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Metabolomic signatures of corals thriving across extreme reef habitats reveal strategies of heat stress tolerance

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Anthropogenic stressors continue to escalate worldwide, driving unprecedented declines in reef environmental conditions and coral health. One approach to better understand how corals can function in the future is to examine coral populations that thrive within present day naturally extreme habitats. We applied untargeted metabolomics (gas chromatography–mass spectrometry (GC–MS)) to contrast metabolite profiles of *Pocillopora acuta* colonies from hot, acidic and deoxygenated mangrove environments versus those from adjacent reefs. Under ambient temperatures, *P. acuta* predominantly associated with endosymbionts of the genera *Cladocopium* (reef) or *Durusdinium* (mangrove), exhibiting elevated metabolism in mangrove through energy-generating and biosynthesis pathways compared to reef populations. Under transient heat stress, *P. acuta* endosymbiont associations were unchanged. Reef corals bleached and exhibited extensive shifts in symbiont metabolic profiles (whereas host metabolite profiles were unchanged). By contrast, mangrove populations did not bleach and solely the host metabolite profiles were altered, including cellular responses in inter-partner signalling, antioxidant capacity and energy storage. Thus mangrove *P. acuta* populations resist periodically high-temperature exposure via association with thermally tolerant endosymbionts coupled with host metabolic plasticity. Our findings highlight specific metabolites that may be biomarkers of heat tolerance, providing novel insight into adaptive coral resilience to elevated temperatures.

1. Background

Warming sea surface temperatures (SSTs) are expected to accentuate the current decline of coral reefs worldwide [1–4]. Coral populations that can cope with, or recover from, increasingly frequent and intense stress events are critical to the persistence of tropical reefs, and recent efforts to predict the future structure and functioning of reefs have logically focussed on resolving the nature of coral tolerance to acute heating events [5–10]. One approach has been to examine coral populations that have adapted to present day extreme environments; for example, warm lagoons [11,12], intertidal pools [13,14] and thermally extreme seas [15,16]. Profiling coral biology from these so-called 'natural laboratories' has shown how numerous factors contribute to high heat tolerance, including rapid modifications in gene expression related to

extracellular matrix formation and oxidative stress [17,18], heat-shock protein upregulation [19], but also rapid recovery to a pre-disturbance baseline level of these differentially expressed genes [20]. Other mechanisms include associations with heat-tolerant algal symbionts [5,15,21,22], and specific bacterial communities that may mitigate bleaching susceptibility [23].

Corals thriving in mangrove lagoons adjacent to tropical reefs are characterized by particularly extreme environmental conditions. Waters within reef-neighbouring mangrove lagoon habitats can reach 33°C (Woody Isles, Great Barrier Reef) in summer [24], which is well above the tolerance threshold for many reef corals [3], but also experience extensive diel acidification (pH range 7.2–8.2) and deoxygenation (oxygen range 0.6–7.33 mg l^{-1}) [11,25]. Despite these relatively extreme lagoonal conditions, coral populations can thrive through associations with different microbial communities, including both micro-algal endosymbionts (family: Symbiodiniaceae) and bacteria [24,26,27], and exhibit very different metabolic properties compared to the reef corals [12,25,27]. For example, we recently demonstrated that populations of the coral *Pocillopora acuta*—which can be both heat-sensitive [28,29] and heat-tolerant [30,31]—are prevalent across mangrove lagoons on the Great Barrier Reef, but associate with more thermally tolerant Symbiodiniaceae and more taxonomically diverse bacterial communities [26]. However, as a trade-off for survival, coral-associated Symbiodiniaceae in mangrove environments provide less nutrition to their hosts [27], which may be supplemented through increased heterotrophy [32]. Changes in both symbiont associations and shifts in emergent metabolic capabilities suggest that inherent metabolic coupling between host and symbiont most likely re-adjusts the compatibility of the holobiont to different environmental optima [33]. However, we still do not know how these corals shift their metabolism to survive in these extreme environments. Resolving this gap in knowledge could identify important—yet unresolved—pathways that prevent the breakdown of symbiosis under stress exposure.

Maintaining functional symbioses is essential for reef-building corals to meet the energetic and metabolic demands required for growth, respiration and calcification [34–36]. Elevated temperatures cause a disruption in coral–Symbiodiniaceae symbiosis and in turn, coral central metabolism [37,38], which has major implications for host energy budgets and biosynthesis pathways such as lipogenesis [39], and homeostatic responses [40–42]. However, not all corals appear to exhibit the same metabolic response to thermal stress. Recent results revealed that metabolite profiles of thermally stressed corals differ among conspecific coral taxa [43,44], which may reflect different levels of holobiont tolerance to stress. Ultimately, corals persisting within thermally extreme mangrove lagoons can provide important insights into the underlying mechanisms of holobiont tolerance to thermal stress, but the upper thermal limits of these coral populations have not been characterized yet. We therefore used gas chromatography–mass spectrometry (GC–MS)-based untargeted metabolomics to contrast the metabolite profiles produced by colonies of *P. acuta* in both a mangrove lagoon and a neighbouring reef habitat when exposed to both ambient and transient heat stress conditions. Pools of metabolites from both symbiont and host fractions were examined to identify the metabolic strategies used by mangrove corals to retain stable symbioses.

2. Material and methods

(a) Coral collection

Sampling locations for coral collection were chosen based on our previous characterization of these sites. Briefly, Woody Isles mangrove lagoon is subjected to constant diurnal changes in temperature (up to 7.7°C), pH (up to 1.3), O_2 (up to 7.33 mg l^{-1}) and salinity (up to 15.5), while physiochemical conditions at Low Isles reef remain relatively constant in comparison [25]. Temperature, pH, oxygen and salinity were previously monitored over a 9-month period up until the day of collection (see [26]) for this current study.

