A BI-1 mediated cascade improves redox homeostasis during thermal stress and prevents oxidative damage in a preconditioned reef-building coral

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Preprint: BioRxiv

Classification: Biological Sciences, Cell Biology

Keywords: Coral Bleaching, Oxidative Stress, siRNA gene knockdown, Acclimatization, Antioxidants

Abstract

Global coral reef decline is driven by the breakdown of the coral-algal symbiosis during temperature stress. Corals can acclimatize to higher temperatures on intra-generational timescales, but the complex cellular processes that underlie this ability and its trade-offs are poorly understood. We show that preconditioning-based improvements in thermal tolerance in *Pocillopora acuta* are accompanied by host increases in glutathione reductase (GR) activity and expression, which support a reducing intracellular environment that facilitates reactive oxygen scavenging and prevents DNA damage. We found a strong correlation between *GR* and *BI-1* (Bax-inhibitor 1) expression in heat-stressed preconditioned corals and discovered an antioxidant response element (ARE) in the *GR* promoter, suggesting BI-1 could regulate *GR* expression in corals through the Nrf2/ARE pathway. To fortify this link, we developed an siRNA-mediated gene knockdown protocol and targeted the coral BI-1 gene. BI-1 knock-down decreased glutathione reductase expression, glutathione reductase activity and increased oxidative DNA damage in heat-stressed preconditioned corals, showing that enhanced regulation of antioxidant response during acute heat stress is a key mechanism that prevents oxidative DNA damage after preconditioning. These results describe the manipulation of an important molecular cascade at the core of symbiosis maintenance under thermal stress and show that 'induced' symbiosis stability does not impact DNA integrity.

Significance Statement

Coral bleaching is a fundamental threat to reef ecosystems, but the molecular drivers of this process remain poorly understood. We show that corals pre-exposed to sublethal thermal stress

gain tolerance through their ability to process reactive oxygen species, a critical cellular toxin during coral bleaching. This ability is due to overexpression of glutathione reductase, an enzyme which stabilizes the reducing environment of the cell and buffers oxidative stress. Importantly, knock-down of genes that influence glutathione reductase expression initiates a molecular cascade that leads to DNA damage, a hallmark of oxidative thermal stress in corals. This work expands our understanding of symbiosis ecology and the importance of thermal acclimatization in corals.

Introduction

Healthy coral reefs support nearly one-third of marine species and provide shelter, nursery habitat and coastal protection across the tropical oceans (1–3). The foundation of this ecosystem is the coral-algal symbiosis, which is disrupted by thermal stress and is critically threatened by climate change. Climate induced mass bleaching has impacted most of the world's reefs and is predicted to increase in frequency and intensity, threatening the long-term persistence of these ecosystems (4–6). A deeper understanding of the cellular and molecular mechanisms underlying coral-algal symbiosis maintenance is critical for modern conservation and management (7, 8).

Corals are energetically dependent on their intracellular algal symbionts (family *Symbiodiniaceae*), which provide photosynthetically synthesized sugars and receive shelter and a supply of inorganic molecules from the host. During thermal stress, symbionts release increased reactive oxygen species (ROS), which are believed to trigger molecular cascades resulting in coral bleaching and can lead to the eventual death of the coral host organism (9–17); however, definitive proofs for this hypothesis are still missing (18).

Under normal conditions, ROS are scavenged by antioxidant systems in the host and symbionts to reduce damage to cell membranes, lipids and nucleic acids (19, 20). Under increased temperatures, ROS become elevated and the coral holobiont activates first-line enzymatic antioxidants such as catalase or superoxide dismutase and maintain a reducing intracellular environment via the glutathione redox cycle (10, 12, 14, 16, 21–24). These systems are well-studied in *Symbiodiniaceae*, where genera with different thermal resilience vary in antioxidant gene expression (12, 16, 23) and thermally resilient algae generally produce more antioxidants,

which are able to better maintain cellular homeostasis. Conversely, the role of host-derived antioxidants in bleaching and thermal resilience remains disputed. While pioneering studies show antioxidant activation in adult coral host tissue and larvae under heat stress (16, 25–29), more recent experiments have failed to find this pattern (10, 22). However, the coral host control of the level of oxygen radicals and ROS is likely a major component in the dynamics of coral-algal symbiosis maintenance (18, 30).

Intra-generational flexibility in coping with stress is critical for the long-term persistence of coral reefs, 'buying time' for adaptive change (31). Corals are sessile invertebrates unable to avoid stressful environmental change, highlighting the importance of acclimatization. Corals can improve their thermal tolerance after pre-exposure to sublethal temperatures (32–39), but the molecular triggers and consequences of this process remain poorly understood.

In this work, we show that preconditioned *P. acuta* prevents heat-induced oxidative DNA damage via increased activity of glutathione reductase. This glutathione reductase activity is highly correlated with coral BI-1 (Bax-inhibitor 1) gene expression in heat-stressed corals. We developed and conducted siRNA-mediated gene knockdown experiments in living adult corals to investigate the link. BI-1 downregulation leads to a decreased glutathione reductase expression and decreased glutathione reductase activity and results in an accumulation of oxidative DNA damage in coral tissue upon acute heat stress. The evidence from vertebrate models and the discovery of an antioxidant response element (ARE) in the coral glutathione reductase gene putative promoter region suggest that BI-1 likely impacts coral antioxidant system through the Nrf2/ARE signaling pathway.

Results

Thermal preconditioning improves bleaching susceptibility in P. acuta

This study uses samples from the same coral colonies described in the preconditioning experiment of Majerova et al. (39). When exposed to acute heat stress (32°C), preconditioned (PC) corals showed substantially increased symbiosis stability when compared to non-preconditioned (NPC) corals (Fig. 1B). After 3 days of heat stress, NPC corals were visibly bleached while PC corals resembled control, non-treated corals. This difference was more pronounced after 5 days of heat stress. However, PC corals still lost symbionts, but significantly more slowly than in NPC corals ($p < 0.001$) (39).

Preconditioning selectively increases the activity of host-derived glutathione reductase

The activity of glutathione reductase in *P. acuta* is influenced by acute heat stress (ANOVA, activity~treatment*time + (1|coral, p(time) = 0.0078) and was higher in PC corals than NPC corals in both ambient conditions (timepoint 0h) and under heat stress ($p_{(treatment)} < 0.001$, Fig. 2A). Interestingly, the activity of peroxide-scavenging antioxidants was dynamic ($p_{(time)} = 0.0017$) but did not differ between PC and NPC corals except at initial conditions ($p_{(0h)} = 0.0258$). Initially, PC corals had increased peroxidase activity, but this difference was lost immediately after the beginning of heat stress. Increased activities of two different peroxidases - catalase and glutathione peroxidase – have been detected in stressed corals (16, 21, 25, 27), but the catalase activity kit used here may detect any enzyme with peroxidase activity (Bioassay Systems, personal communication). Thus, we separately tested the activity of glutathione peroxidase with

a specific kit (Bioassays). There was no glutathione peroxidase activity in our samples, so we conclude that the peroxidase activity is primarily catalase, which has previously been observed in stressed corals.

