Identifying Disease-Resistant and Thermal-Tolerant Genotypes in the Threatened Staghorn Coral, Acropora cervicornis

Morgan V. Hightshoe
Nova Southeastern University, mh2120@nova.edu

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Morgan V. Hightshoe

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Approved:
Thesis Committee

Major Professor: Nicole Fogarty
Committee Member: Steven Miller
Committee Member: Esther Peters

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HALMOS COLLEGE OF NATURAL SCIENCES AND OCEANOGRAPHY

IDENTIFYING DISEASE-RESISTANT AND THERMAL-TOLERANT GENOTYPES IN THE THREATENED STAGHORN CORAL, ACROPORA CERVICORNIS

By

Morgan V. Hightshoe

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Abstract

Since the 1970s, loss of herbivores, coral bleaching, pollution, and disease epidemics have reshaped the ecological framework of coral reefs. Staghorn coral, *Acropora cervicornis*, was a major reef-building scleractinian coral found throughout Florida and the Caribbean that experienced unprecedented population declines primarily due to disease and coral bleaching. These two stressors are coupled; the highest coral disease prevalence occurs after periods of thermal stress caused by increased sea surface temperature. Previous research documented three disease-resistant *A. cervicornis* genotypes in Panama, but it is unknown if disease-resistant genotypes exist in the Florida Keys. Thermal tolerance has been found to be variable among different species of corals and is relatively unknown in *A. cervicornis*. To investigate disease resistance and thermal tolerance in corals collected from the Florida Keys, pathogen transmission, thermal tolerance experiments, and coral outplanting studies were conducted, along with histological work to assess the condition of coral tissues. Corals were challenged *in situ* with exposure to rapid tissue loss (RTL) and bleaching resistance was evaluated *ex situ* in temperature-controlled seawater tanks, using 39 *A. cervicornis* genotypes. Disease and bleaching were further characterized in the wild using outplanted colonies. In a pathogen transmission pilot study, 7 out of 39 genotypes developed signs of rapid tissue loss. An expanded transmission experiment that used 12 potentially disease-resistant genotypes (based on anecdotal information and results from the pilot study), all genotypes developed signs of RTL. However, susceptibility was variable but not statistically different among genotypes (*p*>0.05), ranging from 40–100% transmission. Histological analyses revealed significant (*p*<0.01) differences in tissue characteristics between samples with and without visible signs of RTL following exposure to diseased fragments in the transmission experiments, largely a result of differences in the health of epidermal mucocytes. Coral fragments exposed to elevated temperature stress (32°C) responded similarly to controls maintained at 28°C (*p*>0.05) related to photosynthetic efficiency and tissue condition metrics. No significant differences in mortality, disease, or predation were found between disease-resistant and disease-susceptible genotypes in outplanting experiments (*p*>0.05). This study reports the first evidence that disease resistance is present in Florida *A. cervicornis* genotypes. The variability of disease resistance found within genotypes suggests that genotype is not the only factor influencing pathogen transmission. Short-term exposure to thermal stress revealed heat tolerant *A. cervicornis* genotypes, which corroborates with recent published studies. Taken together, these results provide insights into how Caribbean *Acropora* and other scleractinian species persist through multiple disease and coral bleaching events.

Keywords: *Acropora cervicornis*, disease resistance, rapid tissue loss, temperature stress, histology, outplanting
Chapter 1 – Introduction

1.1 Importance of Coral Reefs

Coral reefs are essential to coastline protection, critical to recreational and commercial activities, and offer a level of biodiversity that rivals that of tropical rainforests (Reaka-Kudla 1997, 2005, Knowlton et al. 2010). Approximately 95,000 species have been identified, making up 35% of marine species and 5% of the world’s known diversity (Reaka-Kudla 1997, 2005). Studies suggest that only 5–10% of coral reef species have been identified with estimates of total diversity ranging from 500,000–10 million species (Reaka-Kudla 1997, 2005, Small et al. 1998, Bouchet 2006, Chapman 2009).

At the heart of these immensely diverse ecosystems are the corals themselves. Scleractinians, stony corals, are essential to creating the complex structural foundation of the coral reef ecosystem, thus creating habitat for a wide range of marine flora and fauna (Sheppard 2018). The symbiotic relationship between the coral polyp and the dinoflagellate algae (zooxanthellae) within their tissues has allowed these organisms to thrive in oligotrophic marine environments (Sheppard 2018). The coral polyp provides a stable environment for the zooxanthellae, in turn the algae generates ~ 90% of the polyp’s energy requirements, allowing for sustained growth of the coral and secretion of a calcium carbonate skeleton (Sheppard 2018). The complex structure of the coral skeleton also facilitates niche diversification, which drives evolution and speciation (Moberg and Folke 1999). In addition, the physical barriers created by coral reefs create suitable environments for seagrass and mangrove ecosystems. These three ecosystems interact with one another to form important spawning, nursery, breeding, and feeding areas for many organisms (Moberg and Folke 1999). In Florida and the Greater Caribbean, the scleractinian genus Acropora has been a significant contributor to shallow reef accretion for the past 500,000 years (Jackson 1992, 1994, Pandolfi 2002). The high linear growth rates and intricate three-dimensional structure of Acropora cervicornis and Acropora palmata creates vital habitat for a variety of fish, turtles, and invertebrate species (Bruckner 2007).
Beyond their stunning biodiversity, coral reefs provide a myriad of ecosystem goods and services that support communities and economies worldwide. These include tourism, local fisheries, coastline protection, building materials, pharmaceutical products, and many others (Moberg and Folke 1999). Reef related fisheries account for approximately 9–12% of the world’s total fisheries (Smith 1978), and coral reef-generated coastline protection is a key service for many tropical coastal communities. Cesar (1996) estimated between $820–1,000,000 USD per km of Indonesian coastline was lost due to decreased coastline protection resulting from coral destruction. Conservation International (2008) assessed the global value of coral reefs at approximately $30 billion annual USD, although other studies estimated that value is more likely to be in the hundreds of billions (Edwards and Gomez 2007, Stoeckl et al. 2011). More than 450 million people in 109 countries live in coral reef-supported coastal communities around the world (Pandolfi et al. 2011). The goods and services of the Florida Keys (Riegl et al. 2009) and Caribbean reefs (Burke and Maidens 2004) have each been valued between $3.1–$4.6 billion USD per year, with most of that value generated by tourism and recreation.

1.2 Reef Decline

A combination of local (overfishing, sedimentation, eutrophication, habitat destruction, and predation) and global stressors (disease, increasing ocean temperature, ocean acidification, and storms) have led to the recent decline of coral reefs around the world. Approximately 60% of global coral reefs have been degraded or lost and are directly impacted by these stressors, and one-third of all reef-building corals are at risk of extinction (Carpenter et al. 2008, Jackson 2008, Burke et al. 2011). Pandolfi et al. (2003) modeled the ecological histories of 14 coral reef ecosystems across the world and found that while there was variation in reef decline among sites, the overall historical trajectory of reef degradation was markedly linear. Even the world’s most effectively managed reef system, the Great Barrier Reef, has been subject to significant coral decline due to large scale climate- and human-induced disturbances (Pandolfi et al. 2003, Aronson and Precht 2006, De’ath et al. 2012, Hughes et al. 2018b). Like all other ecosystems, disturbance plays a key role in sustaining coral reef biodiversity and providing opportunities for
colonization and succession (Rogers 1993), but the increasing frequency and scale of these disturbances has made natural recovery unlikely and pushed many reef ecosystems from coral to algal-dominated states (Hughes 1994, Scavia et al. 2002, Hughes 2003, Mumby 2007, Jackson et al. 2014).

Since at least the 1970s, a combination of global and local stressors have played a major role in reshaping the ecological and physical framework of Caribbean coral reefs (Aronson and Precht 2006, Baker et al. 2008, Jackson et al. 2014). From 1970–2011, average coral cover for the wider Caribbean declined from 34.8% to 16% based on data from 88 survey locations (Jackson et al. 2014). A meta-analysis of 65 Caribbean coral cover studies, encompassing 263 sites, revealed an 80% decline in Caribbean coral cover from 1977–2001 (Gardner et al. 2003). A variety of causes may be responsible for these declines, but research has identified increasing ocean temperatures and infectious microorganisms as the two most severe threats to corals (Harvell et al. 1999, 2004, Eakin et al. 2009, National Marine Fisheries Service 2015).

The majority of reef-building corals thrive in seawater temperatures between 25–29°C (Wells 1957, Stoddart 1969). Water temperatures exceeding this threshold for extended periods of time can result in coral bleaching, a stress response in which corals expel their symbiotic zooxanthellae (Brown 1997) or lose the algal pigments as the zooxanthellae die within the gastrodermal cells (Glynn et al. 1985). Since the 1980s, bleaching has been reported from almost every region that supports coral reefs (Baker et al. 2008). In addition, mass bleaching events have increased in frequency and severity as global sea surface temperatures continue to rise (Hoegh-Guldberg 1999, Baker et al. 2008, Eakin et al. 2009, Pandolfi et al. 2011, Hughes et al. 2018a). Historical temperature measurements from Florida Keys’ coral reef habitats document approximately 0.8°C increase in SST in the last century (Kuffner et al. 2014), and the current models of global climate change predict a mean increase in SST of 0.027°C per year (Bopp et al. 2013). Less common but still deleterious is the coral response to extremely low temperatures; a cold-water event in 2010 caused the worst coral mortality on record for the Florida Reef Tract (Lirman et al. 2011).

Simultaneously, there has been a recent increase in the prevalence and severity of bacterial, fungal, and viral diseases affecting coral species, especially in the Caribbean
(Harvell et al. 1999, Aronson and Precht 2001, Harvell et al. 2004). The first published record of a major coral-disease outbreak was in 1975 in the upper Florida Keys (Dustan 1977), and since then, reports of coral diseases such as white band, white plague, white pox, and aspergillosis have increased in number, causing significant declines in live coral cover (Harvell et al. 2004). Between the years 1996–1998, Porter et al. (2001) reported an increase in disease prevalence at 160 survey stations throughout the Florida Keys. Results showed substantial increases in the number of locations exhibiting disease (404% increase), the number of species affected (218%), and the rate of coral mortality (60% at some locations). In the United States Virgin Islands, 19 scleractinian corals were affected by disease that ultimately resulted in an average coral cover loss of 61% from 2005–2007 (Miller et al. 2009). In 2014, a white-plague disease outbreak affected 61% of at least 13 coral species at 14 sites along the southeast Florida coast, where some species were reduced to <3% of their initial population densities (Precht et al. 2016).

Most of the loss in Caribbean coral cover has been due to the severe decline of Acropora species (80–90%), caused by an increase in white-band disease (WBD) infections starting in the 1970s (Gladfelter 1982, Aronson and Precht 2001, Bruckner 2007). Until the 1980s, A. cervicornis and A. palmata were the primary ecological and geological contributors at many reefs throughout the Caribbean (Aronson and Precht 2001, Bruckner 2007). In 1983, an unknown pathogen caused the widespread loss of Diadema antillarum, a major consumer of macroalgae (Lessios et al. 1984). The loss of this key herbivore, combined with the increase in WBD, led to an increase in macroalgal dominance and significant declines in Acropora coral cover during a ten-year period (Carpenter 1990, Aronson and Precht 2001, Bruckner 2007).

