J Exp Biol Advance Online Articles. First posted online on 15 December 2016 as doi:10.1242/jeb.150391
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.150391

# Warm-preconditioning protects against acute heat-induced respiratory dysfunction and delays bleaching in a symbiotic sea anemone

Thomas D. Hawkins*, Mark E. Warner*

School of Marine Science and Policy, College of Earth, Ocean and Environment, University of Delaware, Lewes, Delaware, USA.

*Corresponding authors: tom.hawkins.photo@gmail.com, mwarner@udel.edu.

**Summary Statement**:

This article investigates the effect of thermal history on the activity of aerobic respiratory enzymes in a model symbiotic cnidarian exposed to acute heat-stress.



Journal of Experimental Biology • Advance article

**Abstract**

Preconditioning to non-stressful warming can protect some symbiotic cnidarians against the high temperature-induced collapse of their mutualistic endosymbiosis with photosynthetic dinoflagellates (*Symbiodinium* spp.), a process known as bleaching. Here, we sought to determine if such preconditioning is underpinned by differential regulation of aerobic respiration. We quantified *in vivo* metabolism and mitochondrial respiratory enzyme activity in the naturally symbiotic sea anemone *Exaiptasia pallida* preconditioned to 30°C for > 7 weeks as well as anemones kept at 26°C. Preconditioning resulted in increased *Symbiodinium* photosynthetic activity and holobiont (host + symbiont) respiration rates. Biomass-normalised activities of host respiratory enzymes (citrate synthase and the mitochondrial electron transport chain [mETC] complexes I and IV) were higher in preconditioned animals, suggesting that increased holobiont respiration may have been due to host mitochondrial biogenesis and/or enlargement. Subsequent acute heating of preconditioned and "thermally naïve" animals to 33°C induced dramatic increases in host mETC complex I and *Symbiodinium* mETC complex II activities only in thermally naïve *E. pallida*. These changes were not reflected in the activities of other respiratory enzymes. Furthermore, bleaching in preconditioned *E. pallida* (defined as the significant loss of symbionts) was delayed by several days relative to the thermally naïve group. These findings suggest that changes to mitochondrial biogenesis and/or function in symbiotic cnidarians during warm-preconditioning might play a protective role during periods of exposure to stressful heating.

**Keywords**: Coral bleaching, *Aiptasia*, *Exaiptasia pallida,* thermal stress, respiration, cnidarian-dinoflagellate symbiosis, acclimation.

## Introduction

Scleractinian corals (Cnidaria, Anthozoa) form the structural basis of coral reefs, and depend on photosynthetically fixed carbon from their symbiotic dinoflagellates (*Symbiodinium*) to sustain growth, calcification, and reproduction (Davy et al., 2012). Rising ocean temperatures are driving global coral reef degradation, notably by destabilising this symbiotic relationship – a process known as "coral bleaching" (Ainsworth et al., 2016). Despite much effort (see Weis et al., 2008 and Lesser, 2011 for reviews) our understanding of coral bleaching at the cellular level remains incomplete. Substantial evidence points to the thermal inhibition of *Symbiodinium* photosynthesis resulting in an over-production of pro-oxidant reactive oxygen species (ROS), ROS influx into the host and resultant "oxidative stress" (Lesser, 2006; Lesser, 2011). Consequently, bleaching can occur *via* host and *Symbiodinium* cell necrosis (Dunn et al., 2004), apoptosis (Dunn et al., 2007; Tchernov et al., 2011; Hawkins et al., 2013) and/or host cell autophagy (Dunn et al., 2007; Downs et al., 2009). However, the roles of host and *Symbiodinium* in initiating the cellular bleaching cascade are being reconsidered (Ralph et al., 2001; Downs et al., 2009; Paxton et al., 2013; Krueger et al., 2015; Lutz et al., 2015). For example, recent work reported heat stress-induced host mitochondrial degradation independent of *Symbiodinium* dysfunction (Dunn et al., 2012; Lutz et al., 2015). With mitochondria being a major source of ROS in animal cells (see below; Cadenas and Davies, 2000), it is surprising that few studies have explicitly quantified mitochondrial function in bleaching cnidarians (although see Agostini et al. (2016) for recent efforts). This represents a significant gap in our mechanistic models of bleaching.

An additional area of debate concerns differential bleaching susceptibility (Baird et al., 2008; van Oppen et al., 2009; Weis, 2010; Grottoli et al., 2014) and the mechanisms by which corals acquire increased thermal tolerance (Hoegh-Guldberg et al., 2002; Baker et al., 2004; Bay and

Palumbi, 2015; Camp et al., 2016). In some cases, bleaching resistance is conferred by heat-tolerant *Symbiodinium* species (Rowan et al., 1997; Berkelmans and van Oppen, 2006; Silverstein et al., 2014), but it may also derive from environmental variability (Oliver and Palumbi, 2011; but see Camp et al., 2016) or "preconditioning" to moderate, non-stressful warming (Middlebrook et al., 2008; 2012b; Bellantuono et al., 2012a; Bay and Palumbi, 2015; Ainsworth et al., 2016). Thermal preconditioning in marine ectotherms often involves altered carbohydrate metabolism and aerobic respiration (Sokolova and Pörtner, 2003; Sommer and Portner, 2004; Kraffe et al., 2007; Pörtner et al., 2007; Oellermann et al., 2012; Chung and Schulte, 2015), notably through the regulation of mitochondrial function (Somero and Hochachka, 2002). For example, increasing mitochondrial densities and higher aerobic capacity is a common adaptive response to cold conditions (polychaetes - Sommer and Portner, 1999; polar marine invertebrates - Pörtner, 2001; Peck, 2002; Pörtner et al., 2007), while warm-acclimation often correlates with decreased mitochondrial densities/aerobic capacity (rainbow trout - Kraffe et al., 2007; polychaetes - Chakravarti et al., 2016) and reduced sensitivity to short-term heating (killifish - Chung and Schulte, 2015). Warm-preconditioning in corals may occur through similar processes. For example, Castillo & Helmuth (2005) noted an effect of thermal history on respiration in *Montastraea* (= *Orbicella*) *annularis* corals undergoing a subsequent thermal challenge. Furthermore, Bay & Palumbi (2015) and Dixon et al. (2015) observed increased heat-tolerance correlating with altered expression of genes associated with carbohydrate metabolism and mitochondrial function, respectively. Dixon et al. (2015) further hypothesised that this phenomenon could have evolutionary benefits *via* the transfer of thermally resilient mitochondria from parent to offspring. The findings of Putnam & Gates (2015) point to a similar hypothesis; they noted an effect of maternal warm-preconditioning on $O_2$ consumption by *Pocillopora damicornis* larvae. While interesting, these data have limitations, in that transcriptional changes do not always translate to changes at the functional

protein/enzyme level (Evans, 2015), and live coral $O_2$ consumption reflects host and *Symbiodinium* respiration as well as *Symbiodinium* chlororespiration (Tytler and Trench, 1986; Roberty et al., 2014). Thus, there is a need to characterise functional enzyme-level changes in symbiotic cnidarian respiration during thermal preconditioning, as well as separate the responses of host and *Symbiodinium*.

Key steps in eukaryotic aerobic respiration include the tricarboxylic acid (TCA) cycle (also known as the citrate cycle or Krebs' cycle) and oxidative phosphorylation (Fig. 1, Table 1). The TCA cycle progressively oxidises glycolysis-derived carbon-rich substrates and transfers their electrons to $NAD^+$ and FAD (Berg et al., 2002; Somero and Hochachka, 2002). While oxygen is not directly involved, the TCA cycle is dependent on regeneration of $NAD^+$ and FAD by the mitochondrial electron transport chain (mETC) where $O_2$ is the terminal electron acceptor (Berg et al., 2002; Martinez-Cruz et al., 2012). With the TCA cycle acting as the "hub" of cellular aerobic metabolism (Somero and Hochachka, 2002), biomass-normalised activity of its gate-keeper enzyme (citrate synthase [CS]) is a useful marker of tissue mitochondrial density and aerobic capacity (e.g. Srere, 1969; Urschel and O'Brien, 2008; Vigelsø et al., 2014; Hawkins et al., 2016a). TCA cycle-derived NADH and $FADH_2$ drive oxidative phosphorylation at the mETC, which comprises several multi-protein complexes embedded in the inner mitochondrial membrane (IMM; see Fig. 1 for details). The mETC is the main site of ATP synthesis in mitochondria and its function depends on the effective transfer of electrons to molecular oxygen by cytochrome *c* oxidase (CCO, Fig. 1). This prevents the over-reduction of mETC components upstream of CCO (Turrens, 2003; McDonald et al., 2009), consequences of which can include heightened superoxide ($O_2^-$) generation (Abele et al., 2007; Murphy, 2009). $O_2^-$ is a potentially harmful ROS implicated in coral bleaching (Lesser, 2006; Weis, 2008). Ordinarily, it is rapidly detoxified by cellular antioxidants including the superoxide

dismutase (SOD) enzyme (Sies, 1997; Cadenas and Davies, 2000). However, $O_2^-$ fluxes during abiotic stress can necessitate SOD upregulation or exhaust the cell's protective responses (Sies, 1997; Cadenas and Davies, 2000; Turrens, 2003; Lutz et al., 2015). In many symbiotic cnidarians, host SOD activity is sensitive to acute changes in temperature, irradiance, and tissue $O_2$ concentrations (Dykens and Shick, 1982; Lesser et al., 1990; Richier et al., 2003; Agostini et al., 2016).

The aim of this study was to investigate plasticity in aerobic capacity and mitochondrial enzyme activity in a symbiotic cnidarian (the sea anemone *Exaiptasia pallida*) undergoing thermal preconditioning and/or bleaching. We hypothesised that – as for many marine ectotherms undergoing warm-acclimation (Sommer and Portner, 1999; Sommer and Portner, 2004; Martinez-Cruz et al., 2012; Chung and Schulte, 2015) – preconditioning of *E. pallida* and its *Symbiodinium* correlates with declining mitochondrial density / aerobic capacity. Second, we hypothesised that preconditioning has a measurable influence on the acute heating-response of host and symbiont mitochondrial respiration, and protects against excessive superoxide generation and thermal bleaching.

## Materials and Methods

*Reagents*

2,6-dichlorophenolindophenol (DCPIP), 5,5-dithio-bis-(2-nitrobenzoic acid), acetyl coenzyme A, citrate synthase (from porcine heart), cytochrome *c* (from equine heart), decylubiquinone (DUB), ubiquinone$_1$ (Ub$_1$), malonic acid, NADH, oxaloacetate, potassium cyanide (KCN), sodium succinate, sodium dithionite, Triton X-100, xanthine and xanthine oxidase (from bovine milk) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rotenone was purchased from Cayman Chemical (Ann Arbor, MI, USA). All other reagents were purchased from Fisher Scientific (Waltham, MA, USA). Detailed procedures for reagent preparation and storage are described in the accompanying supplementary information (S.I.).

*Warm-preconditioning and acute heating of* E. pallida

Specimens of *Exaiptasia pallida*, naturally symbiotic with ITS2-type A4 *Symbiodinium* (Hawkins et al., 2016b), were collected from Key Largo, FL (FWCC permit no. DD-J2T15642566). Anemones were maintained at 26°C in a 35-L flow-through tank (flow 0.5 L min$^{-1}$) supplied with recirculating 1 µm-filtered and UV-treated natural seawater (FSW) sourced from a 400-L sump. Photosynthetically active radiation (PAR) was provided by cool-white LEDs (12 h: 12 h light: dark cycle, 90 µmol photons m$^{-2}$ s$^{-1}$, Cree XP-G2; LED Supply, Randolph, VT, USA). Animals were fed weekly with freshly hatched *Artemia* nauplii and maintained under these conditions for > 6 months. One month prior to treatment, similar-sized anemones (~5 mm oral disk diameter) were randomly transferred to glass bowls (n = 5 replicate bowls per treatment with six anemones per bowl) evenly distributed across three 35-L tanks supplied with flow-through FSW (0.5 L min$^{-1}$). After two weeks, one anemone from each bowl was sampled (see below) and the temperature of one tank was increased by 0.5°C day$^{-1}$ over 13 days to 30°C ("preconditioned"; mean 29.8 ± 0.64°C [s.d.], Fig. 2A). This temperature is

slightly below the annual maximum experienced by these anemones in their natural habitat (mean annual temperature range ≈ 17°C-31°C; https://www.nodc.noaa.gov/dsdt/cwtg/all_meanT.html). The other two groups ("thermally naïve" and "control") remained at ~26°C (26.2 ± 0.2°C [s.d.] and 26.2 ± 0.4°C [s.d.], respectively, Fig. 2A). These conditions were maintained for seven weeks before anemones were randomly sampled from each bowl (see below). Due to a malfunction in the heating apparatus approximately four weeks into the preconditioning period, the temperature of the preconditioning treatment briefly (< 2 h) exceeded 33°C (Fig. 2A). At least another four weeks passed before these anemones were heated further, so we are confident that this brief period of heating did not compromise their subsequent thermal responses. To simulate a high-temperature anomaly that might induce bleaching, preconditioned and thermally naïve anemones were heated by ~0.9°C $day^{-1}$ – closer to the maximum heating rate associated with bleaching events in the field (Middlebrook and Anthony, 2010). Heating was staggered such that the two groups reached maximum temperature (33.0 ± 0.3°C [s.d.]) simultaneously. Anemones were sampled 10 days after initial ramping (upon reaching 33.0°C) and again after a week at 33°C. Temperature for the control group, and irradiance for all three treatments, remained unchanged. Anemones were fed weekly with freshly hatched *Artemia* nauplii and bowls were regularly moved within each treatment.

The maximum quantum yield of *Symbiodinium* photosystem II (PSII; $F_v/F_m$) was measured using a Diving PAM fluorometer (Walz, Effeltrich, Germany) 30 min after lights-off, in all animals, every 2-3 days during ramping and every day once the heated groups had reached 33°C. Further sampling was conducted as described in Figure 2A, always at least four days after feeding. Briefly, one anemone was removed from each replicate bowl (n = 5 per

treatment), and photosynthetic and respiratory $O_2$ fluxes were quantified using sealed glass chambers and oxygen sensors with the intact symbiosis ("holobiont") (Hawkins et al., 2016a). Irradiance during the 20-min illumination period was set at 200 µmol photons $m^{-2}$ $s^{-1}$ (slightly below saturating irradiance for these anemones under control conditions). Gross photosynthesis ($P_{Gross}$) was calculated by subtracting net photosynthesis and dark respiration rates ($R_D$). The gross photosynthesis/respiration ratio (P:R) was calculated using a light period of 12 h and photosynthetic and respiratory carbon quotients of 1.1 and 0.9, respectively (Muscatine et al., 1981). Each anemone was then washed with FSW, transferred to a screw-cap vial, snap-frozen in liquid nitrogen and stored at -80°C.

