Coral reef symbioses under a changing climate: an integrative approach

Thesis submitted by

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AUTHORSHIP DECLARATION

The chapters constituting this thesis are based on four manuscripts; Chapter 2 is published in *Marine Biology*, Chapter 3 is published in *Molecular Ecology*; Chapter 4 and 5 are in preparation to be submitted for publication.

Chapter	**Publication**	**Author contributions**
1	General Introduction	EM wrote the chapter; PWL, DB and NSW revised it.
2	Microbiome-mediated mechanisms contributing to the environmental tolerance of reef invertebrate species	EM performed the literature search and wrote the first draft of the manuscript. All authors contributed to the study concept and reviewed the manuscript.
3	Life-stage specificity and cross-generational climate effects on the microbiome of a tropical sea urchin (Echinodermata: Echinoidea)	NSW, SU and EM designed the research. SU and FP performed the research. EM collected the samples, performed laboratory work and analysed the data. EMM assisted with the statistical analyses. EM wrote the manuscript and generated the figures. PWL, DGB and NSW revised the manuscript and made substantial contributions to its intellectual content.
4	Destabilization of mutualistic interactions shapes the early heat stress response of the coral holobiont	EM, NSW, DGB and PWL conceived and designed the experiment. EM and NSW provided funding for the experiment. EM and JYQL performed the experiment. EM performed the laboratory work. EM analysed the data, with assistance of PWL, PB and MT. EM wrote the original draft of the manuscript, and NR, PWL and DGB made substantial contributions to its form.
5	Integrating host, Symbiodiniaceae and host responses to heat stress in a tropical sponge symbiosis	EM, NSW, DGB and PWL conceived and designed the experiment. EM and NSW provided funding for the experiment. EM performed the experiment, laboratory work and analysed the data. EM wrote the original draft of the manuscript, and PWL, DGB and NSW made substantial contributions to its form.
6	General Discussion	EM wrote the chapter; PWL and DB revised it.

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GENERAL ABSTRACT

The ecological success of many coral reef invertebrates relies on their metabolic exchanges with microbial symbionts. These essential host-microbe relationships, however, can be altered under increasing environmental pressures, with climate change posing one of the major threats to reef holobiont (i.e. host and symbiont) health. Understanding how future climate will shape host-symbiont dynamics, and its consequences on coral reef ecosystem functioning, is therefore critical for developing and applying effective reef conservation strategies. In this thesis, I explored the stability and molecular mechanisms underpinning host-symbiont interactions within coral reef invertebrates under a changing climate, using integrated analyses that combine physiological and omics-based tools (16S rRNA gene sequencing and transcriptomics). Through an extensive literature search on established model systems, I proposed direct and indirect microbiome-mediated mechanisms potentially beneficial for host tolerance under climate stressors, with a particular focus on nutrient regulation, oxidative stress and host defensive mechanisms. As emerging evidence suggests transgenerational effects of climate change may also influence host-microbe dynamics, I experimentally tested whether parental exposure to climate conditions may alter offspring microbiome in a coral reef species (sea urchin). Although microbial specificity within each life stage was maintained across urchin generations, parental exposure to climate treatments influenced the offspring microbiome, demonstrating that climate cross-generational effects can contribute to the restructuring of the microbial community. My thesis findings reinforce the importance of considering host-microbe interactions in coral reef conservation initiatives to generate realistic predictions of reef ecosystem health under future climate. In addition to prokaryotic microbial communities, many reef species are also associated with Symbiodiniaceae, which can strongly influence their host thermotolerance. To identify the key metabolic processes that can impact holobiont survival, I have explored how increasing temperatures affect these mutualistic interactions in two reef taxa, the scleractinian coral *Porites lutea* and the sponge *Cliona orientalis*, through an integrative approach. Through host and Symbiodiniaceae transcriptomic analyses combined with physiological measures, I was able to identify an altered exchange of limited nutrients as a critical factor underpinning the initial destabilization of the symbiosis in both species. While the alteration in the availability of both carbon and nitrogen was associated with the destabilisation of the coral symbiosis, only carbon limitation was altered within mutualistic interactions between the host sponge and its associated symbionts. Given the sponge *C. orientalis* showed a higher tolerance to high temperatures compared to the coral species, differences in symbiotic nutrient cycling may underlie the distinct thermotolerance of these two ecologically important reef taxa. In addition, microbial community changes coincided with the breakdown of the symbiosis in the coral species, but not in the sponge, which maintained a stable microbial community over increasing temperature, highlighting how multiple members of the holobiont can contribute to stability during thermal stress responses, and confirming microbial responses to heat stress are species-specific within reef invertebrates taxa. Overall, the work