To examine if the observed increases in enzyme activities were connected to host or symbiont response, we analyzed the expression of glutathione reductase (GR) and catalase (CAT) genes in separate host and symbiont extractions (Fig. 2B). Host glutathione reductase expression changed over time and between treatments (ANOVA, expression~treatment*time + (1|coral), $p_{(time)} < 0.001$, $p_{(treatment)} = 0.0098$). Host glutathione reductase expression increased in PC corals shortly after the beginning of the heat stress ($p_{(1h)} = 0.0736$) and peaked at 3 hours, when the expression was ~ 2-fold higher compared to NPC corals ($p_{(3h)} = 0.0015$). Host catalase gene expression varied significantly over time ($p_{(time)} = 0.0198$) but was not different between treatments ($p_{(treatment)} = 0.4743$).

Symbiont catalase and glutathione reductase expression were not significantly different between treatments ($p_{(sKat)} = 0.7359$, $p_{(GR)} = 0.8099$) and did not change over time ($p_{(sKat)} = 0.5487$, $p_{(GR)} = 0.1485$). This suggests that glutathione reductase dynamics are driven by the coral host; however, there was no significant correlation between protein activity and gene expression for either enzyme in host or symbiont cells (Fig S1).

Increased activity of antioxidants protects DNA from oxidative damage

Glutathione reductase helps stabilize the reducing environment of the cell, thus enhancing its ROS scavenging ability and preventing cellular stress such as oxidative DNA damage

(19, 40). To clarify whether the increase in glutathione reductase activity improves ROS protection in heat-stressed corals, we analyzed the level of oxidized guanine species (8-OHdG), markers of oxidative DNA damage (19). There was a clear difference between PC and NPC corals in time (Two-Way ANOVA, 8-OHdG~conditioning*time with Tukey post-hoc testing, $p_{(time:conditioning)} = 0.0098$, Fig. 3). Unlike in PC corals, we observed an accumulation of 8-OHdG in NPC corals after 24 hours of the acute heat stress (p(NPC) = 0.0991, p(PC) = 0.4082, $p_{(NPC\text{-}PC,\ 24h)} = 0.0310$).

To fortify the connection between glutathione reductase activity and 8-OHdG, we treated NPC corals with 10mM mannitol during acute heat stress. Mannitol protects plants and algae from ROS and is considered a non-enzymatic antioxidant (41) that prevents general DNA damage in coral tissue aggregates (20). Again, NPC corals accumulated marks of oxidative DNA damage during heat stress (One-way ANOVA, $p_{(treatment)} = 0.0044$), but the addition of 10mM mannitol eliminated this increase (One-Way ANOVA, $p_{(no\ treatment:mannitol)} = 0.0298$, $p_{(control:mannitol)} = 0.6869$).

pa-BI-1 controls the expression of glutathione reductase in preconditioned corals.

BI-1 (BAX inhibitor 1) is an anti-apoptotic protein that – among others – promotes cell survival by increasing the production of antioxidants through the activation of Nrf2 transcription factor in human cells (42, 43). We previously showed that PC corals increase the expression of pa-*BI-1* during acute heat stress compared to NPC corals (39). Since these observations were made on the same set of samples as our measurements of the expression of glutathione reductase (pa-*GR*), we examined the correlation between the gene expressions of pa-*BI-1* and pa-*GR*. Surprisingly, we observed a strong positive correlation in PC (Pearson = 0.948, $p_{(lm)}$ =

0.004) but not NPC (Pearson = 0.126, $p_{(lm)}$ = 0.4679) corals (Fig. S2). The strongest correlation occurred during the first 3 hours of the heat stress, where we observed a significant overexpression of both genes in PC but not NPC corals ((39) and Fig. 2), suggesting coral BI-1 can regulate the expression of antioxidant genes upon stress conditions.

To confirm this hypothesis, we developed a protocol for siRNA-mediated gene knockdown in living adult corals and inhibited the expression of pa-*BI-1* in heat-stressed PC corals (Fig. 4). We optimized the timing between siRNA administration, the beginning of a heat stress and coral sampling to reach the most significant knock-down of pa-BI-1 during the first hours of the acute heat stress when it was most strongly overexpressed in PC corals (39). We successfully inhibited pa-*BI-1* expression in 8 out of 17 corals (we set a threshold of 86% gene expression as a successful knock-down) ranging from 13% to 86% expression compared to siNTC – corals treated with control siRNA (52.02% ± 26.23, mean ± SD).

As expected, we observed an overexpression of both pa-*BI-1* and pa-*GR* in heat-stressed PC corals (Fig. 4A, siNTC) when compared to control corals (PC corals at ambient temperature). After siBI-1 knockdown (siBI-1), the expression of both genes decreased (Fig. 4A, Wilcoxon test, $p_{(BI-1)}$ = 0.0078, $p_{(GR)}$ = 0.03125) but was still significantly higher than in control corals (Wilcoxon test, $p_{(BI-1)}$ = 0.03906, $p_{(GR)}$ = 0.02344). There was a strong correlation between pa-*BI-1* expression and pa-*GR* expression following the gene knockdown (lm, GR~BI-1, p = 0.0011, r^2 = 0.51), indicating the inhibition of pa-*BI-1* expression leads to a decrease in pa-*GR* expression.

Decrease in glutathione reductase gene expression results in a decrease in enzyme activity

Since changes in gene expression are not always directly mirrored in the protein level and/or activity, we measured the activity of glutathione reductase in coral host tissue after siBI-1 gene knockdown. The decrease in pa-*GR* expression after 3 hours of heat stress is followed by a significant decrease in the enzymatic activity at 24 hours post stress (Paired t-test, $p_{(activity)}$ = 0.0391, Fig. 4B). There was a rapid decrease in the glutathione reductase activity in 5 corals, and a very slight increase in 2 corals (Fig. 4B dotted lines connecting siNTC-siBI-1 pairs), suggesting that decreased pa-*GR* expression does result in decreased pa-GR activity, but there may be other genotype-specific effects.

