

Estimating the heritability of thermal tolerance in *Acropora cervicornis* and the physiological
basis of adaptation that correlates to survival at elevated temperatures

by

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ABSTRACT

Human activities have substantially increased the atmospheric concentrations of greenhouse gases, resulting in warmer ocean temperatures that are having a negative impact on reef corals, which are highly susceptible to changes in temperature. Understanding the degree to which species vary in their tolerance to elevated temperatures and whether this variation is heritable is important in determining their ability to adapt to climate change. In order to address this, *Acropora cervicornis* fragments from 20 genetically distinct colonies were kept at either ambient or elevated temperatures, and mortality was monitored for 26 days. Heritability of thermal tolerance was estimated using a clonal method comparing the difference in lifespan within and among clones in a one-way ANOVA, as well as a marker based method using the program MARK (Ritland 1996) to estimate relatedness between colonies. To understand the physiological basis of thermal tolerance, tissue samples from both treatments were taken after 12 hours to investigate gene expression associated with sub-lethal temperature stress at both the mRNA and the protein level. The results revealed that this population of *A. cervicornis* has a relatively high amount of total genetic variation in thermal tolerance ($H^2 = 0.528$), but low additive genetic variation for this trait ($h^2 = 0.032$). In addition, both gene expression and protein expression among colonies were highly variable and did not show consistent patterns related to differences in thermal tolerance among colonies. These results reveal that this population of *A. cervicornis* may have a limited capacity to respond to projected increases in ocean temperatures. In addition, the results suggest that the molecular basis of thermal tolerance in this species is complex and that there are potentially many genotypic combinations that can result in a heat-tolerant phenotype.

INTRODUCTION

Human activities have substantially increased the atmospheric concentrations of greenhouse gases, resulting in warmer air and ocean temperatures that are having a negative impact on marine ecosystems worldwide (IPCC 2013). This is a dire threat to reef corals and their algal endosymbionts, which can both suffer from heat stress from changes as small as 1°C above long-term regional maximum temperatures (Goreau and Hayes 1994; Coles and Brown 2003; Oliver and Palumbi 2011). Higher ocean temperatures and irradiance levels (Brown et al. 2000; Takahashi and Murata, 2008) have repeatedly been reported to disrupt the symbiosis between corals and their algal endosymbionts, leading to higher occurrences of expulsion of the endosymbionts from the coral tissue, resulting in coral bleaching and mortality (Glynn 1996; Hoegh-Guldberg 1999; Douglas 2003; Csaszar et al. 2009). Corals rely on their endosymbionts for nutrients, which supply up to 95% of the coral host's energy requirements (Muscatine 1990; Jones et al. 2008); if average temperatures continue to increase above their temperature threshold, coral populations will be less likely to recover and will suffer increased mortality.

There are a number of ways in which corals can respond to a changing thermal environment. Corals can respond to temperature changes within a single generation by adjusting their thermal tolerance through acclimatization or by shifting the concentration of different *Symbiodinium* clades to better suit their environment (Jones et al. 2008; Sampayo et al. 2008; Baums et al. 2014). Corals can also respond to temperature changes through short term cross generation processes in which individual colonies inherit greater tolerance from parents who have been previously exposed to stress, likely through epigenetic inheritance (Hoegh-Guldberg 2014). Finally, colonies with greater tolerance to increasing sea surface temperature possess alleles that lead to increased survival and reproductive success, thereby increasing the

frequencies of these beneficial alleles and leading to local adaptation in coral populations (Hoegh-Guldberg 2014). However, evolution by means of natural selection is possible only if genetic variation exists within a population for traits that affect fitness (Fisher 1930). Therefore, in order to determine whether coral populations are likely to have the ability to adapt to predicted changes in climate, estimates of adaptive genetic variation in traits related to thermotolerance are needed. It is also essential to investigate differences in gene expression between colonies exposed to thermal stress in order to understand what loci may underlie differences in thermal tolerance.

Several studies have shown that gene expression changes within coral individuals when under heat stress. While most studies have been able to confirm that gene expression can differ significantly among different coral genotypes under heat stress (Csaszar et al. 2009; Seneca and Palumbi 2015), and that coral genotype can significantly impact coral thermal tolerance (Jin et al. 2016), only a few studies have focused on quantifying the genetic variation present in these traits (Csaszar et al. 2010; Dixon et al. 2015). In many cases, it can be difficult to investigate gene expression differences and differentiate between acclimatization in the short term and adaptive genetic variation in the long term. Previous studies have relied on reciprocal transplantation (Barshis et al. 2010; Kenkel and Matz 2016), or, in the case of other organisms, multiple generations of controlled breeding experiments, to successfully estimate the amount of genetic variation present in a trait, or heritability. Controlled breeding experiments are difficult to accomplish in a reasonable time frame with the time to sexual maturity and the long lifespans of many coral species (Chamberland et al. 2016). Some studies have overcome these constraints by crossing adults of different genotypes and investigating genetic variance in the larvae produced (Polato et al. 2013), but the phenotypes expressed in the planktonic larval stage may

not translate well to a sessile adult stage. Fortunately, with the advent of molecular genotyping and the ability of corals to be fragmented to propagate clones, studies can be designed to incorporate the effects of coral genotype on gene expression and the phenotype of thermal tolerance (Baums et al. 2005, 2009, 2010; Carlon et al. 2011; Csaszar et al. 2009, 2010; Jin et al. 2016; Ladd et al. 2017).

With the threat of global warming, much research has focused on not only identifying and measuring genetic variation in thermal tolerance, but also on understanding the underlying physiological mechanisms of thermal tolerance and coral bleaching. Previous work has shown consistent changes in the expression of ubiquitin (Barshis et al. 2010), ferritin (Csaszar et al. 2009), Hsp70 (Csaszar et al. 2009), and genes regulating apoptosis, ribosomal RNA and mRNA processing (Bay and Palumbi 2014; Seneca and Palumbi 2015) in either coral from different thermal environments or from different experimental temperature treatments. While many of these studies have been able to connect temperature stress with patterns of gene expression, these studies have not been able to connect gene expression following a short term temperature stress to life span during a long term temperature exposure. This is truly important when trying to quantify the potential for adaptation, since differences in survival can have implications for reproductive success.

In order to understand the ability of corals to adapt to warming ocean temperatures, the amount of genetic variation in thermal tolerance among nursery colonies of the staghorn coral *Acropora cervicornis* was estimated, and patterns of gene expression at both the mRNA and protein level were measured and tested for correlations to survival under thermal stress. This species was selected for study due to its rapid population decline and threatened status under the United States Endangered Species Act (2006) (NOAA 2006). Populations of *A. cervicornis* have

declined by up to 98% since 1980 throughout their natural range, and this has mainly been attributed to disease outbreaks as well as temperature and salinity variation, bleaching, human impacts, and low genetic diversity (NOAA 2006). While a recent study has determined that there is high genetic diversity at neutral genetic markers among *A. cervicornis* along the Florida Reef Tract (Drury et al. 2016), the amount of genetic variation in traits related to thermal tolerance as well as biomarkers correlated to survival have yet to be measured in this species. Determining whether there is adaptive genetic variation of thermal tolerance present within these nursery populations will provide information on whether outplanting can be used to promote adaptation in wild populations (van Oppen et al. 2015).

MATERIALS AND METHODS

Collection of Corals and Heat-Stress Experiments

A total of 13 fragments ~8-9 cm in length were obtained from each of 20 genetically distinct colonies of the staghorn coral *A. cervicornis* from the Coral Restoration Foundation's Tavernier offshore nursery (24°58'58.8"N 80°26'07.8"W by 24°58.51.6"N 80°26'07.8"W by 24°55'04.8"N 80°26'15.0"W by 24°58'58.8"N 80°26'15.0"W). Colonies were fragmented and maintained at the nursery for a minimum of 30 days to heal before being transported to the Keys Marine Laboratory located in Layton, FL. Fragments were then given 48 h to acclimate at ambient temperature (27 – 29 °C) to lab conditions. One fragment per genotype was immediately flash frozen for genotyping of the coral host. The remaining fragments were then randomly assigned to positions within each of six separate tanks, such that each tank contained two fragments per genotype from all 20 genotypes. Following acclimation, temperature was increased over the course of six hours to 32 °C ($\pm 1^\circ\text{C}$) in three of the tanks, while three of the

tanks were kept as controls and were maintained at ambient temperature. After 12 hours of elevated temperature exposure, one fragment per genotype was removed from each tank and was flash frozen for molecular analysis of short term stress. The survival of the remaining fragments was measured for the remainder of the experiment for a total of 26 days. Pulse amplitude modulated (PAM) fluorometry was used to measure the Fv/Fm for each fragment twice daily at approximately 0730 and 1930. Mortality was determined once Fv/Fm scores for individual fragments reached < 0.2 for two consecutive time periods and confirmed by total loss of coral tissue. Previous studies have shown that these scores correspond to severe bleaching and mortality (Rasher and Hay 2010).