presented in this thesis largely advance the current state of knowledge within coral reef symbioses, and demonstrates how transgenerational effects, species specificity and host-symbiotic interactions can all contribute to response to increasing climate conditions. A greater understanding of how these complex interactions manifest within reef invertebrates is invaluable for targeting reef conservation and intervention strategies into the future.

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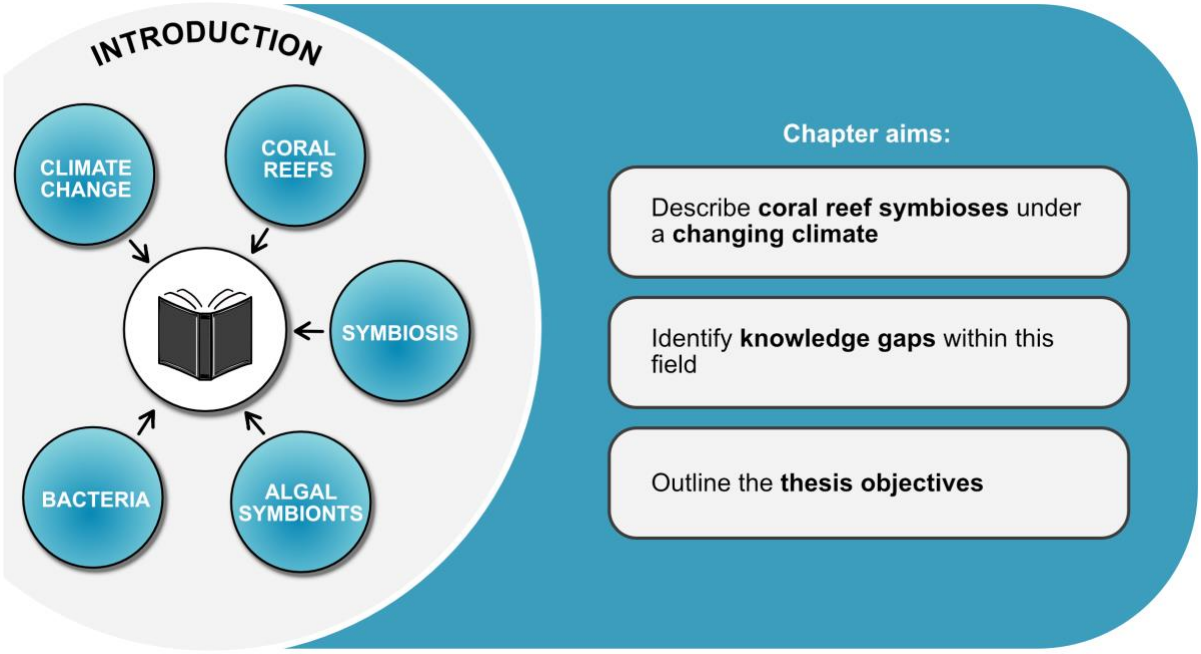
CHAPTER 1 : GENERAL INTRODUCTION