1.3 Coral Disease

Disease is defined as any impairment of vital body functions, systems, or organs, and involves the interaction between a host, a pathogen, and the environment (Peters 2015). A disease may be caused by biotic (e.g., bacteria or protozoa) or abiotic (e.g., virus, prion, radiation, toxicant) pathogens, or a combination of the two types (Peters 2015). The study of coral disease is extremely challenging because the environment (composed of potentially abiotic pathogens) continuously affects the host and biotic
pathogens simultaneously. The coral polyp microbiome includes a complex community of bacteria, viruses, fungi, dinoflagellates, and endolithic algae (Kline and Vollmer 2011), making it extremely challenging to identify whether a pathogenic microorganism is causing a disease. To date, approximately 20 coral diseases have been described from the Caribbean and Indo-Pacific (Sutherland et al. 2004, Gignoux-Wolfsohn and Vollmer 2015). Of those diseases, only five (white plague II, acroporid serratiosis, aspergillosis, and two types of bacterial bleaching associated with the *Vibrio* family) of their respective etiologic agents have been identified through the satisfaction of Henle-Koch’s postulates, a series of criteria used to determine if a specific microorganism is the cause of a disease (Koch 1890, Sutherland et al. 2004, 2016). Researchers suggest that many coral diseases are likely caused by the interactions of a consortium of bacteria influenced by specific environmental conditions (Kline and Vollmer 2011, Gignoux-Wolfsohn and Vollmer 2015).

Tissue loss diseases caused massive region-wide mortality of *A. cervicornis* and *A. palmata*, starting in the early 1980s (Harvell et al. 1999, Aronson and Precht 2001, Porter et al. 2001, Gardner et al. 2003, Harvell et al. 2004, Vollmer and Kline 2008). White pox and rapid tissue loss contributed to the decline (Patterson et al. 2002, Williams and Miller 2005, Miller et al. 2014), however, WBD was the primary cause (Gladfelter 1982, Aronson and Precht 2001, Porter et al. 2001). WBD is the field-identification name given to a particular pattern and rate of tissue loss recognized as affecting Caribbean *Acropora* taxa (Peters 1984, Precht et al. 2002, Sutherland et al. 2004), and can be further subdivided into type I (WBD I) and type II (WBD II). WBD I is characterized by sloughing of pigmented tissue off the skeleton, leaving a band of denuded white skeleton. WBD II exhibits the same signs, but also includes a margin of bleached tissue preceding the margin of tissue loss (Ritchie and Smith 1998). The tissue loss usually proceeds from the base of a branch to the tip, more rarely beginning in the middle of a branch or from the tip toward the base. It eventually may lead to colony death, although the tissue loss may stop and the margin heals (Gladfelter 1982, Smith 2013).

The disease termed rapid tissue loss (RTL) displays similar characteristics to WBD, which has led researchers to speculate that WBD and RTL may be the same disease (Williams and Miller 2005, Miller et al. 2014). The etiologic agent for RTL is
also unknown, and the disease is characterized by a less uniform tissue loss margin and an increased progression rate of ~ 1 cm per day compared to 1–2 mm per day for WBD. The lesions can appear quickly along branches and will often coalesce as they enlarge and the *A. cervicornis* colony dies (Miller et al. 2014). While RTL may be widespread, little is known about the disease. A similar but unnamed condition was documented in Curacao in 1980, but there is no evidence to confirm the disease as WBD or RTL (Bak and Criens 1981). Miller et al (2014) found no histological differences between WBD- and RTL-affected colonies, which suggests that the different tissue loss patterns may be due to exposure to different biotic and abiotic stressors. These results combined with the lack of uniformity among the various WBD descriptions suggest that many documented cases of WBD may in fact be RTL (Williams and Miller 2005, Miller et al. 2014). Until the pathogen for each of these diseases is identified, it is useful to identify the disease using observable signs. Several infectious bacteria have been proposed as etiologic agents of WBD I, but no distinct pathogen has been confirmed (Peters et al. 1983, Ritchie and Smith 1998, Casas et al. 2004, Gil-Agudelo et al. 2006, Kline and Vollmer 2011, Miller et al. 2014, Sweet et al. 2014, Gignoux-Wolfsohn and Vollmer 2015). Some researchers hypothesize that WBD is a result of infection by microbes already present in *Acropora* coral tissue (Randall and van Woesik 2015). Other studies have shown that WBD is potentially caused by drastic changes in the coral microbiome that involve an increase in multiple disease-associated pathogens acting together as a consortium, rather than a single pathogen (Sweet et al. 2014, Gignoux-Wolfsohn and Vollmer 2015). However, these hypotheses are difficult to confirm because the composition of the microbial communities associated with diseased colonies have been inconsistent across studies (Casas et al. 2004, Sweet et al. 2014, Gignoux-Wolfsohn and Vollmer 2015).

Complicating things further, *rickettsia*-like organisms (RLOs), a group of obligate intracellular parasites suspected as a WBD pathogen, have been consistently found to be associated with apparently healthy and visibly diseased *Acropora* coral species (Peters et al. 1983, Casas et al. 2004, Miller et al. 2014, Shaver et al. 2017).

Exposure to suspected WBD pathogens has resulted in the disease signs appearing in apparently healthy corals following direct contact with affected colonies, the water column, or biological vectors, such as the corallivorous snail *Coralliophila abbreviata*.
As a result, disease can spread rapidly through areas of high *Acropora* density. However, during WBD episodes unaffected colonies can sometimes persist immediately adjacent to diseased ones, and newly affected colonies develop signs of disease at distant locations beyond the vectors’ territory or distribution ability (Gladfelter 1982, Gignoux-Wolfsohn et al. 2012). This suggests that some *Acropora* genotypes are resistant to WBD. In WBD pathogen transmission assays, 3 out of 49 genotypes of *A. cervicornis* were found to be resistant developing the disease in Panama after 3 days of direct tissue exposure and in wild colony surveys (Vollmer and Kline 2008). These results provided the first evidence for host disease resistance in scleractinian corals.

1.4 Combined Impacts of Disease and Temperature


Like many diseases caused by biotic pathogens, WBD prevalence increases with rising ocean temperature (Muller et al. 2008, Randall and van Woesik 2015). This is supported by evidence of increased WBD outbreaks following bleaching events (Aronson and Precht 2001, Porter et al. 2001, Muller et al. 2008), and the strong relationship between high temperatures and WBD occurrence (Randall and van Woesik 2015). Using eight historical and contemporary metrics of SST, Randall and van Woesik (2015) developed models to characterize the relationship between ocean warming and recent outbreaks of WBD. For both Caribbean *Acropora* species, their results showed that
increases in WBD prevalence are strongly coupled with increasing thermal stress due to climate change.

1.5 Restoration and Conservation

Due to major population declines throughout their range, Caribbean Acropora corals were listed as “Threatened” under the United States Endangered Species Act in 2006 (NOAA 2006) and as “Critically Endangered” by the International Union for Conservation of Nature (IUCN) in 2008. In 2015, the National Oceanic and Atmospheric Administration (NOAA) developed an Acropora recovery plan to identify strategies for rebuilding and ensuring the long-term viability of A. palmata and A. cervicornis coral populations in the wild (NMFS 2015). In response to Acropora coral declines and the emphasis from NOAA, considerable efforts throughout Florida and the Caribbean are underway to grow Acropora species in coral nurseries and to outplant genetically diverse colonies to depauperate reefs (Johnson et al. 2011). This technique, known as coral gardening (Rinkevich 1995), has had variable success and while there are over 60 Acropora restoration projects throughout Florida and the Caribbean, there is limited published literature on the process (Johnson et al. 2011, Young et al. 2012). Much of the literature focuses on methods, such as harvesting, site selection, nursery structures, outplanting techniques, and success based on growth rates and survival (Young et al. 2012). There is a paucity of information focused on the performance of individual genotypes related to disease and thermal stress. Although evidence of disease-resistant wild A. cervicornis colonies exists (Vollmer and Kline 2008) and informal observations suggest disease-resistant and thermal-tolerant genotypes are present in nursery populations (Coral Restoration Foundation pers. comm.), no study has experimentally tested for these qualities.

1.6 Importance and Goals of this Study

The goal of this research was to expand on previous disease resistance work (Vollmer and Kline 2008, Kline and Vollmer 2011), and to experimentally identify disease-resistant (WBD or RTL) and thermal-tolerant genotypes maintained in the Coral
Restoration Foundation’s (CRF) nursery, located in the Florida Keys. The work included three objectives:

1. **Identify disease-resistant A. cervicornis genotypes using in situ pathogen transmission assays.**
2. **Identify thermal-tolerant A. cervicornis genotypes using ex situ temperature stress experiments.**
3. **Outplant resistant and non-resistant genotypes in replicated multi-genotypic clusters to identify field performance based on genotypic diversity.**

Results from this work will help assess recovery potential and resistance in the natural population. Additionally, the results will help inform and potentially increase the efficacy of future management and conservation strategies of Acropora populations throughout the Florida Keys and the Caribbean, with the ultimate goal of restoring densities to where natural recovery can occur through sexual reproduction. For coral restoration programs such as the CRF, maintaining the highest level of genetic diversity in nurseries and during outplanting efforts is the most prudent long-term recovery strategy, and the inclusion of resistant genotypes in such outplanting efforts has the potential to enhance survival rates. These results will also help researchers predict how wild populations will respond to the combination of microbial pathogens and rising ocean temperatures.
Chapter 2 – Publication

2.1 Introduction

Since the 1970s, disease epizootics have played a major role in reshaping the ecological framework of Caribbean coral reefs. In only four decades, average coral cover in the Caribbean declined from 35% to 16% (Jackson et al. 2014). Over the same time period populations of the previously abundant reef building Acropora corals declined 80-90%, primarily due to white band disease (WBD) outbreaks (Gladfelter 1982, Aronson and Precht 2001, Porter et al. 2001, Bruckner 2007, Gardner et al. 2003). The disease is thought to only affect Acropora taxa (Peters 1984, Precht et al. 2002, Sutherland et al. 2004) and is characterized by the sloughing of pigmented tissue off the skeleton that leaves behind a band of denuded white skeleton. Tissue loss usually proceeds from the base of a branch to the tip and can eventually lead to colony death (Gladfelter 1982, Smith 2013). While several infectious bacteria have been proposed as etiologic agents of WBD, no distinct pathogen has been identified (Peters et al. 1983, Ritchie and Smith 1998, Casas et al. 2004, Gil-Agudelo et al. 2006, Kline and Vollmer 2011, Miller et al. 2014, Sweet et al. 2014, Gignoux-Wolfsohn and Vollmer 2015).

Until the 1980s, A. cervicornis and A. palmata were the primary ecological and geological contributors to many reefs throughout the Caribbean for thousands of years (Aronson and Precht 2001, Bruckner 2007). While WBD is cited as the primary cause of Caribbean Acropora population loss, other diseases have also contributed that have similar signs. In particular, the recently identified condition termed rapid tissue loss (RTL) was identified as responsible for declines in these species (Williams and Miller 2005, Miller et al. 2014). RTL displays similar characteristics to WBD, but with a less uniform tissue loss margin and a faster tissue-loss rate (Williams and Miller 2005, Miller et al. 2014). No histological differences exist between these lesions, leading researchers to speculate that many documented cases of WBD may in fact be RTL (Miller et al. 2014).

