Gene networks governing the response of a calcareous sponge to future ocean conditions reveal lineage-specific *XBP1* regulation of the unfolded protein response

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Keywords: Climate change, Calcarea, UPR, *XBP1*, gene duplication

**Abstract**

Marine sponges are predicted to be winners in the future ocean due to their exemplary adaptive capacity. However, while many sponge groups exhibit tolerance to a wide range of environmental insults, calcifying sponges may be more susceptible to thermo-acidic stress. To describe the gene regulatory networks that govern the stress response of a calcareous sponge, *Leucetta chagosensis* (class Calcarea, order Clathrinida), individuals were subjected to warming and acidification conditions based on the climate models for 2100. Transcriptome analysis and gene co-expression network reconstruction revealed that the unfolded protein response (UPR) was activated under thermo-acidic stress. Among the upregulated genes were two lineage-specific homologs of X-box binding protein 1(*XBP1*), a transcription factor that activates the UPR. Alternative dimerization between these *XBP1* gene products suggests a clathrinid-specific mechanism to reversibly sequester the transcription factor into an inactive form, enabling the rapid regulation of pathways linked to the UPR in clathrinid calcareous sponges. Our findings support the idea that transcription factor duplication events may refine evolutionarily conserved molecular pathways and contribute to ecological success.

**Introduction**

Marine calcifying organisms that build calcite or aragonite skeletons are potentially vulnerable to changes in seawater carbonate chemistry (Harvey, et al. 2013). Historical data show that increasing ocean *p*CO2 over the past 40 000 years has caused the notable decline in calcification of the dominant coccolithophore, *Emiliania huxleyi* (Beaufort, et al. 2011). Exposure to acidified conditions (pH 7.4) resulted in arrested spine development and impaired biocalcification in the juvenile sea urchin, *Heliocidaris erythrogramma* (Wolfe, et al. 2013). Ocean warming and acidification (31oC, pH 7.55) negatively affected the net calcification rates of scleractinian corals, *Pocillopora damicornis* and *Stylophora pistillata*, due to reduced calcifying fluid pH and aragonite saturation state (Guillermic, et al. 2021). Despite these alarming reports, responses of other key marine calcifiers, such as calcareous sponges, remain poorly understood.

Sponges (phylum Porifera) are sessile aquatic invertebrates characterized by a simple filter-feeding bauplan. They are one of the oldest multicellular animals, having diverged from other metazoans during the Precambrian around 600 MYA (Yin, et al. 2015). Due to their early-branching position, sponges have been instrumental in elucidating the origins and early evolution of animal complexity (Taylor, et al. 2007; Pick, et al. 2010; Philippe, et al. 2011). Marine sponges are critical members of the benthos, which play major functions in ecological processes such as in ecosystem structuring, reef consolidation, bio-erosion, and nutrient cycling (Bell 2008). A comprehensive review of climate change-associated studies of abundant benthic groups proposed that sponges are to be winners under future climate scenarios (Bell, et al. 2018). Their remarkable ecological success is attributed to their simple yet highly adaptable body plan (Carballo and Bell 2017). There are four morphologically distinct extant sponge classes (Demospongiae, Hexactinellida, Homoscleromorpha, and Calcarea) (Van Soest, et al. 2012). Calcareans are conspicuously distinguished from other sponge groups by their synapomorphic calcified structures (Sethmann and Wörheide 2008). They are grouped into two monophyletic subclasses: the Calcinea (composed of order Clathrinida) and the Calcaronea (composed of order Baeriida, Leucosolenida, and Lithonida) (Manuel, et al. 2003). Class Calcarea is comprised of 675 recognized extant species, divided among 75 genera and 22 families (Boury-Esnault, et al. 2014).

Although calcareous sponges are thought to be more vulnerable under thermo-acidic stress due to their calcium carbonate skeletal elements, lower microbiome diversity, and rapid suppression of host immune functions under stress (Posadas, et al. 2021), other recent findings show that some calcaronean calcisponges such as *Leucosolenia complicata* and *Paraleucilla magna* can tolerate low pH conditions (Peck, et al. 2015; Ribeiro, et al. 2023). To date, however, only two studies have examined the combined effects of warming and acidification stress in the microbiome and spicules of a calcareous sponge (Ribeiro, et al. 2020; Posadas, et al. 2021). This underscores the need to conduct further experiments to investigate the compounded impacts of thermo-acidic stress on other calcarean representatives and to identify other processes that may underpin the stress response and adaptability of calcareous sponges.

To describe the molecular mechanisms underlying the stress response of a calcareous sponge under future ocean conditions, we sequenced the transcriptomes of *Leucetta chagosensis* (class Calcarea, order Clathrinida, family Leucettidae) that were subjected to elevated temperature and reduced pH treatments. This revealed activation of the unfolded protein response (UPR) network under thermo-acidic stress and the potential role of alternative dimerization of two lineage-specific homologs of the X-box binding protein 1(*XBP1*) in the rapid regulation of UPR in clathrinid calcareous sponges. Findings of the present study highlight how gene duplication events may fine-tune evolutionarily conserved molecular pathways and contribute to ecological success.

**Materials and Methods**

**Sponge collection and culture**

The work described here is part of a larger study investigating the response of the *L. chagosensis* holobiont to thermo-acidic stress (Posadas, et al. 2021). Six specimens of *L. chagosensis* were collected from the Bolinao-Anda Reef Complex in Pangasinan, northwestern Philippines (16.296° N, 120.014° E) with permission from the Philippines Department of Agriculture (Gratuitous Permit No. 0169-19). Sponge identities were confirmed by their morphology (Hooper and Van Soest 2002) and 28S ribosomal RNA (rRNA) gene analyses (Posadas, et al. 2021). Donor sponges were cut into 12 (≈1 cm3) fragments using a sterile razor and allowed to heal *in situ* for 30 days. Healed fragments were brought to the Bolinao Marine Laboratory and reared for seven days in aquaria receiving flow-through seawater under ambient conditions (pH 8.0 and 28 °C).

**Stress response experiments**

Stress response experiments were conducted as previously described (Posadas, et al. 2021). Briefly, conditions were designed to simulate the present day and predicted 2100 Representative Concentration Pathway (RCP) 6.0 and 8.5 scenarios (Pachauri, et al. 2014). Treatment conditions include: (i) pH 8.0, 28 °C (Present Day)­, (ii) pH 7.6, 28 °C (Acidification), (iii) pH 8.0, 32 °C (Warming), (iv) pH 7.8, 30 °C (RCP 6.0), and (v) pH 7.6, 32 °C (RCP 8.5). Temperatures were regulated using 300 W submersible heaters (EHEIM GmbH & Co. KG, Baden Wurttemberg, Germany), levels of injected CO2 were manipulated using a mass flow controller, and illumination was provided by daylight LED lamps following a 12:12 light:dark photoperiod. Monitoring of physico-chemical parameters in treatment tanks is described in Supplementary Methods.