General introduction

Coral reef symbioses

Symbiosis is a major driver of the complexity of life on earth, shaping organisms' biological adaptation and ecological diversification (Henry et al., 2021; Joy, 2013; Moran, 2007). Given the ecological success of many mutualistic interactions, highly specialized symbioses have arisen in both terrestrial and aquatic environments among evolutionary distant organisms. The bidirectional nutrient exchange observed in some of these intimate associations can represent a significant ecological advantage. For example, aphids are associated with the endosymbiotic bacteria *Buchnera*, forming a highly integrated metabolism where both partners contribute to the production of essential amino acids. This is particularly important for the host as it compensates for the aphid's limited access to these compounds through its phloem sap-based diet (Hansen & Moran, 2011). In freshwater systems, the association between *Hydra* and algae of the genus *Chlorella* ensures host energy reserves are maintained via the transfer of photosynthetically derived carbon from the algae to the host, and, in turn, host-biosynthesized glutamine is utilized by *Chlorella* as a primary nitrogen source (Hamada et al., 2018). To maintain symbiotic partner fidelity across host generations, symbionts can be acquired through vertical (i.e. parental) or horizontal (i.e. environmental) transmission (Hartmann et al., 2017; Webster & Reusch, 2017). In vertical transmission, symbionts are heritable and generally taken up from maternal eggs (Engelberts et al., 2022); while horizontal transmission relies on host-symbiont mediated mechanisms for recognition and uptake of specific symbionts from the surrounding environment (Davy et al., 2012; Nyholm & McFall-Ngai, 2021). In the highly specific squid-*Vibrio* association, *Vibrio fisheri* colonization involves multiple biochemical mechanisms occurring in both host and symbiont to enable the initial engagement, with the symbiosis maintained over time via physiological and biochemical communication between the two partners (Nyholm & McFall-Ngai, 2021; Visick et al., 2021). Given the metabolic co-dependency and partner fidelity frequently observed between multicellular host and associated microbial symbionts, there has been ongoing debate whether to consider the holobiont (i.e. host and associated symbionts) as a distinct biological entity governed by natural selection (Douglas & Werren, 2016; Rosenberg & Zilber-Rosenberg, 2018).

In the holobiont, the integrated metabolism between the symbiotic partners can enable the multicellular host to expand into new ecological niches (Joy, 2013). One prominent example is represented by coral – Symbiodiniaceae associations, where endosymbiotic dinoflagellates (Symbiodiniaceae) provide their coral host with critical nutrients that allow the coral to thrive in tropical oceans, which are highly oligotrophic systems. Specifically, Symbiodiniaceae support 95% of the coral metabolism through the provision of carbon-based compounds (i.e. glucose), in return for nitrogen-based compounds and

carbon dioxide (CO_2) (Cui et al., 2023; Muscatine & Porter, 1977; Thies et al., 2022). The metabolic interdependency between reef-forming corals and symbionts constitutes the foundation of coral reef ecosystems, accounting for greater than 30% of total marine biodiversity (Fisher et al., 2015). However, the family Symbiodiniaceae is genetically diverse, and different symbiont genera and species provide the coral host distinct physiological advantages (LaJeunesse et al., 2018; Stat et al., 2008; Suggett et al., 2017). The association with *Cladocopium* sp., for example, appears to be more beneficial for the coral host than *Symbiodinium* sp., as *in vitro* release of carbon was found to be higher in this Symbiodiniaceae genus (Stat et al., 2008). Although most of the literature focusses on cnidarian – algal symbiosis, Symbiodiniaceae are also associated with other reef species, including foraminifera, bivalve molluscs (giant clams) and sponges of the Clionaidae family, where they participate in C and N assimilation (Achlatis et al., 2018; Mies et al., 2017). The obligate symbiosis between Symbiodiniaceae and multiple heterotrophic hosts demonstrates the critical role of these dinoflagellates for reef ecosystem functioning.

