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The Influence of Symbiont Identity on the Proteomic and Metabolomic Responses of the Model Cnidarian Aiptasia to Thermal Stress

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ABSTRACT

We examined the effects of symbiont identity and heat stress on the host metabolome and proteome in the cnidarian–dinoflagellate symbiosis. *Exaiptasia diaphana* ('Aiptasia') was inoculated with its homologous (i.e., native) symbiont *Breviolum minutum* or a heterologous (i.e., non-native) symbiont (*Symbiodinium microadriaticum*; *Durusdinium trenchii*) and thermally stressed. Integrated metabolome and proteome analyses characterised host thermal responses between symbioses, with clear evidence of enhanced nutritional deprivation and cellular stress in hosts harbouring heterologous symbionts following temperature stress. Host metabolomes were partially distinct at the control temperature; however, thermal stress caused metabolomes of anemones containing the two heterologous symbionts to become more alike and more distinct from those containing *B. minutum*. While these patterns could be partly explained by innate symbiont-specific differences, they may also reflect differences in symbiont density, as under control conditions *D. trenchii* attained 60% and *S. microadriaticum* 15% of the density attained by *B. minutum*, and at elevated temperature only *D. trenchii*–colonised anemones bleached (60% loss). Our findings add to a growing literature that highlights the physiological limits of partner switching as a means of adaptation to global warming. However, we also provide tentative evidence for improved metabolic functioning with a heterologous symbiont (*D. trenchii*) after sustained symbiosis.

1 | Introduction

The success of coral reefs is based on the nutritional exchange between the cnidarian host and its photosynthetic dinoflagellate endosymbionts (Muscatine and Hand 1958; Burriesci et al. 2012). The host facilitates the transport of inorganic carbon, nitrogen, and phosphorus compounds from the water column or derived from host metabolism to the symbiont, whereas the endosymbiont releases photosynthetically derived organic compounds to the host (Davy et al. 2012; Cunning et al. 2017). The cnidarian endosymbionts are dinoflagellates that belong to the family Symbiodiniaceae, with 11 genera and numerous species (LaJeunesse et al. 2018, 2021; Pochon and LaJeunesse 2021; Nitschke et al. 2020). Characteristics such as thermal tolerance, photosystem function, and inorganic carbon acquisition vary widely among taxa, and many species of cnidarians form a symbiosis with only one or a limited range of symbiont species, with distinct patterns of host–symbiont specificity (Poland and Coffroth 2017; Putnam et al. 2012; Nitschke et al. 2022).

The cnidarian–dinoflagellate symbiosis is highly sensitive to environmental change. Thermal stress or pollution can result in coral bleaching, where the dinoflagellates are lost from the host's tissues (Weis 2008; Fitt et al. 2001). Coral bleaching is contributing to the precipitous decline of coral reefs around the world (Mellin et al. 2024). Exact cellular mechanisms for bleaching are only partially understood, although oxidative stress in both the symbiont cell and host tissues is a major contributor (Oakley and Davy 2018). Oxidative stress resulting from photodamage in symbiont chloroplasts has been proposed to result in the diffusion of oxidants into host cells, triggering expulsion or degradation of the symbionts (Smith et al. 2005; Lesser 1996; Warner and Suggett 2016). However, while bleaching poses a large threat to coral health, it may also provide a window of opportunity for altering the host's symbiont community composition to better accommodate changing environmental conditions (Fautin and Buddemeier 2004; Buddemeier and Fautin 1993; Claar et al. 2020; Cunning et al. 2015; Cunning 2021). Alterations in endosymbiont types that reside in the cnidarian host might occur in one of two ways: symbiont 'shuffling' or 'switching' (Baker 2003). Shuffling occurs when the relative abundances of different symbiont species already present in the host's tissues are 'rebalanced', whereas switching involves the uptake and establishment of a new symbiont partner from the environment.

Harbouring thermally tolerant symbionts (whether Symbiodiniaceae or other microbes) allows the holobiont (i.e., whole symbiosis) to tolerate higher temperatures than the native cnidarian–endosymbiont association (Stat and Gates 2011; Kemp et al. 2014; Boulotte et al. 2016; van Oppen and Blackall 2019). However, bleaching may also allow less beneficial symbionts to invade immune-compromised host tissues (Stat and Gates 2011; Thornhill et al. 2009; Abrego et al. 2008). Experimental tests of inter-partner switching have demonstrated that symbiosis with a heterologous (i.e., non-native) symbiont can result in a suboptimal nutritional relationship, and even cause tissue stress and host mortality (Weis et al. 2001; Dunn and Weis 2009; Gabay et al. 2018; Matthews et al. 2017; Mashini et al. 2022). However, how the benefit versus cost of symbioses with homologous (i.e., native) and heterologous symbionts changes upon exposure to thermal stress has not been established at the cellular level (proteins and metabolites); this aspect of the association is particularly important given the implications of partner switching in response to climate change.

This work focused on examining the combined effect of symbiont genotype and thermal stress on the proteome and metabolome of the sea anemone *Exaiptasia diaphana* ('Aiptasia'). Aiptasia was colonised with its homologous symbiont *Breviolum minutum* or one of two heterologous species, *Durusdinium trenchii* or *Symbiodinium microadriaticum*. Both *D. trenchii* and *S. microadriaticum* have a relatively high thermal tolerance in culture (Robison and Warner 2006; Ragni et al. 2010; Hawkins and Davy 2012; Krämer et al. 2012). Despite this tolerance, under non-stressful environments, *D. trenchii* provides a reduced nutritional benefit to the host and may increase oxidative stress in Aiptasia compared to its homologous partner (Matthews et al. 2017; Sproles et al. 2020). This potentially explains why, even following exposure to thermal stress, *D. trenchii* (as well as *S. microadriaticum*) is 'outcompeted' by *B. minutum* when the symbionts are simultaneously inoculated into Aiptasia, and why this cnidarian exhibits a strong specificity for *B. minutum* over its entire range in the Indo-Pacific (Gabay et al. 2018, 2019; Thornhill et al. 2013). How these symbionts respond to thermal stress at the cellular level when they have already populated Aiptasia is therefore crucial for understanding the drivers of host–symbiont specificity and the potential for adaptive bleaching via partner switching. A combination of metabolomic and proteomic analyses used in this study addresses this knowledge gap.

2 | Experimental Procedures

2.1 | Experimental Organisms

Specimens of the sea anemone Aiptasia ($n = 1000$, clonal line NZ1) were rendered aposymbiotic using a menthol bleaching protocol (Matthews et al. 2016) combined with weekly cold shocks, as described in the Supporting Information. Aposymbiotic anemones with an oral disc diameter of 4–5 mm were placed in sterile 400-mL plastic jars (LabServ, approximately 30 per jar) containing 250 mL 35 ppt artificial seawater (Aquaforest Marine).

Three Symbiodiniaceae cultures were used: the homologous species *B. minutum* (culture ID FlAp2, originating from Aiptasia in Long Key, Florida, USA; Matthews et al. 2017; Mashini et al. 2024), and the heterologous species *S. microadriaticum* (culture ID CCMP2467, originating from the Gulf of Aqaba, the Red Sea) and *D. trenchii* (culture ID Ap2, originating from Okinawa, Japan). These cultures were grown in an incubator at 25°C on a 12:12 h light:dark cycle and an irradiance of 30 μmol photons $m^{-2} s^{-1}$ (Phillips, 18 W 840) in silica-free f/2 culture medium (AusAqua Pty, South Australia, Australia).