Tissue loss resembling WBD and RTL can occur in apparently healthy colonies after direct contact with tissue from affected colonies, biological vectors (e.g. Coralliophila abbreviata), and through the water column (Gladfelter 1982, Williams and
Miller 2005, Gignoux-Wolffsohn et al. 2012). As a result, disease spreads actively through areas of high Acropora density. However, unaffected colonies can persist immediately adjacent to diseased ones (Gladfelter 1982, Gignoux-Wolffsohn et al. 2012), leading to the hypothesis that some Acropora genotypes are disease-resistant, meaning that they can withstand the impacts of pathogenic agents (Stedman 2016). Vollmer and Kline (2008) found experimentally that 3 out of 49 genotypes of A. cervicornis in Panama were resistant to WBD transmission, providing the first evidence of host disease resistance in scleractinian corals.

Like many diseases caused by microbial pathogens, WBD prevalence has increased with rising ocean temperature (Aronson and Precht 2001, Porter et al. 2001, Muller et al. 2008, Randall and van Woesik 2015). The majority of reef-building corals thrive in seawater temperatures between 25–29°C (Wells 1957, Stoddart 1969); however, corals in the Persian Gulf, northwestern Australia, and American Samoa can tolerate much higher thermal extremes (Oliver and Palumbi 2011, Riegl et al. 2012, Palumbi et al. 2014, Schoepf et al. 2015). Water temperatures exceeding this threshold for extended periods of time can result in coral bleaching, a stress response in which corals expel their symbiotic zooxanthellae (Brown 1997) or lose the algal pigments as the endosymbiont dies within the gastrodermal cells (Glynn et al. 1985). Since the 1980s, bleaching has been reported from almost every region that supports coral reefs (Baker et al. 2008). Increasing water temperatures simultaneously increase pathogen virulence and weaken the coral immune system (Bruno 2015, Harvell et al. 1999, Lesser et al. 2007, Muller and van Woesik 2012, Porter et al. 2001, Randall and van Woesik 2015). Increases in WBD prevalence in both Caribbean Acropora species is shown to be strongly coupled with increasing thermal stress (Muller et al. 2008, Randall and van Woesik 2015).

The loss of these critical species has resulted in their listing as “threatened” under the United States Endangered Species Act in 2006 (NOAA 2006), and has led to the development of restoration programs throughout Florida and the Caribbean (Young et al. 2012). The goals of these projects are to conserve the genetic diversity of these threatened species through the use of coral nurseries; combat declines using active restoration, known as outplanting (Johnson et al. 2011, Young et al. 2012); and restore populations so recovery can occur through sexual reproduction. There is a paucity of
information focused on the performance of individual genotypes related to disease and thermal stress and these data will likely be valuable information for improving the success of both the nursery and outplanting techniques of these projects. Continued research on the effectiveness of different techniques is prudent to improving the efficacy of these restoration efforts. Confirmation of disease-resistant and thermal tolerant individuals in additional geographic locations may help explain the continued presence of wild populations and colonies that survive WBD/RTL epizootics or mass bleaching events.

With the increasing frequency of global bleaching events and disease outbreaks, developing an understanding of scleractinian coral response to these climate-induced stressors has become a major research priority. Through a combination of pathogen transmission and thermal tolerance experiments, histological investigations, and outplanting studies, all conducted using colonies from the Florida Keys, the goals of this research were to: (1) identify disease-resistant *A. cervicornis* genotypes, (2) identify thermal-tolerant *A. cervicornis* genotypes, and (3) characterize disease resistance in the wild. While disease-resistant *A. cervicornis* genotypes are known to exist, it is unknown whether or not they exist in the Florida Keys. Results from this work may help inform coral restoration strategies in the Florida Keys and may also help explain the persistence of the wild population, despite repeated tissue loss and bleaching events.

### 2.2 Materials and Methods

#### 2.2.1 Study Species and Genotype Selection

The staghorn coral, *Acropora cervicornis*, is a major reef-building scleractinian coral found throughout Florida and the Caribbean, and was a significant contributor to shallow reef accretion for the past 500,000 years (Jackson 1992, 1994, Pandolfi 2002). *Acropora cervicornis* is a fast-growing branching coral that forms dense thickets and thrives in intermediate water depths of 5–20 meters (Bruckner 2007). The intricate three-dimensional structure created by the species provides habitat for a variety of marine organisms including fishes, invertebrates, and sea turtles (Bruckner 2007).

*Acropora cervicornis* corals were sampled from the CRF Tavernier nursery (24.98222° N, 80.43633° W) in the upper Florida Keys. The nursery contains
approximately 105 unique genotypes of *A. cervicornis*, of which 48 (Appendix A) were selected based on anecdotal CRF data of lower disease and bleaching prevalence.

2.2.2 *Genotyping target Acropora cervicornis colonies*

Microsatellite genotyping was conducted after the pathogen transmission study to confirm genotypic identity of the 48 genotypes. A small fragment (< 1 cm) was collected from each colony and preserved in 96% molecular grade ethanol. Samples were trimmed into smaller pieces and transferred into CHAOS solution (4M guanidine thiocyanate, 0.1% N-lauroyl sarcosin sodium, 25 mM Tris pH8, 0.1M 2-mercaptoethanol, ultra-pure water) for tissue digestion. DNA was extracted using a magnetic bead protocol. For each sample 50 µl of digested coral tissue was mixed with 10 µl of Agencourt AMPure XP (magnetic beads), and 80 µl of 100% isopropyl. Samples were placed on a magnetic plate for 10 min, and then drained. The samples were then rinsed with 200 µl of cold 70% EtOH, drained, and air dried for 60 minutes. Next, 50 µl of 1 X TE buffer was added to each sample and placed on a shaker for 60 minutes. The samples were removed from the shaker and returned to the magnetic plate for 10 minutes, and then 50 µl of supernatant was pipetted out. DNA was quantified using a microplate spectrophotometer (ThermoFisher Scientific). DNA for each coral sample was then PCR amplified at five microsatellite loci [loci 166, 181, 182, 187, and 201 (Baums et al. 2005)] using protocols described in Fogarty (2010) and Fogarty et al. (2012). Briefly, each of the five microsatellite loci was PCR amplified separately using a typical PCR cocktail and a locus-specific cycle (e.g., 94°C for 2 min followed by 94°C for 30s, 46°C for 30s, and 72°C for 45s for 30 cycles followed by a final extension of 72°C for 3 min). PCR products of all five primers were multiplexed using HiDI Foramide (12.5 µl) and 0.5 µl Genescan 400 Rox. Two separate multiplexes were run based on primer amplification color (multiplex 1: 166, 181, 187; multiplex 2: 182, 207) and sent for fragment analysis to Florida State University. Samples that did not amplify were re-run individually. Peaks for each amplified locus were binned and analyzed using Genemapper 5. Lastly, the Excel microsatellite toolkit (Park 2001) was used to confirm the number of unique genotypes (Appendix A).
2.2.3 *In situ pathogen transmission pilot study (Experiment 1a)*

During the summer of 2016, sixteen PVC trees (Fig. 1) were constructed and installed in the designated research area at the CRF Tavernier nursery. The trees were divided into groups of four and assigned to one of the following treatments: diseased fragment application (D), asymptomatic fragment attached (C1), cable tie only attached (C2), or nothing attached (C3) (Appendix B). An individual tree held 12 fragments, such that each treatment contained all 48 genotypes, with no treatment being replicated (Fig. 2). To avoid the risk of unintentional pathogen transmission, diseased fragment-carrying trees were installed down-current from controls (based on the prevailing direction of currents at the site). For each treatment, one healthy fragment (10 cm) from each of the 48 putative genotypes (n = 192) was clipped from a source colony within the nursery, attached to a tree using monofilament loops, and given 13 days to acclimate. Three days before the transmission experiment, active disease was identified by marking diseased colonies within the nursery with a cable tie at the margin of tissue loss. Active disease was confirmed after three days if the tissue loss continued away from the cable tie at a rate of least 1 cm per day.

This experiment followed the WBD-pathogen transmission methods established by Vollmer and Kline (2008). Disease resistance was tested *in situ* by attaching a single 7-cm fragment with an active disease margin to an apparently healthy fragment using a beaded cable tie for a 4.5-day period (6/9–6/14/16). WBD is distinguished by a characteristic tissue margin where the zooxanthellae-bearing tissue is removed from the skeleton (Gladfelter 1982, Miller et al. 2014). RTL shows a similar but less uniform margin of sloughing tissue with a patchy distribution of lesions and a much faster progression rate of 1 cm per day (Williams and Miller 2005, Miller et al. 2014). WBD was rare during disease sampling; therefore, fragments displaying signs of active RTL were selected.

All fragments were monitored daily for presence/absence of disease, disease progression (cm), and general condition, and photos were taken with a ruler for scale. HOBO Pendant data loggers (Onset) were placed on each tree to record temperature. After the experimental period, fragments were photographed and samples were preserved for histological analysis (see Histology section). This experiment served as an initial
screening (no replication) of the 48 genotypes. A second experiment (Experiment 1b) was conducted with replication that used genotypes that appeared to be disease resistant based on this pilot study. All pathogen transmission experiments followed the guidelines established in the contamination safety section (Appendix C). Briefly, all disease fragments were handled separately using disposable gloves and designated tools, which were bleached after each use.

2.2.4 Replicated pathogen transmission study (Experiment 1b)

In July of 2017, a replicated pathogen transmission experiment was conducted using similar methods as described in Experiment 1a. Twelve potentially disease-resistant genotypes from the original 48 were selected based on results from Experiment 1a, a pathogen transmission experiment using grafting and homogenate treatments on a subset of the 48 genotypes conducted in November of 2016 (Bock 2018), as well as anecdotal disease and bleaching prevalence data from CRF. Six new PVC trees were installed, including one designated as the control (only used asymptomatic fragment attached control based on results of the pilot study) and five trees for diseased treatment (Fig. 3). Five healthy fragments (10 cm) of each of the twelve genotypes (n = 60) were collected and randomly distributed so that each tree contained a single replicate of each genotype. Previously marked fragments with active RTL were attached to the apparently healthy treatment fragments, and an apparently healthy fragment of the same genotype was attached to the apparently healthy control fragments. The fragments were monitored at intervals of 1, 4, 5, 7, 9, and 11 days after the experiment began and concluded on day 11 (7/10–7/21/17). Pre and post-experimentation histology samples were collected for all fragments on days 0 and 11, respectively.

2.2.5 Histology

Prior to all pathogen transmission experiments, a ~ 2 cm piece of each experimental fragment and C1 control fragment were collected and preserved in a Z-Fix Concentrate solution (1:4 dilution in seawater) or 4% paraformaldehyde solution for histological analysis to determine the baseline tissue quality. Post-experiment samples were collected from the same fragments for comparison. Additionally, a subset of samples from the attached diseased fragments (from each experiment) were collected to
provide a histological comparison using colonies exhibiting active disease. Histoslides were prepared from decalcified samples in the Histology Laboratory at Nova Southeastern University’s Oceanographic Center (NSUOC) based on protocols identified in Miller et al. (2014), developed by Dr. Esther Peters (George Mason University). Samples were trimmed to 2 cm fragments using a Dremel tool and diamond-coated-tile-cutting blade, being sure to include the tissue-loss margin wherever present. Samples with such a margin were enrobed in 1.5% agarose and decalcified using multiple solutions of 10% disodium dihydrate ethylenediaminetetraacetic acid (EDTA) at pH 7. When decalcified, enrobed samples were rinsed with freshwater, trimmed into 2–3 mm slices and placed in cassettes. Samples lacking a tissue-loss margin were decalcified using 5% hydrochloric acid (HCL)/EDTA solution. All samples were then processed through a graded series of ethanols, cleared, and infiltrated with molten Paraplast Plus®, and embedded in Paraplast Xtra®. Sections (4μm thick) were obtained using a Leica RM 2125 microtome, mounted on clean microscope slides, stained with Harris’s hematoxylin and eosin, and examined using an Olympus BX43 light microscope at 4–60x magnification. Photomicrographs were taken with the attached Olympus DP21 digital camera.