Corals with inhibited expression of pa-BI-1 are more prone to oxidative DNA damage

To examine the connection between antioxidant system and oxidative DNA damage in heat-stressed corals, we analyzed the level of oxidative DNA damage using an 8-OHdG marker in corals with pa-*BI-1* knockdown and subsequent decrease in glutathione reductase activity. As shown in Fig. 4C, siBI-1 treated corals accumulate significantly more oxidized guanines in DNA during acute heat stress (24 hours at 32 °C, Paired t-test, p = 0.0181) when compared to the PC corals treated with control siRNA. This observation supports the hypothesis that during heat stress, corals use antioxidants to protect important cellular structures from oxidative damage.

An antioxidant responsive element (ARE) lies within the promotor of coral glutathione reductase gene

In mammals, BI-1 can activate Nrf2 transcription factor that in turn regulates gene expressions through the *cis*-acting elements in the Nrf2 target gene promoters called antioxidant

responsive elements (ARE) (42, 44). Nrf2 gene has not yet been discovered and described in reef-building corals but we searched the promoter region (lies within NW_020843386.1) of the glutathione reductase gene (XM_027196629.1) in *Pocillopora damicornis* (45) and found an ARE-similar sequence 5'-TGACTTAGC-3' (44, 46) 557 bp upstream of the predicted beginning of the gene ORF. This ARE was first discovered and described in the promoter of glutathione peroxidase in human liver carcinoma cells (HepG2) at positions -76 and -387 with respect to +1 transcription start site (46). This striking resemblance suggests that the Nrf2/ARE pathway might be evolutionary more conserved than has been previously thought.

Discussion

Like most organisms, corals have the ability to acclimatize to stress conditions after pre-exposure and recent studies have confirmed that acclimatization reduces the severity of bleaching and mortality (32–36, 39, 48). However, this natural phenomenon may be genotype-specific (37) and could be lost under future climate-change scenarios (38). One of the main setbacks to the application of this strategy for conservation (7, 8, 31) is our limited understanding of molecular and cellular mechanisms behind acclimatization and/or adaptation to increased temperatures. Here we show that preconditioning-based acclimatization is mediated by the interaction of the pro-life gene *BI-1* and antioxidant response, which impacts cellular phenotypes such as DNA damage.

Transcriptomic studies in acclimatized corals shed light on the main gene families and cellular pathways that play a role in the bleaching process, but functional studies have been largely missing in reef-building corals (32, 33, 35, 49). Recently, we showed that preconditioning

in *Pocillopora acuta* leads to improved thermal tolerance due to modulations in the programmed cell death pathway (PCD), most likely via autophagy/symbiophagy (39). However, the primary signals or molecular consequences of such a prolonged symbiosis maintenance under thermal stress are unclear. During heat stress, the coral host and symbionts release increased reactive oxygen species (ROS) (10, 11, 13, 14, 18, 24, 50) which can activate an array of regulatory pathways, often depending on the level of ROS accumulation (51, 52). For example, low doses of ROS activate cell survival signaling pathways such as UPR (unfolded protein response) or Nrf2, while high doses of ROS activate PCD (52). We thus hypothesized that after preconditioning, the level of ROS signaling molecules was reduced, which resulted in changes in PCD signaling.

We find that preconditioned (PC) corals with higher tolerance to thermal stress and reduced bleaching rate (39) have higher activity of glutathione reductase but not peroxide-scavenging enzymes in the host tissue when compared to non-preconditioned (NPC) corals (Fig. 1A). Gene expression analysis suggests that the increase in glutathione reductase activity derives from the host cells but is probably dependent on more factors than just expression rate. While we observe an increase in host-derived glutathione reductase (pa-GR) expression at one hour after the beginning of the heat stress, peaking at three hours and then returning to the same level as NPC corals, the activity of the antioxidant is higher in PC corals even in ambient conditions (time 0). We hypothesize that while posttranscriptional and posttranslational modifications may positively impact the activity, turnover rate or localization of the antioxidant in PC corals in ambient conditions, the rapid induction of pa-*GR* gene expression is a major contributor to the observed activity differences upon acute heat stress.

Glutathione is a non-enzymatic antioxidant that exists in reduced (GSH) and oxidized (GSSG) form in the cell (reviewed in 30). GSH neutralizes ROS while being oxidized to glutathione disulfide (GSSG); this oxidized state is converted back to the reduced state by glutathione reductase (GR). Under normal conditions, > 90 % of glutathione pool is maintained as GSH by GR activity. GR is thus directly responsible for maintaining the reducing environment of the cell and for ROS scavenging. The level of GSH and/or the activity of enzymes involved in the glutathione redox cycle are inversely associated with the oxidative DNA damage (53, 54). Moreover, heat exposure induces oxidative stress and DNA damage in mice, humans, plants and fish (55–60), and heat acclimation led to a decreased accumulation of 8-OHdG markers in blood cells of navy boiler tenders exposed to high heat during work (61). Finally, increased DNA damage was observed in coral tissue explants exposed to elevated temperatures or to direct sunshine (20, 62).

We tested the level of oxidized guanine species, markers of oxidative DNA damage, in PC and NPC corals after 24 hours of heat stress and found that while NPC corals accumulate these markers, PC corals do not (Figure 3). Previous experiments showed that the addition of exogeneous antioxidant mannitol can reduce DNA damage in heat-stressed corals (20), so we tested this hypothesis on a new set of NPC corals. NPC corals suffered DNA damage upon heat stress unless 10mM mannitol was added (Fig. 3), clearly implicating antioxidant activity in differences in DNA damage between PC and NPC corals.

DNA damage is – besides ROS signaling – another trigger of diverse PCD pathways, including autophagy (63), supporting our previous results showing preconditioning improves coral thermal tolerance via modulations in the autophagy pathway (39). BI-1 (BAX-1 inhibitor), a pro-survival PCD gene involved in the regulation of PCD pathways, was shown to reduce ROS

accumulation in vertebrates and to activate Nrf2, which is a transcription factor of various antioxidants, including glutathione reductase (42, 64). Interestingly, we saw an upregulation of pa-*BI-1* in PC corals upon heat stress, peaking at 3 h after the stress start (39), which parallels host glutathione reductase expression (Fig. 1B). We compared the expression rates of pa-*BI-1* and pa-*GR* in PC and NPC corals and strikingly, they are highly correlated in PC corals in the early phase of the heat stress response (< 6hours), but not in NPC corals (Fig. S2). We hypothesize preconditioning may enable pa-BI-1 to effectively regulate the expression of pa-*GR* through epigenetic modifications of the pa-*GR* regulatory elements. DNA methylation patterns vary between corals living in different environments and is dynamic over time in corals exposed to environmental changes, very likely enabling gene expression fine tuning in response to various conditions (65–68).

