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Symbiosis and Immunity at the Tri-kingdom Interface

Stephen Alexander Lazar

SYMBIOSIS AND IMMUNITY AT THE TRI-KINDGOM INTERFACE

By

STEPHEN ALEXANDER LAZAR

B.M., University of Miami
B.S., Florida Institute of Technology
M.S., Florida Institute of Technology

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A DISSERTATION

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STEPHEN ALEXANDER LAZAR

Approved as to style and content by:

_____
Andrew G. Palmer, Ph.D. Chairperson
Associate Professor, Ocean Engineering and Marine Sciences

_____
Robert Van Woesik, Ph. D.
Professor, Ocean Engineering and Marine Sciences

_____
Alan Leonard, Ph.D.
Professor, Biomedical and Chemical Engineering and Sciences

_____
Eric Guisbert, Ph.D.
Assistant Professor, Biomedical and Chemical Engineering and Sciences

_____
Richard Aronson, Ph.D.
Professor and Head, Ocean Engineering and Marine Sciences

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ABSTRACT

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by Stephen Alexander Lazar, B.M., University of Miami; B.S., Florida Institute of Technology; M.S., Florida Institute of Technology

Chairperson of Advisory Committee: Andrew G. Palmer, Ph.D.

The rise in global sea-surface temperatures drastically and adversely impacts coral reefs worldwide through coral bleaching, diseases, and subsequent coral mortality. Corals become bleached by the expulsion of endo-symbiotic dinoflagellates, which reduces host pigmentation. Coral bleaching can also lead to enhanced disease prevalence immediately after thermal-stress and during the associated re-uptake of symbiotic dinoflagellates. This aspect has yet to be thoroughly investigated. A model system is necessary to study the relationships between symbiosis and disease that has sufficient flexibility to include a variety of host symbionts as well as pathogens. For this dissertation project, *Exaiptasia pallida* was investigated as a model system to evaluate the relationship between hosts, their symbionts, and pathogens. This system tested representative species of symbiotic dinoflagellates and the pathogen *Serratia marcescens.*

This study showed that the pathogen *S. marcescens* maintained viable culture densities in a closed saltwater system, but decreased when co-cultured with

symbiotic dinoflagellates. There was no sign of antimicrobial activity, suggesting symbiotic dinoflagellates are able to outcompete *S. marcescens* for resources.

The effect of symbiotic state on disease resistance was investigated in a 24-well plate assay by exposing the model host *E. pallida,* in either a stable symbiotic or aposymbiotic state, to the pathogen *S. marcescens*. There were differences in host mortality between 10^8 CFU/ml and 10^7 CFU/ml concentrations of pathogen, however there were no differences in host mortality based on symbiotic state. Since being in a stable bleached or unbleached state had no effect on host mortality, an increase in disease prevalence might be a consequence of thermal stress or transition to a symbiotic state.

To investigate the effect of transitions between symbiotic state on disease susceptibility, bleached *E. pallida* were exposed to the symbiotic dinoflagellate *Breviolum minutum* from 6 hours to 14 days. Disease resistance increased at early time points, decreased at 7 days, and increased at 14 days. Other species of symbiotic dinoflagellates, *Symbiodinium microadriaticum, Cladocopium goreaui, and Durusdinium trenchii,* were introduced to aposymbiotic hosts for either 7 or 14 days. At 7 days, disease resistance significantly increased for the *D. trenchii* treatments. At 14 days, *S. microadriaticum* and *B. minutum* treatments were more disease resistant than symbiotic or aposymbiotic controls, and treatments with *C. goreaui* and *D. trenchii* were even more disease resistant. Yet in natural systems *E. pallida* associates with *S. microadriaticum* and *B. minutum*, whereas it is not

known to associate with *C. goreaui* or *D. trenchii*. Rejecting incompatible symbionts might prime the innate-immune system. We hypothesize that symbiotic compatibility confers no disease resistance to the host, whereas symbiotic incompatibility elicits an immune response by the host that confers temporary disease resistance.

An unidentified bacterium appeared in some of the pathogen control experiments. The number of culturable unidentified microbes increased with exposure to *S. marcescens*, whereas the density of culturable *S. marcescens* decreased. Aposymbiotic anemones were inoculated at 10^8 CFU/ml of the unknown, and produced mortality in excess of *S. marcescens* exposure, with complete sample mortality within 4 days. When *E. pallida* was exposed to *E. coli* HB101 at these concentrations there was no mortality, supporting the possibility that specific interactions exist between the disease causing bacteria and the host, rather than a general increase in bioload. The severity of disease state appears mediated by host exposure to symbiotic dinoflagellates, which varies across time and by symbiont species. On coral reefs, it is probable that the appearance of certain disease states might be induced by foreign bacteria that disrupt the host's microbial community. These interactions are complex, however it should be possible to use *E. pallida* in future efforts to understand key factors responsible for coral disease.

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CHAPTER I
INTRODUCTION

CORAL SYMBIOSIS

Coral reefs are one of the most biodiverse ecosystems on the planet. They occupy roughly 0.1% of the surface of the earth, yet harbor anywhere between an estimated ~600,000 – 9,000,000 species (Reaka-Kudla et al., 1996). This makes coral reefs one of the most biologically diverse ecosystems on the planet, on par or even exceeding the diversity of tropical rain forests (Small et al., 1998). Coral reefs are also economically important, providing food, opportunities for ecotourism, and protection to coastlines from storm damage and flooding (Moberg and Folke, 1999). With most of the species on coral reefs requiring a very narrow range of environmental conditions, disturbances can be detrimental to reef-building animals and associated organisms (Loya et al., 2001).

Yet, diverse coral reefs occur in remarkably nutrient poor waters. In fact when nutrient levels become chronically high, reef corals are unable to compete with macroalgae for substrate space (Vermeij et al., 2010), which underscores the delicate nature of these ecosystems. Corals have however evolved in nutrient-limited waters by forming symbiotic relationships with photosynthetic dinoflagellates, in the family Symbiodiniaceae.

BENEFITS TO HOST AND SYMBIONT

Coral colonies host symbiotic dinoflagellates within cellular vacuoles, called symbiosomes, in their gastrodermal tissue. These symbiosomes are an arrested form of phagosomes, which translocate material captured by the host to digestive lysosomes (Fitt and Trench, 1983). If the symbionts do not function properly, coral hosts can resume digestion of symbionts by fusing symbiosomes with lysosomes for symbiont degradation (Chen et al., 2004).

In exchange for shelter and resources, symbiodinium contribute up to 40% of the total carbon they fix through photosynthesis to their coral host, primarily in the form of glycerol (Muscatine, 1967). What makes this partnership important is that symbionts provide up to 90% of the energy required for the Cnidarian host (Falkowski et al., 1984). Therefore, this coral-symbiont relationship is critical in nutrient poor waters where prey is scarce. In return, the host invertebrate provides a steady supply of organic and inorganic nutrient excrements, such as CO_2, NH_3 and PO_4^{3-} to the symbionts (Yellowlees et al., 2008).

In contrast to many other unicellular microorganisms, Symbiodiniaceae are slow growing by nature. As fast growth under nutrient limited conditions can lead to unstable populations, slow growth is likely a beneficial trait in nutrient poor waters. Coral hosts are able to control the population density of symbiodinium within their tissue by fine-tuning the release of nutrients (Rees, 1991), and thereby maintain stable populations of symbionts.

ESTABLISHMENT AND SELECTION

Symbiosis is established in individual coral colonies either vertically or horizontally (Stat et al., 2006). In vertical transmission symbionts are transferred from a host into eggs, planulae larvae, or clones. By contrast, in horizontal transmission symbionts are acquired from the local environment. There is some experimental evidence suggesting that while a host can maintain a varied population of Symbiodiniaceae, initial symbiont species selection is biologically fixed, although some coral hosts are flexible with their initial acquisition of symbionts (Little et al., 2004). Coral species that rely on horizontal transmission are generally more tolerant to a wider variety of symbiodinium associations than those which rely on vertical transmission (Fabina et al., 2012). However, the relative population distribution of symbionts can change over the lifetime of the host, especially after thermal-stress events (Silverstein et al., 2015).

Symbiont identification and selection is thought to be mediated by lectin-glycan interactions. Glycoproteins on the surface of symbiotic dinoflagellates interact with host lectins, mediating the effectiveness of host infection (Lin et al., 2000). Experimental alteration of the dinoflagellate cell wall by trypsin or *N*-glycosidase reduces the ability of the symbiont to colonize a potential host (Wood-Charlson et al., 2006).

Symbiont selection can have a long-term and substantial impact on a host as not all symbionts yield identical benefits. While some populations of endosymbiont

can provide the host with carbon photosynthates for optimal growth, other populations are more tolerant to thermal-stress events but provide a reduced pools of photosynthates (Little et al., 2004). Indeed, the composition of the host-symbiont association may ultimately be a consequence of where the host settles and the availability of optimal symbiotic partners (Rowan and Powers, 1991).

DIFFERENCES IN SYMBIOTIC ORGANISMS

Variability in the success of host-symbiont association is not surprising given the diversity of the Symbiodiniaceae family. They each have their own photosynthetic requirements and thrive in different ecological niches within the reef environment. There are Symbiodiniaceae species which are tolerant of different temperature and light levels, most notably dinoflagellates of the genus *Durusdinium* sp. which are resistant to thermal stress (Jones and Berkelmans, 2012). A few coral species are capable of harboring multiple dinoflagellate species simultaneously and the distribution of symbionts within host tissue mirrors the favored conditions of individual dinoflagellate species (Rowan et al., 1997). Host-symbiont pairings are also driven by the micro environment within host tissue, where closely related species can have different niche distributions with different coral hosts in similar habitats (Lewis et al., 2019).

Different species of symbiotic dinoflagellates also have varying growth rates, photosynthetic efficiencies and mechanisms for photoadaptation (Chang et al., 1983). Temperature, which regulates both photosynthetic efficiency and

photoadaptation, is a strong component of symbiont selection by hosts (Brener-Raffalli et al., 2018). Symbiotic dinoflagellates of the genus *Durusdinium* have photosynthetic machinery that, although less efficient than other genera, are stable within their hosts at high temperatures (Silverstein et al., 2017). In populations of *Exaiptasia pallida* from two distinct thermal conditions harboring either *Symbiodinium* sp. (warmer) or *Breviolum* sp. (cooler) dinoflagellates, *Symbiodinium* sp. dinoflagellates were also more thermotolerant to photodamage and reactive oxygen species production than *Breviolum* sp. Infecting *E. pallida* from cooler habitats with *Symbiodinium* sp. increased the thermal tolerance of the host (Goulet et al., 2005).

The thermal tolerance of the symbiotic dinoflagellate population is particularly important for hosts acclimated to areas where the average sea surface temperature is steadily increasing. Symbionts which are less thermally tolerant have a greater chance of a dysfunctional relationship with their host under chronic high-temperature conditions, which in the long term can have severe negative consequences on the host populations.

BREAKDOWN IN SYMBIOSIS

When the symbiotic relationship between coral host and symbiotic dinoflagellate becomes dysfunctional, such as during thermal-stress events, the coral host loses pigmentation. This phenomenon is commonly referred to as bleaching. The loss of pigment can occur in many ways, from a decrease in

chlorophyll concentrations in symbiotic dinoflagellates to more extreme events when symbiotic dinoflagellates are expelled, along with damaged cells from the host tissue (Gates et al., 1992). These responses are a consequence of a buildup of reactive oxygen species (ROS) during the breakdown of the photosynthetic process. Indeed, the buildup of products of oxidative damage, such as protein carbonyl and lipid peroxidation levels, are positively correlated with visible coral bleaching (Downs et al., 2002).

PHOTOSYNTHESIS

Within dinoflagellates, and plants that host chloroplasts, ROS are normally generated at two stages in the photosynthetic process. Singlet oxygen is generated in the initial stage of photosynthesis, in PSII, which is responsible for the initial harvesting of light and the splitting of water. This protein complex absorbs light at 680 nm allowing the 'special pair' (P680) of chlorophyll molecules to be oxidized, passing electrons along the photosynthetic electron transport chain. Reduction of the special pair is driven by the oxidation of water molecules, at the oxygen-evolving complex. In addition to providing electrons to regenerate the special pair, this process releases H^+ and singlet oxygen ($^1O_2$) into the lumen of the thylakoid membranes of the chloroplast. The former helps build a proton gradient for the production of ATP, whereas two molecules of the latter quickly combine to form molecular oxygen (O_2) (Krieger-Liszkay, 2005).

Electrons are transferred from PSII to photosystem I (PSI) via the cytochrome b6f complex. When PSI is energized by light, it again excites electrons harvested from molecular oxygen (O_2), creating superoxide (O_2^-) which is quickly reduced by the enzyme superoxide dismutase into hydrogen peroxide. Hydrogen peroxide is then reduced to water by the enzyme catalase. The harvested electrons are used in the production of ATP and NADPH, which are then used to assemble carbohydrates from CO_2.

The Calvin cycle assembles carbohydrates from CO_2, independently of light *in vitro,* and is driven by ATP and NADPH produced during the "light reactions" described above. Although this enzyme activity and synthesis are frequently called "dark reactions", they are however governed by pH gradients generated by the light reactions and illuminance and are therefore more appropriately called "carbon reactions" (Buchanan, 2016).

The reporting of photosynthetic activity to the host cell is mediated through ROS generated in PSI and PSII. The efficiency of photosystem management is largely related to the ability of these molecules, or signaling intermediaries, to access the nucleus to generate responses, which differs greatly between photosynthetic species (Foyer, 2018).

PHOTOSYNTHETIC DECOUPLING

Symbiotic breakdown is ultimately dependent on the efficiency of photosynthesis in Symbiodiniaceae. Photoinhibition of the symbiont is directly

proportional to the rate of symbiont expulsion directed by the host organism (Perez et al., 2001). Chemically induced inefficiency of photosynthesis by glycolaldehyde or potassium cyanide only causes bleaching when reactive oxygen is generated (Hill et al., 2014). Although breakdown of symbiosis is related to the generation of ROS, nitric oxide (NO), a free radical which is important as an intracellular signaling molecule in the host, is also involved in dysymbiosis (Perez and Weis, 2006). NO is generated in the host through various nitrous oxide synthases (NOS), which convert arginine, NADPH, and O_2 into citrulline, $NADP^+$, and NO. NO has some initial functions that are beneficial to the host under oxidative stress, such as the ability to react to and mitigate ROS toxicity (Wink and Mitchell, 1998). A series of experiments by Perez and Weiss (2006) investigated the role of NO signaling in cnidarian bleaching by examining this phenomenon in the model *E. pallida*. During heat stress in symbiotic *E. pallida*, the host is responsible for NO production. NO is not produced in aposymbiotic individuals under heat stress. While high levels of NO are present during bleaching, the exogenous production of NO leads to expulsion of Symbiodiniaceae from host tissue. The addition of exogenous NO in the form of sodium nitroprusside induces bleaching at room temperature (Perez and Weis, 2006).

Increased levels of ROS are associated with the decoupling of photosynthesis. The decoupling of photosynthesis in PSII begins with the D1 subunit, which is central to the water splitting complex and electron excitation. D1

is routinely damaged by normal irradiance during the photosynthetic process, inactivating the entire photosystem complex. Experimentally PSI, does not degrade with increases in temperature and light stress in symbiotic dinoflagellates (Hoogenboom et al., 2012). Most photosynthetic organisms can repair the PSII complex by replacing the D1 subunit rather than synthesizing and constructing an entirely new complex (Sass et al., 1997). Under optimal conditions, to maintain photosynthetic efficiency, this protein maintains a high turnover rate to match the rate of damage. When exposed to extreme illumination, damaged forms of D1 are generated faster than they are replaced.

When D1 is not being actively replaced, energized PSII does not pass on electrons through normal photosynthetic pathways. At low levels of damage there are nonphotochemical quenching mechanisms which accept electrons from excited PSII (Demmig-Adams and Adams, 1992). When electron excitation frequency exceeds the capacity of nonphotochemical quenching for protection, molecular oxygen becomes an acceptor for excited electrons and ROS, principally superoxide, are generated (Nishiyama et al., 2005).

PHOTOSYSTEM REPAIR

While the damage to D1 and subsequent inactivation of PSII is light driven, the repair mechanisms in some coral-symbiont pairings are highly sensitive to temperature (Takahashi et al., 2004). The reduction in repair capacity and variability in response is also found in cultured symbiodinium (Takahashi et al.,

2009). Both extremes in high and low temperature can cause breakdown in the signaling required for D1 synthesis and incorporation into PSII. Turnover of the D1 subunit of PSII in response to temperature varies greatly between species of symbiotic dinoflagellates, which plays greatly into bleaching sensitivity (Warner et al., 1999). While damage to PSII can stimulate the production of excess D1 protein (Hill et al., 2011), there is variability between species in their ability to be incorporated in actual repair (Hill and Takahashi, 2014).