Jars with anemones were randomly chosen for inoculation with one of the three Symbiodiniaceae cultures and inoculated by applying the symbionts at a cell density of ~3 million cultured algal cells mL^{-1} mixed with freshly hatched *Artemia* nauplii (to stimulate feeding) to the oral disc. This process was performed once *per* week for 4 weeks. Once weekly, three individual anemones *per* symbiont species were removed from the experimental setup and checked for colonisation success by confocal microscopy (IX81, Olympus New Zealand; 635 nm laser, 655–755 nm emission filter). During and after inoculation, anemones were maintained at the control temperature of 25°C and exposed to 100 μmol photons $m^{-2} s^{-1}$ on a 12:12 h light:dark diel cycle (GE Lighting T5 F54W/840). These conditions were identical to those experienced by anemones in our rearing tanks. Anemones were left for 85 days (approximately 12 weeks) to enable symbiont populations to establish fully within host tissues (Gabay et al. 2018).

2.2 | Experimental Treatments

Eighty-five days after inoculation, samples were taken for symbiont genotyping, and anemones were divided into two groups, each maintained in a water bath, with one maintained at the 'control' temperature (25°C) and the other 'heat stressed' ($n = 5$ jars per treatment per symbiont species). The heat-stressed condition involved ramping the temperature up by 1°C day^{-1} for 7 days to a peak of 32°C, which was maintained for 3 days.

2.3 | Sampling and Processing

Ninety-four days after inoculation, anemone samples were collected and flash-frozen in liquid nitrogen to quench metabolism (~10 s). The remaining anemones were used to measure the dark-adapted Photosystem II quantum yield (F_v/F_m; Diving PAM, Heinz Walz GmbH, 20 min dark adaptation, full details in Supporting Information). Sample processing was performed according to previously published methods (Matthews et al. 2017). In brief, after the anemones were homogenised, aliquots were taken for symbiont genotyping and cell density measurements, with the latter normalised to host protein content. To produce a clean host fraction (i.e., devoid of symbiont contamination), host and symbiont fractions were separated by centrifugation (twice at 2500g). Host fractions were freeze-dried prior to metabolite extraction (Matthews et al. 2017), followed by protein extraction using a filter-aided sample preparation protocol on the remaining host material (Oakley et al. 2022). Full processing and symbiont genotyping details are provided in the Supporting Information.

2.4 | Metabolite and Protein Profiling

Details of the metabolomic and proteomic analyses of the host fraction are provided in the Supporting Information. Briefly, gas chromatography–mass spectrometry (GC–MS)–based metabolite profiling was conducted on polar and semipolar free metabolites (Matthews et al. 2017) at Metabolomics Australia (Melbourne, Australia). Chromatograms and MRMs were evaluated using the Shimadzu GC–MS browser and LabSolutions Insight software. Tryptic peptide samples were profiled using liquid chromatography–tandem mass spectrometry (LC–MS/MS) in a Thermo Fisher Orbitrap Fusion Lumos Tribrid mass spectrometer with an UltiMate 3000 RSLCnano LC system at Victoria University of Wellington (Oakley et al. 2022). Proteins were identified and their abundance levels quantified from the peptide raw spectra using MaxQuant (1.6.10.43; Cox and Mann 2008; Cox et al. 2014), which identifies the proteins associated with the peptides based on predicted gene models from the Aiptasia genome (Baumgarten et al. 2015) followed by annotation of protein sequences to UniProtIDs using DiamondBLAST (Buchfink et al. 2015).

2.5 | Data Analysis and Visualisation

Full description of data analysis can be found in the Supporting Information. In brief, metabolites were identified using limma (Ritchie et al. 2015), with a false discovery rate (FDR) threshold of $q < 0.05$. Only metabolites with a $\log_2$-fold change in their absolute value that was larger than 0.5 are reported. For the proteomic analysis, identified proteins and label-free quantification intensities were analysed in Perseus (1.6.15.0; Tyanova et al. 2016). After removing known false detections and contaminants, the samples were normalised to total intensity values and $\log_2$-transformed in R. To minimise the effect of missing protein abundance values on the data analyses, we conservatively imputed missing values with a constant non-zero value just below the detection threshold in the dataset. Significant differentially abundant proteins (DAPs) between treatments were identified using the Miss test in PolySTest (Schwämmle et al. 2020) with an FDR threshold of $q < 0.05$, and only those annotated proteins with a $\log_2$-fold change absolute value larger than 0.25 were reported. PCAs were generated for both host metabolomes and proteomes using R. One-way ANOVAs were used to test if PCA axes could significantly separate the samples based on thermal treatment or symbiont species.

Integrated pathway analysis was performed using the joint pathway analysis function in MetaboAnalyst (version 5.0; Pang et al. 2022). Input data consisted of $\log_2$-fold change values of DAPs and $\log_2$-fold change values in the relative abundance of differentially abundant metabolites (DAMs). The model organism selected for the integrated pathway analysis was *Homo sapiens*, as this showed the most matches to identified proteins. Pathways were considered significant if the FDR was < 0.05 and the pathway impact, a topological assessment that measures how central the identified compounds are to the pathway (Pang et al. 2022), was > 0. Pathways with fewer than three genes and/or metabolites were filtered out.

In order to identify which proteins and metabolites were most representative of the host thermal response when hosting different symbiont species, we performed an integrated metabolome and proteome analysis using the DIABLO function in mixOmics (Le Cao et al. 2016). As *S. microadriaticum* only achieved low cell densities, to better identify a clear bleaching signature in the host proteome and metabolome, data from anemones hosting *S. microadriaticum* were omitted from the DIABLO analysis (see Figure S1 and Tables S1 and S2). Using a 0.9 correlation matrix, initial model tuning indicated that two components using centroid distance were sufficient to optimise the model. To enable this analysis, missing protein intensities were imputed with a positive integer below the lowest observed intensity.

3 | Results

3.1 | Symbiont Cell Density and Photosynthetic Health

Endosymbiont genotyping and confocal microscopy confirmed the successful colonisation of Aiptasia by *B. minutum*, *D. trenchii* and *S. microadriaticum*. There were large differences in densities of the symbiont populations under control conditions, with *S. microadriaticum* and *D. trenchii* reaching only about 15% and 60%, respectively, of the density attained by *B. minutum* (Figure 1A). The lower population densities in Aiptasia achieved by the heterologous endosymbionts had been previously observed (Gabay et al. 2018; Mashini et al. 2022; Matthews et al. 2018; Sproles et al. 2019).

Only anemones populated with *D. trenchii* significantly bleached in response to heat treatment, losing 60% of their symbionts (Figure 1A). PAM fluorometry showed a significant decrease in maximum Photosystem II quantum efficiency in response to

