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RESEARCH ARTICLE

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

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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 whether 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 naive' animals to 33°C induced dramatic increases in host mETC complex I and *Symbiodinium* mETC complex II activities only in thermally naive *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 naive 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.

KEY WORDS: 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, 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, 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., 2015), 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; Bellantuono et al., 2012a,b; 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 Pörtner, 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 density and aerobic capacity is a common adaptive response to cold conditions (polychaetes: Sommer and Pörtner, 1999; polar marine invertebrates: Pörtner, 2001; Peck, 2002; Pörtner et al., 2007), while warm acclimation often correlates with decreased mitochondrial density/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 and Helmuth (2005) noted an effect of thermal history on respiration in *Montastraea*

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List of abbreviations

AOX	alternative oxidase
CCO	cytochrome *c* oxidase
CoQ	Coenzyme Q
CS	citrate synthase
Cyt *c*	cytochrome *c*
FSW	fresh seawater
F_v/F_m	maximum quantum yield of *Symbiodinium* photosystem II
IMM	inner mitochondrial membrane
mETC	mitochondrial electron transport chain
NQO	NADH:coenzyme Q oxidoreductase
O_2^-	superoxide
P:*R*	gross photosynthesis to respiration ratio
P_{gross}	gross photosynthesis
R_D	dark respiration
ROS	reactive oxygen species
SOD	superoxide dismutase
TCA	tricarboxylic acid

(=*Orbicella*) *annularis* corals undergoing a subsequent thermal challenge. Furthermore, Bay and Palumbi (2015) and Dixon et al. (2015) observed increased heat tolerance correlated 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 and 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), the 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,

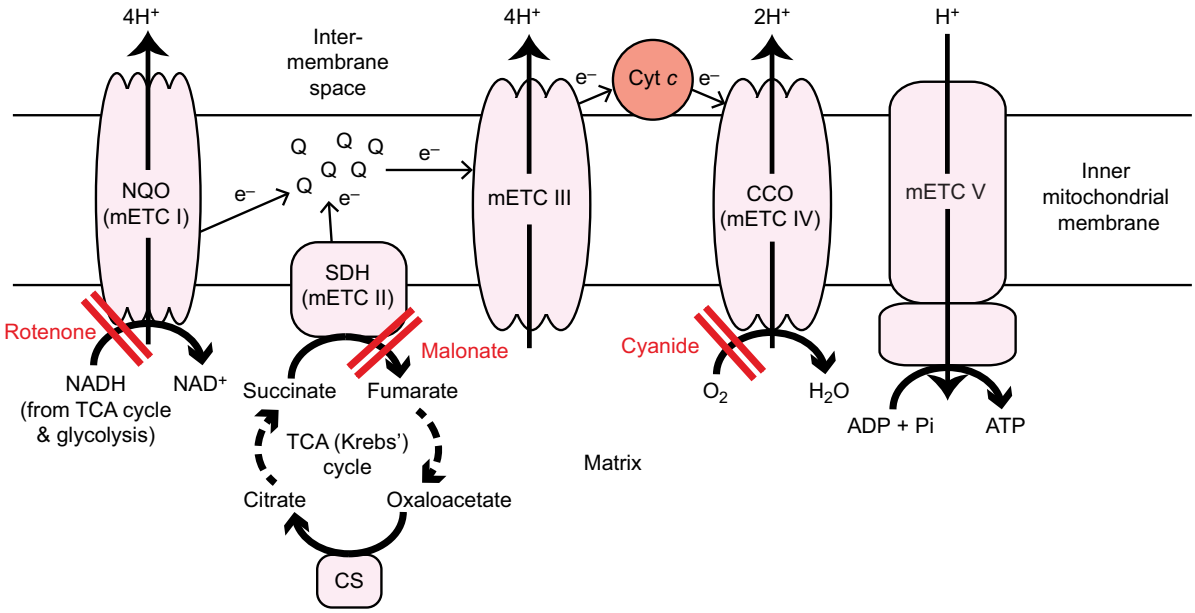

Fig. 1. Simplified conceptual model of the mitochondrial electron transport chain (mETC). Carbon-rich glycolysis-derived organic substrates are oxidised through the tricarboxylic acid (TCA) cycle (omitted steps indicated by dashed lines), transferring electrons (e^-) to NAD^+ and FAD. NADH is oxidised 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 of complexes I, III and IV drives ATP 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.