Some coral-symbiont pairings are less able to tolerate photosystem damage than other pairings and much of these differences seem to be attributed to increased temperature. At 31˚C, *Montipora digitata* is unable to fully recover within 24 hours from damage caused by high illumination. By contrast, *Pavona divaricata* is able to recover fully over 24 hours at the same temperature. Incomplete photosystem recovery results in increased susceptibility to subsequent photosystem damage, causing chronic photoinhibition that results in coral bleaching (Yakovleva and Hidaka, 2004).

The severity of bleaching is largely dependent on both the species of symbiotic dinoflagellates and the ability of the host to tolerate the increase in ROS (Baird et al., 2009). The loss of pigment can occur in many ways (Gates et al., 1992), and corals that do not completely bleach during stress events experience a decrease in carbon contribution from their Symbiodiniaceae (Porter et al., 1989).

Bleaching can be mitigated by several physiological mechanisms, and the ability to provide these mitigating responses in both host and symbiont are likely responsible for differences in thermal tolerance. The first line of defense are heat shock proteins (HSPs), which maintain protein stability under thermal stress. For example, the coral *Porites cylindrica* naturally expresses higher levels of HSP 70 before the introduction of thermal stress than *Stylophora pistillata*. These differences in protein expression could account for differences in bleaching susceptibility with *P. cylindrica* considerably more tolerant than *S. pistillata* (Fitt et al., 2009), even when coral hosts are harboring the same species of symbiont. During the 1997-1998 El Niño event in Belize, different species of *Agaricia* showed differential mortality after bleaching, which correlated with differential expression levels of HSC 70 and HSP 90 (Robbart et al., 2004).

Oxidative stress from ROS generated by photosynthetic decoupling can be lessened by numerous antioxidant scavenging molecules. These antioxidants can be generated by both the host and the symbiotic dinoflagellates. The generation of both ROS and antioxidant molecules in symbiotic dinoflagellates are closely related to thermal tolerance of dinoflagellates (Krueger et al., 2014; McGinty et al., 2012). Scavenging of ROS is primarily undertaken by the enzymes superoxide dismutase, ascorbate peroxidase, and catalase, but can also include microsporine-like amino acids (Rosic and Dove, 2011), carotenoids, tocopherols (Krieger-Liszkay, 2005), and fluorescent proteins (Palmer et al., 2009).

TEMPERATURE MEDIATED BLEACHING

Coral bleaching is triggered by intense illumination causing photoinhibition, which if prolonged can cause damage to the photosystems which will not recover in the dark. Such chronic photoinhibition is intensified by deviation from the optimal photosynthetic temperature for Symbiodiniaceae, which varies between species. Coral bleaching is frequently considered only as a response to temperatures near the upper limits of physiological tolerance. Yet the phenomenon of bleaching is a consequence of both illumination and temperature and occurs at both upper and lower limits of thermal tolerance (Roth et al., 2012).

Oxidative stress also occurs under cold temperature conditions. While there is no increase in the enzymatic ability of oxidation quenching, this cold-temperature stress may be because of decreases in the efficiency of enzymes at low temperature. Coupled with dysfunction of photosynthetic electron transfer, cold stress can lead to bleaching (Higuchi et al., 2015). Much like the high temperature counterpart, low-temperature photoinhibition and damage to photosystems is also mediated by light exposure (Hoegh-Guldberg et al., 2005), and increasing illumination can enhance the effects of cold-induced bleaching and stress (Saxby et al., 2003).

EFFECTS OF BLEACHING

Bleaching can result in a multitude of downstream issues for the affected individual coral colonies. While the obvious terminal condition is mortality,

bleaching also affects coral reproduction. Declines in successful fertilization have been observed following bleaching events, which are thought to be a consequence of reduced sperm motility (Omori et al., 2001). In *Acropora palmata*, increased temperature is associated with higher developmental anomalies, higher mortality, and lower settlement rates. *Oculina* corals in the Red Sea experience reduced fecundity and a reproductive decline after exposure to a single thermal-stress event, but subsequent annual events see surviving individuals returning to normal gametogenesis (Armoza-Zvuloni et al., 2011).

BLEACHING MITIGATION

Mass bleaching events have been documented since the 1980s (Glynn, 1984), but are now recurring with increased frequency and intensity. Mass bleaching events are primarily characterized by chronic exposure to elevated illumination and elevated temperatures, in some locations are initiated by an increase of only 1˚C above the local seasonal maximum (Brown, 1997; Hughes et al., 2003; Manzello et al., 2007). It is estimated that at some point between 2020 and 2035 mass coral bleaching will become an annual occurrence at most localities around the globe (Manzello, 2015). Extensive bleaching can alter coral cover, community composition, and can cause local extinction of vulnerable populations (Loya et al., 2001).

Biological traits of the host can have drastic effects on bleaching severity. Branching corals tend to be much more susceptible than massive corals to thermal-

stress events. This can be attributed to surface area exposed to warmer temperatures, the thickness of tissue, and/or differences in mass transfer based on colony morphology (Loya et al., 2001). Indeed, both thicker tissue and massive corals are inherently more resistant to thermal, but are more susceptible to reflected irradiance by light colored sandy substrate (Ortiz et al., 2009). Bleaching sensitivity is also driven by symbiont composition (Berkelmans and van Oppen, 2006), which react differently to temperature and illumination stress.

Ecological traits also temper the bleaching response. Shallow water coral colonies are generally exposed to more extreme illumination and temperature stress than deep water coral colonies. Yet, coral colonies in high-flow environments generally suffer less bleaching and mortality than coral colonies in low-flow environments (Nakamura et al., 2003). Similarly, turbidity can reduce illumination and thereby moderate bleaching (Sully and van Woesik, 2020).

Repeated exposure to thermal-stress events can also select for increased thermal tolerance in surviving individuals. *Pocillopora*, *Acropora*, and *Porites* species on the Great Barrier Reef were less susceptible to bleaching in 2002 than in 1998 (Maynard et al., 2008). Habitats which naturally experience high variability in temperature also tend to select for bleaching resistant individuals (McClanahan et al., 2007). However, repeated thermal-stress events and the resultant coral mortality can drastically alter the composition and assemblages.

In the Caribbean, coral cover has declined by up to 80% since the 1980s (Gardner et al., 2003). Indeed, in modern times the Caribbean has seen declines in a key reef-building species, *Acropora cervicornis*, to a level unprecedented for the last 3000-4000 years in the geological record (Aronson and Precht, 2001). In the past, *A. cervicornis* has persisted despite variations in local climate, and recent declines are primarily attributed to anthropogenic stressors (Greer et al., 2009; Cramer et al., 2012). These anthropogenic stressors have driven present day coral mortality in the form of coral bleaching and disease (Goreau, 1992; Harvell et al., 1999).

While coral bleaching is clearly related to high illumination and thermal stress, it is also related to an increase in the prevalence of some coral diseases. The importance of thermal stress has been linked to increased incidence of coral disease in a number of recent studies (Selig et al., 2013; Randall and van Woesik, 2015), and disease combined with bleaching can severely increase tissue loss and mortality during thermal-stress events (Brodnicke et al., 2019). Population density also appears to link with the severity of disease outbreaks (Bruno et al., 2007), but many diseases do not seem to follow any sort of contagion model (Muller and van Woesik, 2012). There is also evidence that disease outbreaks occur after high-temperature stress events, mediated by preceding winter temperatures (Heron et al., 2010).

The field of coral diseases is still maturing, and there is opportunity for further research into underlying causes of disease including processes that influence disease symptoms, disease onset, disease incidence, disease transmission, disease resistance, and disease prevalence. One particularly confounding aspect of coral disease is that a coral colony exists as a holobiont. The holobiont is the community of coral host, symbiotic dinoflagellates, and microbial community. Diseases can affect the host coral, its symbiotic dinoflagellates, or even its associated bacterial community. Shifts or perturbations in environmental conditions which disrupt any of these populations can result in disease.

Diseases of particular interest in the Caribbean are the "white" diseases, which include white-band disease, white-pox disease, and white-plague disease. White-band disease progresses with either a band of necrotic tissue adjacent to receding but normally pigmented coral tissue (White-band disease type 1) or a margin of bleached tissue between pigmented and necrotic tissue (White-band disease type 2) (Ritchie and Smith, 1998). This disease is caused by pathogenic bacteria (Kline and Vollmer, 2011) and can be transmitted from coral to coral through the water column or by animal vectors (Gignoux-Wolfsohn et al., 2012). White-band disease has caused massive mortality in both *Acropora palmata* and *Acropora cervicornis* in the Caribbean (Aronson and Precht, 2001) and, along with other diseases, has contributed to both coral species being listed as a threatened (Aronson et al., 2008a, 2008b).

White-plague disease is a broad term for several different diseases with similar progression, which is a white band of dead tissue that starts at the edge of a colony and quickly migrates (Roder et al., 2014). There are specific pathogens associated with White-plague disease type 2 (Denner et al., 2003) and an outbreak in the Red Sea (Thompson et al., 2006), though there is a strong possibility that other cases are caused by shifts in the microbial community (Kellogg et al., 2013; Roder et al., 2014).

White-pox disease is specific to the coral *Acropora palmata* and first appeared off the Florida Keys (Holden, 1996). It presents as irregular lesions of exposed skeleton that can appear simultaneously over the entire coral colony (Patterson et al., 2002). White-pox disease was originally associated with the pathogen *Serratia marcescens*, however this bacteria only appears in approximately 25% of white pox lesions (Joyner et al., 2015). Outbreaks of white-pox disease are thought to be associated with sewage water, as *S. marcescens* is an enterobacteria which can be associated with the human gut (Sutherland et al., 2010).

Temperature stress can exacerbate disease by increasing the growth rate and activity of potential pathogens (Toren et al., 1998), and by causing stress to coral colonies. Some diseases are known to increase after thermal-stress events. Diseases such as white-plague disease and white-pox disease have appeared weeks after thermal-stress events and subsequent coral bleaching (Miller et al., 2009; Precht et

al., 2016), after acute stress to the coral holobiont, and after temperature-associated increases in pathogen load and virulence (Tout et al., 2015).

One potential mediator of the temperature-related effect could be the interaction of coral hosts with their symbiotic dinoflagellates, which are partially or completely expelled during thermal-stress events. The loss of symbionts can potentially influence diseases because of their contributions to their host, which are not entirely nutritional. This is underscored by a number of studies which have found that (i) the loss of symbionts reduces disease resistance in the host (Muller et al., 2018), (ii) community shifts in symbionts affect disease resistance (Rouzé et al., 2016), and (iii) that re-uptake of symbionts compromises the host immune response (Merselis et al., 2018).

My research focuses on the importance of the association of symbiotic dinoflagellates on disease resistance. The model organism *Exaiptasia pallida* is used in this study as it can be maintained long-term both in a bleached state (aposymbiotic) with supplemental feeding and in a non-bleached state (symbiotic). This model system allows decoupling of the physiological stress associated with the phenomenon of 'bleaching' from the actual absence of the symbiont for the purpose of studying the contributing factors to disease resistance. *E. pallida* has been established as a model for both cnidarian-dinoflagellate symbiosis (Berner et al., 1993; Biquand et al., 2017; Bucher et al., 2016; Detournay et al., 2012; Dunn et al., 2002; Emilie-Fleur et al., 2017; Gates et al., 1992; Lehnert et al., 2012; Perez

and Weis, 2006; Perez et al., 2001; Poole et al., 2016; Sawyer and Muscatine, 2001; Voolstra, 2013; Wolfowicz et al., 2016) and disease (Bailey et al., 2019; Divya et al., 2018; Krediet et al., 2014, 2013; Poole et al., 2016; Zaragoza et al., 2014). By being able to maintain *E. pallida* in a bleached state with supplemental feeding, it is possible to keep the experimental animal alive without considering the confounding problems of starvation. Animals can be maintained long-term and exposed to specific strains of symbiotic dinoflagellates. Using *E. pallida* as a model also facilitates experiments in small volumes of liquid in 24-well plates. Such experiments can establish reliably repeatable experimental conditions while minimizing resource usage.

This dissertation systematically investigates three primary objectives.

I. To determine the role symbiotic state plays in disease resistance.

The establishment of symbiosis requires either a dampening of the immune response or an evasion of the innate-immune system. If the immune system is evaded by symbiotic dinoflagellates, there should be no impact on disease resistance. If maintaining a symbiotic state relies on dampening the immune response of the host, then disease resistance should decrease. These hypotheses are tested by exposing *Exaiptasia pallida* in either a symbiotic or aposymbiotic state to *Serratia marcescens*, a pathogen.

II. To determine how disease resistance is affected by the amount of time a prospective host is exposed to symbiotic dinoflagellates.

Since some coral diseases have increased prevalence following bleaching events, recolonization of the host with symbiotic dinoflagellates may play a role in disease resistance. After bleaching, the host can acquire symbiotic dinoflagellates from the environment and go through a period of sorting in which it determines the acceptability of the symbiont. The amount of time the host has available to go through the recognition and sorting process may affect disease resistance. This hypothesis is tested by exposing *Exaiptasia pallida* to the dinoflagellate *Breviolum minimum* for time periods ranging from 6 hours to two weeks before being challenged with the pathogen *Serratia marcescens*.

III. To determine the effect that different species of symbiotic dinoflagellates have on the host resistance to disease.

Colonization of a host with specific species of symbiotic dinoflagellates not only seems to modulate the ability of the host susceptibility to bleaching, it also correlates with different levels of disease prevalence in the wild (Correa et al., 2009; Rouzé et al., 2016). By using a healthy but bleached host, specificity in symbiont species can be assured without interference from background species either in host tissue or the environment. The modulation of host disease resistance may be based on which symbiotic dinoflagellate species that is associated with the host. This hypothesis is tested by exposing *E. pallida* to *Symbiodinium microadriaticum*, *Breviolum minimum*, *Cladocopium goreaui*, or *Durusdinium*

trenchii for a period of one week before being challenged with the pathogen *Serratia marcescens*.

CHAPTER II
CHARACTERIZING BEHAVIOR OF SERRATIA MARCESCENS

INTRODUCTION

Serratia marcescens is responsible for acroporid serratiosis, also known as white-pox disease (Patterson et al., 2002). *S. marcescens* is an opportunistic pathogen that presumably enters sea water through sewage discharge (Sutherland et al., 2010). This microorganism can survive in saline environments (Looney et al., 2010), however it is primarily found in damp, warm terrestrial environments (Petersen and Tisa, 2013). *S. marcescens* is an opportunistic pathogen of mammals, invertebrates, and plants which rarely causes an infection on its own (Grimont and Grimont, 1978). However, the pathogen will take advantage of a compromised host, such as those with an existing infection or who are otherwise already immunocompromised. The pigment prodigiosin is characteristic of *S. marcescens*, giving it a red coloration. There are natural mutants that do not produce this pigment and appear white, although this alteration of color does not affect the pathogenic properties (Zhou et al., 2016).

Previous work with coral disease using *Exaiptasia pallida* has included the pathogen *S. marcescens*. This work includes experimentation on: (i) the modulation of swarming behavior and biofilm formation by coral-associated microbiota (Alagely et al., 2011), (ii) the ability of *S. marcescens* to colonize cnidarian mucous

(Krediet et al., 2013), (iii) establishing *E. pallida* as a susceptible model for various coral diseases (Zaragoza et al., 2014), (iv) interactions between pathogenic and non-motile *S. marcescens* with *E. pallida* (Krediet et al., 2014), and (v) potential immune recognition by *E. pallida* (Poole et al., 2016). While overall interactions with the host and some of its associated microbiota have been characterized, there is a general lack of studies directly dealing with the symbiotic state and the importance of species of symbiotic dinoflagellates influencing the pathogenic susceptibility of the host. In this chapter, I fill some of these gaps in the literature by investigating the growth behavior in *S. marcescens* in a saltwater environment and examining interactions between the pathogen and dinoflagellates in the family Symbiodiniaceae.

