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Early molecular responses of coral larvae to hyperthermal stress

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Abstract

Most of the work on the impact of elevated temperature and light on *Symbiodinium*-invertebrate symbioses have focused primarily on how the photosynthetic (algal) partner is impacted. Understanding how the same stresses affect the invertebrate host, however, is in its infancy. In this study, we re-examined the direct effect of elevated temperatures on the invertebrate host exploring the early transcriptional response of aposymbiotic (without algal symbionts) coral larvae. The temperatures tested in the experimental design were 24 °C (ambient seawater temperature), 28 °C and 31 °C; and the sampling points were 3 and 10 h after temperature exposure. We explored relative changes in transcription using a cDNA microarray constructed for the scleractinian coral, *Acropora millepora*, and containing 18 142 expressed sequence tag (EST) clones/8386 unigenes. Our study identified 29 genes that were significantly up- and down-regulated when *A. millepora* coral larvae were exposed to elevated temperatures. Down-regulation of several key components of DNA/RNA metabolism was detected implying inhibition of general cellular processes. The down-regulation of protein synthesis, however, was not simple and random, which suggested that the stress response was a more complicated adjustment of cellular metabolism. We identified four significant outcomes during the very early hours of the transcriptional response to hyperthermal stress in coral larvae. First, the expression of heat-shock proteins increased rapidly (within 3 h) in response to hyperthermal stress. Second, a fluorescent protein homologue, DsRed-type FP, decreased its expression in response to elevated temperature reinforcing a potential role as a molecular marker for monitoring hyperthermal stress in nature. Third, the down-regulation of a coral mannose-binding C-type lectin under elevated temperature suggests that heat stress might compromise some components of the coral immune defence and therefore might bring about susceptibility to pathogenic diseases. And last, genes involved in protecting cells against oxidative stress showed little response at the early hours to heat stress, supporting the proposal that up-regulation of cnidarian host oxidative stress genes may require reactive oxygen species generated by stressed algal symbionts.

Keywords: climate change, coral larvae, coral stress response, ecological genomics, microarray

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Introduction

Reef-building corals are sensitive to small increases in seawater temperature (>1 °C) above the long-term summer maxima (Brown 1997; Hoegh-Guldberg 1999). These small changes result in the disintegration of the symbiosis between corals and dinoflagellates, severely constraining the energy available to the host. This, in turn, reduces growth and reproduction, and increases the risk of disease and starvation (Brown 1997; Douglas 2003; Bruno *et al.* 2007; Hoegh-Guldberg *et al.* 2007; Muller *et al.* 2008). Projections of sea temperature even under moderate climate change soon will exceed the

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known thermal thresholds for corals, leading to the prediction that coral dominated reef systems will disappear as bleaching and death from thermal stress becomes chronic and annual (Hoegh-Guldberg 1999; Hoegh-Guldberg *et al.* 2007).

Our understanding of the cellular and physiological mechanisms that occur during thermal stress is currently biased towards the algal symbiont, where considerable work has been done (Iglesias-Prieto *et al.* 1992; Lesser 1996; Warner *et al.* 1996, 1999; Jones *et al.* 1998; Hoegh-Guldberg 1999; Hill *et al.* 2004; Tchernov *et al.* 2004; Hill & Ralph 2006, 2008; Venn *et al.* 2008). By comparison, the impacts of thermal stress on any cellular machinery of the host cell are less well described. The use of molecular tools, such as DNA microarrays and other molecular markers (Downs *et al.* 2000; Edge *et al.* 2005; Morgan *et al.* 2005; Perez & Weis 2006; Richier *et al.* 2006, 2008; Dunn *et al.* 2007; Foret *et al.* 2007; DeSalvo *et al.* 2008; Schwarz *et al.* 2008), has provided recently new opportunities to answer some of these fundamental questions associated with thermal stress and its impacts on the coral host. These results are likely to be crucial to our ability to form accurate models of the cellular events that lead to symbiosis dysfunction and coral bleaching (Weis 2008; Weis *et al.* 2008).

One of the earliest steps in the physiological dysfunction that ultimately leads to coral bleaching is disruption of the biochemical steps resulting in light capture, with the subsequent production of reactive oxygen species (ROS; Lesser 2006; Weis 2008). Sudden increases of ROS, such as those that occur during thermal stress-related photosynthetic dysfunction, can overwhelm these antioxidant enzyme systems, leading to a buildup of ROS and damage to cellular components (Lesser 2006; Weis 2008). Eventually, ROS might also diffuse into host tissue where the damage continues leading to bleaching. This hypothesis is supported by recent gene expression studies, where DeSalvo *et al.* (2008) have shown that thermal stress in the symbiotic coral host *Montastrea faveolata* caused an oxidative stress response suggesting a straight effect of ROS on host cell. In addition, they postulated that oxidative stress in thermal stress corals causes a disruption of the host Ca^{2+} homeostasis, which in turn leads to changes in cytoskeletal structures and cell adhesion, and the initiation of host cell death via apoptosis and necrosis. Eventually, algal cells leave the coral tissues, either enclosed in host cells or as free dinoflagellate cells (Gates *et al.* 1992).

Although DeSalvo and colleagues showed indirect evidence of the presence of ROS affecting host cells, it was not clear whether the ROS involved were derived from the host (from dysfunctional mitochondria) or algal symbionts. The understanding of the origin of these ROS is crucial component of any model planning to explain the cellular events that lead to symbiosis breakdown (Weis 2008). Equally, it is important to understand the role of the host in defining the pattern of processes that occur during bleaching. In this regard, the importance of the host cell has been highlighted by a number of authors (e.g. Enriquez *et al.* 2005; Dove *et al.* 2006; Ainsworth *et al.* 2008; Baird *et al.* 2009). There is abundant evidence that indicates host corals might influence the thermal stress response, and thus the bleaching outcome, through a number of physiological mechanisms including production of protective fluorescent pigments and mycosporine-like amino acids, antioxidants and heat-shock proteins (Baird *et al.* 2009).

Describing the individual responses of each partner within algal endosymbiosis is inherently difficult given the intimate association of the two partners. Here, we begin to explore the host response in isolation to the response of the algal symbiont using aposymbiotic (lacking algal symbionts) larvae from the coral *Acropora millepora* from Great Barrier Reef. Using this type of experimental system allows us the opportunity to answer the question as to whether or not temperature can trigger an oxidative response in host cells, or whether it necessarily requires the presence of dysfunctional symbionts. In this study, we report the gene expression response in aposymbiotic coral larvae to elevated temperatures using of cDNA microarrays with a specific focus on genes associated with the oxidative stress pathways within the host tissues.