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Table 1. The mitochondrial respiratory enzymes quantified in this study

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

TCA, tricarboxylic acid; CoQ, coenzyme Q.
*Mitochondrial matrix. ‡Inner mitochondrial membrane.

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 concentration (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. First, we hypothesised that – as for many marine ectotherms undergoing warm acclimation (Sommer and Pörtner, 1999, 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 (CS; from porcine heart), cytochrome *c* (Cyt *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 Table S1.

Warm preconditioning and acute heating of *E. pallida*

Specimens of *Exaiptasia pallida* (Agassiz in Verrill 1864), naturally symbiotic with ITS2-type A4 *Symbiodinium* (Hawkins et al., 2016b), were collected from Key Largo, FL, USA (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 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 disc 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 2 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±s.d. 29.8±0.64°C; Fig. 2A). This temperature is slightly below the annual maximum experienced by these anemones in their natural habitat (mean annual temperature range ~17–31°C; https://www.nodc.noaa.gov/dsdt/cwtg/all_meanT.html). The other two groups ('thermally naive' and 'control') remained at ~26°C (mean±s.d. 26.2±0.2 and 26.2±0.4°C, respectively; Fig. 2A). These conditions were maintained for 7 weeks before anemones were randomly sampled from each bowl (see below). Because of a malfunction in the heating apparatus approximately 4 weeks into the preconditioning period, the temperature of the preconditioned treatment briefly (<2 h) exceeded 33°C (Fig. 2A). At least another 4 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 naive anemones were heated by ~0.9°C day^{-1} – closer to the maximum heating rate associated with bleaching events in the field (Middlebrook et al., 2010). Heating was staggered such that the two groups reached maximum temperature (mean±s.d. 33.0±0.3°C) 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 (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 Fig. 2A, always at least 4 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 dark respiration (R_D) from net photosynthesis. The gross photosynthesis to 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-capped vial, snap-frozen in liquid nitrogen and stored at −80°C.

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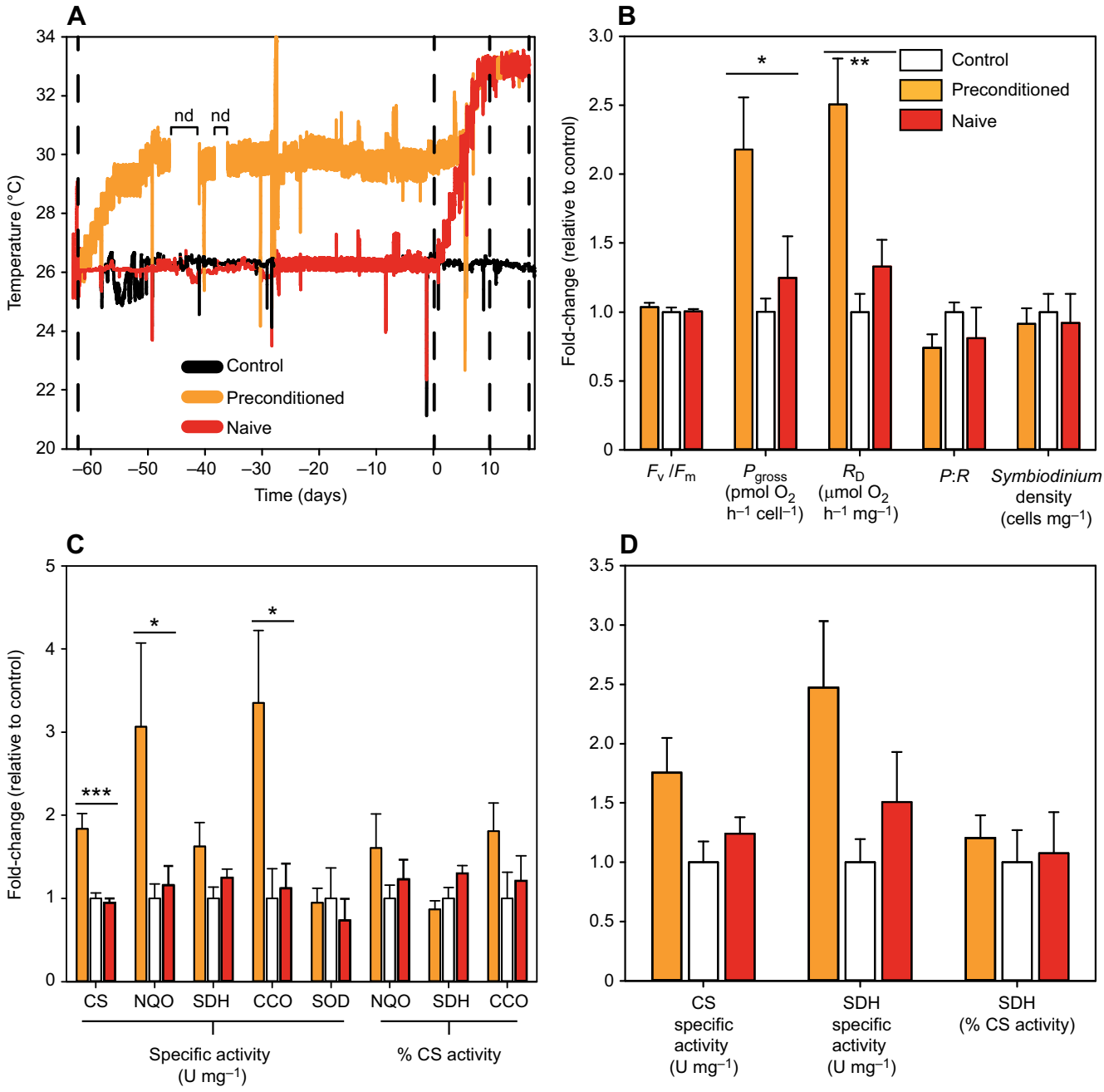