While members of the Symbiodiniaceae have developed interdependent relationship with specific reef building organisms, a broader range of coral reef invertebrates are associated with other microeukaryotes, Bacteria, Archaea and virus, constituting a highly complex microbial community. Prokaryotic microbial (i.e. 'microbes' hereafter) associations with reef invertebrates are often highly diverse, and contribute to host development and metabolism through the provision of essential compounds and metabolic pathways (Robbins et al., 2021; Song et al., 2021; van Oppen & Blackall, 2019). In the sponge *Amphimedon queenslandica*, for example, the biosynthesis of arginine by specific Proteobacteria is essential for initiating sponge larval settlement, and thus, for completing the host's life cycle (Song et al., 2021). Microbes also provide their hosts with critical compounds for holobiont fitness; the production of B-vitamins and essential amino acids, for example, are postulated to fuel the host metabolism in specific sponge and coral species (Robbins et al., 2019; Thomas et al., 2010). Determining the functional role of multiple holobiont members within the holobiont framework is therefore critical for understanding the key mechanisms underpinning holobiont health. Yet, few studies have attempted to characterize reef species functioning at the holobiont level due to technological challenges associated with disentangling the functional capabilities of each individual member.

Reef symbiosis under a changing climate

Anthropogenic climate change is driving a degradation of coral reef ecosystems worldwide (Hughes et al., 2017a). Climate stressors (e.g. high temperature and pCO_2 levels) affect the physiology of reef organisms and their associated symbionts, which frequently require metabolic adjustments to cope with the changing environmental conditions. The resulting metabolic changes, however, may impair the

nutritional balance underpinning functional symbiosis, shifting symbiosis from mutualistic towards more commensal relationships (Baker et al., 2018). If heat stress is severe, a complete breakdown of symbiosis may occur, resulting in widespread bleaching or mortality events (Hoegh-Guldberg, 1999). An increase in frequency and severity of coral bleaching events has been observed in recent years due to heat stress anomalies (Hughes et al., 2021; Oliver et al., 2018), with model predictions suggesting a further increase in bleaching occurrences as global warming continues (McWhorter et al., 2022). This climate-driven breakdown of the coral symbiosis translates into a decrease in energy supply for the coral metabolism, resulting in lower coral growth, fecundity and fitness (Gold & Palumbi, 2018; Hughes et al., 2019a). Under prolonged heat stress periods, coral starvation leads to coral mass mortality events which, in conjunction with a decline in larval recruitment (Hughes et al., 2018a, 2019a), impact the reef ecosystem as a whole and all interdependent neighbouring systems (e.g. seagrass meadows (Ainsworth & Mumby, 2015; Hughes et al., 2017a; Saunders et al., 2014)). Increasing pCO_2 levels (i.e. ocean acidification) further alters coral reefs as the lower calcium carbonate saturation state can impact calcifying organisms, such as reef building corals and echinoderms, resulting in reduced growth rates (Doney et al., 2009). Reef conservation strategies require an in-depth understanding of reef species responses to a changing climate (Duarte et al., 2020; Kenkel & Wright, 2022; van Woesik et al., 2022).

To generate realistic predictions of the biological and ecological consequences of future climate on coral reef systems, organisms' responses should be assessed at the holobiont level. From the host perspective, genetic adaptation and environmental acclimatisation may allow coral reef species to persist under changing environmental conditions (Barshis et al., 2013; Bay & Palumbi, 2014; Kenkel & Matz, 2016; Torda et al., 2017). While genetic adaptation acts over relatively long-time scales, acclimatisation can occur within one generation through phenotypic changes (Bellantuono et al., 2012; Savary et al., 2021). For instance, corals have shown signs of acclimatisation to high temperatures through changes in gene expression following short-term exposure to heat stress (Bay & Palumbi, 2015), which may help corals tolerate altered environmental conditions if the required phenotypic adjustments are within the physiological thresholds of the species. It has also been demonstrated that exposure to altered environmental conditions in one generation can sometime influence the performance of the next generation (transgenerational acclimatisation) (Putnam & Gates, 2015; Uthicke et al., 2021). However, the mechanisms underlying acclimatisation within and between generations are poorly understood among coral reef species (Torda et al., 2017), further confounded by the complex interactions among host and symbiont members.

