Exploring the mitochondrial response to oxidative DNA damage in octocorals

Gaurav G Shimpi [1], **Sergio Vargas** [1], **Gert Wörheide** Corresp. [1, 2, 3]

[1] Department of Earth and Environmental Sciences, Palaeontology & Geobiology, Ludwig-Maximilians-Universität München, Munich, Germany

[2] GeoBio-CenterLMU, Ludwig-Maximilians-Universität München, Munich, Germany

[3] SNSB –Bavarian State Collections of Palaeontology and Geology, Munich, Germany

Corresponding Author: Gert Wörheide
Email address: woerheide@lmu.de

Mitochondrial response to oxidative stress is intricately related to cellular homeostasis due to the high susceptibility of the mitochondrial genome to oxidative damage. Octocoral mitogenomes possess a unique DNA repair gene, *mtMutS,* potentially capable of counteracting the effects of oxidative stress induced mtDNA damage. Despite this unique feature, the response of octocoral mitochondria to increased oxidative stress remains unexplored. Here we explore the response of the octocoral *Sinularia* cf. *cruciata* to elevated temperature and low-pH stress and its ability to reverse acute oxidative mtDNA damage caused by exogenous agents like hydrogen peroxide (H_2O_2). The differential transcriptional response to these climate change-related stresses was recorded for two mtDNA-encoded genes and three stress biomarkers. Only *HSP70* was significantly upregulated during thermal stress whereas significant reduction in the expression levels of *HSP70*, *GPX*, and *COI* was observed along with an increased number of *mtMutS* transcripts during low-pH stress. Damage to mtDNA was evident, accompanied by changes in mtDNA copy number. Damage caused by H_2O_2 toxicity was reversed within 5 hours and initial mtDNA copy number apparently influenced damage reversal. Our results indicate that different stress-specific resilience strategies are used by this octocoral species and its mitochondria to reverse oxidative stress and associated mtDNA damage. These experiments provide the first account on the response of octocoral mitochondria with its unique gene repertoire among animals to different stressors and highlight its potential role in conferring resilience to the host cells during different climate change scenarios.

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[1]Department of Earth and Environmental Sciences, Palaeontology & Geobiology, Ludwig-Maximilians-Universität München, Richard-Wagner Str. 10, 80333 Munich, Germany

[2]GeoBio-Center[LMU], Richard-Wagner Str. 10, 80333, Munich, Germany

[3]SNSB –Bavarian State Collections of Palaeontology and Geology, Richard-Wagner Str. 10, 80333 Munich, Germany

Corresponding Authors:

Gert Wörheide[123]

Email address: woerheide@lmu.de

Abstract

Mitochondrial response to oxidative stress is intricately related to cellular homeostasis due to the high susceptibility of the mitochondrial genome to oxidative damage. Octocoral mitogenomes possess a unique DNA repair gene, *mtMutS,* potentially capable of counteracting the effects of oxidative stress induced mtDNA damage. Despite this unique feature, the response of octocoral mitochondria to increased oxidative stress remains unexplored. Here we explore the response of the octocoral *Sinularia* cf. *cruciata* to elevated temperature and low-pH stress and its ability to reverse acute oxidative mtDNA damage caused by exogenous agents like hydrogen peroxide (H_2O_2). The differential transcriptional response to these climate change-related stresses was recorded for two mtDNA-encoded genes and three stress biomarkers. Only *HSP70* was significantly upregulated during thermal stress whereas significant reduction in the expression levels of *HSP70*, *GPX*, and *COI* was observed along with an increased number of *mtMutS* transcripts during low-pH stress. Damage to mtDNA was evident, accompanied by changes in mtDNA copy number. Damage caused by H_2O_2 toxicity was reversed within 5 hours and initial mtDNA copy number apparently influenced damage reversal. Our results indicate that different stress-specific resilience strategies are used by this octocoral species and its mitochondria to reverse oxidative stress and associated mtDNA damage. These experiments provide the first account on the response of octocoral mitochondria with its unique gene repertoire among animals to different stressors and highlight its potential role in conferring resilience to the host cells during different climate change scenarios.

Background

Since the advent of aerobic life on planet Earth nearly 2.5 Gyr ago(Falkowski et al. 2004), oxidative stress exerted by the production of cellular reactive oxygen species (ROS) has been associated with biological systems (Cadenas 1989). The excessive accumulation of ROS in the cellular environment results in damage to lipids, proteins and most importantly the DNA (Lesser 2006). Global climate change has long been implicated in imposing greater oxidative stress upon marine organisms (Lesser 2006). The rapid changes in the climate apparently induced by anthropogenic activities are contributing to the warming and acidification of the oceans and cause increased stress on coral reef communities. These environmental perturbations, especially increased sea surface temperatures, affects coral productivity and growth, resulting in partial or complete colony mortality during so called “coral bleaching” events, and ultimately leading to the loss of these “rainforests of the ocean” (Hoegh-Guldberg et al. 2007). However, despite an ever-increasing knowledge about the biology and the ecological implications of climate change-induced stress on corals, a precise understanding of the impact on the cellular powerhouse, the mitochondrion, and their response to these stressors remains unknown. This is at odds with the pivotal role of mitochondria in energy production as well as its involvement in other important cellular processes, such as apoptotic programmed cell death (Ott et al. 2007).

The energy status of an organism, including corals, determines its performance under stressful conditions and is crucial for survival (Lesser 2013). Mitochondria generate ATP, the cellular energy currency, through oxidative phosphorylation (OXPHOS), which involves series of electron transfer by the electron transport chain (ETC). Leakage of highly reactive electrons during this transfer leads to generation of ROS. Thus, being an energy hub, mitochondria are a major source of ROS, and consequently a main site of oxidative damage in animals, including corals (Blackstone 2009). Mitochondrial DNA (mtDNA) is particularly prone to such damage

(Sawyer et al. 2001) and the integrity of the mitochondrial genome is constantly threatened by the production of endogenous ROS, which in addition can elicit pre-apoptotic protein signaling cascades ultimately leading to cell death (Ott et al. 2007). Hence, the response of mitochondria during and after oxidative stress caused by any disturbances in the environment is crucial in deciding the fate of a cell and ultimately of the organism (Dunn et al. 2012).