Methods

Larval collection

Ten colonies of *Acropora millepora* were collected prior to spawning on the southeast of the Heron Island reef flat (Lat. 23º33′ S, Long. 151º54′ E), Great Barrier Reef, Australia in November 2006 and were maintained in tanks with running seawater at the University of Queensland's Heron Island Research Station. The corals spawned on 13 November, and the collected egg and sperm bundles from the 10 colonies were mixed into a 20-L container to allow fertilization to occur. Fertilized eggs were then washed twice in filtered seawater (0.5 μm FSW) and were maintained in the 2-L containers filled with FSW during larval development at densities of 3–5 larvae/mL. All planulae were transferred daily to new containers filled with fresh FSW. The larvae were kept in these containers until the 10th day after fertilization, and no settlement was observed as settlement substrata were not provided.

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Experimental design

Gene expression changes associated with coral larval development is very dynamic, as has been recently documented by Grasso *et al.* (2008). However, around 6–7 days after fertilization, swimming larvae slow down their development until adequate settlement substrata is available, which then induces major metamorphosis changes and lead to the attachment to the substrata and the development of the first polyp. In order to prevent settlement, we did not to provide substrata to the swimming planulae and kept the larvae in the swimming stage up to 10 days after fertilization to minimize major developmental changes. These 10-day-old swimming larvae were used in the experiment and split among three temperature treatments—each treatment contained four 1.5-L beakers with larvae. These beakers represented our sampling unit. Approximately 1500 larvae were placed in each of these beakers. The four beakers in each of the three treatments were then placed in three different 500-L water bath bins set in three distinct temperatures: 24 °C (Control), 28 °C (Moderate temperature) and 31 °C (High Temperature), which represented the temperature treatments. This was done so that the temperature in all the beakers from each water bath could be kept exactly at the same treatment temperature. While the beakers in each treatment were not fully independent as they were heated within the same water bath, the water contained in the beakers was never in contact among them. The ambient temperature on the reef at the time of the experiment was 24 °C. Temperatures were monitored during the experiments in all the treatments using underwater temperature loggers (HOBO PRO V2) set to record the temperature every 20 min. A total of 12 beakers and 18 000 larvae were included in the experimental design. For gene expression analyses, samples of ~500 larvae from each beaker were taken at time 0, 3 and 10 h after the beginning of the experiment. Larvae, in each sampling time, were also collected for respirometry measurements as explained below. The survivorship of coral larvae was also monitored for the duration of the experiment, by examining under the light microscope four subsamples of the larval culture (3 mL each) from each beaker and for every sampling point. The number of live swimming and dead nonswimming larvae were recorded.

Larval respiration rate through the time-course of the experiment

Respiration rates of the planula larvae of *A. millepora* were measured using an OXY-4 respirometer system (PreSens). Thirty larvae were transferred from each beaker to 2-mL precombusted (400 °C, 4 h) glass vials filled with FSW. The sensors were put into the vials without any air bubbles. The oxygen consumptions were measured for 15–40 min just before the experiment started (time 0), and then 3 and 10 h after the experiment began in each temperature treatment. New larvae were used every time for each sampling measurement. Two-way analysis of variance (ANOVA) was used to test for significant differences in oxygen consumption in larvae exposed to different temperature conditions across the sampling points. The normality was checked and the homogeneity of variances was examined using Bartlett's test prior to ANOVA analysis. A Tukey HSD's *post hoc* test was used to examine significant differences.

Use of Microarray technology to follow gene expression

The microarrays used in this experiment consisted of 18 142 spots derived from the same amount of cDNA clones, including 290 spots representing positive and negative controls. These microarrays are the third generation cDNA microarrays designed for *A. millepora* by the groups of coral genomics in both the Australian National University and James Cook University (Foret *et al.* 2007). The selection of clones, methodological approach for the cDNA libraries construction and the fabrication of microarrays are explained in Grasso *et al.* (2008).

Hybridization of arrays

For probe construction, total RNA was extracted from each sample using Trizol (Invitrogen) and RNeasy Kit (QIAGEN). The integrity and quality of total RNA were assessed using a Bioanalyzer (Agilent Technology). Only samples showing intact RNA were used for probe construction. cDNA probe synthesis was performed from 500 ng total RNA using Superscript Reverse Transcripase (Invitrogen) and the Genisphere 3DNA-900 microarray kit according to the manufacturers' instructions. Slides for hybridization were chosen randomly from the batch of high quality printed arrays. The arrays were prehybridized and hybridized with the labelled samples following the manufacture's instructions of Genisphere and using a dynamic hybridization system (MAUI; BioMicro Systems). Following posthybridization washes, slides were scanned using a GenePix® 4200 scanner (Axon Instruments) and image acquisition was performed using the software GenePix® Pro 5. Correction was made on the median data by subtracting the background signal.

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Experimental design and statistical analysis of microarray data

We applied a reference microarray design for the multi-factorial experiment outlined in the study, including two factors: Temperature (three levels: 24, 28 and 31 °C) and Time (two levels: 3 and 10 h). Samples from time 0, which was the time when the pool of the 18 000 larvae were split and placed in the respective beakers and treatments, were used to generate the reference sample for the microarray hybridization experiments. Only three replicates out of the four available per treatment were used in the hybridizations giving a total of 18 microarrays used for the entire experiment (3 replicates × 3 temperature levels × 2 time levels). The microarray experiments have been submitted to the Gene Expression Omnibus Database (NCBI; GSE16351). Ratio-intensity (RI) plots were constructed for each array data to explore whether or not intensity dependence of log ratios, which appears as curvature, was present. The assumption from cDNA microarray data is that most genes are not differentially expressed among treatments, and therefore most points in the RI plots should fall along a horizontal line centred on 0. Because curvatures were detected in a few of the arrays, an rLowess curve fitting transformation (Yang *et al.* 2002) was applied to the data. The transformation was applied to all the arrays to keep consistence in the whole data set as suggested by Cui *et al.* (2003). Quantile normalization was also applied to mean log-intensities in order to make the distributions essentially the same across arrays. To detect differentially expressed genes among treatment through the course time of the experiment, a two-way ANOVA model was fitted to the log-transformed intensity data using the microarray analysis software GeneSpring (Agilent Technology). Factors were considered fixed, and an interaction term was also fitted to the ANOVA model. To correct for type I error derived from multiple testing, the Benjamini and Hochberg method was applied as a false discovery rate (FDR) approach (Wu *et al.* 2003). *K*-mean cluster analysis was used to search for clusters of expression profiles in the data. The clustering analysis was produced from 100 interactions and carried out using the software R/MAANOVA (Churchill's laboratory; Jackson Laboratory).

