Expression pattern of potential heat stress genes under warm and cold episodes in scleractinian coral (*Dipsastraea matthaii*) in the hottest sea, Persian Gulf

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Abstract

Shallow coral reefs face significant threats due to anthropogenic ocean warming, which occasionally surpasses their thermal tolerance limits. However, specific hard coral species exhibit thermal resilience and can rapidly adapt to anomalous thermal stress events by modulating the expression of their heat stress-related genes. In a comparative analysis of microarray data, we identified candidate genes associated with temperature stress. Subsequently, to validate these findings, we selected four genes—namely, Cell Division Cycle 16 (CDC16), Ubiquitin C (UBC), Heat Shock Protein 90 Beta Family Member 1 (HSP90B1), and Histone acetyltransferase p300 (EP300)—as responsive to temperature stress in the scleractinian coral *Dipsastera matthaii*, collected from the Persian Gulf. Real-time PCR analysis during warm and cold periods confirmed significant changes in UBC, HSP90B1, and EP300 expression in response to heat stress. Notably, EP300 enhancement resulted from transcription activation via binding to transcription factors. A crucial aspect of this response involved the upregulation of molecular chaperones, including HSP90B1, due to the activation of heat shock factor 1 (HSF1). Additionally, HSF1 played a role in activating UBC, which facilitates the removal of critically digested proteins through the ubiquitin proteolysis system. These findings contribute to identifying potential candidate genes that could serve as valuable biomarkers for the selection of heat-tolerant genotypes.

Keywords: Thermal stress, Gene expression, Reef-building coral, Thermally resilient coral, Persian Gulf

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Introduction

Coral bleaching occurs due to either the loss of a coral's symbiotic algae (known as zooxanthellae) or the degradation of the algae's photosynthetic pigments (Glynn 1993; Hoegh-Guldberg 1999). Primarily, coral bleaching happens in response to thermal stress, often triggered by elevated sea temperatures (Glynn 1993, 1996). Besides heat, other factors such as light exposure, pollution, and nutrient imbalances can also contribute to coral bleaching (Glynn 1993, 1996). When corals experience thermal stress, their gene expression patterns change significantly (Seneca *et al*., 2010; Barshis *et al*., 2010; Weis 2010; Kenkel and Matz 2016; Moghaddam *et al*., 2021). Specific genes are upregulated or downregulated in response to stress. These changes in gene expression play a crucial role in the coral's ability to cope with environmental challenges. Researchers have identified several genes associated with heat stress response in corals. For example, the heat shock protein 90 beta family member 1 (HSP90B1) is one such gene that becomes more active during bleaching events (Rodriguez-Lanetty *et al*., 2009). Other genes involved in protein synthesis, metabolism, and cellular defense also play critical roles (Cleves *et al*., 2020; Moghaddam *et al*., 2021). By measuring the expression levels of specific genes, researchers can gauge the severity of heat-light stress experienced by corals. Notably, the expression of genes like Hsp16 and actin can serve as indicators of coral health during bleaching events (Kenkel *et al*.,

2011). A gene expression biomarker is expected to encapsulate the intricate interplay of gene expression, function, and regulatory mechanisms. These genes exhibit swift upregulation in response to external stimuli, yet their expression declines even if the environmental change continues to persist. For example, antioxidant genes, Cytochrome P450 isoforms, and heat shock proteins can act as biomarkers of optimal gene expression for early detection of imminent coral bleaching (Louis *et al*., 2017).

The thermal history of corals, or stress memory, appears to be an influential protective factor when they are exposed to extreme heat stress (Barshis *et al*., 2010; Weis 2010; Seveso *et al*., 2020). It is believed that the response of corals to thermal stress is partly determined by their heat exposure history (Roche *et al*., 2018). Although certain coral species (e.g., shallow water corals) have undergone extreme and persistent warming episodes in different hot seas (e.g., the Gulf of Aqaba, the Kimberley coastal area, Ofu Island, the Persian Gulf), they have successfully survived (Barshis *et al*., 2010; Grottoli *et al*., 2017; McCulloch *et al*., 2017; Burt *et al*., 2019). Such superior thermal-tolerant species are capable of adapting to unusual thermal stresses which are fatal for other species (Camp *et al*., 2018). Coral species with the highest recognized bleaching thermal threshold occur in the Persian Gulf (Howells *et al*., 2016; Riegl *et al*., 2011; Smith *et al*., 2017). This sea is characterized by an arid subtropical climate with the excess

of evaporation over precipitation and river runoff in the northwestern sector (Sheppard *et al.*, 2010). Despite harsh environmental conditions concerning high salinity (45 ppt) and remarkably large SST annual cycle (i.e., approximately 12.8°C in winter and 36.8°C in summer) (Reynolds, 1993), several coral species have surprisingly survived and adapted to these conditions (Hume *et al.*, 2013). In boreal summer, these corals are exposed to long-lasting thermal stresses (>34°C) (Riegl *et al.*, 2011). However, several widespread mass coral die-off episodes (i.e., 1996, 1998, 2017) have affected the corals in the Persian Gulf leading to 80% disappearance of corals with the entire elimination of *Acropora* spp. corals (Burt *et al.*, 2019; Riegl *et al.*, 2018; Riegl and Purkis, 2015; Shokri and Mohammadi, 2021; Shuail *et al.*, 2016). Over the past decades, the warming trend across the Persian Gulf has been higher than the global average (Oliver *et al.*, 2018). Such severely warm conditions are predicted for Indo-Pacific corals in the late twenty-first century and under strong greenhouse emission scenarios (Heron *et al.*, 2016). Given the sensitivity of coral symbionts and their rapid response to thermal stress, early detection of any disorder in their health and other performance factors, before the occurrence of visible symptoms, is very beneficial for the protection and restoration of these delicate ecosystems (Kenkel *et al.*, 2011, 2014; Traylor-Knowles and Palumbi 2014; Wright *et al.*, 2017; Parkinson *et al.*, 2020). Over the past recent decades, a molecular

approach, termed Gene Expression Biomarkers (GEB), has been successfully developed and implemented in the early detection and quantification of stress in corals (Louis *et al.*, 2017). Such molecular toolkits, which are widely used in biomedical research and clinical practices, measure the resilience of species and enhance the possibility of restoration (Parkinson *et al.*, 2020; Rivera *et al.*, 2021).

In transcriptomic responses of corals and their coexisting algae to heat stress, the most genes evaluated are heat shock proteins (Rosic *et al.*, 2011; Meyer *et al.*, 2011; Leggat *et al.*, 2011; Kenkel *et al.*, 2011; Zhang *et al.*, 2017; Seveso *et al.,* 2020). Heat shock proteins are molecular chaperones and play key roles in protein metabolism. Chaperones are protected proteins that are widely expressed in the presence of stress (Whitley *et al.*, 1999). Heat shock proteins, which act as molecular protectors, are expressed in a wide range of stressors (Schmitt *et al.*, 2007). During a stress event, for example, heat stress, incorrect protein folding, accumulation, or disruption of the regulation and separation of multiple protein complexes can lead to Signaling pathway changes (Li, 2004).