*Sample processing and determination of* Symbiodinium *cell density.*

Anemones were thawed on ice and 0.8-1.4 mL lysis buffer (50 mM $KP_i$, pH 7.8, 1 mM EDTA, 10% [v/v] glycerol) and two 5-mm stainless steel beads (Qiagen, Hilden, Germany) were added to each vial. Anemones were homogenised as described previously (Hawkins et al., 2016a), and the homogenate was centrifuged for 10 min at 700 × g. The supernatant (host fraction) was aspirated, split into 110-µL aliquots and snap-frozen in liquid nitrogen. The *Symbiodinium* pellet was re-suspended in a known volume of FSW and a 100-µL aliquot was removed, fixed with 5 µL 8% (w/v) glutaraldehyde and stored at 4°C for subsequent cell counts. The remaining algal sample was snap-frozen in liquid nitrogen and stored at -80°C. Frozen *Symbiodinium* cell suspensions were thawed on ice, washed and lysed in 400 µL lysis buffer as described previously (Hawkins et al., 2016a). Lysates were centrifuged (700 × g, 10 min) and the supernatant aspirated, split into aliquots, snap-frozen in liquid nitrogen and stored at -80°C. The soluble protein of the host fraction and *Symbiodinium* lysate were determined using a linearized Bradford assay (Ernst and Zor, 2010). *Symbiodinium* cell counts were performed

using epifluorescence microscopy and digital image analysis using ImageJ (NIH, Bethesda, MD, USA) following the methods of Hawkins et al. (2016a). Algal cell numbers were normalised to host soluble protein.

*Quantification of host and* Symbiodinium *mitochondrial respiratory enzyme activity.*

Citrate synthase (CS) activities of host fractions and *Symbiodinium* lysates were determined using the methods of Srere (1969) modified for use with cnidarians and *Symbiodinium* (Hawkins et al., 2016a). The amount of protein added to each reaction (in triplicate) was standardised at 10 μg (host fraction) and 3 μg (*Symbiodinium* lysate). Additionally, specific activities of host NQO, host and *Symbiodinium* SDH, and host CCO (see Table 1) were assessed spectrophotometrically (Spinazzi et al., 2012) in quartz cuvettes using a UV-VIS spectrophotometer (Evolution 201, ThermoFisher, Waltham, MA, USA). NQO activity was quantified after adding 50 μL host fraction (~30 μg protein) to 332 μL 18.2-MΩ water and incubating at 27°C for 1 min. This hypotonic treatment further disrupts mitochondrial membranes and solubilises the NQO complex (Frazier and Thorburn, 2012; Spinazzi et al., 2012). Reaction mixtures were 500 μL, containing 50 mM $KP_i$ pH 7.5, 0.3 mM KCN, 3 mg $mL^{-1}$ BSA, 60 μM $Ub_1$, 0.2 mM NADH, and 1% (w/v) ethanol. After mixing by inversion, NADH oxidation was monitored at 340 nm for 3 minutes. Rotenone-sensitive NADH oxidation (NQO specific activity) was determined by repeating the procedure with 10 μM rotenone (1 mM stock solution in ethanol). Reaction rates were determined over the 30-120 seconds after mixing, and the rotenone-sensitive activity ([Δ340 nm – rotenone] – [Δ340 nm + rotenone]) was calculated using $\varepsilon^{NADH}_{340\ nm} = 6.2\ mM^{-1}\ cm^{-1}$.

SDH activity was determined using 50 µL host fraction (~30 µg protein) or 100 µL *Symbiodinium* lysate (5-15 µg protein). Final reaction conditions (500 µL) were 25 mM $KP_i$ pH 7.5, 0.3 mM KCN, 1 mg $mL^{-1}$, BSA, 75 µM DCPIP, 20 mM succinate, and 50 µM DUB. Samples were incubated with succinate for 10 min prior to adding DUB, and baseline Δ600-nm absorbance was measured over the final 1 min. Change in 600-nm absorbance following the addition of DUB was measured for a further 3 min (rate determined over the 30-120 s after mixing). Respective baseline and "+ DUB" Δ600-nm rates were subtracted and SDH activity was calculated using $\varepsilon^{DCPIP}_{600\ nm}$ = 19.1 $mM^{-1}$ $cm^{-1}$.

CCO activity was determined in 50 µL host fraction (~45 µg protein), with final reaction conditions of 25 mM $KP_i$ pH 7.0 and 50 µM reduced cyt *c* in 500 µL. Cyt *c* oxidation was then determined by recording the decrease in 550-nm absorbance for 2 min before and after the addition of host fraction. CCO activity was calculated using $\varepsilon^{Cyt\ c}_{550\ nm}$ = 18.5 $mM^{-1}$ $cm^{-1}$. All enzyme assays took place at 27°C, and one unit of enzyme activity was defined as the oxidation of 1 µmol substrate $min^{-1}$ (NQO and CCO) or the reduction of 1 µmol DCPIP $min^{-1}$ (SDH). Assay linearity was tested across a range of sample protein concentrations (0.1-2.0 mg/mL) and specificity of the SDH and CCO assays were confirmed by using the specific inhibitors malonate (10 mM) or KCN (300 µM), respectively. Changes in tissue aerobic capacity can stem from changes in mitochondrial size and/or density as well as altered function of individual mitochondria (Somero and Hochachka, 2002; Urschel and O'Brien, 2008). Thus, in addition to calculating biomass-normalised (specific) enzyme activity, we normalised all mETC complex activities to that of CS, a reliable enzymatic indicator of mitochondrial density (Holloszy et al., 1970; Spinazzi et al., 2012; Vigelsø et al., 2014).

*Determination of host and* Symbiodinium *superoxide dismutase (SOD) enzyme activity.*

Total SOD activities of host fraction and *Symbiodinium* lysate were quantified using a xanthine/xanthine oxidase-nitroblue tetrazolium assay in a 96-well plate format. Briefly, 30 µL host fraction or *Symbiodinium* lysate were added to 210 µL reaction buffer (prepared such that reagent concentrations after addition of 10 µL xanthine oxidase solution were 50 mM KPi pH 7.8, 0.1 mM EDTA, 0.1% [w/v] BSA, 0.025% [v/v] Triton X-100, 0.14 mM NBT, 0.1 µM xanthine). Blank reactions (n = 18 wells) were prepared with 30 µL lysis buffer in place of experimental samples. Plates were incubated for 5 minutes at 27°C prior to the addition of xanthine oxidase (0.15 mU total activity per well). Linear rates of NBT-formazan dye generation were determined spectrophotometrically over 5 min ($\lambda$ = 550 nm; Fluostar Omega microplate reader, BMG Labtech, OrtenBerg, Germany). One unit of SOD activity was defined as the inhibition of NBT-formazan generation by 50%. Assay validation was conducted by running a standard curve of 500 to 0.05 U SOD enzyme (from bovine erythrocytes) per reaction.

*Statistical analysis*

Respiratory enzyme/complex activities were normalised to the soluble protein contents of host fractions and *Symbiodinium* lysates, respectively. Validation of CS, NQO, SDH, and CCO activities as correlates of holobiont respiration was conducted by Pearson's correlation analysis of natural log-transformed total host enzyme activity data from individual anemones (S.I. Figs. S1, S2; R ver. 3.2.2; R Development Core Team, 2015). Relationships between total host CS and NQO, SDH, and CCO activities were analysed similarly.

Initial physiological states were compared between the three groups of *E. pallida* using multivariate analysis of variance (MANOVA) in R. Data were tested for equal variance and normality with Levene's and Shapiro-Wilk tests, respectively, and were transformed where appropriate. This analysis was repeated for samples collected after the warm-preconditioning period, with univariate tests conducted using the summary.aov([MANOVA]) function in order to identify variables that differed significantly between treatment groups.

Effects of acute heating were investigated using linear mixed-effects analysis of variance (LM-ANOVA) (R package "nlme" (Pinheiro et al., 2016)). Null models were initially constructed to include only the random effect of "Replicate". "Day", "Treatment" and "Day × Treatment" effects were added sequentially. Akaike Information Criteria (AIC) were compared between models and F-statistics (for the best-fitting model) obtained with the anova([LM-ANOVA]) function. When there was no significant interaction ($p > 0.05$), the model was re-analysed with only the main effects. Model validity was assessed further by fitting a normal distribution to the residuals. When a significant "Day × Treatment" interaction was noted, further pair-wise *post hoc* analysis compared treatment groups on each day using the glht() function in R package "multcomp" (Hothorn et al., 2015). Additionally, the influence of treatment on relationships between host NQO and CCO specific activities was investigated using multiple regression and Pearson's correlation analyses ("NQO specific activity" and "Treatment" as linear predictors and correlates of CCO activity, respectively). *Symbiodinium* SOD activity could not be quantified for all anemones due to limited protein yields. These data were not analysed with LM-ANOVA.

## Results

*Physiological responses to prolonged thermal preconditioning in* E. pallida *and its* Symbiodinium

Prior to starting the experiment, no differences were noted in the initial physiological states of *E. pallida* or their symbionts (MANOVA of *in vivo* variables and mitochondrial/SOD enzyme specific activities: $F_{2,12} = 4.45$, $p = 0.08$; MANOVA of CS-normalised mETC enzyme activities: $F_{2,12} = 0.441$, $p > 0.1$). Significant responses to thermal preconditioning included increased holobiont $R_D$ and *Symbiodinium* gross photosynthesis cell$^{-1}$ (Table 2, Fig. 2B). Preconditioning had no effect on $F_v/F_m$, *Symbiodinium* densities or holobiont P:R (Table 2, Fig. 2B). Host CS activity (mg protein$^{-1}$) was almost two-fold higher in preconditioned anemones than in either the "control" or "thermally naïve" groups (Table 2, Fig. 2C). Host NQO (mETC I) and CCO (mETC IV) specific activities were approximately 3-fold higher in preconditioned animals than in those kept at 26°C (Table 2, Fig. 2C), but were not significantly different when normalised to CS activity (to control for the effects of changes in mitochondrial size or density; see above). There was no difference in host SDH (mETC II) or SOD specific activities between treatment groups. *Symbiodinium* CS and SDH activities were also similar across treatments (Table 2, Fig. 2D), although a weak trend for slightly higher CS and SDH specific activities in preconditioned *E. pallida* was apparent ($p < 0.08$).

*Responses of* E. pallida *and* in hospite Symbiodinium *to acute heating.*

*Symbiodinium* $F_v/F_m$ declined significantly in the thermally naïve anemones exposed to 33°C (Days 9-16 in Fig. 3A). This decline was delayed by 3-4 days in the preconditioned group (Fig. 3A). After six days at 33°C (Day 16), $F_v/F_m$ in preconditioned and thermally naïve anemones were ~ 50% lower than that of the control group. Holobiont P:R (Fig. 3B) and *Symbiodinium*

cell density (Fig. 3C) declined within thermally naïve *E. pallida* after initial heating (Day 10) but did not change significantly in preconditioned anemones. However, after a further 7 days, *Symbiodinium* densities and P:R in preconditioned animals were intermediate between those of the thermally naïve and control groups (Fig. 3B,C). Changes in $P_{Gross}$ per symbiont cell followed a different pattern, with exposure to 33°C causing a transient increase in $P_{Gross}$ $cell^{-1}$ in thermally naïve animals, but a uniform decline in $P_{Gross}$ $cell^{-1}$ in the preconditioned group (Fig. 3D). $P_{Gross}$ $cell^{-1}$ was similar across treatments at the end of the experiment (Fig. 3D). Responses of holobiont $R_D$ to acute heating also differed according to thermal history (Table 3, Fig. 3E); preconditioned animals displayed slightly reduced $R_D$ following heating to 33°C, while the opposite response (albeit transient) was noted for the thermally naïve group. As for $P_{Gross}$ $cell^{-1}$, no treatment-effects were evident in holobiont $R_D$ at the end of the experiment (Fig. 3E).

*Symbiodinium* CS activity in preconditioned anemones exposed to acute heating was similar to the control group's (Fig. 4A). CS activity in the symbionts of thermally naïve anemones under the same conditions, however, was ~4-fold lower than that of the other two groups (Fig. 4A). *Symbiodinium* SDH specific activity in preconditioned anemones showed no response to acute heating, but notably increased in thermally naïve anemones under the same conditions (Fig. 4B). Indeed, when normalised to that of CS, *Symbiodinium* SDH activity at the end of the experiment was approximately five times higher in thermally naïve *E. pallida* than in the preconditioned or control groups (Fig. 4C). An increasing trend was noted for *Symbiodinium* SOD activity in thermally naïve anemones subjected to acute heating (Fig. 4D), and it appeared that preconditioning was not associated with changes to SOD activity. However, as noted above, limitations in the amount of *Symbiodinium* material obtained from some anemones prevented the complete analysis of these data with LM-ANOVA.

The effects of acute heating on host CS and mETC complex activities differed according to thermal history as well as between specific enzymes/mETC complexes (Table 4). In preconditioned anemones, specific activities of host CS, NQO and CCO declined during initial heating to 33°C (Day 10), and no further changes were noted (Figs. 3F, 5A, E). Few changes were seen in host SDH specific activity (Fig. 5C) or in CS-normalised NQO, SDH, or CCO activities in these animals (Fig. 5B, D, F). In contrast, thermally naïve anemones displayed a > 2-fold increase in host NQO specific activity during exposure to 33°C (Fig. 5A), but no corresponding increase in CS activity (Fig. 3F). Thus, CS-normalised NQO activity was much higher in this group than in preconditioned anemones under the same conditions, or in the control group (Fig. 5B). This increase was not evident in CS-normalised SDH or CCO activities, which remained similar to those of preconditioned and control animals (Fig. 5D, F).

The relationship between host NQO and CCO activities was influenced by thermal history (Predictor: NQO [U $mg^{-1}$], Dependent: CCO [U $mg^{-1}$]; $F_{NQO \times Treatment} = 3.85_{(2, 38)}$, p = 0.030). Specifically, biomass-normalised NQO and CCO activities in control as well as preconditioned animals undergoing acute heating showed a significant positive correlation (Pearson's $r$ = 0.703 [p = 0.003] and $r$ = 0. 553 [p = 0.033], respectively; Fig. 6A). However, no relationship between NQO and CCO activities was apparent in thermally naïve anemones during acute heating ($r$ = 0.022 [p = 0.938]; Fig. 6A). We also did not observe any change in host SOD specific activity between treatment groups (Fig. 6B; LM-ANOVA, p > 0.2).

## Discussion

Aerobic respiration is critical to metazoan physiology and is highly sensitive to the abiotic environment (Somero and Hochachka, 2002; Clarke, 2003; Martinez-Cruz et al., 2012). In symbiotic cnidarians such as *E. pallida* and reef corals, regulation of aerobic respiration could be important in determining their sensitivity to ocean warming (Dunn et al., 2012; Dixon et al., 2015; Lutz et al., 2015; Jin et al., 2016). Here, we describe, for the first time to our knowledge, the effects of warm-preconditioning on mitochondrial enzyme activity in a symbiotic cnidarian. Furthermore, we noted a significant effect of thermal history on the heat-sensitivity of host and *Symbiodinium* mitochondrial enzymes and the intensity of thermal bleaching.

### *Preconditioning increases host aerobic capacity in* E. pallida.

Several weeks of preconditioning to elevated temperature induced notable physiological changes in *E. pallida* and its symbionts. Holobiont dark respiration ($R_D$) and *Symbiodinium* gross photosynthesis ($P_{Gross}$) increased significantly. Increased respiration following warm-preconditioning has been reported by Yakovleva and Hidaka (2004), who compared four reef corals exposed to moderate, non-stressful heating. However, Castillo & Helmuth (2005) found the opposite pattern in the coral *Orbicella annularis*, with colonies from lower-temperature environments showing higher $R_D$ than warm-acclimated colonies. These inconsistencies may result from *in vivo* $R_D$ reflecting the combined metabolic activities of all symbiotic partners. Furthermore, temperature-induced changes in $R_D$ reflect the effect of heating on enzyme kinetics (Schulte et al., 2011; Martinez-Cruz et al., 2012) as well as the active regulation of respiratory enzyme expression or activity (Clarke, 2003; Sommer and Portner, 2004; Pörtner et al., 2007). Thus, quantifying $R_D$ alone is inadequate if the aim is to determine which

mechanisms or symbiotic partner(s) are driving the observed response. A targeted biochemical approach, as applied here, can be more informative.