Genotyping of Coral Hosts

A total of 15 microsatellite loci were used to genotype each of the coral colonies by using primers developed in *Acropora palmata* by Baums et al. (2005, 2009), which have been previously shown to amplify in *A. cervicornis* (van Oppen et al. 2000; Vollmer and Palumbi 2002; Baums et al. 2005, 2009, 2010). Fragments of approximately 2 – 3 cm² of tissue with skeleton from each colony were digested in CHAOS solution [4 M guanidine thiocyanate, 0.1% N-lauroyl sarcosine sodium, 10 mM Tris pH 8, 0.1 M 2-mercaptoethanol (Fukami et al. 2004)] for seven days at room temperature and then stored at – 80°C until extraction. The DNA extractions were performed following the protocol outlined in Levitan et al. (2011) and the resulting DNA was then stored at – 20°C until ready for use in PCR reactions. The PCR reaction mixture consisted of 2.5 µL of 10X PCR buffer (Invitrogen), 1.5 µL of 50 mM MgCl₂, 0.5 µL of 10 mM dNTPs, 0.2 µL of Taq (Invitrogen), 0.2 µL of forward primer (10 µM), 0.4 µL of reverse primer (10 µM), 1.5 µL of DNA template, and nuclease free water to bring to a total volume of

26.5 μ L. The PCR amplification was carried out with an initial denaturation step at 95°C for 5 min followed by 35 cycles of 95°C for 20 s, annealing at 50 – 55°C for 20 s, and 72°C for 30 s, followed by a final extension at 72°C for 30 min on a BioRad C1000 Touch Thermal Cycler. The PCR products were sent to UF ICBR Gene Expression & Genotyping for sequencing using an AB 3730 with an internal size standard (LIZ600) for determining fragment size. Electropherograms were analyzed with GeneMarker software V2.7.0 (SoftGenetics).

Heritability Estimation

Broad sense (H^2) and narrow sense (h^2) heritability of thermal tolerance in *A. cervicornis* were estimated in this study utilizing distinct methods. Broad sense heritability was estimated following the clonal method described in Csaszar et al. (2010) and Falconer and MacKay (1996) where phenotypic variance is partitioned into genetic and environmental components based on estimates of among and within coral clone variation in survival at elevated temperatures using an analysis of variance (ANOVA). Thermal tolerance in this study was defined as the difference between the mean lifespan of fragments maintained at elevated temperatures and the mean lifespan of fragments from the same colony maintained at ambient temperatures. Therefore, colonies with a smaller difference in lifespan between elevated temperature and control were considered to have a higher thermal tolerance, while colonies with larger differences were considered to have lower thermal tolerance. Broad sense heritability was estimated using a one-way ANOVA (IBM SPSS Statistics 25) with thermal tolerance as the dependent variable and coral genotype as the main effect. The ANOVA allows the estimation of the amount of variance in thermal tolerance within fragments from the same colony (V_E) as well as the amount of variance in thermal tolerance among colonies of different genotype (V_G). The summation of

these variance components provides an estimate of V_P , the total phenotypic variance of a trait, and allows H^2 to be calculated using the below equation (Falconer and MacKay 1996):

$$H^2 = \frac{V_G}{V_P}$$

Narrow sense heritability was estimated using a marker based computer program, MARK (Ritland 1996), which uses a mathematical regression model developed by Lynch and Ritland (1999) to calculate the relatedness between individual fragments based on their multi-locus genotype. The program then compares the relatedness coefficient to an estimate of phenotypic similarity (in this case thermal tolerance) in order to calculate the narrow sense heritability (h^2) of the trait in question. High heritability is indicated by more related individuals possessing a similar phenotype and less related individuals possessing dissimilar phenotypes. One hundred bootstraps were performed on the estimate of h^2 and a bootstrap percentile test was used to determine if the estimate of h^2 was significantly different from bootstrapped values at $\alpha = 0.05$. Narrow sense heritability is based on the proportion of additive genetic variance in a trait, which is the only form of genetic variance that can respond to selection (Lande and Shannon 1996).

Gene Expression

Three fragments per genotype per treatment were flash frozen 12 hours after the initiation of the experiment to look at gene expression at both the mRNA and the protein level. For both investigations, the three most thermal tolerant genotypes and the three least thermal tolerant genotypes, as determined by the time of mortality following heat stress (see Results), were used to determine the effect of expression level or concentration of stress biomarkers on thermal tolerance. Three control and three heated samples were included for each genotype for a total of 36 samples. In addition, for expression at the protein level, all fragments were included in a

correlation analysis to determine if there were significant correlations between biomarker concentrations and difference in lifespan following a long term thermal stress (N=120).

For mRNA expression, the entire transcriptome was analyzed in order to identify potential loci that may be involved in thermal tolerance in this species, whereas for protein expression, relative concentrations and activity were measured for the following four molecules: catalase, heat shock protein 70 (Hsp70), ubiquitin, and 4-hydroxynonenal (4HNE). These biomarkers were chosen due to their responses under elevated temperature stress in other coral species (Csaszar et al. 2009; Barshis et al. 2010; Seneca et al. 2010; Ross et al. 2013). Elevation in reactive oxygen species due to thermal stress often results in an upregulation of catalase, an enzyme that breaks down H_2O_2 into O_2 and H_2O , and a greater abundance of 4HNE, a molecule produced by reactive lipid peroxides (Halliwell and Gutteridge 1999; Halliwell 2006; Barshis et al. 2010; Fisher et al. 2012; Ross et al. 2013). Ubiquitin is a cellular protein tag that marks proteins for degradation, and higher levels can be indicative of elevated levels of stress (Hawkins 1991; Barshis et al. 2010; Jin et al. 2016), and Hsp70 is a molecular chaperone that is often found to be upregulated in response to thermal stress and aids in maintaining protein structure and function following heat related denaturation (Feder and Hofmann 1999; Barshis et al. 2010, 2013; DeSalvo et al. 2010).

For mRNA expression, total RNA was extracted from a small portion (< 1 cm) of each fragment using a TRI Reagent extraction (ThermoFisher Scientific), then purified using RNeasy columns (Qiagen) following the methods of Bay et al. (2009). Quality and concentration of total RNA was determined via spectrophotometer readings at 260 and 280 nm. The total RNA was then fragmented by incubating at 95 °C for 15 minutes and used to synthesize first-strand cDNA with adapters at both 5' and 3' ends (Matz et al. 1999; Meyer et al. 2011) using a PrimeScript

First Strand cDNA synthesis kit (Clontech) per the manufacturer's instructions. The second strand of cDNA was synthesized and amplified using the following PCR reaction: 0.5 μ L of dNTPs (2.5 mM each), 2.5 μ L of 10x PCR buffer, 0.5 μ L each of 10 μ M of both adapter sequences, 0.5 μ L of Klentaq1 polymerase (DNA Polymerase Technology), and 10 μ L of first strand cDNA in a total volume of 30 μ L. The PCR amplification was carried out on a BioRad C1000 Touch Thermal Cycler and carried out as follows: an initial denaturation step at 94°C for 2 min followed by 15 cycles of 94 °C for 30 s, 63 °C for 1 min, and 68 °C for 2 min. Each cDNA sample was labelled with a specific combination of two barcodes and gel-extracted using a MinElute Gel Extraction kit (Qiagen) to purify the size fraction (400 – 500 bp) for sequencing.

Barcoded samples were pooled for sequencing on the Illumina HiSeq 2500 at the Case Western Reserve University School of Medicine Genomics Core and the libraries were sequenced with single-end 50 bp read lengths, resulting in a total of 608,536,472 raw reads. A custom perl script provided by M.V. Matz was used to discard reads sharing the same sequence as the read and degenerate adaptor (PCR duplicates) and to trim the leader sequence from remaining reads (Kenkel and Matz 2016). The fastx_toolkit was then used, following the protocol of Kenkel and Matz (2016), to trim reads after a homopolymer run of 'A' \geq 8 bases was encountered, to retain reads with a minimum sequence length of 20 bases, and to quality filter, requiring PHRED of at least 20 over 90% of the read. After the removal of PCR duplicates and quality trimming, 720,646 reads remained. The *A. cervicornis* transcriptome (Libro et al. 2013), consisting of 95,389 transcripts with a N50 of 977, was downloaded from NCBI and annotated using custom perl scripts and protocol provided by M.V. Matz. Filtered reads were mapped to this reference transcriptome with Bowtie2, and read counts were assembled by isogroup, groups of sequences potentially originating from the same gene, or with sufficiently high sequence

similarity to justify the assumption that they serve the same function, using a custom perl script provided by M.V. Matz (Kenkel and Matz 2016). Reads mapping to multiple isogroups were discarded. A total of 301,333 transcripts mapped against the transcriptome, with an overall alignment rate of 41.81%, and these unique reads mapped to 20,471 coral isogroups.

Differential expression (DESeq2) was carried out in the R statistical environment (v 3.4.0, R Foundation for Statistical Computing) following the methods of and using the R scripts provided by Kenkel and Matz (2016) for TagSeq analysis. Low-expression genes (those with less than a mean count of 3) were removed from the dataset, leaving 188 genes for DESeq2 analysis. Gene counts were normalized and log-transformed using a regularized log transform with the command `rlog()` in DESeq2 for subsequent analyses. A principal coordinate analysis (PCoA) was performed in order to cluster samples by treatment (control vs heated) and by tolerance (tolerant vs susceptible), as well as to allow statistical tests to verify significance of the observed groupings. A matrix of pairwise “Manhattan” distances (sum of log-fold differences across all genes) among the 36 samples was constructed for PCoA, and the results plotted using principal coordinates one and two.

Differences in gene expression among samples were analyzed using a two-way factorial ANOVA with genotype and treatment as main effects, followed by an additional two-way factorial ANOVA with tolerance and treatment as main effects. Each of these models were included in the DESeq2 analysis for determining differentially expressed genes (DEGs). The likelihood ratio test (LRT) was used for factors with greater than two levels (genotype, genotype by treatment interaction, tolerance by treatment interaction), while the Wald test was used for factors with two levels (treatment, tolerance). For significant DEGs in relation to thermal tolerance, the nucleotide sequences were extracted from the *A. cervicornis* transcriptome (Libro

et al. 2013) and each sequence was aligned against the NCBI protein database using blastx (NCBI) so as to establish likely function. In order to determine whether gene expression of the identified DEGs had a significant effect on thermal tolerance, the average gene counts for each significant DEG were compared among genotypes and between tolerance groups using either a two-way ANOVA or Scheirer-Ray Hare extension for Kruskal Wallis if assumptions for normality were not met (IBM SPSS Statistics 25).