To fortify this functional correlation, we developed a protocol for siRNA-mediated gene knockdown in living adult corals and inhibited pa-*BI-1* gene expression (Fig. 4A). We manipulated the gene in 8 out of 17 corals and hypothesize that the mucus that corals excrete when disturbed likely interfered with the siRNA transfection in some individuals. In the 8 corals with efficient pa-*BI-1* knockdown, pa-*GR* expression decreased, leading to a decline in pa-*GR* activity (Fig. 4B). This proves that pa-BI-1 can regulate gene expression of pa-GR in preconditioned corals. In vertebrate models, BI-1 was found to regulate expression of genes coding for antioxidants through Nrf-2 transcription factor (42, 43). Even though Nrf2 has not yet been described in reef-building corals, a homolog of the Nrf-2 gene was identified and annotated in *Nematostella vectensis* (GenBank KU746947.1, (69)) We thus hypothesized this transcription factor could also connect the two genes in *Pocillopora acuta*. Nrf2 regulates the expression of antioxidant genes via binding to the

so-called ARE (antioxidant response element) *cis*-elements located upstream of the transcription start site (44). We found an ARE similar to one described in the promoter of the glutathione peroxidase gene in human liver cells (46), suggesting the Nrf2/ARE pathway is conserved in Cnidarians, where it controls the expression of antioxidant genes during environmental stress response.

The decreased expression of pa-*GR* was mirrored in the lower activity of glutathione reductase antioxidant in siBI-1 corals, and these corals accumulated more oxidative damage than corals treated with a control siRNA (siRNA with no known target in *P. acuta*) (Fig. 4B, C), supporting the hypothesis that antioxidants prevent cellular oxidative damage in corals.

In summary, we propose a model where in non-preconditioned heat-stressed corals, the amount of ROS exceeds the ability of the corals to scavenge it, which leads to oxidative cellular damage, activation of programmed cell death pathway (autophagy/symbiophagy), symbiosis disruption and coral bleaching. After preconditioning, heat-stressed corals improve the ability to maintain cellular redox homeostasis through BI-1-mediated glutathione reductase overexpression which prevents the accumulation of oxidative stress markers, avoids the activation of programmed cell death and results in prolongated coral-algal symbiosis. This work describes the cellular and molecular principles of coral symbiosis maintenance under heat stress and how it is modulated during acclimatization. We also show that prolongated symbiosis maintenance during heat stress in preconditioned corals does not impair genome stability in short-term period, but the long-term consequences still need to be analyzed.

Materials and Methods

Collections, experimental setup and preconditioning

Seven colonies of *P. acuta* were collected in spring 2018 at different sites and depths ranging between 1 to 4m across Kāneʻohe Bay, Hawaiʻi to maximize genetic diversity. Experimental treatments followed in Majerova et al. (39). Briefly, corals were fragmented and allocated into preconditioning (PC) and control (NPC) treatments and PC corals were exposed to a 29°C for 72h before returning to ambient while NPC corals were maintained at 26°C (Fig 1A). Fragments were then clipped into 5cm nubbins and reallocated into heat stress treatments. After two weeks at 26°C, corals were exposed to a 32°C treatment or control and sampled at 0, 1, 3, 6, 12, and 24 hours. Fragments were stored immediately at -80°C. Bleaching rate was assessed as the shift in symbiont-to-host signal ratio with time-lapse confocal microscopy (Zeiss LSM-710) as described in Majerova et al.(39).

Antioxidant activity assays

Samples previously stored at -80°C were homogenized in ice cold extraction buffer (100mM Tris-HCl, 20mM EDTA, pH 7.5) in Qiagen TissueLyser ($30s^{-1}$ for 20s) with acid-washed glass beads (Sigma). The symbiont and host cells were separated with low-speed centrifugation (800g, 5min, 4°C) and the supernatant was then sonicated for 3 mins. Cell debris were pelleted by centrifugation (14,000g, 10min, 4°C) and whole cell protein extract concentration was measured using Qubit Protein Assay Kit (Thermo Fisher). Protein extracts were immediately used for EnzyChrom Catalase Assay Kit (1340ng) and EnzyChrom Glutathione Reductase Assay Kit (300ng; BioAssay Systems), respectively. The working protein concentration was optimized prior

to the experiment to ensure the measured activity was within the range of kit detection limits. We calculated the normalized enzymatic activity of each sample relative to the activity of the non-preconditioned coral at timepoint 0. Each analysis included one sample per timepoint per colony (n=7).

The Catalase Assay Kit is not specific to catalase and measures activity of all peroxide-scavenging antioxidants, so we used the EnzyChrom Glutathione Peroxidase Assay Kit (BioAssay Systems) to distinguish activity of different peroxide-scavenging antioxidants. All assays (extract concentration 2 – 10µg) were below the detection limit of the kit, so we concluded the activity of Glutathione Peroxidase in *P. acuta* corals used in this experiment is insignificant.

Gene expression analysis

Gene expression was analyzed as described in Majerova et al.(39). Briefly, RNA was extracted via RiboZol RNA Extraction Reagent (VWR Life Science) with DNase I step between phenol-chloroform extraction and ethanol precipitation. 1µg of RNA was reversely transcribed with High-Capacity cDNA reverse transcription kit (ThermoFisher Scientific). Reverse transcription quantitative PCR (RT-qPCR) reactions were run in 12 µl with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) for 40 cycles. Profiles of four genes for each organism (host and symbiont) under different treatments were compared with Best Keeper software and pa-*EF-1* (elongation factor 1a, *P. acuta*), and s*SAM* (S-adenosyl L-methionine synthetase, *Symbiodinium sp.)* showed the highest expression stability upon heat stress and were chosen as the reference genes. All primer sequences are listed in Table S1. Expression of target genes was calculated relative to the non-preconditioned coral at time 0 with ΔΔCt method.

$$\Delta\Delta\mathrm{Ct} = 2^{-[(Ct_{target,treated} - Ct_{ref,treated}) - (Ct_{target,control} - Ct_{ref,control}]}$$

Each analysis included one sample per timepoint per colony (n=7).

Oxidative DNA damage assay

To assess the impact of oxidative stress on DNA, we analyzed the level of 8-Hydroxy-2'-deoxyguanosine (8-OHdG) in PC and NPC corals as a proxy for cumulative oxidative DNA damage. 2 fragments from 6 corals in each treatment were sampled prior to and after 24 hours of thermal stress (as described above), frozen and kept at -80°C.

