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Examining the Effect of Climate Change on the Upper Mesophotic Coral Montastrea cavernosa (Linnaeus 1767)

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Examining the Effect of Climate Change on the Upper Mesophotic Coral *Montastrea cavernosa* (Linnaeus 1767)

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John Skutnik

Abstract

Coral reefs are under increasing pressure from global climate change. In particular, ocean warming is having a deleterious effect on many of the world's shallow reefs. Some authors suggest that acute exposure is more detrimental than chronic, versus others who indicate the opposite. However, little knowledge exists regarding heat induced stress on deeper mesophotic reefs. Here, I examined the effect of acute (72 hrs.) and chronic (480 hrs.) heat stress using laboratory experiments on coral *Montastraea cavernosa* (Linnaeus 1767) collected from an upper mesophotic (~30 m) reef off Islamorada Florida. I examined a variety of putative immune and stress genes as a proxy for response to heat stress. The acute experiment (Heat and Heat + Oil (Deep Horizon Oil) increased from 27 °C to 33 °C over six hours whereas the chronic experiment (Heat) increased from 27 °C at 1.5 °C increments every 72 hours until temperatures reached 33 °C. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on six genes including *RpL9*, *RpS7*, *BCL-2*, *HSP90*, *catalase*, and *cathepsin L1*, resulting in two distinct gene expression profiles (rapid transcript upregulation and variable transcript expression). A generalized linear mixed model (GLMM) with a Markov chain Monte Carlo (MCMC) sampling scheme was used to model the expression of genes under treatment conditions. Acute heat exposure resulted in an increase in *catalase*, *BCL-2*, and *HSP90* at all time points whereas Heat + Oil yielded a strong increase in *catalase* activity from hour 24 to 48. Fewer genes were up-regulated in the chronic experiment until hour 28 (30 °C) where 5 of 6 genes were up-regulated, three of which were significantly up-regulated. Overall, both acute and chronic heat stress elicited a significant response in gene expression relative to control samples. Acute exposure resulted in the activation and upregulation of an oxidative protective enzyme,

molecular chaperone, and anti-apoptotic protein. Chronic heat exposure elicited a physiological response at 30 °C which I propose as a heat-stress threshold for *M. cavernosa* at this depth. In conclusion, *M. cavernosa* at the upper mesophotic zone is susceptible to increased ocean temperature and should be regarded as a sensitive ecosystem.

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Abbreviations

AWRI	Annis Water Resources Institute
AUV	Autonomous underwater vehicle
$CaCO_3$	Calcium carbonate
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon Dioxide
COA	Certificate of analysis
DH	Deep Horizon
ET	Experimental tank
FKCMS	Florida Keys Coral Reef Marine Sanctuary
GLMM	Generalized linear mixed model
HV	Highly variable
IC	Incubation chamber
LN_2	Liquid nitrogen
MCE	Mesophotic coral ecosystem
MCMC	Markov Chain Monte Carlo
MV	Moderately variable
PAR	Photosynthetically active radiation
PMC	Plastic milk crate
PP	Peristaltic pump
qRT-PCR	Quantitative real-time polymerase chain reaction
RIN	RNA integrity number

RNA-Seq	Whole transcriptome sequencing
ROV	Remotely operated vehicle
SCUBA	Self-contained underwater breathing apparatus
WHOI	Woods Hole Oceanographic Institute
WJ	Water jacket

Chapter 1

Introduction:

Earth is composed of a variety of ecosystem types, complexities, and ecosystem functions. Globally, ecosystems face a daunting challenge due to an abrupt change in climate (on a geological time scale) despite being a natural component of earth's history. In this context, it may not be the change of climate but rather the rate at which change is occurring. The rate of climate change has increased with the corresponding use of fossil fuels as a primary energy source. Fossil fuel use increased during the industrial revolution where it was used and combusted for heat and energy. It is widely hypothesized that heat-energy combustion led to the start of a dramatic shift in earths atmospheric composition with a staggering increase in gases such as carbon dioxide (CO_2) and carbon monoxide (CO), to name a couple. Collectively, these combustion by-products, called green-house gases, contributed to climate change, and have been shown to have a direct and negative impact on terrestrial, marine, and freshwater ecosystems. Shifts in ecosystem function and diversity are becoming apparent across all ecosystems [1, 2]. Variations in migration patterns and plant developmental cycles have been observed to be influenced by the shift in seasons due to climate warming [1, 2]. Some species are becoming tolerant/resistant to climate forcings while a larger percentage of species are susceptible and less likely to survive.

All ecosystems are at risk of warming due to the insulating effect greenhouse gases have on the earth's atmosphere. Greenhouse gases are potent absorbents of heat and have reflective properties that can emit heat back toward earth's atmosphere. In the absence of these greenhouse gases, energy obtained from the sun would bounce off earth's surface into space in the form of heat, maintaining a relative energy equilibrium in earths system. The excess energy in earth's

system acts to warm the atmosphere as well as marine and freshwater ecosystems [3]. Due to the relatively rapid warming in these ecosystems, most organisms are unable to cope with the rapid changes and are beginning to show signs of shifting physiologies, changing morphologies, or succumbing to death [4]. Marine and freshwater ecosystems are at risk of acidification from excessive atmospheric CO_2 reacting with water with forms carbonic acid [5]. However, climate change is not always detrimental to an organism. Forests can benefit from the excess CO_2 in the atmosphere through elevated levels of carbon sequestration (i.e. growth) and that some areas of land will benefit and become agricultural, where prior to a warming climate, that was not possible [6, 7]. Although some organisms and geographic areas may thrive in an altered climatic state, the result of climate change generally causes a loss of species diversity and an overall weakening of terrestrial, marine, and freshwater global ecosystems.

Dramatic effects from earths changing climate are already being observed in terrestrial ecosystems. For instance, precipitation patterns have changed where some ecosystems are getting more rain versus others are receiving less [8], agricultural growth zones have increased northward and southward toward the poles [9], and polar ice shelves/caps are becoming substantially reduced in size [10, 11]. Not only are organisms and their ecosystems at risk from climate change, humans that rely on various ecosystems for food are also affected. Climate change is already displacing island communities as the sea level rises from melting polar ice caps, and many communities near sea level are in danger as climate change continues [12]. Similar to terrestrial ecosystems, an increase in sea level may also result in negative consequences for aquatic systems by, for instance, “drowning” coral through light attenuation [5].

Marine ecosystems have increasingly become affected by climate change as illustrated by massive coral bleaching events (e.g. disruption of coral-algal symbiosis; [13], increasing temperature in the deep ocean [14], and a reduction in ocean pH (e.g. 0.7 units; [15]). Organisms living at their thermal threshold/tolerance are becoming less physiologically capable of coping with climate stress, and calcifying organisms are finding it difficult to accrete and maintain the integrity of the calcium carbonate structures essential for their survival [16]. As climate change continues to increase in severity, additional stressors will act synergistically increasing the overall stress placed on these organisms and the habitats in which they live.

There is a growing concern that the effect of a broad range of climate change induced stressors across all ecosystems is taking place. Here, I focus on the effect of climate change induced heat stress on the coral ecosystem, specifically, the upper mesophotic coral ecosystem. Coral generally grow between 1 – 2,000 m depths. The mesophotic zone, and the focus of this study is defined as a depth between 30 – 150 m in depth [17, 18] and is generally an extension of shallow water reefs. Mesophotic zones have received increasing attention in recent years as a potential refuge for coral, where coral from these depths may be used to "reseed" reefs at shallower depths. Much of the shallow water coral ecosystems are already under severe duress and likely will not survive further climate change exacerbation [19].

Purpose:

The purpose of this research is to examine the response of the upper mesophotic Scleractinian coral *Montastraea cavernosa* (Linnaeus 1767) to induced heat stress that mimics climate change. Currently, there is a lack of understanding of how corals in this depth range respond to climate change, particularly heat stress. As climate change progresses, it is believed that this ecosystem, being on the threshold of shallow and mesophotic depth zones, is the next most likely to be influenced by climate change [20, 21]. It is also likely that the refugia hypothesis [17, 22], which states that mesophotic coral ecosystems might serve as a refuge for reseeding shallow coral reefs, will depend on the response of these upper mesophotic coral to changing sea surface temperatures. Due to the proximity of the upper mesophotic zone to the shallow reefs, it is plausible that the upper reef may be best suited to repopulate shallow reefs. Hence, my study will assess how one particularly dominant coral (*M. cavernosa*) in the upper mesophotic zone will respond to climate change. Because larvae of coral have a similar temperature tolerance as the parent population, tangentially, my study will be important to reef managers determining if "offspring" can be used to reseed a reefal habitat.

Scope:

The scope of this thesis is limited to the coral species *M. cavernosa* collected from the Florida Keys, sampled at the upper mesophotic reef, and exposed to acute and chronic temperatures they would naturally experience in a field setting. Mesophotic coral ecosystems are distributed globally and represent an increasing percentage of global coral reefs yet to experience extreme heat stress. Climate change, recognized as a global problematic occurrence with increasing severity, will thus likely affect all mesophotic coral ecosystems to a varying degree, depending on their species composition and locations.

Assumptions:

During the completion of this thesis, all experiments will be conducted under the assumption that environmental conditions will be mirrored in the laboratory-controlled experimental process. This assumes that laboratory conditions are the best approximation to *in situ* conditions to assess corals physiological response to heat stress. All reagents used will be adequately used and disposed of following the manufacturer's recommendations as per the certificate of analysis (COA) provided. It will be assumed that all corals will respond similarly to heat stress and that no colony specific response will occur. It is also assumed that all coral colonies sampled harbor the same *Symbiodinium* clade, based on sampling proximity, such that no *Symbiodinium* specific effect will be observed in the study.

Hypothesis:

The hypothesis is based on the specific study organism, *Montastraea cavernosa*, and thus the results of the experiment are only interpreted and extended to that species.

Null: *Montastraea cavernosa* exposed to heat stress or heat + oil stress will show no differential gene expression compared to control samples at ambient temperature.

Alternative: *Montastraea cavernosa* exposed to heat stress or heat + oil stress will express statistically significant differential gene expression profiles compared to control samples at ambient temperature.

Research Questions:

How will *Montastraea cavernosa* collected from an upper mesophotic coral reef zone located in Florida respond to climate change induced heat stress? How will *Montastraea cavernosa* respond to both acute and chronic heat stress? How will *Montastraea cavernosa* respond to acute heat + oil stress?

Significance:

The laboratory controlled experiments conducted in partial fulfillment of the Master of Science thesis will help characterize the effect of climate change (i.e. heat stress) on coral collected from an upper mesophotic reef zone. The significance of my study is that such coral may play a vital role in "re-seeding" shallow bleached reef habitat. As such, this study will provide the first data necessary for reef managers to understand how coral from deeper habitats respond to heat stress, potentially preserving a multimillion dollar tourist industry in Florida.

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Chapter 2 (Manuscript – PLOS One)

Full Title:

Examining the Effect of Climate Change on an Upper Mesophotic Threshold Community: *Montastraea cavernosa*

Short Title:

Climate Change and the Upper Mesophotic Zone

John E. Skutnik

Abstract:

Coral reefs are under increasing pressure from global climate change, particularly shallow reefs exposed to acute and chronic ocean warming. However, little knowledge exists regarding heat induced stress on deeper mesophotic reefs. Here, I examined the effect of acute (72 hrs.) and chronic (480 hrs.) heat stress using laboratory experiments on coral *Montastraea cavernosa* (Linnaeus 1767) collected from an upper mesophotic (~30 m) Florida reef. I examined a variety of stress genes as a proxy for coral response to stress. The acute experiment (Heat and Heat + Oil (Deep Horizon Oil)) increased from 27 °C to 33 °C, over six hours, whereas the chronic experiment (Heat) increased from 27 °C at 1.5 °C increments every 72 hours until 480 hours. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the genes *RpL9*, *RpS7*, *BCL-2*, *HSP90*, *catalase*, and *cathepsin L1*, resulting in two distinct gene expression profiles. A generalized linear mixed model (GLMM) with a Markov chain Monte Carlo (MCMC) was used to model the expression of genes under treatment conditions. Acute heat exposure resulted in an increase in *catalase*, *BCL-2*, and *HSP90* at all time points whereas Heat + Oil yielded a strong increase in *catalase* activity from hour 24 to 48. Fewer genes were up-regulated in the chronic experiment until hour 28 (30 °C) where 3 of 6 genes were significantly up-regulated. Overall, both acute and chronic heat stress elicited a significant response in gene expression relative to control samples. Acute exposure resulted in the activation of an oxidative protective enzyme, molecular chaperone, and anti-apoptotic protein. Chronic heat exposure elicited a physiological response at 30 °C which I propose as a heat-stress threshold for *M. cavernosa* at this depth. In conclusion, *M. cavernosa* at the upper mesophotic zone is susceptible to increased ocean temperature.

INTRODUCTION:

Climate change has consistently increased in severity over the past two centuries and is expected to continue to increase, further threatening both terrestrial and aquatic global ecosystems, specifically the coral reef ecosystems [1]. A product of the industrial revolution, continuous fossil fuel combustion has rapidly transformed earth's climate by altering atmospheric chemistry leading to significant terrestrial and marine ecosystem changes [2]. Climate change has been a natural part of earth's history, however, the rate and magnitude at which change is occurring is cause for concern [3]. Considered a product of both natural and anthropogenic sources, the primary anthropogenic effect causing climate change is derived from fossil fuel combustion. Fossil fuel combustion produces a variety of gases and in particular, carbon dioxide; collectively all of these gases contribute to the 'greenhouse effect [4]. When carbon dioxide reacts with seawater it forms carbonic acid, reducing the oceans pH, making it difficult for calcifying organisms to accrete their skeletal structures [1]. Carbon dioxide and other combustion byproducts also form an "insulating layer", generating a greenhouse effect in the atmosphere that traps energy in the earth's atmospheric system resulting in global net warming [5, 6]. Atmospheric warming helps increase sea surface temperatures (SST); SSTs are generally measured using satellites, buoys, and on research vessels [7]. Ocean warming has significant implications for shallow reef coral communities by stressing a corals thermal limit often causing bleaching [8]. This is referred to as "bleaching" due to the loss of *Symboidinium*, which provides coral most of its color, revealing the translucent flesh of the coral and underlying white skeleton [9].

Bleached coral are generally affected by either acute and/or chronic thermal stress, which may also be coupled to a variety of other stressors (e.g. disease, lack of nutrients, etc.). In this

study, my focus is on coral stress caused by heat, specifically acute and chronic. Historically, chronic heat stress is considered to be more detrimental to coral survival than acute warming, indicating that the duration of elevated temperature exposure is pivotal to a coral's survival [10]. Unlike chronic heat stress, transient acute events (cold and warm) can be caused by storms or abnormal weather events [11]. Chronic heat stress usually occurs at the latter part of the summer where water temperatures are at the seasonal peak due to extended months of elevated atmospheric temperatures and pose the greatest risk for coral injury [12]. Scleractinian coral, sometimes called 'reef-building' coral, form an intimate symbiosis with an intracellular dinoflagellate *genus* called *Symbiodinium* [13, 14, 15] (colloquially known as "zooxanthellae"). The coral-algal symbiosis is instrumental for most corals health and fitness and allows the holobiont (coral, symbiont, bacteria) to thrive in oligotrophic water by being the beneficiary of the symbionts photosynthetic capacity [16, 17]. Heat stress disrupts the symbiotic association between coral and algae causing the dinoflagellate algae to dissociate, ending the relationship between the host and the symbiont. During the bleaching process, coral growth, reproduction, and the ability to fight disease are severely hindered [1, 18, 19, 20, 21]. Coral can survive aposymbiotic (coral without symbiont) periods caused by bleaching, but the length of time varies by species and does not promote the survival and growth of the species [22, 23, 24].