Fig. 2. Physiological response of *E. pallida* and *Symbiodinium* following thermal preconditioning. (A) Temperature profiles used for control (26°C), preconditioned (26°C to 30°C then to 33°C) and thermally naive (26°C to 33°C) treatments. The dashed line on day 0 indicates the start of acute heating and when samples in B–D were collected, while other dashed lines indicate additional sampling days, and 'nd' refers to periods where temperature data are not available because of a malfunction of the temperature logging equipment. (B) Maximum quantum yield of *Symbiodinium* photosystem II (F_v/F_m), gross photosynthesis per symbiont cell (P_{gross}), holobiont dark respiration (R_D), holobiont P_{gross} to R_D ratio (*P*:*R*), and *Symbiodinium* cell density. (C) Specific activities of host citrate synthase (CS), NQO, SDH, CCO and superoxide dismutase (SOD) enzymes alongside CS-normalised NQO, SDH and CCO activities. (D) Variables as for C, but measured from *Symbiodinium* lysate. Values in 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).

Sample processing and determination of *Symbiodinium* cell density

Anemones were thawed on ice and 0.8–1.4 ml lysis buffer [50 mmol l^{-1} potassium phosphate (KH_2PO_4), pH 7.8, 1 mmol l^{-1} 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

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 of 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 mmol l^{-1} KH_2PO_4, pH 7.5, 0.3 mmol l^{-1} KCN, 3 mg ml^{-1} BSA, 60 µmol l^{-1} Ub_1, 0.2 mmol l^{-1} NADH and 1% (w/v) ethanol. After mixing by inversion, NADH oxidation was monitored as the change in absorbance at 340 nm (ΔA_{340}) for 3 min. Rotenone-sensitive NADH oxidation (NQO specific activity) was determined by repeating the procedure with 10 µmol l^{-1} rotenone (1 mmol l^{-1} stock solution in ethanol). Reaction rates were determined over the 30–120 s after mixing, and the rotenone-sensitive activity ($\Delta A_{340-rotenone}$–$\Delta A_{340+rotenone}$) was calculated using $\varepsilon_{NADH,340nm}$=6.2 mmol l^{-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 mmol l^{-1} KH_2PO_4 pH 7.5, 0.3 mmol l^{-1} KCN, 1 mg ml^{-1} BSA, 75 µmol l^{-1} DCPIP, 20 mmol l^{-1} succinate and 50 µmol l^{-1} DUB. Samples were incubated with succinate for 10 min prior to adding DUB, and baseline ΔA_{600} was measured over the final 1 min. ΔA_{600} 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' ΔA_{600} rates were subtracted and SDH activity was calculated using $\varepsilon_{DCPIP,600nm}$=19.1 mmol l^{-1} cm^{-1}.

