

Gene expression profiles during short-term heat stress in the red sea coral *Stylophora pistillata*

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Abstract

During the past several decades, corals worldwide have been affected by severe bleaching events leading to widespread coral mortality triggered by global warming. The symbiotic Red Sea coral *Stylophora pistillata* from the Gulf of Eilat is considered an opportunistic ‘r’ strategist. It can thrive in relatively unstable environments and is considered a stress-tolerant species. Here, we used a *S. pistillata* custom microarray to examine gene expression patterns and cellular pathways during short-term (13-day) heat stress. The results allowed us to identify a two-step reaction to heat stress, which intensified significantly as the temperature was raised to a 32 °C threshold, beyond which, coping strategies failed at 34 °C. We identified potential ‘early warning genes’ and ‘severe heat-related genes’. Our findings suggest that during short-term heat stress, *S. pistillata* may divert cellular energy into mechanisms such as the ER-unfolded protein response (UPR) and ER-associated degradation (ERAD) at the expense of growth and biomineralization processes in an effort to survive and subsequently recover from the stress. We suggest a mechanistic theory for the heat stress responses that may explain the success of some species which can thrive under a wider range of temperatures relative to others.

Keywords: coral, gene expression, microarray, stress response

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Introduction

Coral reefs are among the most spectacular and diverse marine ecosystems on the planet and contain hundreds and thousands of species, many of which remain enigmatic to science (Moberg & Folke, 1999). Over the past several decades, corals throughout the world have been affected by global warming. Since the onset of the Industrial Revolution and the increased burning of fossil fuels, atmospheric carbon dioxide levels have increased from approximately 280 to 390 ppm, and projections suggest an additional increase of up to 800 ppm by the year 2100 [Intergovernmental Panel on Climate Change (IPCC), 2007]. Correspondingly, the sea surface temperature (SST) has risen by approximately 0.4 °C since the 1950s (Levitus *et al.*, 2009). Frequent episodes of higher SST induce severe coral bleaching events, which typically lead to coral death (Brown, 1997; Hoegh-Guldberg, 2010). Mass coral bleaching events have increased in frequency, intensity and geographical extent over the last three decades, affecting up to 100% of coral cover across reefs, regions and countries (Hoegh-Guldberg, 2010).

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Coral bleaching is associated with the mass expulsion of unicellular photosynthetic dinoflagellate symbionts (Brown, 1997), the suppression of pigment synthesis or both (Weis *et al.*, 2008). Previous studies have established a link between coral bleaching and episodes of exposure to environmental stresses, which are mainly associated with elevated water temperatures (Coles & Brown, 2003). Bleaching threshold temperatures and mortality vary worldwide and are dependent on geographic location and the mean annual and monthly sea surface temperature conditions (Steen & Muscatine, 1987).

The development of complementary DNA (cDNA) microarrays for non-model organisms has enabled major advances in coral genomics and bioinformatics research, enabling large scale studies of expression patterns for thousands of genes simultaneously. RNA microarrays have been used to study the transcriptomic response to heat stress in the corals *Montastraea faveolata* (DeSalvo *et al.*, 2008, 2010), *Acropora palmata* (DeSalvo *et al.*, 2010), *Porites astreoides* (Kenkel *et al.*, 2013), the larvae of *Montastraea faveolata* (Polato *et al.*, 2010), *Acropora millepora* (Rodriguez-Lanetty *et al.*, 2009) and *Acropora palmata* (Portune *et al.*, 2010). Other coral studies have utilized this technique to detect responses to darkness (Desalvo *et al.*, 2012), ultraviolet radiation

(Aranda *et al.*, 2011), and to study coral-algal symbiosis (Voolstra *et al.*, 2009) and phenotypic plasticity (Bay *et al.*, 2009). Several of these stress studies have revealed cellular processes that are active in corals during heat stress, including cytoskeleton reorganization, calcification, Ca²⁺ homeostasis, cell death, metabolic modifications and antioxidant and chaperone protein synthesis. However, to date, there is no scientific consensus on the cellular mechanisms underlying bleaching and no distinct pathway has been described (Reaser *et al.*, 2000).

Stylophora pistillata (Esper, 1797) is a scleractinian hermatypic coral commonly occurring in Indo-Pacific coral reefs. Red Sea *S. pistillata* is an important frame builder in the Gulf of Eilat coral reefs due to its wide abundance that spans from the physically controlled reef flats to environmentally predicted deep reefs. In contrast to the majority of Eilat's scleractinian corals, *S. pistillata* has a wide range of life history characteristics indicating it is an opportunistic 'r' strategist. It is a pioneer species, colonizing new environments and unpredictable habitats, it develops rapidly, has a large population turnover, reproduces early, has a small body size, short life span, density-independent mortality (often catastrophic) and poor competitive ability (Loya, 1976). Thus, Eilat's Red Sea *S. pistillata* is known as a stress-tolerant species that thrives in diverse and dynamic environments (Loya, 1976). Consequently, these traits allow for easy maintenance of *S. pistillata* in aquaria and highlight its potential as a suitable model organism (Weis, 2008), particularly when studying aspects of global change. *S. pistillata* has been studied for various research topics, including phenotypic plasticity (Shaish *et al.*, 2007), molecular and cellular biology (Weis, 2008), calcification (Gattuso *et al.*, 1999), photosynthesis (Yamashiro, 1995; Gattuso *et al.*, 1999), physiology (Rinkevich & Loya, 1985) and coral-algae symbiosis (Davy *et al.*, 2012).

