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Mitochondrial DNA damage, repair and copy number dynamics of *Sclerophytum* sp. (Anthozoa: Octocorallia) in response to short-term abiotic oxidative stress

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ABSTRACT

As a consequence of global climate change, the increasing frequency of environmental disturbances and surplus oxidative stress experienced by coral reefs will likely contribute to phase shifts from stony to soft corals. Mitochondrial response to reactive oxygen species (ROS) -induced oxidative damage appears pivotal for bioenergetic adaptation and recovery during environmental stress, partly governed by mitochondrial DNA copy number. Unlike other animals, octocorals possess unique mitogenomes with an intrinsic DNA mismatch repair gene, the *mtMutS*, that is likely to have a role in mitochondrial response and mtDNA damage recovery. Yet, there is a general lack of stress response studies on octocorals from a mitochondrial perspective. Here we evaluate the mitochondrial response of the octocoral *Sclerophytum* sp. subjected to acute elevated temperature and low pH, and its putative competence to reverse oxidative mtDNA damage caused by exogenous agents like hydrogen peroxide (H_2O_2). Temporal changes in mtDNA copy number and mtDNA damage and recovery were monitored. Both short-term thermal and low pH stress applied independently instigated mtDNA damage and affected mtDNA copy number differently, while *mtMutS* gene was significantly upregulated during low pH stress. mtDNA damage caused by H_2O_2 insult was observed to be promptly reversed in *Sclerophytum* sp., and a higher mtDNA copy number was associated with lower mtDNA damage. These findings provide insights into the potential role of *mtMutS* gene in conferring resilience to octocorals, the relevance of mtDNA copy number, and emphasize the importance of better understanding the mitochondrial stress response of cnidarians in the context of climate change.

1. Introduction

Anthropogenic climate change leading to elevated sea surface temperatures and ocean acidification have been linked to oxidative stress in corals (Lesser, 1997; Szabó et al., 2020). The response of reef-building/stony or scleractinian corals to these stressors has been extensively studied (DeSalvo et al., 2008; Doering et al., 2023; Lesser, 2011; Lesser, 2006; Samshuri et al., 2023; Soriano-Santiago et al., 2013), as oxidative stress may impact coral reef productivity and growth, and lead to large-scale coral mortality (Carpenter et al., 2008; Hughes et al., 2017). However, several studies exploring the physiological and transcriptomic response of octocorallian soft corals under thermal and pH stress found that octocorals are remarkably resilient to these stresses (Haguenauer et al., 2013; Lõhelaid et al., 2014; Parrin et al., 2016; Sammarco and Strychar, 2013; Steinberg et al., 2022; Teixeira et al., 2013; Vargas et al., 2022; Woo et al., 2014), likely possessing an advantage over stony corals under changing climate conditions. Consequently, coral reefs will likely experience phase shifts from the currently dominating reef-building

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scleractinian corals to other groups of organisms including algae, sponges, and soft corals (Done, 1992; Fung et al., 2011; Norström et al., 2009; Quattrini et al., 2020; Schmitt et al., 2019). Even though multiple physiological and genetic adaptations could be attributed to the resilience and success of octocorals, the role of the bioenergetic response governed by mitochondria for stress mitigation and recovery may be critical, yet seldom investigated.

Mitochondria are constantly sensing and responding to environmental changes through energy production and signaling (Eisner et al., 2018). They fuel all cellular activities in non-photosynthetic aerobic eukaryotes by producing adenosine triphosphate (ATP) using oxidative phosphorylation (OXPHOS). They are also a major source of reactive oxygen species (ROS) resulting from the same process, and consequently a main site of oxidative damage in animals (Tzameli, 2012). Mitochondrial dysfunction, a consequence of excessive stress, exacerbates the release of ROS in the cellular environment and results in damage to lipids, proteins, and most importantly DNA (Duarte-Hospital et al., 2021; Halliwell, 2007). Animal mitochondrial DNA (mtDNA) is particularly prone to ROS-induced damage as it lacks nucleotide excision repair (NER) ability that efficiently corrects DNA damage resulting from UV or other environmental insults in the case of nuclear DNA (Rong et al., 2021). Threats to mtDNA integrity by endogenous ROS not only lead to mitochondrial degradation and removal of compromised mitochondria through mitophagy but can also elicit pre-apoptotic protein signaling cascades ultimately leading to cell death (Van Houten et al., 2016). Hence, the response of mitochondria during (and after) oxidative stress caused by disturbances in the environment, such as thermal and low pH stress, is crucial in determining cell fate and organismal physiology (Dunn et al., 2012; Nadalutti et al., 2022).

Cnidarian mitochondrial genomes are highly variable and deviate substantially from generally conserved animal mitochondrial genome organization found in bilaterian animals. This includes circular or linear chromosomes (Anthozoa vs. Medusozoa), 19 genome rearrangements (Feng et al., 2023), variable genome sizes, genetic codes, substitution rates, differences in the number of mitochondrial tRNAs, and the presence of additional genes, among others (Kayal et al., 2012; Lavrov and Pett, 2016). Most octocorals display unusually slow rates of mtDNA sequence evolution, which is often attributed to the unique presence of an additional, mitochondrially-encoded DNA mismatch repair gene (*mtMutS*) (Hellberg, 2006; Pont-Kingdon et al., 1995; Shearer et al., 2002) that was laterally acquired from giant viruses (Bilewitch and Degnan, 2011). The role of *mtMutS* in maintaining low levels of sequence variation and its involvement in mtDNA repair and gene rearrangements in the octocoral mitochondrial genome has been proposed (Muthye et al., 2022; Shimpi and Bentlage, 2023), although the exact function of this gene has yet to be experimentally demonstrated. While most studies to date examined nuclear DNA damage caused by ROS and other agents in scleractinian corals (Lesser and Farrell, 2004; Nesa et al., 2012; Schwarz et al., 2013; Svanfeldt et al., 2014; Nesa and Hidaka, 2008), none have explored the potential of mtDNA damage recovery in octocorals despite their mitogenomes harboring a DNA repair gene, *mtMutS*, that may partially explain octocoral resilience to environmental stress.