Each treatment was represented by four independent replicate aquaria containing three fragments, with each fragment originating from a different sponge donor (n = 12 fragments per treatment). Temperature and pH levels were changed gradually (temperature: +1°C/day, pH: −0.5/day) until the desired conditions were reached. Treatment conditions were maintained for two days then the experiment was terminated because tissue necrosis had begun to manifest in some fragments, with visible whitening and disintegration of tissues. Surviving sponges were washed with ultraviolet-filtered seawater, any necrotic tissues were excised, and the remaining healthy tissues were flash-frozen in liquid nitrogen for transport, then stored at −80 °C.

**Transcriptome sequencing, assembly, and annotation**

Transcriptome assembly for *L. chagosensis* was initially reported in (Posadas, et al. 2021). Total RNA of sponge individuals that were collected after two days of sustained exposure to the treatments was extracted from 50 to 100 mg of tissues using TRIzol (Invitrogen, Waltham, MA, USA) following the manufacturer’s protocol. Libraries were prepared from three samples per treatment, except for the Warming and RCP 6.0 treatments, for which only two samples each had high-quality RNA extracts. Replicate samples for each treatment were selected from different donor sponges. Barcoded libraries were prepared at Macrogen, South Korea, using the Truseq RNA Library Preparation Kit (Illumina, Inc.). mRNA-enriched libraries were sequenced on the Novaseq 6000 platform (Illumina, Inc.) to generate 100 bp paired-end reads. Raw sequence reads can be accessed from the NCBI Short Read Archive database under BioProject PRJNA689294.

*De novo* transcriptome assembly was carried out using Trinity (Haas, et al. 2013). The non-redundant reference transcriptome was generated through sequence clustering (90% similarity) and isoform selection (see Supplementary Methods). Quality and completeness of the assemblies were assessed through Bowtie 2 v2.3.5.1 (Langmead and Salzberg 2012), Detonate (Li, et al. 2014), TransRate v1.0.3 (Smith-Unna, et al. 2016), and BUSCO v3.1.0 (Simao, et al. 2015). The reference transcriptome was then annotated by aligning against the UniProtKB/Swiss-Prot database (April 2020) with an e-value cut-off of 1x10-5. The reference transcriptome used in this study is available at DDBJ/EMBL/GenBank under the accession number GIYV00000000.

Peptides were predicted using the Transdecoder package in Trinity and annotated by alignment against the GenBank non-redundant (nr) sequence and UniProtKB/Swiss-Prot databases with an e-value cut-off of 1x10-5. The top Blastp hit for each peptide was used as input into OmicsBox (BioBam, Valencia, Spain) (Conesa and Gotz 2008) to predict gene ontology (GO) annotations. Protein domains were identified by mapping the peptide sequences against Pfam 32.0 database (Finn, et al. 2014) using HMMER v3.3 (Eddy 1998). The top Blastx and Blastp hits in UniProtKB/Swiss-Prot database and Pfam annotations for each transcript were then used as input into Trinotate v3.2.2 (Haas 2015) to generate a comprehensive annotation report. GO annotations generated in OmicsBox and Trinotate were merged to improve the annotation rate.

**Sequence similarity, ortholog analysis, and gene content comparison**

Pairwise sequence comparisons between *L. chagosensis* and other sponge species were performed using Blastp alignments at an e-value cutoff of 1 × 10−5. A total of 18 sponge species representing the four poriferan classes were included in the analysis (Table S1). These include demosponges (*Amphimedon queenslandica* Hooper & van Soest, 2006 (Srivastava, et al. 2010), *H. tubifera* (Guzman and Conaco 2016a), *Petrosia (Petrosia) ficiformis* (Poiret, 1789) (Riesgo, et al. 2014), *X. testudinaria*, *S. carteri* (Ryu, et al. 2016), *Aplysina aerophoba* (Nardo, 1833), *Dysidea avara* (Schmidt, 1862) (Pita, et al. 2018), *Ephydatia muelleri* (Lieberkühn, 1856) (Kenny, et al. 2020), *Neopetrosia compacta* (Ridley & Dendy, 1886) (Posadas, et al. 2021)), calcareans (*Sycon ciliatum*, *L. complicata* (Montagu, 1814) (Fortunato, et al. 2014), *Grantia compressa* (Fibricius, 1780), *Pericharax orientalis* van Soest & De Voogd, 2015, *Clathrina sp.* (Voigt, et al. 2021)), homoscleromorphs (*Oscarella carmela* Muricy & Pearse, 2004, *Corticum candelabrum* Schmidt, 1862), and a hexactinellid (*Aphrocallistes vastus* (Schulze, 1886)). Gene repertoire of *L. chagosensis* were compared with other sponge species (Supplementary Methods).

Orthologous gene families in the transcriptome of *L. chagosensis* and in the genomes or transcriptomes of other sponge species were identified using OrthoFinder (Emms and Kelly 2019). Intersections of orthologous groups (OGs) across different species were visualized using the UpSetR package in R (Conway, et al. 2017).

**Identification of gene regulatory elements in *L. chagosensis***

Transcription factors (Bahrami, et al. 2015), as well as epigenetic modifiers, including histone modifying enzymes (Lee and Workman 2007; Kooistra and Helin 2012; Seto and Yoshida 2014; Yi, et al. 2015) and components of DNA methylation machinery (de Mendoza, et al. 2019), were identified in the *L. chagosensis* transcriptome based on their characteristic domains and top Blastp hit (e-value < 1x10-5) against the UniProtKB/Swiss-Prot database.

**Differential gene expression analysis**

Transcript abundance was estimated by mapping reads to the reference transcriptomes using RNA-Seq by Expectation Maximization (Li and Dewey 2011) with bowtie2 alignment (Langmead and Salzberg 2012). DEGs were identified using the edgeR (Robinson, et al. 2010) package in R. Generalized linear model functionality for likelihood ratio testing method, which is recommended for datasets with few replicates (Robinson, et al. 2010), was applied. Expected counts were converted to counts per million (CPM) and only genes (n = 22 417) with > 2 CPM in at least two libraries were included in edgeR analysis. This filtering step was done to remove lowly expressed genes (< 10 counts). Genes were considered differentially expressed if upregulation or downregulation was ≥ 4-fold relative to the controls with a False Discovery Rate (FDR) ≤ 0.05. Pairwise comparisons were conducted between the Present Day samples and samples subjected to the other treatments.

**Gene co-expression network analysis**

Gene co-expression networks were reconstructed using rlog-transformed expected counts through WGCNA (Langfelder and Horvath 2008) implemented in R. WGCNA identifies sets of genes, termed as modules, which have similar expression patterns within treatments across experimental samples. Lowly expressed genes (< 10 count in at least two libraries) were filtered out prior to the analysis. Only 22 417 *L. chagosensis* genes were used for network construction, with the following parameters: softPower = 30 and minModuleSize = 30. To capture both putative activators and repressive factors, a combination of signed and unsigned methods csuWGCNA (Dai, et al. 2019) was implemented in the network analysis. Further clustering (cutHeight=0.60) was applied to generate the final set of modules.

The general expression pattern of gene sets can be represented by the module’s eigengene. Eigengene can then be correlated with external traits. In the present study, significant correlations were used to identify modules that are associated with the five treatments to capture the full set of co-expressed genes representing molecular pathways involved in the response of *L. chagosensis* to warming and acidification stress. Hub genes, which are significantly associated with a specific treatment (gene significance (GS) ≥ |0.2|) and are highly co-expressed (module membership (MM) > |0.8|) within a module, were identified and used in downstream analyses. GS is the absolute value of the correlation between an individual gene and the treatment of interest, while MM is the correlation of the individual gene’s expression with the module eigengene (Langfelder and Horvath 2008).

