Nutritional fluxes in the cnidarian- dinoflagellate symbiosis and the adaptability of corals to climate change

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Abstract

Symbiont diversity could be the answer to ensuring survival of coral reefs in light of a changing climate: changes in symbiont community composition could allow the host to adjust to prevailing environmental conditions. However, this is limited by sub-optimal performance when hosts are paired with non-preferred, i.e. heterologous, symbionts. The potential for cellular integration between host and heterologous symbiont is currently unknown. Here, I explore whether symbiosis function can improve over time when the model cnidarian *Aiptasia* (*Exaiptasia pallida*) is inoculated by heterologous symbionts, and the effect of symbiont diversity on host function in juvenile corals (*Acropora tenuis*). A combined omics approach (proteomics and metabolomics) and stable isotopic labelling were used to establish the potential for improved host-symbiont integration, by elucidating the physiological responses of the hosts, and nutritional exchange between the partners.

In Chapter 2, I characterised the host metabolome and proteome of *Aiptasia* under thermal stress, when in symbiosis with the homologous *Breviolum minutum*, or one of the heterologous, but more thermally tolerant symbiont species *Durisdinium trenchii* and *Symbiodinium microadriaticum*, over three months in symbiosis. The host metabolome and proteome were symbiont-specific under control conditions, and under thermal stress a clear divide between the host metabolome and proteome of homologous vs. heterologous symbiont-colonised anemones appeared. Differences included reduced carbohydrate stores and increased nitrogen metabolism in hosts paired with heterologous symbiont species compared to *B. minutum*-colonised hosts, and these were further exacerbated under heat stress. Moreover, heterologous symbionts induced much higher expression of host heat stress proteins (HSPs) and oxidative stress proteins, but suppressed host apoptosis-related proteins. These differences indicate the stressed state of the host when colonised by heterologous symbionts.

In Chapter 3, I investigated the effects of time and repeated thermal stress on the *Aiptasia* metabolome and proteome with these same symbionts, to assess whether time in symbiosis led to improved symbiosis function. Anemones were left in symbiosis for a total of seven months, while half of the population was exposed to repeated thermal stress and recovery. After this time, no convergence of the host metabolome or proteome was observed between anemones colonised by the heterologous and homologous symbionts. Patterns of protein and metabolite abundance were similar to those described in Chapter 2, with two interesting exceptions. First, anemones containing *D. trenchii* or *S. microadriaticum* expressed Niemann-Pick type C (NPC) proteins after three and seven months in symbiosis, respectively; these proteins have previously only been reported in the presence of homologous symbionts. Secondly, *S. microadriaticum*-colonised hosts contained more glycerol than those containing *B. minutum* at seven months, perhaps indicating improved metabolic benefit, or alternatively, cellular stress. Symbiosis with these heterologous symbionts therefore showed tentative signs of improvement over time, but only in the absence of thermal stress.

In Chapter 4, I assessed the flux of autotrophic carbon from symbiont to host, and heterotrophic nitrogen from host to symbiont, to determine if time in symbiosis or repeated heat stress events could alter these patterns. Two complementary mass spectrometry methods were used: NanoSIMS, to measure nutrient fluxes at the cellular scale, and IRMS, to measure bulk nutrient fluxes at the holobiont scale. *B. minutum*-colonised anemones displayed no changes in carbon or nitrogen enrichment with time or thermal regime, even though the cell densities of *B. minutum* decreased over time (between four and ten months after initial colonisation). By comparison, *D. trenchii* and *S. microadriaticum* translocated similar amounts of autotrophic carbon to the host at the cellular scale, but on the holobiont scale did not provide the hosts with as much carbon as *B. minutum* due to the much higher population density of the homologous symbiont. Additionally, *D. trenchii* translocated less photosynthate to the host than *S. microadriaticum* on a cell-specific basis, with this characteristic becoming more pronounced under thermal stress despite photosynthetic function and nitrogen consumption of *D. trenchii* being unaffected. Thus, *D. trenchii* seemed to display more opportunistic traits, with these becoming even more pronounced under thermal stress. By comparison, *S. microadriaticum* could be a more favourable symbiotic partner for *Aiptasia*, though no evidence was found of improved cellular integration over time, while its consistently low population density limited its overall benefit to the host.

In Chapter 5, I used the coral *Acropora tenuis* to investigate the effects of symbiont identity and thermal stress on the host proteome, thereby expanding on the model systems approach used elsewhere. Juvenile *A. tenuis* were inoculated with one of two homologous symbionts: *D. trenchii* and *Cladocopium* C1. Four weeks after colonisation, both symbiont species induced increases in host proteins associated with apoptosis, heat stress, and increased metabolic activity under control conditions. Despite these similarities, however, *Cladocopium* C1 induced a larger change in the host proteome than *D. trenchii*, with heat stress proteins (HSPs) and proteins involved in apoptosis being more abundant, and cell proliferation-related proteins being less abundant in *Cladocopium* C1-colonised corals. Under heat stress, the response of *A. tenuis* was again symbiont-specific. In particular, while *D. trenchii* is often considered as a thermally tolerant symbiont, corals containing *D. trenchii* exhibited the largest proteomic response to increased temperature. Indeed, my results suggest that *Cladocopium* C1 better prepares its host for thermal stress.

Overall, my results provide new insights into the symbiont-specific responses to symbiosis establishment and thermal stress. Additionally, I show the limited possibilities of improved host-symbiont integration over time or in response to repeated thermal stress events, when hosts are paired with heterologous symbiont species. Ultimately, host-symbiont specificity is a severely limiting factor when considering the adaptive potential of corals in response to climate change, highlighting the urgent need for the development of tools for managing the Coral Reef Crisis.

Contributions and publications

This thesis is written as a series of manuscripts in preparation for submission in the near future. As a result, there is some repetition of themes in between chapters, particularly in the “introduction” and “material and methods” sections. Tables that were too large for the printed document are available online as indicated in the Supplementary Data. This thesis is the intellectual and analytical work of the author, with assistance as described below.

Chapter 2: This chapter is formatted as an independent manuscript:

Lust B., Oakley C.A., Lewis R.E., Mendis H., Grossman A.R., Weis V.M., Davy S.K. Metabolomic and proteomic changes in response to symbiont identity and heat stress in the model cnidarian *Aiptasia*.

R.E. Lewis assisted with laboratory work. C.A. Oakley assisted with protein preparations, experimental design, and writing. GC-MS analysis was undertaken at Metabolomics Australia, The University of Melbourne, Australia. A. Lutz, D. de Souza and H. Mendis assisted with sample preparation and GC-MS analysis. V.M. Weis and A.R. Grossman assisted with experimental design. S.K. Davy assisted with experimental design and writing.

Chapter 3: This chapter is formatted as an independent manuscript:

Lust B., Oakley C.A., Lewis R.E., Mendis H., Grossman A.R., Weis V.M., Davy S.K. Temporal and thermal effects on the host metabolome and proteome of a model cnidarian when in symbiosis with homologous and heterologous symbionts.

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Chapter 4: This chapter is formatted as an independent manuscript:

Lust B., Oakley C.A., Escrig, S., Bury S., Grossman A.R., Meibom A., Weis V.M., Davy S.K. The influence of symbiont identity and thermal stress on inter-partner nutrient exchange in a model cnidarian-dinoflagellate symbiosis.

GC-MS analysis was performed at the National Institute of Water and Atmospheric Research, the Environmental and Ecological Stable Isotope Facility, Wellington, New Zealand. S. Bury, J. Delgado and J. Brown assisted with GC-MS sample preparation and analysis. NanoSIMS analysis was undertaken at the École Polytechnique Fédérale de Lausanne, Switzerland. S. Escrig and A. Meibom assisted with NanoSIMS preparation and analysis. V.M. Weis, A.R. Grossman and C.A. Oakley assisted with experimental design. S.K. Davy assisted with experimental design and writing.

Chapter 5: This chapter is formatted as an independent manuscript:

Lust B., Oakley C.A., Grossman A.R., Harii S., Weis V.M., Davy S.K. The effects of symbiont identity and acute heat stress on host proteome in juvenile *Acropora tenuis*.

Experimental work was conducted at the Tropical Biosphere Research Center of the University of the Ryukyus, on Sesoko Island, Okinawa, Japan. C.A. Oakley assisted with protein preparations, experimental design, and writing. S. Harii, F. Sinniger, V.M. Weis and A.R. Grossman assisted with experimental design. S.K. Davy assisted with experimental design and writing.

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Tūwhitia te hopo, mairangatia te angitū.

- Feel the fear, and do it anyway.

Table of contents

Abstract.....	3
Contributions and publications	5
Acknowledgements.....	7
List of figures.....	14
List of tables.....	17
List of abbreviations	18
Chapter 1 General introduction	21
1.1 Symbiosis.....	21
1.2 The cnidarian-dinoflagellate symbiosis	22
1.2.1 The cnidarian host.....	22
1.2.2 The dinoflagellate symbiont.....	23
1.2.3 The holobiont	24
1.2.3.1 Uptake, establishment, and regulation of the symbiosis	25
1.2.3.2 Host-symbiont specificity	27
1.3 Nutritional exchange within the symbiosis	27
1.3.1 Host supplied inorganic nutrients.....	28
1.3.2 Symbiont supplied photosynthate	29
1.4 The cnidarian-dinoflagellate symbiosis in a changing world.....	31
1.4.1 Cellular mechanisms of dysbiosis.....	32
1.4.2 Opportunities for climate change mitigation <i>via</i> symbiont diversity.....	33
1.5 The use of model systems	35
1.5.1 The sea anemone <i>Aiptasia</i>	35
1.5.2 The coral <i>Acropora tenuis</i>	36
1.6 Aims and objectives.....	37
Chapter 2 Metabolomic and proteomic changes in response to symbiont identity and heat stress in the model cnidarian <i>Aiptasia</i>	38
2.1 Abstract.....	38
2.2 Introduction.....	38

2.3	Methods.....	40
2.3.1	Experimental organisms.....	40
2.3.2	Inoculation	41
2.3.3	Experimental treatments and sampling	41
2.3.4	Homogenisation and separation of fractions.....	42
2.3.5	Confirmation of symbiont genotype	42
2.3.6	Metabolite extractions.....	43
2.3.7	Metabolite profiling	43
2.3.8	Protein extractions.....	44
2.3.9	Protein profiling	45
2.3.10	Data analysis and visualisation	45
2.4	Results.....	46
2.4.1	Symbiont cell densities & bleaching.....	46
2.4.2	Omics results.....	47
2.5	Discussion	57
2.5.1	TCA cycle, glycolysis, and carbohydrate metabolism.....	59
2.5.2	Nitrogen metabolism.....	60
2.5.3	Stress physiology	61
2.5.4	Other proteins of interest.....	63
2.6	Conclusion	64
Chapter 3	Temporal and thermal effects on the host metabolome and proteome of a model cnidarian when in symbiosis with homologous and heterologous symbionts	65
3.1	Abstract.....	65
3.2	Introduction.....	66
3.3	Methods.....	68
3.3.1	Experimental organisms and inoculation	68
3.3.2	Experimental treatments	68
3.3.3	Sampling, homogenisation, and separation of host and symbiont fractions	69
3.3.4	Confirmation of symbiont genotype and cell counts	69

3.3.5	Metabolite extractions.....	70
3.3.6	Metabolite profiling	70
3.3.7	Protein extractions.....	71
3.3.8	Protein profiling	72
3.3.9	Data analysis and visualisation	72
3.4	Results.....	73
3.4.1	Host growth and symbiont population density.....	73
3.4.2	Symbiosis-specific responses over time and thermal treatment in host metabolome and proteome	75
3.4.3	Inter-symbiosis differences in the host metabolome and proteome	84
3.5	Discussion	91
3.5.1	Symbiont population density and host growth.....	92
3.5.2	TCA cycle, glycolysis, and carbohydrate metabolism.....	94
3.5.3	Nitrogen metabolism.....	96
3.5.4	Stress physiology	99
3.5.5	NPC proteins.....	101
3.6	Conclusion	102
Chapter 4 The influence of symbiont identity and thermal stress on inter-partner nutrient exchange in a model cnidarian-dinoflagellate symbiosis.....		103
4.1	Abstract.....	103
4.2	Introduction.....	104
4.3	Methods.....	107
4.3.1	Experimental organisms.....	107
4.3.2	Inoculation with symbionts.....	107
4.3.3	Experimental treatments	108
4.3.4	Preparation of isotope solutions.....	108
4.3.5	Incubation and sampling	109
4.3.6	Preparation of samples for NanoSIMS	109
4.3.7	NanoSIMS image analysis.....	111
4.3.8	Preparation of samples for IRMS.....	111

4.3.9	IRMS sample analysis.....	112
4.3.10	Host growth, symbiont cell densities and genotyping	112
4.3.11	Statistical analysis of results	113
4.4	Results.....	114
4.4.1	Host growth and symbiont cell density.....	114
4.4.2	Autotrophic carbon enrichment	115
4.4.3	Heterotrophic nitrogen enrichment	119
4.5	Discussion	122
4.5.1	Autotrophic carbon translocation.....	123
4.5.2	Heterotrophic nitrogen translocation.....	125
4.6	Conclusion	126
Chapter 5	The effects of symbiont identity and acute heat stress on the host proteome in juvenile <i>Acropora tenuis</i>	128
5.1	Abstract.....	128
5.2	Introduction.....	128
5.3	Methods.....	132
5.3.1	Collection of corals and coral spawning	132
5.3.2	Larval maintenance and settling.....	132
5.3.3	Inoculation with symbionts.....	132
5.3.4	Experimental treatments	133
5.3.5	Sampling and processing	134
5.3.6	Confirmation of symbiont genotype	134
5.3.7	Protein extraction	135
5.3.8	Protein profiling.....	135
5.3.9	Data analysis and visualisation	136
5.4	Results.....	136
5.4.1	Response to symbiotic state	136
5.4.2	Response to thermal stress	141
5.5	Discussion	145

5.5.1	Effects of symbiosis establishment on the host proteome.....	145
5.5.2	Effects on heat stress and symbiont identity on the host proteome	149
5.6	Conclusion	151
Chapter 6	General discussion	153
6.1	Summary	153
6.2	Implications for the future of coral reefs.....	154
6.2.1	Will corals be able to adapt to climate change through adaptive bleaching?.....	154
6.2.2	What information can be taken from these studies to develop tools to aid in coral survival?	160
6.3	Limitations and future research.....	163
6.4	Concluding remarks	164
References.....		165
Supplementary data.....		207

List of figures

Figure 1.1	Organisation of a symbiotic cnidarian	24
Figure 1.2	Summary schematic of nutritional acquisition and exchange in the cnidarian-dinoflagellate symbiosis	29
Figure 1.3	Mechanisms of breakdown of symbiosis	33
Figure 1.4	Model organisms Aiptasia and <i>Acropora tenuis</i>	36
Figure 2.1	Cell density and PAM data for Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> , under control and heat stress	46
Figure 2.2	Photographs of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i>	47
Figure 2.3	PCA of host metabolome and proteome of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> comparing between species under control <i>versus</i> heat stress	48
Figure 2.4	PCA of host metabolome and proteome of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> comparing control <i>versus</i> heat stress <i>per</i> symbiont species	49
Figure 2.5	Relative host metabolite abundance of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> under control and heat stress	50-51
Figure 2.6	Differentially expressed proteins <i>per</i> category between Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> under control and heat stress	53
Figure 2.7	Heat map of differentially expressed proteins between Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> under control and heat stress	54
Figure 2.8	Metabolic pathway representation of heat stressed Aiptasia in symbiosis with <i>Durusdinium trenchii</i> or <i>Symbiodinium microadriaticum</i> compared to <i>Breviolum minutum</i>	58
Figure 3.1	Visual representation of experimental treatments and timing of sampling	69
Figure 3.2	Increase in total host protein content and symbiont cell densities of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> from three to seven months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last four months	74
Figure 3.3	Symbiont cell densities of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at three and seven months in	

	symbiosis and during heat stress events, having been maintained at control temperatures or exposed to repetitive heat stress for the last four months	75
Figure 3.4	PCA of host metabolome and proteome of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at three and seven months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last four months	77
Figure 3.5	Relative host metabolite abundance of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at three and seven months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last four months	78-79
Figure 3.6	PCA of host metabolome and proteome of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at seven months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last four months	85
Figure 3.7	Heat map of differentially expressed proteins between Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at seven months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last four months	87
Figure 3.8	Metabolic pathway representation of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at seven months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last four months	92
Figure 3.9	Metabolic pathway representation of Aiptasia in symbiosis with <i>Durusdinium trenchii</i> or <i>Symbiodinium microadriaticum</i> compared to <i>Breviolum minutum</i> at seven months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last four months	93
Figure 4.1	Visual representation of experimental treatments and timing of sampling	108
Figure 4.2	Increase in total host protein content and symbiont cell densities of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> from four to ten months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last six months	115
Figure 4.3	Symbiont cell densities of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at four and ten months in symbiosis and during heat stress events, having been maintained at control temperatures or exposed to repetitive heat stress for the last six months	116
Figure 4.4	Carbon enrichment of host and symbiont measured via NanoSIMS and IRMS for Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or	

	<i>Symbiodinium microadriaticum</i> at four and ten months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last six months	117
Figure 4.5	NanoSIMS images of carbon enrichment of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at four and ten months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last six months	118
Figure 4.6	Nitrogen enrichment of host and symbiont measured via NanoSIMS and IRMS for Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at four and ten months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last six months	120
Figure 4.7	NanoSIMS images of nitrogen enrichment of Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at four and ten months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last six months	121
Figure 4.8	NanoSIMS symbiont nitrogen enrichment over host carbon enrichment in Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at four and ten months in symbiosis, having been maintained at control temperatures or exposed to repetitive heat stress for the last six months	123
Figure 5.1	Fluorescence microscope images of <i>Acropora tenuis</i> colonised by <i>Cladocopium</i> C1 or <i>Durusdinium trenchii</i>	133
Figure 5.2	Host proteome PCA scores plot with 95% confidence intervals for <i>Acropora tenuis</i> in symbiosis with <i>Cladocopium</i> C1, <i>Durusdinium trenchii</i> , or aposymbiotic	138
Figure 5.3	Cell densities and PAM data for <i>Acropora tenuis</i> in symbiosis with <i>Cladocopium</i> C1 or <i>Durusdinium trenchii</i> under control and heat stress	141
Figure 5.4	Differentially expressed proteins per category for <i>Acropora tenuis</i> in symbiosis with <i>Cladocopium</i> C1 or <i>Durusdinium trenchii</i> under control and heat stress	142
Figure 5.5	Host proteome PCA scores plot with 95% confidence interval for <i>Acropora tenuis</i> in symbiosis with <i>Cladocopium</i> C1 or <i>Durusdinium trenchii</i> under control and heat stress	143
Figure 6.1	Summary of cellular processes in different host-symbiont pairings after prolonged time in symbiosis	157

List of tables

Table 2.1	Number of differentially expressed features between control and heat-stressed Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i>	48
Table 2.2	Differentially expressed host proteins in Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> in response to heat stress	52-53
Table 2.3	Differentially expressed host proteins between Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> , under control and heat stress	55-57
Table 3.1	Number of differentially expressed features in Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> over time in symbiosis and thermal regime, and between Aiptasia in symbiosis with these symbiont species at seven months in symbiosis under control or repetitive heat stress conditions	76
Table 3.2	Differentially expressed host proteins in Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> in response to time in symbiosis and thermal regime	81-83
Table 3.3	Differentially expressed host proteins between Aiptasia in symbiosis with <i>Breviolum minutum</i> , <i>Durusdinium trenchii</i> , or <i>Symbiodinium microadriaticum</i> at seven months in symbiosis under control conditions or exposed to repetitive heat stress	88-90
Table 4.1	Number of symbiont cells analysed via NanoSIMS per replicate anemone	111
Table 5.1	Differentially expressed proteins in <i>Acropora tenuis</i> induced by symbiosis with <i>Cladocopium</i> C1 and <i>Durusdinium trenchii</i> compared to aposymbiotic <i>A. tenuis</i>	139
Table 5.2	Differentially expressed proteins in <i>Acropora tenuis</i> in response to symbiosis establishment with <i>Cladocopium</i> C1	140-141
Table 5.3	Differentially expressed proteins in <i>Acropora tenuis</i> in symbiosis with <i>Cladocopium</i> C1 or <i>Durusdinium trenchii</i> in response to heat stress	144
Table 5.4	Differentially expressed proteins between heat stressed <i>Acropora tenuis</i> in symbiosis with <i>Cladocopium</i> C1 versus <i>Durusdinium trenchii</i>	145

List of abbreviations

¹³ C	Isotopically-labelled carbon
¹⁵ N	Isotopically-labelled nitrogen
AA	Amino acid
AAT	Aspartate aminotransferase
ABH	Adaptive bleaching hypothesis
ACN	Acetonitrile
AKR7A2	Aflatoxin B1 aldehyde reductase
AL4A1	Delta-1-pyrroline-5-carboxylate dehydrogenase
ALDOC	Fructose-bisphosphate aldolase X
ANOVA	Analysis of variance
APA	Alkaline phosphatase
ApoLp	Apolipoprotein
BHMT	Betaine-homocysteine S-methyltransferase
BLAST	Basic local alignment search tool
BME	Beta-mercaptoethanol
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CA	Carbonic anhydrase
CALUB	Calumenin-B
CCM	Carbon concentrating mechanism
DEM	Differentially expressed metabolite(s)
DEP	Differentially expressed protein(s)
DHW	Degree heating weeks
DIC	Dissolved inorganic carbon
DIN	Dissolved inorganic nitrogen
DOM	Dissolved organic nitrogen
EPFL	École Polytechnique Fédérale de Lausanne
FA	Fatty acid
FDR	False discovery rate
FP	Fluorescent protein
FUCA	Alpha-L-fucosidase
GABA	Gamma aminobutyric acid
GC-MS	Gas chromatography mass spectrometer
GFP	Green fluorescent protein
GLNA	Glutamine synthetase

GLUT	Glucose transporter
Hpcal4	Hippocalcin-like protein 4
HRF	Host release factor
HSP	Heat shock protein
IAA	Iodoacetamide
IRMS	Isotope-ratio mass spectrometry
ITS2	Internal transcribed spacer unit 2
Klp6	Kinesin-like protein 6
LC-MS	Liquid chromatography mass spectrometer
MAMP	Microbe-assisted molecular pattern
MDH	Malate dehydrogenase
MLS	Malate synthase
MRM	Multiple reaction monitoring
MTA	5'-methylthioadenosine
MtnN	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase-like isoform X1
NanoSIMS	Nanoscale secondary ion mass spectrometry
NPC	Niemann-Pick type C
PCA	Principal component analysis
PEPCK	Phosphoenolpyruvate carboxykinase
POM	Particulate organic nitrogen
PRR	Pattern recognition receptors
PSAT	Phosphoserine aminotransferase
RFP	Red fluorescent protein
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SGLT	Sodium-coupled glucose transporter
SST	Sea surface temperature
TCA	Tricarboxylic acid cycle
TMCS	Trimethylchlorosilane
TSR	Thrombospondin-type-1 repeat
VG	Vitellogenin

Chapter 1 General introduction

1.1 Symbiosis

In 1878, Anton de Bary defined symbiosis as “the living together of two unlike organisms” (Oulhen et al., 2016), to the benefit of at least one of the organisms. The symbiotic relationship can take on several forms and can be seen as a spectrum, varying on a gradient of benefit and complexity, as it ranges from parasitism to mutualism (Leung & Poulin, 2008). Parasitism occurs where one of the organisms benefits from the relationship at the cost of the other, for example a tapeworm in the digestive tract of a mammal (Tsai et al., 2013). Commensalism is the term given when one of the organism benefits at no cost to the other organism, like a barnacle living on the shell of a turtle (Zardus et al., 2014), whereas mutualism is the relationship that benefits both partners of the symbiosis, for example a clownfish living between the tentacles of an anemone (Litsios et al., 2012).

A symbiotic relationship is classified as ectosymbiotic when the symbiotic partners remain outside of each other, or endosymbiotic when the smaller of the partners (the symbiont) is located within the tissues of the other partner (the host). Together the symbiotic partners of an endosymbiosis are termed the holobiont. Mitochondria and plastids, such as the chloroplast, are the oldest known examples of endosymbiosis. Endosymbiotic theories, or symbiogenesis, explain how these organelles stem from prokaryotes being taken up by an early eukaryote, thus giving rise to eukaryotic cells as we know now (Sagan, 1967; Margulis, 1970; Lane & Martin, 2010; Martin et al., 2015). The main reason why these prokaryotes were not digested by the eukaryotic host is the benefit they provided to the host, allowing them to escape host digestion. Millions of years of evolution then led to establishment and tight integration of the symbiosis, including the movement of DNA from the endosymbiont genome to host genome (Allen, 1993, 2003), and the symbiotic partners have become so integrated that now, they seem to consist of just one entity.

A mutualistic symbiosis is not always equally beneficial to both partners. Facultative mutualists can survive and/or reproduce in isolation of the symbiotic partner, whereas obligate mutualists cannot survive for long periods in isolation (Starr, 1975). A mutualistic symbiosis offers a competitive advantage to the symbiotic partners, often due to the acquisition of a novel capability (Douglas, 1994). This is most likely in the form of nutrient synthesis or recycling between the partners. Therefore, a mutualistic symbiosis offers the partaking organisms new possibilities, such as overcoming limits of growth, or being able to spread to otherwise uninhabitable locations. A good example of this is a lichen, in which the cooperation of fungus and algae allows the holobiont to spread to one of the most uninhabitable conditions, the surface of bare rock (Honegger, 1991). The symbiosis allows them to

thrive there, while on their own neither of the symbiotic partners would have been able to survive this environment.

1.2 The cnidarian-dinoflagellate symbiosis

The symbiosis between cnidarians and dinoflagellates, such as in corals, is one of the oldest and most widespread obligate mutualistic endosymbioses. Coral reefs have existed for millions of years, and play numerous vital roles in ecosystem services (Hughes et al., 2017). They are considered one of the most complex ecosystems in the world, and the intricate system forms the base for several ecosystem processes, such as being spawning, nursery, breeding and feeding grounds for many marine species. Coral reefs are considered a biodiversity hotspot (McAllister, 1991); providing habitat for at least a quarter of all known marine species (Fisher et al., 2015). Coral reefs display high connectivity with other ecosystems, such as mangroves and seagrass beds, and many fish species found in these areas rely on coral reefs for at least one of their life-stages (Moberg & Folke, 1999). Mankind benefits from coral reefs in numerous ways, primarily in terms of fisheries, tourism, and coastal protection (Ruitenbeek et al., 1999; Kunkel et al., 2006; Spalding et al., 2017). Although it is difficult to express the value of these benefits in a comprehensible way, the value of such key ocean assets has been estimated as at least US\$ 24 trillion (Hoegh-Guldberg, 2015).

The basis of success of the cnidarian-dinoflagellate symbiosis is found in the exchange of nutrients between the symbiotic partners, which has long been a topic of interest (Muscatine & Hand, 1958; Muscatine & Lenhoff, 1963; Von Holt & Von Holt, 1968; Trench, 1971; Burriesci et al., 2012; Sproles et al., 2020). Principally, the symbionts are able to take up and use the cnidarian host's metabolic end products, in the form of inorganic nitrogen and carbon, for photosynthesis, and in return supply the cnidarian host with photosynthetic products. The endosymbiotic algae are essential for the existence of coral reefs, as they provide the animal with the majority of its required metabolic carbon (Muscatine et al., 1981), and facilitate nitrogen cycling and conservation (Wang & Douglas, 1998). Additionally, calcification rates are enhanced by the translocation of photosynthetic products from symbiont to host (Colombo-Pallotta et al., 2010; Holcomb et al., 2014; Bertucci et al., 2015), as well as by favourable changes in seawater and tissue carbonate chemistry by the photosynthetic consumption of CO₂ (Raybaud et al., 2017). This explains why coral reefs have vastly expanded and are highly productive in oligotrophic waters (Muscatine & Porter, 1977).

1.2.1 The cnidarian host

Cnidarians are some of the simplest multicellular organisms, such as jellyfish, sea anemones, and corals. Characteristic for cnidarians is the simple, radially symmetrical body plan. They are diploblastic, and

the tissue layers are organised around a central body cavity, the coelenteron. The outer tissue layer contains nematocysts, or stinging cells. A significant number of cnidarians are found in symbiosis with unicellular microalgae, which are located within the gastrodermal tissue layer (Figure 1.1). For reef-building corals, this is an obligate symbiosis. The origin of photosymbiosis in reef building corals occurred some 200 million years ago, during the Triassic period (Muscatine et al., 2005; Stanley Jr, 2006), and during the mid-Jurassic period there was an enormous diversification period (Leinfelder, 2001; Lathuilière & Marchal, 2009), giving rise to the many species of morphologically diverse corals we know today.

1.2.2 The dinoflagellate symbiont

Dinoflagellate symbionts within the family Symbiodiniaceae are a large and diverse group of unicellular algae, 6-12 µm in size. The family Symbiodiniaceae was previously perceived to be one global species, *Symbiodinium microadriaticum* (Freudenthal, 1962), primarily based on morphology. The advancement of molecular technologies allowed for the division of this one species into one genus, *Symbiodinium*, containing nine different clades with numerous sub-clades, also considered as ‘types’. The distinction between clades was made by sequencing of the nuclear internal transcribed spacer unit 2 (ITS2; Sampayo et al., 2009; LaJeunesse & Thornhill, 2011). However, since this division, new molecular evidence has established that these clades or types, can actually be classified as separate genera within the family Symbiodiniaceae (LaJeunesse et al., 2018), with currently eleven genera described within the family: *Symbiodinium*, *Breviolum*, *Cladocopium*, *Durusdinium*, *Effrenium*, *Fugacium*, *Freudenthalidium*, *Gerakladium*, *Halluxium*, *Miliolidium*, and *Philozoon* (LaJeunesse et al., 2018, 2021; Nitschke et al., 2020; Pochon & LaJeunesse, 2021). These genera and the species they contain continue to be expanded on (Fujise et al., 2021; González-Pech et al., 2021). It is now clear that these genera are the product of a long evolutionary history starting in the mid-Jurassic period, coinciding with the adaptive radiation of calcifying corals (Leinfelder, 2001; Lathuilière & Marchal, 2009).

Some genera within the Symbiodiniaceae are fully free-living, while others are mainly symbiotic, e.g. the genus *Breviolum* is primarily associated with cnidarians (LaJeunesse et al., 2018). Other genera that are found in association with cnidarians include *Symbiodinium*, *Cladocopium*, *Durusdinium*, *Gerakladium* and *Philozoon*. Between genera, there are large differences in gene composition, as well as host-range diversity, and biogeographic distribution (LaJeunesse, 2005; LaJeunesse et al., 2010, 2018, 2021; Nitschke et al., 2020; Pochon & LaJeunesse, 2021). The molecular diversity within the Symbiodiniaceae is reflected in their physiological diversity (LaJeunesse et al., 2018; Pochon & LaJeunesse, 2021). Some of the genera capture this physiological diversity; for example, the genus *Durusdinium* contains a lot of “extremophiles” in terms of temperature and turbidity tolerance (LaJeunesse et al., 2018). However, even within genera, species can be physiologically diverse, for

example with respect to photosystem function, inorganic carbon acquisition and stress tolerance (LaJeunesse et al., 2018, 2021; Nitschke et al., 2020; Pochon & LaJeunesse, 2021). Physiological diversity within the Symbiodiniaceae can in turn be translated to host physiology when in symbiosis (Matthews et al., 2017; Gabay et al., 2018), though different symbionts do not always cause metabolic differences in a host (Matthews et al., 2020).

1.2.3 The holobiont

The cnidarian-dinoflagellate symbiosis is an endosymbiosis, and the symbionts are located within host-derived vacuoles – “symbiosomes” – in the cytoplasm of the cnidarian host’s gastrodermis (Figure 1.1). The symbionts are surrounded by several layers of dinoflagellate-derived membranes and the outermost layer is a host-derived membrane, called the symbiosome membrane. Together, these layers form the “symbiosome membrane complex” (Roth et al., 1988; Wakefield & Kempf, 2001). The symbiosome membrane complex is the interface for molecular exchange between host and symbiont, and is important for the maintenance of symbiosis. Approximately one to six symbiont cells *per* host gastrodermal cell is housed in the cnidarian host, which has been termed the “cell specific density” (CSD; Muscatine et al., 1998).

Figure 1.1 (A) The sea anemone *Exaiptasia pallida* (‘Aiptasia’) with dinoflagellate endosymbionts (red). (B) Schematic representation of the tissue layers of a cnidarian showing the ectodermis (E), mesogloea (M), and gastrodermis (G) within which the symbionts (S) are located. (C) Transmission electron micrograph of a symbiont cell within *Aiptasia* gastrodermal tissue, surrounded by the symbiosome membrane (see arrow).

1.2.3.1 Uptake, establishment, and regulation of the symbiosis

Symbionts can be transmitted to offspring either horizontally or vertically. Horizontal transmission indicates that offspring need to acquire symbionts directly from the environment, while in vertical transmission, offspring acquire symbionts from the parent, usually *via* the egg (Padilla-Gamiño et al., 2012; Quigley et al., 2017). In horizontal transmission, it has been shown that initial uptake is nonspecific (Little et al., 2004), but horizontal transmitters can still display high specificity and symbiont fidelity (Trench et al., 1981; Coffroth et al., 2001). In order for this to occur, a sorting mechanism has to discriminate between different symbiont species intracellularly.

Uptake of symbionts usually occurs by feeding, as Symbiodiniaceae from the environment can be taken up along with food particles (Davy et al., 1997, 2012; Schwarz et al., 1999; Hambleton et al., 2014). Therefore, the symbionts enter the host through the gastrovascular cavity. From there, similar to food particles, they are taken up into the gastrodermis by endosomes destined for lysosomal degradation. The next step required for establishment of symbiosis is some sort of host-symbiont recognition in order to avoid host digestion by these lysosomes. This process, called “winnowing”, involves molecular signalling and cell-surface interactions, and it is suggested that markers released by the symbiont play a role in the required host-symbiont recognition (Weis et al., 2008).

Studies into pathogenic infection of cnidarian hosts and their immunity have inspired studies into the establishment of symbiosis, as evidence indicate that the symbiont is able to manipulate the host’s immune system and in this way avoid the maturation of endosomes into lysosomes and thus escape digestion (Chen et al., 2003, 2004, 2005; Hong et al., 2009; Neubauer et al., 2017; Jacobovitz et al., 2021). How Symbiodiniaceae manipulate the cnidarian host’s immune system is not exactly determined yet, but pattern recognition receptors (PRRs) that recognise microbe-associated molecular patterns (MAMPs) on microbe cell surfaces are suspected to be involved. For example, glycans are often found on the surface of Symbiodiniaceae cells, and glycan-binding proteins in the host, called lectins, could play a role in symbiont recognition and persistence (Wood-Charlson et al., 2006; Wood-Charlson & Weis, 2009; Logan et al., 2010; Bay et al., 2011). However, recent research has established that differences in glycan patterns between very diverse Symbiodiniaceae species are subtle and alteration of these patterns does not explain colonisation success between these species (Parkinson et al., 2018).

Another potential candidate in Symbiodiniaceae recognition is the Niemann-Pick protein complex (NPC). Studies have found an increase in host expression of these proteins when in symbiosis, and one of these proteins (AvNOC2-d) is associated with the symbiosome (Dani et al., 2014, 2017). In vertebrates, these proteins are involved in sterol trafficking in late endosomes and lysosomes, plus NPC proteins are thought to be involved in pathogen recognition and entry (Shi et al., 2012; Hambleton et

al., 2019; Rosset et al., 2021). A different study has found another possible candidate for recognition in the cnidarian-dinoflagellate symbiosis: thrombospondin-type-1 repeat (TSR) domain proteins. Up- or down-regulation of such TSR proteins has been found to either promote or dramatically reduce colonisation success in *Exaiptasia pallida* (commonly known as ‘Aiptasia’), respectively (Neubauer et al., 2017). Nonetheless, there is no consensus on exactly which protein complexes are involved in the recognition and successful establishment of this symbiosis.

As mentioned before, there is high diversity within the Symbiodiniaceae family, and hosts can take up a plethora of symbiont species, even if they are specialists (see Section 1.2.3.2). Any symbionts that do not get recognised properly in the winnowing process will be submitted to either host cell apoptosis, or are unable to prevent the maturation of endosomes into lysosomes and are therefore digested by the host (Chen et al., 2005; Dunn & Weis, 2009; Davy et al., 2012).

Once the symbiont population is established in the host, symbiont proliferation must be regulated. Symbionts seem to be quiescent in the G₁ phase of the cell cycle (Smith & Muscatine, 1999), at which point photosynthesis is highest. As the host grows, the symbionts must be stimulated to progress through cell division to maintain a constant host-symbiont biomass ratio; therefore proliferation may be regulated through feedback loops (McAuley, 1985; Fitt, 2000; Rodriguez-Lanetty et al., 2006; Detournay & Weis, 2011). Compared to cultured Symbiodiniaceae populations, cell division rates of symbiotic Symbiodiniaceae are strongly reduced, as otherwise the symbionts would grow faster than their host coral cells (Muscatine & Pool, 1979; Chang et al., 1983; Davy et al., 1996, 2012). To prevent this, the host exerts control over symbiont growth rate, and thus cell division, as well as operates mechanisms to rid its tissues of symbionts. There is evidence that the host may limit the algal symbiont’s growth by nutrient limitation (Cook & D’Elia, 1987; Hoegh-Guldberg & Smith, 1989; Muscatine et al., 1989; Jackson & Yellowlees, 1990; Rees, 1991; Muller-Parker et al., 1994; Goiran et al., 1996; Smith & Muscatine, 1999; Leletkin, 2000; Koop et al., 2001; Davy et al., 2012; Xiang et al., 2020). Surplus symbiont numbers are then removed from the host by one or more of the following mechanisms: (1) apoptosis, in which the host cell is destroyed along with its resident symbiont cells (Dunn et al., 2004, 2007a; Richier et al., 2006; Fuess et al., 2017); (2) autophagy, in which the symbiosome that contains the symbiont matures into a lysosome after all (Dunn et al., 2007a; Fuess et al., 2017); or (3) exocytosis or vomocytosis (Jacobovitz et al., 2021), in which the symbiont cell is expelled to the external environment. Additionally, symbionts can be removed by host cell detachment or necrosis (Gates et al., 1992; Weis, 2008). Stress can also induce termination of symbiosis *via* these mechanisms.

1.2.3.2 Host-symbiont specificity

The specificity of a symbiosis refers to the taxonomic range with which an organism associates (Silverstein et al., 2012). Within the diversity of cnidarian-dinoflagellate symbioses, generalists and specialists exist, in both host and symbiont species. A specific host will only tolerate one species of symbiont, whereas a specific symbiont will only pair with one host species, even if that host species contains multiple symbiont species (Belda-Baillie et al., 2002; Baker, 2003; Goulet, 2006; Putnam et al., 2012; Poland & Coffroth, 2017). Many corals typically form stable mutualisms with one or a very few dominant Symbiodiniaceae species, but new molecular techniques have revealed that they can also host several compatible background species (Carlos et al., 2000; Baker, 2003; Apprill & Gates, 2007; Loram et al., 2007; Micog et al., 2007; Correa et al., 2009; McGinley et al., 2012; Grégoire et al., 2017), and that community composition of symbionts is usually highly stable over time within a cnidarian host (Arif et al., 2014). The symbionts that persist in a host in the absence of stress, i.e. native symbionts, are referred to as being homologous (as opposed to heterologous, or non-native symbionts). The specificity in the cnidarian-dinoflagellate symbiosis suggests that co-adaptation of host and symbiont to environmental conditions has taken place (Trench, 1997), thus fine-tuning their symbiosis over a long evolutionary time period. Matthews et al. (2020) showed that the host metabolome of the coral *Montipora capitata* was the same for hosts paired with either *Cladocopium* spp. or *Durusdinium glynnii*, which are both considered as homologous symbionts for *M. capitata*. On the contrary, heterologous symbionts are known to induce many changes in the host proteome and metabolome, and affect host fitness in terms of reduced growth and impaired nutrient exchange (Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2019). Therefore, especially with regards to the work presented in this thesis, the pairing between host and homologous symbiont is taken to symbolise the ‘optimal state’ for host functioning, i.e. patterns of inter-partner nutritional exchange and communication are reflective of the most advantageous situation for both partners.

1.3 Nutritional exchange within the symbiosis

The metabolic exchange between symbiont and host forms the foundation of their relationship and this is therefore an important area of research when investigating the effects of climate change and symbiont diversity. The coral is dependent on its photosynthetic endosymbionts for its provision of nutrients, which is why dysbiosis can result in death of the coral (McClanahan, 2004). Millions of years in the oligotrophic waters where coral reefs thrive has led to tight nutrient cycling between host and symbiont. The cnidarian host supplies the symbionts with inorganic carbon and nitrogen, which the symbionts use for photosynthesis and respiration (Muscatine & Porter, 1977), while the symbiotic dinoflagellates provide a diverse array of photosynthetic products to the host. This bilateral exchange of nutrients is summarised in Figure 1.2.

1.3.1 Host supplied inorganic nutrients

As can be seen from Figure 1.2, the cnidarian host supplies the symbiotic cells with three essential elements: carbon, nitrogen and phosphorus. Non-symbiotic Symbiodiniaceae normally take up such inorganic nutrients from the external environment, i.e. the seawater, however, as the symbiont is encapsulated by the symbiosome membrane complex within the host tissues, required nutrients need to be transported from host tissues across this membrane complex to the symbionts.

Inorganic carbon is essential for photosynthesis and the symbionts receive this as: dissolved inorganic carbon (DIC) from the seawater, in the form of carbonate (HCO_3^-) or carbon dioxide (CO_2) (Furla et al., 2000; Furla, Galgani, et al., 2000); CO_2 from host and symbiont respiration (Harland & Davies, 1995); or CO_2 formed as a by-product during coral calcification (Ware et al., 1991). Any carbon that is supplied to the symbiont in a form other than CO_2 , needs to be transformed to CO_2 so that the symbionts can use it in photosynthesis. Thus, carbon concentrating mechanisms (CCM) and transport pathways need to be in place to facilitate the movement of inorganic carbon from the external environment through the host to the symbiont (Allemant et al., 1998). Both the dinoflagellate symbiont and the cnidarian host use carbonic anhydrases (CAs), proteins that aid in the conversion of bicarbonate to CO_2 , as part of the CCM (Weis, 1991; Bertucci et al., 2013). CA expression and activity in the host is dependent on symbiotic state (Weis, 1991; Ganot et al., 2011). Additionally, the host acidifies the symbiosome vacuole, which drives the pH dependent equilibrium towards the conversion of carbonate or bicarbonate to CO_2 , and thus allows inorganic carbon to be concentrated as CO_2 , which consequently helps to increase photosynthesis (Barott et al., 2015).

Inorganic nitrogen is sourced as dissolved inorganic nitrogen (DIN) from seawater in the form of nitrate (NO_3^-) or ammonium (NH_4^+) (Kopp et al., 2013, 2015), as well as from host metabolic end products that stem from heterotrophic feeding in the form of ammonium (Porter, 1974; Steen, 1986; Anthony & Fabricius, 2000), and from nitrogen fixation by bacterial colonies within the holobiont (Lesser et al., 2007; Freeman et al., 2016). Transport enzymes for nitrate and ammonium have been discovered in the genomes and transcriptomes of Symbiodiniaceae (Leggat et al., 2007; Sproles et al., 2018). Lastly, phosphorus is also supplied to the host *via* active transport of phosphate (Jackson & Yellowlees, 1990; Godinot et al., 2009; Ferrier-Pagès et al., 2016).

Once carbon and other inorganic nutrients have been taken up by the symbionts, it is fixed through the Calvin-Benson cycle (the C_3 pathway), and a range of organic molecules are synthesised. It can then be metabolised or stored by the algae, but it will mainly (potentially up to >99%) be translocated to the host (Muscatine & Hand, 1958; Muscatine & Cernichiaro, 1969; Davy et al., 2012; Tremblay et al., 2014).

Figure 1.2 Schematic diagram summarising the bilateral exchange of nutrients between host and symbiont in the cnidarian-dinoflagellate symbiosis. (1) Dissolved inorganic carbon (DIC) uptake from the surrounding seawater. (2) Photosynthesis; CO₂ is fixed by the symbiont through the Calvin-Benson cycle (the C₃ pathway), with the symbiont synthesising a range of organic compounds, such as amino acids. (3) Translocation of some of these compounds to the host. (4) Reverse translocation of organic compounds from the host to symbiont. These could arise either from host metabolism or be in the same forms as originated from the symbiont. (5) Host metabolism of received organic compounds, alongside dissolved organic matter (DOM) and particulate organic matter (POM) taken up from the seawater. The catabolism of nitrogenous compounds leads to the generation of nitrogenous metabolic end products, i.e., ammonium, which can be assimilated by the symbiont leading to (6) nitrogen recycling between host and symbiont. (7) Nitrate assimilation and (8) phosphate assimilation after uptake from seawater. Only the symbiont can convert nitrate to ammonium for subsequent assimilation. In addition to pathways shown, uptake of nutrients can also occur from the ambient seawater *via* the epidermis. Redrawn from Davy et al. (2012).