Colonies (smaller than 10 cm) of the coral *P. acuta* were collected in February 2019 from Woody Isles mangrove lagoon and Low Isles reef ($n = 5$ colonies per habitat) (as described in [26]) and were then immediately transported in plastic containers filled with native reef water, and installed with small pumps for continued aeration, to James Cook University (Cairns). This coral species was selected based on its abundance at both sampling sites and because of the large amount of physiological and microbiological data available [24–26]. Within 2 h of arrival, colonies were fragmented (6 per colony) and mounted onto egg crates in flow-through outdoor aquaria with locally sourced seawater. Coral colonies were sampled during peak summer temperatures, which reached a maximum of 35.3°C for the mangrove environment and 31.2°C for the adjacent reef environment (electronic supplementary material, figure S1), to ensure acclimatization and to avoid a heat shock response under thermal treatments. Small fragments (approx. 3 cm) were removed from each colony and snap frozen to verify species identification as *P. acuta* (as per [26]).

(b) Tank setup/experimental design

Coral fragments were distributed across 8 experimental aquaria (30 L) and acclimated for 10 days under shaded conditions (maximum midday photosynthetic active radiation (PAR) approximately 650 µmol photons m^{-2} s^{-1}) at an ambient temperature of approximately 28°C. Continuous flow through seawater (1 L min^{-1}) was provided from a 33 048 L holding tank, with additional water motion provided by submersible pumps in each aquarium. Circulating seawater was filtered using biological and mechanical filtration, including live rock, protein skimmers, algae scrubbers and a 200 µm filtration sock. Following 10 days of acclimatization, coral tanks containing mixed fragments of reef and mangrove corals were assigned to either control or heat stress conditions ($n = 4$ replicate tanks per control and heat stress treatment), whereby half of each colony was distributed to each treatment (figure 1). Control tanks were sustained at an average temperature of 28.7 ± 0.03°C (mean ± s.e.), while temperature in heat stress tanks was gradually increased by approximately 1°C per day [45] until reaching an average temperature of 32.7 ± 0.06°C and a peak daytime temperature of approximately 35°C (figure 2*a*), resembling conditions measured in the mangrove during a non-marine heat wave day (electronic supplementary material, figure S1). Temperature was maintained (with daily fluctuations) using aquarium bar heaters and recorded every 10 min with HOBO pendant data loggers in each tank. Maximum yield of photosystem II (PSII) photochemistry (F_v/F_m, dimensionless) was measured daily (before noon) as a diagnostic response to heat stress over time [46], using pulse amplitude modulation (PAM) fluorometry (Diving PAM, Walz, Effeltrich, Germany) as described previously [47]. Fluorescence data were analysed for statistical differences over time and between treatments with repeated-measures ANOVA (rmANOVA) in SPSS v.24 (IBM Corporation, New York).

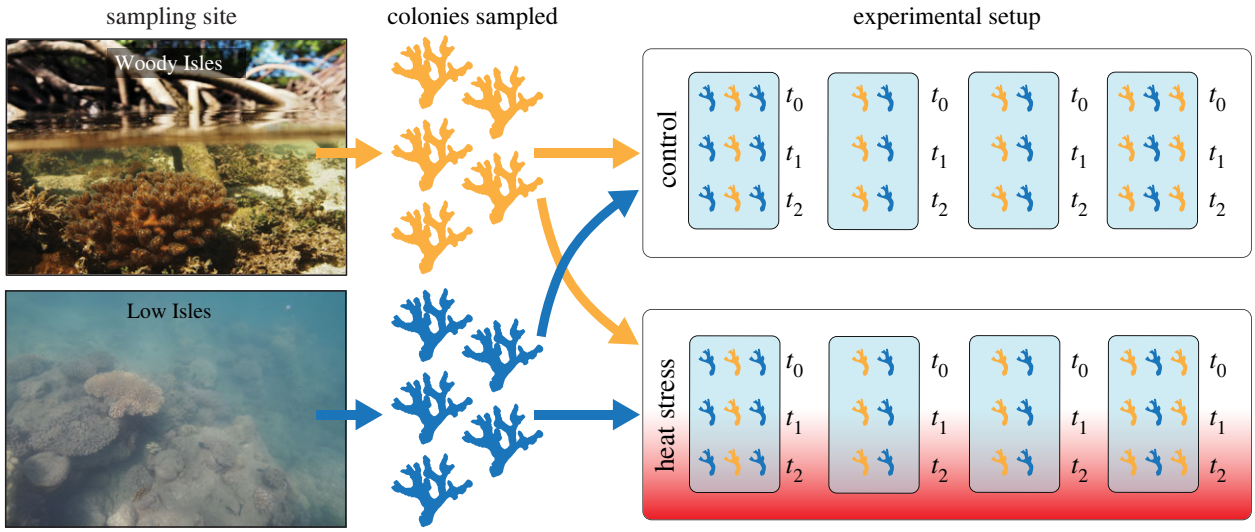

Figure 1. Sampling sites and experimental setup. Coral colonies were sampled from Woody Isles mangrove lagoon (yellow) and Low Isles reef crest (blue); sampling sites were approximately 500 m apart. Whole colonies ($n = 5$ per site) were sampled and split into 6 fragments, whereby 3 fragments were distributed into control tanks and 3 fragments were distributed into heat-treated tanks. One fragment from each colony and one from each treatment were sampled at each timepoint; t_0 = before heat ramping, t_1 = beginning of heat stress and t_2 = end of heat stress.