In recent years, other perturbations affecting coral have included unintended oil spills. The recent Deep Horizon (DH) oil spill demonstrated the potential for these accidents and the large scale at which they can occur. Oil from the DH oil spill was found as far east as Key Largo Florida and throughout the Gulf of Mexico where coral reefs are prevalent [25]. The extent to which climate change coupled to oil exposure affects coral is not well understood and very little information exists. Based on the little evidence that does exist involving coral exposure to

hydrocarbons (i.e. gasoline), short duration of exposure has been observed as tolerable [26]. Despite coral's sensitivity to climate change and environmental perturbations, environmental managers recognize the importance of saving reefs due to the extremely productive and diverse nature of these ecosystems which are rivaled only by terrestrial rainforests.

Coral reef ecosystems provide significant global economic and ecological benefits. Reefs provide protection from storms by mitigating coastal erosion, are a foci for tourism for many coastal countries (e.g. more than $1 Billion dollars is generated via Australia's coral reef tourism), provide the means for subsistence and industrial fisheries, and act as shelter and feeding grounds for many organisms [1, 12, 27]. Costanza *et al*. [28] suggest that the total value of global ecosystem services per annum is ~$20.2 trillion dollars and coral reefs in the form of tourism, coastal erosion protection, and storm mitigation for instance, contribute $11.9 trillion dollars. The estimated coral reef economic loss is ~58.9% of the entire global total loss. Considering the tiny ecological footprint (0.1% ocean surface) coral reefs occupy, the ecosystem service loss per unit area is far greater than any other ecosystem on earth. The global reliance on coral reefs, from subsistence fishing to tourism, will become increasingly noticeable as climate change stressors, such as heat stress, become more prevalent and severe.

Much of the work examining the effect of climate change, specifically heat stress, has focused on shallow water coral communities due to their economic and ecological importance, obvious signs of distress, and ease of access [1]. Climate change threats to coral found in the deep ocean (defined here as being > 300 m) and mesophotic reefs (defined as photosynthetically active reefs ranging from 30 – 150 m depth) are relatively minimal [29, 30]. Some researchers [see 30, 31, 32] propose that deeper mesophotic reefs have the potential to serve as a refuge and repopulate shallow depleted reefs affected by climate change stressors but there remains caution

as to its likelihood of success. Little work, however, has been done to assess how deeper communities might respond to environmental stimuli such as thermal stress. Here, I examined the coral *Montastraea cavernosa* from the upper most depth zone of the 'mesophotic ecosystem' and simulated acute and chronic heat stress. The objectives of my experiments were to (1) Examine the effect of elevated temperature on coral ≥ 30 m through gene expression analyses of selected putative immune and stress genes; (2) Identify any potential relationship with the duration of stress on gene expression; (3) Characterize gene-gene interaction caused by heat stress; (4) Distinguish between any similarities or differences in the response to acute versus chronic exposure to elevated temperatures; and (5) Assess whether oil coupled with heat stress caused an increased/decreased response compared to heat stress alone. Based upon the outlined experimental objectives, I hypothesize that (1) both acute and chronic heat stress will elicit a significant physiological response observed as an increase in the expression of select putative stress and immune genes; (2) there will be noticeable differences in the gene expression profile of coral exposed to either acute or chronic heat stress; (3) time will be a significant factor in the response to elevated temperatures; and (4) oil coupled to heat stress will elicit a stronger gene expression profile.

METHODOLOGY

Study Organism:

Montastraea cavernosa (Linnaeus 1767) is an ideal coral species to study when examining how climate change stressors affect a wide depth range of coral communities. *M. cavernosa* is known as a depth generalist broadcast spawning coral species distributed between 3 - 91m found in the Caribbean, Gulf of Mexico, Florida, the Bahamas, and Bermuda [33, 34, 35]. *M. cavernosa* may contribute to genetic connectivity across reef depths, based on their broadcast spawning reproductive characteristics, revealing the potential to study the response to climate change on the same genotype across depths [36]. *Symbiodinium* found intracellularly produced most of the host coral's primary nutritional needs, however, when zooxanthellae are compromised, *M. cavernosa* can supplement its nutritional needs *via* heterotrophic feeding [35].

Coral Collection Site & Sample Identification:

All *M. cavernosa* samples were collected off the coast of Tavernier Florida at Conch Reef, specifically, at the base of Conch Wall (Fig 1) which falls under the jurisdiction of the Florida Keys National Marine Sanctuary (FKNMS). This location was chosen because of its access (permitted to collect coral at that site, Permit # FKNMS-2014-088), depth, and location. The base of Conch Wall is between 30 – 34 m with a moderate SW to NE current (at the time of sampling). This was the most easily accessible coral below 25 m in the northern Florida Keys area. All sampling was considered opportunistic, as coral were sampled based on being the "most accessible" meeting a specific size (25.4 cm^2) standard.

Figure 1. Sampling Location. *Montastraea cavernosa* sampling took place off the coast of Islamorada Florida near the Conch Reef Research area on Conch Wall. To the north and northeast of the sampling area was a marine protected area/sanctuary. The red box

highlights the sampling area immediately south of the east-most buoy (black circle with white outline) where the research vessel was moored. Photo credit Google Earth.

Collection Site Environmental Parameter Measurements: (PAR & Temperature)

Photosynthetically active radiation (PAR) was measured using a Li-193 Underwater Spherical Quantum Sensor (LiCor Inc.) on a clear sunny day with relatively strong currents. Measurements were made at 5 m increments from 5 – 25 m (depth limited by instrument cord length) by suspending the instrument over the side of the research vessel. Measurements were recorded every 5 s (total recorded values per depth; n = 3) at each depth. Due to the current at this location, depth values are estimated to be slightly less than intended due to drag caused by the current and thusly no less than 7 m above the sampling depth. Temperature was continuously logged on each dive with an affixed HOBO data logger (Onset Computer Corporation) to each diver. This data was used to help define laboratory treatment tank conditions upon returning to the Annis Water Resources Institute (AWRI) as well as used in a monitoring effort to help understand daily temperature fluctuations at the reef site.

Coral Collection and Fragmentation:

At the base of Conch Wall southwest of the nearest buoy located near the Florida Keys Coral Reef Marine Sanctuary (FKCRMS; see Fig 1, – Lat: 24.9580667, Lon: -80.45243), divers using SCUBA (Self Contained Underwater Breathing Apparatus) performed exploratory dives (30 – 33 m) to locate colonies of *M. cavernosa.* A series of dives to collect the coral were then planned and completed between July 13 – 16, 2014. All collected coral samples were at the base of Conch Wall and were within a linear 100 – 150 m transect set-up away from the FKCRMS as stipulated in the research collection permit (FKNMS-2014-088) issued for this study. The size of the coral heads were approximately 25.4 cm^2. A rock saw, hammer and chisel were used to

free coral heads during sampling. Coral were then placed into black milk crates covered with shade cloth and remained at depth to help reduce any stress they may have experienced during collection. Prior to the departure to Michigan, all coral were then collected at depth, transported to the surface and at the research vessel, rinsed with pre-chilled seawater (at a temperature equivalent to the collection depth; 28.0°C), placed in coolers containing pre-chilled seawater aboard our research vessel and transported to the shore where a research van was waiting; 189 L (50 gal) drums were also partially filled and chilled to be used to flush the cooler water during transport. Seawater in both the coolers housing the coral and the 189 L drums were aerated using portable battery operated bubblers. Transport from the Florida Keys to the AWRI located in Muskegon Michigan took ~30 hrs; every 6 hrs, ~25% of the cooler water was replaced with drum water (containing sea water from the collection site) to help reduce the concentration of secondary metabolites and other contaminants excreted by the coral.

Immediately upon arrival to AWRI, all coral were placed in holding tanks both to allow the coral to acclimate and to depurate; corals were held for ~ 4 days. Coral colonies were than fragmented into ~ 2.54 cm^2 cubes using a model C-40 band-saw (Gryphon Corporation) with a specially enhanced diamond blade for coral fragmenting; sectioning occurred over seven days where I established an arbitrary requirement of at least three polyps per fragment. During the fragment process, care was taken to try and not disturb or destroy any whole polyps. Prior to any experimentation, all "frags" (= fragmented coral) were then placed in holding tanks to help minimize the stress of fragmentation giving them time (~ two weeks) to depurate.

Experimental Design and Analysis:

Experimental Tank Design:

Each experimental tank (ET) used in this study consisted of a 17.3 L incubation chamber (IC) and a 45 L water jacket (WJ) (Fig 2). Each ET was independent of each other regarding the water jacket, heating element, shade cloth, and submersible pumps. Due to space limitation, however, the control tanks used filtered seawater from one 208 L reservoir, all heat treatment tanks drew seawater from a different 208 L reservoir, and all Heat + Oil treatment tanks from a third 208 L reservoir (in the acute experiment only). During the chronic heat treatments, ETs used seawater from two 208 L reservoirs; five tanks on the left-hand side were considered "reservoir #1 versus five tanks on the right-hand side were considered "reservoir #2" (see Fig 2). Thus, both control and treatment tanks randomly used seawater from the same reservoirs in the chronic experiment. To create flow for fluid exchange through the IC's, artificial seawater was pumped using two peristaltic pumps (COLE PARMER Masterflex 7519-15) from the respective reservoirs at a rate of 2.3 L/hr. One peristaltic pump (PP) added water to each ET from reservoir #1 whilst the other ETs used seawater from reservoir #2. The outflow (i.e. waste seawater) from each ET was removed from the system *via* gravity through grommets in the IC and WJ into a waste channel leading to a floor drain. Mixing inside the IC was accomplished by use of a single submersible aquarium pump to help evenly distribute heat and mix incoming seawater. Another submersible pump was placed in the WJ to circulate heat and maintain the IC at a constant temperature. To maintain and manipulate temperature, a 50 W or 250 W drop-in aquarium heater was placed in the WJ.

Figure 2. Incubation chamber (IC) and water jacket (WJ) setup. An exampe of an individual IC (A), WJ(B), and double layered shade cloth (C). The WJ was used to maintain and manipulate temperature in the IC as is evident by the drop-in aquarium heater (D).

Maintaining adequate light for the coral was achieved by using four double lamp T5 high output fixtures with a 54W pure actinic and 54W AquaBlue^{+} light (ATI Aquaristik, Hamm Germany) to simulate wavelengths found at a depth of 30 – 34 m. To reduce the potential of light stress, sections of neutral density screen were excised, doubled, and placed on top of both control and treatment tanks. Light was measured under the shade cloth prior to the start of the experiment for each tank which was below the detected PAR and intensity found *in situ*.

Each ET was equipped with a white egg crate lighting panel (Home Depot, commonly used to cover fluorescent light fixtures) beneath the IC to allow water to flow underneath the IC for better heat distribution. Each IC tank was set-up with a HOBO tag (Onset Computer Corporation, Bourne, MA) that continuously monitored and logged temperature and light intensity every 20 minutes. Additionally, temperature was manually logged prior to sampling at each sampling interval. It should be noted that prior to the addition of any coral to the tanks, irradiance and temperature were monitored for 48 h to ensure reproducibility between tanks.

Chronic Treatment: A matched pair design was used in this treatment randomizing experimental ETs as follows: five tanks were placed on a top shelf and five tanks on a bottom shelf (n = 10 tanks); each pair (one top and one bottom tank) was either a control or treatment and decided by flipping a coin; this was done for all five treatment pairs. Coral fragments were randomly selected from the main holding tank and placed randomly in ETs predetermined by using an Excel© randomization function. Forty-eight coral fragments (total) were placed in each of the ten IC tanks.

Acute Treatment: Similar to the chronic treatment, ten tanks (total) in a multi-level design were set-up such that three control tanks, four heat treatment tanks, and three Heat + Oil treatment tanks could be examined simultaneously (Fig 3). To randomize which tanks received

one of three treatments, the following numbers: 1, 1, 1, 1, 2, 2, 2, 3, 3, and 3, were input into Excel and then randomized, where 1 corresponded to Heat, 2 to Control, and 3 to Heat + Oil. The order in which they were generated (top to bottom in Excel) then corresponded to tanks one to ten. Coral fragments were randomly selected from the primary holding tank and randomized amongst the Control IC, Heat treatment IC, or Heat + Oil treatment IC. Six coral fragments (total) were placed in each IC tank. Corals were allowed to acclimate to the new experimental tanks for 48 h prior to the first sample taken.

Figure 3. Example of the experimental tank (ET) setup (A) Denotes 2 of the 3 the reservoir tanks, (B) shows the drainage system leading to a floor drain, (C) shows how the overflow feeds through an overflow tube in the incubation chamber (IC) through the water jacket (WJ) down to the PVC drainage system. Lights were hung with equal distance from the top of the IC for both levels.

The analysis of a physiological response to treatment conditions for both chronic and acute experiments was monitored using quantitative real time polymerase chain reaction (qRT-PCR) on six immune/stress-related genes: *RpL9* (ribosomal protein L9), *RpS7* (ribosomal protein S7), *BCL-2* (proto-oncogene B-cell lymphoma 2), *HSP90* (heat shock protein 90), *catalase*, and *cathepsin L1*. Examining gene expression transcript levels of these six genes during the heat stress study when compared to control samples is a good indicator of a physiological change/response.

Chronic Heat Stress Exposure

The duration of the chronic experiment was 20 days with incremental increases in temperature at set time intervals. Initial conditions for both control and heat treatment tanks were 27 °C under identical light and tank setup. Time intervals and corresponding temperature increases were as

follows: Hour 0 – 72 (27.0 °C), Hour 96 – 192 (28.5 °C), Hour 216 – 288 (30.0 °C), Hour 312 – 384 (31.5 °C), and Hour 408 – 480 (33.0 °C). Control tanks were maintained at 27 °C throughout the duration of the experiment. Sampling order (i.e. tank) and fragment number were randomized prior to starting any treatments. Duplicate coral fragment samples were sacrificed each day (11:00 am) from Hour 0 to Hour 480 and immediately flash frozen and stored at –80 °C until downstream processing occurred. Ten samples from each tank were used in downstream analysis: one sample at the onset of a new temperature and one sample at the last day of that particular temperature block (i.e. the first and last day of a particular temperature). This was intended to capture the response of the coral throughout an entire temperature block.

Acute Heat Exposure and Gulf of Mexico Oil Exposure

The temperature regime applied to the acute treatment was as follows: all tanks started at 27 °C during which the temperature in the control tank was maintained throughout the study ±0.5 °C; both Heat and Heat + Oil ETs were increased to 33 °C from the onset of the experiment over six hours using submersible aquarium heaters inside the WJ. A single coral fragment was collected from each IC tank at 11:00 am starting on 9/29/2014 and every 24 hours until 10/2/2014 for a total exposure of 72 hours (n = 4 samples/tank). Samples were immediately flash frozen at each sampling interval and placed at –80 °C for downstream processing.

