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Examining the Effect of Heat Stress on *Montastraea cavernosa* (Linnaeus 1767) from a Mesophotic Coral Ecosystem (MCE)

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Abstract: Coral reefs are under increasing pressure from global warming. Little knowledge, however, exists regarding heat induced stress on deeper mesophotic coral ecosystems (MCEs). Here, we examined the effect of acute (72 h) and chronic (480 h) heat stress on the host coral *Montastraea cavernosa* (Linnaeus 1767) collected from an upper MCE (~30 m) in Florida, USA. We examined six immune/stress-related genes: ribosomal protein L9 (RpL9), ribosomal protein S7 (RpS7), B-cell lymphoma 2 apoptosis regulator (BCL-2), heat shock protein 90 (HSP90), catalase, and cathepsin L1, as a proxy for coral response to heat stress. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to evaluate the gene expression. Overall, both acute and chronic heat stress treatments elicited a response in gene expression relative to control samples. Acute heat exposure resulted in up-regulation of catalase, BCL-2, and HSP90 at all time points from hour 24 to 48, suggesting the activation of an oxidative protective enzyme, molecular chaperone, and anti-apoptotic protein. Fewer genes were up-regulated in the chronic experiment until hour 288 (30 °C) where catalase, RpL9, and RpS7 were significantly up-regulated. Chronic heat exposure elicited a physiological response at 30 °C, which we propose as a heat-stress threshold for *Montastraea cavernosa* (*M. cavernosa*) collected from an MCE.

Keywords: mesophotic coral ecosystems (MCEs); coral bleaching; global warming; immunity; stress-genes; qRT-PCR

1. Introduction

Global warming is a natural component of the Earth's history, shifting between warming and cooling over thousands and millions of years driven by orbital and axial variations as it circles the sun (described as Milankovitch cycles) [1]. Toggweiler and Lea [2] and Keeling and Whorf [3] describe these warming and cooling events as occurring approximately every 100,000 years. The Open Source Systems, Science, Solutions Foundation [4] suggests that we should currently be experiencing a cooling trend evidenced by normalizing data of total solar irradiance accumulated over the last three decades and Pacific Decadal Oscillations from 1979–2010 [5,6]. Coinciding with the beginning of the Industrial Revolution (~1850), however, atmospheric concentrations of CO_2 have steadily increased warming rather than cooling [7]. Following this logic, when the Earth cycles out of a cooling trend, Earth's heating trend will be compounded by temperatures already above the expected "normal". Such warming has considerable implications for reef communities by stressing host corals and their associated symbiont's

thermal limit, resulting in "bleaching" [8] caused by the loss of color and/or loss of the number of cells, revealing the underlying white skeleton of a coral [9].

Bleached coral is affected by either acute and/or chronic thermal stress, which may also be coupled to a variety of other stressors such as disease, lack of nutrients, and light levels. Chronic heat stress may 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]; however, other variables such as location, radiation, and colony/species physiology may also play a significant role in coral susceptibility to heat stress. 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 [11], albeit late in the summer season, high radiation may also be affecting a coral's physiology. Unlike chronic heat stress, transient acute events (cold and warm) can be caused by storms or abnormal weather events [12]. Scleractinian coral, called 'reef-building' coral, can form an intimate symbiosis with intracellular dinoflagellates (e.g., from the genus *Symbiodinium* [13] (colloquially known as "zooxanthellae") previously Clade A) or establish multiple symbioses with genera belonging to the Symbiodiniaceae family. The coral-algal symbiosis is instrumental for health and fitness and allows the holobiont to thrive by being the beneficiary of the symbiont's photosynthetic capacity [14,15]. 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 [16–20]. 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, growth, or reproductive success of the species [21–23].

Much of the work examining the effect of heat stress has focused on shallow water coral communities due to their economic and ecological importance, obvious signs of distress, and ease of access [24,25]. Heat stress affecting coral found in the deep ocean (defined here as being >300 m) and mesophotic coral ecosystems (MCEs; defined as photosynthetically active reefs ranging from 30–150 m depth) [26,27] are relatively minimal [28–30] because of the lack of access. However, MCEs are considered as widespread and diverse as shallow-water reefs. Kosaki et al. [31] suggests that coral reef fish endemism on MCEs increases with latitude reaching percentages as high as 92%. Others [32,33] propose that MCEs have distinct communities that exist no-where else and need to be protected [34–38]. Lindfield et al. [39] suggest that a large number of species share shallow-water and MCEs habitats. We speculate that the sharing of habitats amongst coral species more likely occurs between the upper MCE habitat (≥30 m) than at deeper depths. Gress et al. [40] suggest that MCEs should be considered marine protected areas given their similarly high levels of productivity and shared number of species with shallower reef ecosystems.

Some authors believe that MCEs at 30–150 m depths experience fewer ecological disturbances, less wave energy, and fewer temperature anomalies resulting in environments more stable than shallow reef habitats [41], albeit Pinheiro et al. [42] suggest that disturbance may be beneficial to survivorship. Haslan et al. [43] who studied *Montastraea cavernosa* by moving species from shallow to deep habitats (and vice versa) suggest disturbance promotes better heat tolerance. Here, we examined *M. cavernosa* from an upper MCE (≥30 m) and simulated acute versus chronic heat stress. We chose the upper MCE as it is more conceivable that species at this depth are more likely to interact with shallower water species than coral at a much deeper depth along the MCE gradient. We also chose this species because it is a depth generalist capable of living at shallow to deep depths, is remarkably adaptive and as a consequence, affords good comparative analyses along a wide environmental gradient [43,44]. The objectives were to: (1) Examine the effect of elevated temperature on coral in the upper MCE (≥30 m) through gene expression analyses of selected putative immune/stress genes; (2) Identify any potential relationship with the duration of stress on gene expression; (3) Distinguish between any similarities or differences in the response to acute versus chronic exposure to elevated temperatures. We hypothesize (1) that coral from the upper MCE are highly susceptible to the effects of heat stress displayed via increased gene expression through time, and (2) are more stressed during acute versus chronic exposure.

2. Materials and Methods

2.1. Materials Study Organism, Collection, and Preparation

Montastraea cavernosa (Linnaeus 1767) is known as a depth generalist. It is a broadcast spawning species generally distributed between 3–91 m found in the Caribbean, Gulf of Mexico, Florida, the Bahamas, and Bermuda [45–47]. *M. cavernosa* may contribute to genetic connectivity across reefs, based on their broadcast spawning reproductive characteristics, revealing the potential to study the response to heat stress on the same genotype across depths [48]. Symbiodiniaceae found intracellularly produce most of the host coral's primary nutritional needs; however, when symbiosis is compromised, *M. cavernosa* can supplement its nutritional needs via heterotrophic feeding [47].

Divers using SCUBA (Self Contained Underwater Breathing Apparatus) performed exploratory dives (30–33 m) off the coast of Tavernier Florida at Conch Reef (Florida, USA) during July (Summer). Since permits were only granted to collect coral from one location, transects were established at the base of Conch Wall southwest of the nearest buoy located near the Florida Keys National Marine Sanctuary (FKNMS; Figure 1A). At that location, divers established a series of 150 m linear transects, identifying 40 colonies of *M. cavernosa* meeting a specific FKNMS permit size standard (≤254 cm^2; e.g., Figure 1B). Along each transect, photosynthetically active radiation (PAR) was measured using a Li-193 Underwater Spherical Quantum Sensor (LiCor Inc., Lincoln, NE, USA) at 5 m increments. Measurements were recorded every 5 s (total recorded values per time interval; n = 3) at each incremental depth. Temperature was continuously logged on each dive with an affixed HOBO data logger (Onset Computer Corporation, Bourne, MA, USA) to each diver.