**Pfam, gene ontology, and pathway enrichment analysis**

Pfam and GO enrichment analyses for DEGs and module members were performed using a script (github.com/fle1/canolab\_scripts/blob/master/Pfam\_enrichment.R) and the topGO package (Alexa and Rahnenführer 2009) implemented in R. Only Pfam domains and GO terms with *p*-value < 0.05 were considered significantly enriched.

Predicted peptides of *L. chagosensis* were searched against the human proteome v.11.5 from STRING v.11 database (Mering, et al. 2003) with an e-value cut-off of 1x10-5. Blastp top hits for each hub gene were used as input in the KEGG pathway enrichment analysis. Protein–protein interactions of genes involved in the identified enriched pathways (score > 0.400 and FDR < 0.01) were retrieved from the STRING v.11 database (Mering, et al. 2003). Interaction networks were visualized using Cytoscape v.3.7.2 (Shannon, et al. 2003). Relative expression of sponge gene homologs in RCP 8.5 relative to the Present Day control was computed as the average sum of transcripts per million (TPM).

**Phylogenetic analysis of *XBP1* homologs**

*HAC1*/*bZIP60*/*XBP1* homologs were identified from the sponge predicted peptides, yeast (*Saccharomyces cerevisiae*), plant (*Arabidopsis thaliana*), and other metazoans by searching for Blastp matches (e-value ≤1 × 10−5) with *XBP1* sequences in UniProtKB/Swiss-Prot database. Metazoan *XBP1* sequences included in the analysis are from ctenophores (*Mnemiopsis leydii* and *Pleurobrachia bachei*), placozoan (*Trichoplax adhaerans*), coral (*Acropora digitifera*), sea anemone (*Nematostella vectensis*), freshwater polyp (*Hydra magnipapillata*), leech (*Helobdella robusta*), polychaete (*Capitella telata*), limpet (*Lottia gigantea*), water flea (*Daphnia pulex*), red flour beetle (*Tribolium castaneum*), fruit fly (*Drosophila melanogaster*), nematodes (*Pristionchus pacificus* and *Caenorhabditis elegans*), sea urchin (*Strongylocentrotus purpuratus*), sea squirt (*Ciona intestinalis*), lancelet (*Branchiostoma floridae*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), cattle (*Bos taurus*), and human (*Homo sapiens*). Predicted peptide sequences were downloaded from Ensembl Metazoa.

Amino acid sequences corresponding to the bZIP\_1 domain (PF00170) of *HAC1*/*bZIP60*/*XBP1* homologs were used for phylogenetic comparisons. Multiple sequence alignment was performed using Clustal Omega (Madeira, et al. 2019) and the aligned sequences were manually trimmed (Data S1). The best-fit substitution model (LG+I+G) was identified based on Bayesian Information Criterion using prottest v3.4.2 (Darriba, et al. 2011). Bayesian inference analysis was performed in MrBayes v.3.2 (Ronquist and Huelsenbeck 2003) with two-independent MCMC runs and four chains per run. The analysis was sampled every 100 trees until the average standard deviation of split frequencies was < 0.01. The first 25% of trees were discarded as burn-in. Expression of *XBP1* homologs in untreated adult tissues of the leucettids, *L. chagosensis* and *P. orientalis,* was determined based on TPM values from transcriptome data (Voigt, et al. 2021).

**Identification of *XBP1* dimer pairs**

Leucine zipper heptads of *LchaXBP1, PoriXBP1,* and *CspXBP1* homologs were manually evaluated to identify possible dimerization pairs. Heptads (L0-L5) were grouped (*gabcdef*) to visualize amino acids in the *a*, *d*, *e*, and *g* positions, which regulate dimerization stability and specificity of bZIP TFs (Fig. S1). The complementary *a* ↔ *a’* and *d* ↔ *d’* interactions create a hydrophobic core that is essential for dimer stability (Thompson, et al. 1993) while electrostatic interactions between *g* ↔ *e’* pair can either be attractive or repulsive (Cohen and Parry 1990). Attractive basic-acidic interactions include Arg ↔ Glu and Lys ↔ Glu while Glu ↔ Arg, Glu ↔ Lys, Asp ↔ Arg, and Asp ↔ Lys are attractive acidic-basic interactions. Glu ↔ Glu, Glu ↔ Asp, Glu ↔ Gln, and Gln ↔ Glu form acidic repulsive interactions, whereas basic repulsive interactions include Lys ↔ Lys, Arg ↔ Lys, Gln ↔ Lys, Arg ↔ Gln, and Lys ↔ Gln (Vinson, et al. 2002).

**Prediction of DNA binding potential of *XBP1* dimer pairs**

Predicted 3D models of *XBP1* dimer pairs were built through SWISS-MODEL (Waterhouse, et al. 2018). Best-hit structural homologs based on the QMEANDisCO score were used as templates for predicting the protein structure. QMEANDisCO is a composite scoring function derived from the entire structure and per residue quality estimates (0, lowest - 1, highest). The binding potential of the predicted dimer pairs was then tested through *in silico* docking experiments. The CRE DNA substrate containing ‘aureo-box’, TGACGT, (Banerjee and Mitra 2020) from CREB structure PDB ID: 1DH3 (Schumacher, et al. 2000) was used as the ligand in the docking simulations through pyDockDNA (Rodriguez-Lumbreras, et al. 2022). *XBP1* preferentially binds to CRE-like elements in which the core “ACGT” is highly conserved (Clauss, et al. 1996). The best-scoring complex structures based on pyDock (Rodriguez-Lumbreras, et al. 2022) and FTDOCK (Gabb, et al. 1997) algorithms were visualized and post-processed in Pymol (DeLano 2002) and PDBePISA (EMBL‐EBI) to identify *XBP1* - CRE interfaces.

**Visualization**

All visualizations were done using ggplot2 (Wickham 2016) in R. Phylogenetic trees were edited in iTOL (Letunic and Bork 2019).

**Results**

To describe the responses of a calcareous spongeto future ocean conditions, *L. chagosensis* individuals were subjected to stress response experiments with warming and acidification treatment conditions based on the climate models for 2100: (i) pH 8.0, 28 °C (Present Day)­, (ii) pH 7.6, 28 °C (Acidification), (iii) pH 8.0, 32 °C (Warming), (iv) pH 7.8, 30 °C (RCP 6.0), and (v) pH 7.6, 32 °C (RCP 8.5). As described in our previous study, *L. chagosensis* exhibited visible tissue necrosis (i.e., whitening and disintegration of tissues) under the Warming, RCP 6.0, and RCP 8.5 conditions, but not in the Acidification only treatment, and only 25% (3 out of 12) of the *L. chagosensis* fragments survived in the RCP 8.5 treatment after just two days of sustained exposure (Posadas, et al. 2021).