METHODS

BACTERIAL CULTURE. Cultures of *S. marcescens* were grown in a saltwater lysogeny broth (SWLB). SWLB is composed of 10 g/L tryptone and 5 g/L yeast extract dissolved in artificial saltwater (ASW), a solution of Instant Ocean® Sea Salt (Spectrum Brands) dissolved to manufacturer specifications in deionized water (dH_2O). This is a modification of standard lysogeny broth (LB) media, which is made by dissolving 10 g/L tryptone, 5 g/L yeast extract mixed, and 10 g/L of sodium chloride in dH_2O. Unless otherwise noted, all bacterial assays used cells grown to log-phase at 37˚C, as verified by absorbance readings at 590 nm. Cells were harvested, pelleted, washed with sterile ASW to remove excess

media, then resuspended in ASW in concentrations necessary for the given experiment.

Whenever new freezer stocks of *S. marcescens* were made, the culture was tested for purity using multiple assays. These differential tests included Triple Sugar Iron (TSI) (Sulkin and Willett, 1940), Mannitol Salt Agar (MSA) (Chapman et al., 1938), DNase (Jeffries et al., 1957), Gelatinase stab, and Eosin Methylene Blue (EMB) assays (Holt-Harris and Teague, 1916). For *S. marcescens*, a positive TSI assay in a slant culture is indicated by an alkaline slant, acid butt, no gas production, and no hydrogen sulfide production. A positive Mannitol Salt assay does not show any growth and maintains red coloration on the plate. A positive DNase assay shows a clearing of green pigment around colonies on the agar plate. A positive Gelatinase assay liquifies a gelatin slant at room temperature over a week. A positive EMB assay produces pink, non-metallic colonies (Figure 2.1).

SYMBIODINIACEAE DINOFLAGELLATE CULTURE. Cultures of different species of Symbiodiniaceae were grown in filter sterilized (Thermo Scientific™ Nalgene™ Rapid-Flow™ 0.2µm 50mm Filter Unit) ASW enriched with Guillards's (f/2) marine water enrichment solution (MilleporeSigma®).

Cultures were grown in a shaded greenhouse in ambient lighting (approximately 1400 lux at noon in June subject to seasonal variation measured with a Dr. Meter LX1330B) and temperature conditions (max 24˚C) and were

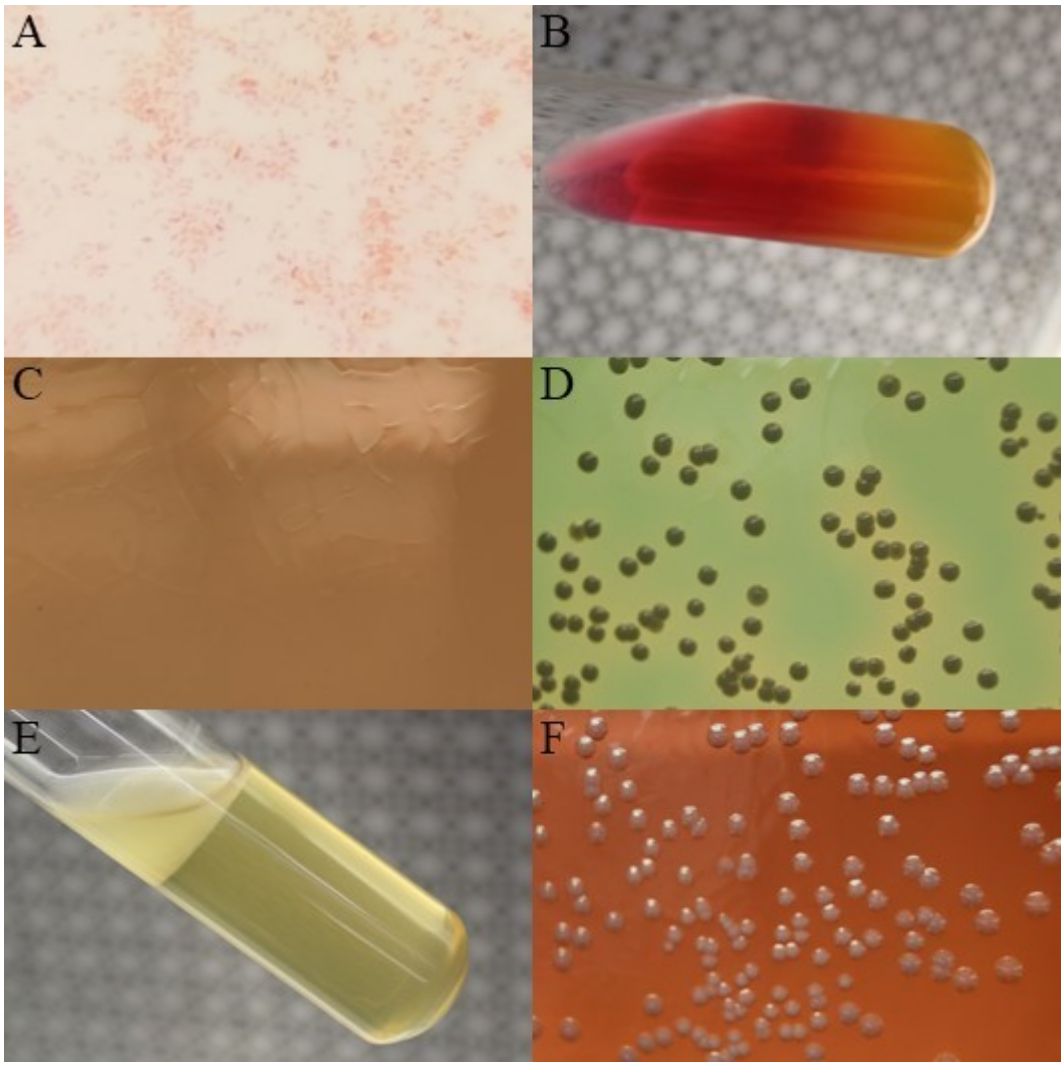

Figure 2.1. *Serratia marcescens* microbial assays. A. Negative gram stain, B. TSI stab with red alkaline slant, orange acid butt, and no gas or hydrogen sulfide production, C. Mannitol salt agar with no growth present, D. DNase agar with zones of green pigment clearing around colonies, E. Gelatinase stab with liquified gelatin, F. EMB agar with pink, non-metallic colonies.

subject to seasonal variations (Figure 2.2). Cultures showing signs of bacterial contamination were supplemented with an antibiotic solution resulting in a final concentration of 0.2 g/L streptomycin, 0.1 g/L kanamycin, 0.02 g/L neomycin, 0.8 mg/L gentamycin, and 1.2 mg/L tetracycline (modified from (Polne-Fuller, 1991). Every two weeks cultures were centrifuged and resuspended in fresh media.

SERRATIA MARCESCENS VIABILITY IN SALTWATER. To investigate the ability of *S. marcescens* to persist in a pure ASW media, with no additional nutrient source, the bacteria were incubated in 10 ml ASW at a concentration of 10^8 CFU/ml for 20 days in 15 ml polypropylene culture tubes in a Precision™ 818 low temperature illuminated incubator at 28˚C with a 12/12 light cycle of 880 Lux, measured with a Dr. Meter LX1330B. Log phase *S. marcescens* cultures were used for the initial inoculation in three replicates. 100 µl fractions were taken daily from the cultures and dilutions were plated in triplicate. The average number of CFU from the three plates was used to determine the number of viable *S. marcescens* cells per ml per culture.

SERRATIA MARCESCENS AND SYMBIODINIACEAE DINOFLAGELLATE CO-CULTURE. To identify interactions between *S. marcescens* and species of Symbiodiniaceae, 10 ml of ASW was inoculated to a final concentration of 10^8 *S. marcescens* cells and 10^5 cells of either *Symbiodinium microadriaticum*, *Breviolum minutum*, or *Cladocopium goreaui*. The concentration

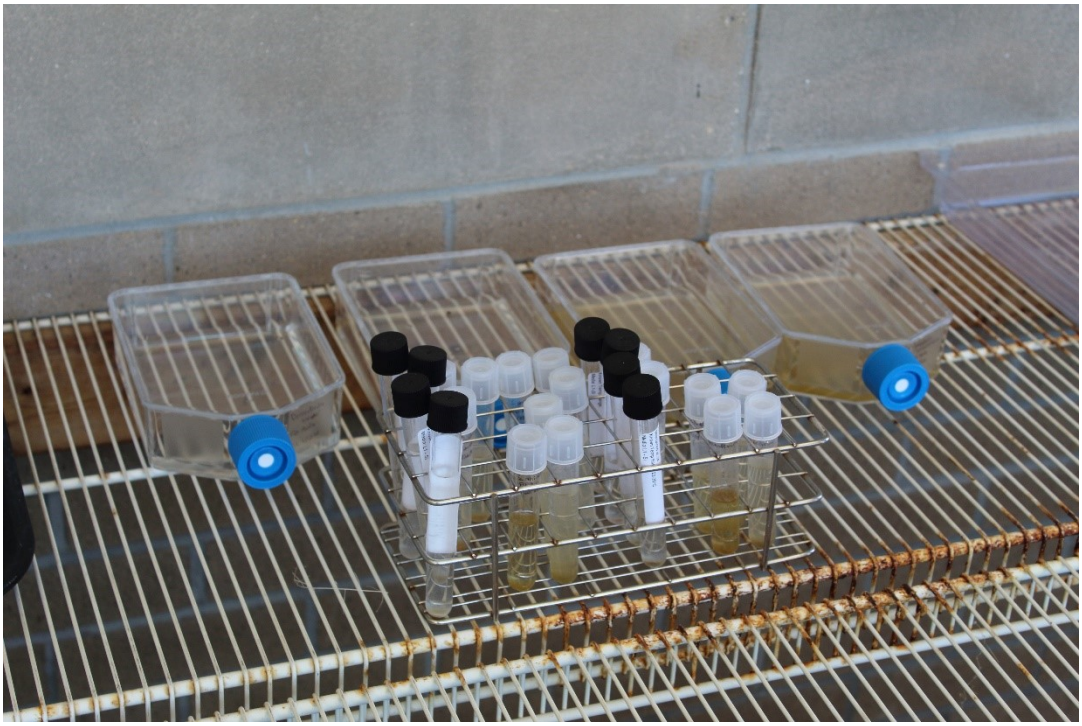

Figure 2.2. Symbiotic dinoflagellate cultures grown in Guillard's f/2 media in a greenhouse in indirect lighting under ambient conditions.

of Symbiodiniaceae cells was determined by the average of three measurements using a hemocytometer. Cultures were maintained in 15-ml polypropylene culture tubes in an illuminated incubator at 28˚C with a 12/12 light cycle. Samples of 100 µl were taken at 0, 3, 5, 10, 15, and 20 days from the cultures and dilutions were plated in triplicate. The average number of CFU from the three plates was used to determine the number of viable *S. marcescens* cells per ml per culture. At Day 20, the number of Symbiodiniaceae cells was also measured in each culture tube via hemocytometer.

ZONE OF CLEARING ASSAY. To identify any possible antibiotic properties of Symbiodiniaceae species, zone of clearing assays were performed with plates spread with 10^6 CFU of *S. marcescens*. After spreading the bacteria, diffusion disks made of sterilized filter paper were placed on the plate and 5 µl of stationary cultures of either *S. microadriaticum*, *B. minutum*, or *C. goreaui* were placed on the diffusion disks. As a positive control a diffusion disk was spotted with 5 µl of 1x modified antibiotic solution from Pulne-Fuller (1991). Three replicate plates were incubated at 28˚C in a Precision™ 818 low temperature illuminated incubator at 28˚C with a 12/12 light cycle of 880 Lux for 24 hours. Zones of clearing were measured at the end of this time period.

RESULTS

BACTERIAL CULTURE. To examine the relationship between the number of *S. marcescens* CFU with absorbance, three cultures were grown, sampled hourly,

and then dilutions were plated in triplicate. From this information, a line of best fit was generated over log-phase growth with the equation CFU = $7.23x10^{7}*e^{2.0013(abs)}$ (Figure 2.3). The doubling rate of *S. marcescens* during log-growth is approximately 74.4 minutes at 37˚C in SWLB. The exponential growth constant (k) of 0.805 (gen/hr) was calculated by dividing the log of the number of CFU at the end of the exponential growth period (B_n) minus the log of the number of CFU at the beginning of the growth period (B_0) by the total time of exponential growth (t) times the log of 2. The log phase was identified by graphing the number of CFU as they increased over time as $k = (\log B_n - \log B_0) / (t \times \log 2)$ (Figure 2.4 and Figure 2.5). The generation time is the inverse of the experimental growth constant, g = 1/k, which is 1.24 hours/generation. Exponential growth occurs between the absorbance values of 0.476 – 1.335 at 590 nm.

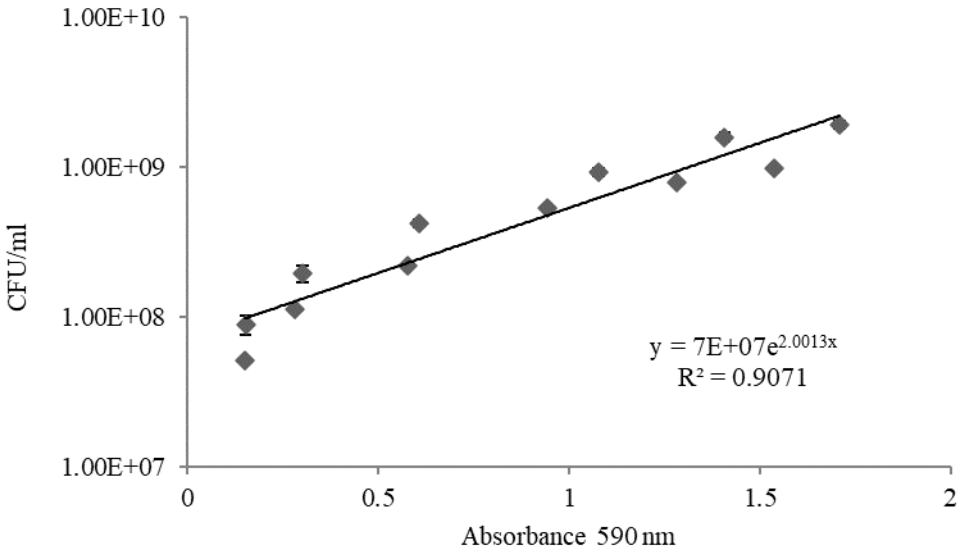

Figure 2.3. Absorbance vs CFU in *Serratia marcescens*. Cultures were grown in SWLB at 37˚C and agitated at 100 RPM, n=12 with each sample being plated in triplicate.

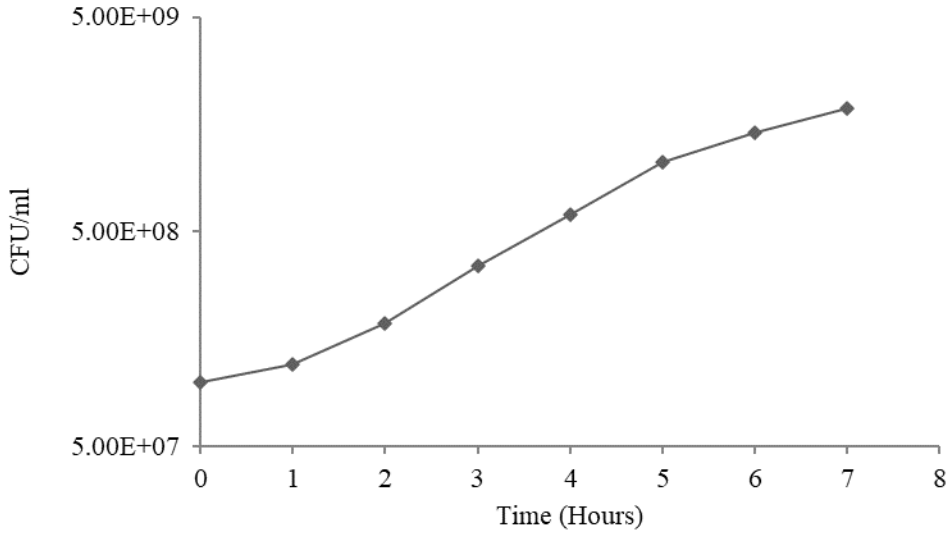

Figure 2.4. Growth curve of *S. marcescens*. A culture of *S. marcescens* was started at an absorbance of 0.157 at 590 nm and observed over 7 hours. The optical density was recorded, and triplicate plating of culture dilutions were performed every hour. Samples were agitated at 100 RPM and incubated at 37˚C in SWLB.