The 208 L treatment reservoirs were heated with a drop-in aquarium heater to help facilitate the target temperature. The Heat + Oil treatment tank required the addition of an oil slurry directly to the reservoir tank. Oil was obtained by Dr. James Cervino (Woods Hole Institute of Oceanography; WHOI) from deltas immediately south of New Orleans *via* upwelling from the Deep Horizon (DH) Oil Spill. A high-powered submersible pump was used to maintain a homogenous suspension. Prior to the addition of the oil solution, “stock oil”, which consisted

of a slurry of beach sand and rock covered in DH oil, was added to beakers of Milli-Q water and mixed (shaken and stirred) thoroughly for 72 hours to re-suspend the oil. Dilution volumes were calculated following Sammarco *et al*. [25] who described the average suspension of oil in solution (i.e. mixed in with seawater; 0.047 ppm) immediately after the Gulf of Mexico DH oil spill from the Gulf of Mexico to the Florida Keys; I added 41.6 mL of concentrated oil solution to 208 L of seawater to mimic the oil spill concentration of DH Oil Spill in the Gulf of Mexico [25].

Sample Processing & RNA Extraction:

Flash frozen samples were removed from the –80 °C and crushed with a mortar and pestle. Each set of mortar and pestles were cleaned with a residue-free detergent, rinsed with de-ionized water, sprayed with RNaseZAP (Sigma Aldrich), washed in Milli-Q water, and placed in a –20 °C freezer to cool. Liquid nitrogen (LN_2) was continuously used throughout the crushing process to maintain the mortar, pestle, metal spatula and sample as cold as possible. Caution was used when pouring LN_2 into the mortar as not to propel and lose any crushed sample from the mortar. Each sample was crushed to a fine powder, scooped into 2 mL micro centrifuge tubes (number of tubes 3 – 5 depending on the size of the subsample), and stored at –80°C until the RNA extraction phase.

Prior to RNA extraction, a crushed sample was transferred to a new "RNA extraction ready" tube containing the appropriate sample weight. During the weighing process, all materials, including coral, metal spatulas and the "RNA extraction ready" tubes, were continuously maintained in LN_2 to retain RNA integrity. The crushed sample weight ranged between 100 – 130 mg. After samples were aliquoted they were returned to –80°C freezer until all samples were weighed.

RNA extractions were performed in batches of 12 or 24. The RNA extraction method used was a modified Trizol/RNeasy Mini protocol. An initial Trizol step was used to lyse the coral cells followed by a chloroform addition to isolate total RNA. The top aqueous phase was then placed into an RNeasy Mini Kit for subsequent cleanup and purification. All RNA samples were eluted with 30 µL RNase/DNase free water. Samples were quantified using a NanoDrop 1000 and qualitatively assessed on Agilent's BioAnalyzer 2100 and then placed in a –80 °C freezer until cDNA synthesis.

cDNA Synthesis:

Prior to cDNA synthesis, total RNA from all samples were DNased with DNase I (Invitrogen) to remove any contaminating genomic DNA. From the total RNA, a total of 300 ng of RNA per reaction was reverse transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio) in a 30 µL reaction volume. Oligo d(T) was the only primer used in reverse transcription. Thermo-cycle conditions were followed per the manufacturer's instruction: 15 minutes at 37 °C, 5 minutes at 85 °C, and held at 4 °C indefinitely. After cDNA synthesis, a cold ammonium acetate precipitation was performed to purify total cDNA; cDNA was then normalized to 5 $ng \cdot uL^{-1}$.

Primer Validation and Efficiency

cDNA from random samples was taken and pooled to produce stock cDNA. The stock cDNA was then used for PCR amplifications to validate that primers amplified a single product in the expected size range. *Taq* polymerase (Invitrogen, Thermo Fisher Scientific Inc.) was used in the amplification reaction with PCR conditions as follows: initial denaturation at 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 15 seconds, 57 °C for 30 seconds, 72 °C for 1 minute, with a final extension of 5 minutes at 72 °C followed by 4 °C indefinitely. All PCR

products were run on a 2 % agarose gel. Primers that yielded a single band from the gel were then validated with qRT-PCR for melt curve analysis. rEVAlution 2X Master Mix (Empirical Bioscience) was used for all qRT-PCR reactions with a thermo cycler profile following: 2 minutes at 95 °C, followed by 40 cycles of 5 seconds at 95 °C, 7 seconds at 57 °C, 25 seconds at 72 °C followed by a melt curve analysis for 1 minute at 95 °C, 30 seconds at 57 °C, and 30 seconds at 95 °C.

Stock cDNA was two-fold serially diluted to produce a dilution series for primer efficiency analysis. Each primer set was run in triplicate for each dilution under the same thermal conditions prior qRT-PCR runs. Primers were considered validated if demonstrating an efficiency between 95% – 105%; primer sequences for all genes analyzed are provided in Table 1.

Table 1: Primer Sequence for all Genes Analyzed

Primer	Sequence
RpL9	F – 5'-GCTCTTTCCTTCCATCCTTCG-3'
	R – 5'-GTAACTTCACGGCCCCTTAG-3'
RpS7	F – 5'-TCCCAAGTGAAATTGTTGGA-3'
	R – 5'-CTTTGCCTGTGAGCTTCTTG-3'
BCL-2	F – 5'-GCACGAAGCGTTATGAAAA-3'
	R – 5'-CCCAGTTGATACCTGTGCTG-3'
Catalase	F – 5'-GACCCTGAAGCATCTTATCT-3'
	R – 5'-CGCTGATACAAGTTGGAAAG-3'
HSP90	F – 5'-CAGAAGGTGGAGACTGATAA-3'
	R – 5'-CCAGATGACAAGAGAGAGG-3'

Cathepsin L1	F – 5'-GGGACCTGTCACTTCAAT-3'
	R – 5'-CACCTTCGTCTCCACTTT-3'

Statistical Analysis:

In lieu of normalization using highly stable reference genes, the R package mcmc.qpcr was developed to analyze data without reference genes [37]. This approach was used to model the expression of six different genes assayed in this study (Table 1). The mcmc.qpcr package uses a Poisson-lognormal distribution to allow for the inclusion of data where no 'ct' value was observed (i.e. no fluorescence/cycle threshold was detected by qRT-PCR). In addition, this method of analysis uses a Bayesian Markov Chain Monte Carlo algorithm to estimate the effects of experimental factors on gene expression. The model was set to hold the factors 'Time + Treatment' and the 'Time:Treatment' interaction fixed while calling for the variable 'Sample' to be random. 'Sample' is defined as every technical replicate pair (qRT-PCR technical replicates) of coral fragments used in the experiment. Seawater replenishment rates pumped through through the IC removed secondary metabolites that had potential to alter the physiology or response of neighboring coral frags. Based on the flow rate and continuous replenishment of IC water on a daily basis I felt it was sufficient to classify samples originating from the same IC as independent.

Descriptive statistics of the gene and time point most affected in each study was determined by summating the absolute value of the fold change of each gene across all time points and each time point across all genes. This provides insight into the gene and time point producing the largest deviation from control samples and also paired to a particular temperature. The largest value for gene and time point was thusly determined as most affected.

RESULTS:

Acute Experiment:

Significant Gene Expression Response:

All genes analyzed (6 genes) for each time point sampled were estimable under the model employed to assess gene expression. Significant differential gene expression was observed across treatments relative to control, and ranged between 1.60 and –2.16 fold (Table 2**)**. When exposed to only heat, expression of *RpL9* decreased –2.11 fold after 24 hours ($p = 0.05$). After 72 hours, expression of *BCL-2* increased 1.60 fold ($p < 0.01$). Additionally, *RpS7* expression decreased –1.52 fold and *HSP90* increased 1.90 fold after hours 24 and 72, respectively, but differences were not significant ($p = 0.07$). Three genes exhibited significant variation in expression when exposed to the Heat + Oil treatment: catalase expression decreased –2.16 fold ($p = 0.04$) and *RpL9* decreased –1.32 fold ($p = 0.05$) upon initial exposure (hour 0). In contrast, *BCL-2* initially decreased –1.70 fold ($p < 0.01$) but then increased 1.31 fold ($p = 0.01$) at hour 72. No other genes approached significance during the Heat + Oil treatment.

Table 2: Statistical Significance Observed in Acute Exposure Study

Gene	Treatment	Time Point	Fold Change	*p*-value
RpL9	Heat	Hour 24	–2.11	0.05
BCL-2	Heat	Hour 72	1.60	<0.01
BCL-2	Heat + Oil	Hour 0	–1.70	<0.01
Catalase	Heat + Oil	Hour 0	–2.16	0.04
RpL9	Heat + Oil	Hour 0	–1.32	0.05
BCL-2	Heat + Oil	Hour 72	1.31	0.01

Trends in Individual Gene Response:

Gene abundance (log_2(Abundance)) for all treatment groups, control, heat, and heat + oil, is provided in Fig 4 by time point, treatment, and gene.

Figure 4: Gene Abundance During Acute Heat Exposure. Gene Abundance by Time and Treatment with Reference Genes. Gene abundance is provided for each estimable gene by treatment type. The x-axis denotes time and the y-axis log_2(abundance).

Heat Exposure:

BCL-2 and *HSP90* expression increased as the duration of heat stress increased (hour 24 – 72). *Catalase* showed a similar slight linear increase in expression from hour 0 – 72, whereas *cathepsin L1* expression was reduced at all time points except hour 72. Ribosomal protein *RpL9* and *RpS7* expression patterns for these two genes were virtually identical, but with a slight reduction in expression at hour 24 followed by a linear increase to hour 72. Gene expression relative to control is provided in Fig 5 as fold changes, corresponding to Table 2.

Figure 5: Heat Treatment – Gene Expression Fold Change Relative to Control. Expression of each gene relative to control is provided as a fold change at each time point measured. Asterisks denote a statistically significant difference relative to control, at that particular time point. Fold change is on the y-axis and time is represented on the x-axis. Each cluster of bars represents a single gene over the course of 72 hours.

Heat + Oil Exposure:

BCL-2 expression was low relative to control and heat treated samples at hour 0 but increased at all time points following hour 0. *Catalase* expression increased sharply from hour 24 to 48 before stabilizing. *HSP90* and *cathepsin L1* were expressed similarly with low expression concentration at hour 0, sharply elevated expression at hour 24 and a reduction in expression at

hour 48. *RpL9* and *RpS7* showed similar expression patterns with control and heat treatment groups over the course of the experiment. A slight deviation occurred at hour 72 from increased expression of *RpL9* whereas *RpS7* expression decreased. Gene expression relative to control is provided in Fig 6 as fold changes. Significant expression differences relative to control are reference in Table 2.

Figure 6: Heat + Oil Treatment – Gene Expression Fold Change Relative to Control. The Expression of each gene relative to control at each time point is provided as a fold change. Asterisks denote a statistically significant expression difference between treatment and control. Fold change is represented on the y-axis and time on the x-axis. Each cluster of bars represents a single gene over the course of 72 hours.

Gene Expression: Most Influenced Time Point and Gene

The time point where the largest effect on gene expression occurred during the acute experiment for both Heat and Heat + Oil treatments was hour 24 (33.0 °C). Exposure to Heat only revealed that *HSP90* was the most affected gene, whereas in the Heat + Oil treatment, *catalase* was found to be most affected followed closely by *BCL-2*.

Chronic Experiment:

Significant Gene Expression:

All genes analyzed (6 genes) for each time point sampled were estimable under the model employed. Significant differential expression was observed in heat treated coral ranging from -6.6 to 3.4-fold change. Twelve significant gene expression differences were observed, relative to control, (Fig 7) corresponding to the following genes: *catalase* (2), *cathepsin L1* (1), *HSP90* (4), *RpL9* (2), and *RpS7* (3). Three of twelve (25%) genes were significantly up-regulated, all occurring at hour 288 at 30 °C (Table 3**)**

Figure 7: Gene Expression Fold Change of Heat Treated Samples Relative to Control. The Expression of each gene relative to control at each time point is provided as a fold change. Asterisks denote a statistically significant expression difference between treatment and control. Fold change is represented on the y-axis and time on the x-axis. Each cluster of bars represents a single gene over the course of 480 hours.

Table 3: Statistical Significance Observed in Chronic Exposure Study

Gene	Treatment	Time Point	Fold Change	*p*-value
Catalase	Heat	Hour 72	–6.6	<0.01
Catalase	Heat	Hour 288	3.4	0.02
Cathepsin L1	Heat	Hour 216	–2.7	0.03
HSP90	Heat	Hour 72	–2.8	0.02
HSP90	Heat	Hour 192	–3.4	0.01
HSP90	Heat	Hour 216	–4.4	0.01
HSP90	Heat	Hour 312	–4.6	<0.01
RpL9	Heat	Hour 192	–1.5	0.03
RpL9	Heat	Hour 288	1.8	0.01
RpS7	Heat	Hour 192	–1.5	0.04
RpS7	Heat	Hour 288	2.2	<0.01
RpS7	Heat	Hour 480	–1.7	<0.01

There were statistically significant intra-gene expression differences within all six genes analyzed. All significant intra-gene expression differences are provided as fold changes with corresponding *p*-values in Supplementary Table 1. Eighty-nine interactions in the heat treatment

group, across all six genes and all ten time points were found to be significant. There was also a total of one hundred and three significant time interactions across all six genes and time points in the control treatment group.

Supplementary Table 1: Intra-gene Statistically Significant Interactions: Statistically significant interactions were observed within individual genes. The gene and time points corresponding to statistically significant gene expression is provided.

Trends in Gene Expression:

The gene expression profile is (Fig 8**)** described as the $\log_2$ abundance of each genes expression concentration over time for both control and heat treatment groups. An overall downward trend in gene expression was observed from hour 0 to hour 480 in both treatment groups. *RpS7* was the least affected gene in both treatment groups. Throughout the duration of the experiment, *RpL9* and *RpS7* consistently revealed the highest transcript concentration in both control and treatment samples. Substantial intra-gene variation in expression was observed in heat samples, and to a lesser extent, in control samples. Gene expression in both treatment groups revealed a similar trend in expression at most time points analyzed (i.e. all/most genes up- or down-regulated at a particular time point).

Figure 8: Chronic Heat Treatment - Gene Abundance. Gene abundance is represented as the $\log_2$ on the y-axis and time represented on the x-axis. Each point estimate is provided a standard error bar generated by the Bayesian Z-test. The left panel provides control gene abundance and the right panel shows heat treatment gene abundance.

In the control group, all genes maintained a similar expression concentration across nearly all time points (hours 96 – 408). The heat treatment group resulted in much less intra-gene expression stability over the course of the experiment. Gene expression stability of

ribosomal proteins *RpL9* and *RpS7*, between the control and treatment group, was loosely maintained over the time period studied. Ribosomal proteins *RpL9* and *RpS7* responded to heat exposure over time in a seemingly parallel fashion (i.e. *RpL9*:*RpS7* ratio of expression was similar over time; Fig 8).

C*atalase* expression showed the largest intra-variation over time, followed closely by *HSP90* and *cathepsin L1* after hour 192. *Bcl-2* expression decreased in response to heat exposure at every temperature increase (hours 96, 216, and 312), with the exception of time points beyond hour 384 at 33 °C. The magnitude of change in *Bcl-2* concentration was relatively small compared to other genes analyzed (Fig 7**)**.

Overall Treatment Effect on Gene and Time Point

Similar to results observed during the acute heat treatment study, chronic heat exposure had a large effect on *HSP90*, which was highly down-regulated at hours 72, 192, 216, and 312, with a total fold change difference relative to control at +23.38 (calculated as the sum of the absolute value of fold change at each time point). The time point where the largest total fold change across all genes occurred was hour 72 (27.0 °C) with the second largest occurring at hour 312 (31.5 °C). Hour 72 is largely influenced by a substantial down regulation of *catalase*. Additionally, hours 192 (28.5 °C) and 288 (30.0 °C) had the highest frequency of genes showing statistically significant differences in expression (3 genes each).

