EFFECTS OF TEMPERATURE ON ZOOXANTHELLAE STRAINS IN CORAL SPECIES *Rhodactis rhodostoma*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Effects of temperature on Zooxanthellae strains in coral species Rhodactis rhodostoma.
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Coral reefs are among the most diverse and fragile ecosystems in the ocean. Corals living in lighted zones can have a mutualistic symbiosis with algal dinoflagellates known as Zooxanthellae, specifically the genus *Symbiodinium.* In the event of stress from the environment (e.g. greater water temperature, acidity, or light permeation), coral can expel Zooxanthellae from their tissues in a process called bleaching. Bleaching is reversible, and it is possible for corals to repopulate with Zooxanthellae strains that are genetically distinct from the original symbiont and thus potentially better suited for the stressing environment. To determine whether this process of bleaching and repopulation could be studied in a small volume captive recirculating system, I took tissues samples from the soft coral *Rhodactis rhodostoma* held in the Great Aggie Reef, a 135 gallon reef display tank in Texas A&M University's Biology Department, before and after exposure to increased temperature stress. To determine whether Zooxanthellae were expelled, I used a polymerase chain reaction-based technique to detect the presence of the symbiont-specific ITS1 gene in coral tissue. Whereas more rapid temperature increases were fatal to corals, a gradual increase to 32 degrees was successful in inducing a loss of ITS1 gene expression in surviving corals. These results suggest that this recirculating system may be suitable for further study of coral bleaching and repopulation with Zooxanthellae under controlled conditions.

DEDICATION

This Undergraduate Research Scholar's thesis is dedicated to those who have supported my project, including the MacKenzie lab, family, and friends.

ACKNOWLEDGEMENTS

Thank you to my research advisor and mentor, Dr. Duncan MacKenzie, for the support, leadership, and assistance provided to me with this project. Thank you also to Dr. Richard Jones and Dr. Bill Cohn for their support and guidance throughout this project, as well as Lewis Weil for his inspiration and support.

CHAPTER I

INTRODUCTION

The symbiosis between cnidarians and Zooxanthellae algae is a cornerstone of the formation coral reefs. Zooxanthellae (more specifically of the genus *Symbiodinium*) are dinoflagellates that live within the gastrodermal cells of soft and hard bodied corals, anemones, and other invertebrates. These organisms assist in the nutrition of the coral, providing the products of photosynthesis for host consumption as the host provides protection and inorganic nutrients to the symbiont[1]. The algal symbiont now partnering with corals is likely evolved from a single adaptive radiation[2]. As a result, *Symbiodinium* is composed of several clades, each with characteristic species. The symbiotic dinoflagellate genus *Symbiodinium* is genetically diverse, containing eight divergent lineages (clades A–H). Corals predominantly associate with clade C *Symbiodinium* and to a lesser extent with clades A, B, D, F, and G[3]. Different clades usually live at different depths and light intensities, and multiple clades of these symbionts are known to inhabit the same coral species[4].

This relationship between corals and their symbionts can change dramatically in response to environmental stimuli. Corals are known to "bleach", a process in which the coral expels the Zooxanthellae in response to environmental stressors. If the coral goes too long without its symbiont it will die. The most common stressor in natural ecosystems is temperature. Accordingly, the potential impacts of climate change on coral reef ecosystems are numerous, but the most devastating effects to date have been large-scale and severe episodes of coral bleaching[5,6]. As the temperature of surface ocean water increases above yearly maximums, the

corals bleach by expelling their symbionts[7]. Increasing frequency and severity of mass bleaching events is linked to climate change, making the longer-term future of coral reefs concerning[8,9]. Coral bleaching, however, is reversible. Zooxanthellae from the environment are capable of repopulating corals and reestablishing the symbiotic relationship, potentially with environmentally better-suited clades[10-12]. Little is known about this process of repopulation. For example, it is not known if the same strain of Zooxanthellae repopulates a coral, or if clade diversity is introduced during repopulation. If the corals within a system are able to repopulate with a Zooxanthellae strain that is better adapted to function in their altered environment, the coral may not bleach as easily and the coral may thus be better able to survive increased environmental temperatures. However, few controlled experimental studies of coral bleaching have been conducted to examine this question because of the challenges associated with keeping these animals in captivity.