CCO activity was determined in a 50 µl host fraction (~45 µg protein), with final reaction conditions of 25 mmol l^{-1} KH_2PO_4, pH 7.0 and 50 µmol l^{-1} reduced Cyt *c* in 500 µl. Cyt *c* oxidation was then determined by recording the decrease in A_{550} for 2 min before and after the addition of host fraction. CCO activity was calculated using $\varepsilon_{Cyt\,c,550nm}$=18.5 mmol l^{-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^{-1}) and specificity of the SDH and CCO assays was confirmed by using the specific inhibitors malonate (10 mmol l^{-1}) and KCN (300 µmol l^{-1}), 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* SOD enzyme activity

Total SOD activities of host fraction and *Symbiodinium* lysate were quantified using a xanthine/xanthine oxidase–nitroblue tetrazolium (NBT) assay in a 96-well plate format. Briefly, 30 µl host fraction or *Symbiodinium* lysate was added to 210 µl reaction buffer [prepared such that reagent concentrations after addition of 10 µl xanthine oxidase solution were 50 mmol l^{-1} KH_2PO_4, pH 7.8, 0.1 mmol l^{-1} EDTA, 0.1% (w/v) BSA, 0.025% (v/v) Triton X-100, 0.14 mmol l^{-1} NBT, 0.1 µmol l^{-1} xanthine]. Blank reactions (n=18 wells) were prepared with 30 µl lysis buffer in place of experimental samples. Plates were incubated for 5 min 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 (λ=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 500–0.05 U SOD enzyme (from bovine erythrocytes) per reaction.

Statistical analysis

Respiratory enzyme/complex activities were normalised to the soluble protein content of host fractions and *Symbiodinium* lysates. 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 (Figs S1, S2; R v. 3.2.2, http://www.R-project.org). 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', https://CRAN.R-project.org/package=nlme). 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) were 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' (https://CRAN.R-project.org/package=multcomp). Additionally, the influence of treatment on relationships between host NQO and CCO specific activities was investigated using multiple regression

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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 because of limited protein yields. These data were not analysed with LM-ANOVA. All R scripts used in this study are provided in Script S1.

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 its symbiont (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* P_{gross} cell^{-1} (Table 2, Fig. 2B). Preconditioning had no effect on F_v/F_m, *Symbiodinium* cell density or holobiont *P*:*R* (Table 2, Fig. 2B). Host CS activity (mg^{-1} protein) was almost 2-fold higher in preconditioned anemones than in either the control or thermally naive 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).

Table 2. Effects of prolonged thermal preconditioning in *Exaiptasia pallida* and its *Symbiodinium*

Variable	Control group (mean±s.d.)	$F_{2,12}$	P
F_v/F_m	0.512±0.038	0.445	0.651
P_{gross} (pmol O_2 h^{-1} cell^{-1})	0.395±0.084	4.788	**0.030**
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
Symbiont lysate			
CS specific activity (mU mg^{-1})	59.336±23.059	3.320	0.072
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

F_v/F_m, maximum quantum yield of *Symbiodinium* photosystem II; P_{gross}, gross photosynthesis; R_D, dark respiration; *P*:*R*, gross photosynthesis/respiration ratio.

Data are the means of dependent variables in the *E. 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 naive). Statistics reflect the outcome of two MANOVA analyses comparing the effect of treatment on (i) CS-normalised mETC complex activities; and (ii) all other variables.§$\log_{10}$-transformed data; ‡square root-transformed data; *inverse square root-transformed data; n=5.

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

Symbiodinium F_v/F_m declined significantly in the thermally naive 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 6 days at 33°C (day 16), F_v/F_m in preconditioned and thermally naive anemones was ∼50% lower than that of the control group. Holobiont *P*:*R* (Fig. 3B) and *Symbiodinium* cell density (Fig. 3C) declined within thermally naive *E. pallida* after initial heating (day 10) but did not change significantly in preconditioned anemones. However, after a further 7 days, *Symbiodinium* density and *P*:*R* in preconditioned animals were intermediate between those of the thermally naive 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 naive 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 naive 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 that of the control group (Fig. 4A). CS activity in the symbionts of thermally naive 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 naive 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 naive *E. pallida* than in the preconditioned or control groups (Fig. 4C). An increasing trend was noted for *Symbiodinium* SOD activity in thermally naive 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 naive 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].