In general, corals exposed to heat stress or other stresses that lead to bleaching increase the expression level of heat shock proteins as a defense mechanism (Louis *et al.*, 2017; Traylor-Knowles *et al.*, 2017; Seveso *et al.*, 2020). These proteins are the core of balancing cell death and life and also protect cells against apoptosis and stress

(Nollen and Morimoto 2002; Das *et al.*, 2019). An HSF1 transcription factor is lost or suppressed when activated. HSF1 eventually binds to promoters of heat stress genes (Jolly and Morimoto 2000; Pirkkala *et al.*, 2001). Thermal stress proteins are named according to their molecular weight and function (e.g., HSP60, HSP70, HSP90) (Dubey *et al.*, 2015). Using the real-time-PCR technique, the increase in HSP90 expression in *Montastraea cavernosa* coral was explored as an indicator for protein denaturation (based on renaturing function) (Skutnik *et al.*, 2020). Skutnik *et al.* (2020) further argued that this might be considered an indicator of host stress and immediate response to control negative consequences and control homeostasis. In adult *Porites astreoides* colonies under laboratory heat and light stress, HSP90 expression increased 6-fold after 3 hours of exposure to 36-35 ° C, which is (7-8 °Cwarmer than control conditions and 10 times more light intensity of control medium) (Kenkel *et al.*, 2011). In another study, an increase in the expression of molecular chaperones from the HSP90b1 family, HSP26 / 42, and DNAJ in *Montipora aequituberculata* corals were recorded under thermal stress of 29.5 and 32 ° C compared to controls at 27 ° C (van de Water *et al.*, 2018). Ubiquitin C (UBC) is one of the four genes encoding ubiquitin in the mammalian genome. The UBC gene is a stress-protecting gene that is overexpressed under a variety of stressful conditions, including exposure to UV radiation, heat shock, oxidative stress, and translational disorders. Using Western blotting, certain protein biomarkers including Hsp70, Ubiquitin-conjugate, and MnSOD were selected in *Porites lobata* in Ofu Island, Samoa (USA), among which Ubiquitin-conjugates had higher levels in the colonies in warmer back reefs than in the colonies in the cooler fore reef (Barshis *et al.*, 2010). Another crucial process for activating heat-responsive genes involves the elevation of the enzyme histone acetyltransferase. The histone acetyltransferase p300 (EP300) gene acts as a transcriptional inhibitor with intrinsic lysine acetyltransferase activity, regulating gene transcription and expression through various mechanisms. Notably, p300 controls the stability of numerous transcription factors via acetylation, ultimately activating heat shock and stress response proteins (Raychaudhuri *et al.*, 2014).

The Persian Gulf as a semi-enclosed sea surrounded by dry landmasses is characterized by relatively harsh environmental conditions concerning high temperature and salinity (Sheppard *et al.*, 2010). The coral communities in this sea have been formed under harsh environmental conditions that kill conspecifics elsewhere (Hume *et al.*, 2013; Price *et al.*, 1993). As such, these unique coral communities are attracting attention for their ability to inform how coral reefs globally are adapting to projected increases in water temperatures over the next century (Burt *et al.*, 2012; Hume *et al.*, 2015; Ziegler *et al.*, 2017).

Despite the progress that has been made in understanding the causes and implications of coral bleaching across the northern Persian Gulf, it remains unclear, as a direct indication of adaptability, how the expression of thermal stress-related genes in native corals change under severe heat stress. In the present study, the changes in the expression of potential heat stress genes [i.e., Cell Division Cycle 16 (CDC16), Ubiquitin C (UBC), Heat Shock Protein 90 Beta Family Member 1 (HSP90B1), Histone acetyltransferase p300 (EP300)] in scleractinian massive coral "*Dipsastera matthaii*", as a heat stress tolerator (Edinger and Risk 2000) collected off Hengam Island, in the northeastern Persian Gulf, were investigated. In doing so, the seasonal pattern of change in the expression of selected genes was explored between warm and cold periods in both shallow and deep waters. Accordingly, structural analysis of the cognate network was used to select the most important genes involved in the temperature stress pathway. In this study, four series of microarray transcriptome and RNA-SEQ data were analyzed. The aim was to use gene expression studies related to molecular processes involved in temperature stress conditions and genes involved in responding to the organism's thermal stress to construct a gene network and subsequent analyses. Likewise, according to the results of co-expression network analysis, the expression of four genes (i.e., 12 CDC16, UBC, HSP90B1, EP300) with the highest rank was investigated as important genes of temperature stress paths (plural) in *D. matthaii* corals.

Materials and methods
Study area and sampling
The specimens of scleractinian coral, *Dipsastraea matthaii* (Vaughan, 1918) (Merulinidae), were collected off Hengam Island located in the Strait of Hormuz in the northeast of the Persian Gulf (Fig. 1).
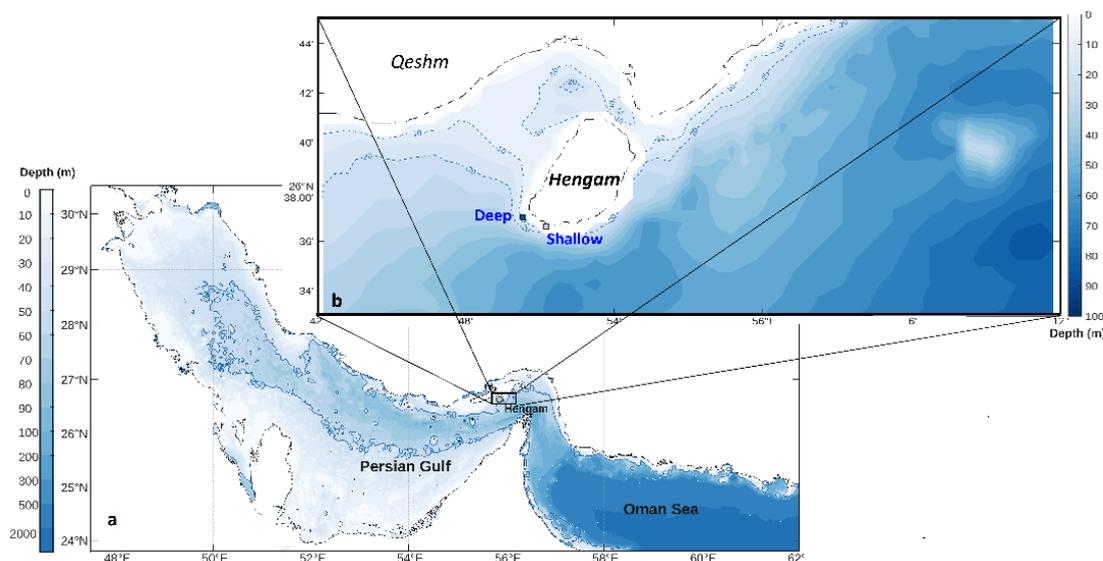
Figure 1: Topographic map of seafloor in the Persian Gulf and Gulf of Oman (a) as well as the shallow water area near Hengam Island (b). Contours in (a) indicate the 50 m depth whereas they denote 10 m and 20 m depth in (b). Shown dark (light) square in (b) is the location of the deep (shallow) corals used in this study. Note that (a) and (b) have different color scales. The bathymetry data was taken from the latest version of the global bathymetric provided by the General Bathymetric Chart of the Oceans (GEBCO; www.gebco.net).