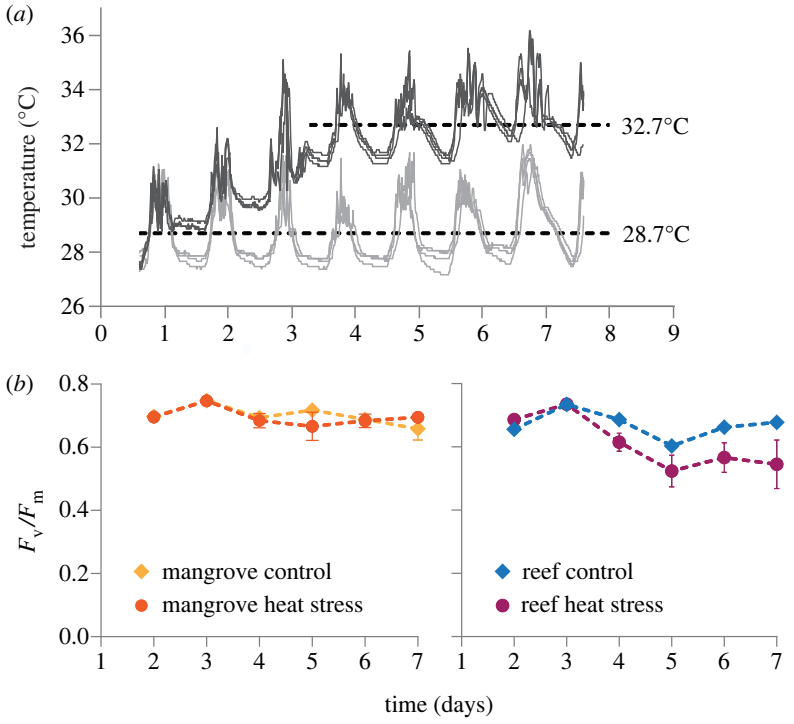

Figure 2. Tank temperatures and photophysiology. (*a*) Measured temperature of each individual tank during heat ramping (days 1–4) and during acute heat stress (days 4–8) for heat stress tanks (dark grey), $n = 4$, and control tanks (light grey), $n = 4$. Dashed lines represent average temperatures for heat stress tanks (32.7°C) and control tanks (28.7°C). (*b*) Daily photophysiology measurements ($n = 5$) measured as Fv/Fm using PAM fluorometry in both heat-stressed and control mangrove and reef corals.

(c) Sampling for internal transcribed spacer (ITS2) and metabolomics analysis

Sampling of all colony replicates for metabolomics and ITS2 analysis was conducted on the day prior to heat ramping (t_0), after which further samples were collected following 4 days of heat ramping (t_1) and a further 5 days of heat stress (t_2) (one fragment per colony and per treatment for control or heat stress fragments; figure 1). Coral fragments were removed from the aquaria before midday, wrapped in aluminium foil, immediately snap frozen in liquid nitrogen and stored at −80°C for later analysis.

(d) DNA extractions and ITS2 sequencing

Small coral fragments (less than 2 cm) were air brushed into 2 ml of artificial seawater (sea salt mix) and then centrifuged at 8000 g for 10 min, the supernatant was discarded and pellets were frozen at −80°C prior to subsequent analysis. DNA was extracted using a modified phenol-chloroform protocol as previously for this coral species [26].

To characterize the Symbiodiniaceae communities among *P. acuta* colonies at t_0 and t_2, extracted DNA was amplified using 1.5 µl of the primers ITSintfor2 5′–TCGTCGGCAGCGTCAGATGTG TATAAGAGACAGGAATTGCAGAACTCCGTG–′3 and ITS2-

reverse 5′–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGGATCCATATGCTTAAGTTCAGCGGGT–′3 (Illumina adaptors underlined), 12.5 µl of Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA, USA) and diluted template DNA (10–20 ng l^{-1}) for a 25 µl reaction. PCR conditions for the ITS2 region were as follows: 98°C for 30 s, 25 cycles of 98°C for 10 s, 55°C for 15 s, 72°C for 30 s, and a final extension at 72°C for 2 min. Resulting amplicons were sent to Australian Genomic Research Facility (Victoria, Australia), where library preparation was performed using the workflow outlined by the manufacturer (Illumina, USA). Amplicons were purified using calibrated AMPure XP beads (Beckman Coulter, USA). Purified DNA was indexed using the Nextera XT Kit (Illumina, USA) in standard PCR conditions, and the indexed amplicons were then pooled together and sequenced on the Illumina MiSeq platform (2 × 300 bp).

(e) Sequencing analysis

DNA reads from FASTQ files were analysed using the SymPortal analytical framework [48], which uses the intragenomic ITS2 diversity associated with microalgal symbionts and provides ITS2-type profiles representative of putative Symbiodiniaceae taxa. Sequences were quality controlled with Mothur 1.39.5 [49], BLAST + suite of executables [50] and minimum entropy decomposition [51]. Differences between Symbiodiniaceae communities over time and between treatments were tested using a permutational multivariate analysis of variance (PERMANOVA) with Bray–Curtis Dissimilarity matrix in the PRIMER-E + PERMANOVA package v.1.0.6.

(f) Sample processing and intracellular metabolite extraction

Sample processing protocols for coral and symbiont fraction separation and intracellular metabolites extraction were adapted from Matthews *et al.* [52]. Briefly, coral tissue was removed from the skeleton via airbrush into 5 ml chilled MilliQ at 4°C. Symbionts were pelleted by centrifugation (3000 g for 5 min at 4°C) and the host supernatant transferred and diluted with 5 ml of cold (4°C) MilliQ water, followed by vigorous vortexing for 1 min and a second centrifugation (3000 g for 5 min at 4°C) to remove residual symbionts cells. The remaining symbiont fraction was vortexed for 1 min followed by a second centrifugation (3000 g for 5 min at 4°C), and the supernatant discarded. The host and remaining symbiont fractions were frozen at −80°C for 1 h and lyophilized at −105°C for 18 h.

The semi-polar metabolites were extracted in 1 ml 100% MeOH (−20°C) from 30 mg and 15 mg of lyophilized host and symbiont material, respectively, and with the addition of the internal standard D-sorbitol-6-^{13}C at a final concentration of 10 µM. Polar metabolites were extracted with 1 ml 50% MeOH (−20°C) and combined with the semi-polar extracts. The total extract was then further centrifuged at 16 000 g for 15 min at 4°C to ensure the removal of all particulates. Aliquots (4 × 50 µl) were then concentrated under vacuum (Eppendorf Concentrator 5301) at 30°C until dry. All remaining host and symbiont cell debris were frozen at −20°C for protein quantification, as determined by a modified Bradford colorimetric method [53].

(g) Metabolite data extraction, pre-processing, normalization and statistical analysis

Concentrated samples were derivatised and analysed as per Matthews *et al.* [54]. GC–MS analysis was performed on an Agilent 7890 gas chromatograph equipped with Gerstel MPS2 multipurpose sampler and coupled to an Agilent 5975C VL mass selective detector, run in splitless mode, with an injection volume of 1 µl of each sample and a technical replicate of two injections per sample. Instrument control was performed with Agilent G1701A Revision E.02.01 ChemStation software, with settings as described in Matthews *et al.* [54]. Compounds were identified using an in-house mass spectral library and retention-time standard mixtures comprised n-alkanes.