The mtDNA copy number is a crucial proxy for mitochondrial function, as it is directly linked to cellular energy budget, mitochondrial membrane potential, and oxidative stress (Castellani et al., 2020; Clay Montier et al., 2009; Filograna et al., 2021). mtDNA copies are tuned according to the metabolic needs of cells and tissues and result from the homeostasis between mtDNA replication and turnover. The function of a gene like *mtMutS* intrinsic to the mtDNA could be particularly advantageous for maintaining a healthy quantity and quality of mtDNA copies. Despite increasing knowledge about the physiological mechanisms and ecosystem-level implications of climate change-induced stress on corals, a thorough understanding of climate change impacts on mitochondria is lacking. This is at odds with the pivotal role of mitochondria in energy production, its regulation as well as signaling in response to environmental disturbances.

To address this knowledge gap, we studied mtDNA damage, recovery, and mtDNA copy number changes in the octocoral *Sclerophytum* sp. in response to acute abiotic stressors. We utilized a sensitive quantitative real-time PCR-based approach to assess the extent of mtDNA damage during two climate change-associated stressors, i.e., elevated seawater temperature and reduced seawater pH, along with differential gene expression analysis of two mitochondrial genes (cytochrome *c* oxidase subunit I and *mtMutS*). Moreover, mtDNA damage and recovery from exposure to an exogenous ROS (hydrogen peroxide, H_2O_2) was assessed. Quantification of mtDNA copy number was performed to understand copy number dynamics during the mitochondrial recovery process following stress. By integrating gene expression and quantification of mtDNA damage/repair, we shed light on the ability of octocoral mitochondria to mitigate oxidative stress events, laying the foundation for understanding the mechanism of mitochondrial stress tolerance in octocorals.

2. Materials and methods

2.1. Experimental model and aquaria setup

Clonal explants were obtained from a *Sclerophytum* sp. (Octocorallia: Sarcophytidae) colony that had been maintained in a closed circuit seawater aquarium at the Molecular Geo- and Palaeobiology lab, Department of Earth and Environmental Sciences Palaeontology & Geobiology, LMU, Munich under controlled conditions for several years (25 ± 1 °C, pH 8.2 ± 0.1) with a biweekly exchange of 50% fresh artificial seawater. LED lights (GHL Mitras LX 6200-HV) at a light intensity of 14 ± 2 kLux in a 12 h light / 12 h dark cycle were used. A similar light regime was used for both the control and experimental systems described below.

2.2. Experimental oxidative stress and DNA damage treatments

To determine the effect of oxidative stress on mtDNA and gene expression, approximately 5 cm × 5 cm nubbins of *Sclerophytum* sp. were exposed to elevated seawater temperature, decreased pH (both acute, and sub-lethal), and hydrogen peroxide (H_2O_2) in the water (acute toxicity). Due to aquarium facility limitations, replication of treatment and control conditions was limited. A single tank was used per condition for thermal and low pH stress experiments, resulting in limitations to correct for sample-dependence effects; H_2O_2 treatment was temporally replicated (Cornwall and Hurd, 2016; Hurlbert, 1984). Our goal was to assess the mitochondrial response (mtDNA damage/repair mtDNA copy number and gene expression) as a function of the initial mitochondrial state (steady-state). The study aimed to understand spatiotemporal changes in mtDNA damage/repair, mtDNA copy number, and gene expression below the level of the individual, where the mitochondrion was the observational unit while *Sclerophytum* sp. nubbin was the experimental unit rather than the aquarium tank (Lazic et al., 2018; Schank and Koehnle, 2009).

2.2.1. Thermal stress

Three *Sclerophytum* sp. nubbins were placed in an experimental ~10 l tank and the temperature in the tank was raised gradually from 26 °C to 34 °C for 2 h and was maintained at 34 °C for 6 h thereafter. Three control nubbins were maintained in a similar tank but the temperature was maintained at 26 °C throughout the experiment.

2.2.2. Low pH stress

Three *Sclerophytum* sp. nubbins were exposed to low seawater pH by pumping carbon dioxide (CO_2) into the seawater of a ~ 10 l experimental tank to maintain a pH of 7.5. The pH was first reduced to 7.5 for 2 h and then maintained at this level for 24 h. The pH was recorded throughout the experiment to ensure it remained constant at 7.5.

Sclerophytum sp. nubbins were sampled after 24 h exposure. Control samples were maintained under normal conditions (pH 8.2); seawater temperature in treatment and control tanks was maintained at 26 °C during the experiment.

2.2.3. Hydrogen peroxide treatment

To evaluate the capacity of mtDNA to recover from severe mtDNA damage in *Sclerophytum* sp., hydrogen peroxide (H_2O_2), a principal mediator of oxidative stress and one of the reactive oxygen intermediates generated in mitochondria, was used as a DNA damaging agent due to its natural occurrence and longer stability in seawater as well as high membrane permeability (Lesser, 2011) allowing it to diffuse freely throughout the cell and causing DNA damage via a Fenton reaction (Henle et al., 1996). Recently Ousley et al. (2022) observed H_2O_2 bursts at the coral polyp surface of two scleractinian corals indicating its dynamic presence in the proximity of corals. Three independent DNA damage trials were performed at different times on individual *Sclerophytum* sp. nubbins. Temporal replication was chosen for this experiment due to limited availability of genetically identical, branched coral nubbins. A cutting was taken from the donor colony and reared until it reached approximately 5–7 cm in size and had developed branches. This process was repeated three times independently, creating the temporal replicates used for the experiment. During each trial, tissue biopsies were taken from one nubbin that was used as a control (time zero), while the other nubbins were exposed to 30% *v*/v H_2O_2 (Sigma-Aldrich) in a 2 l experimental tank containing artificial seawater to achieve a final concentration of 5 mM H_2O_2. Corals were kept in this solution for 30 min after which treatment tissues were subsampled. Following treatment, corals were returned to initial control conditions for recovery. During recovery, three small tissue samples (0.5 cm × 0.5 cm) were taken after 1 h, 5 h, and 48 h post-treatment from the tip of different branches of each nubbin.