Coral specimens were collected from shallow depth (~2.5 m, 15 m from the shore, 26˚36.707' N, 55˚51.441' E) and intermediate-depth (~9 m, 250 meters from the shore, 26˚37.055 'N, 55˚ 50.725' E). These sites were referred to as shallow and deep corals, respectively. Three colonies of the same size were selected and marked at each sampling site (i.e., shallow and deep sites). Sampling was performed by SCUBA diving during midday (14:00–16:00 local time) on the 17th of August as a warm period and on the 3rd of December 2018 as a cold period. Using a hammer and chisel, three small fragments with an approximate size of 5 cm were collected from each colony providing a total of nine replicates for each site at each warm and cold period. By fragmenting single colonies unwanted sources of genetic and biological variability derived from

colony size, shapes, and thermal/light life histories were eliminated (Granados-Cifuentes *et al*., 2013; Hemond *et al*., 2014; Parkinson *et al*., 2015). The coral fragments were immediately frozen in liquid nitrogen and kept at -80 °C in the laboratory until the RNA extraction. Two HOBO Pendant data loggers were lodged at each shallow and deep site and the water temperature was recorded from August 17, 2018, to December 31, 2018, with a 30-minute interval in both shallow and deep waters.

Identification and selection of appropriate expression stress experiments in D. matthaii

Using the keyword "Heat stress", the expression data of coral transcripts on the NCBI were examined in the GEO DataSets section and the Array Express section of the EBI. Finally, to identify

the genes responding to temperature stress, three sets of microarray data and one set of RNA-Seq data in the NCBI database were obtained from the GEO section. The data set used in this study with access codes GSE41435, GSE47779, GSE16151, and PRJNA177515 were obtained in the GEO section of the NCBI database (Table 1S). The various datasets were examined from various aspects, including the number of samples, and by considering these factors, the appropriate data set was selected. Based on the analysis of transcripts data, candidate genes were selected to the expression pattern of these genes was explored within warm and cold periods.

RNA extraction and cDNA synthesis

RNA extraction was carried out according to the RNeasy Mini kit (Qiagen), by following the manufacturer's instructions. RNA quantity was assessed using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000). RNA integrity was checked through agarose gel electrophoresis and evaluated based on clear 28S and 18S ribosomal RNA bands. cDNA synthesis was carried out using the QuantiTect reverse transcription kit (Qiagen).

Primer design

Since no genomic/transcriptomic data for *D. matthaii* were available, the RNAseq data of *Dipsastraea favus and Dipsastraea sp.* as close species to *D. matthaii*, were utilized. In doing so, the BlastStation2 software

(https://www.blaststation.com/ intl/en/blaststation2.php) was used. The local database was created from the assembled file of *D. favus and D. sp.* The sequences of genes were blasted against the database and sequences with the highest identity and the lowest E-Value as the best hit for each gene were considered. Additionally, selected sequences were blasted with other species of Porites corals by using the database provided at http://reefgenomics.org/ blast. Sequences were aligned using Clustal W of MEGA. Primers were designed based on conserved regions. For coral's algal symbiont, the sequences of the genes in the Symbiodiniaceae were blasted against the database in Santos Lab (http://webhome.auburn.edu/*santosr/ symblast.htm). Primers were designed based on conserved regions using MEGA. The selection of genes was based on the analysis of macroarray data. The genes involved in responding to the organism's thermal stress were selected as candidate genes.

The primers were designed based on protected areas between the two species, and the best pair of primers were selected for PCR analysis. The properties of the designed primers such as melting temperature, GC percentage, and the possibility of forming secondary structures were investigated using OligoAnalyzer online tools. Finally, the specificity of the primers was checked using the Primer-BLAST program. PCR efficiency and coefficient of determination (r^2) for each primer pair were determined using LinRegPCR

software. The sequence of designed primers is presented in Table 1.

Table 1: The sequence of designed primers for the selected genes.

Gene	Primer sequence (5′–3′)	Size (bp)	Biological process
EP300	F-TCAAGAATACGGAAGCGAATGTG R-GGAGGACAAGCCCAAATATGAG	186	transcriptional co-activator
HSP90B1	F- AGGTTGCTGTAGAGGTGTTAGG R- GCACAAACCCTGGAGAAACATC	126	Heat Shock Protein
UBC	F- AGGATGGGATGTCACGTTTGG R- GCTGAGCCTGTCTATGGATTG	158	ubiquitination
CDC16	F -GCAAGCACGGAGGTATTTCAG R- GTGACATCGAGGCATCAGTTTG	154	ubiquitination
EF1A	F- CAGCATCACCCGACTTCAC R -ATATGCTCCTGTGTTGGATTGC	131	Control gene

Quantitative real-time PCR

The quantitative real-time PCR was carried out in a total volume of 10.0 µL, containing 5 µL of 2x SYBR Green Master Mix (Amplicon), 1.0 µL of the cDNA, 1 µL of each primer (10 PM), and 3 µL of DEPC-water for 35 cycles. Three technical replicates for each colony were performed within each run. Reactions were run in standard mode on a Real-Time PCR System (Rotor gene-RG6000).

Data analysis

The samples collected in December from the deep site, termed as Deep-December, were considered control samples. The relative fold changes in other groups were evaluated concerning the control samples. Given the fact that variation in water temperature tends to be smaller for the deep corals, therefore, samples from the control group were not exposed to the environmental thermal stress when sample collection was carried out (T \ 26 °C; see Fig. 4). First Ct values of the target genes were normalized by using the reference gene. In all sample groups, this value was compared with the control group as the reference group. Gene expression values were computed according to the fold change (2-DDCt). Besides, the ANOVA statistical test was implemented to estimate the statistical confidence level of the gene expression differences for warm versus cold periods, shallow versus deep sites, acclimation experiments, and deep-recovery fragments (Table S1). This analysis was done using Genex v. 6 and SPSS v. 21. Results were visualized using GraphPad Prism 8.

Differential expression analysis and observation of sample distribution

When samples understudy has been extracted from independent biological sources, the variability of their variables is usually modeled by associated statistical distributions. Limma is a widely used software package that uses an application model to analyze microarray results. In this linear model,

the normal distribution is used as the (distribution) basis to model the microarray test expressions.

Statistical test of hypothesis using an R programming language

In the first part of the present study, using the expression matrix created from the results of the previous step (preprocessing and expression calculation step) and by knowing the information about the content of the samples, an object in a special R class was created. In the expression matrix of each row representing an ENSEMBL gene identifier or any other identifier ID, each column displays the samples tested in the experiment and each score represents the expression of the genes.