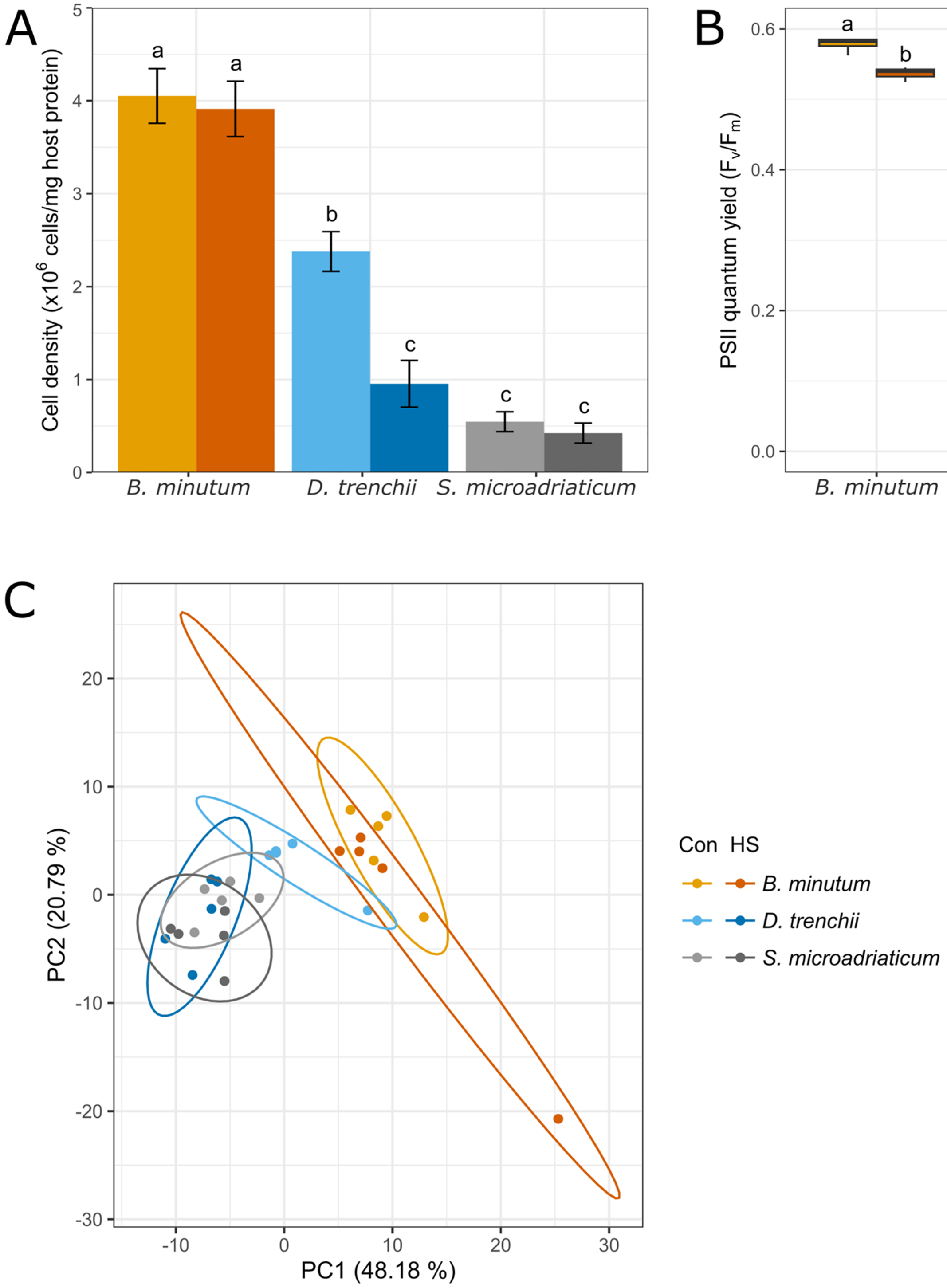

FIGURE 1 | (A) Symbiont cell densities (±SE) in Aiptasia colonised by different symbiont species (i.e., *Breviolum minutum*, *Durusdinium trenchii* or *Symbiodinium microadriaticum*) under control (25°C) or heat treatment (ramping to 32°C, then held for 3 days). Letters indicate statistically significant groups. (B) Dark-adapted quantum yield of Photosystem II (F_v/F_m) of *B. minutum* and *D. trenchii* under control temperature and heat stress; data are omitted for *S. microadriaticum* due to low symbiont cell densities. Letters indicate statistically significant groups. (C) Principal component analysis of the full host metabolome of Aiptasia in symbiont–host pairings at control temperature and heat treatment. Ellipses represent the 95% confidence intervals.

heat stress in both *B. minutum*– and *D. trenchii*–colonised hosts (Figure 1B), although in both cases this value remained > 0.5. Fluorometry data could not be reliably collected for *S. microadriaticum*–colonised anemones because of their low symbiont population density.

3.2 | Proteome and Metabolome Results

A total of 93 unique metabolites and 3768 unique proteins were identified across all samples (full details provided in Tables S3–S5). The DAMs and DAPs are shown in Figures 2 and

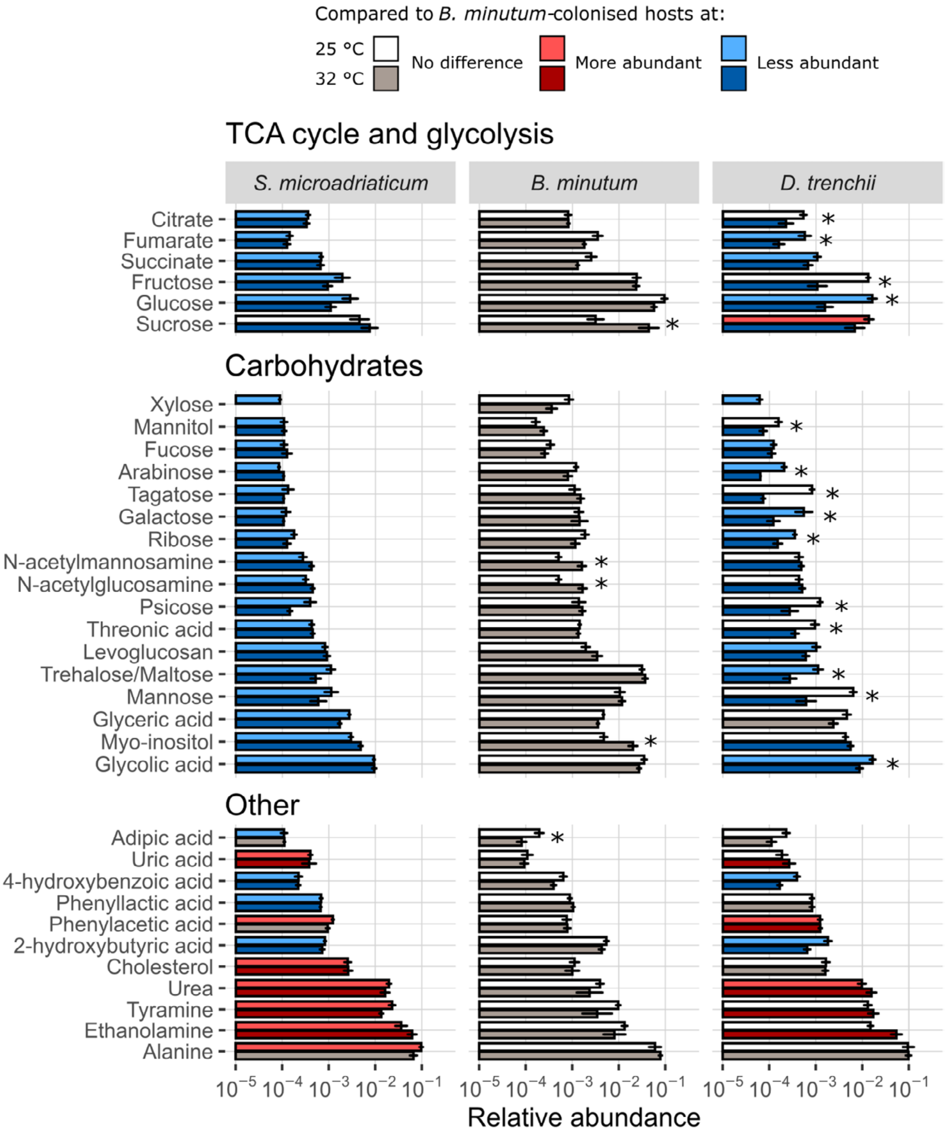

FIGURE 2 | Relative quantities of differentially abundant metabolites (DAMs) *per* metabolic category in Aiptasia host tissue colonised by different symbiont species (i.e., *Breviolum minutum*, *Durusdinium trenchii* or *Symbiodinium microadriaticum*) under control (25°C) and heat stress conditions (ramping to 32°C, then held for 3 days). Asterisks indicate significant differences within species caused by heat stress, whereas colouring of bars indicates significant differences from *B. minutum*–colonised anemones (red means significantly more abundant while blue means significantly less abundant when in symbiosis with the heterologous symbionts). Only DAMs that were differentially expressed in at least two comparisons are shown.