Validation by real-time quantitative PCR

Specific primers amplifying ~100–200 bp PCR products were designed for the genes chosen for validation of the microarray data as indicated later in the text (Table S1 in Supporting Information). cDNAs from each of the samples from all treatments were synthesized from 1000 ng of total RNA and then diluted in 1:6. Two microlitres of diluted cDNA was used in triplicate 10 μL quantitative PCR reactions with 200 nM primers and Sybr Green (Applied Biosystem) mix for a total of 40 cycles, using an Applied Biosystem thermocycler. The comparative delta CT method corrected for the actual PCR efficiency was used to determine relative quantities of mRNA transcripts from each sample. The PCR efficiency was determined using LinRegPCR (Ramakers *et al.* 2003). For normalization purposes, we used multiple house-keeping genes and calculated a normalization factor from the geometric mean of their expression levels, following the methodology described in Rodriguez-Lanetty *et al.* (2008). To identify house-keeping gene controls, we selected 20 genes from our microarray platform that did not show significant differences among the treatments (*F*-test, corrected *P*-values > 0.05) and had the ratio of expression among the treatment not different to a value of 1. These putative house-keeping genes were then tested for their expression stability using geNorm (Vandesompele *et al.* 2002). The three most stable genes were used to calculate the normalization factor for each of the cDNA samples (Table S1). These three housekeeping genes were *Prefoldin subunit 4* (NCBI Acc. no.: GQ423563), *Calmodulin* (NCBI Acc. no.: GQ423564) and a *lipoma HMGIC fusion partner-like 3* (NCBI Acc. no.: GQ427199). Prefoldin is a family of proteins used in protein folding complexes. It is classified as a heterohexameric molecular chaperone in both archaea and eukarya, including humans, and considered a very important protein to keep the homeostasis process in the generation of nascent proteins (Vainberg *et al.* 1998). The second house-keeping gene, Calmodulin (CaM) is a calcium-binding protein expressed in all eukaryotic cells. It can bind to and regulate a number of different protein targets, thereby affecting many different cellular functions (Stevens 1983). Many of the proteins that CaM binds are unable to bind calcium themselves, and as such use CaM as a calcium sensor and signal transducer (Chin & Means 2000). While DeSalvo *et al.* (2008) found a homologue of this gene to be differentially regulated in intact adult/dinoflagellates symbiosis as function of temperature, our study in aposymbiotic coral larvae showed that CaM did not vary with hyperthermal stress and its expression was very stable among all treatments. This suggests that the differential modulation of the CaM homologue detected in DeSalvo *et al.* (2008) might have been directly affected by the thermally compromised endosymbionts. Lastly, the third housekeeping gene used was the Lipoma HMGIC fusion partner-like 3. We decided to include this gene in our Q-RT-PCR analysis, even knowing that its function is still unclear, because it showed a very stable gene expression among all treat-

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ments. Statistical analysis was conducted using permutation *F*-test (Perm. #: 500) on the normalized data (R Project).

Results

Metabolic response of coral larvae to thermal stress

The respiration rate of *Acropora millepora* planulae increased as the temperature rised (two-way ANOVA, Temperature: $F = 14.782$, $P = 0.0001$; Time: $F = 0.057$, $P = 0.944$) showing significant changes at 31 °C after 3 and 10 h of exposure compared to the control (24 °C) and moderate temperature (28 °C) treatments (Tukey HSD test, $P < 0.007$; Fig. 1). There was not significant interaction between temperature and time within the two-factorial experiment (two-way ANOVA, Time × Temperature: $F = 2.720$, $P = 0.056$). The highest oxygen consumption, 3.49 ± 0.81 nmol O_2/larval/h, was detected at 31 °C after 10 h. We also detected 5% of larval mortality at treatment of 31 °C after 10 h of exposure (no mortality was observed in the other treatments). These results revealed a clear increase in the metabolic response of coral larvae to the elevated temperature indicating a sensing reaction to heat-associated stress condition.

Gene expression as a function of thermal stress

Microarray analyses revealed significant changes of gene expression in 10-day-old *A. millepora* larvae as a response to the increase in seawater temperature. A total of 55 EST clones present in the 18K cDNA coral

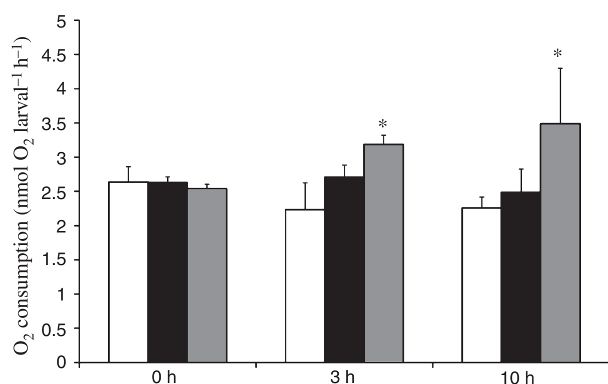

Fig. 1 Respiration (oxygen consumption) of 10-day-old coral larvae of *Acropora millepora* as a function of hyperthermal stress. Temperature treatments were 24 °C (Control; white bar), 28 °C (black bar) and 31 °C (gray bar). Measurements were done at the beginning of the experiment time 0; 3 and 10 h after hyperthermal exposure. Error bars represent the standard error of the mean. Statistical significance is shown above the bars (Tukey HSD test; *$P < 0.01$).

microarray were either up- or down-regulated as a function of temperature ($P < 0.001$, FDR-corrected), including changes driven by both temperature and time. These 55 EST cDNA clones grouped into 29 unique contigs from which seven are currently unknown genes (with no annotations), two are hypothetical/predicted proteins and 20 showed significant homology to other proteins deposited in the NCBI protein database (Table 1). From the factorial two-way ANOVA analysis, we also found that a total of 74 unique contigs (133 EST clones) were differentially expressed uniquely as function of time but not temperature ($P < 0.01$, FDR-corrected) and only one gene showed a statistically significant interaction effect between time and temperature. Considering that Grasso *et al.* (2008) reported 1084 genes that changed gene expression in *A. millepora* larvae as function of developmental stages, the group of genes (74 contigs) from our study that changed uniquely, as function of time was relatively small. This supports the assumption taken in our experimental design that 10-day-old coral larvae slow down their developmental process until recruitment substrata are available to the larvae. The developmental-responding genes not affected by temperature were not followed in this study as the focus and interest were on temperature-responding genes.