Results

Changes in water temperature

Water temperature in the deep site in August ranged from ~29°C to ~33°C. The water temperature declined notably right after the high tide suggesting that the corals had remarkably (~1.8 °C) cooled down by the displacement of cold subsurface water associated with tidal motion, as previously noted in the study area (Vajed Samiei *et al*., 2014). In the same period, shallow corals experienced a high temperature, typically warmer

than 33°C. Unlike the deep site, the temperature drop accompanied by the tidal cycle was not observed in the shallow site. During the sampling on August 17, 2018, between 14:00 and 16:00, the water temperature in the shallow and deep sites was 34.2°C and 32.7°C, respectively. During the *in situ* sampling in August, the sunshine was very intense and the sky was clear with almost no cloud coverage. The water was also clear. Thus, the observed thermal stress in the shallow site seems to be closely related to high light intensity. The severe heat stress conditions remained for several consecutive days. During the sampling on December 3, 2018, between 14:00 and 16:00, the water temperature was about 25.7 °C in both shallow and deep sites. In December 2018, the daily cycle of temperature was much smaller than that measured in the summer. The temperature difference between deep and shallow sites was minimal compared with summertime indicating that the stratification was very minimal and the mixed layer depth reached the deep site. The connection between deep site temperature and tidal motion, as observed in August, was broken down (Fig. 2a, b).
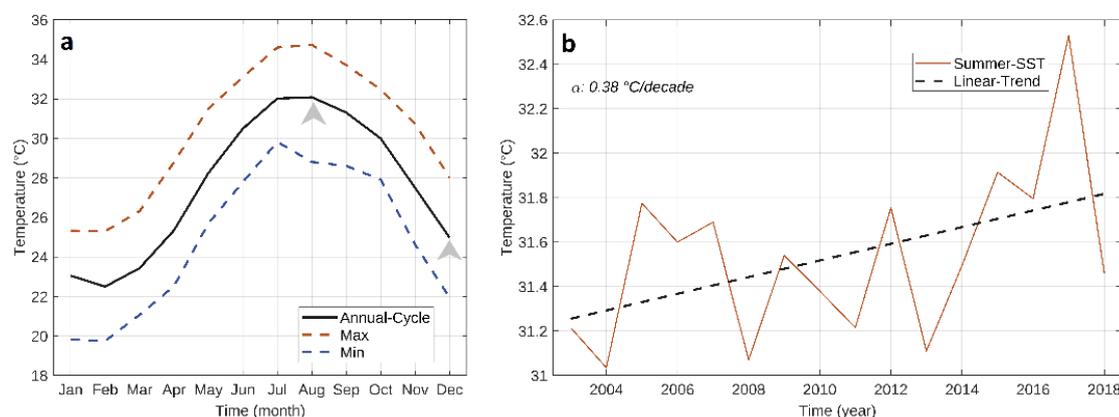
Figure 2: The annual cycle of SST (a; °C) obtained from the areal averaged over 55.7°E-56.0°E, 26.55°N-26.70°N, taken as the measure for the climatic seasonal cycle of Hengam Island (See Figure 1). Blackline in (a) displays the long-term (2003-2018) climatological monthly mean. The Red (blue) dash-line in (a) shows the warmest (coldest) observed temperature over (2003 - 2018). Redline in (b) shows the time series of the boreal summer (June-August) SST average. The back dash-line in (b) shows the trend line fitted to the SST time series. The trend line is obtained from the room-mean-square regression. The slope of the trend line is denoted by α in the upper-left corner of the panel (b). SST data were taken from the daily product of MODIS-Aqua (https://podaac.jpl.nasa.gov/dataaccess). Grey arrows in panel (a) indicate the calendar month of the sampling dates.

Changes in the expression of thermal stress-related genes

The data set used in this study with access codes GSE41435, GSE47779, GSE16151, and PRJNA177515 were obtained in the GEO section of the NCBI database (Table 1S). The results of the preprocessing process can be examined by various diagrams. For example, a box diagram shows the possibility of evaluating the distribution of data in different arrays. Usually, the difference in the shape and center of the boxes in the diagram indicates that the specimens need to be normalized. In this section, we show examples of the GSE41435 dataset before and after normalization by the Quantile method. Graphing was done by ggplot bundle. It should be noted that this section uses the raw data for each experiment as input. As can be seen from the graphs, the samples became more comparable after the normalization process (Fig. 3a, b). The normalization processing was done for each sample (Data not shown).
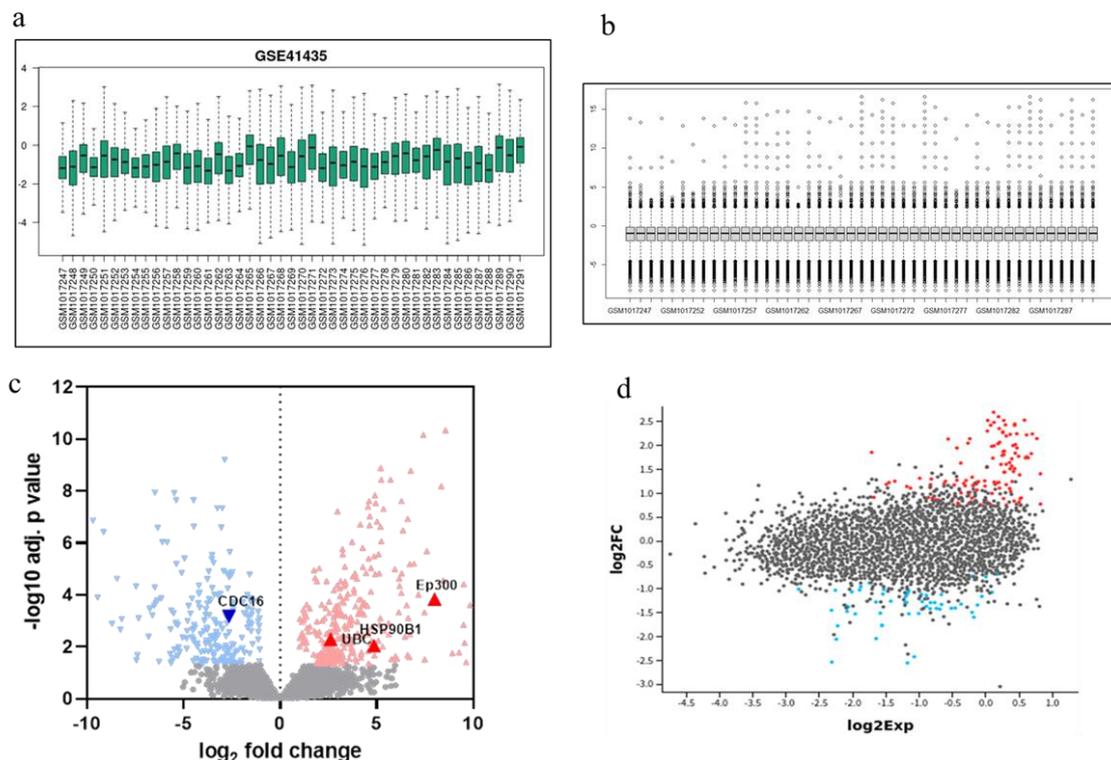
Figure 3: GSE41435 dataset before normalization (preprocessing) (a), GSE41435 dataset after normalization process (preprocessing) (b), volcano diagram showing genes with significant expression change, blue dots of genes with decreased expression and red dots of genes with significant expression (c), and M.A. diagram for GSE4143 dataset (d).