The pathological changes in cells and tissues were analyzed using a semi-quantitative approach developed by Dr. Peters. Tissue parameters were scored using a modified rubric (Miller et al. 2014, Appendix E) that rates the condition or severity/intensity of tissue changes compared to normal (0 = Within Normal Limits, to 5 = Severe). Figure 4 shows examples of Acropora samples in excellent and very poor condition. Eight parameters were scored: epidermal mucocytes, costal tissue loss, surface body wall (SBW) zooxanthellae, cnidoglandular band (CNGB) mucocytes, CNGB degeneration, mesenterial filaments, basal body wall (BBW) gastrodermis, and calicodermis. Scores from each parameter were summed to generate an overall tissue condition score.

2.2.6 *Ex situ* thermal stress tolerance study (Experiment 2)

Thermal tolerance experiments were conducted *ex situ* in the SEACOR experimental system at Nova Southeastern University’s Oceanographic Center in Dania
Beach, FL in October 2016 (10/5–10/15/18). Six fragments (~ 10 cm) from each of the 48 genotypes (n = 288) were collected from CRFs Tavernier nursery and transported (wrapped in seawater-soaked plastic bubble wrap, immersed in seawater-filled coolers, maintained at 28°C) to NSU’s SEACOR experimental tank system. The fragments were then glued to ceramic plugs, placed in labeled egg crate racks, and distributed to 12 control tanks and 12 stress-treatment tanks (Fig. 5). All tanks were maintained at ambient temperature (28°C) while fragments acclimated for 4 days. Temperature in the thermal stress tanks (+2°C) was then increased to 30°C at 1°C/day. Photosynthetic efficiency of the zooxanthellae was measured using pulse amplitude modulation (PAM) chlorophyll fluorometry (Diving-PAM, Walz Germany). PAM fluorometry measures the light adapted effective quantum yield [(Fv/Fm or ∆F/Fm)] by applying a saturation pulse of light, and then determining yield from the ratio of initial fluorescence (F) to maximum fluorescence (Fm) (Turner 2016). As photosynthetic efficiency varies among individuals, percent change of yield values from the beginning to the end of the experiment were calculated to generate an appropriate measure of change in photosynthetic efficiency. PAM readings were taken during a 30-minute window at dawn (15 min before, 15 min after) to ensure differences in photosynthetic efficiency were not due to changes in light intensity. All stress replicates and one control replicate received two readings at different locations along the fragment to capture an average value of photosynthetic efficiency. Initial readings were taken before experimental temperature was increased, and then at day 0 and 3 during treatment. Salinity, dissolved oxygen, and pH was monitored daily throughout the experiment using a YSI ProDSS meter. Corals were monitored and photographed next to a coral health color chart (CoralWatch, Siebeck et al. 2006) every day.

Treatment temperature was maintained at 30°C for 5 days. On day 6, temperature was increased at 1°C/day for two days and then maintained at 32°C for 72 hours. PAM readings and color chart health photos were again taken during this period using the same protocols as in days 1–4. Histology samples were collected for all stress replicates and one control replicate at the end of the temperature stress treatment.

Hurricane Matthew passed along the coast of Florida during the temperature stress experimentation period. Although data collection was inhibited due the facility
being inaccessible for two days, there was no loss of power or damage to the experimental system during this time.

2.2.7 Multi-genotypic cluster outplanting study (Experiment 3)

Performance of resistant and non-resistant genotypes was evaluated in multi-genotypic outplant clusters at three established outplant reef sites throughout the upper Florida Keys: Molasses (25.00788° N, 80.37708° W), Pickles (24.98450° N, 80.41663° W), and Little Conch (24.94222° N, 80.47457° W). Based on the results of the preliminary pathogen transmission and thermal stress experiments, and CRFs anecdotal genotype performance data, six genotypes (three high performing: 004, 005, 017 and three poor performing: 034, 037, 006). A total of 36 multi-genotypic clusters of *A. cervicornis* were outplanted to the three sites (12 per site). Each multi-genotypic cluster contained 3 colonies (~15 cm diameter) placed ~10 cm apart, with two variations (Fig. 6). Distance between clusters was ~1 m and colonies were attached with a 2-part marine epoxy (Magic-Sculpt). Each cluster was identified with a unique numbered cattle tag and colonies were marked using individual genotype tags (Fig. 7).

The multi-genotypic clusters were outplanted in May 2017 and were monitored monthly for four months. Monitoring data included performance of each cluster, as well as each individual genotype within the cluster. Surveys documented: disease prevalence (WBD or other tissue loss) by recording each colony as either affected or unaffected; disease incidence for each outplant cluster by recording the number of newly diseased colonies during each survey interval; bleaching prevalence; predation prevalence; and percent mortality (estimated visually and attributed as either predation, disease, or undefined). Predation impacts caused by snails, fireworms, or fish were not managed (by removing snails or fireworms) but were documented during the surveys if present.

Monitoring was limited to 4 months due to impacts from hurricane Irma in September of 2017. A post-hurricane survey conducted in October 2017 recorded a 99% loss of the multi-genotypic outplants.
2.2.8 Statistical Analyses

All statistical tests were conducted using the statistical software package R (V 3.4.3). For in situ pathogen transmission data, a survival analysis (log-rank test) was used to determine any association between genotype and time to appearance of tissue loss, whereas a frequency analysis (chi-square test, Fisher exact test) tested the association between genotype and apparent tissue loss. Histological and PAM fluorescence data were tested for normality and homogeneity of variances using a Shapiro-Wilk test and Bartlett’s test, respectively. Data that met the assumptions of normality and homogeneity of variances were tested using parametric tests (t-test, one-way or two-way ANOVA). Log transformed data that did not meet the parametric assumptions were analyzed using non-parametric tests (one or two sample Wilcoxon test, Kruskal-Wallis test). Outplanting data were analyzed using a combination of t-tests, ANOVAs, frequency analyses, and survival analyses.

2.3 Results

2.3.1 Genotyping target Acropora cervicornis colonies

Microsatellite genotyping revealed that 13 of the putative unique genotypes were clones (100% match at five loci) for a new total of 39 unique genotypes. The experimental genotypes were updated based on the microsatellite results (Appendix A). The presence of clones among the original 48 selected genotypes altered the experimental design of the pathogen transmission pilot study by creating unexpected replicates.

2.3.2 In situ pathogen transmission pilot study (Experiment 1a)

During the pathogen transmission screening, 7 of the 39 (18%) experimental fragments showed visible signs of tissue loss. These included genotypes 015, 018, 019, 022, 026, 034, and 037 (Fig. 8). Two C1 (control 1 = asymptomatic fragment attached) fragments also developed signs of RTL (genotypes 006, 024). Tissue loss continued at an average rate of 0.43 cm/day with maximum and minimum rates of 1.2 cm/day and 0.2 cm/day, respectively (Fig. 9). Attachment of the diseased fragment to the apparently healthy fragment had no significant effect on time to start of tissue loss (Log-rank test, p>0.05). When comparing control and diseased treatment samples, no association was
found between the condition of the attached fragment (RTL, no RTL) and the resulting condition (diseased, apparently healthy) of the post-exposure fragment (Chi-square test, p>0.05).

Histology

Tissue condition was determined based on the semi-quantitative scale in which lower numbers represent healthier condition or lower intensity/severity of changes and higher numbers represent unhealthier condition or more intense/severe changes. Histological analyses of overall tissue condition scores (sum of all eight individual tissue parameter scores) revealed a significant degradation in tissue condition from pre- to post-exposure treatment samples (Fig. 10, Kruskal-Wallis test, p<0.001). There was no difference between the tissue condition scores of pre-exposure fragments that eventually developed tissue loss (n = 8) and those that did not, but post-exposure fragments with signs of RTL (n = 7) had tissues that were in significantly poorer condition than post-exposure fragments with no signs of RTL (n = 17) (Fig. 10, Kruskal-Wallis test, p<0.001).

Additional analysis was conducted on overall tissue condition scores of apparently healthy fragment attached (control) versus diseased fragment attached (treatment) post-exposure samples (Fig. 11). There was no significant difference between the tissue condition scores of control and treatment samples that did not develop visible RTL. Treatment samples developing visible RTL were not different from controls that also developed RTL, but were in significantly poorer condition than both control and treatment samples with no visible RTL (Kruskal-Wallis test, p<0.001). Although tissue condition scores of control samples with signs of RTL were relatively higher than controls with no RTL, this difference was not significant. This is most likely due to the low sample size for control, RTL visible samples (n = 1).

To further evaluate tissue health, additional analysis was performed on the individual parameters. Pre-exposure results revealed that gastrodermal architecture of the basal body wall (GA-BBW) was in significantly poorer condition in pre-exposure fragments that showed eventual signs of tissue loss than in pre-exposure fragments with no eventual signs of RTL (Fig. 12, Wilcoxon test, p=0.01). Post-exposure fragment parameter analysis found fragments with visible signs of tissue loss had significantly
poorer (higher) tissue condition scores in seven out of the eight parameters (Fig. 13, Wilcoxon tests, p<0.01). Cnidoglandular band epithelium mucocytes (CNGB-M) was the only parameter with no significant difference, mucocytes appeared normal with pale staining mucus and variable distributions in the CNGB. There was a significant difference between epidermal mucocyte tissue condition scores of fragments with and without visible signs of RTL (Fig. 13, Wilcoxon tests, p<0.01). Fragments with no RTL developing had minimal to mild tissue conditions and were characterized by an abundance of hypertrophied mucocytes, and in some cases these appeared uneven or misshapen. Mucocytes in fragments with RTL showed marked to severe atrophy or were completely absent, which was evident in sections near the tissue loss margin. A discriminant analysis identified this parameter as the best predictor (highest absolute value) of whether fragments showed signs of RTL or not, with 100% correctness.

2.3.3 Replicated pathogen transmission study (Experiment 1b)

The replicated pathogen transmission experiment found all 12 genotypes tested showed gross signs of tissue loss, although the proportion of fragments developing tissue loss varied between and within genotypes (Fig. 14). The average proportion of fragments showing signs of RTL was 70%, with a minimum and maximum of 40% (017) and 100% (004, 012), respectively. No significant association was found between genotype and disease signs (Fisher exact test, p>0.05) or between genotype and time to start of tissue loss (Log-rank test, p>0.05). Genotype also had no significant effect on the rate of tissue loss (Kruskal-Wallis test, p>0.05), which was an average of 0.83 cm/day with minimum and maximum of 0.26 cm/day and 1.46 cm/day, respectively.

2.3.4 Ex situ thermal stress tolerance study (Experiment 2)

The percent change of photosynthetic efficiency yield values (ΔF) were calculated from days 0–9 of the temperature stress experiment. These readings were then analyzed to determine whether fragments exposed to elevated temperature stress (32°C) displayed greater change in fluorescence compared to control fragments maintained at ambient temperature (28°C). There was no significant difference in the ΔF values for the control and elevated temperature (Fig. 15, Wilcoxon test, p>0.05). These results were further
supported by the histological analysis, which showed no significant difference in overall tissue condition scores between post-experiment samples from ambient (28°C) and elevated (32°C) temperature treatments (Fig. 16, one-way ANOVA, p>0.05). Samples taken before the thermal stress experiment had significantly higher tissue condition scores than samples taken after both the ambient and elevated temperature treatments.