In order to examine how temperature impacts coral symbiont diversity we must first be able to distinguish the existence of, and differences between, individual Zooxanthellae strains before, during, and after coral bleaching. This can be done by sequencing the DNA of Zooxanthellae coding for conserved ribosomal proteins. I proposed to utilize the van Oppen et al.[13] protocol for the ITS1 internal spacer gene associated with ribosomal proteins to characterize the changes in symbiont presence and diversity in coral in response to an environmentally-realistic temperature increase in a controlled system, the Great Aggie Reef aquarium in the Biological Sciences Building East at Texas A&M University. This aquarium system provides a unique opportunity to determine whether a controlled, captive environment can be successfully employed to examine effects of coral bleaching on Zooxanthellae diversity.

The objectives for my study were to confirm first that techniques for describing coral symbiont presence developed for wild populations can be applied to coral in the Great Aggie Reef. I hypothesized that I would be able to establish the presence of Zooxanthellae within coral tissues using established molecular techniques for identifying the ITS1 ribosomal subunit gene. From this, I further hypothesized that initial experimental coral samples would indicate a presence of ITS1 ribosomal subunit gene. I then examined whether a temperature increase results in loss of symbionts, whether this loss was associated with coral pigmentation loss, and whether Zooxanthellae would successfully repopulate the coral after bleaching.

I hypothesized that with heat treatments coral bleaching could be induced in my system and that this bleaching would cause a loss in coral pigmentation. Finally, I hypothesized that using the molecular techniques I would find no symbiont presence in the experimentally bleached coral tissue but confirm symbiont presence after recovery. I also hoped to examine whether *Symbiodinium* diversity changed during repopulation by determining which clades of *Symbiodinium* were present before and after bleaching.

The Great Aggie Reef aquarium is a temperature controlled 135 gallon marine system that has continuously sustained specimens of the soft coral *Rhodactis* for over 20 years. Evaluating the diversity of Zooxanthellae strains in aquarium corals promises insights into the general stability of the coral-algal relationship[14]. If the present study showed a repopulation event with a significant difference in strains the results could be useful in suggesting approaches for restoring bleached areas of coral reef ecosystems with better suited clades or strains of *Symbiodinium*.

CHAPTER II

METHODS

Coral Bleaching

The Great Aggie Reef, a 135 gallon reef tank containing both vertebrate and invertebrate species, is held at 25˚C and between 32-35 ppt salinity, and is fed and cleaned daily. *Rhodactis* coral specimens residing in the Great Aggie Reef tank were removed by hand and transferred into adjacent 5 gallon experimental and 10 gallon control tanks. Both tanks contained a 50:50 mix of Reef tank water and fresh Instant Ocean salt water to maintain nutrients from the main tank and allow clean water for the corals. Temperature in all tanks was controlled by JAGER 3604 aquarium heaters. For the initial protocol, the control tank was maintained at 25˚C for the duration of the experiment, whereas the experimental tank began at 34˚C. The second heat treatment protocol began with both tanks held at 25˚C. After an 18 hour acclimation period, the control tank was kept at 25˚C for the remainder of the experiment and the experimental tank was increased 2˚C every 24 hours until reaching 32˚C, which was maintained until coral showed pigmentation loss. The final heat treatment protocol began with both tanks held at 25˚C for an 18 hour acclimation period. The control tank was kept at 25˚C for the remainder of the experiment while the experimental tank was increased 1˚C every 24 hours until 32˚C was reached and maintained for 48 hours, a timeline established by Strychar et al.[15] After 48 hours the water temperature was decreased by 1˚C until 25˚C was reached and the coral, now termed the recovered coral, was returned to the main tank. Coral health was visually assessed throughout all three protocols, checking for the discoloration or folding of the main body of the coral that precedes coral death, which is distinguished by loss of body structure and movement.