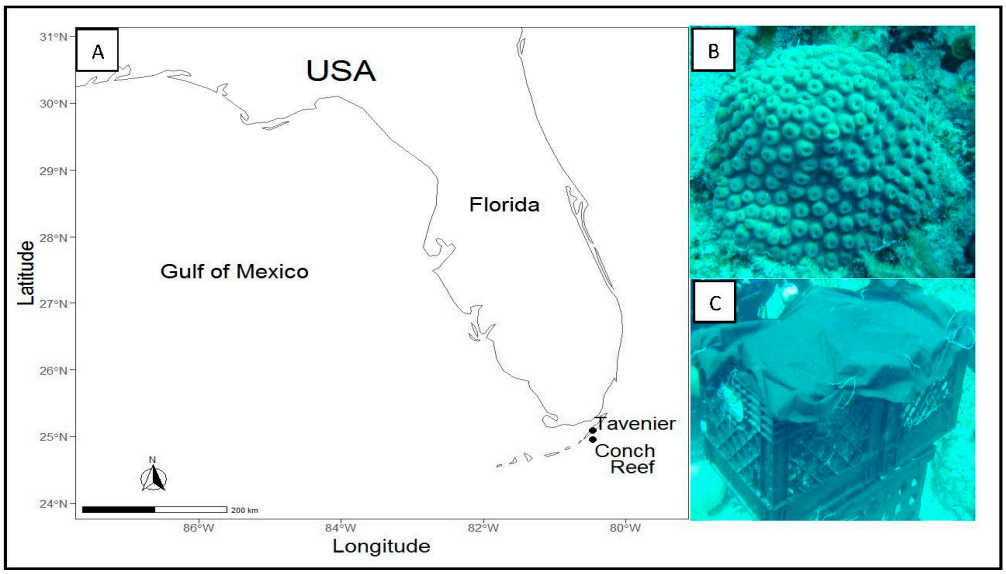

Figure 1. (**A**) Coral were collected from Conch Reef within the Florida Keys National Marine Sanctuary (FKNMS) located near Tavenier (Lat: 24.96, Long: −80.45) Florida, USA. (**B**) Colonies of *Montastraea cavernosa* were collected and placed in (**C**) black crates covered with shade cloth to help reduce stress experienced during collection.

Once all coral needed for experimental analyses were tagged and the respective environmental conditions recorded, a rock saw, hammer and chisel were used to free the coral colonies from the benthic substrate. Coral were then placed into black crates, covered with shade cloth (Figure 1C), and remained at depth for 48 h to help reduce any stress they may have experienced during collection. All coral at depth were then collected, transported to the surface and at the research vessel, rinsed with pre-chilled seawater (at a temperature equivalent to the collection depth; 28 °C), placed in coolers containing pre-chilled seawater and transported to the shore where a research vehicle was waiting for transport to our laboratory.

Aboard the research vessel, we also collected 756 L of seawater, which was maintained at the same temperature the coral were collected. The 756 L of aerated seawater was then used to flush the coral during transport from shore to the research laboratory to help reduce the concentration of secondary metabolites and other contaminants excreted by the coral. Immediately upon arrival to the research institute (Annis Water Resources Institute, located in Muskegon, Michigan, USA), all coral were placed in holding tanks both to allow the coral to acclimate and to depurate; corals were held for ~4 days. Forty coral colonies, each ~25.4 cm^2 were than fragmented into ~2.54 cm^2 cubes using a model C-40 band-saw (Gryphon Corporation, Sylmar, CA, USA) with an enhanced diamond blade (each coral colony produced approximately ten 2.54 cm^2 fragments); voucher specimens were not preserved as per the FKNMS permit stating any fragments not used for experimental purposes would be returned to the collection site. During the fragment process, each fragment was arbitrarily defined as requiring 3 polyps minimum; care was taken to not disturb or destroy any whole polyps. All "frags" (i.e., fragmented coral) were then placed in holding tanks for two weeks to help minimize the stress of fragmentation. During the two-week holding phase, tanks were visually monitored for water level, algae growth, and water chemistry each day. Phosphate, nitrate, calcium, and carbonate were monitored using Aquarium Pharmaceuticals® API Saltwater Liquid Master Test Kit (USA). Any results deviating from manufacturer recommendations resulted in a 50% water change. In addition, corals were fed biweekly using PhytoPlex Phytoplankton (Kent Marine, Franklin, WI, USA) and micronutrient supplements (Kent Marine Essential Elements, Franklin, WI, USA) per the manufacturer's recommendation. We recognize that feeding the coral may introduce a bias. Experiments done with filtered natural seawater will have dissolved matter which can be utilized by the coral. However, artificial seawater lacks any such benefit, hence we fed biweekly. Temperature was maintained between 26 °C–27 °C using a 250 W submersible aquarium heater; this temperature was observed during sample collection and is known to be well below stress thresholds.

2.2. Experimental Design and Analysis

Within each 45 L experimental tank (ET), a 17.3 L incubation chamber (IC) was insulated by water (Figure 2). Each ET was independent of each other regarding the heating element (HE), shade cloth (SC), and submersible pumps (SP). Due to space limitation, the control tanks used filtered artificial seawater from one 208 L reservoir and all heat treatment tanks drew seawater from a different 208 L reservoir (Figure 2); during the chronic heat treatments, ETs used seawater from two 208 L reservoirs. To create flow for fluid exchange through the IC's, artificial seawater was pumped using two peristaltic pumps (Cole Parmer Masterflex 7519-15, Vernon Hills, IL, USA) from the respective reservoirs at a rate of 2.3 L/h. One peristaltic pump (PP) added water to each ET from reservoir #1 whilst the other ETs used seawater from reservoir #2. The outflow (Figure 2) from each ET was removed from the system via gravity (Figure 2). Mixing inside the IC was accomplished by the use of a single submersible aquarium pump to help evenly distribute heat and mix incoming seawater. Another submersible pump was placed in the ET to circulate heat and maintain the IC at a constant temperature. To maintain and/or manipulate temperature, an aquarium heater was placed in the ET. Maintaining adequate light for the coral was achieved by using four double lamp T5 high output fixtures with a 54 W pure actinic and 54 W AquaBlue light (ATI Aquaristik, Hamm, Germany) to simulate wavelengths found at the depths of the collected coral; SC was placed on top of both control and treatment tanks. Light was measured under the SC prior to the start of the experiment for each tank, which was at or slightly below the detected PAR and intensity found at the MEC. Each ET was also equipped with a white plastic egg crate (PEC) panel beneath the IC to allow water to flow underneath allowing better heat distribution (Figure 2). Each IC tank was set-up with a HOBO (Onset Computer Corporation, Bourne, MA, USA) that continuously monitored and logged temperature and light intensity every 20 min. Temperature was also manually logged prior to sampling at each sampling interval.

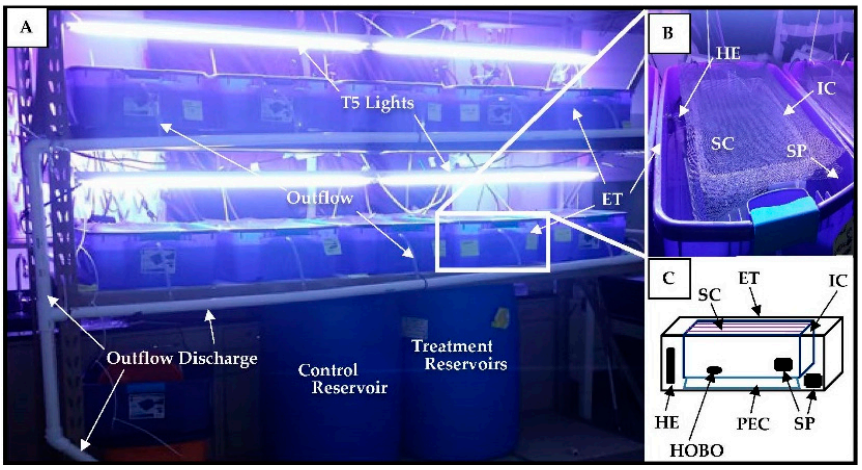

Figure 2. (**A**) Experimental tank design. (**B**) Treatment tank design. (**C**) Schematic of treatment tanks. Control Reservoir = control tank water, ET = experiment tank, HE = heating element, HOBO = temperature and light meter, IC = incubation chamber, Outflow = IC water drainage, Outflow discharge = discharged water, PEC = plastic egg crate, SC shade cloth, SP = submersible pump, T5 lights = 54 watt lights.