The higher biomass-normalised host CS, NQO, and CCO activities in warm-preconditioned *E. pallida* suggest that their increased $R_D$ reflected the up-regulation of aerobic respiratory pathways and not just the effects of heating on enzyme kinetics. Moreover, the constancy of NQO or CCO activities relative to CS activity indicates that this heightened $R_D$ emerged from increasing host mitochondrial densities or sizes, rather than changes in the mETC function of individual mitochondria (Holloszy et al., 1970; Spinazzi et al., 2012; Vigelsø et al., 2014). Given that warm-acclimation in better-studied marine ectotherms such as teleosts, annelids, and molluscs often associates with reduced mitochondrial activity (Pörtner, 2001; Kraffe et al., 2007; Martinez-Cruz et al., 2012; Chung and Schulte, 2015), our findings might seem surprising. However, when one considers the presence of photosynthetic symbionts within *E. pallida*, we should not expect this organism to respond to thermal preconditioning in the same way as the non-symbiotic organisms examined in previous investigations. Here, for example, host CS, NQO, and CCO specific activities increased with rising symbiont photosynthesis ($P_{Gross}$) during preconditioning, and declined with falling $P_{Gross}$ upon greater heating. These changes could reflect the availability of translocated carbon-rich material from the symbionts, since nutritional input directly effects respiration (Båmstedt, 1980; Clarke and Walsh, 1993; Holcomb et al., 2014). Indeed, host CS and CCO activities in the reef coral *Stylophora pistillata* decreased with reduced irradiance (Gattuso et al., 1993), and *Symbiodinium* density and host mitochondrial electron transport rates or CS activities were positively correlated in six coral species (Agostini et al., 2013) and *E. pallida* (Hawkins et al., 2016a).

Since carbon transfer from symbiont to host was not directly measured, we cannot definitively attribute increased host mitochondrial enzyme activity in preconditioned *E. pallida* to higher carbon translocation. For instance, additional fixed carbon may have been consumed by symbiont respiration. Thus, alternative explanations for the correlation between *Symbiodinium* photosynthesis and host aerobic capacity should be considered. One possibility is that heightened symbiont photosynthesis might place greater demands on host carbonic anhydrases (Bertucci et al., 2013) or other inorganic carbon delivery pathways, requiring additional energy expenditure and respiratory activity. Higher $O_2$ concentrations from increased symbiont photosynthesis could also have stimulated host aerobic capacity (Shick, 1990; Rands et al., 1992; Shashar et al., 1993; Holcomb et al., 2014), likely through mitochondrial biogenesis or enlargement as a protective strategy against local hyperoxia within individual mitochondria (Abele et al., 2007; Martinez-Cruz et al., 2012). Equally, increased host respiration, perhaps related to the mobilisation of energy stores during preconditioning (Grottoli et al., 2014), may have stimulated *Symbiodinium* photosynthesis *via* increased tissue $CO_2$ concentrations.

*Preconditioning dampens thermal sensitivity of host and* Symbiodinium *mitochondrial function and delays bleaching.*

Responses of *Symbiodinium* $P_{Gross}$ and holobiont $R_D$ to acute heating were significantly influenced by thermal history. Preconditioned *E. pallida* actually displayed modest reductions in symbiont $P_{Gross}$ and $R_D$ (relative to Day 0), while transient increases were noted in thermally naïve anemones under the same conditions. Given that *Symbiodinium* densities and -CS activities were declining in thermally naïve animals at this time, their increased $R_D$ (Day 10) was likely driven predominantly by host physiology. Yet, host CS and mETC complex activities displayed no increases that could explain this rise in $R_D$ relative to the control group.

Thus, we suggest that increased $R_D$ of thermally naïve *E. pallida* heated to 33°C resulted from the effects of heating on *in vivo* enzyme kinetics rather than changes in mitochondrial activity, density, or size. The corresponding increase in *Symbiodinium* $P_{Gross}$ was somewhat surprising, as it occurred while $F_v/F_m$ dropped. This could reflect the releasing of remaining *Symbiodinium* cells from carbon limitation as *in hospite* symbionts declined (Hoadley et al., 2015). Equally, it might have resulted in part from positive effects of heating on the rate of *Symbiodinium* A4 Rubisco activity (Galmés et al., 2015).

Heating-induced changes in symbiont $P_{Gross}$ and holobiont $R_D$ of thermally naïve anemones were transient and both variables were similar between groups at the end of the experiment. However, this similarity masked fundamental differences in both the anemones' internal environment and host- and *Symbiodinium* mitochondrial function. Notably, the ~80% decline in *Symbiodinium* densities – and no compensatory increase in *Symbiodinium* $P_{Gross}$ $cell^{-1}$ – would have depressed host tissue oxygen tensions in thermally naïve *E. pallida* relative to those of preconditioned or control animals (Rands et al., 1992; Richier et al., 2005). Moreover, activity of the TCA cycle and mETC in host and symbiont mitochondria became increasingly unbalanced in thermally naïve anemones undergoing bleaching. For example, CS-normalised SDH activity in the *Symbiodinuim* increased > 5-fold, a change driven by declining CS activity and increasing SDH activity. As the TCA cycle is the primary source of NADH for mETC function (Berg et al., 2002) and assuming no increase in $NAD^+$ reduction by compensatory mechanisms, the decline in TCA-cycle activity suggests potential NADH-limitation and a reliance on SDH-generated $FADH_2$ as the source of electrons for the mETC. In other organisms, succinate-dependent respiration can promote ROS generation through altered SDH activity (Jardim-Messeder et al., 2015), the autoxidation of partially reduced coQ (Abele et al., 2007), reverse electron flow through NQO (Turrens and Boveris, 1980; Grivennikova et al.,

2007) and – since SDH does not pump protons (Fig. 1) – changes to the IMM polarisation state. While total *Symbiodinium* SOD activity reflects combined mitochondrial, cytosolic and chloroplast-localised SOD and should be interpreted with caution due to the low sample sizes, it is suggestive of growing oxidative challenge in *Symbiodinium* of thermally naïve *E. pallida* during prolonged heat stress.

In the host mitochondria of thermally naïve *E. pallida* undergoing bleaching, an increase in NQO activity unmatched by changes in CS, SDH, or CCO activities indicated that equilibrium between the initial mETC reducing complex (NQO) and the terminal oxidising complex (CCO) had broken down. Increasing NQO enzyme activity accords with the changes in NADH:coenzyme Q oxidoreductase subunit 1 protein abundance (protein ID: NDUFS1) noted in a recent study of *Aiptasia pulchella* exposed to acute heating (C. Oakley, personal communication). However this contrasts with reduced transcript abundance for a gene encoding NADH:ubiquinone oxidoreductase in thermally stressed *Orbicella* (=*Montastrea*) *faveolata* corals (Desalvo et al., 2008). These apparent contradictions probably reflect taxonomic differences in the regulation/inhibition of mETC complex activity. For instance, Desalvo et al. (2008) suggested that the downregulation of *O. faveolata* NQO was caused by nitric oxide- and superoxide-derived peroxynitrite (Riobó et al., 2001). However, peroxynitrite does not have a significant role in the thermal bleaching of *Exaiptasia pallida* (Hawkins and Davy, 2013), and we noted little evidence of host SOD upregulation in the present study. The precise driver of increased NQO activity and the source(s) of the necessary NADH are not entirely clear. It is likely not increased by $NAD^+$ reduction *via* the TCA cycle, since host CS activity remained unchanged. Glycolysis is an alternative pathway for NADH production (Berg et al., 2002), and a build-up of glycolytic products has been noted for this species of sea anemone during heat-stress (Hillyer et al., 2015). Whatever the compensatory source of

NADH, its increased oxidation by NQO could shift the cellular $NAD^+$/NADH balance, with potential consequences for cell viability (Ying, 2008; Santidrian et al., 2013).

As we did not measure mETC III activity we cannot precisely characterise changes in the *E. pallida* mETC redox state downstream of complexes I and II. However, as noted above, the dramatic loss of photo-symbionts from thermally naïve anemones would likely have reduced tissue $O_2$ concentrations significantly (Rands et al., 1992; Shashar et al., 1993). In the absence of increased CCO activity (to sustain adequate rates of cyt *c* oxidation in a less oxidative environment), higher NQO activity could result in the progressive over-reduction of the CoQ pool as well as an altered IMM polarisation state (Abele et al., 2007). These phenomena are common features of heat- or hypoxia-induced stress and, in addition to inhibiting ATP synthesis (Bagkos et al., 2014; Forkink et al., 2014), they promote superoxide generation through mETC III activity, ubiquinol autoxidation, and/or NQO dysfunction (Boveris and Chance, 1973; Turrens and Boveris, 1980; Miwa and Brand, 2003; Yin et al., 2010). However, we observed no corresponding increases in host SOD activity, suggesting that a) no $O_2^-$-driven oxidative challenge arose, and/or b) constitutive SOD abundance was sufficiently protective. The first possibility raises questions about the fate of electrons transferred to CoQ, if not to generate $O_2^-$ or $H_2O$ (the latter through CCO activity), while the second raises doubts about the implied necessity for superoxide accumulation to drive cnidarian bleaching (Lesser, 2006; Hawkins and Davy, 2013; Hawkins et al., 2015; Krueger et al., 2015; Agostini et al., 2016). Here, any excess $O_2^-$ was likely consumed by other antioxidant systems, including the reduced CoQ pool (Ernster and Forsmark-Andrée, 1993; Jin et al., 2016). Equally, alternative oxidase (AOX) could have prevented superoxide overproduction by shuttling excess electrons from ubiquinol to oxygen, generating $H_2O$ (McDonald et al., 2009). Symbiotic cnidarians and their dinoflagellates are thought to possess AOX (McDonald et al., 2009; Oakley et al., 2014), but

we know little about its role in maintaining mETC equilibrium. Certainly, any protective effects of AOX activity would likely come at the cost of reduced ATP synthesis, since AOX does not translocate $H^+$ and cannot buffer against the changes in IMM polarisation state induced by NQO/CCO disequilibrium (McDonald et al., 2009).

Importantly, preconditioned *E. pallida* showed no signs of mETC disequilibrium, even under acute heating. The mETC/CS ratios did not increase and responses to heating were fairly uniform for all mETC complexes quantified. Indeed, the function of host and *Symbiodinium* mitochondria in preconditioned anemones at 33°C, as quantified here, was similar to that of anemones kept at 26°C. We hypothesise that the protective effect of mild warming on symbiotic cnidarian mitochondrial thermal resilience occurred through multiple inter-dependent processes. These include a stimulatory effect of preconditioning on *Symbiodinium* photo-physiology, potentially aided by increased host respiratory activity lifting local $CO_2$ concentrations (see above). The relatively hyperoxic internal environment (and possibly increased carbohydrate availability) resulting from heightened symbiont photosynthesis could have promoted the enlargement and/or multiplication of mitochondria within host cells (and possibly *Symbiodinium*). Rather than increasing the thermal susceptibility of respiratory apparatus, a heightened aerobic capacity may have allowed preconditioned *E. pallida* to avoid heating-induced disequilibrium in mETC function experienced by thermally naïve animals. Specifically, in response to acute heating, preconditioned anemones down-regulated biomass-normalised mitochondrial enzyme activities but maintained equilibrium between the TCA cycle and the mETC and between different mETC components. A similar pattern was observed by Loftus (2012), who noted buffering of acute heating-induced increases in NQO activity in warm acclimated killifish.

It is important to note that while preconditioning to higher temperature had a protective influence on mitochondrial function and symbiosis integrity (similar to that noted by Middlebrook et al., 2008; 2012b; Bellantuono et al., 2012a), preconditioned *E. pallida* eventually bleached under acute heating. Clearly, reduced thermal sensitivity of host and symbiont mETC function is not sufficient to prevent bleaching. Moreover, we applied a photic regime unlikely to induce excessive light stress within the *Symbiodinium* (ambient irradiance approximately 50% saturating; data not shown). Higher light intensities could have exaggerated the warm-preconditioning responses of host respiration via increased symbiont carbon fixation/translocation (Anthony and Hoegh-Guldberg, 2003). Yet, very high irradiance exacerbates the effects of heating on symbiosis stability (Lesser et al., 1990; Hawkins et al., 2015). Under such conditions, the relationship between *Symbiodinium* photosynthesis and host aerobic capacity might not be robust. Given that symbiotic cnidarians routinely experience fluctuating irradiances in the field, the links between light exposure, *Symbiodinium* autotrophy and host mitochondrial function should be explored further.

Our data present an incomplete picture of holobiont metabolism, and application of histological analyses (Dunn et al., 2012) and "omics" techniques at the post-translational level (Drake et al., 2013; Hillyer et al., 2015; Oakley et al., 2015; Weston et al., 2015) are needed to confirm or refute the hypothesised changes in aerobic metabolic pathways and mitochondrial densities, respectively. Notwithstanding these limitations, this investigation provides some of the first evidence for significant effects of thermal preconditioning on the heat sensitivity of symbiotic cnidarian and *Symbiodinium* mitochondrial activity. Given the importance of mitochondria for cellular energetics and the determination of cell fate (Kroemer and Reed, 2000; Berg et al.,

2002; Somero and Hochachka, 2002), additional work should focus on linking cnidarian mitochondrial function with better-known mechanisms of bleaching, such as the apoptosis, autophagy and the widespread disruption of cellular redox homeostasis (Weis, 2008; Lesser, 2011).

**Acknowledgements**

We thank Dr. Kenneth Hoadley and Julia Hagemeyer for their assistance with animal husbandry and maintenance of aquarium facilities. We also thank Dr. Jonathan Cohen for providing assistance with spectrophotometric enzyme assays.

**Competing Interests**

No competing interests declared.

**Author Contributions**

TH conceived the study questions and experimental design. TH carried out the experimental work with assistance from MW. TH conducted all biochemical assays and analysed the data. TH wrote the manuscript with guidance from MW.

**Funding**

This research was funded by the National Science Foundation (grant no. 1316055).