Tissue Collection and RNA Extraction

Tissue sample and TRIzol (Ambion, NY USA) reagent RNA extraction were done according to a combination of Rosic[16] and MacKenzie lab protocol[16,17]. Tissue samples, approximately 2.5 cm by 0.5 cm, were excised from the main body of both experimental and control corals using sterile scissors and placed directly in to a mortar cooled to -80˚C. Enough liquid nitrogen was poured directly into the mortar to submerge the sample, which was then ground into a very fine powder with a chilled pestle. The powdered sample was placed into centrifuge tube containing 1 ml of Instant Ocean saltwater and centrifuged twice for 30 min at 2,000 rpm discarding the supernatants after each round [18]. One ml of TRIzol reagent (Ambion, NY USA) was added to the final pellet of each sample, which was then incubated at room temperature for 5 minutes. Chloroform (0.2 ml) was added and the samples were shaken by hand for fifteen seconds followed by 3 minutes of room temperature incubation. Samples were then centrifuged at 14,000 rpm for 15 minutes to separate the phases. The upper phase was removed and placed into a centrifuge tube containing 0.5 ml of isopropyl alcohol and incubated at room temperature for 10 minutes. The samples were then centrifuged at 14,000 rpm for 10 minutes and the supernatant was discarded. The sample pellet was washed with 0.5 ml of 100 ethanol then vortexed. Samples were centrifuged at 14,000 rpm for 5 minutes, the ethanol was decanted, and they were allowed to air dry for 10 minutes. The sample pellets were then washed with DEPC water (10-15 µL) until dissolved. Concentration of RNA present in sample was calculated using Nanodrop technology (Thermo Scientific, Asheville, NC).

Reverse-transcription -PCR

Samples were treated with DNAse before reverse transcription using a recombinant DNAse I kit (Ambion, NY USA). For each sample 2 µg of RNA was added to 1 µL of DNAse and 1 µL of DNAse buffer. DEPC-treated H_2O was added to a total volume 12 µL for each sample. This mixture was incubated at 37˚C for 30 min then 3 µL of EDTA was added. The samples were then incubated in a 70˚C water bath for 10 minutes before use in reverse transcription. DNAse-treated RNA was converted into complementary DNA (cDNA) with a high capacity cDNA reverse transcription kit (Invitrogen NY, USA) using the manufacture's protocol: 5 µL 10X RT buffer, 2 µL 25X dNTP mix, 8 µL 10X RT random primers, 2.5 µL oligo dT, RNA equal to 2 µL, 2.5 µL reverse transcriptase, and sufficient autoclaved H_2O to equal 50 µL. The Applied Biosystems 2720 Thermocycler PCR protocol was 25˚C for 10 minutes, 37˚C for 120 minutes, 85˚C for 5 minutes, and a 4˚C hold. Samples were then stored at -20˚C.

PCR

To amplify the Zooxanthellae ITS1 region, I utilized dinoflagellate-specific primers designed by van Oppen et al.[13]: symITSFP (5'-CTCAGCTCTGGACGTTGYGTTGG-3') forward and symITSRP (5'-GCTGCGTTCTTCATCGATGC-3') reverse. These primers have been used to amplify my ITS1 target region previously in relevant literature [13]. All primers were ordered from Integrated DNA Technologies (Coralville, IA). For the PCR reaction, each sample was prepared using 10 µL GoTaq from the GoTaq Green kit (Promega, WI, USA), 2 µL of the sample RT reaction, 2 µL random primers, and 4 µL of forward and reverse ITS1 primers. The mixture was brought up to 20 µL with ddH_2O. A no template control was used with 2 µL ddH_2O in place of cDNA. The thermocycler settings were: 1 cycle of 95˚C for 1 minute; 40 cycles of 95˚C for 30

minutes, annealing temperature of 52˚C for 30 seconds, and 72˚C for 60 seconds; 1 cycle of 72˚C for 5 minutes; and hold at 4˚C for up to 4 hours, then store at -20˚C.