**Generating a reference transcriptome for *L. chagosensis***

Here, we sequenced the transcriptome of *L. chagosensis* (Fig. 1A) on the NovaSeq 6000 platform, generating an average of 19 299 672 clean 100 bp paired-end reads (Table S2). *De novo* transcriptome assembly rendered 248 731 total transcripts. The non-redundant transcriptome, following filtering through isoform selection and sequence clustering, is composed of 91 886 (N50 = 1 409; Ex90N50 = 2 463) transcripts (Table S3). The largest contig is 46 605 bp while the smallest contig is 300 bp long. The transcriptome has 45.94% GC content, 99.20% of all bases are covered by reads, and 87.29% of reads mapped back to the assembly. Assembled transcripts were translated into 44 538 peptides (Table S4). Ortholog benchmarking indicates that the transcriptome contains 93.40% and 90.80% of the eukaryotic and metazoan core genes, respectively.

The gene repertoire of *L. chagosensis* is comparable to other calcareous sponges (Table S5) with 538 orthogroups (904 *L. chagosensis* genes) present among calcareans, and 412 (1 433 *L. chagosensis* genes) and 455 (2 332 *L. chagosensis* genes) orthologous gene sets shared among clathrinids and leucettids, respectively (Fig. 1B). 1 401 putative species-specific genes, assigned to 205 orthogroups, were also identified in the assembly. LDA-LEfSe and homolog search revealed that calcarean genomes or transcriptomes are relatively enriched for genes involved in transcriptional control (Fig. 1C-D, Table S6-9, Fig. S2-3, Supplementary Results).

**Transcriptome of *L. chagosensis* is dynamic under stress**

Principal component analysis (PCA) and pairwise comparisons of transcriptome profiles of control (Present Day) and treated samples revealed that the host transcriptome of *L. chagosensis* is dynamic under stress (Fig. 2A). Two PC axes explained 70.50% of variance, with the first axis (42.12%) and second axis (28.38%) correlating to the effects of pH and temperature, respectively. The number of differentially expressed genes (DEGs) across treatments varied, with the highest in RCP 8.5 (n = 1 194), followed by RCP 6.0 (n = 766), Warming (n = 454), and Acidification (n = 80) (Fig. 2B). A notable number of upregulated genes across the four treatments have detectable paralogs (Acidification = 48, Warming = 163, RCP 6.0 = 244, RCP 8.5 = 270) (Fig. 2C) and are species-specific (Acidification = 12, Warming = 80, RCP 6.0 = 122, RCP 8.5 = 170) (Fig. S4).

**Gene regulatory networks governing the sponge stress response**

To describe gene regulatory networks (GRNs) that govern the calcareous sponge stress response, highly co-expressed genes (i.e., gene modules) in *L. chagosensis* were identified through correlation network analysis (Fig. 2D). Four gene modules were positively correlated with the tested conditions (Fig. 2E). Modules L3 and L5 were significantly associated with Present Day (R2 = 0.76, *p*-value= 0.002) and RCP 8.5 (R2 = 0.61, *p*-value = 0.03), while modules L1 and L4, although not statistically supported, were correlated with RCP 6.0 (R2 = 0.16, *p*-value = 0.6) and Warming (R2 = 0.52, *p*-value = 0.07), respectively. Eigengene expression for each module shows that L3 (n = 7 582) and L5 (n = 5 803) are sets of genes that were generally downregulated and activated with stress, respectively (Fig. S5). Module L1 (n = 5 282) and L4 (n = 2 121) are comprised of genes that are most expressed at RCP 6.0 and Warming treatments.

Hub genes, which are genes that show significant association with a treatment (gene significance (GS) ≥ |0.2|) and are highly co-expressed with other module members (module membership (MM) > |0.8|), likely serve as regulatory components of transcriptional networks (Fabina, et al. 2013). Module L3 has 2 577 hub genes, L5 consists of 1 849 hub genes, L1 has 316 hub genes, and module L4 is composed of 398 hub genes (Fig. 2E, Table S10). Of the hub genes, L3 = 1 584 (61.46%), L5 = 1 346 (72.08%), L1 = 111 (35.13%), and L4 = 176 (44.22%) were annotated with STRING v.11 database (Table S10). We focused on these hub genes in subsequent analyses.

**Pathways activated or repressed under stress**

KEGG pathway enrichment analysis revealed that L3 (downregulated) hub genes are enriched for players involved in cell growth and death (hsa4110: cell cycle), replication and repair (hsa3030: DNA replication), transport and catabolism (hsa4142: lysosome, has4144: endocytosis), glycan metabolism (hsa531: glycosaminoglycan degradation), lipid metabolism (hsa600:sphingolipid metabolism), and cellular community (hsa4510: focal adhesion, hsa4520: adherens junction) (Fig. 2F). Repression of these basic cellular and metabolic processes, as well as ECM components, suggest that *L. chagosensis* is susceptible to prolonged exposure to thermo-acidic stress, as predicted for other calcareous sponges (Smith, et al. 2013; Guzman and Conaco 2016b; Bell, et al. 2018). In fact, genes implicated in transcriptional control, cell proliferation and extracellular matrix maintenance, biocalcification, metabolism, stress response, and innate immunity were differentially regulated under RCP 8.5 (Table S11). These include downregulation of epithelial and focal adhesion components [collagen (*CO4A1*, *CO1A2*,*CO6A5*, *CTHR1*, *COLL2*, and *COLL5*), integrin (*ITB6*), fibrillin (*FBN2* and *FBN3*), and mucin (*MUC5B* and *MLP*)], along with biocalcification genes, such as carbonic anhydrases (*CAH1*, *CAH2*, and *CAH7*) and bicarbonate transporter proteins (Table S12-13), which may have compromised the integrity of *L. chagosensis* physical defenses and structural support under thermo-acidic conditions.

L5 (upregulated) hub genes are enriched for pathway components engaged in transport and catabolism (hsa4137: mitophagy and hsa4140: autophagy), translation (hsa970: aminoacyl-tRNA biosynthesis), and protein folding, sorting, and degradation (hsa4141: protein processing in endoplasmic reticulum, hsa3050: proteasome, hsa4120: ubiquitin-mediated proteolysis) (Fig. 2F). These pathways are part of or linked to the unfolded protein response (UPR), a well-orchestrated network of processes aimed to restore normal endoplasmic reticulum (ER) functions under ER stress (ERS) (Karagoz, et al. 2019). The ER serves as a protein-folding factory, where secretory proteins undergo chaperone-assisted folding to acquire their appropriate 3D conformations (Schwarz and Blower 2016). Protein folding is a highly error-prone process (Hebert and Molinari 2007), which is aggravated under stressful conditions when the demands for protein folding exceed the capacity of the system (Malhotra and Kaufman 2007; Liu and Howell 2010). The UPR detects the build-up of mutant proteins and enforces adaptive mechanisms by optimizing rates of protein synthesis and folding, as well as setting off ER-associated protein degradation (ERAD) pathways (Walter and Ron 2011).

ERS is implicated in the development of several human pathologies (Marciniak and Ron 2006; Lin, et al. 2008). Intriguingly, we found that along with the components of UPR, genes and pathways that are associated with multiple neurodegenerative diseases are enriched in module L5 (Fig. S6, Supplementary Results). This coordinated activation may be further explored to point out ancestral molecular targets linking environmental etiologies to the development and progression of neurodegenerative diseases.