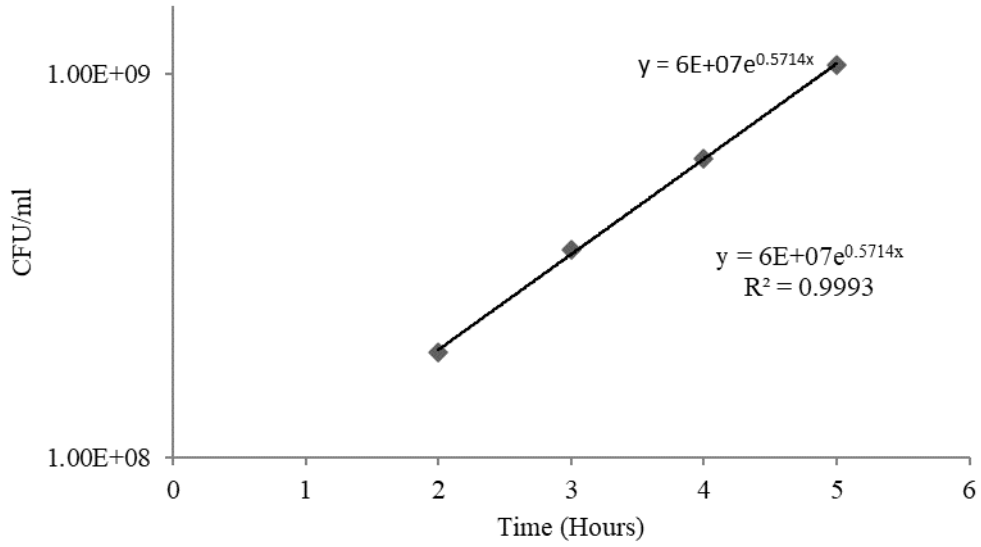

Figure 2.5. Exponential growth of *S. marcescens* graphed on a log scale. In order to show that the time-frame of 2 to 4 hours from the growth curve was a period of exponential growth, a linear line of best fit was generated with an R value of 0.9993. Samples were agitated at 100 RPM and incubated at 37˚C in SWLB.

SERRATIA MARCESCENS VIABILITY IN SALTWATER. Culture densities of *S. marcescens* were maintained over the course of the experimental condition in ASW, with no additional nutrient supplimentation. When a culture of 10^8 CFU/ml of *S. marcescens* was moved from SWLB to ASW, there is an initial spike in the number of CFU/ml lasting for about three days (Figure 2.6). This density then dropped over the course of the next two days, and the culture density stayed between 1.25 x 10^8 and 1 x 10^8 from Days 5 through 15. After 15 days, the number of CFU in an ASW culture starts to drop below the initial inoculation load. However, the final CFU concentration after 20 days is within 50% of the initial inoculation load.

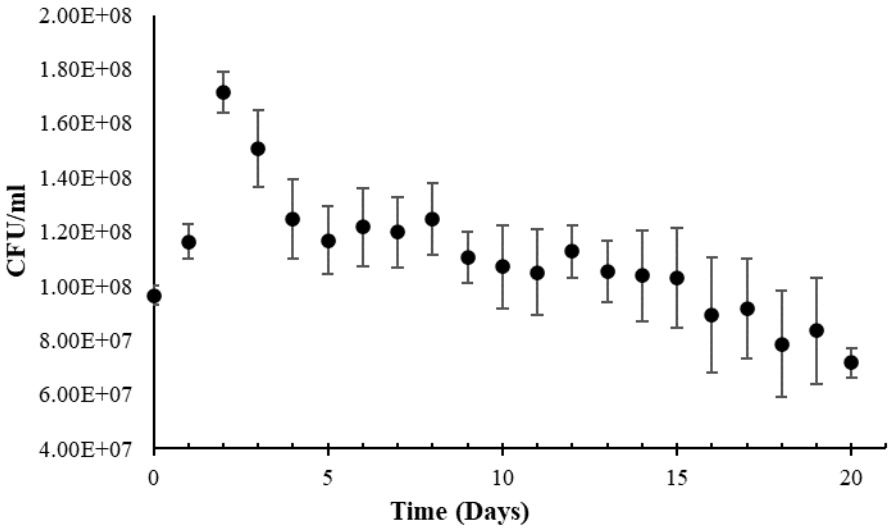

Figure 2.6. *Serratia marcescens* in a closed saltwater culture. The number of CFU increase through to Day 3, then stays slightly elevated until Day 15, then drops just under the starting value. Three continuous cultures were sampled daily. The bacterial cultures were grown in sterile ASW at 28˚C with no agitation.

SERRATIA MARCESCENS AND SYMBIODINIACEAE DINOFLAGELLATE CO-CULTURE. Without an external nutrient source, which would normally be provided by LB or Guillard's media, the dinoflagellates *S. microadriaticum*, *B. minutum*, and *C. goreaui* persisted in culture with *S. marcescens* and increased in concentration from starting values (Table 2.1). At the same time, the number of viable *S. marcescens* cells dropped in a logarithmic fashion, to the point where they are below 100 CFU/ml or no longer present after 20 days of co-culture (Figure 2.7).

Table 2.1. Growth of symbiotic dinoflagellates co-cultured with *S. marcescens*. Each species was incubated at an initial culture density of $1x10^5$ cells/ml in ASW with $1x10^8$ CFU *S. marcescens* for 20 day. The ending cell count is the average of three replicates and includes the standard error.

Species	Ending cell count (cells/ml)
S. microadriaticum	$3.1x10^5 \pm 8.7x10^4$
B. minutum	$5.9x10^5 \pm 1.1x10^5$
C. goreaui	$5.0x10^5 \pm 1.4x10^5$

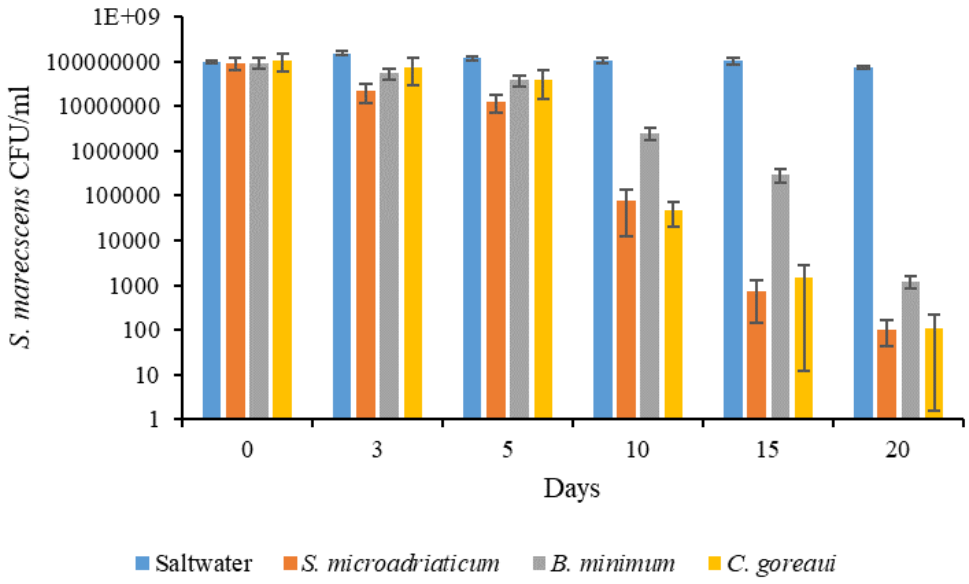

Figure 2.7. Effect of co-culture of *S. marcescens* with species of Symbiodiniaceae. Culture of symbiotic dinoflagellates with the opportunistic pathogen *S. marcescens* in salt water with no additional nutrients reduces the number of CFUs over time, regardless of species. Error bars represent standard error. Each bar consists of the data from 3 replicates.

ZONE OF CLEARING ASSAY. No zones of clearing for *S. marcescens* were produced by the symbiotic dinoflagellate species *A. microadriaticum*, *B minutum*, or *C. goreaui*. A zone of clearing was produced by the modified antibiotic solution modified from Folne-Puller (1991) (Figure 2.8). The small bacteria-free zones right at the edges of the filter paper are also present in additive-free controls and when tetracycline is spotted (data not shown), and is likely an artifact of placement of the filter paper on the plates after they have been spread.

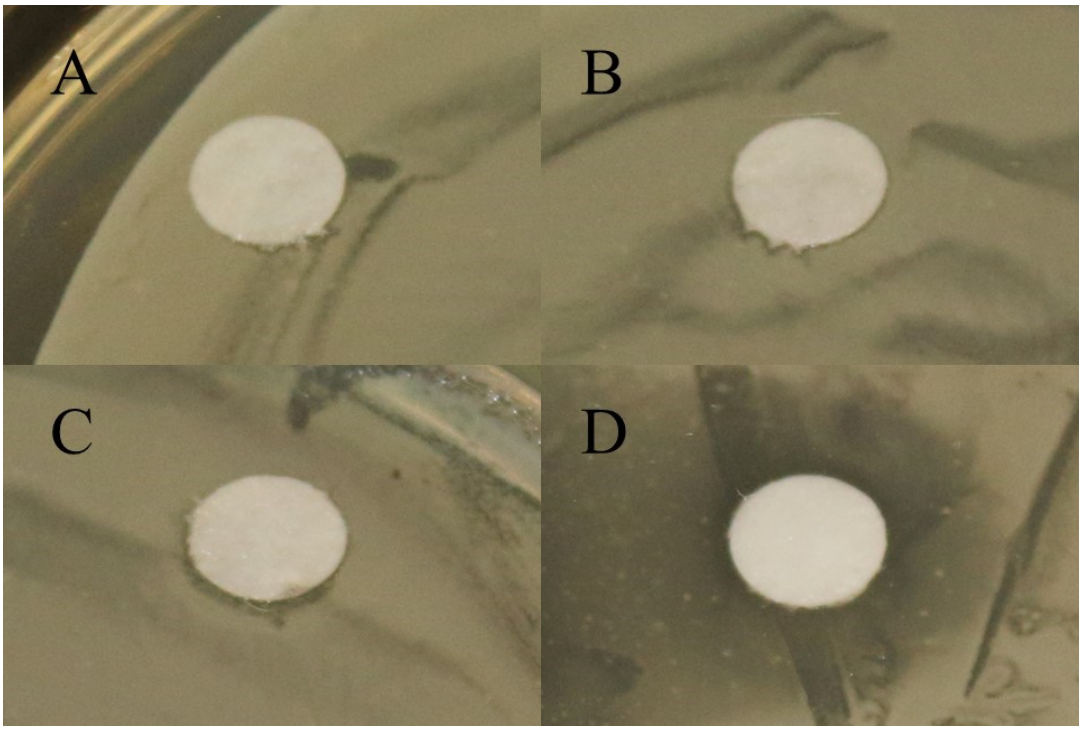

Figure 2.8. Zone of Clearing Assay. No zones of clearing were observed for *S. marcescens* when exposed to the dinoflagellates (A) *S. microadriaticum*, (B) *B. minutum*, or (C) *C. goreaui*. A zone of clearing was observed when exposed to the modified antibiotic solution (D) from Polne-Fuller (1991).

DISCUSSION

The growth characteristics and viability of *S. marcescens* was examined in a saline environment. While it may be a common practice to grow *S. marcescens* in standard LB media before transfer into the experimental conditions, the shock of entering a higher salt or different pH environment could affect viability. Considering the potential for a salt and pH shock, *S. marcescens* was grown in a saltwater augmented media (SWLB) rather than in a standard LB media before the transfer to ASW. The generation time of *S. marcescens* in SWLB, at 74.4 minutes, is longer than other reported values. Generation times of 49 minutes in LB media at 37˚C without agitation (Fedrigo et al., 2011) and 22 minutes in Difco nutrient broth at 37˚C without agitation have been reported (Jennson, 1935). The differences in generation times however may be highly strain dependent. These results indicate that the extra salt in the media either had a slowing effect on the growth rate of *S. marcescens* or was stressful enough to increase the mortality rate. Cultures were also grown in closed ASW conditions without being supplemented with LB nutrients.

In a closed system, *S. marcescens* is capable of maintaining near initial inoculation numbers in saltwater without supplements over the course of 20 days. There was a spike in the number of CFU at Day 3, which is most likely the time where excess nutrient uptake from the initial nutrient supplemented culture broth is utilized. From that point on, there was a steady but slow decrease in the number of

viable cells that appears to happen in an exponential manner. This decrease is most likely because of cell turnover and recycling of nutrients from expired *S. marcescens*, since it has been previously reported that the life expectancy of *S. marcescens* in saltwater is roughly 15 hours (Looney et al., 2010). In terms of infection experiments, this means that the inoculation load should stay within $5x10^7$ of the initial number of CFU for the course of the experiment. Bacterial concentrations could be potentially affected by *E. pallida*, either positively or negatively. This could happen by one of two processes. Firstly, mucus could contain nutritional components that sustain the bacteria and allow them to grow to infectious concentrations (Silveira et al., 2017). Secondly, the mucus of some cnidarians contain antibiotic compounds produced by commensal bacteria (Fu et al., 2013). As long as these bacterial communities are not disturbed, pathogenic bacteria are kept in check outside the organism and should not be able to reach host tissue and therefore encounter host defenses.

When *S. marcescens* is co-cultured with symbiotic dinoflagellates, it fails to compete declining to almost negligible numbers over 20 days. In some cases, there were undetectable levels of *S. marcescens* in the culture after this time period. Since there was no noticeable zone of clearing produced by spotting *S. marcescens* plates with cultures of symbiotic dinoflagellates, this is most likely due to out-competition for resources by the dinoflagellates. Since *S. marcescens* survives alone in salt water, as cells perish the nutrients are most likely used by living cells

to maintain culture populations. When cultured with symbiotic dinoflagellates, there is competition for these nutrients. If the dinoflagellates absorb environmental nutrients more readily than *S. marcescens* in a saltwater environment, than the growth of the dinoflagellates in tandem with the decline in *S. marcescens* CFUs is expected. Given the autotrophic nature of the dinoflagellates, their added carbon production might be giving them a competitive edge even though they are slower growing organisms.

CHAPTER III
INTERACTIONS OF BACTERIA WITH APOSYMBIOTIC OR SYMBIOTIC EXAIPTASIA PALLIDA

INTRODUCTION

Increases in disease prevalence in coral reefs is frequently observed during thermal-stress events, which normally coincides with coral bleaching. Disease severely increases coral mortality and tissue loss associated with thermal-stress (Brodnicke et al., 2019). While the increase in disease following thermal-stress is clear, the underlying cause of this increase in mortality is a subject of debate because heat stress could (i) cause stress to the host (Lesser et al., 2007) and (ii) increase growth of pathogenic bacteria (Toren et al., 1998; Tout et al., 2015).

In *Acropora cervicornis*, resistance to white-band disease decreases drastically after the loss of symbiotic dinoflagellates, caused by thermal stress (Muller et al., 2018). By contrast, it has also been hypothesized that coral bleaching confers “a transient immunological advantage to the coral host” (Merselis et al., 2018), however this hypothesis has not been thoroughly investigated. Due to the multiple effects of thermal stress on the coral reef environment, it would be advantageous to perform studies in its absence.

Using *E. pallida*, I am able to take the variable of increased temperature, which leads to host stress, increased host-associated microbial growth, and long-term resource depletion out of the potential causes and observe what effect

symbiotic state alone might have on disease resistance. In this chapter, I test the hypothesis that being in a stable symbiotic or aposymbiotic state influences disease resistance when *E. pallida* is exposed to *S. marcescens*.

METHODS

EXAIPTASIA PALLIDA HUSBANDRY. *E. pallida* strain CC7 anemones were obtained from the Pringle Lab, Stanford, CA (Sunagawa et al., 2009) and were maintained in a greenhouse in aerated 38 L aquaria (Figure 3.1). Symbiotic anemones were kept in ambient lighting of approximately 1400 lux at noon in June, subject to seasonal variation (measured with a Dr. Meter LX1330B) and temperature conditions (maximum 24˚C), subject to seasonal variations. Aposymbiotic anemones were kept in light-protected foil-wrapped aquaria and were maintained symbiont-free for a period of at least one year prior to experimentation. Aposymbiotic anemones placed back into light did not regenerate symbiont populations within a 30-day period. Feeding was done twice weekly with newly hatched *Artemia* (brine shrimp). Water changes of 50% total volume were performed with artificial sea water (ASW), a solution of Instant Ocean® Sea Salt (Spectrum Brands) mixed to manufacturer specifications, the day after feedings to remove excess food from the systems.