3, respectively (see also Figures S2–S4). Integrated pathway analysis showed a total of 19 differentially regulated pathways across this study (Table 1; full details provided in Tables S6 and S7). Under control conditions, the most differentially regulated pathways were between the anemones colonised by *S. microadriaticum* and those colonised by *B. minutum* (nine pathways; Table 1 and Figure 4; see also Figure S1). Fewer pathways were differentially impacted, and to a lesser extent, when *D. trenchii*– and *B. minutum*–colonised anemones were compared (five pathways; Table 1). Heat stress led *D. trenchii*– and *S. microadriaticum*–colonised host pathways to become more similar to each other, and less similar to *B. minutum*–colonised host pathways. This pattern was also reflected in the spatial separation of the different host metabolomes by PCA. PC1 significantly separated all symbiont-specific host metabolomes under control conditions (Figure 1C). However, upon heat stress, PC1 could not separate *S. microadriaticum*– and *D. trenchii*–colonised hosts from each other, but the hosts of these two associations could be separated from the *B. minutum*–colonised hosts (Figure 1C). A schematic overview of processes affected by symbiont identity under control conditions, and the effects of heat stress within host–symbiont pairings are presented in Figure 5.

3.3 | Integrated Proteome and Metabolome Results

The two latent components of the proteome and metabolome datasets were strongly correlated (Figure 4A). All conditions were well defined in Component 1, while *B. minutum* at 32°C was strongly separated from the other conditions in Component 2. A total of 275 proteins and three metabolites, all sugars, had non-zero loadings in Component 1 (Figure 4B).

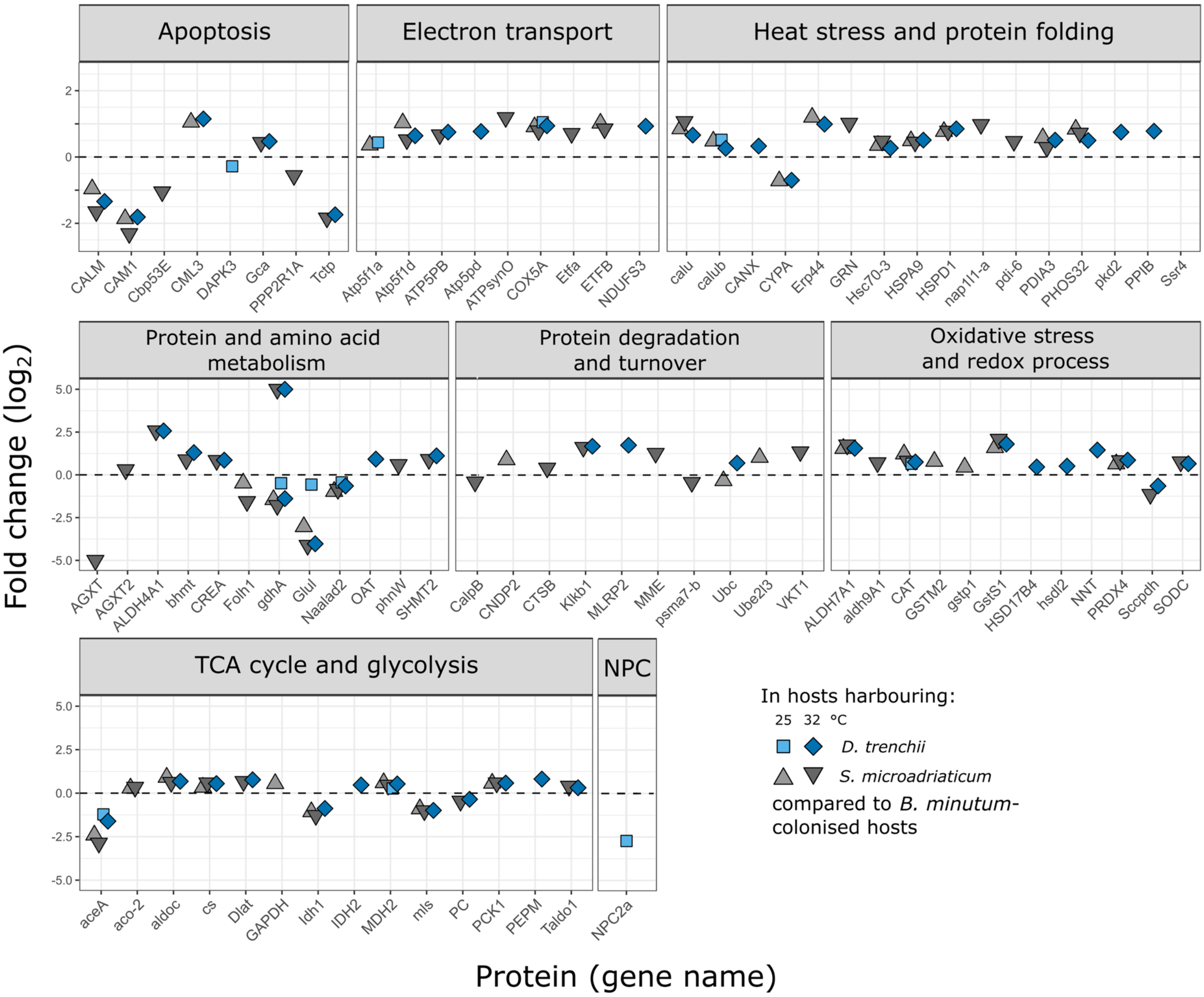

FIGURE 3 | Log$_2$-fold changes of differentially abundant proteins (DAPs; by gene name, full protein names can be found in Table S5) in Aiptasia containing heterologous symbiont species (i.e., *Symbiodinium microadriaticum* or *Durusdinium trenchii*) compared to Aiptasia symbiotic with *Breviolum minutum* under control (25°C) and heat stress conditions (ramping to 32°C; *n* = 5).

Glucose, arabinose, and xylose were signatures of *B. minutum* at 25°C. We were particularly interested in which proteins and metabolites contributed to the distinct response of *B. minutum* at 32°C, which was less affected by high temperature than *D. trenchii* (Figure 4C). Several protein chaperones, including calumenin and HSP90, were strongly distinctive of the host response to high temperature with *B. minutum*. Metabolites that implied a beneficial thermal response in *B. minutum* were elevated myo-inositol, the glucose derivatives *N*-acetylglucosamine and *N*-acetylmannosamine, and sucrose (Figure 4C). Full loadings of proteins and metabolites are available in Tables S8 and S9.

4 | Discussion

4.1 | TCA Cycle, Glycolysis, and Other Carbohydrates

Integrated pathway analysis showed significant differences between both *S. microadriaticum*– and *D. trenchii*–colonised hosts compared to those containing *B. minutum* related to core metabolism (‘pyruvate metabolism’, ‘citrate cycle’, and ‘glyoxylate and dicarboxylate metabolism’; Table 1). Additional pathways impacted in *S. microadriaticum*– versus *B. minutum*–colonised hosts included ‘glycolysis or gluconeogenesis’, ‘glucagon signalling pathway’, and ‘amino sugar and nucleotide sugar metabolism’ (Table 1). *S. microadriaticum*–colonised hosts displayed significantly lower abundances of DAMs associated with the TCA cycle and glycolysis (e.g., citrate and fumarate) than *B. minutum*–colonised hosts, as well as another 17 carbohydrate compounds (Figure 2). We observed increased abundance of several core metabolism–related proteins (e.g., malate dehydrogenase and citrate synthase; Figure 3), and reduction of others (e.g., malate synthase and isocitrate dehydrogenase) in hosts containing *S. microadriaticum* compared to those with *B. minutum*. When comparing *D. trenchii*– to *B. minutum*–colonised hosts, DAMs included increased relative levels of sucrose, but less citramalate, succinate and glucose, and reduced levels of an additional eight compounds involved in carbohydrate metabolism in hosts harbouring *D. trenchii* (at 25°C; Figure 2). In terms of DAPs, in comparison, one was found at higher abundance (malate dehydrogenase; Figure 3) and one at lower abundance (isocitrate lyase) in *D. trenchii*–colonised anemones.