Metabolite data were extracted, analysed and normalised as described in Matthews *et al.* [54]. Spectral components in each sample were separated, detected and identified during deconvolution using automated mass spectral deconvolution and identification system (AMDIS), with the application of retention indices and alkane standard runs. To test for overall differences in metabolite pools between environments (mangrove and reef) and treatments (control and heat-stress), multivariate analyses were performed with PERMANOVA as described for the ITS2 analysis above. Further statistical analyses were then performed using MetaboAnalyst 4.0 [55], where data were tested for normality and homogeneity and $\log_2$-transformed where necessary. Data were then autoscaled, and concentrations of both the host and algal symbiont fractions were evaluated by principal component analysis (PCA). Significance analysis of microarrays (and metabolites) (SAM; [56]) was performed to identify individual metabolites in the host and algal symbiont fractions that were differentially abundant between the treatment groups. Differentially abundant individual metabolites were determined based on a false discovery rate (FDR) corrected significance value ($p_{adj} < 0.05$).

3. Results

(a) Experimental treatments

Elevated temperatures in heat stress tanks reached a maximum of 36.2°C during the final day of sampling (t_2), while the control tanks reached a maximum of 32.2°C (mean ± SE, 28.7 ± 0.04°C; (figure 2*a*). All other environmental conditions (pH, oxygen and salinity) were maintained at ambient conditions throughout the experimental process (electronic supplementary material, table S1).

(b) Thermal stress and bleaching indicators

Values of F_v/F_m after thermal ramping (t_1), and following 5 days of exposure to daily mean (± SE) temperatures of 32.7 ± 0.06°C (t_2), exhibited minor variations that were not statistically different, for both heat stressed and control mangrove corals (rmANOVA, $p > 0.05$, figure 2 and electronic supplementary material, table S2). By contrast, exposure to elevated temperatures resulted in a significant drop in F_v/F_m compared to control for reef corals (rmANOVA, $p < 0.001$), which also coincided with visible signs of bleaching (i.e. pigment colour loss) by the end of the experimental period for heated reef but not mangrove corals (electronic supplementary material, table S3 and electronic supplementary material, figure S2).

(c) ITS2 community characterization

Sequencing of the ITS2 marker confirmed that mangrove and reef corals hosted different Symbiodiniaceae communities (PERMANOVA, $F = 43.7$, $p < 0.005$, electronic supplementary material, table S4). Mangrove corals ($n = 10$) were exclusively associated with species of the genus *Durusdinium*, but comprised two ITS2 type profiles (D1bt/D4–D6–D1–D1cf–D1bs–D1ce) and (D4–D1–D6–D1bu–D1h–D2.2–D6b–D1cd; figure 3). Reef corals ($n = 10$) were almost exclusively associated with species of the genus *Cladocopium* (with the exception of

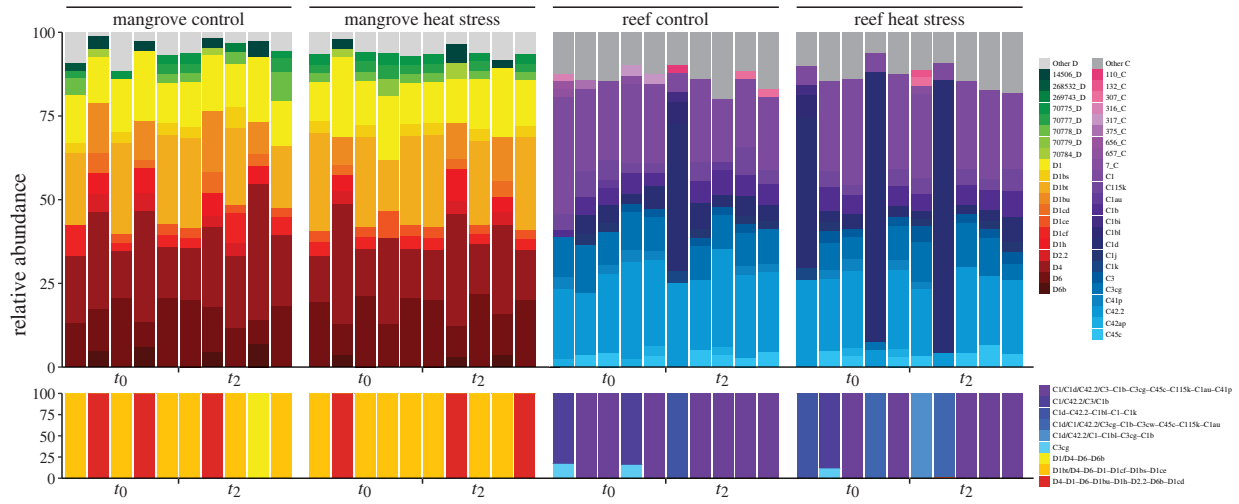

Figure 3. Relative abundance (%) of Symbiodiniaceae communities associated with the coral *P. acuta* from mangrove and reef environments under both control and heat stress conditions over time; t_0 = during acclimation and t_2 = end of heat stress. Symbiodiniaceae taxonomy is based on the ITS2 sequence types (upper bars) and predicted major ITS2 type profiles (lower bars).