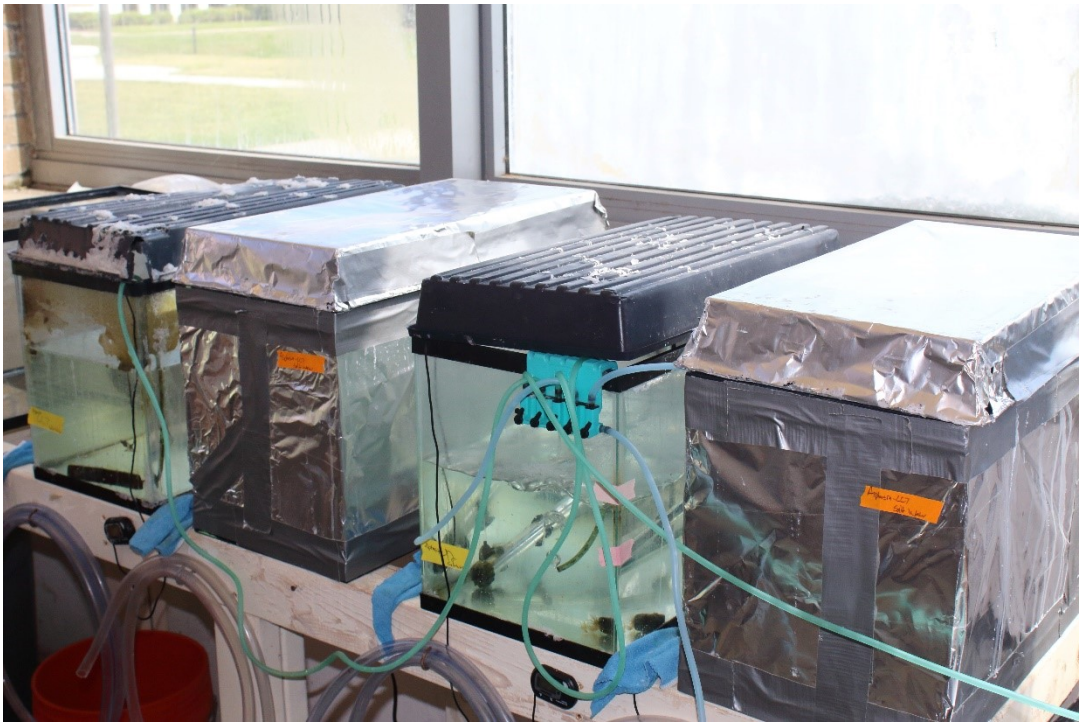

Figure 3.1. *E. pallida* husbandry. Aiptasia were maintained in a greenhouse under ambient light and temperature. Aposymbiotic anemones were maintained in foil-wrapped aquaria to eliminate light exposure.

EXAIPTASIA PALLIDA INFECTION ASSAYS. Prior to infection assays, anemones with a 2-3 mm foot were transferred to 100 ml culture jars and acclimated to test conditions in a Precision™ 818 low temperature illuminated incubator (880 Lux) over a period of a week. This size was selected because it offered the most consistent selection of similarly sized individuals. These sizes represent approximately 1-month old pedal lacerations, where older anemones vary greatly in size. Anemones acclimated to test conditions of 28˚C were transferred individually to wells of a 24-well plate in 1 ml of ASW and subjected to either no bacteria, 1 x 10^7, or 1 x 10^8 CFU/ml of *S. marcescens*. Anemones were subjected to test conditions over a period of 20 days and were observed for mortality. Mortality was determined to occur either by the dissolution of tissue or by complete loss of tissue. Evaporation was minimized by placing excess dH_2O reservoirs to block direct air flow and increase ambient humidity (Figure 3.2).

RESULTS

SERRATIA MARCESCENS EXPOSURE TO SYMBIOTIC OR APOSYMBIOTIC E. PALLIDA. To test the hypothesis that a stable symbiotic state produces symbiotic hosts that are less resistant to disease, mortality in a 24-well plate assay was determined by visual observation of *E. pallida* anemones. An anemone was determined to be deceased when it lost cohesion, which was normally preceded by a clear state of stress where the tentacles of the anemone were either partially or fully retracted (Figure 3.3).

Figure 3.2. Illuminated incubator configuration. In order to minimize evaporation and increase local humidity, water filled trays were placed on (from top to bottom) racks 2, 3, and 4. Tests were conducted on rack 5. Lights were mounted inside the door of the incubator.

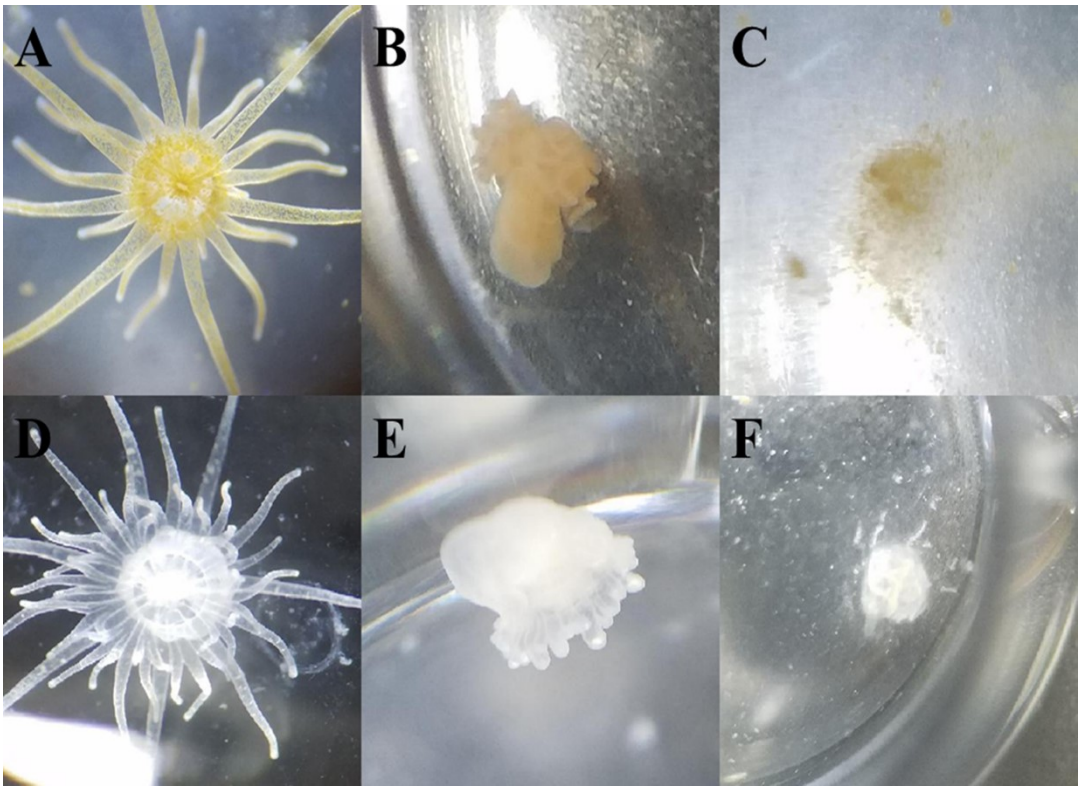

Figure 3.3. Stages of *Exaiptasia pallida* mortality. A. Healthy symbiotic anemone, B. Stressed symbiotic anemone, C. Dead symbiotic anemone, D. Healthy aposymbiotic anemone, E. Stressed aposymbiotic anemone, F. Dead aposymbiotic anemone.

No mortality was observed in the control experiments where no bacteria were added to the anemones, in culture plates containing either symbiotic or aposymbiotic *E. pallida* (Figure 3.4). Two different concentrations of *S. marcescens* were used, 10^7 and 10^8 CFU/ml, based on experiments from other groups which showed limited mortality at concentrations below 10^7 CFU/ml (Krediet et al., 2013). Initial experiments with 10^9 CFU inoculations produced extreme mortality by Day 3. Three days was not sufficient time to observe more minor changes in disease resistance in later experiments, so the inoculation load of 10^9 CFU was not pursued further (data not shown). The temperature used for all subsequent assays was 28˚C, below the limit of thermal stress, to approximate the temperature at which disease starts to appear rather than 21˚C, which is the temperature used in the existing literature. Experimentation with inoculation load was necessary to identify potential differences in host response and an appropriate standard infection load.

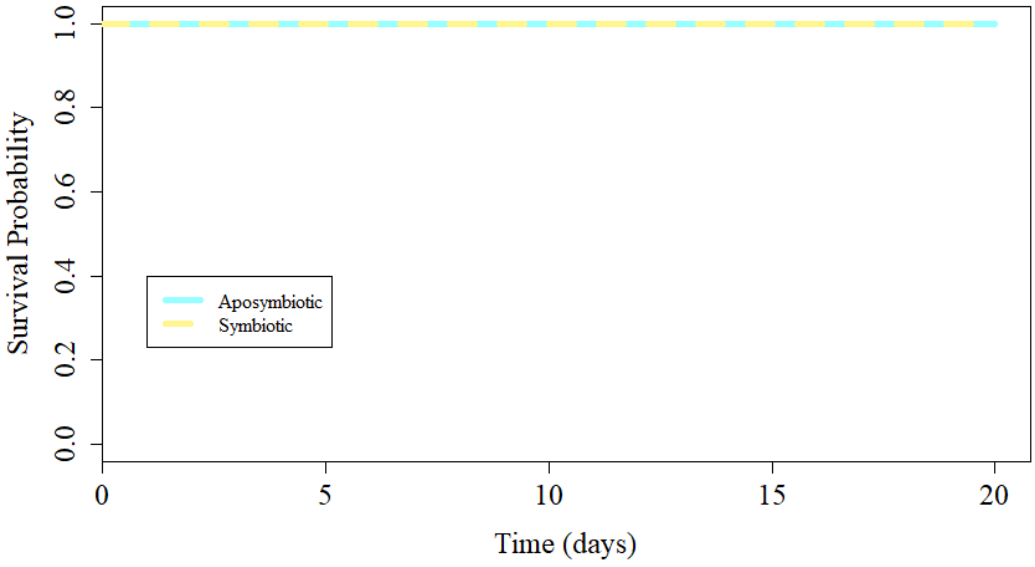

Figure 3.4. Kaplan-Meier survival plot of aposymbiotic (blue) and symbiotic (yellow) *E. pallida* in test conditions without the addition of bacteria. Anemones were acclimated to test conditions for 1-week prior to experimentation, after which they were placed in 1 ml of saltwater in individual wells of a 24 well plate. n=30 for each condition. Wells were checked daily for mortality.

When comparing chronic aposymbiotic and symbiotic *E. pallida,* there was no significant difference in mortality between the two states when infected with either 10^7 CFUs ($p = 0.32$) or 10^8 CFUs ($p = 0.93$) CFUs/ml of *S. marcescens* (Figure 3.5 and Figure 3.6). Between infection loads an order of magnitude apart, there is no obvious benefit for *E. pallida* to be in either a symbiotic or in an aposymbiotic state. When comparing between concentrations within symbiotic state, there was a significant difference between inoculation of 10^7 and 10^8 in aposymbiotic ($p < 0.0001$) and symbiotic ($p < 0.0001$) anemones (Figure 3.7 and Figure 3.8). The number of *S. marcescens* CFU used for infection had a distinct effect on the susceptibility of *E. pallida* to mortality, whereas symbiotic state did not have a distinct effect on the susceptibility of *E. pallida* to mortality. An infection load of 10^8 CFUs/ml of *S. marcescens* was chosen for symbiont exposure experiments. This load had an early onset of mortality at approximately 4 days, with ongoing die-off until Day 9. Within the 20-day time frame of the experiment, this would allow for more modulation in growth curve shape than the 10^7 CFU infection load, in which mortality was not observed until 11 days into the experiment.

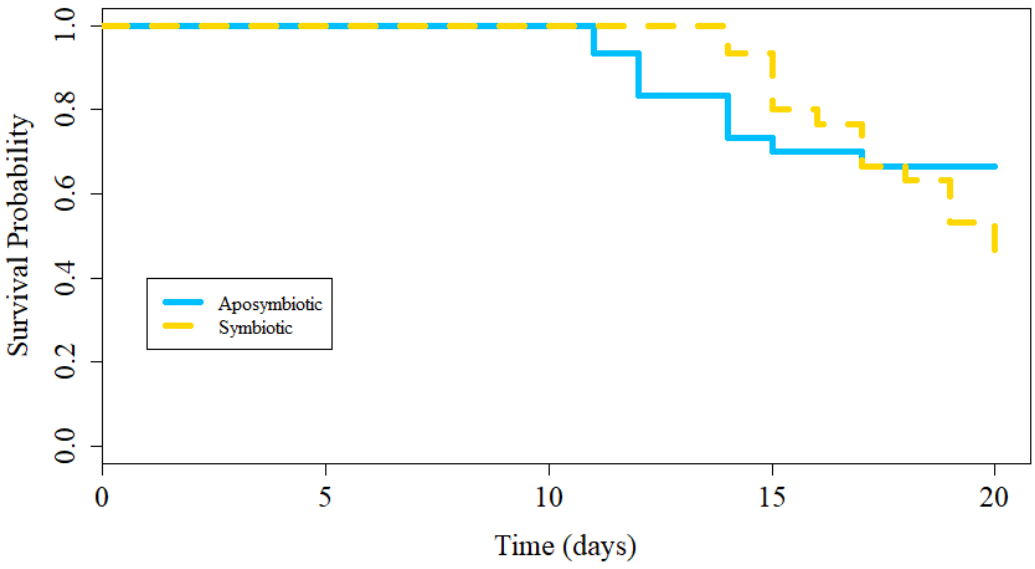

Figure 3.5. Kaplan-Meier survival plot of aposymbiotic (blue) and symbiotic (yellow) *E. pallida* exposed to 10^7 CFUs of *S. marcescens*. Anemones were acclimated to test conditions for 1 week prior to experimentation, after which they were placed in 1 ml of saltwater in individual wells of a 24 well plate. n=30 for each condition. Wells were checked daily for mortality.

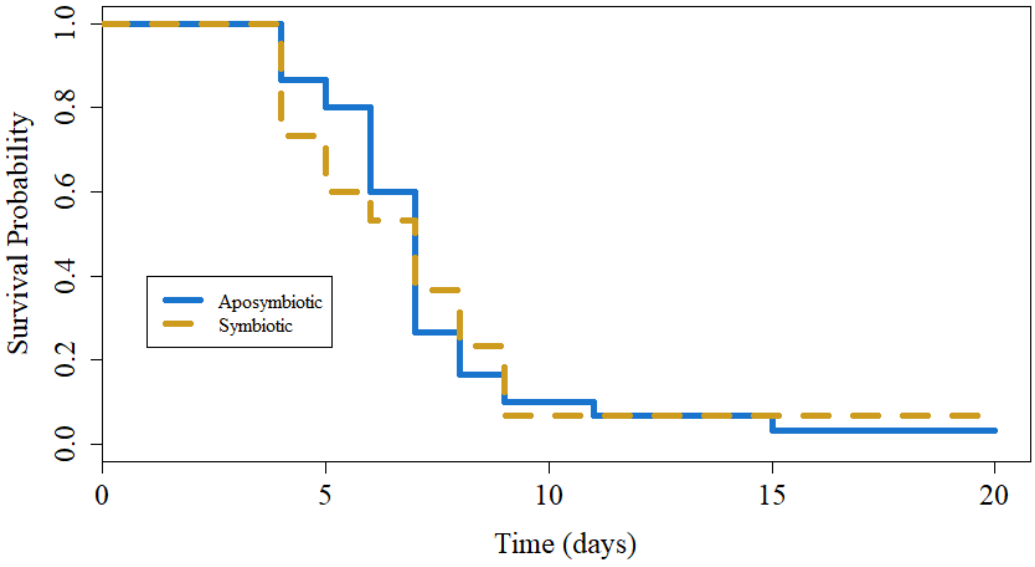

Figure 3.6. Kaplan-Meier survival plot of aposymbiotic (blue) and symbiotic (yellow) *E. pallida* exposed to 10^8 CFUs of *S. marcescens*. Anemones were acclimated to test conditions for 1 week prior to experimentation, after which they were placed in 1 ml of saltwater in individual wells of a 24 well plate. n=30 for each condition. Wells were checked daily for mortality.