Previous studies of Red Sea *S. pistillata* have shown that this species is relatively resistant to stress conditions (Loya, 1976) and that its thermal threshold is approximately 34 °C (Shaish *et al.*, 2007). Therefore, in the present research, we focused on the cellular heat stress pathways activated by exposure to a temperature of 34 °C for 13 days. We present, for the first time, a broad synchronous expression of genes of known cellular functions, namely, the endoplasmic reticulum (ER) unfolded protein response (UPR), ER-associated degradation (ERAD) and ubiquitin-mediated proteolysis. Transcriptome data were analyzed using various bioinformatics tools, and protein interaction networks were constructed based on the up- and down-regulated genes. Additionally, we identified potential 'early warning genes,' i.e. those expressed at temperatures up

to 32 °C, as well as a second group of 'severe heat-related genes' that are expressed at temperatures above 32 °C. These genes can serve as a tool for predicting bleaching events and monitoring subsequent reef recovery worldwide.

Materials and methods

Coral sampling and experimental design

During May 2012, a colony of the scleractinian *Stylophora pistillata* was collected by SCUBA diving at a depth of 10 m in the Gulf of Eilat (Red Sea). The top of the colony was split into 42 fragments approximately 5 cm in size. By fragmenting a single colony, we established duplicate 'micro-colonies,' eliminating unwanted sources of biological variability that are associated with corals derived from colonies of different sizes, shapes and thermal/light life histories (Tambutté *et al.*, 1995; Brown *et al.*, 2002).

Following a 1 month acclimation period, the fragments were placed into two aquariums; the control aquarium was maintained at 24 °C, and the experimental aquarium was subjected to an increase of 1 °C per day from 24 to 34 °C. Three fragments were sampled at the same time of day from both the control and the heat treatment aquariums at time points corresponding to 28, 32 and 34 °C, i.e. days 5, 10 and 13, respectively (sampling was conducted 24 h after temperature elevation) (see Figure S1). Data S1.

PAM

An imaging pulse amplitude modulation (IPAM) fluorometer (Heinz Waltz GmbH, Germany) was used to evaluate the photosynthetic efficiency of photosystem II in the algal symbionts. The fluorescence of three *S. pistillata* fragments was measured following 30 min of darkness-acclimation at each sampling point – 28, 32 and 34 °C – corresponding to days 0, 5, 10 and 13. The effective quantum yield (Fv/Fm) was calculated for each sample by determining the dark-level fluorescence yield (F0) and the maximum fluorescence yield (Fm) when all PSII reaction centers were photochemically reduced [Fv/Fm = (Fm–F0)/Fm]. The effective quantum yield helped in monitoring the photosynthetic performance during the experiment, which is a putative indicator of thermal stress (Fitt *et al.*, 2001; Ainsworth *et al.*, 2008).

RNA extraction and microarray hybridization

Total RNA was extracted from each fragment using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the methods presented in (Levy *et al.*, 2007, 2011), and the samples were further purified using an RNA Clean and Concentrator kit (Zymo Research Corp., Irvine, CA, USA). Total RNA was labeled and hybridized against a custom *S. pistillata* microarray. An Agilent two-color gene expression microarray platform with 8 × 15 K probes per slide was used. Oligonucleotide probes (60-mers) were designed based on the

approximately 12 000 genes that are predicted to encode proteins retrieved from a recent *de novo* assembly of 454-sequenced EST libraries of *S. pistillata* (Karako-Lampert *et al.*, 2014). Labeling and hybridization were conducted using the Agilent Low Input Quick Amp Labeling Kit according to the manufacturer's instructions. The intensity of the emitted fluorescence from a target spot on the array was detected using an Agilent G2565BA microarray scanner. The raw data as well as the processed data of the microarray were deposited under accession number GSE47779 (NCBI GEO, please see; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=dxmjvgeyeogkabq&acc=GSE47779>). *Stylophora pistillata* EST data are also stored at the Cnidarian Database of Centre Scientifique de Monaco: <http://data.centrescientifique.mc/CSMdata-home.html> (Data S1).

Microarray data analysis

The data from all arrays were first subjected to background correction and LOESS within-array normalization using Agilent Feature Extraction software (version 9.5.1.1 Agilent Technologies, Santa Clara, CA, USA). The remaining analyses were performed in Partek® Genomics Suite software (version 6.6, Copyright©2012, Partek Inc., St. Louis, MO, USA). Data from three biological replicates and two to four technical replicates were used to perform a one-way ANOVA. The normalized data were analyzed to identify genes with significantly up- or down-regulated expression [FDR (false discovery rate) $P < 0.05$] with an arbitrary cutoff of at least a two fold change. We generated networks of highly interconnected proteins using the STRING (Search Tool for the Retrieval of Interacting Genes, Heidelberg, Germany) 9.0 database (Szklarczyk *et al.*, 2011). The clustering of protein interactions with high confidence scores (at least 0.7) was examined and subsequently exported to Cytoscape software 2.8 (Smoot *et al.*, 2011) for graphical editing. We focused on main stress-relevant clustering protein groups that consist of multi-interactions connections, as provided by the STRING database. The Gene Ontology (GO) cellular component and biological processes associated with the up- and down-regulated proteins were retrieved from the STRING database and compared between the controls and heat stress treatments. To validate the microarray results, quantitative real-time polymerase chain reaction (qRT-qPCR) assays were performed for four selected genes: two up-regulated and two down-regulated genes. Complementary DNAs were synthesized from the total RNA and the selected genes were amplified using specific qRT-PCR primers. The comparative delta CT method was used to calculate fold changes. Data S1.