The holobiont is a highly dynamic system, and environmental conditions can influence both Symbiodiniaceae and microbial community structures. Within the symbiont community, shuffling (i.e. changes in relative abundance) and switching (i.e. acquisition of new symbiont) events may positively contribute to host fitness if symbiotic restructuring provides beneficial traits under the altered

environmental conditions (Boulotte et al., 2016; Cunning et al., 2018; Quigley et al., 2022; Webster & Reusch, 2017). For example, shuffling from a dominance of *Cladocopium* sp. towards *Durusdinium* sp. was shown to confer a higher thermotolerance to the coral *Acropora millepora*, suggesting the thermotolerant *Durusdinium* sp. may provide an advantage to its coral host in warming oceans (Berkelmans & Van Oppen, 2006). Similarly, it has been recently inferred that microbial plasticity may also influence host environmental tolerance (Webster & Reusch, 2017) through a wide range of mechanisms enhancing host metabolism and immunity (Chapter 2, published as Marangon et al., 2021). It is not yet known to what extent microbes may contribute to host thermotolerance in coral reef species, but recent findings confirm the potential of microbiome-mediated acclimatisation in cnidarians (Avila-Magaña et al., 2021; Baldassarre et al., 2022). Parental exposure to altered environmental conditions may also influence the offspring symbiont community, and, if beneficial symbionts are transmitted between host generations, could even facilitate transgenerational acclimatisation (Baldassarre et al., 2022; Luter et al., 2020; Quigley et al., 2019; Webster & Reusch, 2017). To predict reef ecosystems functioning under climate change, it is critical to understand whether the restructuring of symbiont communities will shift towards beneficial or detrimental associations, and if transgenerational exposure to altered environmental conditions may affect symbiotic responses.

Understanding holobiont response to environmental stress through an integrative approach

When elucidating reef invertebrate responses to future climate, most functional studies focus on either single holobiont members or on host-Symbiodiniaceae interactions (Barshis et al., 2013; Cunning & Baker, 2020; Pinzón et al., 2015), with several Symbiodiniaceae studies performed on *ex hospite* cultures (Chakravarti et al., 2020; Levin et al., 2016; Stat et al., 2008). Despite the important functional insights derived from these culture-based studies, it is now clear that properties of organisms living in symbiosis are distinct to biological traits governing their free-living state (Bellantuono et al., 2019; Mohamed et al., 2020; Pogoreutz et al., 2022). For example, Symbiodiniaceae-host associations are governed by a fine equilibrium, with downregulation of symbiosis-related genes and alterations in nitrogen / carbon availability representing early signs of heat stress (Cleves et al., 2020a; Rädecker et al., 2021; Xiang et al., 2020); however, microbes are likely involved in the maintenance of this equilibrium given their exchange of essential nutrients with both host and Symbiodiniaceae (Maire et al., 2021; Matthews et al., 2020; Robbins et al., 2019). When integrating these three holobiont compartments, Avila-Magaña et al. (2021) demonstrated that microbial communities may provide alternative metabolic pathways under heat stress, which may support holobiont tolerance to changing conditions. Characterizing this integrated holobiont metabolism can therefore advance our

understanding on the mechanisms underlying reef holobiont functioning and climate-driven symbiosis breakdown.

Thesis aims

In my PhD thesis, I used an experimental approach to explore the effects of future climate on complex reef symbiotic interactions (Fig. 1.1). Firstly, I reviewed the current state of knowledge about potential microbiome-mediated mechanisms beneficial for the environmental tolerance of reef invertebrate species (Chapter 2). Next, I tested the potential for microbiome-mediated acclimatisation in a model reef invertebrate species (sea urchin) exposed to predicted future climate conditions across host life stages and multiple generations (Chapter 3). Thirdly, I characterized host-Symbiodiniaceae-microbe integrated functional responses to increasing temperatures in two ecologically important reef invertebrate species to identify the mechanisms underlying symbiotic breakdown under thermal stress (Chapters 4 and 5). Finally, I discussed the ecological relevance of my findings for coral reef conservation strategies, identifying critical and contemporary research questions within this field and suggesting future research directions (Chapter 6).