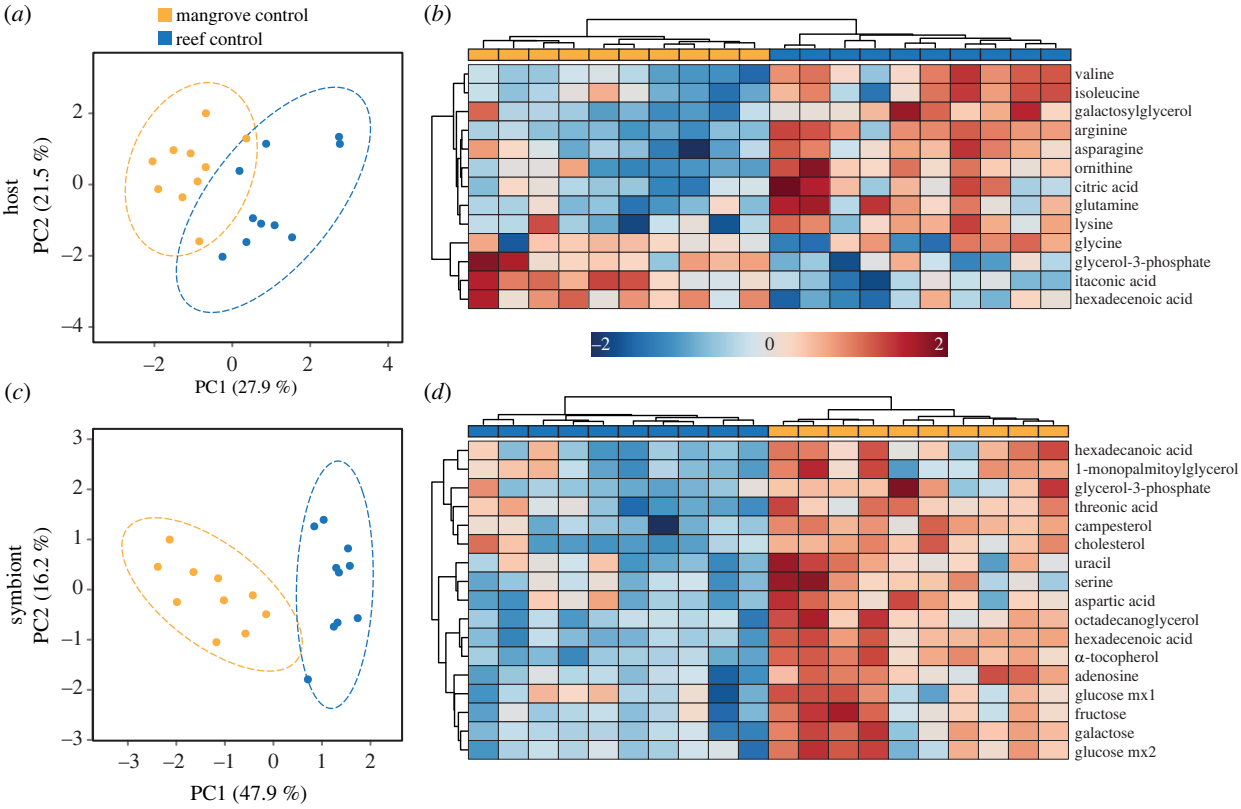

Figure 4. Differences in metabolite profiles between mangrove and reef *P. acuta* prior to heat stress. (*a*) PCA plot of differences between mangrove and reef host metabolite pools. (*b*) Heatmap of significantly different compounds present in mangrove and reef host fractions. (*c*) PCA plot of differences between mangrove and reef symbiont metabolite pools. (*d*) Heatmap of significantly different compounds present in mangrove and reef symbiont fractions.

one replicate that had < 2% relative abundance of *Durusdinium*) and 4 distinct ITS2 type profiles were detected (C1/C1d/C42.2/C3–C1b–C3cg–C45c–C115k–C1au–C41p, C1/C42.2/C3/C1b, C1d–C42.2–C1b1–C1–C1k and C1d/C1/C42.2/C3cg–C1b–C3cw–C45c–C115k–C1au; figure 3). Following 5 days of heat treatment (t_2), Symbiodiniaceae communities were unchanged for either reef or mangrove corals (PERMANOVA, $F = 1.6$, $p > 0.05$, electronic supplementary material, table S5), and hence reef *P. acuta–Cladocopium* associations were pre-disposed to greater thermally sensitivity compared to mangrove *P. acuta–Durusdinium* associations.

(d) Metabolite pools in mangrove and reef corals before heat stress

Non-targeted GC–MS analysis identified a total of 66 annotated metabolites, all present in both mangrove and reef *P. acuta* host tissues, including a suite of amino acids, organic acids, carbohydrates, fatty acids and sterols (electronic supplementary material, table S6). Metabolite profiles were significantly different between habitats (PERMANOVA, $F = 4.5$, $p < 0.001$; figure 4*a*), with 13 differentially abundant metabolites identified (SAM, FDR < 0.5). These included the amino acids arginine,

