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9A Coral bleaching and climate change

# Thermal stress-related gene expression in corals with different *Symbiodinium* types

**Melissa M Rocker**[1,2], Bette L Willis[1,3], Line K Bay[4]

[1]School of Marine and Tropical Biology, James Cook University, Townsville, QLD 4811 Australia
[2]AIMS@JCU, Australian Institute of Marine Science, James Cook University, Townsville, QLD 4811 Australia
[3]ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville QLD 4811 Australia
[4]Australian Institute of Marine Science, PMB 3, Townsville MC, QLD, 4810 Australia
Corresponding author: melissa.rocker@my.jcu.edu.au

**Abstract.** The endosymbiotic relationship between scleractinian corals and *Symbiodinium* spp underpins the biodiversity and productivity of coral reefs worldwide. The genetic and physiological characteristics of *Symbiodinium* have large effects on coral host physiology and thermal tolerance; however, the degree to which molecular responses to thermal and oxidative stress vary among corals with different symbiont types is still not well understood. We examine gene expression in response to laboratory-based thermal stress in 1 year-old juveniles of *Acropora millepora* hosting different dominant *Symbiodinium* types. We detected significant changes in symbiont dominance through time, with 59.7% of coral juveniles changing their symbiont type during a 12-month growth period in the wild and 22% hosting two types simultaneously. Only three of 50 genes with a putative role in heat and oxidative stress were differentially expressed. Heat Shock Proteins 70 and 90 were expressed at higher levels in juveniles hosting multiple symbiont types during early stages of heat stress, whereas a NOS-interacting gene (a gene regulating nitric oxide production) was up-regulated concurrently with a decline in maximum quantum yield during heat stress. Our results support an important role for symbiont complements in the transcriptomes of corals and highlight high variability among individuals.

**Key words:** gene expression, juvenile corals, thermal stress, oxidative stress, *Acropora millepora.*

## Introduction

The symbiotic relationship between scleractinian corals and *Symbiodinium* spp that underpins the biodiversity and productivity of coral reefs is under threat from local and global stressors, particularly increasing ocean temperatures and acidity (Anthony et al. 2011). The thermal and physiological flexibility of *Symbiodinium* may provide mechanisms for scleractinian corals to adapt and survive in the face of mounting stressors (Berkelmans & van Oppen 2006). Therefore, greater understanding of molecular stress responses in relationships between corals and their symbionts could enhance understanding of the recovery potential of these important ecosystems.

The dominant *Symbiodinium* type hosted by corals can greatly affect their physiology and stress tolerance, but little is known about the underlying molecular mechanisms. *Symbiodinium* type has been shown to affect coral growth rates, which can be increased when corals associate with metabolically efficient symbiont types (i.e. ITS1 type C1; Little et al. 2004, Jones & Berkelmans 2010), possibly through more efficient translocation of photosynthates (Cantin et al. 2009). Conversely, thermal tolerance is often greater when corals associate with ITS1 type D (Mieog et al. 2009a). Associating with multiple *Symbiodinium* types may provide the option of shuffling symbiont types to temporarily increase thermal tolerance (e.g. Berkelmans & van Oppen 2006). Therefore, assessing the physiological effects of *Symbiodinium* complements on coral hosts is essential to further understand the responses of differing symbiont types to bleaching and global climate change.

Analysis of the molecular mechanisms associated with coral bleaching, i.e. the breakdown of endosymbiosis between scleractinian corals and their photosymbionts in response environmental stressors (Coles & Brown 2003), suggests that many heat and oxidative stress-related genes are involved in stress responses. In particular, heat shock proteins (HSPs), green florescent protein (GFP), and other oxidative stress-related proteins (DeSalvo et al. 2008, Voolstra et al. 2009) have been implicated. Changes in the expression of genes typically occur prior to visual physiological damage (Hohmann 2002), which enables earlier assessment of stress. Understanding qualitative differences in responses between short-term stress, which typically yields a down-regulation of gene expression, and long-term stress, which typically results in up-regulation of expression, could provide valuable information on the nature of stress.