2.3. Total RNA extraction and cDNA synthesis

Tissue samples for RNA extraction were flash-frozen in liquid nitrogen and stored at −80 °C until further processing. Total RNA was extracted from control and treatment samples exposed to thermal and pH stress using TRIzol (Invitrogen, USA) following the manufacturer's instructions. Contaminating DNA was eliminated from RNA extracts with the help of RQ RNase-free DNase (Promega, USA) according to the manufacturer's protocol. RNA was further purified using sodium acetate-ethanol precipitation. The purity of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). RNA samples with absorbance at OD260/280 and OD260/230 ratio ~ 2.0 were used for further analysis. RNA integrity was also verified by 1% agarose gel electrophoresis and using a Bioanalyzer 2100 (Agilent Inc., USA). RNA extracts with a RIN score ≥ 7.5 were used for cDNA synthesis. For each sample, ~1 μg of total RNA was reverse transcribed using the ProtoScript First Strand cDNA Synthesis Kit (NEB, Germany) with an anchored oligo-(dT) primer in 20 μl reactions according to the protocol provided with the kit.

2.4. Quantitative real-time PCR for gene expression

Quantitative Real-time PCR (qPCR) was performed on a Rotor-Gene Q 2plex system (Qiagen, Germany) using KAPA SYBR FAST universal mastermix (Peqlab, Germany) in 15 μl reactions containing 1 μl diluted cDNA, 7.5 μl 2× mastermix, and 250 to 400 nM each primer. A two-step qPCR including an initial denaturation step of 3 min at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. A non-template control was included in each assay. Melting curve analysis was performed at the end of each qPCR to confirm amplification specificity and amplification products were confirmed by agarose gel electrophoresis after each assay. The primers used for thermal and pH stress qPCRs can be found in Table 1.

2.5. Semi-long run quantitative real-time PCR for mtDNA damage

Extraction of total DNA from ethanol-preserved H_2O_2 toxicity control and treatment tissues as well as all tissue samples from thermal and low pH stress experiments was performed using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions. DNA quantity and purity were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), which indicated high-quality DNA extracts (A260/A280 ≥ 1.8).

To quantify mtDNA damage a semi-long run quantitative real-time PCR (SLR-qPCR) was performed as described by Rothfuss et al., 2010. Briefly, a large (1057 bp) and a small DNA (100 bp) fragment of the same mitochondrial region (*COII*-igr-*COI*) was amplified using the KAPA SYBR FAST universal mastermix (Peqlab, Germany) in 15 μl reactions containing 1× mastermix, and 500 nM of each, forward and reverse primer and 5 ng total DNA. The cycling conditions consisted of an initial denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s for the small fragment, and 95 °C for 10 s, 58 °C for 20 s and 72 °C for 30 s for the large fragment. The mitochondrial regions, primers, and PCR efficiencies are listed in Table 2. Each sample was assayed in triplicates and the amplicon specificity was monitored by melting curve analysis and gel electrophoresis. Quantification cycle (Cq) values and mean PCR efficiency (E) for the primer pairs were obtained using the computer program LinRegPCR (Ramakers et al., 2003). DNA isolated from the control sample was used as a reference (no DNA

Table 1
Description of gene-specific qPCR primers used for gene expression analysis.

No.	Gene	Gene Name	Primer Sequences (5′ to 3′)	Product Size (bp)	Amplicon Tm (°C)	*E*
	Reference genes primers					
1	*ACTB*	β-Actin	for: CCAAGAGCTGTGTTCCCTTC rev: CTTTTGCTCTGGGCTTCGT	107	83.8	1.97
2	*TUBB*	β-Tubulin	for: ATGACATCTGTTTCCGTACCC rev: AACTGACCAGGGAATCTCAAGC	115	80.5	1.99
3	*RPL12*	Ribosomal protein L12	for: GCTAAAGCRACTCAGGATTGG rev: CTTACGATCCCTTGSTGGTTC	142	80.5	1.97
4	*SRP54*	Signal recognition partical 54	for: TGGATCCTGTCATCATTGC rev: TGCCCAATAGTGGCATCCAT	184	79.5	1.97
	Target gene primers					
4	*COI*	Cytochrome c oxidase subunit 1	for: ACGGCTTGATACACCTATGTTGTGG rev: TACCGAACCAATAGTAGTATCCTCC	200	78.7	1.99
5	*mtMutS*	Mitochondrial *muts* homolog	for: GCATGAGCCCGATACTTCTAGT rev: ACGAAGCAACTTGTTCAATGG	119	81.7	1.98

E represents PCR efficiency.

Table 2
Description of qPCR primers used for mtDNA damage and mitochondrial copy number quantification.