To cope with the DNA damage, cells posses a number of DNA repair mechanisms. However, the variety, the fidelity and the efficacy of DNA repair appears to be different for nuclear (nDNA) and mtDNA (Boesch et al. 2011). mtDNA damage persists longer (Yakes & Van Houten 1997) and the accumulation of mutations is 10 times faster in mtDNA than nDNA in animals (Brown et al. 1979), which has been attributed to less efficient DNA repair mechanisms for mitochondria (Boesch et al. 2011). Interestingly, the high mtDNA mutation rate observed among most animals is not present in non-bilaterian metazoans, such as anthozoan corals and sponges, which exhibit unusually slow rates of mtDNA sequence evolution (Huang et al. 2008; Shearer et al. 2002), and show unique features in terms of mitogenome organization and gene content. A typical animal mitochondrial genome encodes 13 protein coding genes, 22 tRNA and 2 ribosomal RNA genes. In addition, non-bilaterians harbor group I introns (Szitenberg et al. 2010; van Oppen et al. 2002), additional protein coding genes and/or unknown ORFs and gene duplications (Park et al. 2011; Pont-Kingdon et al. 1995), among other novelties. No DNA repair or oxidative stress related protein-coding genes have been reported in animal mitochondrial genomes so far. However, the octocoral mitogenomes encode a mismatch DNA repair gene (*mtMutS*) (Pont-Kingdon et al. 1995). Although the exact function of this gene remains to be determined, its role in maintaining low levels of sequence variation and its involvement in mtDNA repair and gene rearrangement in the octocoral mitogenome has been proposed (Bilewitch & Degnan 2011;

Brockman & McFadden 2012). While most studies have examined the damage to nuclear DNA, in response to oxidative stress and other DNA damaging agents, in scleractinian corals (Lesser & Farrell 2004; Schwarz et al. 2013; Svanfeldt et al. 2014), the impact of ROS on mtDNA and the potential of mtDNA recovery in octocorals has not been explored, despite their mitogenomes harboring a unique DNA repair gene.

Oxidative stress in marine ecosystems is a well-known phenomenon with adverse effects on marine organisms (Lesser 2006). Thermal and pH stress have long been implicated in inducing oxidative stress in corals and the response of reef-building scleractinian corals to increased temperature has been extensively studied (Lesser 2006; Lesser 2011). Studies exploring the physiological and transcriptomic response of octocorals/soft corals to such environmental stresses have recently started to emerge (Lõhelaid et al. 2014; Pratlong et al. 2015). However, a combined assessment of effect of climate change-related oxidative stress on mitogenome integrity of octocorals, its potential for recovery, and the response of *mtMutS* and other stress biomarkers genes is yet lacking.

Here, we aim to explore how octocoral mitochondria respond to different abiotic stressors and an exogenous DNA damaging agent. We use a sensitive quantitative real-time PCR based approach to assess the extent of mtDNA damage caused by common climate change-related stressors such as high seawater temperature and reduced pH, as well as an exogenous DNA damaging agent, hydrogen peroxide (H_2O_2), and the capacity of octocoral *Sinularia* cf. *cruciata* to repair damaged mtDNA. We followed the dynamics of mtDNA copy number to understand the associated changes during the mitochondrial recovery process. In addition, the differential gene expression of two mitochondrial genes (cytochrome c oxidase subunit I and *mtMutS*) and three nuclear genes involved in oxidative stress response, namely, heat shock protein 70 *(HSP70),* glutathione

peroxidase *(GPX)* and Cu/Zn superoxide dismutase *(CuZnSOD)* was assessed. This is one of the first attempts to integrate gene expression and mtDNA damage/repair quantification to explore the ability of octocorals to mitigate and resist climate change-induced oxidative stress events, and represents a first step towards developing fundamental/mechanistic mitochondria-centric models of stress tolerance in octocorals.

Materials and Methods

Coral collection and maintenance

Coral colonies were obtained from a commercial source. They were subsequently cut into several pieces that were allowed to grow independently in a closed circuit seawater aquarium at the Molecular Geo- and Palaeobiology lab, Department of Earth and Environmental Sciences Palaeontology & Geobiology, LMU, Munich. The corals were kept under controlled conditions (25 ± 1 °C, pH 8.2 ± 0.1) with a biweekly exchange of 50% fresh artificial seawater (Red Sea, Germany). All the corals were maintained on a 12 h light / 12 h dark light-regime provided by LED light (GHL Mitras LX 6200-HV) at a light intensity of 14 ± 2 kLux. A similar light regime was used for both control and experimental systems mentioned below.

Gene identification, sequencing and qPCR primer design:

Sequences of stress-related genes *GPX* and *CuZnSOD* were obtained from shallow transcriptomic data (unpublished), their identities were confirmed by BLASTn, and BLASTp and these sequences were used for qPCR primer design. *HSP70* and reference genes primers were obtained from a previous study (Shimpi et al. 2016). Mitochondrial gene primers were

designed using mitochondrial genome of *S* cf. *cruciata* (GenBank accession: KY462727) (Shimpi et al. 2017).

For semi-long run qPCR (SLR-qPCR) and mtDNA copy number assays (see below), a large (1057 bp) fragment spanning the mitochondrial *COI*-igr-*COII* genes was sequenced using previously reported primers (McFadden et al. 2011) and a new primer binding 100 bp upstream the 3' end of this large fragment was designed to yield a short internal fragment of the same region. Nuclear *ACTB* gene primers were used to determine mtDNA copy number; these primers were same as those used for gene expression. Primer design was performed using Primer3 (Untergasser et al. 2012) and Geneious 6.1 (Kearse et al. 2012) was used for all sequence analyses. Melting curves (see Fig. S2), gel electrophoresis and sequencing of the amplification products of a primer pair confirmed the specificity of all the primers used. Newly obtained sequences were submitted to European Nucleotide Archive (ENA Accession No.: LT717245, LT717246).