Results

Stylophora pistillata fragments were subjected to heat stress using a gradual increase in temperature from 24 to 34 °C over a period of 13 days. Fragments were sampled from both the treatment and control aquariums (the latter was kept at 24 °C) when the temperature in the treatment aquarium reached 28, 32 and 34 °C; the

corresponding samples were referred to as t1, t2 and t3 and c1, c2 and c3. Using Partek Genomics Suite™ software, we created a principle component analysis (PCA) that displays the variance of the samples and captures 65.3% of the total variability for different temperatures and replicates. The PCA revealed that replicate samples were grouped into clusters. All control samples that were sampled throughout the experiment, were grouped together close to t1, whereas the t2 and t3 replicates were grouped separately from the controls as well as from each other (Figure S2).

Each treatment sample was normalized to its control data set, and the resulting RNA expression pattern data were analyzed. Using Partek Genomics Suite™ software, we constructed a Venn diagram (Figure S3) of all differentially expressed genes (both up- and down-regulated genes with an FDR $P < 0.05$) to highlight common genes shared among the three treatment time points. The t1 (28 °C) vs. c1 and t2 (32 °C) vs. c2 comparisons produced lower numbers of differentially expressed genes (963 and 805, respectively) than did the t3 (34 °C) vs. c3 comparison (one-way ANOVA FDR $P < 0.05$). The transcriptome reaction at the third time point was more intense and included a total of 2229 up- (1127 genes) or down- (1102 genes) regulated genes. Moreover, the 34 °C time point yielded 1481 differentially expressed genes that were neither up- nor down-regulated in the previous treatments. A total of 163 genes were either up- or down-regulated at all three sampled temperatures – common genes (Table S1). These results are similar to those acquired from a preliminary experiment that was conducted on short-term heat-stressed *S. pistillata*, when temperatures were elevated from 24 °C to a maximum of 32 °C. The 28 °C preliminary treatment did not yield a significant heat stress reaction, and the reaction to the 32 °C treatment was mild. Two up-regulated and two down-regulated candidate genes were evaluated for the validation of the microarray results using quantitative real-time polymerase chain reaction (qPCR) assays. The calculated fold changes were found to be in the same direction as and consistent with those in the microarray data, thus confirming the microarray results (as described in the supporting information (Figure S4)).

The visual color intensity of the heat-stressed coral fragments appeared to fade as the experiment was prolonged, and bleaching was maximized at 34 °C. The photosynthetic efficiency measured using IPAM showed that the Fv/Fm values for the control fragments remained constant throughout the experiment, whereas the heat-stressed fragments showed a significant decrease in the Fv/Fm values at 34 °C (Fig. 1a and b). Results from the Fv/Fm data were subjected to *post hoc* Tukey's test with significance for $P < 0.01$.

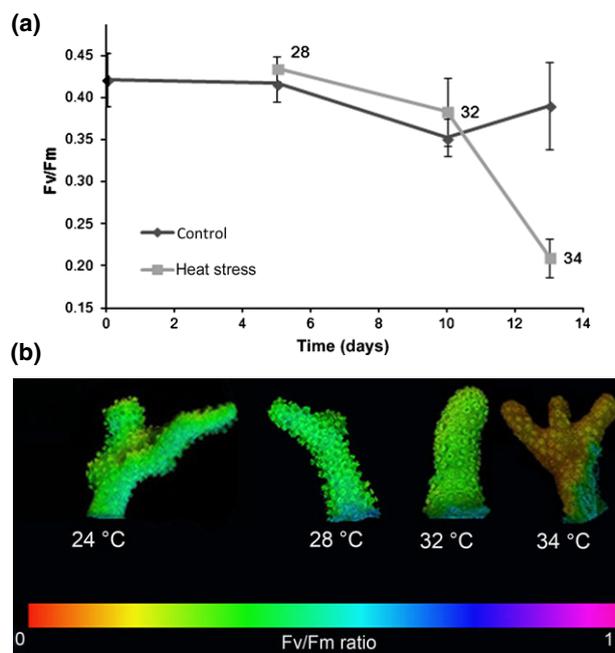


Fig. 1 Photosynthetic efficiency of *Stylophora pistillata* fragments. (a) Effective quantum yield (Fv/Fm) values throughout the experiment for heat-stressed and control fragments. (b) Representative images of effective quantum yield measurements at 24, 28, 32 and 34 °C. The relative values of the Fv/Fm ratio, ranging from 0 to 1, are displayed according to the provided color scale.

To examine the cellular processes that occurred in *S. pistillata* cells during severe heat stress, we investigated the interactions among the proteins encoded by genes that were up- or down-regulated at 34 °C. As shown in Fig. 2a, a large group of proteins related to ER stress and protein processing in the ER was up-regulated. This cluster is connected to clusters of up-regulated proteins responsible for cell cycle regulation, cell death and cell death regulation. Proteins involved in ubiquitin-mediated proteolysis also interact with the aforementioned groups of cell death and cell death regulation proteins. Furthermore, cell stress-response proteins, including those involved in sensing and repairing DNA damage, were found to be up-regulated in the 34 °C heat-stress sample. Nine genes responsible for the regulation of cell-cycle arrest were also up-regulated in this experiment.