No.	Gene fragments	Gene	Primer Sequences		Size (bp)	Product Tm (°C)	*E*
			Forward (5′ to 3′)	Reverse (5′ to 3′)			
1	Small mt-fragment	*COI*	TAATTCTACCAGGATTTGG	ATCATAGCATAGACCATACC	97	75.8	1.95
2	Large mt-fragment	*COII-COI*	CCATAACAGGACTAGCAGCATC	ATCATAGCATAGACCATACC	1057	82.3	1.76
3	Nuclear fragment	*ACTB*	CCAAGAGCTGTGTTCCCTTC	CTTTTGCTCTGGGCTTCGT	107	83.5	1.96

E represents PCR efficiency. The reverse primer was common for both small and large mitochondrial fragments.

damage) whereas Cqs of the large and small mitochondrial fragments were used for DNA damage quantification. Cq values were efficiency-corrected using the (Kubista et al., 2007) equation,

$$Efficiency\ corrected\ Cq = Cq\left(\frac{log\ (E)}{log\ (2)}\right)$$

Efficiency-corrected Cq values were used to calculate mitochondrial lesion frequency (MLF) following (Rothfuss et al., 2010).

$$Lession\ rate\ [lesions\ per 10kb\ DNA] = \left(1 - 2^{-\left(\Delta Cq_{long} - \Delta Cq_{short}\right)}\right) \times \frac{10000\ [bp]}{size\ of\ long\ fragment\ [bp]}$$

where ΔCq is the difference between the quantification cycle of untreated versus treated condition of the respective long and short fragments calculated using $2^{-\Delta\Delta CT}$ method expressed as the ratio of intact DNA (Rothfuss et al., 2010).

2.6. Determination of mtDNA copy number

To determine the extent of damage of experimental treatments to mitochondria, the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) ratio was calculated before and after treatment using qPCR; this ratio provides relative mitochondrial copy number per cell. Equal amounts of total DNA was used to amplify a nuclear (*ACTB*) and a mitochondrial gene (*COI*) in control and treatment samples and the ratios of mtDNA/nDNA ratios were obtained using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001); the non-treated sample served as control and the *ACTB* Cq values as reference. Primer details are listed in Table 2.

2.7. Data analysis

The raw fluorescence data obtained after qPCR was baseline corrected using LinRegPCR (Ramakers et al., 2003), and Cq values and amplification efficiencies for each amplification curve were calculated using this program. These Cq values were used for mtDNA damage repair, mtDNA copy number, and gene expression analyses.

Gene expression analysis was performed using the method described by (Pfaffl, 2001). Multiple, treatment-specific reference genes were used for normalization (Bustin et al., 2009; Vandesompele et al., 2002). Fold changes in the expression of target genes (*COI and mtMutS*) were calculated using *RPL12, SRP54,* and *ACTB* during thermal stress, and *ACTB, TUBB,* and *SRP54* during pH stress, as reference genes (see (Shimpi et al., 2016) for details on reference genes selection). Statistical significance for gene expression was tested using a one-way ANOVA with Tukey's post-hoc test used to calibrate normalized relative quantities, as described previously (Pfaffl, 2001), after having checked that data are normally distributed with a Shapiro-Wilk test. Data were represented as mean ± SE and Tukey's $p < 0.05$ was considered as a threshold indicating statistical significance. The present study conforms to the Minimum Information for Publication of Quantitative Real-Time PCR guidelines (Bustin et al., 2009).

Statistical data analyses for the H_2O_2 stress experiment were performed with the JASP 0.18.3 software (Love et al., 2019). The combined quantitative data from H_2O_2 experimental trials was analyzed using a non-parametric Friedman's test followed by the Conovor post-hoc test to determine differences between groups. The correlation between mtDNA damage and mtDNA copy number represented as mtDNA/nDNA ratio was examined using Pearson's correlation coefficient (*r*).

3. Results

3.1. Effect of thermal and pH stress on mtDNA (Sub-lethal treatment)

Significant mtDNA damage was detected during both sub-lethal thermal and acidification stress. During the elevated seawater temperature treatment (6 h exposure), *Sclerophytum* sp. exhibited 1.29 lesions per 10 kb DNA ($p < 0.05$) (Fig. 1A). DNA damage was higher (3.22 lesions per 10 kb DNA; $p < 0.01$) after 24 h exposure to lowered seawater pH (Fig. 1A). mtDNA copy number decreased (mtDNA/nDNA = 0.68, $p < 0.05$) in response to thermal stress and increased (mtDNA/nDNA = 1.57, $p < 0.01$) during pH stress (Fig. 1B). *COI* gene expression varied, though overall showed upregulation (2.17 folds increase) in response to thermal stress. Similarly, the *mtMutS* gene thought to be involved in DNA damage repair (Muthye et al., 2022; Shimpi and Bentlage, 2023) was also upregulated with a 1.44-fold increase in transcript abundance during thermal stress (Fig. 1C). Acidification (low pH) stress resulted in the downregulation of mitochondrial *COI* (−2.56 fold; $p = 0.47$) but *mtMutS* was upregulated (1.37 fold, $p = 0.050$) (Fig. 1C).