TABLE 1 | Integrated pathway analysis between Aiptasia in symbiosis with different species of symbionts (i.e., *Breviolum minutum*, *Durusdinium trenchii* or *Symbiodinium microadriaticum*) in response to heat stress (ramping to 32°C), and compared to each other at control (25°C) and heat stress ($n = 5$). Colour scale and numbers indicate pathway impact (from highest in red, through lesser impact in white, to lowest in blue), with * indicating the level of significance (*FDR < 0.05, **FDR < 0.01, ***FDR < 0.001), and values in parentheses represent the percentage of compounds (metabolites and proteins) involved in the pathway.

Pathway affected per species in response to heat stress in	***B. minutum***	***D. trenchii***	***S. microadriaticum***
Glycolysis or gluconeogenesis		0.49* (5.1%)	
Alanine, aspartate and glutamate metabolism		0.23* (6.3%)	
Protein processing in endoplasmic reticulum	0.01** (2.3%)		

	S. microadriaticum* versus *B. minutum		***D. trenchii* versus *B. minutum***		***S. microadriaticum* versus *D. trenchii***	
Pathway affected between species at treatment	**25°C**	**32°C**	**25°C**	**32°C**	**25°C**	**32°C**
Pyruvate metabolism	0.60*** (10%)	0.75*** (10%)	0.21* (4.3%)	0.75*** (10%)	0.40** (5.7%)	
Citrate cycle (TCA cycle)	0.66*** (10%)	0.82*** (12%)	0.39* (6%)	1.09*** (14%)	0.41* (6.0%)	
Glyoxylate and dicarboxylate metabolism	0.35*** (6.5%)	0.56*** (8.7%)	0.10* (3.3%)	0.39** (6.5%)		
Alanine, aspartate and glutamate metabolism	0.42* (6.3%)	0.23*** (9.4%)		0.20* (6.3%)		
Glycolysis or gluconeogenesis	0.23* (5.1%)				0.49*** (5.1%)	
Tyrosine metabolism	0.11* (4.4%)					
Glucagon signalling pathway	0.08* (3.8%)					
Propanoate metabolism	0.06* (4.9%)					
Amino sugar and nucleotide sugar metabolism	0.08* (3.2%)					
Glycine, serine and threonine metabolism		1.09*** (7.8%)				
Starch and sucrose metabolism			0.07* (4.1%)			
Phenylalanine metabolism			0.03* (3.9%)			
Oxidative phosphorylation				0.01** (4.7%)		

(Continues)

TABLE 1 | (Continued)

	S. microadriaticum* versus *B. minutum		***D. trenchii* versus *B. minutum***		***S. microadriaticum* versus *D. trenchii***	
Pathway affected between species at treatment	**25°C**	**32°C**	**25°C**	**32°C**	**25°C**	**32°C**
Parkinson's disease				0.01* (3.8%)		
Galactose metabolism				0.10* (5.2%)		
Non-alcoholic fatty liver disease (NAFLD)				0.03* (3.3%)		
Nicotinate and nicotinamide metabolism				0.36* (4.3%)		
Pentose phosphate pathway					0.05* (4.6%)	

Most DAMs, DAPs, and differentially regulated pathways between symbioses were related to core metabolism and, to a lesser extent, carbohydrate metabolism (Figure 5), in which hosts paired with heterologous symbionts showed lower relative abundances of energy stores such as carbohydrates. It seems probable that this was linked to lower quantities of photosynthate translocated from symbionts to hosts. This is very likely due, in part, to the markedly lower symbiont cell densities of the heterologous symbionts (Hoogenboom et al. 2010), although previous work has suggested that *D. trenchii* also has a lower cell–specific potential to provide photosynthetically fixed carbon to Aiptasia than does *B. minutum* (Matthews et al. 2017; Sproles et al. 2020). This could also be the case for *S. microadriaticum*: host carbohydrate reserves were greatly reduced in *S. microadriaticum*– versus *B. minutum*–colonised hosts, which is further supported by the differentially regulated pathways when comparing these two symbioses. These results are also supported by recent findings showing that *D. trenchii* and *S. microadriaticum* induce altered expression of symbiosome-associated metabolite transporters in Aiptasia, and that these differences are most pronounced in *S. microadriaticum*–colonised anemones (Mashini et al. 2022).

A previous metabolomic analysis of Aiptasia colonised by *B. minutum* or *D. trenchii* for 5 weeks, in which symbiont densities were similar to one another, showed increased mobilisation of carbohydrate and lipid stores, as well as increased catabolism of proteins and amino acids in *D. trenchii*–colonised hosts (Matthews et al. 2017), suggesting that this symbiont was unable to fulfil the host's nutritional requirements. The current study showed that, after more than 5 months in symbiosis, there was an increase in sucrose in *D. trenchii*–colonised anemones but a decrease in many other carbohydrates compared to *B. minutum*–colonised anemones. It is uncertain why sucrose increased in relative abundance, particularly in light of the lower population density of *D. trenchii*, but it could be related to other functions of this compound such as its osmotic properties that aid in protein folding during stress (Gouffi et al. 1998; Khan et al. 2010; Street et al. 2006). Indeed, increased concentrations of osmolytes have been suggested to increase thermal resistance in Aiptasia (Gegner et al. 2019).

In response to heat stress, hosts containing *S. microadriaticum* displayed no changes in their metabolome or proteome, possibly because they were already stressed when in symbiosis with this heterologous symbiont, whereas hosts containing *B. minutum* showed some significant changes (Figure 5). Specifically, heat-stressed *B. minutum*–colonised anemones displayed an increase in three sugars (*N*-acetylmannosamine, *N*-acetylglucosamine, and myo-inositol; Figures 2 and 4) and DAPs involved in core metabolism (e.g., sorbitol dehydrogenase, isocitrate lyase; Figure S3). In contrast, heat stress had a significant effect on the pathway 'glycolysis or gluconeogenesis' in *D. trenchii*–colonised hosts. There was a decreased abundance of metabolites involved in the TCA cycle and glycolysis, and 10 other metabolites associated with carbohydrate metabolism (Figure 2). Similarly, there was an upregulation of numerous proteins involved in core metabolism in the presence of *D. trenchii* (e.g., succinate–semialdehyde dehydrogenase, citrate synthase; Figures S3 and S4). Additionally, several proteins involved in the electron transport chain (i.e., increased respiration within the mitochondria) were found in increased abundance. Four other pathways were significantly upregulated with *D. trenchii* versus *B. minutum* under heat stress: 'oxidative phosphorylation', 'Parkinson's disease', 'non-alcoholic fatty liver disease', and 'nicotinate and nicotinamide metabolism'. Most compounds identified in these pathways overlapped among all four pathways (see Table S7). Functionally, these terms could indicate increases in oxidative phosphorylation, that is, increased respiration within the mitochondria, likely due to higher metabolic demands on host carbohydrate reserves (Figure 5).

The stronger response of *D. trenchii*–colonised hosts to heat stress is consistent with previous work which suggested that *D. trenchii* is more thermally sensitive when in symbiosis with Aiptasia