**Activation of the unfolded protein response adaptive phase under stress**

The interaction network of genes involved in folding, sorting, and degradation (hsa4141, hsa3050, and hsa4120), referred hereafter as the “UPR interaction network”, is part of the adaptive or pro-survival phase of the UPR (Fig. 2G). Upregulation of molecular chaperones (*HSP90B1*, *HSPA5, CALR*, *P4HB*, *HSP90AA1, PDIA6, BCAP31*, and *HYOU1*) and co-chaperones (*DNAJA2, DNAJB12, DNAJA1, DNAJB11, DNAJC10, DNAJC5B, DNAJC3, HSPH1,* and *SIL1*), may serve to enhance the capacity for protein folding (Walter and Ron 2011). While generally linked to protein maturation, folding, structural maintenance, and transport, chaperones also play critical roles in eliminating mutant proteins. For instance, *DNAJB12* and *DNAJB11* bind directly to unfolded proteins and divert them to ERAD pathways (Jin, et al. 2009).

N-linked oligosaccharides attached to nascent proteins also function in protein folding and serve as tags for selection of ERAD substrates (Moremen and Molinari 2006). Module L5 is enriched with genes involved in N-glycan processing (GO6491) and protein glycosylation (GO6486) (Table S14). These include dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunits (*RPN1, RPN2, STT3A, STT3B,* and *DDOST*), which tag nascent polypeptide chains with triglycosyl moiety (Glc3Man9GlcNAc2), as well as glucosidases (*PRKCSH* and *GANAB*) (Fig. 2G), which convert triglycosylated to monoglycosylated proteins (GlMan9GlcNAc2) (Xu and Ng 2015). Protein folding is facilitated by repeated interactions of monoglycosylated proteins with lectin chaperones such as calreticulin (*CALR*) (Ruggiano, et al. 2014; Tax, et al. 2019), which also increased in expression. *CALR*, along with other upregulated lectins (e.g., protein ERGIC-53 (*LMAN1*) and vesicular integral-membrane protein VIP36 (*LMAN2*)), can recognize specific N-linked oligosaccharide structures in improperly folded glycoproteins for ERAD (Ruggiano, et al. 2014; Tax, et al. 2019). Activation of mannosyl-oligosaccharide 1,2-alpha-mannosidase (*MAN1A2* and *MAN1B1*) and ER degradation-enhancing alpha-mannosidase-like proteins (*EDEM1, EDEM2,* and *EDEM3*) (Table S15), which facilitate mannose trimming and substrate transfer to EDEM, signal the initiation of ERAD mechanisms (Oda, et al. 2003; Nishikawa, et al. 2005).

Proteins triaged as terminally misfolded are retrotranslocated into the cytosol where they undergo a series of ubiquitination steps and are escorted into the proteasome for degradation (Smith, et al. 2011). Enrichment of Ubiquitin-Proteosome System (hsa4120 and hsa3050) genes in module L5 (Fig. 2F), along with increased expression of translocation channel protein (*DERL2*) and its linker selenoprotein S (*VIMP*) (Fig. 2G), indicates active dislocation and proteolysis of misfolded proteins. Module components include a ubiquitin-activating enzyme E1 (ubiquitin-like modifier-activating enzyme 1 (*UBA1*)), ubiquitin-conjugating enzymes E2 (*UBE2M*, *UBE2G1*, *UBE2D2*, *UBE2J1*, *UBE2M*, *UBE2N*, *UBE2F*, *UBE2G1*, *UBE2D2*, *UBE2E3*, and *UBE2J1*), and ubiquitin ligase enzymes E3 (*STUB1*, *SYVN1*, *UBE3C*, *HUWE1*, *BIRC6*, *UBR5*, *PIAS2*, and *TRAF6*). Differential regulation of E3 adapter and core components (e.g., upregulated S-phase kinase-associated protein 1 (*SKP1*), cullin-5 (*CUL5*), F-box/WD repeat-containing proteins (*FBXW11* and *FBXW7*), elongin (*TCEB1* and *TCEB2*), and downregulated protein sel-1 homolog 2 (*SEL1L2*)), which facilitate substrate recognition and positioning (Smith, et al. 2011), indicates stringent substrate targeting.

Polyubiquitinated substrates and 26S proteasome binding through UV excision repair protein (*RAD23B*) and ubiquilin-1 (*UBQLN1*), may facilitate active proteasomal degradation of mutant proteins, as evidenced by increased levels of the 20S (*PSMA1*, *PSMA2*, *PSMA3*, *PSMA4*, *PSMA5*, *PSMB2*, and *PSMB6*) and 26S proteasome subunits (*PSMC1*, *PSMC3*, *PSMC4*, *PSMD1*, *PSMD3*, *PSMD4*, *PSMD8*, *PSMD11*, and *PSMD14*). *Leucetta chagosensis* may further extend its capacity to degrade misfolded proteins and damaged ER by mounting lysosome-dependent pathways such as autophagy and mitophagy (Chipurupalli, et al. 2021) (Fig. 2F).

Apart from enhancing protein folding capacity and clearance via ERAD, the UPR also relieves ERS by decreasing the flux of nascent proteins through global repression of translational initiation (Pavitt and Ron 2012). Under ERS, phosphorylated protein kinase-R-like ER kinase (*PERK*) typically converts eukaryotic translation initiation factor 2 subunit 1 (*EIF2S1*) into a global protein synthesis inhibitor to facilitate translation attenuation (Walter and Ron 2011). Notably, the UPR interaction network of *L. chagosensis* includes two homologs of *EIF2S1* (Fig. 2G). Moreover, previous studies have proposed that activation of specific amino acid biosynthetic pathways and their cognate tRNA synthetases is, in fact, an adaptive mechanism to support protein synthesis demands of UPR-activated genes (Gonen, Meller, et al. 2019; Gonen, Sabath, et al. 2019). Components of aminoacyl-tRNA biosynthesis (hsa970) are enriched in module L5 (Fig. 2F), along with other players involved in protein targeting and trafficking (i.e., translocon-associated protein subunits (*SSR1, SSR1, SSR1,* and *SSR1*) and protein transport proteins (*SEC23A*, *SEC24C, SEC31B,* and *SEC61A1*)), which suggests some degree of active translation. Interestingly, five out of 11 tRNA synthetases (*SARS*, *NARS2*, *QARS*, *CARS*, and *GARS*) in module L5 (Fig. 2G) are cognates of the UPR amino acid signature (Ser, Asn, Glu, Cys, and Gly, respectively) (Gonen, Meller, et al. 2019).

If ERS remains unmitigated, the UPR transitions from the adaptive to the proapoptotic phase through the PERK (*via* cAMP dependent TF ATF-4 (*ATF4*) and C/EBP Homologous Protein(*CHOP*) TF activities) or *IRE1*-*CASP2* (*via* Regulated inositol-requiring enzyme 1ɑ (*IRE1*)-Dependent Decay (RIDD)) pathways (Walter and Ron 2011; Chen and Brandizzi 2013). GO enrichment analysis of module L5 revealed enrichment of genes linked to programmed cell death (GO12501) (Table S14). These include *CASP2*, an initiator protease essential for apoptosis execution (Vakifahmetoglu-Norberg and Zhivotovsky 2010), as well as both activators (Bcl-2 homologous antagonist/killer (*BAK1*) and SAP30-binding protein (*SAP30BP*)) and suppressors (*BCL2*, Bax inhibitor 1 (*TMBIM6*), and baculoviral IAP repeat-containing protein 6 (*BIRC6*)) (Fig. 2G). Presence of apoptotic regulatory and effector proteins in the L5 gene module indicates that the UPR is in transition from adaptive to proapoptotic phase. Switching and balancing between these two phases necessitates strict regulatory mechanisms, which may determine cell survivorship under ERS (Chan, et al. 2015).