1.3.2 Symbiont supplied photosynthate

The first findings that confirmed the translocation of photosynthate from symbiont to host tissues used ¹⁴C radio-labelling methods (Muscatine & Hand, 1958; Von Holt & Von Holt, 1968). It was first thought that photosynthate is translocated mainly as glycerol (Muscatine, 1967; Battey & Patton, 1984), however more recent studies have confirmed the main compound to be glucose (Ishikura et al., 1999; Burriesci et al., 2012; Hillyer et al., 2018). Up to >99% of photosynthetically-fixed carbon is translocated from symbiont to host, contributing 50-100% of the host's daily carbon requirement in terms of respiration, maintenance and growth, depending on host species and light regime (Muscatine et al., 1981; Edmunds & Davies, 1986; Davies, 1991; Davy et al., 1996). It has been suggested that a 'host release factor' (HRF) stimulates translocation from symbiont to host (Muscatine et al., 1972; Gates

et al., 1999; Davy & Cook, 2001), but there is no agreement on the identity of this compound, or indeed whether it even exists (Gates et al., 1995; Grant et al., 1998; Cook & Davy, 2001). Nitrogen limitation by the host is another mechanism proposed to regulate both symbiont proliferation (Muscatine et al., 1989; Cook & Davy, 2001; Yellowlees et al., 2008), and carbon translocation (Wang & Douglas, 1998; Smith & Muscatine, 1999; Cook & Davy, 2001; Jiang et al., 2014); nitrogen limitation in the symbionts leads to the accumulation of carbon rich energy stores, such as lipid bodies, which may in turn be made available to the host.

Other compounds that are translocated from the symbiont to the host include other sugars, lipids, amino acids and glycoproteins (Papina et al., 2003; Burriesci et al., 2012; Kopp et al., 2015; Hillyer et al., 2016; Sproles, 2017). For example, several essential amino acids as well as omega-3 and omega-6 fatty acids (FAs), which cannot be synthesised by the cnidarian host, have been found to be translocated from symbiont to host tissues (Wang & Douglas, 1999; Hillyer et al., 2017). Fatty acids are precursor molecules to complex lipids, and are important in energy storage, membrane structure, cell signalling and homeostasis (Díaz-Almeyda et al., 2011; Dunn et al., 2012). Lipids have also been found to act as co-directors of phagocytosis (Heung et al., 2006; Steinberg & Grinstein, 2008), and thus could play a role in the establishment and/or maintenance of symbiosis.

While translocated carbon-rich compounds are mainly used for host respiration and energy stores, nitrogen-rich compounds, such as proteins and amino acids, received from the symbionts are important contributors to host calcification, growth and reproduction (Tanaka et al., 2006; Kopp et al., 2015). Thus, the translocation of fixed carbon from the symbiont to the host allows the host to conserve nitrogen-rich compounds, in a process termed “nitrogen conservation” (Wang & Douglas, 1998; Tanaka et al., 2006, 2017). Ultimately, the catabolism of nitrogenous compounds by the host leads to the generation of NH_4^+ as metabolic end products, which can then be assimilated by the dinoflagellates back into amino acids, completing the process of “nitrogen recycling” (Wang & Douglas, 1999; Tanaka et al., 2006, 2015; Davy et al., 2012; Pernice et al., 2012). The turnover rate of nitrogen within the holobiont is very slow, taking more than one year to complete (Tanaka et al., 2018). Nitrogen conservation and recycling are crucial aspects of the cnidarian-dinoflagellate symbiosis, given the oligotrophic nature of the waters that they regularly frequent (Rädecker et al., 2015).

The transport of large molecules across the symbiosome membrane requires the incorporation of active transport molecules. Two families of glucose transporter proteins have been identified in *Aiptasia*, the facilitated glucose transporter (GLUT) and the sodium-coupled glucose transporter (SGLT) (Baumgarten et al., 2015; Sproles et al., 2018). Of these two protein families, many more sequences have been identified in the GLUT family in cnidarians (Sproles et al., 2018). Gene expression analysis has indicated that one of these transporters, GLUT8, is upregulated in the symbiotic state compared to

the non-symbiotic state (Lehnert et al., 2014), thus suggesting that this transporter family is involved in the transport of glucose across the symbiosome membrane complex.

1.4 The cnidarian-dinoflagellate symbiosis in a changing world

Stress can induce the termination of symbiosis between the cnidarian host and the photosynthetic symbiont. This is also called dysbiosis or, in corals, bleaching, named so due to the loss of colour in the corals and the tissues appearing white, or bleached, due to the visibility of the coral skeleton (Lesser, 1997; Hoegh-Guldberg, 1999; Douglas, 2003; Weis, 2008). Bleaching can be triggered by several types of stress, for example extremes of temperature or high irradiance. In the era of the Anthropocene, coral reefs experience a variety of local stressors. These include pollution, sedimentation, nutrient enrichment, over-fishing of reef species, or outbreaks of the crown-of-thorn starfish (*Acanthaster planci*), oftentimes several of these taking place concurrently (Hughes, 1999; Hoegh-Guldberg et al., 2007; Carilli et al., 2009; Russell et al., 2009). Nevertheless, one threat common to all coral reefs is global anthropogenic climate change (Hoegh-Guldberg, 1999; Dove et al., 2013; Hughes et al., 2018). Although the susceptibility to bleaching is dependent on the interplay between host tolerance and Symbiodiniaceae genetic variation (Krueger et al., 2014; Hawkins et al., 2016; Sampayo et al., 2016), however, once bleached, if the coral cannot regain a symbiosis with Symbiodiniaceae, it will die (McClanahan, 2004; Grottoli et al., 2014).

As corals live very close to their upper thermal tolerance, an increase of only 1 °C can exert immense stress on the coral and, if this small temperature increase is prolonged, it can induce bleaching. Due to this, a measure has been established that indicates the severity and duration of thermal increases on a coral reef or region, namely ‘degree heating weeks’ (DHW; Gleeson & Strong, 1995). DHW is a measure of accumulated temperature stress over a rolling period of 12 weeks for a given region, thus adding up the temperature anomalies exceeding the maximum of the monthly sea surface temperature (SST) above the seven-year climatology. For example, 1 DHW indicates that the temperature on the reef has been higher than the average temperature by 1 °C for one week during the last 12 weeks. 4 DHW indicates that the temperature has been higher either by 4 °C for one week, by 1 °C for four weeks, or anything in between. Therefore, DHW measures cumulative thermal stress experienced by reefs, and has been found to be a good indicator of coral bleaching (Liu et al., 2003, 2006, 2013; Eakin et al., 2010; Spillman et al., 2013; Kayanne, 2017; Hughes et al., 2018).

Hughes et al. (2018) found that coral reefs display mortality from three to four DHW, with typical losses of more than 50% from four to five DHW. Eight months after bleaching, heavy transformations in the functional-trait composition of assemblages of coral reefs were found, indicating a shift away from fast growing, branching and tabular species that provide most of the three-dimensionality of reefs.

Additionally, many of the corals that survive bleaching and regain their photosynthetic symbionts still continue to die slowly due to severe loss of tissue, fragmentation or injury, and because of their increased susceptibility to disease (Muller et al., 2008; J. Miller et al., 2009). This is alarming, as replacement of dead corals by larval recruitment and coral growth will take at least a decade, even for fast-growing, highly fecund coral species (Kayanne et al., 2002; Gilmour et al., 2013), and much longer than that for longer-lived, slow-growing species. Lack of connectivity between coral reef ecosystems will only complicate such recovery, as well as the presence of macroalgae, sponges or other organisms that can exclude and outcompete corals (Aronson et al., 2000; Roff & Mumby, 2012). Moreover, reefs that experience local stresses concurrently with thermal stress have less resilience to withstand bleaching (Carilli et al., 2009). This has disastrous consequences. If the human population continues to emit greenhouse gases at the current rate of increase, coral reefs are projected to disappear by 2050 (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007; Veron, 2008; Burke et al., 2011; Frieler et al., 2013).

1.4.1 Cellular mechanisms of dysbiosis

The cellular mechanisms that trigger the coral host to initiate dysbiosis are not yet fully resolved even after over three decades of research (reviewed in: Weis, 2008; Oakley & Davy, 2018; Suggett & Smith, 2020). Increased temperature can cause failure of the photosynthetic mechanisms in Symbiodiniaceae. Failure of photosystem II leads to build-up of reactive oxygen species (ROS) in the chloroplast membranes of the algae. If any ROS leak out of the symbiont cells, this leads to oxidative stress in the host cells, which can then initiate pathways that lead to the removal of the initial cause of this stress, the symbionts. Although ROS are thought to play a role in bleaching, it is unsure whether this is a primary cause of bleaching or a secondary consequence. In particular, expelled symbionts are usually photosynthetically viable, which supports the hypothesis that ROS are only a secondary consequence rather than an initial cause (Ralph et al., 2001; Hill & Ralph, 2007). Second, the host might experience oxidative stress before the symbiont does, as indicated by the research of Hawkins et al. (2014), Krueger et al. (2015), and Hillyer et al. (2017), who all found evidence of increased ROS as well as antioxidant upregulation in the host, without such upregulation in symbionts after thermally induced bleaching.

Once triggered, the removal of symbionts from the host tissues can occur in several ways (Figure 1.3). Symbionts can be lost by *in situ* degradation, in which the symbiosome matures into a late lysosome, or symbionts can be expelled to the external environment *via* exocytosis or vomocytosis (Jacobovitz et al., 2021). In severe states of stress the host cells are also affected by the dysbiosis, and symbionts are expelled *via* host cell detachment, apoptosis or necrosis (Dunn et al., 2002, 2004; Bieri et al., 2016).

Figure 1.3 Mechanisms by which symbiont cells (S) can be lost from host (H) tissues; this can happen as *in situ* degradation, or expulsion of symbiont cells *via* exocytosis. Hosts cells have also been observed undergoing apoptosis, necrosis, or detachment. M stands for host mesogloea. Figure redrawn from Weis et al. (2008).

Factors that affect metabolic stability influence bleaching sensitivity in corals. For example, susceptibility to bleaching is increased on reefs exposed to eutrophication (D'Angelo & Wiedenmann, 2014; Donovan et al., 2020), or deoxygenation (Alderdice et al., 2021). Indeed, destabilisation of inter-partner nutrient exchange has been observed prior to the onset of bleaching (Rädecker et al., 2021). Like the occurrence of ROS, the exact cellular processes behind dysbiosis due to metabolic instability remain to be resolved. A suggested mechanism is the host control mechanism of symbiont proliferation through nutrient limitation (Smith & Muscatine, 1999; Cui et al., 2019). Increased nutrient levels in the seawater increase symbiont cell densities in host tissues (Blank & Muscatine, 1987; Wooldridge, 2016, 2017). When symbiont cell density is higher than optimal, the onset of bleaching occurs earlier (at lower temperature) and the coral will bleach more severely (Cunning & Baker, 2014). The impact of a nutrient spike on symbiont cell density can last up to several months, leaving the corals sensitive to bleaching at mild thermal stress even after the nutrient levels have returned to normal for the coral reef environment (Falkowski et al., 1993).

1.4.2 Opportunities for climate change mitigation *via* symbiont diversity

It has been suggested that bleaching increases the survival of the host by providing an opportunity to alter its symbiont community, described in the adaptive bleaching hypothesis (ABH; Buddemeier & Fautin, 1993). This can potentially occur by two mechanisms, namely symbiont “shuffling” and

symbiont “switching”. Shuffling occurs when the relative abundance of the species of symbionts present changes, e.g. a species that was present in low numbers now becomes the dominant symbiont species. In the case of symbiont switching, the present symbiont species is completely replaced by a different symbiont species. These repopulation mechanisms could provide a short-term solution to increased thermal stress, by taking in symbionts with a higher thermal tolerance (Buddemeier & Fautin, 1993; Baker, 2001; Baker et al., 2004; Rowan, 2004; Berkelmans & Van Oppen, 2006; Mieog et al., 2007; Silverstein et al., 2017). However, specificity is likely to play a restrictive role in the host’s ability for symbiont switching and/or shuffling (Baker, 2003; Goulet, 2006; Gabay et al., 2019).

Previous research has shown that certain Symbiodiniaceae species are more resistant to elevated temperature compared to others (Rowan, 2004; Howells et al., 2012; LaJeunesse et al., 2014; Silverstein et al., 2017). For example, *Cladocopium thermophilum* (previously known as *Symbiodinium thermophilum*) has been found to be the most heat resistant symbiont species (Hume et al., 2013, 2015; Howells et al., 2020). Additionally, *Durusdinium trenchii* (previously known as *Symbiodinium trenchii*, LaJeunesse et al., 2018), also has a higher thermal tolerance compared to many other species (Toller et al., 2001; Mieog et al., 2007; Mostafavi et al., 2007; Silverstein et al., 2017). This translates to the host’s thermal tolerance: corals containing *D. trenchii* have been found to be more resistant to bleaching, extending their host’s thermal tolerance by 1-2 °C (Leal et al., 2015; Silverstein et al., 2017). In support of the ABH, several studies have found the abundance of *D. trenchii* on coral reefs to increase after bleaching events (Baker et al., 2004; Jones et al., 2008; LaJeunesse et al., 2009; Boulotte et al., 2016).

Although such non-native, or ‘heterologous’, symbiont species could provide benefits to the host in periods of thermal stress, when temperatures return to normal, symbiont communities often do too (LaJeunesse et al., 2009). Previous research has shown that heterologous symbionts, such as *D. trenchii*, are less beneficial to the host when no stress is experienced (Jones & Berkelmans, 2011; Pernice et al., 2014; Starzak et al., 2014; Matthews et al., 2017; Gabay et al., 2018), leading to reduced host growth and reproduction, as well as smaller energy reserves. Therefore, it is possible that *D. trenchii* is not necessarily a beneficial species in times of stress, but rather a more opportunistic symbiont, invading the coral tissue when its immune system is compromised due to heat stress (Stat & Gates, 2011). Model simulations by Ortiz et al. (2013) concluded that *D. trenchii* might actually diminish the capacity of the reefs to recover in response to bleaching events due to this reduced host growth and reproduction when stressful conditions are absent. Of note, however, in these models they did not include the possibility that reefs might switch or shuffle symbiont communities back to the homologous species, which often occurs (LaJeunesse et al., 2009).

Another interesting point is raised by the research of Sachs & Wilcox (2006), who showed that the symbiont transmission mechanism in the jellyfish *Cassiopeia xamachana* could alter cooperative traits

of its symbiont community. Their results showed that horizontal transmission allows the symbiont community to become more opportunistic, while vertically transmitted symbionts show selection for cooperation with the host, measured as increased host reproduction and growth. This suggests that a selection mechanism exist in the host, and thus vertical transmission could present the host with an opportunity for building a more cooperative symbiosis, i.e. to choose more favourable symbionts over less favourable ones to pass on to the next generation, and therefore optimise the symbiosis over time.

In summary, the cnidarian-dinoflagellate symbiosis can be more or less mutualistic depending on the partners involved: it can be considered as a spectrum, ranging from more mutualistic to less mutualistic, and perhaps even parasitic (Abrego et al., 2008; LaJeunesse et al., 2009; Wooldridge, 2010; Baker et al., 2018). With climate change, insurmountable challenges are posed to the optimal state of the cnidarian-dinoflagellate symbiosis, i.e. the pairing between cnidarian host and homologous symbiont. Therefore, future research should focus on the metabolic exchange between heterologous species and the host, to investigate the limits that heterology poses, i.e. to outline the possibility of *D. trenchii* and other thermally tolerant species to mitigate the negative effects of thermal stress, thus offering corals some hope in this rapidly changing world.

1.5 The use of model systems

1.5.1 The sea anemone *Aiptasia*

The sea anemone *Exaiptasia pallida*, widely known as ‘Aiptasia’ (Figure 1.4A; Grajales & Rodríguez, 2014) has been globally adopted as a model system for the study of cnidarian-dinoflagellate symbioses (Weis et al., 2008; Dunn et al., 2012; Starzak et al., 2014; Grawunder et al., 2015; Matthews et al., 2016; Oakley et al., 2016; Hillyer et al., 2017; Sproles et al., 2020). Aiptasia is easier to maintain in laboratory settings than many coral species and has a much higher growth rate. It is possible to establish clonal lines, which enables the study of host processes in the absence of biological variation. Although the Aiptasia-dinoflagellate symbiosis lacks some key components of a coral-dinoflagellate symbiosis, such as a hard calcareous skeleton, it resembles the symbiosis in many ways. Aiptasia is commonly found in association with *B. minutum* (Lajeunesse et al., 2012; Thornhill et al., 2013a; Starzak et al., 2014), but can be rendered and maintained aposymbiotic, i.e. without symbionts, and subsequently recolonised by a range of different symbiont species (Weis et al., 2008; Matthews et al., 2016; Gabay et al., 2018). This includes species with which it does not normally form a symbiotic relationship in nature. Additionally, many genetic resources are available for the study of Aiptasia (Sunagawa et al., 2009; Baumgarten et al., 2015). For these reasons, Aiptasia provides an ideal system for the examination of the cellular mechanisms involved in the onset and maintenance of symbiosis with new partners. For this thesis, the clonal line NZ1 was used.

1.5.2 The coral *Acropora tenuis*

The genus *Acropora* contains some of the world's major reef-building corals, making it a keystone taxon (Veron, 2000; Wilson et al., 2019), and is characterised by high growth rates as well as high bleaching susceptibilities (Baird & Marshall, 2002). *Acropora tenuis* (Figure 1.4B) is widely used in coral symbiosis research (Van Oppen et al., 2001; Bay et al., 2011; Yuyama et al., 2012; Yamashita et al., 2013; Quigley et al., 2017; Yorifuji et al., 2017; Seveso et al., 2018), as it transmits its symbionts vertically, meaning that larvae and juveniles can be maintained symbiont-free and colonised with specific symbiont species. It is a step up from *Aiptasia* in complexity, and thus comes with more challenges in its study. Unlike *Aiptasia*, *Acropora* species do not survive long aposymbiotically, so the natural occurrence of symbiont-free juveniles is useful in the study of the onset and maintenance of symbiosis. Additional benefits in the use of *A. tenuis* relate to the ability to readily induce metamorphosis with a synthetic peptide (Iwao et al., 2002), which allows for the establishment of synchronised populations of juveniles. Finally, valuable molecular resources are available for *A. tenuis*, such as fully a published genome (Liew et al., 2016; Yoshioka et al., 2020), as well as an unpublished transcriptome assembly (Matz lab, 2012).

Figure 1.4 Model systems used for the study of the cnidarian-dinoflagellate symbiosis: (A) The sea anemone *Exaiptasia pallida* ('Aiptasia') of the clonal line NZ1, and (B) a fragment of *Acropora tenuis* (right), here seen glued back onto the reef after the spawning event of 2018 at Sesoko island, Okinawa, Japan.

1.6 Aims and objectives

The primary aim of this study was to establish the combined effect of symbiont identity, thermal stress, and time in symbiosis on host physiology. To achieve this aim, the project consisted of the following specific objectives and hypotheses:

1. To establish the effect of symbiont identity and thermal stress on the host proteome and metabolome in *Aiptasia*. Hypotheses: a) host proteomic and metabolomic profiles will differ with symbiont identity; b) host proteomic and metabolomic profiles will be significantly affected by thermal stress and differ depending on symbiont identity.
2. To establish the effects of symbiont identity and time in symbiosis, including repeated thermal stress events, on the host proteome and metabolome in *Aiptasia*. Hypotheses: a) symbiont identity will have a significant effect on the host proteome and metabolome; b) the host proteome and metabolome will converge over time in anemones colonised by heterologous symbionts to more closely resemble those of anemones colonised by homologous symbionts; and c) repeated thermal stress events will promote this convergence through selection processes.
3. To establish the effect of symbiont identity, thermal stress, and time in symbiosis on nutritional exchange between the host and symbiont in *Aiptasia*. Hypotheses: a) symbiont identity will significantly affect nutritional exchange; b) increased time in symbiosis will lead to nutritional exchange in anemones colonised by heterologous symbionts to more closely resemble that of anemones colonised by homologous symbionts; and c) repeated thermal stress will facilitate this temporal convergence through selection processes.
4. To determine the effect of symbiont identity and thermal stress on the host proteome in the coral *A. tenuis*. Hypotheses: a) the host proteome will differ in response to symbiont identity; b) acute heat stress will affect the host proteome differently depending on symbiont identity.

Ultimately, this thesis sought to elucidate and expand our understanding of how thermally tolerant symbionts might aid adaptation to climate change through host-symbiont shuffling or switching.

Chapter 2 Metabolomic and proteomic changes in response to symbiont identity and heat stress in the model cnidarian *Aiptasia*

2.1 Abstract

This study investigated the effect of symbiont identity and heat stress on the *Aiptasia* metabolome and proteome to establish how heterologous yet more thermally tolerant symbionts affect host function under stress in a model organism. Three species of symbionts were used: the homologous *Breviolum minutum*, and the heterologous *Symbiodinium microadriaticum* and *Durisdinium trenchii*. Three months after colonisation with one of these symbionts, *Aiptasia* were either maintained at the control temperature of 25 °C or ramped to 32 °C to induce thermal stress. Symbionts did not reach similar cell densities, with *D. trenchii* reaching only 60% and *S. microadriaticum* reaching only 15% of the density established by *B. minutum*. Under control conditions, results show that the host metabolome and proteome of *D. trenchii*-colonised hosts is intermediate to those with *B. minutum* and *S. microadriaticum*. Differences between the associations show that *S. microadriaticum*-colonised hosts have reduced carbohydrate stores, increased nitrogen metabolism, and much higher expression of HSPs and oxidative stress proteins. In contrast, *D. trenchii*-colonised host metabolome and proteome resembles the association with *B. minutum* more closely, as it displays fewer reductions in carbohydrate stores. However, under elevated temperature, hosts containing *B. minutum* or *S. microadriaticum* do not experience stress caused by symbionts, where those in symbiosis with *D. trenchii* do. In fact, holobionts containing *D. trenchii* were the only association that displayed bleaching. Heat stress caused a clear divide between heterologous associations and the homologous state. Under heat stress, heterologous associations show large increases in nitrogen and carbohydrate metabolism, resulting in largely depleted carbohydrate stores, as well as large increases in expression of HSPs, oxidative stress proteins, and a reduction in host apoptosis-related proteins.

2.2 Introduction

Symbiosis is the living together of two unlike organisms (Oulhen et al., 2016), and ranges from a spectrum of benefit for both partners (mutualism), to benefit for one partner and disadvantage for the other (parasitism). The cnidarian-dinoflagellate symbiosis is a mutualism between photosynthetic microalgae and a cnidarian host. The nutrient exchange between these partners is the base of their successful cooperation (Muscatine & Hand, 1958; Von Holt & Von Holt, 1968; Trench, 1971; Burriesci et al., 2012; Sproles et al., 2020), and has led to the widespread distribution of coral reefs in coastal oligotrophic waters (Muscatine & Porter, 1977; Cuning et al., 2017).

The vast majority of symbiotic dinoflagellates belong to the family Symbiodiniaceae, with eleven genera and numerous species currently being described (LaJeunesse et al., 2018, 2021; Nitschke et al., 2020; Pochon & LaJeunesse, 2021). Characteristics such as thermal tolerance, photosystem function, and inorganic carbon acquisition vary widely between the many genera, species and subspecies that comprise this family, and many species of cnidarians ‘prefer’ to form a symbiosis with only one or a limited range of symbiont species, creating distinct patterns of host-symbiont specificity (Belda-Baillie et al., 2002; Baker, 2003; Goulet, 2006; Putnam et al., 2012; Poland & Coffroth, 2017).

The cnidarian-dinoflagellate symbiosis is highly sensitive to disturbance. For example, thermal stress or pollution can result in coral bleaching, where the dinoflagellates are lost from the host’s tissues (Weis, 2008). This process is contributing to the massive decline of coral reefs around the world (Hughes et al., 2018). The exact cellular mechanisms behind bleaching remain unresolved, however, though it is thought that oxidative stress in both the symbiont and host tissues plays a major role (Oakley & Davy, 2018). In particular, oxidative stress resulting from photodamage in the symbionts’ chloroplasts has been proposed to lead to the leakage of reactive oxygen species (ROS) into the host’s tissues. This then causes oxidative stress in the host (Lesser, 1996; Warner et al., 1996; Smith et al., 2005; Warner & Suggett, 2016) that may trigger exocytosis or apoptotic pathways in the host, and eventually lead to the expulsion or degradation of the symbionts (Weis, 2008). Nevertheless, while bleaching poses a huge threat to coral health, it has been suggested that it may also provide a window of opportunity for changing the symbiont community composition to one better suited to the new environmental conditions (Buddemeier & Fautin, 1993; Fautin & Buddemeier, 2004; Cunning et al., 2015; Claar et al., 2020). This might happen in one of two ways: symbiont ‘shuffling’ or ‘switching’ (Baker, 2003). Shuffling occurs when the relative abundance of different symbiont species that are already present in the host’s tissues shifts, whereas switching indicates the uptake and establishment of a new symbiont partner from the environment.

There is some evidence for shuffling in the field, with some studies showing that an increased thermal tolerance in the symbiont is able to translate to the host, thus allowing the holobiont (i.e., whole symbiosis) to withstand higher temperatures (Stat & Gates, 2011; Kemp et al., 2014; Boulotte et al., 2016; Silverstein et al., 2017). This is not always the case, however, as bleaching can provide an opportunity for less beneficial symbionts to invade the tissues of an immune-compromised host (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011). In contrast to shuffling, there is little evidence for switching partners in the field, once again highlighting a high level of host-symbiont specificity despite the potential adaptive benefits of changing partners. Indeed, experimental tests of inter-partner switching have demonstrated that symbiosis with a heterologous (i.e., non-native) symbiont species can result in a sub-optimal nutritional relationship, and potentially even host cellular stress and mortality (Weis et al., 2001; Belda-Baillie et al., 2002; Dunn & Weis, 2009; Starzak et al., 2014; Gabay et al.,

2018; Matthews et al., 2018). However, how the symbiotic interaction state (i.e., benefit *versus* cost) of homologous and heterologous symbionts changes under thermal stress has not been established at the cellular level.

The aim of this chapter was therefore to establish the combined effect of symbiont identity (i.e., homologous *versus* heterologous) and thermal stress on the host proteome and metabolome, using the sea anemone *Exaiptasia pallida* ('Aiptasia'), a model system for the cnidarian-dinoflagellate symbiosis. Aiptasia is easily rendered aposymbiotic (i.e. symbiont-free), and can be colonised with different species of symbionts (Weis et al., 2008). For this study, Aiptasia was colonised with its homologous symbiont species *Breviolum minutum*, and either of two heterologous species, *Durisdinium trenchii* or *Symbiodinium microadriaticum*. Both *D. trenchii* and *S. microadriaticum* are considered to have a relatively high thermal tolerance (Robison & Warner, 2006; Ragni et al., 2010; Hawkins & Davy, 2012; Krämer et al., 2012). Moreover, *D. trenchii* has been found in increased densities in coral tissues during and after bleaching events, consistent with its reputation as an opportunist (Toller et al., 2001; Glynn et al., 2001; Baker et al., 2004; Berkelmans & Van Oppen, 2006; Jones et al., 2008; Correa et al., 2009; LaJeunesse et al., 2009; Stat & Gates, 2011; Cooper et al., 2011; Silverstein et al., 2012; Kemp et al., 2014; Kennedy et al., 2015; Boulotte et al., 2016). Despite this, however, under experimental conditions, *D. trenchii* provides less nutritional benefit and causes increased oxidative stress in Aiptasia compared to the homologous *B. minutum* (Matthews et al., 2017; Sproles et al., 2020). This potentially explains why, even under thermal stress, *D. trenchii* (and indeed *S. microadriaticum* as well) is always 'over-run' by the homologous *B. minutum* when both symbionts are inoculated into Aiptasia simultaneously, and why this cnidarian is so specific for *B. minutum* throughout its Indo-Pacific range (Thornhill et al., 2013a; Gabay et al., 2018, 2019). How these symbionts respond to thermal stress at the cellular level when in Aiptasia is therefore crucial for understanding the drivers of host-symbiont specificity and the limitations for adaptive bleaching *via* partner switching. The powerful combination of metabolomics and proteomics used in this study addresses this knowledge gap.

2.3 Methods

2.3.1 Experimental organisms

Specimens of the sea anemone Aiptasia (n ~ 1,000, clonal line NZ1) were bleached using a menthol bleaching protocol based on Matthews *et al.* (2016). In brief, anemones were kept in 2 L beakers in 0.22-µm filtered seawater (FSW), to which menthol (20% w/v in ethanol) was added at a final concentration of 0.19 mmol L⁻¹, for 8 h *per* day, 4 days *per* week. DCMU (100 mmol L⁻¹) was added to the tank at a final concentration of 5 µmol L⁻¹ to inhibit photosynthesis of any remaining symbionts. FSW was replaced daily. In addition to this, anemones were exposed to a weekly cold shock by placing

the beaker on ice for a period of 3 h. After anemones were confirmed as being aposymbiotic using confocal microscopy (IX81, Olympus New Zealand; 635 nm laser, 655-755 nm emission filter), they were kept in the dark and fed twice weekly with freshly hatched *Artemia* brine shrimp until experimental manipulation.

Three Symbiodiniaceae cultures were used: the homologous species *B. minutum* (previously known as ITS2 type B1; culture ID FlAp2, originating from Long Key, Florida, USA), and the heterologous species *S. microadriaticum* (previously known as ITS2 type A1; culture ID CCMP2467, originating from the Gulf of Aqaba, the Red Sea) and *D. trenchii* (previously known as ITS2 type D1a; culture ID Ap2, originating from Okinawa, Japan). Cultures were grown in an incubator at 25 °C on a 12 h:12 h light:dark cycle with an irradiance of 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Phillips, 18W 840), in silica-free f/2 culture medium (AusAqua Pty, SA, Australia) and refreshed once a month.

2.3.2 Inoculation

Symbiodiniaceae cultures were refreshed three days before each inoculation. Anemones with an oral disc diameter of 4-5 mm (approximately 30 per jar) were placed in sterile 400 mL plastic jars (LabServ) containing 250 mL artificial seawater (ASW; Aquaforest Marine sea salt dissolved in distilled water to 35 ppt) one day before the first inoculation to allow them to settle. Jars were randomly allocated to one of the three Symbiodiniaceae cultures, and then a drop of this culture, containing 3 million cells mL^{-1} , mixed with some freshly-hatched *Artemia* nauplii was applied to the oral disc of the anemones to stimulate a feeding response. This was repeated once weekly for a total of four weeks. Throughout the entire experiment, anemones were fed freshly hatched *Artemia* twice weekly.

2.3.3 Experimental treatments and sampling

Anemones were maintained at a control temperature of 25 °C under an irradiance of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (GE Lighting T5 F54W/840). Three months after initial inoculation, anemones were split into two groups, each held in a water bath: control and heat stress ($n = 5$ jars *per treatment per species*). The heat stress consisted of a ramping by 1°C day^{-1} for seven days, reaching a peak temperature of 32 °C, which was maintained for three days. On the sampling day, algal photosynthetic ‘health’ was measured using a Pulse Amplitude Modulated (PAM) fluorometer (Diving PAM, Heinz Walz GmbH), measuring light adapted photosystem II quantum yield (F_v/F_m ; four measurements (anemones) *per jar*, five jars *per treatment per species*). PAM settings were maintained at: measuring light 6, saturation intensity 8, saturation width 1 s, gain 6 and damping 2. Jars were then removed from the water baths, ASW was gently discarded, and each jar was immediately sealed and flash frozen in liquid nitrogen to quench metabolism (~10 s). Jars were stored at -80 °C for 24 h, after which the anemones were quickly removed

and transferred to 2 mL Eppendorf tubes ($n = 5$ replicates *per* treatment, with 30 anemones *per* replicate). These were then stored at $-80\text{ }^{\circ}\text{C}$ until further processing.

2.3.4 Homogenisation and separation of fractions

Samples were homogenised in 500 μL cold ($4\text{ }^{\circ}\text{C}$) milliQ water using a mechanical saw-tooth homogeniser at a mid-speed setting for 1 min. A 5 mL aliquot of cold milliQ water was added after homogenisation, thoroughly vortexed, and a 500 μL sub-sample was taken for cell counts and protein quantification, after which the homogenate was centrifuged for 5 min at $2500 \times g$ to pellet the symbionts. The host fraction was decanted and spun again to ensure the removal of all symbionts, frozen for at least one hour at $-80\text{ }^{\circ}\text{C}$, and then freeze-dried using a lyophiliser (FreeZone® Plus™ 4.5L Cascade, Labconco, Auckland, New Zealand) for 18 h at $-105\text{ }^{\circ}\text{C}$. The symbiont fraction was resuspended in 500 μL cold milliQ water and gently vortexed to resuspend the cells. A 100 μL aliquot was taken to which 500 μL DNA buffer were added for symbiont identification (Section 2.3.5). The remaining symbiont material was stored at $-80\text{ }^{\circ}\text{C}$.

The 500 μL aliquot for cell counts was also spun down at $2500 \times g$ to separate host and symbiont fractions. The symbiont fraction was resuspended in 500 μL ASW. Cell counts were performed using a haemocytometer (Improved Neubauer) with eight replicate counts *per* sample, and were normalised to protein content determined *via* the Bradford assay (Bradford, 1976). Cell density (symbionts *per* mg host protein) was then calculated.

2.3.5 Confirmation of symbiont genotype

Before inoculation, all cultures were genotyped. DNA from cultures was extracted using a bead-beat protocol. At each sampling time-point, anemones were also checked for symbiont genotype. DNA was extracted according to the CTAB-chloroform protocol of Baker & Cunning (2016). 100 μL symbiont cells in DNAB from each replicate sample was digested overnight at $37\text{ }^{\circ}\text{C}$ using 10 μL 10 mg/mL proteinase K. Thereafter, 200 μL CTAB was added, and samples were incubated at $65\text{ }^{\circ}\text{C}$ for 30-60 min. After cooling down to room temperature, 300 μL chloroform was added, and samples were incubated at room temperature on a rotating platform for 2-3 h. Samples were centrifuged at $10,000 \times g$ for 10 min, and the top 250 μL was drawn off and collected into a new tube. 100% ethanol at twice the sample volume was added and DNA was stored at $-20\text{ }^{\circ}\text{C}$ for a minimum of 2 h to promote DNA precipitation. Samples were then centrifuged again at $10,000 \times g$, after which the ethanol was decanted and remaining DNA was dried under vacuum (Eppendorf Concentrator 5301) before redissolving the DNA in 0.3 M sodium acetate (NaOAc), after which they were ethanol washed, first with 100% ethanol

and again with 70% ethanol, then dried again under vacuum, and finally resuspended in 20 μ L TE buffer.

PCR was performed using 2 μ L extracted DNA for each replicate sample, using the primers ITS1 (forward, 5'-GTGAATTGCAGAACTCCGTG-3') and ITS2rev2 (reverse; 5'-CCTCCGCTTACTTATATGCTT-3'). PCR conditions consisted of an initial stage of 3 min at 95 °C, then 35 rounds of 15 s at 95 °C, 15 s at 56 °C, and 10 s at 72 °C, with a final elongation phase of 5 min at 72 °C, followed by a resting stage at 4 °C. PCR products were stored at 4 °C. Extracted DNA was sent to Macrogen (Seoul, Korea) for purification and sequencing. Sequence results were checked for quality and then BLAST searched against two databases, GeoSymbio and NCBI, to confirm symbiont species.

2.3.6 Metabolite extractions

Metabolite extractions were based on previous work by Matthews et al. (2017). In brief, freeze-dried host material was resuspended in 1 mL cold (-20 °C) 100% methanol with internal standard ($^{13}\text{C}_6$ sorbitol, at a final concentration of 1%), and then sonicated at 4 °C for 30 min in an ultrasonic bath. The material was then spun down at 3000 x g for 30 min, after which the supernatant containing the semipolar metabolites was drawn off and stored at -80 °C. The remaining material was resuspended in cold (-20 °C) 50% methanol and vortexed for 1 min to extract polar metabolites. Samples were centrifuged again at 3000 x g for 30 min, and the supernatant was combined with the first extract. The total pooled extract was centrifuged at 16,000 x g for 15 min at 4 °C to ensure the removal of all particulates. Then, repeatedly 50 μ L were dried under vacuum (Eppendorf Concentrator 5301) in glass vial inserts for MS profiling, making for a total of 400 μ L *per* sample. 50 μ L *per* sample were pooled to create pooled biological controls.

2.3.7 Metabolite profiling

Metabolite profiling was done at Metabolomics Australia (Melbourne, Australia). Dried samples for targeted analysis were prepared by adding 25 μ L of methoxyamine hydrochloride (30 mg/ml in pyridine) followed by shaking at 37 °C for 2 h. Samples were then derivatized with 50 μ L of N,O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA with 1% TMCS, Thermo Scientific) for 1 h at 37 °C. Samples were left for 1 h before 1 μ L was injected onto the gas chromatography column using a hot needle technique. Splitless injections were done for each sample.

The gas chromatography–mass spectrometry (GC-MS) system used was comprised of an AOC6000 564 autosampler, a 2030 Shimadzu gas chromatograph and a TQ8050 quadrupole mass spectrometer

(Shimadzu, Japan). The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). GC-MS was performed on a 30 m Agilent DB-5 column with 1 μ m film thickness and 0.25 mm internal diameter column. The injection temperature was set at 280 °C, the MS transfer line at 280 °C and the ion source was adjusted to 200 °C. Helium was used as the carrier gas at a flow rate of 1 ml min⁻¹ and argon gas was used as the collision cell gas to generate the MRM product ion. Sample analysis was performed under the following temperature program: start at injection 100 °C, held for 4 min, followed by a 10 °C min⁻¹ oven temperature ramp to 320 °C, and a final hold for 11 min. Approximately 520 quantifying MRM targets were collected using the Shimadzu Smart Database along with the qualifier for each target, which covers about 350 endogenous metabolites and multiple ¹³C-labeled internal standards. Both chromatograms and MRMs were evaluated using the Shimadzu GC-MS browser and LabSolutions Insight software. Resulting area responses were normalized to the internal standard ¹³C₆ sorbitol area response.

2.3.8 Protein extractions

Host protein extractions were performed using a filter-aided sample preparation protocol on the pellet remaining after metabolite extraction, based on a protocol by Oakley et al. (in prep). Samples were resuspended in 500 μ L 5% SDC and sonicated for 20 x 2 s pulses at 20% output, followed by a 20 min incubation at 85 °C with 1% β -mercaptoethanol (BME) to denature proteins. Thereafter, samples were washed twice with ethyl acetate, before drying off remaining ethyl acetate under vacuum (Eppendorf Concentrator 5301). The sample was spun in a spin filter at 14,000 x g for 15 min, and then washed three times with 50 mM Tris. Protein content was quantified using Qubit, and protein extractions continued with 100 μ g protein on the filter. Any remaining material was frozen at -80 °C in case of re-analysis. BME was added to a final concentration of 10 mM, and incubated at 37 °C for 10 min. Then, iodoacetamide (IAA) was added to a final concentration of 50 mM, followed by an incubation of 20 min at room temperature. Samples were digested overnight at 37 °C with 2 μ g trypsin.

Following protein digestion, samples were centrifuged at 14,000 x g for 15 min, and any remaining SDC was pelleted with the addition of 1% final concentration formic acid. The following protocol was used to desalt samples: a ZipTip (Agilent Technologies Bond Elute) was activated with 100% acetonitrile (ACN), rinsed with 0.1% formic acid, and peptides were bound to the ZipTip by aspirating the sample 25 times. Thereafter, the ZipTip was rinsed in 0.1% FA three times, and peptides were eluted in 50% ACN by aspirating the sample 15 times. This was repeated once more with elution in 30% ACN. These elutions were then combined and dried down completely (Eppendorf Concentrator 5301). Samples were rehydrated in 0.1% formic acid and incubated at 37 °C for 30 min. Qubit was used again to quantify protein content, in order to run 200 ng peptides for identification and quantification.

2.3.9 Protein profiling

Peptide samples were profiled using liquid chromatography-tandem mass spectrometry (LC-MS-MS). The LC used to separate peptide samples was performed with a linear gradient from 5% to 35% buffer B (buffer A: 0.1% formic acid; buffer B: 80% acetonitrile, 0.1% formic acid) at 300 nL min⁻¹ on an Acclaim PepMap C18, 3 µm, 100 Å column (Thermo Scientific, Auckland, New Zealand) and Ultimate 3000 liquid chromatography system (Dionex, Sunnyvale, CA). Peptide ionisation occurred by electrospray at 1.8 kV, with analysis by an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific). The Orbitrap acquired precursor mass spectra at a resolution of 120,000, rejecting singly-charged ions, while quadrupole isolation was enabled and an automatic gain target of 7.0e5 used. The injection time was set at a maximum of 50 ms. The 20 most intense precursor spectra were fragmented by a higher-energy collision dissociation, and then analysed in the ion trap. The automatic gain target was 5.0e3 and this had a maximum injection time of 300 ms. Dynamic exclusion was enabled with a duration of 60 s.

Protein identification was performed from raw spectrum files using the Andromeda search algorithm in MaxQuant (1.6.10.43, Cox & Mann, 2008; Cox et al., 2014). Proteins were searched against the Aiptasia genome (Baumgarten et al., 2015). Trypsin was set as the digest enzyme, allowing for a maximum of two missed cleavages. Viable modifications included oxidation of methionine, and acetylation of the protein *n*-terminus, while carbamidomethylation of cysteine was considered a fixed modification. The first peptide search tolerance was 20 ppm, main search peptide tolerance 4.5 ppm, and the mass tolerance was 0.5 Da for the ion trap MS2 search. Other settings included enabling of label-free quantification, with a minimum of two unique plus razor peptides for quantification, and a match between runs enabled. Peptide spectrum match and protein false discovery rates were both set to 1%, with a minimum of two peptides required for identification.

2.3.10 Data analysis and visualisation

Cell density data were tested for normality and homoscedasticity, and then tested with a two-way ANOVA in R (version 4.0.3; R Core Team, 2020). Identified metabolites were normalised against the internal ¹³C₆ sorbitol standard, as well as total protein content determined during protein extractions, and finally to total metabolite abundance. Data were log₂ transformed and analysed using the online PolySTest (Schwämmle et al., 2020). Significant metabolites were identified using the Limma test, with an FDR threshold of $q < 0.05$, and only metabolites with a log₂-fold change larger than 0.5 or -0.5 are reported.

For the proteomics, identified proteins and label-free quantification intensities were analysed in Perseus (1.6.15.0, Tyanova et al., 2016) to remove known false detections and contaminants from the analysis. Samples were then normalised to total intensity values and log₂ transformed in R (4.0.3, R Core Team, 2020). Data analysis was done using PolySTest (Schwämmle et al., 2020) online to identify significantly different expressed proteins between treatments. Significant proteins were identified using the Miss test in PolySTest (Schwämmle et al., 2020) with an FDR threshold of $q < 0.05$, and only annotated proteins with a log₂-fold change larger than 0.25 or -0.25 are reported. PCAs were made based on host metabolome as well as proteome profiles using R (version 4.0.3, R Core Team, 2020). ANOVAs were then used to test if the PCA axes were able to significantly separate the samples based on thermal treatment or symbiont species.

2.4 Results

2.4.1 Symbiont cell densities & bleaching

Genotyping indicated successful colonisation by *B. minutum*, *D. trenchii* and *S. microadriaticum*. There was a large difference in symbiont population density between species, with *S. microadriaticum* and *D. trenchii* reaching only about 15% and 60% of the density of *B. minutum*, respectively (Figure 2.1A and Figure 2.2). Only anemones containing *D. trenchii* significantly bleached (two-way ANOVA species x

Figure 2.1 (A) Cell densities ($\pm$ SE) reached by the different symbiont species (i.e. *Breviolum minutum*, *Durisdinium trenchii*, and *Symbiodinium microadriaticum*) in Aiptasia under control temperature (25 °C) and heat stress (ramping to 32 °C over seven days, then held for three days; $n = 5$). (B) Peak photosystem II quantum yield (F_v/F_m) for *B. minutum* and *D. trenchii* under control temperature and heat stress. PAM data are missing for *S. microadriaticum* due to low symbiont cell densities. Letters indicate statistically significant groups.

treatment, $F_{2,30} = 5.450$, $p = 0.0096$; Tukey HSD *post hoc*, $p = 0.0014$) in response to heat treatment, losing 60% of their symbionts (Figure 2.1). PAM data (Figure 2.1B) for *B. minutum*- and *D. trenchii*-colonised anemones showed that, although there was a minor decrease in photosystem II quantum yield in response to heat stress (F_v/F_m ; two-way ANOVA, $F_{1,9} = 11.896$, $p = 0.0073$), it was still > 0.5 for both symbioses, indicating ongoing photosynthetic health. (Note: PAM data could not be reliably collected for *S. microadriaticum*-colonised anemones due to the low symbiont population density).

Figure 2.2 Aiptasia colonised by different species of symbionts, three months after inoculation under control conditions: (A) the homologous *Breviolum minutum*; and the heterologous (B) *Durusdinium trenchii* and (C) *Symbiodinium microadriaticum*. White bars indicate 10 mm.

2.4.2 Omics results

A total of 93 metabolites and 3768 proteins were identified in the samples. Table 2.1 summarises the number of differentially expressed metabolites (DEMs) and differentially expressed proteins (DEPs) *per* comparison. When comparing between symbiont species, the number of DEMs and DEPs show similar patterns (Table 2.1). In particular, under control conditions the largest differences were found between *S. microadriaticum*-colonised anemones and those harbouring the homologous *B. minutum*, while anemones containing *D. trenchii* were intermediate between these. This pattern was also reflected in the spatial separation of the different host metabolomes (Figure 2.3A), though not the host proteomes (Figure 2.3B).

Under control conditions, metabolomic analysis showed that *S. microadriaticum*-colonised hosts had a lower abundance of compounds involved in the TCA cycle and glycolysis (citrate, fumarate, succinate, and fructose), as well as carbohydrate metabolism (16 carbohydrate compounds), compared to *B. minutum*-colonised hosts (Figure 2.5). In comparison, when compared to anemones colonised by the homologous *B. minutum*, *D. trenchii*-colonised anemones contained less citramalate, succinate, and glucose, but increased levels of sucrose; moreover only eight compounds involved in carbohydrate metabolism were present at lower levels in the *D. trenchii*-colonised anemones (Figure 2.5).

Table 2.1 Summary of differentially expressed metabolites and proteins between Aiptasia in symbiosis with different species of symbionts (i.e. *Breviolum minutum*, *Durisdinium 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).