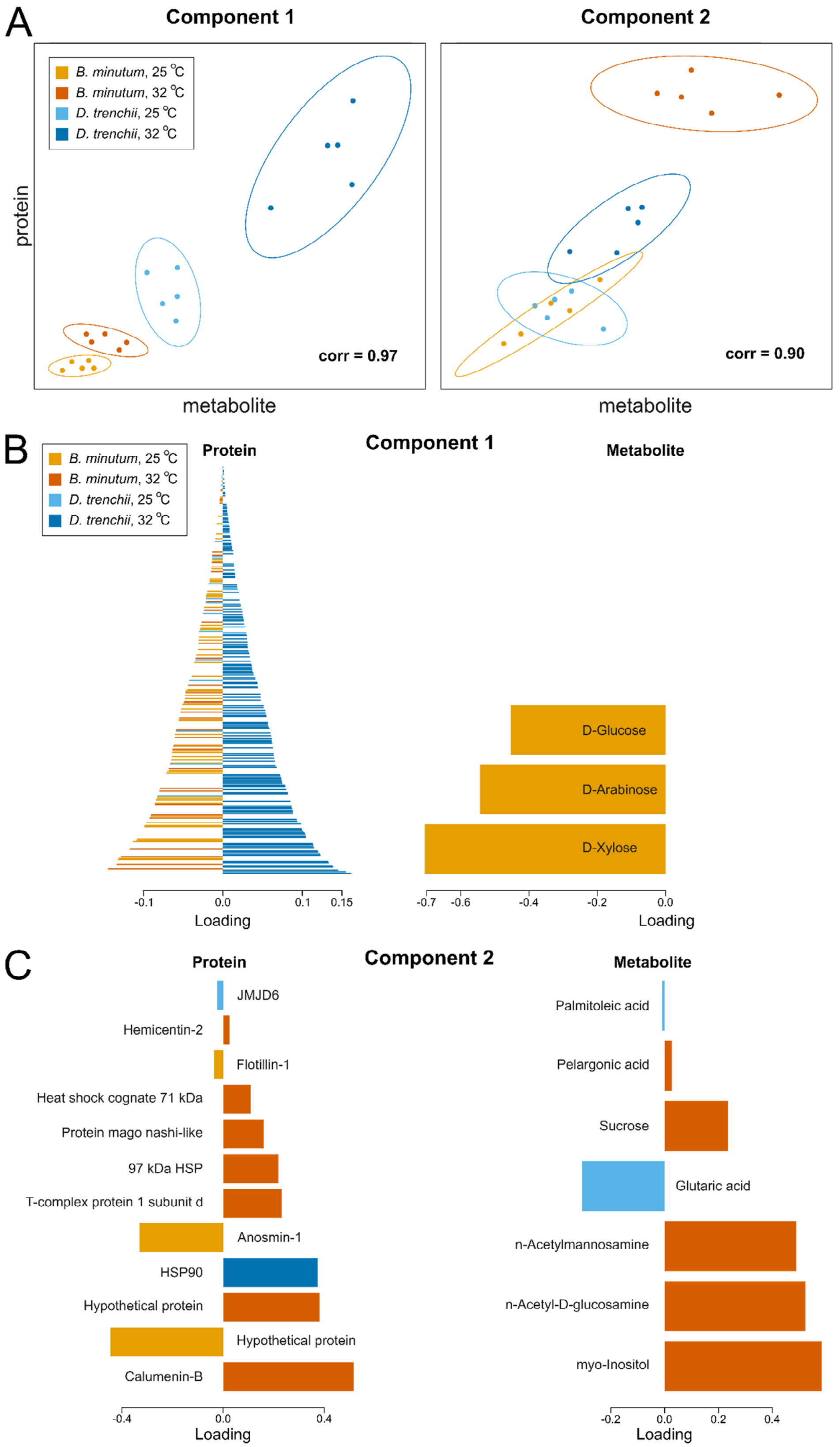

FIGURE 4 | DIABLO integration of metabolome and proteome data of Aiptasia hosting *Breviolum minutum* or *Durusdinium trenchii* to characterise thermal stress responses. (A) Correlation plot of latent Components 1 and 2, representing the correlation values (corr) between the protein and metabolite abundance datasets. 'Components' are artificial, linear variables constructed from the protein/metabolite data to reduce the number of dimensions. Each point represents one sample, ellipses are 95% confidence intervals. (B, C) Loading plot of the proteins or metabolites with non-zero loadings for Component 1 (B) or Component 2 (C). 'Loadings' are coefficients which indicate how much each protein/metabolite contributes to each component. Bar colours indicate the hosted species with which the protein/metabolite has the greatest abundance.

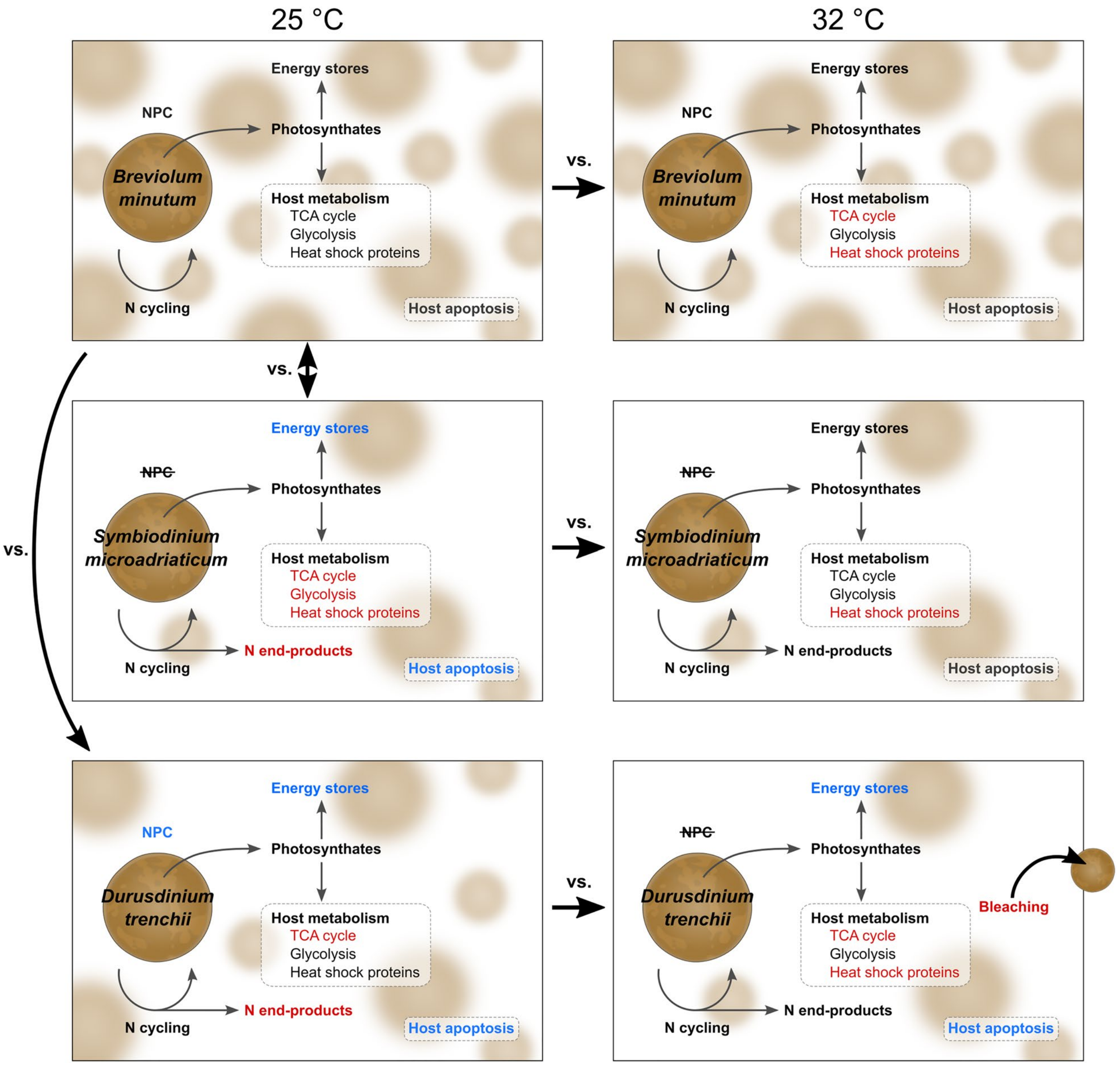

FIGURE 5 | Schematic overview of processes and symbiont densities in host tissues that differed between Aiptasia containing different symbiont species (i.e., *Breviolum minutum*, *Symbiodinium microadriaticum* or *Durusdinium trenchii*) at control temperature (25°C), and how processes changed within host-symbiont pairing in response to heat stress conditions (ramping to 32°C; $n = 5$). Red indicates an increase while blue indicates a decrease in process and/or process compounds. Grey indicates no change, whereas strikethrough indicates that the compound was not detected in this scenario. Mean symbiont cell densities (schematically reflected by brown shading) were 4.05, 0.55, and 2.38×10^6 cells mg^{-1} host protein for *B. minutum*–, *S. microadriaticum*–, and *D. trenchii*–colonised hosts at control temperature, respectively, and 3.91, 0.42, and 0.95×10^6 cells mg^{-1} host protein under heat stress, respectively.

than expected from studies of cultured Symbiodiniaceae (Gabay et al. 2019; Swain et al. 2017; McGinty et al. 2012). *D. trenchii* has previously been shown to provide less photosynthate to Aiptasia than *B. minutum* under non-stressful temperatures (Matthews et al. 2017; Sproles et al. 2020), and this distinction might be exacerbated by symbionts retaining more photosynthate to support their own metabolism under heat stress (Tremblay et al. 2016). In this case, *D. trenchii* would release even less photosynthetically fixed carbon to its host, which would need to further deplete its carbohydrate reserves to support its metabolism; this scenario is consistent with the metabolomic and proteomic changes observed.