For protein expression, fragments were airbrushed with a 50 mmol sodium phosphate buffer containing 0.05 mol/L dithiothreitol and lyophilized. The lyophilized tissue was resuspended in sodium phosphate buffer before use. Catalase activity within coral tissue was measured using the Amplex® Red Catalase Assay Kit (ThermoFisher Scientific) per the manufacturer's instructions, and then adjusted for the total amount of protein (U activity /mg total protein) as measured using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific) per the manufacturer's instructions (Ross et al. 2013).

Hsp70, ubiquitin, and 4HNE levels were first detected in samples through immunoblot assays following the methods of Barshis et al. (2010), and then quantified through ELISA. In each well, 50 µg of total protein per sample were added to 10 µL of 2x Laemmli SDS-PAGE sample buffer (Bio-Rad). Positive controls consisted of 5 – 10 µg of Heat Shocked HeLa Cell Lysate (ADI-LYC-HL101-F, Enzo Life Sciences) for Hsp70 and 4HNE-conjugates, respectively, and Rat Brain Tissue Extract for ubiquitin-conjugates (ADI-LYT-RB100-F, Enzo Life Sciences) following the methods of Barshis et al. (2010). All samples, standards, and positive controls were loaded into Mini PROTEAN TGX precast gels (4-20%) (Bio-Rad), and run for approximately 30 min at 200 V on a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad). Protein

transfer was performed in a Mini TransBlot Cell (Bio-Rad) onto an Immuno-Blot PVDF membrane (Bio-Rad) at 100 V for one hour.

Membranes were blocked overnight in 50 mL of 10% nonfat dry milk in Tris buffer saline (TBS, 0.05 M TrisBase, 0.15 M NaCl, pH 7.6) at 4 °C and then incubated at 4 °C overnight with primary antibody diluted 1:2000 in 1% nonfat dry milk in TBS (Hsp70: Cat. #ADI-SPA-822, Enzo Life Sciences; ubiquitin: Cat. #ADI-SPA-200, Enzo Life Sciences; 4HNE: Cat. #AB5605, EMD Millipore) (Barshis et al. 2010). Following the primary antibody incubation, membranes were incubated at room temperature for 1 hour with secondary antibody diluted 1:10,000 in 1% nonfat dry milk in TBS (anti-mouse alkaline phosphatase (AP) conjugated: Cat #A3562, Sigma-Aldrich; anti-rabbit AP conjugated: Cat #A0418, Sigma-Aldrich; anti-goat AP conjugated: Cat #A7650, Sigma-Aldrich) (Barshis et al. 2010). Afterwards, a BCIP/NBT Alkaline Phosphatase Substrate Kit (Vector Laboratories) was used to visualize detection of the protein biomarkers by the primary antibody, per the manufacturer's instructions. All blot images were recorded using an Amersham Imager 600 (GE Healthcare Life Sciences).

For ELISA, 25 µg total protein per sample were added to 100 µL of 0.2 M sodium carbonate-bicarbonate buffer (pH 9.4) per well on a 96-well plate; 100 µL of phosphate buffer saline (PBS, 0.01 M monobasic NaH₂PO₄, 0.15 M NaCl, pH 7.2 – 7.4) were used as a blank. Both primary and secondary antibody dilutions were 1:10,000 in 1% nonfat dry milk in PBS. A TMB Substrate Kit (ThermoFisher Scientific) was used for the detection of Hsp70, ubiquitin, and 4HNE. Absorbance was measured at 450 nm using a Synergy HT plate reader (BioTek) and analyzed using Gen5 (BioTek). All samples and blanks were run in triplicate.

In order to determine whether the expression of biomarkers at the protein level had a significant effect on thermal tolerance, the mean relative concentrations or the mean activity of each biomarker were compared between the three most thermal tolerant and the three least tolerant genotypes using a two-way ANOVA or Scheirer-Ray-Hare extension for Kruskal Wallis if assumptions for normality were not met (IBM SPSS Statistics 25). A second analysis was performed utilizing all 20 genotypes to determine if mean relative concentrations or mean activity of each biomarker correlated with thermal tolerance using either a Pearson correlation or Spearman's Rho if assumptions for normality were not met (IBM SPSS Statistics 25).

RESULTS

Heritability Estimation

During the long term temperature stress, it was discovered that one of the heated tanks had elevated temperatures of 1 °C higher than the other two heated tanks for a period of approximately 12 hours. When a two-way ANOVA was performed, there was a significant tank effect due to this temperature difference among heated tanks ($F_{2,38} = 3.963$, $p = 0.027$) which disappeared once the aberrant tank was removed from the data set ($F_{1,19} = 0.416$, $p = 0.527$). Therefore, all subsequent analyses involving difference in lifespan were conducted without the inclusion of that tank.

The lifespan of *A. cervicornis* coral fragments was significantly affected by temperature. While all of the control fragments survived the entire length of the experiment (612 hours) and showed no decrease in health according to Fv/Fm scores, all fragments maintained at elevated temperature showed signs of decline in Fv/Fm after 204 hours (Figure 1), and heated fragments had a significantly reduced lifespan compared to the controls, with fragments surviving 474.15

hours on average under thermal stress ($F_{1,118} = 768.508$, $p < 0.001$). However, this reduction in lifespan relative to the controls was not consistent across all coral genotypes ($F_{1,19} = 3.15$, $p = 0.008$) (Figure 2). The average difference in lifespan between control and treatment fragments varied from 72 to 216 hours among genotypes, with genotypes U33 ($p = 0.041$) and M7 ($p = 0.041$) having significantly lower thermal tolerance than genotype M5 according to a Tukey's HSD post hoc test (Figure 2). The three genotypes that had the lowest average difference in lifespan and therefore the highest thermal tolerance were M3, M5, and U58, while the three genotypes that had the highest average difference in lifespan and therefore the lowest thermal tolerance were M7, U1, and U33. These genotypes differed by at least 90 hours of lifespan differential. These six genotypes were then used for comparisons of mRNA and protein expression between tolerant and susceptible genotypes in later analyses (see below). The significant differences in lifespan among genotypes resulted in a significant broad sense heritability of temperature tolerance ($H^2 = 0.528$). However, the narrow sense heritability estimate was much lower ($h^2 = 0.032$), and was not significantly different from bootstrapped values.

Gene Expression

Although the three most thermal tolerant and the three most susceptible genotypes differed greatly in their long term survival to a thermal stress, there was a great deal of overlap in overall gene expression at the mRNA level between both treatments (control vs heated) and both tolerance groups (tolerant vs susceptible) following 12 hours of elevated temperature exposure (Figure 3). When testing the effects of genotype, treatment, and their interaction, it was found that overall patterns of gene expression did not differ significantly due to treatment ($F_{1,24} = 0.182$,

$p = 0.182$), while both genotype ($F_{5,24} = 2.2310$, $p = 0.001$) and the genotype by treatment interaction ($F_{5,24} = 1.5544$, $p = 0.010$) had significant effects on differences in gene expression (Table 1).

Since patterns of gene expression differed significantly among genotypes, we tested whether these differences were associated with thermal tolerance using three different models: 1) inclusion of all six genotypes separated into tolerant and susceptible groups; 2) inclusion of only the three genotypes that significantly differed in lifespan differential (M5, tolerant vs. U33 and M7, susceptible); and 3) the same tolerant and susceptible groups as in model 2 with the inclusion of the other three genotypes in an intermediate group. Neither tolerance, treatment, nor the interaction between tolerance and treatment had a significant effect on the differences in overall gene expression among samples for any of the three models tested ($p > 0.10$) (Table 1).

Since there were no differences in the results among these three different scenarios, the first model was included along with the genotype, treatment, and genotype by treatment interaction model in the DESeq2 analysis for determining differentially expressed genes (DEGs) due to these different factors. When examining the effect of treatment, genotype, genotype by treatment interaction, and tolerance on DEGs among these six genotypes, treatment resulted in no significant DEGs, genotype had a significant effect on 64 DEGs, the genotype by treatment interaction had a significant effect on seven DEGs, and tolerance had a significant effect on four DEGs. The tolerance DEGs were selected to investigate the effect of differences in gene expression on long term thermal tolerance, while the genotype by treatment interaction DEGs were selected to investigate how genotypes differed in their response to thermal stress and whether there were consistent patterns that distinguished thermotolerant and susceptible genotypes.

When examining the four DEGs that differed significantly among corals with different thermal tolerances, these DEGs aligned with the following proteins in the NCBI database: peridinin-chlorophyll a-binding protein, cytochrome C, and an uncharacterized protein from *Symbiodinium microadriaticum*, and an uncharacterized protein from *Acropora digitifera*. For all four of these DEGs, gene counts were highly variable among genotypes and they were not consistent between tolerance groups (Figure 4). Neither the uncharacterized protein (Two-way ANOVA, $F_{5,29} = 2.122$, $p = 0.091$) nor peridinin-chlorophyll a-binding protein (Scheirer-Ray-Hare, $F_{5,29} = 0.939$, $p = 0.471$) from *S. microadriaticum* significantly varied in their expression level among these six genotypes. While the expression of the uncharacterized protein from *A. digitifera* was significantly different among genotypes (Scheirer-Ray-Hare, $F_{5,29} = 3.152$, $p = 0.022$), and the expression of cytochrome C was marginally significant among genotypes (Scheirer-Ray-Hare, $F_{5,29} = 2.533$, $p = 0.051$), only cytochrome C showed a significant difference between tolerance groups when genotypes were averaged together (Scheirer-Ray Hare, $F_{1,33} = 9.307$, $p = 0.004$); this was mainly due to intolerant genotypes U1 and U33 having higher cytochrome C expression on average compared to all three tolerant genotypes (Figure 4a).