A similar analysis performed on cluster groups of down-regulated interacting proteins yielded different results (Fig. 2b). A large core group of proteins that affect the negative regulation of cell death were down-regulated in the 34 °C heat-stress treatment sample. Proteins in the Wnt and Notch signaling pathways as well as four oxidative phosphorylation-related genes were down-regulated in this treatment. Large clusters

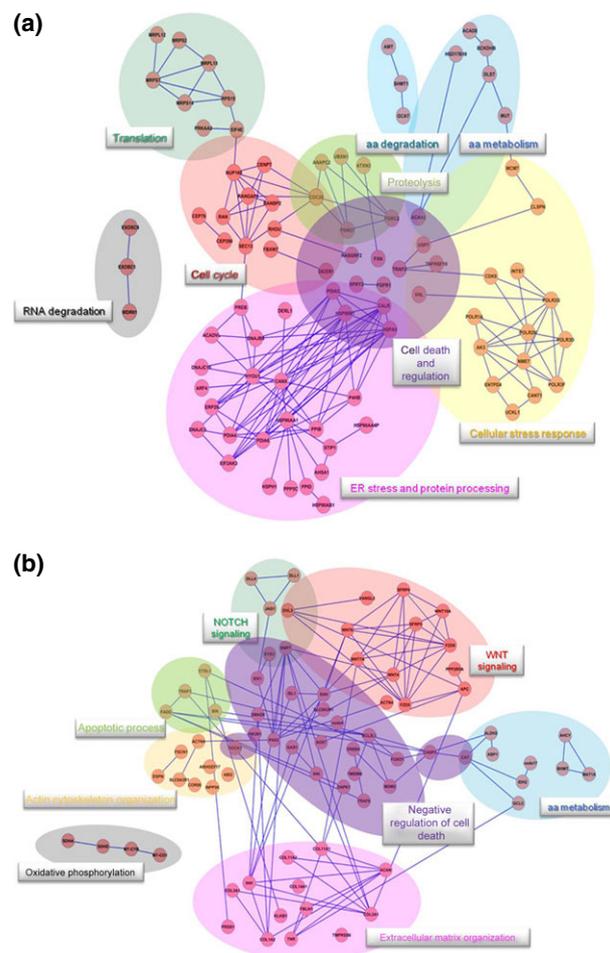


Fig. 2 Protein interaction network of the genes that were up-regulated (a) and down-regulated (b) at 34 °C. The t3 vs. c3 data set was uploaded to the Search tool for the retrieval of interacting genes (STRING) database and edited in Cytoscape software. Protein interactions with high confidence scores are categorized based on their Gene Ontology classifications (aa, amino acids; proteolysis, ubiquitin-mediated proteolysis).

of down-regulated proteins with functions in extracellular matrix organization, including several collagen types, were also observed. Actin cytoskeleton organization-related proteins were also among the core group of down-regulated proteins that were associated with the negative regulation of cell death.

Although the strongest cellular reaction to heat stress was observed at the highest temperature treatment, the reaction appears to intensify gradually from 28 to 34 °C, as indicated by the fold change values of the selected up-regulated genes (Figure S5a and b). For example, there was a gradual increase in the up-regulation of genes from the groups involved in ER stress and protein folding in the ER and proteasomal ubiquitin-mediated proteolysis (Figure S5a). Furthermore, genes in relevant down-regulated groups, such as

extracellular matrix organization, Wnt and Notch signaling and actin cytoskeleton organization, showed gradual changes in the intensity of their fold changes as the experiment progressed and the temperature increased (Figure S5b).

The aforementioned comparison of the protein interaction networks between the t3 vs. c3 treatments revealed interesting connections of common pathways among the up-regulated genes, which were absent or significantly reduced in quantity and intensity in the t2 vs. c2 and t1 vs. c1 treatments. We focused on the largest cluster of up-regulated proteins, which comprised of multiple interconnections between these proteins, ER stress and protein processing in the ER. We identified a total of 62 up-regulated genes in three cellular pathways based on KEGG database annotations: protein processing in the ER, ubiquitin-mediated proteolysis and proteasome function. A graphical representation of these pathways is presented in Fig. 3.

As the temperature increased from 28 to 34 °C and heat stress was prolonged, the fold changes of the affected genes increased dramatically, and additional genes were added to the same clustering groups that were detected at 32 °C. As shown in Fig. 4, nine genes related to ER stress and protein folding in the ER were up-regulated in the 28 °C sample. That number increased to 18 in the 32 °C sample and, following an increase of only 2 °C, an additional 12 genes were added. Similar trends were observed for the proteasomal ubiquitin-mediated proteolysis, extracellular matrix organization and Wnt signaling clusters.