We used a custom extraction protocol to extract DNA from host cells after symbiont and host cells were separated as described above. After the low-speed centrifugation, SDS (0.5% final concentration) was added to the supernatant containing host cells and nuclei and incubated at 58°C for 15 min. Then, proteinase K (0.5 mg/ml final concentration) was added and incubated at 58°C for 2h. After incubation, samples were cooled to room temperature and KAc was added to a final concentration 0.5M. Samples were centrifuged (14,000g, 10min, 4°C) and the supernatant was precipitated by isopropanol (1:0.7 ratio). After precipitation, the DNA pellet was resuspended in water and the sample was treated by RNAse A for 15 min. DNA was purified by phenol-chloroform extraction followed by ethanol precipitation (O/N, -20°C) and resuspended in water. 500ng of total genomic DNA in 20µl was denatured at 95°C for 5 min, rapidly cooled down on ice, digested to single nucleotides by 20U of Nuclease P1 (NEB) at 37°C for 30 min and dephosphorylated by 1U of Shrimp Alkaline Phosphatase (NEB) for 30 min at 37°C. All the enzymes were inactivated at 75°C for 10 min. DNA was then diluted 1:5 in 1x Assay buffer and processed according to the kit instructions (DNA Damage Competitive ELISA Kit, Invitrogen). The

initial DNA concentration used in the assay was optimized to ensure the resulting values fall within the optimum range of kit detection limits (between Std3 and Std6). We used MyCurveFit.com software to model the standard curve and to predict result values. The analysis was conducted in 2 samples per timepoint per colony using 6 colonies in total. The results from biological replicates were averaged before statistical analysis.

Due to the results indicating that corals with increased glutathione reductase activity are less prone to oxidative DNA damage accumulation, we decided to test the impact of exogenous antioxidants to heat-stressed non-preconditioned corals. For this analysis, a new set of corals was collected from Kāne'ohe Bay in spring 2020 and prepared as describe above. This new set of corals was used for the following mannitol experiment and all the siRNA-involved experiments (see below).

To analyze the impact of exogenous antioxidant to oxidative DNA damage, we treated NPC nubbins with 10mM mannitol (20) or a seawater control for the duration of the heat stress experiment (6 NPC and 6 PC corals, 2 nubbins per colony per treatment). All coral fragments were sampled after 24h of acute heat stress (32°C), frozen and kept at -80°C. DNA damage assay was conducted as described above.

siRNA-mediated knockdown

PC coral fragments (~ 3 cm long) were placed into 20 ml cultivation vials, completely submerged into flow-through tanks with ambient temperature seawater and left to acclimatize overnight. The next day, vials were moved into precise temperature-controlled water bath, and the seawater was carefully removed without disturbing corals and the siRNA transfection was

carried out according to the manufacturer's instructions (INTERFERin® transfection reagent, Polyplus). siRNA transfection mix (20 µl of 10nM siRNA and 16 µl INTERFERin® reagent in 250 µl 0.2nm filtered seawater) was pipetted directly on the exposed coral and after ~ 2 minutes, 5 ml of 0.2 nm filtered seawater was added to fully cover the nubbin and coral was incubated at ambient temperature for 6 hours. After this time, vials were fully submerged into a flow-through tank with ambient temperature seawater for two days. 48 hours after the beginning of the siRNA transfection, corals were exposed to an acute heat stress (32°C, ramping speed of ~1°C per 10 mins). At 3 hours post-stress, each coral was sampled for the gene expression analysis and at 24h hours post-stress for glutathione reductase activity and DNA damage assay. This sampling consumed the fragment.

Statistical analyses

All statistical analyses were run in RStudio on data assembled in Microsoft Excel. Datasets were tested for normality (histogram plot) and heteroscedascity (Levene test). Where needed, data were normalized to logarithmic scale or using BestNormalize function in R (package BestNormalize).

We analyzed antioxidant activity after normalizing data to the initial control activity (NPC, time 0) for each colony. We used a generalized mixed model with treatment and time as main effects and colony as a random effect with a Tukey post-hoc testing for each timepoint.

We analyzed gene expression after normalizing data to the initial control expression for each colony. We used a generalized mixed model to examine normalized expression of each gene separately, using conditioning treatment and time as main effects and colony as a random effect.

We estimated least-squares means and compared between treatments for each timepoint using bonferroni adjusted p-values.

To analyze DNA damage, we ran a two-way ANOVA to compare the level of 8-OHdG (pg per µl DNA) of PC and NPC corals with time and conditioning as main effects. The differences between timepoints and conditioning were tested using Tukey post-hoc analysis. The effect of mannitol treatment to DNA damage was tested by one-way ANOVA with treatment (control, heat stress, heat stress with mannitol) as variable followed by a Tukey post-hoc testing.

We calculated Pearson correlation coefficients and variance explained using linear regression for the relationships between a) gene expression and enzymatic activity and b) BI-1 and glutathione reductase expression.

The effect of siRNA-mediated knockdown was analyzed using paired samples Wilcoxon test on data normalized to control (ambient temperature, non-treated) coral samples. Antioxidant activity data were also first normalized to control coral samples and then subjected to paired T-test. DNA damage analysis in siRNA-treated corals was analyzed with paired t-test on raw data.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank Shayle Matsuda and our interns Fiona C. Carey, Filip Blaštík, and Vojtěch Prokůpek for their help with coral collection and siRNA knock-down optimization tests. We thank

the To-Bo Lab (HIMB) for the access to their qPCR instrument. We dedicate this manuscript to Ruth Gates, who mentored us and inspired us to use molecular tools to understand the coral bleaching crisis. This work was funded by the Paul G. Allen Family Foundation and the Annenberg Foundation. Corals were collected under SAP-2020-25 to HIMB. This is HIMB contribution xx and SOEST contribution xx.

Author Contributions

EM conceived the experiments, conducted research, analyzed data and wrote the manuscript. **CD** analyzed data and wrote the manuscript. Both authors approved the final version.