2.3.5 Multi-genotypic cluster outplanting study (Experiment 3)

Monitoring data on the multi-genotypic outplants from May–September (4 months) showed no significant difference in mortality, disease, or predation between cluster variations (Fig. 17, Wilcoxon tests, p>0.05, Chi-square tests, p>0.05). “Mortality” refers to the number of 100% dead colonies per cluster; “disease” refers to the number of colonies showing signs of tissue-loss disease per cluster; and “predation” refers to the number of colonies showing signs of predation per cluster. Cluster variation and genotype were also found to have no significant effect on time to start of tissue loss (Log-rank tests, p>0.05) or on percent mortality (Wilcoxon test, Kruskal-Wallis test, p>0.05).

2.4 Discussion

Candidate disease-resistant coral genotypes showed a high degree of variability in the replicated pathogen transmission experiment, both among and within genotypes. Disease resistance was also found to vary among fragments of the same colony. These results provide evidence that disease resistance exists in Acropora cervicornis genotypes found in the Florida Keys, and helps explain why colonies of staghorn corals persist during tissue-loss disease events that kill large numbers of corals. However, disease resistance and mortality did not vary significantly among different multi-genotypic clusters that were outplanted to the reef, most likely due to the limited monitoring period. While preliminary and speculative, results from the ex situ thermal tolerance experiment were not significantly different from controls, which might indicate that the tested genotypes have some heat tolerant properties. With the continued increase in the frequency and severity of coral bleaching events (Baker et al. 2008, Hughes et al. 2018a) and widespread tissue-loss disease throughout the Florida Keys in the past and present (Aronson and Precht 2001, Porter et al. 2001, Gardner et al. 2003, Harvell et al. 2004,
Precht et al. 2016), resistance to increasing temperature and infectious agents that has a genetic basis will play an important role in the survival of natural populations, as well as in restoration projects.

Monoclonal variation to disease susceptibility was evident in both pathogen transmission experiments. In the pilot study, two of the seven genotypes (018 and 022) had fragments that developed RTL and others that had no visible signs of RTL at the end of the experiment. Genotype 024, (n = 6) developed tissue loss only on the control fragment and none on the experimental fragments, possibly due to prior pathogen exposure. This same genotype was later used in the replication experiment and had one of the lowest proportions of RTL (40%). Tissue loss in the replication experiment was variable across and within genotypes, ranging from 40–100%. Only two of the genotypes had 100% of the fragments develop tissue loss, suggesting that 10 out of the 12 tested genotypes showed some resistance to developing this disease. Variability of disease resistance within individual genotypes was documented in other A. cervicornis pathogen transmission studies (Vollmer and Kline 2008, Bock 2018), as well as in a nursery population (Goergen 2018). Vollmer and Kline (2008) found that the percent of fragments that developed disease within an individual genotype ranged from 0–80%, with the majority between 30–50%. In an ex situ pathogen transmission experiment three out of twelve genotypes showed signs of tissue loss following exposure to a homogenate treatment (waterborne) compared to ten genotypes in a grafting (direct contact) treatment, with the proportion of diseased fragments ranging from 0–100% (Bock 2018). Given that the etiologic agents have yet to be identified for these tissue loss diseases it was impossible to standardize the pathogen used in the transmission experiments. This factor may also play a role in the variable responses seen among genotypes as different pathogens may display similar observable signs but differing levels of virulence.

Variation in disease resistance also occurred among apical fragments from the same colony. A similar result was reported within individual colonies of octocorals (Harvell and Fenical 1989, Dube et al. 2002, Ward 2007). The sea fan, Gorgonia ventalina, exhibited greater disease resistance in younger blade edge tissue compared to older tissue in the center of the fan, based on the measured activity of antifungal metabolites (Dube et al. 2002, Ward 2007). Other octocorals have also shown similar
patterns of defense compounds occurring at greater concentrations in younger edge tissue (Harvell and Fenical 1989). The condition of mucous secretory cells, a key component of the coral immune system (Mullen et al. 2004, Reed et al. 2010), were observed to vary throughout an individual colony (Peters 1984). Our results suggest that scleractinian immunological defenses may also be heterogeneous within an individual colony, but the mechanisms controlling this variation are unclear.

One possible explanation for the variation we observed within colonies is the somatic mutation theory of clonality, where the accumulation of somatic mutations in a clonal organism over time will result in a genetically heterogeneous individual (Klekowski 1997, Devlin-Durante et al. 2016). The rate of somatic mutation can also differ among individuals due to varying exposure to environmental stress (Haag-Liautard et al. 2007, de Witte and Stocklin 2010, Conrad et al. 2011). However, genotypic differences from somatic mutations within a colony can also occur (Levitan et al. 2011, K. Olsen unpubl. data.). Epigenetic modifications based on varying environmental pressures could also play a role in monoclonal heterogeneity (van Oppen et al. 2015, Devlin-Durante et al. 2016). Gene expression is shown to vary by up to 1,024 fold in A. cervicornis colonies of the same clone when exposed to temperature stress (Parkinson et al. 2018). Such high variation could also be present in response to exposure to infectious pathogens. Differences in responses may be due to a combination of environmental (previous disease stress events) and biological (gene expression, antimicrobial response, age of the organism, and nutritional status) factors (Mullen et al. 2004).

In addition to quantifying disease resistance through gross observations, development of tissue loss following exposure to apparently healthy or diseased fragments was also investigated at the microscopic tissue level using histological techniques. Although no difference in overall tissue condition occurred between pre-exposure fragments, analysis of tissue condition scores for the individual parameters found the gastrodermal architecture of the basal body wall in fragments that eventually developed signs of RTL had significantly higher scores (were in worse condition or had more severe or intense alterations, i.e., less healthy) than fragments that did not eventually develop RTL. In unhealthy coral fragments tissue degeneration begins interiorly, the basal body wall cells slowly die as surface body wall and oral region cells
proliferate (E. Peters pers. comm.). Preliminary degeneration of this tissue could indicate that colonies with less healthy internal tissue structures, such as the gastrodermis, are more susceptible to pathogen transmission.

A worsening tissue condition was clearly documented, moving from pre- to post-diseased-fragment exposure. This indicates that the application of the diseased fragment had a negative impact on tissue health for both fragments that elicited tissue loss and those that did not. The decline in tissue health in post-exposure fragments that did not develop tissue loss is most likely due to stress involved with the attachment of another fragment, as post-exposure control fragments had statistically similar declines in health. Results also showed that post-exposure samples that had tissue loss had significantly higher tissue condition scores than post-exposure treatment samples that did not develop disease. This indicates that the exposure to diseased tissue by direct contact had severe impacts on tissue health. The condition differences were also apparent in the analyses of seven out of eight parameters that were found to have significantly higher scores (less healthy) in samples with visible signs of tissue loss. The majority of fragments with no tissue loss had a mild abundance of hypertrophied mucocytes, whereas diseased samples were characterized by marked to severe atrophy of the epidermis, with either misshapen or a complete loss of mucocytes. These mucous secretory cells are typically abundant in the epidermis as mucus production is central to a coral’s innate immune system. The epidermal mucociliary system of corals gives these sedentary organisms the ability to remove sediment and trap or expel potential pathogens at their surfaces (Mullen et al. 2004, Reed et al. 2010). Degeneration of these cells is detrimental to a coral and indicates severe stress.

Photosynthetic efficiency and tissue condition did not vary between corals exposed to ambient and elevated water temperatures. However, histological samples taken before the thermal stress experiment had significantly less healthy tissue samples than those taken after the ambient and elevated temperature treatments. This result may be attributed to rapid tissue degeneration occurring between time of sample collection and histological preservation caused by collection and transport stress. Work on experimental acclimation in A. cervicornis shows a significant decline in overall tissue health directly after sampling and a recovery of epidermal mucocytes and zooxanthellae
in the surface body wall after nine days (Bock 2018). Temperature acclimation and the short heat stress exposure period may explain the lack of algal symbiont photosynthetic or tissue alteration responses between control and treatment corals in our experiment. Sea surface temperatures during the 2016 summer months (Jun-Sep) prior to experimentation ranged from 29–31°C and the bleaching threshold for the Florida Keys is 30.6°C (NOAA Coral Reef Watch 2018). During a reciprocal transplant study, colonies of Pacific A. hyacinthus from different reef locations were shown to develop increased bleaching resistance when exposed to increased temperatures for a period of 27 months (Palumbi et al. 2014). Yetsko 2018 exposed A. cervicornis genotypes from the same CRF nursery to heat stress (32°C) and saw a decline in photosynthetic efficiency beginning at day 8. Our study only exposed corals to 32°C for 3 days, which likely did little to evoke a stress response. A less likely explanation for the lack of variation in heat stress is that the tested genotypes harbor symbionts with greater heat tolerance properties. Symbiodinium in clade D resist increased temperatures (Rowan 2004, Baker et al. 2008), although A. cervicornis typically harbors species from clade A (Thornhill et al. 2006). Research has yet to determine if A. cervicornis has the ability to alter its symbiont community, but it has been documented in corals in the Caribbean and other parts of the world in response to bleaching stress (Glynn et al. 2001, Baker et al. 2004, Rowan 2004, Berkelmans and van Oppen 2006).

In the multi-genotypic outplanting experiment, no differences in mortality, disease prevalence, and predation were found between disease-resistant and disease-susceptible clusters. The short 4-month monitoring period, during which there was a lack of disease and mortality likely explain this result. An A. cervicornis outplant study monitored from 2013 to 2015 found higher percent survival in multi-genotypic clusters compared to monoclonal clusters, but found no difference in disease prevalence between cluster types (Ware 2015). Although no advantage was found using different multi-genotypic outplant designs in this study, a key conservation practice is to enhance the genetic diversity of a population, thus diversifying response to stress within that population (Baums 2008). If certain colonies within a population are disease-resistant or possess disease-resistant characteristics, then the likelihood of an individual becoming diseased is reduced based on the concept of herd immunity (Fine et al. 2011). Outplanting
a genetically diverse population of coral colonies can help increase the potential resistance within a population and reduce the likelihood of widespread mortality due to a single stress event.

As bleaching events and tissue-loss disease outbreaks continue to increase in severity and frequency (Baker et al. 2008, Hughes et al. 2018a), understanding Acropora cervicornis response to these stressors is critical to forecasting the future and developing robust management plans for this threatened species and other scleractinian corals. The results of this study showed that disease resistance and possibly thermal tolerance are present within multiple genotypes of A. cervicornis in Florida. The identification of genotypes with these traits could also be used to help inform A. cervicornis restoration projects. Furthermore, this is the first study to identify within-colony disease resistance variability in scleractinian corals. These results help explain the persistence of natural populations of A. cervicornis in Florida and elsewhere, and may indicate that individuals possess sufficient variability to survive increasing climate stressors.
Figure 1. Installed PVC tree with fragments.
Figure 2. (A) The pathogen transmission pilot study PVC tree layout consisted of sixteen trees, divided into four groups to test the 48 genotypes (12 genotypes per group). Each of the four trees represents one of three controls or the pathogen transmission treatment, denoted by the abbreviations C1, C2, C3, and D. (B) Each tree consisted of four PVC arms suspended at a depth of 7.6 m, with a duckbill anchor and top-float for support. 12 genotypes represented by 12 fragments were attached per tree, denoted by the red X. Fragments were attached to the PVC arms via monofilament line. Genotype location was randomly assigned for each tree.
Figure 3. The replicated pathogen transmission included six PVC trees, five disease treatment and one control. Each tree contained one replicate of the 12 candidate disease-resistant genotypes. One control method was used, asymptomatic fragment attached. The trees were suspended at a depth of 7.6 m, with a duckbill anchor and top-float for support. Genotype location was randomly assigned for each tree.