Response to heat stress <i>per</i> species	Differentially expressed metabolites		Differentially expressed proteins	
<i>B. minutum</i>	5		26	
<i>D. trenchii</i>	21		84	
<i>S. microadriaticum</i>	0		26	
	Differentially expressed metabolites		Differentially expressed proteins	
Between species	Control	Heat stress	Control	Heat stress
<i>S. microadriaticum</i> vs. <i>B. minutum</i>	43	33	126	173
<i>D. trenchii</i> vs. <i>B. minutum</i>	20	33	30	137
<i>S. microadriaticum</i> vs. <i>D. trenchii</i>	19	0	83	22
Shared features heterologous vs. homologous	15	30	21	80

Figure 2.3 PCA based on host metabolome (A, B) and proteome (C, D) of Aiptasia in symbiosis with different symbiont species, i.e. *Breviolum minutum*, *Durisdinium trenchii* or *Symbiodinium microadriaticum*, at (A, C) control temperature (25° C), and (B, D) under heat stress (ramping to 32 °C). 95% confidence intervals are shown, and PC axes in bold have been found to be significantly different when separating the groups (significance level: ** p < 0.01; *** p < 0.001).

Heat stress had the largest effect on *D. trenchii*-colonised hosts (26 DEMs and 84 DEPs), whereas hosts containing *B. minutum* or *S. microadriaticum* only showed a few changes (5 DEMs and 26 DEPs, vs. 0 DEMs and 26 DEPs, respectively). Host metabolome and proteome profiles showed significant spatial separation in response to elevated temperature for *D. trenchii*-colonised anemones (Figure 2.4). Relative metabolite abundance in host tissues is presented in Figure 2.5, and DEPs in response to heat stress for each symbiotic association are listed in Table 2.2.

Figure 2.4 PCA based on host metabolome (A, B, C) and proteome (D, E, F) of *Aiptasia*, comparing control (25 °C) and heat stress (ramped to 32 °C) for hosts in symbiosis with the different symbiont species. (A, D) *Breviolum minutum*, (B, E) *Durisdinium trenchii*, and (C, F) *Symbiodinium microadriaticum*. Ellipses represent 95% confidence intervals, and PC axes in bold have been found to be significantly different when separating the groups (significance level: ** $p < 0.01$; *** $p < 0.001$).

Changes in the relative abundance of metabolites in response to elevated temperature included an increase in three sugars (N-acetylmannosamine, N-acetylglucosamine, and myo-inositol), and a decrease in adipic acid in anemones containing the homologous symbiont *B. minutum*. Anemones in symbiosis with *S. microadriaticum* did not show any significant changes in response to elevated temperature. *D. trenchii*-colonised anemones, on the other hand, showed a decrease in the relative abundance of metabolites involved in the TCA cycle and glycolysis (citrate, fumarate, fructose and glucose), as well as 10 other carbohydrates (Figure 2.5), in response to heat stress. The only metabolite that increased in abundance in *D. trenchii*-colonised anemones at elevated temperature was ethanolamine. Proteomics data supported these observations, with only a couple of proteins involved in metabolism upregulated in anemones containing *B. minutum* in response to elevated temperature, while

Heat shock

TCA cycle and glycolysis

Carbohydrate metabolism

Amino acid metabolism

Figure 2.5 Relative abundance of metabolites *per* metabolic category in Aiptasia host tissue colonised by different symbiont species (i.e. *Symbiodinium microadriaticum*, *Breviolum minutum*, or *Durudinium trenchii*) under control (25 °C) and heat stress conditions (ramping to 32 °C). Asterisks indicate significant differences within species caused by heat stress (red means upregulated, blue means downregulated in the heat stressed condition). Hatching of bars indicates significant differences from *B. minutum*-colonised anemones (red means upregulated while blue means downregulated when in symbiosis with the heterologous symbionts).

many proteins involved in core metabolism (i.e. the TCA cycle and glycolysis) and nitrogen metabolism were upregulated in *D. trenchii*-colonised anemones in response to elevated temperature (Table 2.2). Additionally, all hosts proteomes showed similar upregulation of proteins involved in heat stress and protein folding: 10 DEPs for hosts containing *B. minutum*, 12 for those containing *D. trenchii*, and eight for hosts paired with *S. microadriaticum*. The largest upregulation (i.e. 5 to 7 log₂-fold) was found in

the expression of the chaperone calumenin-B (CALUB) for all host-symbiont pairings. A full table of all DEPs can be found in the Supplementary Data.

Table 2.2 Differentially expressed proteins in response to heat stress (ramping to 32 °C) for *Aiptasia* containing different symbiont species (i.e. *Breviolum minutum*, *Durudinium trenchii* or *Symbiodinium microadriaticum*; n = 5). Colour scale indicates up- (red) or downregulation (blue) in the heat stressed host compared to control.

<i>B. minutum</i>	<i>D. trenchii</i>	<i>S. microadriaticum</i>	Annotation	UniProt	Gene Name	E value
Core metabolism (TCA cycle and glycolysis)						
	0.63		Succinate-semialdehyde dehydrogenase, mitochondrial	Q3MSM3	SSDH	6.00E-176
0.58			Sorbitol dehydrogenase	Q58D31	DHSO	1.90E-123
	0.62		Alpha-enolase	P06733	ENOA	8.30E-199
	0.57		Citrate synthase, mitochondrial	Q72VY5	CISY	9.10E-190
	0.54		Triosephosphate isomerase	P00940	TPIS	1.30E-94
0.53			Multifunctional protein ADE2	P38024	PUR6	9.30E-141
	0.5		Fructose-bisphosphate aldolase C	P53448	ALDOC	3.60E-138
	0.48		Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	Q5R5J1	PCKGC	1.40E-236
	0.46		Dihydrolipoylysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	Q8BMF4	ODP2	5.10E-135
	0.26		Transaldolase	Q9EQS0	TALDO	2.70E-124
-1.13			Isocitrate lyase	Q9K9H0	ACEA	6.00E-164
Nitrogen metabolism						
	1.88		Betaine--homocysteine S-methyltransferase 1	Q5XGM3	BHMT1	2.40E-146
	1.48		Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	A7YWE4	AL4A1	4.50E-172
	0.67		Aspartate aminotransferase, cytoplasmic	P13221	AATC	1.20E-137
		0.52	Aspartate aminotransferase, mitochondrial	P12345	AATM	4.70E-169
	-0.85		Glutamate dehydrogenase	P94598	DHE3	8.20E-172
	-3.58		Glutamine synthetase	P15105	GLNA	1.40E-153
Lipid metabolism						
	8.00	3.06	Enoyl-[acyl-carrier-protein] reductase [NADPH] FabL	P51831	FABG	7.60E-19
	-0.46		Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	P48818	ACADV	2.60E-208
Heat stress and protein folding						
6.61	5.92	4.91	Calumenin-B	B5X4E0	CALUB	1.90E-64
1.03	0.99	0.92	Protein disulfide-isomerase A4	P13667	PDIA4	3.90E-191
0.75	0.6	0.88	Calreticulin	P29413	CALR	1.80E-76
	0.82		Endoplasmic reticulum resident protein 44	Q9D1Q6	ERP44	5.00E-86
0.72	0.82	0.71	Endoplasmic	P08110	ENPL	1.80E-271
0.48	0.79		Protein disulfide-isomerase A3	P30101	PDIA3	2.60E-139
0.60	0.73	0.79	Protein disulfide-isomerase A6	Q11067	PDIA6	2.30E-142
0.74	0.61	0.76	Heat shock protein HSP 90-alpha 1	O57521	HS90B	1.20E-297
0.29	0.67		Stress-70 protein, mitochondrial	Q3ZCH0	GRP75	1.40E-252
0.52	0.58	0.65	78 kDa glucose-regulated protein	P29844	BIP	1.80E-295
	0.51		97 kDa heat shock protein	Q94738	HSP97	8.90E-220
	0.51		60 kDa heat shock protein, mitochondrial	P18687	CH60	1.60E-220
0.45		0.25	Heat shock cognate 71 kDa protein	A2Q0Z1	HSP7C	1.80E-289
Immune response						
	0.87		Cytosolic 5-nucleotidase 3A	Q5ZID6	5NT3A	1.00E-54
	-0.34		Adenosylhomocysteinase	P27604	SAHH	1.30E-203
Apoptosis						
	-0.35		Calmodulin	P25071	CML12	8.70E-49
	-1.02		Calmodulin	P04352	CALM	1.50E-13
	-1.31		Calmodulin-like protein 3	P53440	CALMF	8.80E-06

Table 2.2 continued

<i>B. minutum</i>	<i>D. trenchii</i>	<i>S. microadriaticum</i>	Annotation	UniProt	Gene Name	E value
Oxidative stress and redox process						
	0.90		Peroxiredoxin-4	Q9BGI2	PRDX4	7.00E-104
	0.73	0.43	Alpha-aminoadipic semialdehyde dehydrogenase	Q2KJC9	AL7A1	6.50E-216
	0.44	0.42	Superoxide dismutase [Cu-Zn]	P81926	SODC	1.30E-57
0.34			Synaptic vesicle membrane protein VAT-1-like	Q8JFV8	VAT1	1.20E-91
		-0.30	Catalase	P04040	CATA	7.20E-209
Protein degradation and turnover						
	0.49		Proteasome subunit alpha type-7-A	Q9PVQ1	PSA7B	6.20E-108
	0.27		Aminopeptidase N	Q32LQ0	AMPE	4.80E-193
	-0.32		Cathepsin B	P83205	CATB	4.00E-115
Electron transport						
	0.70		ATP synthase subunit delta, mitochondrial	P35434	ATPD	4.30E-30
	0.61		Succinate dehydrogenase [ubiquinone] flavoprotein subunit B, mitochondrial	Q801S2	SDHAB	2.60E-268
0.42			ATP synthase subunit alpha, mitochondrial	Q03265	ATPA	7.30E-255
Iron binding						
0.47	0.63	0.48	Serotransferrin	P56410	TRFE	5.70E-48

Comparison ■ *S. microadriaticum* vs. *B. minutum* ■ *D. trenchii* vs. *B. minutum* ■ *S. microadriaticum* vs. *D. trenchii*

Figure 2.6 Number of differentially expressed proteins *per* category when comparing Aiptasia in symbiosis with different symbiont species (i.e. *Symbiodinium microadriaticum*, *Breviolum minutum*, or *Durisdinium trenchii*) under control temperature (25 °C, left) and under heat stress (ramping to 32 °C, right). Only categories with a minimum of three significant proteins for at least one of the comparisons are shown.

These effects of heat stress caused the metabolomes of hosts associated with heterologous symbionts to converge, i.e., become more similar to each other, and more different from anemones containing the homologous symbiont. Host metabolomes showed significant spatial separation over PC axis 1 at both control and elevated temperatures (Figure 2.3A, B), whereas host proteome profiles did not

Figure 2.7 Heat maps of differentially expressed proteins (DEP; $q < 0.05$) between hosts in symbiosis with different symbiont species (i.e. *Breviolum minutum*, *Durusdinium trenchii* and *Symbiodinium microadriaticum*) under (A) control conditions (25 °C, 146 total DEPs) and (B) heat stressed conditions (ramping to 32 °C, 231 total DEPs). Rows are proteins, columns are biological replicates ordered by hierarchical clustering of protein expression. The colour scale is $\log_2$ -fold change relative to the protein's mean. The tree is a hierarchical clustering of proteins based on Pearson's correlation of their expression across samples.

(Figure 2.3C, D). Again, DEMs between the hosts containing different symbiont species are mainly involved in carbohydrate metabolism, core metabolism, and amino acid metabolism (Figure 2.5), with a downregulation of the compounds involved in the first two categories, and an upregulation of compounds involved in the latter. Relative expression of DEPs is presented as heat maps in Figure 2.7, while Figure 2.6 shows the number of DEPs *per* functional category when comparing the host proteomes in response to symbiont identity. It shows that most differences occurred in structural proteins, followed by proteins involved in translation, core metabolism, and heat stress and protein folding. The most relevant DEPs (Table 2.3) reflected the DEM patterns, with many changes in proteins

involved in core metabolism and electron transport, as well as nitrogen and lipid metabolism. Oxidative stress proteins appeared to be upregulated when colonised by heterologous symbionts relative to the homologous *B. minutum*, especially under heat stress (Table 2.3). A table of all DEPs can be found in the Supplementary Data.

Table 2.3 Differentially expressed proteins and log₂-fold changes in *Aiptasia* containing different symbiont species (i.e. *Symbiodinium microadriaticum*, *Breviolum minutum*, or *Durudinium trenchii*) under control (25 °C) and heat stress conditions (ramping to 32 °C; n = 5).

<i>S. micro-adriaticum</i> vs. <i>B. minutum</i>		<i>D. trenchii</i> vs. <i>B. minutum</i>		<i>S. micro-adriaticum</i> vs. <i>D. trenchii</i>		Annotation	UniProt ID	Gene name	E value
Con	HS	Con	HS	Con	HS				
Core metabolism (TCA cycle and glycolysis)									
0.91	0.62		0.68	0.57		Fructose-bisphosphate aldolase C	P53448	ALDOC	4.00E-138
			0.81			Phosphoenolpyruvate phosphomutase	P56839	PEPM	1.00E-145
	0.67		0.77			Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	Q8BMF4	ODP2	5.00E-135
0.60	0.46	0.27	0.53			Malate dehydrogenase, mitochondrial	P00346	MDHM	2.00E-120
0.34	0.59		0.55			Citrate synthase, mitochondrial	Q7ZVY5	CISY	9.00E-190
0.57	0.59		0.58	0.49		Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	Q5R5J1	PCKGC	1.00E-236
				0.57		Triosephosphate isomerase	P00940	TPIS	0
0.55				0.42		Glyceraldehyde-3-phosphate dehydrogenase	Q05025	G3P	0
			0.47			Isocitrate dehydrogenase [NADP], mitochondrial	Q4R502	IDHP	5.00E-175
	0.40		0.31			Transaldolase	Q9EQS0	TALDO	3.00E-124
0.29	0.34					Putative aconitate hydratase, mitochondrial	P34455	ACON	0.00E+00
	-0.46		-0.35			Pyruvate carboxylase, mitochondrial	Q29RK2	PYC	0.00E+00
				-0.85		Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	O19069	SUCA	0
-0.90	-1.01		-0.99			Malate synthase	P95329	MASY	1.00E-143
-1.07	-1.28		-0.88			Isocitrate dehydrogenase [NADP] cytoplasmic	P41562	IDHC	3.00E-182
-2.41	-2.87	-1.22	-1.61			Isocitrate lyase	Q9K9H0	ACEA	6.00E-164
Nitrogen metabolism									
	5.00		5.00			Glutamate dehydrogenase	P94598	DHE3	4.00E-162
	2.57		2.57			Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	A7YWE4	AL4A1	5.00E-172
	0.88		1.3			Betaine--homocysteine S-methyltransferase 1	Q5XGM3	BHMT1	2.00E-146
	0.90		1.11			Serine hydroxymethyltransferase, mitochondrial	P34897	GLYM	1.00E-192
			0.92			Ornithine aminotransferase, mitochondrial	Q3ZCF5	OAT	5.00E-176
	0.84		0.86			Creatinase	P38487	CREA	3.00E-107
	0.60					2-aminoethylphosphonate--pyruvate transaminase	B1HPR6	PHNW	2.00E-87
				0.37		Aspartate aminotransferase, cytoplasmic	P13221	AATC	0
	0.30					Alanine--glyoxylate aminotransferase 2, mitochondrial	Q9BYV1	AGT2	3.00E-161
-1.45	-1.79	-0.49	-1.39	-0.96		Glutamate dehydrogenase	P94598	DHE3	8.00E-172
-3.03	-4.12	-0.57	-4.03	-2.46		Glutamine synthetase	P15105	GLNA	1.00E-153
	-5.00					Serine--pyruvate aminotransferase, mitochondrial	P31029	SPYA	3.00E-115

Table 2.3 continued

<i>S. micro-adriaticum</i> vs. <i>B. minutum</i>		<i>D. trenchii</i> vs. <i>B. minutum</i>		<i>S. micro-adriaticum</i> vs. <i>D. trenchii</i>		Annotation	UniProt ID	Gene name	E value
Con	HS	Con	HS	Con	HS				
Lipid metabolism									
	5.00		5.00			Enoyl-[acyl-carrier-protein] reductase [NADPH] FabL	P51831	FABG	8.00E-19
			0.55			Enoyl-CoA hydratase, mitochondrial	Q8BH95	ECHM	1.00E-100
Heat stress and protein folding									
1.2			0.99			Endoplasmic reticulum resident protein 44	Q9D1Q6	ERP44	5.00E-86
0.85	1.07		0.66	0.47	0.41	Reticulocalbin-3	Q28BT4	CALU	9.00E-43
	1.02					Granulin	P28799	GRN	2.00E-44
	0.98					Nucleosome assembly protein 1-like 1	Q4U0Y4	NPL1A	6.00E-96
0.77	0.78		0.85			60 kDa heat shock protein, mitochondrial	P18687	CH60	2.00E-220
0.84	0.72		0.5	0.52		Universal stress protein YxiE	Q8VYN9	PHO32	1.00E-07
			0.78			Peptidyl-prolyl cis-trans isomerase B	P24367	PPIB	4.00E-81
			0.75			Polycystin-2	Q6IVV8	PKD2	4.00E-218
0.58	0.29		0.51	0.38		Protein disulfide-isomerase A3	P30101	PDIA3	3.00E-139
0.48		0.52				Calumenin-A	B5X4E0	CALUB	0
0.49	0.45		0.51			Stress-70 protein, mitochondrial	Q3ZCH0	GRP75	1.00E-252
0.35	0.48		0.27			78 kDa glucose-regulated protein	P29844	BIP	2.00E-295
	0.47					Protein disulfide-isomerase A6	Q11067	PDIA6	2.00E-142
			0.33			Calnexin	P27824	CALX	4.00E-18
			0.26			Calumenin-B	B5X4E0	CALUB	4.00E-77
-0.72			-0.70			Peptidyl-prolyl cis-trans isomerase	P54985	PPIA	9.00E-76
-5.00				-5.00		Translocon-associated protein subunit delta	Q62186	SSRD	0
Apoptosis									
1.05			1.15			Calmodulin	Q0IQB6	CML3	3.00E-29
	0.44		0.47			Sorcin	Q8VC88	GRAN	5.00E-42
		-0.28		0.23		Death-associated protein kinase 3	O43293	DAPK3	0
	-0.56					Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	P54612	2AAA	2.00E-89
	-1.05					Secretagogen	P41044	CAB32	4.00E-42
-0.96	-1.65		-1.34			Calmodulin	P04352	CALM	2.00E-13
	-1.84		-1.74			Translationally-controlled tumor protein-like	Q7QCK2	TCTP	3.00E-11
-1.86	-2.31		-1.81	-1.41		Calmodulin-like protein 3	P53440	CALMF	9.00E-06
Immune response									
1.99						Interferon-induced protein 44-like	Q53G44	IF44L	0
1.10						Guanylate-binding protein 6	Q5R9T9	GBP6	0
			0.82			Cytosolic 5-nucleotidase 3A	Q5ZID6	5NT3A	1.00E-54
NPC proteins									
-5.00	-5.00	-2.74	-5.00			Protein NPC2-like	Q9VQ62	NPC2	1.00E-18
-5.00	-5.00		-5.00			Epididymal secretory protein E1	P79345	NPC2	1.00E-15
Oxidative stress and redox process									
1.59	2.08		1.8			Glutathione S-transferase	P46428	GST	3.00E-26
1.54	1.75		1.55	0.50		Alpha-aminoadipic semialdehyde dehydrogenase	Q2KJC9	AL7A1	7.00E-216
			1.45			NAD(P) transhydrogenase, mitochondrial	Q13423	NNTM	0.00E+00
1.22	0.8	0.65	0.75	0.57		Catalase	P04040	CATA	7.00E-209
0.64	0.83		0.86	0.64		Peroxisredoxin-4	Q9BG12	PRDX4	7.00E-104
0.79						Glutathione S-transferase Mu 2	Q9TSM4	GSTM2	0
	0.76		0.65			Superoxide dismutase [Cu-Zn]	P81926	SODC	1.00E-57
	0.71					Betaine aldehyde dehydrogenase	P56533	AL9A1	5.00E-173
			0.51			Hydroxysteroid dehydrogenase-like protein 2	Q6P5L8	HSDL2	1.00E-120
			0.46			Peroxisomal multifunctional enzyme type 2	P51659	DHB4	6.00E-230
0.45						Putative glutathione S-transferase 6	Q8JF22	GSTP1	0
	-1.14		-0.65			Saccharopine dehydrogenase-like oxidoreductase	Q6AY30	SCPDL	3.00E-106

Table 2.3 continued

<i>S. micro-adriaticum</i> vs. <i>B. minutum</i>		<i>D. trenchii</i> vs. <i>B. minutum</i>		<i>S. micro-adriaticum</i> vs. <i>D. trenchii</i>		Annotation	UniProt ID	Gene name	E value
Con	HS	Con	HS	Con	HS				
Protein degradation and turnover									
			1.74			Transmembrane protease serine 9	B3EWZ6	MLRP2	4.00E-89
	1.62		1.68			Plasma kallikrein	P14272	KLKB1	2.00E-35
	1.36					Kunitz-type protease inhibitor 2	W4VSH9	VKT1	9.00E-23
	1.27					Neprilysin	P08049	NEP	2.00E-110
1.03						Ubiquitin-conjugating enzyme E2 L3	P68037	UB2L3	0
0.89						Cytosolic non-specific dipeptidase	Q5R432	CNDP2	0
-0.33			0.70			Polyubiquitin-B	Q63429	UBC	2.00E-92
	0.41					Cathepsin B	P83205	CATB	4.00E-115
	-0.43					Proteasome subunit alpha type-7-A	Q9PVQ1	PSA7B	6.00E-108
-0.48						Glutamate carboxypeptidase 2	P70627	FOLH1	0
	-0.50					Calpain-B	Q9VT65	CANB	3.00E-157
-0.97	-0.85	-0.44	-0.65			Glutamate carboxypeptidase 2	Q9CZR2	NALD2	4.00E-91
	-1.56					Glutamate carboxypeptidase 2	O35409	FOLH1	4.00E-46
Electron transport									
	1.19					ATP synthase subunit O, mitochondrial	Q24439	ATPO	3.00E-35
0.91	0.79	1.05	0.94			Cytochrome c oxidase subunit 5A, mitochondrial	B0VYY3	COX5A	2.00E-38
1.03	0.52		0.64			ATP synthase subunit delta, mitochondrial	P35434	ATPD	4.00E-30
1.02	0.85					Electron transfer flavoprotein subunit beta	P38117	ETFB	8.00E-97
			0.93			NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	P23709	NDUS3	3.00E-62
			0.77	-0.35		ATP synthase subunit d, mitochondrial	P31399	ATP5H	1.00E-20
	0.67		0.75			ATP synthase F(0) complex subunit B1, mitochondrial	P24539	AT5F1	3.00E-30
	0.71					Electron transfer flavoprotein subunit alpha, mitochondrial	Q99LC5	ETFA	5.00E-123
0.36		0.44				ATP synthase subunit alpha, mitochondrial	Q03265	ATPA	0
Iron binding									
1.21	1.28		1.28			CDGSH iron-sulfur domain-containing protein 1	Q3ZBU2	CISD1	2.00E-25
0.90	0.98					Heme oxygenase 2	P43242	HMOX2	3.00E-60
0.68	0.69	0.62	0.77			Serotransferrin	P56410	TRFE	6.00E-48
			0.59			Melanotransferrin	O93429	TRFE	3.00E-47
	0.45					Soma ferritin	P42577	FRIS	2.00E-65
	-0.89					V-type proton ATPase catalytic subunit A	P31400	VATA	4.00E-308

2.5 Discussion

The different symbiont species were not equally successful at establishing a symbiosis with *Aiptasia*. After nearly three-and-a-half months under control conditions, *D. trenchii* reached a density approximately 60% that of *B. minutum*, whereas *S. microadriaticum* reached a density of about only 15% that of *B. minutum*. These lower colonisation rates of heterologous species have been observed in previous research with this same model system (Starzak et al., 2014; Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2019). Consistent with the symbiont population density, when comparing host metabolomes and proteomes, the symbiosis with *D. trenchii* appeared intermediate between the symbioses with *S. microadriaticum* and *B. minutum* under control conditions. Heat stress increased the

distinction between the host proteome and metabolome in the presence of homologous *versus* heterologous symbionts, while the proteome and metabolome with the two heterologous symbionts converged with heat treatment. The differences in the host metabolites and proteins involved in these reactions in the different symbioses are visualised in Figure 2.8. This figure highlights the increased catabolism in the presence of heterologous symbionts under heat stress, with highly-diminished carbohydrate reserves, plus decreased quantities of some TCA cycle products. Amino acid metabolism showed signs of protein degradation, indicated by the higher relative abundance of amino acids, as well as the increased prevalence of nitrogenous metabolic end products such as urea and uric acid.

Figure 2.8 Changes in metabolic pathways in heat-stressed (ramping to 32 °C) *Aiptasia* containing two different heterologous symbiont species (i.e. *Symbiodinium microadriaticum* or *Durisdinium trenchii*) compared to the homologous symbiont species (*Breviolum minutum*). Proteins identified using proteomics are indicated in *italics* by gene name, as found in Table 2.3, with the colour indicating up- (red) or downregulation (blue) of the protein.

2.5.1 TCA cycle, glycolysis, and carbohydrate metabolism

Given that most of the proteomic and metabolomic differences between these different symbioses related to core metabolism (TCA cycle and glycolysis), and carbohydrate metabolism, it seems likely that they were linked, at least in part, to lower quantities of photosynthate translocated from symbiont to host. This is likely due to the lower symbiont cell densities of the heterologous symbionts (Hoogenboom et al., 2010), though previous work has concluded that *D. trenchii* also has a lower cell-specific potential to provide photosynthetically-fixed carbon to *Aiptasia* than *B. minutum* does (Matthews et al., 2017; Sproles et al., 2020). This seems to be the case for *S. microadriaticum* as well: although elevated temperatures had no effect on the host metabolome in *S. microadriaticum*-colonised anemones, host carbohydrate reserves were strongly reduced in the association with *S. microadriaticum* compared to *B. minutum*-colonised hosts.

In a previous metabolomic analysis of *Aiptasia* when colonised by *B. minutum* or *D. trenchii* for five weeks (rather than more than three months, as in the current study), the symbiont population density showed a similar trend to that seen here and *D. trenchii* was unable to fulfil the host's nutritional requirements, leading to increased mobilisation of carbohydrate and lipid stores in *D. trenchii*-colonised hosts, as well as increased catabolism of proteins and amino acids (Matthews et al., 2017). The current study showed that, after more than three months of colonisation, 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, but it could be related to other functions of this compound, such as its osmolytic properties that aid in protein folding during stress (Gouffi et al., 1998; Street et al., 2006; Khan et al., 2010). Some of the most thermally tolerant corals are found in the hottest and most saline waters (Hume et al., 2013; D'Angelo et al., 2015; Bellworthy & Fine, 2017; Krueger et al., 2017; Osman et al., 2018), and recently, this link between osmolytes and heat stress has been made in corals too (Gegner et al., 2019).

D. trenchii-colonised anemones showed the largest response to heat stress of all three host-symbiont combinations. It was the only symbiosis that showed significant signs of bleaching, accompanied by the most changes in the host metabolome (21 DEMs) and proteome (84 DEPs). It was also the only symbiont species that induced a significant spatial separation of the host metabolome and proteome over a PCA axis in response to heat stress. This is consistent with the previous work by Gabay et al. (2019), who concluded that *D. trenchii* is more thermally sensitive when in symbiosis with *Aiptasia* than was to be expected from studies with cultures (McGinty et al., 2012; Swain et al., 2017). Metabolomic changes due to elevated temperature in *D. trenchii*-colonised hosts were largely associated with decreased pools of carbohydrates and some decreases in TCA cycle products such as citrate and fumarate (Figure 2.8), while proteomic changes indicated the upregulation of several

proteins involved in the electron transport chain (i.e., increased respiration within the mitochondria). It therefore appears that *D. trenchii*-colonised anemones were receiving less photosynthetically-fixed carbon from their symbionts than the anemones colonised by the other two symbiont species. *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), while this distinction might be exacerbated by the fact that symbionts retain more photosynthate to support their own metabolism under heat stress (Tremblay et al., 2016). In this case, this would cause *D. trenchii* to release even less photosynthetically fixed carbon to its host, meaning that the host would need to deplete its carbohydrate reserves to support its metabolism; a scenario entirely consistent with the metabolomic and proteomic changes observed.

2.5.2 Nitrogen metabolism

The reduced supply of photosynthetically generated carbohydrate to the host could have caused the observed increase in nitrogen metabolism. Wang & Douglas (1998) showed that having a supply of organic carbon, either from heterotrophic feeding or from symbiont-supplied photosynthate, enables the host to conserve nitrogen, i.e., a lower requirement for catabolism of proteins and amino acids. On the contrary, both symbioses with heterologous symbionts showed an increased relative abundance of urea and uric acid - nitrogenous metabolic end products from amino acid breakdown – in the host's tissues under control conditions as well as under heat stress. The proteomic data further supported increased protein degradation and turnover, and a reduced capacity for nitrogen assimilation, most obviously in heat-stressed *D. trenchii*-colonised anemones. For example, some proteins involved in glutamate metabolism were upregulated in response to elevated temperature, such as delta-1-pyrroline-5-carboxylate dehydrogenase (AL4A1) and aspartate aminotransferase (AAT). Both enzymes lead to the production of glutamate, AL4A1 through the conversion of ornithine or proline (Hu et al., 1996) and AAT through the conversion of aspartate or cysteine (Pavé-Preux et al., 1988). Glutamate is the by-product of amino acid breakdown, while the host proteome also showed signs of increased protein degradation and turnover through increased expression of proteases and peptidases. Conversely, glutamine synthetase (GLNA), which produces the amino acid glutamine from ammonia and glutamate, was strongly downregulated in heat-stressed *D. trenchii*-colonised anemones. Inorganic nitrogen assimilation in both host and symbiont is thought to be primarily regulated through GNLA (Miller & Yellowlees, 1989; Wang & Douglas, 1998; Pernice et al., 2012). These changes in nitrogen metabolism indicate 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 limitation of carbon supply and stores.

2.5.3 Stress physiology

There were no indications of bleaching in *B. minutum*-colonised anemones, with the algae being photosynthetically healthy throughout. Consistent with this, the host metabolome showed relatively few changes in response to elevated temperature, while proteomic changes mainly consisted of increased expression of heat stress proteins (HSPs) and molecular chaperones in charge of protein folding. This increased HSP and chaperone expression occurred irrespective of symbiotic partner, suggesting a core response by the cnidarian host to heat stress. Despite this response, or perhaps because of these defences, as HSPs have been shown to be capable of dealing with oxidative stress (Ikwegbue et al., 2018), anemones containing *B. minutum* did not display any signs of oxidative stress in either the proteome or metabolome.

With regards to the symbioses with the heterologous symbionts, *S. microadriaticum*-colonised anemones showed similar changes in the proteome to anemones containing *B. minutum*, however the metabolome was unaffected by heat stress. Consistent with this, there was no sign of bleaching even though this symbiont only achieved low cell densities in the first place. This could be due to the relatively high thermal tolerance of *S. microadriaticum* (Robison & Warner, 2006; Ragni et al., 2010; Hawkins & Davy, 2012; Krämer et al., 2012; Gabay et al., 2019). Alternatively, however, cellular stress induced by the presence of the heterologous *S. microadriaticum* might have preconditioned the host for dealing with further stress. In particular, anemones containing *S. microadriaticum* showed a large increase in HSPs and oxidative stress proteins under control conditions compared to anemones harbouring the homologous symbiont, which could have become beneficial to the host when temperatures started to rise. Such pre-conditioning has been seen before in the coral *Montipora digitata*, where a naturally high abundance of antioxidants affords a relatively high degree of bleaching resistance (Krueger et al., 2015). In comparison, *D. trenchii*-colonised anemones only showed increased expression of one HSP (calumenin-A) in comparison to *B. minutum*-colonised anemones under control conditions. Under heat stress, this changed to 11 HSPs, indicating that the symbiosis with *D. trenchii* needed to compensate for the initial lack of HSP expression. Although symbiont identity might play a role in expression levels, thermal sensitivity may ultimately depend on host expression of antioxidants and HSPs.

In terms of apoptosis, under control conditions, one calmodulin protein was strongly upregulated in *S. microadriaticum*-colonised anemones, while two others (calmodulin and calmodulin-like protein 3) were strongly downregulated in comparison to anemones containing the homologous symbiont. Calmodulins activate apoptosis by increasing calcium uptake into mitochondria, which then activates various pathways leading to apoptosis (Berchtold & Villalobo, 2014). In *D. trenchii*-colonised anemones only one apoptosis-related protein (death-associated protein kinase 3) was downregulated

compared to *B. minutum*-colonised hosts. Symbiotic dinoflagellates typically induce suppression of apoptosis in host cnidarian tissues (Rodriguez-Lanetty et al., 2006; Pernice et al., 2011, Gabay et al., submitted). This suppression of host immunity is thought necessary for persistence of the symbionts inside the host cells, and inoculation of *Aiptasia* with heterologous symbiont species has been shown to rapidly increase apoptosis under normal temperatures (Dunn & Weis, 2009; Gabay et al., submitted). In the current study, however, the heterologous symbionts induced a stronger suppression of apoptosis in the host than the homologous *B. minutum*, even though they never attained the cell densities achieved by *B. minutum*. This was unexpected, though it must be remembered that these symbioses had been established for more than three months, much longer than the associations used in previous studies. Interestingly, heat stress caused a stronger downregulation of proteins involved in apoptosis in the *D. trenchii*-colonised hosts, even though there was an increase in oxidative stress proteins. Bleaching and oxidative stress are associated with increased activation of apoptotic pathways (Dunn et al., 2007a; Weis, 2008; Suggett & Smith, 2020). Inhibition of host apoptosis by apicomplexans has also been observed (reviewed in Chakraborty et al., 2017). Apicomplexans are a group of parasites closely related to the Symbiodiniaceae (Lenaers et al., 1991; Coats, 2002; Adl et al., 2012). The strong decreases in the expression of calmodulins and calmodulin-like proteins in the current study therefore offers support to the theory that *D. trenchii* is able to manipulate the host's immune response, including when the host's immune system is compromised in response to heat stress. This is consistent with the widespread view that *D. trenchii* is an opportunist or even parasite, which can become dominant on reefs after bleaching episodes (Stat & Gates, 2011; Lesser et al., 2013; Pettay et al., 2015; Silverstein et al., 2017; Gabay et al., 2018).

This study found an increase in the methionine-synthesising enzyme betaine-homocysteine S-methyltransferase (BHMT) when *Aiptasia* was in symbiosis with the two heterologous symbionts compared to *B. minutum*-colonised anemones, but only under heat stress. Previous research found an increase in the expression of homologs of BHMT in *Aiptasia* colonised by *D. trenchii* five weeks after initial colonisation under control conditions (Sproles et al., 2019), and suggested that the upregulation of BHMT when harbouring a symbiont was an indication that the symbiont could not provide enough methionine to the host, requiring the host to synthesise methionine itself. Methionine, besides playing an important role in protein synthesis, is a precursor for the methyl donor S-adenosylmethionine (SAM) and the antioxidants glutathione and dimethylsulfoniopropionate (DMSP). DMSP is strongly correlated with photoinhibition and oxidative stress, and has been found in increased concentrations in response to acute heat stress (Raina et al., 2013) and osmotic stress (Aguilar et al., 2017), and has also been shown to depend on symbiont identity (Borell et al., 2016). The fact that BHMT was not upregulated during control conditions, in contrast to the findings of Sproles et al. (2019), could be an indication that the oxidative stress initially experienced by the host after establishment of symbiosis with a heterologous species (Matthews et al., 2017; Sproles et al., 2019, 2020), dissipates over time. This idea

is strengthened when considering the expression of other oxidative stress proteins in the longer term. For example, although *S. microadriaticum*-colonised Aiptasia had significantly higher expression of six proteins involved in oxidative stress compared to *B. minutum*-colonised anemones under control conditions, in *D. trenchii*-colonised anemones, only catalase was upregulated relative to *B. minutum*-colonised anemones.

All three symbioses also showed a significant upregulation of an iron-binding protein, serotransferrin, in response to heat stress. Iron is a micronutrient involved in photosynthesis (Ferrier-Pagès et al., 2001; Twining & Baines, 2013) and antioxidant protection (Krueger et al., 2015), and a lack of iron during thermal stress has been shown to increase bleaching susceptibility (Shick et al., 2011; Ferrier-Pagès et al., 2018). Heterotrophic feeding is the main source of iron in corals (Ferrier-Pagès et al., 2018), and hosts were fed throughout this experiment. Hosts containing *D. trenchii* showed the largest increase in serotransferrin expression, consistent with evidence that these holobionts were experiencing the largest response to heat stress, so requiring the most marked physiological response.

These results add to the finding that the symbiosis is more stress-tolerant when Aiptasia associates with its native symbiont rather than heterologous ones (Matthews et al., 2017; Gabay et al., 2019). It seems that *D. trenchii* was unable to confer any heat tolerance to the host, but the heterologous symbionts showed that they are able to manipulate the host, and therefore symbiont proliferation and survival, to a certain extent even under heat stress.

2.5.4 Other proteins of interest

When comparing the host proteome in the presence of the different symbiont species, most proteins that differed were those involved in the cytoskeleton, structure and muscle, such as actin, myosin, and actin-binding proteins (see Supplementary Data). The establishment of symbiosis is accompanied by large changes in the cytoskeleton and cellular structure to allow for the presence of symbiont cells within host cells (Fitt & Trench, 1983a; Wakefield & Kempf, 2001), so these changes are thought to reflect the structural reorganisation that occurred over time to accommodate symbiont proliferation.

Niemann-Pick type C (NPC) proteins are usually only detected in associations with homologous symbionts (Sproles et al., 2019). However, the present study identified two NPC proteins, epididymal secretory protein E1 and protein NPC2-like, and both proteins were detected at low quantities in several samples of hosts containing the heterologous *D. trenchii* under control conditions. NPC proteins play a role in cell recognition and immunity processes in mammals (Shi et al., 2012; Dani et al., 2014), and thus have been suggested to play a role in successful symbiosis establishment (Dani et al., 2014, 2017; Rosset et al., 2021). In cnidarians, they are also thought to be involved in sterol transport between the

symbiotic partners across the symbiosome membrane complex (Infante et al., 2008; Storch & Xu, 2009; Dani et al., 2014, 2017; Lehnert et al., 2014; Baumgarten et al., 2015; Oakley et al., 2016; Hambleton et al., 2019). Why NPC proteins were observed here in a novel host-symbiont partnership, in contrast to previous work, is unknown, but could be related to the longer period of time (nearly 3.5 months *versus* five weeks) over which these partners were in symbiosis compared to the earlier study; more work is needed to test whether this is the cause.

2.6 Conclusion

Under control conditions, the host proteome and metabolome varied with symbiont identity. *S. microadriaticum*-colonised anemones displayed depleted carbon stores and increased nitrogen metabolism, while the metabolome of *D. trenchii*-colonised anemones was intermediate between that of *B. minutum*-colonised and *S. microadriaticum*-colonised anemones. However, under stressful conditions, the differences between anemones colonised by the two heterologous symbionts largely disappeared, forming a more generalised response with a clear distinction between hosts colonised by homologous *versus* heterologous symbionts. Heterologous symbionts caused a depletion of carbohydrate stores, which was further exacerbated under thermal stress and, in turn, a need for increased protein degradation and nitrogen metabolism, whether due to their lower population densities or inherent, species-specific attributes. Such changes were not seen in the presence of the homologous symbiont, with this greater thermal tolerance *in hospite* perhaps helping to explain the relative success of *B. minutum* when inoculated simultaneously into *Aiptasia* with either *D. trenchii* or *S. microadriaticum* and exposed to elevated temperature (Gabay et al. 2019). In contrast, *D. trenchii* showed more opportunistic tendencies, consistent with previous research. However, there were signs that cellular stress diminished over time during sustained colonisation by a heterologous symbiont, as the host proteome ultimately showed fewer signs of oxidative stress than in previous, more short-term studies. The expression of NPC proteins in the presence of heterologous symbionts was also notable, as this has not been observed in shorter-term studies and perhaps suggests an improving level of host-symbiont integration over time. Future research should therefore focus on whether symbiosis function can improve over time upon colonisation by a heterologous symbiont, and if so, consider the implications for the adaptive potential of partner switching in light of such a functional shift.

Chapter 3 Temporal and thermal effects on the host metabolome and proteome of a model cnidarian when in symbiosis with homologous and heterologous symbionts

3.1 Abstract

This study measured the effects of symbiont identity, time in symbiosis, and repetitive heat stress on the metabolome and proteome of the sea anemone *Exaiptasia pallida* ('Aiptasia') to determine the level of flexibility in Aiptasia's relationship with its homologous symbiont, and whether initially less compatible, heterologous symbionts can ultimately shift to a more compatible state. Three species of symbionts were used; the homologous *Breviolum minutum*, and two heterologous symbionts, the dinoflagellates *Durisdinium trenchii* and *Symbiodinium microadriaticum*. Three months after initial colonisation with these symbiont species, time-zero samples were taken and the remaining holobionts allocated to one of two thermal conditions: control or repetitive heat stress (RHS) treatment. Heat stress events consisted of a ramping of 1 °C day⁻¹ for seven days, reaching a peak temperature of 32 °C, which was held for three days. After that, temperatures were allowed to drop back to 25 °C and a six-week recovery period followed, before the process was repeated once more. Samples for metabolomics and proteomics were taken again after four months, allowing for a total of seven months since initial colonisation. *B. minutum*-colonised anemones displayed more changes over time than in response to thermal regime, in both the metabolome and proteome. In *B. minutum*-colonised anemones, changes in the host metabolome indicated decreased reliance on host carbohydrate reserves, regardless of thermal regime, allowing for the accumulation of lipids over time. Additionally, the host proteome indicated upregulation of oxidative stress and apoptosis-related proteins, while cell densities of *B. minutum* decreased over time, again, regardless of thermal regime. In contrast, the anemones containing the two heterologous symbionts exhibited less change in their metabolome over time, and differed little from each other at the end of the experiment, especially when exposed to RHS. Both *D. trenchii*- and *S. microadriaticum*-colonised anemones continued to impose higher carbohydrate and nitrogen metabolic demands on their hosts compared to *B. minutum*-colonised hosts, and proteomics results showed higher expression of HSPs and oxidative stress proteins, especially in the anemones exposed to RHS. Additionally, apoptosis-related proteins were downregulated in anemones containing heterologous symbionts compared to the homologous *B. minutum*, indicating continued suppression of host apoptosis by their symbionts. However, *S. microadriaticum*-colonised anemones maintained under control conditions showed signs that could indicate improved host-symbiont integration over time, such as higher host glycerol content than *B. minutum*-colonised hosts, and increased lipid content. Additionally, low expression of NPC proteins were found at the final time-point in *S. microadriaticum*-colonised hosts maintained under control conditions. These results indicate that, while Aiptasia and *B. minutum* are able to integrate and establish a metabolic balance over time, the symbiosis between Aiptasia and *D. trenchii* is more fixed and seems unable to improve, irrespective of thermal regime, likely due to the

opportunistic nature of this symbiont species. The association between *Aiptasia* and *S. microadriaticum* perhaps has a limited capability of changing to a more beneficial state for the host, but only in the absence of stress. These results imply that host specificity is a severely limiting factor in symbiont shuffling as thermal stress relief mechanism.

3.2 Introduction

Coral reefs, one of the world's largest ecosystems, are founded on the successful cooperation between two symbiotic partners: corals and their endosymbiotic dinoflagellate microalgae. The symbiosis is under severe threat of collapse from anthropogenic global warming (Hoegh-Guldberg, 1999; Dove et al., 2013; Hughes et al., 2018). During ever-increasing extreme heat wave events, dysbiosis, i.e. the loss of symbionts from the host, ensues, which can ultimately result in the death of the coral (McClanahan, 2004; Grottoli et al., 2014). Although the molecular basis of dysbiosis remains to be fully resolved, research that could aid coral survival in the face of climate change is a priority.

One critical research avenue relates to the potential for symbiont diversity to offer increased thermal resilience to the cnidarian host, initially described in the 'adaptive bleaching hypothesis' almost three decades ago (Buddemeier & Fautin, 1993; Fautin & Buddemeier, 2004). The microalgae that form symbioses with reef-building corals and many other cnidarian species belong to the family Symbiodiniaceae (LaJeunesse et al., 2018; Nitschke et al., 2020; Pochon & LaJeunesse, 2021), which is comprised of many genera, species, and subspecies. Crucially, these diverse taxa display a range of physiological differences with respect to, e.g. thermal tolerance, photosystem function and inorganic carbon acquisition (Baker et al., 2013; Starzak et al., 2014; Hawkins et al., 2016; McIlroy et al., 2016). Moreover, thermal tolerance of the dominant symbiont species within a host has been shown to translate to thermal tolerance of the holobiont (i.e., the intact symbiosis) (Stat & Gates, 2011; Kemp et al., 2014; Boulotte et al., 2016; Silverstein et al., 2017).

