologies). Each of the four time points was run on a separate Ion 318 Chip v2 (Life Technologies), which included all experimental treatments from that time point. The Ion Torrent PGM (Life Technologies) performed 200 base-read sequencing using the Ion PGM Sequencing 200 Kit v2 (Life Technologies). Ion Torrent PGM sequences were filtered using Torrent Suite v4.2 software to remove polyclonal and low quality sequences.

Identification of differentially expressed genes

The FASTQ files generated by the Ion Torrent server were imported into the public Galaxy server (usegalaxy.org, Goecks et al. 2010, Giardine et al. 2005). These files are publically available in under project ID PRJNA286280 in the NCBI's Sequence Read Archive. Seasonal (field) samples and laboratory experiment samples were analyzed independently. Ion Torrent .fastq files were groomed using the FASTQ

Groomer (Blankenberg et al. 2010), and aligned to the *P. strigosa* transcriptome (http://people.oregonstate.edu/~meyere/data.html) using Bowtie2 (Langmead and Salzberg 2012). Cufflinks v. 2.1.1 (Trapnell et al. 2010) was used to assemble transcripts. Data sets were merged using Cuffmerge, after which Cuffdiff was used to detect significant differences in gene expression using a 5% false discovery rate (Trapnell et al. 2010). Significantly differentially expressed genes were manually curated using previous annotations (http://people.oregonstate.edu/~meyere/data.html), GO terms, literature, and protein databases. Genes were then categorized according to molecular pathway associations. Only genes that were significantly differentially expressed as determined by Cuffdiff using a 5% false discovery rate are reported.

Results

Differential gene expression

From the Ion Torrent analysis, a total of 29,706,020 raw reads were obtained, 16,375,032 of which passed the Torrent Suit™ quality control check. For seasonal field sampling all barcodes contained between 1,409,577 and 2,558,998 reads, while all laboratory experiment barcodes contained between 164,383 and 805,588 reads (Table 3.1). For all samples, 37-58% of *P. strigosa* reads and 40-64% of the *D. labyrinthiformis* reads mapped to the *P. strigosa* genome (http://people.oregonstate.edu/~meyere/data.html). Average read length for all barcodes ranged from 113 to 137 bp. A total of 689 transcripts (0.14% of all transcrips) were identified as significantly different (Cuffdiff) between seasonal field samples (Appendix A, Table A1), 359 of which were unique (i.e. some transcripts were significantly different

for more than one comparison). Of the unique transcripts, 83 matched known proteins, 59 matched putative proteins, and 217 had no known matches (see "Putative?" column in Appendix A, Tables A1 and A2) according to the mapped *P. strigosa* annotations (http://people.oregonstate.edu/~meyere/data.html). A total of 571 transcripts (1.47% of all transcripts) were identified as significantly different between laboratory experiments (Appendix A, Table A2), 261 of which were unique. Of the unique transcripts, 57 matched known proteins, 47 matched putative proteins, and 157 had no known matches. The total number of genes significantly up or down regulated among all seasonal samples and all experimental samples are summarized in Table 3.2 and Table 3.3, respectively. Transcripts matching known proteins were grouped into the following categories: cell regulation and metabolism, stress induced, cell structure and movement, protein regulation and metabolism, lipid and fatty acid metabolism, carbohydrate metabolism, and other/function unknown (Figure 3.1). Only significant differentially expressed genes between each species within the same season and each species between seasons (Table 3.4), and each species within the same treatment and each species between treatments (Table 3.5), are discussed below.

Cell regulation and metabolism

Within the data set associated with seasonal variation on the reef (field sampling), 11 genes associated with cell regulation and metabolism were differentially expressed. In general, *D. labyrthinthiformis* tended to upregulate genes associated with cell cycle and ion transporters in the summers, while *P. strigosa* upregulated genes such as tumor necrosis factors (TNF3 and TNFR1) and transcription factor TenA in the summers. Only

one transcript associated with ATP metabolism was significantly different between the two species in Summer 2013, and was upregulated in *P. strigosa*. In the laboratory experiments, *P. strigosa* had more differentially expressed genes (seven) than *D. labyrinthiformis* (one). These include genes for folate synthesis, Ran GTPase, and progranulin.

Stress induced

Sixteen genes associated with stress were differentially expressed seasonally on the reef. Differentially expressed genes of interest for *P. strigosa* include those for DNA damage response, cell membrane repair, hypoxia response lectins, a negative regulator of excessive innate immune response, heat shock protein (HSP) 67B2, oxidative stress resistance and dehydration response proteins. *Diploria labyrinthiformis* differentially expressed very few stress related genes, including a lectin binding protein and a possible immune associated proline rich protein.

In the laboratory experiments, eleven genes associated with stress were differentially expressed. Interestingly, *D. labyrinthiformis* exhibited no significantly different genes between the Control and Temperature groups, and only two transcripts (nitric oxide synthesis) were upregulated in the Temperature + pH group compared to the Temperature group. *Pseudodiploria strigosa* displayed more differentially expressed genes in laboratory experiments, including responses to oxidative stress, temperatures stress, and hypoxia. Comparing species within the control group, two genes associated with response to oxidative stress were upregulated in *P strigosa* and C3 was upregulated in *D. labyrinthiformis*.

Cell structure and movement

A total of 19 genes associated with cell structure and movement were differentially expressed seasonally, 13 for *D. labyrinthiformis* and six for *P. strigosa*. Of the 17 genes differentially expressed between summer and winter, 16 were upregulated in the summer. Particular genes of interest include those associated with collagen, cytoskeletal proteins (such as alpha tubulin and dynein), cell adhesion proteins, titin, clustering/fusing of late endosomes, and vesicular trafficking. In the laboratory experiments, *D. labyrinthiformis* exhibited no differentially expressed genes (associated with cell structure and movement) between the three treatments. *Pseudodiploria strigosa* had four differentially expressed genes between treatments, and two differentially expressed genes between species, all associated with extracellular vesicular exosomes.

Protein regulation and metabolism

Seasonally, 16 genes associated with protein regulation and metabolism were significantly different in the two coral species. In particular, two protein folding chaperones, and a proteasome complex protein were upregulated in the summers for *D. labyrinthiformis*, and *P. strigosa* upregulated a folding chaperone, a protease inhibitor, and an extracelluar metalloprotease in summer. Experimentally, 16 genes were differentially expressed as well. *Diploria labyrinthiformis* tended to upregulate metalloendoproteases, while *P. strigosa* upregulated a response to unfolded proteins (ERAD). Calumenin was differentially expressed between the two species in the Control group (upregulated in *P. strigosa*).

Lipid and fatty acid metabolism

Each species had four seasonally differentially expressed genes (8 total). For *D. labyrinthiformis* genes for allene oxide biosynthesis and annexin were upregulated in the summer, and for *P. strigosa* those for fatty acid and phospholipid biosynthesis. One gene associated with fatty acid metabolism and synthesis was significantly downregulated in Summer 2014 (i.e., upregulated in Winter) for both species. Only four lipid and fatty acid metabolism genes were seen to be differentially expressed in the experimental data.

Carbohydrate metabolism

In general, very few genes associated with carbohydrate metabolism were differentially expressed. Only one gene associated with carbohydrate metabolism (a glutamate transporter) was seasonally differentially expressed, and six were differentially expressed in the experimental data. Of particular interest, *P. strigosa* upregulated genes associated with pyruvate and the citric acid cycle in the Temperature + pH treatment when compared to *D. labyrinthiformis*.

Other/function unknown

All transcripts that matched proteins that were either unknown or did not fall into the above categories were considered "other/function unknown". Seasonally, there were 35 differentially expressed genes in the category "other/function unknown". Genes of interest include those for crystalline, which was upregulated in *P. strigosa* in Summer 2013, and trefoil factor 2, which was downregulated in *P. strigosa* but upregulated in *D. labyrinthiformis* in both summers. The experimental data included 42 genes that were

classified as “other/function unknown”, including a green fluorescent protein and E3 ubiquinin ligases.

Discussion

Relatively few genes (3-14) were differentially expressed between the two species within the same season (summer) for the field studies or for the experimental Temperature treatment, indicating that the two species respond similarly to temperature changes. However, 51 genes were differentially expressed when the two species were exposed to lowered pH, suggesting they may respond differently to ocean acidification. For the majority of these 51 genes (80%), the function was unknown (according to mapped *P. strigosa* annotations), shedding little light as to why these two species responded differently to the decrease in pH. In all seasonal time points (field data) and experimental treatments (laboratory data), *P. strigosa* had a greater number of differentially expressed genes (compared to control or winter samples) than *D. labyrinthiformis*, suggesting that *P. strigosa* is more responsive and/or sensitive to environmental stressors and corroborating the findings of Barshis et al. (2013) in which there were fewer differentially expressed genes in the more resilient *A. hyacinthus*.

Interestingly, *D. labyrinthiformis* tended to differentially express genes (in both field and laboratory data) associated with innate immunity. These included genes for complement (C3), nitric oxide synthase (also associated with thermal and oxidative stress; DeSalvo et al. 2008), and allene-oxide synthase-lipoxygenase (AOS-LOX). The protein AOS-LOX is of particular interest, as it is a catalyst necessary for the formation of clavulone I, which is thought to have anti-viral and anti-cancer roles (De Luna *et al*.

2013). The first coral AOS-LOX, described in *Plexaura homomalla* (Caribbean sea whip) by Oldham et al. (2005), is known to be upregulated in white band infected *A. cervicornis* (Libro et al. 2013) and has been directly associated with wound healing in the soft coral *Capnella imbricata* (Lõhelaid et al. 2014).

Mydlarz et al. (2006) hypothesize that environmental conditions can compromise innate immune response of coral. The hypothesis is supported by the current study, in which *P. strigosa* upregulated a negative regulator of innate immune response both summers. The suppression of the immune system is particularly concerning, as this indicates that *P. strigosa*'s innate immune system may be compromised when coral disease peaks (Rosenberg and Ben-Haim 2002; Muller et al. 2008; Sharp and Ritchie 2012). However, it appears that the innate immune system of *D. labyrinthiformis* was not suppressed in the summer. It is possible that the effect of environmental changes on the innate immune system may vary by species, contributing to the observed differences in disease susceptibility.

Transcripts coding for lectins were upregulated in both species in Summer 2013, and in *P. strigosa* in Summer 2014. These results oppose the trend observed in *A. hyacinthus* (Barshis et al. 2013) and *A. millepora* larvae (Rodriguez-Lanetty et al. 2009), in which lectins were downregulated in thermally stressed individuals in manipulative experiments. Alternatively, lectins were found to be upregulated in *Acropora cervicornis* with white band disease (Libro et al. 2013), thermally stressed *S. pistillata* (Maor-Landaw et al. 2014) and naturally bleached *A. millepora* (Seneca et al. 2010). While lectins often have innate immune roles such as pathogen recognition, they are also

associated with *Symbiodinium* uptake (Davy et al. 2012), as well as many other functions. Combined, these studies provide evidence that the expression of lectins is dependent upon more than temperature alone, innate immunity, or *Symbiodinium* uptake alone, confounding any clear patterns that might otherwise be observed.

While *D. labyrinthiformis* differentially expressed genes associated with innate immunity in both summers, *P. strigosa* upregulated more genes associated with environmental stress, such as those associated with responses to hypoxia, dehydration, oxidative stress, DNA and cell membrane damage, and temperature stress. Curiously, transcripts associated with DNA damage, oxidation stress, and hypoxia (different transcripts than those upregulated in the summers), were upregulated in the winter. The response of *P. strigosa* to stressors appeared to be more unpredictable than *D. labyrinthiformis*, perhaps reflecting that it is the more sensitive of the two species in terms of temperature changes, also seen in the Control group, where oxidative, temperature, and hypoxia responses were also upregulated. Hypoxia response has also been observed to be upregulated in dark-induced bleached *A. palmata* (DeSalvo et al. 2012), and oxidative stress genes are commonly upregulated in thermally stressed corals (DeSalvo et al. 2008; Rodriguez-Lanetty et al. 2009). It is particularly interesting in the present study that *D. labyrinthiformis* did not differentially express any of these stress-related genes at any point.

Similarly, heat shock proteins (specifically HSP67B2) were only differentially expressed in *P. strigosa* and were upregulated each summer. Upregulation in folding chaperones was detected in both species in either Summer 2013 or 2014. Heat shock

proteins (HSPs) are commonly the focus of cnidarian thermal stress studies (Leggat et al. 2011), however they may not tell the entire story, as they are absent in several studies of coral heat stress (the present study and Barshis et al. 2013), and have been shown to decrease in expression levels a mere 10 hours after the initial temperature change (Rodriguez-Lanetty et al. 2009). Heat shock proteins can also be unexpectedly downregulated, for example HSP90 was downregulated in *Porites astreoides* (Kenkel et al. 2013). Sharp et al. (1997) reported a downregulated in HSP70 in *Goniopora dljiboutiensis*. Since folding chaperones were observed upregulated in both species (the present study), they may provide a reliable marker of long-term thermal stress across coral species.

In all cases, protein transport associated transcripts were upregulated in the summer and in the experimental aquaria that experienced a temperature increase. There was no clear pattern in the upregulation of metalloproteases, protease inhibitors, or degradation of glycoproteins, or endoplasmic-reticulum-associated-protein degradation (ERAD). Although Maor-Landaw et al. (2014) hypothesized that ERAD is an early warning sign of thermal stress, in the present study it was only upregulated under experimental conditions, and not in a consistent manner. In terms of acidification, this study did not match the results of Moya et al. (2012), which examined gene expression of *A. millepora* under acidification. Primarily, they observed an increase in extracellular peptidases and metalloproteases. Only one metalloprotease was significantly different (in *D. labyrinthiformis*), which was down regulated. Additionally, Moya et al. (2012) suggest that metabolism is suppressed by acidification, yet the present study demonstrated that *P.*

strigosa had an increase in amino acid and methionine biosynthesis genes. The differences in protein metabolism and regulation genes between these studies may lie in the fact that the present study increased temperature and acidification together, while Moya et al. (2012) examined acidification alone.

Calumenin is an interesting protein which was upregulated in the *P. strigosa* Control group. Calumenin is a calcium binding protein associated with protein folding and sorting, the coagulation system, wound healing, and has loosely been tied to lung fibrosis and arterial pulmonal hypertension diseases in humans (Honoré 2009). In corals, calumenin is attributed to other roles, such as calcification (Libro et al. 2013) and the endosymbiosis of *Symbiodinium* (Davy et al. 2012). Additionally, it has been found to be upregulated in white band infected *A. cervicornis* (Libro et al. 2013) and thermotolerant *A. millepora* (Bellantuono et al. 2012). While the specific roles calumenin plays in corals remains vague, its implication in several processes crucial to coral health make it clear this gene should be a target of interest in future studies.