## References


**Abele, E., Philip, E., Gonzalez, P. M. and Puntarulo, S.** (2007). Marine invertebrate mitochondria and oxidative stress. *Frontiers in bioscience : a journal and virtual library* **12**, 933-946

**Agostini, S., Fujimura, H., Hayashi, H. and Fujita, K.** (2016). Mitochondrial electron transport activity and metabolism of experimentally bleached hermatypic corals. *Journal of Experimental Marine Biology and Ecology* **475**, 100-107.http://dx.doi.org/10.1016/j.jembe.2015.11.012

**Agostini, S., Fujimura, H., Fujita, K., Suzuki, Y. and Nakano, Y.** (2013). Respiratory electron transport system activity in symbiotic corals and its link to calcification. *Aquatic Biology* **18**, 125-139.10.3354/ab00496

**Ainsworth, T. D., Heron, S. F., Ortiz, J. C., Mumby, P. J., Grech, A., Ogawa, D., Eakin, C. M. and Leggat, W.** (2016). Climate change disables coral bleaching protection on the Great Barrier Reef. *Science* **352**, 338-342.10.1126/science.aac7125

**Anthony, K. R. N. and Hoegh-Guldberg, O.** (2003). Variation in coral photosynthesis, respiration and growth characteristics in contrasting light microhabitats: An analogue to plants in forest gaps and understoreys? *Functional Ecology* **17**, 246-259

**Bagkos, G., Koufopoulos, K. and Piperi, C.** (2014). ATP synthesis revisited: new avenues for the management of mitochondrial diseases. *Current pharmaceutical design* **20**, 4570-4579

**Baird, A. H., Bhagooli, R., Ralph, P. J. and Takahashi, S.** (2008). Coral bleaching: The role of the host. *Trends in Ecology and Evolution* **24**, 16-20

**Baker, A. C., Starger, C. J., McClanahan, T. R. and Glynn, P. W.** (2004). Corals' adaptive response to climate change. *Nature* **430**, 741

**Båmstedt, U.** (1980). ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. *Journal of Experimental Marine Biology and Ecology* **42**, 267-283.http://dx.doi.org/10.1016/0022-0981(80)90181-1

**Bay, R. A. and Palumbi, S. R.** (2015). Rapid acclimation ability mediated by transcriptome changes in reef-building corals. *Genome Biology and Evolution* **7**, 1602-1612.10.1093/gbe/evv085

**Bellantuono, A. J., Hoegh-Guldberg, O. and Rodriguez-Lanetty, M.** (2012a). Resistance to thermal stress in corals without changes in symbiont composition. *Proceedings of the Royal Society B-Biological Sciences* **279**, 1100-1107.10.1098/rspb.2011.1780

**Bellantuono, A. J., Granados-Cifuentes, C., Miller, D. J., Hoegh-Guldberg, O. and Rodriguez-Lanetty, M.** (2012b). Coral thermal tolerance: Tuning gene expression to resist thermal stress. *PLoS ONE* **7**, e50685

**Berg, J. M., Tymoczko, J. L. and Stryer, L.** (2002). Biochemistry, 5th Edition. New York: W H Freeman.

**Berkelmans, R. and van Oppen, M. J. H.** (2006). The role of zooxanthellae in the thermal tolerance of corals: A 'nugget of hope' for coral reefs in an era of climate change. *Proceedings of the Royal Society B-Biological Sciences* **273**, 2305-2312.10.1098/rspb.2006.3567

**Bertucci, A., Moya, A., Tambutté, S., Allemand, D., Supuran, C. T. and Zoccola, D.** (2013). Carbonic anhydrases in anthozoan corals—A review. *Bioorganic & Medicinal Chemistry* **21**, 1437-1450.http://dx.doi.org/10.1016/j.bmc.2012.10.024

**Boveris, A. and Chance, B.** (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochemical Journal* **134**, 707-716

**Cadenas, E. and Davies, K. J.** (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med* **29**, 222-230

**Camp, E. F., Smith, D. J., Evenhuis, C., Enochs, I., Manzello, D., Woodcock, S. and Suggett, D. J.** (2016). Acclimatization to high-variance habitats does not enhance physiological tolerance of two key Caribbean corals to future temperature and pH. *Proceedings of the Royal Society of London B: Biological Sciences* **283**.10.1098/rspb.2016.0442
**Castillo, K. D. and Helmuth, B. S. T.** (2005). Influence of thermal history on the response of Montastraea annularis to short-term temperature exposure. *Marine Biology* **148**, 261-270.10.1007/s00227-005-0046-x
**Chakravarti, L. J., Jarrold, M. D., Gibbin, E. M., Christen, F., Massamba-N'Siala, G., Blier, P. U. and Calosi, P.** (2016). Can trans-generational experiments be used to enhance species resilience to ocean warming and acidification? *Evolutionary Applications*.10.1111/eva.12391
**Chung, D. J. and Schulte, P. M.** (2015). Mechanisms and costs of mitochondrial thermal acclimation in a eurythermal killifish (Fundulus heteroclitus). *Journal of Experimental Biology* **218**, 1621-1631.10.1242/jeb.120444
**Clarke, A.** (2003). Costs and consequences of evolutionary temperature adaptation. *Trends in Ecology & Evolution* **18**, 573-581.http://dx.doi.org/10.1016/j.tree.2003.08.007
**Clarke, M. E. and Walsh, P. J.** (1993). Effect of nutritional status on citrate synthase activity in *Acartia tonsa* and *Temora longicornis*. *Limnology and Oceanography* **38**, 414-418.10.4319/lo.1993.38.2.0414
**Davy, S. K., Allemand, D. and Weis, V. M.** (2012). The cell biology of cnidarian-dinoflagellate symbiosis. *Microbiology and Molecular Biology Reviews* **76**, 229-261
**Desalvo, M. K., Rvoolstra, C., Sunagawa, S., Schwarz, J. A., Stillman, J. H., Coffroth, M. A., Szmant, A. M. and Medina, M.** (2008). Differential gene expression during thermal stress and bleaching in the Caribbean coral *Montastraea faveolata*. *Molecular Ecology* **17**, 3952-3971.10.1111/j.1365-294X.2008.03879.x
**Dixon, G. B., Davies, S. W., Aglyamova, G. A., Meyer, E., Bay, L. K. and Matz, M. V.** (2015). Genomic determinants of coral heat tolerance across latitudes. *Science* **348**, 1460-1462.10.1126/science.1261224
**Downs, C. A., Kramarsky-Winter, E., Martinez, J., Kushmaro, A., Woodley, C. M., Loya, Y. and Ostrander, G. K.** (2009). Symbiophagy as a cellular mechanism for coral bleaching. *Autophagy* **5**, 211-216
**Drake, J. L., Mass, T., Haramaty, L., Zelzion, E., Bhattacharya, D. and Falkowski, P. G.** (2013). Proteomic analysis of skeletal organic matrix from the stony coral Stylophora pistillata. *Proceedings of the National Academy of Sciences* **110**, 3788-3793.10.1073/pnas.1301419110
**Dunn, S. R., Schnitzler, C. E. and Weis, V. M.** (2007). Apoptosis and autophagy as mechanisms of dinoflagellate symbiont release during cnidarian bleaching: Every which way you lose. *Proceedings of the Royal Society B-Biological Sciences* **274**, 3079-3085.10.1098/rspb.2007.0711
**Dunn, S. R., Thomason, J. C., Le Tissier, M. D. A. and Bythell, J. C.** (2004). Heat stress induces different forms of cell death in sea anemones and their endosymbiotic algae depending on temperature and duration. *Cell Death and Differentiation* **11**, 1213-1222
**Dunn, S. R., Pernice, M., Green, K., Hoegh-Guldberg, O. and Dove, S. G.** (2012). Thermal stress promotes host mitochondrial degradation in symbiotic cnidarians: Are the batteries of the reef going to run out? *PLoS ONE* **7**, e39024
**Dykens, J. A. and Shick, J. M.** (1982). Oxygen production by endosymbiotic algae controls superoxide dismutase activity in their animal host. *Nature* **297**, 579-580
**Ernst, O. and Zor, T.** (2010). Linearization of the Bradford protein assay. *Journal of Visualised Experiments* **38**, e1918.10.3791/1918
**Ernster, L. and Forsmark-Andrée, P.** (1993). Ubiquinol: an endogenous antioxidant in aerobic organisms. *The Clinical Investigator* **71**, S60-S65.10.1007/bf00226842

**Evans, T. G.** (2015). Considerations for the use of transcriptomics in identifying the 'genes that matter' for environmental adaptation. *The Journal of Experimental Biology* **218**, 1925-1935.10.1242/jeb.114306
**Forkink, M., Manjeri, G. R., Liemburg-Apers, D. C., Nibbeling, E., Blanchard, M., Wojtala, A., Smeitink, J. A., Wieckowski, M. R., Willems, P. H. and Koopman, W. J.** (2014). Mitochondrial hyperpolarization during chronic complex I inhibition is sustained by low activity of complex II, III, IV and V. *Biochim Biophys Acta* **1837**, 1247-1256.10.1016/j.bbabio.2014.04.008
**Frazier, A. E. and Thorburn, D. R.** (2012). Biochemical Analyses of the Electron Transport Chain Complexes by Spectrophotometry. In *Mitochondrial Disorders*, vol. 837 (ed. L.-J. C. Wong), pp. 49-62. New York: Springer Science & Business Media.
**Galmés, J., Kapralov, M. V., Copolovici, L. O., Hermida-Carrera, C. and Niinemets, Ü.** (2015). Temperature responses of the Rubisco maximum carboxylase activity across domains of life: phylogenetic signals, trade-offs, and importance for carbon gain. *Photosynthesis Research* **123**, 183-201.10.1007/s11120-014-0067-8
**Gattuso, J. P., Yellowlees, D. and Lesser, M. P.** (1993). Depth- and light-dependent variation of carbon partitioning and utilization in the zooxanthellate scleractinian coral *Stylophora pistillata Marine Ecology Progress Series* **92**, 267-276
**Grivennikova, V. G., Kotlyar, A. B., Karliner, J. S., Cecchini, G. and Vinogradov, A. D.** (2007). Redox-Dependent Change of Nucleotide Affinity to the Active Site of the Mammalian Complex I. *Biochemistry* **46**, 10971-10978.10.1021/bi7009822
**Grottoli, A. G., Warner, M. E., Levas, S. J., Aschaffenburg, M. D., Schoepf, V., McGinley, M., Baumann, J. and Matsui, Y.** (2014). The cumulative impact of annual coral bleaching can turn some coral species winners into losers. *Global Change Biology* **20**, 3823-3833.10.1111/gcb.12658
**Hawkins, T., Krueger, T., Wilkinson, S., Fisher, P. and Davy, S.** (2015). Antioxidant responses to heat and light stress differ with habitat in a common reef coral. *Coral Reefs* **34**, 1229-1241.10.1007/s00338-015-1345-4
**Hawkins, T. D. and Davy, S. K.** (2013). Nitric oxide and coral bleaching: Is peroxynitrite generation required for symbiosis collapse? *Journal of Experimental Biology* **216**, 3185-3188
**Hawkins, T. D., Bradley, B. J. and Davy, S. K.** (2013). Nitric oxide mediates coral bleaching through an apoptotic-like cell death pathway: evidence from a model cnidarian-dinoflagellate symbiosis. *FASEB Journal* **27**, 4790-4798.10.1096/fj.13-235051
**Hawkins, T. D., Hagemeyer, J. C. G., Hoadley, K. D., Marsh, A. G. and Warner, M. E.** (2016a). Partitioning of Respiration in an Animal-Algal Symbiosis: Implications for Different Aerobic Capacity Between *Symbiodinium* spp. *Frontiers in Physiology* **7**, 128.10.3389/fphys.2016.00128
**Hawkins, T. D., Hagemeyer, J. C. G. and Warner, M. E.** (2016b). Temperature moderates the infectiousness of two conspecific *Symbiodinium* genotypes isolated from the same host population. *Environmental Microbiology* **In Press**
**Hillyer, K. E., Tumanov, S., Villas-Bôas, S. and Davy, S. K.** (2015). Metabolite profiling of symbiont and host during thermal stress and bleaching in a model cnidarian-dinoflagellate symbiosis. *Journal of Experimental Biology* **219**, 516-527.10.1242/jeb.128660
**Hoadley, K. D., Rollison, D., Pettay, D. T. and Warner, M. E.** (2015). Differential carbon utilization and asexual reproduction under elevated pCO2 conditions in the model anemone, *Exaiptasia pallida*, hosting different symbionts. *Limnology and Oceanography* **60**, 2108-2120.10.1002/lno.10160
**Hoegh-Guldberg, O., Jones, R. J., Ward, S. and Loh, W. K.** (2002). Is coral bleaching really adaptive? *Nature* **415**, 601-602

**Holcomb, M., Tambutté, E., Allemand, D. and Tambutté, S.** (2014). Light enhanced calcification in Stylophora pistillata: effects of glucose, glycerol and oxygen. *PeerJ* **2**, e375.10.7717/peerj.375
**Holloszy, J. O., Oscai, L. B., Don, I. J. and Molé, P. A.** (1970). Mitochondrial citric acid cycle and related enzymes: Adaptive response to exercise. *Biochemical and Biophysical Research Communications* **40**, 1368-1373.http://dx.doi.org/10.1016/0006-291X(70)90017-3
**Hothorn, T., Bretz, F., Westfall, P., Heiberger, R. M., Schuetzenmeister, A. and Scheibe, S.** (2015). Package "multcomp": Simultaneous Inference in General Parametric Models. *CRAN Repository*
**Jardim-Messeder, D., Caverzan, A., Rauber, R., de Souza Ferreira, E., Margis-Pinheiro, M. and Galina, A.** (2015). Succinate dehydrogenase (mitochondrial complex II) is a source of reactive oxygen species in plants and regulates development and stress responses. *The New phytologist* **208**, 776-789.10.1111/nph.13515
**Jin, Y. K., Lundgren, P., Lutz, A., Raina, J.-B., Howells, E. J., Paley, A. S., Willis, B. L. and van Oppen, M. J. H.** (2016). Genetic markers for antioxidant capacity in a reef-building coral. *Science Advances* **2**, e1500842.10.1126/sciadv.1500842
**Kraffe, E., Marty, Y. and Guderley, H.** (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout Oncorhynchus mykiss: roles of membrane proteins, phospholipids and their fatty acid compositions. *Journal of Experimental Biology* **210**, 149-165.10.1242/jeb.02628
**Kroemer, G. and Reed, J. C.** (2000). Mitochondrial control of cell death. *Nature Medicine* **6**, 513-519
**Krueger, T., Hawkins, T. D., Becker, S., Pontasch, S., Dove, S., Hoegh-Guldberg, O., Leggat, W., Fisher, P. L. and Davy, S. K.** (2015). Differential coral bleaching—Contrasting the activity and response of enzymatic antioxidants in symbiotic partners under thermal stress. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **190**, 15-25.http://dx.doi.org/10.1016/j.cbpa.2015.08.012
**Lesser, M. P.** (2006). Oxidative stress in marine environments. *Annual Reviews of Physiology* **68**, 253-278
**Lesser, M. P.** (2011). Coral bleaching: Causes and mechanisms. In *Coral Reefs: An Ecosystem in Transition*, eds. T. J. Dubinsky and J. S. Stamler), pp. 405-419. Berlin: Springer.
**Lesser, M. P., Stochaj, W. R., Tapley, D. W. and Shick, J. M.** (1990). Bleaching in coral reef anthozoans: Effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. *Coral Reefs* **8**, 225-232.10.1007/BF00265015
**Loftus, S.** (2012). Analysis of Complex I Activity Within and Among Populations of *Fundulus heteroclitus*, vol. PhD Thesis. Miami: University of Miami.
**Lutz, A., Raina, J.-B., Motti, C. A., Miller, D. J. and van Oppen, M. J. H.** (2015). Host coenzyme Q redox state is an early biomarker of thermal stress in the coral *Acropora millepora*. *PLoS ONE* **10**, e0139290.10.1371/journal.pone.0139290
**Martinez-Cruz, O., Sanchez-Paz, A., Garcia-Carreño, F., Jimenez-Gutierrez, L. and Muhlia-Almaza, A.** (2012). Invertebrates Mitochondrial Function and Energetic Challenges. In *Bioenergetics*, (ed. K. Clark): InTech.
**McDonald, A. E., Vanlerberghe, G. C. and Staples, J. F.** (2009). Alternative oxidase in animals: unique characteristics and taxonomic distribution. *J Exp Biol* **212**, 2627-2634.10.1242/jeb.032151
**Middlebrook, R. and Anthony, K. R.** (2010). Heating rate and symbiont productivity are key factors determining thermal stress in the reef-building coral *Acropora formosa*. *Journal of Experimental Biology* **213**, 1026-1034