DNA Gel Electrophoresis

For a 1% gel, 0.5g of agarose (GeneMate, bioexpress.com) was dissolved in 49 mL of ddH_2O and 1 mL of 50XTAE (Tris Acetic Acid EDTA). After cooling, 2 µL ethidium bromide was added and swirled to mix. The mixture was poured into an ethanol sterilized and ddH_2O rinsed 8x10 cm gel tray with comb and left at room temperature for 45 minutes to solidify. The gel tray was then placed into 500 mL of TAE running buffer in a sterilized gel box. The comb was removed gently so as not to tear the wells. PCR samples were thawed by hand and the entire 20 µL volume was loaded into the gel. The DNA Ladder was mixed with 1 µL of 100bp ladder (New England BioLabs, MA, USA), 2 µL of 6XDNA loading buffer (New England, MA, USA), and 9 µL ddH_2O. Gels were run at 94V until the loading dye was at the bottom of the gel. The gel tray was removed from the gel box and visualized under UV light.

CHAPTER III

RESULTS

Coral Bleaching

Rhodactis control coral showed no visual signs of bleaching or distress (Figure 1A), and was successfully returned to the Great Aggie Reef tank. During the initial heat treatment protocol, the experimental coral died within 24 hours of 34˚C exposure. In the second heat treatment experiment, the experimental coral (Figure 1B) visually lost color after 72 hours in 32˚C water (Figure 1C), and died from suspected heat stress while returning to 25˚C before post stress tissue samples could be obtained. In the third heat treatment protocol the experimental coral (Figure 2A) did not lose visible pigmentation after 48 hours of 32˚C water (Figure 2B), but did exhibit signs of expelling Zooxanthellae. I observed a white, opaque stream of slime from its tissues and a visible green cast to the aquarium water. The coral was then successfully returned to 25 ˚C and transferred back to the main tank after 96 hours, with samples obtained from the recovered coral 13 days later for RNA extraction.

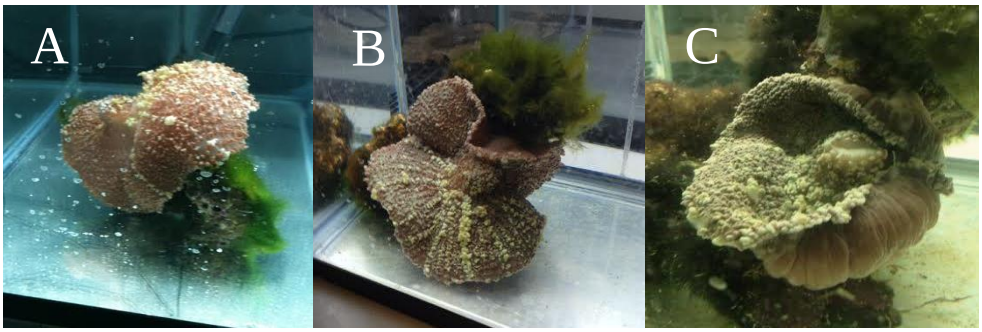

Figure 1. Effects of 2˚C daily temperature change on coral appearance. A. Control coral. B. Initial experimental coral before heat treatment of 2˚C temperature increase per day to 32˚C. C. Experimental coral after heat treatment of 2˚C temperature increase per day to 32˚C for 72 hours.