Ion transporters have been found to be differentially expressed in thermally stressed corals. They have been found to be upregulated in *Acropora millepora* larvae subject to heat stress (Meyer et al. 2011) and downregulated in heat stressed *O. faveolata* (DeSalvo et al. 2008). In other studies, expression of ion transporters was found to be varied (Barshis et al. 2013). In the present study, ion transporters were upregulated in *D. labyrinthiformis* in both summers, while *P. strigosa* did not upregulate ion transporters in either summer. Of particular interest, no genes associated with ion transport were significantly different in the acidification experiment, as was found in with *A. millepora*

larvae (Moya et al. 2012). It has been hypothesized that calcification may be tied to ion transport, and that acidification may affect this process (Moya et al. 2012, Allemand et al. 2011). However, in this experiment only seasonal temperature changes affected the expression of ion transporters.

Rearrangement of the cytoskeleton in warmer temperatures is a common theme found across the majority of coral transcriptome studies, although there is not always a consistent pattern in the up or downregulation of these genes. In increased temperatures, cytoskeletal associated genes were downregulated in adult and larval *O. faveolata* (DeSalvo et al. 2008; Polato et al. 2010), *A. millepora* embryos (Portune et al. 2010), and *S. pistillata* (Maor-Landaw et al. 2014). However, they were found to be upregulated in active white band disease of *A. cervicornis* (Libro et al. 2013). In the present study, cytoskeletal regulation in response to stressors appears to be important for both *D. labyringthiformis* and *P. strigosa*, as cytoskeletal associated genes were upregulated in summers. There are several hypotheses as to why cytoskeletal associated genes are differentially expressed with thermal stress. First, remodeling of host skeleton, manipulation of endosomal trafficking and the inhibition of phagosome/lysosome fusion are associated with the phagocytosis of *Symbiodinium* and creation of a symbiosome (Davy et al. 2012). As bleaching (the loss of *Symbiodinium*) is a response to thermal stress, the differentially expressed cytoskeletal genes may reflect a shift in the coral's interaction with the symbiosome. Similarly, cytoskeleton rearrangement could be a sign of apoptotic or phagocytosis activities (Libro et al. 2013). Cytoskeleton regulation may be associated with vesicular transport unrelated to *Symbiodinium*, as vesicles use the

cytoskeleton in order to move about the cell. Whatever the reason, the present study reinforces the idea that cytoskeletal rearrangement is a distinct response to thermal stress, and should be targeted for further investigative transcriptomic studies.

The gene expression for a few seemingly random proteins may also provide insight into the mechanisms of disease and bleaching resistance. For example, the upregulation of collagen-associated proteins has been found in more thermally-tolerant individuals (Barshis et al. 2013, Kenkel et al. 2013). However, in the present study collagen was upregulated for both species in Summer 2013, suggesting that collagen does not play a role in the different susceptibilities of *D. labyrinthiformis* and *P. strigosa*. Genes assosciated with E3 ubiquitin ligase (UB3), which plays roles in many pathways, has been noted to be differentially expressed in several studies, including this one. Gene expression of UB3 has been found to be downregulated in partially bleached *P. astreoides* (Kenkel et al. 2013), and the ubiquitin conjugating enzyme E2 was downregulated in larval *A. millepora* subject to thermal stress (Meyer et al. 2011). However in the present study, UB3 was found to be upregulated in only *P. strigosa* in the Temperature + pH treatment. The implications of UB3 upregulation are unknown, and may be a response to acidification. Lastly, Tumor Necrosis Factor 3 (TNF3), which regulates apoptosis and innate immune functions, was upregulated in Summer 2013 in *P. strigosa*, which has also been seen in thermally stressed *Acropora hyacinthus* (Barshis et al. 2013) and yellow band diseased *O. faveolata* (Closek et al. 2014). The TNF Receptor 1 (TNFR1) is also of interest, as it was upregulated in *P. strigosa* in Summer 2014. The TNFR1 was also upregulated in white band diseased *A. cervicornis* (Libro et al. 2013).

The upregulation of TNFs and associated genes may be an ideal stress response indicator, as it appears to be upregulated consistently with the association of stress.

Very few genes associated with carbohydrate metabolism were differentially expressed in this study. However, consistent upregulation of fatty acid and lipid metabolism in response to temperature and pH stressors was seen in both species. In contrast, for white band diseased *A. cervicornis*, the genes associated with the breakdown of lipids were upregulated and fatty acid synthesis was downregulated (Libro et al. 2013). Kenkel et al. (2013) showed that inshore *P. astreoites* may have upregulated lipid metabolism compared to offshore corals, implying that the more thermally intolerant individuals may rely upon lipid metabolism. In the present study there were no significant differences between *P. strigosa* and *D. labyrinthiformis,* as fatty acid biosynthesis tended to be upregulated in Summer 2013 and downregulated in Summer 2014 for both species, possibly indicating a higher level of stress in 2014.

During the period of the present study, none of the *in situ* corals sampled bleached or became diseased. However, two weeks after the August 2014 samples were taken, a severe bleaching event occurred that affected the entire Florida Reef Tract. (It is not known whether the corals sampled in this study bleached during this event.) This is very interesting because, for both species, Summer 2013 had many more differentially expressed genes (vs. Winter) than Summer 2014, and is potentially indicative of a response to thermal (and possibly UV) stress at the time of sampling, which was then manifested two weeks later as a mass bleaching event on these reefs. Therefor, we

hypothesize that bleaching may be predicted by a weak gene response to seasonal temperatures and/or a failure to respond to environmental stressors.

It must be considered that all of the gene expression discussed above, shown to be statistically significantly up- or downregulated in the transcriptomic analysis, does not necessarily mean that there was complete translation of the proteins they code. The RNA-Seq technique is limited by the number of known genes, and it is possible protein homologues may have functions different than those assigned, as function is commonly inferred through homology. Despite these limitations, this study has shown that RNA-Seq provides valuable information for gaining insight into molecular mechanisms of corals.

In this study we found differential gene expression between two closely related coral species indicating a transcriptomic response to thermal and acidification stress. In all conditions, *P. strigosa* had more differentially expressed genes compared to either the Control or Winter samples, possibly indicating a higher sensitivity to stressors. In support of this hypothesis, *P. strigosa* tended to differentially express genes associated with stress compared to *D. labyrinthiformis*. Of the two species, *D. labyrinthiformis* is more disease resistant, which is corroborated by the consistent upregulation of innate immunity genes in both Summers. The patterns in gene expression observed in this study provide the first insight into the gene expression of two closely related species with varying susceptibility to disease and bleaching, and can be used in future studies to aid in identifying resilient coral species and genotypes.

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Table 3.1 Number of raw reads, quality reads, aligned reads, and average read length for all samples. Each Ion 318 Chip had 2 barcodes for seasonal data, and 6 barcodes for experimental data.

Sample	Number of Raw Reads per Ion 318 Chip	Number of Quality Reads (QC > 20) per Barcode	Number of Aligned Reads	Percent of Aligned Reads	Average bp Length
Summer 2013 *D. labyrinthiformis*	8,408,039	1,931,703	1,230,293	63.69%	113
Summer 2013 *P. strigosa*		2,558,998	1,486,167	58.08%	116
Winter *D. labyrinthiformis*	7,408,020	2,325,722	927,298	39.87%	135
Winter *P. strigosa*		1,510,875	563,976	37.33%	121
Summer 2014 *D. labyrinthiformis*	8,408,039	3,363,351	1,862,709	55.38%	135
Summer 2014 *P. strigosa*		1,409,577	686,500	48.70%	137
Control (25°C, pH 8.2) *D. labyrinthiformis*	6,153,249	662,689	302,630	45.67%	128
Temperature (31°C, pH 8.2) *D. labyrinthiformis*		164,383	73,575	44.72%	122
Temperature + pH (31°C, pH 7.7) *D. labyrinthiformis*		718,567	424,445	59.07%	132
Control (25°C, pH 8.2) *P. strigosa*		329,494	190,838	57.92%	127
Temperature (31°C, pH 8.2) *P. strigosa*		260,934	129,548	49.65%	127
Temperature + pH (31°C, pH 7.7) *P. strigosa*		805,588	399,404	49.58%	135
Total	**29,706,020**	**16,375,032**	**8,277,383**	**50.55% (average)**	**127.3**

Table 3.2 Number of differentially expressed genes between all coral species (*P. strigosa* and *D. labyrinthiformis*) and seasons (Winter, Summer 2013, and Summer 2014). A total of 689 genes were differentially expressed between all comparisons. Arrows indicate up- and down-regulation in relation to the samples listed in the column at the left.

	***D. labyrinthiformis* Winter**	***D. labyrinthiformis* Summer 2013**	***D. labyrinthiformis* Summer 2014**	***P. strigosa* Winter**	***P. strigosa* Summer 2013**
***D. labyrinthiformis* Summer 2013**	91 (46↑, 45↓)	-	-	-	-
***D. labyrinthiformis* Summer 2014**	29 (18↑, 11↓)	25 (4↑, 21↓)	-	-	-
***P. strigosa* Winter**	3 (3↑, 0↓)	93 (37↑, 56↓)	27 (10↑, 17↓)	-	-
***P. strigosa* Summer 2013**	78 (36↑, 42↓)	14 (2↑, 12↓)	45 (24↑, 21↓)	103 (59↑, 44↓)	-
***P. strigosa* Summer 2014**	33 (21↑, 12↓)	57 (19↑, 38↓)	13 (6↑, 7↓)	40 (23↑, 17↓)	38 (15↑, 23↓)

Table 3.3 Number of differentially expressed genes between all coral species (*P. strigosa* and *D. labyrinthiformis*) and treatment groups (Control- 25°C, pH 8.2 vs. 31°C, pH 8.2 and 31°C, pH 7.7). A total of 571 genes were differentially expressed between all comparisons. Arrows indicate up- and down-regulation in relation to the samples listed in the column at the left.

	D. labyrinthiformis **Control 25°C, pH 8.2**	***D. labyrinthiformis*** **31°C, pH 8.2**	***D. labyrinthiformis*** **31°C, pH 7.7**	***P. strigosa*** **Control 25°C, pH 8.2**	***P. strigosa*** **31°C, pH 8.2**
D. labyrinthiformis **31°C, pH 8.2**	24 (17↑, 7↓)	-	-	-	-
D. labyrinthiformis **31°C, pH 7.7**	78 (36↑, 42↓)	11 (9↑, 2↓)	-	-	-
P. strigosa **Control 25°C, pH 8.2**	10 (5↑, 5↓)	64 (10↑, 54↓)	106 (32↑, 74↓)	-	-
P. strigosa **31°C, pH 8.2**	47 (23↑, 24↓)	6 (5↑, 1↓)	8 (2↑, 6↓)	85 (50↑, 35↓)	-
P. strigosa **31°C, pH 7.7**	3 (3↑, 0↓)	28 (10↑, 18↓)	51 (33↑, 18↓)	15 (13↑, 2↓)	35 (18↑, 17↓)

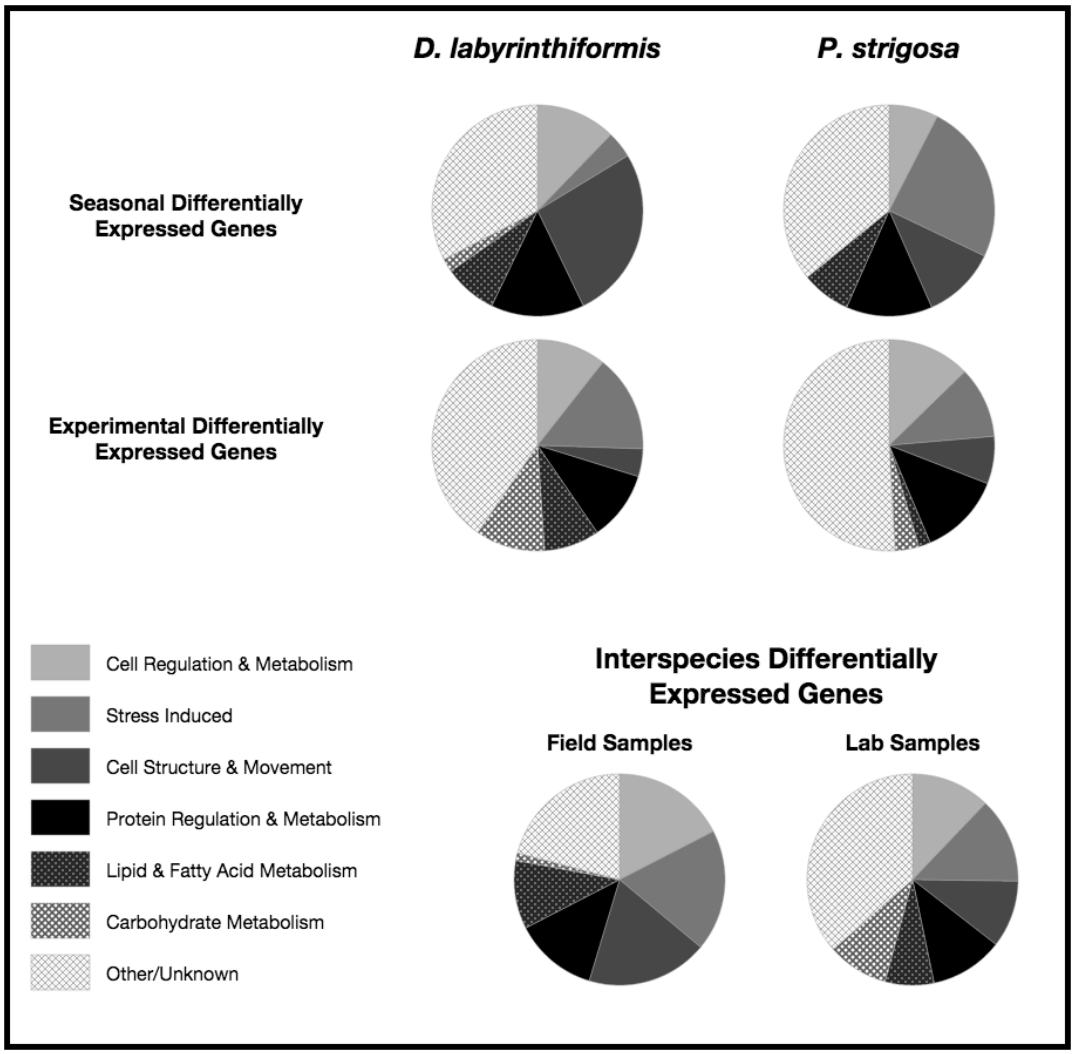
Table 3.4 Number of differentially expressed genes between seasons within the same species, and between species within the same season. Comparisons are between three seasons (Summer 2013, Summer 2014, and Winter), and two coral species (*P. strigosa* and *D. labyrinthiformis*). Differentially expressed genes are categorized according to molecular pathway associations. No match indicates the transcript did not match any known proteins.