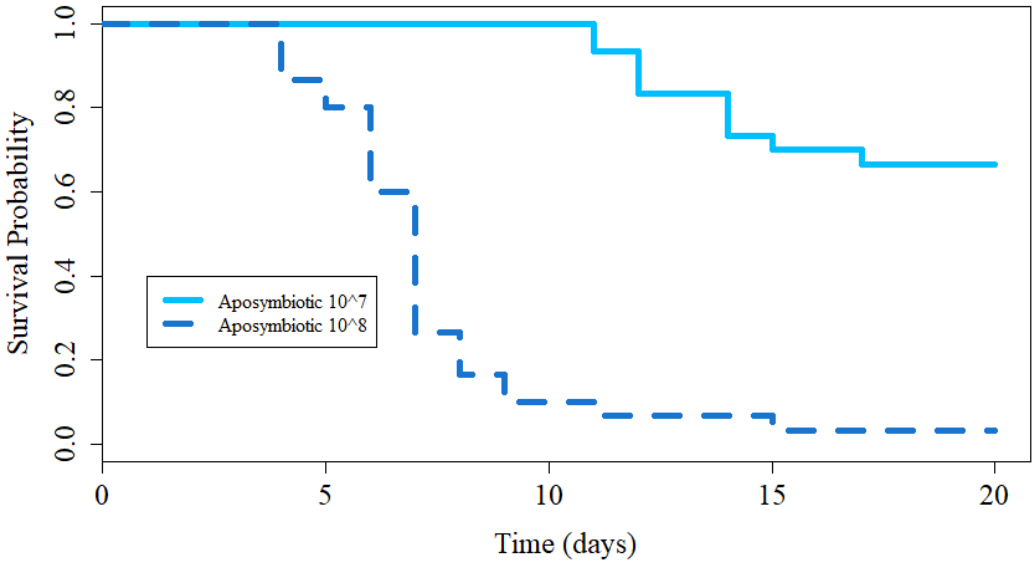

Figure 3.7. Kaplan-Meier survival plot of aposymbiotic *E. pallida* exposed to 10^7 (light blue) or 10^8 (dark blue) CFUs of *S. marcescens*. Anemones were acclimated to test conditions for 1 week prior to experimentation, after which they were placed in 1 ml of saltwater in individual wells of a 24 well plate. n=30 for each condition. Wells were checked daily for mortality.

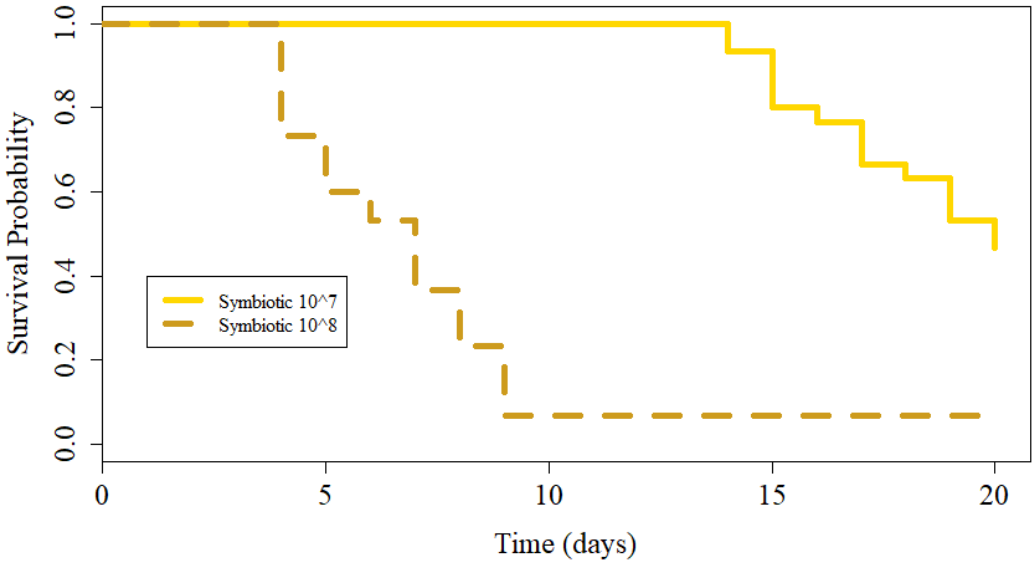

Figure 3.8. Kaplan-Meier survival plot of symbiotic *E. pallida* exposed to 10^7 (light yellow) or 10^8 (dark yellow) CFUs of *S. marcescens*. Anemones were acclimated to test conditions for 1 week prior to experimentation, after which they were placed in 1 ml of saltwater in individual wells of a 24 well plate. n=30 for each condition. Wells were checked daily for mortality.

DISCUSSION

SERRATIA MARCESCENS EXPOSURE IN SYMBIOTIC OR APOSYMBIOTIC EXAIPTASIA PALLIDA.

No mortality occurred when *E. pallida* was subjected to the conditions of the infection assay without the addition of pathogenic bacteria. Incubation in 1 ml of ASW in a 24-well plate under a 12:12 illumination cycle at 28˚C is not inherently deadly to the anemones, and starvation of individuals did not appear to be an issue over the course of experiments. One limitation that appeared and was dealt with during the course of testing was well evaporation. As the assay was being developed there was significant evaporation, which required wells in the plates to be topped off daily with dH_2O. This led to constantly fluctuating conditions where salts, waste, and pathogen were quickly and constantly changing. A spare well with dH_2O was used to measure evaporation and inform the volume to top off anemone containing wells, which reached as high as 8% of the well volume per day. In order to reduce this water loss due to evaporation, water filled trays were used to buffer humidity and block direct air flow in the testing chamber. The total water loss after addition of the trays, over the 20-day experimental period, was less than 10% of the initial volume.

There was a significant difference in disease mortality between an infection of 10^7 and 10^8 CFUs of *S. marcescens* in both states. This difference is not surprising, as an increase in pathogen should cause less time to be present between

initial infection and presentation of disease. These initial values were chosen because previous literature used inoculations of approximately this scale (Krediet et al., 2013; Poole et al., 2016). In those studies, a significantly lower survival rate was observed compared with the values I obtained, but they were achieved at 21˚C rather than at 28˚C, and the sample size was 6 instead of 30. These animals are poikilothermic and body temperature is reliant on their environment. Since they are tropical animals, either that low temperatures can potentially be outright stressful to *E. pallida* or that low temperature could make *E. pallida* physiology sluggish, rendering them susceptible to infection. On the other hand, *S. marcescens* grows faster at higher temperatures to which it is more suitable. In salt water, the survival time of *S. marcescens* is almost tripled when the temperature is increased from 30˚C to 35 ˚C (Looney et al., 2010). I decided to use 10^8 CFUs for further study because I assumed that, given the shape of the curves for both aposymbiotic and symbiotic individuals, slight differences would be more pronounced in the shape and overall survival outcome for *E. pallida* under different conditions.

Aposymbiotic *E. pallida* exhibited similar survival trends when compared with symbiotic *E. pallida* across both low and high CFU *S. marcescens* inoculation loads. This means that the observed increase in disease occurrence after bleaching is not directly related to the symbiotic state of the animal, but rather to either changing environmental conditions or changes in symbiotic state. While transition states occur at both the onset and recovery from bleaching, the onset of bleaching is

acute and short lived. Merselis et al. (2018) present a hypothesis where transient bleaching confers an immunological edge to the host organism. This hypothesis does not appear to be supported by the current results, as being in a bleached state offers no difference in disease susceptibility than being in a symbiotic state. Perhaps there is a different explanation for increases in prevalence of disease state following bleaching in the wild. The process of recovery is more prolonged, sometimes lasting months (Cunning et al., 2016). During this time the host deals with shuffling of different symbiont populations before a stable holobiont is reformed. If the transition from an aposymbiotic state to a stable symbiotic one makes the host less disease resistant than when it is in a consistent stable state, then this time period to reach a stable system may be critical.

CHAPTER IV
DISEASE RESISTANCE OF EXAIPTASIA PALLIDA DURING BLEACHING RECOVERY AND MODULATED BY SPECIES SPECIFIC SYMBIODINIACEAE EXPOSURE

INTRODUCTION

Coral hosts associate with different species of symbiotic dinoflagellates, with many of these associations being host specific. Hosts that are capable of association with more than one species of symbiont over the course of their life show differences in how fast they grow (Little et al., 2004) and their ability to tolerate thermal-stress events (Berkelmans and van Oppen, 2006). While there is some experimental evidence showing that even though bleaching exacerbates disease, different strains of the same species of symbiotic dinoflagellate do not have an effect on disease resistance (Muller et al., 2018). There is, however, evidence that different species of symbionts can pre-dispose the host to a disease state (Rouzé et al., 2016).

In this study, the modulation of disease resistance was investigating by (i) changing the amount of time a host was exposed to symbiotic dinoflagellates, along with the effect of withholding food before exposure, and (ii) changing the species of symbiotic of symbiotic dinoflagellate the host was exposed to before being challenged with *S. marcescens*.

METHODS

BLEACHING RECOVERY. The Symbiodiniaceae strain used to study how incubation time with symbiotic dinoflagellates affects disease resistance in *E. pallida* was *Symbiodinium minimum* (now *Breviolum minutum*), CCMP 830 from the Bigelow National Center for Marine Algae and Microbiota (East Boothbay, Maine). Symbiont culture density was determined with a hemocytometer and the desired cell count was harvested, pelleted, and resuspended in fresh ASW. For each trial, aposymbiotic anemones were incubated with a total of 1×10^5 cells/ml for either 0.25, 0.5, 1, 3, 7, or 14 days. Anemones were fed prior to incubation in order to prevent mortality at extended exposure times. After the incubation period recovering anemones had their media replaced with sterile ASW and were used for infection assays with 10^8 CFU/ml *S. marcescens*, as described in the methods section of Chapter 2.

STARVATION BEFORE RECOVERY. To determine the effects of withholding food from *E. pallida* before symbiotic dinoflagellate exposure, aposymbiotic anemones were held without food for 7 days. After this period, they were exposed to *B. minimum* as described above for 7 days, washed with fresh ASW, and then exposed to 10^8 CFU/ml *S. marcescens*. No additional feeding occurred during exposure to *B. minimum*.

SPECIES SPECIFIC SYMBIODINIACEAE EXPOSURE. The Symbiodiniaceae strains used to study the effects on disease resistance of specific

strains of symbiotic dinoflagellates exposed to *E. pallida* were *S. minimum* (now *Breviolum minutum*), *S. goreaui* (now *Cladocopium goreaui*), *S. trenchii* (now *Durusdinium trenchii*), and *S. microadriaticum* (CCMP 830, CCMP 2466, CCMP 3428, and CCMP 2464 respectively). All strains were acquired from the Bigelow National Center for Marine Algae and Microbiota. Symbiont culture density was determined with a hemocytometer and the desired cell count was harvested, pelleted, and resuspended in fresh ASW. For each trial, aposymbiotic anemones were incubated with a total of 1 x 10^5 cells/ml for either 7 or 14 days. After the incubation period recovering anemones had their media replaced with sterile ASW and were used for further assays.

RESULTS

BLEACHING RECOVERY. The first experiment in this chapter was a time course study with *B. minutum* incubation with *E. pallida* to identify when and if a shift in disease resistance happens when the host is exposed to symbionts and then infected with 10^8 CFUs of *S. marcescens*. *B. minutum* was used because it is a symbiont originally extracted from *E. pallida*. *E. pallida* was exposed to *B. minutum* for either 1, 3, 7, or 14 days (Figure 3.1). An exposure time of 7 days did not show any significant difference from *E. pallida* that was either symbiotic or aposymbiotic (Table 3.1). Exposure times of 1 day, 3 days, and 14 days were not

significantly different from one another, but expressed higher resistance to disease than 7-day exposure and symbiotic/aposymbiotic *E. pallida*.

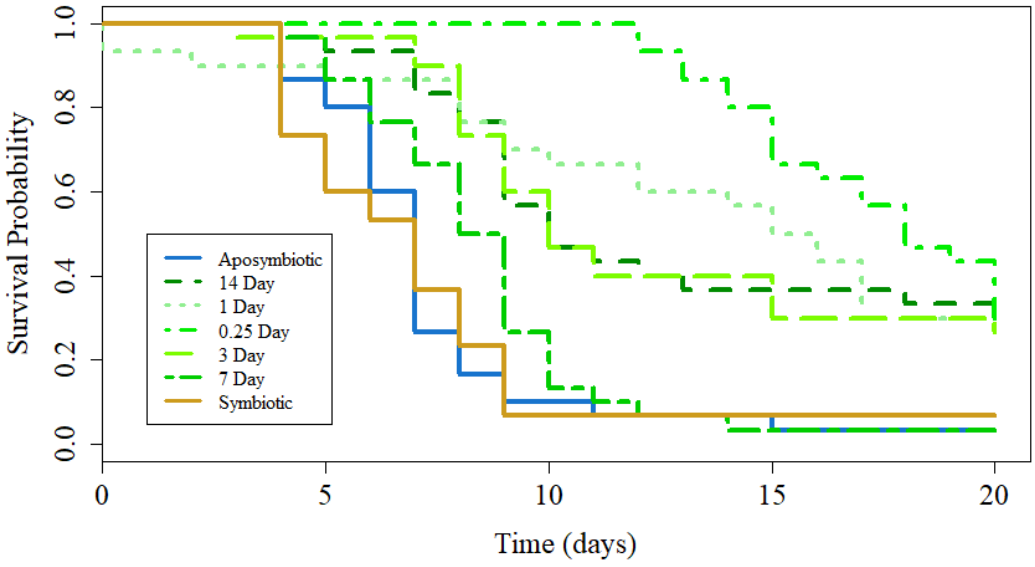

Figure 4.1. Infection time course of *E. pallida* exposed to *B. minutum*. Anemones were incubated with *B. minutum* for the indicated time period, after which they were placed in 1 ml of ASW in individual wells of a 24 well plate with 10^8 CFUs of *S. marcescens*. Aposymbiotic anemones are depicted in blue, symbiotic anemones in brown, and 1-14 day *B. minutum* exposures in increasingly dark shades of green. Wells were checked daily for mortality. For each condition n=30. Conditions split into two groupings of significance based on pairwise χ^2 comparison: either aposymbiotic, symbiotic, and 7 days of exposure or 1 day, 3 day, and 14 days of exposure.

Table 4.1. Significance in survival after *B. minutum* Exposure. Pair-wise significance comparisons between p-values from Kaplan-Meier curves of time exposure trials with 10^8 CFU inoculation, also compared with stable symbiotic and aposymbiotic *E. pallida*. For each trial n=30.

	Symbiotic	**Aposym-biotic**	***B. minutum* 1 days**	***B. minutum* 3 days**	***B. minutum* 7 days**
***B. minutum* 14 days**	$p < 0.0001$	$p < 0.0001$	$p = 0.81$	$p = 0.77$	$p = 0.00079$
***B. minutum* 7 days**	$p = 0.054$	$p = 0.059$	$p < 0.0001$	$p = 0.00065$	
***B. minutum* 3 days**	$p < 0.0001$	$p < 0.0001$	$p = 0.49$		
***B. minutum* 1 day**	$p < 0.0001$	$p < 0.0001$			
Aposymbiotic	$p = 0.93$				

STARVATION BEFORE RECOVERY. There was a significant difference when *E. pallida* was starved for 7 days then exposed to *B. minimum* when compared to 7-day exposure with a full gut ($p < 0.0001$) and 14 day exposure with a full gut ($p = 0.0024$) (Figure 4.2). This indicates that gut content plays a role in how *E. pallida* responds to ingested *B. minimum*. Starvation for 7 days with 7 days of *B. minimum* exposure has the same time from last feeding to *S. marcescens* exposure as 14 days of *B. minimum* exposure.