Figure 4. (A) An *Acropora* baseline (Looe Key, 1976) histology sample with labeled anatomical features of interest representing a tissue condition score of 0 (normal). (B) An *A. cervicornis* sample with RTL representing a tissue condition score of 40 (severe). Both images were captured at 40x magnification.
Figure 5. The thermal tolerance experiment consisted of twelve control (28°C) and twelve treatment (30-32°C) tanks. Treatment tanks temperatures were maintained at 30°C for five days and then increased to 32°C for 3 days. Each of the 48 genotypes had three control and three treatment replicates. Genotype location was randomized within each tank.

Figure 6. Layout of variations 1 and 2 of multiclonal outplants. A) Variation 1: 2 poor performers (x), 1 high performer (o). B) Variation 2: 1 poor performer (x), 2 high performers (o).
Figure 7. A newly outplanted multi-genotypic cluster with individual genotype tags (white) and a cluster ID tag (yellow).
Figure 8. Spatial distributions of genotypes on experimental trees. Genotypes that developed gross signs of tissue loss are highlighted red.
**Figure 9.** Daily tissue loss rate on experimental fragments showing signs of disease. Average rate of tissue loss was 0.43 cm/day with maximum and minimum rates of 1.2 cm/day and 0.2 cm/day, respectively.

**Figure 10.** Overall tissue condition scores (sum of all 8 parameter scores) of pre- and post-exposure samples in the pathogen transmission pilot study. All pre-exposure samples were collected on day 0 and had no visible signs of disease. Fragments that eventually developed RTL during the experiment are designated as “RTL Visible”, and “RTL Not Visible” designates fragments that did not develop RTL. Letters denote significant difference between samples (Kruskal-Wallis test, p<0.01). Tissue condition scores range from 0 (healthy) to 40 (unhealthy).
Figure 11. Overall tissue condition scores for post-exposure samples in the pathogen transmission pilot study. Kruskal-Wallis test, $p<0.01$. Tissue condition scores ranged from 0 (healthy) to 40 (unhealthy).

Figure 12. Tissue condition scores for disease treatment, pre-exposure samples in the pathogen transmission pilot study. Each parameter was tested using a separate t-test (Wilcoxon tests, * = $p<0.05$). Parameter key: epidermal mucocytes (EM), costal tissue loss (CTL), zooxanthellae in surface body wall (Z-SBW), cnidoglandular band epithelium mucocytes (CNGB-M), cnidoglandular band degeneration (CNGB-D), mesenterial filament dissociation (MFD), gastrodermal architecture of the basal body wall (GA-BBW), calicodermis condition (CC). Tissue condition scores range from 0 (healthy) to 5 (unhealthy).
**Figure 13.** Tissue condition scores for disease treatment, post-exposure samples in the pathogen transmission pilot study. Each parameter was tested using a separate t-test (Wilcoxon tests, * = p<0.01). Parameter key: epidermal mucocytes (EM), costal tissue loss (CTL), zooxanthellae in surface body wall (Z-SBW), cnidoglandular band epithelium mucocytes (CNGB-M), cnidoglandular band degeneration (CNGB-D), mesenterial filament dissociation (MFD), gastrodermal architecture of the basal body wall (GA-BBW), calicodermis condition (CC). Tissue condition scores range from 0 (healthy) to 5 (unhealthy).

**Figure 14.** The number of fragments per genotype (n = 5) showing visible signs of RTL during the replicated pathogen transmission study.
Figure 15. The distribution of percent change values of ΔF/Fm for fragments in control (28°C) and stress temperatures (32°C) from day 0-10 of the thermal stress tolerance experiment. Wilcoxon test, p>0.05.

Figure 16. Overall tissue condition score distributions for pre-experiment, control (28°C) post-experiment, and elevated (32°C) post-experiment samples in the temperature stress experiment. Tissue condition scores range from 0 (healthy) to 40 (unhealthy). One-Way ANOVA, p<0.01.
Figure 17. The average number of colonies in each cluster with mortality, disease, and predation in the multiclonal outplanting experiment. Variation 1 included two poor performing genotypes and one high performing, and variation 2 included one poor performing genotype and two high performing. There are three colonies in each cluster and 18 clusters per variation (n=36). Bars represent standard error. Wilcoxon tests, p>0.05.
Chapter 3 – Discussion

Globally, coral reefs have been severely affected by the relatively recent rise in climate-induced stressors, most notably increasing SST and biotic pathogens. The goal of this research was to experimentally identify disease-resistant and thermal-tolerant properties among genotypes of *A. cervicornis* within a Florida Keys’ nursery population. While disease resistance has previously been identified in an *A. cervicornis* population in Panama (Vollmer and Kline 2008), recent research on the association between increasing temperature and increasing disease has created a need to better understand the synergistic impacts of biotic and abiotic pathogens. Using a combination of pathogen transmission and thermal stress experiments, this study has confirmed the presence of disease resistance and thermal tolerance characteristics and identified candidate genotypes that may be more likely to withstand continued environmental pressures.

Resistance is defined as the ability of an organism to maintain immunity or resist the effects of an antagonistic agent (Stedman 2016). Corals have been shown to possess innate and adaptive-like immunological responses (Mullen et al. 2004, Reed et al. 2010). An innate response refers to a generalized ability to react to potentially pathogenic agents. A coral’s innate response can include chemical secretion, mechanical or physical barriers, bioactive compound production, microorganism removal via phagocytosis, and the ability to move, shed, or expel pathogens (Cotran et al. 1999, Mullen et al. 2004, Reed et al. 2010). Adaptive-immunological responses involve an organism’s ability to recognize specific biotic pathogens and make adjusted responses based on previous infection experience (Reed et al. 2010). Corals were previously not thought to possess adaptive responses, but studies of self-/non-self-recognition and immunological memory have offered evidence to the contrary (Hildemann et al. 1977, Rosenberg et al. 2007). The documented responses do not meet all of the criteria that define vertebrate adaptive immunity, and therefore authors have designated these responses as ‘adaptive-like’ (Reed et al. 2010). The mechanisms that cause immunological response in corals are still poorly understood. Mullen et al. (2004) noted that responses may be due to a combination of environmental (previous disease-resulting stress events) and biological (genotype, gene expression, antimicrobial response, age of the organism, and nutritional status) factors.
The results from the pathogen transmission experiments indicated that no tested genotypes were completely resistant to developing tissue loss. They do show that disease resistance is present with a high amount of variability among and within genotypes, and even within a colony. Variability of disease susceptibility within individual genotypes is not rare. Vollmer and Kline (2008) used *in situ* WBD pathogen transmission experiments combined with field surveys and found that the percent of fragments that developed disease within an individual genotype ranged from 0–80%, with the majority between 30–50%. When combining the transmission and field surveys, they also found that some genotypes were resistant to developing tissue loss *in situ* and not in the field surveys and vice versa. An *ex situ* pathogen transmission experiment using direct contact with diseased fragments and homogenized diseased fragment tissue application found variable genotypic response among 12 unique genotypes from the CRF nursery (Bock 2018). Only three genotypes showed signs of tissue loss following exposure to the homogenate treatment (waterborne) compared to 10 genotypes in the grafting (direct contact) treatment, with the proportion of diseased fragments ranging from 0–100%. Monoclonal variation of disease prevalence was also documented in *A. cervicornis* nursery genotypes in Florida (Goergen 2018). The results of these studies help to validate the wide-ranging genotypic responses found here.

The coral holobiont is a complex association between animal, plant, and suite of microorganisms. The interactions of all these components make it difficult to isolate what factors influence a coral colony’s response to different stressors. All of the pathogen transmission experimental fragments (control or treatment) were collected from the same colony, meaning that the variability in response was not only present within a single genotype but within a single colony. Our results may suggest that scleractinian immunological defenses are also heterogeneous within an individual colony, but the mechanisms controlling this variation are unclear. Previous research has also found disease resistance to be heterogeneous within individual colonies of octocoral species (Harvell and Fenical 1989, Dube et al. 2002, Ward 2007). A study of aspergillosis, a fungal disease affecting *Gorgonia ventalina*, found younger blade edge tissues versus older center tissues had greater pathogen resistance, measured as a function of antifungal metabolite concentration (Ward 2007). Variation of chemical defenses within a single
individual is a well-documented characteristic in terrestrial plants (McKey 1974). Optimal defense theory predicts that greater chemical defenses are allocated to tissues with greater fitness value (Rhoades 1979). Extending this theory to corals is challenging because it is difficult to designate the value of a coral’s tissue. The concept of fitness suggests greater value should be given to reproductive areas, which for branching corals like the Caribbean acroporids are the older central branch tissues, but the apical tips of the branches are responsible for growth, and like plant leaves, are likely to be more productive than older tissues (McKey 1974, 1979). Because all experimental fragments were from branch tips, optimal defense theory is an unlikely explanation for the variability. More probable explanations include somatic mutations and epigenetic modifications (van Oppen et al. 2015, Devlin-Durante 2016). Somatic mutations refer to changes in the DNA of a cell (excluding germ cells, sperm and eggs) occurring at any point in cell division, which are frequently caused by environmental factors (Klekowski 1997). The somatic mutation theory of clonality asserts that an accumulation of somatic mutations in a clonal organism over time will lead to divergent cell lineages resulting in a genetically heterogeneous individual (Klekowski 1997, Devlin-Durante 2016). Devlin-Durante (2016) reported 342 unique mutations in 147 genets of A. palmata colonies throughout Florida and the Caribbean. Research shows that the rate of somatic mutation can differ among individuals due to varying exposure to environmental stressors (Hagg-Liautard et al. 2007, de Witte and Stocklin 2010, Conrad et al. 2011). Epigenetic modifications based on varying environmental pressures could also play a role in monoclonal heterogeneity (van Oppen et al. 2015, Devlin-Durante et al. 2016).

Epigenetics describes external modifications in genes, without changes in the gene sequence, that result in different expression levels of those genes (van Oppen et al. 2015). These changes have commonly been documented as DNA methylation, histone tail modification, chromatin remodeling, and altered regulatory mechanisms of small noncoding RNAs (Danchin et al. 2011). Gene expression is shown to vary by up to 1,024 fold in A. cervicornis colonies of the same clone when exposed to temperature stress (Parkinson et al. 2018). Such high variation could also be present in response to disease stress. The mechanisms causing immunological defense variation of scleractinian corals
are unknown, but these hypotheses may offer some explanation for the variability found in this study.