3.2. Effect of acute H_2O_2 stress on mtDNA damage and recovery dynamics

The response of octocoral mtDNA to acute DNA damage induced by a high concentration of H_2O_2 was highly variable, and separate experimental trials showed markedly variable results. However, DNA damage was observed in trials following treatment. While initial mtDNA damage differed across trials, the treatment appeared to elicit DNA damage repair, with negative lesion frequencies indicating extensive and excessive repair indicated by an increase in mtDNA copy number 5 h into recovery. The first trial showed the most dramatic effect of H_2O_2 treatment on mtDNA integrity, inducing 8 lesions per 10 kb. This damage was completely reversed after 1 h of recovery with excessive repair indicated by a negative lesion frequency of −11.64 lesions per 10 kb and − 13.61 per 10 kb after 5 h recovery (Fig. 2A). During the second trial mtDNA damage was relatively low with only 2.3 lesions per 10 kb observed after 30 min of H_2O_2 exposure; lesions increased (3.2) during initial recovery but were reverted by 5 h into recovery (−2.3) (Fig. 2A). During the third trial, the observed lesion frequency was minimal (0.4 lesions per 10 kb) after treatment, followed by damage reversal during recovery. All nubbins survived the treatment for longer than 48 h, except one during trial 2, which was unable to recover likely due to factors other than the treatment. The 48 h post-treatment readings for that sample were considered outliers and were removed from statistical analyses. Despite observed differences in the magnitude of mtDNA damage and subsequent repair, mtDNA damage was detected in all trials, and excessive repair was observed during recovery (especially 5 h recovery time point) suggesting an effective response to mtDNA damage in *Sclerophytum* sp. (Fig. 2A). The Friedman test could not detect any significant effect of the treatment when results of the temporally replicated trials were analyzed together, $\chi 2 = 7.200$ ($df = 4$, $n = 3$), $p = 0.126$. Post hoc analysis using Conovor's test also indicated no significant difference

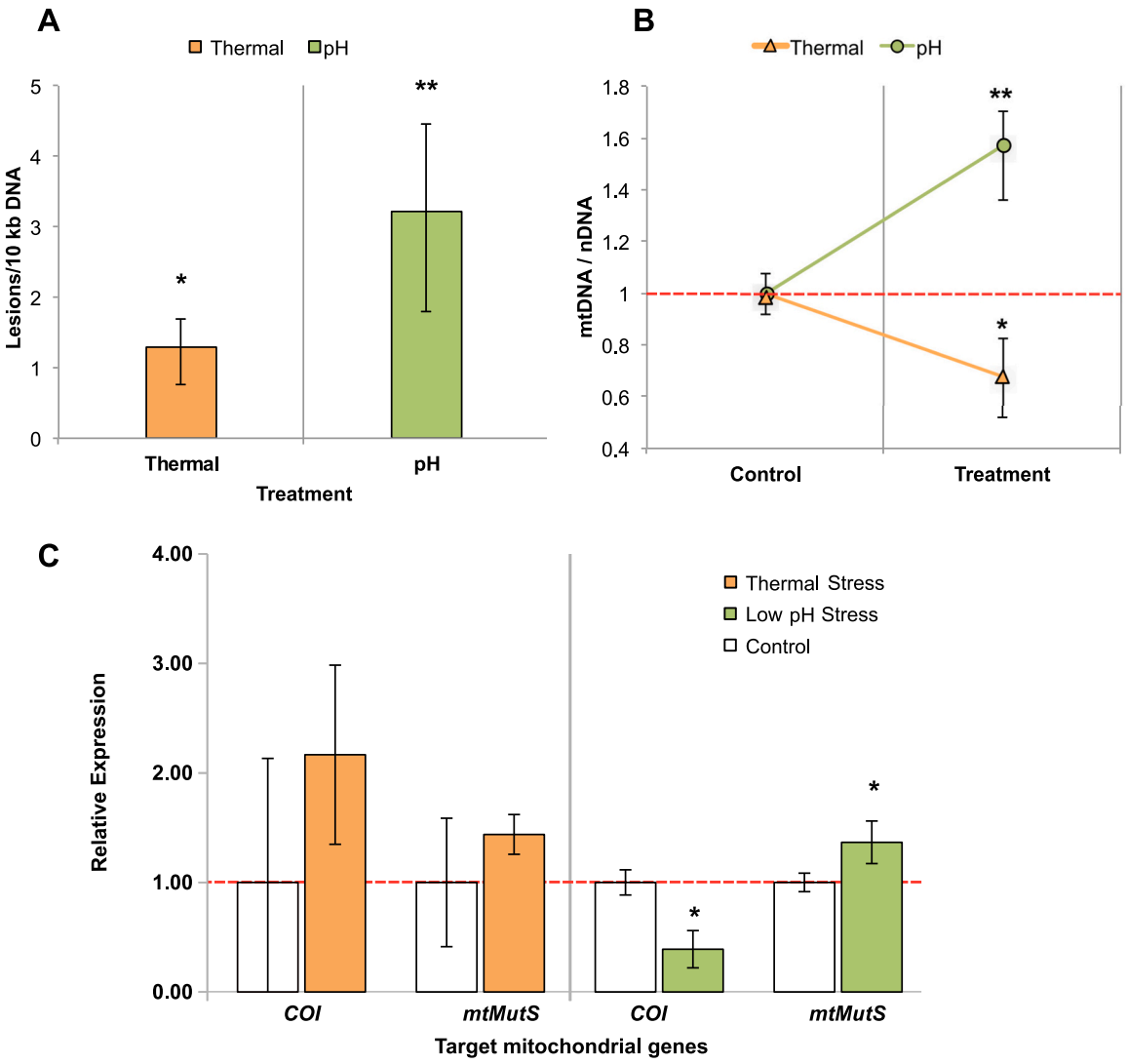

Fig. 1. Thermal and low pH stress induced mtDNA damage, recovery dynamics, and mitochondrial DNA copy number variation. (A) Quantification of mtDNA lesion frequency (MLF) per 10 kb DNA by SLR-qPCR amplification of total DNA from *Sclerophytum* sp. exposed separately to elevated temperature (34 °C) for 6 h and reduced pH for 24 h. (B) In parallel, the mitochondrial copy number was determined by amplifying one mitochondrial fragment and normalized using one nuclear fragment. Untreated controls (26 °C or pH 8.2) were used as reference during respective experiments. Data represents the mean ± SE. * Statistical significant at $p \leq 0.05$; ** Statistically significant at $p \leq 0.01$. (C) Relative expression of mitochondrial genes post thermal and low-pH stress. Changes in transcript levels of 2 mitochondrial genes *COI* and *mtMutS* were assessed. Normalization was performed using validated sets of three reference genes namely *ACTB* and *SRP54* during either and *RPL12* and *TUBB* during thermal and pH stress, respectively. Bars represent the mean expression value (fold change ± SE) relative to untreated controls (26 °C or pH 8.2) *Asterisks* (*) denote significantly higher or lower expression relative to respective controls (Tukey's $p \leq 0.05$).

between groups, except between treatment and recovery after 5 h ($p = 0.046$) (Supplementary Material, Table S1-S2).