The analysis of a physiological response to treatment conditions for both acute and chronic experiments was monitored using quantitative real time polymerase chain reaction (qRT-PCR) on 6 immune/stress-related genes following Hauff-Salas et al. [49] who similarly used six genes to characterize coral stress. In this study, we chose ribosomal protein L9 (RpL9), ribosomal protein S7 (RpS7), B-cell lymphoma 2 apoptosis regulator (BCL-2), heat shock protein 90 (HSP90), catalase, and cathepsin L1. We chose RpL9 and RpS7 as these ribosomal proteins (RPs) have various functions including protein biosynthesis, transcription and repair, cell proliferation and growth, and the regulation/suppression of apoptosis and disease [50,51], indicating they may contribute to pro-survival functions [52]. We also chose BCL-2 as it plays an important role in promoting cellular survival inhibiting pro-apoptotic actions. Localized to the outer mitochondria membrane, BCL-2 helps in the release of cytochrome C and reactive oxygen species (ROS), which are known to play roles in the signaling pathway of apoptosis [53]. HSP90, a chaperone protein, is one of the most common heat related proteins up-regulated to stabilize other proteins against heat stress [54]. Catalase is an important enzyme and each molecule of catalase can convert millions of molecules of hydrogen peroxide each second [55] to oxygen and water, protecting the cell from damage caused by ROS. Cathepsin L is a peptidase up-regulated during stressful events as it has the capacity to degrade nearly all proteins including enzymes and receptors [56]. Cathepsin L plays an important role in the normal physiological functioning of a cell and helps in disease resistance.

2.3. Acute versus Chronic Heat Treatments

The acute treatment consisted of seven tanks on a top shelf and seven tanks on a lower shelf. Seven tanks were control tanks while seven were treatment tanks. The location of either treatment or control tank was randomized between the top and bottom shelves. Coral fragments were selected from the primary holding tank and randomized (an Excel randomization function was used) amongst the Control IC or Heat treatment IC (e.g., Figure 2). A total of six coral fragments taken from 40 different colonies were placed in each IC tank; this was done to reduce any within and between colony variation. Corals were allowed to acclimate to the new experimental tanks for 48 h prior to the first sample taken for analysis. 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. Heat in the seven ETs was increased to 33 °C from the onset of the experiment over six hours using submersible aquarium heaters; we recognize that a 1 °C acute change in temperature is unlikely to be observed naturally in a MCE, but wanted to observe a distinct difference (if any) between the acute

vs. chronic study (this temperature ramp-up equated to a 0.0125 °C change per hour). A single coral fragment was collected from each IC tank at 11:00 a.m. and every 24 h until a total exposure of 72 h occurred. At each time interval (4 total; i.e., 0 = start, 1 = 24 h, 2 = 48 h, 3 = 72 h), seven fragments were collected (one from each control tank (n = 7) and one from each treatment tank (n = 7)). Samples were immediately flash frozen at each sampling interval and placed at −80 °C for downstream processing.

Conversely, in the chronic treatment, 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 coral placed in these tanks was decided randomly using a random generating method using an Excel© (Microsoft Corporation, Redmond, WA, USA) randomization function. Fifty coral fragments (total) were placed in each of the 10 IC tanks. The duration of our chronic experiment was 20 days representing a good contrast compared to the acute treatment in this study; we recognize, however, environmentally chronic conditions may last several months to perhaps years. In this chronic study, incremental increases in temperature were 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 °C), Hour 96–192 (28.5 °C), Hour 216–288 (30 °C), Hour 312–384 (31.5 °C), and Hour 408–480 (33 °C) representing a 0.0125 °C change per hour. 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 from each treatment (n = 5) and each control (n = 5) tank every day (11:00 a.m.) from Hour 0 to Hour 480 and immediately flash frozen and stored at −80 °C until downstream processing occurred.

2.4. Sample Processing and RNA Extraction

Flash frozen samples were removed from the −80 °C freezers 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, St. Louis, MO, USA), washed in Milli-Q water, and placed in a −80 °C freezer to cool. Liquid nitrogen (LN2) 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 LN2 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 (ribonucleic acid) 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 RNA extraction process, all materials, including coral, metal spatulas and the RNA extraction ready tubes, were continuously maintained in LN2 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 (complementary DNA) synthesis.

2.5. cDNA Synthesis

Prior to cDNA synthesis, total RNA from all samples were treated with DNase I (Invitrogen, USA) to remove any contaminating genomic DNA (deoxyribonucleic acid). From the total RNA, 300 ng of RNA per reaction was reverse transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio, Mountain View, CA, USA) 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 min at 37 °C, 5 min 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/uL.

2.6. 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., Waltham, MA, USA) was used in the amplification reaction with PCR conditions as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 57 °C for 30 s, 72 °C for 1 min, with a final extension of 5 min 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, Grand Rapids, MI, USA) was used for all qRT-PCR reactions with a thermo cycler profile following: 2 min at 95 °C, followed by 40 cycles of 5 s at 95 °C, 7 s at 57 °C, 25 s at 72 °C followed by a melt curve analysis for 1 min at 95 °C, 30 s at 57 °C, and 30 s 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 analyzed genes are shown in Table 1.

Table 1. Forward (F) and reverse (R) primer sequences for ribosomal protein L9 (RpL9), ribosomal protein S7 (RpS7), B-cell lymphoma 2 apoptosis regulator (BCL-2), Catalase, heat shock protein 90 (HSP90), and Cathepsin L1 used in this study.

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′

2.7. Statistical Analysis

In lieu of normalization using highly stable reference genes, the R package mcmc.qpcr was developed to analyze data without reference genes following Matz et al. [57]. 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 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, we classified samples originating from the same IC as independent. Descriptive statistics of the gene and time point most affected in each study were 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 thus determined as most affected.

Formatted qPCR data as molecule count data was log transformed to meet normality assumptions and analyzed using mixed models (fixed and random effects) procedure in SAS 9.4. Note, mixed models, rather than assuming there is a single intercept for the population, assume that there is a distribution of intercepts. Every sample/tank's intercept is a random variable from a shared normal distribution. A random intercept for log molecule count means that there is some average log molecule count in the population, but there is variability between samples/tanks.

For acute heat stress experiments, molecule count for each treatment at ten subsequent time molecules was at 0 h, 24 h, 48 h, and 72 h. The population model for the linear relationship between Log Molecule Count and Time that includes different intercepts ($\beta 2$) and different slopes ($\beta 3$) for Heat and Control is as follows: $Y = \beta 0 + \beta 1X1 + \beta 2X2 + \beta 3X1\ X2 + \varepsilon$, where the condition is that the ε are independent and each from a N (0, σ_ε) distribution. This model allows one to determine if Mean Log Molecule Count differs between the two treatments when controlling for Time and also determine if an average rate of increase in log Molecule Count for every unit increase in Time differs between the two treatments. Significant development overtime for BCL2, for example, depends on treatment (Table 2). Note, predicted Log Molecule count = 2.4722 − 0.003 Time and predicted Log Molecule count = (2.4722 + 0.011) + (−0.003 + 0.0063)Time for Control and Heat respectively.

Table 2. *Montastrea cavernosa* mixed linear model showing the effects of acute heat stress on Log Molecule Count over 72 h. StdErr = Standard Error, DF = Degrees of Freedom, tValue = t-statistic, Lower = 95% Lower Limit for parameter estimate, Upper = 95% Upper Limit for parameter estimate.