	D. labyrinthiformis			***P. strigosa***			**Interspecies Comparison (*D. labyrinthiformis* vs. *P. strigosa*)**		
	Winter vs. Summer 2013	Winter vs. Summer 2014	Summer 2013 vs. Summer 2014	Winter vs. Summer 2013	Winter vs. Summer 2014	Summer 2013 vs. Summer 2014	Summer 2013	Summer 2014	Winter
Cell Regulation and Metabolism	5	1	0	3	1	0	1	0	0
Stress Induced	2	0	0	5	5	3	0	1	0
Cell Structure and Movement	10	2	1	3	2	1	0	0	0
Protein Regulation and Metabolism	5	3	1	4	1	2	0	0	0
Lipid and Fatty Acid Metabolism	3	1	0	3	1	0	0	0	0
Carbohydrate Metabolism	1	0	0	0	0	0	0	0	0
Other/Function unknown	11	0	2	11	6	4	1	0	0
No Match	54	22	21	74	24	28	12	12	3
Total	91	29	25	103	40	38	14	13	3

Table 3.5 Number of differentially expressed genes between experimental treatments within the same species, and between species within the same treatment. Comparisons are between three treatments (Control (25°C, pH 8.2) vs. Temperature (31°C, pH 8.2) and Temperature + pH (31°C, pH 7.7)), and two coral species (*Pseudodiploria strigosa* and *Diploria labyrinthiformis*). Differentially expressed genes are categorized according to molecular pathway associations. No match indicates the transcript did not match any known proteins.

	D. labyrinthiformis		***P. strigosa***		**Interspecies Comparison (*D. labyrinthiformis* vs. *P. strigosa*)**		
	25°C, pH 8.2 vs. 31°C, pH 8.2	31°C, pH 8.2 vs. 31°C, pH 7.7	25°C, pH 8.2 vs. 31°C, pH 8.2	31°C, pH 8.2 vs. 31°C, pH 7.7	25°C, pH 8.2	31°C, pH 8.2	31°C, pH 7.7
Cell Regulation and Metabolism	0	1	6	1	0	0	0
Stress Induced	0	2	5	0	3	1	0
Cell Structure and Movement	0	0	2	2	0	0	2
Protein Regulation and Metabolism	1	1	6	4	1	0	3
Lipid and Fatty Acid Metabolism	1	0	1	0	0	0	2
Carbohydrate Metabolism	0	0	1	1	1	0	3
Other/Unknown	6	0	12	8	3	1	12
No Match	16	7	52	19	2	4	29
Total	24	11	85	35	10	6	51

Figure 3.1 Differentially expressed genes for the two coral species *D. labyrinthiformis* and *P. strigosa* between seasons (Summer 2013, Winter 2013, and Summer 2014), and experimental treatments (Control- 25°C, pH 8.2, vs. 31°C, pH 8.2, and 31°C + pH 7.7). Genes are arranged by functional category.



CHAPTER 4

MICROBIAL ASSOCIATES OF TWO SCLERACTINIAN CORAL SPECIES WITH DISSIMILAR DISEASE AND BLEACHING SUSCEPTABILITIES DURING ELEVATED TEMPERATURE AND LOWERED pH STRESS

Abstract

Coral bleaching and disease are two common occurrences which are contributing to global coral cover decline. Understanding the interactions between the coral animal and its microbial associates, and how they may change in the presence of stressors such as warming and acidification, is a crucial component to understanding resistance to disease and bleaching. In this study, the bacterial communities of the surface mucopolysaccharide layer, tissue, and skeleton of two closely related coral species with different disease and bleaching susceptibilities (*Diploria labyrinthiformis* and *Pseudodiploria strigosa*) were examined *in situ* on the Florida Reef Tract for 18 months (sampled every six months), and experimentally in the laboratory. *In situ* sampling included seasonal temperature variation, with an anomalously high summer temperature on these reefs. The laboratory study involved experimental manipulation of both temperature increase and lowered pH. Bacterial communities of both coral species were highly similar in the winter, and shifted differently in each summer, suggesting that coral may harbor "winter" and "summer" microbiota. Differences between the two coral species were primarily caused by Clostridia, Gammaproteobacteria, and rare species, with *P. strigosa* harboring more rare species than *D. labyrinthiformis.* It is these rare species, such as *Desulfovibrio,* that may contribute to the relatively higher disease susceptibility of *P. strigosa* when compared to *D. labyrinthiformis.*

Introduction

Coral reef degradation is occurring world wide, and is believed to be largely the result of anthropogenic impacts (Pandolfi et al. 2011). The health of the coral itself is dependent

upon all members of the coral holobiont, which is composed of the coral animal, algal endosymbionts (*Symbiodinium*), viruses, bacteria, archaea, and fungi (Rohwer et al. 2002; Reshef et al. 2006; Rosenberg et al. 2007). The bacterial members of the holobiont are particularly crucial, fulfilling roles such as disease resistance through production of antimicrobials (Ritchie 2006), niche occupation believed to prevent invasion by pathogens (Reshef et al. 2006), and nutrient biogeochemical cycling such as nitrogen fixation, nitrification, and sulfate reduction (Beman et al. 2007; Raina et al. 2009; Kimes et al. 2010; Lema et al. 2012; Bourne et al. 2013; Pratte 2013). Given the importance of the coral-associated bacterial community, it is essential to understand how the bacterial community might contribute to coral health, disease, and resilience in the presences of anthropogenic stressors.

Coral susceptibility to disease and bleaching (the loss of the symbiotic *Symbiodinium* from the coral tissue) is thought to be exacerbated by anthropogenic impacts such as warming and acidification (Pandolfi et al. 2011). These susceptibilities are variable between different coral species, as well as among colonies of the same coral species (Sutherland et al. 2004; Grottoli et al. 2006; Hughes et al. 2010; Pandolfi et al. 2011). On reefs of the wider Caribbean, two major reef-building corals, *Pseudodiploria strigosa* and *Diploria labyrinthiformis*, display different bleaching and disease susceptibilities, despite being closely related. *Pseudodiploria strigosa* tends to be more susceptible to diseases, while *D. labyrinthiformis* bleaches more readily (reviewed in Pratte and Richardson 2014). Comparing the associated microbial communities of these two coral species can reveal differences in the bacterial communities, and when assessed

in response to stressors such as temperature increase and acidification, may lead to insights into possible mechanisms of both bleaching resilience and resistance to disease. For example, the coral probiotic hypotheses states that individual bacterial members of the coral holobiont are responsible for evolving protection mechanisms against potential pathogens, thereby protecting the entirety of the coral holobiont (Reshef et al. 2006). Therefore the presence of specific antimicrobial bacteria associated with *D. labyrinthiformis* may contribute to its relative disease resistantance.

Examining the eukaryotic (coral and zooxanthellae) and prokaryotic components of the holobiont in parallel is crucial to understand how multiple components of the holobiont interact, particularly when exposed to warming and acidification. Therefore, the present study is presented in conjunction with chapter three in which the response of the coral transcriptome to these environmental stressors was examined. Here the microbial component of the study is presented, in which the bacterial communities of the two coral species *D. labyrinthiformis* and *P. strigosa* were compared seasonally for 18 months (*in situ*), and experimentally in the presence of warming and acidification, for the purpose of identifying differences in the bacterial populations which may contribute to bleaching or disease resistance.

Materials and Methods

Field sample collection

Six pairs of coral colonies (with each pair comprised of one *D. labyrinthiformis* colony and one *P. strigosa* colony) were identified in the Florida Keys National Marine

Sanctuary. Three pairs were located at Horseshoe Reef (N 25' 08.362 W 80' 17.641) and three at Algae Reef (N 25' 08.799 W 80' 17.579). Within each pair, the two colonies were less than 3 meters apart, to limit variation of environmental conditions. Small samples (1 cm^2) that included coral tissue, the surface mucopolysaccharide layer (SML), and underlying skeleton were taken from each coral head using a hammer and chisel, and immediately placed in individual Whirlpak bags, followed by patching the sample site with non-toxic modelling clay. Samples were then promptly brought to the surface, put into 1 ml of RNA later® (Life Technologies) in a 2 ml microcentrifuce tube, and placed directly on dry ice. Samples were then transported to the laboratory where they were kept at -80°C until further processing. Parallel (separate) samples were collected for transcriptomic analysis at the same time (chapter three). All samples were collected at three time points: August 2013 and 2014, to investigate the effects of warmer temperatures, and February 2014 (winter on these reefs). Hereafter, these time points will be referred to as Summer 2013, Summer 2014, and Winter. Temperature upon the time of collection was 30°C in each Summer, and 25°C in the Winter.

Laboratory experiments

Laboratory experiments were carried out to compare coral bacterial associates in the presence of acidification (pH 7.7) and warming (31°C) in synergy under controlled conditions. Three colonies of each species were collected from the Florida Keys National Marine Sanctuary Coral Nursery between November 2012 and October 2013 and fragmented into approximately 3 cm^2 pieces. Fragments were set onto small cement pedestals, and allowed to recover a minimum of 3 weeks before experimentation.

Experimental aquaria were set up identically, according to Pratte and Richardson (2014) and chapter two. Briefly, three 19 liter experimental aquaria contained a recirculating filter, live rock, and artificial sea water and were maintained on a 12:12h light:dark cycle. All aquaria were allowed to establish for two weeks before coral introduction. The temperature treatment group (referred to as Temperature) was subject to increasing temperature from 25-31°C over a period of six weeks at a rate of 1°C per week. The second treatment group (referred to as Temperature + pH) included both increasing temperature as described above and acidification from pH 8.2 to 7.7 at a rate of 0.1 units per week. The change in pH was implemented using a CO_2 injection system (AZOO, Taiwan). The Control group was maintained at 25°C and a pH of 8.2. All aquaria were set up in duplicate, with 2-5 fragments of each species in each aquarium, depending upon availability. A total of five *D. labyrinthiformis* fragments and eight *P. strigosa* fragments were used for each treatment. At the termination of the experiment, skeleton, tissue, and SML samples were collected as described above for the *in situ* study, and stored at -80°C until further processing. As with the field analysis, samples were collected at the same time for transcriptomic analysis (chapter three).

DNA extraction, processing, and analysis

Samples frozen at -80°C in RNAlater® (Life Technologies) were allowed to thaw on ice. Once thawed, the sample of SML, tissue, and skeleton was placed in a bead beating column provided by FastDNA™ Spin Kit for Soil (Qbiogene, Vista, CA, USA) and genomic DNA extracted according to the manufacturer's protocol. DNA was then quantified using the Qubit® 2.0 Fluorometer (Life Technologies) and pooled according

to species for each treatment or time point. All pooled genomic DNA samples were diluted to a concentration of 20 ng/µl. To identify the bacterial population associated with samples, the V4 and V5 region of the 16S rRNA gene was amplified via PCR using primers F563/BSR926 (Claesson et al. 2010). The concentration used for all PCR reactions was 1x PCR Buffer, 2.5 mM $MgCl_2$, 0.5 µM of each forward and reverse primer, 0.25 U GoTaq® Hot Start Polymerase (Promega), and 10 ng genomic DNA. The total volume was brought up to 20 µl with DNA-grade sterile water. The PCR amplifications were conducted in a Peltier Thermal Cycler (PTC-200, MJ Research, Waltham, MA, USA) under the following conditions: 94°C for 8 min, followed by 35 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute, with a final soak at 72°C for 10 minutes. All reactions were run in duplicate, and products verified on a 1.8% TBE agarose gel with GelRed™ (Biotium). Total DNA was quantified using the Qubit® 2.0 Fluorometer (Life Technologies, Foster City, CA, USA). The DNA samples were pooled according to species for each treatment or time point collected. Each species and/or treatment was given a separate barcode using the Ion Xpress RNA-Seq Barcode 01-16 Kit (Life Technologies). Each of the three time points from seasonal sampling, and all experimental samples, were run on a separate Ion 314 Chip v2 (Life Technologies). The Ion Torrent PGM (Life Technologies) performed 200 base-read sequencing using the Ion PGM Sequencing 200 Kit v2 (Life Technologies). Ion Torrent PGM sequences were filtered using Torrent Suite v4.2 software to remove polyclonal and low quality sequences. All raw .fastq files are publicly accessible via the MG-RAST metagenomics analysis server (project ID 12497).

Data analysis

The .fastq files generated by the Ion Torrent server were imported into the MG-RAST server for analysis (Meyer et al. 2008). All sequences were screened for quality control (QC) a second time within MG-RAST. Only sequences which passed QC and were of bacterial origin (as determined by the default settings of MG-RAST) were further analyzed. The 16S rRNA gene sequences were sorted according to class and exported for further statistical analysis using PRIMER 6 software (Primer-E Ltd). Only bacterial classes with a minimum of five reads were included in the analysis. Data were square root transformed and non-metric multi-dimensional analysis (NMDS) was performed using Bray-Curtis similarity matrices (Clarke 1993; Clarke and Gorley 2001). The SIMPROF (similarity profile) analysis was performed to identify significantly different communities, and the SIMPER (similarity percentages) analysis was used to perform dissimilarity contributions (Clarke 1993; Clarke et al. 2008). Shannon-Weiner diversity indices (H' log e) were also calculated using PRIMER-E. Additionally, individual operational taxonomic units (OTUs) at the genus and species level were manually curated for OTUs of interest.

Results

Next generation sequencing

Altogether 1,475,367 raw reads were obtained, 1,440,516 of which passed the Torrent Suit™ (QC) check, and 1,023,141 of which passed the MG-RAST QC control check. After QC, all barcodes for seasonal field sampling contained between 138,108 and

247,238 reads, and laboratory experiment barcodes contained between 6,416 and 123,660 reads (Table 4.1). For all samples, GC content ranged between 43-50%. Average read length after QC for all barcodes ranged from 165 to 205 bp.