DISCUSSION:

The objective of this study was to evaluate the response of the upper mesophotic (30 – 50 m) coral, *M. cavernosa*, to acute and chronic heat stress, and heat stress plus oil. There are different definitions of acute and chronic associated with heat stress (i.e. climate change). Dove and Ortiz (2006) described acute heat stress to be less than 24 hours whereas Kenkel et al. [38] suggest acute as persisting for four days. The definition of chronic, however, ranges from approximately seven days to six weeks or longer [10, 39,]. Here, I define acute stress as a 72 hour (3 day) exposure and chronic stress as a 480 hour (20 day) exposure. Acute exposure of both Heat and Heat + Oil revealed a striking difference in the abundance of most genes analyzed when compared between each other and to control samples. Additionally, when comparing the acute (both treatments) to the chronic response, different gene expression profiles were observed.

Coral Response to Acute Experimental Treatments

Corals had a milder response to acute heat stress than to the combination of Heat + Oil treatments which was expected as oil is an additional stressor. The Heat treatment elicited a similar pattern of expression to that of the control samples (in terms of concentration) with slightly elevated concentrations indicating a lack of substantial stress on the coral host. The magnitude of change in most genes (fold change relative to control) when comparing heat treatment to control was similar to other coral species (e.g. *Orbicella faveolata* (formerly *Montastrea cavernosa*)) subjected to thermal stress at similar temperatures tested here (31.5 °C) [40]. There was a greater effect on coral gene abundance both in terms of fold change (relative to control) and intra-gene variance observed between time points in both Heat and Heat + Oil treatments compared to the control.

The expression of *RpL9* and *RpS7* revealed a consistent gene concentration ratio at each time point across all treatments (except for Heat + Oil at hour 72) which was expected as they are both required in the synthesis of protein products as structural constituents of the small and large ribosomal subunits [41, 42]. Throughout the acute study for all treatment types, it was speculated that the production of proteins would not be substantially affected using the expression of *RpL9* and *RpS7* as a proxy for ribosomal activity.

The only genes to remain relatively constant were ribosomal proteins *RpL9* and *RpS7*, which was likely attributable to the continuous production of proteins required to sustain homeostasis over the short duration of stress [43]. The relatively constant expression of *RpL9* and *RpS7* is not surprising as [44, 45] have used these proteins as reference genes in qRT-PCR experiments relying on their constant and similar expression concentrations. The relative expression of ribosomal proteins to control samples indicates that the treatment had an effect on the coral as seen by a reduction in expression concentration. However, it must be noted that the concentrations of all transcripts at hour 0 varied between treatment and control which may account for the observed treatment effect (down-regulation during early time points). The cause of variation is unknown and perplexing as there were numerous steps taken to ensure even template loading at all phases of the gene expression analysis.

The trend of gene upregulation relative to the control, for *BCL-2*, *catalase*, and *HSP90* at all observed time points, indicated a cell-survival response where an increase in anti-apoptotic factors prevent premature apoptosis, anti-oxidant enzymes mitigating host damage from oxygen radicals, and the molecular chaperone is employed to maintain protein integrity by re-folding heat denatured proteins. The overall production of proteins can be speculated as decreasing with the down-regulation of *RpL9* and *RpS7*, as these proteins are essential for the proper functioning

of the ribosomal unit as a whole. Although total protein production was considered down-regulated, the increased concentration of *BCL-2, catalase, and HSP90* may have occurred because these genes are precursors to the expression of other stress response genes (e.g. *superoxide dismutase, HSP70, Caspase-3*) which help reduce any stress a host coral is experiencing.

In the control samples, the change of gene expression was not significant over time which was to be expected as no stress was applied to these corals. Starting and ending at hour 0 to hour 72, gene concentrations remained highly similar in abundance in all genes in the control samples except *HSP90* which was somewhat elevated potentially indicating the occurrence of protein denaturation (based on the renaturation function of *HSP90*).

Coral Response to Chronic Experimental Heat Stress

Montastraea cavernosa exposed to chronic heat stress revealed a markedly different response compared to acute heat stress. The difference may be due to the duration of stress which has similarly been observed by [10, 46, 47]. In both control and treatment samples examined here, I observed a reduction in transcript concentrations from the onset to conclusion of the experiment. There was, however, significant intra-gene variation (up- and down-regulation) relative to consecutive time points for all genes assayed. Unlike the acute study, the concentrations of all genes in the control and treatment samples were highly similar at hour 0.

RpL9 and *RpS7* remained relatively constant throughout the treatment. Constant expression maintained over time suggests a need/demand by the coral to produce ribosomal proteins for the continued production of other intracellular proteins required for a stress response/normal physiological function. Time and treatment had negligible effect on the production of *RpL9* and *RpS7* except at hour 288 (30 °C) and 480 (33.0 °C). The strong

upregulation of other stress response genes at hour 288 and 480 likely required elevated levels of ribosomes which may explain the upregulation of *RpL9* and *RpS7* at these time points. There were few instances where transcript concentrations surpassed those measured at hour 0 and raw values are not reported here. The majority of time points that did surpass hour 0 concentrations fell during hours 288 and 408 which indicates a substantial physiological response (based on the rarity of transcript abundances surpassing hour 0 values). I speculate that the response of up-regulation at hour 288 represents a physiological tipping point as five of the six analyzed gene concentrations increased. The putative functions of the genes analyzed indicate a physiological stress has occurred as there is little to no benefit of having excess intracellular concentrations during a non-stressed physiological state. In addition to the relative up-regulation, the direction of transcript concentrations was opposite to that of control samples for all genes at hours 288 and 408 (i.e. from hour 216 to 288, control transcript levels were decreasing while heat treatment transcript levels increased).

Multiple genes (*catalase*, *HSP90*, *RpL9*, and *RpS7*) were observed significantly differentially expressed at temperatures between 27 – 28.5 °C (hours 0 – 192), which is considered a sub-lethal temperature [48, 49]. These differential responses at sub-lethal or ambient temperatures are likely normal physiological processes which may account for the high degree of variability within a gene as coral are known to have a high degree of gene expression variability even within the same colony [50, 51].

Significant differences were observed within and among genes in the same treatment group (control and heat). Control samples had a higher number of significant differences within the control group when comparing between genes and time points. For instance, two time points analyzed in the control group represent 75% of the time interactions (e.g. see *cathepsin L1*)

within that gene which is different than the heat treatments where a variety of time points are different to one another. It is plausible that the higher number of time points contributing to a significant time interaction represent a physiological response to stress. Another words, a higher number of intra-gene significant time point differences may demonstrate a continuous physiological change indicating a dynamic response to stress. These particular time points may be outliers skewing the data and misleading the influence that duration has regarding gene expression under ambient conditions. Alternatively, the heat treatments may have elicited a higher number of time points contributing to the overall significant time interaction representative of a worsening stress to the coral. Although fewer significant intra-gene points of time in the heat treatments were observed, more heterogeneity exists when compared to control samples. This observation suggests that temperature and time had a significant effect throughout the entire treatment period compared to control samples where time was the only factor.

Hour 288 at 30.0 °C revealed a substantial physiological response indicated as an abrupt increase in gene concentration during heat treatment compared to control. Based on the influence of heat at Hour 288 to the entire suite of genes analyzed, the significant increase of gene concentration may be a critical physiological turning point in host survivorship. Because a number of genes (5) were simultaneously up-regulated relative to the control, it is plausible that coral exposed to 30 °C for 72 hours (plus previous heat exposures of 27 °C and 28.5 °C) experienced significant physiological stress, representing a chronic temperature threshold. Although it has been observed that coral can survive temperatures exceeding 30.0 °C [8], there is a lack of heat stress data regarding coral communities below 30 m. I speculate that the deeper coral sampled here (i.e. mesophotic coral) have yet to experience significant heat anomalies

rendering them more susceptible to temperature thus lowering the temperature needed to cause physiological stress [47, 52].

BCL-2 was limited in the number of statistically significant differential time points relative to the control, but it was observed to be highly responsive to changes in temperature with a down- then up-regulation at the beginning and end of each temperature increase. This observation was consistent from time points hour 0 through hour 384 at 31.5 °C. At each time point where temperature was first increased, the expression of *BCL-2* relative to control decreased. When the next successive time point was measured, the expression of *BCL-2* increased. The increase in expression was observed after 72 hours of exposure at each new temperature until hour 384. The increase in expression after 72 hours associated with increased temperature I hypothesize is likely a cell salvaging mechanism preventing the premature apoptosis of coral host cells. Host coral cells that have not been subjected to high levels or long periods of heat stress to cause mortality show signs of preventing apoptosis and ultimately recover when conditions return to a favorable state [53].

Acute versus Chronic Exposure to Stress:

Comparisons of acute versus chronic exposures to heat stress showed no significant intra-gene differences between time points during acute heat treatments. The lack of significant differences in the acute treatments may be due to the lower number of coral fragments in the tank compared to chronic treatments (possible coral fragment interaction with densely populated IC), or possibly the duration of the treatments (72 hours versus 480 hours). The lack of significant differences in the acute experiment is indicative that the duration of exposure has a significant effect on the physiology of the coral host. Because the final temperatures were identical in each of the

treatments (i.e. 33 °C), the length of exposure (i.e. duration) contributed to elevated physiological stress observed in the chronic experiment.

A primary difference between the gene expression patterns observed in the chronic versus acute treatment was the continual decline in total transcript abundance from Hour 0 in the chronic experiment, over the entire course of the experiment but was variable in the acute study (some higher and/or lower than Hour 0). This discrepancy may be due to the short duration of the acute treatment. Based on the highly volatile expression of *catalase* in the chronic experiment, it is most probable that coral exposed to chronically elevated temperatures had substantially more oxidative stress than acute coral samples exposed to acute treatments. *HSP90* was up-regulated in all but one time point in the acute experiment in both Heat and Heat + Oil samples but was significantly down-regulated at most time points in the chronic experiment.

CONCLUSION:

In this study, *M. cavernosa* coral exposed to the acute heat treatments showed less physiological response than those exposed to the Heat + Oil treatments. Although the heat stress treatments did elicit an internal physiological response, *M. cavernosa* showed no symptoms of an external physiological stress over the short duration. The internal physiological response observed included relatively small but consistent increases in stress response genes (i.e. *catalas*e, *HSP90*, and *BCL-2*). Heat + Oil exposure produced a much different response, observed as large intra-gene gene abundance fluctuations between time points compared to control and heat alone. In general, the pattern of gene expression produced by the host coral fluctuated likely due to various biotic (e.g. normal physiological variation) and abiotic (e.g. oil exposure & heat) factors. It is likely that a 72 hour exposure at 33 °C was not sufficient to cause coral mortality. To confirm that a 72 hour exposure in elevated temperatures (with the addition of oil) is survivable for upper mesophotic coral, a post-treatment (i.e. recovery phase) gene expression analysis should be conducted in future studies. It is also plausible that a chronic heat treatment would have been more revealing and more comparable to the chronic heat treatment in this study. It is also unclear what constituent(s) of the oil slurry contributed to the elevated corals stress when exposed to both Heat + Oil. Future studies should examine individual chemical components of oil, particularly those from and oil spill like that in the Gulf of Mexico and/or the chemicals used to clean-up the oil (e.g. Corexit), and how individually and synergistically they contribute to the overall stress of coral at mesophotic depths.

Chronic exposure to temperatures at 30.0 °C for 72 hours may be considered as a physiological tipping point for *M. cavernosa* corals sampled on Conch Reef at upper mesphotic depths (30 m), as observed by the up-regulation of five of six genes assayed. The up-regulation

of these five genes (*BCL-2*, *Catalase, Cathepsin L1, RpL9, & RpS7*) most likely represents a physiological threshold. All of the up-regulated genes are involved with host physiological function including anti-apoptotic effects, proteolytic capacity, ribosomal protein structure, and anti-oxidant properties. These are important functions in preserving the well-being of the host through normal protein production, maintaining protein integrity, and recycling old or potentially hazardous cellular components.

A noticeable trend of transcript abundance decline from Hour 0 was observed for both control and chronic heat treatments. The decline of transcript abundance from Hour 0 may be in response to elevated temperature as a larger decline was similarly observed in heat treated samples. It is also plausible that general captivity had a negative effect on transcript concentration. It is unlikely, however, that general captivity would influence the experimental data because in all cases, the experimental tanks and the fragments of all coral were randomized.

Overall, the acute and chronic heat treatments elicited coral responses that showed highly different trends in gene expression. Coral exposed to chronic heat revealed more dramatic intra-gene fluctuations whereas the acute exposure was less volatile. A physiological "tipping point," (i.e. physiological processes/cascades leading to cell death) for the coral host was not observed in the acute study, perhaps due to the short duration of the experiment, but I suggest based upon the chronic heat treatments, that at a minimum exposure of 72 hrs at 30 °C, at time point Hour 288, mesophotic coral will show significant symptoms of stress. In this study, *M. cavernosa* coral sampled from Conch Reef at an upper mesophotic depth seem to respond similarly to those found in shallow water communities [40] and are likely to be equally threatened as climate change continues to warm seawater.

FUTURE WORK:

Additional work is needed to further refine the coral host's physiological response to heat stress at depths exceeding 30 meters. Additional gene classes should be included such as cellular homeostasis genes, energy utilization genes, and reproductive related genes. Further investigation should be applied to detect differences in the response of shallow water coral to those of mesophotic or even deeper environments where *M. cavernosa* exists. Additional depth generalist species might offer the best solution when examining the response of coral at different depths and also provide insight as to how deeper coral may survive transplant or repopulating shallow reefs.