**Middlebrook, R., Hoegh-Guldberg, O. and Leggat, W.** (2008). The effect of thermal history on the susecptibility of reef-building corals to thermal stress. *Journal of Experimental Biology* **211**, 1050-1056
**Miwa, S. and Brand, M. D.** (2003). Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochem Soc Trans* **31**, 1300-1301.10.1042/
**Murphy, Michael P.** (2009). How mitochondria produce reactive oxygen species. *Biochemical Journal* **417**, 1-13.10.1042/BJ20081386
**Muscatine, L., R. McCloskey, L. and E. Marian, R.** (1981). Estimating the daily contribution of carbon from zooxanthellae to coral animal respiration. *Limnology and Oceanography* **26**, 601-611.10.4319/lo.1981.26.4.0601
**Oakley, C., Hopkinson, B. and Schmidt, G.** (2014). Mitochondrial terminal alternative oxidase and its enhancement by thermal stress in the coral symbiont Symbiodinium. *Coral Reefs* **33**, 543-552.10.1007/s00338-014-1147-0
**Oakley, C. A., Ameismeier, M. F., Peng, L., Weis, V. M., Grossman, A. R. and Davy, S. K.** (2015). Symbiosis induces widespread changes in the proteome of the model cnidarian Aiptasia. *Cellular Microbiology* **18**, 1009-1023.10.1111/cmi.12564
**Oellermann, M., Pörtner, H. O. and Mark, F. C.** (2012). Mitochondrial dynamics underlying thermal plasticity of cuttlefish (Sepia officinalis) hearts. *Journal of Experimental Biology* **215**, 2992-3000.10.1242/jeb.068163
**Oliver, T. A. and Palumbi, S. R.** (2011). Do fluctuating temperature environments elevate coral thermal tolerance? *Coral Reefs* **30**, 429-440.10.1007/s00338-011-0721-y
**Paxton, C. W., Davy, S. K. and Weis, V. M.** (2013). Stress and death of host cells play a role in cnidarian bleaching. *Journal of Experimental Biology* **216**, 2813-2820.10.1242/jeb.087858
**Peck, L. S.** (2002). Ecophysiology of Antarctic marine ectotherms: limits to life. In *Ecological Studies in the Antarctic Sea Ice Zone: Results of EASIZ Midterm Symposium*, eds. W. E. Arntz and A. Clarke), pp. 221-230. Berlin, Heidelberg: Springer Berlin Heidelberg.
**Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S. and Van Willigen, B.** (2016). Package "nlme". *CRAN Repository*
**Pörtner, H.** (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Die Naturwissenschaften* **88**, 137-146.10.1007/s001140100216
**Pörtner, H. O., Peck, L. and Somero, G.** (2007). Thermal limits and adaptation in marine Antarctic ectotherms: an integrative view. *Philosophical Transactions of the Royal Society B: Biological Sciences* **362**, 2233-2258.10.1098/rstb.2006.1947
**Putnam, H. M. and Gates, R. D.** (2015). Preconditioning in the reef-building coral *Pocillopora damicornis* and the potential for trans-generational acclimatization in coral larvae under future climate change conditions. *The Journal of Experimental Biology* **218**, 2365-2372.10.1242/jeb.123018
**Ralph, P. J., Gademann, R. and Larkum, A. W. D.** (2001). Zooxanthellae expelled from bleached corals at 33oC are photosynthetically competent. *Marine Ecology Progress Series* **220**
**Rands, M. L., Douglas, A. E., Loughman, B. C. and Ratcliffe, R. G.** (1992). Avoidance of Hypoxia in a Cnidarian Symbiosis by Algal Photosynthetic Oxygen. *The Biological Bulletin* **182**, 159-162
**Richier, S., Furla, P., Plantivaux, A., Merle, P. L. and Allemand, D.** (2005). Symbiosis-induced adaptation to oxidative stress. *Journal of Experimental Biology* **208**, 277-285.10.1242/jeb.01368
**Richier, S., Merle, P. L., Furla, P., Pigozzi, D., Sola, F. and Allemand, D.** (2003). Characterization of superoxide dismutases in anoxia- and hyperoxia-tolerant symbiotic cnidarians. *Biochim Biophys Acta* **1621**, 84-91

**Riobó, N. A., Clementi, E., Melani, M., Boveris, A., Cadenas, E., Moncada, S. and Poderoso, J. J.** (2001). Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. *Biochemical Journal* **359**, 139-145
**Roberty, S., Bailleul, B., Berne, N., Franck, F. and Cardol, P.** (2014). PSI Mehler reaction is the main alternative photosynthetic electron pathway in Symbiodinium sp., symbiotic dinoflagellates of cnidarians. *New Phytologist* **204**, 81-91.10.1111/nph.12903
**Rowan, R., Knowlton, N., Baker, A. and Jara, J.** (1997). Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. *Nature* **388**, 265-269
**Santidrian, A. F., Matsuno-Yagi, A., Ritland, M., Seo, B. B., LeBoeuf, S. E., Gay, L. J., Yagi, T. and Felding-Habermann, B.** (2013). Mitochondrial complex I activity and NAD+/NADH balance regulate breast cancer progression. *The Journal of Clinical Investigation* **123**, 1068-1081.10.1172/JCI64264
**Schulte, P. M., Healy, T. M. and Fangue, N. A.** (2011). Thermal Performance Curves, Phenotypic Plasticity, and the Time Scales of Temperature Exposure. *Integrative and Comparative Biology* **51**, 691-702.10.1093/icb/icr097
**Shashar, N., Cohen, Y. and Loya, Y.** (1993). Extreme Diel Fluctuations of Oxygen in Diffusive Boundary Layers Surrounding Stony Corals. *Biological Bulletin* **185**, 455-461.10.2307/1542485
**Shick, J. M.** (1990). Diffusion Limitation and Hyperoxic Enhancement of Oxygen Consumption in Zooxanthellate Sea Anemones, Zoanthids, and Corals. *Biological Bulletin* **179**, 148-158.10.2307/1541749
**Sies, H.** (1997). Oxidative stress: oxidants and antioxidants. *Experimental Physiology* **82**, 291-295.10.1113/expphysiol.1997.sp004024
**Silverstein, R. N., Cunning, R. and Baker, A. C.** (2014). Change in algal symbiont communities after bleaching, not prior heat exposure, increases heat tolerance of reef corals. *Global Change Biology* **21**, 236-249.10.1111/gcb.12706
**Sokolova, I. M. and Pörtner, H.-O.** (2003). Metabolic plasticity and critical temperatures for aerobic scope in a eurythermal marine invertebrate (Littorina saxatilis, Gastropoda: Littorinidae) from different latitudes. *Journal of Experimental Biology* **206**, 195-207.10.1242/jeb.00054
**Somero, G. N. and Hochachka, P. W.** (2002). Biochemical Adaptation: Mechanism and Process in Physiological Evolution. New York: Oxford University Press.
**Sommer, A. and Portner, H. O.** (1999). Exposure of Arenicola marina to extreme temperatures: adaptive flexibility of a boreal and and a subpolar population. *Marine Ecology Progress Series* **181**, 215-226
**Sommer, A. M. and Portner, H. O.** (2004). Mitochondrial function in seasonal acclimatization versus latitudinal adaptation to cold in the lugworm Arenicola marina (L.). *Physiol Biochem Zool* **77**, 174-186.10.1086/381468
**Spinazzi, M., Casarin, A., Pertegato, V., Salviati, L. and Angelini, C.** (2012). Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nat Protoc* **7**, 1235-1246.10.1038/nprot.2012.058
**Srere, P. A.** (1969). Citrate synthase. *Methods in Enzymology* **13**, 3-11
**Tchernov, D., Kvitt, H., Haramaty, L., Bibby, T. S., Gorbunov, M. Y., Rosenfeld, H. and Falkowski, P. G.** (2011). Apoptosis and the selective survival of host animals following thermal bleaching in zooxanthellate corals. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 9905-9909.10.1073/pnas.1106924108
**Team, R. D. C.** (2015). R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org.

**Turrens, J. F.** (2003). Mitochondrial formation of reactive oxygen species. *The Journal of Physiology* **552**, 335-344.10.1113/jphysiol.2003.049478

**Turrens, J. F. and Boveris, A.** (1980). Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* **191**, 421-427

**Tytler, E. M. and Trench, R. K.** (1986). Activities of enzymes in beta-carboxylation reactions and of catalase in cell-free preparations from the symbiotic dinoflagellates *Symbiodinium* spp. From a coral, a clam, a zoanthid and two sea anemones. *Proceedings of the Royal Society of London B: Biological Sciences* **228**, 483-492.10.1098/rspb.1986.0065

**Urschel, M. R. and O'Brien, K. M.** (2008). High mitochondrial densities in the hearts of Antarctic icefishes are maintained by an increase in mitochondrial size rather than mitochondrial biogenesis. *Journal of Experimental Biology* **211**, 2638-2646.10.1242/jeb.018598

**van Oppen, M. J., Baker, A. C., Coffroth, M. A. and Willis, B. L.** (2009). Bleaching resistance and the role of algal endosymbionts. In *Coral Bleaching: Patterns, Processes, Causes and Consequences*, eds. M. J. van Oppen and J. M. Lough), pp. 83-96. Heidelberg, Germany: Springer-Verlag.

**Vigelsø, A., Andersen, N. B. and Dela, F.** (2014). The relationship between skeletal muscle mitochondrial citrate synthase activity and whole body oxygen uptake adaptations in response to exercise training. *International Journal of Physiology, Pathophysiology and Pharmacology* **6**, 84-101

**Weis, V. M.** (2008). Cellular mechanisms of cnidarian bleaching: Stress causes the collapse of symbiosis. *Journal of Experimental Biology* **211**, 3059-3066

**Weis, V. M.** (2010). The susceptibility and resilience of corals to thermal stress: Adaptation, acclimatization or both? *Molecular Ecology* **19**, 1515-1517.10.1111/j.1365-294X.2010.04575.x

**Weston, A. J., Dunlap, W. C., Beltran, V. H., Starcevic, A., Hranueli, D., Ward, M. and Long, P. F.** (2015). Proteomics links the redox state to calcium signalling during bleaching of the scleractinian coral *Acropora microphthalma* on exposure to high solar irradiance and thermal stress. *Molecular & Cellular Proteomics* **14**, 585-595.10.1074/mcp.M114.043125

**Yakovleva, I. and Hidaka, M.** (2004). Different Effects of High Temperature Acclimation on Bleaching-Susceptible and Tolerant Corals. *Symbiosis* **37**, 87-105

**Yin, Y., Yang, S., Yu, L. and Yu, C.-A.** (2010). Reaction Mechanism of Superoxide Generation during Ubiquinol Oxidation by the Cytochrome bc1 Complex. *Journal of Biological Chemistry* **285**, 17038-17045.10.1074/jbc.M110.104364

**Ying, W.** (2008). NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid Redox Signal* **10**, 179-206.10.1089/ars.2007.1672

**Figures**

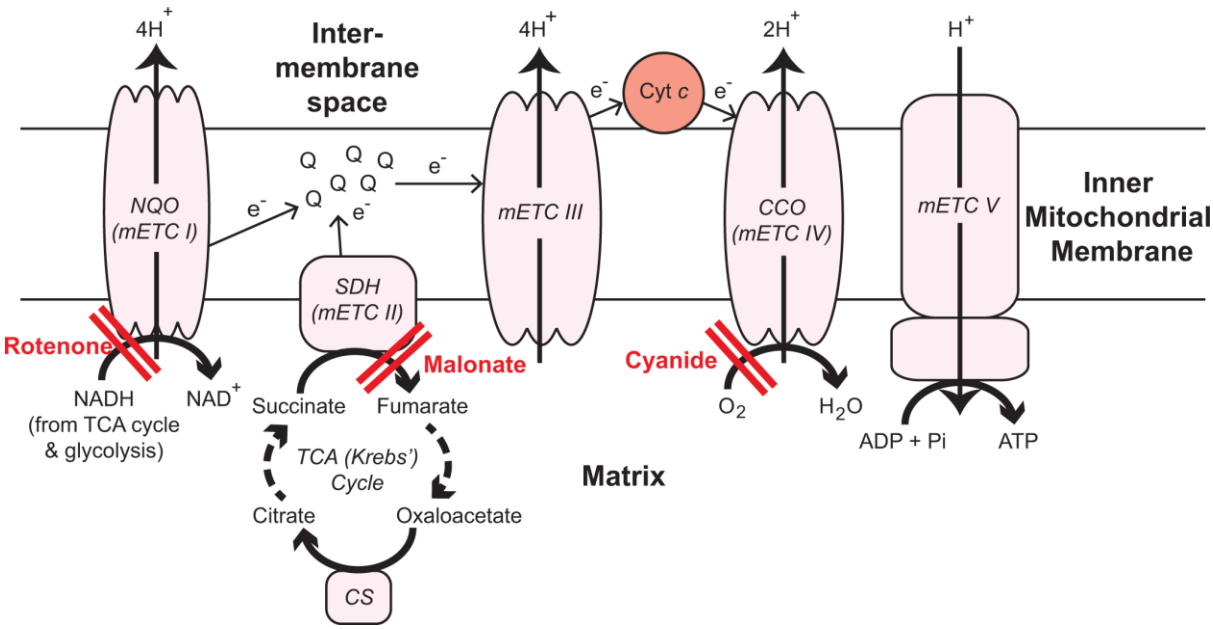


**Figure 1.** Simplified conceptual model of the mitochondrial electron transport chain (mETC). Carbon-rich glycolysis-derived organic substrates are oxidised through the tricarboxylic acid (TCA) cycle (omitted steps indicated by dashed lines), transferring electrons ($e^-$) to $NAD^+$ and FAD. NADH is oxidized by the first complex of the mETC – NADH:coenzyme Q oxidoreductase (NQO, mETC I) – which transfers electrons to the carrier molecule coenzyme Q (CoQ, also known as ubiquinone). Further reduction of CoQ to ubiquinol ($QH_2$) is achieved by the additional transfer of elections (from succinate and $FADH_2$) by succinate dehydrogenase (SDH, mETC II). Coenzyme Q:cytochrome *c* oxidoreductase (mETC III) regenerates oxidised CoQ by transferring electrons from $QH_2$ to cytochrome *c* (cyt *c*). The latter is finally oxidised by cytochrome *c* oxidase (CCO, mETC IV), with its electrons transferred to $O_2$ (Berg et al., 2002). The proton gradient generated by the activities complexes I, III, and IV drives ATP

Journal of Experimental Biology • Advance article

production by ATP synthase, or mETC complex V (Berg et al., 2002; Somero and Hochachka, 2002). mETC complex inhibitors applied in this study are indicated in red.