Out of the 29 unique genes that responded to temperature, 14 had increased and 15 had decreased the expression levels with the increase in temperature (Table 1). *K*-mean cluster analyses grouped these genes into five clusters based on their similarities in the pattern of gene expression (Fig. 2). Within the genes that showed a higher expression with the increase in temperature, there were three heat-shock protein genes (*hps70*, *hsp90* and Chaperone *gp96*) and one hypothetical protein all displaying the same pattern of gene expression (see *K*-cluster 1, Fig. 2a). Interestingly, the expression of these genes increased with temperature at 28 and 31 °C after 3 h but later the transcriptional responses dropped in both thermal treatments at 10 h. Nevertheless, the transcript levels of *hsp70* and *gp96* remained significantly higher at 31 °C after 10 h compared to the control 24 °C treatment (Tukey HSD test, $P < 0.001$). The expression profile was validated for one gene, *hsp70*, within this cluster, using another gene expression assay Q-RT-PCR (Fig. 3).

K-cluster 2 grouped the largest number of genes, however only five genes are annotated, including *histone H2B*, *B-cell receptor-associated protein 31* (*BCAP31*), *NAPDH oxidoreductase*, *B-cell translocation gene1* (*BTG1*) and *Egln2-prov protein gene* (Fig. 2b). The expression of these genes to elevated temperature increased gradually, and was significantly different from the controls (i.e. 24 °C) after 10 h at 28 °C (Tukey HSD, $P < 0.001$).

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Table 1 Differentially expressed genes within aposymbiotic larvae of the coral *Acropora millepora* as a function of exposure to elevated temperatures. Known (Annotated) unigenes were identified based on significant blast hits (E-values < 10^{-4}) from GenBank (NCBI). The unknown genes are labelled with the prefix NA (no annotated) followed by the representative clone name in parenthesis

K-cluster/ regulation	Gene name	Contig name in 18K cDNA microarray	*E*-value	FDR-corrected *P*-value
1/up	Hypothetical protein (A037-E9)	C_mge-A037-E9-post68-T	3×10^{-19}	0.00896
1/up	Heat-shock protein 90 (D016-C6)	C_mge-A021-G8-post62-T	9×10^{-66}	0.00985
1/up	Heat-shock protein 70 (A017-C4)	C_mge-A017-C4-post26-T	1×10^{-155}	0.00292
1/up	Protein gp96 (A050-C8)	C_D038-G5_39	1×10^{-156}	0.00636
2/up	B-cell receptor-associated protein 31 [BCAP 31] (A043-H6)	C_mge-A043-H6-post47-T	9×10^{-44}	0.00245
2/up	NA (C019-E2)	C_mge-C019-E2-pre12_T3	No hit	0.00215
2/up	NA (D043-E9)	S_D043-E9_69	No hit	0.00011
2/up	NADPH quinone oxidoreductase (A014-E11)	S_mge-A014-E11-post84-T3	3×10^{-7}	0.00220
2/up	Histone H2B (B024-F2)	C_mge-B044-B2-prawn9_T	1×10^{-44}	0.00854
2/up	Hypothetical protein (B016-H8)	C_mge-A044-E12-post92-	2×10^{-66}	0.00095
2/up	B-cell translocation gene 1 (B023-C3)	C_mge-B023-C3-prawn18_	7×10^{-22}	0.00543
2/up	Egln2-prov protein (A016-E3)	S_mge-A016-E3-post20-T3	1×10^{-13}	0.00245
2/up	NA (C012-E7)	C_mge-C012-E7-pre52_T3	No hit	0.00842
2/up	NA (D037-G10)	C_D037-G10_79	No hit	0.00046
3/down	RNA binding protein (D016-D12)	C_D016-D12_92	6×10^{-16}	0.000261
3/down	DsRed-type fluorescent protein (GS01PC04)	C_mge-C006-A7-pre48_T3	3×10^{-57}	0.00704
3/down	RNA binding protein (GS01SG09)	S_GS01SG09.b1.ab1	8×10^{-4}	0.00001
3/down	Ribonucleoprotein A3 (B035-G2)	C_mge-B015-E7-prawn52_	5×10^{-19}	0.00985
4/down	RNA binding protein (B014-A2)	C_mge-B028-A11-prawn80	1×1^{-34}	0.00245
4/down	Hypothetical protein (B023-G6)	S_mge-B023-G6-prawn46_T3	1×1^{-51}	0.0017
4/down	NA (GS01WG04)	S_GS01WG04.b1.ab1	No hit	0.000537
4/down	RNA binding protein (D040-B2)	C_D040-B2_10	4×10^{-14}	0.00113
4/down	RNA binding protein (C002-C8)	C_D016-D12_92	5×10^{-12}	0.00193
5/down	Splicing factor 4 (B042-B11)	C_mge-B042-B11-prawn81	5×10^{-42}	0.00543
5/down	P68 RNA helicase (B013-A8)	C_mge-B013-A8-prawn56_	3×10^{-77}	0.00985
5/down	Splicing factor 4 (B027-E10)	C_mge-B028-B6-prawn41_	4×10^{-40}	0.00985
5/down	NA (A044-G1)	C_GS01QD09.b1.ab1	No hit	0.00207
5/down	NA (GS01WE03)	S_GS01WE03.b1.ab1	No hit	0.00706
5/down	C-Type Lectin [+Mannose-binding site] (GS01UH10)	C_GS01UH10.b1.ab1	2×10^{-25}	0.00015

The expression level at 28 °C following 10 h were equivalent to changes already detected at 31 °C after 3 h for most of the genes from these two clusters.

With regards to the 15 genes that showed lower expression with the increase of thermal stress, 11 genes showed significant sequence homology with other proteins found in the genetic databases (Table 1). Based on the expression profiles, these down-regulated genes were grouped in three different *K*-mean clusters. We detected various genes involved in nucleic acid metabolisms that showed a down-regulation with elevated temperature, such as several RNA binding proteins (Fig. 2c, d), a nuclear ribonucleoprotein A3 (Fig. 2c) and splicing factor genes (Fig. 2e). In *K*-cluster 3, we also detected a down-regulation at 28 °C after 10 h of exposure of a GFP-homologue gene, *DsRed-type FP* (Fig. 2c). The drop at 31 °C after 10 h was even more substantial for this and the other genes present in this cluster—fivefold lower than the control treatment (Tukey HSD, $P < 0.0001$). Moreover, the same down-regulation profile as a function of temperature for *DsRed-type FP* was also confirmed using Q-RT-PCR data (Fig. 3).