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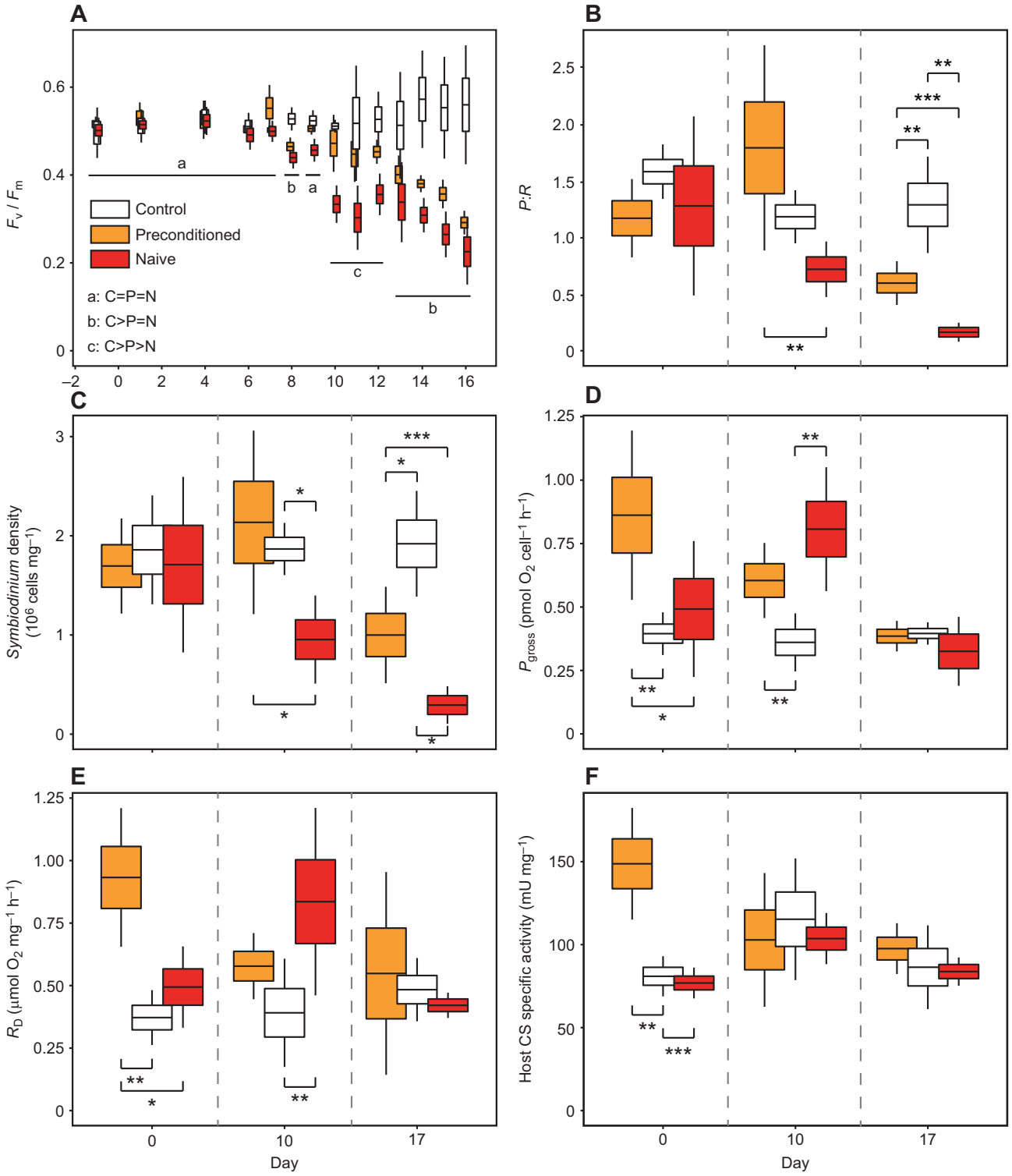

Fig. 3. Responses of *E. pallida* to acute heating. (A) Maximum quantum yield of *Symbiodinium* photosystem II (F_v/F_m). Asterisks indicate statistically significant differences between the three treatment groups: control (C), preconditioned (P) and naive (N). (B) Holobiont *P*:*R* ratio. (C) *Symbiodinium* cell density (per mg host protein). (D) P_{gross} per *Symbiodinium* cell. (E) Holobiont R_D. (F) Host 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 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).

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 naive 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).

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Table 3. Effects of acute heating on *E. pallida* and its *Symbiodinium*

Variable	Effect	Model AIC	$F_{d.f.}$	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 P_{gross} (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**
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**

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 R_D and *Symbiodinium* 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 and 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 Pörtner, 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 density or size, 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 is often associated 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, as nutritional input directly affects 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).

As 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), probably 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 naive anemones under the same conditions. Given that *Symbiodinium* densities and CS activities were declining in thermally naive animals at this time, their increased R_D (day 10) was probably 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 naive *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 release 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).