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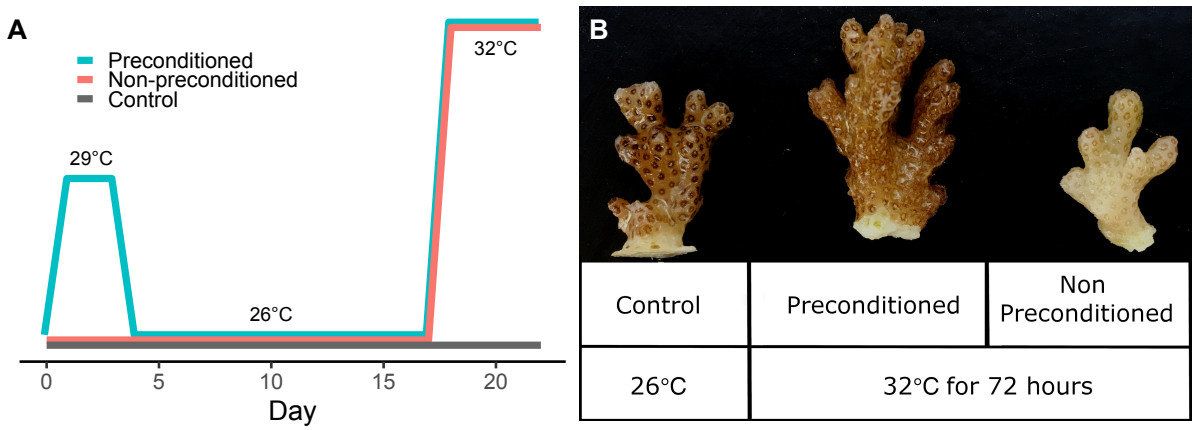

Figure 1 – Experimental design. A) Preconditioning profile. Preconditioned corals (PC) were exposed to sublethal 29°C for 72 hours and returned to 26°C for additional two weeks before undergoing acute thermal stress (32°C). Non-preconditioned (NPC) corals were exposed to acute thermal stress with no previous preconditioning. B) Different response to acute heat stress (32°C for 72 hours) in preconditioned (PC) and non-preconditioned (NPC) corals (right). While PC corals resembled control corals, NPC corals were visibly bleached.

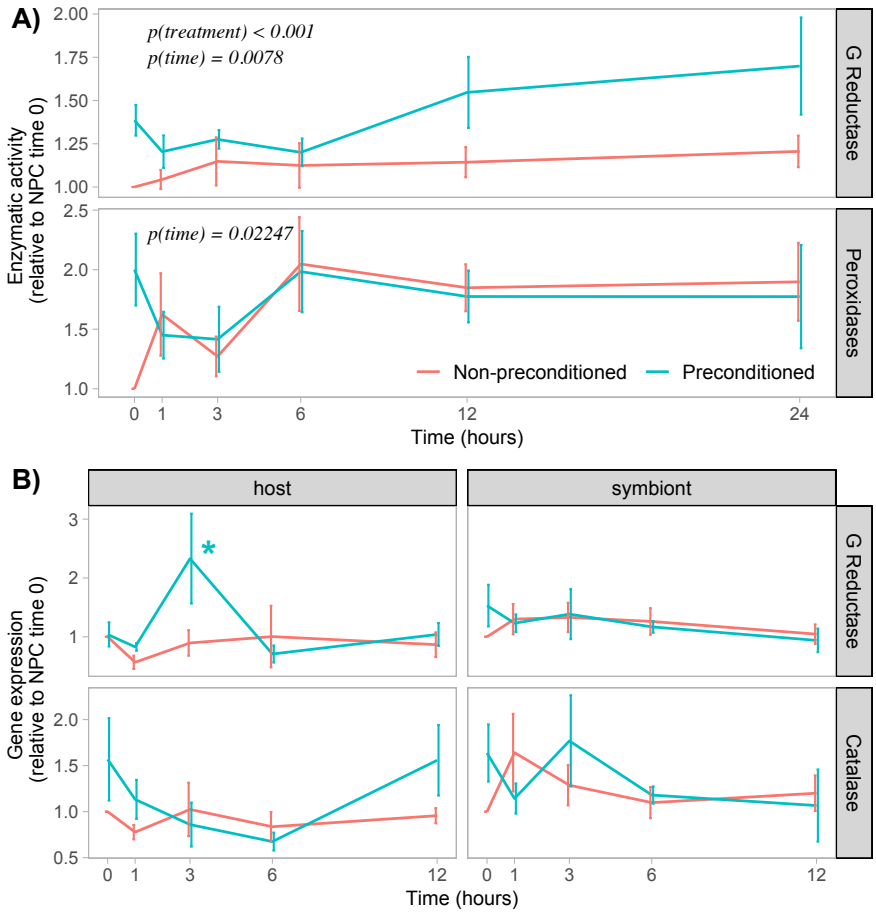

Figure 2 – *Enzymatic activity and gene expression during thermal stress*

A) Thermally *PC* corals *have higher* activity of glutathione *reductase than NPC,* but do not differ in the activity of peroxide-scavenging enzymes. Activity was measured in host cell extracts. *B)* Gene expression pattern of respective antioxidants in host and symbiont cells. Gene expression of host-derived glutathione reductase significantly increased at 3 hours of acute heat stress in *PC corals, but not NPC* corals. The remaining gene expression patterns are not different between treatments. Graphs depict means with standard errors, n=6.

Figure 3

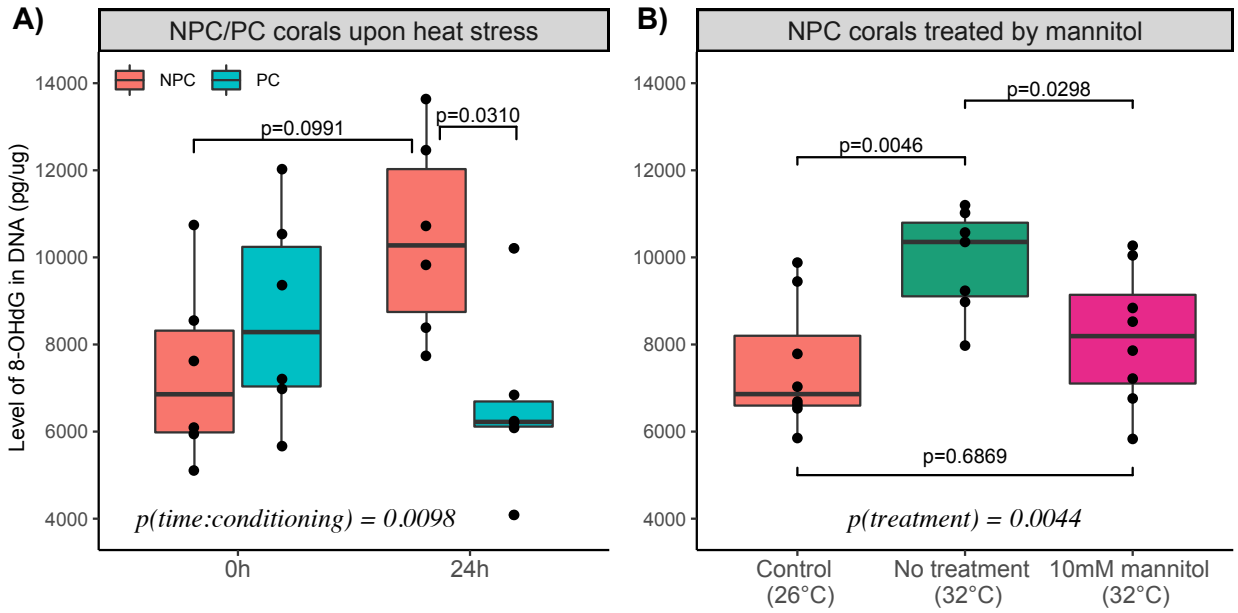

Figure 3 – Oxidative DNA damage during heat stress.
A) Non-preconditioned corals accumulate DNA damage (the oxidized guanine species, 8-OHdG) but preconditioned corals do not. After 24 hours of the stress, there is a significant difference in the level of 8-OHdG between the treatments. n = 6 B) The accumulation of 8-OHdG in non-preconditioned corals during acute heat stress is prevented by the addition of 10 mM mannitol, a non-enzymatic antioxidant. n = 8