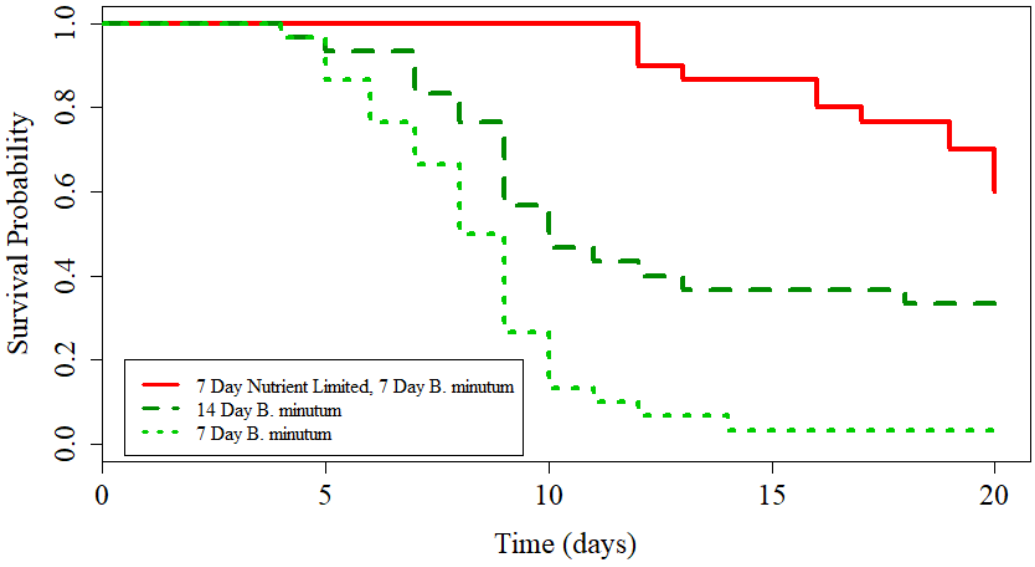

Figure 4.2. Effect of nutrient limiting on disease resistance before symbiotic dinoflagellate exposure. Being held in sterile seawater for 7 days after feeding, then exposed to *B. minutum* for 7 days before S. marcescens infection increased disease resistance both for 7-day exposure and 14-day treatments when *E. pallida* was exposed to *B. minutum* on a full gut. 14-day exposure has the same timeframe from last feeding until *S. marcescens* exposure as the starvation treatment.

SPECIES SPECIFIC SYMBIODINIACEAE EXPOSURE. At 7 days, there is no statistical difference between disease resistance and *S. marcescens* exposure to different 3 different species of symbiont (Figure 4.3). However, *E. pallida* exposed to *D. trenchii* were the only treatment that showed significant differences with *S. microadriaticum* ($p < 0.0001$), *B. minutum* ($p < 0.0001$), and *C. goreaui* ($p < 0.0001$). Survival was significantly greater for *E. pallida* exposed to *D. trenchii* than *E. pallida* exposed to any other symbionts. None of the conditions, with the exception of *D. trenchii*, were significantly different from the previous stable symbiotic or aposymbiotic *E. pallida* inoculated with 10^8 CFUs of *S. marcescens* (Table 4.2).

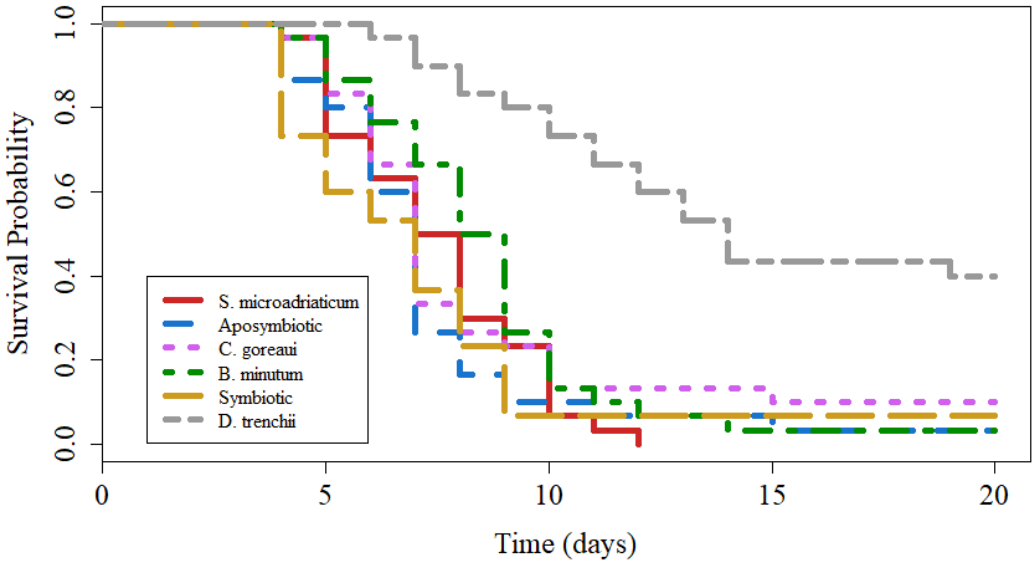

Figure 4.3. Kaplan-Meier plot of disease resistance after seven days of symbiont exposure. The only difference in host resistance appears after exposure to *D. trenchii*. Except for anemones maintained chronically symbiotic or aposymbiotic, anemones were incubated with the indicated species of symbiont for 1 week prior to experimentation, after which they were placed in 1 ml of saltwater in individual wells of a 24 well plate with 10^8 CFUs of *S. marcescens*. Wells were checked daily for mortality. For each condition, n=30.

Table 4.2. Significance in survival after 7 days of symbiont exposure. Pair-wise comparisons between p-values from Kaplan-Meier of 7-day symbiont exposure trials and 10^8 CFU inoculation of stable symbiotic and aposymbiotic *E. pallida*. For each trial, n=30.

	Symbiotic *E. pallida*	**Aposymbiotic *E. pallida***	***D. trenchii***	***C. goreaui***	***B. minutum***
S. microadriaticum	p = 0.38	p = 0.57	p < 0.0001	p = 0.53	p = 0.15
B. minutum	p = 0.054	p = 0.059	p < 0.0001	p = 0.62	
C. goreaui	p = 0.27	p = 0.27	p < 0.0001		
D. trenchii	p < 0.0001	p < 0.0001			
Aposymbiotic *E. pallida*	p = 0.93				

When *E. pallida* was exposed to symbionts for 14 days instead of 7 days, disease resistance increased across all conditions. Within symbiont species, this increase was significant in *S. microadriaticum* ($p < 0.0083$), *B. minutum* ($p = 0.00079$), *C. goreaui* ($p < 0.0001$), and *D. trenchii* ($p = 0.0033$). When comparing 14-day trials to one another across species, the results were split into three groups of responses. The two species to exhibit the highest level of disease resistance were *D. trenchii* and *C. goreaui*. The next group of resistance, at a slightly lower level were *S. microadriaticum* and *B. minutum*. Finally, the least disease resistant in comparison with all other symbiont incubations were the stable aposymbiotic and symbiotic anemones, with the lowest level of disease resistance (Figure 4.4). All 14-day symbiont exposure trials were significantly more resistant to disease than either symbiotic or aposymbiotic *E. pallida* inoculated with 10^8 CFUs of *S. marcescens* (Table 4.3). *E. pallida* had a difficult time surviving in culture with *S. microadriaticum*, and out of 50 attempts to expose *E. pallida* to this symbiont for 14 days only 19 individuals survived to *S. marcescens* infection.

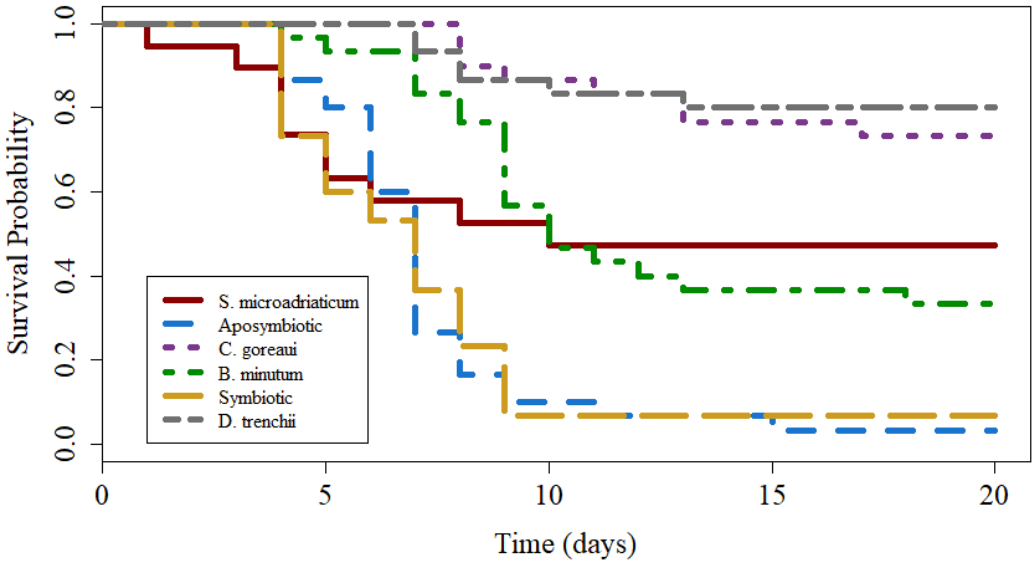

Figure 4.4. Kaplan-Meier plot of disease resistance after 14 days of symbiont exposure. Exposure conditions split into three groups: *D. trenchii* and *C. goreaui*, *S. microadriaticum* and *B. minutum*, or aposymbiotic and symbiotic. Except for anemones maintained chronically symbiotic or aposymbiotic, anemones were incubated with the indicated species of symbiont for 2 weeks prior to experimentation, after which they were placed in 1 ml of saltwater in individual wells of a 24 well plate with 10^8 CFUs of *S. marcescens*. Wells were checked daily for mortality. For each condition, n=30 except *S. microadriaticum*, where n=19.

Table 4.3. Significance in survival after 14 days of symbiont exposure. Pair-wise comparisons between p-values from Kaplan-Meier of all 14-day symbiont exposure trials and 10^8 CFU inoculation of stable symbiotic and aposymbiotic *E. pallida*. For each trial, n=30 except *S. microadriaticum* where n=19.

	Symbiotic	**Aposym-biotic**	***D. trenchii* 14 day**	***C. goreaui* 14 day**	***B. minutum* 14 day**
***S. microadriaticum* 14 day**	p = 0.016	p = 0.012	p = 0.0057	p = 0.031	p = 0.93
***B. minutum* 14 day**	p < 0.0001	p < 0.0001	p = 0.00038	p = 0.002	
***C. goreaui* 14 day**	p < 0.0001	p < 0.0001	p = 0.45		
***D. trenchii* 14 day**	p < 0.0001	p < 0.0001			
Aposymbiotic	p = 0.93				

DISCUSSION

BLEACHING RECOVERY. The amount of time that a host is exposed to a symbiont may present different levels of disease resistance in terms of identifying and progressing through bleaching recovery. To attempt to find a time period for which disease resistance in *E. pallida* changes in relation to exposure time to symbiotic dinoflagellates, a range of time points were used, including 1, 3, 7, and 14 days. It has been reported that maximum environmental uptake occurs under experimental condition within the first 18 hours of symbiont exposure, and recolonization reaches the end of exponential growth between 7 and 15 days (Berner et al., 1993). All time points tested, other than 7 days, increased disease resistance. The 7-day time point was not significantly different from either stable symbiotic or stable aposymbiotic *E. pallida*. These data are particularly interesting because they do not indicate that the immune response of *E. pallida* is being suppressed in any way, but in fact is being increased. When exposed to symbiotic dinoflagellates, the organism could ramp up an immune response to reject the symbionts, with a lull at Day 7. This Day 7 point could potentially be where the host decides whether it is going to ultimately keep or reject the symbiont.

It should be noted that within 14 days pigment recovery in the host is limited. Some individuals appear lightly pigmented and some still appear bleached to the naked eye. However, interactions between an aposymbiotic host and the symbiont does occur, and it is those interactions which are of primary concern.

STARVATION BEFORE RECOVERY. While gut content plays a role in *E. pallida*'s response to *B. minimum*, what is surprising about this experiment is that there is a difference between 7 days of starvation followed by *B. minimum* exposure and 14 days of *B. minimum* exposure with a full gut. The amount of time *E. pallida* takes before *Artemia* DNA is no longer detectable is approximately 6 days, though there is a sharp drop in DNA content in the first 3 days (Leal et al., 2014), which should make the starvation and 14-day exposures equivalent. A possible explanation for this phenomenon is that during the 14-day exposure to *B. minimum*, *E. pallida* may be consuming the dinoflagellates along with the full gut of *Artemia* rather than attempting uptake, reducing the number of viable cells left in culture once digestion is complete. Waiting until prey is digested before exposure to *B. minimum* presents *E. pallida* with an appropriate symbiont without the complication of existing gut contents, and therefore interaction between symbiont and host is maximized.

An alternative to this has to do with the effect of nutrient limiting on the relationship between the host and symbiotic organisms. In the model organism *Hydra viridissima*, algal cell division is regulated by the nutritional state of the host. When the host is starved, cell division of the symbiotic algae *Chlorella* is halted (McAuley, 1985). In *Aiptasia pulchella*, host starvation is associated with decreased growth and chlorophyll content (Smith and Muscatine, 1999). In both the *E. pallida* and *H. viridissima* systems, endosymbionts are reliant on the host to

supplement their nutrition. If the host is hosting a symbiont that is not efficiently providing carbon because the host isn't reciprocating with their metabolic byproducts, the host may potentially be detecting the relationship as parasitic which could be stimulating an immune response.

SPECIES SPECIFIC SYMBIODINIACEAE EXPOSURE. After exposure to one of four strains of symbiotic dinoflagellates, the only strain to show a difference in survival was *D. trenchii*. *D. trenchii* is considered an opportunistic symbiont, which is able to colonize hosts during warming events, after which it is again displaced by the host (LaJeunesse et al., 2009). While this may be advantageous to short-term survival, it is accompanied by reduced levels of growth and fecundity (Cunning et al., 2015; Jones and Berkelmans, 2011). Surprisingly, no significance difference in survival from stable aposymbiotic or symbiotic state was observed for any *E. pallida* exposed to symbionts. This is perhaps because of the specific timeframe, so both longer and shorter exposure periods were tested.

After 14 days of exposure to symbionts, disease resistance was increased in all treatments, with *D. trenchii* and *C. goreaui* having significantly higher resistance than other treatments. There was also a second grouping of symbionts, *S. microadriaticum* and *B. minutum*, which had disease resistance that was less pronounced than *D. trenchii* and *C. goreaui*. These results reinforce the trend that there is a distinct difference in host response between 7 days and 14 days of symbiont exposure in all treatments, and that the Day 7 timepoint is one of

particular interest. Paradoxically, these results of disease resistance inversely correspond with symbiont compatibility. Traditionally, Clade C (*C. goreaui*) and D (*D. trenchii*) symbionts are not associated with *E. pallida*, whereas clades A (*S. microadriaticum*) and B (*B. minutum*) are normally found in Caribbean *E. pallida*. In fact, while *B. minutum* was isolated from *E. pallida*, the strain used in this experiment traditionally harbors an unnamed clade A strain (Sunagawa et al., 2009). We hypothesize that symbiotic compatibility confers no disease resistance to the host, whereas symbiotic incompatibility elicits an immune response by the host that confers temporary disease resistance. Perhaps the opportunist nature of the symbiont affects the host response to disease, potentially by the extent of photosynthate contribution. Notably, it is remarkably more difficult to grow both *C. goreaui* and *D. trenchii* in culture than *S. microadriaticum* or *B. minutum*. In fact, it was difficult to expose *E. pallida* to *S. microadriaticum* for the two-week incubation period. Of a total of 50 anemones, only 19 survived to the point where infection with *S. marcescens* was initiated. In every other incubation treatment there were no difficulties with the host anemone surviving until *S. marcescens* exposure, so the symbionts may produce a compound toxic to the anemones when they are in high culture concentration.

CHAPTER V
COMPLICATIONS OF DISEASE STATE CAUSED BY NATIVE MICROBIOTA

INTRODUCTION

The stability of the holobiont to coral colonies is important in maintaining a healthy organism, and associated microbiomes are implicated in disease resistance through their interaction with potentially pathogenic organisms (Krediet et al., 2013). The composition of the microbiome shifts in response to elevated temperature both before and past the threshold of a thermal-stress event (Mao-Jones et al., 2010). The microbiome of coral colonies during thermal-stress events undergo drastic changes in the composition of bacteria and the species of symbiotic dinoflagellates (Gardner et al., 2019). These shifts also include an increase in the abundance of known pathogenic bacteria such as *Vibrio sp.* (Tout et al., 2015).

One of the conundrums with coral diseases is the lack of a reliably identifiable pathogen (Bourne et al., 2009). Perhaps shifts in the population dynamics of the native microbiota, rather than the introduction of a specific disease organism, could potentially be the cause of some diseases. Running control experiments during this study revealed dynamic shifts in the population numbers of at least one native microbe, and while outside the main scope of this dissertation some preliminary work was done to both classify and determine if elevated

population numbers induced a disease state in the absence of the opportunistic pathogen *Serratia marcescens*.