4.2 | Protein and Amino Acid Metabolism and Nitrogen Availability

Under control conditions, symbiont identity also affected the metabolism of proteins and amino acids. Integrated pathway analysis showed that both 'alanine, aspartate and glutamate metabolisms' and 'tyrosine metabolism' differed in hosts colonised by *S. microadriaticum* versus *B. minutum* (Table 1), with higher abundances of alanine and tyramine in the presence of *S. microadriaticum* (Figure 2). When comparing *D. trenchii*– to *B. minutum*–colonised anemones, the pathway 'phenylalanine metabolism' was significantly different (Table 1), with an increased abundance of phenylacetic acid (Figure 2), a catabolite of the essential amino acid phenylalanine. DAPs related to these various pathways were generally found at lower abundances in hosts containing *D. trenchii* or *S. microadriaticum* than *B. minutum* (e.g., glutamate carboxypeptidase, Figure 3).

Heat stress led to significant changes in the 'alanine, aspartate and glutamate metabolism' pathway in anemones containing *D. trenchii* (Table 1), highlighted by a significant increase in the abundance of ethanolamine (Figure 2) and several DAPs (e.g., betaine-homocysteine *S*-methyltransferase 1 and delta-1-pyrroline-5-carboxylate dehydrogenase; Figure S3). These changes contributed to the partial convergence of the metabolomes and proteomes of anemones containing the two heterologous symbionts when exposed to heat stress, and their increasing dissimilarity to anemones containing the homologous *B. minutum* (Figure 4).

A reduced supply of photosynthetically generated sugars/carbohydrates from symbiont to host could have caused the observed increase in nitrogen-rich metabolites in the anemones associated with *D. trenchii* and *S. microadriaticum*, particularly during heat stress. An organic carbon supply, either from heterotrophic feeding or symbiont-supplied photosynthate, enables the host to conserve nitrogen (Wang and Douglas 1998; Cui et al. 2019), that is, it requires less catabolism of proteins and amino acids, and ammonium generated can be incorporated into organic compounds through GS-GOGAT (glutamine synthase–glutamine oxoglutarate aminotransferase) of Aiptasia. In contrast, both of the hosts with heterologous symbionts were significantly impacted in metabolic pathways related to amino acids. In particular, they displayed an increase in the relative abundance of urea and uric acid compared to *B. minutum*–colonised hosts (Figure 2), that is, elevated end-products from amino acid breakdown, in host tissues under both control and heat stress conditions. The proteomic data further supported increased protein degradation and turnover in the host, and a reduced capacity for nitrogen assimilation, which is most prominent in heat-stressed *D. trenchii*–colonised anemones (Figure 3). For example, some proteins involved in glutamate metabolism were elevated at higher temperatures, including delta-1-pyrroline-5-carboxylate dehydrogenase (ALDH4A1) and aspartate aminotransferase (GOT1). Both enzymes lead to glutamate production; ALDH4A1 through the conversion of ornithine or proline (Hu et al. 1996) and GOT1 through the conversion of aspartate or cysteine (Pavé-Preux et al. 1988). Glutamate is a by-product of protein breakdown, and protein breakdown and turnover were also inferred by the increased accumulation of proteases and peptidases. Conversely, glutamine synthetase (GLUL), which utilises glutamate and ammonium to catalyse the synthesis of glutamine, was much less abundant in heat-stressed *D. trenchii*–colonised anemones. Inorganic nitrogen assimilation in both host and symbiont is thought to be primarily regulated through GLUL (Wang and Douglas 1998; Miller and Yellowlees 1989; Pernice et al. 2012). These changes in protein and amino acid metabolism suggest a decrease in nitrogen assimilation and an increase in nitrogen catabolism, consistent with physiological stress and the utilisation of nitrogenous resources, as might be expected under extreme carbon supply limitation arising from photosynthetic dysfunction and/or a low symbiont population density.

4.3 | Stress Proteins

All host proteomes showed similar abundance patterns for proteins involved in heat stress and protein folding in response to elevated temperature: 10 DAPs for anemones containing *B. minutum*, 12 for anemones with *D. trenchii*, and eight for anemones with *S. microadriaticum* (Figure 3 and Figure S3). The protein that increased the most (5–7 $\log_2$-fold) for all host–symbiont pairings was the chaperone calumenin-B (CALUB). The accumulation of heat shock proteins (HSPs) and chaperones occurred irrespective of the symbiotic partner, which indicates a core response by the cnidarian host to heat stress (Rodriguez-Lanetty et al. 2009; Leggat et al. 2011). Nevertheless, this response did not necessarily predict whether or not bleaching occurred. In particular, while the abundance of HSPs increased from one to 11 in *D. trenchii*–colonised hosts in response to elevated temperature, this was insufficient to prevent bleaching (Figure 5). In contrast, *B. minutum*–colonised anemones underwent a similar increase of HSPs and molecular chaperones (assembly of complexes and protein folding; Figure S3) under thermal stress, accompanied by an upregulation of the 'protein processing in the endoplasmic reticulum' pathway (Table 1), yet their metabolome remained largely unchanged and they did not bleach. Anemones harbouring *S. microadriaticum* also did not bleach, despite their low initial symbiont population density. Of note, under control conditions these latter anemones possessed a broadly greater abundance of HSPs and proteins involved in oxidative stress responses than did anemones containing *B. minutum* (eight DAPs, Figures 3 and 5). These results are similar to several studies that reported oxidative stress proteins to be upregulated in aposymbiotic versus symbiotic anemones (Lehnert et al. 2014; Oakley et al. 2017; Ganot et al. 2011), indicating that very low symbiont cell densities, as for *S. microadriaticum* here, may render the host 'functionally aposymbiotic'. While *S. microadriaticum* has a relatively high thermal tolerance (Robison and Warner 2006; Ragni et al. 2010; Hawkins and Davy 2012; Krämer et al. 2012; Gabay et al. 2019), this high 'baseline' abundance of HSPs and oxidative stress proteins may explain why *S. microadriaticum*–colonised anemones did not bleach following exposure to heat stress. Such pre-conditioning has been observed in the coral *Montipora digitata*, in which a naturally high abundance of antioxidants may afford a relatively high degree of bleaching resistance (Krueger et al. 2015). It is clear from these results that the cellular processes underlying the differential bleaching sensitivity of different host–symbiont pairings are highly complex.