3.3. *mtDNA copy number variation and its impact on DNA damage repair dynamics*

The accumulation of lesions in mtDNA beyond a threshold level blocks the transcription and replication ultimately leading to mtDNA degradation (Alexeyev et al., 2013). We evaluated the impact of H_2O_2-driven mtDNA damage on mtDNA replication (and expression) after treatment and its recovery as an indicator of the extent of mtDNA damage. Mitochondrial DNA copy number relative to nuclear DNA was quantified and compared to pre-treatment controls during each independent trial to understand the impact of H_2O_2 treatment on mtDNA copy number and its correlation with DNA damage. During the first trial, the mtDNA copy number decreased to nearly half compared to the control after 30 min H_2O_2 exposure, indicating degradation of severely damaged mtDNA during the treatment. This was rapidly reversed after 1 h recovery to 2-fold excess repair relative to the time-zero control. mtDNA copy number remained high (1.6× control) after the remaining recovery period (Fig. 2B). During the second trial however, mtDNA copy number increased during the 30 min treatment. It remained 1.5 fold higher after 1 h recovery and subsequently returned to a value equivalent to the pre-treatment control indicating no mitochondrial degradation (Fig. 2B). During the third trial, there was no detectable increase or decrease during the treatment or the recovery period, and the mtDNA copy number ranged between 0.98 and 1.3 (Fig. 2B). Results from three independent trials when analyzed together using Friedman's test to identify differences between groups indicated no statistical significance $\chi 2 = 5.627$ ($df = 4$, $n = 3$), $p = 0.229$; and no significant pairwise difference was observed by Conovor's post hoc test (Supplementary Material, Table S3-S4).

We observed a negative correlation between mtDNA copy number and mtDNA damage (Pearson's correlation coefficient $r = -0.664$, $p = 0.010$) (Fig. 3), indicating a higher mtDNA copy number would likely result in a more robust stress response, as shown previously for various

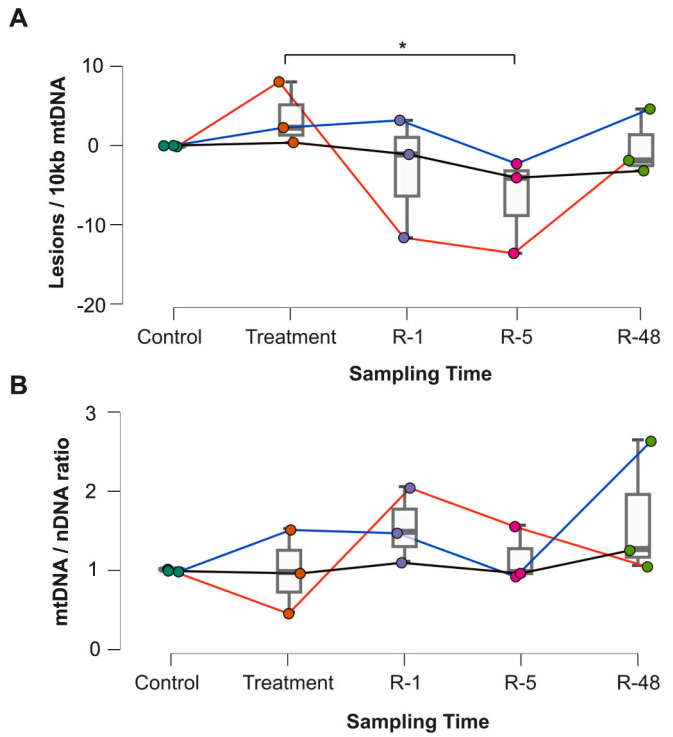

Fig. 2. Hydrogen peroxide-induced mtDNA damage, recovery, and mitochondrial copy number variation. Box plots with individual data points where trend line colors orange, blue and black denote trial 1, trial 2, and trial 3, respectively. (A) Quantification of mtDNA lesion frequency (MLF) per 10 kb DNA by SLR-qPCR amplification of total DNA from *Sclerophytum* sp. exposed to 5 mM H_2O_2 for 30 min (indicated as 'Treatment') followed by recovery for 1 h, 5 h, and 48 h (designated as 'R-1', 'R-5', and 'R-48', respectively). A significant difference identified by Conovor's post hoc analysis is highlighted by asterisks. (B) Mitochondrial copy number changes were determined by amplifying a mitochondrial fragment and normalized using a nuclear fragment. Three independent trials were performed temporally. Each box corresponds to the 25% and 75% percentile while the line across the box represents the median. The whiskers indicate the maximum and minimum values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

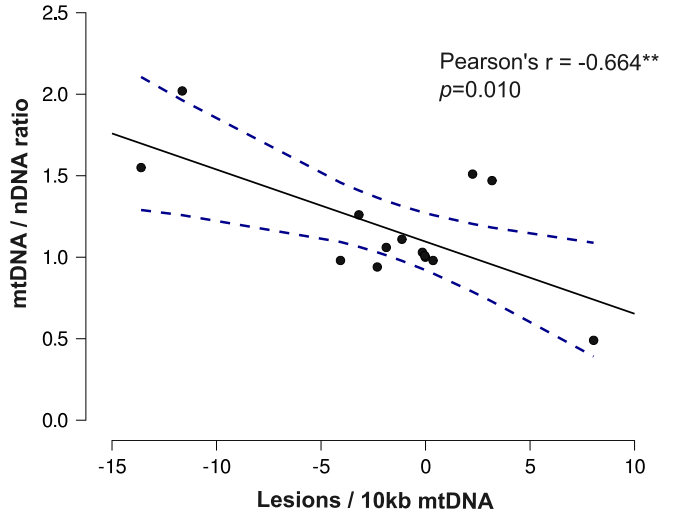

Fig. 3. The scatter plot showing the relationship between mtDNA damage and mtDNA copy number. Pearson's correlation analysis ($n = 14$) with a trend line accompanied by a 95% CI denoted by dashed line.

human cell lines (Dannenmann et al., 2017).