METHODS

SERRATIA MARCESCENS SURVIVAL WITH EXAIPTASIA PALLIDA. *E. pallida* anemones were exposed to *S. marcescens* as described for aposymbiotic anemones exposed to 10^8 CFU/ml of *S. marcescens*. Wells were sampled at 0, 3, 5, 10, 15, and 20 days to identify viable CFU of *S. marcescens* in the water column. Plates containing *E. pallida* were shaken at 100 RPM on an orbital shaker before water samples were taken. Water samples were plated on SWLB media supplemented with tetracycline in order to attempt to select for *S. marcescens*.

MICROBIAL CHARACTERIZATION OF AN UNKNOWN BACTERIUM. An unknown bacterium was identified during the *S. marcescens* survival with *E. pallida* assay. It grew on tetracycline supplemented plates meant to isolate *S. marcescens,* and was culturable from both tank water and from homogenized samples of *E. pallida*. It was subjected to the same characterization tests as *S. marcescens*, outlined in Chapter 2, which include TSI, MSA, DNase, Gelatinase, and EMB assays. In addition, VP-MR, XLD, Citrate, and SIM media were used to investigate additional properties to attempt identification.

MORTALITY OF EXAIPTASIA PALLIDA WHEN EXPOSED TO HIGH CONCENTRATION OF THE UNKNOWN BACTERIUM. In order to see if the

unknown bacterium affected the mortality of *E. pallida*, this bacterium was exposed to aposymbiotic *E. pallida* at 10^8 CFU/ml. Exposure conditions are identical to the aposymbiotic *E. pallida* exposure conditions with *S. marcescens* at 10^8 CFU/ml as described in Chapter 3.

RESULTS

SERRATIA MARCESCENS SURVIVAL WITH EXAIPTASIA PALLIDA

The number of CFU/ml of *S. marcescens* decreased over time when incubated with *E. pallida* (Table 5.1). At 10 days, an unknown bacterium grew to the point where it obscured signs of *S. marcescens* growth, though the extent of growth was unpredictable. This lead to dilutions of the culture being difficult to plan out properly, and very few accurate growth plates were generated in the time available for experimentation. Because of this data generated from day 10 on is not presented. If the anemone died during the experiment the number of CFU's of the unknown grew denser than those in wells where the anemone was still alive, and in most cases grew a bacterial lawn on the media plates. The unpredictable nature of the growth of the unknown made accurate plating and number counts after Day 5 impractical to count accurately within the time constraints of the project.

Table 5.1. *Serratia marcescens* survival with *Exaiptasia pallida*. As time increases the number of CFU's of *S. marcescens* declines while an unknown bacterium spikes in number before declining. S. marcescens growth is completely obscured after 5 days and the unknown reacts unpredictably, either declining or increasing in the number of CFU's.

	Days		
	0	3	5
Bacterium	Colony Forming Units		
S. marcescens	$1.22 \times 10^8 \pm 2.80 \times 10^6$	$1.63 \times 10^7 \pm 5.09 \times 10^6$	$4.34 \times 10^6 \pm 2.01 \times 10^6$
Unknown	47.8 ± 9.88	$2.87 \times 10^6 \pm 2.55 \times 10^6$	$1.38 \times 10^6 \pm 1.28 \times 10^6$

MICROBIAL CHARACTERIZATION OF AN UNKNOWN BACTERIUM. The unknown bacterium is a gram-negative rod which produces orange, button-like colonies. In the TSI assay, it produces an alkaline slant with no anaerobic growth. It grows on MSA agar without mannitol fermentation. There is no growth for DNase or EMB assays, and it produces no gelatinase (Figure 5.1). There were also additional assays used as the previous tests did not provide enough information to make an educated guess on the bacterial species. These included MR-VP, Citrate, XLD, and SIM media. Unfortunately, the unknown bacterium did not grow on any of these differential media and did not provide additional information needed to narrow down potential bacterial species.

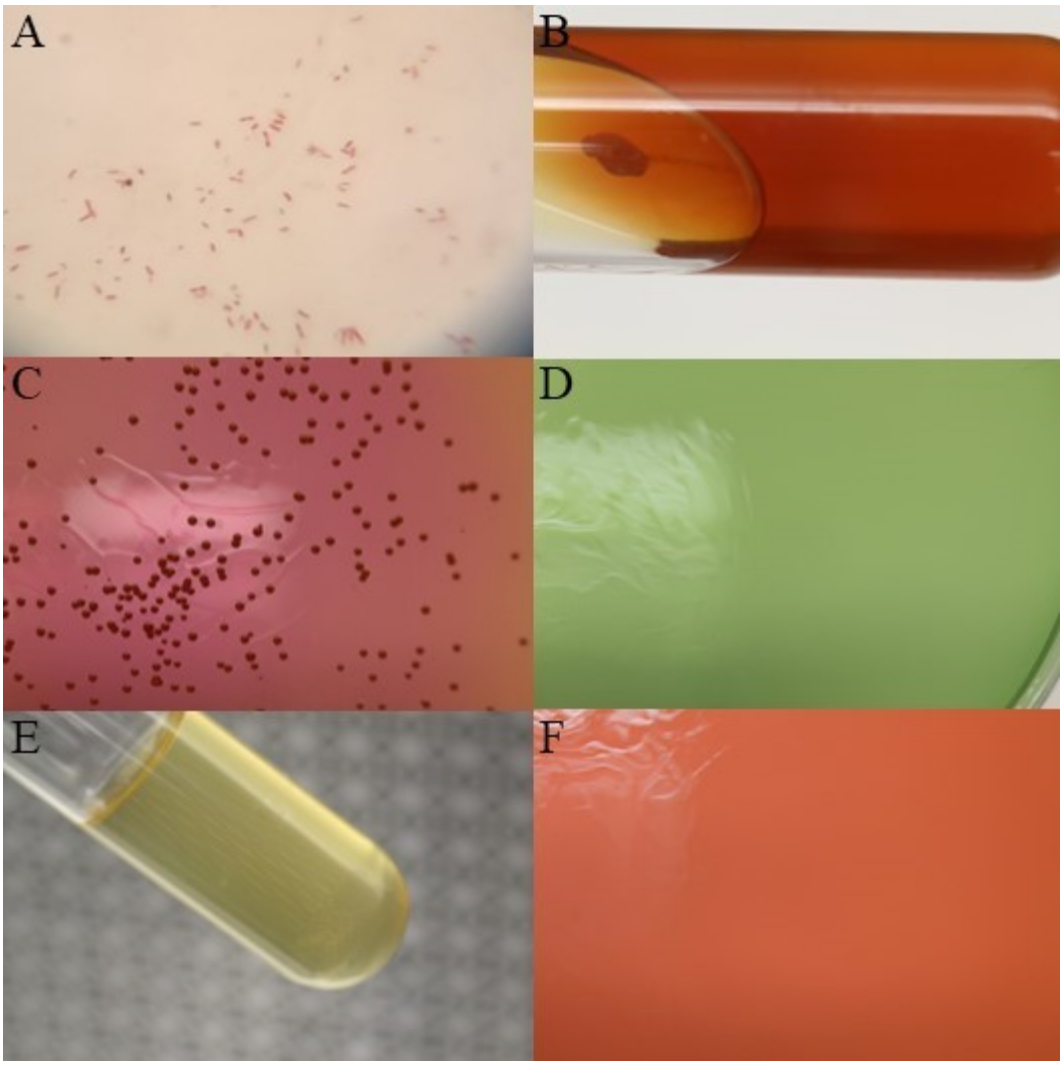

Figure 5.1. Microbial assays for an unknown bacterium. A. Negative gram stain, B. TSI stab with red alkaline slant, unchanged butt, and no gas or hydrogen sulfide production, C. Mannitol salt agar with growth present and no yellow clearing, D. DNase agar with no growth, E. Gelatinase stab with no growth, F. EMB agar with no growth.

MORTALITY OF EXAIPTASIA PALLIDA WHEN EXPOSED TO HIGH CONCENTRATION OF THE UNKNOWN BACTERIUM. When *E. pallida* is exposed to the unknown bacterium at CFU concentration identical to *S. marcescens*, it produces a significant lower probability of survival ($p < 0.0001$). Mortality begins at Day 2 and is complete by Day 4 across 30 replicates (Figure 5.2).

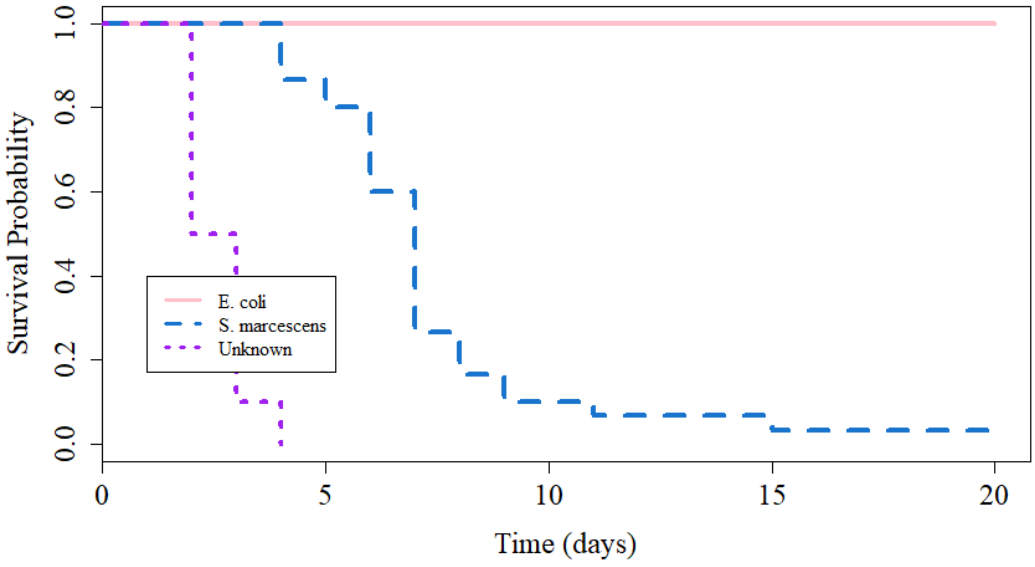

Figure 5.2. Kaplan-Meier survival plot of *E. pallida* exposed to 10^8 CFU of three different bacterial species. Aposymbiotic anemones were acclimated to test conditions for 1 week prior to experimentation, after which they were placed in 1 ml of saltwater in individual wells of a 24 well plate. n=30 for each condition. Wells were checked daily for mortality.

DISCUSSION

When *E. pallida* is exposed to elevated levels of *S. marcescens* it destabilized the existing holobiont. This is evident in the chaotic growth behavior of the tetracycline resistant unknown bacterium that outgrew *S. marcescens* when it was incubated with aposymbiotic *E. pallida* anemones. These numbers further increased upon anemone death. Background CFU's of this bacteria increase slightly from single digits their rearing aquaria, where the ratio of water to anemone is very high, to about 50 CFU/ml when *E. pallida* is maintained in a small volume of water, supporting this bacterium as being closely associated with the animal itself. Clearly, it does not normally grow to significant concentrations in the absence of an external stimulus.

While the unknown failed to grow sufficiently on selective media plates to confirm its identity, there is enough difference in activity to say that it is not an errant strain of *S. marcescens* that contaminated the culture. At present, my assignment is limited to a Gram-negative rod. To make sure that the media itself was viable, *S. marcescens* was subjected to the same tests. *S. marcescens* grew and provided appropriate results, so some aspect of the media itself was not conducive to survival of the unknown. Given the selective media studies were incapable of better characterizing this unknown, 16S RNA sequencing is likely the best way to identify this microbe.

In *E. pallida* exposure assays conducted with the unknown bacterium, it induced severe mortality at 108 CFU/ml at a level similar to *S. marcescens* at 10^9 CFU/ml. When *E. pallida* is exposed to *E. coli* at 10^8 CFU/ml, there is no mortality at all but it remains to be seen if this exposure increases the growth of the unknown bacterium. It is possible that *S. marcescens* increases the growth of this unknown by (i) the release of nutrients as *S. marcescens* dies, or (ii) by releasing molecular signals which promote increased virulence in the unknown so it acquires nutrients from the host anemone. Since the addition of *E. coli* does not induce mortality in *E. pallida*, and the addition of the unknown bacterium alone severely increases mortality, it would be more likely that signaling rather than direct nutrient input to the system is involved.

These findings support a model in which the disruption of the innate microbiome can induce disease. The microbial composition in some disease states is fairly conserved and different than that of healthy individuals (Kellogg et al., 2013). The entire holobiont serves as a complete organism, with a functional microbial community being necessary for the health of the coral host (Gates and Ainsworth, 2011). Indeed, coral hosts which have their mucous antibiotically cleared of their microbiome show increased levels of bleaching and necrosis, and shedding of the mucosal layer can aid in clearing pathogens (Glasl et al., 2016).

It could be that some of the differential effects on survival for exposure to symbiotic dinoflagellates does not completely fall on the modulation of immune

function in the host. Potentially, the host may try to optimize its microbial community based on nutrient cycling, of which the specific wastes released change depending on species of dinoflagellate the host is exposed to and how likely it is to "feed" them. It has been observed that community composition of the microbiome is largely based on the specific carbon sources available (Kelly et al., 2014), so at the times when the microbiome is changing the host could be open to a potential disease state. There is also evidence that specific carbon sources can lead directly to pathogenic activity in planktonic marine bacteria (Cárdenas et al., 2018). The complexity of the entire holobiont has many factors that can cause drastic changes, and the importance of whole holobiont health is critical to the future of the health of the coral reef ecosystem.

The interactions between host and symbiont are complex, but fail to explain the decrease in disease resistance observed after bleaching events (Figure 5.3). Being in a stable symbiotic or aposymbiotic state has no differential effect on disease resistance, but pathogen density modulates onset and severity of disease. For up to 14 days of symbiont exposure starting from a bleached state, *E. pallida* does not have any significant decrease in disease resistance. In fact, for all conditions tested disease resistance either was no different than a stable symbiotic or aposymbiotic state or was increased. While it may still be possible for long-term association with an inappropriate symbiont to cause a decrease in disease resistance, short-term exposure increased disease resistance over appropriate

symbiont species. This means there is another explanation for increases in disease prevalence following thermal-stress events. Alterations to the microbiome, as mentioned above, are a likely candidate. Infecting *E. pallida* with a host-associated bacteria at the same density as *S. marcescens* produced significantly higher probability of mortality than *S. marcescens.* This bacteria grows in population after the addition of *S. marcescens* to *E. pallida*, potentially to the point where it becomes pathogenic. It is not unreasonable to assume that other factors could affect competition and population dynamics with the host-associated microbiome as symbiotic dinoflagellates do interact with *S. marcescens*, and likely other microorganisms. Research aimed at changes in microbiome composition and how stress to the host affects disease resistance are the appropriate next step in following up on the research presented in this dissertation.

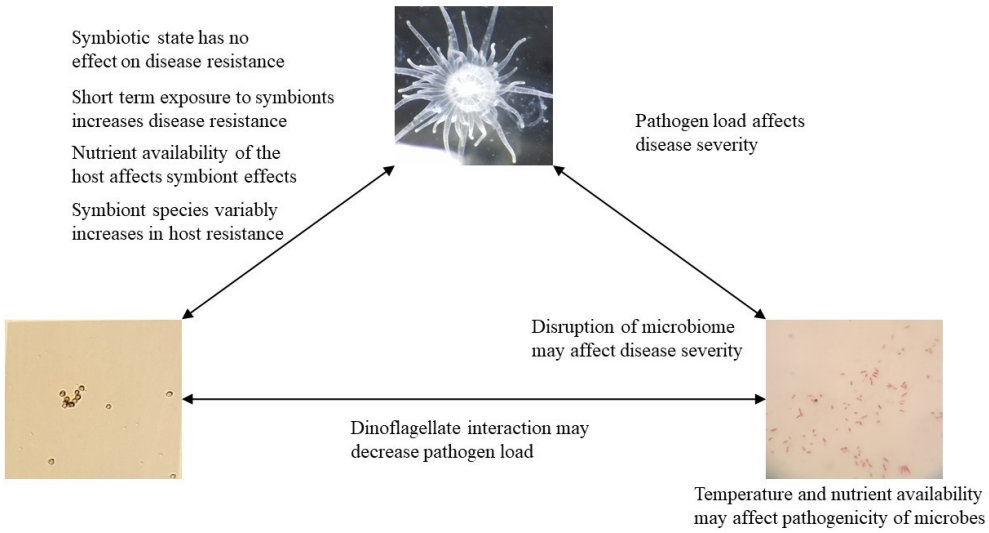

Figure 5.3. Summary of observed interactions with the holobiont. The interplay of the host, symbiotic dinoflagellates, and both associated and pathogenic bacteria are more complex than assumed by initial hypotheses.

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