When examining the seven significant genotype by treatment interaction DEGs, five of these genes aligned as follows: dimethylsulfoniopropionate (DMSP) lyase, 40s s6-like ribosomal protein, soma ferritin, a plectin like isoform, and an uncharacterized protein, all found in *A. digitifera*. For the last two significant DEGs, one had an ambiguous origin as it aligned with an uncharacterized protein found in multiple species, particularly in protists, plants, bacteria, and fungi, while the other did not align with any proteins, and there were no conserved domains found in this sequence.

For the 40s s6-like ribosomal protein ($F_{5,24} = 4.286$, $p = 0.006$), soma ferritin ($F_{5,24} = 5.552$, $p = 0.002$), and the uncharacterized protein from *A. digitifera* ($F_{5,24} = 3.539$, $p = 0.015$), tolerant genotypes typically upregulated these genes in the heated treatment, while they tended to be downregulated in the susceptible genotypes (Figure 5a-c). This pattern, however, was not consistent across all tolerant and susceptible genotypes. For example, tolerant genotype U58 did not change expression between treatments for the 40s s6-like ribosomal protein, and it downregulated expression of soma ferritin and the uncharacterized protein in the heated treatment (Figure 5a-c). Meanwhile, susceptible genotype U1 upregulated these same three genes in the heated treatment (Figure 5a-c). Although DMSP lyase ($F_{5,24} = 2.825$, $p = 0.038$), the plectin-like isoform ($F_{5,24} = 4.747$, $p = 0.004$), and the unaligned protein ($F_{5,24} = 4.892$, $p = 0.003$) all showed significant genotype by treatment interactions on gene expression, there was no significant genotype by treatment interaction on expression of the uncharacterized protein of unknown origin ($F_{5,24} = 2.553$, $p = 0.055$) (Figure 5d-g). No apparent patterns between thermal tolerance and gene expression existed at any of these four loci (Figure 5d-g).

Antibody recognition of proteins from *A. cervicornis* tissues was observed for ubiquitin-conjugated and 4HNE-conjugated proteins, but not for Hsp70 (Figure 6); Hsp70 was therefore not included in the following analyses. When investigating the effect of treatment on protein expression differences among the three most thermal tolerant and the three least tolerant genotypes, elevated temperature was found to have no effect on either the activity of catalase (Schierer-Ray-Hare, $F_{1,29} = 0.285$, $p = 0.598$) or the relative absorbance of ubiquitin (Two-way ANOVA, $F_{1,29} = 0.615$, $p = 0.439$) or 4HNE (Two-way ANOVA, $F_{1,29} = 0.281$, $p = 0.6$) (Figure 7). Genotype, on the other hand, was found to have a significant effect on the activity of catalase (Schierer-Ray-Hare, $F_{5,29} = 10.6$, $p < 0.001$) and the absorbance of 4HNE (Two-way ANOVA,

$F_{5,29} = 35.569$, $p < 0.001$), but not the absorbance of ubiquitin, which varied little among samples (Two-way ANOVA, $F_{5,29} = 0.629$, $p = 0.679$) (Figure 7).

When examining the effect of tolerance on biomarker activity or relative concentration, tolerant genotypes tended to have higher catalase activity on average, although this difference was only significant for U58 according to a Tukey's HSD post hoc test ($p < 0.05$ when compared to genotypes M7, U1, and U33) (Figure 7a). The absorbance of 4HNE tended to be lower in tolerant genotypes compared to susceptible genotypes, although this was not statistically significant between tolerance groups due to genotype U58, which had the highest 4HNE absorbance measured here (Figure 7c). Since the absorbance of ubiquitin did not differ among genotypes, there were no trends observed due to thermal tolerance (Figure 7b).

To determine if there were significant correlations between protein biomarkers and thermal tolerance, lifespan differential for the three most thermal tolerant and the three least tolerant genotypes was compared to biomarker activity or relative concentration three different ways: 1) inclusion of only the biomarker and lifespan data for the heated fragments of each genotype; 2) the average of the biomarker data for the control fragments for each genotype; and 3) the difference in average biomarker activity or relative concentration between the heated and control fragment for each genotype. The only significant correlation with lifespan differential for these genotypes was with catalase activity in the heated treatment (Table 2). A Model I Linear Regression analysis was performed using catalase activity as the independent predictor variable and difference in lifespan as the dependent variable (Figure 7d). Using this model, catalase activity following a temperature stress explained 45.02% of the difference in lifespan among these genotypes ($F_{1,10} = 8.197$, $p = 0.017$) (Figure 7d).

When including all 20 genotypes in the analyses, treatment again had no significant effect on either the activity of catalase (Scheirer-Ray Hare, $F_{1,79} = 0.385$, $p = 0.537$) or the absorbance of ubiquitin (Scheirer-Ray-Hare, $F_{1,79} = 0.013$, $p = 0.908$) or 4HNE (Scheirer-Ray-Hare, $F_{1,79} = 0.349$, $p = 0.557$). Significant differences among genotypes, on the other hand, were found for all three biomarkers (catalase activity: Scheirer-Ray-Hare, $F_{19,79} = 8.279$, $p < 0.001$; ubiquitin: Scheirer-Ray-Hare, $F_{19,79} = 11.389$, $p < 0.001$; 4HNE Scheirer-Ray-Hare, $F_{19,79} = 13.163$, $p < 0.001$). The mean difference in lifespan for each genotype was also compared to the mean biomarker activity or concentration for the control samples, the heated samples, or the difference in activity or concentration between control and heated samples. However, these analyses also resulted in no significant correlations (Table 2) (Figure 8).

DISCUSSION

Broad sense heritability was estimated at 0.528, meaning that 52.8% of the variation in thermal tolerance observed in this study can be attributed to differences in coral genotype, suggesting a high degree of genetic variation in thermal tolerance present in this nursery population. However, narrow sense heritability was estimated to be only 0.032, and it was not significantly different from bootstrapped values, suggesting that most of the genetic variation observed in thermal tolerance is due to non-additive sources such as dominance or epistatic effects. Since additive variation is the only portion of total genetic variation that can respond to natural selection (Lande and Shannon 1996), this population of *A. cervicornis* would appear to have a limited capacity to respond to projected increases in ocean temperatures. In addition, while there appeared to be a great deal of variation among genotypes in the gene expression patterns investigated here, there were very few consistent differences between tolerant and

susceptible genotypes. The few patterns that did arise, such as higher average catalase activity overall and upregulation of ferritin following temperature stress in tolerant genotypes, were not consistent across all tolerant or susceptible genotypes tested here. This suggests that the molecular basis of thermal tolerance is complex and that there are potentially many genotypic combinations that can result in a heat-tolerant genotype.

There have been few previous studies that estimate either broad sense or narrow sense heritability of thermal tolerance in corals, which limits the comparisons that can be made to our current study. Our estimate of broad sense heritability was similar to the broad sense heritability estimate of growth rate under thermal stress in *Acropora millepora* ($H^2 = 0.59$) (Csaszar et al. 2010). In their study, Csaszar et al. (2010) estimated the heritability for a number of traits during a thermal stress from the symbiont, the coral host, and the coral holobiont. They found higher genetic variation in symbiont-derived traits, such as photosystem efficiency ($H^2 = 0.50$), and the holobiont-derived trait of growth rate, compared to host-derived traits, such as manganese superoxide dismutase production ($H^2 = 0.18$), suggesting higher adaptive potential of the symbionts compared to the host in this coral species. On the other hand, our broad sense heritability estimate of thermal tolerance was much lower than the estimate for the heritability of thermal tolerance in *A. millepora* reported by Dixon et al. (2015), who estimated a broad sense heritability of 0.87. This suggests that different species will likely vary in their amount of genetic variation in thermal tolerance, specifically regarding survival following a thermal stress. It has been noted that the mode of reproduction in *A. cervicornis* along the Florida Reef Tract is largely asexual (Gilmore and Hall 1976), which may result in lower genetic diversity in thermal tolerance traits relative to other species. Conversely, our estimate of narrow sense heritability

was quite low compared to estimates of narrow sense heritability for morphological traits (Carlon et al. 2011) and settlement cues (Meyer et al. 2009) in *A. millepora*.

The estimation of narrow sense heritability is highly dependent on the markers utilized and the amount of actual variation in relatedness of the samples utilized (Ritland 1996). The variation in relatedness was estimated at -0.144 ± 0.083 (mean \pm SD) for the nursery population used in this study. According to Ritland (1996), zero or negative values for variance in relatedness are typically either due to sampling individuals that are all related to similar extents, or to utilizing too few markers or markers with few alleles. However, the 15 microsatellite loci used in this study as well as the number of alleles per locus (6.667 ± 0.803 , mean \pm SE) are well within the range recommended by Ritland (1996). This suggests that the negative actual variance in relatedness is more likely due to a similar degree of relatedness among the colonies sampled. Ritland (1996) recommends that the number of pairwise comparisons should be as large as possible (10^4 or 10^5 if feasible), and in our study there were only 780 pairwise comparisons between fragments due to sampling constraints. Therefore, the estimate of narrow sense heritability reported here should be interpreted with caution, and further studies will be needed utilizing a wider range of *A. cervicornis* colonies with a higher variance in actual relatedness to more accurately estimate narrow sense heritability.