There was an interesting pattern of gene expression associated with *K*-cluster 5. This was a gene that encodes a C-type lectin protein containing a mannose-binding site (GS01UH10) (Fig. 2e, Table 1). The expression of this gene dropped significantly at 28 °C after 3 h of exposure (Tukey HSD, $P < 0.001$). The initial increase was short lived, however, with transcript levels returning back to the same levels as the control treatment after 10 h of exposure to 28 °C. At 31 °C, the initial reduction of transcripts after 3 h of exposure to heat stress was significantly lower than those already observed at 28 °C (Tukey HSD, $P = 0.006$), and

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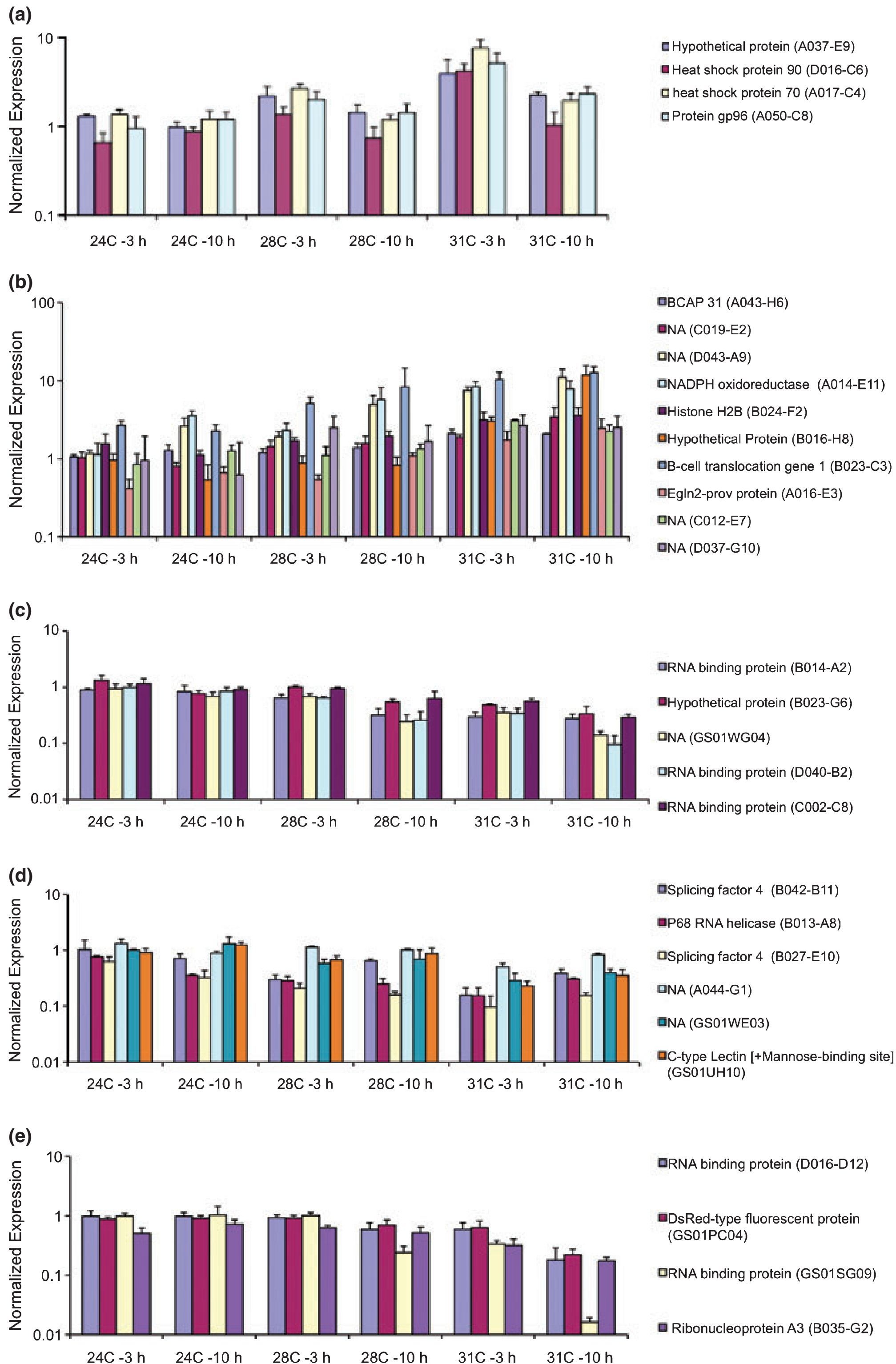

Fig. 2 *K*-mean clusters of the 29 genes differentially expressed a function of temperature within aposymbiotic larvae of the coral *Acropora millepora* based on their profile of expression. The genes that were up-regulated grouped within the *K*-mean cluster 1 (a) and *K*-mean cluster 2 (b). The other three *K*-mean clusters grouped the genes that were down-regulated (c–e). The clustering correlation factor within each group was higher than 90%. Normalized expression represents normalized Log Ratio of Test sample/Reference sample. Error bars represent the standard deviation of the mean. The *X*-axes values represent: 24 °C at 3 h (24 °C-3h), 24 °C at 10 h (24 °C-10h), 28 °C at 3 h (28C-3h), 28 °C at 10 h (28C-10h), 31 °C at 3 h (31C-3h) and 31 °C at 10 h (31C-10h).

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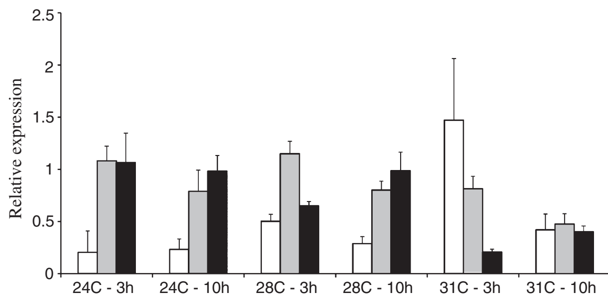

Fig. 3 Relative expression of selected genes within aposymbiotic larvae of the coral *Acropora millepora* using Q-RT-PCR data. The expression levels of *hsp70* (white bar), *DsRed-type FP* (gray bar) and *C-type Lectin* [*+mannose-binding site*] (black bar) are displayed. The Q-RT-PCR data for these genes were normalized using house-keeping genes indicated in Table S1. Error bars represent the standard deviation of the mean.

remained low even after 10 h—threefold lower than the controls (Tukey HSD, $P < 0.0001$). This gene showed a similar pattern of expression when assayed through Q-RT-PCR (Fig. 3). This suggest that the level of this lectin protein at homeostasis was able to recover 7 h after the initial decline caused by the increase in temperature at 28 °C, however, this recovery was not detected during the same period of time at the elevated temperature of 31 °C.