Effect	Treatment	Estimate	StdErr	DF	tValue	Pvalue	Lower	Upper
BCL2								
Intercept		2.4722	0.1147	5	21.55	<0.0001	2.1773	2.7672
Treatment	Heat	0.01100	0.1468	43	0.07	0.9406	−0.2850	0.3070
Treatment	Control	0						
Time		−0.00299	0.001896	43	−1.58	0.1223	−0.00681	0.000835
Time*Treatment	Heat	0.006261	0.002478	43	2.53	0.0153	0.001263	0.01126
Time*Treatment	Control	0						
Catalase								
Intercept		3.1663	0.1148	5	27.57	<0.0001	2.8712	3.4615
Treatment	Heat	0.03873	0.1446	43	0.27	0.7902	−0.2530	0.3304
Treatment	Control	0						
Time		−0.00087	0.002418	43	−0.36	0.7206	−0.00575	0.004005
Time*Treatment	Heat	0.004607	0.003164	43	1.46	0.1526	−0.00177	0.01099
Time*Treatment	Control	0						
CathepsinL1								
Intercept		2.4525	0.2016	5	12.17	<0.0001	1.9343	2.9708
Treatment	Heat	−0.2616	0.2538	43	−1.03	0.3085	−0.7734	0.2503
Treatment	Control	0						
Time		0.000603	0.004299	43	0.14	0.8891	−0.00807	0.009273
Time*Treatment	Heat	0.002503	0.005626	43	0.44	0.6586	−0.00884	0.01385
Time*Treatment	Control	0						
HSP90								
Intercept		2.2331	0.2346	5	9.52	0.0002	1.6300	2.8362
Treatment	Heat	0.1241	0.2954	43	0.42	0.6766	−0.4716	0.7198
Treatment	Control	0						
Time		0.001310	0.005003	43	0.26	0.7947	−0.00878	0.01140
Time*Treatment	Heat	0.004564	0.006548	43	0.70	0.4895	−0.00864	0.01777
Time*Treatment	Control	0						
RpL9								
Intercept		3.9631	0.1255	5	31.57	<0.0001	3.6405	4.2858
Treatment	Heat	−0.2770	0.1580	43	−1.75	0.0867	−0.5957	0.04164
Treatment	Control	0						
Time		−0.00259	0.002677	43	−0.97	0.3385	−0.00799	0.002807
Time*Treatment	Heat	0.004375	0.003503	43	1.25	0.2184	−0.00269	0.01144
Time*Treatment	Control	0						
RpS7								
Intercept		3.4121	0.09987	5	34.16	<0.0001	3.1553	3.6688
Treatment	Heat	−0.1526	0.1257	43	−1.21	0.2316	−0.4061	0.1010
Treatment	Control	0						
Time		−0.00021	0.002130	43	−0.10	0.9227	−0.00450	0.004087
time*Treatment	Heat	0.001249	0.002787	43	0.45	0.6562	−0.00437	0.006870
time*Treatment	Control	0						

3. Results

3.1. Gene Expression Changes During Acute Treatment

All six genes analyzed at each time point sampled were estimable under the model employed to characterize heat stress in *M. cavernosa*. Differential gene expression was observed across all treatments relative to the control, ranging from 1.60 to −2.16-fold change (Figure 3). When exposed to heat, expression of RpL9 decreased −2.11-fold after 24 h ($p = 0.05$; Table 3). After 72 h, expression of BCL-2 increased 1.60-fold ($p < 0.01$; Figure 3; Table 3). Additionally, RpS7 expression decreased −1.52-fold and HSP90 increased 1.90-fold after hours 24 and 72, respectively, but differences were not significant ($p = 0.07$; Figure 3). BCL-2 and HSP90 expression increased as the duration of heat stress increased from 24 to 72 h (Figure 3). Catalase showed a similar linear increase in expression from hour 0–72, whereas cathepsin L1 expression was reduced at all time points except hour 72 (Figure 4). Ribosomal protein RpL9 and RpS7 expression patterns for these two genes were very similar, but with a slight reduction in expression after 24 h followed by a linear increase at 72 h. The time point where the largest effect on gene expression occurred during the acute heat stress experiment occurred at hour 24 (33 °C).

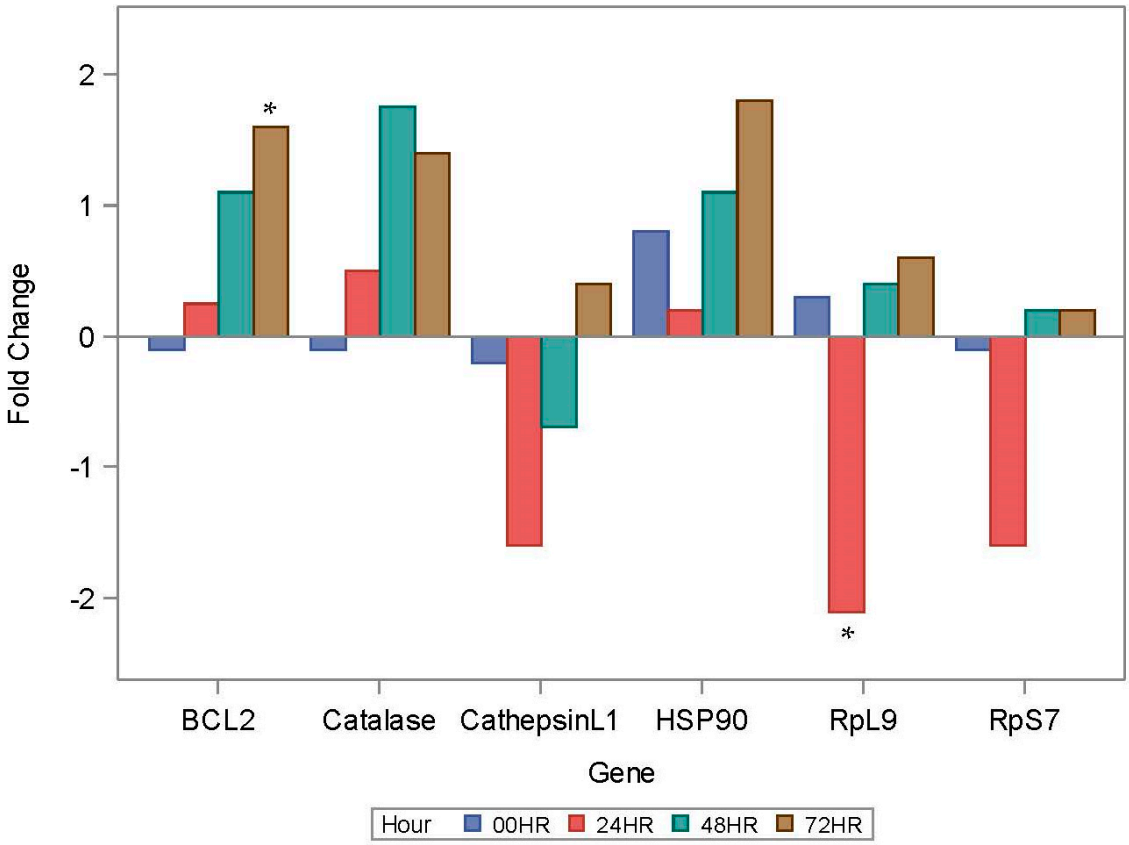

Figure 3. Acute heat exposure showing gene abundance (B-cell lymphoma 2 apoptosis regulator (BCL-2), Catalase, CathepsingL1, heat shock protein 90 (HSP90), Ribosomal protein L9 (RpL9), and ribosomal protein S7 (RpS7)) expressed over a 72-h period. Data expressed as 'fold change' defined as the concentration difference between the control and the particular gene being examined. Star "*" denotes statistically significant ($p \leq 0.05$) data.

Table 3. Statistical significance of gene expression observed at a particular time point (hour) during acute and chronic heat treatments; only statistically significant data are shown. Negative numbers show a decrease in gene concentration. Fold change is defined as the quantity of change between the control and the expressed gene.