Differential expression analysis

In this study the main criterion for identifying candidate genes is the modified p-value, and genes with a q-value or so-called FDR of less than 0.05 will be considered genes with expressive differentiation and the candidate genes with the highest percentage difference are given in Table 2. Out of 191 genes, the key genes (CDS16, UBC, HSP9031, and EP300) were selected in the pathway of response to stress and the significance of their expression (Fig. 3c).

The results related to the analysis of DEG genes revealed that the changes in the expression of candidate genes were significant. The M.A. plot diagram (Fig. 3d) generally shows the logarithm of the expression ratio versus the logarithm of

the mean expression. This diagram shows the expressive differences measured between the samples. The X-axis represents the average of the expressions in the whole samples and the Y-axis shows their expressive differences. The candidate genes are marked with a marked difference in expression in red and blue.

Table 2: The final parts of the differential expression test.

ID	log2FoldChange	padj
UBC	2.61	0.005
CDC16	-2.64	0.0006
EP300	7.99	0.0001

Gene network

In this study, the STRING database contains a great deal of information on

protein-protein interactions which, by submitting genes identified as DEGs in it, the protein-protein interaction network was drawn. Due to the relative size of the constructed network, the PPI network was divided into smaller modules using the MCL algorithm and each of them was examined and interpreted separately. Figure 4 illustrates the constructed PPI network.
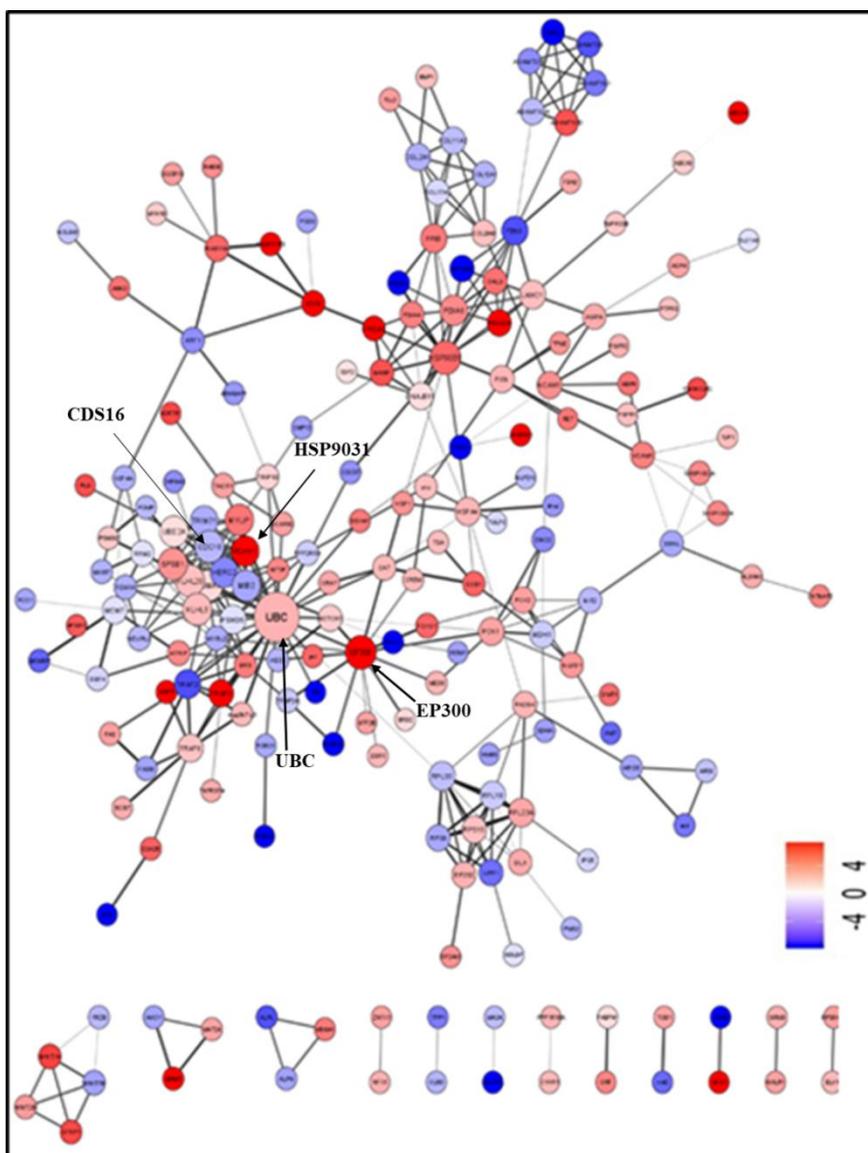

Figure 4: **Protein-protein interaction network for key proteins involved in temperature stress, mapped with Cytoscape software.**

The output file containing protein interactions was then entered into Cytoscape 3.8.2 software. Using the CytoHubba plug-in in addition to the MNC algorithm, highly connected genes were introduced as hubs. In the Cytoscape, the values of the above parameter were obtained for all nodes and the genes that contained the maximum values for this parameter were considered effective genes (CDS16,

UBC, HSP9031, and EP300 proteins were indicated by arrows in Figure 4. Figure 5 shows the degree of genes being studied. In addition, the genes (CDS16, UBC, HSP9031, and EP300) that had the most interaction (degree) in the network have been demonstrated in the expression change diagram. Accordingly, only hub genes and genes with a log FC greater than 1 or less than -1 have been combined in various studies and used in the network.
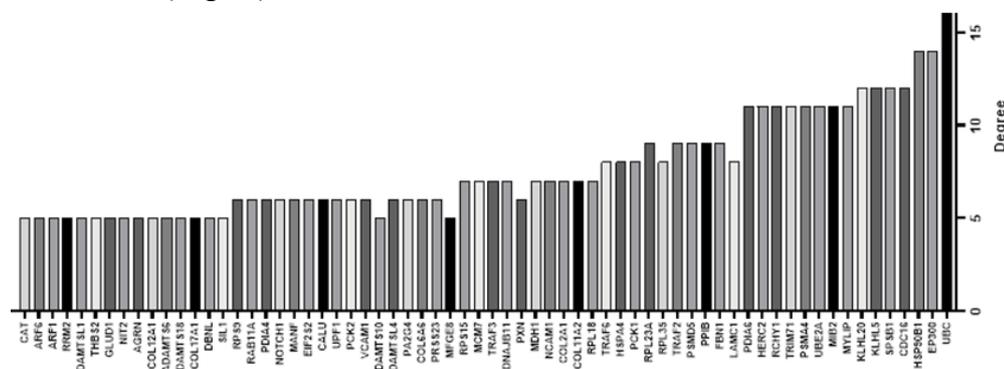

Figure 5: The degree of communication (degree) of genes in the network interacting using Cytoscape software.

Functional review of modules
The employed algorithm resulted in the formation of about 32 modules for the PPI network. Here, Module 1 was chosen, because it had the most important and largest number of genes. An overview of the network and its available communications for this module in the STRING database are presented in Figure 6. Some of the most important processes involved for the components in this module are listed in Table 3. Module 2 also had a high genetic diversity and is second in terms of the number of genes (Table 4).