Stress-driven changes in a host's symbiont community composition towards a more thermally tolerant one have been observed in the field (Baker et al., 2004; Berkelmans & Van Oppen, 2006; Jones et al., 2008; Kemp et al., 2014; Cunning, et al., 2015; Boulotte et al., 2016; Silverstein et al., 2017). However, despite the potential thermal benefits of establishing a new partnership, a symbiosis between a host and a 'heterologous' symbiont – that is, a symbiont that does not typically form a persistent symbiosis with the host under regular conditions (*versus* a 'homologous' symbiont) – may lead to a sub-optimal state, resulting in reduced host growth and/or fecundity (Weis et al., 2001; Belda-Baillie et al., 2002; Dunn & Weis, 2009; Gabay et al., 2018; Matthews et al., 2018). In contrast, different species of homologous symbionts in the same host species have been shown to induce indistinguishable host metabolic profiles (Matthews et al., 2020). There therefore seems to be a trade-off between thermal tolerance and host

fitness (Cunning et al., 2015a, b), and after stress events, the symbiont community usually returns to the pre-disturbance community composition (LaJeunesse et al., 2009). This change back to a homologous symbiont community is largely due to the limits posed by host-symbiont specificity, i.e. the capability of a host or symbiont to establish a symbiosis with a different partner, with partners classified as specialists to generalists (Belda-Baillie et al., 2002; Baker, 2003; Goulet, 2006; Putnam et al., 2012; Boulotte et al., 2016; Poland & Coffroth, 2017).

Currently, there have been no experimental studies to test the capacity for novel cnidarian host-dinoflagellate pairings to shift towards a more stable, beneficial nutritional state over a longer timescale. Such changes have been observed in bacterial mutualisms, evolving from parasitic lineages through vertical transmission of symbionts over host generations (Weeks et al., 2007; Bright & Bulgheresi, 2010; Sachs et al., 2011), as well as in the jellyfish *Cassiopea xamachana* which harbours Symbiodiniaceae (Sachs & Wilcox, 2006). The aim of this chapter was thus to establish the effects of symbiont identity, time in symbiosis, and repeated thermal stress events on the host metabolome and proteome of the model sea anemone *Exaiptasia pallida* ('Aiptasia'; Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2019). Aiptasia was used as a model organism as it can easily be rendered and maintained aposymbiotically (i.e., symbiont-free) (Weis et al., 2008), and subsequently colonised with various species of symbionts, including those it does not naturally form a symbiosis with. For this study, the symbiont species used included the species homologous to Aiptasia across the Indo-Pacific region, *Breviolum minutum*, and two heterologous species with a suspected higher thermal tolerance, *Durisdinium trenchii* (Toller et al., 2001; Glynn et al., 2001; Baker et al., 2004; Berkelmans & Van Oppen, 2006; Jones et al., 2008; Correa et al., 2009; LaJeunesse et al., 2009; Stat & Gates, 2011; Cooper et al., 2011; Silverstein et al., 2012; Kemp et al., 2014; Kennedy et al., 2015; Boulotte et al., 2016) and *Symbiodinium microadriaticum* (Robison & Warner, 2006; Ragni et al., 2010; Hawkins & Davy, 2012; Krämer et al., 2012). Both *D. trenchii* and *S. microadriaticum* have been shown to be more energetically costly to the host when inoculated into Aiptasia (Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2019, 2020), while *D. trenchii* also has a reputation for being opportunistic and less beneficial in corals (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Matthews et al., 2017; Sproles et al., 2020). Thus, it was expected that the host metabolome and proteome would differ with symbiont identity, with more evidence for cellular stress in hosts containing heterologous symbionts. However, over time and following thermal stress, it was hypothesised that the different metabolomes and proteomes would converge to more closely resemble the profiles seen during colonisation by the homologous symbiont, thereby providing a means by which coral reefs may adapt to our changing climate.

3.3 Methods

3.3.1 Experimental organisms and inoculation

Aiptasia bleaching and colonisation were done as described previously in Chapter 2. In brief, Aiptasia ($n \sim 3,000$; culture ID NZ1) were rendered aposymbiotic using the menthol bleaching method of Matthews et al. (2016), combined with a weekly cold-shock. Anemones were transferred to sterile 400 mL plastic jars (LabServ) containing 250 mL artificial seawater (ASW; Aquaforest Marine sea salt dissolved in distilled water at 35 ppt), with approximately 30 anemones *per* jar, one day before initial inoculation. For inoculation, the same algal cultures were used for this experiment as in Chapter 2: the homologous species *B. minutum* (culture ID: FlAp2), and the two heterologous species *D. trenchii* (Ap2) and *S. microadriaticum* (CCMP2467). These were maintained in an incubator at 25 °C on a 12 h:12 h light:dark cycle with an irradiance of 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Phillips, 18W 840), in silica-free f/2 culture medium (AusAqua Pty, SA, Australia) and the culture medium refreshed once a month, for more than ten years. Three days prior to inoculation, the culture medium was refreshed once more. As in Chapter 2, inoculation was performed once weekly for four weeks, by application of a drop of symbiont culture at 3 million cells mL^{-1} mixed with freshly hatched *Artemia* sp. nauplii to the oral disc of the anemones. A total of 18 jars were colonised *per* symbiont species.

3.3.2 Experimental treatments

Aiptasia were fed twice weekly with freshly hatched *Artemia* sp. nauplii for the duration of the experiment. Jars were kept in water baths maintained at 25 °C, under an irradiance of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (GE Lighting, T5 F54W/840). After three months under these conditions, one third of the anemones were sampled for the first time-point (6 jars *per* symbiont species), after which the remaining anemones were randomly assigned to one of two groups: control or repetitive heat stress treatment (RHS; Figure 3.1). The control group was kept at 25 °C for the remainder of the experiment, while the RHS treatment was exposed to a series of heat stress events and recovery periods. The heat stress event consisted of a ramping by 1°C day⁻¹ for a period of seven days, reaching a peak temperature of 32 °C, which was maintained for three days. A temperature of 32 °C has been shown to induce cellular stress and bleaching in Aiptasia (Hillyer et al., 2016, 2017). At the end of the third day at 32 °C, anemones were allowed to cool to 25 °C, and this formed the start of a six-week recovery period. After six weeks, the next heat stress event was performed in the same way. Final sampling was conducted seven months after initial colonisation, i.e. four months after the first sampling point, at which point the RHS treatment had gone through two heat stress events and recovery periods.

Figure 3.1 Visual representation of experimental treatments: control was maintained at 25 °C for the entire duration of the experiment, while the repetitive heat stress (RHS) treatment was exposed to two heat stress events and recovery periods. A heat stress event consisted of ramping by 1 °C *per* day to a peak temperature of 32 °C, which was held for three days. Temperatures were allowed to drop to 25 °C and a six-week recovery period followed. Sampling points are indicated in the timeline.

3.3.3 Sampling, homogenisation, and separation of host and symbiont fractions

On sampling days, jars were removed from the water baths, ASW was gently discarded, and the jars sealed and flash-frozen in liquid nitrogen to quench metabolism (~10 s). Jars were stored at -80 °C for 24 h, after which the anemones were quickly removed from the jars and transferred to 2 mL Eppendorf tubes ($n = 6$ replicates *per* treatment, with 30 anemones *per* replicate). These were then stored at -80 °C until further processing.

Further processing included homogenisation of samples in 500 μ L cold (4 °C) milliQ water, for which a mechanical saw-tooth homogeniser was used at a mid-speed setting for 1 min. A 5 mL aliquot of cold milliQ water was added, vortexed briefly, and a 500 μ L sub-sample taken for cell counts and protein quantification. The sample was then centrifuged for 5 min at 2500 $\times$ g to pellet the symbionts. The fractions were separated and collected into separate tubes, and the host fraction spun again to ensure the removal of all symbionts, frozen for at least one hour at -80 °C, and then freeze-dried using a lyophiliser (FreeZone® PlusTM 4.5L Cascade, Labconco, Auckland, New Zealand) for 18 h at -105 °C. The symbiont fraction was resuspended in 500 μ L cold milliQ water and gently vortexed to resuspend the cells. A 100 μ L aliquot was taken to which 500 μ L DNA buffer (DNAB) were added for symbiont identification. The remaining symbiont material was stored at -80 °C.

3.3.4 Confirmation of symbiont genotype and cell counts

Before inoculation, all cultures were genotyped. DNA from cultures was extracted using a bead-beat protocol. At each sampling time-point, anemones were also checked for symbiont genotype. DNA was extracted according to the CTAB-chloroform protocol of Baker & Cuning (2016), as described in Chapter 2. PCR was performed on all extracted DNA using the primers ITSd (forward, 5'-GTGAATTGCAGAACTCCGTG-3') and ITS2rev2 (reverse; 5'-CCTCCGCTTACTTATATGCTT-

3'). PCR conditions consisted of an initial stage of 3 min at 95 °C, then 35 rounds of 15 s at 95 °C, 15 s at 56 °C, and 10 s at 72 °C, with a final elongation stage of 5 min at 72 °C, followed by a resting stage at 4 °C. PCR products were stored at 4 °C. Extracted DNA was sent to Macrogen (Seoul, Korea) for purification and sequencing. Sequence results were checked for quality and then BLAST searched against two databases, GeoSymbio and NCBI, to confirm symbiont identity.

The 500 µL aliquot for cell counts was spun down at 2500 x g to separate host and symbiont fractions. The symbiont fraction was resuspended in 500 µL ASW. Cell counts were performed using a haemocytometer (Improved Neubauer) with eight replicate counts *per* sample, and were normalised to protein content determined *via* the Bradford assay (Bradford, 1976). Cell density (symbionts *per* mg host protein) was then calculated.

3.3.5 Metabolite extractions

Of the six replicate samples *per* treatment, five were used for metabolite and protein profiling, with the sixth sample being a spare ($n = 5$ *per* treatment). The freeze-dried host material was resuspended in 1 mL cold (-20 °C) 100% methanol containing the internal standard ($^{13}\text{C}_6$ sorbitol, at a final concentration of 1%), and then sonicated at 4 °C for 30 min in an ultrasonic bath. The material was then spun down at 3000 x g for 30 min, after which the supernatant containing the semipolar metabolites was drawn off and stored at -80 °C. The remaining material was resuspended in cold (-20 °C) 50% methanol and vortexed for 1 min to extract polar metabolites. Samples were centrifuged again at 3000 x g for 30 min, and the supernatant combined with the first extract. The total pooled extract was centrifuged at 16,000 x g for 15 min at 4 °C to ensure the removal of all particulates. Then, repeatedly 50 µL were dried in glass vial inserts for MS profiling under vacuum (Eppendorf Concentrator 5301, Global Science, Auckland, New Zealand), making for a total of 400 µL dried extract *per* sample. A total of 50 µL *per* sample were combined to create pooled biological controls, of which a total of 400 µL were again dried down.

3.3.6 Metabolite profiling

Metabolite profiling was performed at Metabolomics Australia (Melbourne, Australia), as described in Chapter 2. In brief, dried samples were redissolved in 25 µL of methoxyamine hydrochloride (30 mg/ml in pyridine), and derivatized with 50 µL of N,O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA with 1% TMCS, Thermo Scientific). Samples were allowed to rest for 1 h before 1 µL was injected onto the gas chromatography column using a hot needle technique (Grob & Biedermann, 2002). Splitless injections were performed for each sample.

The gas chromatography–mass spectrometry (GC-MS) system used was comprised of an AOC6000 564 autosampler, a 2030 Shimadzu gas chromatograph and a TQ8050 quadrupole mass spectrometer (Shimadzu, Japan). The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). GC-MS was performed on a 30 m Agilent DB-5 column with 1 μ m film-thickness and 0.25 mm internal diameter column. The injection temperature was set at 280 °C, the MS transfer line at 280 °C and the ion source was adjusted to 200 °C. Helium was used as the carrier gas at a flow rate of 1 ml min⁻¹ and argon gas was used as the collision cell gas to generate the MRM product ion. Sample analysis was performed under the following temperature program: start at injection 100 °C held for 4 min, followed by a 10 °C min⁻¹ oven temperature ramp to 320 °C, and a final hold for 11 min. Approximately 520 quantifying MRM targets were collected using the Shimadzu Smart Database along with the qualifier for each target, which covers about 350 endogenous metabolites and multiple ¹³C-labeled internal standards. Both chromatograms and MRMs were evaluated using the Shimadzu GC-MS browser and LabSolutions Insight software. Resulting area responses were normalized to the internal standard ¹³C₆ sorbitol area response.

3.3.7 Protein extractions

Host protein extractions were performed using a filter-aided sample preparation protocol on the pellet remaining after metabolite extraction, as described in Chapter 2. In brief, samples were resuspended in 500 μ L 5% SDC and sonicated for 20 x 2 s pulses at 20% output, followed by a 20 min incubation at 85 °C with 1% β -mercaptoethanol (BME) to denature proteins. Thereafter, samples were washed twice with ethyl acetate, before drying off remaining ethyl acetate under vacuum (Eppendorf Concentrator 5301, Global Science, Auckland, New Zealand). The sample was spun in a spin-filter (Amicon Ultra, Millipore) at 14,000 x g for 15 min, and then washed three times with 50 mM Tris. The protein content was quantified using Qubit, and protein extractions continued with 100 μ g protein on the filter. Any remaining material was frozen at -80 °C in case of re-analysis. BME was added to a final concentration of 10 mM, and incubated at 37 °C for 10 min. Then, iodoacetamide (IAA) was added to a final concentration of 50 mM, followed by an incubation of 20 min at room temperature. Samples were digested overnight at 37 °C with 2 μ g trypsin.

Following protein digestion, samples were centrifuged at 14,000 x g for 15 min, and any remaining SDC was pelleted with the addition of 1% final concentration formic acid. A Ziptip protocol was followed for desalting the samples, which were then eluted in 50% and 30% acetonitrile, combined, and dried down completely. Samples were rehydrated in 0.1% formic acid and incubated at 37 °C for 30 min. Qubit was used again to quantify the protein content, to run 200 ng peptides for identification and quantification.

3.3.8 Protein profiling

Peptide samples were profiled using liquid chromatography-tandem mass spectrometry (LC-MS-MS). To separate peptide samples, the LC was performed with a linear gradient from 5% to 35% buffer B (buffer A: 0.1% formic acid; buffer B: 80% acetonitrile, 0.1% formic acid) at 300 nL min⁻¹ on an Acclaim PepMap C18, 3 µm, 100 Å column (Thermo Scientific, Auckland, New Zealand) and Ultimate 3000 liquid chromatography system (Dionex, Sunnyvale, CA). Peptide ionisation was performed by electrospray at 1.8 kV and analysed by an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific). The Orbitrap acquired precursor mass spectra at a resolution of 120,000, rejecting singly-charged ions, while quadrupole isolation was enabled and an automatic gain target of 7.0e5 set. The injection time was set to a maximum of 50 ms. The 20 most intense precursor spectra were fragmented by higher-energy collision dissociation, and then analysed in the ion trap. The automatic gain target was 5.0e3 and this had a maximum injection time of 300 ms. Dynamic exclusion was enabled with a duration of 60 s.

Protein identification was performed from raw spectrum files, using the Andromeda search algorithm in MaxQuant (1.6.10.43, Cox & Mann, 2008; Cox et al., 2014). Proteins were searched against the *Aiptasia* genome (1.1, Baumgarten et al., 2015). The digest enzyme was set to trypsin, allowing for a maximum of two missed cleavages. Oxidation of methionine, and acetylation of the protein n-terminus were considered viable modifications, while carbamidomethylation of cysteine was considered a fixed modification. The first peptide search tolerance was 20 ppm, the main search peptide tolerance was 4.5 ppm, and the mass tolerance was 0.5 Da for the ion trap MS2 search. Label-free quantification was enabled, with a minimum of two unique plus razor peptides for quantification, and match between runs enabled. Peptide spectrum match and protein false discovery rates were both set to 1%, with a minimum of two peptides required for identification.

3.3.9 Data analysis and visualisation

Total anemone protein content for the start time-point was averaged and subtracted from the total protein content for the end-points, to estimate growth as the increase in total protein content *per* anemone. Data were tested for normality and homoscedasticity, and then tested with a two-way ANOVA in R (version 4.0.3; R Core Team, 2020). Cell density data were tested for normality and homoscedasticity, and then tested with a series of ANOVAs in R; a one-way ANOVA to compare between species for the start-point, a two-way ANOVA with the factors ‘treatment’ and ‘species’ to compare between the species for the end-point, as well as a one-way ANOVA within each species to measure the effect of time and treatment. For this last comparison, a combined factor of ‘treatment and time-point’ was made to compensate for the lack of a full factorial design. To correct for multiple

testing, a conservative approach was adopted by applying a Bonferroni correction: dividing α by the number of tests. Therefore, $\alpha = 0.05/3 = 0.0167$. Cell density data for the heat stress events were tested separately, using a one-way ANOVA with the factor ‘treatment’ *per* symbiont species *per* time-point, to establish if heat stress reduced cell density, i.e., caused bleaching.

Identified metabolites were normalised against the internal $^{13}\text{C}_6$ sorbitol standard, as well as total protein content determined during protein extractions, and finally to total metabolite abundance *per* sample. As PCAs constructed in R showed potential influential outliers, an outlier detection method was performed using PCA loadings. Outliers were identified using a conservative Median Absolute Deviation (MAD) approach (Leys et al., 2013), meaning if datapoints were more than six MAD away from the median, they were removed from the analysis. This led to the removal of three individual replicates, all from different treatment groups, so a minimum of $n = 4$ was maintained. To detect differentially expressed metabolites (DEM) between treatment groups, data were $\log_2$ transformed and analysed using the online PolySTest (Schwämmle et al., 2020). DEM were identified using the Limma test, with an FDR threshold of $q < 0.05$, and only metabolites with a log fold-change larger than 0.5 or smaller than -0.5 are reported.

For the proteomics, identified proteins and label-free quantification intensities were analysed in Perseus (1.6.15.0, Tyanova et al., 2016) to remove known false detections and contaminants from the analysis. Samples were then normalised to total intensity values *per* sample and $\log_2$ transformed in R (4.0.3, R Core Team, 2020). Data analysis was done using PolySTest (Schwämmle et al., 2020) online to identify significantly differentially expressed proteins between treatments. Significant proteins were identified using the Miss test in PolySTest (Schwämmle et al., 2020) with an FDR threshold of $q < 0.05$, and only annotated proteins with a log fold-change larger than 0.25 or -0.25 are reported. PCAs were made based on proteome profiles using R (version 4.0.3, R Core Team, 2020), and showed no sign of influential outliers, so $n = 5$ remained for the proteomics data. One-way ANOVAs were then used to test if the PCA axes were able to significantly separate the samples based on treatment or species.

3.4 Results

3.4.1 Host growth and symbiont population density

Host growth, measured as increase in total protein *per* anemone (Figure 3.2), was significant for the interaction between species and treatment (two-way ANOVA, $F_{2,26} = 3.82$, $p = 0.0351$). Anemones containing the homologous symbiont increased most in size, without any significant effects of RHS (Tukey HSD *post hoc*, $p = 0.9832$). As for anemones harbouring heterologous symbionts, those containing *D. trenchii* grew similarly to *B. minutum*-colonised hosts under control conditions, but RHS

caused the anemones to grow significantly less than *B. minutum*-colonised anemones (Tukey HSD *post hoc*, $p = 0.0027$). In comparison, *S. microadriaticum*-colonised anemones kept under both control and thermally stressful conditions grew significantly less than *B. minutum*-colonised anemones under either control or elevated temperature (Tukey HSD *post hoc*, $p < 0.05$ for all comparisons). By comparison, under control conditions, *S. microadriaticum*-colonised anemones grew less than *D. trenchii*-colonised anemones (Tukey HSD *post hoc*, $p = 0.0243$), though this difference was not apparent under RHS (Tukey HSD *post hoc*, $p = 0.9999$).

Figure 3.2 Increase in total host protein content ($\pm$ SE, *per anemone*) for anemones in symbiosis with different species of Symbiodiniaceae (*Breviolum minutum*, *Durisdinium trenchii*, or *Symbiodinium microadriaticum*) from three months to seven months in symbiosis, having been maintained either at control temperature (Con), or exposed to repetitive heat stress (RHS) ($n = 6$ *per treatment*).

With respect to symbiont cell density (Figure 3.3), *B. minutum* averaged 6.65×10^6 ($\pm 5.68 \times 10^5$ SE) cells *per mg* host protein three months after symbiosis establishment, which reduced by 70% over the remainder of the experiment, regardless of treatment (one-way ANOVA, $F_{2,14} = 45.89$, $p = 4.05 \times 10^{-7}$). Both heterologous symbiont species had significantly lower cell densities at the start of the experiment compared to *B. minutum* (one-way ANOVA, $F_{2,15} = 82.75$, $p = 7.89 \times 10^{-9}$, Tukey HSD *post hoc*, $p < 0.0001$ for both comparisons). However, while *D. trenchii* displayed a steep drop similar to that seen for the homologous symbiont between the three-month time-point and the end-point, i.e. four months later, under both control and RHS conditions (one-way ANOVA, $F_{2,14} = 44.04$, $p = 9.12 \times 10^{-7}$), the population of *S. microadriaticum* remained stable, though at very low densities (one-way ANOVA, $F_{2,13} = 1.597$, $p = 0.24$). At the final time-point, both heterologous species still had significantly lower

cell densities than *B. minutum* (two-way ANOVA, $F_{2,27} = 158.25$, $p = 1.23 \times 10^{-15}$), but there was no effect of thermal treatment (two-way ANOVA, $F_{1,27} = 2.93$, $p = 0.0983$). During the heat stress events, only *D. trenchii*-colonised anemones showed significantly reduced symbiont cell densities (HS1: one-way ANOVA, $F_{1,10} = 18.62$, $p = 0.0015$; HS2: one-way ANOVA, $F_{1,10} = 11.2$, $p = 0.0074$).

Figure 3.3 Cell densities ($\pm$ SE) of the different symbiont species (*Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*) throughout the experiment for control (Con) and repetitive heat stress (RHS) treatment (ramping to 32 °C over seven days, then held for three days). Side legend indicates timing and temperatures of sampling points; start and end-points were all sampled at control temperature, i.e. at the end of the recovery period for the RHS treatment, while HS1 and HS2 were sampled at the peak of the heat-stress event to establish bleaching rates. The time is in months since initial inoculation with symbionts. * indicates significant differences ($p < 0.05$) between control and RHS during heat-stress events, while letter groups indicate significant differences between and within species for the start and end sampling points ($n = 5$).

3.4.2 Symbiosis-specific responses over time and thermal treatment in host metabolome and proteome

B. minutum-colonised anemones showed a large response over time in both their metabolome and proteome, though thermal treatment did not have a large effect on these responses (Table 3.1). Both symbioses with the heterologous symbionts displayed fewer changes over time, and also few differences at the end of thermal treatment (i.e., control vs. RHS). Only the host metabolome of *B. minutum*-colonised anemones was significantly separated over the primary PC axis in response to thermal regime (Figure 3.4), with small changes seen in all functional categories (Figure 3.5). For the TCA cycle and glycolysis, these changes included a downregulation of fumarate (-2.39 log₂-fold and -1.87 log₂-fold

for control and RHS, respectively) and succinate (-1.23 and -1.25 log₂-fold for control and RHS, respectively), and an upregulation in citrate (0.84 and 0.97 log₂-fold for control and RHS, respectively) for both control and RHS treatment, while GABA and sucrose were only upregulated over time under control conditions (1.15 and 1.72 log₂-fold, respectively). With respect to carbohydrate metabolism, some carbohydrates increased (mannitol; 1.85 and 1.74 log₂-fold increase in control and RHS, respectively) or decreased (glycolic acid, -0.82 and -0.85 log₂-fold; and glycerol, -1.93 and -1.18 log₂-fold change in control and RHS, respectively) in relative abundance over time regardless of thermal treatment, while psicose and threonic acid increased only in the RHS treatment (1.24 and 0.86 log₂-fold, respectively).

Table 3.1 Summary of differentially expressed metabolites and proteins between Aiptasia in symbiosis with different species of symbionts (*Breviolum minutum*, *Durisdinium trenchii*, or *Symbiodinium microadriaticum*) in response to time in symbiosis maintained either under control conditions (con, 25 °C), or exposed to repetitive heat stress (RHS) events (ramping to 32 °C, followed by six weeks recovery), and compared to each other at the end-point of this experiment after either control or RHS treatment.

Within species over time	Differentially expressed metabolites			Differentially expressed proteins		
	Start - End con	Start - End RHS	End con - End RHS	Start - End con	Start - End RHS	End con - End RHS
<i>B. minutum</i>	20	17	0	89	76	10
<i>D. trenchii</i>	2	0	0	33	30	3
<i>S. microadriaticum</i>	14	1	2	38	22	68
Between species at final time point	Differentially expressed metabolites		Differentially expressed proteins			
	Con	RHS	Con	RHS		
<i>S. microadriaticum</i> vs. <i>B. minutum</i>	23	19	86	116		
<i>D. trenchii</i> vs. <i>B. minutum</i>	21	22	59	48		
<i>S. microadriaticum</i> vs. <i>D. trenchii</i>	0	0	26	22		

In terms of differentially expressed proteins (DEPs; Table 3.2) in *B. minutum*-colonised anemones, very few changes were apparent in the host's core metabolism, with only an upregulation in triosephosphate isomerase (0.36 log₂-fold) and a downregulation of succinyl-CoA ligase (-0.42 log₂-fold) over time under control conditions being apparent. Nitrogen metabolism showed increases in aspartate aminotransferase at the end-point *versus* start for both the control and RHS treatment (0.53 log₂-fold and 0.41 log₂-fold, respectively), while several proteins involved in the electron transport chain were downregulated in both the control and RHS treatment (ATP synthase subunit β, -0.42 and -0.44 log₂-fold; ATP synthase subunit O, -0.36 and -0.65 log₂-fold; and ATP synthase subunit d, -0.57 and -0.47 log₂-fold change different under control and RHS conditions, respectively). Additionally, there was a

decrease in the expression of NPC proteins between the beginning and end of the experiment under both control and RHS conditions (epididymal secretory protein E1, -0.45 and -0.50 $\log_2$ -fold change; and protein NPC2-like, -1.33 and -1.19 $\log_2$ -fold change in the control and RHS treatment, respectively). Conversely, two apoptosis-related proteins were upregulated in both the control and RHS treatment as the experiment progressed (death-associated protein kinase 3, 0.73 and 0.76 $\log_2$ -fold; and calmodulin, 0.50 and 0.45 $\log_2$ fold-change in the control and RHS treatment, respectively), while another was downregulated (programmed cell death 6-interacting protein, -0.37 and -0.30 $\log_2$ -fold change, respectively). A full table of all DEPs can be found in the supplementary information.

Figure 3.4 PCAs of host metabolome (A, B, C) and proteome (D, E, F) for Aiptasia in symbiosis with different species of symbionts: *Breviolum minutum* (A, D), *Durudinium trenchii* (B, E), or *Symbiodinium microadriaticum* (C, F) containing individual replicates of the different time-points of the experiment, at the start and end, having gone through either the control treatment (Con) or the repetitive heat stress treatment (RHS). Ellipses represent 95% confidence intervals, and axes in bold represent significant separation of groups (significance level: ** p < 0.01).

In comparison to the metabolome of anemones containing the homologous symbiont, *D. trenchii*-colonised anemones exhibited very few differences in their metabolome over time (Figure 3.5). The only differences were increased expression of beta-alanine (1.68 $\log_2$ -fold) and a decrease in phenylacetic acid (-1.22 $\log_2$ -fold) under control conditions. The proteome (Table 3.2) also showed some of the fewest changes, with the main differences being the downregulation of heat-stress and protein-folding proteins, more so under control conditions than in the RHS treatment (eight DEPs under

Figure 3.5 Relative abundance of metabolites *per* metabolic category in Aiptasia host tissue colonised by different symbiont species (*Symbiodinium microadriaticum*, *Breviolum minutum*, or *Durudinium trenchii*) at start and end of the experiment, having being maintained under control conditions or exposed to the repetitive heat stress (RHS) treatment. * indicates significant differences between the start-point and end control treatment, while # indicates significant differences between the start-point and end RHS treatment, with the colour indicating up- (red) or downregulation of the metabolite at the end-point compared to the start. Hatching of bars indicates significant differences from *B. minutum*-colonised anemones and is indicated for the end time-points only. Colours of the bars indicate up- (red) or downregulation in anemones harbouring the heterologous symbionts.

control conditions, of which one was upregulated, *versus* four DEPs under RHS, of which two were upregulated). Additional temporal changes included a strong downregulation of glutamine synthase (-1.59 and -1.47 log₂-fold for the control and RHS treatment, respectively), and increased expression of

two oxidative stress proteins for the RHS treatment alone (catalase and superoxide dismutase; 0.37 and 0.35 log₂-fold upregulated, respectively). Additionally, the iron-binding protein, melanotransferrin, showed strongly increased expression over time under both control and RHS conditions (1.29 and 1.25 log₂-fold, respectively).

When in symbiosis with *S. microadriaticum*, the host metabolome showed increased amino acid metabolism over time under control conditions (Figure 3.5), with an increased relative abundance of the amino acids proline (2.36 log₂-fold), serine (1.60 log₂-fold), threonine (1.16 log₂-fold), alanine (0.96 log₂-fold), leucine (0.93 log₂-fold), and aspartate (1.31 log₂-fold). Furthermore, there was a temporal increase in GABA (1.10 log₂-fold) under control conditions in *S. microadriaticum*-colonised anemones. Under RHS, the only difference between the start and end-point of the experiment was the reduced abundance of 3-hydroxyisovaleric acid (-1.52 log₂-fold). Two DEMs were identified between the control and RHS treatment at the final sampling point, with hosts that were subjected to RHS having a reduced abundance of palmitoleic acid and glycerol than those maintained under control conditions (-1.88 and -1.57 log₂-fold, respectively). In contrast to the metabolite data for *S. microadriaticum*-colonised anemones, DEPs for these anemones were most different between the control and RHS treatment at the final sampling point (Table 3.2). Most of these differences showed that RHS caused increased expression of heat stress proteins (HSPs; six DEPs, of which five were upregulated). By contrast, core metabolism proteins remained unchanged across time under RHS, though under control conditions DEPs included the core metabolic proteins malate synthase, fructose-bisphosphate aldolase C, and malate dehydrogenase (0.64, -0.44 and -0.44 log₂-fold change, respectively).

Table 3.2 Differentially expressed proteins for hosts in symbiosis with different species of symbionts (*Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*), compared between the different time-points and treatments (i.e. start: three months after initial colonisation under control conditions; end con: seven months after initial colonisation under control conditions; end RHS: seven months after initial colonisation, having gone through two heat stress events and recovery periods since the start time point; n = 5).

Breviolum minutum			Durusdinium trenchii			Symbiodinium microadriaticum			Annotation	UniProt ID	Gene name	E value
End con - Start	End RHS - Start	End RHS - End con	End con - Start	End RHS - Start	End RHS - End con	End con - Start	End RHS - Start	End RHS - End con				
Core metabolism												
						0.64			Malate synthase	P95329	MASY	1.1E-143
						-0.44		0.41	Fructose-bisphosphate aldolase C	P53448	ALDOC	3.6E-138
0.36									Triosephosphate isomerase	P00940	TPIS	1.3E-94
-0.42									Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	O19069	SUCA	2.7E-140
		-0.31		-0.28					Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	Q5R5J1	PCKGC	1.4E-236
						-0.44			Malate dehydrogenase, mitochondrial	P00346	MDHM	1.9E-120
Nitrogen metabolism												
0.53	0.41								Aspartate aminotransferase, cytoplasmic	P13221	AATC	1.2E-137
							0.41		Serine hydroxymethyltransferase, mitochondrial	P34897	GLYM	1.8E-174
						-0.41			Glutamate carboxypeptidase 2	P70627	FOLH1	5.1E-158
							-0.53		Aspartate aminotransferase, mitochondrial	P12345	AATM	4.7E-169
			-1.59	-1.47					Glutamine synthetase	P15105	GLNA	1.4E-153
Heat stress and protein folding												
			-0.30			-1.18		0.95	Calumenin-A	B5X4E0	CALUB	8.9E-61
0.85	0.32							0.62	Peptidyl-prolyl cis-trans isomerase B	P24367	PPIB	3.9E-18
								0.59	Calnexin	P27824	CALX	3.8E-18
								0.56	Calreticulin	P29413	CALR	1.8E-76
0.53	0.37								Heat shock protein HSP 90-alpha 1	O57521	HS90B	1.2E-297
			0.46	0.38		-0.61		0.52	Heat shock 70 kDa protein	P02827	HSP70	4.3E-237
				0.47					Universal stress protein YxiE	Q8VYN9	PHO32	9.9E-8
		-0.33							Endoplasmic	P08110	ENPL	1.8E-271
			-0.36						60 kDa heat shock protein, mitochondrial	P18687	CH60	1.6E-220
			-0.42	-0.41		-0.37			78 kDa glucose-regulated protein	P29844	BIP	1.8E-295
						-1.17	-0.44		Reticulocalbin-3	Q28BT4	CALU	8.6E-43
-0.58	-0.47		-0.53					-1.51	Peptidyl-prolyl cis-trans isomerase	P54985	PPIA	8.9E-76
			-0.53						Protein disulfide-isomerase A6	Q11067	PDIA6	2.3E-142
			-0.59						10 kDa heat shock protein, mitochondrial	P61603	CH10	1.0E-29
			-0.98	-0.72					Calumenin-B	B5X4E0	CALUB	4.3E-77

Table 3.2 continued

<i>Breviolum minutum</i>			<i>Durusdinium trenchii</i>			<i>Symbiodinium microadriaticum</i>			Annotation	UniProt ID	Gene name	E value
End con - Start	End RHS - Start	End RHS - End con	End con - Start	End RHS - Start	End RHS - End con	End con - Start	End RHS - Start	End RHS - End con				
-0.97	-0.86								B-cell receptor-associated protein 31	Q5R8H3	BAP31	9.7E-34
Apoptosis												
0.73	0.76					0.25	0.39		Death-associated protein kinase 3	O43293	DAPK3	4.2E-40
						-0.75		0.76	Caspase-7	P55214	CASP7	4.0E-56
0.50	0.45							-0.75	Calmodulin	P04352	CALM	1.5E-13
								0.48	Reticulon-4	Q9NQC3	RTN4	9.5E-34
				0.35					Sorcini	Q8VC88	GRAN	5.3E-42
-0.37	-0.30								Programmed cell death 6-interacting protein, partial	Q9W6C5	PDC6I	8.9E-25
							-0.91	-1.07	Calmodulin-like protein 3	P53440	CALMF	8.8E-6
Immune response												
			0.40				0.68	0.48	Major antigen			0
NPC proteins												
						-0.56		0.35	Protein NPC2-like	Q9VQ62	NPC2	1.6E-23
-0.45	-0.50								Epididymal secretory protein E1	P79345	NPC2	1.2E-15
-1.33	-1.19								Protein NPC2-like	Q9VQ62	NPC2	1.2E-18
Oxidative stress and redox process												
0.70	0.30								Peroxiredoxin-4	Q9Z0V5	PRDX4	1.0E-83
0.64	0.44								Putative glutathione S-transferase 6	Q8JFZ2	GSTP1	8.3E-9
								0.63	Superoxide dismutase [Mn], mitochondrial	Q5FB30	SODM	3.0E-90
0.61									Glutathione S-transferase Mu 2	Q9TSM4	GSTM2	3.5E-52
0.34	0.38			0.37					Catalase	P04040	CATA	7.2E-209
				0.35					Superoxide dismutase [Cu-Zn]	P81926	SODC	1.3E-57
Protein degradation and turnover												
1.46	1.11								Ubiquitin-conjugating enzyme E2 L3	P68037	UB2L3	7.1E-67
				1.27		1.17			Transmembrane protease serine 3	Q8K1T0	TMPS3	1.1E-45
								0.76	Blastula protease 10	P55112	NAS4	2.1E-34
0.27						0.51			Calpain-B	Q9VT65	CANB	2.9E-157
			-0.37						Cathepsin B	P83205	CATB	4.0E-115
								-0.57	Putative aminopeptidase W07G4.4	Q27245	YH24	5.5E-41
-1.63	-1.10								Transmembrane protease serine 9	B3EWZ6	MLRP2	3.9E-89

Table 3.2 continued

Breviolum minutum			Durusdinium trenchii			Symbiodinium microadriaticum			Annotation	UniProt ID	Gene name	E value
End con - Start	End RHS - Start	End RHS - End con	End con - Start	End RHS - Start	End RHS - End con	End con - Start	End RHS - Start	End RHS - End con				
Electron transport												
								0.38	ATP synthase subunit delta, mitochondrial	P35434	ATPD	4.3E-30
						0.27			ATP synthase subunit alpha, mitochondrial	Q03265	ATPA	7.3E-255
-0.42	-0.44		-0.32						ATP synthase subunit beta, mitochondrial	Q5ZLC5	ATPB	6.0E-233
-0.36	-0.65								ATP synthase subunit O, mitochondrial	Q24439	ATPO	2.5E-35
-0.57	-0.47		-0.72						ATP synthase subunit d, mitochondrial	P31399	ATP5H	1.0E-20
				-1.08					Cytochrome c oxidase subunit 6A, mitochondrial	O13085	COX6A	5.0E-20
Iron binding												
			1.29	1.25				0.78	Melanotransferrin	O93429	TRFE	2.9E-47
0.65									V-type proton ATPase catalytic subunit A	P31400	VATA	3.7E-308
-0.51		0.58				-0.73			Soma ferritin	P42577	FRIS	1.5E-65
						-0.62		0.38	Serotransferrin	P56410	TRFE	5.7E-48

3.4.3 Inter-symbiosis differences in the host metabolome and proteome

This study focused on the longer-term development of symbiosis with different symbiont species, and will therefore not report on the host metabolome and proteome of the different symbiotic associations at the start of the experiment (i.e., three months after initial colonisation), as these differences were the focus of Chapter 2. Nevertheless, there is value in comparing the start of the current experiment with the control samples in Chapter 2, to gauge the degree of consistency. In this respect, there were minor differences in both the host metabolome and proteome. *B. minutum*-colonised anemones possessed three DEMs (3-hydroxyisovaleric acid, 1.45 log₂-fold; glycerol 1.42 log₂-fold; and beta-alanine, -2.00 log₂-fold different from the host metabolome in Chapter 2) and three DEPs (acyl-CoA-binding protein, 0.63 log₂-fold; 60S acidic ribosomal protein, 0.88 log₂-fold; and an unknown protein, 0.66 log₂-fold different from the host proteome in Chapter 2). *D. trenchii*-colonised hosts possessed no DEMs and seven DEPs (60S acidic ribosomal protein P1, 0.62 log₂-fold; myophilin, 0.51 log₂-fold; peptidyl-prolyl cis-trans isomerase, 0.28 log₂-fold; neutral and basic amino acid transport protein rBAT, -0.67 log₂-fold; tubulin polymerisation-promoting protein family member 2, -0.27 log₂-fold; 14-3-3 protein epsilon, -0.28 log₂-fold; and sorcin, -0.34 log₂-fold different from the host proteome in Chapter 2). *S. microadriaticum*-colonised anemones possessed one DEM (3-hydroxyisovaleric acid, 1.29 log₂-fold different from host metabolome in Chapter 2) and no DEPs.

At the end of the experiment, the host metabolome when colonised by the homologous symbiont was significantly separated from that when colonised by either of the heterologous symbionts, explaining over 40% of the variation seen within the profiles, under both control conditions and the RHS treatment (Figure 3.6A, B). No differences in the host metabolome were found between *D. trenchii*- and *S. microadriaticum*-colonised anemones either under control or RHS conditions, though similar numbers of DEMs were measured in these anemones to the number measured in *B. minutum*-colonised anemones (Table 3.1). Changes in relative metabolite abundance are presented in Figure 3.5, which shows that most changes occurred in carbohydrate metabolism (12 DEMs), followed by the TCA cycle and glycolysis (six DEMs). These categories of metabolites showed the same trends: downregulation under both control and RHS treatment in the presence of heterologous versus homologous symbiont species. Amino acid metabolism exhibited increased activity, with an upregulation of tyramine (1.43 log₂-fold in *S. microadriaticum*-colonised anemones under control conditions, versus 2.07 and 1.56 log₂-fold in *D. trenchii*-colonised anemones under control and RHS conditions compared to *B. minutum*-colonised anemones, respectively). Moreover, there was an increased abundance of the nitrogenous metabolic end products urea (1.85 and 1.32 log₂-fold in *S. microadriaticum*-colonised and *D. trenchii*-colonised anemones under control conditions compared to *B. minutum*-colonised anemones) and uric acid (1.43 log₂-fold in *S. microadriaticum*-colonised hosts under RHS; 1.47 log₂-fold in *D. trenchii*-colonised anemones under control conditions compared to *B. minutum*-colonised

anemones). In contrast, the host proteome at the end of the experiment was not significantly separated over any PC axes and instead showed considerable overlap (Figure 3.6C, D), though clustering of the samples based on DEPs showed a clear grouping of the host proteome by symbiont identity in the RHS treatment (Figure 3.7). The DEPs supported the metabolomic evidence for increased carbohydrate metabolism in the symbioses with both *S. microadriaticum* and *D. trenchii* compared to the association with *B. minutum*, with upregulation of core metabolism and electron transport proteins (Table 3.3).

Figure 3.6 PCA based on host metabolome (A, C) and proteome (C, D) of *Aiptasia* in symbiosis with different symbiont species (*Breviolum minutum*, *Durisdinium trenchii* or *Symbiodinium microadriaticum*), at the end of the experiment, having being maintained under control conditions (A, C), or exposed to the repetitive heat stress treatment (B, D). Ellipses represent 95% confidence intervals, and axes in bold represent significant separation of groups (significance level: *** p < 0.001).

Additionally, the proteome revealed increased protein degradation and turnover, nitrogen metabolism, HSPs, chaperones, and oxidative stress proteins, as well as proteins involved in maintaining redox homeostasis in the presence of the heterologous symbionts when compared to *B. minutum*-colonised anemones, under both control and RHS conditions. Furthermore, *S. microadriaticum*-colonised anemones alone exhibited an upregulated immune response compared to *B. minutum*-colonised

anemones (interferon-induced protein 44-like, 1.05 log₂-fold increase under control; adenosylhomocysteinase, 0.26 and 0.49 log₂-fold increase under control and RHS, respectively; major antigen, 0.46 log₂-fold increase under RHS). Only one NPC protein was expressed in anemones associated with *S. microadriaticum* under control conditions (protein NPC2-like, -2.26 log₂-fold compared to *B. minutum*-colonised hosts), whereas hosts containing *D. trenchii* showed expression of NPC proteins, although at significantly lower levels than *B. minutum*-colonised hosts (epididymal secretory protein E1, -2.05 versus -1.72 log₂-fold; NPC2-like protein, -1.75 versus -2.19 log₂-fold under control versus RHS compared to *B. minutum*-colonised anemones, respectively). Apoptosis-related proteins were mainly downregulated with the heterologous symbionts under control conditions, but at the end of the RHS treatment, various apoptosis-related proteins showed a mix of up- and downregulation in both *D. trenchii*- and *S. microadriaticum*-colonised anemones compared to *B. minutum*-colonised anemones (see Table 3.3).

Figure 3.7 Heat maps of differentially expressed proteins (DEP; $q < 0.05$) between Aiptasia in symbiosis with different symbiont species (*Breviolum minutum*, *Durudinium trenchii* and *Symbiodinium microadriaticum*) for seven months, having being maintained under (A) control conditions (125 total DEPs) or (B) repetitive heat stress treatment (142 total DEPs) for the final four months. Rows are proteins, columns are biological replicates ordered by hierarchical clustering of protein expression. The colour scale is log₂ fold-change relative to the protein's mean. The tree is a hierarchical clustering of proteins based on Pearson's correlation of their expression across samples.

Table 3.3 Differentially expressed proteins between Aiptasia containing different symbiont species (*Breviolum minutum*, *Durudinium trenchii*, or *Symbiodinium microadriaticum*), after seven months in symbiosis. Anemones exposed to either control conditions (Con) or repetitive heat stress (RHS) for the final four months (n = 5).