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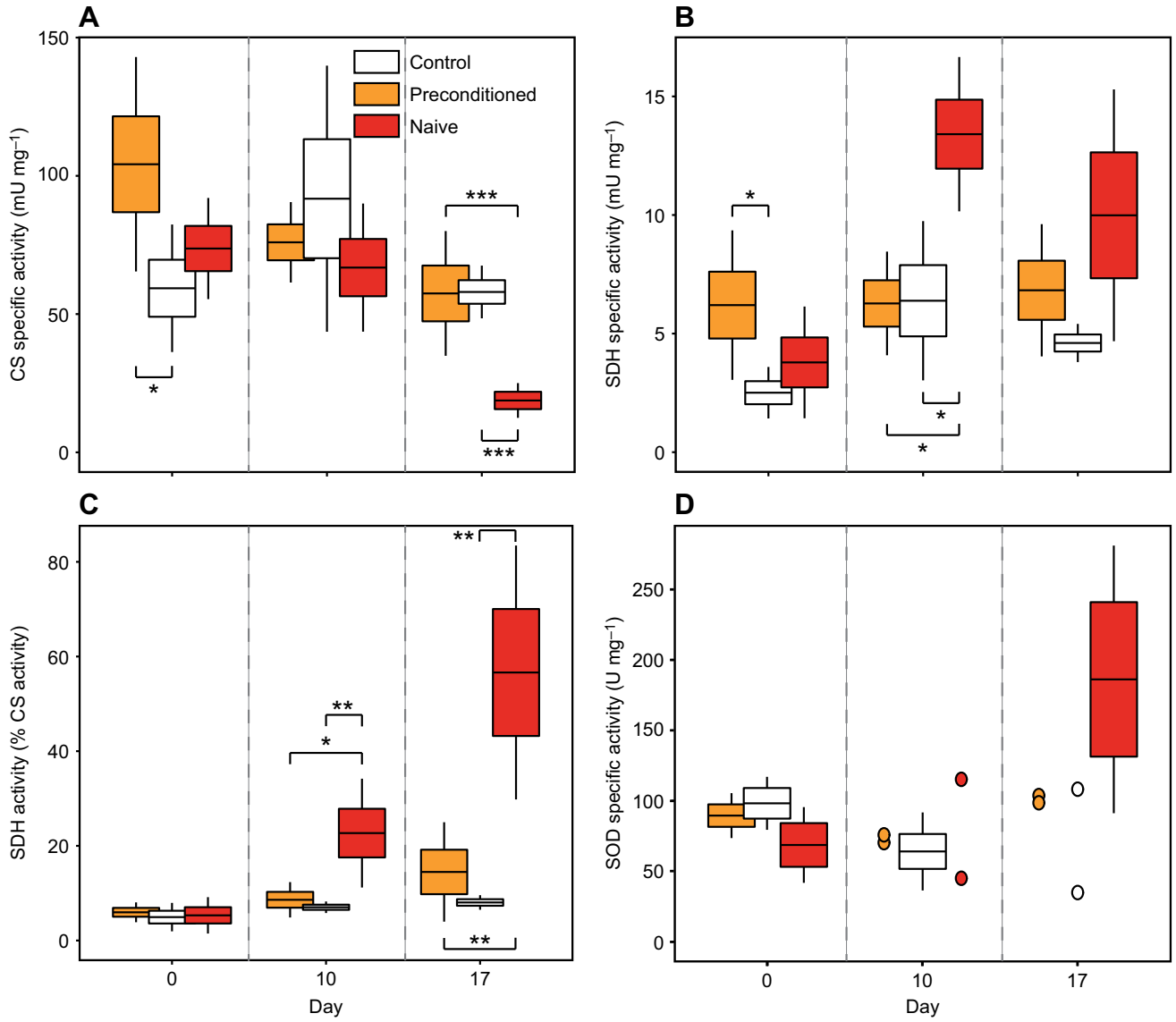

Fig. 4. Responses of *in hospite Symbiodinium* mitochondrial enzyme and SOD activity to acute heating. (A) CS specific activity. (B) SDH specific activity. (C) CS-normalised SDH activity. (D) SOD specific activity. Boxes represent means±1 s.e.m. and whiskers denote 1 s.d. of the mean (n=5 per treatment group per day). Asterisks in 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 D represent raw data where n<3 for treatment groups on respective days; LM-ANOVA could therefore not be conducted on the SOD data.