In this study, we examine the gene expression response of 1 year-old juveniles of *A. millepora* that had been experimentally inoculated with C1 or D *Symbiodinium* types, grown in a common field location for one year, and then exposed to laboratory-based thermal stress. Our aim was to determine if *Symbiodinium* type affects the expression of genes involved in responses to oxidative and thermal stress. Our findings depict the highly variable nature of individuals, and provide evidence for the important role that environmental factors (i.e. thermal stress) and symbionts play in shaping coral transcriptomes.

## Material and Methods

*Experimental procedures*

*Acropora millepora* juveniles used in this experiment were produced during the coral spawning season in November 2006 at Magnetic Island, Australia (19°10'S, 146°50'E). Briefly, gametes from locally sourced coral colonies were mixed, fertilized, and allowed to develop into planula larvae following methods described in Abrego et al. (2008). When competence was attained, coral larvae were settled onto autoclaved, pre-conditioned terracotta tiles and equal numbers were inoculated with one of two types of freshly isolated *Symbiodinium*. *Symbiodinium* ITS1 type C1 cells were obtained from *A. tenuis* and ITS1 type D from *A. millepora*; donors were collected from Magnetic Island. *Symbiodinium* types of both freshly extracted material and infected coral juveniles were verified using PCR and SSCP (described below). Coral juveniles were grown on the reef flat in Nelly Bay, Magnetic Island for approximately one year. The tiles were collected in December 2007 and transported to Orpheus Island Research Station (18°38'S, 146°30'E), where a thermal stress experiment was conducted (described in Littman et al. 2010). Briefly, tiles with C1 and D inoculated coral juveniles were randomly placed in six aquaria (three ambient control temperature aquaria and three experimental heat stress aquaria) and acclimated to 28°C for three days prior to commencing the experiment. Temperatures in the heat treatment aquaria were raised 0.5°C every six hours until water temperature reached 32°C (target heat stress temperature) one day prior to the official start of the experiment. During the subsequent 14 days of the experiment, C1 and D inoculated juvenile colonies of *A. millepora* were sampled every second day, from both the control and elevated temperature treatments. A diving PAM fluorometer (Walz) was used to measure the health of photosystem II (PSII). Dark-adapted measurements of corals inoculated with *Symbiodinium* C1 or D were taken every second day, for both the ambient and heat stress treatments (n=10/day/treatment).

*Molecular laboratory procedures*

To determine the *Symbiodinium* types in corals, DNA was extracted from a $2mm^2$ portion of juveniles using a modified CTAB extraction protocol. PCR was used to amplify the 18S nuclear ribosomal DNA internal transcribed spacer 1 region (ITS1) and was carried out using primers and conditions described in van Oppen et al. (2001). PCR products were diluted in a formamide buffer, denatured for 5 min at 95°C, and snap-cooled on ice. One µL of each product was electrophoretically separated following Fabricius et al. (2004) using a GelScan 3000. *Symbiodinium* type in our samples was determined by comparison to samples with known ITS1 types. However, this technique is not able to detect cells if they have an abundance of less than 5-10% (Mieog et al. 2009b).

Total RNA was extracted from the remaining samples (approximately 4 $mm^2$) using an RNAqueous® Kit (Ambion) and treated with TURBO DNA-*free*™ DNase (Ambion) to eliminate any residual DNA from the samples. The concentration and purity of RNA was determined in each sample using a Bioanalyzer RNA 6000 Pico Kit (Agilent Technologies). 69 samples had high RNA quality and were diluted to 15.0ng/µL in 10mM Tris-HCL (pH 8.0) for further analysis. Three samples had degraded RNA and were removed from the analysis.

Reverse Transcription qPCR (RT-qPCR) was used to quantify gene expression levels in coral juveniles exposed to thermal stress using the GeXP multiplex technique (Souter et al. 2011). Two assays, each containing 25 target genes with putative roles in oxidative stress, thermal stress and bleaching in corals, 4 control genes and one spike-in control were used to examine the molecular response of corals with different symbiont types to thermal stress (Seneca et al. in prep). Amongst others, genes examined included 7 heat shock protein (HSP) genes, 3 florescent protein genes (FP), 3 nitric oxide synthase (NOS) genes, 3 peroxidase genes, 3 peroxidasin genes, and 3 superoxide dismutase genes (complete gene lists, sequences, and primer concentrations are available from the authors upon request). RNA was reverse transcribed to cDNA then PCR amplified and run on a CEQ™ 8800 capillary sequencer (Beckman Coulter) following Souter et al. (2011). Three technical replicates of each sample were conducted per assay; reverse transcriptase (RT) enzyme and template controls included to identify possible RNA or DNA contamination were always clean. GenomeLab™ Genetic Analysis System software (Beckman Coulter) was used to image electropherograms and estimate gene expression levels. GeNorm was used to identify the three most stable control genes (RPL9, RPS7, and CTG 1913)