Another factor to consider is the environmental history experienced by the colony. If corals do possess adaptive immunological responses, then previous pathogen exposures resulting in disease could potentially improve a colony’s ability to resist subsequent infections. The bacterium *Vibrio shiloi* was identified as the causative agent of bacterial bleaching in the Mediterranean coral *Oculina patagonica* (Kushmaro et al. 1997). Infections are triggered by an increase in seawater temperature (Kushmaro et al. 1998); at high temperatures, the bacteria are attracted to chemicals in the coral mucus. Once the bacterium penetrate the coral’s cells, it multiplies and produces a toxin that inhibits photosynthesis of the symbiotic algae, which ultimately leads to bleaching (Rosenberg and Falkowitz 2004, Rosenberg et al 2007). During eight consecutive years of summer bleaching, researchers isolated *V. shiloi* from bleached corals and then used the bacteria to trigger bleaching in healthy colonies. However, in year nine the bacteria could no longer be isolated from bleached or healthy corals and inoculation of healthy corals with *V. shiloi* no longer resulted in bleaching (Rosenberg et al. 2007). Further investigation revealed that resistant corals were able to lyse the intracellular *V. shiloi* on inoculation.

Similarly, in the Florida Keys, the isolated bacterial pathogen that caused the white plague disease outbreak in 1995 no longer infected corals in subsequent inoculation experiments (Richardson and Aronson 2002). These studies have led to the introduction of the ‘coral probiotic hypothesis, which states that a dynamic relationship exists between symbiotic microorganisms and corals under different environmental conditions that selects for the most advantageous coral holobiont (Reshef et al. 2006, Rosenberg et al. 2007). Disease outbreaks in the CRF nursery are common and consistent (K. Nedimyery, pers. comm.). Nursery-raised corals could be exhibiting similar adaptive-like responses due to constant pathogen exposure. The concept of environmental memory has been proposed to persist for at least ten years in corals (Brown et al. 2015), and epigenetic modifications may be a driving factor.

The disease resistance experiments provide a gross identification of fragments with visible signs of RTL, but are limited in their ability to characterize the host response at the tissue and cellular level. Histology allows for a detailed observation of the different
coral cells and tissues. With the methodology used, a quantitative rating of tissue health was generated from observational characterizations of different cells and tissues. While disease resistance was variable among genotypes, the tissue conditions associated with diseased-fragment exposure were consistent throughout the histological analysis. Results showed worsening tissue health from pre- to post-diseased-fragment exposure samples, indicating that the application of the diseased fragment had a negative impact on tissue health for both fragments that developed tissue loss and those that did not. Similar to the histopathological observations of apparently healthy samples in Miller et al. (2014), all pre-exposure samples in our study were generally characterized by numerous, mildly hypertrophied mucocytes, minimal costal tissue loss, a thick layer of well-stained algal symbionts, and minimal degeneration of cnidoglandular bands and mesenterial filaments. Differences in the gastrodermal architecture of the BBW were observed between samples that eventually developed tissue loss and samples that did not. The BBW of samples with no eventual disease appeared similar to normal samples, with minimal swelling in the gastrodermis and slight atrophy of calicoblasts. Samples that eventually showed signs of RTL displayed mild to moderate hypertrophy and cell necrosis of the gastrodermis. This cell necrosis was caused by an accumulation of water in the cells, known as cellular swelling, and is one of the early signs of cellular degeneration in response to injury when membrane integrity is compromised (Kumar et al. 2017). Other histological studies have documented similar patterns of tissue degeneration beginning and progressing from interior to exterior tissues in the acroporid corals (E. Peters, pers. comm.). Preliminary degeneration of this tissue layer may indicate that colonies with less healthy internal BBW tissue structures are more susceptible to developing overt tissue loss.

Here, post-exposure samples with tissue loss were found to be significantly in poorer condition than samples that did not develop disease, meaning that the presence of disease reflected severe effects on tissue health. This difference was significant for seven out of eight individual parameters and was particularly evident in the epidermal mucocytes. Diseased samples were characterized by marked to severe atrophy of the epidermis, with either misshapen mucocytes or a complete loss of them, and necrosis becoming more apparent closer to the tissue loss margin. Mucus production is central to a coral’s immune defense system and is regarded as the primary form of innate immune
response (Mullen et al. 2004). Mucous secretory cells are typically abundant in the epidermis, and the release of large amounts of mucus combined with the activity of apical cilia on epidermal supporting cells gives these sedentary organisms the ability to remove sediment and trap or expel potential pathogens at their surfaces (Mullen et al. 2004, Reed et al. 2010). A study on the inhibitory properties of *A. palmata* mucus found greater antibacterial activity in mucus from unbleached colonies compared to bleached colonies. Mucus collected prior to bleaching inhibited human bacterial growth, but mucus collected from the same colonies during a bleaching event showed no inhibitory effect against the same bacteria (Ritchie 2006). A decline in the health and effectiveness of the mucociliary system is likely detrimental to a coral’s immune system and indicative of exposure to stress.

A coral’s response to elevated temperature stress depends on environmental pressures, colony genotype, and the species of *Symbiodinium* present in the tissue (Baker et al. 2008). This study found no difference in photosynthetic efficiency or histological tissue conditions between corals exposed to ambient and elevated water temperatures. These results might indicate that the tested genotypes harbor the same or similar distributions of *Symbiodinium* clades, and these symbionts possess greater heat tolerance properties, or that the corals have acclimated to increased temperatures due to repeated exposure. *A. cervicornis* has been found to host *Symbiodinium* from clades A, C, and D, but typically associates with species from clade A3: *S. ‘fitti’* (Thornhill et al. 2006). The predominance of a clade is largely dependent on depth (Baker et al. 1997, Baums et al. 2009, Lirman et al. 2014). In *A. cervicornis* colonies sampled from different reef locations along the Florida reef tract, clade A was found to dominate in deeper, forereef (12.7 m) colonies, whereas inshore (4.3 m) colonies hosted all three clades but were dominated by clade D (Baums et al. 2009). Shifting symbiont communities to more heat-tolerant species (clade D) has been documented in corals in the Caribbean and other parts of the world in response to bleaching stress (Glynn et al. 2001, Baker et al. 2004, Rowan 2004, Berkelmans and van Oppen 2006) and has been proposed as a mechanism of adaptation (Baker et al. 2004). Research has yet to determine if *A. cervicornis* can alter its symbiont community. All corals were collected from the CRF nursery where colonies are maintained on PVC trees at a depth range of 6–7.5 m, meaning that all colonies have
likely experienced very similar environmental conditions since the nursery was started in 2007. With the increasing frequency and severity of bleaching events, it is possible that these nursery corals are undergoing ‘experience moderated tolerance’ (Brown et al. 2000) by altering their symbiont communities in favor of more heat-resistant *Symbiodinium*. However, in a study of measured gene expression using RNA sequencing, colonies of *A. cervicornis* exposed to periodic temperature shocks showed greater temperature-based variation of gene expression in the host compared to the symbiont, but ultimately the symbiont strain was no more likely than the host genotype to predict expression levels (Parkinson et al. 2018). A more likely explanation is that the experimental corals were acclimated to elevated water temperatures due to increasingly warm summers. During a reciprocal transplant study, colonies of Pacific *A. hyacinthus* from different reef locations were shown to develop increased bleaching resistance when exposed to increased temperatures for a period of 27 months (Palumbi et al. 2014). Sea surface temperatures during the 2016 summer months prior to experimentation ranged from 29–31°C, and temperatures during the 2015 summer months ranged from 28–32°C (NOAA Coral Reef Watch 2018). Yetko 2018 exposed *A. cervicornis* genotypes from the same CRF nursery to heat stress (32°C) and saw a decline in photosynthetic efficiency beginning at day 8. The experimental temperature maximum (32°C) and short exposure period (3 days) likely did little to evoke a stress response.

The multi-genotypic outplanting experiment found no differences in mortality, disease prevalence, and predation between the different cluster designs, although the lack of variation may be attributed to the short 4-month monitoring period. An *A. cervicornis* outplant study monitored from 2013–2015 found higher percent survival in multi-genotypic clusters compared to monoclonal clusters, but found no difference in disease prevalence between cluster types (Ware 2015). Although no advantage was found in using a particular multi-genotypic outplant design in this study, a key practice in conservation and restoration work is to enhance the genetic diversity of a population, thus diversifying the population’s response to stress (Baums 2008). If the responses of the 12 genotypes from the replicated pathogen transmission experiment are pooled to represent a population (n = 60), then 37% of the population was resistant to developing tissue-loss disease. The greatest proportion of disease resistance within an individual genotype was
60% and the lowest was 0%. While multi-genotypic disease resistance was not as high as some of the individual genotypes, this is only a measure of one attribute and doesn’t include other characteristics such as bleaching, growth rates, survival, reproduction, etc., which are all critical factors when measuring coral performance. It will likely be advantageous for coral restoration programs to use genotypes with disease resistance, thermal tolerance, and high growth rates, but emphasis should remain to collect, maintain, and outplant a genetically diverse population of coral colonies to increase the potential disease resistance within the population and reduce the likelihood of widespread mortality due to a single disease outbreak event.

Caribbean Acropora corals are commonly found in large, high density thickets, with multiple colonies growing directly next to or in contact with one another. This growth pattern creates an intricate reef structure but also provides an easy pathway for contagious pathogens to spread to different coral colonies, especially as may be the case in tissue loss diseases like WBD and RTL. The within-colony variability demonstrated in our experiments could indicate that while certain colonies may be susceptible to developing disease they are still likely to survive a disease-outbreak event because some parts of the colony are resistant. Future work should begin to investigate resistance variability of different fragment types from the same colony. Some portions of a colony or its branches may experience disease-related mortality while other portions do not, which could serve the purpose of arresting tissue loss and allowing for the continued survival of the colony and genotype.

The results of this study have provided the first evidence of disease resistance in Florida Keys A. cervicornis populations and help to explain the persistence of these and other acroporid corals during increasingly prevalent disease epizootics and bleaching events. Continued research into the etiologic agent(s) of RTL and other tissue-loss diseases is imperative as pathogen identification is a key component to understanding disease. The high degree of disease resistance variability found within and among genotypes suggests that genotype is not the only factor influencing stress responses. Additionally, the lack of variation between corals exposed to ambient and elevated seawater temperatures could indicate that these genotypes have begun to develop more heat-tolerant properties. Future investigations of disease and bleaching resistance will
benefit from incorporating measurements of gene expression and somatic mutations, as epigenetics is now shown to play an integral role in an organism’s response to varying environmental pressures. Identifying the mechanisms that control within-colony variability will be critical in understanding how future disease and bleaching events will affect wild populations, thus guiding more effective management practices.

Coral reefs are among the most important marine ecosystems, both from a biological and economic perspective. Their persistence will depend on a combination of global and local action to reduce anthropogenic-related stressors, maintain genetic diversity, and properly manage and restore natural populations using scientifically based decision making.
Literature Cited


Levitan, D. R., Fogarty, N. D., Jara, J., Lotterhos, K. E., & Knowlton, N. 2011. “Genetic, spatial, and temporal components of precise spawning synchrony in reef building


### Appendix A

CRF genetic IDs and updated experimental IDs based on microsatellite analysis.