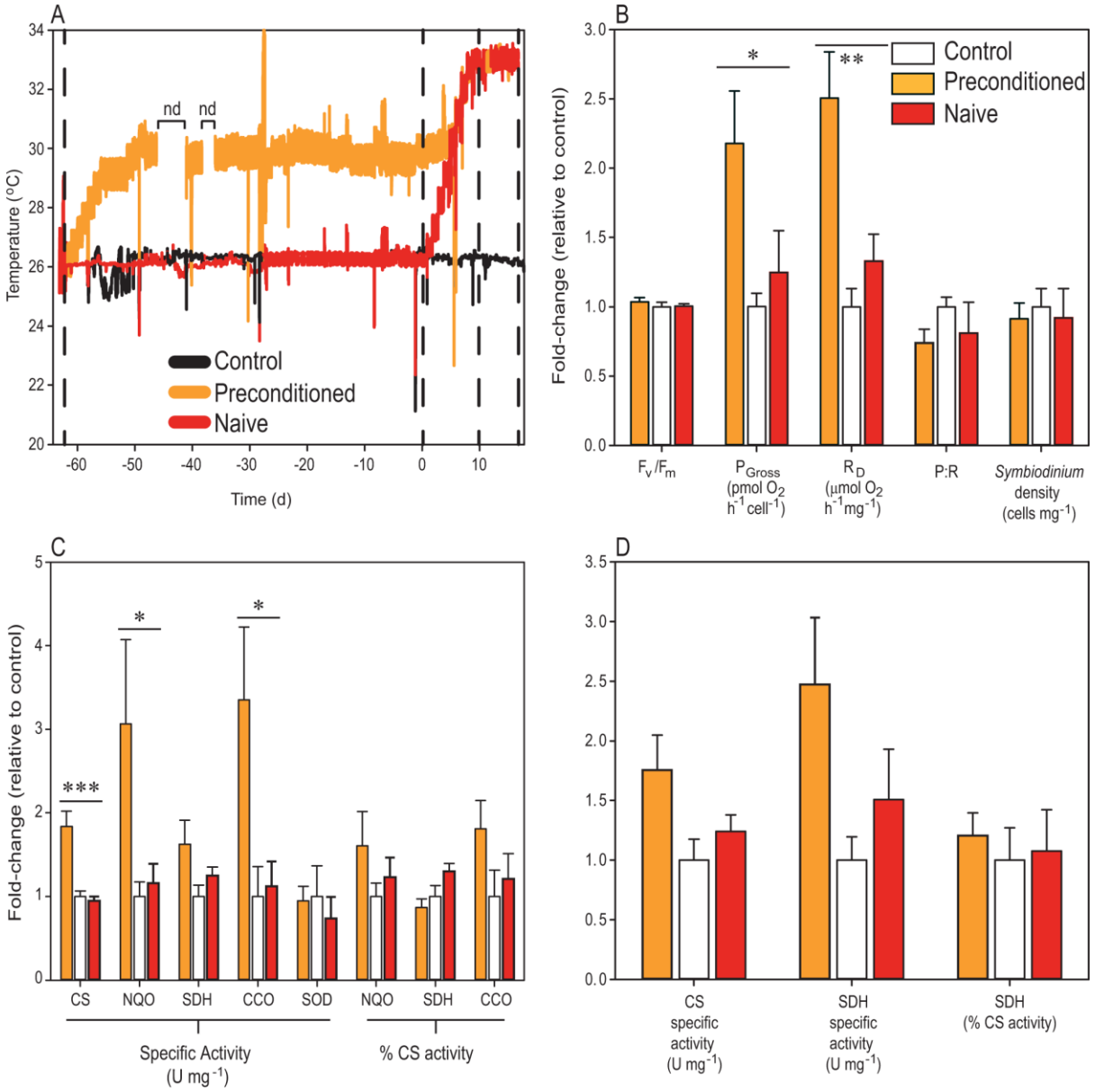


**Figure 2**. Panel **A)** Thermal treatments of E*xaiptasia pallida* anemones: "Control" (26°C), "Preconditioned" (26°C to 30°C then to 33°C) and "Thermally Naïve" (26°C to 33°C). The solid red line indicates Day 0 of the acute heating experiment, dashed lines indicate additional sampling days, and "nd" refers to periods where temperature data are not available due to

Journal of Experimental Biology • Advance article

malfunctions of temperature logging equipment. **B)** Maximum quantum yield of *Symbiodinium* PSII ($F_v/F_m$), gross photosynthesis per symbiont cell ($P_{Gross}$), holobiont dark respiration ($R_D$), holobiont $P_{Gross}$ / $R_D$ ratio, and *Symbiodinium* cell density. **C)** Specific activities of host citrate synthase (CS), NADH:coenzyme Q oxidoreductase (NQO), succinate dehydrogenase (SDH), cytochrome *c* oxidase (CCO) and superoxide dismutase (SOD) enzymes alongside CS-normalised NQO, SDH, and CCO activities. **D)** Variables as for panel (C), but measured from *Symbiodinium* lysate. Values in panels **B-D** are means ± s.e.m. (n = 5) relative to the mean of the control group. Significant differences between treatment groups were identified using univariate tests conducted within multivariate analyses of variance (MANOVA, * $p < 0.05$, **, $p < 0.01$, *** $p < 0.001$.

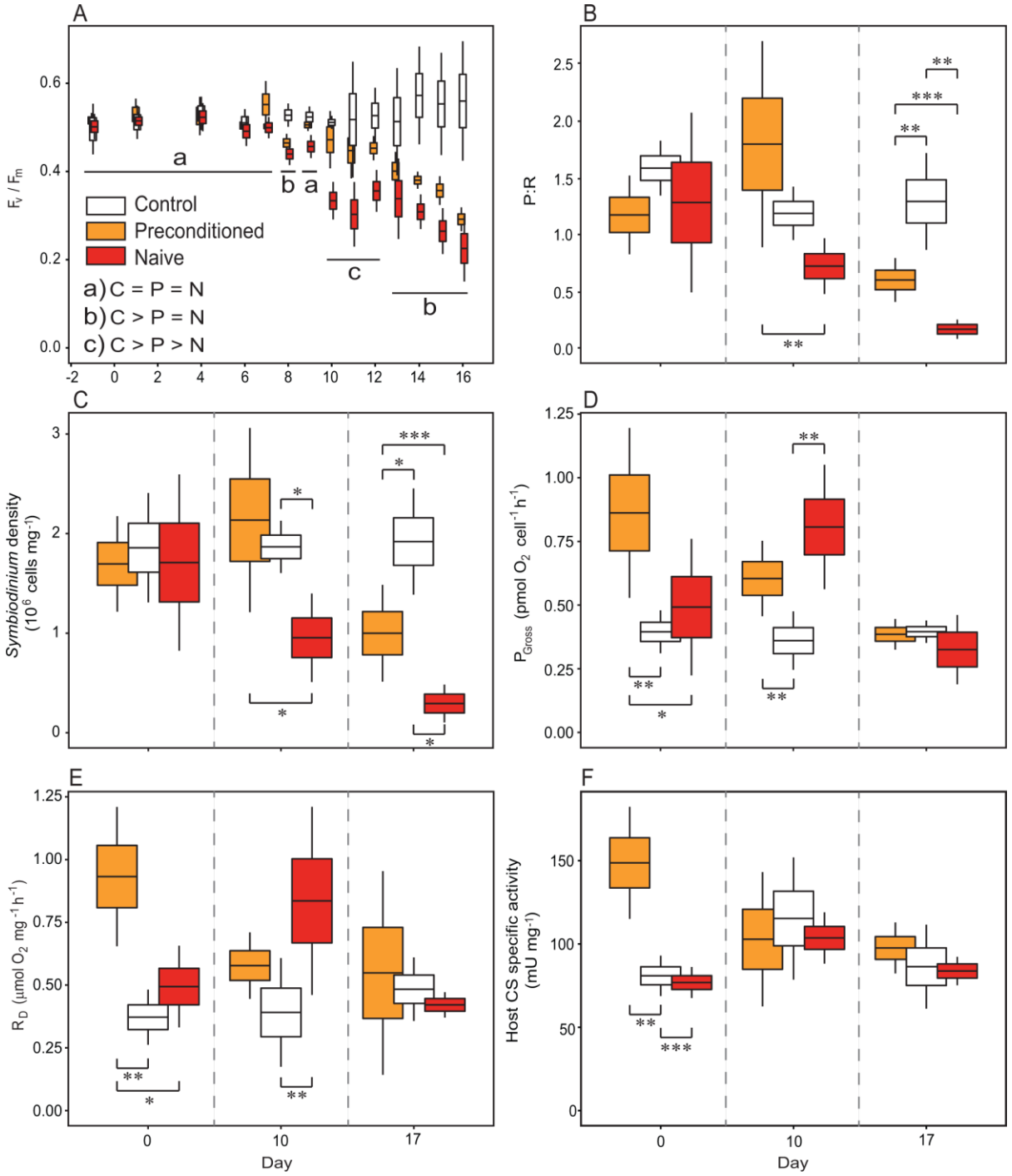


**Figure 3.** Responses of *Exaiptasia pallida* to acute heating. Panel **A**) Maximum quantum yield of *Symbiodinium* PSII ($F_v/F_m$). Asterisks indicate statistically significant differences between

the three treatment groups: “Control” (C; 26°C), “Preconditioned” (P; 30°C to 33°C) and “Naïve” (N; 26°C to 33°C); **B**) Holobiont gross photosynthesis / respiration ratio (P:R); **C**) *Symbiodinium* cell density per mg host protein; **D**) Gross photosynthesis per *Symbiodinium* cell; **E**) Holobiont dark respiration rate; **F**) Host citrate synthase (CS) specific activity. Boxes represent means ± 1 s.e.m. and whiskers denote 1 s.d. of the mean (n = 5 per treatment group per day). Asterisks in panel **B-F** indicate significant differences between treatment groups on each day (LM-ANOVA, Tukey *post hoc* tests,*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$).

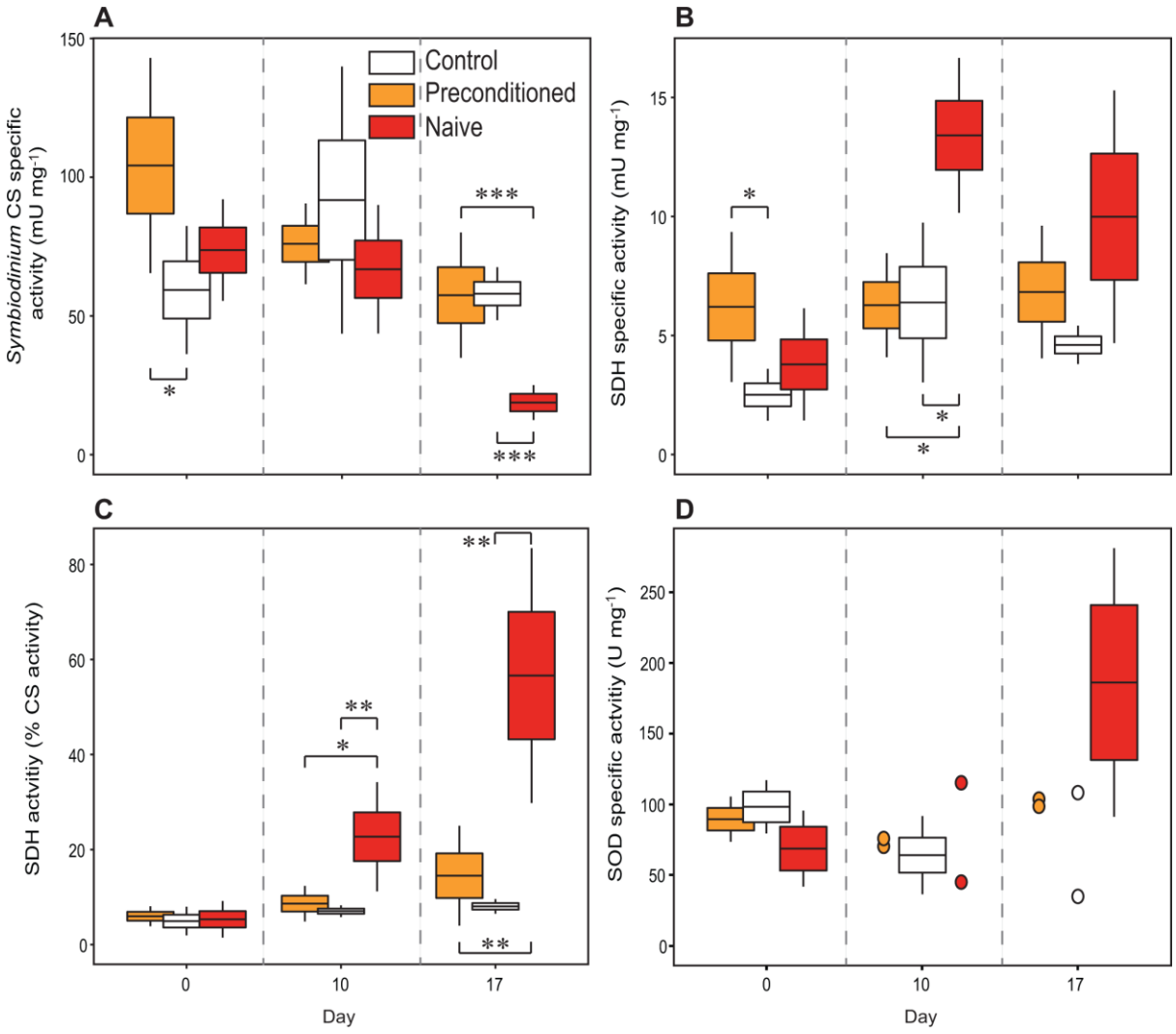


**Figure 4**. Responses of *in hospite Symbiodinium* mitochondrial enzyme and superoxide dismutase (SOD) activity to acute heating. Panel **A**) Citrate synthase (CS) specific activity; **B**) Succinate dehydrogenase (SDH) specific activity; **C**) CS-normalised SDH activity; **D**) SOD specific activity. Boxes represent means ± 1 s.e.m. and whiskers denote 1 s.d. of the mean (n = 5 per treatment group per day). Asterisks in panel **A-C** indicate significant differences between treatment groups on each day (LM-ANOVA, Tukey *post hoc* tests,*p < 0.05, ** p <

0.01, ***p < 0.001). Markers in panel **D** represent raw data where n < 3 for treatment groups on respective days; LM-ANOVA could therefore not be conducted on the SOD data.