<i>S. microadriaticum</i> vs. <i>B. minutum</i>		<i>D. trenchii</i> vs. <i>B. minutum</i>		<i>S. microadriaticum</i> vs. <i>B. minutum</i>		Annotation	UniProt ID	Gene name	Eval
End Con	End RHS	End Con	End RHS	End Con	End RHS				
Core metabolism									
0.37	0.80	0.52	0.59			Fructose-bisphosphate aldolase C	P53448	ALDOC	3.6E-138
0.41	0.79		0.54			Glyoxylate reductase/hydroxypyruvate reductase	Q9UBQ7	GRHPR	8.1E-97
	0.72					Phosphoenolpyruvate phosphomutase	P56839	PEPM	1.2E-145
0.69				0.51		Fructose-1,6-bisphosphatase 1	P00636	F16P1	1.1E-117
0.27	0.62				0.45	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	Q5R5J1	PCKGC	1.4E-236
	0.57					Isocitrate dehydrogenase [NADP], mitochondrial	Q4R502	IDHP	4.6E-175
0.48	0.55				0.42	Alpha-enolase	P06733	ENOA	8.3E-199
	0.53					Citrate synthase, mitochondrial	Q7ZVY5	CISY	9.1E-190
		0.33				Malate dehydrogenase, mitochondrial	P00346	MDHM	1.9E-120
		-0.34			0.32	Glyceraldehyde-3-phosphate dehydrogenase	Q05025	G3P	5.7E-146
-0.64						Triosephosphate isomerase	P00940	TPIS	1.3E-94
Nitrogen metabolism									
	5.00					Glutamate dehydrogenase	P94598	DHE3	3.7E-162
1.94	2.09					Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	A7YWE4	AL4A1	4.5E-172
	0.82					S-adenosylmethionine synthase isoform type-2	Q91X83	METK1	1.2E-118
0.46	0.81					Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	Q17M80	MMSA	2.5E-206
	0.66				0.49	Serine hydroxymethyltransferase, mitochondrial	P34897	GLYM	1.8E-174
	0.52					3-hydroxyisobutyrate dehydrogenase, mitochondrial	Q99L13	3HIDH	1.3E-105
-0.77	-0.46	-0.79	-0.86			Glutamate dehydrogenase	P94598	DHE3	8.2E-172
-0.98	-0.61	-1.23				Asparagine synthetase [glutamine-hydrolyzing], partial	Q5ZJU3	ASNS	1.3E-190
-2.47	-2.83	-2.05	-1.92			Glutamine synthetase	P15105	GLNA	1.4E-153
Lipid metabolism									
0.92						Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	P00348	HCDH	6E-57
0.62	0.81					Propionyl-CoA carboxylase beta chain, mitochondrial	P79384	PCCB	3.1E-226
	0.81					Enoyl-CoA hydratase, mitochondrial	Q8BH95	ECHM	1.1E-100
Heat stress and protein folding									
0.79	-0.83			1.18		Peptidyl-prolyl cis-trans isomerase	P54985	PPIA	8.9E-76
	1.16					Endoplasmic reticulum resident protein 44	Q9D1Q6	ERP44	5E-86
	1.11				0.61	Peptidyl-prolyl cis-trans isomerase B	P24367	PPIB	3.9E-81

Table 3.3 continued

<i>S. microadriaticum</i> vs. <i>B. minutum</i>		<i>D. trenchii</i> vs. <i>B. minutum</i>		<i>S. microadriaticum</i> vs. <i>B. minutum</i>		Annotation	UniProt ID	Gene name	Evalue
End Con	End RHS	End Con	End RHS	End Con	End RHS				
			0.96			Thrombospondin-1	Q28178	TSP1	3.5E-16
	0.95		0.68			Reticulocalbin-3	Q28BT4	CALU	8.6E-43
0.81						Protein NDRG1	Q66IG4	NDRG1	8.5E-33
	0.68					Calnexin	P27824	CALX	3.8E-18
	0.62		0.63			Universal stress protein YxiE	Q8VYN9	PHO32	9.9E-8
	0.58					Peptidyl-prolyl cis-trans isomerase FKBP3	O46638	FKBP3	3.6E-62
0.56	0.57	0.44				60 kDa heat shock protein, mitochondrial	P18687	CH60	1.6E-220
	0.48	-0.53		0.45		Endoplasmic	P08110	ENPL	1.8E-271
	0.47	0.32				Protein disulfide-isomerase A3	P30101	PDIA3	2.6E-139
	0.35					Protein disulfide-isomerase	P09103	PDIA1	2E-161
	0.29					78 kDa glucose-regulated protein	P29844	BIP	1.8E-295
					0.25	Heat shock protein HSP 90-alpha 1	O57521	HS90B	1.2E-297
-0.48				-0.38		Heat shock 70 kDa protein	P02827	HSP70	4.3E-237
	-1.22					Reticulocalbin-2	O35783	CALU	1.3E-8
Apoptosis									
	1.18		0.87			Calmodulin	Q0IQB6	CML3	3.1E-29
	0.67		0.28		0.39	Caspase-7	P55214	CASP7	4E-56
	0.64		0.61			Sorcin	Q8VC88	GRAN	5.3E-42
	-0.77	-0.61	-0.72	0.54		Calmodulin	P04352	CALM	1.5E-13
-0.40	0.33					EF-hand domain-containing protein D2	Q9D8Y0	EFHD2	9E-43
-0.40	-0.28	-0.42				Death-associated protein kinase 3	O43293	DAPK3	4.2E-40
-0.53		-0.30				Reticulon-4	Q9NQC3	RTN4	9.5E-34
	-1.44	-1.18	-0.93			Calmodulin-like protein 3	P53440	CALMF	8.8E-6
Immune response									
1.05						Interferon-induced protein 44-like	Q53G44	IF44L	7.1E-31
0.26	0.49					Adenosylhomocysteinase	P27604	SAHH	1.3E-203
	0.46					Major antigen	0	0	0
NPC proteins									
					0.31	Protein NPC2-like	Q9VQ62	NPC2	1.6E-23
-5.00	-5.00	-2.05	-1.72			Epididymal secretory protein E1	P79345	NPC2	1.2E-15
-2.26	-5.00	-1.75	-2.19			Protein NPC2-like	Q9VQ62	NPC2	1.2E-18

Table 3.3 continued

<i>S. microadriaticum</i> vs. <i>B. minutum</i>		<i>D. trenchii</i> vs. <i>B. minutum</i>		<i>S. microadriaticum</i> vs. <i>B. minutum</i>		Annotation	UniProt ID	Gene name	Eval
End Con	End RHS	End Con	End RHS	End Con	End RHS				
Oxidative stress and redox process									
1.32	1.82					Alpha-aminoadipic semialdehyde dehydrogenase	Q2KJC9	AL7A1	6.5E-216
	1.31					NAD(P) transhydrogenase, mitochondrial	Q13423	NNTM	0
	1.16					Superoxide dismutase [Mn], mitochondrial	Q5FB30	SODM	3E-90
0.76	0.67	0.63	0.91			Superoxide dismutase [Cu-Zn]	P81926	SODC	1.3E-57
0.73	0.76	0.55	0.64			Catalase	P04040	CATA	7.2E-209
0.59						Hydroxysteroid dehydrogenase-like protein 2	Q6P5L8	HSDL2	9.7E-121
	-0.90					Pyridoxal 5-phosphate synthase subunit SNZERR	Q8WPW2	PDX1	2.8E-113
Protein degradation and turnover									
1.93	1.83	1.84	1.69			Transmembrane protease serine 9	B3EWZ6	MLRP2	3.9E-89
	1.01					Xaa-Pro dipeptidase	Q5I0D7	PEPD	4.9E-194
	0.81					Ubiquitin-fold modifier 1	Q7PXE2	UFM1	5.5E-37
	0.57					Cytosolic non-specific dipeptidase	Q5R432	CNDP2	1.9E-135
0.53						Neprilysin	P08049	NEP	2.3E-110
-0.47	-0.41	-0.42				Cathepsin B	P83205	CATB	4E-115
			-0.59			Small ubiquitin-related modifier 1	Q8QGH2	SUMO1	8.6E-28
Electron transport									
	0.77	1.13				ATP synthase F(0) complex subunit B1, mitochondrial	P24539	AT5F1	2.8E-30
	0.99					Cytochrome c oxidase subunit 5B, mitochondrial	Q5REG2	COX5B	2.5E-15
		0.89				Cytochrome c oxidase subunit 6A, mitochondrial	O13085	COX6A	5E-20
	0.68					Succinate dehydrogenase [ubiquinone] flavoprotein subunit B, mitochondrial	Q801S2	SDHAB	2.6E-268
	0.64					ATP synthase subunit delta, mitochondrial	P35434	ATPD	4.3E-30
		0.57				Cytochrome b-c1 complex subunit Rieske, mitochondrial	P13272	UCRI	3.5E-77
		0.46				Cytochrome c oxidase subunit 5A, mitochondrial	B0VYY3	COX5A	2.1E-38
0.40	0.44		0.38			ATP synthase subunit alpha, mitochondrial	Q03265	ATPA	7.3E-255
0.33				0.34		ATP synthase subunit beta, mitochondrial	Q5ZLC5	ATPB	6E-233
Iron binding									
	0.66					Melanotransferrin	O93429	TRFE	2.9E-47
		0.61				Soma ferritin	P42577	FRIS	1.5E-65
-0.28						Serotransferrin	P56410	TRFE	5.7E-48
-0.57		-1.10				V-type proton ATPase catalytic subunit A	P31400	VATA	3.7E-308

3.5 Discussion

The three symbioses showed metabolomic and proteomic differences over time, both under control conditions and heat treatment i.e., two consecutive heat stress events and recovery periods. Changes in the host metabolome of the individual associations over time and in response to heat stress are summarised in Figure 3.8. *B. minutum*-colonised anemones showed a variety of temporal changes, though there were few differences in response to temperature at the end of the experiment. *B. minutum*-colonised anemones displayed both up- and downregulation of compounds involved in carbohydrate metabolism, glycolysis and the TCA cycle, irrespective of thermal conditions. They also exhibited various changes associated with nitrogen metabolism, including an upregulation of glycine, beta-alanine and putrescine, and a decrease in urea. Thermal regime had little effect on host metabolome of anemones colonised by the heterologous symbionts. The only changes observed in the host metabolome over time were in *S. microadriaticum*-colonised anemones maintained under control conditions, where there was an upregulation of many amino acids and some metabolites related to fatty acid and lipid metabolism. The lack of change in the host metabolome of *Aiptasia* associated with the two heterologous symbiont species resulted in a similar pathway comparison of heterologous *versus* homologous symbiont species to that reported in Chapter 2, which is summarised in Figure 3.9.

The proteomic data largely corroborated the metabolomic information, and additionally showed that oxidative stress and apoptosis-related proteins are mainly upregulated in *B. minutum*-colonised anemones over time, regardless of thermal regime, while proteins involved in the electron transport chain and NPC proteins were downregulated in *B. minutum*-colonised anemones. The host proteome of *D. trenchii*-colonised anemones mainly exhibited altered expression of HSPs; anemones that remained at control temperature showed an overall decrease in HSP expression, while those that underwent RHS showed a mixture of up- and downregulation. *S. microadriaticum*-colonised anemones had the most DEPs of the three symbioses between the control and RHS conditions, including marked upregulations of HSPs, apoptosis-related proteins, and iron-binding proteins under the heat stress regime. Interestingly though, only two DEMs were observed for this latter symbiosis under control *versus* RHS conditions.

Figure 3.8 Pathway reconstruction of changes in host metabolites over time for Aiptasia in symbiosis with different species of symbionts (*Breviolum minutum*, *Durudinium trenchii*, or *Symbiodinium microadriaticum*). Symbioses established for a total of seven months, with anemones either maintained under control conditions or exposed to RHS treatment for the final four months.

3.5.1 Symbiont population density and host growth

Cell densities of *B. minutum* reduced over time, to approximately 30% of initial (i.e., 3-month colonisation) levels regardless of treatment. Previous studies have also reported that symbiont cell densities peak and decline after establishment (Gabay et al., 2018), though there are no reports of what happens beyond the first few months of colonisation with the homologous symbiont to compare with the current study. Typically, *B. minutum* can achieve peak density in Aiptasia after four to six weeks (Starzak et al., 2014; Gabay et al., 2018), and this likely contributed to the lack of any observable effect from RHS in this symbiosis, given that it had six weeks to recover from the heat stress. By comparison, densities of *D. trenchii* and *S. microadriaticum* in Aiptasia were only ~30% and 10% of those reached by *B. minutum* at the start, agreeing with the findings of previous research (Starzak et al., 2014;

Figure 3.9 Metabolic pathway reconstruction of changes in host metabolome between Aiptasia containing heterologous symbiont species (*Symbiodinium microadriaticum* or *Durisdinium trenchii*) compared to the homologous association with *Breviolum minutum* at the final time point of this study (seven months post-colonisation), having being held under control conditions or exposed to repetitive heat stress (RHS) for the final four months.

Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2019). Here, similarly to *B. minutum*, cell densities of *D. trenchii* dropped over time to <35% of original levels irrespective of thermal treatment, while the density of *S. microadriaticum* was consistently low across time. None of these previous studies tracked the success of the symbionts after 12 weeks, but the temporal decline of the homologous symbiont and *D. trenchii* does suggest ongoing ‘sorting’ and moderation of the symbiont population beyond the timeframe usually studied (Davy et al., 2012).

In terms of host growth, Aiptasia containing *B. minutum* and *D. trenchii* grew at similar rates, however *B. minutum*-colonised anemones were unaffected by RHS while *D. trenchii*-colonised anemones suffered reduced growth. This indicates that *D. trenchii* is able to provide enough resources to Aiptasia

under non-stressful conditions but not under thermal stress. These results both agree and are in contrast to previous work by Gabay et al. (2018), who showed that symbiont identity affected host growth in *Aiptasia*, with *B. minutum*-colonised anemones growing significantly more than either *D. trenchii*- or *S. microadriaticum*-colonised anemones after eight weeks in symbiosis. It is interesting to see that over a longer time period, anemones hosting *D. trenchii* are able to ‘keep pace’ with the homologous association in the absence of stress, as this could indicate a potential of the host to sort symbionts over time to improve host-symbiont integration and holobiont fitness. If such host-symbiont integration is possible over time, this could have a significant implications for the possibilities of corals to adapt to climate change through a change in symbionts.

3.5.2 TCA cycle, glycolysis, and carbohydrate metabolism

Anemones in association with *B. minutum* displayed many changes in the host metabolome over time, associated with the TCA cycle and glycolysis, as well as carbohydrate metabolism, regardless of thermal treatment. In particular, there was an increase in citrate, and a decrease in succinate and fumarate, while under control conditions, increased levels of sucrose and GABA were observed (Figure 3.8). Carbohydrate metabolism also showed increases in mannitol, but decreases in glycolic acid and glycerol under both control and thermal stress conditions, and under RHS there was also an increase in psicose and threonic acid. While the precise function of these compounds is unknown, and there are no studies with which compare these longer time-scale changes in a homologous association, it is easy to speculate that increased carbohydrate reserves are beneficial to the host. This is supported by proteomics data, as DEPs indicated that proteins involved in the electron transport chain decreased in abundance over time, regardless of thermal treatment. These data suggest that hosts in symbiosis with *B. minutum* have reduced energy requirements over time, presumably due to the fine-tuned balance in nutrient cycling between host and symbiont.

In comparison, *D. trenchii*-colonised anemones showed no DEMs involved in the TCA cycle, glycolysis, or carbohydrate metabolism in response to time in symbiosis, regardless of thermal regime, while *S. microadriaticum*-colonised anemones kept under control conditions showed an increase in GABA and a decrease in sorbitol (Figure 3.8). At the final time-point, these changes in *S. microadriaticum*-colonised hosts led to similar expression of GABA and sorbitol in *S. microadriaticum*-colonised hosts and *B. minutum*-colonised hosts, regardless of thermal treatment. In contrast, *S. microadriaticum*-colonised hosts that remained under control conditions had significantly higher levels of glycerol than *B. minutum*-colonised hosts (Figure 3.9). Glycerol is an important metabolic fuel to symbiotic cnidarians (Colombo-Pallotta et al., 2010; Holcomb et al., 2014), and it is one of the compounds translocated from symbiont to host (Davy et al., 2012; Lohr et al., 2019). Previous research in *Aiptasia* has shown that glycerol is accumulated in host tissues at greater rates when the host is paired

with its homologous symbiont (Starzak et al., 2020). The increase of glycerol in host tissues of *S. microadriaticum*-colonised hosts might therefore indicate that the host receives increased benefits from the symbiont over time, i.e. the symbiosis between Aiptasia and *S. microadriaticum* is capable of moving along the symbiotic state spectrum to a slightly more beneficial state seven months after initial establishment of symbiosis. However, glycerol is also an important osmolyte (Burg & Ferraris, 2008), and is upregulated in response to thermal stress in Aiptasia (Hillyer et al., 2017). Additionally, upregulated expression of osmolytes has been found to increase thermotolerance (Gegner et al., 2017). Therefore, the increase in glycerol could instead be related to cellular stress and/or homeostasis in *S. microadriaticum*-colonised anemones maintained under control conditions, stress that is perhaps induced by the symbiont. Future research should focus on the nutritional exchange between these symbiotic partners to resolve this question, for example *via* nanoSIMS analysis using stable isotopes, which allows for the localisation of stable isotopes within tissues (Pernice et al., 2014; Sproles et al., 2020; Räddecker et al., 2021).

Like to the metabolomic changes, the proteome of *S. microadriaticum*-colonised anemones kept under control conditions showed changes related to carbohydrate metabolism. An increase in malate synthase (MLS), and a decrease in malate dehydrogenase (MDH) were found over time. MLS is involved in coupling gluconeogenesis and lipid synthesis, thus allowing the host to turn carbohydrates into lipids (Voet & Voet, 2004), the main energy reserves of anemones (Giese, 1966; Blanquet et al., 1979; Oakley et al., 2016) and corals (Pearse & Muscatine, 1971; Davies, 1991). MDH is one of the key enzymes in energy anabolism, and has previously been found to be downregulated in response to symbiosis in Aiptasia, even in response to heterologous symbionts (Medrano et al., 2019). The metabolite data showed an increase in the two fatty acids palmitoleic acid and elaidic acid in *S. microadriaticum*-colonised anemones compared to *B. minutum*-colonised anemones under control conditions at the end of the experiment. This, in combination with the upregulation of MLS and downregulation of MDH and aldolase C (ALDOC), which is a key glycolytic protein involved in catabolism of fructoses and ATP synthesis (Lebherz & Rutter, 1969; Arakaki et al., 2009), shows that *S. microadriaticum*-colonised anemones have reduced energy requirements over time and are even able to build up lipid reserves from carbohydrates, perhaps from the glycerol that was found in increased quantities in *S. microadriaticum*-colonised host tissues compared to *B. minutum*-colonised hosts. This reduced energy requirement could be due to the lower respiratory rate of the holobiont caused by lower symbiont cell density, given the close, positive co-relationship between the rates of photosynthesis and respiration in the cnidarian-dinoflagellate symbiosis (Harland & Davies, 1995); alternatively, it could be another sign that anemones containing *S. microadriaticum* are capable of moving to a more beneficial state over time.

DEPs for *D. trenchii*-colonised anemones showed only a slight decrease in phosphoenolpyruvate carboxykinase (PEPCK) by the end of the RHS treatment. This decrease was insufficient to cause a

significant difference in expression of PEPCK between *D. trenchii*- and *B. minutum*-colonised anemones under any circumstances. However, *S. microadriaticum*-colonised anemones had much higher expression levels of PEPCK than *B. minutum*-colonised anemones at the final time-point, under both control and RHS conditions. PEPCK is involved in the irreversible conversion of oxaloacetate to phosphoenolpyruvate, and is a rate-limiting enzyme in gluconeogenesis (Pilkis & Granner, 1992). It has previously been observed to be upregulated in response to bleaching in the coral *Porites astreoides* (Kenkel et al., 2013), as well as starvation in mammals (Tilghman et al., 1974; Jitrapakdee & Wallace, 1999). Establishment of symbiosis with a homologous symbiont typically causes downregulation of PEPCK (Ganot et al., 2011). Therefore, PEPCK is upregulated when energy requirements increase, i.e., when carbohydrate stores need to be utilised for energy production. Another study found a downregulation of PEPCK in *Aiptasia* colonised by *D. trenchii* (Medrano et al., 2019), which is in agreement with the decrease in PEPCK in *D. trenchii*-colonised hosts over time when exposed to RHS in the current study and supports a degree of nutritional benefit provided by this heterologous symbiont. In contrast, it seems that *S. microadriaticum* provided little metabolic support to *Aiptasia*, whether due to poor host-symbiont cellular integration or, more likely, its very low population density. Indeed, when considered alongside the evidence described above, it seems that colonisation by *S. microadriaticum* is associated with a capacity for increased storage of energy-rich compounds, potentially due to reduced metabolic demands in the absence of a highly productive symbiont population, and the mobilisation of these stores to support host metabolism.

3.5.3 Nitrogen metabolism

Under control conditions, *B. minutum*-colonised anemones showed increases in glycine, nicotinic acid, beta-alanine and putrescine, as well as decreases in glutaric acid, and 3-hydroxyisovaleric acid over time, regardless of thermal regime (Figure 3.8). Under control conditions, there was also a decrease in urea, while under RHS, a decrease in 2-hydroxyglutaric acid was observed in this symbiosis. Glycine is one of the most abundant free amino acids in symbiotic cnidarians, such as the anemone *Anemonia viridis* (Roberts et al., 2001), the zoanthid *Zoanthus* sp. (Von Holt, 1968), and the coral *Isopora palifera* (Wang et al., 2012). Additionally, it is a major component of collagen, the single most abundant protein within the animal kingdom (Lodish et al., 2000). Increased dietary glycine has been shown to enhance growth in pigs (Wang et al., 2014), a species of bird (Taylor et al., 1994), and coriander plants (Mohammadipour & Souri, 2019), and increased concentrations of glycine have also been shown to stimulate collagen synthesis in cartilage cell lines (De Paz-Lugo et al., 2018). Therefore, the increase in glycine in *B. minutum*-colonised hosts could be indicative of a successful symbiosis, allowing for, or even stimulating, host growth. Nicotinic acid, also known as niacin or vitamin B3, is a precursor of both NAD and NADP (Vaquero, 2020). While NAD is mainly involved in oxidation reactions, i.e. catabolism, NADP plays an important role in anabolism of fatty acids (Seubert et al., 1968; Sauer et al.,

2004). Additionally, the non-essential amino acid beta-alanine can be converted to malonate and subsequently enter the fatty acid biosynthesis pathway, or diverted into pantothenic acid and coenzyme A, which also has a notable role in fatty acid synthesis and oxidation (Berg et al., 2002). These results thus suggest that *B. minutum*-colonised anemones are able to divert some energy towards growth and synthesis of fatty acids and lipids, which, again, are the main energy reserves of anemones (Giese, 1966; Blanquet et al., 1979; Oakley et al., 2016) and corals (Pearse & Muscatine, 1971; Davies, 1991), reaffirming the success of the association between *Aiptasia* and *B. minutum*. The decrease in urea in *B. minutum*-colonised anemones under control conditions agrees with previous results and could indicate a mechanism by which the host is able to control symbiont cell densities, i.e. nitrogen limitation (Wang & Douglas, 1998; Grover et al., 2002; Yellowlees et al., 2008; Matthews et al., 2017), however cell densities also decreased under RHS, and no decrease in urea was found in this treatment. Alternatively, the decrease in urea in *B. minutum*-colonised hosts maintained under control conditions could indicate an increase in efficiency of nitrogen cycling between host and symbiont (O’Neil & Capone, 2008; Rådecker et al., 2015; Cunning et al., 2017), thereby leading to a decrease in metabolic end products. The increased metabolic demands posed by RHS could have led to a build-up of nitrogenous metabolic end products in these hosts (Chapter 2).

As for the heterologous symbionts, *D. trenchii*-colonised anemones only showed an increase in beta-alanine under control conditions (Figure 3.8), while proteomics data showed a decrease in glutamine synthetase (GLNA) over time under both control conditions and thermal stress. At the final time-point, *D. trenchii*-colonised anemones displayed reduced expression of glutamate dehydrogenase (GDH) and GLNA compared to *B. minutum*-colonised anemones under all conditions. GLNA, which catalyses the formation of glutamine from glutamate and ammonia, was previously seen to be strongly downregulated in heat-stressed anemones containing *D. trenchii* (Chapter 2), indicative of reduced nitrogen assimilation and increased nitrogen catabolism in the host. The reduced expression of both GLNA and GDH in *D. trenchii*-colonised anemones compared to *B. minutum*-colonised hosts at seven months after symbiosis establishment could be explained by lower quantities of glutamate, the substrate required for both enzymes. Glutamate is one of the main nitrogen-compounds translocated from symbiont to host in *Aiptasia* (Swanson & Hoegh-Guldberg, 1998). This is in agreement with a previous study that showed that *D. trenchii* is more selfish than *B. minutum* in *Aiptasia* in terms of nitrogen translocation to the host (Sproles et al., 2020). These results indicate that the anemones in association with *D. trenchii* are increasingly nitrogen-starved over time, while the symbionts themselves do not seem to be nitrogen-limited, as the abundance of urea in *D. trenchii*-colonised host tissues is increased compared to *B. minutum*-colonised hosts. Nitrogen cycling between *Aiptasia* and *D. trenchii* seems to remain sub-optimal, which is perhaps even exacerbated over time. As nitrogen cycling is one of the key traits of the cnidarian-dinoflagellate symbiosis (O’Neil & Capone, 2008; Rådecker et al., 2015; Cunning et al., 2017), the lack of improvement in nitrogen cycling within the association between *D. trenchii* and

Aiptasia indicates poor host-symbiont integration over the time frame used in this study, i.e. seven months of symbiosis.

In *S. microadriaticum*-colonised anemones, multiple nitrogen-metabolism related changes were observed in host metabolome. In contrast to *B. minutum*- or *D. trenchii*-colonised hosts, the host metabolome of *S. microadriaticum*-colonised anemones showed that the amino acids alanine, serine, proline, threonine and leucine became increasingly abundant over time under control conditions (Figure 3.8). However, few changes occurred in the host metabolome under the RHS treatment in the presence of *S. microadriaticum*. The increased abundance of these free amino acids could be indicative of increased nitrogen catabolism, i.e. breakdown of proteins to fulfil the nutritional requirements of the host, consistent with the relatively high quantities of urea in *S. microadriaticum*-colonised anemones, and the evidence for increased protein degradation and turnover through increased expression of a protease (transmembrane protease serine 3) and calpain-B, a calcium-regulated protease (Jékely & Friedrich, 1999; Croall & Ersfeld, 2007), over time under control conditions. Compared to *B. minutum*-colonised anemones, three DEPs were involved in protein degradation and turnover, of which two were upregulated and one downregulated in *S. microadriaticum*-colonised anemones maintained under control conditions.

In comparison, protein degradation and turnover also increased in *S. microadriaticum*-colonised anemones exposed to RHS (five DEPs, of which four were upregulated), but without the associated increase in free amino acid levels. Free amino acids (FAAs) have previously been considered as ‘host release factors’ (HRFs), i.e. compounds that stimulate photosynthate release from the host (Wang & Douglas, 1997; Gates et al., 1999; Davy & Cook, 2001), which could explain the increase in glycerol in these hosts if viewed from a beneficial, nutritional perspective (Section 3.5.2). However, no conclusive evidence for the existence of HRFs *in hospite* has ever been presented, and the current opinion is that they are an experimental artefact (Davy et al., 2012). Alternatively, FAAs can act as osmolytes in cellular homeostasis (Pierce, 1982). Increased osmolyte expression has been shown to aid in thermal resistance in Aiptasia (Gegner et al., 2017), so perhaps osmolyte expression also increases in response to cellular stress. If so, these results might indicate that *S. microadriaticum* and/or a low symbiont population density continue to induce cellular stress in the host under control conditions, but that the host nevertheless still has the capacity to physiologically respond, compared to when under thermal stress when no such response was seen. Further work is needed to establish the exact role of these FAAs, as well as whether the observed changes in FAA pools under control *versus* RHS conditions are due to changes in host and/or symbiont physiology. Only then can we conclusively establish whether the increased FAA pools over time under control conditions represent a positive or negative development for symbiosis function.

3.5.4 Stress physiology

Differences that developed over time in *B. minutum*-colonised anemones included upregulation of apoptosis-related proteins and oxidative stress proteins, irrespective of thermal regime. Apoptosis-related proteins include calmodulin, which activates various apoptosis pathways by increasing calcium uptake into mitochondria (Berchtold & Villalobo, 2014), and death-associated protein kinase 3 (DAPK3), which induces morphological changes in cells that initiate the disruption of the cell membrane, i.e. blebbing, which eventually leads to the degradation of cellular structure and thus apoptosis (Cohen et al., 1999; Usui et al., 2012; Wickman et al., 2013). Oxidative stress proteins that were upregulated in *B. minutum*-colonised anemones over time were aimed at removal of peroxides, through peroxiredoxin-4 (Rhee et al., 2001), catalase (Chelikani et al., 2004), and a putative glutathione S-transferase 6, which conjugates the highly toxic end-products of lipid peroxidation formed during oxidative stress (Leaver & George, 1998). This upregulation is consistent with the increased cell densities that *B. minutum* achieves in Aiptasia compared to the heterologous symbiont species, and the likely accompanying elevated rate of photosynthesis, and hence generation of intracellular oxygen and ROS. This results in an elevated need for detoxification and thus apoptosis, as oxidative stress and apoptosis are tightly linked (Dunn et al., 2007a; Weis, 2008; Suggett & Smith, 2020). Additionally, this increased apoptotic activity could be linked to a greater need to manage symbiont proliferation, becoming increasingly important as the symbiosis reaches a fully-established state (Lecoite et al., 2016). Apoptosis has previously been suggested to play a central role in symbiont population control, both under non-stressful conditions as well as in response to stress, i.e. bleaching (Gates et al., 1992; Dunn et al., 2007; Weis, 2008; Dunn & Weis, 2009). The upregulation seen in these oxidative stress and apoptosis-related proteins therefore likely represents the increased photosynthetic activity within the homologous association, as well as a mechanism by which the host can manage its symbiont population.

In *D. trenchii*-colonised anemones, one apoptosis-related protein (sorcin) and two oxidative stress proteins (catalase and superoxide dismutase) were upregulated under RHS but not under control conditions. In *S. microadriaticum*-colonised anemones by comparison, there were no changes in the expression of oxidative stress proteins, though the apoptosis-related DAPK3 was upregulated over time irrespective of temperature, while the expression of caspase-7, one of the key proteins during the demolition phase of apoptosis (Walsh et al., 2008), was reduced in anemones maintained at the control temperature, and calmodulin-like protein 3 was reduced under RHS. At the end of the experiment, this resulted in similar patterns of expression of oxidative stress and apoptosis-related proteins within the two associations with heterologous symbionts, as reported also in Chapter 2. As mentioned before, *B. minutum*-colonised hosts showed increased expression of calmodulin and DAPK3 over time. Compared to *B. minutum*-colonised anemones, both symbioses with the heterologous symbionts showed decreased

expression of apoptosis-related proteins, including calmodulin and DAPK3, as well as several others. Reticulon-4, which when downregulated increases a cell's resistance to calcium-induced apoptosis (Jozsef et al., 2014; Kawaguchi et al., 2018), and EF-hand domain-containing protein D2 (EFHD2) – of which the function has not yet been fully resolved, but has been shown to be involved in apoptosis (Huh et al., 2015; Vega, 2016) – were downregulated in anemones containing heterologous *versus* homologous symbionts under control conditions. In comparison, anemones containing heterologous symbionts that were exposed to RHS increased their expression of proteins involved in apoptosis. In particular, calmodulin, caspase-7 and sorcin were all upregulated in both *D. trenchii*- and *S. microadriaticum*-colonised anemones compared to anemones harbouring *B. minutum*. In Chapter 2, I suggested that heterologous symbionts were able to suppress host apoptosis to enhance their proliferation within the host's tissues, and here it seems that over a longer timeframe under control conditions, heterologous symbionts continue this activity, while the host continues to exhibit clear signs of oxidative stress. This could again be indicative of a more opportunistic, or even parasitic nature, or that the mismatch between host and symbiont causes inadequate host responses that eventually are disadvantageous for the host (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Matthews et al., 2017; Sproles et al., 2020).

In terms of HSP expression, with *B. minutum* there was 'balanced' up- and downregulation of HSPs over time under all conditions: two proteins were upregulated (peptidyl-prolyl cis-trans isomerase B and HSP 90-alpha 1), while two others were downregulated (peptidyl-prolyl cis-trans isomerase and B-cell receptor-associated protein 31). With heterologous symbionts, both symbioses exhibited reduced HSP activity under control conditions – but not thermal stress - compared to the start of the experiment (*D. trenchii*: seven HSPs downregulated *versus* one upregulated; *S. microadriaticum*: four HSPs downregulated). The reduced expression of HSPs over time in *D. trenchii*- and *S. microadriaticum*-colonised anemones maintained under the control temperature could be an indication that the symbioses became less stressful over time. Reduced stress could, in part, be explained by a reduced cell density, and thus photosynthetic activity and ROS production, though such a decline was only seen with *D. trenchii*. Alternatively, the decline in HSPs could be a sign of improved host-symbiont function over time, though it is difficult to reconcile this with, e.g. the changes in FAA pools discussed above. Indeed, despite the decline in HSP expression, ongoing stress induced by the heterologous symbionts was indicated by the fact that HSP expression in anemones harbouring these symbiont species was consistently higher than that in anemones containing the homologous symbiont, consistent with the shorter-term experiment described in Chapter 2, as well as previous studies of cellular stress and immune responses in *Aiptasia* (Chen et al., 2003, 2004, 2005; Hong et al., 2009; Matthews et al., 2017; Neubauer et al., 2017; Jacobovitz et al., 2021).

The iron-binding protein melanotransferrin was significantly upregulated over time regardless of thermal conditions in *D. trenchii*-colonised anemones, while iron-binding proteins were reduced in expression only under control conditions in *S. microadriaticum*-colonised anemones. Chapter 2 showed that *D. trenchii*-colonised anemones had the highest increase in the iron-binding protein serotransferrin in response to heat stress, in combination with the largest response to elevated temperature. The increase in melanotransferrin over time under all conditions in the current study once again indicates that iron plays an important role in maintenance of the symbiosis between Aiptasia and *D. trenchii*. As iron is involved in photosynthesis, antioxidant protection, and bleaching resistance (Ferrier-Pagès et al., 2001, 2018; Shick et al., 2011; Twining & Baines, 2013; Krueger et al., 2015), results seem to indicate that *D. trenchii* is the most stressful to the host of the three symbiont species used in this study. This reaffirms the less beneficial nature of *D. trenchii* (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Matthews et al., 2017; Sproles et al., 2020).

3.5.5 NPC proteins

With *B. minutum*, expression of NPC proteins decreased over time, regardless of thermal regime. In mammals, NPC proteins are involved in cell recognition and immunity processes (Shi et al., 2012; Dani et al., 2014), and they have been suggested to be associated with successful establishment of the cnidarian-dinoflagellate symbiosis (Dani et al., 2014, 2017; Rosset et al., 2021). In cnidarians, they are also thought to be involved in sterol transport between the symbiotic partners across the symbiosome membrane complex (Infante et al., 2008; Storch & Xu, 2009; Dani et al., 2014, 2017; Lehnert et al., 2014; Baumgarten et al., 2015; Oakley et al., 2016; Hambleton et al., 2019). As NPC proteins are associated with the symbiosome, the decrease of NPC proteins in *B. minutum*-colonised anemones over time could be directly correlated to the reduction in symbiont cell density. In Chapter 2, it was found that *D. trenchii* was able to induce expression of NPC proteins in Aiptasia, in contrast to a previous, shorter time-frame experiment (Sproles et al., 2019), while no such proteins were detected in the symbiosis between Aiptasia and *S. microadriaticum*. In this current study, seven months after the establishment of the symbiosis, *D. trenchii*-colonised anemones continued to express NPC proteins, still at much lower quantities than found in the presence of *B. minutum*. This lower expression could once again be linked to the relatively low symbiont density of *D. trenchii*, but notably, the expression of these proteins did not change over time even though the density of *D. trenchii* declined. It may therefore be that the lower expression is, in part, also a species-specific response, perhaps reflecting a reduced degree of host-symbiont integration with the heterologous symbiont (Sproles et al., 2019). As for *S. microadriaticum*, although NPC proteins were not detected in anemones containing this symbiont upon exposure to RHS, one NPC protein was found in *S. microadriaticum*-colonised anemones that were held under control conditions. Once again, this NPC protein was present at low levels, potentially due to the same reasons given for *D. trenchii*-colonised anemones. This study is the first to detect these

proteins in hosts with heterologous symbionts, likely due to the shorter timeframes of those studies compared to this one. This temporal difference provides tantalizing evidence for improved symbiosis function over time with these heterologous symbionts, warranting further exploration.

3.6 Conclusion

When *Aiptasia* was colonised by the heterologous symbionts *D. trenchii* and *S. microadriaticum* there was little evidence of functional change over time, either under control conditions or under RHS, though the expression of NPC proteins (contrary to shorter-term studies) and the reduced HSP expression over time under control conditions, in particular, may suggest a slight functional improvement. Overall though, the host metabolome and proteome did not converge markedly to more closely resemble those found when anemones were colonised by the homologous *B. minutum*. Indeed, while *B. minutum*-colonised anemones displayed changes over time that indicated a progressively lower reliance on host carbohydrate reserves for metabolic support, heterologous symbionts continued to induce cellular stress in the host. This study therefore suggests that host-symbiont specificity, underpinned by cellular integration, limits the capacity for change, even over more prolonged periods or under repeated stress, when selective pressure might conceivably lead to enhanced symbiont and holobiont performance. Further research is necessary, however, to assess the degree of phenotypic and/or genotypic diversity in the symbiont population to better assess the potential for selection, as well as test whether even longer timeframes offer an opportunity for improved host-symbiont integration and hence holobiont function. Only then we will be able to conclusively determine the potential for partner switching to provide a mechanism by which corals can adapt to climate change and hence survive into the future.

Chapter 4 The influence of symbiont identity and thermal stress on inter-partner nutrient exchange in a model cnidarian-dinoflagellate symbiosis

4.1 Abstract

This study determined the effects of symbiont identity, time in symbiosis, and repetitive heat stress on the inter-partner translocation of autotrophic carbon and heterotrophic nitrogen in a model symbiotic cnidarian, the sea anemone *Exaiptasia pallida* (Aiptasia), using nanoscale secondary image mass spectrometry (NanoSIMS) and bulk isotope-ratio mass spectrometry (IRMS). Symbiont species used were the homologous *Breviolum minutum*, and the heterologous *Durussdinium trenchii* and *Symbiodinium microadriaticum*, with the aim of testing whether nutritional fluxes in the presence of heterologous, physiologically sub-optimal symbionts, can shift to a more optimal nutritional state over time, so facilitating coral reef survival through the establishment of novel, potentially more thermally-resistant partnerships. Four months after initial colonisation with these symbiont species, time-zero samples were taken and the remaining anemones allocated to one of two conditions: control temperature (25 °C) or repetitive heat stress treatment (RHS) consisting of repeated ramps (1 °C day⁻¹) to a peak of 32 °C, holding this temperature for three days, and a six-week recovery period. Anemones were sampled after six months of these conditions, i.e., 10 months after initial colonisation, during which the heat stress event and recovery period were repeated for a total of three times. *B. minutum*-colonised anemones displayed no changes in carbon enrichment with time or thermal regime, while cell densities of this symbiont decreased over the final six months regardless of thermal treatment. Additionally, there was no effect of thermal regime on symbiont nitrogen enrichment in *B. minutum*-colonised hosts. Cell densities of *D. trenchii* decreased similarly to *B. minutum* over time, regardless of thermal regime, while cell densities of *S. microadriaticum* remained stable throughout time and regardless of thermal exposure. In terms of nutrient translocation, NanoSIMS showed that *D. trenchii* and *S. microadriaticum* are capable of translocating similar amounts of autotrophic carbon to the host on a local tissue-scale. However, on a holobiont-scale, i.e. determined *via* IRMS, these symbiont species did not provide their hosts with similar amounts of carbon as *B. minutum*. Additionally, *D. trenchii* translocates less photosynthate to the host than *S. microadriaticum* on a cell-specific basis. On the contrary, in terms of nitrogen enrichment, *S. microadriaticum* maintained at control conditions displayed lower enrichment of nitrogen *per cell* than *B. minutum* exposed to RHS at the final time-point, contrary to the expectation that inter-cellular nutrient competition would be less at the lower symbiont cell density. Nitrogen enrichment of *D. trenchii* remained the same relative to host nitrogen enrichment regardless of thermal regime. Therefore, *D. trenchii* displayed more opportunistic traits, which were further amplified in anemones exposed to RHS. The reduced consumption of nitrogen by *S. microadriaticum*, combined with the more beneficial sharing of carbon products, indicates that *S. microadriaticum* could be a more suitable symbiotic partner for Aiptasia than *D. trenchii*, however there was no evidence for improved

host-symbiont integration in this symbiosis over time or thermal regime. These findings suggest that partner switching is unlikely to benefit host cnidarians in the long run, though further studies with more symbiont species and over longer timeframes, with corals, are needed to confirm this.

4.2 Introduction

Some of the world's largest ecosystems, coral reefs, are founded on the successful cooperation between two symbiotic partners: corals and their endosymbiotic dinoflagellate microalgae. Corals are widespread in nutrient-poor waters (Muscatine & Porter, 1977; Cunning et al., 2017), and the success of this symbiosis is based on the nutritional exchange between host and symbiont (Muscatine & Hand, 1958; Von Holt & Von Holt, 1968; Trench, 1971; Burriesci et al., 2012; Sproles et al., 2020). Principally, the endosymbiont uses the cnidarian host's metabolic end products, e.g. inorganic nitrogen and carbon, for photosynthesis and growth, and returns organic carbon compounds to the host. This photosynthate is an important nutritional source for the host, meeting up to 100% of the host's metabolic carbon requirements (through the provision of energy-rich compounds such as glucose, glycerol and lipids), as well as providing amino acids for growth (Muscatine & Hand, 1958; Muscatine & Cernichiar, 1969; Muscatine et al., 2005; Burriesci et al., 2012; Davy et al., 2012; Tremblay et al., 2014; Hillyer et al., 2018). Additionally, nitrogen cycling and conservation are important aspects of the symbiosis (O'Neil & Capone, 2008; Rädicker et al., 2015; Cunning et al., 2017). Nitrogen is taken up from the environment as dissolved inorganic nitrogen (DIN) or by host feeding in the form of dissolved or particulate organic matter (DOM and POM, respectively). The translocation of fixed carbon from the symbiont to the host allows the host to conserve nitrogen-rich compounds, which is termed "nitrogen conservation" (Wang & Douglas, 1998; Tanaka et al., 2006, 2017), while the catabolism of nitrogenous compounds by the host leads to the generation of NH_4^+ as the metabolic end-product, which can then be assimilated by the dinoflagellate into amino acids, completing the process of "nitrogen recycling" (Wang & Douglas, 1999; Tanaka et al., 2006; Davy et al., 2012; Pernice et al., 2012; Tanaka et al., 2015).

The symbiotic dinoflagellates that form symbioses with cnidarians belong to the family Symbiodiniaceae, of which currently eleven genera and numerous species have been described (LaJeunesse et al., 2018, 2021; Nitschke et al., 2020; Pochon & LaJeunesse, 2021). Characteristics such as thermal tolerance, photosystem function, and inorganic carbon acquisition vary widely between the many genera, species and subspecies that comprise this family. Traits like these have contributed to the establishment of distinct host-symbiont specificities, as many species of cnidarians 'prefer' to form a symbiosis with only one or a limited range of symbiont species (Belda-Baillie et al., 2002; Baker, 2003; Goulet, 2006; Putnam et al., 2012; Poland & Coffroth, 2017). The cellular mechanisms behind host-

symbiont specificity remain to be resolved, but several innate immune pathways have been shown to be involved (Hambleton et al., 2014; Matthews et al., 2017; Chapter 2).

Host specificity is an important aspect when considering coral adaptability to climate change; the symbiosis between these two partners is highly sensitive to disturbances, and environmental perturbations such as thermal stress events cause the collapse of the symbiosis (Hoegh-Guldberg, 1999; Dove et al., 2013; Hughes et al., 2018), also known as coral bleaching or dysbiosis. During bleaching, the bilateral nutrient exchange between host and symbiont becomes derailed; symbionts have been shown to retain photosynthate during thermal stress (Tremblay et al., 2016; Baker et al., 2018; Räddecker et al., 2021), while hosts metabolic demands increase both due to their reduced supply of photosynthate and increased demands to produce antioxidant and protein folding chaperones (Hawkins et al., 2015; Krueger et al., 2015; Gardner et al., 2017; Hillyer et al., 2017; Baker et al., 2018; Räddecker et al., 2021; Chapter 2). However, while bleaching poses a huge threat to coral health, it has been suggested that it may also provide a window of opportunity for changing the symbiont community composition to one better suited to the new environmental conditions (Buddemeier & Fautin, 1993; Fautin & Buddemeier, 2004; Cunning et al., 2015; Claar et al., 2020), *via* symbiont ‘shuffling’ or ‘switching’ (Baker, 2003). Shuffling refers to a change in the relative abundance of different symbiont species or genotypes within the host’s tissues, while switching occurs *via* the uptake and establishment of a new symbiont partner from the environment, with much more evidence for shuffling than switching in the field (but see Boulotte et al., 2016). Of particular interest, increased abundances of the more thermally tolerant symbiont species *Durussdinium trenchii* have been found in host tissues during and after bleaching events (Toller et al., 2001; Glynn et al., 2001; Baker et al., 2004; Berkelmans & Van Oppen, 2006; Jones et al., 2008; Correa et al., 2009; LaJeunesse et al., 2009; Stat & Gates, 2011; Cooper et al., 2011; Silverstein et al., 2012; Kemp et al., 2014; Kennedy et al., 2015; Boulotte et al., 2016). The greater thermal tolerance of *D. trenchii* can translate to the host, thus allowing the holobiont (i.e., whole symbiosis) to withstand higher temperatures (Stat & Gates, 2011; Kemp et al., 2014; Boulotte et al., 2016; Silverstein et al., 2017).

However, housing a more thermally tolerant symbiont type can come at a great cost to the host; symbiont shuffling and/or switching has been shown to cause a trade-off with respect to host growth, reproduction, and metabolite exchange (Jones & Berkelmans, 2010, 2011; Baker et al., 2013; Pernice et al., 2014; Starzak et al., 2014; Matthews et al., 2017, 2018; Cunning et al., 2018; Gabay et al., 2018; Sproles et al., 2019; Chapter 2). As mentioned before, within the Symbiodiniaceae family, species show tremendous diversity, including with respect to the amounts of fixed carbon that they provide the host, as well as the amount of nitrogen they assimilate both in symbiosis and culture (Grover et al., 2008; Baker et al., 2013; Pernice et al., 2014). Under non-stressful conditions, the symbiont species that typically persist in the host – in this work defined as ‘homologous’ symbionts – are characterised by

higher translocation rates of autotrophic carbon to the host than those seen in the presence of stress-induced symbiont community compositions (Pernice et al., 2014; Sproles et al., 2020), i.e. the host receives less benefit from these non-native, or heterologous, symbiont species. Additionally, work with the model sea anemone *Exaiptasia pallida* ('Aiptasia') has shown that the heterologous symbiont *D. trenchii* acquires a larger proportion of heterologous nitrogen from the host than the homologous symbiont *Breviolum minutum* (Sproles et al., 2020), i.e. it potentially incurs a higher cost to the host. Therefore, it has been posed that bleaching provides an opportunity for usually less abundant, less beneficial symbionts to proliferate in the tissues of an immune-compromised host (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011). More promisingly, however, there are tentative indications that the symbiotic interaction state (i.e. benefit *versus* cost) of initially sub-optimal, heterologous symbionts could change over time in symbiosis (Chapter 3), perhaps even converging with that of homologous symbionts in the long-run. Indeed, it is notable that the metabolite profile of the coral *Montipora capitata* was unaffected by its symbiont community composition, even when it was dominated on rarer occasions under regular circumstances by the thermally-tolerant but potentially less beneficial *Durussdinium glynnii* rather than the more common but more thermally-sensitive *Cladocopium* spp. (Matthews et al., 2020). However, whether the nutritional fluxes with a heterologous symbiont can shift over time has not been investigated.