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Figures (Uploaded as separate files to PLOS One)

Figure 1: Sampling Location Map (Lat: 24.9580667, Lon: -80.45243)

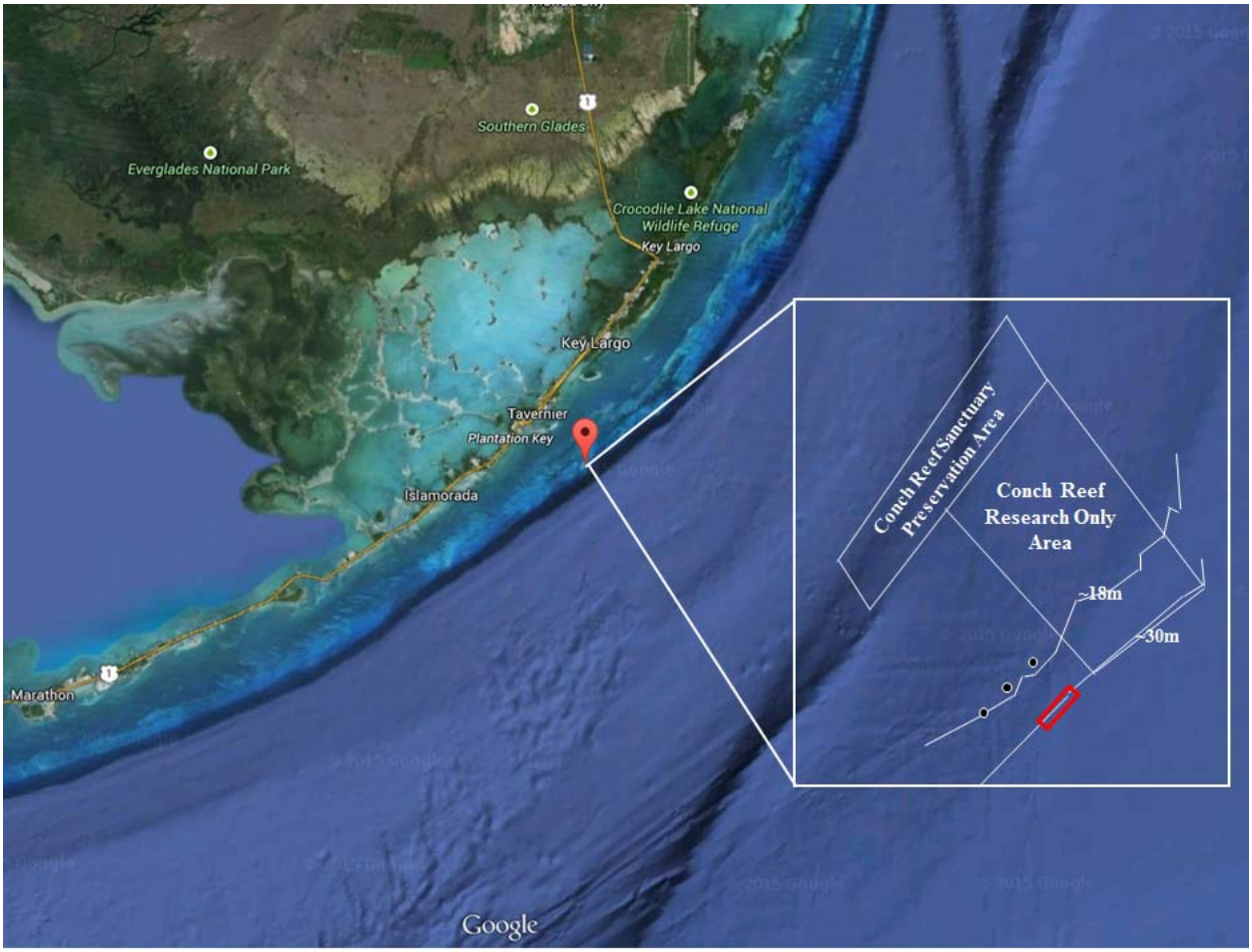


Figure 2: Incubation Chamber and Water Jacket Setup

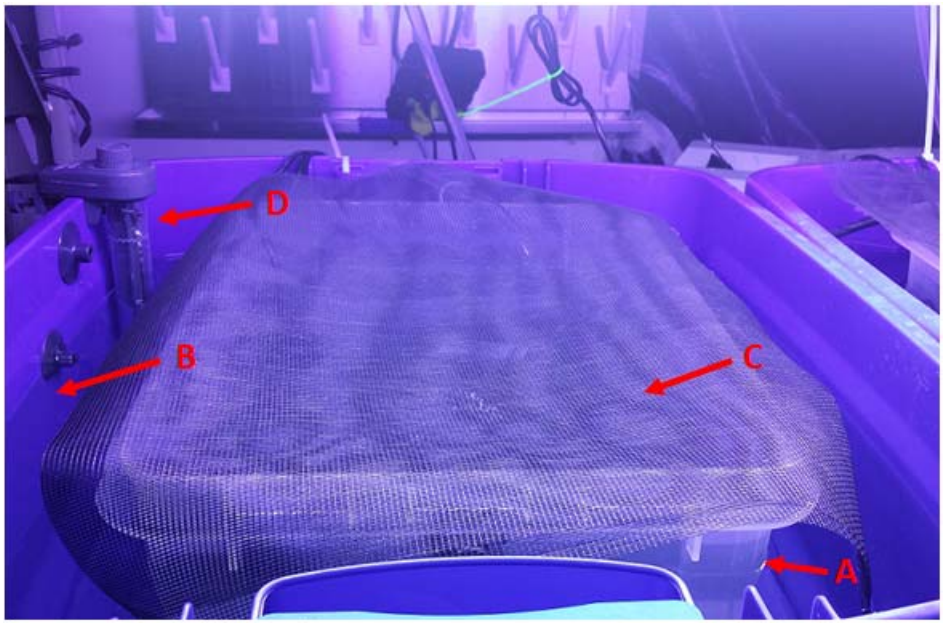


Figure 3: Example of the experimental tank setup

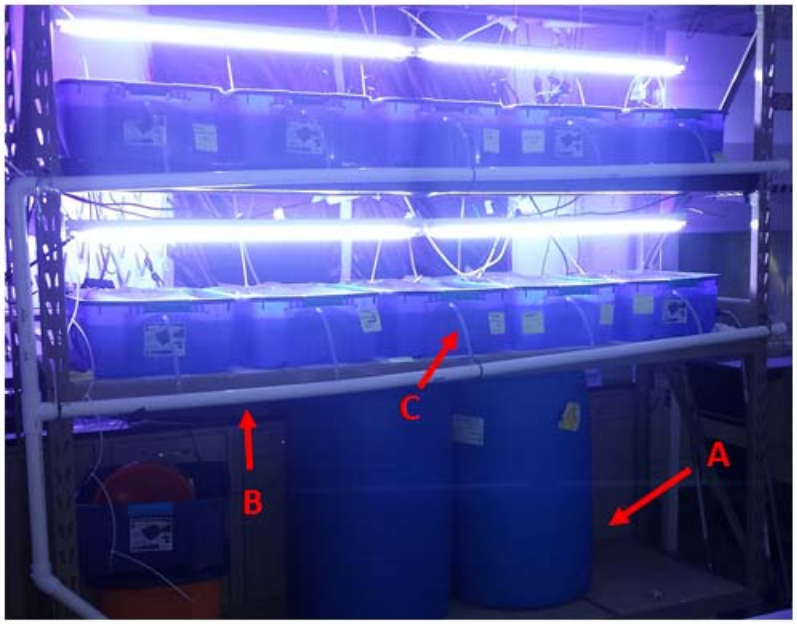


Figure 4. Gene Abundance during Acute Heat Exposure

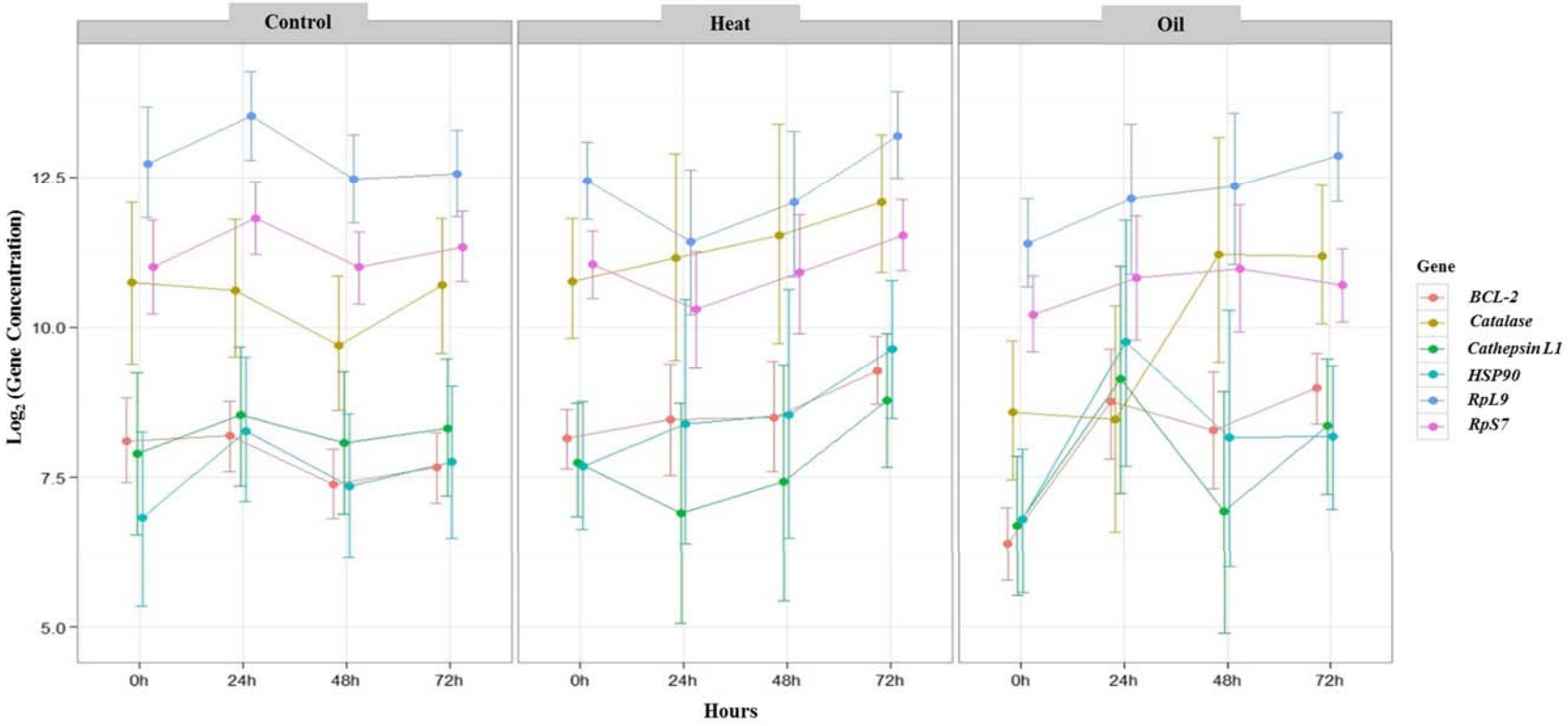


Figure 5: Heat Treatment – Gene Expression Fold Change Relative to Control.

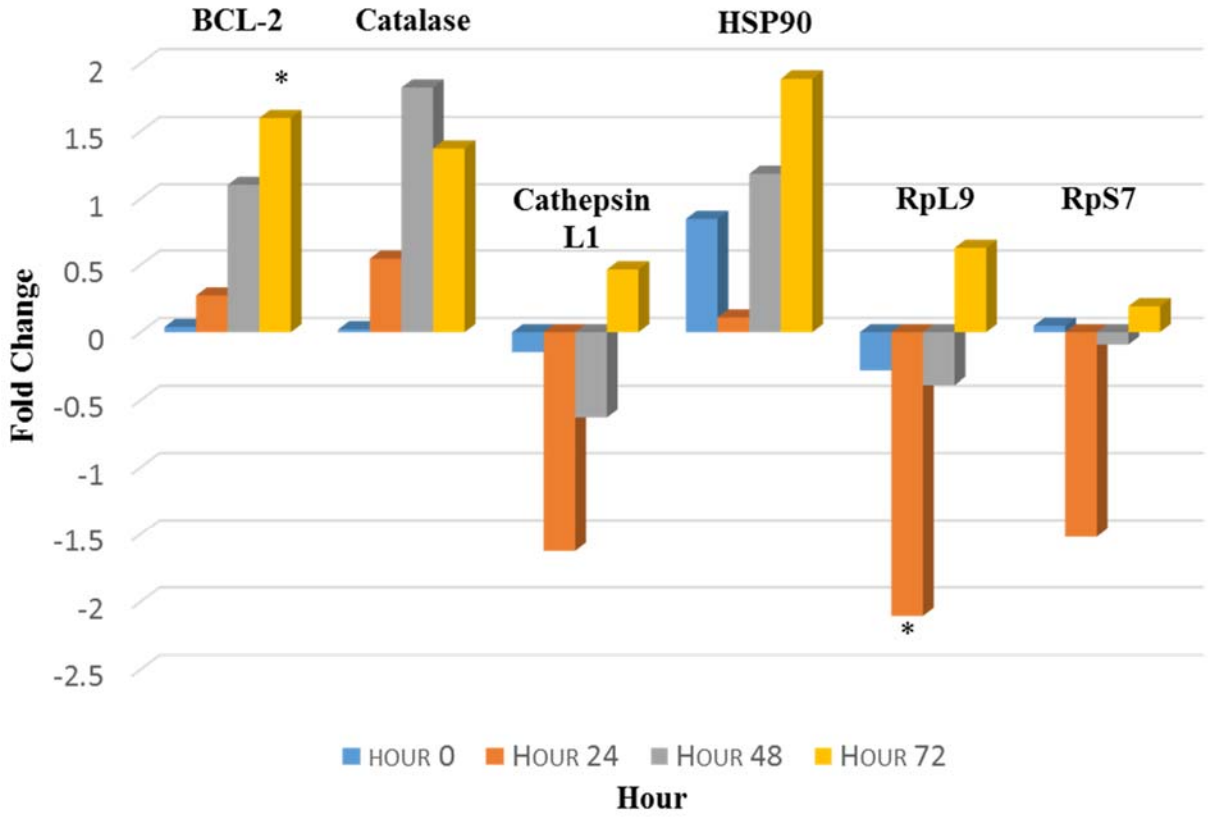


Figure 6: Heat + Oil Treatment – Gene Expression Fold Change Relative to Control.

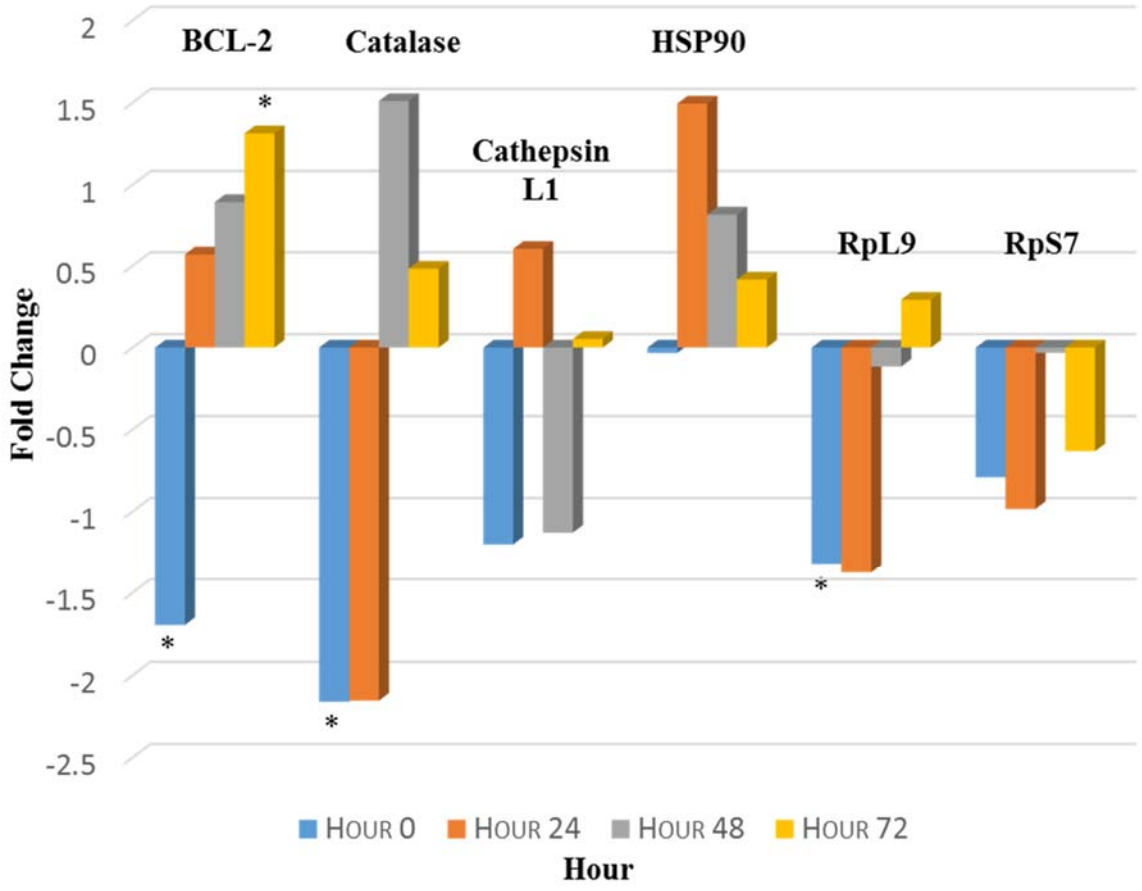


Figure 7: Gene Expression Fold Change of Heat Treated Samples Relative to Control