NMDS and Shannon-Wiener indices

For each barcode, the number of bacterial ribosomal reads detected by MG-RAST ranged from 93,550 to 147,854 for field samples, and 3,454 to 56,993 for experimental samples (Table 4.1). These reads were clustrered according to bacterial class and used to create an NMDS plot to compare bacterial communities. For the field data, the Winter samples of both species cluster closely, while the Summer samples show no clear pattern for either coral species (Figure 4.1). The SIMPER analysis indicated that the Winter bacterial communities of each species were 92.88% similar, while in Summer 2013 they were 64.00 % similar for *P. strigosa* and 80.1% similar for *D. labyrinthiformis* (Table 4.2). In the NMDS plot using data from the laboratory experiment, there was no clear pattern regarding species or treatment (Figure 4.2). The SIMPER analysis showed that between species Control bacterial communities were 85.1% similar, Temperature bacterial communities were 74.4% similar, and pH + Temperature Communities were 87.9% similar (Table 4.3). Shannon-Weiner diversity indices ranged from 1.694-2.042 for all seasonal data, and 1.826-2.134 for experimental data (Table 4.1). With the exception of the Temperature treatment, *P. strigosa* demonstrated a higher diversity index than *D. labyrinthiformis* (within the same treatment or season), largely because of rare species. Although it is possible the higher abundance of rare taxa in *P. strigosa* samples is as a result of contamination from DNA extraction and amplification (Salter et

al. 2014), this is unlikely, as both species were processed in random order using only one kit per time point. However, diversity indices need to be examined and the bacterial genera and species level for multiple individual corals in order to verify that *P. strigosa* has a slightly higher diversity index than *D. labyrinthiformis.*

Bacterial communities

A total of 32 bacterial classes were detected within all experimental and seasonal (*in situ*) communities. *Pseudodiploria strigosa* Summer 2013 was represented by 27 classes (Figure 4.3), and was the only seasonally statistically different bacterial community ($p < 0.05$). All other seasonal bacterial communities were comprised of 10-13 classes (Figure 4.3). Accordingly, *P. strigosa* Summer 2013 was not included in calculated averages and standard deviations of classes. Spartobacteria, Opitutae, Planctomycetia, Lentisphaerae, Gemmatimonadetes, Chrysiogenetes, and Solibacteres were found only in the *P. strigosa* Summer 2013 bacterial community. Bacilli (26.0 ± 2.9%), Gammaproteobacteria (23.9 ± 2.9%) , and Clostridia (28.0 ± 8.3%) were most abundant within all field samples, with the exception of *P. strigosa* Summer 2013, in which Alphaproteobacteria were the most abundant group (29.8%). All other seasonal bacterial communities contained an average of 1.9 ± 1.3% Alphaproteobacteria. Differences in Alphaproteobacteria contributed the most dissimilarity between Summer 2013 bacterial communities and all other seasonal bacterial communities (Table 4.3). Cyanobacteria were detected in all field samples, although they were more abundant in *P. strigosa* Summer 2013 (16.7%) as compared to all other communities (2.7 ± 1.7%). The most abundant cyanobacterial genus detected was *Cyanothece*. The classes Fusobacteria

(3.5 ± 1.4%), Actinobacteria (7.1 ± 4.4%), Bacteroidia (4.2 ± 1.7%), Betaproteobacteria (1.0 ± 0.7%) and Deltaproteobacteria (1.5 ± 0.8%) were also detected in all samples. Classes that were only detected in Summer (2013 or 2014) for either species include Chloroflexi, Sphingobacteria, Deinococci, Mollicutes, and Verrucomicrobia (all < 1%). For *D. labyrinthiformis,* differences in Betaproteobacterial abundance contributed the most to dissimilarities between Summer and Winter samples, although dissimilarity contributions were fairly dispersed between all classes (Table 4.2). *Pseudodiploria strigosa* comparisons were much more variable, with the classes Actinobacteria, Alphaproteobacteria, Bacteroidia, Clostridia, and Cyanobacteria contributing the most to seasonal dissimilarities. Alphaproteobacteria, Bacilli, Bacteroidia, Clostridia and Cyanobacteria were the largest contributors to dissimilarity between the two coral species (Table 4.2).

In the laboratory experiment, the Control *D. labyrinthiformis* was only represented by 10 bacterial classes. This low number of classes must be interpreted with caution since there were fewer reads for this sample than all others (Table 4.1). All remaining experimental samples contained between 22 and 32 classes (Figure 4.4). The *D. labyrinthiformis* Temperature treatment clearly shows a different bacterial community, and was statistically different ($p < 0.05$) from all other experimental samples. Therefore this data set was subsequently not included in calculated averages and standard deviations. As with the seasonal bacterial communities, Bacilli (19.9 ± 3.9%), Clostridia (24.9 ± 5.7%), and Gammaproteobacteria (21.4 ± 3.3%) were the most abundant classes. Alphaproteobacteria represented an average of (5.2 ± 2.1%) of sequences for all

treatments, with the exception of the Temperature treatment for *D. labyrinthiformis* which contained 19.9% Alphaproteobacteria. Accordingly, the Alphaproteobacteria had the highest percent dissimilarity between *D. labyrinthiformis* treatments (Supplementary Table 4S). Actinobacteria (11.5 ± 1.7%), Deltaproteobacteria (5.1 ± 0.7%), Planctomycetia (1.9 ± 0.8%) and Bacteroidia (5.9 ± 1.5%) were present in all samples. Cyanobacteria were found in all samples although they were considerably more abundant in Temperature *D. labyrinthiformis* (17.8%) compared to all other samples (2.3 ± 0.8%), largely caused by the species *Thermosynechococcus elongatus* (14.5%). Lentisphaerae, Spartobacteria and Chlorobia were found in low abundance (<1%) only in treatments with raised temperatures (i.e. not the Control). Aquificae was only found in *P. strigosa* in both treatments with raised temperature (<1%). Ktedonobacteria only found in *P. strigosa*, in all treatments (<1%). The remaining classes (Chloroflexi, Cytophagia, Flavobacteria, Fusobacteria, Deinococci, Gemmatimonadetes, Opitutae, Solibacteres, Sphingobacteria, Verrucomicrobiae, Epsilonpreteobacteria, Mollicutes, Spirochaetia and Thermomicrobia) were present low abundance (<1%) for at least three of the six treatment/coral species combinations. Unclassified bacteria drove the largest percent dissimilarity among all experimental bacterial communities.

Individual operational taxonomic units (OTUs) were further mined for trends at the genus and species levels. However these data should be read with caution, as they are limited to the information contained in the M5NR database used by MG-RAST. The M5NR limitation, combined with shorter reads produced by next generation sequencing, may result in incorrect hits at the genus and species level (see Webster et al. 2010).

Additionally, the presence of an OTU does not indicate whether that bacterium is currently in a latent state or not (see Klein 2015). Therefore the following discussion of data should be interpreted as OTUs with similarity to that genus and species, rather than the definite presence of the specified bacteria. The percent similarity and significance values, as well as percent community composition, are given in Appendix B, Table B1.

The cyanobacterial genus *Trichodesmium* was present in all samples (with the exception of *P. strigosa* Temperature), and was more abundant in Winter samples than Summer 2013 and 2014 samples. *Lactobacillus vaginalis* was the primary reason for the large Bacilli presence in all samples, with the exception of *P. strigosa* in Summer 2013. *Reinekea blandensis* composed the majority of Gammaproteobacteria, again with the exception of *P. strigosa* in Summer 2013. *Clostridium botulinum* and *Heliobacterium modesticaldum* were present in all seasonal samples, were more abundant in *D. labyrinthiformis* than *P. strigosa*, and tended to be more abundant in the Winter. *Syntrophus aciditrophicus* was present in all samples and more abundant in both Summers compared to the Winter. The genera *Azotobacter*, *Alcanivorax,* and *Teredinibacter* was only found in *P. strigosa* communities (all experimental treatments and at least one Summer) in low abundance (<1%) except *Teredinibacter* which was 3.8% in Summer 2013. *Desulfovibrio* was only present in experimental treatments with increased temperature, or in summer months, and was more common in *P. strigosa* communities than *D. labyrinthiformis.* At the family level, Desulfobacteraceae, Desulfobulbaceae, Desulfomicrobium, and Desulfohalobiaceae followed the same trend. *Acidovorax* was only present in *P. strigosa* in treatments with increased temperature,

although it was found in both species and all seasons in the field. *Candidatus Puniceispirillum marinum, Sulfitobacter*, *Brucellaceae, Lentisphaerae* and *Deinococcus* were only detected in warm samples (i.e., both Summers and the treatments with increased temperature) for both species at low abundance (<1%).

Discussion

Comparison of coral-associated bacterial communities

Coral are known to maintain distinct bacterial communities that vary among coral species (Rohwer et al. 2002) and/or genera (Littman et al. 2009). In the present study the NMDS plots reveal that the Winter bacterial communities of *D. labyrinthiformis* and *P. strigosa* cluster tightly, indicating that two species of the genera *Diploria* and *Pseudodiploria* maintain similar bacterial communities during Winter (Figure 4.1). However, each summer the bacterial community of each species diverged, and did not cluster according to summer season (2013 or 2014), or to coral species. The seasonally shifting pattern in the coral microbiome is intriguing, as similar patterns were found in the coral transcriptomic data collected at the same time (chapter three). Specifically, the transcriptomes of both coral species were very similar in Winter, while there were many significant differences in the Summers. Upon taxonomic evaluation, the overall structure of the coral-associated bacteria appears to be relatively stable between seasons at the class level (Figure 4.3), but further investigation revealed subtle shifts occurred at the genus and species levels. For instance, OTUs similar to *Desulfovibrio, Candidatus Puniceispirillum marinum, Sulfitobacter*, *Brucellaceae, Lentisphaerae,* and *Deinococcus* were all only detected in warm temperatures, while OTUs similar to *Trichodesmium,*

Clostridium botulinum and *Heliobacterium modesticaldum* were more abundant in the Winter. These data suggest that corals may harbor a "winter microbiome" that may be similar, yet distinct, from that in the summer. Typically, shifts in the coral microbiota are commonly associated with bleaching or disease (Pantos and Bythell 2006; Bourne et al. 2008); however, the present study is the first to use high-throughput sequencing to examine seasonal changes in the coral microbial associates of two closely related coral species. These community shifts may be natural, such as the seasonal shifts shown in the maple sap microbiota (Filteau et al. 2010), rhizosphere microbiota (Smalla et al. 2001), high mountain lake microbiota (Pernthaler et al. 1998), and the microbiota of the Arctic water column (Murray et al. 1998). Seasonal microbial evaluation of three sponges also revealed that although the core microbiota remained relatively stable, minor shifts were detected in microbes present in lower abundance (Taylor et al. 2004), and seasonal patterns in the microbiota associated with several different coral species have also been shown (Ceh et al. 2011; Chiu et al. 2012). It remains to be seen whether the observed shift in seasonal microbiota is a result of changes in environmental conditions, a change in the bacterial source (i.e., water column), or possibly the change in coral gene expression (chapter three) leading to changes in the bacterial community.

The NMDS plots of all experimental treatments of both coral species revealed no clear pattern (Figure 4.2). It has previously been shown that the coral-associated bacterial community shifts dramatically when placed in aquaria (Kooperman et al. 2007; Pratte et al. 2015), possibly explaining the lack of clustering. An NMDS plot of all bacterial communities indicated a very strong clustering according to sample origin (field or

aquarium), with the exception of *P. strigosa* Summer 2013 (data not shown). However, the bacterial class composition was roughly consistent between all experimental samples (Figure 4.4), as well as seasonal (field) samples (Figure 4.3) and those previously published for *P. strigosa* (Rohwer et al. 2002; Cooney et al. 2002), with the exception of two bacterial communities (*P. strigosa* in Summer 2013 and the *D. labyrinthiformis* Temperature treatment) which were more similar to that published by Sunagawa et al. (2010). Each of these bacterial communities is marked by a much higher abundance of Alphaproteobacteria and Cyanobacteria, and lower abundances of Actinobacteria, Clostridia, and Bacilli (Figure 4.1). Diversity indices were also highest for the *P. strigosa* Summer 2013 communities compared to all other field communities, and for the *D. labyrinthiformis* Temperature treatment community compared to all other treatments. Increases in both bacterial diversity and Alphaproteobacteria have been previously associated with coral bleaching (Bourne et al. 2009) and disease (Sekar et al. 2008; Sunagawa et al. 2009), and it may be that these two bacterial communities are representative of a subclinical infection, despite the colonies being visually healthy (Reed et al. 2010). These results emphasize the need to acquire multiple data points when assessing the coral microbial community, to be able to identify anomalies such as those described here.

In the present study, pH had no apparent effect on the bacterial communities of either coral species. In similar studies, Meron et al. (2011) documented an increase in Alphaproteobacteria, Vibrionaceae and Alteromonadaceae when *Acropora eurystoma* was subjected to a pH of 7.3, and Vega Thurber et al. (2009) saw an increase in

Bacteroidetes, Chlorobi, Cyanobacteria, and Spirochaetes in *Porites compressa*. However, none of these shifts were detected in the present study, possibly because of differences in the levels of acidification (7.7 in the current study vs. 7.3 and 6.8 in the mentioned studies). It is also possible that subtle shifts in the microbiota occurred that were too small to be detected or below the cut-off value of number of reads utilized in this study.

Functional significance of differing coral-associated bacterial communities

Direct comparisons between the two coral species reveal that seasonally, *D. labyrinthiformis* had a higher abundance of Clostridia, specifically OTUs similar to *Clostridium botulinum* and *Heliobacteruim modesticaldum*, in all seasons when compared to *P. strigosa.* Clostridia also had the highest percent contribution to the dissimilarities detected between both species. Rohwer et al. (2002) hypothesized that Clostridia in the coral mucus play a role in the fermentative breakdown of polysaccharides, which in turn may suggest that the mucus of *D. labyrinthiformis* is slightly richer in polysaccharides than *P. strigosa*, although this has not been proven. With the exception of the Summer 2013 bacterial community, *P. strigosa* contained a higher percentage of Gammaproteobacteria than *D. labyrinthiformis*, as well as a higher diversity index (Table 4.1). In particular, *Azotobacter, Alcanivorax,* and *Teredinibacter* were all found only in *P. strigosa* bacterial communities. *Azotobacter* and *Teredinibacter* are known nitrogen fixers (Bulen et al. 1965; Distel et al. 2002), while *Alcanivorax* is known for hydrocarbon degradation (Hara et al. 2003). It is curious that two bacterial genera found only in *P. strigosa* are known to fix nitrogen, which may imply that

nitrogen fixation is more prominent in *P. strigosa* than *D. labyrinthiformis.* Additionally, *Acidovorax*, which is known for nitrate-dependent iron oxidation (Carlson et al. 2013), was higher in abundance for *P. strigosa* in all experimental treatments. However, the significance of these findings needs to be validated, as they comprised less than 1% of the overall community, and the percent similarity is low for the majority of these genera and species (see Appendix B, Table B1). Finally, *Desulfovibrio* was more common in *P. strigosa* bacterial communities, particularly in the summer. Although *Desulfovibrio* is present in relatively low abundance, its presence could explain the higher prevalence of black band disease (BBD) in *D. strigosa* (see Pratte et al. 2014) as the initiation of BBD is dependent on production of sulfide by sulfate-reducing bacteria such as *Desulfovibrio* (Richardson et al. 2009; Brownell and Richardson 2014).

The genus *Vibrio* is commonly associated with coral disease, and has been shown to increase in abundance either from primary infection (Ben-Haim et al. 2003; Kushmaro et al. 2001) or with bleaching (Bourne et al. 2008) and in the summer (Koren and Rosenberg 2006). Members of the *Vibrio* genus have also been associated with apparently healthy coral microbial communities (Ritchie 2006; Littman et al. 2011). It is undisputed that vibrios play crucial roles in coral health, in particular in bleaching, and were therefore examined carefully in the present study. *Vibrio* spp. were not present in high abundance (<3%) in any of the experimental treatments. Only in the Summer 2013 *P. strigosa* bacterial community did the relative abundance of *Vibrio* rise above three percent, while all other seasonal samples were <0.5% *Vibrio.* Similarly, Littman et al. (2011) and Vega Thurber et al. (2009) did not detect a significant shift in *Vibrio*

abundance in bleached corals, although it should be considered that virulence genes of *Vibrio* may increase without an increase in the overall population (Vega Thurber et al. 2009). These findings indicate that the relative susceptibility of *D. labyrinthiformis* to bleaching is not likely the result of higher *Vibrio* populations associated with this species in comparison to *P. strigosa*.