Selection of the most important genes involved in thermal stress based on cognate network analysis
According to the results of co-expression and PPi network analysis, in this study, the expression of four genes, such as CDC16 UBC, HSP90B1, and EP300 with the highest ranking, were selected as important genes of temperature stress path in *D. matthaii* corals. The EF1a gene was identified as the reference gene for data analysis in this experiment. During different stages of the experiment and in all samples, the expression of this gene was almost constant and the standard deviation of the internal control gene was calculated to be 0.92. Samples taken in December from the Deep site (Deep-December) were considered as a control group in this study.
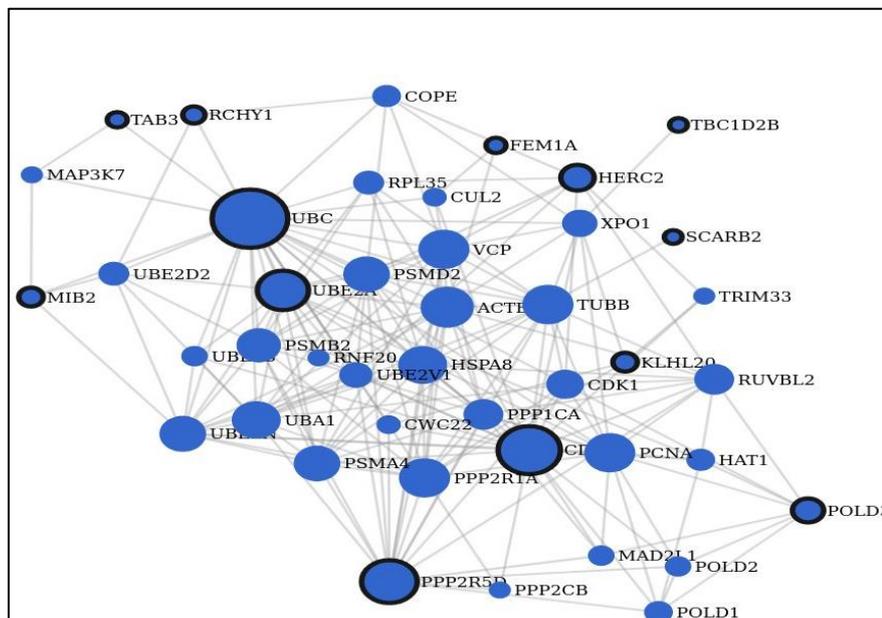
Figure 6*: **The module1 in the KEGG path indicating the pathway of Ubiquitin mediated proteolysis.**

Table 3: Interpretations related to the contents of module 1.

ID for the interpretation provided in the relevant database	Brief explanation for the proposed interpretation	Proposed interpretation category
GO:0016567	Protein ubiquitination	BP
GO:0000209	Protein polyubiquitination	BP
GO:0000151	Ubiquitin ligase complex	CC
GO:0004842	Ubiquitin-protein transferase activity	MF
GO:0061630	Ubiquitin protein ligase activity	MF
hsa04120	Ubiquitin mediated proteolysis	KEGG

Table 4: Suggested interpretations for the contents of module 2.

ID for the interpretation provided in the relevant database	Brief explanation for the proposed interpretation	Proposed interpretation category
GO:0016567	Protein ubiquitination	BP
GO:0000209	Protein polyubiquitination	BP
GO:0000151	Ubiquitin ligase complex	CC
GO:0004842	Ubiquitin-protein transferase activity	MF
GO:0061630	Ubiquitin protein ligase activity	MF
hsa04120	Ubiquitin mediated proteolysis	KEGG

The expression of UBC (1.8), HSP90B1 (3.48), and EP300 (5.6) genes was upregulated in shallow samples in August ($p > 0.05$). Yet, no significant change was recorded in the expression of the CDC16 gene in the shallow samples in August (Fig. 7) (Tables S1, S2, and S3 in supplementary).
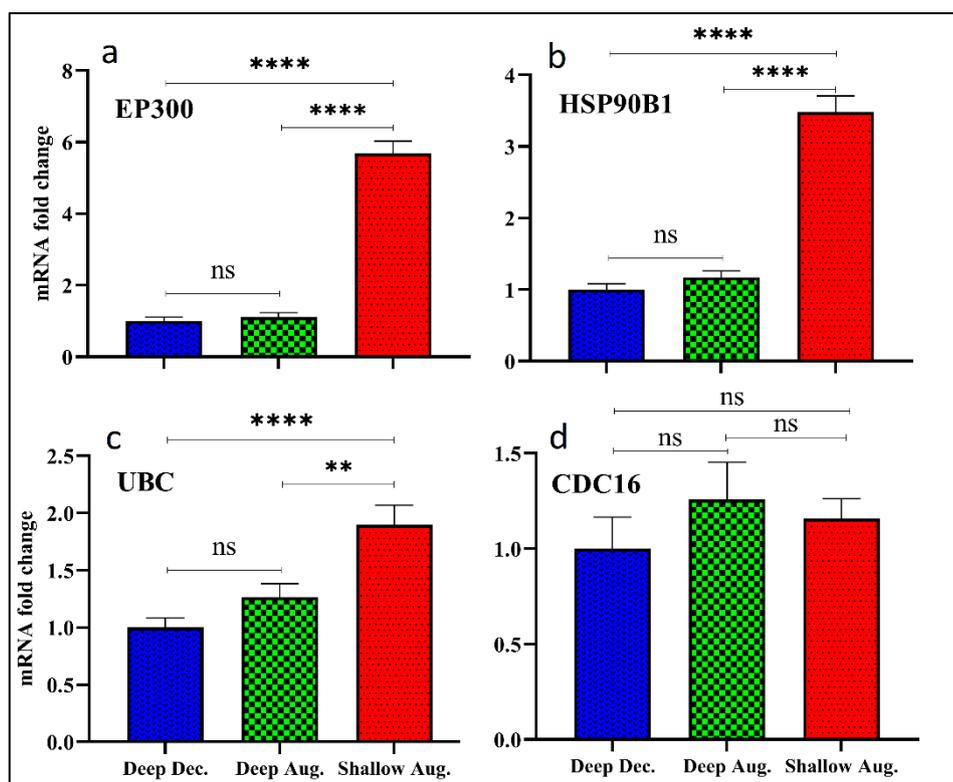
Figure 7*: Changes in the expression of EP300 (a), HSP90B1 (b), UBC (c) and CDC16 (d) genes in shallow and deep water corals in August (red, and green) and December (blue). Further details of these gene expression changes are provided in Table S2.*

Table S1: Summary results of ANOVA testing the difference in expression of selected genes between the warm and cold episodes in scleractinian coral 'Dipsastraea matthaii' .