Discussion

In this study, we explored the 'r' strategist *S. pistillata* coral transcriptome as a case study during short-term heat stress using a microarray technique studying gene expression profiles. A mild heat stress reaction was observed at 28 °C and intensified significantly at 34 °C. The number of up- and down-regulated genes at 28 and 32 °C was similar, whereas a sharp increase in the number of differentially expressed genes was observed at 34 °C (Figure S3). Moreover, variation in the intensity of the heat stress reaction was demonstrated, as the fold change was more pronounced at 34 °C relative to the lower temperatures (Figure S5a and b). Our preliminary results led us to use higher heat levels in subsequent experiments, as the heat stress reaction to a preliminary 32 °C treatment was mild. Further analysis justified this enhancement: the preliminary 28 °C treatment revealed only two up-regulated genes belonging to the GO response to stress category; at 32 °C, 17 genes from this category were up-regulated, whereas the 34 °C treatment yielded 41 genes in this category. A

similar trend was observed in other GOs, such as proteolysis. In addition, a Venn diagram constructed for the preliminary 32 °C, current 32 °C treatment and current 34 °C results showed that the majority of the preliminary 32 °C up-regulated genes were the same as the current 32 °C genes, whereas the differentially expressed genes in the 34 °C treatment were primarily unique (Figure S6).

The cellular heat shock response is characterized by the activation of target genes, such as chaperones and genes related to ubiquitination and proteolysis, defense against oxidative stress, signal transduction, energy generation, vesicular transport and cell wall, cytoskeleton and carbohydrate metabolism (Hahn *et al.*, 2004) (e.g. from a whole yeast genome study). The cellular stress-response genes (Fig. 2) were up-regulated only in response to the 34 °C temperature treatment. Based on examination of the interconnections between the clusters of the protein processes in the ER, Ubiquitin-mediated proteolysis and cell death (Fig. 2), we propose a stress-response pathway (Fig. 3) that highlights protein-encoding genes that were up-regulated following the 34 °C treatment as well as their known functional roles derived from the principles established in model organisms. Essentially, secretory and membrane proteins synthesized in the ER membrane enter the lumen in an unfolded conformation. While in the ER, peptides assemble, fold and undergo posttranslational modifications. Only properly folded proteins will exit the ER toward the Golgi via a transport vesicle comprising SAR1-GTP, Sec23/24 and Sec13/31 (Barlowe, 1998; Springer *et al.*, 1999), which were increased in the 34 °C treatment. Unfolded correctly glycoproteins will undergo cycles of interactions with calreticulin and calnexin as a 'quality control' process (Rajagopalan *et al.*, 1994), and ERP57 chaperone can be recruited to form disulfide bonds (Zapun *et al.*, 1998). When luminal chaperones, such as NEF (HYOU1), Hsp40, GRP94 and Bip (Tyedmers *et al.*, 2003)(Haas, 1994), fail to achieve proper protein recognition and folding, peptides tend to accumulate, leading to ER stress, which triggers the unfolded protein response (UPR). Three molecules orchestrate mammalian signaling crosstalk, and one of these, the kinase PERK, was found to be up-regulated following the 34 °C treatment in our experiment. The dissociation of Bip from the PERK luminal domain leads to PERK activation (Walter & Ron, 2011). PERK inhibits protein translation (Walter & Ron, 2011), and if the ER stress is not relieved, the UPR will eventually induce apoptosis (Hetz, 2012) and PERK will induce cell cycle arrest (Brewer & Diehl, 2000). High expression levels of DUB, a gene that was also up-regulated in our experiment, lead to cell cycle arrest (Zhu *et al.*, 1996). As illustrated in Fig. 2a and b, genes