The aim of this chapter was therefore to establish the effects of symbiont identity, time in symbiosis, and repeated thermal stress events on nutritional exchange between host and symbiont in the model sea anemone Aiptasia. Specifically, the translocation of autotrophic carbon from symbiont to host, and heterotrophic nitrogen from host to symbiont was assessed. Aiptasia was used as a model organism as it can easily be rendered aposymbiotic, and subsequently colonised with various species of symbionts, including those it does not naturally form a symbiosis with (Weis et al., 2008). For this study, the symbiont species included the species homologous to Aiptasia across the Indo-Pacific region, *B. minutum* (Thornhill et al., 2013b), and two heterologous species with a suspected higher thermal tolerance, *D. trenchii* (Baker et al., 2004; Berkelmans & Van Oppen, 2006; LaJeunesse et al., 2009; Stat & Gates, 2011; Silverstein et al., 2017) and *Symbiodinium microadriaticum* (Robison & Warner, 2006; Ragni et al., 2010; Hawkins & Davy, 2012; Krämer et al., 2012; Swain et al., 2017). *D. trenchii* in particular has a reputation for being more thermally tolerant, but also opportunistic and less beneficial to the host (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Pettay et al., 2015; Matthews et al., 2017; Sproles et al., 2020). Both *D. trenchii* and *S. microadriaticum* have been shown to be more energetically costly to the host in Aiptasia (Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2019, 2020), as well as more thermally sensitive than the homologous *B. minutum* (Gabay et al., 2019; Chapter 2). The aim of this chapter was to investigate the possibility of improved nutritional cooperation between host and symbiont over time, both at the cellular and organismal levels, by applying complementary mass spectrometric techniques, including cutting edge NanoSIMS sub-cellular imaging

technology (Pernice et al., 2012, 2014; Räddecker et al., 2018; Sproles et al., 2020). It was hypothesised that host and symbiont enrichment of carbon and nitrogen would depend on symbiont identity, but that, given time in symbiosis, the nutritional fluxes with heterologous symbionts may shift to more closely resemble those with the homologous symbiont due to selective pressure acting at the cellular level leading to improved host-symbiont integration.

4.3 Methods

4.3.1 Experimental organisms

Aiptasia (n ~ 400; culture ID NZ1) were bleached using an adapted menthol bleaching protocol based on Matthews et al. (2016), as described in Chapters 2 and 3. This protocol was combined with a weekly cold shock by placing a beaker containing Aiptasia in 0.22- μ m filtered seawater (FSW) on ice for a period of 3 h. After anemones were confirmed as aposymbiotic using confocal microscopy (IX81, Olympus New Zealand; 635 nm laser, 655-755 nm emission filter), they were kept in the dark and fed twice weekly with freshly hatched *Artemia* sp. nauplii until experimental manipulation.

Three Symbiodiniaceae cultures were used for colonisation, which were comprised of the homologous species *B. minutum* (ITS2 type B1, culture FlAp2), and the heterologous species *S. microadriaticum* (ITS2 type A1, culture CCMP2467) and *D. trenchii* (ITS2 type D1a, culture Ap2). Cultures were grown in an incubator at 25 °C on a 12:12 h light:dark cycle with an irradiance of 30 μ mol photons m⁻² s⁻¹ (Phillips, 80W 840), in silica-free f/2 culture medium (AusAqua Pty, SA, Australia), and refreshed with new medium at least once a month.

4.3.2 Inoculation with symbionts

Symbiodiniaceae cultures were refreshed three days before each inoculation. Anemones were placed in sterile 400 mL plastic jars (LabServ; approximately 10 anemones *per* jar, for a total of 45 jars) containing 250 mL artificial seawater (ASW; Aquaforest Marine seasalt, dissolved in distilled water at 35 ppt) one day before the first inoculation to allow them to settle. Jars were randomly allocated to one of the three Symbiodiniaceae cultures, and then a drop of this culture containing 3 million cells mL⁻¹ mixed with some freshly hatched *Artemia*, was applied to the oral disc of each anemone to stimulate a feeding response. This was repeated once weekly for a total of four weeks, during which time the anemones were fed freshly hatched *Artemia* twice weekly.

4.3.3 Experimental treatments

Anemones were maintained at a constant temperature of 25 °C and light levels of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (GE Lighting T5 F54W/840). These conditions were maintained for the first four months after initial inoculation to allow the symbiont populations to reach stable densities. Throughout the whole experiment, anemones were fed freshly hatched *Artemia* nauplii twice weekly, with water changes the day after feeding.

After four months, Aiptasia were sampled for the first time-point (T0). Thereafter, the remaining jars were randomly divided between control conditions (constant 25 °C) or a repetitive heat stress (RHS) treatment (Figure 4.1). The RHS treatment was then exposed to a series of heat stress events lasting ten days: seven days of heating with increments of 1 °C day⁻¹, and three days at a peak temperature of 32 °C. After ten days, the temperature was reduced back to 25 °C and the anemones allowed to recover for a period of six weeks, after which the next heat stress event started. This was repeated for a total of three heat stress events and recovery periods. Anemones were sampled again after these three consecutive heat stress and recovery periods, making the final time-point ten months after initial colonisation, and six months after the first sampling point. At each heat stress event, samples were taken for the calculation of symbiont population density to assess the extent of bleaching.

Figure 4.1 Visual representation of experimental conditions: control was maintained at 25 °C for the entire duration of the experiment, while the repetitive heat stress (RHS) treatment was exposed to three heat stress and recovery periods. A heat stress event consisted of ramping by 1 °C *per* day to a peak temperature of 32 °C, which was held for three days. Temperatures were then allowed to drop back to 25 °C and a six-week recovery period followed. Sampling points are indicated in the timeline as defined in the key.

4.3.4 Preparation of isotope solutions

¹³C-labelled seawater was prepared by first ridding ASW of existing carbon, by lowering to < pH 3 using HCl, and continually aerating the water for 2 h. ¹³C in the form of sodium bicarbonate (NaH¹³CO₃;

99 atom %, Cambridge Isotope Laboratories, Tewksbury, Massachusetts, USA) was added to create a final concentration of 3 μM , after which 1 M NaOH was added to return the seawater to pH 8.

^{15}N -labelled culture medium (f/2) was prepared with a culture medium kit (Bigelow Laboratory, MA) and ^{15}N was added in the form of sodium nitrate ($\text{Na}^{15}\text{NO}_3$; 98 atom %, Sigma Aldrich, Auckland) to a final medium concentration of 0.882 μM (Guillard, 1975). *Tetraselmis* culture was grown in this culture medium for several months, with refreshing of the medium every two weeks. Freshly hatched *Artemia* sp. nauplii were fed this *Tetraselmis* culture daily for a total of 14 days, after which they were frozen and stored at $-80\text{ }^\circ\text{C}$ until used for sampling. Total enrichment of *Artemia* was determined using NanoSIMS, by fixing *Artemia* tissue as described in Section 4.3.5 and sectioning as described in Section 4.3.6.

4.3.5 Incubation and sampling

The day before each sampling, anemones were moved to separate jars under the same light and temperature conditions (see above), to allow the anemones to reattach to the new jars. At 9 am on the sampling day, the ASW in each jar was replaced with ^{13}C -labelled ASW, and the anemones fed one ^{15}N -labelled *Artemia per* anemone, by pipetting the nauplius onto the oral disc. After a 6-h incubation period (Sproles et al., 2020), anemones for nanoscale secondary ion mass spectrometry (NanoSIMS, $n = 3$ *per* treatment) were transferred to a ‘relaxation solution’ (1:1 mixture of 0.37 M MgCl_2 and 0.22 μM FSW) for 10 min, while anemones for bulk ratio-isotope mass spectrometry (IRMS) were snap-frozen in liquid nitrogen and stored for further analysis. Each replicate IRMS sample ($n = 5$ *per* treatment) consisted of eight pooled anemones to ensure enough biomass for analysis (determined from preliminary trials). One separate anemone *per* container ($n = 5$ *per* treatment) was snap-frozen for symbiont genotyping, symbiont cell counts, and host protein measurement. After 10 min in the ‘relaxation solution’, anemones for NanoSIMS analysis were fixed in a solution of 2.5% glutaraldehyde, 0.5% paraformaldehyde, 0.1 M phosphate and 0.6 M sucrose, and kept at room temperature for 2 h, followed by 22 h at $4\text{ }^\circ\text{C}$. After 24 h, samples were washed 3 x 30 min with Sørensen phosphate buffer, followed by three rinses of 5 min in MilliQ water. Samples were then stored at $4\text{ }^\circ\text{C}$ until further processing.

4.3.6 Preparation of samples for NanoSIMS

Fixed anemones were cut to remove tentacles, which were placed in fresh fixative (see above) in glass 20 mL vials and left overnight. After this, fixative was removed with two washes of FSW. Tentacle tissue was then stained with black osmium solution (1% osmium tetroxide, 1% potassium ferrocyanide, 1:4 parts FSW) for 1 h. Tissue was repeatedly rinsed with FSW until all osmium was removed, and then

dehydrated *via* an ethanol series. Tissue was left in a rotary mixer (Pelco R2) for 10 min, with the following ethanol concentrations: 15%, 30%, 50%, 70%, 80%, and 90%. Two rounds of 20 min followed with 100% ethanol and acetone. Two final rounds of 10 min with propylene oxide were then performed. Next, samples were placed in a mixture of 1:1 propylene oxide:resin (ProSciTech, Australia), with the lids of the vials left on for 10 min, and then off for the following 18 h, so maximizing air flow over the samples. After this, samples were placed in 100% resin, left on the rotator for 1 h, transferred to embedding molds containing fresh resin, and placed at 60 °C overnight to harden.

Samples embedded in resin were cut into semi-thick sections (500 nm) using an ultramicrotome, and adhered to glass coverslips of 10 mm diameter (ProSciTech, Australia). Regions of interest (ROIs) were identified under a light microscope (40x magnification) for each coverslip. Samples were analysed by NanoSIMS at École Polytechnique Fédérale de Lausanne (EPFL), Switzerland.

At EPFL, coverslips were coated with 7 nm gold, and ROIs were imaged with a NanoSIMS ion probe (Hoppe et al., 2013). Samples were bombarded with a 16 keV primary ion beam of (1-2 pA) Cs⁺ focused to a spot size of between 100 – 150 nm on the sample surface. Secondary molecular ions ¹²C¹⁴N⁻, ¹²C¹⁵N⁻, ¹²C¹²C⁻ and ¹²C¹³C⁻ were simultaneously collected in electron multipliers at a mass resolution (M/ΔM) of ~ 9000, enough to resolve the ¹²C¹⁵N⁻ and ¹³C¹⁴N⁻ ions from possibly problematic interference, while charge compensation was not necessary. Images of 40 x 40 μm with 256 x 256 pixels were obtained for ¹²C¹⁴N⁻, ¹²C¹⁵N⁻, ¹²C¹²C⁻ and ¹²C¹³C⁻ by rastering the primary ion beam across the sample with a dwell-time of 5 ms. After drift correction, the ¹⁵N/¹⁴N and ¹³C/¹²C maps were obtained by taking the ratio between ¹²C¹⁵N⁻ and ¹²C¹⁴N⁻ images or ¹²C¹³C⁻ and ¹²C¹²C⁻ images, respectively. ¹⁵N and ¹³C enrichments were expressed in the delta notation (δ¹⁵N and δ¹³C in ‰), as calculated by:

$$\delta^{15}N = \left(\frac{N_{sample}}{N_{con}} - 1 \right) \times 10^3$$

In which N_{sample} is the measured ¹⁵N/¹⁴N ratio of the enriched sample and N_{con} is the natural ¹⁵N/¹⁴N ratio measured in unlabelled control anemone samples; and

$$\delta^{13}C = \left(\frac{C_{sample}}{C_{con}} - 1 \right) \times 10^3$$

In which C_{sample} is the measured ¹³C/¹²C ratio of the enriched sample and C_{con} is the natural ¹³C/¹²C ratio measured in unlabelled control anemone samples. Both N_{con} and C_{con} were measured on sections of

unlabelled anemone tissue each day of NanoSIMS analysis to correct for any potential instrument drift. For most treatments, a minimum of 30 symbiont cells were sampled *per* replicate anemone, with three replicate anemones *per* treatment group. However, cell densities of *S. microadriaticum* were very low at all time points, while *D. trenchii* cell densities were very low for anemones exposed to RHS at the end sampling point, leading to a minimum of five cells *per* replicate anemone for these treatment groups (see Table 4.1).

Table 4.1 Number of replicate symbiont cells per replicate anemone (R1-3) imaged using NanoSIMS for Aiptasia in symbiosis with different symbiont species, i.e. Breviolum minutum, Durusdinium trenchii, or Symbiodinium microadriaticum, at start and end points of the experiment (4 and 10 months after colonisation, respectively), having gone through either the control (con) or repetitive heat stress (RHS) treatment (three stress events consisting of ramping to 32 °C, held for three days, followed by a six-week recovery period). * means the replicate sample was removed from analysis due to being classed as extreme outliers (Section 4.3.11).

Symbiont species	Symbiont cells per replicate at								
	Start			End con			End RHS		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
<i>B. minutum</i>	53	31	57	135	87	62	39	92	60
<i>D. trenchii</i>	61	32	46	46	78	33*	20	9	10*
<i>S. microadriaticum</i>	8	8	11	5	45	24	9	7	6

4.3.7 NanoSIMS image analysis

Images obtained from EPFL were analysed using L'IMAGE ® software (version 5-5-2020). Enrichment values for symbiont and host tissues were collected by definition of ROIs within the images: first, algal cells were outlined, and subsequently subtracted from the surrounding host gastrodermis. ROI enrichment values were averaged between replicates for each sample. For *Artemia* samples (i.e., total ¹⁵N enrichment of food), the entire area containing sample was selected as an ROI.

4.3.8 Preparation of samples for IRMS

Frozen anemones (n = 5, with eight anemones *per* replicate) were thawed on ice and homogenized using a saw-tooth homogenizer in 500 µL MilliQ water. Host and symbiont fractions were separated by centrifugation at 1000 x g for 5 min, after which the symbiont fraction was washed of residual host material by one further centrifugation with MilliQ water. Fractions were then frozen at -80 °C for at least 1 h, after which they were freeze-dried (FreeZone® Plus™ 4.5L Cascade, Labconco, Auckland, New Zealand). Freeze-dried samples were processed at the National Institute of Water and Atmospheric Research (NIWA; Wellington, NZ) for IRMS analysis.

4.3.9 IRMS sample analysis

Stable isotope analysis was carried out on a Delta^{Plus} (Thermo Fisher Scientific, Bremen, Germany) continuous flow isotope ratio mass spectrometer linked to an NA1500 elemental analyser with an AS200-LS autosampler (Fisons Instruments, Rodano, Italy). $\delta^{15}\text{N}$, AT% $^{15}\text{N}/^{14}\text{N}$, $\delta^{13}\text{C}$ and AT% $^{13}\text{C}/^{12}\text{C}$ values were calculated by Isodat software (Thermo Fisher Scientific, Bremen, Germany). $\delta^{15}\text{N}$ values from samples where blank contribution was >10% were blank-corrected. Blank-correction of sample isotope values was by the subtraction method, as described by Langel & Dyckmans (2017) and Ohlsson (2013), and was carried out prior to data normalisation. N_2 blank amount and blank $\delta^{15}\text{N}$ were estimated by the indirect method from multiple measurements of two laboratory standards with very different $\delta^{15}\text{N}$ values. CO_2 blank amount and blank $\delta^{13}\text{C}$ were not deemed necessary, so no blank correction of $\delta^{13}\text{C}$ data was applied. Reference materials were used to determine isotopic values following Paul et al. (2007). Sample $\delta^{15}\text{N}$ values were two-point normalised (following blank-correction where applied) using isotopic data from the daily analysis of L-glutamic acid (NIST 8573 USGS40) and ammonium sulphate (NIST 8548 IAEA-N2). Sample $\delta^{13}\text{C}$ values were two-point normalised using isotopic data from the daily analysis of L-glutamic acid (NIST 8573 USGS40) and sucrose (NIST 8542 IAEA-CH-6). Precision was determined by the repeat analysis of the working laboratory standard DL-Leucine (DL-2-Amino-4-methylpentanoic acid, $\text{C}_6\text{H}_{13}\text{NO}_2$, Lot 127H1084, Sigma, Australia). Data from the analysis of glycine (USGS65) were used to check accuracy and precision. Data from the analysis of NIWA's internal laboratory squid standard were used as a further check of precision.

Due to low cell densities of *D. trenchii* and *S. microadriaticum* within Aiptasia tissues, and therefore low biomass of symbiont fractions, separation of symbionts without a relatively large proportion of host tissue contamination could not be guaranteed. Therefore, due to concerns that this led to misleading enrichment results, the IRMS data for symbiont fractions are not considered further (Supplementary Data Figure S1).

4.3.10 Host growth, symbiont cell densities and genotyping

Anemones sampled for genotyping and cell counts were homogenised and separated as described in Section 4.3.8. Each symbiont fraction was split into two separate aliquots, one for cell counts and one for genotyping ($n = 4$ per treatment). Cell counts were performed using a haemocytometer (Improved Neubauer) with eight replicate counts per sample, and were normalised to protein content determined via the Bradford assay (Bradford, 1976). Cell density (symbionts per mg host protein) was then calculated. Protein content from the Bradford assay was also used to determine total protein content per anemone. Total anemone protein content for the start time-point was averaged and subtracted from the

total protein content at the end-point to establish growth, as the increase in total protein content *per* anemone.

Before inoculation, all cultures were genotyped. DNA was extracted using a bead-beat protocol. At each sampling time-point, anemones were also checked for symbiont genotype. DNA was extracted according to the CTAB-chloroform protocol of Baker and Cuning (2016) as described in Chapters 2 and 3. PCR was performed on all extracted DNA using the primers ITSd (forward, 5'-GTGAATTGCAGAACTCCGTG-3') and ITS2rev2 (reverse; 5'-CCTCCGCTTACTTATATGCTT-3'). Extracted DNA was sent to Macrogen (Seoul, Korea) for purification and sequencing. Sequence results were checked for quality and then BLAST searched against two databases, GeoSymbio and NCBI, to confirm symbiont ITS2 type.

4.3.11 Statistical analysis of results

Host growth data were tested for normality and homoscedasticity, and then tested with a two-way ANOVA in R (version 4.0.3; R Core Team, 2020). Cell density data ($n = 5$), and carbon and nitrogen enrichment values for host and symbiont fractions established by NanoSIMS ($n = 3$) and/or IRMS ($n = 5$) were tested for normality and homoscedasticity, and then tested with a series of ANOVAs in R. These consisted of a one-way ANOVA to compare between symbiont species at the start-point, a two-way ANOVA with the factors 'treatment' and 'species' to compare between the symbiont species at the end of the repeated thermal exposure, and a one-way ANOVA within each species to measure the effect of thermal treatment over time. For this last comparison, a combined factor of treatment and time-point was made to compensate for the lack of a full factorial design. To correct for multiple testing, a highly conservative approach was adopted by applying a Bonferroni correction to the significance threshold: dividing α by the number of tests. Every sample group was tested in two ANOVAs, therefore $\alpha = \frac{0.05}{2} = 0.025$. Nitrogen enrichment values for host and symbiont using NanoSIMS, as well as host fractions using IRMS, were $\log_{10}$ transformed. Additionally, two NanoSIMS replicates from separate treatment groups were removed from analysis due to being extreme outliers (> 6 absolute deviations away from the median; Leys et al., 2013). Cell density data for the heat-stress treatment ($n = 4$) were tested separately, *via* one-way ANOVA, using the factor treatment *per* symbiont species *per* timepoint, to establish if heat stress reduced cell density, i.e. caused bleaching. Only cell densities of *S. microadriaticum* for heat-stress events two and three (HS2 and HS3) required $\log_{10}$ transformation to fit normality assumptions.

4.4 Results

4.4.1 Host growth and symbiont cell density

Host growth, measured as increase in total protein *per* anemone (Figure 4.2), tested significant for both thermal treatment (two-way ANOVA, $F_{1,23} = 10.30$, $p = 0.0040$) and symbiont species (two-way ANOVA, $F_{2,23} = 4.64$, $p = 0.0203$). *Post hoc* testing revealed significant pairwise differences between *S. microadriaticum*-colonised anemones exposed to RHS compared to *B. minutum*- and *D. trenchii*-colonised anemones maintained under control conditions (Tukey HSD *post hoc*, $p = 0.0166$ and $p = 0.0081$, respectively), with those colonised by *S. microadriaticum* showing zero to negative growth when exposed to RHS (Figure 4.2).

All three symbiont species had significantly different population densities at the first sampling point (T0; one-way ANOVA, $F_{2,9} = 66.88$, $p = 3.97 \times 10^{-6}$), with *B. minutum* achieving a three-fold higher density than *D. trenchii* and a 48-fold higher density than *S. microadriaticum* (Figure 4.3). Over the subsequent six months, *B. minutum* (one-way ANOVA, $F_{2,11} = 11.9$, $p = 0.0018$) and *D. trenchii* (one-way ANOVA, $F_{2,11} = 27.69$, $p = 5.08 \times 10^{-5}$) decreased in density regardless of thermal treatment, by 43% and 30% respectively, while no significant change in density occurred over this same period for *S. microadriaticum* (one-way ANOVA, $F_{2,11} = 5.07$, $p = 0.0275$). The only bleaching to occur during any of the heat stress events was in *D. trenchii*-colonised anemones, but only in response to the first bout of elevated temperature (one-way ANOVA, $F_{1,6} = 26.00$, $p = 0.0022$; Figure 4.3), while for the next two heat stress events, as well as all heat stress events for *B. minutum*- and *S. microadriaticum*-colonised anemones, no significant changes in cell density were observed ($p > 0.05$ for all comparisons; see Figure 4.3). By the end time-point, significant differences remained between the symbiont species after heat stress (two-way ANOVA, $F_{2,24} = 165.86$, $p = 8.9 \times 10^{-15}$), with *B. minutum* being 10 times more densely populated than *D. trenchii*, and 40 times more than *S. microadriaticum*.

Figure 4.2 Increase in total host protein content ($\pm$ SE, *per anemone*) for anemones in symbiosis with the different species (i.e. *Breviolum minutum*, *Durisdinium trenchii*, or *Symbiodinium microadriaticum*) from start to end points of the experiment, having been maintained either at control temperature (Con, 25 °C), or gone through the repetitive heat stress (RHS) treatment (three heat stress events which consisted of ramping to 32 °C, which was held for three days, then followed by a six-week recovery period at 25 °C). Letters indicate statistically significant groups.

4.4.2 Autotrophic carbon enrichment

Using NanoSIMS, enrichment of autotrophic carbon in host tissues was not significantly different between the different symbioses at the start (one-way ANOVA, $F_{2,6} = 1.70$, $p = 0.26$; Figure 4.4A and Figure 4.5), while IRMS data *did* show significantly different carbon enrichment of host fractions at the start (one-way ANOVA, $F_{2,12} = 44.05$, $p = 2.97 \times 10^{-6}$; Figure 4.4C). In this latter case, *B. minutum*-colonised host tissues were approximately two-fold more enriched than when colonised by *D. trenchii* (Tukey HSD *post hoc*, $p < 0.0001$), and almost 10-fold more enriched than when colonised by *S. microadriaticum* (Tukey HSD *post hoc*, $p = 0.0005$); host carbon enrichment in the presence of *D. trenchii* was 4.6-fold greater than that with *S. microadriaticum* (Tukey HSD *post hoc*, $p = 0.0046$).

Figure 4.3 (A) Cell densities ($\pm$ SE) of the different symbiont species (i.e. *Breviolum minutum*, *Durisdinium trenchii*, or *Symbiodinium microadriaticum*) throughout the experiment for control (Con) and repetitive heat stress (RHS) treatment (ramping to 32 °C over seven days, then held for three days), and (B) timing and temperatures of sampling points; Start and end points are all sampled at control temperature, i.e. at the end of the recovery period for the RHS treatment, while HS1, HS2 and HS3 are sampled at the peak of the heat stress event to establish bleaching rates. The time is in months since initial inoculation with symbionts. * indicates significant differences between control and RHS during heat stress events, while letter groups indicate significant differences between and within species for the start and end sampling points.

In *B. minutum*-colonised anemones, there were no changes in host carbon enrichment in response to time or thermal regime, whether analysed by NanoSIMS (one-way ANOVA, $F_{2,6} = 0.29$, $p = 0.541$) or IRMS (one-way ANOVA, $F_{2,12} = 1.91$, $p = 0.19$). The same was true when anemones harboured *S. microadriaticum* (NanoSIMS, one-way ANOVA, $F_{2,6} = 1.89$, $p = 0.23$; IRMS, one-way ANOVA, $F_{2,12} = 1.70$, $p = 0.224$). In comparison, when anemones contained *D. trenchii*, changes in host carbon enrichment with both time and thermal regime were detected *via* NanoSIMS (one-way ANOVA, $F_{2,6} = 21.32$, $p = 0.0019$) and IRMS (one-way ANOVA, $F_{2,12} = 8.68$, $p = 0.0047$). Specifically, NanoSIMS showed greater carbon enrichment of host tissues at the final time-point under control conditions, when enrichment was approximately three-fold greater than at the start (Tukey HSD *post hoc*, $p = 0.0072$) and seven-fold greater than after the completed RHS treatment (Tukey HSD *post hoc*, $p = 0.0019$).

Figure 4.4 Carbon enrichment ($\pm$ SE) for (A, C) host tissue, and (B) symbiont cells measured via (A, B) NanoSIMS ($n = 3$) or (C) bulk isotope-ratio mass spectrometry (IRMS, $n = 5$), for *Aiptasia* in symbiosis with different species of symbionts (i.e., *Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*), at start and end points of the experiment (4 and 10 months after colonisation, respectively), having gone through either the control (Con) or repetitive heat stress (RHS) treatment (three stress events consisting of ramping to 32 °C, held for three days, followed by a six-week recovery period). * indicate differences within symbiont species over time or treatment, while letters indicate statistically significant differences between species; ¹ is used for differences identified between species at the start, while ² is used for differences between species and treatments at the end point.

Meanwhile, IRMS showed that host carbon enrichment was eight-fold greater at the start of the experiment than after the completed RHS treatment (Tukey HSD *post hoc*, $p = 0.0039$), while at the end of the experiment enrichment was 5.5-fold greater in the control than after heat stress (Tukey HSD *post hoc*, $p = 0.0490$; Figure 4.4C). Given these different responses to RHS, it was unsurprising that host carbon enrichment was different between the different symbioses upon completion of the experiment. However, this was only apparent with IRMS (two-way ANOVA, $F_{2,24} = 62.59$, $p = 3.01 \times 10^{-10}$), where host tissues were approximately five-fold more enriched in the presence of *B. minutum* than with *D. trenchii* or *S. microadriaticum* (Tukey HSD *post hoc*, $p < 1 \times 10^{-7}$ for both comparisons).

For the symbionts, NanoSIMS revealed that cellular enrichment with carbon differed significantly between the three Symbiodiniaceae species at the start-point (one-way ANOVA, $F_{2,6} = 30.83$, $p =$

0.0007; Figure 4.4B). Specifically, *S. microadriaticum* was 2.5-3 times more enriched than either *B. minutum* (Tukey HSD *post hoc*, $p = 0.0016$) or *D. trenchii* (Tukey HSD *post hoc*, $p = 9.69 \times 10^{-4}$). No changes occurred within any symbiont species over time or in response to thermal treatment, so at the final time-point the species were still significantly different, and by the same magnitude (two-way ANOVA, $F_{2,12} = 31.10$, $p = 1.79 \times 10^{-5}$).

Figure 4.5 NanoSIMS images of autotrophic ^{13}C carbon enrichment within symbiotic Aiptasia in symbiosis with different species of symbionts: (A-C) *Breviolum minutum*, (D-F) *Durusdinium trenchii*, or (G-I) *Symbiodinium microadriaticum*, for the different time points and treatments of the experiment, i.e. (A, D, G) start: four months after establishment of symbiosis; (B, E, H) end control: ten months after establishment of symbiosis having been maintained at control temperatures (25°C); and (C, F, I) end RHS: ten months after establishment of symbiosis, having gone through three heat stress events and recovery periods since the start time point. Colour scale indicates values in delta notation, where black signifies no enrichment and white signifies high enrichment. Symbiont cells are outlined in white, other enriched areas represent anemone host tissue. Note, enrichment of host tissues was much lower than symbiont tissues, therefore enrichment of host tissues is not always visible.

4.4.3 Heterotrophic nitrogen enrichment

Artemia nauplii successfully incorporated ^{15}N from the labelled *Tetraselmis* culture ($\delta^{15}\text{N}$: 145,339 ‰). Host tissues in the different symbioses showed the same degree of nitrogen enrichment at the start of the experiment, *via* both NanoSIMS (one-way ANOVA, $F_{2,6} = 0.92$, $p = 0.449$; Figure 4.6A and Figure 4.7) and IRMS (one-way ANOVA, $F_{2,12} = 0.88$, $p = 0.441$; Figure 4.6C). Subsequently, in *B. minutum*-colonised anemones, there was no significant change in host nitrogen enrichment over time or thermal regime when analysed in the direct vicinity of the symbionts by NanoSIMS (one-way ANOVA, $F_{2,6} = 2.09$, $p = 0.205$), contrary to the finding with bulk tissue analysis by IRMS (one-way ANOVA, $F_{2,12} = 51.99$, $p = 1.23 \times 10^{-6}$), where this parameter declined between the beginning and end of the experiment under both control and RHS conditions (Tukey HSD *post hoc*, $p < 1 \times 10^{-5}$ for both comparisons). By comparison, in the presence of *S. microadriaticum* and *D. trenchii*, host nitrogen enrichment was shown to change with time under control and RHS conditions by both NanoSIMS and IRMS analysis (*D. trenchii*-colonised anemones, NanoSIMS: one-way ANOVA, $F_{2,4} = 118.7$, $p = 0.0002$; IRMS: one-way ANOVA, $F_{2,12} = 13.95$, $p = 0.0007$; *S. microadriaticum*-colonised anemones, NanoSIMS: one-way ANOVA, $F_{2,6} = 48.23$, $p = 0.0002$; IRMS: one-way ANOVA, $F_{2,12} = 6.90$, $p = 0.0101$). In both cases, there was a significant decline from the start to the end of the experiment (Tukey HSD *post hoc*, NanoSIMS: $p < 0.001$ for both comparisons; IRMS $p < 0.02$ for both comparisons; Figure 4.6A, C).

At the final timepoint, NanoSIMS showed a significant interaction effect of symbiont species and thermal treatment on host nitrogen enrichment (two-way ANOVA, $F_{2,10} = 7.31$, $p = 0.0111$). In particular, under RHS with *B. minutum*, host tissues were more enriched with nitrogen than when anemones contained *D. trenchii* (Tukey HSD *post hoc*, $p = 0.0172$); they were also more enriched than *S. microadriaticum*-colonised host tissues under control conditions (Tukey HSD *post hoc*, $p = 0.0026$). By contrast, IRMS did not detect any differences in host nitrogen enrichment in response to symbiont species or thermal treatment (two-way ANOVA, $F_{2,24} = 2.96$, $p = 0.0711$ for species; $F_{1,24} = 1.01$, $p = 0.326$ for treatment).

With respect to symbiont nitrogen enrichment, no significant differences were observed *via* NanoSIMS between symbiont species at the start of the experiment (one-way ANOVA, $F_{2,6} = 0.98$, $p = 0.428$; Figure 4.6B), and there was no significant response to either time or thermal regime in *B. minutum* (one-way ANOVA, $F_{2,6} = 0.85$, $p = 0.47$) or *D. trenchii*, though this latter species was approaching significance (one-way ANOVA, $F_{2,5} = 6.36$, $p = 0.0423$, which is above the designated $\alpha = 0.025$ for this analysis). In contrast, a decrease of nitrogen enrichment in *S. microadriaticum* occurred over time (one-way ANOVA, $F_{2,6} = 33.65$, $p = 0.0005$), with the symbionts being less enriched *per cell* at the end than at the start under both thermal regimes (Tukey HSD *post hoc*, $p < 0.002$ for both comparisons). Given these temporal changes, a significant interaction between treatment and species was apparent at

the end of the experiment (two-way ANOVA, $F_{2,11} = 5.80$, $p = 0.019$), with *B. minutum* exposed to RHS being significantly more nitrogen-enriched than *D. trenchii* exposed to RHS, and *S. microadriaticum* maintained under control conditions (Tukey HSD *post hoc*, $p = 0.0250$ and $p = 0.0424$, respectively). Symbiont nitrogen enrichment of *S. microadriaticum* exposed to RHS did not differ significantly from *B. minutum* or *D. trenchii* (Tukey HSD *post hoc*, $p > 0.05$ for all comparisons).

Figure 4.6 Nitrogen enrichment ($\pm$ SE) for (A, C) host tissue, and (B) symbiont cells measured via (A, B) NanoSIMS ($n = 3$) or (C) bulk isotope-ratio mass spectrometry ($n = 5$), for *Aiptasia* in symbiosis with different species of symbionts (i.e., *Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*), at start and end points of the experiment (4 and 10 months after colonisation, respectively), having gone through either the control (Con) or repetitive heat stress (RHS) treatment (three stress events consisting of ramping to 32 °C, held for three days, followed by a six-week recovery period). * indicate differences within symbiont species over time or treatment, while letters indicate statistically significant differences between species; ¹ is used for differences identified between species at the start, while ² is used for differences between species and treatments at the end point.

Figure 4.7 NanoSIMS images of heterotrophic ^{15}N nitrogen enrichment within symbiotic Aiptasia tissue in symbiosis with different species of symbionts, i.e. (A-C) *Breviolum minutum*, (D-F) *Durusdinium trenchii*, or (G-I) *Symbiodinium microadriaticum*, for the different time points and treatments of the experiment, i.e. (A, D, G) start: four months after establishment of symbiosis; (B, E, H) end control: ten months after establishment of symbiosis having been maintained at control temperatures (25 °C); and (C, F, I) end RHS: ten months after establishment of symbiosis, having gone through three heat stress events and recovery periods since the start time point. Colour scale indicates values in delta notation, where black signifies no enrichment and white signifies high enrichment, scales differ between the different time points due to feeding activity. Symbiont cells are outlined in white, other enriched areas represent anemone host tissue.

4.5 Discussion

This study estimated the translocation of autotrophic carbon and heterotrophic nitrogen between host and symbiont at different times of symbiosis, and the effect of repeated heat stress on this dynamic, to assess whether host and symbiont are capable of improved cellular integration over time. Results indicated that *B. minutum*-colonised anemones received the largest supply of photosynthetic carbon out of the three different symbiotic associations, which on a holobiont-scale was stable with time and thermal regime, even though cell densities of *B. minutum* declined. Similarly, nitrogen enrichment *per* cell remained consistent with time and thermal regime in *B. minutum*-colonised anemones. In comparison, *D. trenchii* did not manage to move towards a more mutualistic state with its host, as nitrogen and carbon translocation patterns remained the same over time under control conditions, which compared to *B. minutum* consisted of lower translocation of carbon from symbiont to host, and more nitrogen from host to symbiont (see Figure 4.8). Moreover, heat stress did not aid in this process, and actually reduced the carbon supply from *D. trenchii* to its host, both on a localised tissue-level scale and the overall holobiont-scale, while nitrogen consumption *per* symbiont cell increased. In contrast, *S. microadriaticum*-colonised anemones showed a continuous supply of carbon *per* symbiont cell over time regardless of thermal regime, and a similar rate of nitrogen assimilation *per* symbiont cell in control anemones when compared to *B. minutum* (see Figure 4.8). However, the amount of carbon supplied by *S. microadriaticum* is unlikely to be sufficient to maintain host metabolism on the holobiont-scale due to its very low population density. These findings have implications for the potential success of novel symbioses over prolonged periods, as might be required for adaptation to our changing climate.

Figure 4.8 Cost vs. Benefit to the host; NanoSIMS Symbiont ^{15}N nitrogen enrichment over host ^{13}C carbon enrichment within symbiotic *Aiptasia* colonised with different symbiont species symbionts (i.e., *Breviolum minutum*, *Durisdinium trenchii*, or *Symbiodinium microadriaticum*), at start and end points of the experiment (4 and 10 months after colonisation, respectively), having gone through either the control (CON) or repetitive heat stress (RHS) treatment (three stress events consisting of ramping to 32 °C, held for three days, followed by a six-week recovery period).

4.5.1 Autotrophic carbon translocation

Symbiont identity had no effect on the degree of host carbon enrichment at the start of the experiment (i.e., three months after colonisation), when assessed at the cellular level by NanoSIMS. However, *S. microadriaticum* cells were 2.5-3.5 times more carbon enriched than *B. minutum* or *D. trenchii* at this time-point. This enhanced enrichment is likely a result of the much lower population density of *S. microadriaticum* than these other symbiont species, resulting in more carbon being available *per* symbiont cell due to reduced competition from neighbouring symbionts, and hence more photosynthesis *per* cell (Cook & D'Elia, 1987; Falkowski et al., 1993). This faster photosynthesis was insufficient to counter the much lower cell density of *S. microadriaticum*, however, with IRMS (i.e., holobiont-level) analysis confirming much less carbon enrichment of the host's tissues overall than for both *B. minutum*- and *D. trenchii*-colonised anemones. Similarly, the lower population density of *D. trenchii* than *B. minutum* at the start of the experiment explains, at least in part, the lesser carbon enrichment of those anemones hosting the former symbiont. However, it is also important to note that the density of *B. minutum* was three times that of *D. trenchii* at the start of the experiment, which might be expected to elevate the rate of cell-specific photosynthesis for *D. trenchii* in a similar manner to that discussed for *S. microadriaticum*. This was not observed, suggesting that *D. trenchii*'s photosynthetic performance

in Aiptasia may be sub-optimal at the cellular level, further contributing to its reduced performance at the holobiont level. This is in agreement with a previous study by Sproles et al. (2020), who showed a significantly lower assimilation of carbon in *D. trenchii*-colonised Aiptasia compared to *B. minutum*-colonised anemones at two and 14 days after symbiosis establishment.

These nutritional patterns continued to be apparent over time, even though, as also reported in Chapter 3, the population densities of both *B. minutum* and *D. trenchii* declined over time under both thermal regimes, while the density of *S. microadriaticum* remained at a stable, albeit low level, again regardless of thermal exposure. Interestingly though, at the end of the experiment, the overall carbon enrichment of *S. microadriaticum*-colonised host tissues was similar to that of *D. trenchii*-colonised hosts, even though the population density of *D. trenchii* was four-fold higher than that of *S. microadriaticum*. This provides further evidence that the cell-specific photosynthetic rate of *D. trenchii* in Aiptasia was less than that of the other two symbiont species, consistent with the widespread opinion that this symbiont species is often less beneficial to its host with respect to supporting the host's metabolism, growth and reproduction (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Pernice et al., 2014; Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2020). Indeed, this less beneficial nature was seemingly exacerbated here under thermal stress, where carbon enrichment of the host's tissues declined in the presence of *D. trenchii* relative to the other symbioses, despite its rate of photosynthesis being unaffected. By comparison, the heterologous *S. microadriaticum* appears more beneficial. The cellular and physiological mechanisms underlying these differences are unknown, but the capacity for different symbionts to form an integrated symbiosis is unquestionably important for enabling a functional relationship.

Interestingly, there was a partial disconnect between host tissue carbon enrichment at the holobiont-level and anemone growth, with growth being similar in the presence of *B. minutum*, *D. trenchii* and *S. microadriaticum* under control conditions, despite the lower contribution of photosynthetically fixed carbon by the heterologous symbiont populations. The similarity with *B. minutum*-colonised anemones was also apparent under thermal stress in the symbiosis with *D. trenchii*. This observation contrasts with that of Gabay et al. (2018), who reported greatly reduced growth and pedal laceration (i.e., asexual reproduction) in *D. trenchii*-colonised Aiptasia in a shorter-term (eight-week) experiment. The means by which anemones containing *D. trenchii* maintained their growth rate here is unknown, but it is conceivable that, in the longer term, anemones mobilised reserves to support their growth, reduced their respiratory consumption, or alternatively fed more efficiently to counter the loss of photosynthetic carbon (Hughes & Grottoli, 2013; Hillyer et al., 2017; Bedgood et al., 2020; Matthews et al., 2020). However, if such metabolic or behavioural adjustment occurred, then it was not seen in *S. microadriaticum*-colonised anemones whose growth was negatively impacted under heat stress. Why thermal stress had such a marked effect in the presence of *S. microadriaticum* is unknown given that

RHS did not have an effect on carbon enrichment in this symbiont species. However, it is important to note that carbon enrichment was only measured after a period of recovery from the thermal stress, so events *during* the thermal stress event were not captured. It is therefore possible that *S. microadriaticum* was more susceptible to thermal stress but that it was able to recover in the timeframe given. Consequently, the capacity for the host to build up carbon reserves may have been diminished relative to the other two symbioses, so limiting the ability of this heterologous symbiont to sustain host metabolism. Consistent with this, evidence for reduced host carbon reserves in response to thermal stress, as well as after repeated heat stress and recovery in *S. microadriaticum*-colonised hosts was presented in Chapters 2 and 3. Additionally, while *S. microadriaticum* has a reputation for being thermally tolerant in culture (Robison & Warner, 2006; Ragni et al., 2010; Hawkins & Davy, 2012; Krämer et al., 2012; Swain et al., 2017), Gabay et al. (2019) showed that an association between *Aiptasia* and its homologous symbiont *B. minutum* was more thermally robust than a pairing with either *D. trenchii* or *S. microadriaticum*, highlighting the role that the host-symbiont interaction plays in determining thermal resistance of the holobiont. More regular sampling and analysis of host and symbiont metabolism over the course of thermal stress and recovery would help to elucidate this matter.

4.5.2 Heterotrophic nitrogen translocation

There was a clear effect of time on host nitrogen enrichment, with much higher nitrogen enrichment values in host tissues at the start-point compared to the end-point, regardless of symbiont type or thermal regime; though note that the effect was not significant for *B. minutum*-colonised anemones when analysed by NanoSIMS, most likely due to the considerable variability of the data. It is, however, very possible that this temporal change resulted from differential feeding activity of the anemones at the sampling points, which could not be controlled for. Therefore, this discussion will exclude results *within* each host-symbiont pairing over time, and instead focus on the comparisons *between* species at each individual time-point.

At the start of the experiment, host nitrogen enrichment was similar for all three symbioses, as measured by either NanoSIMS or IRMS. Likewise, symbiont nitrogen enrichment was similar for the three Symbiodiniaceae species when measured by NanoSIMS. Both *D. trenchii* and *S. microadriaticum* were present at lower population densities than *B. minutum*, so it might be expected that nitrogen enrichment *per* symbiont cell would in fact be higher for the two heterologous species due to reduced competition for nitrogenous resources. Moreover, the relatively low supply of photosynthetically fixed carbon in *D. trenchii*- and *S. microadriaticum*-colonised host tissues (see above) could limit the host's ability to re-assimilate nitrogen generated by host catabolism (Xiang et al., 2020), thereby making more nitrogen available to its symbionts. The fact that elevated nitrogen enrichment of the two heterologous symbionts was not observed perhaps therefore reflects a lesser degree of host-symbiont cellular integration and

cooperation. However, it is also notable that, on a *per cell* basis, *D. trenchii* assimilated an equal amount of nitrogen as the other two symbiont species despite its photosynthetic rate being less than might be expected given its low population density (see above), further supporting the notion that this species is sub-optimal for Aiptasia. Certainly, on a cell-specific basis (if not a population level), the heterologous *S. microadriaticum* appears more beneficial, supplying more photosynthetic carbon while assimilating the same amount of nitrogen from the host. This higher enrichment of nitrogen paired with a lower carbon assimilation by *D. trenchii* in Aiptasia was also observed in a NanoSIMS study by Sproles et al. (2020), who assessed nutrient exchange just two weeks after colonisation. Both this shorter-term study and this current longer-term study suggest that *D. trenchii* will likely cause a nutritional deficiency in Aiptasia over time, as it consumes more of the host's nutrient pool than it provides in return.

Further evidence for the sub-optimal nature of *D. trenchii* was apparent at the end of the experiment. Here, *D. trenchii* assimilated less nitrogen *per cell* than the homologous symbiont in response to RHS. This decline in nitrogen enrichment by *D. trenchii* may have arisen from thermally-induced physiological dysfunction. Alternatively, though, it may simply have been a proportional response to the reduced levels of assimilated nitrogen in the gastrodermis, as seen in the NanoSIMS images. Indeed, this second scenario is more likely given that there was no evidence for cell-specific photosynthetic disruption. Crucially, while *D. trenchii* continued to take up a similar *proportion* of nitrogen from the host as under control conditions, it supplied less carbon in return on a *per cell* basis, suggesting that RHS induces increased selfishness in *D. trenchii*. Indeed, *D. trenchii* is a well-known opportunist (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Matthews et al., 2017; Sproles et al., 2020). By comparison, at the end of the experiment, *S. microadriaticum* assimilated significantly less nitrogen *per* symbiont cell under control conditions than *B. minutum* exposed to RHS. This again supports the argument that *S. microadriaticum* does not show opportunistic traits, even if the overall impact of this is likely very small given the consistently low symbiont population density. More work, with greater replication, will help elucidate these species-specific traits further.