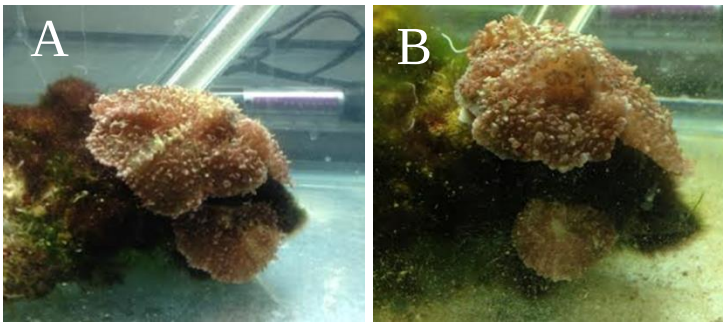

Figure 2. Effects of 1˚C daily temperature change on coral appearance. A. Initial experimental coral before heat treatment of 1˚C temperature increase per day to 32˚C. B. Experimental coral after heat treatment of 1˚C temperature increase per day to 32˚C for 48 hours.

RNA Extraction

RNA yield was calculated using Nanodrop technology. The mean of four measurements of RNA extracted from initial tissue samples was 1.78 for the 260/280 ratio, within the 1.6-2.2 range considered acceptable, and the concentration was 893.4 ng/µL, calculated to 2.2386 µL of sample to equal 2g of RNA[17], yielding enough for reverse transcription. RNA yield for two bleached tissue samples was also calculated using Nanodrop technology. The mean of four measurements of RNA extracted from two bleached tissue samples was 1.68 and 1.65 for the 260/280 ratio, within the 1.6-2.2 range considered acceptable, and the concentrations were 808.25 and1808.75 ng/µL, calculated to 2.49 and 1.12 µL of sample to equal 2g of RNA[17], yielding enough for reverse transcription. RNA yield for two recovered coral tissue samples was also calculated using Nanodrop technology. The mean of four measurements of RNA extracted

from the two recovered tissue samples was 1.72 and 1.61 for the 260/280 ratio, within the 1.6-2.2 range considered acceptable, and the concentrations were 571 and 382.9 ng/µL, calculated to 3.5 and 5.22 µL of sample to equal 2g of RNA[17], yielding enough for reverse transcription.

DNA Electrophoresis and Gel Band Extraction

Using the ITS1 primers, an amplified product of approximately 300bp was expected. The gel of the initial experimental coral sample gel showed a bright, single band at approximately 300bp (Figure 3). The bleached experimental coral sample gel (Figure 4) showed no band at approximately 300bp, while the initial experimental coral tissue showed the appropriate 300bp band as a positive control. The recovered coral sample gel (Figure 5) also showed no 300bp band while having a 300bp band in the positive control.

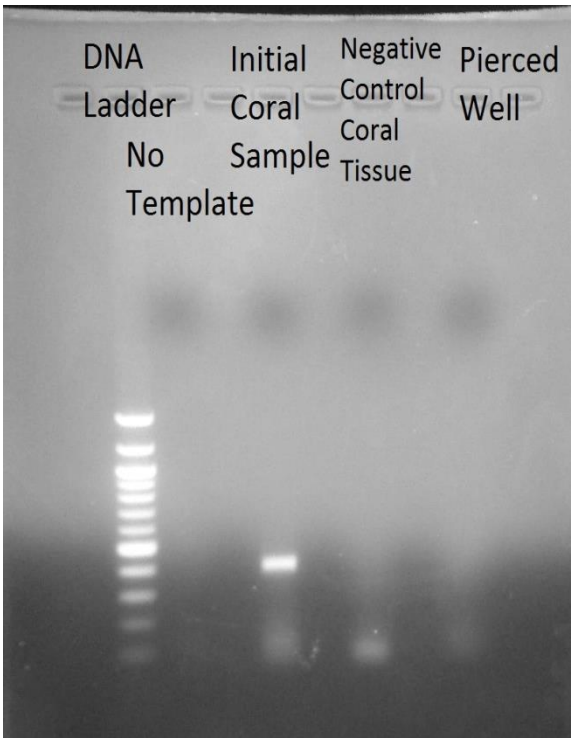

Figure 3. DNA agarose gel showing presence of ITS1 band . Lane 1: DNA ladder. Lane 2: No template control. Lane 4: UV visualized band at approximately 300bp from control *Rhodactis* tissue. Lane 6: Extracted *Rhodactis* tissue for negative control. Lane 8: Pierced well with no sample present.