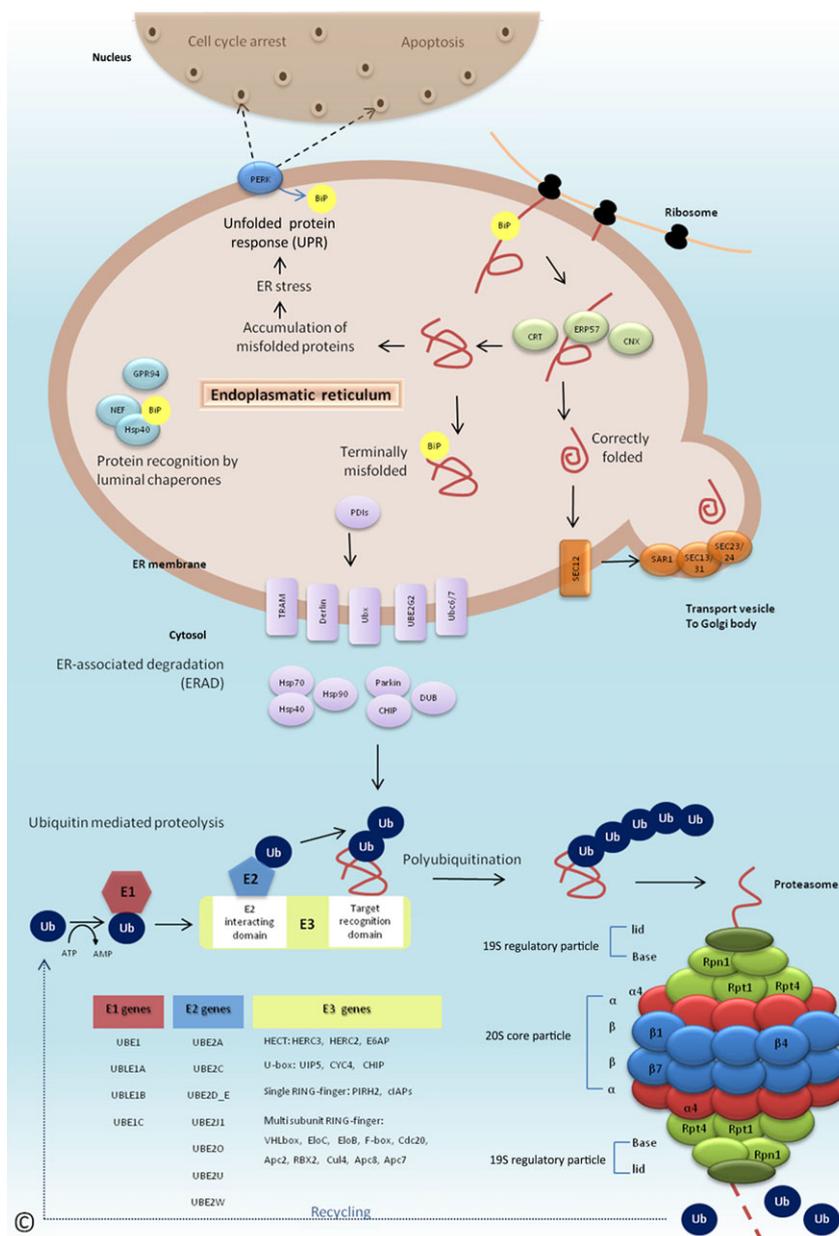


Fig. 3 The endoplasmic reticulum (ER) stress, Unfolded protein response (UPR), ER-associated degradation (ERAD) and ubiquitin-mediated proteolysis pathways. All of the genes indicated in this diagram were up-regulated at 34 °C, with the exception of SEC12. The table in the bottom left corner of the figure shows the E1, E2 and E3 enzymes that were found to be up-regulated in our experiment. Based on principles established in model organisms, newly synthesized peptides enter the ER with assistance of the mediator Bip, in an unfolded conformation, where they will assemble, fold and undergo posttranslational modifications. ER lumen chaperones such as NEF (HYOU1), Hsp40, GRP94 and Bip assist with protein folding. CRT, CNX and ERP57 recognize properly folded proteins. Correctly folded proteins will be shuttled via transport vesicles comprising SAR1-GTP, Sec23/24 and Sec13/31 to the Golgi body for final processing and assembly. Misfolded proteins tend to accumulate in the ER lumen and may trigger the unfolded protein response (UPR); the UPR signal is transduced to the nucleus via PERK, resulting in cell cycle arrest. Eventually, if the ER stress is not mitigated, programmed cell death will be initiated. Terminally misfolded proteins undergo ER-associated degradation (ERAD); following binding to Bip and protein reduction and oxidation by PDIs, the protein exits the membrane through one of several adaptor proteins and dislocation components, such as TRAM. Cytosolic E3-protein ligases, such as PARKIN and CHIP, with the assistance of cytosolic heat shock proteins (HSPs), target proteins for degradation via the attachment of ubiquitin molecules. Finally, ERAD substrates are degraded into small peptides by the multi-catalytic 26S proteasome.

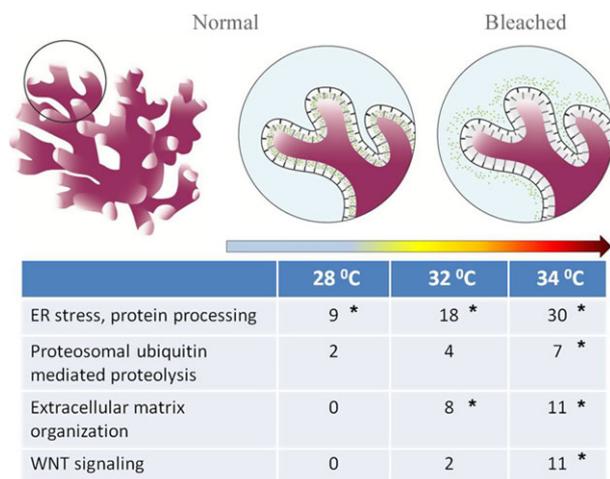


Fig. 4 Increasing numbers of genes involved in cellular pathways. This figure indicates the number of genes involved in endoplasmic reticulum (ER) stress, protein processing in the ER, proteasomal ubiquitin-mediated proteolysis (up-regulated) and extracellular matrix organization and Wnt signaling (down-regulated) that exhibited differential regulation at each stage of the experiment (from normal healthy colony to bleached). The numbers of significant ($P < 0.05$) genes that are within their Gene Ontology category are indicated by asterisks.

responsible for cell cycle arrest were up-regulated following the 34 °C heat treatment, and several genes related to programmed cell death were also expressed. Our results suggest that during severe heat stress at 34 °C, *S. pistillata* cells experience ER stress leading to UPR; if the stress is not mitigated, the cell cycle is arrested and the cell might undergo programmed cell death. Terminally misfolded proteins undergo retrotranslocation to the cytosol and ubiquitin-mediated proteasomal degradation through the ERAD process. Following protein reduction and oxidation by PDIs, the protein exits the membrane through one of several adaptor protein and dislocation components, such as TRAM. Cytosolic E3-protein ligases, such as PARKIN and CHIP, with the assistance of cytosolic heat shock proteins (HSPs), target proteins for degradation via the attachment of ubiquitin molecules (Smith *et al.*, 2011; Kriegenburg *et al.*, 2012). In our experiment, the E3 ligases, as well as the E1 and E2 genes, were up-regulated, as shown in the bottom panel of Fig. 4. Finally, ERAD substrates are degraded into small peptides by the multi-catalytic 26S proteasome. The identification of these up-regulated genes involved in ERAD and ubiquitin-mediated proteolysis supports the concept that *S. pistillata* cells utilize these mechanisms to cope with misfolded proteins that aggregate as a result of the heat.