Response of oxidative stress genes in the early hours of thermal stress

There were no significant changes of the expression of oxidative stress response genes within the experimental time frame of this study (Fig. 4, *F*-test, $P > 0.2$, FDR-corrected). However, there seems to be a slight trend of up-regulation of a few oxidative stress genes between 24 and 31 °C at 10 h. The oxidative stress genes examined here, were *Mn superoxide dismutase*, *glutathione-s-transferase*, *thioredoxin peroxidase*, *cytochrome c-like protein*, *catalase*, *mitochondrial cytochrome c oxidase* and *ferritin* (Fig. 4).

Discussion

There are very few studies, if any, that have examined gene expression of host corals in isolation of the responses and influence of their algal symbiont. This was possible in the present study using the aposymbiotic larvae of the reef-building coral, *Acropora millepora*. This has allowed a unique insight into the host compartment as it experiences thermal stress. The elevated temperatures used in the present study (up to 31 °C) have been shown to cause severe thermal stress and bleaching in many coral genera including *Acropora* on the southern Great Barrier Reef (Jones *et al.* 2000).

Using a cDNA microarray constructed for *A. millepora* containing 18 142 EST clones/8386 unigenes (Foret *et al.* 2007), we detected a total of 29 genes that were up- and down-regulated ($P < 0.01$) in response to increase of temperature. The derived amino acid sequences of seven of these genes failed to show significant homology to existing protein sequences already deposited in the NCBI genetic database. This is not surprising, however, given similar and higher proportions of unknown genes within cnidarian taxa have previously been reported from other genomic studies (Kortschak *et al.* 2003; Rodriguez-Lanetty *et al.* 2006; Richier *et al.* 2008; Sunagawa *et al.* 2009). Our study revealed a fairly small number of genes that responded to elevated temperature within 10 h, as compared to other invertebrates exposed to heat stress, such as in *Drosophila melanogaster* (Sørensen *et al.* 2005). The difference in the number of identified genes involved in thermal stress response could be attributed to several factors including the use of distinct thresholds for statistical significance (alpha), the number of unigenes available for screening spotted on the microarray platform and the actual temperature (level of stress) applied to the biological experimental units. For instance, Sørensen *et al.* (2005) showed that 1222 genes out of 13 000 unigenes were differentially regulated when fruit flies were exposed to an increase

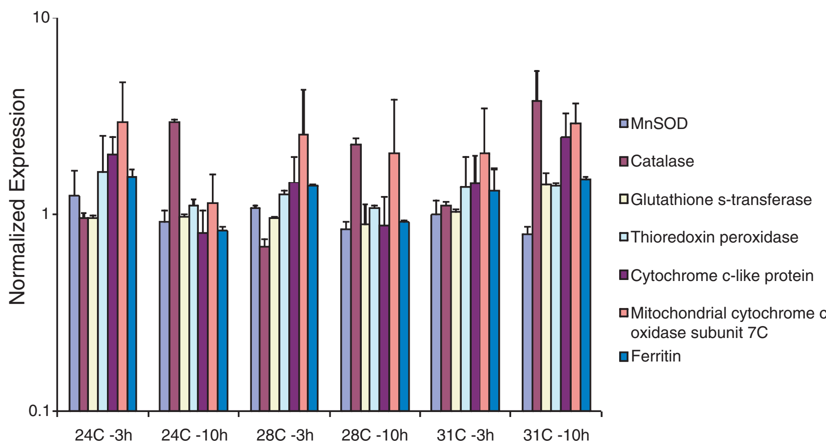

Fig. 4 Normalized expression of oxidative stress genes contained in the 18K *Acropora millepora* microarray. Normalized expression represents normalized Log Ratio of Test sample/Reference sample. Error bars represent the standard deviation of the mean.

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of temperature of 11 °C above ambient temperature, which is a considerably steeper stress profile than the one we carried out in our study. We also used a more conservative significance threshold for detecting change (i.e. $\alpha = 0.01$).

Based on the molecular data presented here, we have shown that the response of coral larvae to thermal stress depends on the intensity and duration of the stressor, as have been documented for Goby fish in previous studies (Buckley & Hofmann 2004). Most of the significant changes occurred in larvae exposed to 31 °C. Interestingly, the response of some genes to moderate temperature stress (28 °C) after 10 h of exposure was similar to changes detected at 31 °C after 3 h of heat exposure, which suggest that similar mechanisms might be in action at higher temperatures but acting more rapidly. Particular groups of genes responded during early stages of heat stress—we explore four major types of these early transcriptional responses to hyperthermal stress by *A. millepora* in the next sections.

Rapid responses by heat-shock proteins to thermal stress

Three heat-shock proteins (*hps70, hsp90* and *gp96*) were up-regulated after 3 h of exposure to 28 °C (4° above the ambient seawater temperature at the time of experiment). The expression of these genes was even higher (threefold change) at 31 °C after the same amount of time. Several studies have already identified and characterized, by using SDS-gel electrophoresis, the response of various members of the heat-shock protein family to elevated temperatures in both corals and sea anemones (Sharp *et al.* 1994, 1997; Black *et al.* 1995; Hayes & King 1995; Fang *et al.* 1997; Tom *et al.* 1999; Choresh *et al.* 2001, 2004, 2007; Hashimoto *et al.* 2004; Downs *et al.* 2005), however, some differences in response were noticed in comparison with our current findings. For instance, while we detected a rapid transcriptional response of heat-shock protein genes from *A. milleopora* larvae exposed to 28 and 31 °C, Black *et al.* (1995) found that coral colonies from *Montastrea faveolota* produced heat-shock proteins only when exposed to temperature higher than 33 °C within a similar time frame of thermal exposure. This absence of expression of heat-shock proteins in temperatures lower than 33 °C observed by Black and collaborator could be attributed to differences in the level of thermal tolerance associated with the ecological background (thermal history) of the species examined between both studies, or perhaps due to a lack of gene expression detection by Black *et al.* (1995). Unlike Black and collaborator' study, our current study measured the levels of gene transcription (mRNA), which provided a more powerful way of detection for gene activation. To be able to detect differential production of proteins through SDS-electrophoresis gels, longer monitoring periods might be required to detect translated proteins from corals exposed to temperatures lower than 33 °C.