Gene	Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F-value	P-value
CDC16	Treatment (between columns)	0.50	2	0.25	0.70	0.51
	Residual (within columns)	8.59	24	0.36		
	Total	**9.09**	**26**	**0.61**		
EP300	Treatment (between columns)	35.61	2	17.80	119.50	0.00
	Residual (within columns)	3.58	24	0.15		
	Total	**39.18**	**26**	**17.95**		
HSP90B1	Treatment (between columns)	17.27	2	8.63	70.14	0.00
	Residual (within columns)	2.95	24	0.12		
	Total	**20.22**	**26**	**8.76**		
UBC	Treatment (between columns)	3.93	2	1.96	15.05	0.00
	Residual (within columns)	3.13	24	0.13		
	Total	**7.06**	**26**	**2.09**		

Table S2: Expression levels of selected genes in scleractinian coral '*Dipsastraea matthaii*' during the warm and cold episodes in shallow and deep sites. Fold change=2$^{-\Delta\Delta Ct}$ was calculated compared to healthy corals collected from deep sites in December.

Gene	Site	August			December		
		N	SD	Fold change	N	SD	Fold change
HSP90B1	Deep site	9	1.17	0.27	9	1.00	0.25
	Shallow site	9	3.48	0.68	-	-	-
UBC	Deep site	9	1.27	0.36	9	1.00	0.25
	Shallow site	9	1.90	0.52	-	-	-
CDC16	Deep site	9	1.26	0.59	9	1.00	0.50
	Shallow site	9	1.16	0.31	-	-	-
EP300	Deep site	9	1.11	0.36	9	1.00	0.32
	Shallow site	9	5.69	1.03	-	-	-

Table S3: The result of the pairwise Tukey test comparing the expression of selected genes between the warm and cold episodes in scleractinian coral '*Dipsastraea matthaii*'. These results are based on fold change and log2 fold change in gene expression.

Gene	Comparison	Mean-diff.	P-value	Summary	Fold-change	Description
CDC16	Deep-August vs deep-November	0.33	0.48	ns	1.26	up: 1.257 fold
	Shallow-August vs deep-November	0.21	0.74	ns	1.16	up: 1.157 fold
	Shallow-August vs deep-August	-0.12	0.91	ns	0.92	down: -1.087 fold
EP300	Deep-August vs deep-November	0.15	0.69	ns	1.11	up: 1.109 fold
	Shallow-August vs deep-November	2.51	0.00	****	5.69	up:5.685 fold
	Shallow-August vs deep-August	2.36	0.00	****	5.13	up: 5.126 fold
HSP90B1	Deep-August vs deep-November	0.23	0.36	ns	1.17	up: 1.173 fold
	Shallow-August vs deep-November	1.80	0.00	****	3.48	up: 3.481 fold
	Shallow-August vs deep-August	1.57	0.00	****	2.97	up: 2.968 fold
UBC	Deep-August vs deep-November	0.34	0.14	ns	1.27	up: 1.265 fold
	Shallow-August vs deep-November	0.92	0.00	****	1.90	up: 1.897 fold
	Shallow-August vs deep-August	0.58	0.01	**	1.50	up: 1.499 fold

-ddct less (more) than 0 indicates a decrease (increase) in gene expression. **** $p<0.0001$, *** $p<0.001$. ** $p<0.01$, * $p<0.05$, ns (non-significant) $p>0.05$.

Discussion

Though the corals appear to have adapted to the relatively harsh environmental conditions (e.g., long extreme warming episodes) in the Persian Gulf, however, they have increasingly experienced bleaching over the past decades. This indicates that they may have been exposed to high temperatures and intense light intensities close to their upper thermal limits for several hours per day during warm

periods (July–August) of the year. In the present study, coral samples were collected in the natural environment and under intense sunlight.

In *D. matthaii* coral, structural analysis of the cognate network was used to select the most important genes involved in the temperature stress pathway. In this study, four series of microarray transcriptome and RNA-SEQ data were analyzed. The aim was to use gene expression studies related to molecular processes involved in temperature stress conditions and genes involved in responding to the organism's thermal stress to construct a network and subsequent analyses. The main goal was to build a network based on the knowledge-based method, which was eventually achieved by using various processes of preprocessing, normalization, and other related calculations. Data were analyzed using Cytoscape software and network centrality criteria were identified to identify genes and important connections in the path of temperature stress. In this study, proteins that have important topological properties in the network were introduced as candidate markers. Likewise, according to the results of co-expression network analysis, the expression of four genes (i.e., CDC16, UBC, HSP90B1, EP300) with the highest rank was investigated as important genes of temperature stress path in *D. matthaii* corals. Among the studied genes, two genes, UBC and CDC16, play important roles in protein ubiquitination/protein degradation. The polybiobicotin C gene is a stress-

protective gene under various stressful conditions, including exposure to UV radiation, heat shock, oxidative stress, and translational disorders. The UBC gene has also been reported to help maintain stable ubiquitin levels under physiological conditions. This is probably due to the increased demand for ubiquitin to remove critically folded proteins (Radici *et al*., 2013).

Consistent with the results of previous studies (Downs *et al*., 2000; Barshis *et al*., 2010, 2018; Maor-Landaw *et al*., 2014), the expression UBC gene was upregulated in corals collected from the shallow site in August and in tidal conditions and at the same time with the maximum daily temperature. This response may be due to increased stress levels due to protein damage. However, non-injury-related functions related to the transport and maintenance of cellular protein homeostasis may also lead to similar increases in gene expression (Hawkins 1991; Welchman *et al*., 2005).

The increase in ubiquitin in the corals of the shallow site compared to the deep site indicates higher stress levels, which are caused by fluctuations in the environmental conditions of the shallow site. Corals that are exposed to high circadian temperature changes are often more heat tolerant and more resistant to bleaching than their counterparts that live in stable environments (Barshis *et al*., 2018). Ubiquitin expression levels have been established as a cellular marker for protein health and response to cellular heat stress (Downs *et al*., 2000; Downs 2003). According to Barshis *et*

al. (2010, 2018), the increase in ubiquitin concentration observed in the back reef corals indicates higher stress levels resulting from dynamic fluctuations in the environmental conditions in the front reef. The UBC gene, on the other hand, has so far been identified as a gene responding the heat shock and therefore may be used as the target gene for HSF1. Increased transcription under prototoxic conditions (as a result of heat stress or proteasome inhibition) is mainly due to the binding of HSF1 to known HSEs in the UBC gene promoter. UBC gene transcription occurs during stress and provides the extra ubiquitin needed to destroy unwrapped proteins (Ryu *et al*., 2007; Bianchi *et al*., 2018). Heat shock elements within the UBC gene promoter have been identified that induce the UBC gene as a result of heat shock factor transcription and are activated by proteotoxic stress (Bianchi *et al*., 2018). Therefore, this gene can be reliable as a candidate gene (Vihervaara *et al*., 2013; Crinelli *et al*., 2015). The CDC16 protein belongs to the anaphase complex (APC), which controls cell progression through mitosis.

In the present study, no significant changes were observed in the expression of the CDC16 gene. Zhou and Rigaud (2001) suggested that decreased expression of the CDC16 gene after oxidative stress may delay the progression of damaged cells in mitosis, leading to a delay in mitotic phase progression. This delay occurs in adapted cells and allows them to repair faster and more efficiently, which

increases tolerance to stressful conditions (Zhou and Rigaud 2001). The high association of the CDC16 gene with other genes in the interaction network and the role of ubiquitin ligase protein, make this gene a key gene and can be used as an important candidate for gene studies and mechanisms of susceptibility of corals under stress.