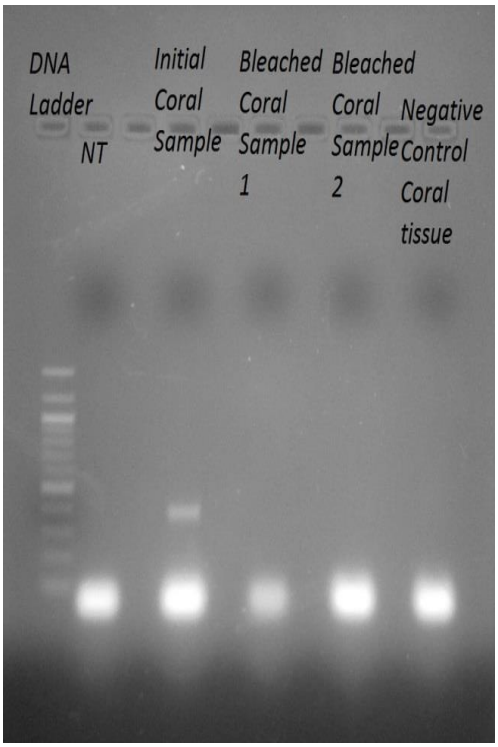

Figure 4. DNA agarose gel showing disappearance of ITS1 band following heat stress.. Lane 1: DNA ladder. Lane 2: No Template control. Lane 4: UV visualized band at approximately 300bp from control *Rhodactis* tissue. Lane 6: Experimentally bleached coral tissue sample 1 showing no ITS1 band. Lane 8: Experimentally bleached coral tissue sample 2 showing no ITS1 band. Lane 9: Purified *Rhodactis* tissue negative control.

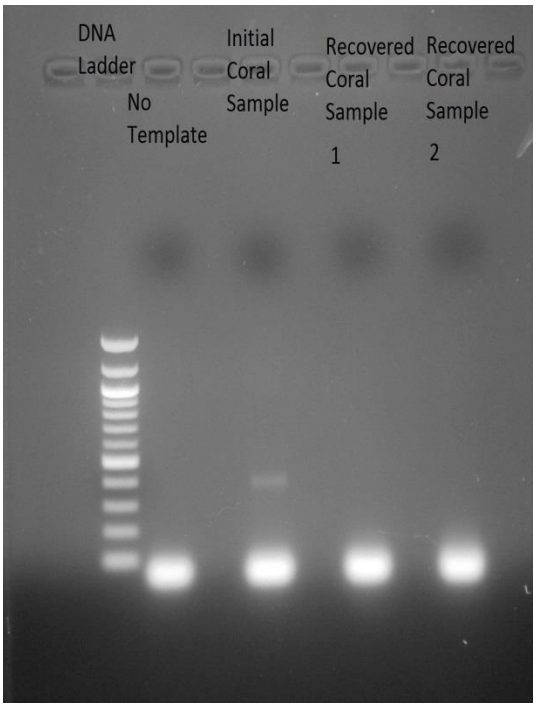

Figure 5. DNA agarose gel showing the continued loss of ITS1 band following 13 day recovery after heat-stress. Lane 1: DNA ladder. Lane 2: No Template control. Lane 4: UV visualized band at approximately 300bp from control *Rhodactis* tissue. Lane 6: Experimentally recovered coral tissue sample 1 showing no ITS1 band. Lane 8: Experimentally recovered coral tissue sample 2 showing no ITS1 band. Lane 9: Purified *Rhodactis* tissue negative control.