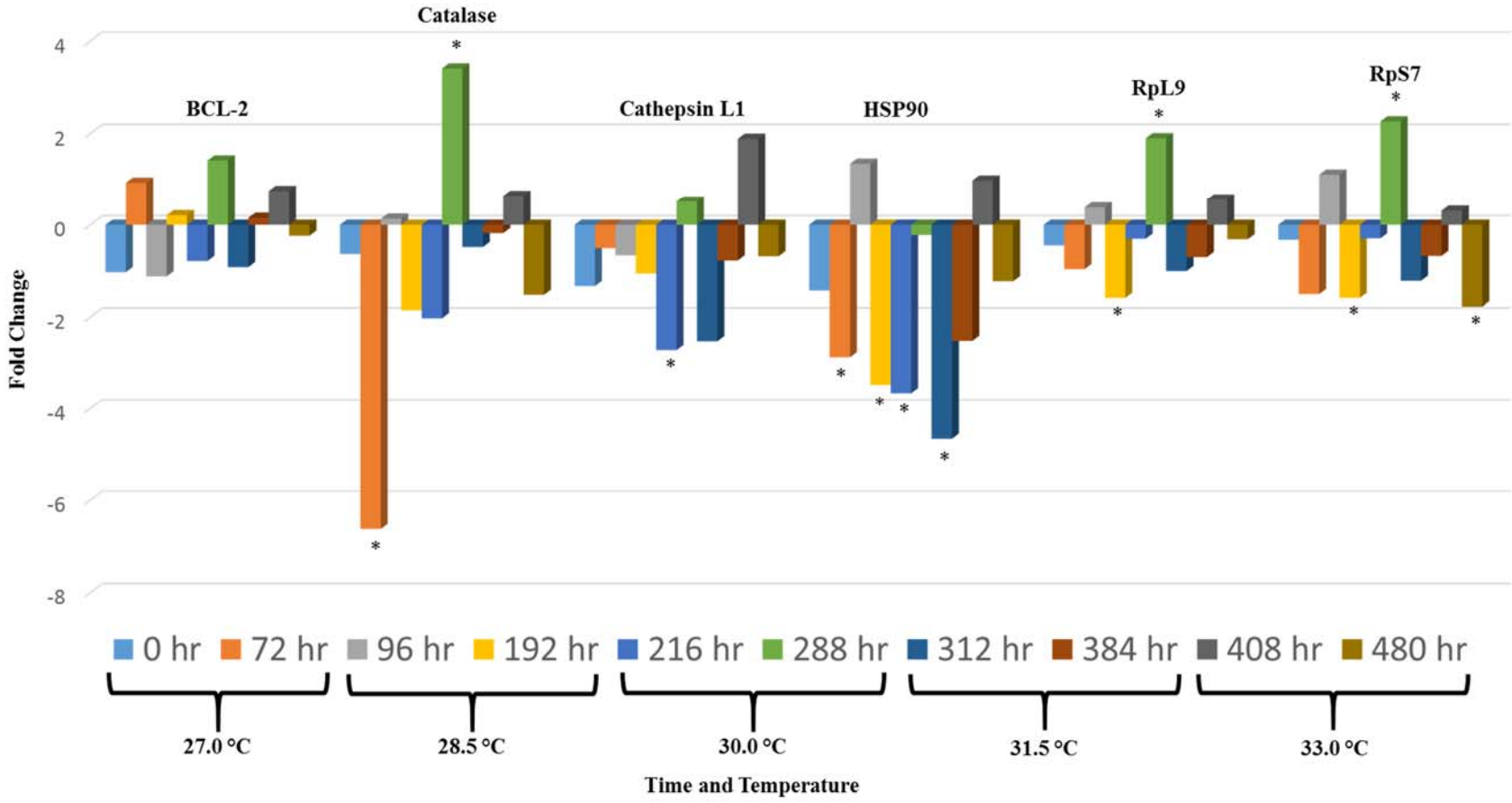
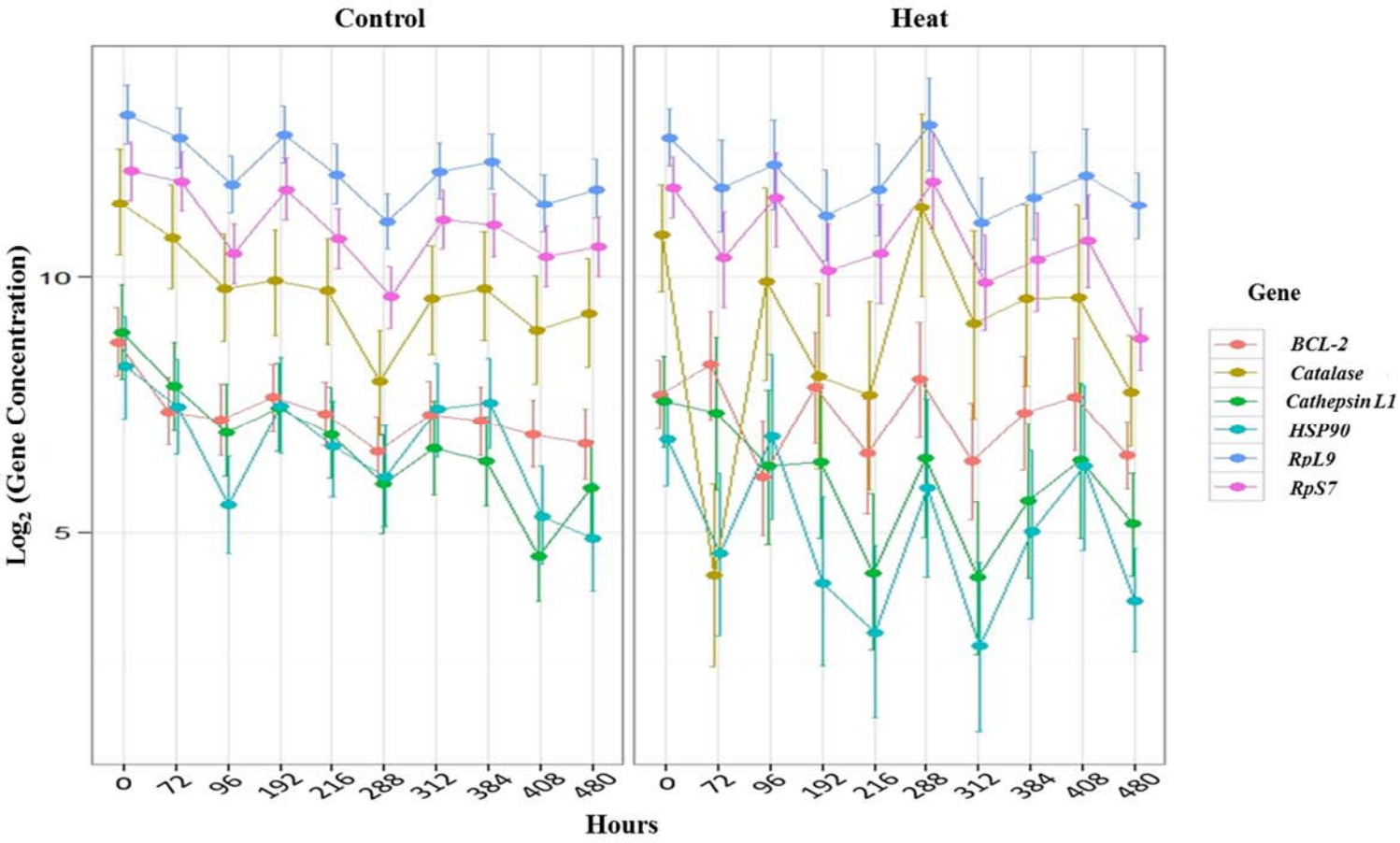


Figure 8: Chronic Heat Treatment – Gene Abundance



SUPPLEMENTARY MATERIAL:

Maintaining Coral Husbandry: Main Holding Tank

The main coral holding tank was visually monitored for water level and algae growth daily and assayed for fluid chemistry on a weekly basis. Phosphate, nitrate, calcium, and carbonate were monitored using Aquarium Pharmaceuticals® API Saltwater Liquid Master Test Kit. Any results deviating from manufacturer recommendations resulted in a 50% water change. In addition, the coral was fed (twice a week) using PhytoPlex Phytoplankton (Kent Marine) and micronutrient supplements (Kent Marine Essential Elements) per the manufacturer's recommendation. Temperature was maintained between 26 – 27 °C using a 250W submersible aquarium heater; this temperature was observed during sample collection and is known to be well below stress thresholds.

Supplementary Table 1: Significant Intragene Time Point Interactions

Gene	**Time Points Revealing Significant Difference**	**Treatment**
Bcl-2	Hour 0 - Hour 96	Heat
Bcl-2	Hour 72 - Hour 96	Heat
Bcl-2	Hour 72 - Hour 216	Heat
Bcl-2	Hour 72 - Hour 312	Heat
Bcl-2	Hour 72 - Hour 480	Heat
Bcl-2	Hour 96 - Hour 192	Heat
Bcl-2	Hour 96 - Hour 288	Heat
Bcl-2	Hour 96 - Hour 408	Heat
Bcl-2	Hour 192 - Hour 312	Heat

Bcl-2	Hour 288 - Hour 312	Heat
Bcl-2	Hour 288 - Hour 480	Heat
Catalase	Hour 0 - Hour 72	Heat
Catalase	Hour 0 - Hour 192	Heat
Catalase	Hour 0 - hour 216	Heat
Catalase	Hour 0 - Hour 480	Heat
Catalase	Hour 72 - Hour 96	Heat
Catalase	Hour 72 - Hour 192	Heat
Catalase	Hour 72 - Hour 216	Heat
Catalase	Hour 72 - Hour 288	Heat
Catalase	Hour 72 - Hour 312	Heat
Catalase	Hour 72 - Hour 384	Heat
Catalase	Hour 72 - Hour 408	Heat
Catalase	Hour 72 - Hour 480	Heat
Catalase	Hour 192 - Hour 288	Heat
Catalase	Hour 216 - Hour 288	Heat
Catalase	Hour 288 - Hour 480	Heat
Cathepsin L1	Hour 0 - hour 216	Heat
Cathepsin L1	Hour 0 - Hour 312	Heat
Cathepsin L1	Hour 0 - Hour 384	Heat
Cathepsin L1	Hour 0 - Hour 480	Heat
Cathepsin L1	Hour 72 - Hour 216	Heat
Cathepsin L1	Hour 72 - Hour 312	Heat

Cathepsin L1	Hour 96 - Hour 216	Heat
Cathepsin L1	Hour 96 - Hour 312	Heat
Cathepsin L1	Hour 192 - Hour 216	Heat
Cathepsin L1	Hour 192 - Hour 312	Heat
Cathepsin L1	Hour 216 - Hour 288	Heat
Cathepsin L1	Hour 216 - Hour 408	Heat
Cathepsin L1	Hour 288 - Hour 312	Heat
Cathepsin L1	Hour 312 - Hour 408	Heat
HSP90	Hour 0 - Hour 72	Heat
HSP90	Hour 0 - Hour 192	Heat
HSP90	Hour 0 - Hour 216	Heat
HSP90	Hour 0 - Hour 312	Heat
HSP90	Hour 0 - Hour 480	Heat
HSP90	Hour 72 - Hour 96	Heat
HSP90	Hour 96 - Hour 192	Heat
HSP90	Hour 96 - Hour 216	Heat
HSP90	Hour 96 - Hour 312	Heat
HSP90	Hour 96 - Hour 480	Heat
HSP90	Hour 192 - Hour 408	Heat
HSP90	Hour 216 - Hour 288	Heat
HSP90	Hour 216 - Hour 408	Heat
HSP90	Hour 288 - Hour 312	Heat
HSP90	Hour 312 - Hour 384	Heat

HSP90	Hour 312 - Hour 408	Heat
HSP90	Hour 408 - Hour 480	Heat
RpL9	Hour 0 - Hour 192	Heat
RpL9	Hour 0 - Hour 312	Heat
RpL9	Hour 0 - Hour 384	Heat
RpL9	Hour 0 - Hour 480	Heat
RpL9	Hour 72 - Hour 288	Heat
RpL9	Hour 96 - Hour 312	Heat
RpL9	Hour 192 - Hour 288	Heat
RpL9	Hour 216 - Hour 288	Heat
RpL9	Hour 288 - Hour 312	Heat
RpL9	Hour 288 - Hour 384	Heat
RpL9	Hour 288 - Hour 480	Heat
RpS7	Hour 0 - Hour 72	Heat
RpS7	Hour 0 - Hour 192	Heat
RpS7	Hour 0 - Hour 216	Heat
RpS7	Hour 0 - Hour 312	Heat
RpS7	Hour 0 - Hour 384	Heat
RpS7	Hour 0 - Hour 480	Heat
RpS7	Hour 72 - Hour 288	Heat
RpS7	Hour 72 - Hour 480	Heat
RpS7	Hour 96 - Hour 192	Heat
RpS7	Hour 96 - Hour 312	Heat

RpS7	Hour 96 - Hour 384	Heat
RpS7	Hour 96 - Hour 480	Heat
RpS7	Hour 192 - Hour 288	Heat
RpS7	Hour 192 - Hour 480	Heat
RpS7	Hour 216 - Hour 288	Heat
RpS7	Hour 216 - Hour 480	Heat
RpS7	Hour 288 - Hour 312	Heat
RpS7	Hour 288 - Hour 384	Heat
RpS7	Hour 288 - Hour 480	Heat
RpS7	Hour 384 - Hour 480	Heat
RpS7	Hour 408 - Hour 480	Heat

Chapter 3

EXTENDED LITERATURE REVIEW

Introduction:

Coral reefs are complex and diverse ecosystems that have significant ecological and economic value which are currently being exploited and deteriorated at an alarming rate [1, 2, 3]. Coral reefs are present in a variety of marine environments and structures (fringing, barrier, patch, deep, and atoll), range substantially in depth (0 m (during low tide) to 1000+ m), and are estimated to cover 255,000 km^2 [4, 5].

The physical and biological structure of coral reefs vary between coral, sponge, microbial, algal, and higher trophic level composition in part due to different environmental conditions and the degree of human influence [6, 7, 8, 9, 10]. Although reefs vary between location, five general characteristics are thought to influence their distribution and structure including temperature, salinity, nutrients, light availability, and aragonite saturation [11]. These general characteristics and field observations were modeled [see 11] and provide an estimated global coral distribution showing that the concentration of most shallow water tropical reefs exist between 30°N to 30°S latitudes.