Experimental oxidative stress and DNA damage treatments

To determine the effect of oxidative stress due to rising seawater temperature, decreased pH (both sub-lethal) and presence of hydrogen peroxide (H_2O_2) in the water (acute toxicity) on mtDNA damage, mtDNA copy number and gene expression, nubbins of *Sinularia* cf. *cruciata* were exposed to these conditions (see below). All experiments were performed in biological as well as technical triplicates unless otherwise stated and controls as well as treated sample tissues were preserved in absolute ethanol for DNA extraction or were snap frozen in liquid nitrogen and subsequently stored at -80°C until RNA extraction. Thermal and low-pH stress treatments were performed as described previously (Shimpi et al. 2016). Briefly,

Thermal stress

Three *S.* cf. *cruciata* nubbins of similar size were placed in an experimental 10L tank and the temperature in the tank was raised gradually from 26 °C to 34 °C over a period of 2 h and was maintained at 34 °C for 6 h thereafter. Three controls were maintained in a similar tank as the experimental tank but temperature was kept at 26ºC during the course of the experiment.

Low-pH stress

Three *S.* cf. *cruciata* nubbins were exposed to low seawater pH by pumping carbon dioxide (CO_2) into the seawater of a 10L experimental tank to maintain a stable low pH value of 7.5. The pH was first reduced to 7.5 over a period of 2 h and then maintained at this value for 24 h. The pH value was recorded throughout the experiment and it was observed to be constant at 7.5. Corals were sampled after 24 h exposure. Control samples were maintained under normal condition (pH 8.2) during the course of experiment and the temperature in both tanks was kept constant at 26 °C.

Hydrogen Peroxide treatment:

To evaluate the capability of octocoral mtDNA to recover from severe mtDNA damage, Hydrogen peroxide (H_2O_2), a principle 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 allowing it to diffuse freely throughout the cell and causing DNA damage via Fenton reaction (Lesser 2011). For this treatment, three independent DNA damage experiments were performed (E1, E2, and E3) at different times on independently growing genetically identical coral nubbins. A 5.0 mM H_2O_2 final concentration was used for acute toxicity and extensive DNA damage. The experiments were performed in 2L tanks. Tissue samples were taken at ‘time-zero’ and used as

respective controls/references. Subsequently, 30% v/v H_2O_2 (Sigma-Aldrich) was added to the seawater to achieve a final concentration of 5 mM. Corals were kept in this solution for 30 min after which tissues were subsampled (Labeled as 'Treatment'). After this the corals were kept at initial control conditions for recovery. During recovery two tissue samples were taken after 1h and 5h post-treatment (Labeled as 'Rec-time'). Additionally, two other octocoral species, *Sinularia sp.,* and *Briareum sp*., were treated similarly and the recovery was monitored for 1 h.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from control and treated 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 manufacturer's protocol. This treated RNA was further purified using Sodium Acetate- Ethanol precipitation. 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.). RNA extracts with a RIN value ≥ 7.5 were used for cDNA synthesis (data not shown). 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.

Quantitative Real-time RT-PCR (qPCR)

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 2X mastermix, and 250 to 400 nM each primer. A two-step qPCR including an initial

196 denaturation step of 3 min at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 20 s. A
197 non-template control was always included in each assay. Melting curve analysis was performed
198 at the end of each qPCR to confirm amplification specificity and amplification products were
199 also checked by agarose gel electrophoresis after each assay. Details on the primers used can be
200 found in Table 1.

201 **DNA extraction:**

202 Extraction of total DNA from control and treated coral tissues was performed using the
203 NucleoSpin Tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions.
204 DNA quality and purity was determined using a Nanodrop ND-1000 spectrophotometer (Thermo
205 Fisher Scientific, USA), which indicated a high quality DNA extracts (A260/A280 ≥ 1.8).

206 **Semi-long run qPCR (SLR-qPCR)**

207 To quantify mtDNA damage a semi-long run quantitative PCR (SLR-qPCR) was performed as
208 described previously (Rothfuss et al. 2010). Briefly, a large (1057 bp) and a small DNA fragment
209 (100 bp) of the same mitochondrial region (*COI*-igr-*COII*) was amplified using the KAPA
210 SYBR FAST universal mastermix (Peqlab, Germany) in 15 µl reactions containing 1X
211 mastermix, and 500 nM of each, forward and reverse primer and 5 ng total DNA. The cycling
212 conditions consisted of a pre-incubation step at 95 °C for 3 min followed by 40 cycles of 95 °C
213 for 10 sec, and 60 °C for 20 sec for the small fragment, and 95 °C for 10 sec, 58 °C for 20 sec
214 and 72 °C for 30 sec for the large fragment. The mitochondrial regions, primers and PCR
215 efficiencies are listed in Table 2. Each sample was assayed in triplicates and the amplicon
216 specificity was monitored by melting curve analysis and gel electrophoresis. Cq values and mean
217 PCR efficiency (E) for the primer pair was obtained using the computer program LinRegPCR
218 (Ramakers et al. 2003). Cq values were efficiency-corrected using the formula "efficiency-

corrected-Cq = Cq * (log(E) / log(2))" (Kubista M 2007) and used in the calculation of mitochondrial lesion frequency (MLF) using the formula, "Lesion rate (lesions/10kb) = $(1 - 2^{-(\Delta \text{ long} - \Delta \text{ short})}) \times (10000 \text{ [bp]} / \text{size of long fragment [bp]})$" (Rothfuss et al. 2010). DNA isolated from the non-treated controls was used as reference whereas Cqs of the large and small mitochondrial fragments were used for DNA damage quantification.

Determination of mtDNA copy number:

To determine the extent of damage of experimental treatments to mitochondria, the mtDNA/nDNA ratio (i.e mtDNA copy number) was calculated before and after treatment using qPCR. Equal amount 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 were obtained using Livak's method (Livak & Schmittgen 2001); the non-treated sample served as control and the *ACTB* Cq values as reference. Primer details are listed in Table 2.

For comparison of initial mitochondrial number among all control samples used during each experiment, which were done at different times during the span of 2 years on nubbins obtained from the same colony, a ratio of *COI* versus *ACTB* gene fragment was obtained for each control separately. The geometric mean of these ratios was calculated and each mtDNA/nDNA ratio was divided by this value to obtain relative mitochondrial copy numbers for control samples using REST2009 (single time-zero control for each H_2O_2 treatment experiment and triplicate control samples for thermal and low pH treatment each).