Gene	Time Point (hours)	Fold Change	*p*-Value
Acute Treatment			
RpL9	24	−2.11	0.05
BCL-2	72	1.60	<0.01
Chronic Treatment			
Catalase	72	−6.6	<0.01
Catalase	288	3.4	0.02
Cathepsin L1	216	−2.7	0.03
HSP90	72	−2.8	0.02
HSP90	192	−3.4	0.01
HSP90	216	−4.4	0.01
HSP90	312	−4.6	<0.01
RpL9	192	−1.5	0.03
RpL9	288	1.8	0.01
RpS7	192	−1.5	0.04
RpS7	288	2.2	<0.01
RpS7	480	−1.7	<0.01

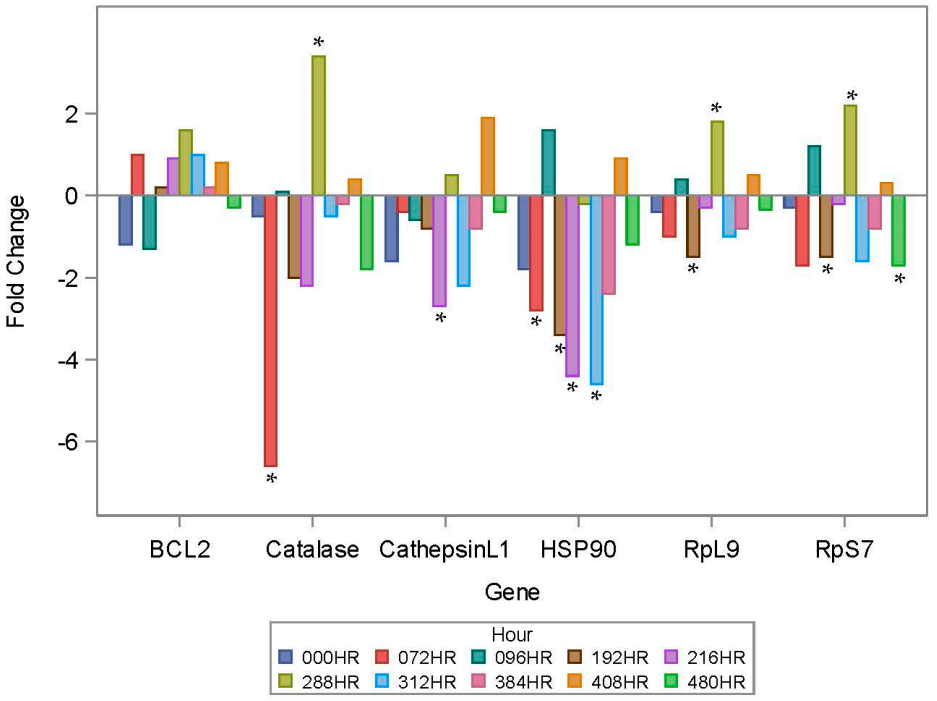

Figure 4. Chronic heat exposure showing gene abundance (B-cell lymphoma 2 apoptosis regulator (BCL-2), Catalase, CathepsingL1, heat shock protein 90 (HSP90), Ribosomal protein L9 (RpL9), and ribosomal protein S7 (RpS7)) expressed over 480 h. Data expressed as 'fold change' defined as the concentration difference between the control and the particular gene being examined. Star "*" = statistically significant ($p \leq 0.05$) data.

3.2. Gene Expression Changes during Chronic Treatment

Differential gene expression was observed in the chronic heat treatment compared with control, ranging from 3.4 to −6.6-fold change (Figure 4). Twelve significant gene expression differences were up-/down-regulated relative to the control (Table 3) corresponding to the following genes: catalase, cathepsin L1, HSP90, RpL9, and RpS7 (all occurring at hour 288 at 30 °C). There were statistically significant intra-gene expression differences within all six genes analyzed relative to the control; all expression differences are provided as fold changes with corresponding p-values (Table 3, Figure 4). Similar to the 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 at 27 °C with the second largest fold change occurring at hour 312 at 31.5 °C. Hour 72 was largely influenced by a substantial down-regulation of catalase. Additionally, hours 192 at 28.5 °C and 288 at 30 °C resulted in the highest frequency of genes showing statistically significant differences in expression (catalase, RpL9, and RpS7). Throughout the duration of the experiment, however, 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-treated samples, and to a lesser extent, in control samples.

In the control group, all genes maintained a similar concentration across nearly all time points (hours 96–408). The heat treatment groups resulted in much less intra-gene expression stability over the course of the experiments. Gene expression stability of ribosomal proteins RpL9 and RpS7, between the control and treatment groups, was generally 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; Figure 4).

Catalase 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 (Figure 4).

3.3. Overall Trends: Treatment Effect on Gene Expression and Time Point

Figures 5 and 6 show the mean log molecule count of each genes expressed for each treatment, (i.e., acute and chronic). The acute heat treatment associated with BCL-2 (Figure 5) showed on average 100.011 = 1.03 more molecules over time versus an average 100.0299 = 1.01 molecule decrease for Control, and an average 100.006261 = 1.02 molecule increase per time period for Heat (Table 2). Catalase (Figure 5) similarly showed an average increase of 100.03873 = 1.09 molecules through time compared to the control, which decreased over time (100.00087 = 1.002 molecule drop per time), and an increase 100.004607 = 1.01 molecule per time period for Heat (see Table 2). Figure 5 shows CathepsinL1 which on average showed a decrease in the number of molecules expressed over time (i.e., 100.2616 = 1.83; Table 2) compared to the control which shows an average increase in gene expression (i.e., 100.000603 = 1.001 molecule increase per time period), and an average 100.002503 = 1.01 molecule increase per time period for Heat. On average, HSP90 showed an increase in the number of molecules expressed (average 100.1241 = 1.33) as did the control (100.00131 = 1.003 molecule increase per time period), and an average 100.004564 = 1.01 molecule increase per time period for Heat (Figure 6; Table 2). On the other hand, RpL9 (Figure 5) was much lower on average (100.2770 = 1.89 molecules) compared to the control (100.00259 = 1.01 molecule drop) during the acute heat treatment; an average 100.004375 = 1.01 molecule increase was observed per time period for Heat (Table 2). Similarly, RpS7 (Figure 5) is on average 100.1526 = 1.42, which reflects a reduced number of molecules compared to the control (which also showed a decrease in the number of molecules expressed (there's an average 100.00021 = 1.0005

molecule drop per time period)), and an average 100.001249 = 1.003 molecule increase per time period for Heat (Table 2).