and target genes were normalized to their geometric mean (Vandesompele et al. 2002).

*Statistical Analysis*

We identified changes in dominant symbiont types after the heat stress experiment had been completed and juveniles had been sampled. Corals were allocated to treatments and their photo-physiology measured based on inoculated, not retained, type. These changes, therefore, affected our statistical design. We used a two-way analysis of variance (ANOVA) in SPSS V20 to compare quantum yields between temperature and day treatments (but not among symbiont types). Gene expression was measured after retained symbiont type had been determined, however, uneven degrees of freedom across factors from symbiont changes required the pooling of early (day 1 – 3), mid (day 5 – 9) and late (day 11 – 13) sampling times. Similarly, pooling of either *Symbiodinium* types or temperature treatments was required to compare expression levels through time for temperature or symbiont type respectively using two-way ANOVAs. To control type I error rates, we used the B-Y algorithm to correct $\alpha$ of significant gene expression ANOVAs (FDR corrected $\alpha = 0.027$; Benjamini & Yekutieli 2001). Time, temperature, and symbiont type were treated as fixed factors for gene expression analyses. ANOVA assumptions were tested using Levene's tests and Q-Q plots. Heterogeneity in dominant symbiont changes was assessed with a chi-squared goodness-of-fitness test.

## Results

*Aquaria Experiments and Photochemistry*

Oxidative and thermal stress-related gene expression during our heat stress experiment was quantified for 72 juveniles of *A. millepora*. 59.7% of the 72 juvenile corals did not retain the *Symbiodinium* type with which they were inoculated and the shift was unequal between corals hosting C1 and D types ($\chi^2$=7.73, p=0.021, *df*=2). 26.4% changed from C1 to D and 12.5% changed from D to C1, resulting in 40.3% ITS1 type C, 37.5% ITS1 type D, and 22.2% mixed ITS1 type C1 and D.

| **Day * Treatment** | | | | |
|---|---|---|---|---|
| Dark-Adapted Yield | *SS* | *df* | F | p |
| Day | 0.188 | 8 | 4.988 | 0.000* |
| Treatment | 0.007 | 1 | 1.53 | 0.218 |
| Day * Treatment | 0.080 | 8 | 2.127 | 0.036* |
| Error | 0.733 | 156 | | |

Table 1: Two-way, factorial analysis of variance (ANOVA) of dark-adapted ($F_v/F_m$) yields of photosystem II among *Acropora millepora* juveniles across time (days of experiment) and between treatments (28°C and 32°C). * denotes significance at $\alpha = 0.05$.

Patterns in dark-adapted photosynthetic yields differed between temperature treatments through time (Table 1). At 28°C, $F_v/F_m$ ratios were mostly at or above 0.6, indicating healthy PSII function, although a drop occurred after 4 days. In contrast, $F_v/F_m$ consistently declined in the 32°C treatment and was below 0.6 after 6 days (Fig. 1). Visible loss of pigmentation was observed on day 9, with some individuals being completely bleached by day 12.

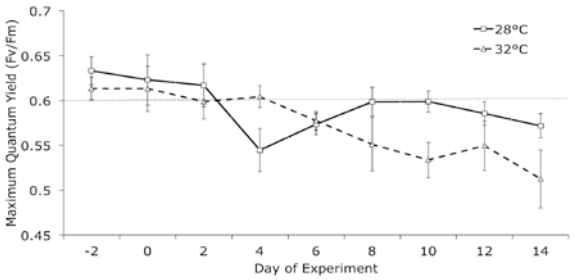


Figure 1: Variation in dark-adapted ($F_v/F_m$) yields of photosystem II among *Acropora millepora* juveniles at 28°C and 32°C. Values are the means +/- SE.