Ecologically, fish and other organisms use coral reefs to reproduce, forage, find shelter and provide an essential food source for higher level predators [12, 13]. Reefs also provide the physical framework for other corals and sessile organisms to settle and colonize. Bacterial assemblages are also commonly associated with coral (in the mucus, internally, and on the calcium carbonate ($CaCO_3$) skeleton) as well as free-living communities in adjacent seawater [9, 13]. The bacterial mucus associated microflora found covering the corals ectoderm are beneficial to coral by suppressing other bacterial growth and provide additional metabolites

which may suppress predation, [14, 15] but may become pathogenic under certain environmental conditions, similar to the commensal bacteria found within the human gut [16, 17]. The microflora growing on coral are of growing interest and it remains to be seen how they influence growth, health, diversity, and function of coral reefs. The importance of symbiosis, however, is well understood in coral. Symbiosis occurs through a relationship with a dinoflagellate microalgal species called *Symbiodinium* sp. Commonly called zooxanthellae, *Symbiodinium* provides the coral host a mechanism to flourish in oligotrophic waters by photosynthesizing and translocating a variety of high energy molecules (e.g., glycerol, amino acids, & lipids) to the host [18]. Collectively, the coral, microbial consortia, and *Symbiodinium* constitute what is referred to as the coral holobiont.

The economic value of coral reefs globally (e.g. Caribbean, Australia, South Pacific islands) is estimated to exceed one billion of dollars, mostly in the form of tourism [19, 20]. Reefs provide an immeasurable capacity for potentially therapeutic compounds, mitigate coastal degradation from storms, provide food for local residents, and are the source of an increasing aquarium trade [1, 3].

In the past decade there have been studies examining mesophotic coral ecosystems to better understand the potential benefits these deeper reefs could provide shallow reefs [8, 21, 22]. Much of the work has been mapping and characterizing these ecosystems by videography using remotely operated vehicles (ROV) or handheld video cameras to investigate substrate type, coral, fish, and algae composition. The following review is organized in the following sections: (1) **a broad understanding of climate change and organismal response, (**2**) coral ecology, and** (3) **methods of detecting physiological responses to climate change.**

Climate Change:

Climate change is a process that has been intimately linked with earth's history. Scientists have been able to explore earths past climatic conditions using deep sea sediment core samples, continental shelf fauna, and ice core samples that all essentially take a snapshot of earth's climate [23]. Although earth's climate is continually in flux, its rate and magnitude of change is alarming. Factors influencing climate change began to increase during the industrial revolution and based on pre-industrial revolution atmospheric measurements, gases like carbon dioxide have increased > 25% and continue to rise [24]. The industrial revolution has led to many scientists referring to this period of climate change as anthropogenic climate change. Humans have not only increased atmospheric gases from fossil fuel combustion, but as an increasing population the demands for more energy, food, and urbanization have led to the depletion of many natural resources.

The combustion of fossil fuel produces a variety of gaseous byproducts including carbon dioxide, carbon monoxide, nitrogen oxides, and clorofluorocarbons [25, 26]. In addition, some animals, such as cows, are a source of a highly potent gas, methane contributing upwards of 50 Tg of methane emission annually [27]. These gases, among others, are collectively called 'greenhouse gases'. Greenhouse gases contribute to the 'greenhouse effect', which is the process by which long-wave radiation, reflected off earth's surface, is absorbed or maintained in earth's atmosphere [24]. As concentrations of these greenhouse gases rise, the 'greenhouse effect' also rises creating a warmer atmosphere [24]. The atmospheric-ocean and freshwater interactions provides a means for some atmospheric heat to be absorbed by aquatic ecosystems. In the 20th century, the warming atmosphere increased the average ocean temperature 0.74 °C and freshwater systems are increasing temperature similarly [28, 29].

Response to climate change:

The health of coral reefs has declined well over the past 100 years and is accelerating at an alarming rate due to harmful environmental (e.g. ocean temperature, ocean pH, ocean chemistry) and anthropogenic (e.g. coastal development, fossil fuel combustion, over fishing) influences [30]. Factors affecting reef structure and health include life history, water temperature, light intensity, pH, salinity, and fluid chemistry, predation, inter species competition (trophic level competition), disease, and over fishing to name a few [12, 31, 32, 33]. The most common environmental hazards leading to the decline in coral health includes light intensity, water temperature, pH, disease, and anthropogenic pollution [34, 35]. Increased ocean temperature can cause coral bleaching, which is the dissociation of coral host and symbiont leading to mortality, and when coupled with high irradiance, increases lethality [36, 37, 38]. During this bleaching process, the symbiont is either digested or expelled by the coral host cell [39].

Barhshis *et al*. [40], Bellantuono *et al*. [41], and Haslun *et al*. [42] observed certain coral species (e.g. *Montastraea cavernosa*) having previous experience with thermal disturbances are more resistant to subsequent thermal stress than those who have had no prior exposure [40, 41]. Bellantuono *et al* [41] found that when exposing coral to a sublethal but still elevated temperature, termed “preconditioning”, compared to a non-preconditioned coral, the former did not bleach whereas the latter did. These authors also observed little to no difference in the types of genes expressed between preconditioned and non-preconditioned coral and rather, the magnitude of the expressed genes varied significantly (i.e., preconditioned coral gene expression fold change was much less than non-preconditioned coral expression; [41]). Observations by Bellantuono *et al* [40] are consistent with a study of Barshis *et al* [41] who examined naturally disturbed/variable (fluctuating temperature conditions) coral populations. Barshis *et al* [41]

collected corals from highly variable coral community (HV) experience fluctuating temperature and irradiance and a moderately variable coral community (MV) where conditions remained relatively unchanged. Both communities were exposed to a simulated bleaching event and found that the differentially expressed genes were similar to studies by Bellantuono *et al* [40]; the HV community gene expression fold increase was much less (i.e. higher constitutive expression) than the MV gene fold increase. The HV coral having a lower expression fold change survived the bleaching event where as the MV coral did not. These observations indicate that pre-exposure of coral reefs to particular perturbations (e.g. heat stress) may be paramount to survivorship.

Coral Ecology:

Mesophotic Coral Ecosystem

Mesophotic coral are photosynthetic holobionts ranging from 30 – 150 m [43, 44]. Early work by Vaughn [45] observed deep corals in Hawaii followed later by Goreau [46] who observed hermatypic corals in Jamaica at depths of 70 m. The intermediate depth of mesophotic coral ecosystems (MCEs) has historically been very difficult to access for observation and sampling purposes. Traditional recreational SCUBA limits did not allow researchers to access the deeper depth range of MCE's (recreational underwater diving limit is ~35 m) and as such would require the use of expensive remote operated vehicles (ROV) and/or autonomous underwater vehicles (AUV). More recently, however, researchers are using technical SCUBA diving techniques (e.g. tri-mix, rebreathers) which can extend the depth and duration of dives at these sites (to date, most MCE's have been described using video monitoring with sleds, ROV's, or technical SCUBA; [22, 47]).

Considered a natural extension of shallow-water reefs, MCEs coral diversity declines with depth, however, the architecture and the flora and fauna associated with these reefs are

similarly very abundant [44]. In the Hawaiian archipelago for example, scleractinian coral cover was highest between 50 and 60 m whereas macroalgae abundance was highest at 70 m [8, 22, 48]. In the Bahamas, Aponte & Ballantine [6] reported macroalgae zonation having a slight overlap with one another and, as observed by [22, 49], percent coral cover decreased with depth. There are many organisms (fish, corals, sponges etc.) specific to these depths as well as organisms that extend over shallow and mesophotic depths [48, 50]. Such "depth generalist" coral species, those that span shallow and deep water, may be prime candidates for reseeding shallow depleted reefs however more research is needed on their metabolic plasticity.

Mesophotic coral ecosystems (MCE's) are generally categorized into zones (i.e. zonation) based upon depth (i.e. upper mesophotic = 30 – 60 m; lower mesophotic = 100 – 150 m). Zonation is characterized by overall organismal species diversity, percent live benthic cover, and morphology [22]. Rooney *et. al.* [22], for example, analyzed data on MCEs of the Hawaiian archipelago and suggested three distinct mesophotic coral "zones"; (1) the upper mesophotic zone between 30 – 50 m, (2) branching/plate coral MCE's between 50 – 80 m, and (3) the Leptoseris MCE's between 80 – 130 m.

One important factor of coral survival is the mechanism in which corals receive their metabolic needs. Much of the energy required by corals is recognized to be a consequence of symbiosis with *Symbiodinium* [51, 52]. Different clades (evolutionary lineages) of *Symbiodinium* have shown to yield particular advantages to their host (e.g. thermo-tolerance, high irradiance tolerance) which contributes to the zonation observed along MCE depth gradients [53, 54]. Mesophotic corals are also able to ingest food heterotrophically fulfilling 20 – 90% of their metabolic needs which occurs more often at greater depths [8, 55].

Mesophotic coral ecosystems are hypothesized as being refuges for reseeding (i.e. providing coral larvae) shallow depleted reefs, those that have succumbed to environmental or anthropogenic disturbance, due to their proximity and depth distribution [56, 57, 58]. Riegl [59] has proposed two models to help characterize why deeper reefs (e.g. MCEs) may be refugias: (1) the top 10 m of the water column cannot support coral growth due to warming and (2) eventually the top 20 m of seawater will become "inhospitable" [59]. Riegl and Piller [59] speculated that corals at deeper depths will play a significant role in "buffering" MCEs from detrimental environmental and anthropogenic influences. For example, the coral *Seriatopora hystrix* was decimated and considered "locally extinct" from shallow reefs off the coast of Okinawa Japan due to bleaching events in 1998 and 2001 [60]. Upon investigating deeper reefs off the coast of Okinawa, it was discovered [61] that numerous large and healthy colonies of *Seriatopora hystrix* between 35 – 47 m. These observations strengthen the proposal that MCE's are buffered from some disturbance events better than shallow water coral reefs and may serve as a refugia for distressed shallow reefs. Researchers have recently undertaken an effort to characterize the genetic connectivity between shallow and mesophotic reefs [48, 67]. Genetic connectivity is essential and will either help to support or refute the refugia hypothesis. Menza [63] and Bongaerts et al [64] suggest, howeverm that these ecosystems are equally susceptible to environmental and anthropogenic stressors. In this study, I have shown evidence that coral at the mesophotic depth are as susceptible to heat stress similarly to shallow water coral. In addition, there is recent evidence, reviewed by Loya et al [65] showing a lack of genetic connectivity between shallow water habitats and MCEs.

***Symbiodinium*:**

Symbiodinium sp. are an important component to most healthy reef building corals in the photic zone. *Symbiodinium* found within symbiosome cells of a host coral provide ~30 – 100% of carbon metabolites important for coral survivorship, generally in the form of high energy carbohydrates [51, 52]. In return, the coral host provides protection from herbivory, a static placement in the photic zone, and nutrients for *Symbiodinium* metabolism. *Symbiodinium* species are categorized into evolutionary clades (A – I) [66, 67] and hundreds of subclades and subtypes [68]. Determining cladal differences within and between coral species are important in decoding the potential resistance a holobiont may have to disturbance (e.g. climate change, ocean acidification). Some *Symbiodinium* clades are specialized in surviving at higher irradiance levels while others can tolerate warmer ocean temperatures [54, 69]. As a consequence, depending on the type of *Symbiodinium* existing within a host coral, the holobiont may have a reduced or higher level of fitness.

Symbiont specificity substantially affects MCE coral structure (i.e., diversity, feeding strategy, and morphology etc). Upper MCE's are similar to shallow water ecosystems, but change drastically with depth due to changes in symbiont type [22, 48, 70]. For example, in shallow water reefs, coral bleaching events have caused shifts in symbiont populations of affected corals from less thermo-tolerant clades (C) to a more thermo-tolerant clade (D) [69, 71]. Similarly, coral who harbored clade D prior to a bleaching episode were less affected compared to corals harboring other clades, e.g. clade C [72]. As such, corals appear to possess a degree of flexibility for harboring different *Symbiodinium* that have different physiological capabilities. In mesophotic zones, reduced irradiance limits coral growth and requires symbionts that are specialized in low light regimes [50]. Lesser et al [50] found that the majority of corals sampled

(*M.cavernosa*) harbored *Symbiodinium* clade C, however, this study only examined one coral species at one reef and likely doesn't reflect the MCE *Symbiodinium* population overall.

Gene expression as a measure of physiological response

Three commonly used gene expression methods to better understand the physiological response of an organism to stress are currently being used. For instance, (1) transcriptomic sequencing (RNA-Seq) has provided researchers a better mechanism to determine stress by examining an organism's entire set of expressed genes at a single point in time [73]. A less exhaustive methodology uses (2) microarray technology [74]. Microarrays allow a researcher to survey thousands of genes for which a particular sequence is already known and can be used as a starting point of reference. A third method (3) is known as quantitative real-time polymerase chain reaction (qRT-PCR), which allows a researcher to analyze the expression of a single gene with high precision at relatively low cost [75]. qRT-PCR allows the researcher the ability to scale up or down the number of genes to analyze and also has the benefit of measuring genes with very low abundances.

Conclusion:

Coral and other reef organisms are essential in maintaining complex reef habitats that have ecological and economic importance. Although there are many biotic and abiotic factors that contribute to reef structure and function, one of the most important facets is symbiosis. *Symbiodinium* sp. help determine a coral's fitness to certain environmental conditions and both diminish and confer resistance to the holobiont during a changing environment. Since the industrial revolution, which has led to the increased combustion of fossil fuels releasing volatile gases into the atmosphere, increasing atmospheric and oceanic temperatures are having catastrophic effects on marine organisms. Increased ocean temperatures have led to mass

bleaching events worldwide and continue to threaten marine ecosystems. However, some researchers hypothesize that deeper photosynthetically dependent reefs, termed mesophotic coral ecosystems (MCEs), may serve as refugias for shallow depleted reefs. Currently, there is evidence both supporting and refuting this hypothesis, yet moving coral from deep to shallower depths may be the best possible opportunity for coral to survive. Through the molecular characterization of coral physiological stress response, it is possible to identify tolerant species, which cellular mechanisms are most affected by stress, and how managers can protect those areas with exceptionally tolerant species.

EXTENDED METHODOLOGY:

Coral Sampling and Husbandry

Coral collection was planned using SCUBA divers at depths of ~30 m. Prior to any diving conducted, each participant carefully planned, discussed and rehearsed each field trip; dives were conducted by Dr. Kevin Strychar and John Skutnik between July 13 –16, 2014. Prior to the collection of any coral, two prior dives were conducted to survey the location, identify the presence and abundance of *M. cavernosa*, and assess the feasibility of sampling at the site. It was not only important to collect coral for this study, but to ensure that the collection and removal would have a negligible impact on the habitat and other surrounding coral. Once the collection site was identified, underwater transect lines were implemented. Samples were then removed from their affixed location using a hammer and chisel and with a few instances, using a rock saw.

Four plastic milk crates (PMC) were used to secure and transport coral from depth. Each coral "head" was fastened to one side on the inside of a PMC with small diameter metal wire. Each PMC contained between 3 and 5 coral heads depending on size and were left at depth (at site of collection) until the second to last day where the PMC were brought to the base of the mooring line (~20 m depth). To avoid possible light induced stress, shade cloth was affixed to the top of each PMC underwater and the coral were left to "de-stress" overnight.