4. Discussion

Octocorals are peculiar members of the phylum Cnidaria, and Metazoa in general, because of their unique mitochondrial genomes encoding a ~ 3 kb DNA mismatch repair gene, the *mtMutS*, discovered nearly three decades ago (Pont-Kingdon et al., 1995). While our understanding of coral responses related to heat and acidification stress has vastly improved in recent years (Cziesielski et al., 2019; Dixon et al., 2020; Voolstra et al., 2021), the investigations focusing on mitochondrial responses to climate change-associated environmental stress scenarios are lacking, despite the pivotal role of mitochondria in stress adaptation through energy production and signaling pathways (Eisner et al., 2018; Picard et al., 2018). In addition, the role of the *mtMutS* DNA repair gene is poorly understood in octocoral mitochondria. Here, we explored the extent and recovery of mtDNA damage upon exposure to different stressors and discovered that mitochondria in our soft coral model are capable of reversing extensive oxidative damage to mtDNA within a relatively short recovery time, which could be attributed to the function of the *mtMutS* gene.

Using sea anemone *Aiptasia pulchella* as a model Dunn et al. (2012) observed compromised mitochondrial integrity of the coral host during thermal stress, indicating that its mitochondria were among the cellular targets of heat stress. The decrease in mtDNA copy number and the presence of mtDNA lesions in our study corroborates that observation. However, the lack of adverse effects on gene expression of mtDNA-encoded *COI* and *mtMutS* suggests that mitochondrial function was minimally compromised in response to acute thermal stress in *Sclerophytum* sp. In contrast, during low-pH stress, the significant reduction in *COI* gene transcripts indicates that the treatment severely compromised mitochondrial function. This is further supported by the higher number of mtDNA lesions relative to the thermal stress treatment (Fig. 1A). Changes in seawater pH result in changing carbonate chemistry thereby elevating oxidative stress and increasing the possibility of DNA damage in marine organisms (Lesser, 2006; Wang et al., 2009). Moreover, prolonged exposure to oxidative stress results in reduced expression of mitochondrial genes in model animals (Austin et al., 1998; Crawford et al., 1998; Morel and Barouki, 1999; Schwarze et al., 1998). Marine organisms also exhibit metabolic suppression in response to elevated CO_2 (Kaniewska et al., 2012; Pörtner, 2008). Hence, a decrease in *COI* gene expression despite the observed increase in mtDNA copy number during acidification stress is anticipated (Fig. 1 B, C).

As a consequence of animal mitochondrial genome organization, transcription of genes on the same strand leads to the formation of an equal amount of pre-mRNA for each gene, yet the steady-state levels of mature mRNA can vary due to posttranscriptional regulation of mRNA abundance (Kotrys and Szczesny, 2020; Mercer et al., 2011). However, the disparity between expression levels of *COI* and *mtMutS,* and the significant upregulation of the latter during both thermal and pH stress (Fig. 1C) hints at its importance as a likely mtDNA repair protein and part of the octocoral stress response toolkit. Here it is interesting to note that despite the damage induced by the low pH treatment, mitochondria were retained and replicated, as implied by the increase in mtDNA copies (Fig. 1A-B). mtDNA copy number and gene expression are important biomarkers of mitochondrial biogenesis (Popov, 2020). When the mutation load is low, damaged mitochondria can recover after undergoing complementation by mitochondrial fusion (Kazak et al., 2012), a process that increases the mtDNA copy number in budding yeast (Hori et al., 2011). Thus, the *mtMutS* is likely involved in countering mtDNA damage caused by acidification stress and thereby helping cells avoid mitochondrial degradation and/or supporting their biogenesis. While *mtMutS* upregulation is evident in both thermal and pH stress response, the disparity in gene expression patterns, mtDNA lesion frequency, and copy numbers between treatments indicate different strategies employed by *Sclerophytum* sp. to mitigate the impacts of these two

distinct stressors on their mitochondria. This difference could either be attributed to the nature or duration of the stress or both and further studies will be required to understand the specific impact and response of octocoral mitochondria to these distinct climate change-associated stressors.