Symbiotic dinoflagellates typically suppress apoptosis in host cnidarian tissues (Rodriguez-Lanetty et al. 2006; Pernice et al. 2011; Gorman et al. 2022), which is thought to be necessary for the symbionts' persistence. Here, under control conditions, one calmodulin protein (calmodulin-like protein 3, CML3) was much more abundant in *S. microadriaticum*–colonised anemones, while two others (CALM and CAM1) were much less abundant than in anemones containing the homologous symbiont (Figure 3). Calmodulins activate apoptosis by increasing calcium uptake into mitochondria, which then activates various pathways leading to apoptosis (Berchtold and Villalobo 2014). Thermal bleaching and oxidative stress are associated with increased activation of apoptotic pathways (Weis 2008; Dunn et al. 2007; Suggett and Smith 2020). In *D. trenchii*–colonised anemones only one apoptosis-related protein (death-associated protein kinase 3) was less abundant than when *B. minutum* was present under control conditions. Heat stress seemed to strengthen suppression of host apoptosis in anemones hosting *D. trenchii*, which is an opportunistic species that can become dominant on reefs after bleaching episodes (Stat and Gates 2011; Gabay et al. 2018; Pettay et al. 2015; Silverstein et al. 2017; Lesser et al. 2013). Several DAPs involved in apoptosis, including calmodulins and calmodulin-like proteins, were found at lower abundance in heat-stressed *D. trenchii*–colonised hosts compared to *B. minutum*–colonised hosts, even though heat stress was accompanied by an increase in oxidative stress proteins for all associations. Inoculation of cnidarians with heterologous symbionts has been shown to rapidly increase apoptosis under normal temperatures (Dunn and Weis 2009; Matthews et al. 2017). In contrast, our proteomic changes suggest that heterologous symbionts suppress host apoptosis more strongly than the homologous *B. minutum* nearly 3.5 months after symbiosis establishment (Figures 3 and 5). This was unexpected, although the symbioses described here were sustained for a much longer time than in previous work (Matthews et al. 2017). Intracellular symbionts necessarily interact with their host's immune mechanisms, and calmodulin acts upstream to promote the activity of the central immune function transcription factor NF-κB via multiple pathways in mammals (Ishiguro et al. 2006; Lilienbaum and Israël 2003). Aiptasia NF-κB proteins are similar to those of vertebrates (Wolenski et al. 2011) and NF-κB expression has been shown to be inhibited by symbiosis (Mansfield et al. 2017), providing a potential mechanism linking calcium sensing via calmodulin to immune function. These results perhaps reflect improved integration between the host and a heterologous symbiont over time or manipulation of the host's apoptotic and immune responses by heterologous symbionts, though these hypotheses require more study.

This study also identified an increase in the methionine-synthesising enzyme betaine-homocysteine *S*-methyltransferase (BHMT) when Aiptasia was in symbiosis with the two heterologous symbionts, but only when heat-stressed (Figure S3). Previous research found an increase in the expression of BHMT homologues in aposymbiotic Aiptasia (Oakley et al. 2016) as well as in Aiptasia colonised by *D. trenchii* 5 weeks after initial inoculation (Sproles et al. 2019). This suggests host methionine limitation when hosting heterologous symbionts. Methionine, besides playing an important role in protein synthesis, is a precursor for the methyl donor *S*-adenosylmethionine (SAM) and the antioxidants glutathione and dimethylsulphoniopropionate (DMSP). DMSP levels are strongly correlated with photoinhibition and oxidative stress, and increase in response to acute heat (Raina et al. 2013) and osmotic stress (Aguilar et al. 2017); levels are also influenced by symbiont identity (Borell et al. 2016) and the coral-associated microbiome (Raina et al. 2013). The finding that BHMT did not increase under control conditions, in contrast to previous findings (Sproles et al. 2019), could indicate that the oxidative stress initially experienced by the host in response to colonisation by the heterologous symbiont (Matthews et al. 2017; Sproles et al. 2020, 2019), dissipates as the duration of the symbiosis increases. This idea is supported by the decreased accumulation of other oxidative stress-related proteins over time. These results support the findings that the symbiosis is more stable when Aiptasia associates with its native rather than a heterologous symbiont (Matthews et al. 2017; Gabay et al. 2019), and while the symbiosis with *D. trenchii* stabilises over time, it does not appear to confer thermal tolerance to the host.

4.4 | NPC Proteins

This study is the first to identify the presence of a Niemann-Pick type C2-like protein (NPC2a, Figure 3) in the pairing between Aiptasia and *D. trenchii*; it was detected in several samples in low quantities under control conditions. In contrast, NPC proteins were not previously detected in the association between Aiptasia and *D. trenchii* in a shorter-duration (5-week) study (Sproles et al. 2019), although they have been observed in a pairing between Aiptasia and *B. minutum*, which in that case was considered as a heterologous symbiont due to the clonal line of Aiptasia (CC7) used, which is not commonly associated with *B. minutum* (Cziesielski et al. 2022). NPC proteins play a role in cell recognition and immunity in mammals (Dani et al. 2014; Shi et al. 2012), and thus have been suggested to be important for the successful symbiosis establishment (Dani et al. 2014, 2017; Rosset et al. 2021). In cnidarians, these proteins are also thought to be involved in sterol transport between the symbiotic partners (Baumgarten et al. 2015; Lehnert et al. 2014; Oakley et al. 2016; Dani et al. 2014, 2017; Hambleton et al. 2019). The appearance of NPC proteins in a novel association could therefore be cautiously interpreted as a sign of improved host–symbiont integration over time, as it seems related to associations that have continued over longer time periods (e.g., nearly 3.5 months here vs. 5 weeks in the earlier studies of Sproles et al. 2019). More work is needed to test whether this is the case.

5 | Conclusion

Under control conditions, the Aiptasia host proteome and metabolome varied with symbiont identity. The extent to which this variability was influenced by the markedly different population densities of the symbiont species versus their innate physiological characteristics remains unclear, but the implications for the success and persistence of the symbiotic association are considerable. *S. microadriaticum*–colonised anemones displayed depleted carbon stores and increased turnover of proteins and amino acids. The metabolomic profile of *D. trenchii*–colonised anemones displayed fewer differences to *B. minutum*–colonised anemones than *S. microadriaticum*–colonised anemones, but under stressful conditions the differences between the anemones colonised by the two heterologous symbionts largely disappeared, revealing a more generalised response that is clearly distinct from anemones hosting their native symbionts. Hosts containing the heterologous symbionts exhibited depleted carbohydrate stores and exacerbation of thermal stress responses, which in turn may result in a potential increase in protein degradation and nitrogen metabolism (a consequence of lower population densities and/or inherent species-specific attributes). Such changes were not observed in the association with the homologous symbiont, with greater thermal tolerance *in hospite* potentially contributing to the relative success of *B. minutum* when inoculated simultaneously into Aiptasia with either *D. trenchii* or *S. microadriaticum* and exposed to elevated temperatures (Gabay et al. 2019). The expression of NPC proteins in the presence of the heterologous *D. trenchii* symbiont was notable, as this has not been observed in shorter-term studies, and may suggest improved physiological integration with time. Future research should confirm whether this is in fact the case, as, if so, it would have significant implications for the adaptive potential of the symbiosis and the potential survival of coral reefs.

Author Contributions

Bobby Lust: conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualization, writing – original draft. **Jennifer L. Matthews:** formal analysis, writing – review and editing. **Clinton A. Oakley:** conceptualization, formal analysis, funding acquisition, methodology, supervision, visualization, writing – review and editing. **Robert E. Lewis:** investigation. **Himasha Mendis:** formal analysis. **Lifeng Peng:** formal analysis. **Arthur R. Grossman:** conceptualization, methodology, writing – review and editing. **Virginia M. Weis:** conceptualization, methodology, writing – review and editing. **Simon K. Davy:** conceptualization, funding acquisition, methodology, resources, supervision, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data generated or analysed during this study are included in this published article and its Supporting Information. Protein mass spectrometry data are publicly available at the ProteomeXchange Consortium (Deutsch et al. 2017) via the PRIDE repository (Perez-Riverol et al. 2019) with identifier PXD055908. Metabolite mass spectrometry data are available from the MetaboLights repository (Yurekten et al. 2024) with the reference MTBLS11107. Sequencing data are available through Zenodo (Lust et al. 2025a) with reference 14708781. Supporting Information, figures and tables are available through Zenodo (Lust et al. 2025b) with reference 14863121.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.