Data Analysis

The raw, non-baseline corrected fluorescence data obtained after qPCR was baseline corrected using LinRegPCR (Ramakers et al. 2003) and Cq values and amplification efficiency 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 of (Pfaffl et al. 2002) implemented in REST2009. Multiple, treatment-specific reference genes (Bustin et al. 2009) exhibiting stable expression in *Sinularia* cf. *cruciata* during thermal and low-pH stress were used for qPCR normalization (Shimpi et al. 2016). Fold changes in the expression of target or stress-related genes (*HSP70, GPX, CuZnSOD, COI and mtMutS*) were calculated using *RPL12, SRP54* and *ACTB* during thermal stress, and *ACTB, TUBB,* and *SRP54* during pH stress, as reference genes. Statistical significance for gene expression was tested using randomization and bootstrapping with 10000 iterations, and standard errors were calculated with the Taylor algorithm implemented in REST 2009. Data is represented as mean ± SE and REST'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).

Results

Effect of thermal stress on gene expression and mtDNA (Sub-lethal treatment):

The effect of thermal stress was apparent on gene expression with *HSP70* expression showing strong induction (>7 folds change; $p < 0.05$) during thermal stress while the expression of *GPX* decreased (-6.1 folds) and that of *CuZnSOD* was less affected (-1.4 fold). *COI* expression varied greatly with an overall upward trend (2.2 folds increase). Similarly, the *mtMutS* expression was also upregulated with a 1.4 fold increase in transcript abundance (Fig. 1A).

Significant mtDNA damage was detected in response to elevated seawater temperature treatment (6 h exposure) represented by 1.29 lesions per 10 kb DNA (p <0.05). The mtDNA copy number was decreased (mtDNA/nDNA = 0.68, p <0.05) in response to this stress (Fig. 2).

Effect of low-pH stress on gene expression and mtDNA (Sub-lethal treatment):

Low-pH stress resulted in the downregulation of *HSP70* (-1.87 fold, p <0.05), *GPX* (-1.71 fold, p <0.05) and *CuZnSOD* (-1.11 fold) expression. The mitochondrial *COI* was also downregulated (-2.8 fold; p <0.05) but, in contrast, the *mtMutS* was significantly upregulated (1.25 fold, p <0.05) after this treatment. The difference in expression between these two mitochondrial genes amounts to a fold change of 4 (p <0.001) (Fig. 1B).

The damage of higher magnitude was detected (3.22 lesions per 10 kb DNA; p <0.01) after 24 h exposure to lowered seawater pH (Fig. 1A). The mtDNA copy number exhibited increase, with respect to the controls, (mtDNA/nDNA = 1.57, p <0.01) during low-pH stress (Fig. 2).

Effect of acute H_2O_2 stress on mtDNA and recovery dynamics:

The response of octocoral mtDNA to excessive acute DNA damage induced by a high concentration of H_2O_2 (5 mM) was variable in magnitude and three independent experiments separated by 4 to 8 months time span, performed on independently grown nubbins showed dramatic changes under corresponding physiological state.

Magnitude of damage induction by 30 min H_2O_2 treatment differed during each independent experiment performed. Lesions incurred were 9.0, 2.4, and 0.4 per 10 kb DNA after treatment for E1, E2 and E3, respectively. The observed differences are likely due to differing mtDNA/nDNA ratios of time-zero controls used in each independent experiment (discussed below).

Recovery dynamics was likely dependent on the initial mtDNA damage and mtDNA copy numbers. Hence, the lesion frequencies quantified after 1 hr recovery were -9.6, 3.4, and -1.1 for E1, E2, and E3, respectively. Recovery after 5 hr from the end of treatment clearly exhibited uniform mtDNA damage reversal indicated by negative number of lesions per 10 kb DNA suggesting an excess repair and/or increased mtDNA copies. Observed lesion frequencies for E1, E2 and E3 were -6.6, -2.4 and -4.2, respectively (Fig. 3A).

Despite the difference in magnitude in all experiments, mtDNA damage was detected and was reversed, and an excess repair was observed within 5 h after treatment (Fig. 3A). An additional experiment performed together with the two other soft corals, *Sinularia sp.* and *Briareum sp.,* also exhibited mtDNA damage followed by a partial damage reversal after 1 h recovery (see Fig. S1).

mtDNA copy number variation upon acute H_2O_2 induced mtDNA damage:

The accumulation of lesions in the mtDNA beyond a threshold level results in blockage of the transcription as well as replication leading to mtDNA degradation (Alexeyev et al. 2013). We evaluated the impact of H_2O_2 driven mtDNA damage on mtDNA replication after treatment and its recovery as a proxy for adversity of damage. Mitochondrial DNA copy number relative to nuclear DNA was monitored to understand the recovery kinetics and its correlation to the DNA damage extent compared to time-zero reference. Dramatic changes in the mtDNA copy number were observed during E1, where a decreased to almost half with respect to the time-zero control after 30 min H_2O_2 exposure, likely indicated degradation of severely damaged mtDNA during the treatment. This was rapidly reversed after 1 h recovery to 2 folds excess copies. mtDNA copy number remained high (1.7 X control) after 5 hr recovery period (Fig. 3B). During the second

experiment 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 time-zero reference. No degradation was observed. During the third experiment, there was no detectable increase or decrease during the treatment or the recovery period and mtDNA copy number ranged between 0.97 and 1.1 during the course of the experiment (Fig. 3B).

Comparison of mtDNA copy number among experimental control/reference samples:

To further understand the reasons for the observed differential responses of mtDNA damage and mtDNA copy number of genetically identical corals under similar initial conditions at different times, the mtDNA/nDNA ratios of the time-zero reference tissues were compared with each other to explore its relation to the differential response. The mtDNA copy number was found lowest for the time-zero reference samples of E1 and highest during the E3 H_2O_2 experiment. The difference between the E1 and E3 experiments was 5.5 fold and a 5.2-fold difference in mtDNA copy number between E1 and E2 experiments was observed. The thermal and low-pH stress initial mtDNA copy numbers were comparable ($p > 0.05$) and found to be similar to the E2 and E3 H_2O_2 experiment rather than to the values observed for the E1 (Fig. 4).

Discussion

Octocorals are notable members of the phylum Cnidaria by virtue of their unique mitochondrial genomes encoding a ~3 kb putative mismatch repair gene likely of bacterial origin (Pont-Kingdon et al. 1995). While most studies on coral stress response focus on the coral-dinoflagellate symbiosis, calcification, bleaching, heat and acidification stress (Hoegh-Guldberg et al. 2007; Lesser 2006; Lesser 2011), investigations on mtDNA damage, repair and gene expression during climate change-associated environmental stress scenarios are lacking.