Heating-induced changes in symbiont P_{gross} and holobiont R_D of thermally naive 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* density – and no compensatory increase in *Symbiodinium* P_{gross} cell^{-1} – would have depressed host tissue oxygen tensions in thermally naive *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 naive anemones undergoing bleaching. For example, CS-normalised SDH activity in the *Symbiodinium* 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 – as 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 because of the low sample sizes, it is suggestive of growing oxidative challenge in *Symbiodinium* of thermally naive *E. pallida* during prolonged heat stress.

In the host mitochondria of thermally naive *E. pallida* undergoing bleaching, an increase in NQO activity unmatched by changes in CS, SDH or CCO activities indicates that the equilibrium

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Table 4. Effects of acute heating on mitochondrial enzyme activity in *E. pallida* and its *Symbiodinium*

Variable	Effect	Model AIC	$F_{d.f.}$	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**

Results of LM-ANOVA of host and symbiont mitochondrial enzyme activities in thermally preconditioned and naive *E. pallida* exposed to acute heating over 17 days, alongside a control group of *E. pallida* maintained at 26°C. Bold type indicates statistical significance (α<0.05). AIC values refer to italicised model terms. *$\log_{10}$-transformed data; ‡square root-transformed data.

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 probably not increased by NAD^+ reduction via the TCA cycle, as 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 naive anemones would probably 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 probably 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 probably come at the cost of

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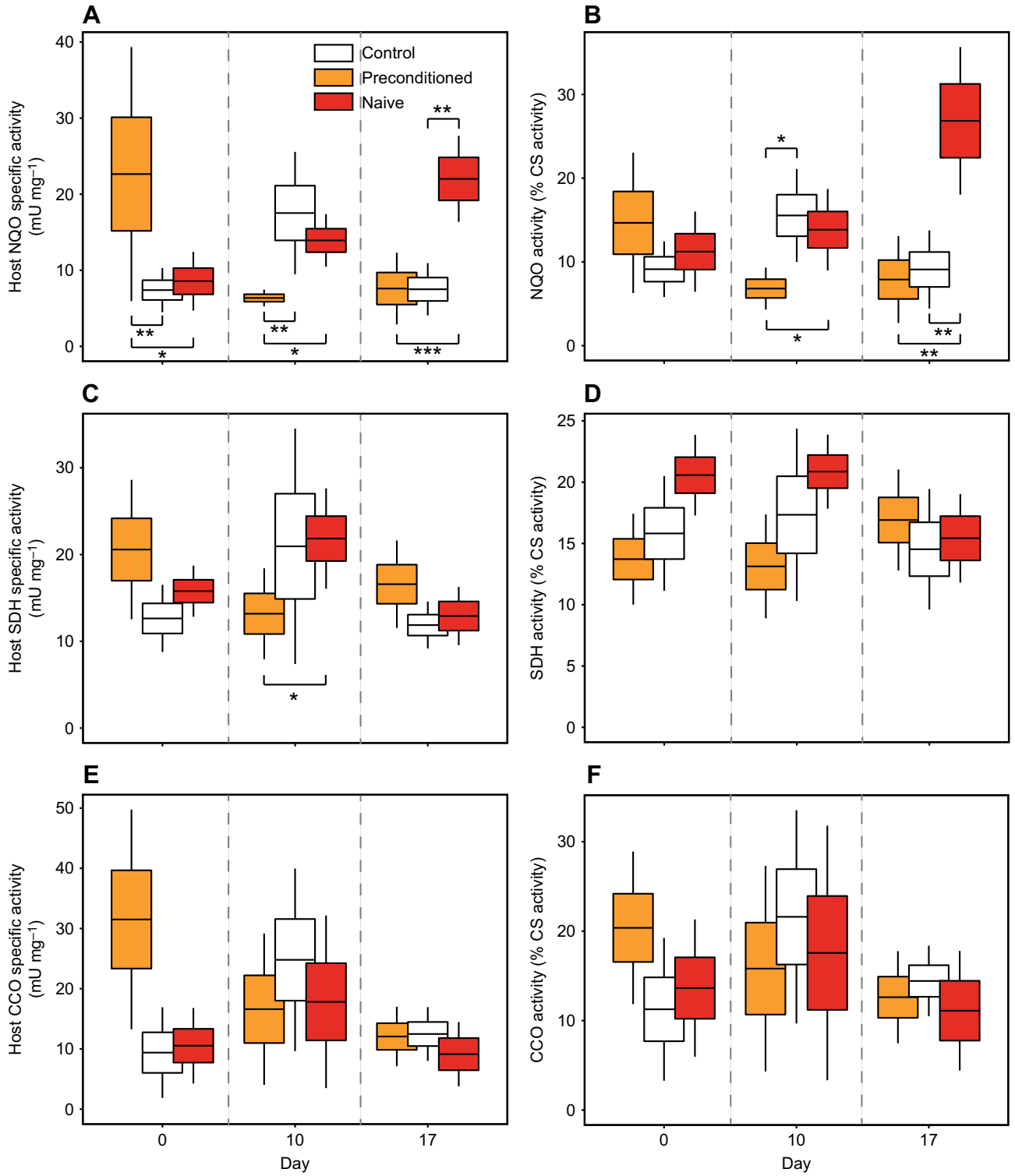