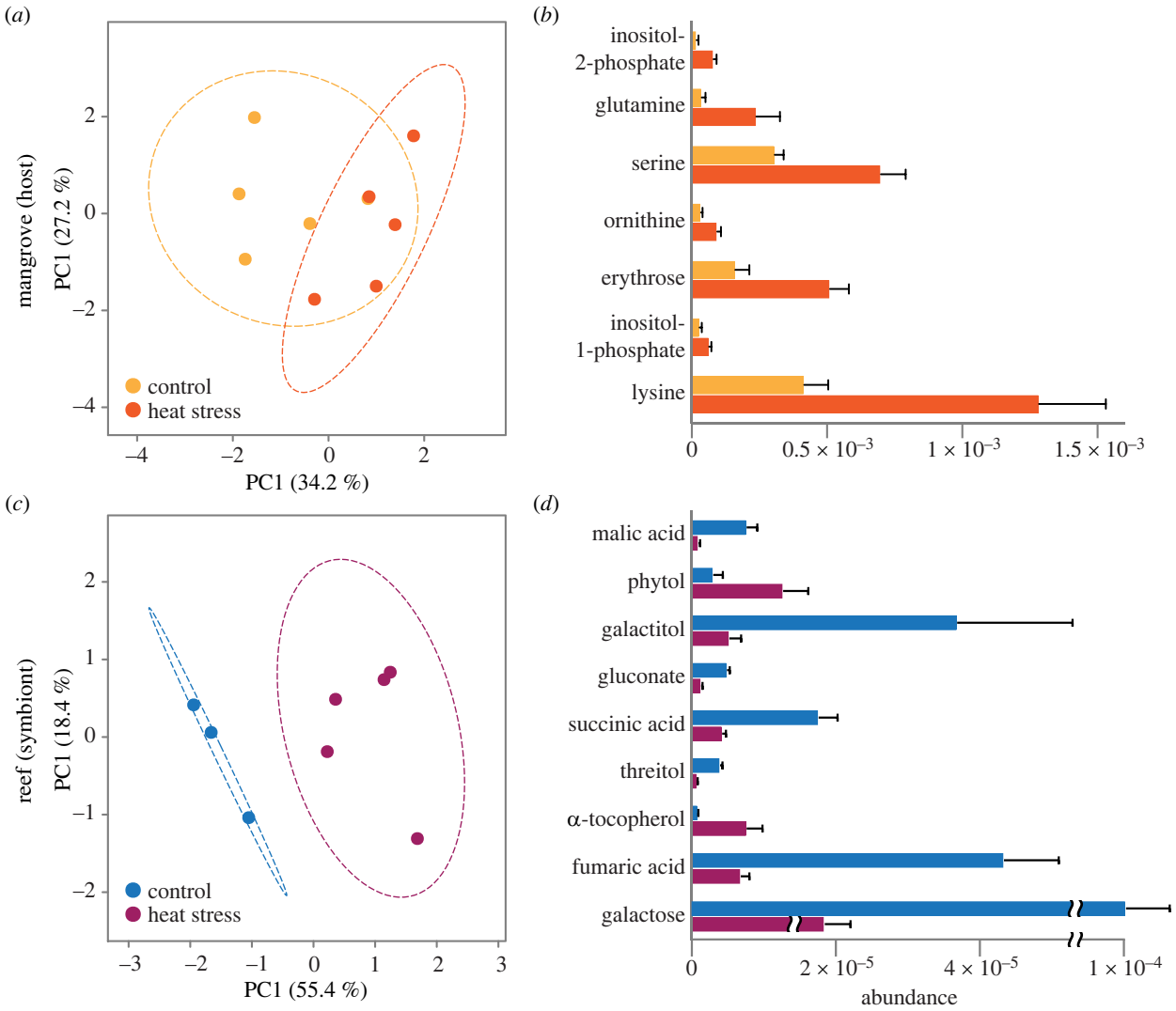

Figure 5. Changes in abundance of host and symbiont metabolite pools in *Pocillopora acuta* under heat stress. (*a*). PCA plot of mangrove host control versus heat stress metabolite pools. (*b*) Statistically different compounds between control and heat-stressed mangrove host fractions (SAM, $p < 0.05$). (*c*) PCA plot of reef symbiont control versus heat stress metabolite pools. (*d*) Statistically different compounds between control and heat-stressed reef symbiont fractions (SAM, $p < 0.05$).

valine, ornithine, glutamine, asparagine, glycine, isoleucine and lysine, which were present in higher relative abundance (2–34 fold) in the host tissues of the reef compared to mangrove corals (figure 4*b*). Notably, the relative abundance of arginine was 34-fold higher in reef corals compared to mangrove corals. By contrast, there was a higher relative abundance (2–3 fold) of the organic acid itaconic acid, the fatty acid palmitoleic acid and glycerol-3-phosphate in mangrove corals (figure 4*b*).

A total of 45 annotated metabolites were identified for mangrove and reef *P. acuta* symbiont cells, consisting of carbohydrates, amino acids, fatty acids and organic acids (electronic supplementary material, table S7). Metabolite profiles were also significantly different between habitats (PERMANOVA, $F = 10.4$, $p < 0.001$; figure 4*c* and electronic supplementary material, table S8), with 16 metabolites significantly more abundant in the symbionts of mangrove corals (SAM, $p < 0.05$; figure 4*d*). Specifically, the organic compounds tocopherol (27-fold higher) and campesterol (8-fold higher) were higher in mangrove coral symbionts than in reef coral symbionts. In addition, there was also larger relative abundance of short- and long-chain fatty acids, organic acids, amino acids and carbohydrates among mangrove coral symbionts (figure 4*d*).

(e) Metabolism under heat stress

While heat-stressed mangrove corals did not exhibit reduced F_v/F_m or bleaching, host metabolite pools were distinct (figure 5*a*) and significantly different between heat-treated and control corals (PERMANOVA, $F = 2.4$, $p < 0.002$, electronic supplementary material, table S9). However, there was no distinctive sample separation of mangrove symbiont fractions in response to heat treatments (electronic supplementary material, figure S3*a*). Analysis of individual metabolites revealed a thermally induced increase in the relative abundance of seven compounds (figure 5*b*), and notably of amino acids. Specifically, a 7-fold increase in glutamine and a 2–4-fold change in ornithine, lysine and serine were recorded in heat-treated compared to the host tissue of control mangrove corals. Small increases in abundance of the carbocyclic sugar inositol and the sugar erythrose were also observed in mangrove host corals in response to heat treatment (figure 5*b*).

Host metabolite profiles in reef corals showed no clustering according to heat-treated and control corals. Furthermore, SAM analysis did not identify any metabolites in the reef host pools that significantly differed in relative abundance between treatments. However, significant shifts in metabolite

pools were observed in symbiont fractions in response to thermal stress (PERMANOVA, $F = 4.5$, $p < 0.002$). Metabolite profiles in symbionts clustered according to their treatment (heat-treated or control; figure 5*c*), with nine metabolites identified as differentially abundant (figure 5*d*). Heat-stressed symbionts displayed significant relative increases in tocopherol (9-fold) and phytol (4-fold). These were coupled with a decline in pools of organic acids involved in the tricarboxylic acid cycle (TCA), such as malic acid, fumaric acid and succinic acid, coupled with declines in carbohydrates and sugar alcohols. Thus, the effect of heat stress on reef corals resulted in strong metabolic changes for the heat-sensitive *Cladocopium*, but not for their host. Conversely, the effect of heat stress on mangrove corals resulted in strong metabolic changes for the host corals but not their *Durusdinium* associates.

4. Discussion

Coral survival under climate change requires rapid adaptation to rising seawater temperatures [57–59], which are predicted to increase 1–4°C above current levels by 2100 [60]. Mangrove lagoons have been hypothesied to harbour more thermally tolerant corals as a result of inherently more extreme environmental conditions [11,32]. However, the superior thermal tolerance of mangrove corals has never been experimentally demonstrated, and the underlying metabolic mechanisms that facilitate their persistence within these extreme environments remain largely unknown. We therefore exposed *P. acuta* colonies originating from both mangrove lagoons and reef habitats to acute thermal stress (5 days) in order to capture the full metabolic changes of both host and algal endosymbionts. Acute thermal stress experiments are considered the most ecologically relevant to understand stress responses of corals originating from shallow lagoons and reef flats, which often experience short-term heat stress from solar heating and low tides [45]. Consistent with our previous observations, Symbiodiniaceae communities differed between reef and mangrove *P. acuta* colonies [26,27], coinciding with distinct metabolite profiles between corals in either habitat. Reef *P. acuta–Cladocopium* colonies were heat-sensitive, exhibiting bleaching and changes to the symbiont (but not host) metabolite pools. By contrast, the mangrove *P. acuta–Durusdinium* colonies were heat-tolerant with no changes to symbiont metabolite pools, but thermally induced changes were detected in the host coral metabolism.