Moreover, the role of this special DNA repair gene is largely unknown in octocoral mitochondria. Here we explore the potential importance of the *mtMutS* gene during stress response and discover that the soft coral mitochondria are capable of reverting extensive oxidative damage to mtDNA within a relatively short recovery time.

The molecular responses to climate change induced oxidative stress are well documented for some members of Phylum Cnidaria, such as members of the genus *Acropora*, and are reported to involve heat shock proteins and antioxidant enzymes (Bhattacharya et al. 2016; Kaniewska et al. 2012; Moya et al. 2015). Studies have shown upregulation of the heat shock protein gene *HSP70* in response to thermal stress in few octocorals as well as other cnidarians, pointing towards the existence of a conserved mechanism among these organisms to mitigate heat stress, alike most other animals (Lõhelaid et al. 2014). The results of the present study corroborate this observation. The very strong induction of *HSP70* during thermal stress in *S.* cf. *cruciata* indicates an important role of this gene in stress mitigation.

The observed significant down-regulation of *HSP70* in acidified seawater is in contrast with previous studies that found either an increase in expression (Moya et al. 2015) or no differential expression of this gene under ocean acidification scenarios in scleractinian corals (Nakamura et al. 2012). Our results imply that the *HSP70* gene induction is not required during low-pH stress, as the external pH changes resulting in acid/base imbalance may not necessarily result in denatured cytoplasmic proteins, unlike heat stress, after which refolding of denatured proteins require assistance from chaperones. During pH stress, the soft coral might try to compensate for the stress by suppressing unnecessary metabolic pathways, investing energy into the important ones to cope with acid-base imbalance, which explains the down-regulation of *HSP70*.

353 *GPX* encodes a glutathione peroxidase, a key antioxidant enzyme that catalyzes the conversion
354 of harmful H_2O_2 to H_2O with the help of reduced glutathione, and plays an important role in
355 ROS detoxification (Halliwell 2006). Surprisingly, *GPX* was downregulated during both thermal
356 and low-pH treatments. Depletion of the glutathione pool during the initial hours of exposure to
357 stress could be the reason for the observed decrease in *GPX* expression (Downs et al. 2000;
358 Sagara et al. 1998). Furthermore, the sea anemone *Nematostella vectensis* genome was found to
359 contain 12 *GPX* isoforms (Goldstone 2008), hence it is also possible that the *GPX* isoform
360 assessed here does not participate in oxidative stress response at this stage and/or under the
361 circumstances studied here. Additionally, another gene involved in antioxidant defense
362 *CuZnSOD,* which occurs predominantly in the cytosol of eukaryotes (Halliwell 2006) remained
363 relatively unaffected during both thermal and low-pH stress conditions. Nonetheless, because
364 three different members of the SOD multigene family have been described in a sea anemone
365 along with several isoforms of *CuZnSOD,* it is likely that other SOD genes or their isoforms are
366 involved in scavenging superoxide radicals under these conditions (Plantivaux et al. 2004).
367 At cellular level, climate change-induced coral bleaching is essentially a consequence of
368 impairment of symbiont's photosynthetic apparatus resulting in excessive leakage of ROS into
369 host cell leading to the breakdown of coral-dinoflagellate symbiosis (Weis 2008). Another major
370 source of ROS inside the host cell due to environmental perturbations is its own mitochondria
371 (Blackstone 2009; Dykens et al. 1992), which may also aggravate the bleaching by contributing
372 to the overall ROS concentration inside the host cell. Mounting of antioxidant defenses by host
373 cell is often insufficient to quench excess concentration of ROS, incurring host protein,
374 membrane and DNA damage (Lesser & Farrell 2004; Richier et al. 2005).

375 Mitochondrial DNA integrity is a prerequisite for cellular homeostasis as it encodes the most
376 crucial component of electron transport chain (ETC) involved in oxidative phosphorylation and
377 energy production. Therefore, it is necessary to appreciate mtDNA damage and the changes in
378 mitochondrial gene expression together during oxidative stress as a proxy to understand its
379 impact at cellular as well as organismal levels in corals. For example, in *Aiptasia sp*., thermal
380 stress adversely affects *ATPase* expression compromising the Complex IV of ETC and leads to
381 adverse changes in mitochondrial structure and functionality (Dunn et al. 2012). Damage to the
382 mitochondria was evident in *S*. cf. *cruciata* as determined by presence of mtDNA lesions and
383 decreased mtDNA copy number observed after thermal stress. However, the absence of a
384 negative effect on *COI* and *mtMutS* gene expression, and the low levels of mtDNA damage,
385 suggest that the mitochondrial integrity was less compromised in response to acute short-term
386 thermal stress in this case.
387 During low-pH stress, the significant reduction in *COI* gene transcripts indicates severely
388 compromised mitochondrial integrity. This is further supported by the higher number of
389 mitochondrial lesions. Changes in seawater pH result in changing the carbonate chemistry
390 thereby elevating the oxidative stress and increasing the possibility of DNA damage in marine
391 organisms (Lesser 2006; Wang et al. 2009). Moreover, prolonged exposure to oxidative stress
392 results in reduced expression of mitochondrial genes similar to the observed *COI* downregulation
393 during pH stress (Morel & Barouki 1999; Schwarze et al. 1998). However, marine organisms are
394 known to exhibiting metabolic suppression in response to elevated CO_2 (Kaniewska et al. 2012;
395 Pörtner 2008). Hence, a decrease in *COI* and other stress-response genes expression during a
396 prolonged and coral host-oriented acidification stress is anticipated. Mitochondrial genes are
397 generally co-expressed with the OXPHOS genes (van Waveren & Moraes 2008) as observed

here during thermal stress. However, the decoupling of expression between *COI* and *mtMutS,* and the significant upregulation of the later during pH stress highlights its importance, likely as an mtDNA repair protein, as a part of an octocoral stress response toolkit. In any case, despite the damage induced by the low-pH treatment, mitochondria were retained and replicated as implied by the increase in mtDNA copies. Damaged mitochondria can recover after undergoing complementation by mitochondrial fusion as observed in animals, occurring when mutational load is low (Kazak et al. 2012). These results point to a likely role of the *mtMutS* gene in counteracting the mtDNA damage imposed and thereby helping the cells to avoid mitochondrial degradation. Taking together, it is evident from the observed differences in mitochondrial as well as stress-related gene expression changes between thermal and pH stress, the octocoral cells as well as their mitochondria exhibit specific strategies to tackle environmental perturbations of different kinds and/or extents. .