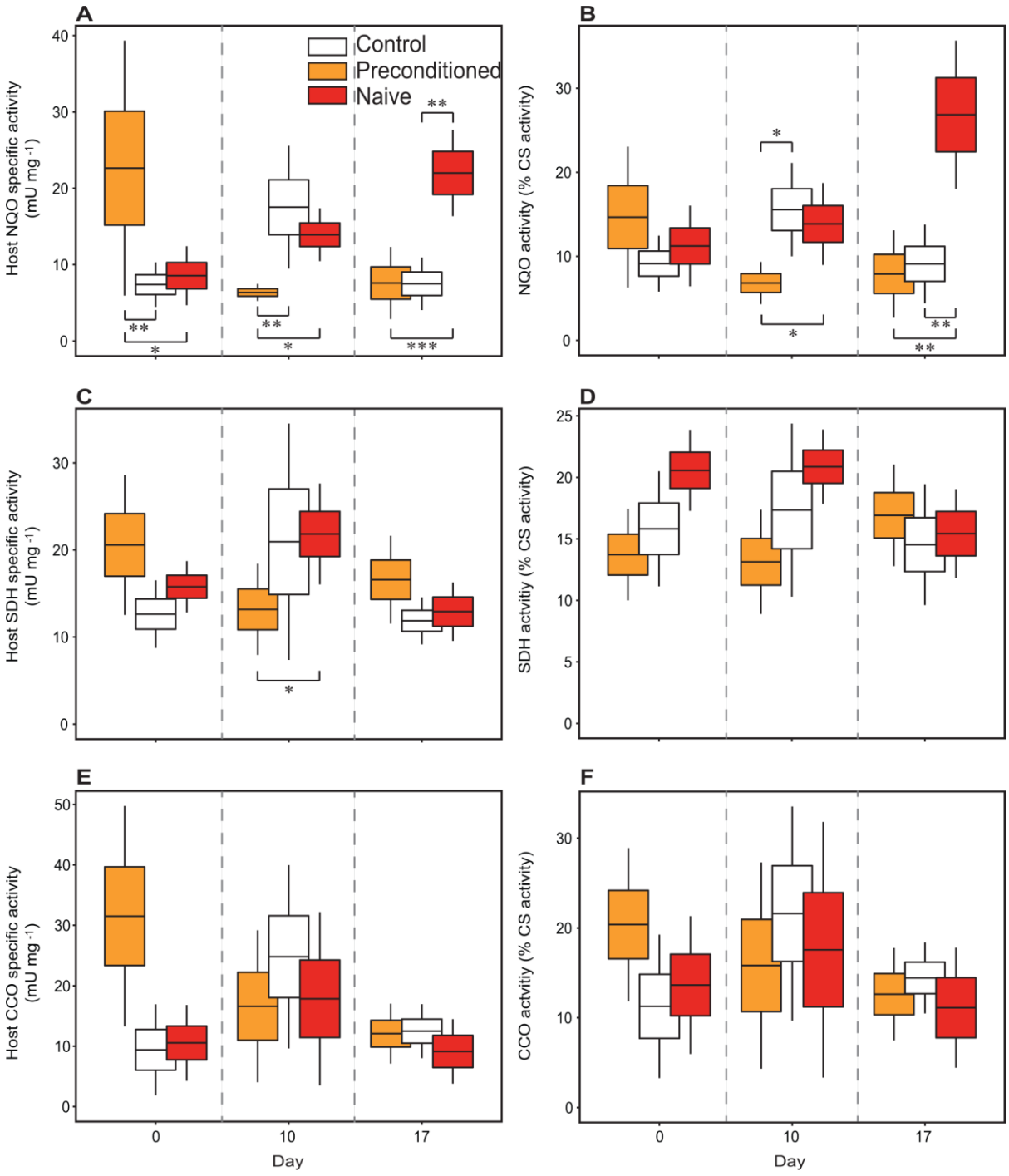


**Figure 5**. Responses of *Exaiptasia pallida* host mitochondrial electron transport chain (mETC) enzyme activity to acute heating. Panels **A, C, E** represent specific (biomass-normalised)

activity, while panels **B, D, F** show enzyme activity normalised to that of citrate synthase (CS). Panels **A, B**) NADH:coenzyme Q oxidoreductase (NQO, mETC I); **C, D**) Succinate dehydrogenase (SDH, mETC II); **E, F**) Cytochrome *c* oxidase (CCO, mETC IV). Boxes represent means ± 1 s.e.m. and whiskers denote 1 s.d. of the mean (n = 5 per treatment group per day). Asterisks indicate significant differences between treatment groups on each day (LM-ANOVA, Tukey *post hoc* tests,*p < 0.05, ** p < 0.01, ***p < 0.001).

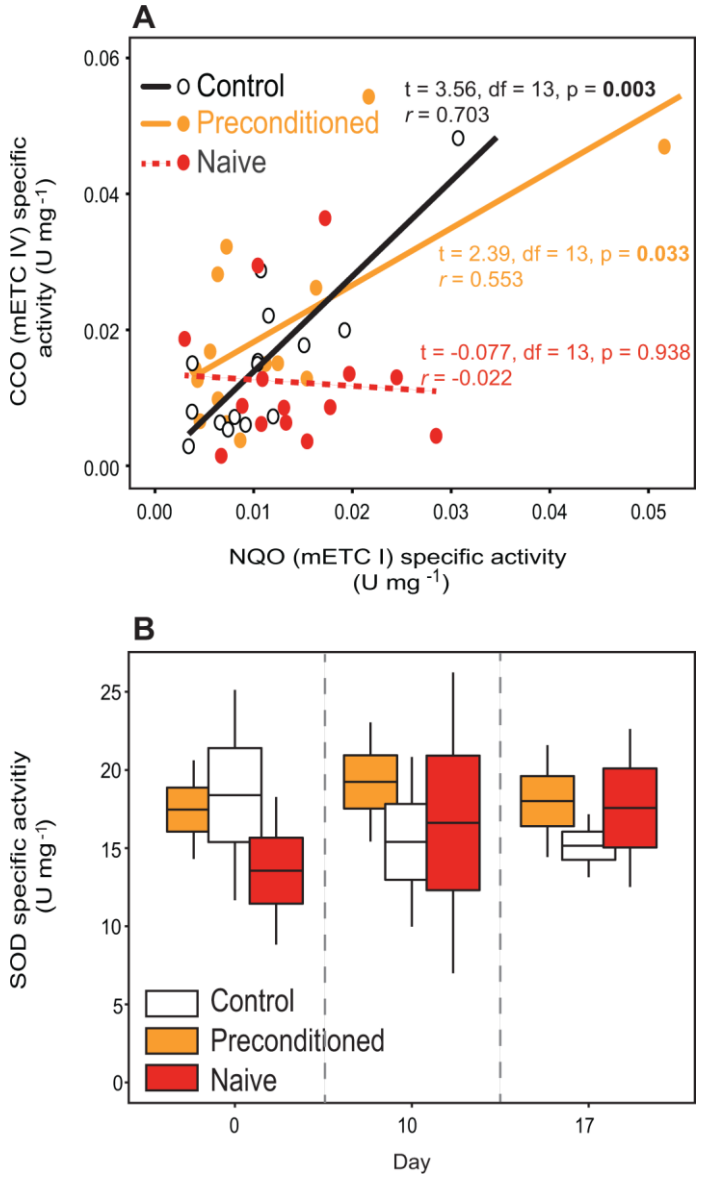


**Figure 6.** Panel **A**) Relationship between specific activities of host NADH:coenzyme Q oxidoreductase (NQO, mETC I) and cytochrome *c* oxidase (CCO, mETC IV) in warm-preconditioned and naïve *Exaiptasia pallida* heated to 33°C, and control animals kept at 26°C. Solid trend-lines indicate a significant relationship (Pearson's correlation analysis, $p < 0.05$), while dashed line denotes no relationship ($p > 0.05$). **B**) Specific activity of host superoxide

dismutase (SOD) in response to acute heating. Boxes represent means ± 1 s.e.m. and whiskers denote 1 s.d. of the mean (n = 5 per treatment group per day). No significant differences were noted between days or treatment groups (LM-ANOVA, $p > 0.2$)

**Tables**

**Table 1**. The mitochondrial respiratory enzymes quantified in this study.

| Component | Abbreviation | Function |
|---|---|---|
| Citrate Synthase[1] | CS | Catalyses first stage in the tricarboxylic acid (TCA) cycle, converting oxaloacetate + acetyl coenzyme A to citrate. |
| NADH:coenzyme Q oxidoreductase / complex I[2] | NQO / mETCI | Transfers electrons from NADH to coenzyme Q (CoQ), reducing CoQ pool. |
| Succinate dehydrogenase / complex II[2] | SDH / mETCII | Sixth stage in the TCA cycle. Converts succinate to fumarate and transfers electrons to CoQ, reducing CoQ pool. |
| Cytochrome *c* oxidase[2] | CCO / mETCIV | Transfers electrons from cytochrome *c* to oxygen, generating $H_2O$. |

1. Mitochondrial matrix
2. Inner mitochondrial membrane

**Table 2.** Mean values (± SD, n = 5) of dependent variables in the *Exaiptasia pallida* control group after a 62-day period in which a second batch of anemones was preconditioned to elevated temperature ("thermally preconditioned") and a third batch was kept under conditions identical to the controls ("thermally naïve"). Statistics reflect the outcome of two MANOVA analyses comparing the effect of treatment on a) CS-normalised mETC complex activities; and b) all other variables.† $Log_{10}$-transformed data. ‡Square root-transformed data. * Inverse square root-transformed data.

| **Source** | **Variable** | **Control group mean (SD)** | **F** (df = 2,12) | **P** |
|---|---|---|---|---|
| | $F_v/F_m$ | 0.512 (0.038) | 0.445 | 0.651 |
| | Gross photosynthesis ($P_{Gross}$; pmol $O_2$ $h^{-1}$ $cell^{-1}$) | 0.395 (0.084) | 4.788 | **0.030** |
| | Dark respiration ($R_D$; nmol $O_2$ $h^{-1}$ $mg^{-1}$) | 0.372 (0.109) | 11.287 | **0.002** |
| | P : R (holobiont)† | 1.586 (0.241) | 1.47 | 0.269 |
| | *Symbiodinium* cell density ($10^6$ cells $mg^{-1}$) | 1.858 (0.549) | 0.093 | 0.912 |
| Host fraction | CS specific activity (mU $mg^{-1}$) | 80.920 (12.100) | 17.924 | **<0.001** |
| | NQO specific activity (mU $mg^{-1}$)‡ | 7.392 (2.905) | 4.947 | **0.027** |
| | SDH specific activity (mU $mg^{-1}$) | 12.636 (3.876) | 2.719 | 0.106 |
| | CCO specific activity (mU $mg^{-1}$)‡ | 9.394 (7.527) | 5.817 | **0.017** |
| | NQO activity (% CS activity) | 9.1 (0.033) | 1.124 | 0.357 |
| | SDH activity (% CS activity) | 15.8 (0.047) | 3.975 | 0.057 |
| | CCO activity (% CS activity) | 11.3 (0.080) | 1.714 | 0.222 |
| | SOD specific activity (U $mg^{-1}$) | 18.392 (6.729) | 1.277 | 0.314 |
| | CS specific activity (mU $mg^{-1}$) | 59.336 (23.059) | 3.320 | 0.072 |

| | | | | |
|---|---|---|---|---|
| Symbiont lysate | SDH specific activity (mU $mg^{-1}$) | 2.508 (1.086) | 3.168 | 0.079 |
| | SDH activity (% CS activity)* | 4.9 (0.030) | 0.583 | 0.573 |

**Table 3.** Results of linear mixed-effects analysis of variance (LM-ANOVA) of photo-physiological and holobiont-level variables in thermally preconditioned and naïve *Exaiptasia pallida* exposed to acute heating over 17 days, alongside a group of *E. pallida* maintained at 26°C. Bold type indicates statistical significance ($\alpha < 0.05$). AIC values refer to italicised model terms. † $Log_{10}$-transformed data.

| **Variable** | **Effect** | **Model AIC** | **$F_{(df)}$** | **P** |
|---|---|---|---|---|
| $F_v/F_m$ | Day | -592.81 | 16.01 $_{(13,156)}$ | < **0.001** |
| | Treatment | | 22.61 $_{(2,12)}$ | < **0.001** |
| | *Day × Treatment* | | 7.82 $_{(26,156)}$ | < **0.001** |
| *Symbiodinium* gross photosynthesis (P; pmol $O_2$ h-1 $cell^{-1}$) † | Day | -37.56 | 7.21 $_{(2,23)}$ | **0.004** |
| | Treatment | | 6.73 $_{(2,12)}$ | **0.011** |
| | *Day × Treatment* | | 5.49 $_{(4,23)}$ | **0.003** |
| Dark respiration ($R_D$; nmol $O_2$ $h^{-1}$ $mg^{-1}$) † | Day | -24.41 | 1.10 $_{(2,23)}$ | 0.350 |
| | Treatment | | 6.35 $_{(2,12)}$ | **0.013** |
| | *Day × Treatment* | | 4.20 $_{(4,23)}$ | **0.011** |
| P : R (holobiont) † | Day | -31.10 | 43.31 $_{(2,23)}$ | < **0.001** |
| | Treatment | | 16.58 $_{(2,12)}$ | < **0.001** |
| | *Day × Treatment* | | 17.21 $_{(4,23)}$ | < **0.001** |
| *Symbiodinium* cell density (cells $mg^{-1}$) † | Day | -8.98 | 12.95 $_{(2,23)}$ | < **0.001** |
| | Treatment | | 14.43 $_{(2,12)}$ | < **0.001** |
| | *Day × Treatment* | | 6.96 $_{(4,23)}$ | < **0.001** |

**Table 4.** Results of linear mixed-effects analysis of variance (LM-ANOVA) of host and symbiont mitochondrial enzyme activities in thermally preconditioned and naïve *Exaiptasia pallida* exposed to acute heating over 17 days, alongside a group of *E. pallida* maintained at 26°C. Bold type indicates statistical significance ($\alpha < 0.05$). AIC values refer to italicised model terms. † Log10-transformed data. ‡ Square root-transformed data.

| Source | Variable | Effect | Model AIC | $F_{(df)}$ | P |
|---|---|---|---|---|---|
| *Host* | CS specific activity (mU $mg^{-1}$) † | Day | -72.94 | 3.96 $_{(2,23)}$ | **0.033** |
| | | Treatment | | 2.72 $_{(2,12)}$ | 0.106 |
| | | *Day × Treatment* | | 7.67 $_{(4,23)}$ | < **0.001** |
| | NQO specific activity (mU $mg^{-1}$) † | Day | -4.58 | 0.62 $_{(2,23)}$ | 0.550 |
| | | Treatment | | 1.80 $_{(2,12)}$ | 0.208 |
| | | *Day × Treatment* | | 12.02 $_{(4,23)}$ | < **0.001** |
| | NQO activity (% CS activity)† | Day | -2.09 | 0.02 $_{(2,23)}$ | 0.979 |
| | | Treatment | | 5.07 $_{(2,12)}$ | **0.025** |
| | | *Day × Treatment* | | 5.58 $_{(4,23)}$ | **0.003** |
| | SDH specific activity (mU $mg^{-1}$) † | Day | -28.65 | 2.21 $_{(2,23)}$ | 0.133 |
| | | Treatment | | 0.59 $_{(2,12)}$ | 0.57 |
| | | *Day × Treatment* | | 3.74 $_{(4,23)}$ | **0.017** |
| | SDH activity (% CS activity)† | *Null model* | -45.22 | - | - |
| | CCO specific activity (mU $mg^{-1}$) ‡ | Day | -143.43 | 1.81 $_{(2,23)}$ | 0.186 |
| | | Treatment | | 1.80 $_{(2,12)}$ | 0.207 |
| | | *Day × Treatment* | | 2.62 $_{(4,23)}$ | 0.061 |
| | CCO activity (% CS activity)† | *Null model* | 17.21 | - | - |
| *Symbiont* | CS specific activity (mU $mg^{-1}$) † | Day | -32.24 | 21.79 $_{(2,23)}$ | < **0.001** |
| | | Treatment | | 4.71 $_{(2,12)}$ | **0.031** |
| | | *Day × Treatment* | | 9.10 $_{(4,23)}$ | < **0.001** |
| | SDH specific activity (mU $mg^{-1}$) † | Day | -8.02 | 13.94 $_{(2,23)}$ | < **0.001** |

| | | | | | |
|---|---|---|---|---|---|
| | | Treatment | | $6.38_{(2,12)}$ | **0.013** |
| | | *Day* × *Treatment* | | $3.34_{(4,23)}$ | **0.027** |
| | SDH activity (% CS activity)† | Day | 4.66 | $20.47_{(2,23)}$ | **< 0.001** |
| | | Treatment | | $13.65_{(2,12)}$ | **< 0.001** |
| | | *Day* × *Treatment* | | $4.83_{(4,23)}$ | **0.006** |

# Key for Datafile "DataSubmission":

## Worksheet "Day0Raw"

Data are raw measurements obtained from anemones sampled prior to the warm-preconditioning period.