(a) Metabolite profiles differ between mangrove and reef *P. acuta* under ambient conditions

Distinct metabolite profiles of mangrove versus reef *P. acuta* colonies prior to heat stress suggest that native environmental conditions between habitats have a considerable influence on metabolic strategies of *P. acuta*. By contrast to reef environments, tidal changes in shallow mangrove lagoons result in highly pronounced changes to water chemistry [11,12], which inherently require adaptive metabolic front-loading to buffer against more dynamic changes [61,62] and/or more continuous regulation of metabolic functioning. For example, lower pH and elevated temperatures during low tide require higher mobilization of energy reserves (or acquisition of additional energy) to counteract internal acidification and oxidative stress [44,63]. By contrast, photosynthesis rates in mangrove compared to reef corals remained unchanged, but importantly this relatively unchanged inorganic carbon uptake is offset by highly active bulk carbon translocation from symbiont to host [27].

We observed evidence for increased levels of specific metabolites in the mangrove symbionts compared to the reef symbionts. Specifically, we detected higher levels of carbohydrates and glycolysis intermediates, such as fructose, along with higher pools of glucose, one of the main photosynthates translocated from the symbiont to the host [54,64,65]. Increased storage of these components could provide a fitness benefit over time [36] and thereby enhance capacity to withstand transient environmental stress. We also cannot rule out the potential role of increased heterotrophy among mangrove *P. acuta*. Even though no additional heterotrophic feeding was provided throughout the duration of this experiment, the presence of various pico-plankton within the experimental aquaria was likely, which could possibly account for some of the elevated levels of metabolism observed. Corals with increased energy reserves are less susceptible to bleaching, and take longer to bleach (or are faster to recover) by sustaining their metabolism in the absence of photosynthetically derived organic compounds [16,66,67]. In addition to the increase in energy sources, pools of the chloroplast-associated antioxidant vitamin alpha-tocopherol were 27-fold higher in the mangrove symbiont cells, which may be linked to the thermal and oxidative tolerance attributed to some species of *Durusdinium* [68–70]. This vitamin has been previously detected in heat-stressed corals [37], along with healthy corals (but lower in diseased corals) [71], and has important functions in the detoxification of pro-oxidants [72] and peroxyl radicals [73]. Considering that these colonies were collected from the mangrove during summer, these metabolite profiles suggest that *P. acuta* elicits potential 'frontloading' as a pre-emptive response in upregulation of heat-stres-related genes for thermal tolerance [8,61]. Frontloading responses in corals have been previously detected for heat shock and antioxidant genes with potential significant effects on coral fitness [74,75].

Pools of the metabolite itaconic acid were also detected in mangrove corals. Itaconic acid is a potent antimicrobial compound that increases in abundance in bivalves during pathogenic challenges [76,77] and was recently discovered to inhibit the growth of the coral pathogen *Vibrio coralliilyticus* [78,79]. Our previous study characterizing the microbiome of mangrove *P. acuta* detected no presence of *Vibrio* sp. in mangrove corals [26], despite the high temperatures to which these corals are exposed in summer in the locations where *Vibrio* sp. often thrives [80,81]. Therefore, it is plausible that mangrove *P. acuta* inherently possesses stronger resistance to potential pathogens compared to reef corals, although this hypothesis would require further investigation. Regardless, our findings highlight the clear differences in metabolite profiles of *P. acuta* between these two habitats and the potentially core tolerance mechanisms to alleviate stress in these extreme mangrove lagoons.

(b) Mangrove corals show resilience to acute thermal stress through metabolomic plasticity

In *Durusdinium*-associated mangrove corals exposed to elevated temperatures (35°C) there was no change to F_v/F_m (a diagnostic of symbiont heat stress sensitivity) and no evidence for symbiosis destabilization in metabolite profiles. Specifically, we did not observe a change to either the identity

or relative abundance of metabolite pools between the control and heat stressed symbiont fraction. Such lack of separation in these metabolite profiles from elevated temperatures is contrary to a number of previous studies [37–39,44] and could indicate that the elevated temperatures used in this experiment were not severe enough to trigger a response in the mangrove Symbiodiniaceae communities. However, our findings of increased thermal tolerance in mangrove corals are in line with previous observations for *P. acuta* associated with *Durusdinium* [68,82]. Biochemical mechanisms for thermal tolerance within the genus *Durusdinium* (e.g. enhanced antioxidant properties) have been documented [70,83,84]. However, considering the importance of maintaining structural integrity of cell membranes [85,86], recent findings suggest that members of *Durusdinium* may also modify and saturate their lipid membrane compositions under stress [40], representing a distinct tolerance strategy. Furthermore, thermally tolerant coral symbionts appear to exhibit higher saturation of betaine lipids compared to historically bleached corals in Kāne'ohe Bay, Hawai'i [62], which in turn may contribute to distinct metabolic diagnostics of thermal tolerance. We therefore hypothesize that mangrove *P. acuta* symbionts could adopt a similar strategy of lipid saturation in aiding tolerance (given the lack of breakdown of symbiont cells compared to reef corals). Our GC–MS-based approach is unable to detect these metabolites, unlike studies that have used LC-MS and mass spectrometry-based lipidomic analysis [40,44,62], and this therefore warrants more detailed investigation.

Despite the lack of metabolite profile shifts for the symbiont under elevated temperatures, we did detect changes in mangrove host metabolite pools, indicating potential biomarkers of thermal tolerance. Such a change suggests that mangrove *P. acuta* exhibits metabolomic plasticity that appears to be lacking in their *Durusdinium* symbionts. Temperature stress treatments triggered an increase in several metabolites, and notably in relative abundance of glutamine and ornithine, known precursors to the antioxidant glutathione, which may represent increased antioxidant capacities [87–89]. Upregulation of genes involved in the expression of the non-enzymatic antioxidant glutathione has been found in the host tissue of *P. acuta* that has a higher tolerance to bleaching [90], suggesting reactive oxygen species detoxification by the host may be particularly important for maintaining stability under stress.