**Transcriptional regulators of calcarean stress response**

To identify key regulatory factors involved in the transcriptional stress response of *L. chagosensis*, we described the expression patterns of epigenetic modifiers and TF genes in the sponge. Some histone modifying enzymes were classified as hub genes of module L5 (Table S16). These include homologs of acetyltransferases (*CBP* and *ELP*), deacetylases (*SIR2* and *SIR6*), methyltransferases (*KMT2E*, *SETB1*, and *EHMT1*), and demethylases (*KDM3B* and *KDM5A*). *CBP-1* in worm, *SIRT3* in mice, and *KDM4B* in human were shown to regulate the activation of UPR (Wang, et al. 2018; Li, et al. 2021; Xiaowei, et al. 2023).

Several TF families (i.e., *Ets*, *bHLH*, *HMG box*, and *Homeobox KN)* were enriched in the set of upregulated *L. chagosensis* genes at RCP 8.5 (Fig. 3A, Table S17). Two of the most highly expressed families, *HMG box* and *bZIP*, also showed a notable increase in expression under the tested conditions. Some of these gene families typically have regulatory functions in mounting stress response programs or confer organismal stress tolerance (Yamamoto, et al. 2004; Reitzel, et al. 2008; Kielbowicz-Matuk 2012; Klotz, et al. 2015; Das, et al. 2019; Klymenko, et al. 2019; Gai, et al. 2022; Ma, et al. 2023). In fact, prolonged exposure of a demosponge *Haliclona tubifera* to thermal stress, actuated various TF families such as *Ets*, *bHLH*, and *bZIP*, along with genes involved in cell repair, apoptosis, signaling, and transcription (Guzman and Conaco 2016b).

To pinpoint key *trans*-acting regulators involved in the UPR of *L. chagosensis*, putative TFs within the reconstructed UPR interaction network (Fig. 2G) were identified. Module L5 includes 20 differentially expressed (FDR ≤ 0.05) TFs with high module membership (MM = 0.81 – 0.95; *p* = 4.89E-07 - 8.39E-04) (Fig. 3B, Table S17). Of these 20 TFs, only the X-box-binding protein 1 (*XBP1*) is integrated into the UPR interaction network (Fig. 2G). *Leucetta chagosensis* has two homologs of *XBP1*: *LchaXBP1\_1* (MM = 0.94; *p* = 1.15E-06) and *LchaXBP1\_2* (MM = 0.92; *p =* 7.15E-06) and both were significantly upregulated at RCP 8.5 (Fig. 3B). *XBP1* isa UPR-specific *bZIP* TF that regulates the production of ER chaperones and ERAD proteins, as well as enzymes involved in lipid biosynthesis (Reimold, et al. 2001; Yoshida, et al. 2001; Chen, et al. 2014). *XBP1* works with *CHOP* and Jun N-terminal kinase (*JNK*) to regulate the balance between the adaptive and proapoptotic phases of UPR (Chan, et al. 2015).

***XBP1* homologs in *L. chagosensis* are lineage-specific**

Most metazoans possess a single copy of *XBP1* (Jindrich and Degnan 2016) whereas calcareans, including *Clathrina* sp., *L. chagosensis*, *P. orientalis*, *L. complicata*, *G. compressa*, and *S. ciliatum,* have at least two copies. Phylogenetic reconstruction indicates that calcarean *XBP1* genes are lineage-specific and group into three major clusters (Fig. 3C). *LchaXBP1\_1* groups with other calcareans (*PoriXBP1\_1*, *CspXBP1\_1*, *LcomXBP1\_1*, *GcomXBP1\_1*, *GcomXBP1\_2*, *ScilXBP1\_1*, and *LcomXBP1\_3* in the Calcarea I cluster), whereas *LchaXBP1\_2* clusters with clathrinid *XBP1*s (*PoriXBP1\_2* and *CspXBP1\_2* in the Calcarea II cluster). *XBP1* sequences from leucosolenids form a separate group (Calcarea III cluster). Homolog distribution suggests that *LchaXBP1\_1* is the conserved ancestral paralog, whereas *LchaXBP1\_2* is a derived copy that later diverged along with other clathrinid *XBP1*s. Expression levels of Calcarea I *XBP1* genes (*LchaXBP1\_1* and *PoriXBP1\_1*) are relatively higher compared to homologs in Calcarea II (*LchaXBP1\_2* and *PoriXBP1\_2*) as observed in untreated adult tissues of *L. chagosensis* and *P. orientalis* (Table S18).

***LchaXBP1* homologs can form homo- and heterodimer pairs**

Similar to other *bZIP* TFs, *XBP1* is composed of a bZIP domain consisting of an N-terminal DNA-binding basic motif and C-terminal dimerization leucine zipper domain (Hurst 1995). A closer investigation of amino acid sequences revealed locus-specific substitutions in the DNA-binding region of *LchaXBP1* homologs (Fig. 4A). Calcarea I members, including *LchaXBP1\_1*, have a conserved His at position 18, whereas a Calcarea II member, *LchaXBP1\_2,* has a species-specific Ile at the same position. A previous study showed that His replacement in the DNA-binding region changes the binding specificity of a *bZIP* TF (Suckow, et al. 1994). An Ile substitution, on the other hand, results in inhibition of DNA binding activity (Nantel and Quatrano 1996). Since *bZIP* TFs need to dimerize in order to bind to dsDNA grooves (Bader and Vogt 2006), it is hypothesized that different dimerization pairs of *LchaXBP1* homologs exhibit different DNA binding properties.

Dimerization partner selectivity can be influenced by environmental stimuli, such as oxidative stress (Amoutzias, et al. 2006), and is a key factor that determines how *bZIP* proteins mediate appropriate responses to specific conditions. By comparing the structures of their leucine zipper heptads, we found that *LchaXBP1* homologs can form both homo- and heterodimer pairs (Fig. 4A). Attractive acidic - basic *g* ↔ *e’* pairs are found in *LchaXBP1\_1* homodimer (2nd heptad), *LchaXBP1\_2* homodimer (5th heptad), and *LchaXBP1* heterodimer (2nd and 5th heptads). *LchaXBP1* homologs also contain Asn in the position *a* of 2nd, 3rd, and 5th heptads, which likely promotes both homo- and heterodimerization (Cohen and Parry 1990). Other heterodimerizing leucine zippers comprising any combination of the three aliphatic amino acids: Ile (e.g., *LchaXBP1\_1*, 4th heptad), Leu (e.g., *LchaXBP1\_2*, 4th heptad), and Val (e.g., 1st heptad) in the *a*position have similar coupling energies (Vinson, et al. 2002). The prevalence of Leu in the *d* position also contribute to dimer stability due to the unique packing interactions of the two Leu and their neighboring amino acids (Cohen and Parry 1990). These homo- and heterodimer interaction signatures were also predicted for *XBP1* homologs in other clathrinids, *P. orientalis* and *Clathrina* sp. (Fig. 4A).