While the overall bacterial communities associated with each coral species in Summer 2013 and Summer 2014 were largely dissimilar (Figure 4.1), there were, in fact, some similarities. For example, Chloroflexi were detected in Summer 2013 for both species, but not Summer 2014. Similarly, Verrucomicrobiae were present for both species in Summer 2013 but not in Summer 2014. The coral transcriptomics study (chapter three) revealed that the transcriptomes of *D. labyrinthiformis* and *P. strigosa* were also different during the Summers of 2013 and 2014. Interestingly, a bleaching event occurred along the Florida Keys reef tract a few weeks after the collection of the Summer 2014 samples. We suggest that bleaching events may be predicted by a reduction in the overall coral gene response to stressors (chapter three), and may also be predicted by specific shifts in the coral microbiota as is seen in this study. Microbial shifts as predictive of coral bleaching have also been proposed by Bourne et al. (2008).

The present study demonstrates that corals may harbor a dynamic "winter microbiota" and "summer microbiota", (as also seen by Ceh et al. 2011; Chen et al. 2011; Chiu et al. 2012). Winter bacterial communities may be more similar between species, and possibly more stable, while the summer bacterial communities are more variable than the winter bacterial communities. *Pseudodiploria strigosa* was more variable in the

summers than *D. labyrinthiformis,* and the variability may contribute to its relative susceptibility to disease. Future studies should focus on identification of a seasonally "normal" shift in the coral bacterial population to serve as a baseline for monitoring microbial shifts that may predict bleaching or disease events. Additionally, it may be useful to target individual coral colonies that demonstrate relatively stable seasonal bacterial communities for coral restoration efforts.

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Table 4.1 Read abundance and quality statistics, and Shannon-Weiner Index (based upon class). Each Ion 314 Chip had 2 barcodes for seasonal data, and 6 barcodes for experimental data.

Sample	**Raw Reads (Ion 314)**	**(QC > 20) (Torrent Suite v4.2)**	**Average bp Length**	**Quality Reads (MG-RAST QC)**	**Average bp Length (MG-RAST QC)**	**% GC**	**Ribosomal Reads(from column 3)**	**Shannon-Weiner Index**
Summer 2013 *D. labyrinthiformis*	397,187	162,094	246 ± 111	126,77	202 ± 92	46 ± 7	126,062	1.812
Summer 2013 *P. strigosa*		221,716	259 ± 112	180,946	205 ± 90	49 ± 8	147,854	2.042
Winter *D. labyrinthiformis*	458,039	210,801	207 ± 97	170,050	168 ± 83	43 ± 8	92,779	1.725
Winter *P. strigosa*		247,238	216 ± 107	192,330	174 ± 86	45 ± 8	140,594	1.734
Summer 2014 *D. labyrinthiformis*	323,978	138,108	248 ± 100	120,386	205 ± 90	48 ± 7	106,559	1.694
Summer 2014 *P. strigosa*		179,723	229 ± 106	149,902	198 ± 91	43 ± 7	93,550	1.717
Control (25°C, pH 8.2) *D. labyrinthiformis*	287,625	6,416	220 ± 111	4,967	169 ± 91	46 ± 7	3,454	1.826
Temperature (31°C, pH 8.2) *D. labyrinthiformis*		123,660	208 ± 106	93,508	165 ± 91	49 ± 7	56,993	2.134
Temp + pH (31°C, pH 7.7) *D. labyrinthiformis*		15,772	213 ± 107	12,120	171 ± 89	50 ± 7	8,285	2.025
Control (25°C, pH 8.2) *P. strigosa*		58,939	218 ± 118	40,080	177 ± 92	48 ± 7	33,581	2.015
Temperature (31°C, pH 8.2) *P. strigosa*		45,818	233 ± 114	36,169	189 ± 92	50 ± 7	30,661	1.951
Temp + pH (31°C, pH 7.7) *P. strigosa*		30,231	227 ± 114	22,683	184 ± 91	50 ± 7	17,775	2.091
Total	1,475,367	1,440,516	227 (average)	1023141	Average 184	Average 47	Average 71,512	Average 1.897

Table 4.2 SIMPER analysis comparing all seasonal bacterial communities. Average similarity between bacterial communities shown in the first row, with the percent contribution of each bacterial class to the differences between bacterial communities shown below (classes contributing < 2% not shown).

	D. labyrinthiformis			***P. strigosa***			**Interspecies Comparison (*D. labyrinthiformis* vs. *P. strigosa*)**		
Season Comparison	Winter vs. Summer 2013	Winter vs. Summer 2014	Summer 2013 vs. Summer 2014	Winter vs. Summer 2013	Winter vs. Summer 2014	Summer 2013 vs. Summer 2014	Summer 2013	Summer 2014	Winter
Average Similarity	88.9	88.6	91.1	60.9	86.0	56.3	67.0	80.1	92.9
	% Contribution to Differences Between Bacterial Communities								
Actinobacteria	3.49	< 2.00	4.56	2.23	24.1	8.83	3.20	17.3	< 2.00
Alphaproteobacteria	12.6	5.34	9.10	20.0	4.11	16.9	15.6	4.81	12.6
Bacilli	< 2.00	5.80	9.48	12.2	< 2.00	11.6	12.3	7.28	6.73
Bacteroidia	6.62	6.60	< 2.00	3.23	17.9	2.13	2.20	9.91	17.8
Betaproteobacteria	13.6	14.5	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	5.18	12.0
Chlorobia	3.94	< 2.00	< 2.00	3.07	< 2.00	2.77	3.27	< 2.00	6.31
Chloroflexi	4.74	< 2.00	5.74	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
Clostridia	4.95	8.97	17.5	9.43	12.6	4.94	11.7	20.5	17.7
Cyanobacteria	9.04	7.10	< 2.00	7.87	19.9	12.7	12.0	7.42	6.38
Cytophagia	5.58	5.54	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
Deinococci	< 2.00	4.51	5.70	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
Deltaproteobacteria	10.0	14.5	< 2.00	5.67	4.79	3.76	3.73	4.14	< 2.00
Flavobacteriia	3.94	3.92	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	6.31
Fusobacteria	8.76	5.63	18.1	4.76	9.46	6.97	7.45	9.99	< 2.00
Gammaproteobacteria	4.25	6.66	< 2.00	2.36	< 2.00	2.38	< 2.00	6.40	6.59
Planctomycetia	< 2.00	< 2.00	< 2.00	2.12	< 2.00	< 2.00	2.26	< 2.00	< 2.00
Sphingobacteriia	< 2.00	4.51	5.70	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00
Verrucomicrobiae	< 2.00	< 2.00	4.21	2.39	< 2.00	2.16	< 2.00	< 2.00	< 2.00
Unclassified	< 2.00	< 2.00	4.21	12.5	< 2.00	11.3	14.3	< 2.00	< 2.00

Table 4.3 SIMPER analysis comparing all experimental bacterial communities. Average similarity between bacterial communities shown in the first row, with the percent contribution of each bacterial class to the differences between bacterial communities shown below (classes contributing < 2% not shown).

	D. labyrinthiformis		***P. strigosa***		**Interspecies Comparison (*D. labyrinthiformis* vs. *P. strigosa*)**		
Treatment Comparison	25°C, pH 8.2 vs. 31°C, pH 8.2	31°C, pH 8.2 vs. 31°C, pH 7.7	25°C, pH 8.2 vs. 31°C, pH 8.2	31°C, pH 8.2 vs. 31°C, pH 7.7	25°C, pH 8.2	31°C, pH 8.2	31°C, pH 7.7
Average Similarity	71.07	74.91	84.67	85.42	85.12	74.42	87.87
	% Contribution to Differences Between Bacterial Communities						
Actinobacteria	5.35	6.02	5.26	4.44	< 2.00	8.86	< 2.00
Alphaproteobacteria	16.12	10.31	3.71	3.52	3.22	12.60	< 2.00
Anaerolineae	< 2.00	< 2.00	< 2.00	4.63	< 2.00	< 2.00	5.50
Aquifacae	< 2.00	< 2.00	3.43	< 2.00	< 2.00	< 2.00	2.24
Bacilli	8.16	5.39	4.98	10.86	5.51	8.29	6.28
Bacteroidia	6.36	< 2.00	2.69	2.71	3.28	6.31	6.26
Chlorobia	2.06	2.20	2.42	< 2.00	< 2.00	< 2.00	3.89
Chloroflexi	< 2.00	< 2.00	< 2.00	< 2.00	3.18	< 2.00	< 2.00
Clostridia	9.84	10.14	12.09	13.34	3.30	< 2.00	< 2.00
Cytophagia	< 2.00	< 2.00	< 2.00	3.25	< 2.00	< 2.00	2.69
Cyanobacteria	16.47	14.27	2.38	4.59	< 2.00	17.39	2.45
Deinococci	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	3.17
Deltaproteobacteria	< 2.00	< 2.00	< 2.00	2.74	3.42	2.16	< 2.00
Epsilonproteobacteria	< 2.00	< 2.00	3.06	2.88	2.59	3.12	< 2.00
Flavobacteriia	< 2.00	< 2.00	< 2.00	< 2.00	5.50	2.64	< 2.00
Fusobacteria	< 2.00	2.37	3.43	< 2.00	2.82	< 2.00	5.94
Gammaproteobacteria	< 2.00	< 2.00	2.81	5.02	5.22	5.63	4.70
Gemmatimonadetes	< 2.00	< 2.00	< 2.00	3.28	< 2.00	< 2.00	< 2.00

Ktedonobacteria	< 2.00	< 2.00	3.55	< 2.00	5.80	< 2.00	2.24
Mollicutes	< 2.00	2.00	< 2.00	3.28	< 2.00	< 2.00	3.89
Opitutae	< 2.00	< 2.00	< 2.00	< 2.00	2.59	< 2.00	< 2.00
Planctomycetia	< 2.00	3.41	6.79	4.60	7.22	< 2.00	< 2.00
Solibacteres	< 2.00	< 2.00	< 2.00	2.88	2.59	< 2.00	2.24
Spartobacteria	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	< 2.00	2.24
Spirochaetia	< 2.00	2.00	3.33	2.67	3.67	< 2.00	3.17
Sphingobacteriia	2.23	< 2.00	2.36	3.28	2.59	2.27	< 2.00
Thermomicrobia	< 2.00	5.81	7.99	< 2.00	8.79	< 2.00	9.49
Verrucomicrobiae	< 2.00	2.16	4.41	2.67	4.85	< 2.00	2.46
Unclassified	14.55	17.84	12.69	8.11	12.52	13.83	20.77

Figure 4.1 Nonmetric Multi-Dimensional Scaling (NMDS) plots of seasonal (Summer 2013, Winter 2014, and Summer 2014) bacterial communities (16S rRNA) associated with the two coral species *Diploria labyrinthiformis* and *Pseudodiploria strigosa.* 2D Stress = 0.

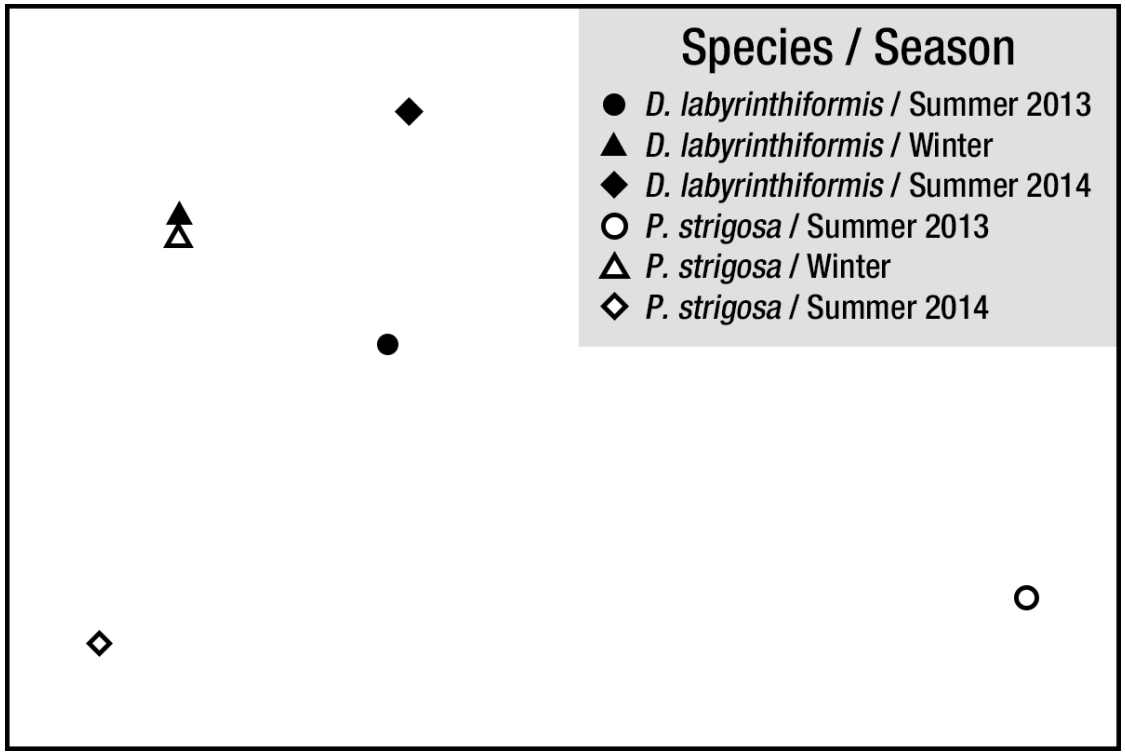


Figure 4.2 Nonmetric Multi-Dimensional Scaling (NMDS) plots of bacterial communities (16S rRNA) associated with the two coral species *Diploria labyrinthiformis* and *Pseudodiploria strigosa* subject to three treatments: Control (25°C, pH 8.2), Temperature (31°C, pH 8.2), and Temperature + pH (31°C, pH 7.7). 2D Stress = 0.