Figure 4

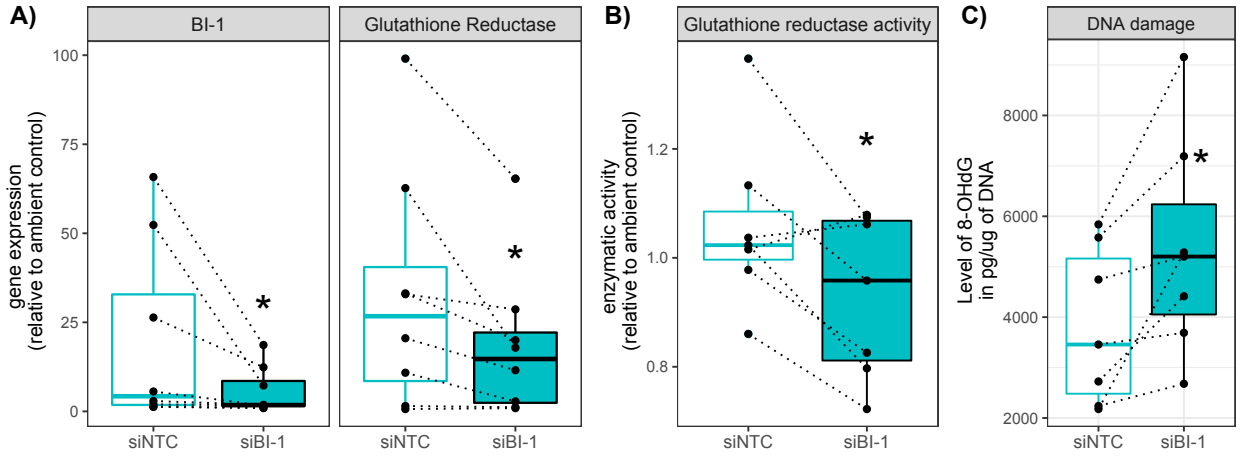

Figure 4 – BI-1 overexpression in heat-stressed preconditioned corals improves DNA damage protection via regulation of glutathione reductase activity. A) Expression of BI-1 and glutathione reductase genes in heat-stressed PC corals is efficiently reduced after siRNA-mediated gene knockdown (siBI-1). siNTC represents PC corals treated with a negative control siRNA. Data are normalized to untreated corals at ambient temperature, n=8 B) The activity of glutathione reductase decreases upon siBI-1 knockdown in PC corals. Data are normalized to untreated corals at ambient temperature, n=7. C) The level of oxidized guanine in coral DNA during heat stress increases after siBI-1 knockdown in PC corals (n = 7). Dotted lines connect paired samples from the same colony.

Figure S1

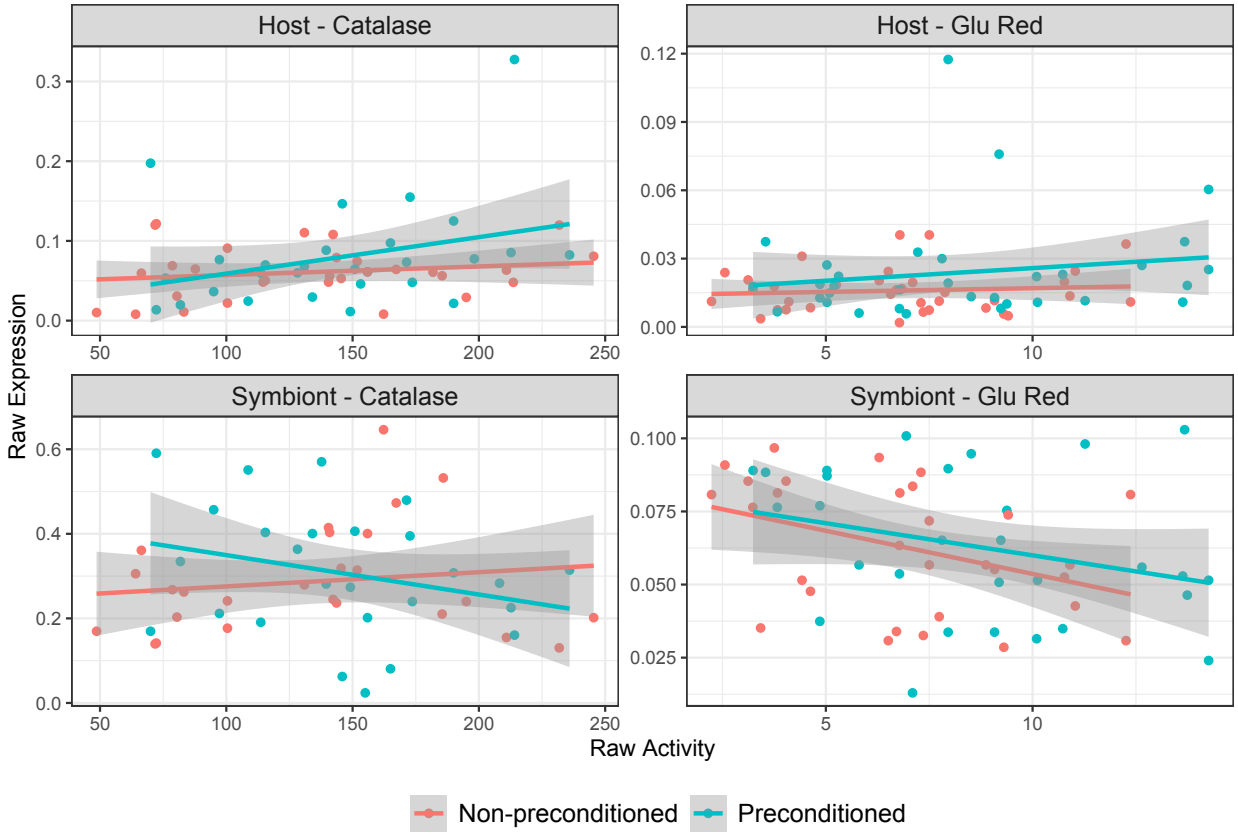

Figure S1 – Correlation of antioxidant activity and gene expression across all timepoints. There is no apparent correlation between the gene expression in host or symbiont cells with the activity of the respective enzyme. Raw expression is calculated as $2^{-\Delta Ct}$;$\Delta Ct = Ct_{(target)} - Ct_{(reference)}$, raw activity is measured in U/L. n = 6.