Bowl: Random factor. Replicate codes "accl 1-5" (acclimation [=preconditioning]), "ctrl 1-5" (control), "ctrl to ht 1-5" (control to high temp [=naïve]).

Treatment: Fixed factor. PC = Preconditioned, CT = Control, NV = Naïve.

P.R: Gross photosynthetic oxygen evolution / holobiont dark respiration (dimensionless)

SymDensity: *Symbiodinium* cell density per mg soluble host protein.

DR: Holobiont dark respiration (μmol $O_2$ per mg host protein per hour)

Pgross: *Symbiodinium* gross photosynthetic oxygen evolution (μmol $O_2$ per cell per hour)

HostCS: Host citrate synthase specific activity (Units per mg protein)

HostSDH: Host succinate dehydrogenase (mETCII) specific activity (Units per mg protein)

HostSDH/CS: Host succinate dehydrogenase (mETCII) specific activity relative to citrate synthase specific activity (dimensionless)

SymCS: *Symbiodinium* citrate synthase specific activity (Units per mg protein)

SymSDH: *Symbiodinium* succinate dehydrogenase (mETCII) specific activity (Units per mg protein)

SymSDH/CS: *Symbiodinium* succinate dehydrogenase (mETCII) specific activity relative to citrate synthase specific activity (dimensionless)

HostNQO: Host NADH:coenzyme Q oxidoreductase (mETCI) specific activity (Units per mg protein)

HostNQO/CS: Host NADH:coenzyme Q oxidoreductase (mETCI) specific activity relative to citrate synthase specific activity (dimensionless)

HostCCO: Host cytochrome *c* oxidase (mETCIV) specific activity (Units per mg protein)

HostCCO/CS: Host cytochrome *c* oxidase (mETCIV) specific activity relative to citrate synthase specific activity (dimensionless)

HostSOD: Host superoxide dismutase specific activity (Units per mg protein)

## Worksheet "PostPreconditioningRelative"

Data are relative to the mean of the control group, measured after the warm-preconditioning period. These data were used for graphing Fig. 1B, C, D.

Column headings are as for worksheet "Day0Raw", with the addition of:

FvFm: Maximum fluorescence yield of *Symbiodinium* photosystem II (dimensionless), measured in the dark.

## Worksheet "PostPreconditioningRaw"

Data are raw values measured after the warm-preconditioning period. These data were analyzed using multivariate analysis of variance (MANOVA).

Column headings are as for worksheet "PostPreconditioningRelative".

## Worksheet "HeatExpt"

Data are raw values measured before, during, and after the experimental exposure of anemones to acute heating.

Column headings are as for worksheet "PostPreconditioningRelative", with the addition of:

Day: Within-subjects factor (Day = 0, 10, 17)

SymSOD: *Symbiodinium* superoxide dismutase specific activity (Units per mg protein)

# R Scripts for statistical analysis

```
library(QuantPsyc)
library(boot)
library(car)
library(ggplot2)
library(devtools)
source_gist("524eade46135f6348140")
library(gdata)
library(phia)
library(nlme)
library(lme4)
library(lsmeans)
library(compute.es);
library(Hmisc);
library(multcomp);
library(pastecs);
library(reshape);
library(WRS)
library(outliers)
#Install packages before running library() commands


#1. Correlation analyses for Supplementary Figs. S1, S2.
data <- read.csv("DATAFILE.csv", header = TRUE) #Read datafile into memory, file contains data for all treatments & time-points
shapiro.test(data$VARIABLE) # Testing for normality
cor.test(data$VARIABLE1,data$VARIABLE2, use = "complete.obs", method = "pearson") #Pearson's correlation analysis, where <VARIABLE1> and <VARIABLE2> are the column-headings of interest.


#2. Multivariate analysis of variance for Figs. 2B, C, D.
data <- read.csv("PostPreconditioningRaw.csv", header = TRUE) #Read datafile into memory, file contains rawdata for post-preconditioning anemones ("PostPreconditioningRaw" worksheet)
```

leveneTest(VARIABLE ~ Treatment, data = data) # Homogeneity of variance test, repeat for each variable.

tapply(data$VARIABLE, data$VARIABLE, shapiro.test) # Testing for normality, repeat for each variable.

#Run the first MANOVA analysis - Raw data

multimod <- manova(cbind(log10(P.R), SymDensity, DR, Pgross, HostCS, sqrt(HostNQO), HostSDH, sqrt(HostCCO), SymCS, SymSDH, HostSOD) ~ Treatment, data = data)

summary(multimod, test = "Pillai")

summary.aov(multimod)

#Run the second MANOVA analysis - mETC complex activities normalised to CS activity.

multimod <- manova(cbind(HostSDH.CS, HostNQO.CS, HostCCO.CS, 1/sqrt(SymSDH.CS)) ~ Treatment, data = data)

summary(multimod, test = "Pillai")

summary.aov(multimod)

#3. Linear Mixed Model Analyses for data in Fig. 3, 4, 5, 6B

data <- read.csv("HeatExptFvFm.csv", header = TRUE) #Read datafile into memory, file contains raw Fv/Fm data for anemones during acute heating experiment ("HeatExptFvFm" worksheet)

data$Day <- as.factor(data$Day) #Set numeric "Day" column as factor

tapply(data$FvFm, data$Day:data$Treatment, shapiro.test) #Test for normality

leveneTest(FvFm ~ Day*Treatment, data = data) #Test for homogeneity of variance

baseline <- lme(FvFm ~ 1, random = ~1|Bowl/Day, data = data, method = "ML", na.action = na.exclude) #Create baseline model, random effect of replicate only, Day defined as within-subjects factor.

dayM <- update(baseline, .~. + Day)

treatmentM <- update(dayM, .~. + Treatment)

day_treatment <- update(treatmentM, .~. + Day:Treatment)

anova(baseline, dayM, treatmentM, day_treatment) #Test model fits

#Run exploratory analysis on model residuals. Plot histogram of residuals to confirm normal distribution.

plot(day_treatment)

plot(day_treatment, SOD1 ~ fitted(.) | Treatment, abline = c(0,1))

```
qqnorm(day_treatment,~resid(.)|Day)

qqnorm(day_treatment,~resid(.)|Treatment)

hist((resid(day_treatment) - mean(resid(day_treatment), na.rm=T)) / sd(resid(day_treatment), na.rm=T), freq=F); curve(dnorm, add = TRUE)

anova(day_treatment) #Get ANOVA table for best-fitting model (lowest AIC value), assuming residuals are normally distributed.

summary(glht(day_treatment, lsm(pairwise ~ Treatment|Day, adjust="tukey"))) #Post hoc pairwise comparisons.

data <- read.csv("HeatExpt.csv", header = TRUE) #Read datafile into memory, file contains raw data for anemones during acute heating experiment ("HeatExptFvFm" worksheet)

#Repeat the Linear Mixed Model analyses for each variable of interest.


#4. Multiple regression analyses testing the effect of treatment on the relationship between host NQO and host CCO (Fig. 6A)

data <- read.csv("HeatExpt.csv", header = TRUE) #Read datafile into memory, file contains raw data for anemones during acute heating experiment ("HeatExpt" worksheet)

        #Subset treatment groups data

preconditioned <- subset(data, Treatment == "PC") #Select data for preconditioned anemones

control <- subset(data, Treatment == "CT") #Select data for control anemones

naive <- subset(data, Treatment == "NV") #Select data for naive anemones

        #Pearson's correlation analyses for Host NQO / Host CCO activity for each treatment group.

cor.test(preconditioned$HostNQO, preconditioned$HostCCO, use = "complete.obs", method = "pearson")

        #Linear regression analyses of HostNQO and Treatment as predictors of Host CCO

regression <- lm(HostCCO ~ HostNQO + Treatment + HostNQO*Treatment, data = data, na.action = na.exclude)

anova(regression)
```

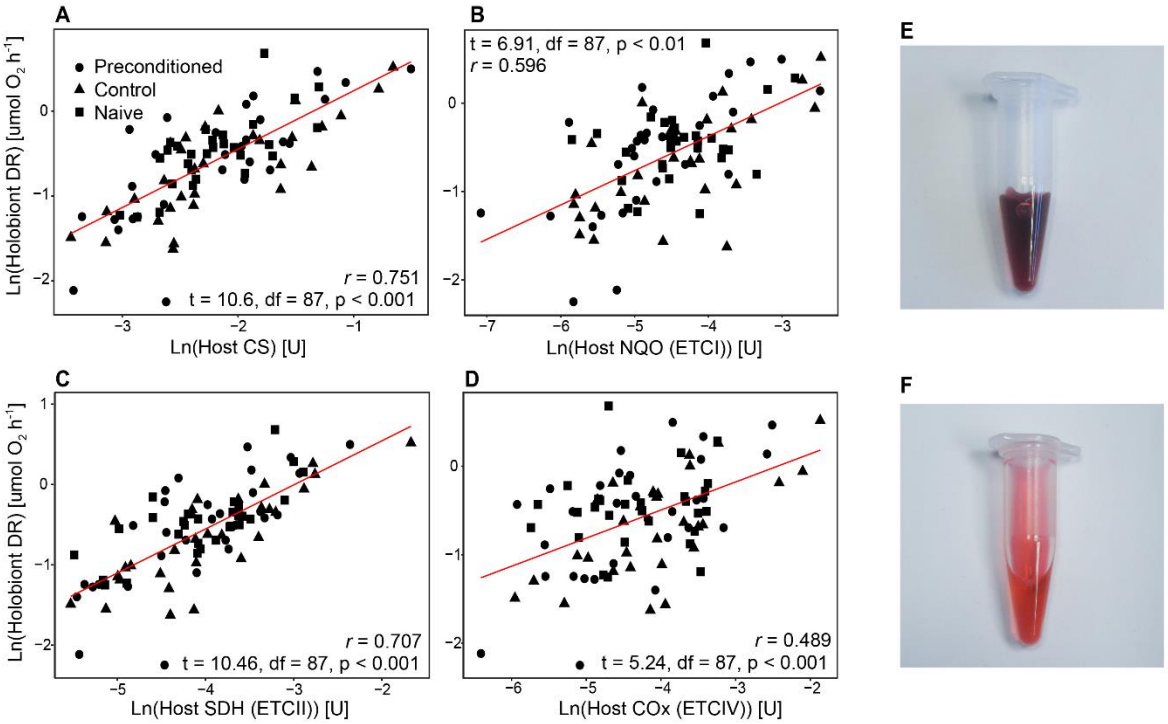


**Fig. S1.** Pearson's correlation analysis of the relationship between holobiont dark respiration (DR) and host (**A**) citrate synthase (CS) activity, (**B**) NADH:coenzyme Q oxidoreductase (NQO) activity, (**C**) succinate dehydrogenase (SDH) activity, and (**D**) cytochrome *c* oxidase (COx) activity in *Exaiptasia pallida* anemones. All data are natural log-transformed. Panel **E**) Oxidised (brown) and (**F**) reduced (pink) cytochrome *c* (1 mM in 20 mM KPi buffer, pH 7.0).

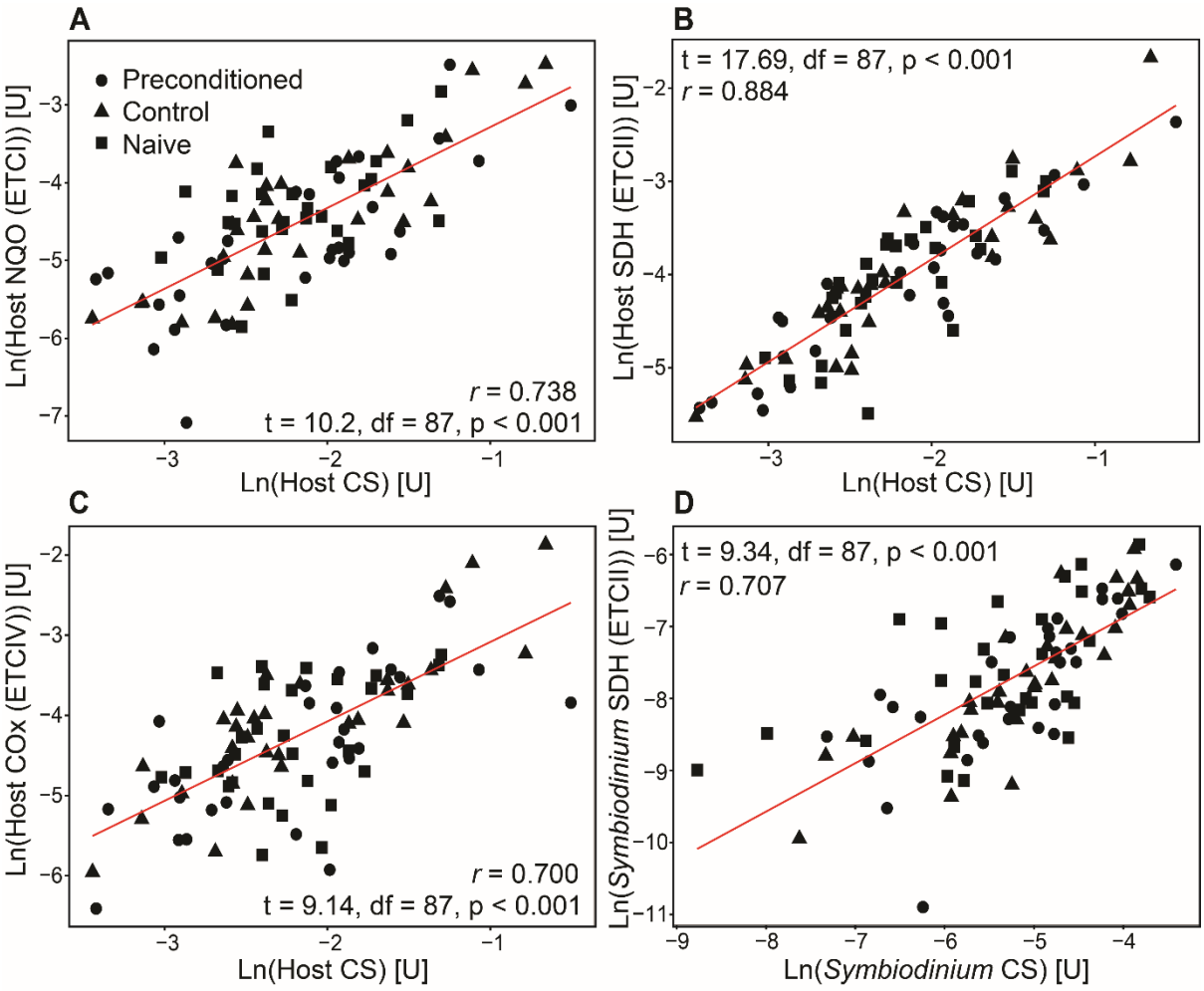


**Fig. S2.** Pearson's correlation analysis of the relationship between host citrate synthase (CS) activity and (**A**) NADH:coenzyme Q oxidoreductase (NQO) activity, (**B**) succinate dehydrogenase (SDH) activity, and (**C**) cytochrome *c* oxidase (COx) activity. Panel **D**) Relationship between *Symbiodinium* CS and SDH activities. All data are natural log-transformed.

**Data Submission**

Click here to Download Data Submission