*Variation in Oxidative Stress Gene Expression among Temperatures and Symbiodinium types*

Only three of 50 genes tested were differentially expressed between either temperature treatments or *Symbiodinium* types through time (Table 2; non-significant results not shown). A NOS-interacting gene (GO000012) was differentially expressed between temperature treatments over time before, but not after, B-Y correction (Table 2). Expression was up-regulated in the middle time period in the stress treatment (32°C; Fig. 2) but remained lower in the temperature control treatment (28°C) until the last sampling time, when expression was up-regulated.

| **Time * Treatment** | | | | |
|---|---|---|---|---|
| NOS-interacting | *SS* | *df* | F | P |
| Time | 0.597 | 2 | 0.471 | 0.629 |
| Treatment | 0.086 | 1 | 0.136 | 0.715 |
| Time * Treatment | 4.23 | 2 | 3.333 | 0.048* |
| Error | 21.575 | 34 | | |
| **Time * Symbiont Type** | | | | |
| HSP70 | *SS* | *df* | F | P |
| Time | 1125.681 | 2 | 4.542 | 0.022** |
| Symbiont Type | 1006.365 | 2 | 4.06 | 0.031* |
| Time * Symbiont Type | 1786.474 | 4 | 3.604 | 0.02** |
| Error | 2850.453 | 23 | | |
| HSP90 | *SS* | *df* | F | P |
| Time | 746.042 | 2 | 4.003 | 0.031* |
| Symbiont Type | 735.029 | 2 | 3.943 | 0.032* |
| Time * Symbiont Type | 1352.429 | 4 | 3.628 | 0.018** |
| Error | 2329.884 | 25 | | |

Table 2: Two-way, factorial analysis of variance (ANOVA) of gene expression across pooled time periods and between treatments (28°C and 32°C) for NOS-interacting gene and between symbiont types for HSP70 and HSP90. * denotes significance at $\alpha = 0.05$ and ** at $\alpha = 0.027$.

Two heat shock proteins, HSP70 (GO000475) and HSP90 (GO003195), displayed significant differences between symbiont types (ITS1 type C1, D, and mixed C1/D) across the three time periods of the experiment

(Table 2), but they were not differentially expressed among temperatures. The expression patterns of HSP70 and HSP90 were almost identical and therefore only HSP90 is shown here (Fig. 3). Expression remained low in coral juveniles with "pure" complements (i.e. only one type >10% abundance) of type C1 and D. Conversely, juvenile corals with a mixed complement of type C1 and D symbionts displayed high heat shock protein expression in the early stage of the experiment, with a large drop to "pure" type levels by the late experimental stage (Fig 3).

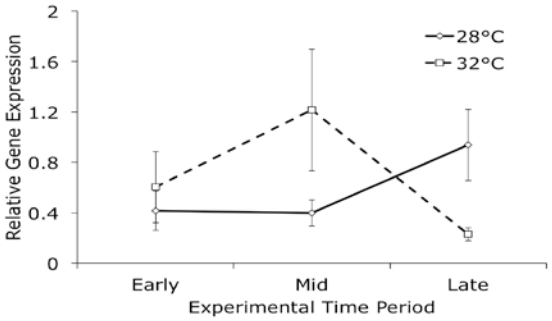


Figure 2: Changes in juvenile *Acropora millepora* gene expression of NOS-interacting gene during a heat stress experiment: experimental treatment (32°C) and control treatment (28°C). Error bars represent +/- SE, n = 3, 7 and 8 for each treatment during early, mid and late times.

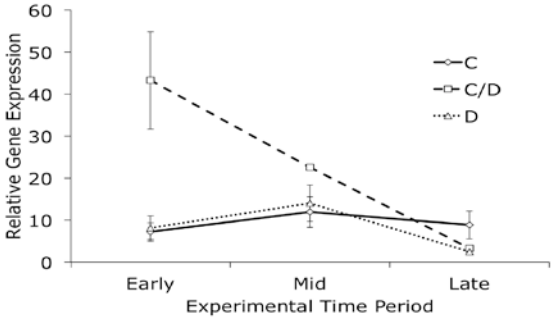


Figure 3: Expression of HSP90 in juvenile *Acropora millepora* hosting different symbiont complements exposed to laboratory heat stress. Error bars represent +/- SE, n ranges between 1 and 8 for each treatment.