Proteolysis, as reported here, is considered a common end result of heat stress, as is the ubiquitination of

proteins designated for degradation. Ubiquitin expression levels have been established as a cellular marker for protein integrity and, hence, cellular heat stress (Downs *et al.*, 2012). Furthermore, increased ubiquitin levels have been documented in heat-stressed cnidarians (Downs *et al.*, 2000, 2002; Yum, 2006). Desalvo *et al.* (2012) performed microarray analyses showing that genes in the UPR GO category were enriched and a PERK homolog was up-regulated in *Acropora palmata* under conditions of constant darkness. Kaniewska *et al.* (Kaniewska *et al.*, 2012) showed that under high CO₂ conditions, calnexin gene expression increased in the coral *Acropora millepora*. However, this study pinpoints on the activities of ER stress, ERAD and UPR processes following heat stress in Anthozoans.

Certain components of the cellular stress response are highly conserved throughout the metazoans with a defined group of proteins indicating a minimal cellular stress proteome (Kültz, 2003). These genes are categorized by their cellular functions, which include redox regulation, DNA damage response, molecular chaperones and energy metabolism. Eleven of the genes from this list were up-regulated in our experiment. Some of these genes were up-regulated in both the 32 and 34 °C samples, and others were unique to the 34 °C sample, meaning that they were not up-regulated in the 32 °C sample or the 28 °C sample. It is clear that genes associated with redox regulation and chaperones were up-regulated at both 32 and 34 °C, whereas DNA damage-related and energy metabolism genes were largely unique to the 34 °C sample (see Table 1). Based on these results, we propose a potential time line for the events occurring in *S. pistillata* cells in response to acute exposure to heat stress: at 32 °C, oxidative damage begins to accumulate in the cells; the thioredoxin expression pattern increases to repair oxidative damage to proteins; and molecular chaperones assist in protein folding. This initial reaction is observed at 32 °C, when the stress is not yet fully realized; thus, these genes can be defined as 'early warning genes.' As the heat is increased to 34 °C, 'severe heat-related genes,' such as those responsible for sensing and repairing DNA damage, are activated. The expression of genes related to energy metabolism increases to satisfy the energetic demands of processes occurring within the cell, such as protein degradation, protein refolding (chaperoning) and DNA repair. Moreover, metabolic enzymes generate reductive elements, such as NADPH and NADH, to address the oxidative damage (Kültz, 2005) that was initiated at lower temperatures (32 °C).

Our transcriptome analysis showed a decrease in actin cytoskeleton- and extracellular matrix organization-related proteins in *S. pistillata* cells during heat stress. Moreover, we have identified 12 significant

Table 1 A list of up-regulated genes common to t2 and t3 or unique to t3. The genes are categorized by function. The common lists serve as early warning heat stress genes

	t2 and t3 common up-regulated genes	t3-unique up-regulated genes
redox regulation	Thioredoxin	
	Peroxiredoxin	
DNA damage		MutS protein
		DNA topoisomerase I
		DNA repair protein RAD51
molecular chaperones	DnaJ	
	Heat shock 70 kDa protein	
energy metabolism	ribosomal RNA methyltransferase	Plasma membrane calcium-transporting ATPase
		Enolase
		Phosphoglucosmutase

(FDR $P < 0.05$) down-regulated genes from (Drake *et al.*, 2013) the proteomic analysis of the skeletal organic matrix of *S. pistillata* (Table S2). Similar changes following thermal stress have been reported in previous studies. For example, the down-regulation of actin homologs in the sea anemone (Richier *et al.*, 2008) and changes in actin cytoskeleton organization and extracellular matrix-related proteins in *Montastraea faveolata* and in *Acropora palmata* (DeSalvo *et al.*, 2008, 2010) have been observed. In contrast, Barshis *et al.* (Barshis *et al.*, 2013) reported increases in the expression levels of collagen and extracellular matrix proteins in thermally tolerant populations of *Acropora hyacinthus* relative to sensitive colonies. It appears that heat stress induced down-regulation of cytoskeleton- and extracellular matrix organization-related proteins impairs the ability to repair the cytoskeleton. However, it has been discussed that heat stress can also directly damage the cytoskeleton's integrity (DeSalvo *et al.*, 2008) or lead to osmotic stress and result in changes in cell volume and fracturing of cytoskeleton elements (Mayfield & Gates, 2007).

Carbonic anhydrase (CA) was up-regulated at 28 °C in our experiment, although several transcripts (CA 2, 6, 12, 13 and 14) exhibited a significant decrease in expression at 34 °C. CA catalyzes the hydration of CO₂ to HCO₃⁻ and is thought to play a significant role in the calcium carbonate assimilation of Scleractinian corals (Tambutté *et al.*, 2006; Bertucci *et al.*, 2011). Several studies have reported the down-regulation of coral CAs following stress (Edge *et al.*,

2005; Desalvo *et al.*, 2012; Kenkel *et al.*, 2013). In addition, a decrease in CA expression was measured in thermally tolerant populations of *Acropora hyacinthus* following a bleaching event. Accordingly, CA was suggested to promote coral resilience against heat stress (Barshis *et al.*, 2013). Thus, CA down-regulation is consistent with the decreased expression of extracellular matrix organization proteins at 34 °C, as both intracellular (i.e. actin cytoskeleton) and extracellular skeletal elements are damaged when the coral is subjected to thermal stress.