***LchaXBP1* homodimers bind with cAMP responsive element while the heterodimer pair does not**

The structure of the *LchaXBP1\_1* and *LchaXBP1\_2* homodimers are similar to structures of the Transcription factor MafB (2wty.1.A) and Transcription factor MafG (3a5t.1.A) homodimers, respectively (Table S19). The *LchaXBP1* heterodimer is comparable to other bZIP dimers, such as the Transcription factor FosB/JunD (5vpd.2) heterodimer. Based on *in silico* docking conformations (Table S20), it is predicted that *LchaXBP1* homodimers can bind to gene promoters through the cAMP responsive element (CRE), whereas the *LchaXBP1* heterodimer does not (Fig. 4B-D). Indeed, *LchaXBP1* homodimers interact with the aureobox “TGACGT” within the CRE, which serves as the *cis*-regulatory element for UPR activation (Clauss, et al. 1996; Glimcher, et al. 2020). In the *LchaXBP1\_1* homodimer bound to CRE (Fig. 4B), residue Arg’42 (C/NH2) forms hydrogen bonds with nucleotides dG’-6(A/O6) and dG’5(B/O6), Ser’49 (D/OG) with dT’-4(B/O4), Arg’54 (D/NH1) with dA’-2(A/O5’), Arg’56 (C/NH2; D/NH2) with dG’-6(A/O3) and dT’-4(B/O3’), and Lys’58 (C/NZ) with DA’-2(B/O3’) (Table S21). In the *LchaXBP1\_2* homodimer bound to CRE (Fig. 4C), residue Arg’33 (D/NH2) forms hydrogen bonds with nucleotide dG’5 (B/O6), Arg’38 (C/NE; D/NH2) with dC’-1(A/O3’) and dG’1 (B/O3’), Arg’45 (C/NE; D/NE) with dA’-2(A/O5’) and dA’-2.B/O3’, and Arg’47 (C/NH2) with dC’-5 (B/O3’) (Table S21). No polar interactions are predicted between *LchaXBP1* heterodimer and CRE (Fig. 4D).

We propose that alternative dimerization of *XBP1* represents a mechanism to maintain the balance between adaptive and proapoptotic phases of UPR (Fig. 4E). *XBP1* homodimer formation promotes adaptive phase UPR through CRE, whereas heterodimer formation reversibly sequesters *LchaXBP1* into an inactive form that then permits apoptosis through the RIDD pathway (Chan, et al. 2015; Hetz, et al. 2020). UPR regulation through this mechanism may be conserved among clathrinids since the *PoriXBP1* and *CspXBP1* heterodimers are also unable to bind to cognate DNA (Fig. S7). A similar mechanism was previously described in rice *bZIP* proteins, where *EmBP-1* and *osZIP-1a* homodimers can both bind with the Em1a element, while heterodimerization of *EmBP-1* with *osZIP-2* prevents binding to DNA (Nantel and Quatrano 1996). Further biochemical testing and functional assays are warranted to verify alternative dimerization of *XBP1* homologs and their effects on UPR regulation in calcareans.

**Discussion**

Extant calcifying members of the reef biome have transcended major upheavals throughout Earth’s history and have had profound roles in shaping marine ecosystems (Hull 2017). However, with steadily increasing emissions of anthropogenic CO2, the oceans are predicted to become more acidic at a rate that is at least 100 times faster than the past hundreds of millennia (MacFarling Meure, et al. 2006). The predicted co-occurrence of acidification and warming is even more detrimental due to the narrowing of organismal thermal tolerance thresholds (Pörtner, et al. 2005). Describing the genomic signatures of adaptability and stress response mechanisms among marine calcifiers can provide insights into their fate in the future ocean. The current study represents the first description of transcriptome-wide changes accompanying the calcarean stress response. Thus, this work contributes to the limited sequence data resource for class Calcarea and, along with the bacterial community shifts and immune responses in *L. chagosensis* described in our previous study (Posadas, et al. 2021), reveals a comprehensive view of the gene regulatory networks underlying the holobiont stress response.

Here, we show that a calcareous sponge activates the UPR under thermo-acidic stress by expressing molecular chaperones, ERAD proteins, and select tRNA synthetases. Activation of the UPR has also been observed in the response of demosponges to elevated temperature stress. In particular, *Spongia officinalis* actuated molecular chaperone genes, as well as other players involved in the ERS and UPR (Koutsouveli, et al. 2020), while *H. tubifera* upregulated genes involved in protein refolding and proteolysis (Guzman and Conaco 2016b). However, unlike the demosponge thermal stress response, *L. chagosensis* under stress also activated the proapoptotic phase of UPR. We propose that the balance between the two UPR phases in *L. chagosensis* is mediated, in part, by reversible sequestration of the bZIP transcription factor, *XBP1,* into an inactive form through alternative dimerization with its paralog. While the UPR is a highly conserved pathway from yeast to human (Wu, et al. 2014), lineage-specific duplication of *XBP1* in clathrinids may have resulted in a mechanism that enables rapid and stricter regulation of pathways linked to the UPR. This mechanism is analogous to the duplication of a stress-responsive TF, MSN2–MSN4, which resulted in adaptive gene expression tuning in yeast (Chapal, et al. 2019). However, it should be noted that our results were inferred from *in silico* experiments and requires further functional validation. Nonetheless, this demonstrates how TF gene duplication promotes evolutionary innovation to further refine or expand the circuitry of transcription networks (Perez, et al. 2014; Voordeckers, et al. 2015). The upregulation of many paralogous genes in *L. chagosensis* under thermo-acidic stress also supports the idea that duplicated genes with roles in organismal stress response are typically selected for retention and exhibit stress-specific transcriptional plasticity (Keane, et al. 2014; Mattenberger, et al. 2017; Kuzmin, et al. 2022).Further characterization of other lineage- and species-specific stress responsive genes in *L. chagosensis* may uncover a greater diversity of novel evolutionary solutions for maintaining organismal health in changing environments.

Population trajectory, breeding success, and known threats are some of the factors that are used to assess species conservation status (Grace 2023). The described transcriptional stress response of *L. chagosensis* may serve as a basis for evaluating the susceptibility of calcareous sponges to different stressors. Molecular targets identified in this study can be leveraged in designing biomarker tools to investigate sublethal impacts that may contribute to population declines over generations. Our findings showing the susceptibility of *L. chagosensis* to future ocean conditions highlight the need to prioritize the conservation and management of calcisponges, which are often underappreciated in studies of reef ecosystem.