Increased detection levels of specific metabolites were also observed in the heat-stressed mangrove corals, via increased expression of compounds involved in central metabolism, and specifically higher accumulations of erythrose, an intermediate in the Calvin Cycle and pentose phosphate pathway. Higher abundance of erythrose present in the heat stressed host fractions thus likely indicates an increase in sugar translocation from symbiont to host as a response to maintain cellular homeostasis [91,92], corresponding with our previous observations for mangrove *P. acuta* exhibiting highly sustained bulk organic carbon translocation despite lower carbon uptake rates [27]. However, erythrose is also an important metabolite for bacterial growth and the biosynthesis of amino acids [93,94]. Symbiotic bacteria in diatoms are provided with erythrose as a fixed carbon source for growth and return fixed nitrogen to their algal partners [94]. Therefore, it is possible that the detected pools of erythrose in heat-stressed mangrove corals may also represent metabolite exchanges between holobiont multiparter networks (e.g. bacteria–Symbiodiniaceae–coral) [95], but this hypothesis remains to be tested.

Potential changes to inter-partner signalling also coincided with changes in the relative abundance of sugars. Inositol has been previously identified in anemones, where it appears extensively upregulated in symbiotic versus aposymbiotic transcriptomes [96,97] and is thought to be involved in coral host–Symbiodiniaceae signalling [37,54]. We detected higher accumulations of inositol phosphates in heat stressed host tissue, suggesting increased inter-partner signalling under elevated temperatures in mangrove *P. acuta*, but this also indicates continued functional symbiosis. Inositol is present in both symbiotic partners [37,98], however, we did not detect any changes in the abundance of this metabolite in the symbiont fraction under heat stress. Another potential role of inositol is disease resistance, where derivatives of this metabolite have been detected in higher abundance in healthy coral mucus compared to diseased corals [99]. However, in our current study it is likely that the inositol originated from the host tissue rather than the surface mucus layer since our coral fragments were thoroughly rinsed prior to processing for metabolite extraction and any external mucus was removed. Together, along with the observed increases in central metabolism and antioxidant capacity, these findings suggest that mangrove *P. acuta* re-adjust their metabolism to enhance resilience to elevated seawater temperature. The apparent metabolic plasticity displayed by mangrove *P. acuta* corals is likely a consequence of the continued exposure to the variable conditions of the mangrove habitat, resulting in various mechanisms allowing these corals to persist in this environment.

(c) Changes in primary metabolites indicate structural changes and cell damage in reef *P. acuta*

By contrast to their mangrove counterparts, reef *P. acuta* associating with *Cladocopium* exhibited reduced signs of symbiont photosynthate translocation, declines in photophysiology and evidence of cellular breakdown under thermal stress. Coral symbiosis is highly susceptible to elevated temperatures (e.g. [33]) and hence numerous studies have attempted to understand the mechanisms of coral bleaching [3,15,74,100–103]. Our findings of thermal susceptibility among reef colonies are perhaps unsurprising since the temperatures used for the heat stress experiment (32–35°C) were above the threshold of most coral reefs [104]. In addition, reduction in photosynthetic efficiency (F_v/F_m) is a consistent response to heat stress-induced photodamage and decline in physiological function of the symbionts (e.g. [46]).

We detected significant changes in metabolite pools of reef *P. acuta* symbionts in response to elevated temperatures in line with previous studies [37,39,40,44,62]. Specifically, we observed a reduction in carbohydrates, along with a reduction in multiple metabolites involved in the TCA cycle (fumaric acid, succinic acid and malic acid), which suggests a significant reduction in the activity of energy-generating networks under heat stress and the onset of bleaching [103,105]. In addition, we observed declines in glucose derivatives and other photosynthetic-related metabolites, likely representing a shift in metabolic function and the departure of efficient carbon fixation in symbionts [106]. Early signs of thermal stress in corals are often characterized by increased activity of energy-generating networks or increases in biosynthesis pathways as a response to maintain cellular homeostasis [37,39,41,42]. However, the overall reduction in metabolic networks observed in our study suggests a much later stage of the thermal stress processes

that has resulted in reduced levels of translocation and subsequent breakdown of symbiosis and bleaching.

5. Conclusion

Understanding the mechanisms enabling corals to persist in present day naturally extreme environmental conditions is a fundamental approach to resolving corals' adaptive capacity to heat (and other) stressors (e.g. [107]). By contrasting heat stress performance of mangrove versus reef populations of the coral *P. acuta*, we show that corals adapted to life in extreme environments exhibit distinct pools of metabolites that appear consistent with changes in emergent physiological properties previously observed (e.g. respiration, photosynthesis and bulk translocation; [25,27]) and exhibit greater resistance to transient heat stress. *P. acuta* exhibited no change to their distinctive Symbiodiniaceae communities from either habitat under heat stress—an observation also consistent where symbiont communities have remained the same when transplanting *P. acuta* between habitats [26]—but heat stress resulted in metabolome re-organization for *P. acuta* mangrove hosts and degradation for *P. acuta* reef-associated symbionts. As such, our data demonstrate that heat tolerance is likely conferred through more heat-tolerant symbionts (with distinct metabolic performance) coupled to greater metabolic plasticity of the host. However, whether the metabolic response of the host reflects an outcome of the altered metabolism of the symbiont (or *vice versa*) remains to be further tested. Our findings of unique metabolite profiles among thermally tolerant corals indicate that specific metabolites could be indicators (and biomarkers) of heat tolerance and thus provide novel insight into metabolic pathways that promote heat tolerance.

Ethics. All field activities were conducted under Great Barrier Reef Marine Park Authority permit numbers G19/42553 and G18/40023.1.

Data accessibility. All raw fastq read files are accessible under NCBI Sequence Read Archive (SRA), under the accession number: PRJNA881016.

The data are provided in electronic supplementary material [108].

Authors' contributions. T.D.H.: conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft; J.L.M.: formal analysis, methodology, supervision, writing—review and editing; J.R.S.: conceptualization, funding acquisition, investigation, supervision, writing—review and editing; J-B.R.: methodology, supervision, visualization, writing—review and editing; J.E.S.: resources; K.C.: resources; E.F.C.: conceptualization, funding acquisition, supervision, writing—review and editing; D.J.S.: conceptualization, funding acquisition, investigation, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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