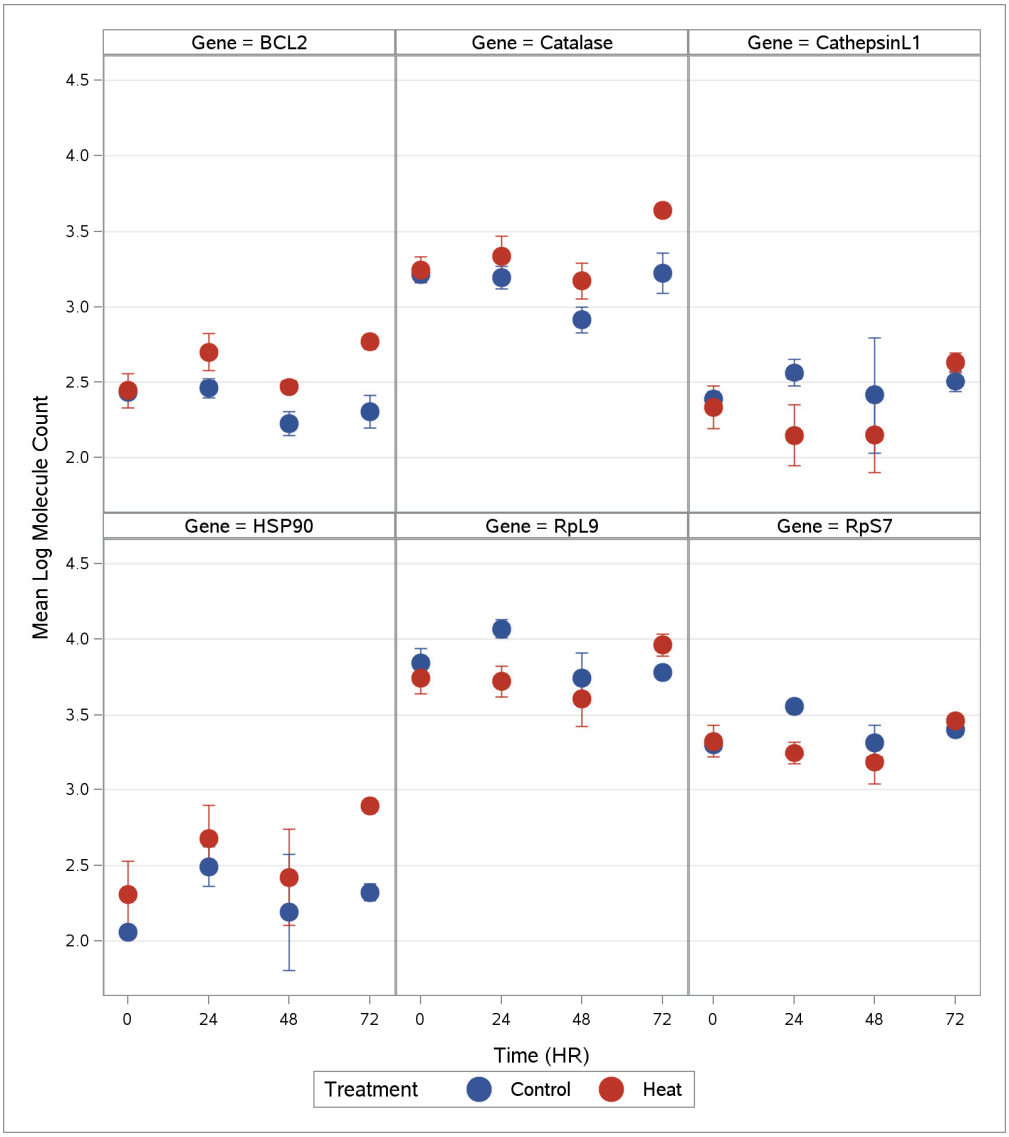

Figure 5. Gene expression of B-cell lymphoma 2 apoptosis regulator (BCL-2), Catalase, CathepsinL1, heat shock protein 90 (HSP90), ribosomal protein L9 (RpL9), and ribosomal protein S7 (RpS7) sampled every 24 h over acute (72 h) time period show mean log molecule count by hour and treatment. Control temperature (●) = 27 °C vs. heat (●) treatments = 33 °C. Error bars show mean ± s.e.m.

For the chronic heat stress experiments, molecule count for each treatment at ten subsequent time molecules was at 0 h, 72 h, 96 h, 192 h, 216 h, 288 h, 312 h, 384 h, 408 h, and 480 h. Significant development overtime for Catalase and RpS7, for example, depends on treatment (Table 4). Note, for Catalase, predicted Log Molecule count = 3.2195-0.00123Time and predicted Log Molecule count = (3.2195 − 0.5883) + (−0.00123 + 0.001771) Time for Control and Heat respectively. Similarly for RpS7, predicted Log Molecule count = 3.5026 − 0.00083 Time and predicted Log Molecule count = (3.5026 + 0.1147) + (−0.00083 + 0.00067) Time for Control and Heat respectively. BCL-2 (Figure 6) produced on average 100.02397 = 1.06 more molecules, while in the control (100.00087 = 1.002), there was a reduction in the expression of this particular gene, and an average 100.000236 = 1.001 molecule increase per time period for Heat (Table 4). Catalase expression also shows an average reduction in molecule expression over time (100.5883 = 3.86 molecules, Table 4; Figure 6), a decrease in the control (100.00123

= 1.003 molecule drop), and an average 100.002 = 1.005 molecule increase per time period for Heat. CathepsinL1 expressed more molecules (100.02818 = 1.07) compared to the control which decreased expression (100.00192 = 1.004; Table 4) per time period (Figure 6), and an average 100.00034 = 1.001 molecule decrease per time period for Heat. HSP90, however, expressed fewer molecules over time (Figure 6; 100.1586 = 1.44 molecules lower, Table 4) and an average 100.00130 = 1.003 molecule drop per time period for Control; an average 100.000379 = 1.001 molecule increase per time period for Heat was also observed (Table 4). Over time, the general trend for RpL9 shows an average 100.07685 = 1.19 (Table 4) more molecules but a 100.00079 = 1.002 molecule decrease per time period for Control (Table 4; Figure 6), and an average 100.00054 = 1.001 molecule drop per time period for Heat. Similarly, RpS7 shows an average 100.1147 = 1.30 increase in expressed molecules (Figure 6; Table 4), an average loss in the control (100.00083 = 1.002; Table 4) per time period for Control, and an average 100.00067 = 1.002 molecule drop per time period for Heat.

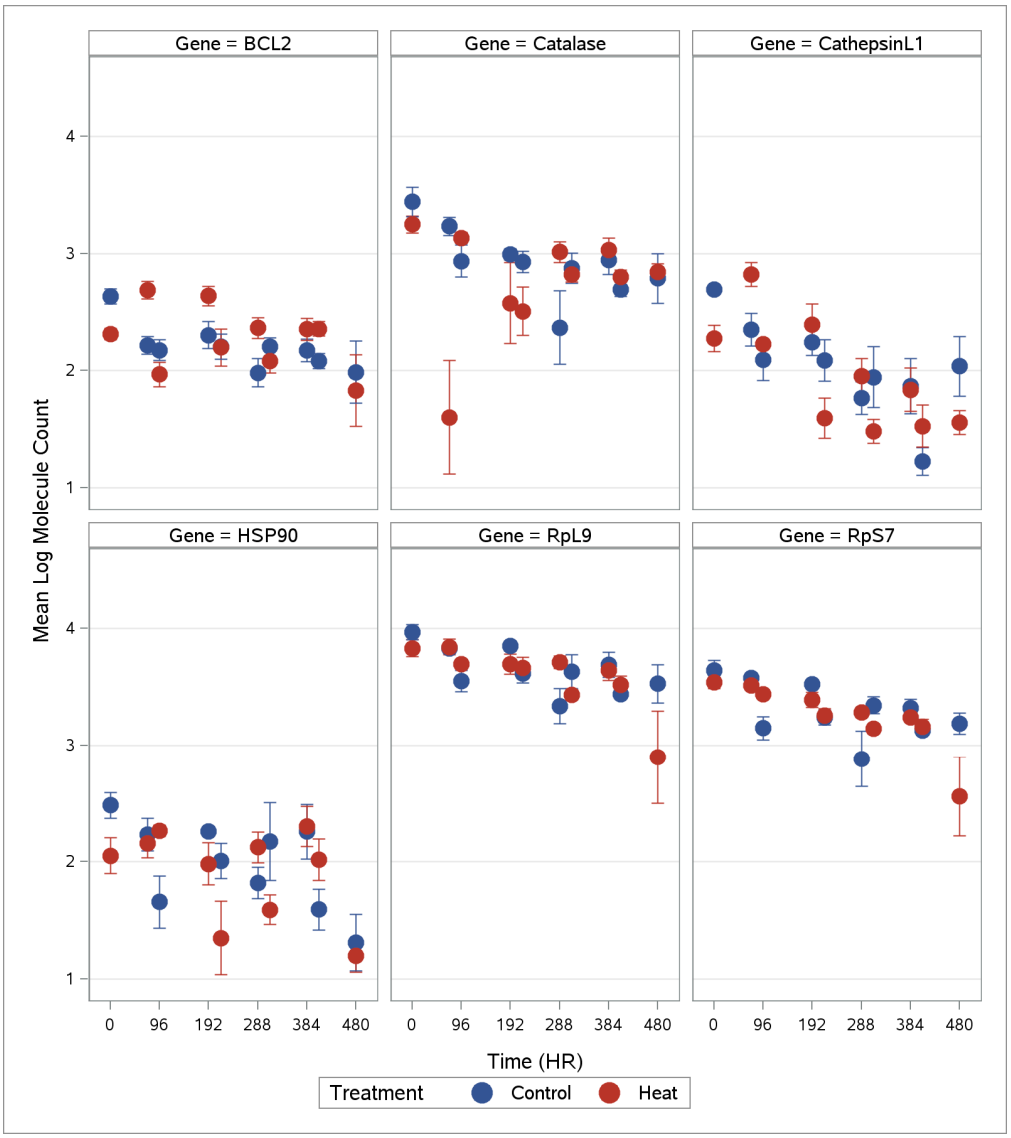

Figure 6. Gene expression of B-cell lymphoma 2 apoptosis regulator (BCL-2), Catalase, CathepsinL1, heat shock protein 90 (HSP90), ribosomal protein L9 (RpL9), and ribosomal protein S7 (RpS7) sampled every 24 h over chronic (480 h) time period show mean log molecule count by hour and treatment. Control temperature (●) = 27 °C vs. heat (●) treatments = 33 °C. Error bars show mean ± s.e.m.