4.6 Conclusion

My results show that *D. trenchii* is less beneficial when in symbiosis with Aiptasia than the homologous symbiont, even exhibiting some opportunistic traits, and RHS did not facilitate the relationship. Similar patterns of translocation were observed four and ten months after establishment of symbiosis as found by Sproles et al. (2020), who measured translocation of carbon in Aiptasia two weeks after establishment of symbiosis. Thus, over time, there seems to be no change in the 'willingness' of *D. trenchii* to share its photosynthate with the host. Chapter 3 showed a similar rigidity in host fitness over time, using metabolomics and proteomics approaches. Many studies have now shown that hosting *D. trenchii* is relatively costly to the host (Jones & Berkelmans, 2011; Baker et al., 2013; Pernice et al.,

2014; Starzak et al., 2014; Matthews et al., 2017; Gabay et al., 2019; Sproles et al., 2020), which has implications for the use of this symbiont species in studies that investigate the adaptability of corals to climate change through changing symbionts. Perhaps a more suitable candidate for this is *S. microadriaticum*, which did not show the same selfish tendencies as *D. trenchii*. In contrast, when maintained under control conditions, *S. microadriaticum* seems more beneficial on a cell-specific basis, though its overall benefits are limited by its low population density. Moreover, the symbiosis with *S. microadriaticum* is not as thermally robust as the other symbioses studied here, with a loss of holobiont biomass under thermal stress despite maintaining a stable but low number of symbionts. However, in the light of the specificity that *Aiptasia* displays for *B. minutum* (Thornhill et al., 2013a; Gabay et al., 2018, 2019), the question remains as to whether *S. microadriaticum* will be able to maintain a symbiosis with *Aiptasia*, or whether it would be immediately displaced by *B. minutum* when given the chance, as seen before in shorter experiments (Gabay et al., 2019). This study adds further evidence that symbiont switching is unlikely to benefit the holobiont due to reduced symbiosis function, however more research with a greater number of symbiont species and over even longer timeframes is necessary to truly confirm how this mechanism could aid the adaptation of corals to climate change.

Chapter 5 The effects of symbiont identity and acute heat stress on the host proteome in juvenile *Acropora tenuis*

5.1 Abstract

This study investigated the effect of symbiotic state, symbiont identity and thermal stress on the host proteome of the juvenile coral *Acropora tenuis*, at a life-stage where it exhibits a lower specificity for its symbionts than seen in the adult coral colony. Two species of symbionts were used to colonise juvenile *A. tenuis*, the homologous (i.e. native) species *Cladocopium* C1 and *Durussdinium trenchii*. Comparisons were made between the host proteome of *A. tenuis* when colonised by these species and aposymbiotic juveniles, and juveniles were then exposed to an acute heat shock of 31.6 °C for two days to measure the effect of thermal stress and symbiont identity on the host proteome. Four weeks after colonisation, both *Cladocopium* C1- and *D. trenchii*-colonised corals showed increases in the expression of apoptosis-related proteins, heat stress proteins, and alkaline phosphatase activity. Additionally, the host proteome of corals colonised by *Cladocopium* C1 showed greater differences from the aposymbiotic host proteome than corals colonised by *D. trenchii*. These differences included increased expression of heat stress proteins (HSPs) and proteins involved in apoptosis, while cell proliferation-related proteins decreased in *Cladocopium* C1-colonised hosts. Under heat stress, the proteomic response of *A. tenuis* was symbiont-specific, with *Cladocopium* C1-colonised corals upregulating their expression of GFP and tetraspanin, while *D. trenchii*-colonised corals show increased expression of lipid transporters and oxidative stress proteins. *D. trenchii* has been suggested to be a more thermally tolerant symbiont, but in response to heat shock, corals containing *D. trenchii* exhibited the largest proteomic response. These results suggest that *Cladocopium* C1 better prepares its host for thermal stress. This benefit is apparent from the reduced proteomic changes that are manifested under thermal stress.

5.2 Introduction

The symbiosis between dinoflagellate microalgae and cnidarians forms the foundation of coral reefs. Coral reefs have existed for millions of years and shape the base of an entire ecosystem, with many species depending on reefs for survival and reproduction. The success of the symbiosis is based on the nutritional exchange between the partners (Muscatine & Porter, 1977), in which the host's inorganic nitrogen and carbon metabolic end-products are assimilated into organic nitrogen and carbon during photosynthesis, and some of these photosynthetic products are transported back to the host. Approximately 90-95% of the host's daily carbon requirement is typically provided by its dinoflagellate symbionts (Muscatine & Hand, 1958; Muscatine & Cernichiaro, 1969; Davy et al., 2012; Tremblay et al., 2014).

The dinoflagellate symbionts that form symbioses with many species of cnidarians belong to the family Symbiodiniaceae (LaJeunesse et al., 2018; Nitschke et al., 2020; Pochon & LaJeunesse, 2021), which comprises many genera, species and subspecies, that display a range of different characteristics with respect to, e.g. thermal tolerance, photosystem functioning and inorganic carbon acquisition (Baker et al., 2013; Starzak et al., 2014; Hawkins et al., 2016; McIlroy et al., 2016). These traits might play a role in the occurrence of host-symbiont specificity, i.e., the capability or “willingness” to form a symbiosis with only one or a few different symbiont species (Belda-Baillie et al., 2002; Baker, 2003; Goulet, 2006; Putnam et al., 2012; Poland & Coffroth, 2017), with the partners ranging from highly specific to generalist.

Host-symbiont specificity indicates that a sorting mechanism must be in place to discriminate between symbiont species upon establishment of symbiosis. Symbionts are taken up from the environment along with food particles, and enter the gastrodermis in endosomes destined for lysosomal degradation. To avoid digestion, symbionts are able to manipulate the host’s immune system and prevent the maturation of endosomes into lysosomes (Chen et al., 2003, 2004, 2005; Hong et al., 2009; Neubauer et al., 2017). It is unclear how exactly this occurs, but proposed mechanisms include roles for cell-surface molecules such as glycans (Wood-Charlson et al., 2006; Wood-Charlson & Weis, 2009; Logan et al., 2010; Bay et al., 2011; but see Parkinson et al., 2018), sterol transporters such as the Niemann-Pick type C (NPC) proteins (Dani et al., 2014; Lehnert et al., 2014; Oakley et al., 2016; Hambleton et al., 2019; Rosset et al., 2021), or thrombospondin-type-1 repeat (TSR) domain proteins (Neubauer et al., 2017).

Although symbionts are able to manipulate the host’s immune system and avoid digestion, stressors can conversely lead to the host’s immune system triggering the removal of its symbionts. Corals, as tropical organisms, live very close to their upper thermal threshold. Exceeding this temperature threshold causes the breakdown of the symbiosis (i.e. dysbiosis), in a process known as coral bleaching. During dysbiosis, the coral’s symbionts are lost from the host’s tissues (Weis, 2008). The individual responses of a host or symbiont to heat stress have been well studied. For the symbiont, this stress response includes decreased photosynthetic activity (Warner et al., 1996; Jones et al., 1998; Hill et al., 2004; Jones et al., 2008; Hawkins et al., 2015; Krueger et al., 2015; Camp et al., 2017; Petrou et al., 2018), photoinhibition and/or photodamage (Warner et al., 1999; Takahashi et al., 2008, 2009; Hill et al., 2011; Petrou et al., 2021), and production of reactive oxygen species, or oxidative stress (Smith et al., 2005). The host response generally includes increased respiration and metabolism (Hillyer et al., 2017a,b; Baker et al., 2018; Rädcker et al., 2021), changes in cell adhesion properties (Gates et al., 1992; Traylor-Knowles, 2019), and antioxidant production (Hawkins et al., 2015; Krueger et al., 2015; Gardner et al., 2017). Proteomic and transcriptomic studies examining the host’s response to thermal stress have reported increases in the production of protein chaperones and antioxidants in *Aiptasia*

(Cziesielski et al., 2018; Chapter 2), *Pocillopora acuta* (Mayfield et al., 2018), *Acropora aspera* (Rosic et al., 2014), and *Acropora millepora* (Petrou et al., 2021). Suppression of immune system functioning is found in both host and symbiont but has been found to last much longer in the host (Pinzón et al., 2015). Despite these general trends, specific responses and outcomes vary greatly over taxa for both host and symbiont species (Takahashi et al., 2008; Hawkins et al., 2014; Krueger et al., 2015; Voolstra et al., 2020).

Ultimately, the combination of host and symbiont identity determines the outcome of stress events (Sampayo et al., 2008; Baird et al., 2009), and although most coral species display a limited range of partner specificity (Belda-Baillie et al., 2002; Baker, 2003; Goulet, 2006; Putnam et al., 2012; Poland & Coffroth, 2017), it has been suggested that changing symbiont community composition offers an opportunity for survival during times of stress (Buddemeier & Fautin, 1993; Baker, 2001; Fautin & Buddemeier, 2004). This can happen in one of two ways; symbiont shuffling or switching. Shuffling means a change in the relative abundance of the species that make up the symbiont community within the host tissue, whereas switching means a replacement of the resident species by a different species taken up from the environment (Baker, 2003).

Such stress-induced changes in symbiont communities have indeed been observed in the field, and one of the symbiont species that has the potential to be more thermally tolerant than other Symbiodiniaceae species is *Durisdinium trenchii* (previously known as *Symbiodinium trenchii*, or ITS2 type D1a, LaJeunesse et al., 2018), as it is found in increased densities in coral tissues during and after bleaching events (Toller et al., 2001; Glynn et al., 2001; Baker et al., 2004; Berkelmans & Van Oppen, 2006; Jones et al., 2008; Correa et al., 2009; LaJeunesse et al., 2009; Stat & Gates, 2011; Cooper et al., 2011; Silverstein et al., 2012; Kemp et al., 2014; Kennedy et al., 2015; Boulotte et al., 2016). Although a heterologous (i.e., non-native) symbiont species may result in a sub-optimal symbiosis (Weis et al., 2001; Belda-Baillie et al., 2002; Dunn & Weis, 2009; Gabay et al., 2018; Matthews et al., 2018), some studies have shown that this increased thermal tolerance translates to the host, allowing the cnidarian host to withstand higher temperatures compared to when it is in symbiosis with other symbionts (Stat & Gates, 2011; Kemp et al., 2014; Boulotte et al., 2016; Silverstein et al., 2017). However, there is also evidence for the contrary, and it has been suggested that *D. trenchii* is a more opportunistic symbiont species rather than a “saviour in distress”, and the increased prevalence of stressed corals is merely an indication of its ability to invade the tissues of an immune-compromised host (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011).

Ultimately, holobiont (i.e., whole symbiosis) functioning depends on the interplay between host and symbiont, and how they respond to environmental change, both individually and in concert. Heterologous (i.e. non-native) symbionts are known to induce different (and potentially negative)

effects than homologous (i.e. native) symbionts in the model sea anemone *Exaiptasia pallida* ('Aiptasia'; Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2019, Chapters 2, 3 and 4). However, more research is needed into how symbiont species, including different homologous species, influence the host at a molecular level in the field, including under thermal stress. This is especially pertinent to reef corals, which often exhibit a greater range of symbiont homology than seen in Aiptasia, particularly in their earlier developmental stages (see earlier chapters). For example, juvenile *Acropora tenuis* host a greater range of symbiont species than do adults (Yuyama et al., 2012; Yamashita et al., 2013; Yorifuji et al., 2017). This more promiscuous life-stage could provide increased survival opportunities to the juveniles by increasing the likelihood of pairings that are better suited to the prevailing environmental conditions. What the physiological implications of hosting these different symbionts are for juvenile corals, however, has not been determined. This knowledge is important for understanding the survivorship of different pairings into adulthood, and hence patterns of host-symbiont specificity on reefs.

The first aim of this chapter was therefore to compare the host proteome of juvenile *Acropora tenuis* in different symbiotic states: aposymbiotic, or in symbiosis with one of two symbiont species, *Cladocopium* C1 and the more thermally-tolerant *D. trenchii*. The second aim of this chapter was to establish the combined effect of symbiont identity and thermal stress on the host proteome of this coral. It was expected that the host proteome would differ based on symbiotic state and symbiont identity; and that acute heat stress would affect the host proteome differently depending on symbiont identity. *A. tenuis* transmits its symbionts horizontally, meaning that larvae can be maintained symbiont-free and colonised with different symbiont species, while it is possible to induce settlement in *A. tenuis* larvae with the use of a synthetic peptide (Iwao et al., 2002). This study was performed in Okinawa, Japan, where juveniles of *A. tenuis* are naturally found in symbiosis with several species of Symbiodiniaceae, including *Symbiodinium*, *Cladocopium* and *Durisdinium* spp. (Yuyama et al., 2012; Yamashita et al., 2013; Yorifuji et al., 2017), while adult *A. tenuis* in this region are dominated by *Cladocopium* C3 (Yorifuji et al., 2017). Additionally, the entire genome of *A. tenuis* has been published (Liew et al., 2016). For these reasons, *A. tenuis* provides an ideal model to study aspects of symbiosis in response to specific symbiont species in an ecologically-relevant context. An untargeted proteomics approach was used as it studies the molecular workings at the posttranslational levels of proteins, and is able to provide insight into a variety of cellular mechanisms (Oakley et al., 2016, 2017; Ricaurte et al., 2016; Sproles et al., 2019).

5.3 Methods

5.3.1 Collection of corals and coral spawning

The experiment was performed at the Tropical Biosphere Research Center of the University of the Ryukyus, on Sesoko Island, Okinawa, Japan. Fragments of coral from twelve colonies were collected June 6th, 2019, from the reef flat (N 26° 39'50.76"; E 127° 52'10.14") at a depth of approx. 5 m, under permit number 31-26 issued by Okinawa Prefectural Government. Corals were kept in outdoor tanks. A cone-shaped trap with mesh was placed above each coral colony to collect egg-sperm bundles, and the colonies were checked for spawning every evening. Spawning occurred on June 12 and June 13, 2019 between 19:30 and 20:00 h. Gamete bundles were collected and transferred to 1 µm filtered seawater (FSW) in the lab. Dissociation and fertilisation were aided by gentle mixing of the FSW, ensuring mixing of gametes from the twelve different colonies to reduce genetic variation effects. FSW was replaced every two hours for the first six hours after spawning, and then replaced twice daily.

5.3.2 Larval maintenance and settling

Larvae became motile three days after spawning, and were maintained in tanks of 5 L FSW, replaced once daily. One week after spawning, the synthetic peptide Hym-248 was used to induce settlement on petri dishes at a final concentration of 10^{-5} mol L⁻¹, settling approximately 120 juveniles *per* petri dish. After a 6 h incubation period, FSW was replaced. From then on, FSW was replaced once every two days.

5.3.3 Inoculation with symbionts

Three days after settlement, juveniles (n = 7,000) were inoculated with one of two species of symbiont: *Cladocopium* C1 (culture name LHI-33, collected at Lord Howe Island, Australia, in 2010) or *D. trenchii* (culture name Ap2, collected at Sesoko Island, Okinawa, Japan, in 2005). *Cladocopium* C1 was not a local culture as local *Cladocopium* cultures were not available. Cultures were grown in an incubator at 25 °C under 30 µmol photons m⁻² s⁻¹ in f/2 culture medium (AusAqua Pty, SA, Australia). A drop of cell culture at a density of 1 million cells *per* mL mixed with homogenised *Artemia* sp. nauplii to induce a feeding response was applied to the oral disc of each juvenile. Seawater was changed six hours after application. The colonisation of juveniles was checked by assessing ten random juveniles every three days under a fluorescence microscope (Olympus BX53, Figure 5.1).

5.3.4 Experimental treatments

After inoculation, juveniles were maintained at a temperature of 26.8 °C (± 0.8 °C SD) and irradiance of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. After four weeks at these conditions, samples were taken of aposymbiotic juveniles, and juveniles colonised with *Cladocopium* C1 or *D. trenchii*, for proteomics ($n = 4$ per treatment). Thereafter, petri dishes were randomly assigned to thermal treatments and either maintained at control temperature of 26.8 °C or exposed to a temperature of 31.6 °C (± 0.4 °C SD) (i.e., acute heat shock). Juveniles were kept at these temperatures for two days, after which they were sampled for proteomics to test the effects of heat stress and symbiont identity ($n = 8$ per treatment). Due to the severe extent of visual bleaching after one day, a Pulse Amplitude Modulated (PAM) fluorometer (Diving PAM, Heinz Walz GmbH) was used to measure algal health and to ensure that symbionts were still present in the tissues. The following PAM settings were maintained: measuring light 8, saturation intensity 8, saturation width 1 s, gain 8 and damping 2.

Figure 5.1 Fluorescence microscope pictures of recolonised *Acropora tenuis* juveniles (A, B) 10 days and (C, D) 26 days after colonisation with (A, C) *Cladocopium* C1 and (B, D) *Durusdinium trenchii*. Green fluorescence is the host's expression of green fluorescent protein (GFP) and red indicates chlorophyll autofluorescence of the symbiont. Magnification (A, B) 10x and (C, D) 4x, white bars indicate 100 μm .

5.3.5 Sampling and processing

To sample, whole petri dishes were snap-frozen in liquid nitrogen, after which they were stored at -80 °C for a minimum of 24 h. Subsequently, frozen polyps were removed from the dish and placed in 2 mL Eppendorf tubes. To ensure sufficient biomass for proteomics, approximately 120 polyps were pooled *per* biological replicate, and five separate polyps *per* replicate were collected in separate tubes to be used for cell counts. Proteomic samples were homogenised by hand using an Eppendorf micropestle. Skeletal fragments were allowed to settle to the bottom of the tube, and the supernatant containing the homogenised host tissue and symbionts was drawn off and centrifuged at 100 x g for 5 min to separate host and symbiont material. Both fractions were centrifuged again (100 x g, 5 min) to ensure full separation of host and symbiont material. Host tissue material was frozen at -80 °C before being freeze-dried using a lyophiliser (Eyela FDU-810). From the symbiont material, a 100 µL aliquot was taken for DNA extraction for symbiont identification and stored in 500 µL 1% SDS in DNAB. The remaining symbiont material was fixed with 10% formalin.

The aliquots containing polyps for cell counts were fixed using 2% paraformaldehyde in 0.1 M sodium cacodylate, and skeletons dissolved with 0.5 M EDTA. After three days, the solution was replaced with 2% formalin. Samples were stored at 4 °C until further processing. Polyp tissue was homogenised with a saw-tooth homogeniser at a mid-speed setting, and cell counts were performed by Neubauer haemocytometer under a light microscope, with a minimum of eight replicate counts *per* sample.

5.3.6 Confirmation of symbiont genotype

DNA extraction of symbiont cells was performed following the CTAB-chloroform protocol of Baker & Cunning (2016) as described in Chapter 2, 3 and 4. In brief, algal cells from each replicate sample of the pooled juveniles in 1% SDS in DNAB were digested using Proteinase K at 37 °C overnight, after which CTAB was added and samples were incubated at 65 °C, followed by an incubation with chloroform at room temperature for 2-3 h. Samples were centrifuged and the top layer discarded. 500 µL 100% ethanol was added and samples were stored at -20 °C to promote DNA precipitation. Samples were then dried under vacuum (Eppendorf Concentrator 5301) before redissolving the DNA in 0.3 M sodium acetate (NaOAc), after which they were ethanol washed, dried again under vacuum, and then resuspended in 20 µL TE buffer.

Extracted DNA was amplified using PCR with Symbiodiniaceae universal primers, ITS1 (forward, 5'-GTGAATTGCAGAACTCCGTG-3') and ITS2rev2 (reverse; 5'-CCTCCGCTTACTTATATGCTT-3'). Thermal cycling included an initial stage of 3 min at 95 °C, then 35 rounds of 15 s at 95 °C, 15 s at 56 °C, and 10 s at 72 °C, with a final final elongation phase of 5 min at 72 °C, followed by a resting

stage at 4 °C. PCR products were stored at 4 °C. Amplified DNA was sent to Macrogen (Seoul, Korea) for purification and sequencing. Sequence results were checked for quality and then BLAST searched against two databases, GeoSymbio and NCBI, to confirm symbiont species.

5.3.7 Protein extraction

Host protein extractions were performed using a filter-aided sample preparation protocol as described in Chapter 2. In summary, freeze-dried samples were redissolved in 1 mL 100% methanol, sonicated at 4 °C for 30 min, and then centrifuged for 30 min at 3000 x g. The supernatant was drawn off, and 1 mL 50% methanol was added to the pellet, vortexed for 1 min and spun down for 30 min at 3000 x g. The pellet was then used for protein extraction, by resuspending in 0.5 mL 5% SDC, ultrasonicated for twenty 2 s pulses at 20% output, and incubating at 85 °C for 20 min with 1% β -mercaptoethanol. Samples were then washed twice with ethyl acetate, after which they were dried and spun through a spin filter (Amicon Ultra, Millipore). They were then washed with 50 mM Tris three times. After protein quantification using Qubit, 100 μ g protein were retained on the filter, incubated with 10 mM BME for 10 min at 37 °C and then with 500 mM acrylamide for 20 min at room temperature, after which they were digested with trypsin at 37 °C overnight. After digestion, samples were desalted with ZipTips, eluted in 50% and 30% acetonitrile, combined and dried, and then rehydrated in 0.1% formic acid. Protein content was quantified again before loading 200 ng protein on the LC-MS (see below) for protein profiling.

5.3.8 Protein profiling

Extracted proteins were profiled using liquid chromatography-tandem mass spectrometry (LC-MS-MS) according to the methods described in Chapter 3. In brief, the LC used to separate peptide samples was performed with a linear gradient from 5% to 35% buffer B (buffer A: 0.1% formic acid; buffer B: 80% acetonitrile, 0.1% formic acid) at 300 nL min⁻¹ on an Acclaim PepMap C18, 3 μ m, 100 Å column (Thermo Scientific, Auckland, New Zealand) and Ultimate 3000 liquid chromatography system (Dionex, Sunnyvale, CA). Peptide ionisation occurred by electrospray at 1.8 kV, with analysis by an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific). The Orbitrap acquired precursor mass spectra at a resolution of 120,000, rejecting singly-charged ions, while quadrupole isolation was enabled and an automatic gain target of 7.0e5 set. The injection time was set at a maximum of 50 ms. The 20 most intense precursor spectra were fragmented by a higher-energy collision dissociation, and then analysed in the ion trap. The automatic gain target was 5.0e3 and this had a maximum injection time of 300 ms. Dynamic exclusion was enabled with a duration of 60 s.

Protein identification was performed from raw spectrum files using the Andromeda search algorithm in MaxQuant (1.6.10.43, Cox & Mann, 2008; Cox et al., 2014). Proteins were searched against the *A. tenuis* genome v0.11 from ReefGenomics.org (Liew et al., 2016). Trypsin was set as the digest enzyme, allowing for a maximum of two missed cleavages. Viable modifications included oxidation of methionine, and acetylation of the protein n-terminus, while carbamidomethylation of cysteine was considered a fixed modification. The first peptide search tolerance was 20 ppm, the main search peptide tolerance was 4.5 ppm, and the mass tolerance was 0.5 Da for the ion trap MS2 search. Other settings included enabling of label-free quantification, with a minimum of two unique plus razor peptides for quantification, and matching between runs enabled. Peptide spectrum match and protein false discovery rates were both set to 1%, with a minimum of two peptides required for identification.

5.3.9 Data analysis and visualisation

Identified proteins and label-free quantification intensities were analysed in Perseus (1.6.15.0, Tyanova et al., 2016) to remove known false detections and contaminants from the analysis. Samples were then normalised to total intensity values and $\log_2$ transformed in R (4.0.3, R Core Team, 2020). Data analysis was performed with PolySTest (Schwämmle et al., 2020) online to identify significantly differentially expressed proteins between treatments. Significant proteins were identified using the Limma test in PolySTest (Schwämmle et al., 2020) with an FDR threshold of $q < 0.05$, and only annotated proteins with a $\log_2$ -fold change larger than 0.25 or smaller than -0.25 are reported. Unannotated differentially expressed proteins with a $\log_2$ -fold change larger than 1 or smaller than -1 were BLAST searched against the NCBI database, and any matches with more than 90% sequence identity are reported. PCAs based on proteomic profiles were constructed in R (version 4.0.3; R Core Team, 2020), after which ANOVAs were used on PC loadings to test if the PC axes were able to separate the groups significantly; a one-way ANOVA to test between symbiotic identity and the aposymbiotic state *versus* a two-way ANOVA to test for effects of symbiont identity and thermal stress.

5.4 Results

Mass spectrometry analysis detected a total of 2492 proteins in the samples, with both symbiotic state and thermal stress affecting protein abundance.

5.4.1 Response to symbiotic state

Genotyping confirmed successful colonisation by *Cladocopium* C1 and *D. trenchii*, with the two species reaching similar population densities (1730.42 ($\pm$ 248.49 SE) and 2188.81 ($\pm$ 164.46 SE) cells *per* polyp, respectively, one-way ANOVA $p = 0.136$). Aposymbiotic polyps failed to produce a band on a

gel electrophoresis after PCR, hence confirming the aposymbiotic state. *Cladocopium* C1- and *D. trenchii*-colonised corals possessed 64 and 24 differentially expressed proteins (DEPs), respectively, when compared to the aposymbiotic state. Of these different proteins, 19 were shared between both symbiont species and hence represented the core difference between the symbiotic *versus* aposymbiotic state (Table 5.1). These 19 proteins are mainly categorised under ‘metabolism’, ‘stress and immune response’, and ‘protein degradation and turnover’, with most proteins in these categories upregulated in symbiosis. The highest upregulation was found in an alkaline-phosphatase-like protein (APA; 4.09 log₂-fold in *Cladocopium* C1-colonised hosts vs. 3.72 log₂-fold in *D. trenchii*-colonised corals), followed by the red fluorescent protein plobRFP (2.31 log₂-fold in *Cladocopium* C1-colonised corals vs. 1.28 log₂-fold in *D. trenchii*-colonised corals).

When comparing the host proteomes of juvenile *A. tenuis* colonised by the different symbiont species, only two DEPs were detected: green fluorescent protein 1 and DBH-like monooxygenase protein 1-like (3.02 log₂-fold and 1.25 log₂-fold upregulated in *Cladocopium* C1-colonised corals, respectively). Of the proteins that were differentially expressed between the two symbiotic states and aposymbiotic state, there were only 5 DEPs for *D. trenchii*-colonised hosts that did not overlap with *Cladocopium* C1-colonised corals. These proteins include a structural protein (Dynein heavy chain, cytoplasmic; 2.28 log₂-fold upregulated in *D. trenchii*-colonised corals), a signalling protein (src kinase-associated phosphoprotein 2; -1.12 log₂-fold downregulated in *D. trenchii*-colonised corals), and three proteins with unknown function or annotation (DUF924 domain-containing protein, and two unannotated proteins; 0.79, 1.01, and -1.02 log₂-fold change different in *D. trenchii*-colonised hosts). For *Cladocopium* C1-colonised corals, 45 DEPs compared to the aposymbiotic state did not overlap with *D. trenchii*-colonised hosts (see Table 5.2). Of these 45 DEPs, 33 were upregulated and 12 were downregulated in *Cladocopium* C1-colonised corals. These DEPs are mainly categorised under ‘metabolism’, and ‘heat stress and protein folding’. A PCA based on host proteome showed a successful separation of aposymbiotic and symbiotic corals on the primary axis (Figure 5.2; one-way ANOVA $p < 0.01$), however it could not separate *Cladocopium* C1- and *D. trenchii*-colonised corals from one another (one-way ANOVA, $p = 0.461$).

Figure 5.2 PCA plot based on host proteomes of the coral *Acropora tenuis*, comparing symbiotic state (i.e., aposymbiotic or in symbiosis with *Cladocopium* C1 or *Durusdinium trenchii*) under non-stressful, control conditions. Ellipses represent 95% confidence intervals. Significance level ** $p < 0.001$.

Table 5.1 Log₂-fold changes of significantly differentially expressed proteins of the coral *Acropora tenuis* when in symbiosis with *Cladocopium* C1 or *Durussdinium trenchii* versus aposymbiotic corals. Unannotated proteins were BLAST searched against the NCBI protein database. The colour scale of the log-fold changes reflects the intensity; red is upregulated in the symbiosis whereas blue is downregulated. Only proteins with a log-fold change >0.25 or <-0.25, and under the FDR threshold of $q < 0.05$ are presented.

Log ₂ -fold change		
<i>Cladocopium</i> C1 vs. aposymbiotic	<i>D. trenchii</i> vs. aposymbiotic	Annotation
Apoptosis		
0.75	0.86	Programmed cell death protein 6
Calcium-binding		
-0.79	-0.73	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1-like
Stress and immune response		
2.31	1.28	Red fluorescent protein plobRFP
1.10	0.90	Chaperonin containing TCP1 subunit 5
1.09	0.88	Heat shock protein, mitochondrial
Iron binding		
1.45	1.01	Ferritin
Metabolism		
1.55	1.48	S-adenosylmethionine synthase
1.70	1.29	Phosphoserine aminotransferase
-0.92	-1.18	Glycine N-methyltransferase
Protein degradation and turnover		
1.35	0.86	Lysosomal aspartic protease
0.75	0.61	Retinoid-inducible serine carboxypeptidase
-0.94	-1.15	Leukocyte cysteine proteinase inhibitor 1
Redox process		
1.19	0.97	Glutaredoxin, CPYC type
Transcription and translation		
-1.06	-0.97	Signal transducer and activator of transcription
0.93	1.05	Ribosomal protein S5
Unannotated: BLAST match; species (reference gene number)		
4.09	3.72	Alkaline phosphatase-like; <i>A. digitifera</i> (30076)
1.56	1.16	Heme-binding protein 2-like; <i>A. digitifera</i> (24742)
1.35	1.05	Mammalian ependymin-related protein 1-like; <i>A. millepora</i> (36640)

Table 5.2 Differentially expressed proteins in the coral *Acropora tenuis* unique to the establishment of symbiosis with *Cladocopium* C1. Unannotated proteins were BLAST searched against the NCBI protein database. The colour scale of the log-fold changes reflects the intensity; red is upregulated in the symbiosis with *Cladocopium* C1 whereas blue is downregulated. Only proteins with a log-fold change >0.25 or <-0.25, and under the FDR threshold of $q < 0.05$ are presented.

Log ₂ -fold change <i>Cladocopium</i> C1 vs. aposymbiotic	Annotation
Apoptosis	
1.01	Cytochrome c, somatic
Calcium-binding	
0.69	Calcyclin-binding protein (Fragment)
-0.96	Flagellar-associated, partial
Cell cycle regulation	
-1.24	Kinesin-like protein KLP6
Cell proliferation	
-1.27	NHL repeat-containing protein 2
Cytoskeleton, structure, and muscle	
0.66	Endophilin-A
-0.60	Beta-adducin isoform X4
-0.74	Coactosin
DNA binding	
0.73	Histone-binding protein Caf1
Stress and immune response	
1.36	bactericidal permeability-increasing protein-like
0.78	T-complex protein 1 subunit eta
0.63	75 kDa glucose-regulated protein (Fragment)
0.54	Heat shock cognate protein 70
0.50	Hypoxia up-regulated protein 1
Metabolism	
1.97	Cystathionine gamma-synthase
1.64	Glutamine synthetase
1.23	L-aminoadipate-semialdehyde dehydrogenase large
1.07	Tissue alpha-L-fucosidase
0.87	Phospholipase B-like
0.81	Beta-galactosidase Bga
0.60	Aflatoxin B1 aldehyde reductase member 2
-0.76	L-tyrosine decarboxylase
Metal ion binding	
1.66	DBH-like monooxygenase protein 1-like
Oxidative stress	
0.94	Glutathione-independent glyoxalase
-0.63	Peroxidasin homolog
Protein degradation and modification	
0.69	Cathepsin L-b
0.54	Acyl-protein thioesterase
Redox process	
0.76	Thioredoxin
Translation	
1.24	40S ribosomal protein S2
1.11	40S ribosomal protein S17
0.70	Eukaryotic translation initiation factor 6

Table 5.2 continued

Log ₂ -fold change <i>Cladocopium</i> C1 vs. aposymbiotic	Annotation
Transport	
2.36	ATP-binding cassette sub-family B MDR/TAP member 1
1.13	Vacuolar proton pump subunit B
0.60	GTP-binding nuclear protein Ran
Unannotated: BLAST match; species (reference gene number)	
1.84	Prosaposin-like; <i>A. millepora</i> (796)
1.15	Zinc finger protein 62 homolog; <i>A. millepora</i> (14300)

5.4.2 Response to thermal stress

Genotyping again confirmed successful colonisation by *Cladocopium* C1 and *D. trenchii*. By this time point, two days since the previous sampling point, *D. trenchii* had reached approximately 25% higher density than *Cladocopium* C1 under control conditions (two-way ANOVA, $p = 0.0017$; Figure 5.3A). Both species bleached significantly in response to heat stress (two-way ANOVA, $p = 0.0002$), with *Cladocopium* C1-colonised corals retaining on average 54% of their symbionts, and *D. trenchii*-colonised corals retaining on average 60% of their symbionts; the extent of bleaching was not significantly different between the two symbiont species (Figure 5.3A). The PAM data showed that, even though the coral experienced bleaching, the remaining symbionts were photosynthetically healthy (Figure 5.3B).

Figure 5.3 (A) Cell densities ($\pm$ SE) and (B) Photosystem II quantum yield reached by *Cladocopium* C1 and *Durisdinium trenchii* in *Acropora tenuis* tissues for control conditions and at heat shock. Letter groups indicate statistically significant differences (two-way ANOVA).

Heat stress had a large effect on the host proteome. In *Cladocopium* C1-colonised corals, 75 proteins responded significantly to heat shock, of which 46 were upregulated and 29 downregulated under heat stress, while 117 proteins did so in *D. trenchii*-colonised corals, of which 82 were upregulated and 35 downregulated under heat stress. The majority of these were categorised as ‘heat stress and protein folding’, ‘protein degradation and turnover’, and ‘calcium-binding’ proteins (Figure 5.4). A total of 38 proteins were shared between *Cladocopium* C1- and *D. trenchii*-colonised corals in response to heat stress, of which the annotated proteins are summarised in Table 5.3. *D. trenchii*-colonised corals showed differential expression of 32 more proteins than corals in symbiosis with *Cladocopium* C1, which were mainly made up of HSPs, oxidative stress proteins, proteins involved in protein degradation and turnover, and proteins that are part of the host’s immune response. A PCA based on the host proteome (Figure 5.5) at the control *versus* elevated temperature showed a significant interaction between those factors on PC1 (two-way ANOVA, $P < 0.001$). *Post hoc* results indicated that PC1 could separate heat stress from control samples ($p < 0.02$), and although it could not separate *Cladocopium* C1- and *D. trenchii*-colonised corals at the control temperature ($p = 0.281$), it could do so under thermal stress ($p < 0.0001$).

Figure 5.4 Number of host (*Acropora tenuis*) proteins *per* category that significantly responded to heat stress when in symbiosis with either *Cladocopium* C1 or *Durusdinium trenchii* (min. three proteins for at least one of the species shown).

A total of 23 annotated proteins differed significantly between the *Cladocopium* C1- and *D. trenchii*-colonised corals in response to heat shock (see Table 5.4). The most notable differences were evident in the expression of GFP, tetraspanin (3.16 and 1.93 log₂-fold upregulated in *Cladocopium* C1-colonised hosts, respectively), and the lipid transporters apolipoprotein and vitellogenin (1.97 and 1.28 log₂-fold upregulated in *D. trenchii*-colonised hosts, respectively). Other DEPs included oxidative stress proteins (peroxidase and peroxidase homolog; 0.56 and 0.63 log₂-fold upregulated in *D. trenchii*-colonised corals, respectively), and the DNA repair protein (DNA photolyase; 0.46 log₂-fold upregulated in *D. trenchii*-colonised corals). Additionally, one of the unannotated proteins, when searched against the NCBI database, came up as a match to 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase-like isoform X1, which showed an upregulation of 3.40 log₂-fold in the *D. trenchii*-colonised corals compared to the *Cladocopium* C1-colonised corals under heat stress.

Figure 5.5 PCA plot based on *Acropora tenuis* proteome when in symbiosis with *Cladocopium* C1 or *Durussdinium trenchii*, under control temperature or heat stress, grouped by significant separation over PC1 (significance level *** $p < 0.001$). Ellipses represent 95% confidence intervals.

Table 5.3 Significantly differentially expressed proteins in the coral *Acropora tenuis* that overlapped between *Cladocopium* C1- and *Durusdinium trenchii*-colonised corals in response to heat stress (n = 8). Colour scale reflects intensity of the fold change (red was upregulated in the heat stress treatment, blue was downregulated).

Log ₂ -fold change (HS:C)		Annotation
<i>Cladocopium</i> C1	<i>D. trenchii</i>	
Calcium binding		
0.40	0.87	Calcyclin-binding protein (Fragment)
0.46	0.45	Calreticulin
-0.60	-0.62	Hippocalcin-like protein 4
-0.85	-0.57	Calbindin-32
Cytoskeleton, structure, and muscle		
0.64	0.52	Tropomyosin beta chain
DNA repair		
-0.32	-0.50	DNA photolyase
Heat stress and protein folding		
0.65	1.00	Heat shock protein 83
0.80	0.82	Heat shock protein 83
0.67	0.81	78 kDa glucose-regulated
0.66	0.80	Protein disulfide-isomerase A4
0.37	0.65	Major heat shock 70 kDa protein Ba
0.41	0.60	Heat shock cognate protein 70
0.56	0.55	Endoplasmic reticulum resident protein 29
0.55	0.52	Protein disulfide isomerase family A member 3
0.50	0.40	Protein disulfide-isomerase A6 homolog
0.34	0.39	Protein disulfide-isomerase
0.29	0.32	Hypoxia up-regulated protein 1
Metabolism		
0.54	0.70	Lysosomal acid lipase/cholesteryl ester hydrolase-like
-0.91	-0.63	Phosphatidylserine decarboxylase
Metal ion binding		
0.51	0.43	1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial
Oxidative stress		
0.74	0.59	Peroxiredoxin-4
Protein degradation and turnover		
0.62	0.99	Proteasome 26S subunit subunit 4 ATPase
0.49	0.52	Asparaginyl endopeptidase
0.29	0.52	Lysosomal aspartic protease
Translation		
0.50	0.61	Translation elongation factor EF-1, subunit alpha
-0.57	-0.57	40S ribosomal protein S17
Transport		
0.46	0.51	Charged multivesicular body protein 4b Chromatin-modifying protein 4b
0.42	0.47	Target of myb1 like 2 membrane trafficking protein
Unannotated: BLAST match; species (reference gene number)		
3.56	9.34	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase-like isoform X1; <i>A. digitifera</i> (4450)
2.31	2.08	Calumenin-B like; <i>A. millepora</i> (21254)
-1.84	-1.50	Alkaline phosphatase-like; <i>A. digitifera</i> (30076)

Table 5.4 Annotated host (*Acropora tenuis*) proteins that significantly differed between *Cladocopium* C1- and *Durussinium trenchii*-colonised corals under heat stress (n = 8). Colour scale reflects intensity of the fold change (red was upregulated in hosts containing *Cladocopium* C1, blue was downregulated).

Log ₂ -fold change <i>Cladocopium</i> C1 vs. <i>D. trenchii</i>	Annotation
Calcium binding	
-0.88	Oncoprotein-induced transcript 3 protein
DNA repair	
-0.46	DNA photolyase
Fatty acid metabolism	
0.36	Enoyl-CoA hydratase
Heat stress and protein folding	
1.93	Tetraspanin
Immune response	
3.16	Green fluorescent protein 1
Lipid transport	
-1.97	Apolipoprotein
-1.28	Vitellogenin
Oxidative stress	
-0.56	Peroxidase
-0.63	Peroxidase homolog
Protein degradation and turnover	
-1.19	Sequestosome-1
Translation	
0.52	Eukaryotic translation initiation factor 6
Transport	
0.64	Canopy FGF signaling regulator 2
Unannotated: BLAST match; species (reference gene number)	
-3.40	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase-like isoform X1; <i>A. digitifera</i> (4450)

5.5 Discussion

5.5.1 Effects of symbiosis establishment on the host proteome

Symbiont identity had a marked influence on the host proteome. Four weeks after colonisation, *Cladocopium* C1 induced a larger response in the host proteome than did *D. trenchii*, despite these symbionts achieving similar population densities. However, there were only two significantly different proteins between the host proteomes in the presence of these different symbionts. This is an indication that the host proteome of juvenile corals containing *D. trenchii* is intermediate between the aposymbiotic state and when colonised by *Cladocopium* C1. This has been seen before in the model cnidarian *Aiptasia*, with respect to the host metabolome when colonised by the homologous *Breviolum minutum* versus the heterologous *D. trenchii* (Matthews et al., 2017). Overall, it seems that establishing a symbiosis in juvenile *A. tenuis* induces changes in the host proteome that are related to host cell proliferation and the reorganisation of cells. Protein turnover and folding are increased, and changes in

metabolism reflect a suite of responses related to cell proliferation, translation and transcription. Both *Cladocopium* C1 and *D. trenchii* were able to induce these responses, however, in addition to these, *Cladocopium* C1 induced a range of other proteomic changes in the coral host. These changes include changes to proteins involved in the stress response, cell cycle proteins, and a protein potentially involved in apoptosis.

Several metabolic proteins responded to the establishment of symbiosis, irrespective of symbiont identity. These included S-adenosylmethionine synthase and glycine N-methyltransferase, whose expression increased and decreased, respectively. S-adenosylmethionine synthase is an enzyme involved in the synthesis of S-adenosylmethionine (SAM), while glycine N-methyltransferase uses SAM to methylate glycine. Given this, the cumulative effect would be for increased SAM synthesis and accumulation in the host's tissues. This is in agreement with the previous research of Lehnert et al. (2014), who recorded an upregulation of four genes responsible for the production of SAM in the model sea anemone *Aiptasia* in comparison to aposymbiotic hosts. Chapter 2 reported an increase in the SAM precursor-synthesising-enzyme BHMT, but only in response to heat stress in hosts containing heterologous symbionts, however, this study was performed three months after initial onset of symbiosis and was not compared to aposymbiotic individuals. SAM is involved in a variety of processes, including transmethylation, transsulfuration, and polyamine synthesis. Transmethylation affects a wide range of processes, from gene expression to membrane fluidity, while transsulfuration can lead to the production of antioxidant precursors and polyamines that are required for normal cell growth (Lu, 2000). The wide range of functions of SAM makes it hard to pinpoint its primary function during symbiosis establishment, but this is worthy of further exploration.

The symbiotic state also induced an increase in the red fluorescent protein plobRFP in host tissues, with this increase being most pronounced in *Cladocopium* C1-colonised corals. PlobRFP is the furthest red-shifted natural fluorescent protein found in scleractinian corals, and has a high pH stability and photostability (Bridges et al., 2020). Fluorescent proteins are involved in a variety of processes, including innate immune responses and photoprotective mechanisms (Kawaguti, 1969; Salih et al., 2000; Dove et al., 2001; Quick et al., 2018). Host cell proliferation is increased in cells that express fluorescent proteins (D'Angelo et al., 2012), and increases in FP have been observed in response to physical damage and pathogen infection in corals (Palmer et al., 2008; D'Angelo et al., 2012). Additionally, multiple studies show that GFP-like proteins may function as antioxidants in corals due to their ROS-scavenging properties (Bou-Abdallah et al., 2006; Palmer, Modi, et al., 2009; Palmer, Roth, et al., 2009; Shikina et al., 2016). Establishment of symbiosis thus seems to be accompanied by oxidative stress, or induces changes that allows the hosts to cope with oxidative stress. This oxidative stress is likely to originate from photosynthesis by the symbionts (Richier et al., 2005).

The expression of numerous unannotated proteins also increased in the symbiotic state. One of these proteins had a log₂-fold change of ~4 in the presence of either *Cladocopium* C1 or *D. trenchii*, so it seems likely that this protein is important for the establishment or maintenance of symbiosis. Using the basic local alignment tool (BLAST), the protein was a 95% predicted match with an alkaline phosphatase-like protein found in *Acropora digitifera*. Phosphatase activity increases in response to phosphorus limitation in free living dinoflagellates (Dyhrman & Palenik, 1999), symbiotic dinoflagellates (Annis & Cook, 2002), and coral hosts (Godinot et al., 2013). This latter study showed that APA activity increases in response to long-term starvation, increased irradiance and bleaching, suggesting that APA is linked to both the metabolic activity of the holobiont and phosphate limitation. Juveniles in my study were not fed after the initial colonisation and yet symbiont cell density increased rapidly thereafter. This symbiont proliferation might therefore cause phosphorus deficiency in the host and thus explain the increase in APA.

Cytochrome *c* is an essential mitochondrial protein that is part of the electron transport chain, and thus involved in energy production, as well as being the initiating factor of a major caspase activation pathway (Liu et al., 1996; Jiang & Wang, 2004). Previous work has shown that caspase activity is increased in response to heterologous symbionts (Gabay, 2018), but this enzyme might also enable the host to control its native symbiont population (Dunn & Weis, 2009). There is a high upregulation of cytochrome *c* in *Cladocopium* C1-colonised hosts, as well as a strong downregulation of kinesin-like protein 6 (*k1p6*), which is required for proper chromosome separation during the anaphase of cell division (Garcia et al., 2002; West et al., 2002). Additionally, a strong downregulation of NHL repeat-containing protein 2 was found, which is likely caused by caspase activity (Nishi et al., 2017). These findings indicate that *A. tenuis* cells in symbiosis with *Cladocopium* C1 are discouraged from proliferating, or that *A. tenuis* has better control of *Cladocopium* C1 than it does of *D. trenchii*. Such symbiont species-specific regulation of symbiont cell densities has been observed previously in *Aiptasia* (Tivey et al., 2020).