Figure S2

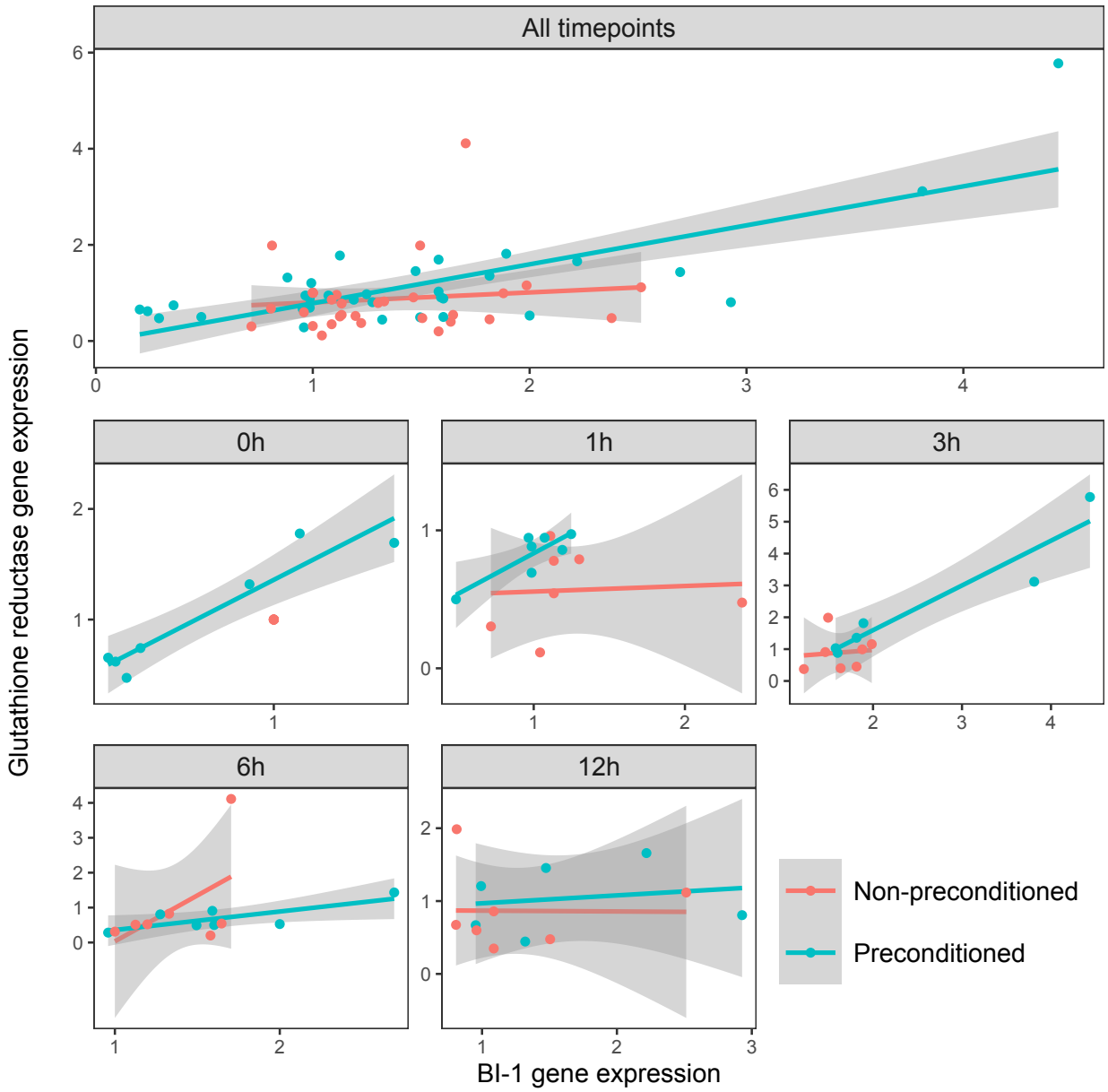

Figure S2 Expressions of BI-1 and glutathione reductase correlate in corals after preconditioning at ambient temperature and during early hours of an acute heat stress. Non-preconditioned (NPC) corals are depicted in red, preconditioned (PC) corals in cyan. All expression levels are normalized to the control coral (NPC at ambient temperature) using $2^{-\Delta\Delta Ct}$ formula.

Table S1

Gene	*Full name*	organism		primer sequence
pa-EF-1a	*elongation factor 1a*	host	fwd	5'- GCTGTGAACAAGATGGACAC -3'
			rev	5'- ATGGGAACAAATACCACGGC -3'
pa-GR	*Glutathione reductase*	host	fwd	5'-TGGAGGGCGGCCAACTGTAC-3'
			rev	5'-GCGATATACCCTGCTCCGAC-3'
pa-Cat	*Catalase*	host	fwd	5'-TTCTGGATGAGATGAGCCAC-3'
			rev	5'-GGTAATGTCATGAGTGACCTC-3'
sSAM	*S-adenosyl L-methionine synthetase*	symbiont	fwd	5'-GCCTACATTTGCCGACAGATG-3'
			rev	5'-AATGGCTTGGCAACACCAAT-3'
sGR	*Glutathione reductase*	symbiont	fwd	5-CGAAGGGCAAGCGTCTGAGC-3'
			rev	5'-CAGGACCCACCATCATCTCG-3'
sKatG	*Catalase peroxidase*	symbiont	fwd	5'-CTTGAGGACCATCAGCAGGC-3'
			rev	5'-GTACCACAAGCAGTTGAAGG-3'
pa-BI-1	*Bax-inhibitor 1*	host	fwd	5' TGGACCTCTGATGGACAATGTG 3'
			rev	5' AAGTTCGTTCCTCTGCCCAC 3'
siBI-1	*siRNA targeting BI-1*	host	sense	5'------GCACUUGAAGAAGGUGUAUGUdTdT--3'
			antisense	3'--TdTdCGUGAACUUCUUCCACAUACA------5'
siNTC	*siRNA with no known target*	host	sense	5'------GGGUUAUCAUACAGGUAGAGUdTdT--3'
			antisense	3'--TdTdCCCAAUAGUAUGUCCAUCUCA------5

Table S1 – List of primers and siRNAs used in the study