## Discussion

### *Symbiont dynamics and effects on coral gene expression*

Dominant symbiont type is a strong predictor of coral holobiont fitness traits, including growth, survival, and thermal tolerance (Mieog et al. 2009a, Csaszar et al. 2010) and is therefore expected to be tuned to local environmental conditions. In our study, 59.7% of juveniles changed their dominant symbiont type, with a greater shift from C1 to D, the type hosted by adults at this location. Abrego et al. (2009) found that *A. millepora* juveniles from Magnetic Island take up type D almost exclusively and retain this type after one year *in situ*. In contrast, we found almost equal numbers of juveniles inoculated with D retained this type or changed to C. In addition, D inoculated juveniles were more than twice as likely to be mixed type compared to C inoculated juveniles. Our study lacks temporal replication and it is possible that differential growth and survival of coral-symbiont associations could have affected our results. This hypothesis is supported by Mieog et al. (2009a), who found that corals hosting *Symbiodinium* C1 had higher survival rates than those with type D at this site.

Gene expression of coral hosts can be influenced by symbiont genotype (e.g. DeSalvo et al. 2010). For example, DeSalvo et al. (2010) found a greater importance of symbiont type than heat stress condition in determining transcriptomic profiles in *Montastraea faveolata*. Our study identified two genes, both HSPs, which were differentially expressed between corals with single or mixed symbiont communities. HSPs are molecular chaperones that stabilize protein structure to enable correct folding and function under homeostasis and stress (Kregel 2002). Corals commonly up-regulate HSPs in response to heat stress (Sharp et al. 1997, DeSalvo et al. 2008). In our study, HSP70 and HSP90 responded similarly and were expressed at higher levels in corals with mixed symbiont communities in the early but not the late sampling period. Our results suggest differences in the need for HSPs under homeostasis or early stress in corals with "pure" vs. mixed symbiont complements, but a similar response to stress between C1 and D types when hosted alone.

### *Effect of heat stress on photophysiology and gene expression*

Coral host transcriptional state, including the response to thermal stress correlates with the photochemical efficiency of their photosymbionts (Warner et al. 2006, DeSalvo et al. 2010). Photochemical efficiency was lower in the stressed condition (32°C) compared to the control (28°C). The significant change in dark-adapted photosynthetic efficiency levels of the stressed juvenile corals occurred mid-experiment (5 – 9 days) and correlated with an increase in the expression of a NOS-interacting gene by juveniles in the 32°C treatment. The NOS-interacting gene regulates nitric oxide production and its up-regulation suggests that corals are experiencing and responding to oxidative stress at 32°C. Despite this, we did not detect differential expression in most of the heat and oxidative stress genes examined, raising the possibility that our experimental treatment of 32°C (and 360 μM photon/$m^2$) was not high enough to thoroughly stress the coral juveniles used. Comparisons of maximum quantum yields from similar experiments corroborate

this conclusion, with our yields 0.25–0.5 units higher than other reports after two weeks (Berkelmans & van Oppen 2006). Our corals were bred from Magnetic Island parents, a relatively warm location with a long-term summer maximum of 30.1°C and inoculated with local *Symbiodinium* genotypes. It is therefore possible that the lower PSII stress detected here was a result of locally warm adapted *Symbiodinium* reducing stress at 32°C (Howells et al. 2012). Alternatively, light levels were too low to cause significant oxidative stress.

*Limitations and Future Research*

Gene expression was highly variable among coral juveniles as has been observed in adult colonies under ambient and stressed conditions (Bay et al. 2009, Souter et al. 2011). However, our assays included three fluorescent protein genes (CFP: AY646070, GFP: AY646067, RFP: AY646073), which showed the expected decline in expression in response to heat stress (Smith-Keune & Dove 2008), but declines were not statistically significant. Future studies aimed at understanding variation in molecular responses to thermal stress among corals hosting different symbiont types should employ high replication across an extended time series.

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