While the estimate for narrow-sense heritability in this study is quite low, this does not necessarily mean that the populations utilized here will have no capacity to adapt to climate change. Thermal tolerance is a complex quantitative trait, which is likely to be due to epistatic interactions among multiple loci (Hoegh-Guldberg 2014). In certain models, it's been shown that epistatic variance can become additive (Cheverud and Routman 1995), and that high epistatic variance can increase additive genetic variance in populations following a population bottleneck

(Cheverud and Routman 1996). This suggests that if a population has substantial epistatic genetic variance, it can lead to a rescue of low additive genetic variance, even following drastic population declines, providing the necessary genetic variation for natural selection to act on. The relatively high broad sense heritability estimate compared to the narrow sense heritability estimate in this study suggests that variation in thermal tolerance may be due more to dominance effects and epistatic interactions instead of additive genetic variance. If the variance in this trait is largely due to epistatic interactions, then this could provide the necessary source of genetic variation for this population to be able to adapt to elevated ocean temperatures with climate change over successive generations.

There are several hypotheses that can explain the differences between thermal tolerant and susceptible colonies. One hypothesis is the frontloading hypothesis, proposed by Barshis et al. 2013, which found that colonies living in warmer conditions constitutively upregulated sets of genes that were otherwise induced only during heat stress, which may better prepare them to circumvent or respond to such stress. Another hypothesis, transcriptome resilience, was proposed by Seneca and Palumbi (2015), under which corals show a wide transcriptome response before bleaching begins, followed by a more rapid return to normal gene expression in more bleaching-resistant colonies. On the other hand, Kenkel and Matz (2016) provide evidence for enhanced phenotypic plasticity leading to higher thermal tolerance rather than the frontloading of stress response genes among populations, noting that which strategy is employed may be due to the frequency at which coral populations are exposed to thermal stress events. Constitutive upregulation would be favored over plasticity if the environment fluctuates more rapidly than the typical response time, which would be analogous to a constant stress environment (Kenkel and Matz 2016). While our study only examined mRNA and protein expression at a single time

point, making it difficult to determine which strategy is being employed in this *A. cervicornis* nursery population, there are a few patterns that are evident, although these patterns were not consistent across all tolerant or all susceptible genotypes.

First of all, there did appear to be constitutive differences between the three most tolerant and the three most susceptible genotypes in terms of catalase activity and the tolerance DEG cytochrome C. Higher catalase activity, regardless of treatment, was associated with longer life spans during a long term thermal stress, which supports the frontloading hypothesis. However, this difference was not consistent across all tolerant genotypes: catalase activity was significantly higher in tolerant genotypes M3 and U58 compared to intolerant genotypes U1 and U33, but there was no significant difference in catalase activity between tolerant genotypes M3 and M5 and intolerant genotype M7. On the other hand, lower cytochrome C expression was associated with longer life spans during a long term thermal stress. Higher upregulation of cytochrome C may be indicative of these colonies experiencing higher stress than their more thermal tolerant counterparts. Higher upregulation of this gene could also lead to higher energetic costs, which may in turn reduce the ability of these colonies to handle a long term thermal stress. However, there was still a great deal of variation in cytochrome C expression among colonies that was not consistent across all tolerant and intolerant colonies: intolerant genotypes M7 and U33 had higher expression on average than intolerant genotype M7, which could be driving the differences observed between tolerant and intolerant genotypes.

Other studies have investigated the roles of catalase and cytochrome C in coral thermal stress responses, and have typically found these molecules to be upregulated in samples following a thermal stress (Rodriguez-Lanetty et al. 2009; Polato et al. 2013; Ross et al. 2013; Rosic et al. 2014). However, our results differ in that a short term thermal stress over 12 hours

did not significantly change expression from control samples maintained at ambient temperature; we only observed differences between tolerant and susceptible colonies. This could be due to a number of factors, including differences in sampling time points, differences in the severity of heat stress, or differences between the species examined. Other studies have confirmed variability in the expression of certain biomarkers, including catalase, between different genotypes (Granados-Cifuentes et al. 2013; Polato et al. 2013). However, this appears to be the first study to attempt to link variability in the activity of these biomarkers after a short term thermal stress to survival under a long term thermal stress.

What is interesting to note is that, while some transcripts obtained aligned to an *A. cervicornis* transcriptome (Libro et al. 2013), other significant DEGs were of *Symbiodinium* origin. Furthermore, the three tolerance DEGs of *Symbiodinium* origin showed high similarities in expression among all six coral genotypes, compared to the one uncharacterized DEG of *Acropora* origin. As has been investigated in other studies, the composition and genotype of *Symbiodinium* are likely to play a major role in overall coral holobiont thermal tolerance (Fisher et al. 2012). Furthermore, different combinations of host and symbiont genotypes are likely to have different responses to thermal stress (Abrego et al. 2008). Along the Florida Reef tract, Baums et al. (2010) found that *A. cervicornis* typically harbors clade A *Symbiodinium* except along inshore environments, in which case it harbors mostly clades C and D *Symbiodinium* instead. Since all 20 genotypes were obtained from the same coral nursery and were therefore raised in the same environment, they are likely to harbor a similar composition of *Symbiodinium* at the clade level. However, *Symbiodinium* still vary greatly at finer scales than the cladal level (Rodriguez-Lanetty et al. 2004), which could further influence coral holobiont thermal tolerance, and these finer scale genotypic differences will need to be investigated in more detail.

Our results also showed that upregulation of three specific DEGs after a thermal stress compared to controls of the same genotype tended to be associated with increased thermal tolerance, whereas downregulation of these same DEGs following thermal stress tended to be associated with increased susceptibility. These DEGs were a 40s s6-like ribosomal protein, soma ferritin, and an uncharacterized protein from *A. digitifera*. However, these differences were not consistent across all tolerant and susceptible genotypes: while this pattern was clearly displayed between tolerant genotypes M3 and M5 compared to intolerant genotype U33, tolerant genotype U58 often showed the opposite pattern. This makes it difficult to determine if this is a consistent pattern between tolerant and susceptible coral colonies, or if this is just a pattern among these three genotypes in general.

Ferritin and ribosomal protein processing genes have shown upregulation in other coral species following a thermal stress (Csaszar et al. 2009; Seneca et al. 2010; Polato et al. 2013; Rosic et al. 2014; Rose et al. 2015). Csaszar et al. (2009) showed that while ferritin tended to be upregulated in response to thermal stress, the degree of upregulation was highly variable, and downregulation did occur in two coral colonies. Here, we show that upregulation shortly after a thermal stress is not only variable among genotypes, but also associated with longer lifespans during a long term thermal stress. In regards to ribosomal protein processing, some studies have shown ribosomal proteins being downregulated in corals following heat stress (DeSalvo et al. 2010; Bellantuono et al. 2012; Bay and Palumbi 2015), while other studies have shown the opposite (Rosic et al. 2014; Rose et al. 2015). Our results here agree with Rosic et al. (2014), which found ribosomal proteins to be upregulated following thermal stress, and our results also show a high degree of variation in expression among different genotypes, which could influence the differences observed among studies.

Overall, our gene expression and biomarker concentration results show that there is a high variability in expression among coral genotypes, and this variability is not consistent across tolerance groups. One thing to note is that, while the three most thermal tolerant genotypes had, on average, longer lifespans compared to the other genotypes used in this study, these differences were only significant for tolerant genotype M5. The same is true for the three most susceptible genotypes: while these three genotypes had shorter lifespans on average compared to the other genotypes used in this study, these differences were only significant for intolerant genotypes M7 and U33. The inclusion of genotypes that did not differ significantly in lifespan may have introduced more variability in our gene and protein expression data, making it difficult to discern significant differences between tolerance groups. Therefore, the data presented here may not technically represent opposite ends of the thermal tolerance spectrum in this species.

While Hsp70 has been extensively studied for its role in thermal stress response and has been shown to be upregulated in response to thermal stress in other coral species and *Symbiodinium* (Barshis et al. 2010, 2013; Leggat et al. 2011; Rosic et al. 2011; Polato et al. 2013), our results show a lack of Hsp70 expression at the protein level in both control and heated samples at the 12 hour sampling time point. No Hsp70 transcripts were identified in the 36 samples utilized for transcriptome analysis either (data not shown), which supports our protein expression data. This is supported by other studies which have noted a sharp decline in Hsp70 in coral samples subjected to thermal stress over time (Hayes and King 1995; Rodriguez-Lanetty et al. 2009). Hayes and King (1995) noted that Hsp70 expression dropped in *Montastrea annularis* between 12 and 24 hours of elevated temperature exposure. Rodriguez-Lanetty et al. (2009) also found that Hsp70 expression decreased in coral larvae of the species *A. millepora* as soon as 3 hours post thermal stress. This suggests that 12 hours post thermal stress may be too late of a

sampling time point to observe Hsp70 expression in this species, and further research utilizing multiple sampling time points will be needed in the future.

In summary, while there is a high amount of genetic variation present in thermal tolerance in the nursery population of *A. cervicornis* sampled here, further research is needed to accurately determine the amount of additive genetic variation present in these genotypes and therefore their adaptive potential. Furthermore, while the gene expression results reported here represent a snapshot of the thermal stress response of *A. cervicornis* under a short term sub-lethal stress, it does bring to light general trends of certain known stress biomarkers with thermal tolerance. The gene expression results also highlight the fact that different sets of genes respond differently to thermal stress, with some being constitutively upregulated or downregulated in tolerant samples, and others being upregulated or downregulated in response to temperature, with the direction differing between tolerant and susceptible samples. Therefore, no one hypothesis for gene expression patterns will likely be suitable for explaining the patterns observed in this species, and further research at multiple time points will be necessary to understand how this species responds at the onset of thermal stress, compared to what we observed after 12 hours, and compared to the onset of temperature-induced bleaching.

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Figure 1. Average maximum quantum yield for all *A. cervicornis* fragments maintained in the heated treatment (32 ± 1 °C) over the course of the experiment. The last fragments were removed at 612 hours. Error bars show standard error.