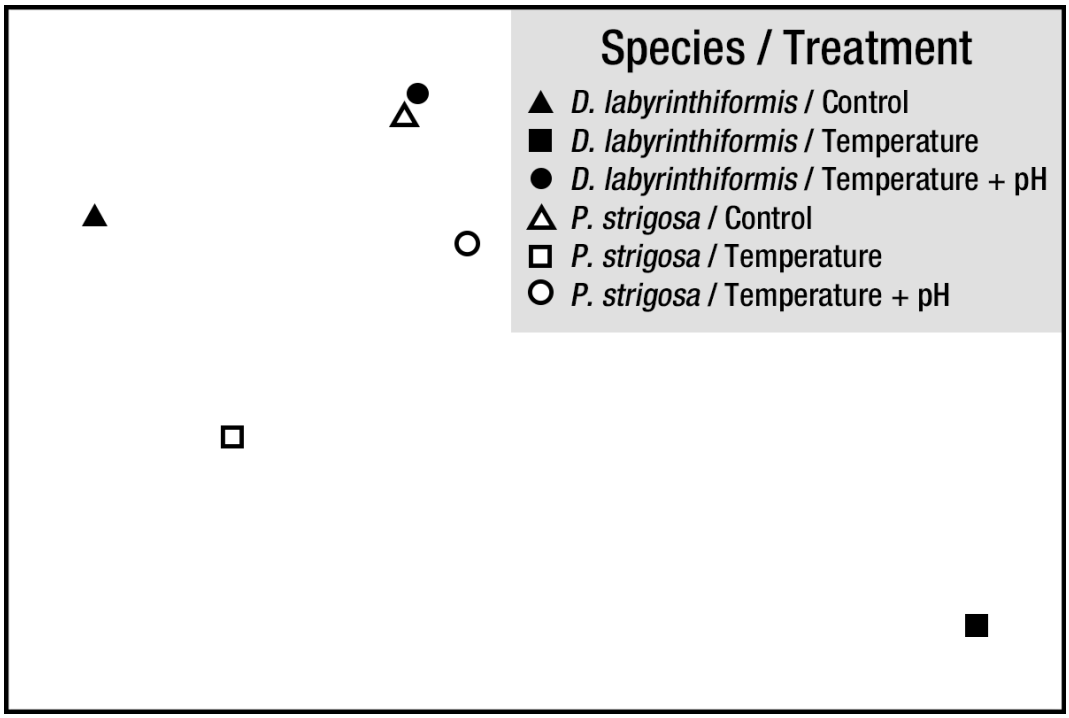


Figure 4.3 Percent composition (by class) of the *in situ* bacterial communities associated with the two coral species *Diploria labyrinthiformis* and *Pseudodiploria strigosa* for three different seasons (Summer 2013, Winter 2014, and Summer 2014).

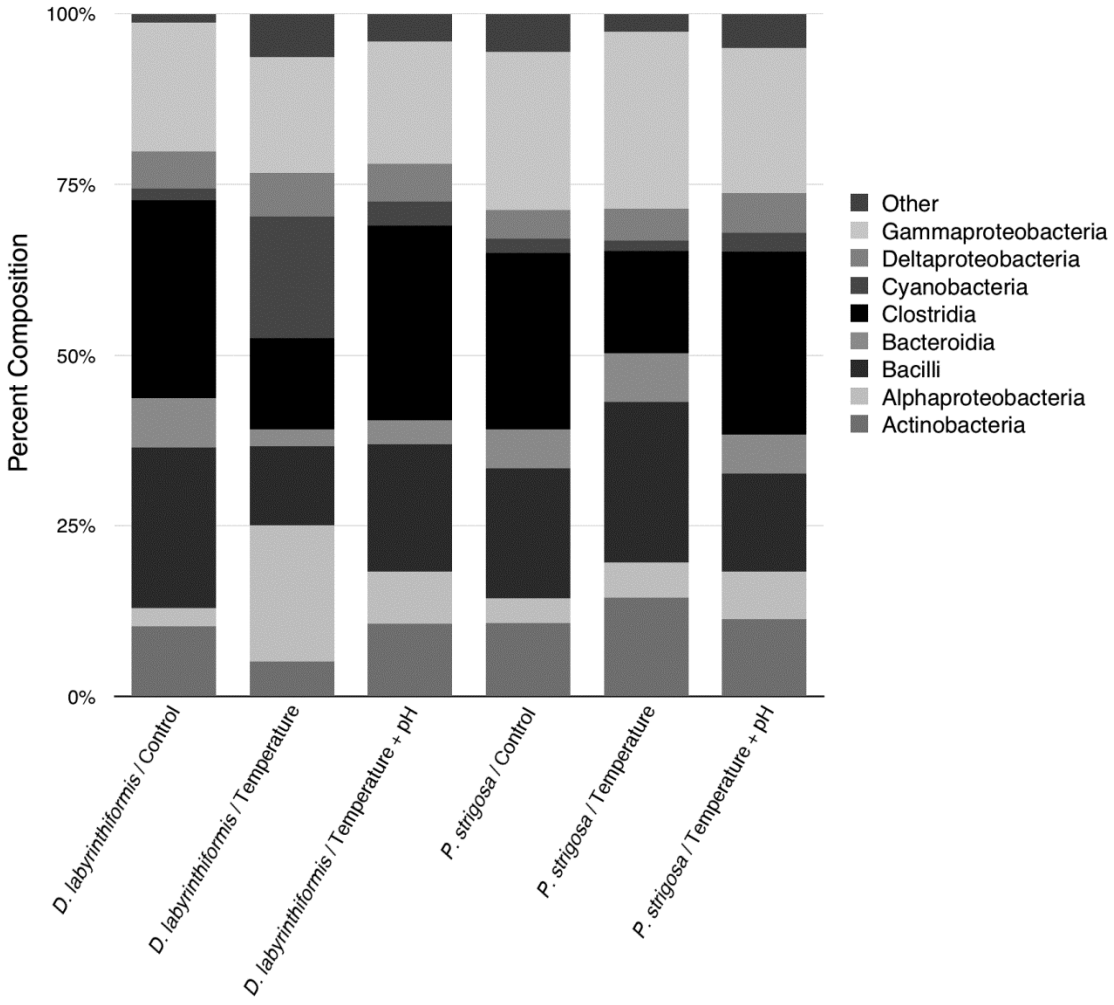
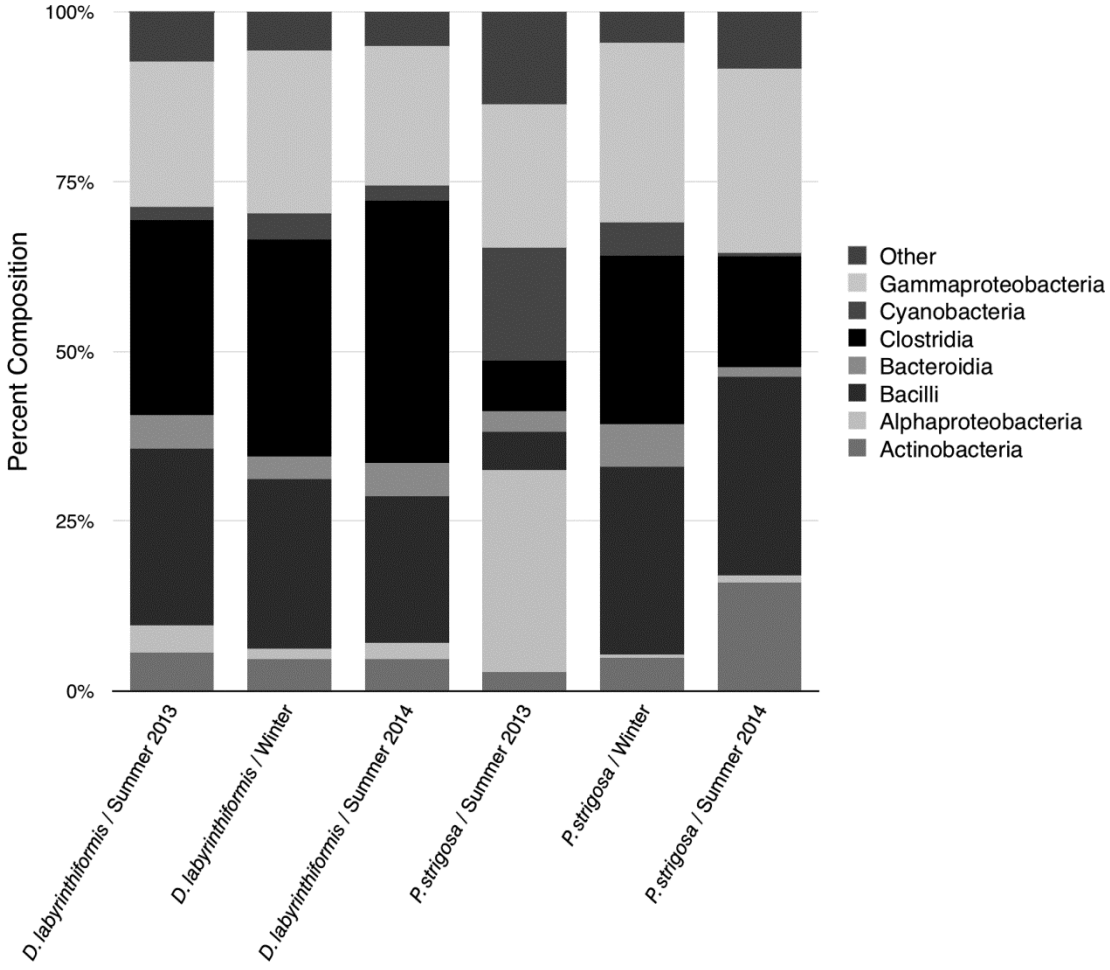


Figure 4.4 Percent composition (by class) of the bacterial communities associated with two coral species, *Diploria labyrinthiformis* and *Pseudodiploria strigosa* for three different treatments: Control (25°C, pH 8.2), Temperature (31°C, pH 8.2), and Temperature + pH (31°C, pH 7.7).



CHAPTER 5

POSSIBLE LINKS BETWEEN WHITE PLAGUE, SCLERACTINIAN CORALS, AND A CRYPTOCHIRIDAE GALL CRAB

Abstract

White plague, a highly destructive coral disease, is known to kill up to 38% of the most susceptible coral species. In the quest to understand this complex disease, it is important to consider both microscopic community members of the coral holobiont (*Symbiodinium*, bacteria, viruses, archaea and fungi), as well as macroscopic members. Small coral-associated gall crabs from the family Cryptochiridae are one such example of macroscopic coral community members. In an aquarium-based study, we examined a small Cryptochiridae crab as a possible disease vector for a white plague-like lesions occurring on the scleractinian corals *Diploria labyrinthiformis* and *Pseudodiploria strigosa*. Removal of the crab from colonies demonstrating white plague-like lesions and placement onto apparently healthy *D. labyrinthiformis* colonies resulted in the development of similar lesions. These lesions were further compared using next generation sequencing of the 16S rRNA gene to profile the bacterial community. Microbial profiling indicated that the microbiota of the crab, crab lesions, and disease lesions were highly similar, while apparently healthy colonies were significantly different. The difference was largely the result of an increase in Alphaproteobacteria, in particular the *Roseobacter* clade. The present study suggests that a potential pathogen of the *Roseobacter* clade may infect the corals *D. labyrinthiformis* and *P. strigosa*, and may be transmitted by the Cryptochiridae gall crab.

Introduction

Coral diseases have been on the rise since their first report in 1965, and are responsible for a large portion of coral cover decline (Sutherland et al. 2004). White

plague is one such coral disease. White plague was originally described in 1977 (Dustan 1977), and in one epizootic event it has been documented to kill up to 38% of the more vulnerable coral species (Richardson et al. 1998a and 1998b). As with the majority of coral disease, white plague is identified on the basis of macroscopic phenotypic characteristics (Ainsworth et al. 2007), primarily a distinct, rapidly progressing boundary between coral diseased tissue and recently exposed (white) skeleton (Dustan 1977; Bythell et al. 2004). Three types of white plague (Types I, II, and III) have been defined by lesion progression rate, prevalence rates, and coral species affected (Richardson 1998b; Richardson et al. 2001; Bythell et al. 2004). However, there is a need to apply microbiological, histological, and cytological methods in addition to physiological characteristics, when describing coral diseases such as white plague (Richardson et al. 2001; Ainsworth et al. 2007; Kellogg et al. 2013; Bourne et al. 2015). Such studies have led to proposed potential pathogens, including *Aurantimonas coralicida* (Denner et al. 2003), and *Thalassomonas loyana* in the Red Sea (Thompson et al. 2006), yet no one pathogen is consistently found in all white plague lesions.

In the hunt for potential pathogens and mechanisms of disease, it is important to consider all aspects of the coral and its many associates. Corals harbor many essential microorganisms including viruses, bacteria, archaea, fungi, and the symbiotic algae *Symbiodinium*, which all play roles in maintaining the health of the coral holobiont (Rohwer et al. 2002; Reshef et al. 2006; Rosenberg et al. 2007; Wegley et al. 2007). Coral also house many macroscopic creatures, such as gall crabs which can spend their entire life on a single coral head (van der Meij 2014). In fact, coral-dwelling crabs are

one of the most abundant members of the macrofauna associated with corals (Stella et al. 2010), occupying up to 25% of coral heads (Simon-Blecher and Achituv 1997). Gall crabs have also been associated with growth anomalies in *Fungia concinna* and *Pocillopora meandrina* (Williams et al. 2011) Yet, the interactions and dynamics between coral-dwelling gall crabs and the microbiota of the coral holobiont are entirely unknown. The vacancy in knowledge is cause for concern, as coral-dwelling organisms may have the potential to act as a vector and spread diseases such as white plague. Therefore white plague-like lesions on the coral *Diploria labyrinthiformis* associated with the Cryptochiridae gall crab were investigated, through coral-crab interaction challenges and observations and microbial assessment via the 16S rRNA microbial profiling.

Materials and Methods

Observation

Two *Pseudodiploria strigosa* colonies (approximately 25 cm^2) were collected from the Florida Keys National Marine Sanctuary Coral Nursery in October, 2013 and placed in a large holding aquarium described in Pratte and Richardson (2014). After one month, white plague-like symptoms were observed on one of the colonies. Five small (2 mm) crabs were discovered on the afflicted coral colony. All crabs were removed and either placed in 95% ethanol to preserve for identification purposes (n = 3), or crushed in RNAlater® (Life Technologies) for microbial analysis (n = 2). In January 2014 a sixth crab was discovered, and kept alive for further experimentation.

Experimental set-up: coral-crab challenges

Previously collected (November 2012 and October 2013) fragments of *D. labyrinthiformis, Montastraea cavernosa,* and *Siderastrea siderea* were also maintained in the large holding tank. All fragments were approximately 3 cm^2 pieces, and set into small cement pedestals. Three *D. labyrinthiformis* fragments were maintained in a smaller isolation aquarium (19 L), which were never exposed to the same water or equipment as the diseased fragments. These three fragments have been termed "naïve". One naïve fragment was placed in a small experimental aquarium (3.8 L) with a recirculating filter under the same lighting conditions. The small live crab collected for experimental purposes was rinsed with sterile sea water and gently patted dry three times with Kim Wipes, and subsequently placed onto the naïve coral fragment to test for transmissibility. Observations were recorded every one to three days. This experiment was repeated 2 more times with the remaining naïve fragments. To compare the disease progression on fragments with and without a crab, two apparently healthy *D. labyrinthiformis* fragments from the aquarium of the original outbreak were placed in one small experimental aquarium (3.8 L) with a fine screen mesh dividing the two fragments. The small crab was rinsed as previously described, and placed on one of the fragments, the screen ensuring the crab did not have access to one fragment. Observations were documented as above. The experiment was repeated a total of three times. As a control, two apparently healthy *D. labyrinthiformis* fragments (non- naïve) were maintained in

identical small experimental aquaria without any crabs, to ensure that aquarium conditions were not the cause for any decline in coral health.