More recently, similar transcriptomic responses of 'heat shock protein' genes to our study have also been documented for other scleractinian corals at adult stage when they harbour the algal symbionts (DeSalvo *et al.* 2008). DeSalvo and collaborators reported up-regulation of *hsp90* and TCP-1 chaperonin in *Montastrea faveolata* with the increase of temperature after 24 h of exposure, which was the earliest sampling point in their study. Moreover, DeSalvo and collaborators did not report a differential change of *hsp70* in their study as we did here, although this could have been due to the absence of this gene from their small microarrays (containing 1310 unigenes) as apposed to a lack of up-regulation. Alternatively, it is also possible that the levels of *hsp70* transcripts returned back to baseline levels after 24 h (the time by which DeSalvo and collaborators made their measurements) as enough *hsp70* proteins might have already been produced. This is consistent with the observation that continuous expression of *hsp70* levels leads to detrimental effects, especially in tissue with high rates of cell division and growth. This arises due to the fact that the induction of *hsp70* is accompanied by a redirection of protein synthesis to heat-shock protein synthesis and degradation of aberrant proteins, and away from the normal protein synthesis of other important proteins (van Straalen & Roelofs 2006). This highlights the importance of monitoring early changes in transcription profiles during thermal stress experiment, as also pointed out by Ainsworth *et al.* (2008).

While an EST clone of *TCP-1* chaperonin was present in our *A. millepora* microarray platform, no significant hybridization was detected in any samples, suggesting that the transcripts of this gene were not expressed at least during the first 10 h after the exposure to hyperthermal stress. This gene may respond to elevated temperature after other heat-shock proteins have already been up-regulated after 24 h or more, as was the case in DeSalvo *et al.* (2008).

The results on HSP90 agree the observations of DeSalvo *et al.* (2008) with the up-regulation of hsp90 and a new member of the HSP90 family, *gp96* (an endoplasmic reticulum chaperone). The roles of *gp96* in protein homeostasis, as well as in cell differentiation and development, are beginning to draw more attention due to rapid development in the structural study of the HSP90 family and the genetics of *gp96* (Yang & Li 2005). An important conclusion from the current study is that the up-regulation of the three heat-shock proteins, *hsp70*, *hsp90* and *gp96*, is rapid and direct, and

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occurs independently of the influence of physiologically challenges to the algal endosymbionts that would normally be living within the cells of the host.

Because of the rapid responses of these genes to thermal stress, heat-shock proteins are clearly key components of thermal tolerance and acclimatization in scleractinian corals. Consistent with this, several studies have correlated a survival response to environmental stress when corals express higher level of these proteins (Robbart *et al.* 2004). Differential expression measured by immunoblotting techniques of heat-shock proteins from two Caribbean scleractinian coral species, *Agaricia agaricites* and *Agaricia tenuifolia*, suggested a correlation between the population recovery following a coral bleaching events and the ability to produce certain heat-shock proteins under a thermal stress scenario (Robbart *et al.* 2004). For instance, *A. tenuifolia* has a lesser ability to produce HSPs for protection against thermal stress and showed higher mortality following the 1998 ENSO event than *A. agaricites*, which showed higher rates of survivorship and an increased production of HSPs under thermal stress conditions.

Thermal effect on fluorescent protein homologues

The tissue of reef-building corals contains a large number of colourful and fluorescent proteins (FP), which are green FP ('pocilloporins'; Dove *et al.* 2001). In this study, transcripts of a *DsRed-type FP* were significantly down-regulated as coral larvae were exposed to hyperthermal stress. The rapid decrease in the expression of DsRed-type FP during thermal stress is consistent with previous studies (using differential display screenings) showing a down-regulation of a colourless GFP-like gene within the first 6 h after exposure to 32 °C in adult colonies of *A. millepora* (Smith-Keune & Dove 2008). It is important to note here that based on new amino acid sequence information currently available from coral FP (Alieva *et al.* 2008), the previous colourless green FP (NCBI Acc. no.: AY650288.1) reported and documented by Smith-Keune & Dove (2008) may be a DsRed type of FP (NCBI Acc. no.: AY646073.1; Alieva *et al.* 2008), which is probably the same gene that we have documented in our study as there is 93% identity between these reported sequences. However, further comparative amino acid sequence analyses are required to confirm the identity of these pigments. Here, we do not only confirm the effect of temperature on this gene but also document that the response is very sensitive—increments of only 4° above ambient temperature but still 3° below summer maxima would affect the transcriptional regulation of this FP-homologue gene in <10 h. Furthermore, we showed that the transcriptional response of this *DsRed-type FP*-homologue is a direct response from the host to hyperthermal stress and not to secondary by-products generated by dysfunctional algal symbionts living inside the host cell.

There is still much to learn about the biological functions of FP in corals. Some studies suggest that cnidarian GFP-homologues may play important roles in photoprotection due to their light absorbing properties shading algal symbionts against high-intensity light fields (Takabayashi & Hoegh-Guldberg 1995; Salih *et al.* 2000; Dove *et al.* 2001; Dove 2004). Other roles may also exist such as the masking of the presence of algal pigments within coral tissues from herbivorous fishes that might otherwise target them (Matz *et al.* 2006). It has only recently been shown that Pocilloporins appear to be sensitive to thermal stress, with at least three studies reporting the down-regulation of coral FP proteins during heat stress (Dove *et al.* 2006; DeSalvo *et al.* 2008; Smith-Keune & Dove 2008). Smith and Dove suggested that reduced transcription of GFP-homologues during thermal stress may be a regulatory mechanism enacted by host corals to prevent wasteful allocation of resources (amino acids) to the production of proteins that may be unable to functionally mature under higher temperatures. This hypothesis is supported by the fact that the ability of immature wildtype GFP to fold correctly has been shown to decrease with increasing temperatures (Lim *et al.* 1995) and it may partly explain this down-regulating trend. This possible explanation is consistent with studies that have shown how heat-shock proteins affect the RNA splicing process during thermal stress with the extrusion of introns sequences from the primary transcripts. This occurs in the spliceosomes, which consequently would reduce the transcription process (Yost & Lindquist 1991). Obviously this process would not affect the transcription process of heat shock genes as many of them lack any introns and so can circumvent the splicing block evoked in the first place by them (van Straalen & Roelofs 2006). This could be one plausible explanation for the down-regulation of the *DsRed-type FP* gene observed just after the up-regulation of heat-shock proteins. What it is still intriguing is that there is not simple down-regulation of overall protein synthesis, as the potential inhibition of the spliceosome does not affect all genes at the same levels as many genes that were not up-regulated were neither down-regulated in our study. This suggests that the response to stress is a more complicated adjustment to the metabolic needs of the cell.