**Acknowledgements**

We thank Dr. Bernard Degnan (University of Queensland), Dr. Lilibeth Salvador-Reyes (University of the Philippines Diliman), and Dr. Ma. Anita M. Bautista (University of the Philippines Diliman) for their comments and suggestions to improve the manuscript; Dr. Ana Riesgo (Museo Nacional de Ciencias Naturales, Madrid, Spain), Dr. Lucia Pita Galan (Institute of Marine Science, Barcelona, Spain), and Dr. Oliver Voigt (LMU München, Germany) for sharing sponge transcriptome datasets; Jake Ivan Baquiran, Michael Angelou Nada, Francis Kenith Adolfo, Robert Valenzuela, and Ronald De Guzman for field and hatchery assistance, and staff of the Bolinao Marine Laboratory for logistical support. This study was funded by the Department of Science and Technology Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (QMSR-MRRD-MEC-295-1449) and UP MSI In-house Research Grant (2020-2022) to CC.

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**Data Accessibility**

The datasets we generated and analyzed are available in Figshare (https://figshare.com/projects/Gene\_regulatory\_networks\_in\_calcareous\_sponge\_thermo-acidic\_stress\_response/195164).

**Author Contributions**

NP and CC designed the research, analyzed data, and wrote the paper. NP performed experiments.

**Conflict of Interest**

The authors declare no conflict of interest.

A close-up of a diagram

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**Fig. 1.** Sponge phylogeny and gene repertoire. **(a)** *Leucetta chagosensis* in its natural habitat. **(b)** Orthologous protein families in *L. chagosensis* and other sponge species. The bar graph represents the number of orthologous peptides that are common amongst sponge species indicated above the dark circles. Only orthogroups with at least 4 members are shown. The species tree was inferred from all genes by the STAG algorithm and rooted using STRIDE (Emms and Kelly 2019). **(c)** Comparison of gene repertoire in *L. chagosensis* and other sponge species. Principal component analysis was based on the relative abundance of peptides with matches to Molecular Functions in the Gene Ontology database for all species. Relative abundance was computed as the percentage of peptides associated with a function relative to the total number of predicted peptides in each species. **(d)** Distinguishing molecular functions in calcareous sponges. Functions that distinguish (LDA score > 2; *p*-value <0.05) between calcareans (red) and other sponge species (grey) were determined using LDA-LEfSe based on relative abundance values. Species abbreviations: *Sycon ciliatum* (Scil), *Grantia compressa* (Gcom), *Leucosolenia complicata* (Lcom), *Pericharax orientalis* (Pori), *Leucetta chagosensis* (Lcha), *Clathrina* sp. (Csp), *Oscarella carmela* (Ocar), *Corticum candelabrum* (Ccan), *Xestopongia testudinaria* (Xtes), *Amphimedon queenslandica* (Aque), *Petrosia ficiformis* (Pfic), *Haliclona tubifera* (Htub), *Neopetrosia compacta* (Ncom), *Stylissa carteri* (Scar), *Ephydatia muelleri* (Emue), *Dysidea avara* (Dava), *Aplysina aerophoba* (Aaer), *Aphrocallistes vastus* (Avas).

A close-up of a diagram

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**Fig. 2.** Transcriptome dynamics in *Leucetta chagosensis* under stress. **(a)** Transcriptome-wide changes visualized through principal component analysis. Color denotes the different treatments. Ellipses were computed using the Khachiyan algorithm (tolerance level = 0.01). **(b)** Number off downregulated (blue) and upregulated (red) genes (log2 Fold change ≥ 2, FDR ≤ 0.05). **(c)** Number of upregulated genes with (dark grey) and without (light grey) detectable paralogs. **(d)** Co-expression network of highly expressed genes in *L. chagosensis*. Each node represents a single gene. Edge distance indicate co-expression strength between genes. Colors denote different gene modules (L1, orange; L3, blue; L4, yellow; L5, red). **(e)** Sets of highly co-expressed genes (modules L1-L5) that correlated with specific treatments (violet, negative correlation; yellow, positive correlation). Values are indicated for significant positive correlations. Bar graph (right) shows the number of genes per module, with hub genes represented by the darker shades. **(f)** KEGG pathway enrichment analysis for module L3 (blue) and L5 (red) components. Gene count (x-axis) and False Discovery Rate (bubble size) for each enriched (FDR < 0.01) pathway are presented. **(g)** Genes in the unfolded protein response (UPR) interaction network including protein folding, sorting, and degradation. Node colors represent upregulation (red) or downregulation (blue) of expression. Node size indicates relative expression value computed as the sum of TPM values at RCP 8.5 relative to the Present Day samples. Edge thickness denotes interaction confidence score. The network is based on human protein–protein interactions in STRING database.

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**Fig. 3.** Stress-responsive transcription factors (TFs) in *Leucetta chagosensis*. **(a)** Bar plot showing the top 10 most abundant TFs and their expression levels across treatments. Asterisks indicate significant Pfam enrichment (*p*<0.05) for upregulated genes in a specific treatment relative to Present Day samples. Colors represent different treatment conditions. **(b)** Differentially expressed TFs in module L5 hub genes. Expression levels of TFs are shown as TPM z-score across all treatments (low, blue; high, red). Asterisks indicate differential expression (FDR < 0.05) relative to the Present Day samples. **(c)** Phylogenetic analysis of *HAC1*/*bZIP60*/*XBP1* homologs in sponges and other organisms. The phylogenetic tree was derived from Bayesian analysis of aligned bZIP sequences (Data S1). Circles on selected branches represent Bayesian posterior probabilities. Color strips mark major *XBP1* clades (red, calcareans; grey, metazoans). Colors of selected branches indicate sponge class.

A diagram of dna and dna molecules

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**Fig. 4.** Dimerization and binding potential of *XBP1* homologs. **(a)** Multiple sequence alignment of bZIP (PF00170) amino acid sequences of *HAC1*/*bZIP60*/*XBP1* homologs in yeast, cress, human, Calcarea I, II, and III clades. The DNA-binding region (light blue) is found within the nuclear localization signal in the N-terminal basic motif. Residues in the L0-L5 heptadpositions are colored if both *g* and *e* contain charged amino acids. Colors indicate type of interactions (green, attractive basic-acidic; orange, attractive acidic-basic; red, repulsive acidic pairs; blue, repulsive basic pairs). Charged residues in positions *g* or *e* are colored (red, acidic; blue, basic) if one of the *g* ↔ *e’* pair is not charged. Polar or charged amino acids in positions *a* and *d* are colored black. **(b-d)** Top predicted conformations for *LchaXBP1*-CRE complexes. Structures of CRE with the **(b)** *LchaXBP1\_1* (cyan)homodimer*,* **(c)** *LchaXBP1\_2* (orange)homodimer, and **(d)** *LchaXBP1* heterodimer generated in the docking experiment were rendered in Pymol (15). All polar interactions between specific residues in *XBP1* dimers and cognate aureobox “TGACGT” DNA sequence within the CRE are indicated for each complex. bZIP-DNA interfaces were predicted using PDBePISA (16). **(e)** Proposed regulation of the UPR in *L. chagosensis* through alternative dimerization of *LchaXBP1* homologs. Detection of unfolded proteins activates the endoribonuclease activity of *IRE-1*, which cleaves XBP1 mRNA (U). Spliced XBP1 mRNA (S) is translated and the peptides form dimers. The *XBP1* homodimers bind to the CRE aureobox to activate pathways linked to the adaptive phase UPR, whereas the heterodimer is unable to bind to the cognate DNA sequence, activating apoptosis through the RIDD pathway (Chan, et al. 2015; Hetz, et al. 2020).