Metagenomic microbial analysis

Skeleton, tissue, and SML samples were collected from three Crab Lesions (lesions associated with the direct presence of the crab) and Disease Lesions (lesions occurring without the direct presence of the crab). Samples were placed into 1 ml of RNAlater® (Life Technologies) and frozen at -80°C until further processing. Two crabs from the original *P. strigosa* colony were also crushed and preserved in 1 ml of RNAlater® (Life Technologies). Data for apparently healthy *D. labyrinthiformis* were used from chapter four.

All DNA extractions proceeded as described in chapter four, using the FastDNA™ Spin Kit for Soil (Qbiogene, Vista, CA, USA). The DNA was then quantified using the Qubit® 2.0 Fluorometer (Life Technologies) and pooled according to sample type. The V4 and V5 region of the 16S rRNA gene was amplified via PCR using primers F563/BSR926 (Claesson et al. 2010). The PCR conditions were according to chapter four. All reactions were run in duplicate, and products verified on a 1.8% TBE agarose gel with GelRed™ (Biotium). Pooled PCR products were then barcoded according to sample type and submitted to the DNA Sequencing Facility at Florida International University. Each sample type was given a separate barcode using the Ion Xpress RNA-Seq Barcode 01-16 Kit (Life Technologies) and run on the Ion 316 Chip v2 (Life Technologies). The Ion Torrent PGM (Life Technologies) performed 200 base-read sequencing using the Ion PGM Sequencing 200 Kit v2 (Life Technologies). Ion Torrent

PGM sequences were filtered using Torrent Suite v4.2 software to remove polyclonal and low quality sequences. All raw .fastq files are publicly accessible via the MG-RAST metagenomics analysis server (project ID 12498).

Data analysis

The .fastq files generated by the Ion Torrent server were imported into the MG-RAST server for analysis (Meyer *et al. 2*008), and screened for quality control a second time. Sequences of bacterial origin (as determined by MG-RAST) were sorted according to class and exported for further statistical analysis using PRIMER 6 software (Primer-E Ltd). Only bacterial classes with a minimum of 5 reads were included in analysis. Data were square root transformed and non-metric multi-dimensional analysis (NMDS) was performed using Bray-Curtis similarity matrices (Clarke 1993; Clarke and Gorley 2001). Statistical significance between communities was tested using similarity profile analysis (SIMPROF, Clarke et al. 2008), and dissimilarity contributions examined using a one-way similarity percentages (SIMPER) analysis (Clarke 1993). Shannon-Weiner diversity indices (H' log e) were also calculated using PRIMER-E. Additionally, individual operational taxonomic units (OTUS) at the genus and species level were manually curated for OTUs of interest.

Results

Observations

The crabs isolated from the original *P. strigosa* coral fragment were identified as being in the Cryptochiridae family (Kropp and Manning, 1987). Specific genus and

species could not be placed, as the crabs were juvenile and distinguishing features were not yet developed. Upon removal of the crabs, the lesion progressed slowly, taking nearly a year to kill the fragment. It maintained the characteristics of white plague, with a distinct boundary of recently exposed skeleton at the lesion front. Coral fragments (all approximately 3 cm^2) residing in the same tank began showing signs of disease approximately 3 months after the introduction of initial *P. strigosa* fragment. Both *P. strigosa* and *D. labyrinthiformis* succumbed to the disease, while *Montastraea cavernosa* and *Siderastrea siderea* remained apparently healthy. Lesions progressed at highly variable rates, independent of species (*D. labyrinthiformis* or *P. strigosa*).

Coral-crab challenges

Upon placing the crab upon naïve *D. labyrinthiformis* fragments (n = 3), lesions developed each time, taking from two weeks to seven weeks to kill the fragment (Figure 5.1). In the screened experiments where the crab did not have access to a fragment, yet shared the same aquarium water, lesions resembling white plague developed on all fragments. In each case (n = 3) the fragment containing the crab died before the fragment which the crab could not access. The two coral fragments (non- naïve) maintained in identical aquaria are still alive and healthy at the time of the submission of this manuscript.

Bacterial Communities

A total of 2,837,430 raw reads were obtained, 2,776,310 of which passed the Ion Torrent QC test, and 2,390,743 of which passed the MG-RAST QC test (Table 5.1).

SIMPROF analysis indicated that the Apparently Healthy bacterial community was significantly different when compared to the rest of the samples ($p < 0.5$). The SIMPER analysis also indicated that the Apparently Healthy bacterial community was the least similar compared to all other samples, while Disease Lesion and Crab Lesion communities were the most similar (Table 5.2). Similarity was verified by an NMDS plot which revealed that bacterial communities of both lesion types as well as the crab cluster together (80% similarity), while the apparently healthy *D. labyrinthiformis* bacterial community clusters independently (Figure 5.2). The SIMPER analysis indicated that for all lesion and crab communities, Alphaproteobacteria was the most dissimilar class when compared to the Apparently Healthy bacterial community (Table 5.2). The Apparently Healthy community was composed of just 3.7% Alphaproteobacteria, while all other samples contained 35.1-25.9% Alphaproteobacteria. Bacili and Clostridia also contributed at least 15% each to the dissimilarities between Apparently Healthy and all other samples. Bacilli were reduced in both lesions (9.9% and 10.5%) compared to Apparently Healthy (19.0%), as well as Clostridia (12.1% and 15.8% compared to 25.8%). The crab microbial community contained 1.4% Fusobacteria, compared to 0.5%-0.9% for all other samples. Gammaproteobacteria were more abundant in the Apparently Healthy community (23.1%) than the other three (17.3%-19.6%) as well as Planctomycetes (2.9% compared to 0.4%-0.6%). Cyanobacteria composed a total of 2.2% for the Crab community, 1.1% for the Crab Lesion community, and 2.4% for the Disease Lesion community.

Comparisons at the genus and species level revealed that *Kineococcus radiotolerans, Trichodesmium erythraeum, Lactobacillus vaginalis, Dethiobacter alkaliphilus,* and *Reinekea blandensis* were all more abundant in the Apparently Healthy bacterial community than both bacterial communities associated with lesions. The genus *Vibrio* was present at 3.4% in the Crab Lesion, and only 0.3% in the Crab, and 0.1% in the Disease Lesion. The genera *Dinoroseobacter, Rhodobacter, Roseobacter, Roseovarius,* and *Ruegeria* were responsible for the increase in Alphaproteobacteria in the lesion and crab samples, in particular an OTU similar to *Roseobacter denitrificans*.

Discussion

Recently, the broad description of white plague lesion rates, diversity of potential pathogens, and range of coral species infected has been suggested to be a result of a variety of causations, rather than a single infectious agent (Ainsworth et al. 2007; Kellogg et al. 2013) as with white syndrome diseases in the Indo-Pacific (Work et al. 2012; Bourne et al. 2015). The present study supports this hypothesis, as *D. labyrinthiformis* and *P. strigosa* fragments were susceptible to this disease, while *M. cavernosa* and *S. siderea* remained apparently healthy. The presence of the gall crab on (and in close proximity to) *D. labyrinthiformis* fragments caused lesions similar to those described for white plague (Bythell et al. 2004) (Figure 5.1). However, the variety of white plague (type I, II, or III) could not be distinguished, as different rates of lesion progression were observed. The mechanism by which the crab caused these lesions is entirely unknown, and could be the result of abrasion, infection, or a combination of these.

The composition of microbial communities was remarkably similar between the Crab, Crab Lesion, and Disease Lesion (Figures 5.2 and 5.3), suggesting that the crab may harbor a microbiota similar to that of a diseased coral, or that the coral itself could have an impact on the crab microbiota. It would be interesting to examine the microbiota of the Cryptochiridae crab inhabiting healthy corals, to determine if the similarities in the coral and crab microbiota exist solely in a diseased state. The bacterial communities of the Crab Lesion and Disease Lesion were over 90% similar, indicating that the lesions initiated by the direct presence of the crab were similar to those indirectly initiated through shared aquarium water. It cannot be determined how much of this similarity is a consequence of opportunistic bacteria taking advantage of the release of nutrients produced by the lysing of coral tissue in all cases. It can be determined, however, that differences between Alphaproteobacteria, Bacilli, and Clostridia accounted for the large majority of dissimilarities detected between the Apparently Healthy and all other bacterial communities (Table 5.2). Higher abundance of Cyanobacteria was expected in association with the crab, as they are thought to cultivate cyanobacteria in their dwellings (Simon-Blecher et al. 1999). However, this does not appear to be the case in the present study.

The OTUs were further examined at the genus and species level. However, extreme caution must be used in interpretation, as the combination of short next generation sequencing reads and a limited M5NR MG-RAST database results in many OTUs with low e values and percent similarity (see Webster et al. 2010 for example). Also, simply because an OTU is present does not indicate that the bacterium is not in a

latent state (see Klein 2015). The information for each OTU discussed can be found in Appendix C, Table C1. Despite these limitations, the data MG-RAST provide at the genus and species level can still prove useful. For instance, the genus *Vibrio* was examined carefully, as it has previously been associated with coral disease (Ben-Haim et al. 2003; Kushmaro et al. 2001) and bleaching (Bourne et al. 2008). The *Vibrio* genus composed 3.4% of the community in the Crab Lesion, 0.3% in the Crab community, and 0.1% in the Disease Lesion. In the Crab Lesion bacterial community, the relatively higher proportion of *Vibrio* was the result of hits similar to *Vibrio harveyi*, and *Vibrio parahaemolyticus. Vibrios* are commonly found in healthy corals (Bourne and Munn 2005; Kellogg et al. 2013), and at such low abundance, it is unlikely that a *Vibrio* species is the pathological agent in this case.

The OTUs similar to *Kineococcus radiotolerans, Trichodesmium erythraeum, Lactobacillus vaginalis, Dethiobacter alkaliphilus, Reinekea blandensis,* were all more abundant in the Apparently Healthy bacterial community than both communities associated with lesions (Appendix C, Table C1). They should be considered a part of the healthy *D. labyrinthiformis* holobiont community, with relative decreases in the abundance of these bacteria possibly indicating disease. In particular OTUs similar to *Reinekea blandensis*, *Dethiobacter alkaliphilus,* and *Lactobacillus vaginalis* composed the majority (53.3%) of the Apparently Healthy community. The genera *Dinoroseobacter, Rhodobacter, Roseobacter, Roseovarius, Ruegeria*, were responsible for the increase in Alphaproteobacteria in the Disease Lesion, Crab Lesion, and Crab

bacterial communities. These genera, and the Rhodobacteraceae family overall, were not found in the Apparently Healthy community.

Many other white plague studies have reported an increase in the Rhodobacterales family as well (Pantos et al. 2003; Pantos and Bythell 2006; Sunagawa et al. 2009; Kellogg et al. 2013; Roder et al. 2014a and 2014b). Rhodobacteraceae is the only class consistently found in higher abundance in these white plague studies. In fact, Rhodobacteraceae, particularly the *Roseobacter* clade, is found in a variety of coral diseases, including black band disease (BBD) (summarized in Mouchka et al. 2010). The majority of these studies attribute the abundance of Rhodobacteraceae in diseased samples to opportunistic heterotrophs, as this fits with the general Rhodobacteraceae life style (Buchan et al. 2005). However, one group (Pantos et al. 2003; Pantos and Bythell 2006) observed that a ribotype similar to the causative agent of juvenile oyster disease (JOD) was present in white band samples of *Acropora palmata*. A similar ribotype was also observed in black band disease (BBD) (Sekar et al. 2006; Sekar et al. 2008). It is now known that *Roseovarius crassostreae* is the etiological agent of JOD (renamed Roseovarius Oyster Disease- Maloy et al. 2007) and belongs to the *Roseobacter* clade (Boettcher et al. 2005). These findings suggest that members of the *Roseobacter* clade may be directly involved in disease etiology.

Several additional studies have come to light suggesting that the *Roseobacter* clade may contain pathogenic members. A bacterial strain (*Ruegeria* sp. R11, later named *Nautella* sp. R11) belonging to Rhodobacteraceae was isolated and shown to infect and cause bleaching of the red marine algae *Delisea pulchra* (Case et al. 2011; Fernandes et

al. 2011). Upon the sequencing of *Nautella* sp. R11, a variety of virulence factors were found including quorum sensing genes (Fernandes et al. 2011). Several studies have shown members of the *Roseobacter* clade to be algacidal and opportunistically pathogenic (Amaro et al. 2005; Imai et al. 2006; Seyedsayamdost et al. 2011). *Roseobacter gallaeciensis* is capable of effectively invading and colonizing the biofilm of the green alga *Ulva australis* under a variety of environmental conditions (Rao et al. 2006). It is logical to wonder if a similar invasion occurs in the surface mucopolysaccharide layer of corals. Most intriguingly, three members of the *Roseobacter* clade are known to create intracellular galls in the marine red algae *Prionitis* (Ashen and Goff, 2000), flourishing between abnormal host cells. In light of these studies, it is not unreasonable to consider members of the *Roseobacter* clade as potentially pathogenic. Given the growing number of algalcidal *Roseobacter* members, future investigations should consider the possibility of *Roseobacter* as a pathogen both to the coral animal, as well as endosymbiotic *Symbiodinium.*

The possibility that gall crabs function as a disease vector should not be ignored, nor has it been definitively proven. Further investigative studies are required to confirm this finding, to establish a mode of transmission, and verify members of the *Roseobacter* clade as pathogenic, either primarily, secondarily or opportunistically. The results of the coral-crab challenge implicate that gall crabs may have a role in white plague etiology, and the results of the microbial analysis suggest that the family Rhodobacteraceae may contain a potential coral pathogen. An abundance of studies need to be conducted before conclusions can be drawn. As specified in Work et al. (2012) and Bourne et al. (2015),

the subsequent step after identifying a potential pathogen in aquarium-based experiments is to isolate that pathogen in the field, and examine the infection at the cellular level and subcellular. The Rhodobacteraceae family needs to be examined beyond 16S rRNA sequencing through culturing and the defining of physiological characteristics (see Richardson et al. 2001). The microbial dynamics between gall crabs and corals needs to be documented. In light of the present study, it is imperative that future investigations consider the possibility of gall crabs acting as a coral disease vector, and that the *Roseobacter* clade may contain potential pathogens.

Acknowledgements

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Table 5.1 Read abundance, quality statistics, and Shannon-Weiner Index (based upon class) for all samples. Data for Apparently Healthy *D. labyrinthiformis* were taken from chapter four.