HSP90 is a chaperone protein that, like other HSPs, plays an important role in the coral's response to heat shock and protein folding. HSP90 also plays a vital role in the cell cycle, signal transduction, and maintenance of cellular homeostasis. In the present study, at low tide conditions with maximum daily temperature, the shallow samples in August show increased expression of HSP90B1 heat shock protein. Previous studies have also reported that under temperature stress conditions, HSP90 mRNA expression was significantly higher than under control conditions (Desalvo *et al*., 2008; Meyer *et al*., 2011; Leggat *et al*., 2011; Kenkel *et al*., 2011; van de Water *et al*., 2018; Skutnik *et al*., 2020).

In the early stages of heat stress, HSP90 gene expression increases due to increased cell damage, which translates to HSP90 adapting to environmental stress (Hao and Gu 2014). In corals exposed to heat stress, it is beneficial for HSP90 to stimulate the mitochondria to produce more ATP, thereby helping to regulate cellular metabolism (Skutnik *et al*., 2020). Similarly, the presence of protective HSP90 during stressful conditions Chloroplasts, possibly associated with symbiosis, allow corals

to achieve a steady state of energy until environmental stress subsides (Skutnik et al., 2020). Along with the production and transport of proteins and carbohydrates, an increase in HSP90 maintains the function of the endoplasmic reticulum during heat tolerance by the Holobiont (Skutnik et al., 2020). According to Skutnik et al. (2020), increasing the concentration of HSP90 is to maintain homeostasis, which indicates the normal functioning of the cell despite the presence of stress. On the other hand, an increase in HSP90 in this study could indicate the occurrence of cell degradation and protein denaturation based on HSP90 renature function (Skutnik et al., 2020).

In the present study, the expression of the EP300 transcription coagulator gene was also increased in shallow samples in August, which could be the response of this gene to severe environmental stress at noon. This increase is related to transcriptional activation by connecting to transcription factors and the transcription system. Important features of this response are the expression of molecular chaperones as a result of activation of the transcription factor HSF1. This involves the acetylation of vital functional lysines during stress, which leads to the activation of HSF1 (Raychaudhuri et al., 2014).

In summary, in corals sampled under midday environmental stress and tidal conditions, the cytosolic response to stress is commonly known as the heat shock response. This response is one of the main mechanisms of cell defense by cells to maintain protein stability (proteostasis) when exposed to prototoxic conditions against heat shock, which activates the HSF1 transcription factor by increasing EP300. HSF1, on the other hand, activates heat shock proteins such as HSP90B1, which are mainly molecular chaperones that prevent the accumulation of malleable proteins and cause them to re-fold or be destroyed. HSF1, on the other hand, activates UBC, which removes badly digested proteins along the path of the ubiquitin proteolysis system. Finally, the nuclear proteasome system, activated by HSF1 degradation, reduces the stress response in a way related to the removal of defective proteins by the ubiquitin-proteasome system.

In addition to the similarity in the communication pattern, the modules tend to have genes with similar functions. The results of the functional analysis confirmed to a large extent that each of the modules that make up the network had similar functional and path patterns between their genes. For example, module 1 in the PPI network constructed is often involved in the Ubiquitin mediated proteolysis process. Most of the terms used in GO for this module are biological processes related to ubiquitin. The results of the GO analysis also confirmed the fact that the different modules each have specific functions and are somehow functionally in separate groups.

It should be noted that corals in the deep site face fewer thermal and light changes in hot periods than shallow water corals. In addition, the displacement of subsurface waters

during high tide causes a significant cooling and decrease in water temperature at this depth. Therefore, this part of the Persian Gulf seems to be a favorable habitat for corals under normal and stable temperature conditions. In the coming decades, heat waves are predicted to be more intense, more frequent, and more continuous than those observed in previous decades (IPCC 2014), a phenomenon that could cause corals to experience environmental stresses that exceed their heat threshold.

Conclusion

The reefs across the northern Persian Gulf live in better environmental conditions including lower temperature, lower salinity, better aragonite saturation, and greater depth that has led to increasing species richness towards the Straits of Hormuz (Riegl and Purkis, 2015). There might be further intrinsic mechanisms that have been developed by each scleractinian coral species to successfully thrive in the less optimal condition of the Gulf. The present study investigated the changes in expression of candidate genes [i.e., Cell Division Cycle 16 (CDC16), Ubiquitin C (UBC), Heat Shock Protein 90 Beta Family Member 1 (HSP90B1), Histone acetyltransferase p300 (EP300)] in a thermally resilient scleractinian coral 'Dipsastraea matthaii' as a conceivable tool for adapting anomalous thermal stress events. The results demonstrated a significant increase in the expression of UBC, HSP90B1, and EP300 genes in response to rising water temperature.

The expression of molecular chaperones such as HSP90B1 occurred as a result of activation of the transcription factor HSF1 leading to UBC activation. Ubiquitin C removes critically digested proteins along the path of the ubiquitin proteolysis system.

Importantly, these findings suggest that gains in the heat tolerance of the studied coral species have a genetic basis. This may further indicate that the thermally resilient scleractinian corals living in the Persian Gulf can facilitate the evolution of coral populations to rapid climate warming. This can be accomplished by interspecific hybridization between the corals from the Gulf with those elsewhere in the world to produce genotypes with enhanced climate resilience.

Data availability

Data are, however, available from the authors upon reasonable request from the corresponding author at Shahid Beheshti
University, Tehran, Iran.

Statements and Declarations

Competing Interests: The authors declare no competing interests.

Highlights

- The coral *Dipsastera matthaii* responds to thermal stress by changing the expression of its genes
- The expression of UBC, HSP90B1, and EP300 was upregulated in response to heat stress
- The EP300 enhancement was triggered by activation through binding to transcription factors
- Anthropogenic ocean warming poses significant threats to shallow coral reefs, occasionally exceeding their thermal tolerance limits.
- Certain hard coral species exhibit thermal resilience and can swiftly adapt to anomalous thermal stress by regulating heat stress-related genes.
- Comparative microarray analysis identified candidate genes associated with temperature stress.
- Four specific genes—Cell Division Cycle 16 (CDC16), Ubiquitin C (UBC), Heat Shock Protein 90 Beta Family Member 1 (HSP90B1), and Histone acetyltransferase p300 (EP300)—were validated as responsive to temperature stress in the scleractinian coral *Dipsastera matthaii* from the Persian Gulf.
- Real-time PCR analysis during warm and cold periods confirmed significant changes in UBC, HSP90B1, and EP300 expression in response to heat stress.

- EP300 enhancement resulted from transcription activation via binding to transcription factors.
- Activation of heat shock factor 1 (HSF1) led to the upregulation of molecular chaperones, including HSP90B1.
- HSF1 also played a role in activating UBC, which aids in removing critically digested proteins through the ubiquitin proteolysis system.
- These findings contribute to identifying potential candidate genes that could serve as valuable biomarkers for the selection of heat-tolerant genotypes.

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