Recent works have highlighted the importance of lectin at the onset of algae-coral symbiosis (Wood-Charlson *et al.*, 2006) and showed a decreased transcript concentration before and during coral bleaching (Vidal-Dupiol *et al.*, 2009). Here, we report a significant (FDR $P < 0.05$) down-regulation of lectin, which intensified with higher temperatures, corresponding to bleaching.

Here, we observed a decrease in the expression of 13 Wnt-related genes at 34 °C. Wnt genes are best known as signaling agents with roles in vertebrate and invertebrate developmental processes and cell-cell interactions. Wnt signaling has been shown to promote osteogenesis (Rodda & McMahon, 2006) and thus may play a role in biomineralization. This outcome is in agreement with previous observations and supports the trend of decreased extracellular matrix assimilation with increasing heat stress. It has also been shown that ER stress can inhibit the Wnt and Notch signaling pathways (Zoltewicz *et al.*, 2009; Aranda *et al.*, 2011), which might provide an additional explanation for the Wnt down-regulation observed in the current study. To date, Lengfeld *et al.* (Lengfeld *et al.*, 2009) are the only authors who have reported the function and localization of Wnt genes in an adult *Cnidaria*. These authors demonstrated the expression of seven Wnt genes in the initial stages of bud formation during asexual reproduction in *Hydra*. If the Wnt cascade has a functional role in asexual budding, it is reasonable to speculate that the Wnt down-regulation that was observed in our experiment may be associated with the inhibition of asexual reproduction processes. Abruptions in gametogenesis and abnormal larval development have been documented following increases in seawater temperature (Szmant & Gassman, 1990; Bassim *et al.*, 2002). In response to stressors, corals can reallocate their resources accordingly; e.g. energy and resources may be diverted from growth and/or reproduction processes to engage in stress response. Moreover, Obura (2009) suggested a model in which corals that invest in reproduction or growth are more sensitive to bleaching at lower stress levels and are more susceptible to mortality, whereas colonies that devote additional resources to the resistance to stress will bleach only at

higher levels of stress and will be more resilient to mortality following bleaching events. The subject of our research, the Red Sea *Stylophora pistillata*, is known for its relative resistance to bleaching and relatively low mortality rates in the Red Sea's Gulf of Aqaba. These characteristics may be consequences of diverting energy toward cellular mechanisms to mitigate stress, such as the ER stress and UPR pathways, with possible negative effects on growth/reproduction and biomineralization processes. Understanding the pathways and cellular mechanisms activated in heat-stressed corals may help improve projections of reef coral survival under the threat of global warming.

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Supporting Information

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

Data S1. Materials and methods.

Figure S1. Graphical illustration of the experimental design.

Figure S2. Principle component analysis (PCA). All the samples and replicates are plotted in a three-dimensional space using three principal components (PC) capturing 65.3% of total variability. This image was constructed using Partek Genomics Suite™ software.

Figure S3. Venn diagram of all differentially expressed genes. The Venn diagram presents differentially expressed genes in the 28 (t1 vs. c1), 32 (t2vs. c2) and 34 °C (t3 vs. c3) trials. The number of genes is indicated within each field.

Figure S4. The relative expression of selected genes assessed via quantitative real-time PCR (ddCt). The expression levels of (A) dnajc3 (DnaJ homolog subfamily C member 3), (B) pdia6 (protein disulfide-isomerase A6) (C) Collagen alpha and (D) Carbonic anhydrase 6, in treatments 1 (28 °C) and 3 (34 °C) (indicted in black) and their corresponding controls (indicted in gray) are shown. Each dCt was normalized to a housekeeping gene: 60S Ribosomal protein L22. Error bars indicate the standard error.

Figure S5. Fold change values of selected up-regulated (A) and down-regulated (B) genes. The fold changes of genes for ER stress and protein folding in the ER and proteasomal ubiquitin-mediated proteolysis (A), and genes for extracellular matrix organization, Wnt signaling, Notch signaling and actin cytoskeleton organization (B) are shown with respect to temperature treatments 1, 2 and 3. The color-scale legend indicating the relative fold change is shown on the right. This figure was constructed using EXPANDER software.

Figure S6. Venn diagram of total differentially expressed genes in preliminary 32 °C and current 32 and 34 °C trials. The number of genes is indicated within each field.

Table S1. A list of 163 differentially expressed genes – 'Common genes' – either up- or down-regulated, at all three sampled temperatures. –1 and 1 indicate significant (FDR $P < 0.05$) down- and up-regulation, respectively.

Table S2. Down-regulated genes from the skeletal organic matrix (SOM) predicted proteins identified by Drake *et al.* 2013 proteomic analysis, in the 28°C (t1 vs. c1), 32°C (t2vs. c2) and 34°C (t3 vs. c3) trials. ↓ and ↑ indicate significant (FDR $p < 0.05$) down-regulated and up-regulated genes, respectively.