H_2O_2 is formed photochemically in seawater under natural conditions and its effects have been studied in relation to metabolic activities on stony corals (Higuchi et al. 2009). The primary aims of H_2O_2 treatment were to induce mtDNA damage and to observe its recovery with respect to the time-zero reference coral. The observance of mtDNA damage, its complete recovery and excess repair observed within 5 h post-treatment are noteworthy. Excess repair is likely when the baseline mtDNA lesions (i.e. those present at time-zero) are also reversed due to an induced process of damage recovery. Increase in mtDNA copy number can also lead to an observed excess repair (e.g. during E1 recovery). We also observed a reduction in mtDNA copy number likely linked to a higher incidences of lesions leading to mtDNA degradation (Shokolenko et al. 2009), and subsequent mitochondrial rescue by the cross-complementation of damaged and undamaged mtDNA along with RNA pool, lipid and protein components through fusion

421 resulting in a maximized oxidative capacity during environmental stress and recovery (Youle &
422 van der Bliek 2012). Efficient mtDNA repair is likely to help in rapid mitochondrial recovery by
423 facilitating mitogenome replication and transcription processes (Li 2008). On the other hand,
424 mtDNA damage below the threshold needed for mitochondrial fusion and/or degradation may
425 have led to the retention of damaged mitochondria (E2 and E3). The mtDNA copy number varies
426 based on energy requirements of the cells and/or oxidative stress conditions (Lee & Wei 2005). It
427 has also been suggested that cells with low mtDNA copy number are more susceptible to
428 mtDNA damage, and that the possession of high copy numbers confers buffering via redundancy
429 (Meyer & Bess 2012). Our observations corroborate these findings and indicate that the higher
430 initial mtDNA/nDNA ratio is likely essential to mitigate the effects of oxidative stress from its
431 onset. Spatiotemporal changes in baseline (normal) physiological conditions among
432 independently growing coral nubbins over a period of time may have resulted in variable initial
433 impact of H_2O_2 as evident from different MLF observed after each independent experiment,
434 which suggests that the baseline physiological status needs to be consider while designing and
435 comparing the experiments. Hence, future studies should be aimed at understanding the factors
436 controlling mtDNA copy number under normal physiological conditions in octocorals to further
437 comprehend mitochondrial dynamics in these non-bilaterian animals. This seems of special
438 importance given the relevance of processes like mitochondrial fission, fusion, and degradation
439 in handling mtDNA damage due to oxidative stress.
440 Finally, it has been shown that the human MutS homolog 5 (hMSH5) protein, which is localized
441 in mitochondria binding to mtDNA, interacts with DNA Polymerase gamma (POLG), and it's
442 overexpression leads to efficient repair of oxidative lesions (Bannwarth et al. 2012). It is
443 tempting to speculate that the elevated transcript levels of *mtMutS* observed after prolonged pH

stress and mtDNA damage observed in our study indicates its role in enhancing the replication fidelity and/or DNA repair capabilities of mitochondria in octocorals. Moreover, the observed slow mtDNA evolution also provides an indirect support to a better mtDNA repair capabilities of these organisms. We are, however, aware that the mtDNA repair observed in the current study is a multifactorial phenomenon involving still unexplored molecular 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, if any, associated proteins involved in mtDNA repair in octocorals. In addition, the gene expression of nuclear encoded mitochondrial genes, such as *POLG*, mitochondrial DNA-directed RNA polymerase (*POLRMT)*, mitochondrial single-stranded DNA binding proteins *(mtSSB)* and mitochondrial transcription factor A (*TFAM)*, along with several other key proteins responsible for the maintenance of mitochondria deserve to be investigated to advance our understanding of the biology of coral stress response.

Conclusions

Here we present a mitochondria-centric view of octocoral stress response and explore its relevance during experimental global climate change scenarios. By investigating mtDNA damage, repair, mtDNA copy number variations coupled with gene expression, we uncover some of the strategies used by octocorals to cope with environmental stressors and the ability of octocoral mitochondria to reverse stress-induced mtDNA damage. Recent data suggest that corals are capable of acclimating to thermal stress via physiological plasticity, transcriptome changes (Bay & Palumbi 2015) and/or by symbiont-switching (Keshavmurthy et al. 2014). In the case of octocorals, their tissues can act as a barrier to resist the adverse effect of low seawater pH (Gabay et al. 2014). The resilience of the octocoral mitochondria observed here offers further hope for octocorals to be, to a certain degree, resilient to changing future oceans.

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Supplemental Information

Figure S1. mtDNA damage and repair in other octocorals species.

Figure S2. Melting curve analysis of target and reference genes (A and B).

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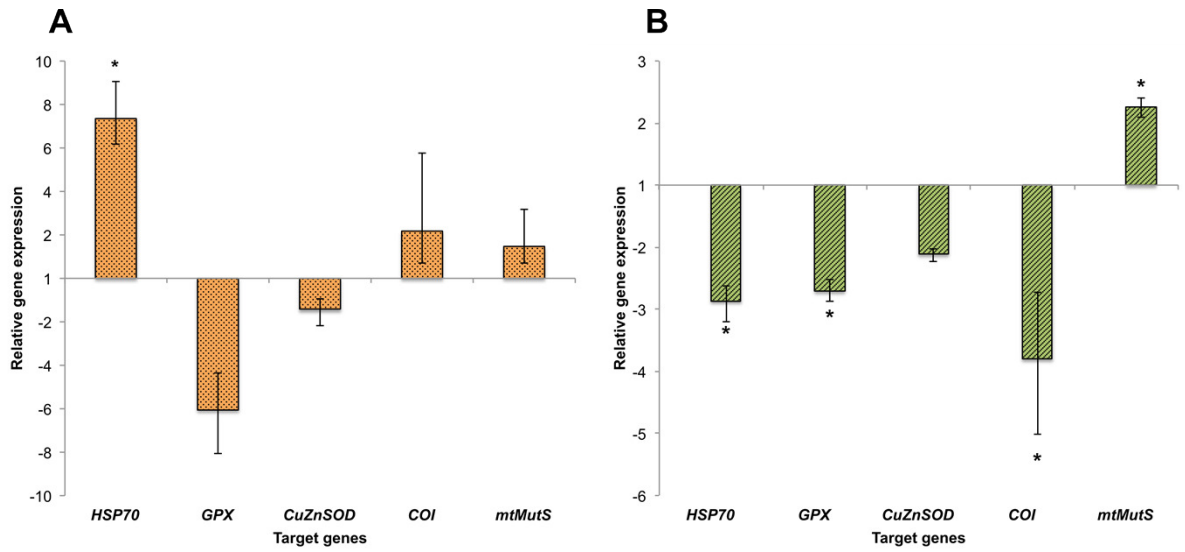
668