Fig. 5. Responses of *E. pallida* host mETC enzyme activity to acute heating. (A,C,E) Specific (biomass-normalised) activity. (B,D,F) Enzyme activity normalised to that of CS. (A,B) NQO (mETC I). (C,D) SDH (mETC II). (E,F) CCO (mETC IV). Boxes represent means±1 s.e.m. and whiskers denote 1 s.d. of the 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).

reduced ATP synthesis, as 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

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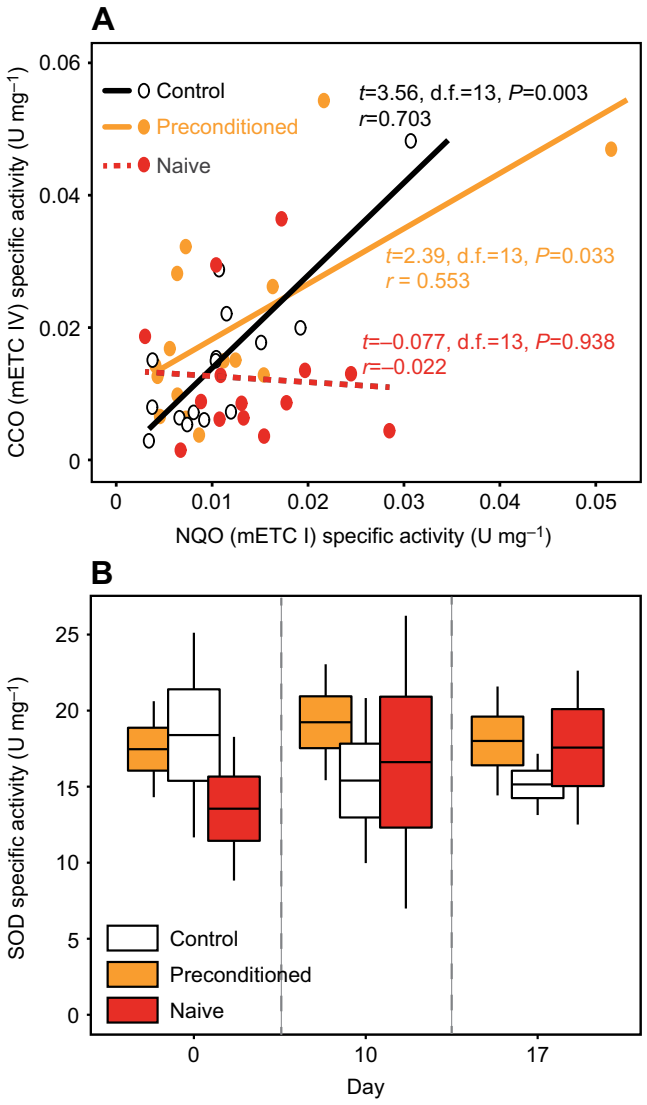

Fig. 6. The relationship between host mitochondrial complex I and IV and SOD with thermal treatment. (A) Specific activities of host NQO (mETC I) and CCO (mETC IV) in warm-preconditioned and naive *E. pallida* heated to 33°C, and control animals kept at 26°C. Solid trend lines indicate a significant relationship (Pearson's correlation analysis, $P<0.05$), while the dashed line denotes no relationship ($P>0.05$). (B) Specific activity of host 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, $P>0.2$).

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 the respiratory apparatus, a heightened aerobic capacity may have allowed preconditioned *E. pallida* to avoid heating-induced disequilibrium in mETC function experienced by thermally naive 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; Bellantuono et al., 2012a,b), 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), respectively, is needed to confirm or refute the hypothesised changes in aerobic metabolic pathways and mitochondrial densities. 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).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
T.D.H. conceived the study questions and experimental design. T.D.H. carried out the experimental work with assistance from M.E.W. T.D.H. conducted all biochemical assays and analysed the data. T.D.H. wrote the manuscript with guidance from M.E.W.

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