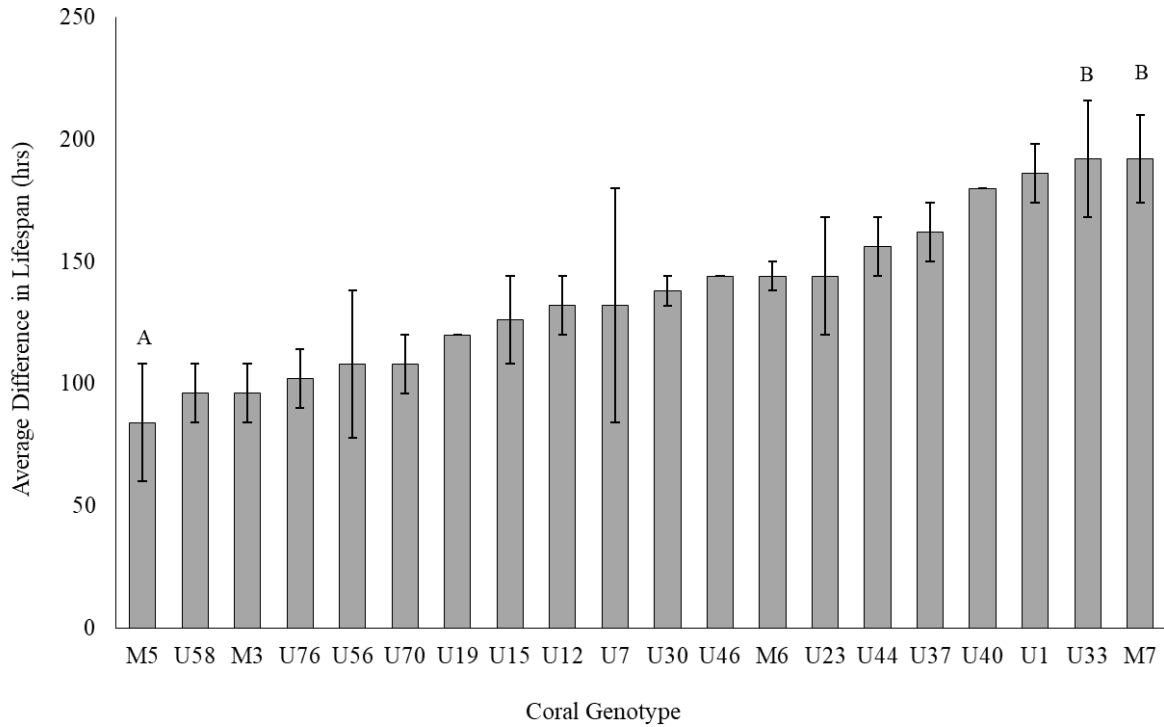


Figure 2. Average difference in lifespan among fragments exposed to elevated temperatures ($32 \pm 1 \text{ }^\circ\text{C}$) and fragments exposed to control temperatures ($28 \pm 1 \text{ }^\circ\text{C}$) over 26 days for 20 *Acropora cervicornis* genotypes. All control fragments survived the duration of the experiment. Significantly different genotypes are indicated by different letters as determined by a Tukey's HSD post hoc test. Error bars show standard error.

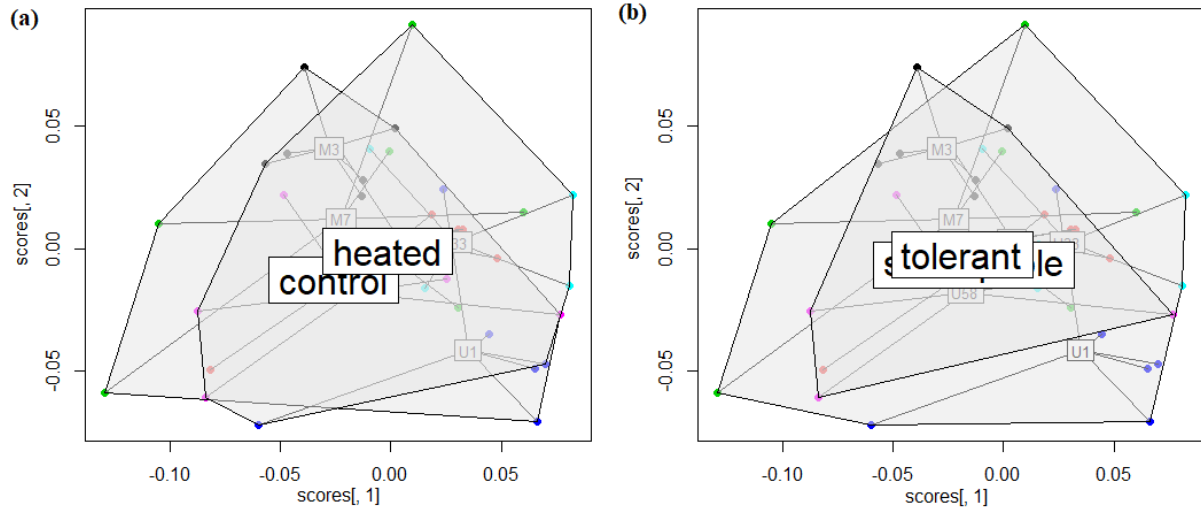


Figure 3. Principal coordinate analysis (PCoA) on the matrix of pairwise Manhattan distances among the 36 samples showing clustering by (a) treatment (control vs heated) and (b) thermal tolerance (tolerant vs susceptible). Results are plotted against principal coordinates 1 and 2. Colored points indicate different genotypes.

Table 1. ANOVA tables on the pairwise Manhattan distance matrices. (a) The first ANOVA investigated the effect of genotype, treatment, and their interaction on overall differences in gene expression among samples. The effect of tolerance, treatment and their interaction was investigated in the following three ways: (a) all samples were included and designated as either tolerant or susceptible; (b) samples that significantly differed in thermal tolerance were designated as tolerant (M5) or susceptible (M7, U33), and all other genotypes were designated as intermediate (M3, U1, and U58); and (c) only the three genotypes that were statistically significantly different in terms of their differences in lifespan were included (M5, M7, and U33)

	df	SS	MS	F	R ²	p
(a) Model: Genotype + Treatment + Genotype × Treatment						
Genotype	5	107060	21412.0	2.2310	0.2522	0.001
Treatment	1	12521	12521.0	1.0377	0.0295	0.182
Genotype × Treatment	5	74592	14918.4	1.5544	0.1757	0.010
Residuals	24	230342	9597.6		0.5426	
Total	35	424515			1.0000	
(b) Model: Tolerance + Treatment + Tolerance × Treatment						
Tolerance	1	16643	16643.3	1.3794	0.0392	0.142
Treatment	1	12521	12521.0	1.0377	0.0295	0.364
Tolerance × Treatment	1	9241	9240.7	0.7659	0.0218	0.694
Residuals	32	386110	12065.9		0.9095	
Total	35	424515			1.0000	
(c) Model: Tolerance + Treatment + Tolerance × Treatment						
Tolerance	2	27182	13591.0	1.1333	0.0640	0.256
Treatment	1	12521	12521.0	1.0441	0.0295	0.330
Tolerance × Treatment	2	25032	12516.0	1.0436	0.0590	0.362
Residuals	30	359780	11993.0		0.8475	
Total	35	424515			1.0000	
(d) Model: Tolerance + Treatment + Tolerance × Treatment						
Tolerance	1	15989	15989.0	1.2915	0.0753	0.177
Treatment	1	6427	6426.7	0.5191	0.0303	0.974
Tolerance × Treatment	1	16504	16503.9	1.3331	0.0778	0.183
Residuals	14	173321	12380.1		0.8166	
Total	17	212240			1.0000	

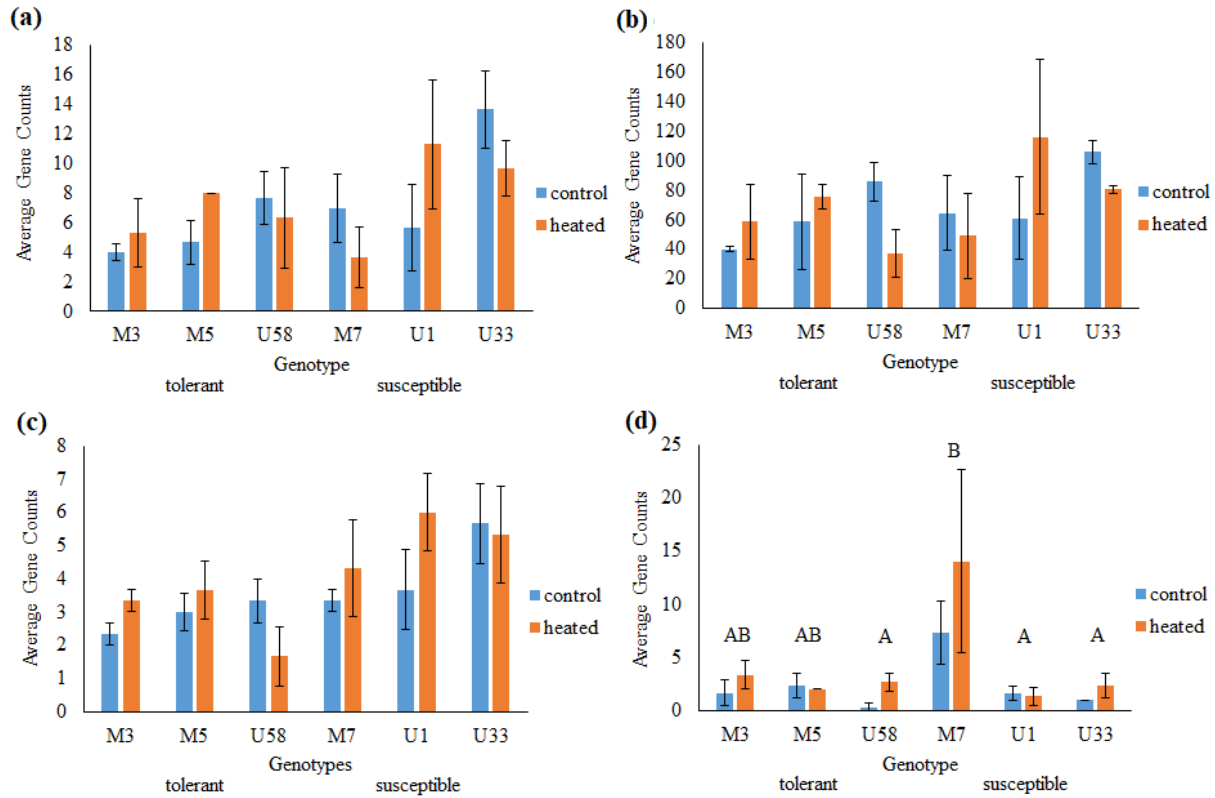


Figure 4. Differences in average gene count for four DEGs identified based on thermal tolerance: (a) an uncharacterized protein from *S. microadriaticum*, (b) peridinin-chlorophyll a-binding protein from *S. microadriaticum*, (c) cytochrome C from *S. microadriaticum*, and (d) an uncharacterized protein from *A. digitifera*. Different letters indicate significant differences according to a Tukey's HSD post hoc test. Error bars show standard error.