Irrespective of the cause, which may be driving the down-regulation of some FP homologue genes, it may be of interest to monitor the expression levels of these FP genes during anomalous thermal episodes in coral reefs. By comparing these expression levels with baseline transcriptional profiles under normal temperature

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conditions, these genes could become stress markers or early indicators of bleaching. This approach could be particularly useful during anomalous hot spots events. There is a need for further investigation using other coral species before these FP genes can be established as potential stress markers.

Down-regulation of a coral mannose-binding lectin and a potential increase in susceptibility to diseases

The gene (GS01UH10; NCBI Acc. no.: EU863781.1) encodes for a protein receptor containing C-type lectin-like domains along with a conserved EPN (mannose-binding) motif site. Both the microarray and Q-RT-PCR results were congruent showing a down-regulation of this gene as a function of heat stress. However, the significant decrease in gene expression from the Q-RT-PCR data was only observed at 31 °C after 3 and 10 h of exposure. While the underlying mechanism for this down-regulation is not clear, we think it may be related to an inhibition of the RNA process that was explained above. The C-type mannose-binding lectin showed a 41% identity in the amino acid sequence level to a variable mannose-binding lectin (NCBI Acc. no.: EU717897.1), which was called millectin (Kvennefors *et al.* 2008). Careful study of millectin has revealed that it has a strong affinity for pathogen and symbionts, and may represent a crucial part of the recognition system, and hence immune defences of corals (Kvennefors *et al.* 2008). Similar to millectin, GS01UH10 is a C-type lectin and has a conserved mannose-binding site (EPN) suggesting that it may recognize various mannose-like carbohydrate structures on nonself cells, such a pathogens, acting in this way as pattern recognition receptor. The down-regulation of GS01UH10 in response to elevated temperature might logically compromise to certain degree the immune defence and increase the susceptibility of the coral to pathogens. However, before this hypothesis can be proved further studies need to look at larger number coral immune genes and determine whether their expression is also affected by thermal stress. If this turns to be the case, then it may provide a molecular basis for the linkage between temperature stress and the frequency of coral disease (Jones *et al.* 2004; Bruno *et al.* 2007; Muller *et al.* 2008). This would imply that the increase of coral susceptibility to disease observed during anomalous rising in seawater temperature may not be an immediate consequence of the symbiotic dinoflagellates loss from the coral host, but a direct and detrimental effect of high temperature on the innate immune system of the coral cell, where immune genes are down-regulated in the coral cells, reducing the coral ability to fight pathogens. The molecular data presented here provides partial evidence supporting the hypothesis stating that coral diseases are opportunistic infections secondary to exposure of thermal–physiological stress that result in reduced host resistance/immunity (Lesser *et al.* 2007).

Response of oxidative stress genes in the early hours under thermal stress

The expression profiles of seven major oxidative stress genes, including *Mn superoxide dismutase*, *glutathione-s-transferase*, *thioredoxin peroxidase*, *cytochrome c-like protein*, *mitochondrial cytochrome c oxidase*, *catalase* and *ferritin*, were also monitored during the early hours of hyperthermal stress, as these genes were known to be present on the microarray platform. Surprisingly, expression of these oxidative stress host genes within the aposymbiotic larvae showed no relationship to thermal stress. Despite the rapid response of other genetic components such as heat-shock proteins, there was no comparable response from the proteins that ultimately deal with ROS during thermal stress.

Previous work has supported a large literature that has shown a response of cellular machinery to ROS produced as a result of photosynthetic dysfunction arising due to thermal stress. The up-regulation of the expression of a number of antioxidant genes, including catalase (Griffin *et al.* 2006; Merle *et al.* 2007), tripeptide glutathione (Sunagawa *et al.* 2008), glutathione-s-transferase (DeSalvo *et al.* 2008) and ferritin (Richier *et al.* 2008) increases during thermal stress. As outlined in the introduction, dramatic increases in ROS can eventually overwhelm defences and trigger apoptosis and cell death in the host, leading ultimately to coral bleaching (Lesser 2006; Weis 2008). Recently, Weis *et al.* (2008) has posed the question on whether the presence and generation of large amount of ROS linked to coral bleaching is produced and derived by the algal symbiont and/or by the host as a response to elevated temperature and light.

Our results suggest that the up-regulation of host oxidative stress genes reported during thermal stress in algal/host symbiosis (Merle *et al.* 2007; Sunagawa *et al.* 2008) may not occur unless directly linked to ROS generated by thermally stressed algal symbionts. Symbiont-generated ROS accumulate rapidly and the antioxidant defence system of the algal symbiont becomes overwhelmed to the point that it cannot detoxify the ROS (Franklin *et al.* 2004). These ROS eventually diffuse into the host tissue triggering a transcriptional oxidative stress response in the host cell. When the amount of ROS is considerably large then the damage continues ultimately leading to coral bleaching, the loss algal symbionts (Weis *et al.* 2008). Host oxidative stress genes might not be suitable bioindicators to monitor the early coral response to hyperthermal stress, as they do not

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seem to respond quickly to the initial increase in seawater temperature unlike heat-shock proteins, which have a rapid response to thermal stress. Moreover, coral host oxidative stress genes may respond when it is already too late as large amounts of ROS generated by the physiologically compromised algal endosymbionts have diffused into the host tissue overwhelming the antioxidant response of host cells. Nevertheless, further studies are required to confirm and evaluate the potential of oxidative stress genes/proteins to monitor the natural thermal response of scleractinian corals.

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M.R.L. uses functional genomics approaches to understand how coral/dinoflagellate symbioses are regulated and what are the adaptive and acclimatization potentials of these symbiotic associations to climate change. S.H. studies the reproductive ecology of scleractinian corals and the dynamics of coral/dinoflagellates in the onset of symbiosis. O.H.G. uses molecular, physiological and ecological approaches to understand how corals respond to climate change.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Table S1 Forward and reverse primers used to amplify the following genes, including house-keeping genes, in Q-RT-PCR assays

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