669 **Figures**

670



671

672 **Fig. 1. Relative expression of stress-related and mitochondrial genes post (A) thermal and**
673 **(B) low-pH stress**. Changes in transcript levels of 3 stress response gene, *HSP70*, *GPX*, and
674 *CuZnSOD*; and 2 mitochondrial genes, *COI* and *mtMutS* were assessed. Normalization was
675 performed using validated sets of three reference genes namely *ACTB* and *SRP54* during either
676 and *RPL12* and *TUBB* during thermal and pH stress, respectively. Bars represent the mean
677 expression value (fold change ± SE) relative to untreated controls (26 °C or pH 8.2) of three
678 biological replicates. *Asterisks* (*) denote significantly higher or lower expression relative to
679 respective controls (REST; $p < 0.05$).

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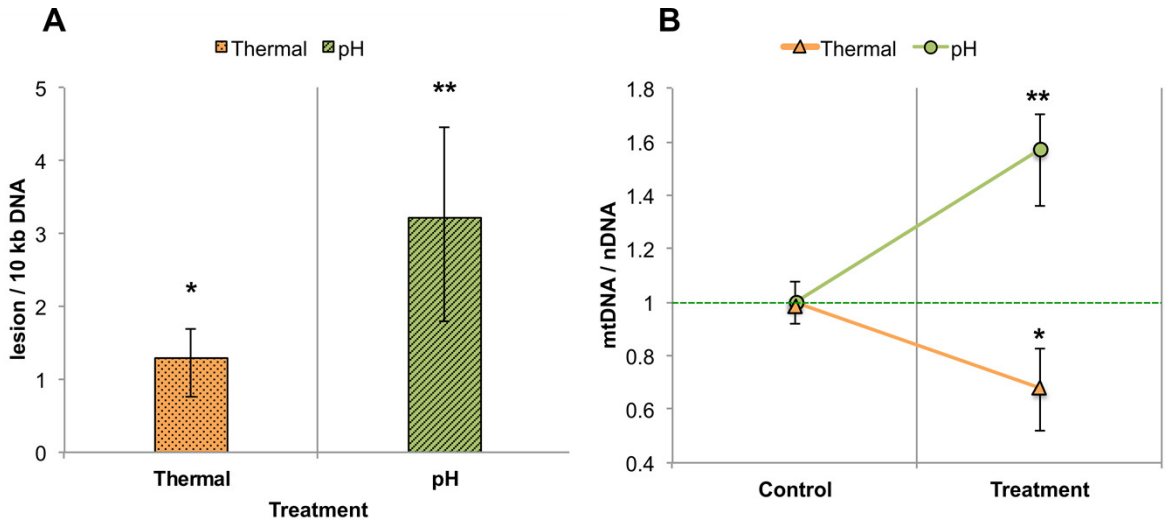

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684 **Fig. 2. Thermal and low-pH stress induced mtDNA damage, recovery kinetics and**

685 **mitochondrial copy number variation**. (A) Quantification of mtDNA lesion frequency (MLF)

686 per 10 kb DNA by SLR-qPCR amplification of total DNA from *Sinularia* cf. *cruciata* exposed

687 separately to elevated temperature (34 °C) for 6 h and reduced pH for 24 h. (B) In parallel,

688 mitochondrial copy number was determined by amplifying one mitochondrial fragment and

689 normalized using one nuclear fragment. Untreated controls (26 °C or pH 8.2) were used as

690 reference during respective experiments. Data represents the mean ± SE of biological triplicates.

691 * Statistical significant at $p < 0.05$; ** Statistical significant at $p < 0.01$.