Table 4. *Montastrea cavernosa* mixed linear model showing the effects of chronic heat stress on Log Molecule Count over 480 h. StdErr = Standard Error, DF = Degrees of Freedom, tValue = t-statistic, Lower = 95% Lower Limit for parameter estimate, Upper = 95% Upper Limit for parameter estimate.

Effect	Treatment	Estimate	StdErr	DF	tValue	PValue	Lower	Upper
BCL2								
Intercept		2.4092	0.09184	8	26.23	<0.0001	2.1975	2.6210
Treatment	Heat	0.02397	0.1301	186	0.18	0.8540	−0.2328	0.2807
Treatment	Control	0						
Time		−0.00087	0.000279	186	−3.11	0.0022	−0.00142	−0.00032
Time*Treatment	Heat	0.000236	0.000394	186	0.60	0.5496	−0.00054	0.001014
Time*Treatment	Control	0						
Catalase								
Intercept		3.2195	0.1273	8	25.30	<0.0001	2.9261	3.5130
Treatment	Heat	−0.5883	0.1793	183	−3.28	0.0012	−0.9421	−0.2344
Treatment	Control	0						
Time		−0.00123	0.000441	183	−2.79	0.0059	−0.00210	−0.00036
Time*Treatment	Heat	0.001771	0.000628	183	2.82	0.0053	0.000532	0.003011
Time*Treatment	Control	0						
CathepsinL1								
Intercept		2.4955	0.1072	8	23.29	<0.0001	2.2484	2.7426
Treatment	Heat	0.02818	0.1518	180	0.19	0.8529	−0.2714	0.3277
Treatment	Control	0						
Time		−0.00192	0.000367	180	−5.22	<0.0001	−0.00264	−0.00119
Time*Treatment	Heat	−0.00034	0.000519	180	−0.65	0.5194	−0.00136	0.000689
Time*Treatment	Control	0						
HSP90								
Intercept		2.3096	0.1211	8	19.07	<0.0001	2.0303	2.5888
Treatment	Heat	−0.1586	0.1716	180	−0.92	0.3567	−0.4971	0.1800
Treatment	Control	0						
Time		−0.00130	0.000419	180	−3.11	0.0022	−0.00213	−0.00048
Time*Treatment	Heat	0.000379	0.000592	180	0.64	0.5228	−0.00079	0.001548
Time*Treatment	Control	0						
RpL9								
Intercept		3.8373	0.09197	8	41.72	<0.0001	3.6252	4.0494
Treatment	Heat	0.07685	0.1303	186	0.59	0.5560	−0.1802	0.3339
Treatment	Control	0						
Time		−0.00079	0.000263	186	−3.00	0.0031	−0.00131	−0.00027
Time*Treatment	Heat	−0.00054	0.000372	186	−1.46	0.1462	−0.00127	0.000191
Time*Treatment	Control	0						
RpS7								
Intercept		3.5026	0.08320	8	42.10	<0.0001	3.3108	3.6945
Treatment	Heat	0.1147	0.1179	186	0.97	0.3317	−0.1178	0.3472
Treatment	Control	0						
Time		−0.00083	0.000239	186	−3.48	0.0006	−0.00130	−0.00036
Time*Treatment	Heat	−0.00067	0.000338	186	−1.99	0.0482	−0.00134	−0.000001
Time*Treatment	Control	0						

4. Discussion

4.1. Acute versus Chronic Heat Stress

There are varying definitions of acute and chronic heat stress used when describing coral bleaching. Dove et al. [58] described acute heat stress to be less than 24 h, Strychar et al. [59] used 48 h, and Kenkel et al. [60] suggest acute as persisting for 96 h. The definition of chronic, however, ranges from ~7 days to 6 weeks or longer [10,61]. Here, we define acute stress as a 72-h (3 day) exposure and chronic stress as a 480-h (20 day) exposure. Corals *M. cavernosa* exposed to acute heat stress had a milder response than those exposed over a much longer time period (i.e., 20 days). This response was unexpected, as *M. cavernosa* is known to be a species sensitive to thermal stress. Haslun et al. [9] show that short exposures (72 h) to stressful heat conditions results in *M. cavernosa* bleaching when coral have not been repeatedly exposed to high heat temperatures (i.e., the more frequent a coral is exposed to high

heat, the more tolerable it is to heat stress). In their study, they compared this species collected from one site (FKNMS) where coral are repeatedly exposed to high temperatures and a second site, where environmental temperatures are relatively stable due to depth (Flower Garden Banks National Marine Sanctuary (FGBNMS). Coral from the FGBNMS were collected at 25 m depths and bleached rapidly versus *M. cavernosa* (collected at 30–33 m in our current study), whose bleaching response was mild in comparison. Since our coral were also collected from FKNMS, we postulate that these coral, too, may have been frequently pre-exposed to comparatively high summer heat stress conditions and as a consequence, were more resistant to bleaching; it is plausible, however, that different results may have been obtained if there was a seasonality component (summer vs. winter) to our study. The concept of ecological/seasonal memory is not new and has also been proposed by, for example, Hughes et al. [62], Torday et al. [63], and Guest et al. [64], who suggest frequent exposure to climate extremes results in some type of memory likely affording species better coping mechanisms. It must be noted, however, that "bleaching" does not necessarily equate to death and simply implies the loss of host/symbiont pigmentation revealing the stony white skeleton below. Cruz et al. [65] raise the question whether bleaching reports of coral worldwide are accurate given that color change may simply be a phenotypic trait associated with stress rather than death. Given this, coupled to our data showing corals responded more favorably to acute heat stressors versus chronic exposure, we followed a study by Csásár et al. [66] and Hauff-Salas et al. [51] who examined specific genes to better describe host health during conditions of heat stress.

4.2. Coral Response to Acute Experimental Heat Stress

During acute heat stress, the only genes to remain relatively constant were ribosomal proteins RpL9 and RpS7. This is likely attributable to ribosome biogenesis and protein translation, which are essential for animal development and cell growth, differentiation and proliferation [67–69]. Impairment of either of these two cellular processes severely reduces cell growth and either hampers animal development or contributes to host death. The expression of RpL9 and RpS7 genes also revealed a consistent gene concentration ratio at each time point across all treatments indicating that when one gene was expressed (e.g., RpL9), the second gene (e.g., RpS7) was immediately up-regulated. This may suggest a consequence of demand, where the host coral up-regulates protein synthesis and cell proliferation [70,71] to keep up with the loss of cells. Ruggero and Pandolfi [72] has shown that the rate at which protein synthesis occurs, cell proliferation is matched. By contrast, if one gene is suppressed, rapid cell proliferation causing a mass of undifferentiated cells may occur, resulting in a host immune response. It is plausible this also occurred as we observed up-regulation of BCL-2, catalase, and HSP90 at all observed time points during the acute heat stress treatment compared with control.

The up-regulation of BCL-2, catalase, and HSP90 at all observed time points likely indicates host stress and an immediate response to curb any negative physiological consequences. For instance, it has been well established that heat stressed coral up-regulate the biological process called apoptosis [73,74]; apoptosis is an essential process in cells involved in the elimination of defective cells. Anti-apoptotic and pro-apoptotic proteins, such as BCL-2, are involved in the regulation of apoptotic cell death by sequestering cysteine proteases called caspases [75,76] and/or prevent mitochondrial apoptosis-inducing factors (AIF) such as cytochrome c and ROS into the cytoplasm. When triggered, AIF and cytochrome c interactions directly activate caspases that permeabilize the mitochondrial membrane to promote apoptosis. Interestingly, BCL-2 also plays a role in the initiation and formation of autophagosomes [77,78], allowing a host to switch between cell death and/or survival mechanisms [79]. Various types of phagocytic cells have been identified in coral in addition to the production of caspases [7], suggesting a host coral's first response to high levels of acute heat may be to trigger apoptosis, killing off the weaker cells. We may assume that the host coral then switches quickly to a survival (autophagy) tactic removing those cells in addition to other harmful/necrotic cells. This mechanism, i.e., switching between death and survival using BCL-2, is not restricted to *Montastraea* spp.

coral and has been similarly observed in *Acropora millepora* [80,81] and *Stylophora pistillata* [82]. *Acropora millepora*, however, appear to be more sensitive than *Montastraea*, as the maximum temperatures used by Pernice et al. [80] were much lower (~30 °C). These authors also suggest that the rate of temperature change may affect whether a coral bleaches or switches to survival mode. Kvitt et al. [82] report that the expression of BCL-2 peaks at 6–24 h when thermally stressed, but declines after 72 h. Contrary to their report, we observed continuous up-regulation of BCL-2. We postulate that when heat stressed is applied, the rate of change, frequency of occurrence, and maximum temperature all contribute to a host coral's response. In order to address which event is more important, sampling of coral will need to occur every few hours in future studies. We also suggest that although BCL-2 plays an integral role in mitigating apoptotic cell death, its role in regulating oxidative stress and ROS production may be more important [83–86].