Cloning and sequencing

Attempts to sequence the ITS1 gene to achieve identification of Zooxanthellae strains within my captive *Rhodactis* tissues using cloning protocols are ongoing, and I hope to be able to identify the Zooxanthellae clades by comparing ITS1 sequences to established clade sequences in GenBank. If successful, the same procedures will be performed on additional bleached corals to attempt to determine diversity of Zooxanthellae within the Great Aggie Reef tank.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The symbiotic relationship between Zooxanthellae and coral is the foundation for entire ecosystems[1], and to address concerns growing about global climate change's effects on the world's coral reefs[9] my study was designed to determine whether bleaching could be studied in captive coral environments containing high animal diversity. I first hypothesized that I would be able to establish the presence of Zooxanthellae within coral tissues using established molecular techniques for identifying the ITS1 ribosomal subunit gene. Next I attempted several heat treatments to determine whether coral tissue bleaching could be induced in my system. Finally, I applied the molecular techniques to determine whether they could be used to establish symbiont presence in the bleached or recovered coral tissue.

My observation of the presence of an amplified ITS1 band in initial coral tissue confirms my hypothesis that I can verify the presence of Zooxanthellae in the tissues of my *Rhodactis* captive species using the van Oppen et al.[13] technique with ITS1 primers. After the first two heat treatments resulted in coral death I attempted a less intense treatment by reducing the temperature change to 1˚C per day and limiting to only 48 hours of exposure to 32˚C water. This heat treatment did not cause the coral to die but it also did not alter the visible pigmentation of the coral tissues, suggesting no Zooxanthellae expulsion. This refutes my hypothesis that the coral will lose coloration as a result increased temperature exposure. However the RT-PCR results showed a loss of the ITS1 band in this heat-exposed tissue and in the 13 day recovered coral tissue, suggesting expulsion had occurred. I am confident in this result, as I included

positive initial coral sample controls and negative purified coral tissue controls on my gels. This suggests that visual changes in coral coloration may be dissociated from the presence of mRNA for ITS1.

Two possibilities may explain the conflicting results between the coral tissue color and molecular data. ITS1 has become an accepted marker for Zooxanthellae presence because it is an internal spacer region for ribosomal RNA which is normally constitutively expressed for survival[13]. The RT-PCR technique I used only detects the presence of mRNA and therefore the possibility exists that at the Zooxanthellae are down-regulating ITS1 gene expression as a response to heat stress. This would cause a disappearance of the 300bp gel band because there would be no ITS1 cDNA for the primers to amplify. Perhaps heat-stressed Zooxanthellae down-regulate ITS1 expression to enter a type of dormant phase without expulsion, but this is currently undocumented. Alternatively, the Zooxanthellae may in fact truly have been expelled without pigmentation loss after 48 hours of expulsion. Alternative heat-resistant algae or bacteria within coral tissues might provide visual pigmentation, but this is also currently undocumented. To resolve these conflicts, I propose directly extracting and detecting Zooxanthellae DNA, as opposed to mRNA that is influenced by expression effects. The tissue sample collected 13 days after the final heat treatment also did not show a 300bp band, indicating the Zooxanthellae symbionts had not yet recovered after this time period. This suggests a 13 day recovery period is insufficient for Zooxanthellae repopulation, or possibly too short to allow reinitiation of expression of the ITS1 gene. Further studies are needed to determine exactly when the captive *Rhodactis* coral will recover its algal population. Unfortunately, gel band Sanger sequencing and

cloning procedures have thus far failed to produce any sequence data, and the diversity aspect of this project is ongoing as a future direction.

In conclusion, I was able to show the existence of Zooxanthellae in my captive corals using molecular techniques developed for wild corals and establish that a gentle temperature increase is necessary to maintain coral viability in captive bleaching experiments. Future directions for this project should focus on developing techniques for direct DNA sequencing of symbionts which would less ambiguously identify the point at which the Zooxanthellae are expelled in relation to color loss during heat treatment. Based on observations of shaded corals in the Great Aggie Reef, light deprivation may also serve as a useful, less destructive method for bleaching in symbiont repopulation studies of *Rhodactis* as it does not kill the coral as rapidly as temperature increase. My results suggest that this captive recirculating reef system can serve to establish techniques to assist the efforts to understand the bleaching process that may potentially contribute to the conservation of coral reefs.

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