Hosts in both associations showed increased expression of three heat shock proteins (HSPs), but hosts containing *Cladocopium* C1 showed upregulation of another five of these proteins compared to hosts in symbiosis with *D. trenchii*. Although the name suggests that HSPs are only upregulated in response to heat stress, they are actually expressed as a general stress response, including cold, UV light, wound healing, or tissue remodelling (Whitley et al., 1999), and changes in the expression levels of HSPs have been used as biomarkers of cell stress in corals (Downs et al., 2000; Brown et al., 2002; Chow et al., 2012; Olsen et al., 2013; Seveso et al., 2016; Louis et al., 2017). Generally, HSPs are involved in protein folding, unfolding, aggregation, degradation, and transport (Sørensen et al., 2003), thereby stabilising cellular structures and processes, and are thus also called molecular chaperones. HSPs are also expressed at low levels in the absence of stress (Chen et al., 2018) and increases in expression of HSPs

prior to stress events are associated with stress tolerance. Previous studies have shown host species-specific expression of HSPs in corals (Robbart et al., 2004; Chow et al., 2009; Fitt et al., 2009; Seveso et al., 2014, 2018), however my results are the first to show that the coral *A. tenuis* shows symbiont species-specific expression of HSPs, even when both symbiont species are homologous to this host. The increased expression of HSPs in *Cladocopium* C1-colonised juveniles indicates that this association could be better primed for future stresses than the association with *D. trenchii*, warranting further research.

A particular metabolic protein of interest is the tissue alpha-L-fucosidase (FUCA), as it might be involved in symbiont cell recognition. FUCA is an enzyme that breaks down fucose, a hexose deoxy sugar, commonly found on N-linked glycans on cell surfaces (Staudacher et al., 1999). Previous research has shown the involvement of fucose on Symbiodiniaceae cells in the establishment of symbiosis (Parkinson et al., 2018; Tortorelli, 2020). Likewise, prosaposin-like protein is involved in the catabolism of glycosphingolipids in lysosomes (Morimoto et al., 1990). Glycosphingolipids are also expressed on the cell surface of most organisms, and have recently been shown to play a role in immune responses (Zhang et al., 2019). The increase in FUCA and prosaposin-like protein in response to symbiosis establishment with *Cladocopium* C1 could indicate increased activation of symbiont cell recognition by the host.

Aflatoxin B1 aldehyde reductase (AKR7A2) was only upregulated during colonisation by *Cladocopium* C1. AKR7A2 offers higher resistance to the toxicity of Aflatoxin B1, a metabolite produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Hartley et al., 1963). In humans, AKR7A2 is involved in neurotransmitter synthesis, and potentially plays a role in detoxification, tumour resistance, and kidney osmolarity regulation (O'Connor et al., 1999; Lyon et al., 2007). Potentially this detoxifying role is why AKR7A2 is more expressed in the symbiosis between *A. tenuis* and *Cladocopium* C1, as this association shows more signs of increased cellular stress in the host. This particular protein has been previously found to be upregulated in symbiosis between the model cnidarian *Aiptasia* and the heterologous *D. trenchii* (Sproles, 2017), which unlike in the current study typically induces signs of cellular stress in this sea anemone (Matthews et al., 2017, 2018; Sproles et al., 2019). It would therefore make an interesting compound for future research.

Overall, it seems that *Cladocopium* C1 incites a greater response in the host proteome during colonisation of *A. tenuis*. As discussed above, many of the proteins involved are part of a stress response, and it seems that *A. tenuis* hosting *Cladocopium* C1 makes more investments in the expression of such proteins, in contrast to when hosting *D. trenchii*. However, upon acute heat shock (see below), the host proteome of *Cladocopium* C1-colonised corals showed fewer changes than that of *D. trenchii*-colonised

corals, perhaps suggesting that the initial investment in expression of stress response proteins induced by hosting *Cladocopium* C1 is outweighed by the long-term benefits.

5.5.2 Effects on heat stress and symbiont identity on the host proteome

D. trenchii has been suggested to be more thermally tolerant than other Symbiodiniaceae species, yet contrasting results have been published on this in *A. tenuis*. Yorifuji et al. (2017) concluded that *A. tenuis* juveniles containing *D. trenchii* perform better in response to heat stress, while others have shown that *Cladocopium* C1 seems to give *A. tenuis* greater thermal resistance than *D. trenchii* (Abrego et al., 2008). My results are in agreement with the latter. In response to thermal stress, both symbioses bleached, however the presence of *D. trenchii* induced a larger response in the host proteome. Heat stress responses that were shared by both *Cladocopium* C1- and *D. trenchii*-colonised corals included upregulated heat stress, oxidative stress and calcium-binding proteins, as well as proteins involved in protein degradation and turnover. Elevated temperature typically causes denaturing of proteins, so the increased expression of molecular chaperones, as well as protein degradation and turnover, was expected, and in agreement with previous proteomic studies of heat stress in corals (Weston et al., 2015; Ricaurte et al., 2016) and *Aiptasia* (Oakley et al., 2017). In addition to these universal stress responses, hosts containing *D. trenchii* displayed more changes in the host proteome than those colonised by *Cladocopium* C1. Proteins that were upregulated uniquely for the symbiosis with *D. trenchii* in response to heat stress were mainly composed of more HSPs, oxidative stress proteins, proteins involved in protein degradation and turnover, and proteins that are part of the host's immune response.

Four calcium-binding proteins exhibited the same thermal response (either up- or down-regulation) in both *Cladocopium* C1- and *D. trenchii*-colonised corals. Of these, calreticulin and calcyclin-binding protein both increased in expression. Calreticulin is a molecular chaperone in the endoplasmic reticulum, but also plays a role in calcium homeostasis which, in turn, is related to apoptosis (Michalak et al., 1999). Calcyclin-binding protein is involved in calcium-dependent ubiquitination of target proteins and their subsequent proteasomal degradation, and additionally acts as an inhibitor of cell proliferation (Ning et al., 2007). The upregulation of these proteins is a clear indication of cellular stress. In contrast, hippocalcin-like protein 4 (Hpcal4) and calbindin-32 were down-regulated in response to heat stress, but the roles of these calcium-binding proteins remain unclear (Reifegerste et al., 1993; Díaz-Balzac et al., 2012; Alvaro et al., 2020).

5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase-like isoform X1 (mtnN), while upregulated in both *Cladocopium* C1- and *D. trenchii*-colonised corals under heat stress, exhibited a much greater increase in the presence of *D. trenchii* (9.34 log₂-fold versus 3.56 log₂-fold). MtnN is involved in the L-methionine biosynthesis pathway. MtnN metabolises both 5'-methylthioadenosine

(MTA) and S-adenosylhomocysteine (SAH), which are both by-products of other metabolic pathways. MTA is a by-product of the polyamine biosynthetic pathway, whereas SAH is a by-product of SAM metabolism. Both products are strong inhibitors of critical cellular processes, such as gene expression, proliferation and apoptosis (Avila et al., 2004; Parveen & Cornell, 2011). As discussed previously, the production of SAM seems to be increased in response to symbiosis, so the upregulation of the enzyme that deals with its inhibitory by-products is not unexpected. However, it is interesting that the upregulation of this enzyme was more marked in heat-stressed corals containing *D. trenchii*, which suggests that this symbiotic association has a higher activity of the SAM pathway. Again, due to the wide range of processes that involve SAM, it is difficult to speculate on the processes that are activated here.

APA expression showed a decrease in response to heat stress in the presence of both species of symbionts, which is in contrast to previous work by Godinot et al. (2013), who observed an increase in APA in adult bleached corals. As mentioned before, the expression of APA increases in response to phosphate deficiency, and the establishment of symbiosis induced increased expression of APA in hosts with either species of symbiont. This is suspected to be caused by the increasing symbiont cell density and growth. In particular, as symbiont growth is reduced by heat stress, the phosphorus limitation within the host might be relieved, thus explaining the decrease in APA.

In addition to these responses to heat stress shown by both symbiotic associations, the symbiont species also induced changes in the host proteome that differed from each other. These included an increased expression of GFP and tetraspanins in *Cladocopium* C1-colonised hosts, whereas hosts containing *D. trenchii* showed increased expression of the lipid transporters apolipophorin and viatellogein.

Corals in symbiosis with *Cladocopium* C1 had a log₂-fold change of 3 in expression of GFP in response to heat stress compared to those harbouring *D. trenchii*. Although no difference in GFP expression was found under control conditions, previous results have shown that *Cladocopium* C1 induces higher GFP expression in *A. tenuis* than *D. trenchii*, although this elevated expression decreased 8-11 weeks after establishment of the symbiosis (Yuyama & Higuchi, 2014). As mentioned before, fluorescent proteins are involved in immune and photoprotective mechanisms, and function as antioxidants. For these reasons, GFP-like proteins have been suggested as markers for coral health (Palmer et al., 2009; Ricuarte et al., 2016; Bridges et al., 2020). While GFP is highly upregulated in *Cladocopium* C1-colonised hosts, *D. trenchii*-colonised hosts displayed increased expression of two other oxidative stress proteins (peroxidase and a peroxidase homolog), which shows that the hosts show a symbiont-specific response to oxidative stress.

Tetraspanins make up a superfamily of proteins that are involved in a wide range of processes, such as cell adhesion, motility, activation and proliferation, as well as metastasis and viral infection (Hemler, 2003, 2005). Much is still unknown about the function of specific tetraspanins, and the tetraspanin found in this study is not defined to type which makes it difficult to speculate on its potential function. Nevertheless, the greater expression of this tetraspanin ($\log_2$ -fold change of nearly two) in corals containing *Cladocopium* C1 versus *D. trenchii* in response to heat stress suggests that it plays an important physiological role.

Apolipoprotein (ApoLp) and vitellogenin (VG) are lipid transporter proteins (Beenackers et al., 1985; Hayward et al., 2010), and my results indicate that the expression of these proteins is significantly upregulated under heat stress in corals containing *D. trenchii* compared to corals colonized by *Cladocopium* C1. VG, while synthesised in somatic tissue, is associated with oocyte development in corals (Hayakawa et al., 2007; Shikina et al., 2013; Tan et al., 2020). It is considered a major yolk protein precursor, and is involved in lipoprotein transport, vitellogenin synthesis, and yolk incorporation into oocytes. ApoLp is derived from a common ancestor as VG (Babin et al., 1999), and was found upregulated in the coral *Porites astreoides* from warmer inshore reefs when compared to cooler offshore reefs, although this study indicated no significant difference in its expression under heat stress (Kenkel et al., 2013). Lipid stores are important for larval survivorship in *A. tenuis* (Harii et al., 2007), and other research has shown that early symbiosis establishment allows larvae to maintain lipid stores (Harii et al., 2010). Therefore, if juvenile *A. tenuis* still have lipid reserves from their larval stages stored, the increase of VG expression could be a sign of mobilisation of these lipid reserves to fuel the metabolic changes required to cope with heat stress. The upregulation of ApoLp, could likewise reflect this lipid store mobilisation.

In future, it would be interesting to include more aspects of host performance; for example, by the addition of metabolomics, or other -omics approaches (Meyer & Weis, 2012; Matthews et al., 2017; Czieiselski et al., 2018). Including such features might unravel more of what is happening at a cellular level, and potentially allow us to identify the processes that proteins with a wide range of functions are involved in.

5.6 Conclusion

Even though both *Cladocopium* C1 and *D. trenchii* are naturally found in symbiosis with juvenile *A. tenuis*, the two symbiont species induced different responses in the host, both under control and thermal stress conditions. Many of the proteins upregulated after colonisation by *Cladocopium* C1 under control conditions included heat stress proteins and apoptosis-inducing proteins, potentially signalling that *Cladocopium* C1 is more physiologically costly to *A. tenuis*. Alternatively, this response could better

prepare the holobiont for future stress events. This was supported by the more marked stress response in the host proteome of *A. tenuis* juveniles colonised by *D. trenchii* in response to elevated temperature. Of course, there is the question of whether it is better to invest in heat stress proteins early and be prepared for future events, or whether it is worth saving that investment for when, or if, a stress event arrives. With the current increases seen in mass bleaching events, it seems likely that some degree of physiological pre-preparation gives the host a crucial advantage; e.g. how the coral species *Montipora digitata* seems ‘prepared’ for dealing with thermal stress compared to some other coral species, attributed to its naturally higher expression of antioxidant responses (Krueger et al., 2015).

Chapter 6 General discussion

6.1 Summary

Currently, major gaps exist in our understanding of symbiosis function when cnidarian hosts are paired with non-native, i.e. heterologous, symbionts, especially in terms of the possibility of host-symbiont integration over longer time-scales. Greater in-depth knowledge is of utmost importance for understanding the role that symbiont diversity could play in the adaptation of coral reefs to environmental change. This thesis expands our knowledge of a number of aspects of host physiology and inter-partner nutrient exchange between host and symbiont, to further our understanding of the potential for partner switching as a mechanism by which corals may survive climate change. Metabolomic and proteomic techniques were used to explore the host's response when paired with heterologous, but more thermally tolerant symbionts in a model symbiosis (*Aiptasia*) in response to heat stress (Chapter 2). Similarly, in a species of coral (*Acropora tenuis*), proteomic techniques were used to determine the host's response to heat stress when colonised by different species of symbionts it naturally harbours (i.e. homologous symbionts; Chapter 5). Proteomic and metabolomic techniques were subsequently used to investigate the host's response when in symbiosis with heterologous species for prolonged periods of time, as well as under a scenario of more frequent occurrences of heat stress events, to test if bleaching provides an opportunity for symbiont selection by the host (Chapter 3). The exchange of carbon and nitrogen between host and symbiont was assessed *via* nanoscale secondary image ion mass-spectrometry (NanoSIMS) and bulk isotope-ratio mass spectrometry (IRMS) in a similar long-term set up (Chapter 4). Several important discoveries were made from this work: most importantly, that symbiont identity affects the heat stress response of a host, both prior to - as well as during - the heat stress event; and that the individual responses of heterologous symbionts on the host metabolome and proteome converge under heat stress towards a more general response (Chapter 2). This corroborates, and adds substantially to, previous research that demonstrated that pairings with homologous symbionts are more robust and capable of dealing with stressors (Gabay et al., 2019).

A further significant discovery arising from my work is that host-symbiont integration over time is highly dependent on the nature of the symbiont species (Chapters 3 and 4). *Durisdinium trenchii* provided little to no opportunity for improved host-symbiont integration over up to ten months in symbiosis. This supports and builds upon evidence that *D. trenchii* is a more opportunistic, less beneficial symbiont (Abrego et al., 2008; LaJeunesse et al., 2009; Thornhill et al., 2009; Stat & Gates, 2011; Kennedy et al., 2015; Matthews et al., 2017; Silverstein et al., 2017; Gabay et al., 2019; Sproles et al., 2020). On the other hand, small changes were observed in the host metabolome and proteome for the association between *Aiptasia* and *Symbiodinium microadriaticum*. While these host metabolome changes could be attributed to the lower respiratory requirements of a near-aposymbiotic anemone, the

changes in the proteome included the presence of NPC proteins, which are believed to be highly important in symbiosis function (Dani et al., 2014, 2017; Baumgarten et al., 2015; Hambleton et al., 2019; Sproles et al., 2019; Rosset et al., 2021). The detection of this protein in an association with a heterologous symbiont could be cautiously interpreted as a sign of improved host-symbiont integration over time. Additionally, although nutritional translocation between *Aiptasia* and *S. microadriaticum* did not become more favourable over time at a cellular level, it was more beneficial to the host than the relationship between *Aiptasia* and *D. trenchii* (Chapter 4). However, any changes that developed over time were small, and many more changes within the host indicated increased cellular stress (Chapters 3 and 4). Additionally, repeated heat stress events did not seem to aid in the process of host-symbiont integration; on the contrary, it seemed to cancel out any room for improvement observed between *Aiptasia* and *S. microadriaticum*, while it seemed to amplify the selfish traits observed by *D. trenchii* (Chapters 3 and 4). This study therefore did not support the hypothesis of improved performance through host selection of individual symbionts as a consequence of thermal stress. However, as clonal symbiont lines were used, the absence of selection could also be due to the lack of genetic diversity within the symbiont population, i.e. there was no, or not enough, variation within the symbiont population, for example in their translocation of nutrients to the host, upon which selection could take place (see below for further discussion).

The findings from this thesis raise two particularly important questions regarding the future survival of coral reefs: (1) Will corals be able to adapt to climate change through adaptive bleaching? And (2) what information can be taken from these studies to develop tools to aid in coral survival? These questions will be considered further here.

6.2 Implications for the future of coral reefs

6.2.1 Will corals be able to adapt to climate change through adaptive bleaching?

The warming of Earth's climate due to greenhouse gas emissions has caused increases in both the severity and frequency of coral mass bleaching events (Hoegh-Guldberg, 1999; Sampayo et al., 2008; Dove et al., 2013), and contributed to global coral reef declines, averaging approximately 30-50% reductions in coral reef cover in as little as 20 years (Selig et al., 2012). Future projections for the survival of coral reefs appear bleak, as model simulations suggest that annual, or even biannual mass bleaching events will be the norm by 2050 (Donner et al., 2005; Frieler et al., 2013; Van Hooidonk et al., 2013; Palumbi et al., 2019). To avoid catastrophic coral reef ecosystem loss, the Intergovernmental Panel on Climate Change has estimated that drastic interventions are required within the next 10 years to limit global temperature increases to 1.5 °C above preindustrial levels. Multiple research avenues have been explored in an attempt to find solutions and enhance coral reef survival (see Section 6.2.2),

including *via* adaptive bleaching (Buddemeier & Fautin, 1993; Baker et al., 2004; Fautin & Buddemeier, 2004; Berkelmans & Van Oppen, 2006; Matthews et al., 2017; Van Oppen et al., 2017; Chapters 2-5).

Adaptive bleaching, i.e. a change in the symbiont community within the host's tissues to allow for the dominant presence of a more thermally tolerant Symbiodiniaceae species, was first proposed as a hypothesis almost 30 years ago (Buddemeier & Fautin, 1993). Since then, many scientific studies have focused on the effects of symbiont shuffling and switching and their implications for host fitness, both in the field and in experimental studies. Field observations have shown that such changes in symbiont communities within coral hosts indeed occur in response to stress (Stat & Gates, 2011; Kemp et al., 2014; Boulotte et al., 2016; Silverstein et al., 2017), though such studies have been unable to provide conclusive evidence that these fluctuations cannot be attributed solely to the opportunistic nature of 'invading' symbionts (Abrego et al., 2008; LaJeunesse et al., 2009; Thornhill et al., 2009; Stat & Gates, 2011; Pettay et al., 2015). In particular, the symbiont species *D. trenchii* may occur in increased densities in coral tissues during and/or after bleaching events (Toller et al., 2001; Glynn et al., 2001; Baker et al., 2004; Berkelmans & Van Oppen, 2006; Jones et al., 2008; Correa et al., 2009; LaJeunesse et al., 2009; Stat & Gates, 2011; Cooper et al., 2011; Silverstein et al., 2012; Kemp et al., 2014; Kennedy et al., 2015; Boulotte et al., 2016), which does not always have optimal outcomes for the host (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Silverstein et al., 2017). Other experimental work has shown that establishing a symbiosis with a thermally tolerant, but heterologous symbiont species seems to come at a great metabolic cost to the host, affecting host fitness and growth (Matthews et al., 2017, 2018; Silverstein et al., 2017; Gabay et al., 2018; Sproles et al., 2020). This thesis aimed to investigate if there was any capacity for improving host-symbiont function over time in these instances, by maintaining a symbiosis between the model cnidarian *Exaiptasia pallida* ('Aiptasia') and a heterologous symbiont species for much longer timeframes than generally used in experimental studies.

As stated previously, this work was founded on the assumption that the algal cultures used displayed enough genotypic and/or phenotypic variation upon which selection could act. However, no assessment of this variation was made, and currently, the extent of variation in Symbiodiniaceae cultures is unknown. Additionally, some of these cultures were assumed as being "clonal", i.e. originating from a single cell, potentially minimising variation in the population. However, Symbiodiniaceae cultures have a high growth rate, i.e. a doubling time of two to three days (Fitt & Trench, 1983b; Kinzie et al., 2001; Taguchi & Kinzie, 2001), and DNA replication is unlikely to occur without any fault on every occasion, thus inherently introducing variance in the population (Lynch et al., 1991; Loxdale et al., 2003). Indeed, while few studies have addressed the issue of genotypic and/or phenotypic variation in dinoflagellate algal cultures, those that have found considerable variation, even in cultures assumed to be clonal (Brand, 1981, 1988; Ogata et al., 1987; Costas, 1990; Lakeman & Cattolico, 2007). Therefore, although

genotypic and phenotypic variation within algal cultures was not measured here, it is likely to have been present.

Studies on microbial symbioses have found evidence that mutualisms have evolved from parasitic lineages through vertical transmission of symbionts over host generations (Weeks et al., 2007; Bright & Bulgheresi, 2010; Sachs et al., 2011; Shapiro & Turner, 2018). Similarly, host fitness of the cnidarian jellyfish *Cassiopea xamachana* increased in response to vertical transmission of symbionts (Sachs & Wilcox, 2006). It is therefore conceivable that Aiptasia and a heterologous symbiont species might be able to move along a spectrum from parasitism to mutualism if permitted a longer time in symbiosis, especially given the likelihood of genotypic and phenotypic variability in the population. However, while slight changes were observed, holobiont-functioning did not seem to improve drastically over the timescales used in these experiments, i.e. a maximum of ten months in symbiosis. Cellular processes within the different host-symbiont pairings at the final time-points of the various studies in this thesis are synthesised in Figure 6.1.

Nutrient exchange between host and symbiont is of paramount importance in the cnidarian-dinoflagellate symbiosis. It forms the basis of its success (Muscatine & Hand, 1958; Von Holt & Von Holt, 1968; Trench, 1971; Burriesci et al., 2012; Sproles et al., 2020), as coral reefs are found in nutrient-poor waters. Previous research has shown that the association of a host with a heterologous symbiont leads to sub-optimal nutrient exchange (Starzak et al., 2014, 2020; Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2020), which was mainly attributed to the selfish nature of the heterologous symbiont species used, e.g., *D. trenchii* (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Pettay et al., 2015; Matthews et al., 2017; Sproles et al., 2020). However, this thesis has presented the first evidence of an association between a host and heterologous symbiont, i.e. Aiptasia and *S. microadriaticum*, showing signs of favourable nutrient exchange between host and symbiont at a cell-specific level (Chapter 4). However, at the holobiont-level, this favourable cellular nutrient exchange was insufficient to support host metabolism to the extent seen with the homologous *B. minutum*, as evidenced by depleted stores of energy-rich compounds such as lipids and carbohydrates, and nitrogen-rich compounds (Chapter 3; Figure 6.1). The same depletion of reserves was found in *D. trenchii*-colonised hosts. These results corroborate and substantiate earlier work by Sproles et al. (2020), who suggested that a symbiosis between Aiptasia and *D. trenchii* would lead to a nutritional deficit in the longer term, as well as Starzak et al. (2014), who showed that heterologous symbiont species in Aiptasia could not provide 100% of the carbon required for the host's daily respiration, in contrast to the homologous *B. minutum*. Furthermore, as energy reserves are of paramount importance for the host's ability to deal with stress events (Rodrigues & Grottoli, 2007; Anthony et al., 2009; Grottoli et al., 2014; Van der Zande et al., 2020), the lack of these reserves explains the disappearance of positive effects of time-in-symbiosis in the association between Aiptasia and the heterologous *S. microadriaticum*.

Figure 6.1 Summary of the cellular processes in different host-symbiont pairings after prolonged time in symbiosis, based on metabolomics, proteomics, nanoSIMS and IRMS data. (A) The homologous symbiont is associated with efficient bilateral nutrient exchange or carbon and nitrogen (1). High symbiont population densities are controlled by host apoptosis (2), and the high photosynthetic rates of a large symbiont population lead to high production of oxidative stress proteins (ox) in the host (3). Hosts are not stressed by other means, and therefore produce few heat stress proteins (HSPs) or osmolytes (osm). Metabolism is driven towards growth and storage (4), shown by large increases in lipid and carbohydrate stores, and nitrogen conservation (N). The homologous symbiont also induces high expression of NPC proteins, which are associated with the symbiosome membrane (5). In comparison, (B) the heterologous symbiont is associated with sub-optimal nutrient exchange, either due to selfish traits of the symbiont in its willingness to share nutrients at a cell-specific scale, or due to low symbiont population densities at a holobiont-scale. Host apoptosis is suppressed by the symbionts (2), and the lower photosynthetic rate due to low symbiont cell densities requires less production of oxidative stress proteins (ox; 3). Hosts experience high levels of cellular stress, indicated by high expression of HSPs and osmolytes, such as glycerol. Metabolism is driven towards cellular maintenance (4), with only limited build-up of lipid stores, and severely depleted carbohydrate stores. Nitrogen-rich compounds, such as proteins and amino acids, are subjected to a high turnover rate, leading to a build-up of nitrogenous metabolic end products, e.g. urea, in host tissues. A low abundance of NPC proteins is evident in the association with a heterologous symbiont (5).

(Chapter 3). The question remains whether host metabolism could be fully supported by *S. microadriaticum* were the symbiont to attain similar densities as *B. minutum*, though this level of population growth seems unlikely.

So why did the population density of *S. microadriaticum* not increase while nutrient exchange seemed favourable to the host? Heterologous symbionts seem to impose a more stressed state on their hosts (Matthews et al., 2017; Gabay et al., 2018, 2019; Chapters 2 and 3), and several mechanisms are in place for removal of incompatible symbionts during stress events: *in situ* degradation and autophagy, exocytosis, host cell apoptosis and/or necrosis, or host cell detachment (Gates et al., 1992; Dunn et al., 2002, 2004, 2007a; Bhagooli & Hidaka, 2004; Tchernov et al., 2011; Bieri et al., 2016; Dani et al., 2016). It is thought that expulsion, or vomocytosis, is the main mechanism employed by the host to rid its tissues of incompatible symbionts (Bieri et al., 2016; Jacobovitz et al., 2021). Alternatively, host apoptosis has been suggested as a mechanism that allows post-phagocytic sorting by the host based on symbiont compatibility (Dunn & Weis, 2009). However, my results oppose the predominant use of apoptosis in symbiont sorting, as both heterologous symbiont species were shown to suppress host apoptosis (Chapters 2 and 3). Suppression of host apoptosis is characteristic of the cnidarian-dinoflagellate symbiosis (Rodriguez-Lanetty et al., 2006; Pernice et al., 2011; Gabay, 2018), and originates from the evolution of mutualisms from parasitisms (Aanen & Hoekstra, 2007; Pannebakker et al., 2007), as it allows for the proliferation of the parasite, or in this case symbiont, within host tissues. In the symbiosis between *Aiptasia* and *B. minutum*, host apoptosis was upregulated over time (Chapter 3) and, paired with the decrease in the cell density of *B. minutum*, was likely the primary mechanism by which the host controlled symbiont proliferation. In comparison, there was little difference in host apoptosis protein expression between the symbioses with *D. trenchii* and *S. microadriaticum*, while only *D. trenchii* decreased in density over time. Thus, while the different expression of host apoptosis-related proteins in response to different symbiont species could be a symptom of host-symbiont incompatibility, it does not explain fluctuations, or lack thereof, in the densities of the heterologous symbiont species. By comparison, the cellular mechanisms behind exocytosis in the cnidarian-dinoflagellate symbiosis are underreported (Weston et al., 2015), but calcium-signalling pathways (Huang et al., 1998; Weston et al., 2012, 2015) as well as HSPs (Zinsmaier & Bronk, 2001) are involved in activation of exocytosis processes. Similarly, involvement of structural proteins is likely important, as exocytosis requires extensive reorganisation of cellular membranes (Kasai et al., 2012). All three of these categories of proteins were differentially expressed in anemones containing a heterologous symbiont (Chapters 2 and 3; Figure 6.1), but specific roles of many of these proteins remain unresolved, especially the structural proteins (Supplementary Data). My work thus helps to establish a baseline of differential protein expression in response to different symbiont species, which is an important first step in elucidating the roles that these various proteins play. Future studies could test the effect of exocytosis

inhibitors (Nüchter et al., 2006; Ivanov, 2014) on symbiont density and the resulting effects on symbiosis function.

Finally, one of the key differences found in this thesis related to the expression of Niemann-Pick type C (NPC) proteins. NPC proteins are sterol-transporters associated with the symbiosome membrane (Dani et al., 2014, 2017; Hambleton et al., 2019; Rosset et al., 2021), and are highly upregulated in response to symbiosis establishment (Oakley et al., 2016). This thesis presented the first observation of this protein in hosts harbouring a heterologous symbiont (Chapters 2 and 3, Figure 6.1), which can be interpreted as a sign that the host and heterologous symbiont were at least making an attempt at improving cellular integration. Interestingly, the association between *Aiptasia* and *D. trenchii* displayed expression of NPC proteins at an earlier time-point than the association between *Aiptasia* and *S. microadriaticum*: *D. trenchii*-colonised hosts expressed NPC proteins three months after initial colonisation, whereas expression was not observed until seven months in *S. microadriaticum*-colonised hosts. Of course, there was no sampling in between these time-points, so I cannot discern when the proteins were first expressed. However, these observations emphasise the importance of time in symbiosis when assessing the capacity for improved host-symbiont integration.

In coral hosts that pair with multiple species of symbionts, i.e. hosts that display less specificity for their symbiont population, the host proteome was also observed to show a symbiont-specific response, both during symbiosis establishment and under thermal stress (Chapter 5). Interestingly, *Cladocopium* C1 seemed to offer greatest degree of thermal resistance, in contrast to expectations that *D. trenchii* is the more thermally tolerant symbiont (Stat & Gates, 2011; Silverstein et al., 2017; Swain et al., 2017). This could be caused by the close phylogenetic relationship between *Cladocopium* C1 and the symbiont species that dominates adult *A. tenuis*, i.e. *Cladocopium* C3 (Yorifuji et al., 2017), thus potentially making *Cladocopium* C1 “more homologous” for juveniles than *D. trenchii*, and thus providing a more thermally robust association than that with *D. trenchii*. Indeed, previous research on *Aiptasia* has shown that a symbiosis with a homologous symbiont is more thermally tolerant than an association with a heterologous, more thermally tolerant, symbiont (Gabay et al., 2018, 2019; Chapters 2-4). Again, elucidating the mechanisms *via* which host-symbiont recognition occurs, and what aspects of host-symbiont communication and integration help determine thermal tolerance, is a priority for future research, to establish the possibilities of partner switching as an adaptive method to circumnavigate the effects of climate change.

In summary, although not inconceivable, it is also not surprising that little change was observed over the timescale used in these experiments. The cnidarian-dinoflagellate symbiosis has adapted over millions of years of evolution (Trench, 1997; Leinfelder, 2001; Lathuilière & Marchal, 2009), during which time many hosts have developed a preference for harbouring specific symbiont species (Belda-

Baillie et al., 2002; Baker, 2003; Goulet, 2006; Putnam et al., 2012; Poland & Coffroth, 2017). The Aiptasia lineage used here, originally from the Indo-Pacific region, similarly shows a high specificity for its homologous symbiont, *Breviolum minutum* (Thornhill et al., 2013a; Gabay et al., 2018, 2019). Host-symbiont specificity thus poses a limiting factor for adaptive bleaching. There is no consensus yet on the molecular basis behind host-symbiont specificity, but several mechanisms have been proposed. These include microbe-associated molecular patterns (MAMPs) and pattern recognition receptors (PRRs), such as glycans expressed on the symbiont cell surface, interacting with glycan-binding proteins, i.e. lectins, of the host (Wood-Charlson et al., 2006; Wood-Charlson & Weis, 2009; Logan et al., 2010; Bay et al., 2011), which another study has since opposed (Parkinson et al., 2018). Other candidates that might play a role in symbiont recognition include Niemann-Pick type C (NPC) proteins, which, as discussed above, were found in small quantities in the symbioses with heterologous symbionts (Dani et al., 2014, 2017). It therefore seems likely that NPC proteins are produced in response to another, symbiont-specific stimulus. It is of crucial importance to establish the molecular basis of host specificity for its symbiont population (and *vice versa*), to investigate the possibilities that adaptive bleaching will aid in coral reef survival in light of current, and future, environmental change. Based on our present knowledge of symbiosis function, adaptive bleaching, and in particular symbiont switching, does not seem like a viable option for corals to adapt to and survive our changing climate given the rapid pace of environmental change. There is therefore now considerable focus on the development of tools, including those that manipulate host-symbiont specificity and symbiosis function, to intervene in the Coral Reef Crisis (Filbee-Dexter & Smajdor, 2019; Rosset et al., 2021).

6.2.2 What information can be taken from these studies to develop tools to aid in coral survival?

As mentioned previously, many research avenues are currently being explored to assess the options for aiding coral survival in the face of climate change; for instance adaptive bleaching (Buddemeier & Fautin, 1993; Baker et al., 2004; Fautin & Buddemeier, 2004; Berkelmans & Van Oppen, 2006; Matthews et al., 2017; Van Oppen et al., 2017; Chapters 2-5), as well as selective breeding of corals and their dinoflagellate symbionts (Van Oppen et al., 2015; Chakravarti et al., 2017; Buerger et al., 2020), coral probiotics (Reshef et al., 2006; Bourne et al., 2016; Peixoto et al., 2017; Rosado et al., 2019), and increasing reef resilience (Anthony et al., 2017; Palumbi et al., 2019; Weis, 2019). Additionally, gene-editing tools such as CRISPR/Cas9 are rapidly increasing in popularity and applicability (Ikmi et al., 2014; Cleves et al., 2018, 2020), raising the possibility of engineering more thermally resistant corals through the manipulation of pathways associated with inter-partner communication and nutrient exchange (Parkinson et al., 2019; Rosset et al., 2021; Chapter 4). There is also considerable interest in the development of tools, such as biomarkers, to monitor coral health quickly and efficiently in the field (Parkinson et al., 2019).

The adaptive bleaching hypothesis (ABH) suggests that bleaching offers an opportunity for coral hosts to change their symbiont community composition to one that is better able to deal with current environmental conditions, such as elevated temperatures (Buddemeier & Fautin, 1993; Baker et al., 2004; Fautin & Buddemeier, 2004; Berkelmans & Van Oppen, 2006). Although the uptake of more thermally tolerant symbionts can result in a higher thermal tolerance in the host, it may come at a cost to the host (Abrego et al., 2008; Thornhill et al., 2009; Stat & Gates, 2011; Silverstein et al., 2017; Matthews et al., 2018; Gabay et al., 2019; Sproles et al., 2020; Starzak et al., 2020; Chapters 2-4). Nevertheless, if tangible evidence for improved symbiosis function over time becomes more available (alongside the tentative evidence provided in this thesis), then engineering and rearing of novel host-symbiont partnerships could be combined with other conservation approaches, such as out-planting of lab-grown coral colonies (Montoya-Maya et al., 2016; Baums et al., 2019).

Selective breeding of corals and/or coral endosymbionts has been considered a form of (human)-assisted evolution, to speed up the adaptive potential of coral reefs to the rapid environmental changes that are occurring (Van Oppen et al., 2017; Baums et al., 2019). For corals, this includes hybridisation of populations to create more genetic diversity within offspring, thereby enhancing adaptive potential and resilience (Van Oppen et al., 2014). However, coral growth is a slow process, which means that the time-span of coral reef formation from new recruits exceeds the time we have to act. Therefore, selective breeding of corals is unlikely to create enough opportunity for adaptation compared to the rapidly changing climate. By contrast, Symbiodiniaceae populations reproduce much quicker: doubling times are often less than three days (Fitt & Trench, 1983b; Kinzie et al., 2001; Taguchi & Kinzie, 2001). Thermal selection experiments on algal cultures have led to increased thermal tolerance in under a year (Chakravarti et al., 2017; Chakravarti & Van Oppen, 2018; Buerger et al., 2020), and, if performed with homologous species of symbionts, would circumvent any issues regarding host specificity, such as highlighted in my work. However, re-introduction of selectively bred symbionts to the host has produced mixed results with regards to the host's thermal tolerance, that is, only in a few cases did the increased thermal tolerance observed in the endosymbionts translate to increased bleaching resistance in the host (Chakravarti et al., 2017; Buerger et al., 2020).

Another form of assistance to coral functioning is the application of marine probiotics. Manipulation of microbiomes has been successfully applied in varied settings, including agricultural developments (Vejan et al., 2016; Porto de Souza Vandenberghe et al., 2017; Woo & Pepe, 2018; Al-Shawi et al., 2020), mitigating effects of pollution (Santos et al., 2011, 2015), and human probiotics (S. Grover et al., 2012). Microbes are associated with corals on several levels: in coral mucus (Krediet et al., 2013), in coral tissues (Work & Aeby, 2014), within the gastric cavity (Agostini et al., 2012), and even in the coral skeleton (Yuen et al., 2013). Therefore, it is not unthinkable that probiotics can have positive effects on host functioning. The development and application of coral probiotics is also a much quicker

process than selective breeding of coral species, and promising results have been obtained in experimental settings (Rosado et al., 2019). However, coral hosts have shown a similar specificity for microbial communities as seen with symbiont preference (Rohwer et al., 2002; Littman et al., 2009; Sunagawa et al., 2010), highlighting again the importance of unravelling host recognition mechanisms for their symbiotic partners, whether algal or microbial.

Thus, for symbiont switching to be considered a successful mechanism *via* which corals can adapt to environmental change, more research is needed into what is necessary for a novel host-symbiont pairing to survive. Crucial to this is unravelling the pathways of symbiont recognition and host-symbiont specificity, as well as inter-partner communication and regulation. Studies on the symbiosome membrane, for instance, could provide valuable insights into key players of such processes, while newly emerging technologies such as CRISPR/Cas9 genome editing will likely prove valuable (Levin et al., 2017; Cleves et al., 2018, 2020; Rosset et al., 2021). CRISPR/Cas9 allows for manipulation of genes, thereby informing us of their function, and has already been applied to sea anemones (Ikmi et al., 2014) and corals (Cleves et al., 2018). There is also great potential to use this technique with the symbiont (Levin et al., 2017), but more difficulty exists in getting this to work in Symbiodiniaceae, not least because of their thick cell wall (Rosset et al., 2021). Once genes that are involved in host specificity and partner recognition, as well as host-symbiont communication and regulation, are identified, gene-editing techniques will be crucial for manipulating symbiotic partnerships between hosts and heterologous symbionts. Such an approach could be invaluable for the engineering of novel, more thermally resistant partnerships, while omics methods such as those described in this thesis (Chapters 2, 3 and 5) will generate much of the foundational, functional information required.

Finally, there is considerable interest in the development of molecular biomarkers to monitor the status of coral colonies in the field (reviewed in Parkinson et al., 2019). Numerous candidates have been proposed as potential biomarkers, such as genomic biomarkers of corals hosts (Bay & Palumbi, 2014; Dixon et al., 2015; Jin et al., 2016; Kirk et al., 2018), gene expression biomarkers (Kenkel et al., 2014; Louis et al., 2017; Bay & Palumbi, 2017; Parkinson et al., 2018), and protein-based biomarkers (Downs et al., 2000; Downs et al., 2005). However, many barriers still exist in the applicability of biomarkers. For instance, expression-based biomarkers, such as suggested by Downs et al. (2000, 2005) revolve around proteins such as HSPs and oxidative stress proteins. Expression of these proteins has been shown to be highly dependent on coral species (Robbart et al., 2004; Chow et al., 2009; Fitt et al., 2009; Seveso et al., 2014, 2018; Krueger et al., 2015), and my work has shown this is also specific within individual hosts in response to symbiont identity (Chapters 2, 3 and 5). For such measures to be useful, extensive development of baselines is required for the correct interpretation of results. Additionally, all biomarkers suggested above require destructive sampling, i.e. to take measurements coral tissue needs to be removed from a colony, which is undesirable in light of coral conservation. Future developments

could look into non-destructive, high-throughput sampling, such as the recently emerged approach of volatilomics (Lawson et al., 2019), which identifies the production of volatile organic compounds in a system.

In conclusion, there is an incredible challenge ahead for coral reef scientists, to intervene in the Coral Reef Crisis before it is too late (Filbee-Dexter & Smajdor, 2019; Parkinson et al., 2019; Rosset et al., 2021). Although climate predictions are looking bleak (Burke et al., 2011; Baker et al., 2013; Frieler et al., 2013; Hughes et al., 2018), many efforts are being made on every possible aspect of coral reef biology to tackle the issue of ensuring the survival of coral reefs into the future. Cooperation between these fronts and research transparency will be highly beneficial in this task. Of high priority is to elucidate the mechanisms that determine inter-partner communication and cooperation, and hence host-symbiont specificity, and ultimately identify possible manipulations that allow for the improvement of host-symbiont function and coral reef survival in our changing world.

6.3 Limitations and future research

Limitations of this study include the use of single-source algal cell cultures. Besides the nutritional complications that arise from working with cultures (Maruyama & Weis, 2021), this work is based on the assumption that these cell lineages express enough genotypic and/or phenotypic variation on which selection could occur. Future research would benefit from the confirmation of the level of genotypic and phenotypic variation within a symbiont population. Additionally, future approaches could include using an even longer time in symbiosis than that used here or mixed populations of symbionts on which selection can act in the host. This could shed more light on the adaptability of cnidarian-dinoflagellate symbioses, and the viability of an association between a host and a heterologous symbiont. Furthermore, the role of vertical transmission has been shown to be important in the evolution of a parasitic symbiosis into a mutualistic one (Sachs & Wilcox, 2006; Weeks et al., 2007; Bright & Bulgheresi, 2010; Sachs et al., 2011; Shapiro & Turner, 2018). Due to the complexity of the required experimental design and analyses, as well as time restrictions, which were further exacerbated by COVID-19, I was unable to include this component in my study. However, *Aiptasia* reproduces clonally through pedal laceration (Clayton, 1985; C. A. Van der Burg & Prentis, 2021), by which the symbionts are transmitted vertically (Muller-Parker & Davy, 2001; Schlesinger et al., 2010). Pedal laceration has been observed even in *Aiptasia* in symbiosis with heterologous symbiont species (Gabay et al., 2018), allowing future work to address how vertical transmission of symbionts might influence symbiosis function.

Furthermore, many scientific studies measure features related to the host, rather than both host and symbiont individually (Matthews et al., 2017; Gabay et al., 2018; Sproles et al., 2019). Future research could include the application of omics techniques on the symbiont cells in addition to host tissues, as

identification of differences in metabolomes or proteomes of homologous *versus* heterologous symbionts could indicate what the host might lack in a symbiosis with a heterologous symbiont. This can then provide more insight into how the symbiosis can be manipulated to become more beneficial to both parties.

Additionally, although much harder to maintain in laboratory settings, future research should include the application of similar experimental setups to coral species, such as *Acropora tenuis* (Chapter 5). *Aiptasia* is a good model system for the cnidarian-dinoflagellate symbiosis (Weis et al., 2008), but, as discussed previously, there are many species of corals across a large spectrum of symbiont specificity, ranging from highly specific to generalist (Baker, 2003; Putnam et al., 2012). It is worthy of investigation how different species of corals, each of course with their own thermal tolerance thresholds irrespective of the symbiont (Loya et al., 2001; McClanahan, 2004; Wooldridge, 2014; Krueger et al., 2015), respond to heterologous symbionts over longer timescales, to identify the cellular, physiological and temporal limits of host-symbiont integration with novel partners.

6.4 Concluding remarks

This thesis aimed to investigate the effect of symbiont identity on host physiology, and the possibility of cellular integration between *Aiptasia* and heterologous symbionts over prolonged time in symbiosis. As cooperation between host and symbiont is required for the symbiosis to persist and propagate, I hypothesised that selection pressure would facilitate a shift towards a more mutualistic relationship over time, so rendering the symbiosis with a heterologous symbiont more similar to that with the homologous symbiont. Additionally, my work sought to test whether such a selective shift might be promoted by exposure to repeated stress events. My results were not always consistent with these hypotheses, however. Although the growth of hosts paired with heterologous symbionts could keep pace with hosts paired with homologous symbionts, cellular changes revealed that the host was typically in a state of stress, and suggested that host and symbiont were only capable of limited change over the timeframes used in these experiments, i.e. a maximum of 10 months after initial colonisation. Further work with coral species, including more host generalist and specialist species, is required to better predict the future directions of coral reefs in response to our changing climate.

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Supplementary data

The following supplementary data can be found below:

Figure S1 Enrichment of ¹³carbon and ¹⁵nitrogen within the symbiont fraction established via bulk isotope-ratio mass spectrometry (IRMS) in *Aiptasia* in symbiosis with different species of symbionts (*Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*), after four and ten months in symbiosis, having been maintained at control conditions or exposed to repetitive heat stress (RHS) for the final six months (Chapter 4).

Other supplementary data can be found at https://github.com/BobbyLust/Thesis_Supplementary and contains the following information:

Table S1 A complete list of differentially expressed proteins in *Aiptasia* in symbiosis with different symbiont species (*Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*) in response to heat stress (Chapter 2).

Table S2 A complete list of differentially expressed proteins between *Aiptasia* in symbiosis with different symbiont species (*Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*) under control conditions and under heat stress (Chapter 2).

Table S3 A complete list of differentially expressed proteins in *Aiptasia* in symbiosis with different symbiont species (*Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*) over time; from start (i.e. three months in symbiosis) to end (i.e. seven months in symbiosis), when maintained either under control conditions or exposed to repetitive heat stress (Chapter 3).

Table S4 A complete table of differentially expressed proteins and their log₂ fold-changes between *Aiptasia* in symbiosis with different symbiont species (*Breviolum minutum*, *Durusdinium trenchii*, or *Symbiodinium microadriaticum*), after seven months in symbiosis. Anemones were exposed to either control conditions or repetitive heat stress (RHS) for the final four months (Chapter 3).

Figure S1 Symbiont (A) carbon and (B) nitrogen enrichment ($\pm$ SE) for symbiont tissue, measured using bulk isotope-ratio mass spectrometry ($n = 5$), for *Aiptasia* in symbiosis with different species of symbionts (i.e., *Breviolum minutum*, *Durisdinium trenchii*, or *Symbiodinium microadriaticum*), at the different time-points and treatments, i.e. start: four months after initial colonisation under control conditions; end: ten months after initial colonisation, having either been maintained under control conditions (con), or exposed to repetitive heat stress (RHS) during the last six months (three stress events consisting of ramping to 32 °C, held for three days, followed by a six-week recovery period).