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Appendix B

Example images of the four treatments used in the pathogen transmission screening experiment

| C1: asymptomatic fragment attached | C2: cable tie only | C3: nothing attached | D: RTL fragment attached |
Appendix C

Contamination Safety Protocol

Precautions were taken to eliminate the potential of researchers becoming vectors of disease. CRF designated a research area along the eastern edge of the nursery that helped isolate the experimental trees and limit the potential for pathogen transmission to nursery corals. All control fragments were handled before all diseased fragments. Diseased treatments were done in separate trees and initiated last. Divers handling diseased corals used surgical gloves; after handling diseased corals, the gloves were removed underwater and placed in watertight zip-lock bags. The bags were transferred to the surface, placed in a second waterproof container, and then disposed onshore. Divers working with diseased corals were not allowed to handle healthy corals the same day, either at the experimental site or in the nursery. Two sets of instruments/materials were designated as “control” and “diseased,” and used on the respective fragments. Diseased fragment-exposed instruments were soaked in a 10% bleach solution after each set of pathogen-transmission experiments. After the experimentation period, remaining fragments not used for histology were disposed of in the nursery’s trash pile where CRF disposes other dead or diseased coral colonies. The PVC trees used for the transmission experiments were removed from the nursery and treated with a 10% bleach solution onshore. Further precautions were taken to sterilize SCUBA equipment with a 10% bleach solution as well.
Appendix D

Glossary of Terms and Definitions (Peters 2016)

**Calicodermis**
The ectodermally derived epithelium that assists in building the calcified skeleton of scleractinians after the planula settles on the substratum.

**Cnidoglandular band**
The distal thickened rim or free margin of a complete mesentery in the gastrovascular cavity below the actinopharynx.

**Basal body wall**
The layer of tissue in contact with the skeleton that includes interior gastrodermis, mesoglea, and calicodermis.

**Epithelium**
A singular sheet of cells packed together (usually hexagonal in cross or transverse section) whose membranes are bound together along their sides by various junctions and cementing substances to provide strength and mediate the exchange of metabolic and messenger molecules and attached along their bases to a basement membrane.

**Gastrodermis**
Inner epithelium that lines the gastrovascular cavity and canals, and contains the zooxanthellae.

**Mesenterial Filaments**
The free rounded edge of a complete mesentery below the actinopharynx that is thickened and modified into a central extremely elongated “ruffled” structure.

**Mesentery**
Internal tissue partitions that extend from the polyp body wall that provide structural support for the polyp.

**Mesoglea**
Primitive connective tissue sandwiched between the epithelia. The mesoglea supports the epidermis and gastrodermis along the surface, or gastrodermis and calicodermis along the base, or gastrodermis and gastrodermis lining the mesenteries.

**Mucocyte**
Unicellular gland cells that secrete mucus through an apical pore to aid in protection, sediment removal, and feeding.

**Surface body wall**
The layer of tissue in direct contact with seawater that includes epidermis, mesoglea, gastrodermis tentacles, oral disc, peristome, and polyp column.

**Epidermis**
The surface of the coral in contact with seawater, including that of the polyps and coenenchyme, covered by a layer of simple columnar or pseudostratified columnar epithelium.
Appendix E

Histology scoring rubric developed by Dr. Esther Peters, Megan Bock, and Morgan Hightshoe. Modified from Miller et al. 2014. Characteristics noted in cells and tissues using light microscopic examination of *A. cervicornis*.

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<tr>
<th>Parameters with “normal” tissue descriptions</th>
<th>Numerical Condition Score</th>
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<td><strong>High Magnification (40-60x)</strong></td>
<td><strong>Minimal</strong></td>
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<td>Epidermal Mucocytes</td>
<td>Slightly hypertrophied, numerous, pale-staining frothy mucus. Ciliated supporting cells still very abundant.</td>
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<tr>
<td>Costal Tissue Loss</td>
<td>Atrophy of epidermis, mesoglea, and calicodermis, but still intact over costae. Minimal costae exposed.</td>
</tr>
<tr>
<td>Parameters with “normal” tissue descriptions</td>
<td>Numerical Condition Score</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Zooxanthellae in SBW (40-60X)</td>
<td></td>
</tr>
<tr>
<td>0 = Gastrodermal cells packed</td>
<td></td>
</tr>
<tr>
<td>with well-stained algal symbionts</td>
<td></td>
</tr>
<tr>
<td>in surface body wall, tentacles;</td>
<td></td>
</tr>
<tr>
<td>scattered algal symbionts deeper in</td>
<td></td>
</tr>
<tr>
<td>gastrovascular canals and absorptive cells</td>
<td></td>
</tr>
<tr>
<td>next to mesenterial filaments.</td>
<td></td>
</tr>
<tr>
<td>0 (No Change)</td>
<td>1</td>
</tr>
<tr>
<td>Similar to 1970s samples, thick layer of</td>
<td>2</td>
</tr>
<tr>
<td>well-stained algal symbionts in</td>
<td>3</td>
</tr>
<tr>
<td>gastrodermis of surface body wall,</td>
<td>4</td>
</tr>
<tr>
<td>tentacles, and scattered cells in</td>
<td>5</td>
</tr>
<tr>
<td>gastrovascular canals and absorptive cells</td>
<td></td>
</tr>
<tr>
<td>next to mesenterial filaments.</td>
<td></td>
</tr>
<tr>
<td>thickness of well-stained algal</td>
<td></td>
</tr>
<tr>
<td>symbionts, but not quite as abundant as in</td>
<td></td>
</tr>
<tr>
<td>1970s samples. Mild atrophy of zoonaxthellae</td>
<td></td>
</tr>
<tr>
<td>and gastrodermis.</td>
<td></td>
</tr>
<tr>
<td>Thal algal symbionts, but not quite as</td>
<td></td>
</tr>
<tr>
<td>abundant as in 1970s samples. Mild atrophy</td>
<td></td>
</tr>
<tr>
<td>of zooxanthellae and gastrodermis.</td>
<td></td>
</tr>
<tr>
<td>Single row of algal symbionts in surface</td>
<td></td>
</tr>
<tr>
<td>body wall gastrodermis and markedly fewer</td>
<td></td>
</tr>
<tr>
<td>in tentacle gastrodermis, some are</td>
<td></td>
</tr>
<tr>
<td>missshapen, shrunken, or have lost</td>
<td></td>
</tr>
<tr>
<td>acidophilic staining as proteins are no</td>
<td></td>
</tr>
<tr>
<td>longer present or nucleus/cytoplasm has</td>
<td></td>
</tr>
<tr>
<td>lysed, accumulation body (vacuole) enlarged</td>
<td></td>
</tr>
<tr>
<td>compared to algal cell or missing.</td>
<td></td>
</tr>
<tr>
<td>No zooxanthellae present in cuboidal</td>
<td></td>
</tr>
<tr>
<td>gastrodermal cells of colony (bleached).</td>
<td></td>
</tr>
<tr>
<td>Cnidoglandular Band Epithelium Mucocytes</td>
<td></td>
</tr>
<tr>
<td>0 = Oral portion lacks mucocytes,</td>
<td></td>
</tr>
<tr>
<td>increasing in number aborally, may be</td>
<td></td>
</tr>
<tr>
<td>abundant with pale mucus; difficult to</td>
<td></td>
</tr>
<tr>
<td>assess significance of appearance</td>
<td></td>
</tr>
<tr>
<td>Less than half the area of</td>
<td></td>
</tr>
<tr>
<td>cnidoglandular band is mucocytes, but could</td>
<td></td>
</tr>
<tr>
<td>be more depending on location along the</td>
<td></td>
</tr>
<tr>
<td>filament, size of mucocytes variable</td>
<td></td>
</tr>
<tr>
<td>(seen in one or a few cnidoglandular bands)</td>
<td></td>
</tr>
<tr>
<td>About half the area is mucocytes, some</td>
<td></td>
</tr>
<tr>
<td>hypertrophied (seen secretions in % of</td>
<td></td>
</tr>
<tr>
<td>cnidoglandular bands)</td>
<td></td>
</tr>
<tr>
<td>About half the area is mucocytes, all</td>
<td></td>
</tr>
<tr>
<td>hypertrophied (seen in % of cnidoglandular</td>
<td></td>
</tr>
<tr>
<td>bands)</td>
<td></td>
</tr>
<tr>
<td>About three quarters of the area is</td>
<td></td>
</tr>
<tr>
<td>mucocytes, mucus production reduced, some</td>
<td></td>
</tr>
<tr>
<td>vacuolation and necrosis present (seen in</td>
<td></td>
</tr>
<tr>
<td>% of cnidoglandular bands)</td>
<td></td>
</tr>
<tr>
<td>Loss of mucocytes, vacuolation and necrosis</td>
<td></td>
</tr>
<tr>
<td>of most cells present (seen in majority of</td>
<td></td>
</tr>
<tr>
<td>cnidoglandular bands)</td>
<td></td>
</tr>
<tr>
<td>Parameters with “normal” tissue descriptions</td>
<td>0 (No Change)</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Degeneration of Cnidoglandular Bands</strong></td>
<td></td>
</tr>
<tr>
<td>0 = Ciliated columnar cells, nematocytes, acidophilic granular gland cells, and mucocytes abundant (but varying with location), tall, thin columnar, contiguous, terminal bar well formed</td>
<td>Mild reduction in cell height in one or a few areas</td>
</tr>
<tr>
<td><strong>Dissociation of Cells on Mesenterial Filaments</strong></td>
<td>Minimal loss of cilia, but will not be present where mucocytes are predominant in one or few areas</td>
</tr>
<tr>
<td>Parameters with “normal” tissue descriptions</td>
<td>Numerical Condition Score</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td><strong>0 (No Change)</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>Gastrodermal Architecture (BBW)</strong></td>
<td>2</td>
</tr>
<tr>
<td>0= Gastrodermis in BBW is uniform, no apparent swelling, scattered zooxanthellae present but not as abundant as SBW (similar to 1976 controls). Thickness of gastrodermis variable based on lipid droplet formation. Swelling indicative of potential intrusion, lysing, necrosis not seen.</td>
<td>3</td>
</tr>
<tr>
<td>None to a few areas of swelling and cell lysing in gastrodermis, scattered zooxanthellae but less than controls</td>
<td>4</td>
</tr>
<tr>
<td>½ of gastrodermis is swollen, cell lysing present, less zooxanthellae and some released into gastrovascular canals</td>
<td>5</td>
</tr>
<tr>
<td>¾ of gastrodermis is swollen, few areas of necrotic tissue, zooxanthellae abundance reduced by ½ or ½ released into gastrovascular canals</td>
<td>¼ of gastrodermis is swollen, necrotic tissue, zooxanthellae abundance reduced by ½ or ½ released into gastrovascular canals</td>
</tr>
<tr>
<td>Entire BBW gastrodermis is necrotic, extreme swelling is visible, few to no zooxanthellae present or majority of zooxanthellae released into gastrovascular canals</td>
<td></td>
</tr>
<tr>
<td><strong>Calicodermis Condition</strong></td>
<td>1</td>
</tr>
<tr>
<td>0 = Calicoblasts numerous both peripherally and internally, squamous but thick cytoplasm</td>
<td>2</td>
</tr>
<tr>
<td>Calicoblasts slightly reduced in height focally (more likely interior of colony, basal body wall) more squamous</td>
<td>3</td>
</tr>
<tr>
<td>About half of calicoblasts atrophied, loss of proteins in cytoplasm. Calicoblasts reduced in number</td>
<td>4</td>
</tr>
<tr>
<td>Most calicoblasts atrophied, fewer in number, spread out thinly on mesogelea, still cuboidal to columnar and active under surface body wall and in apical polyps</td>
<td>5</td>
</tr>
<tr>
<td>Basal and surface body wall calicoblasts severely atrophied or vacuolated, detaching and sloughing, or missing entirely from mesogelea</td>
<td></td>
</tr>
</tbody>
</table>