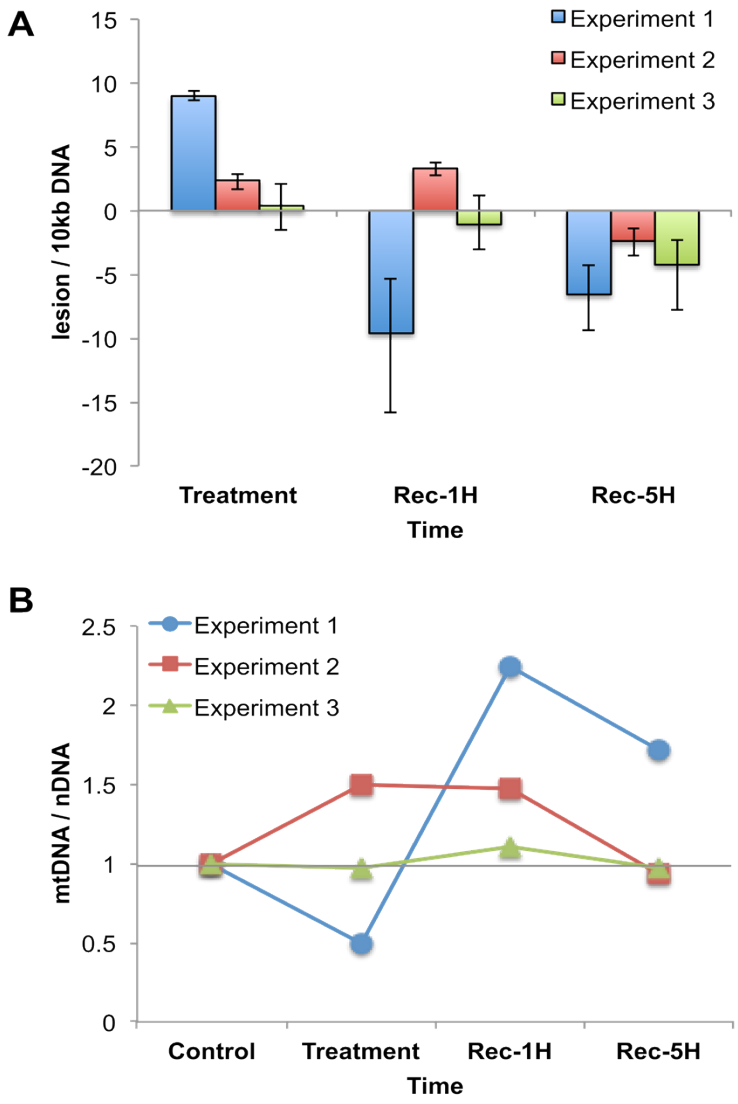
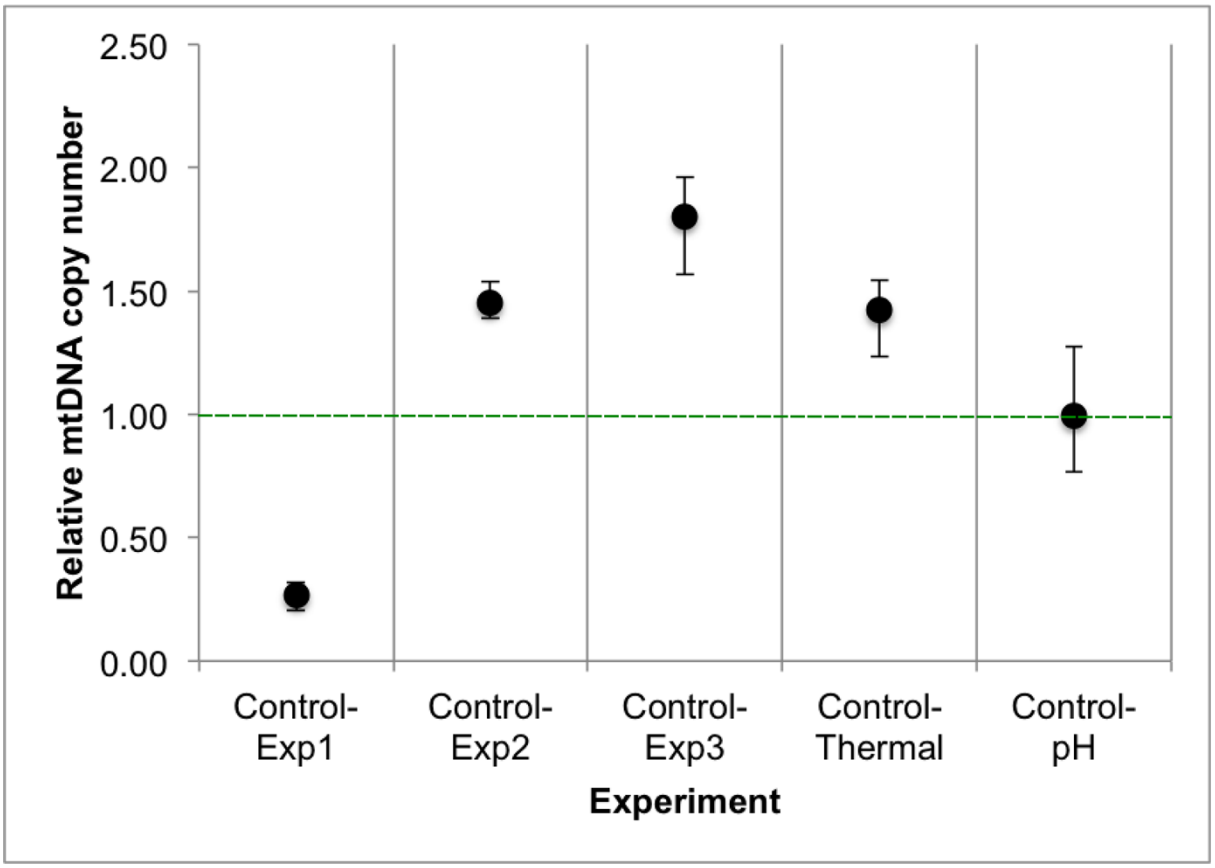


Fig. 3. Hydrogen peroxide induced mtDNA damage, recovery kinetics and mitochondrial copy number variation. (A) Quantification of mtDNA lesion frequency (MLF) per 10 kb DNA by SLR-qPCR amplification of total DNA from *Sinularia* cf. *cruciata* exposed to 5 mM H_2O_2 for 30 min (designated as 'Treatment') followed by recovery for 1 hr and 5 hr (designated as 'Rec-1H' and 'Rec-5H', respectively). Data represents the mean ± s.e.m. of three replicates. (B) In parallel, mitochondrial copy number variation was determined by amplifying a mitochondrial fragment and normalized using a nuclear fragment. Non-treated time-zero corals were used as a reference sample during respective experiments. Three independent experiments performed at different times.



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707

708 **Fig. 4. Comparison of initial mtDNA copy numbers among experimental controls**. Initial
709 mitochondrial copy number variation was determined by amplifying a mitochondrial fragment
710 and normalized using a nuclear fragment. The differences in the Cq values of mitochondrial gene
711 versus nuclear gene were calculated using $2^{-\Delta Cq}$. The geometric mean of the values obtained for
712 all controls served as a baseline (represented by the dotted line in figure), which was used to
713 calculate the ratios. Independent time-zero references for H_2O_2 experiments and untreated
714 controls (in triplicate) for thermal and pH stress were used for comparison. Data represents the
715 mean ± SE.

716

Tables

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					
1	*HSP70*	Heat shock protein 70	for: GGTGTATTTCAACACGGCAAAG rev: CCCCCTTATACTCCACTTCAAC	274	83.5	1.99
2	*GPX*	Glutathione peroxidase	for: TTTCCTTGCAATCAGTTTGG rev: GGCAGTCGTTGGAGAATATC	252	80.0	2.00
3	*CuZnSOD*	Cu-Zn Superoxide dismutase	for: CCAACTGATACAGAGAGGCATG rev: CATCAACACCAGCATGTACCAC	150	80.3	1.99
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 Forward (5' to 3')	Reverse (5' to 3')	Size (bp)	Product Tm (°C)	*E*
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. Reverse primer for small and large mitochondrial fragment are same.