Sample	**Number of Raw Reads per Ion 316 Chip**	**Number of Quality Reads (QC > 20) per Barcode**	**Average bp Length**	**Quality Reads (as determined by MG-RAST QC)**	**Average bp Length (after MG-RAST QC)**	**% GC**	**Number of Ribosomal Reads (from Column 3)**	**Shannon-Weiner Index**
Crab	2,837,430	593,337	256 ± 97	527,016	189 ± 83	47 ± 9	330,619	0.846
Crab Lesion		1,026,498	268 ± 94	878,049	188 ± 85	49 ± 10	639,497	0.8219
Lesion		1,156,475	251 ± 104	985,678	191 ± 87	49 ± 9	669,679	0.8047
Total	2,837,430	2,776,310	258 (average)	2,390,743	189 (average)	48 (av.)	1,639,795	0.8242 (average)

Table 5.2 SIMPER analysis comparing four bacterial communities; the Chryptochiridae gall crab (Crab), lesions caused by the crab (Crab Lesion), lesions caused by possible white plague (Disease Lesion), and Apparently Healthy *D. labyrinthiformis*. Average similarity shown in the first row, with the percent contribution of each bacterial class to differences between bacterial communities shown below (classes contributing < 2% not shown).

	Crab vs Crab Lesion	**Crab vs Disease Lesion**	**Crab Lesion vs Disease Lesion**	**Crab vs Apparently Healthy**	**Crab Lesion vs Apparently Healthy**	**Disease Lesion vs Apparently Healthy**
Average Similarity	88.9	83.6	90.2	70.5	64.83	60.79
	% Contribution to Differences Between Communities					
Actinobacteria	2.61	8.43	11.06	2.79	3.15	5.63
Alphaproteobacteria	32.34	28.62	10.99	39.57	43.26	41.88
Bacilli	17.93	14.16	3.25	15.54	18.63	17.67
Bacteroidia	8.30	12.65	11.64	7.27	3.48	< 2
Clostridia	3.05	13.67	19.26	21.07	18.59	21.64
Deltaproteobacteria	7.95	10.06	7.71	< 2	3.48	5.04
Flavobacteriia	4.33	< 2	4.66	< 2	< 2	< 2
Gammaproteobacteria	3.39	4.65	11.55	< 2	< 2	<2
Unclassified	10.31	< 2	9.25	4.03	< 2	<2

Figure 5.1 A gall crab from the Cryptochiridae family on *D. labyrinthiformis*, creating lesions resembling white plague.

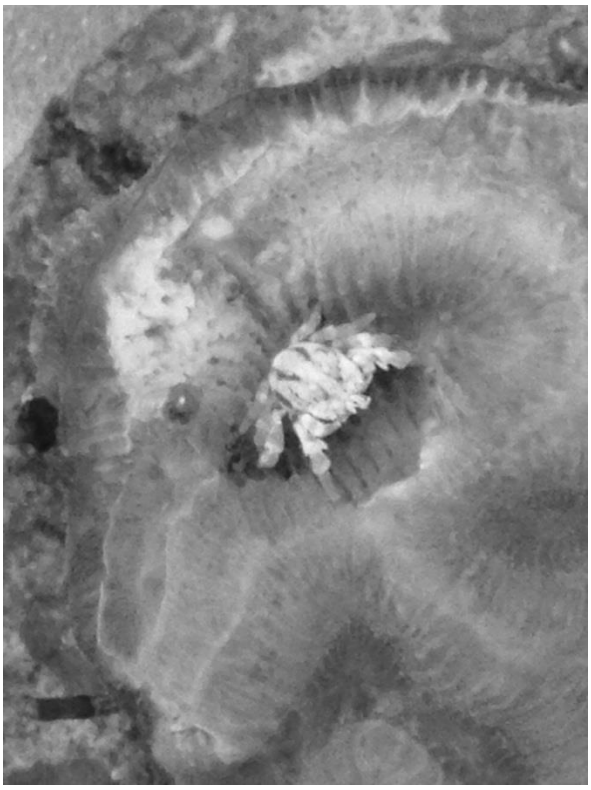


Figure 5.2 Nonmetric multi-dimensional analysis (NMDS) plots of bacterial communities associated with apparently healthy *D. labyrinthiformis* fragments, lesions caused by white plague-like symptoms, lesions caused by the presence of a gall crab, and the bacterial community associated with the gall crab itself. Similarity grouping shown for 70% and 80%.

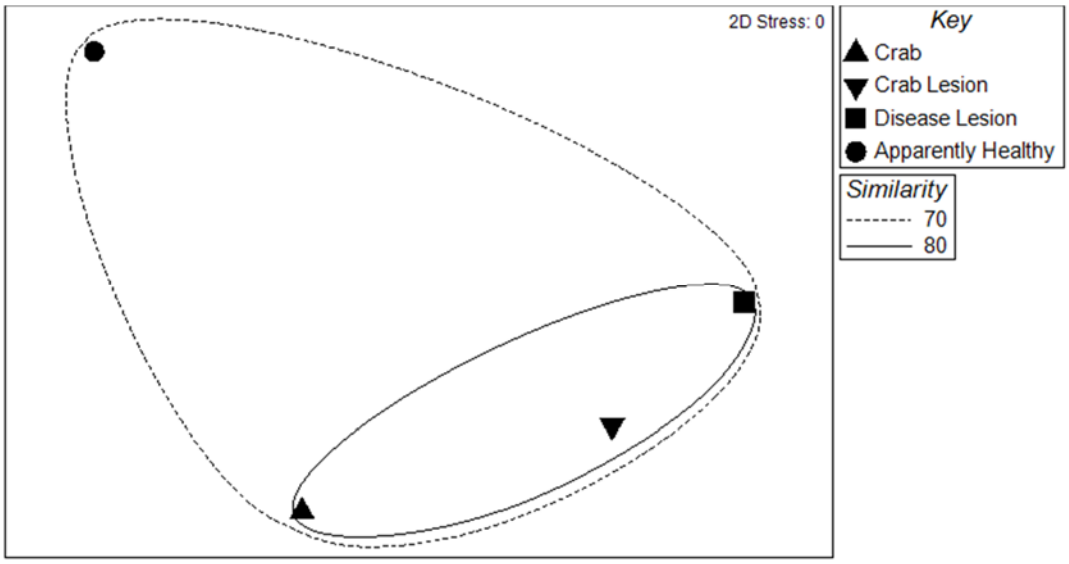
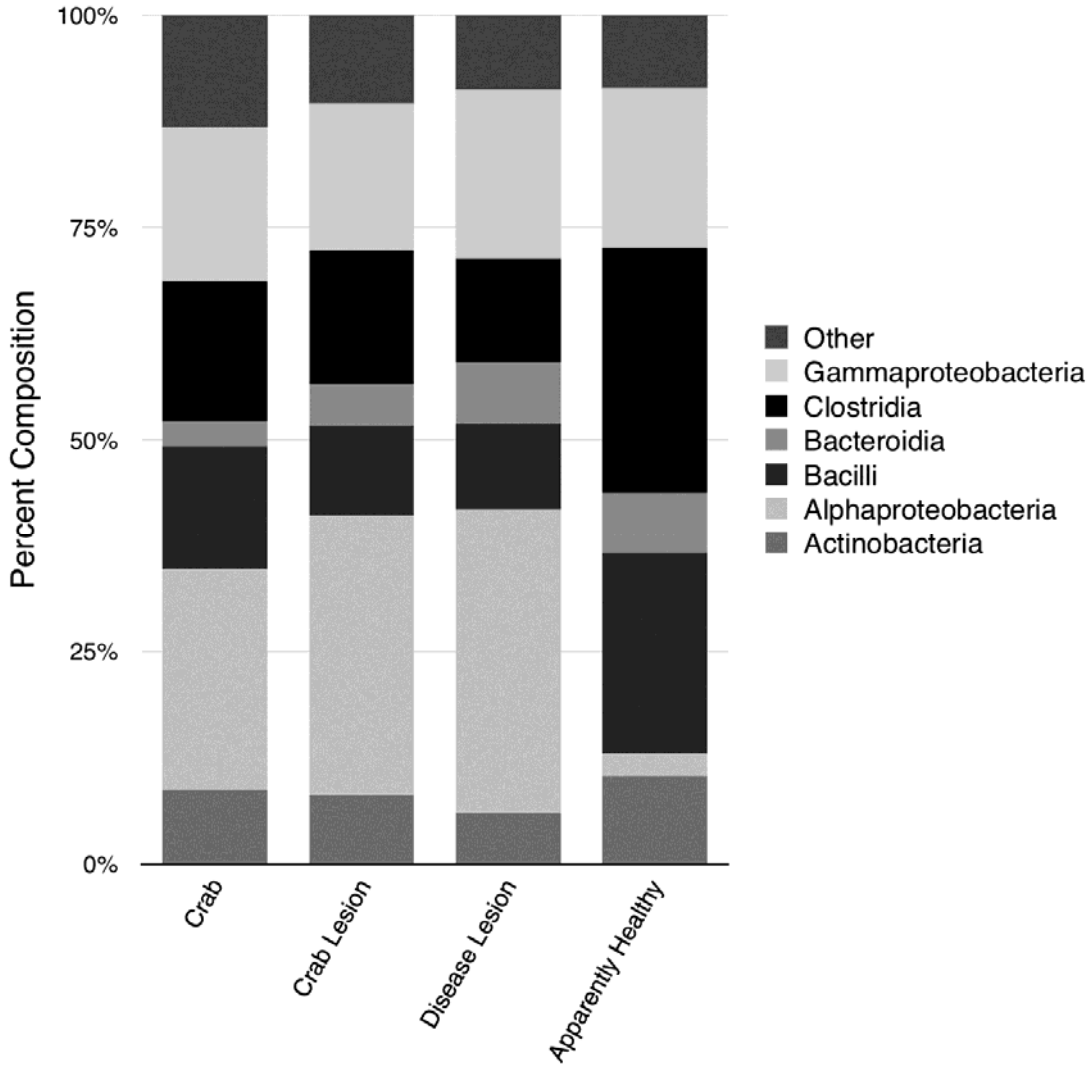


Figure 5.3 Percent composition of four bacterial communities associated with; the gall crab, lesions caused by the crab, lesions caused by possible white plague, and Apparently Healthy *D. labyrinthiformis*.



CHAPTER 6

CONCLUSIONS

The aim of this dissertation was to assess multiple components of the coral holobiont with respect to disease and bleaching resistance and resilience, using *D. labyrinthiformis* and *P. strigosa* as a model system. Chapter two discussed the thinning of the SML as temperature increased, independent of the rate of temperature increase. SML thinning is likely to contribute to the observed increase in coral disease in warmer months (Sharp and Ritchie 2012), as corals are dependent upon the SML as an important defense against potential pathogens (Brown and Bythell 2005). Interestingly, acidification had no detectable effect on SML thickness. Finally, chapter two showed that *D. labyrinthiformis* had a lower mortality rate at 31°C, indicating a higher thermotolerance than *P. strigosa*, which may help contribute to its overall disease resilience.

The third chapter of this dissertation evaluated the coral transcriptome in response to seasonal changes, temperature increase and acidification. Both seasonally and experimentally, *P. strigosa* differentially expressed genes when compared to winter and control samples, particularly transcripts associated with stress. The upregulation of relatively more stress-associated genes indicates that *P. strigosa* is sensitive to environmental changes. This is in agreement with Barshis et al. (2013) who also found more resilient corals had fewer differentially expressed genes. *Diploria labyrinthiformis* also tended to upregulate innate immunity genes in the summer, likely contributing to its relative disease resistance.

As documented in chapter four, bacterial communities of both coral species were highly similar in the winter, and shifted differently in each summer, suggesting that coral may harbor a stable "winter" microbiota and more dynamic "summer" microbiota.

Differences between the microbial communities of the two coral species were primarily due to Clostridia, Gammaproteobacteria, and rare species. *Pseudodiploria strigosa* harbored more rare species (such as *Desulfovibrio*) than *D. labyrinthiformis,* which may contribute to the relatively higher disease susceptibility of *P. strigosa* when compared to *D. labyrinthiformis.*

Chapter five presents an investigation of a white plague-like disease that is associated with both of these coral species, and results suggested that this disease may be caused by a member of the *Roseobacter* clade, facilitated by the Cryptochiridae gall crab. Future directions include histological analysis of these lesions to verify the molecular findings presented here. These results are particularly important, as very few coral disease vectors have been confirmed (Sussman et al. 2003; Nicolet et al. 2013; Chong-Sen et al. 2010). These findings also emphasize the importance of examining all members and associates of the coral holobiont, both macroscopic and microscopic, in the effort to understand coral disease etiology and resistance.

This dissertation demonstrates that although closely related, the two species *D. labyrinthiformis* and *P. strigosa* respond to environmental changes and stressors differently, potentially contributing to their differences in disease and bleaching susceptibility, and affecting their capacity to survive global climate change. It is likely a combination of components, particularly the upregulation of innate immunity genes, the stability of the associated bacteria, and possible variations in the presence of disease vectors such as gall crabs, which enable the relative disease resistance of *D. labyrinthiformis*. The results of this dissertation research are intended to provide crucial

information in the effort toward the restoration, mitigation, and conservation of *Diploria, Pseudodiploria*, and other coral species around the world.

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APPENDICES

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VITA

ZOE A. PRATTE

2005-2009	B.S. Marine Science Minors in Chemistry and Geology Honors Eckerd College St. Petersburg, Florida
2007-2009	Microbiology Lab Intern USGS (Christina Kellogg) St. Petersburg, Florida
2010-2015	Ph.D., Biology Florida International University Miami, Florida
2010-2012	Teaching Assistantship General Microbiology Florida International University
2011, 2012	Professional Development and Research Grant Florida International University
2012	Best Oral Presentation Annual Biology Symposium Florida International University
2012-2015	MBRS RISE Fellow Florida International University

PUBLICATIONS AND PRESENTATIONS

Pratte, Z.A., Richardson, L.L., and Mills, D.E. 2015. Microbiota shifts in the surface mucopolysaccharide layer of corals transferred from natural to aquaria settings. Journal of Invertebrate Pathology, 125:42-44.

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Pratte, Z.A.,* and Richardson, L.L. 2013. The potential impact of global climate change on an essential component of coral innate immunity. AMLC 36th Scientific Meeting, Ochos Rios, Jamaica. June 17-21, 2013. (Oral)

Pratte, Z.A.,* Mills, D.K., and Richardson, L.L. 2013. Microbial population dynamics in scleractinian corals transferred from natural to aquaria settings. Florida Branch ASM Annual Meeting, Islamorada, Florida. April 2013. (Oral)

Pratte, Z.A.*, and Richardson, L.L. 2012. Rising sea surface temperature could cause a reduction in the protective surface mucopolysacharide layer (SML) of scleractinian corals. International Coral Reef Symposium, Australia, Cairns, July 2012. (Oral)