Animal mitochondria are a major source of intracellular ROS. Any minor glitch in electron flow within mitochondria, such as under abiotic stress, can result in higher levels of intracellular ROS (including H_2O_2) and disruption of redox homeostasis. H_2O_2 is formed photochemically in seawater under natural conditions and its effects have been studied in relation to the metabolic activities of scleractinian corals (Higuchi et al., 2009). A recent report indicated H_2O_2 bursts as a regular feature of coral polyp surfaces exhibiting highly elevated levels of H_2O_2 having a dynamic nature (Ousley et al., 2022). It has also been suggested that 500 uM H_2O_2 can exert oxidative stress in the sea anemone *Anemonia viridis* characterized by arrest of cell growth and incomplete recovery after prolonged exposure. However, the effect of exposure was noted to affect endosymbiotic *Symbiodiniaceae* more than the coral host (Cotinat et al., 2022). Spatiotemporal changes in baseline (normal) physiological conditions among independently growing *Sclerophytum* sp. nubbins over a period of time may have resulted in variable initial impact of H_2O_2, as evident from differences in MLFs and mtDNA copy number observed after each independent trial (Fig. 2 A-B). The complete recovery and excess repair observed across trials are noteworthy. An increase in mtDNA copy number contributed to an observed excess repair denoted by negative MLF value (e.g. during first trial recovery). We also observed a reduction in mtDNA copy number in trial 1, likely linked to a higher incidence of lesions leading to mtDNA degradation (Shokolenko et al., 2009), which may have resulted in mitochondrial rescue by the cross-complementation of damaged and undamaged mtDNA along with RNA pool, lipid and protein components through fusion resulting in a maximized oxidative capacity during environmental stress and recovery as discussed previously (Youle and van der Bliek, 2012). The subsequent increase in mtDNA copy number 1 h into the recovery (Fig. 2B) is also consistent with a similar observation in budding yeast upon ROS trigger (Hori et al., 2011). However, mtDNA damage below a certain threshold needed for mitochondrial fusion and/or degradation may have led to the retention of damaged mitochondria (trials 2 and 3). It has been proposed that there is a lower threshold of mtDNA copy number, which triggers replication whereas a higher threshold results in mtDNA degradation (Clay Montier et al., 2009). This critical interplay of mtDNA turnover and biogenesis can also be observed in our study (Fig. 2). Efficient mtDNA repair is likely to help in rapid mitochondrial recovery by facilitating mtDNA replication and transcription processes (Li, 2008).

The mtDNA copy number varies based on the energy requirements of the cells and/or oxidative stress conditions (Lee and Wei, 2005). It has also been suggested that cells with low mtDNA copy numbers are more susceptible to mtDNA damage and that the possession of high copy numbers of mitochondria confers buffering via redundancy (Meyer and Bess, 2012). An increase in ROS level as a result of any environmental stress results in elevated mtDNA replication rates, following a principle of protection by abundance, thus compensating for damaged mtDNA (Dannenmann et al., 2017). Our observations are congruent with this scenario and indicate that the higher initial mtDNA/nDNA ratio is likely essential to mitigate the effects of oxidative stress from its onset. Therefore, the observed differential responses of mtDNA damage and mtDNA copy number of genetically identical corals under similar initial conditions at different times could be attributed to the differences in initial mtDNA copy number before the onset of stress. Future studies aimed at understanding the factors controlling mtDNA copy number under normal physiological conditions in octocorals are required to further comprehend mitochondrial dynamics in these early-branching animals. Given the relevance of processes like mitochondrial fission, fusion, and degradation in handling mtDNA damage due to oxidative stress, this seems of special importance.

The method utilized here to determine mtDNA damage has been used on corals for the first time and may suffer from inherent limitations of the technique (Lehle et al., 2014), such as requiring high copy numbers for accurate estimation of DNA damage incidence. Another limitation was our reliance on clonal replicates that limit biological replication and sample dependence effects, thus limiting generalizations of our results (Lazic, 2016; Lazic et al., 2018). The variability of results observed by us could be a result of treatments not being uniformly applied to a single type of cell. Ideally, cell cultures would be subjected to treatment to ensure uniform application. However, no viable cell lines exist for soft corals at this point. Our observations were made from individuals comprising multiple cell types under different steady-state conditions that may explain some of the variability in our data. For example, sampling more mitochondria-rich myoepithelial cells at polyp-stolon junction in hydroids (Harmata et al., 2015) relative to epithelial cells might result in lower mtDNA lesion frequency and high mtDNA copy number. Nonetheless, considering the combined evidence from previous studies (Hellberg, 2006; Muthye et al., 2022; Shimpi and Bentlage, 2023) and the elevated transcript levels of *mtMutS* after prolonged pH stress and mtDNA damage reversal in our study provide support for *mtMutS* to play a role in enhancing the replication fidelity and/or DNA repair capabilities in octocorals. Additionally, it should also be noted that the mitochondrial response observed in the current study, and stress response in general, is a multifaceted phenomenon involving a still largely unexplored molecular repertoire and mechanisms in need of detailed future investigations. In this respect, functional studies of the *mtMutS* gene are still required, as is the characterization of other potentially associated proteins involved in mtDNA repair in octocorals.

5. Conclusions

By investigating mtDNA damage and repair, and mtDNA copy number variations coupled with gene expression, we provide insights into the mitochondrial stress response of soft coral *Sclerophytum* sp. to cope with environmental stressors associated with anthropogenic climate change. The potential involvement of *mtMutS* gene was evidenced, which could be one of the key elements maintaining mtDNA integrity, aiding mtDNA replication, thus likely supporting a healthy mitochondrial pool for bioenergetic and metabolic adaptation. We show that *Sclerophytum* sp. mitochondria have the ability to reverse stress-induced mtDNA damage. Octocorals have been shown to exhibit remarkable resilience to thermal and pH stress (Gabay et al., 2014; Vargas et al., 2022), also supported by the lability of their skeletal composition to adapt to changing ocean chemistry through evolutionary history (Quattrini et al., 2020), which can also correlate with their general success in the form of observed phase shifts or regime change from scleractinian to octocoral (soft coral) dominated reef systems after chronic environmental disturbances (Edmunds and Lasker, 2016; Lasker et al., 2020). The resilient response of octocoral mitochondria discovered in this study adds to the growing body of these observations and evidence of the potential for acclimation and adaptation of octocorals to changing oceans and provides a new dimension to investigating molecular mechanisms of stress response to understand coral resilience.

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CRediT authorship contribution statement

Gaurav G. Shimpi: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal

analysis, Data curation, Conceptualization. **Sergio Vargas:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Bastian Bentlage:** Writing – review & editing, Visualization, Validation, Methodology, Funding acquisition, Formal analysis, Data curation. **Gert Wörheide:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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