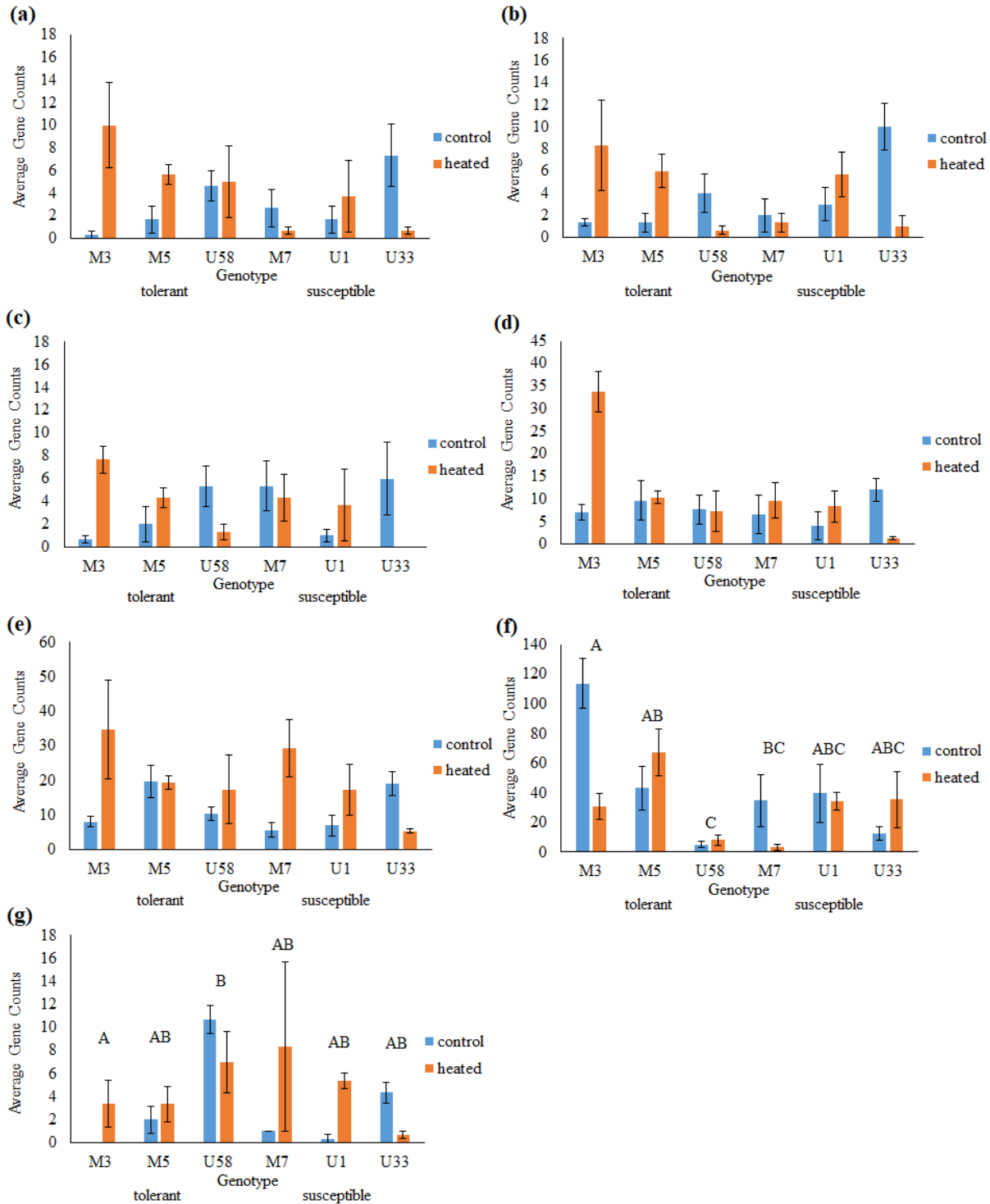


Figure 5. Differences in average gene count for the seven DEGs that were identified as significant due to genotype by treatment interactions: (a) 40s ribosomal protein s6-like, (b) soma ferritin, (c) an uncharacterized protein, (d) DMSP lyase, and (e) a plectin like isoform, all from *A. digitifera*, as well as (f) an uncharacterized protein with ambiguous origin, and (g) isogroup33177, which could not be aligned. Different letters indicate significant differences as determined by a Tukey's HSD post hoc test. Error bars show standard error.

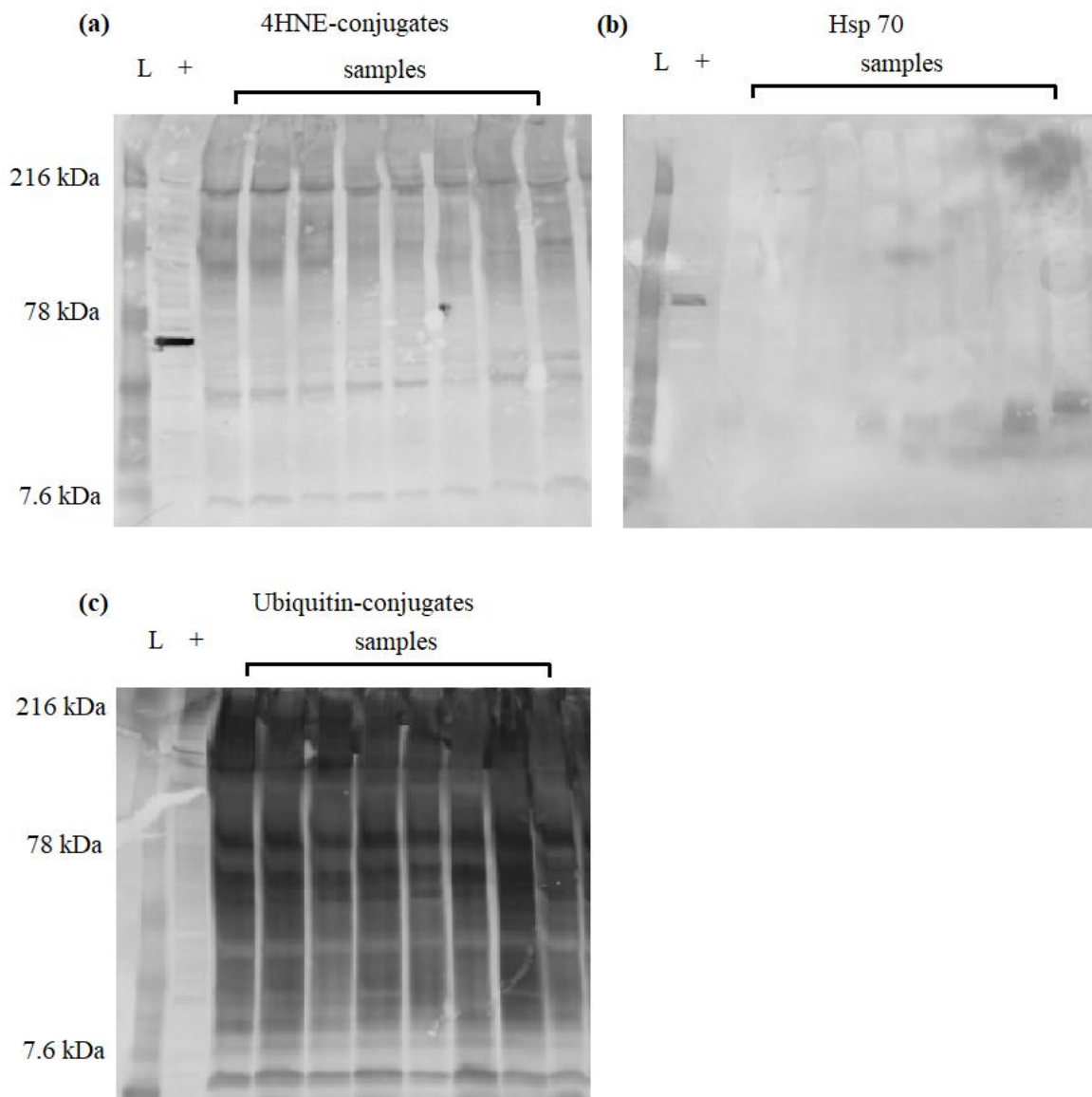


Figure 6. Western blots used for validation of (a) 4-hydroxynonenal-conjugate (4HNE), (b) Hsp 70, and (c) ubiquitin-conjugate antibody specificity against tissue solutions from select control and heated treatment samples of *Acropora cervicornis*. Molecular weights (kDa) are based on the Kaleidoscope standard in lane L. Positive control lanes (+) consisted of 10 μ g of Heat Shocked HeLa Cell Lysate for Hsp 70 and 4HNE-conjugates, and Rat Brain Tissue Extract for ubiquitin-conjugates.