Within cells, there are a number of sources associated with ROS, including mitochondria [84], complex III [87], and the plasma membrane and endoplasmic reticulum [84], although mitochondria are considered the principal source. ROS are essential for everyday physiological functions, including cell-cycle development and progression, cellular migration and differentiation, cell death, maintaining redox equilibrium, cellular signaling pathways, and immune system function [88]. Short lived and considered highly reactive, ROS exists in low to modest concentrations. Excess levels of ROS damage proteins, lipids, nucleic acids and organelles which then pre- or post-transcriptionally trigger BCL-2 to initiate an immune response and removal of those dysfunctional cells [88]. However, as long as ROS exists in low levels, each molecule of catalase (CAT) will convert millions of free radical molecules into hydrogen peroxide helping to produce oxygen and water protecting the cell [55]. Increases in CAT in our study are similar to the observed increases in CAT in studies by [89] who observed up-regulation in both coral (*Acropora formosa, Echinopora lamellosa, Favia favus, Favites halicora, Porites* sp., and *Anacropora forbesi*) and their associated zooxanthellar symbionts. Interestingly, these authors also report that CAT activity was higher in zooxanthellae in four host corals, but relatively equal in *A. formosa* and *F. favus* [89]. Conversely, Teder et al. [90] report that catalase proteins isolated from *Capnella imbricata* were associated with the breakdown of lipids and hydroperoxide metabolism, albeit similarly associated with increasing temperatures from 23 °C to 31 °C. Whilst many authors consider these increases associated with stress signaling pathways, we hypothesize that the observed responses can also be immune-related. Although considered 'primitive' in design, corals possess an innate immune system and even, perhaps, some type of adaptive-like system [7,91–94]. Within that system, toll-like receptors [94,95] and the synthesis of melanin have been identified [96,97] in addition to peroxidase, phenoloxidase, superoxide dismutase, glutathione peroxidase [89,98]. JM van de Water et al. [99] tested 17 immune-related genes, and reported observing increased expression during stress in *Acropora millepora* and reduced response time upon subsequent injury.

In this study, as the heat stress increased, HSP90 continued to increase with time. Considered a chaperone protein, HSP90 helps other proteins fold properly and aids in protecting proteins against elevated temperatures. Schopf et al. [100] suggest that HSP90 is involved in more than a few hundred cellular processes including DNA repair, cell degeneration, and immune responses. Felts et al. [101], Willmund and Schroda [102], and Johnson [103] suggest that this protein can also be observed in mitochondria, chloroplasts, and the endoplasmic reticulum (ER). In coral suffering from heat exposure, it would be advantageous for HSP90 to stimulate the mitochondria to produce more ATP and to help regulate cellular metabolism. Similarly, during stress conditions, the presence of HSP90 protecting chloroplasts likely associated with the symbiont, affords the coral a stable form of energy until the assault (stress) subsides. Associated with manufacturing and transportation of proteins and carbohydrates, increased HSP90 would help to ensure the ER continues to function while the holobiont manages heat stress. Given our observations of increased concentrations of HSP90, we suggest that the host's increase in this protein was an attempt to maintain homeostasis, that is, 'regular' functioning of the cells despite being stressed.

In the control samples, the change of gene expression over time was not significant, which was 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).

4.3. Host Coral Response to Chronic Experimental Heat Stress

M. 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 been similarly observed by Dunn et al. [104], Roth et al. [10], Barshis et al. [105]. In both control and treatment samples, we observed a reduction in gene expression, from the onset to conclusion of the experiment (hour 0 to hour 480). However, there was intra-gene variation (up- and down-regulation) relative to consecutive time points for all genes. 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 or demand by the coral to produce ribosomal proteins for the continued production of other intracellular proteins required for a stress response or normal physiological function. Time and treatment had a negligible effect on the production of RpL9 and RpS7, except at hour 288 (30 °C) and 480 (33 °C). The up-regulation of other stress response genes at hour 288 and 480 likely required elevated levels of ribosomes, which may explain the up-regulation of RpL9 and RpS7 at these time points. There were few instances where gene expression surpassed those measured at hour 0 and raw values are not reported here. The majority of time points that did surpass hour 0 gene expression fell during hours 288 and 408, indicating a substantial physiological response (based on the rarity of transcript abundances surpassing hour 0 values). As five of the six genes have increased gene expression at hour 288 compared to hour 0, we suggest that hour 288 represents a physiological tipping point that indicate physiological stress. 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 differentially expressed at temperatures 27 °C–28.5 °C (hours 0–192), which is considered a sub-lethal temperature [106,107]. 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 [108,109].

Hour 288 at 30 °C revealed a substantial physiological response indicated as an abrupt increase in gene concentration. Based on the influence of heat at hour 288 to the entire suite of genes analyzed, the significant increase in gene expression may be a critical physiological turning point in host survivorship. Because five genes were simultaneously up-regulated relative to the control, it is plausible that coral exposed to 30 °C for 72 h (plus prior heat exposures of 27 °C and 28.5 °C) experienced significant and perhaps compounding physiological stress, representing a chronic temperature threshold. Although it has been observed that coral can survive temperatures exceeding 30 °C [110,111], there is a lack of heat stress data regarding coral communities below 30 m. Since the coral used in our study were sampled from deeper depths (i.e., MCEs), they may have yet to experience significant long-term heat anomalies, rendering them more susceptible to temperature and thus lowering the temperature needed to cause physiological stress [105,112].

BCL-2 was observed to be highly responsive to changes in temperature first represented by down-regulation and then up-regulation at the beginning and end of each temperature increase. The increase in BCL-2 expression after 72 h associated with increased temperature lead us to hypothesize that there is a cell-salvaging mechanism preventing the premature initiation of apoptosis in host coral cells. Guénal et al. [113] report that BCL-2 not only helps in mediating the effect of necrosis on cells, but is also capable of preventing cellular apoptotic death via decreasing mitochondrial morphological

changes. 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 recover when conditions return to a favorable state [80].

5. Conclusions

In this study, 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., catalase, HSP90, and BCL-2). In general, the pattern of gene expression produced by the host coral fluctuated, likely due to normal physiological homeostasis. It is likely that a 72-h exposure at 33 °C was not sufficient to cause coral mortality. To confirm that a 72-h exposure in elevated temperatures is survivable for MCEs, a post-treatment (i.e., recovery phase) gene expression analysis should be conducted in future studies.

Chronic exposure to temperatures at 30 °C for 72 h may be considered as a physiological tipping point for *M. cavernosa* corals sampled on Conch Reef at upper mesophotic depths (30 m), as observed by the up-regulation of five of six stress-related genes assayed. The up-regulation of these five genes (BCL-2, catalase, cathepsin L1, RpL9, and 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 protein production, maintaining protein integrity, and recycling old or potentially hazardous cellular components.

Overall, 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. However, with chronic heat treatments, a minimum exposure of 72 h at 30 °C, at a time point of hour 288, mesophotic coral will show significant symptoms of stress. In this study, *M. cavernosa* coral sampled from an MCE seemed to respond similarly to those found in shallow water communities [114], and are likely to be equally threatened as climate change continues to warm seawater.

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