The following day, each PMC was brought to the surface individually and immediately placed in a large cooler containing chilled seawater (temperature equivalent to bottom depth temperatures where the coral was observed); temperature was set to 21-24°C. Water in each cooler was continually changed while still on site until mucus secretion diminished.

Coral were left affixed to the PMCs inside the cooler to minimize any possible physical damage during transit. Several water flushes occurred during transportation back to the Strychar lab in Muskegon, Michigan; transport took 28 – 32 hours. During the transit from Florida to Michigan, two water changes occurred to minimize metabolite build-up. Temperature was maintained at ~24 – 26°C using pre-chilled seawater collected from the site.

Upon arrival to the research lab in Michigan, corals were slowly acclimated to room temperature by drip acclimation. In holding tanks constructed for the coral, seawater was made by using filtered lake water supplemented with Instant Ocean (Spectrum Brands, Inc.). Lake water had proven a suitable medium for coral growth, having experienced success with growing *M. cavernosa* previously in the Strychar Lab. Temperature and salinity were closely monitored and a shade cloth was placed over the entire holding tank to prevent any possible light stress. After measuring light intensity and PAR reaching the coral within the tank set-up, it was determined that the shade cloth could be removed as light was not inducing any kind of stress. The holding tank contained a mechanical filtration system, protein skimmer, air stone, drop-in aquarium heater, and two submersible pumps to promote mixing.

Coral tank conditions were monitored on every second to third day testing for nitrate, phosphate, calcium, and carbonate concentrations. A refractometer was also used daily to determine the salinity of the holding tank. Salinity was adjusted by slow additional of salt or fresh lake water depending on hypo- or hyper-saline condition.

Coral Fragmentation:

Coral heads were fragmented over the course of five days. A Gryphon rock saw with a diamond blade was used to fragment the coral into ~2.54 cm^2 pieces with an arbitrarily defined 3 polyp minimum per fragment. During fragmentation, care was taken to avoid disrupting any whole

polyp, which resulted in non-uniformly shaped fragments. The time out of water was kept to a minimum and frequent washes with fresh aquarium seawater was conducted to reduce stress and wash the coral of fragmentation debris (i.e. coral skeleton "dust").

Randomization Techniques:

All coral samples and experimental tanks used in this study were randomized multiple times throughout the course of the treatments. First, the tank infrastructure allowed for the inclusion of five tanks on a top shelf and 5 tanks on a lower shelf, corresponding to five "matched pairs". There was a 50% chance that the top or bottom tank could be treatment or control and was decided based on a coin flip. This was done for all 5 pairs in the chronic experiment. The acute experiment randomization technique for determining experimental tank condition was modified, however. Three conditions applied to the acute experiment: (1) Control, (2) Heat, and (3) Heat + Oil. Tanks were arbitrarily numbered 1 – 10. An excel randomization function was used to determine the condition for each tank such that the following numbers were put into excel (1, 1, 1, 2, 2, 2, 3, 3, 3, 3), randomized, and the order in which they were returned corresponded to tanks 1 – 10. The numbers used in Excel corresponded to one of the three treatment conditions where "1" was Control, "2" was Heat + Oil, and "3" was Heat.

The long term "chronic" experiment was conducted first. To help ensure randomization and an unbiased approach, I employed an assistant with no knowledge of coral nor any experience with reef ecology to help determine which coral fragment was chosen for a particular experiment tank. Prior to this selection process, the experimental tanks (numbered 1 – 10; 5 treatment tanks and 5 control tanks) were randomized as to which tank received the coral fragments first, second, and up through tank 10. Further, a grid was created within each experimental tank and numbered 1 – 52. The numbers in the grid were then randomized in excel

to provide the order in which fragments were placed in the experimental tank. This was repeated for all ten tanks in the chronic experiment. The same approach was used in the acute experiment with slight differences (number of fragments per tank and number of treatment conditions) but all other methods were identical.

Coral Sampling During Experiment:

During both the acute and chronic experiments, samples were taken and flash frozen at predetermined sampling intervals. The order in which samples were taken (i.e. from which tank and in what order) was randomized each day to mitigate any bias towards the order of sampling. The randomization function in excel was used to determine the order of tanks sampled as well as which fragments were to be sampled within each experimental tank. Samples were quickly removed from each tank, one by one, photographed next to a labeled piece of aluminum foil, and quickly wrapped placed into LN_2. All samples were kept in LN_2 until the final sample was taken before all were transferred to a –80 °C freezer until down-stream processes occurred.

Coral Homogenization:

Ceramic mortar and pestles (Sigma-Aldrich, USA) were used to crush frozen coral into a fine powder. Temperature, sterility, and endonuclease activity were of the utmost concern during this process. Initially, all mortar and pestles were cleaned with residue-free soap (Alconox© detergent, Sigma Aldrich, USA) and rinsed with Milli-Q water. They were left to air dry before then being sprayed with a RNase inhibitor, following air drying for 15 min, and then rinsed a second time with Milli-Q water. Both the mortar and pestle were chilled with LN_2 prior to the addition of any coral. A metal spatula underwent identical sterilization and nuclease removal steps as the mortar and pestle. Throughout homogenization, LN_2 was constantly applied to the coral homogenate until a fine powder was produced. The length of time to achieve a coral

powder varied between fragments as there were clear signs of morphology differences between samples. Homogenization of all coral samples took ~2-3 months. Once all samples were homogenized, coral powder was placed in sterile RNase/DNase free 2.0 mL Eppendorf tubes and maintained in a freezer at –80 °C until RNA extraction occurred.

RNA extraction:

Before RNA extraction occurred, all coral samples were weighed to ≈100 mg, placed in a clean 1.5 or 2.0 mL Eppendorf tube and kept frozen at –80 °C until all coral had been weighed. During the weighing process, samples were kept in LN_2 to maintain RNA integrity and quickly transferred into clean tubes, weighed, and re-cooled once a measurement was made.

RNA isolation consisted of a modified technique where both an RNeasy Plant Mini Kit (Qiagen) and trizol/chloroform was used. Brifely, Trizol was added to the weighed coral samples (coral sampled were isolated in batched of 12 or 24 samples), per the manufacturers suggestion, and allowed to disrupt cell integrity using a shaker (New Brunswick Scientific) at 400 RPM for 25 mins. Chloroform was then added to each tube (at 1:1 ratio) and centrifuged at 10,000 RPM for 15 mins to cause a phase separation. The organic layer obtained from the phase separation was then loaded into a RNeasy spin column and subsequent steps followed according to the manufacturer. All RNA was quantified on a NanoDrop (Thermo Fisher) and qualitatively assessed on an Agilent's BioAnalyzer (Agilent). An RNA integrity number (RIN) of six or more was deemed high RNA quality to perform qRT-PCR. Those samples that failed to meet this RNA integrity criteria were re-extracted and re-analyzed.

cDNA Synthesis:

cDNA synthesis was conducted using the PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio). A total of 300 ng of RNA was reverse transcribed in 30 µL using poly-A tail

primers. The thermocycler conditions for the reverse transcription reaction are as follows: Initial reaction at 37 °C (reverse transcription) for 15 minutes followed by 85 °C for 5 seconds to inactivate the reverse transcriptase. Samples were held at 4 °C in the thermocycler and later removed and placed at -20 °C.

qRT-PCR Primer Validation:

A total of six genes - *BCL-2*, *catalase*, *HSP90*, *cathepsin L1*, *RpL9*, and *RpS7* - were created using expressed sequence tag data in combination with NCBI BLAST and tested as potential candidates. All genes were initially examined to verify amplification using standard PCR reactions and observed using gel electrophoresis. Primers that failed to amplify were redesigned (maximum 3 times) and the amplification steps repeated. If the primer failed re-amplification three times, the gene was not pursued. All primers that successfully amplified underwent a primer efficiency test using pooled cDNA from various coral samples that had been flash frozen for gene expression analysis. Coral cDNA was serially diluted two-fold with a target efficiency of two (i.e. doubling every cycle). Those that failed the efficiency test were redesigned, tested for amplification using PCR and gel electrophoresis, and tested again for efficiency. A similar threshold (maximum 3 redesigns) was used as a mechanism to screed efficiency.

qRT-PCR:

All qRT-PCR reactions were conducted using a MJ Tetrad (now BioRad) thermocycler. The same instrument was used for all runs to minimize the potential for instrument-instrument differences in sensitivity. A plate control was used to normalize data between runs by pooling cDNA from ~20 samples and running two ribosomal proteins (*RpL9* and *RpS7*). The ribosomal proteins were highest in abundance and did not fail to amplify. All individual sample reactions were run in triplicate to ensure pipetting error did not influence transcript abundance. A 96-well

plate in combination with 8 count PCR tube strips were used to complete all gene expression analysis. All reagents were maintained per the manufacturer's instructions, with special precaution taken for dyes and enzyme solutions.

Statistical Analysis:

Statistical analysis was carried out using R 3.2.1. The R package MCMC.qpcr, developed by Mikhail Matz at the University of Texas, was the primary analysis tool [76]. This package uses a general linear mixed model with a poisson lognormal distribution and a Bayesian Markov Chain Monte Carlo sampling scheme. No prior endogenous control was defined for these analyses. The core of the package relies on raw qRT-PCR data being transformed into count data. The package modeled qRT-PCR data such that no control genes were required although control genes with known stability can be used to improve point estimate precision. Control genes would thus be considered *a-prior* in the model. The model internally normalizes qRT-PCR data to account for template loading error by taking the variation across all samples and using that as the normalizing factor. In this study, the statistical design was a 2-way crossover design with two factors, treatment and time point. Each factor had multiple levels based on the experiment. The acute treatment had three levels ((1) Control, (2) Heat, and (3) Heat + Oil) whereas the chronic experiment only had two levels ((1) Control and (2) Heat). The factor "time" varied in levels from four in the acute treatments to ten in the chronic treatments. The model syntax was generated to assess the effect of time and treatment alone as well as the interaction between the two. Statistics were generated from a Bayesian Z-test which is a 2-sample Z-test.

Appendix: Potential Physiological Response Genes

Prior to performing any experimental procedures, a list of putative stress and immune genes to assay were drafted. The following tables provide gene names with sequences that were tested but ultimately failed either initial amplification with PCR or primer efficiency criteria. Appendix Table 1 provides genes with primer sequences where two sets of primers were generated and tested per gene. Primers are read from left to right corresponding to the 5' and 3' ends.

Appendix Table 1: First Round of Primer Sets Designed

Gene Name	Set	Forward Primer	Reverse Primer
Allene oxide synthase-lipoxygenase	Set #1	ACCTCCGACAAACAAGGAAC	GCTGAGGATTTTCGTCACAA
	Set #2	CCCCTGATGGAGAAAGACAT	GCTGAGGATTTTCGTCACAA
Caspase-3	Set #1	ATTTACAATGACCGCACCAA	CAATGTTGCCATTTCCTTTG
	Set #2	ATTTACAATGACCGCACCAA	CACCCTCTTCACCATGTGTC
Macrophage mannose receptor-1	Set #1	TGCATGACCGCACTACTACA	AGCAACGCAGCAGACAATAC
	Set #2	TGCATGACCGCACTACTACA	GCAACGCAGCAGACAATACT
HSP70	Set #1	TTTACCACTTACGCCGACAA	CAAAGCTGCCAAGAAGATTG
	Set #2	GTGTGTCAGCGGTCGATACT	AGTTTGCATTGTGCCATTTC
Autophagy protein 5	Set #1	CGCATAATCTCCCTTGGAAT	TGATTCGATTGCTTCTTTGC
	Set #2	AATGGAAGGAGATGGAGACG	CCGATCATTTTGAGGTTTGA
Bcl-2	Set #1	CTGTGAGCCGAAGAATGAAA	TAATCGCCTACCAAGTCGTG
	Set #2	GCACGAAGCGTTACTGAAAA	CCCAGTTGATACCTGTGCTG
LITAF	Set #1	AGCTTACCCAGGTCCATCAG	GATAACCTCCTGCCTGCTTT

	Set #2	AGGAGGACCCACAAACTAGC	GGAGGAGCTGGCTGAGTAAC
C3	Set #1	TTTGGTCCTGTGGAAGATCA	GCTGCTTCTCTTGCCTTCTT
	Set #2	TCTGTTTGGTCCTGTGGAAG	GCTGCTTCTCTTGCCTTCTT
LAMP-2	Set #1	CGTACCTTCCCATCACATTG	AGCAACATCAATCCAAGTGC
	Set #2	AGCGTACCTTCCCATCACAT	AGCAACATCAATCCAAGTGC
NOX3	Set #1	GCGTAAAACAGGGGAGAGAG	AATTTCATTTCGCCATAGCC
	Set #2	AGAGGACGCACACCAATACA	CTCTCTCCCCTGTTTTACGC

A second round of primers were generated and tested after success/failure from round one sequences and is provided in Appendix Table 2.

Appendix Table 2: Second Round of Primer Sets Designed

Primer Name	Forward Sequence	Reverse Sequence
Caspase 8	CGGAGTGGAAGATTTGATGAG	GCAATGTCGTCTGTTGATGC
Autophagy-related protein 7	TTCAGCAATCAGTCGGAAACT	CGTAGAAGATGAGGACGCAGA
TRAF3	GCTGATGGGAAACTTGAGACA	AAACGGCACAGAGACAATGA
Protein Kinase C	CTGTCCTGCCATCATCTCG	GTGGAACACCAGACTACATTGC
C3	TGCCAGAAGGAGTGGAACAT	TTGTCTTGGAATCTTGGATGG
MyD88	GTGTAATCCAGGCGGGTAAA	GCTTTAACAGATGTCCAGATGC
HSP90 (set 1)	CAGAAGGTGGAGACTGATAA	CCAGATGACAAGAGAGAGG
HSP90 (set 2)	CGTGACAACAGCACTCTG	CCTCAAGGTCTCCACAATG
Cathepsin L1	GGGACCTGTCACTTCAAT	CACCTTCGTCTCCACTTT

BAX	GGTTGTTACTAAAGACACTGC	TTGACAACACCATCGTTAAAT
ATG5	CATGAGTTGCGTGAAAGAAG	CTGCTTATGATGCTGGATTTG
HSP70	CTTACTCCCGAAGACATTGA	GGCTGTAGGCATACGATT
Al-OX	GACGGCAAGTTTACAGTATTAG	CAGGAAAGCGGGATAGTT
SOD	GCTATTGTTGCTGTGCTG	GGACGAATCTTTCTCCTGAT
Catalase	GACCCTGAAGCATCTTATCT	CGCTGATACAAGTTGGAAAG
Beclin-1	TGTATGGAACTGGAGGATTT	CCACTTTCTCTACTGCTTCT

Appendix Table 3 provides genes and sequences for the final set of primers designed for this thesis.

Appendix Table 3: Third Round of Primer Sets Designed

Primer Name	Set	Forward Sequence	Reverse Sequence
RpS7	Set 1	GTGGATCACAAGCTGGACAC	TGGGAACTCAAACGTCACAT
	Set 2	TCCCAAGTGAAATTGTTGGA	CTTTGCCTGTGAGCTTCTTG
RpL9	Set 1	GCTCTTTCCTTCCATCTTCG	GTAACTTCACGGCCCCTTAG
	Set 2	GCTCTTTCCTTCCATCTTCG	GAATGGTCTTCATGGCTCAA
eIF4E	Set 1	CATGAGATGAACTGCCATCC	GGGAAGTAGCTGGTCTGAGC
	Set 2	ATGAGATGAACTGCCATCCA	GGGAAGTAGCTGGTCTGAGC

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