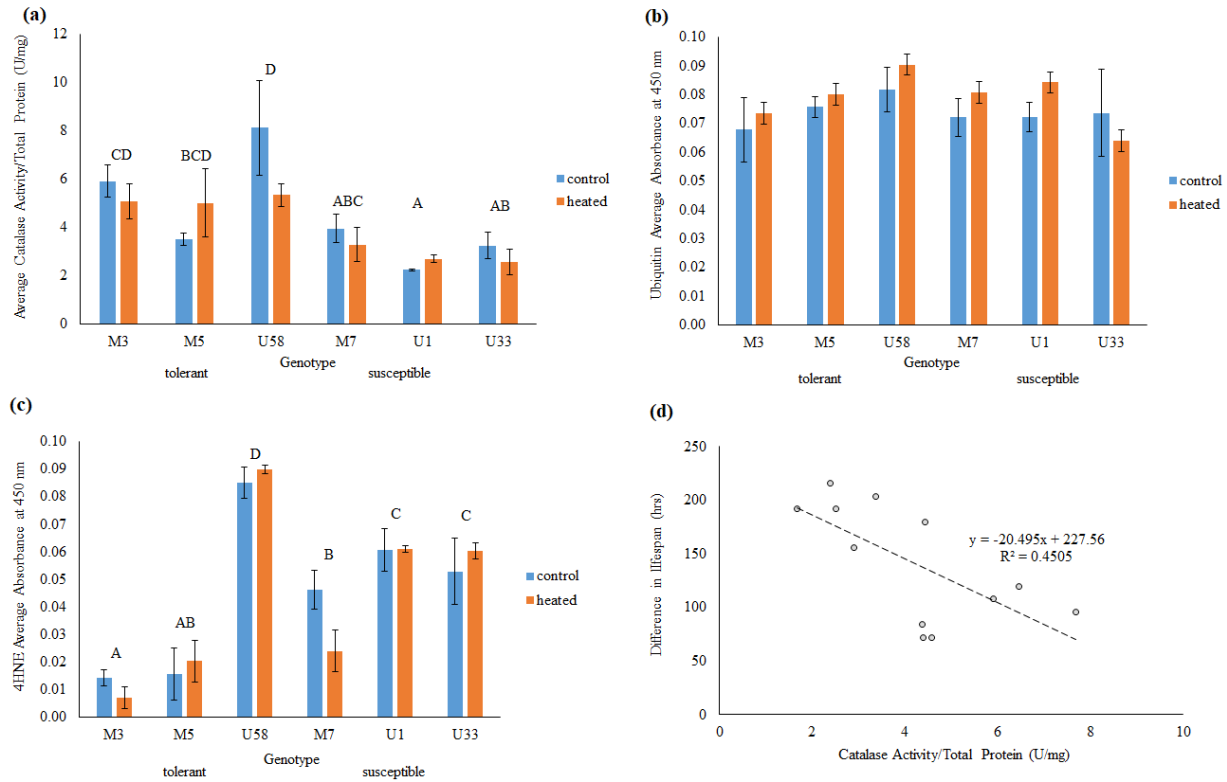


Figure 7. Differences in average (a) catalase activity, (b) ubiquitin absorbance, and (c) 4HNE absorbance between treatments and among coral genotypes for the three most thermal tolerant and three most susceptible genotypes. Different letters indicate significant differences based on Tukey's HSD post hoc test. Error bars show standard error. (d) Model I linear regression showing the relationship of difference in lifespan of the three most tolerant and the three least tolerant genotypes to catalase activity in the heated treatment.

Table 2. Results from correlation analyses between difference in lifespan and biomarker activity or concentration for the following three biomarkers: catalase, ubiquitin, and 4HNE. Each individual fragment was included in correlations for heated fragments only, while the average of biomarker activity or concentration was taken for correlations involving only the control fragments or the biomarker differential. Significant differences are indicated by the bold text and asterisk.

† Pearson correlation

‡ Spearman's Rho

	N	Coefficient	p
Catalase: 3 most tolerant and 3 least tolerant genotypes			
heated treatment x lifespan differential [†]	12	-0.671	0.017*
control treatment x lifespan differential [‡]	6	-0.353	0.492
biomarker differential x lifespan differential [‡]	6	0.265	0.612
Ubiquitin: 3 most tolerant and 3 least tolerant genotypes			
heated treatment x lifespan differential [†]	12	-0.072	0.823
control treatment x lifespan differential [‡]	6	-0.313	0.545
biomarker differential x lifespan differential [‡]	6	-0.265	0.612
4HNE: 3 most tolerant and 3 least tolerant genotypes			
heated treatment x lifespan differential [†]	12	0.131	0.684
control treatment x lifespan differential [‡]	6	0.265	0.621
biomarker differential x lifespan differential [‡]	6	0.706	0.117
Catalase: all 20 genotypes			
heated treatment x lifespan differential [‡]	40	-0.184	0.255
control treatment x lifespan differential [†]	20	-0.186	0.434
biomarker differential x lifespan differential [‡]	20	-0.073	0.759
Ubiquitin: all 20 genotypes			
heated treatment x lifespan differential [‡]	40	-0.072	0.660
control treatment x lifespan differential [‡]	20	-0.111	0.640
biomarker differential x lifespan differential [‡]	20	0.270	0.250
4HNE: all 20 genotypes			
heated treatment x lifespan differential [†]	40	-0.199	0.218
control treatment x lifespan differential [‡]	20	-0.256	0.275
biomarker differential x lifespan differential [‡]	20	0.231	0.328

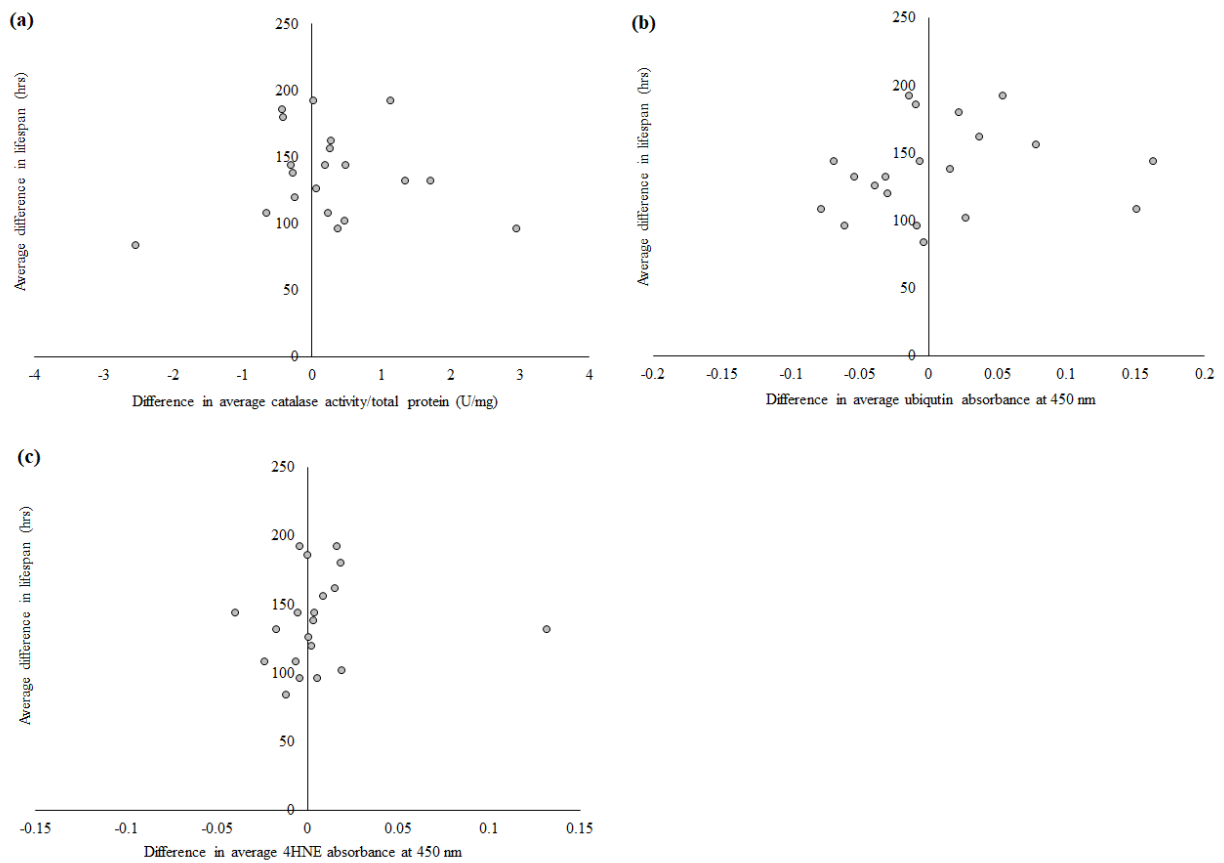


Figure 8. Correlation analyses showing the association of difference in lifespan of all 20 genotypes with difference in relative concentration or activity between heated fragments and control fragments for the following three stress biomarkers: (a) catalase, (b) ubiquitin, and (c) 4HNE.

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K Yetsko, G Sancho. 2015. The effects of salinity on swimming performance of two estuarine fishes, *Fundulus heteroclitus* and *Fundulus majalis*. Journal of Fish Biology, Volume 86, Issue 2, Pages 827-833, DOI: 10.1111/jfb.12590

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