Together, my findings 1) confirm the critical role of microbes in host environmental tolerance, 2) suggest parental exposure to climate stressors can influence the microbiome of the offspring, and 3) reveal thermal stress responses are host-specific, varying also among holobiont members, with host-Symbiodiniaceae functional changes underpinning the symbiosis breakdown under high temperatures. My thesis highlights the importance of cross-generational studies as well as multi-omics approaches for generating realistic predictions on the effects of future climate on reef ecosystem functioning.

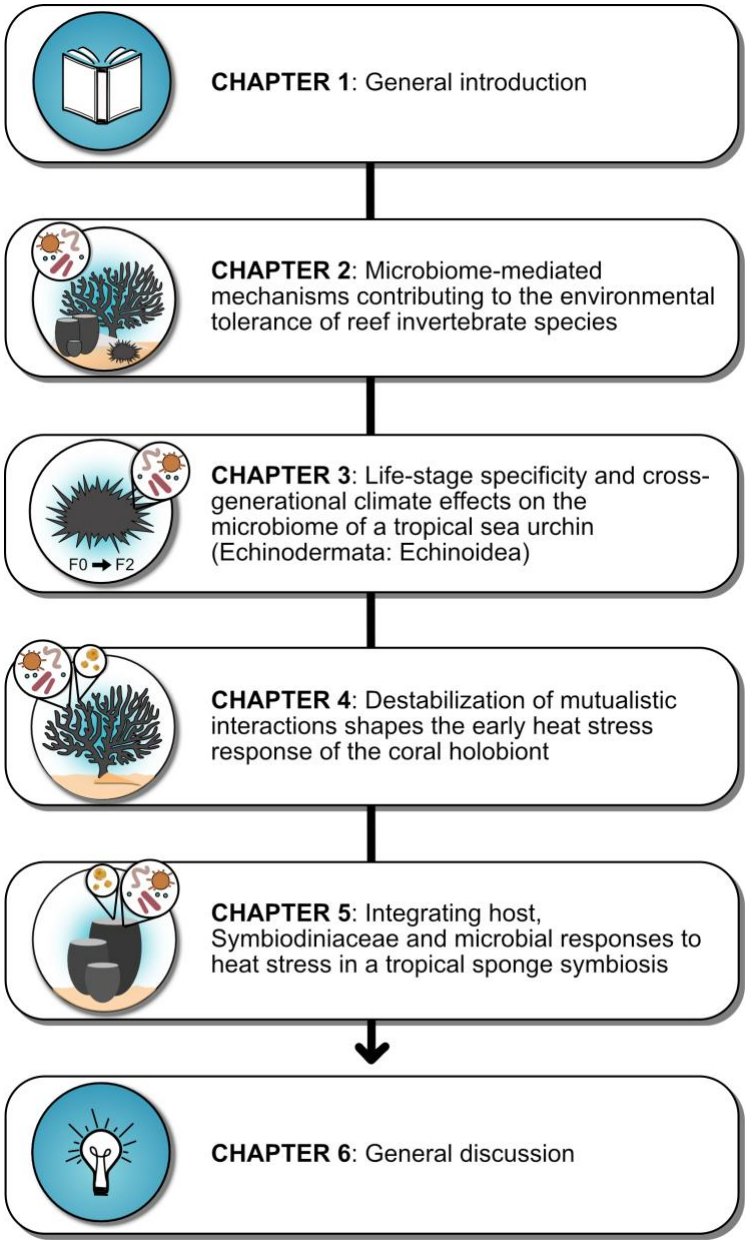
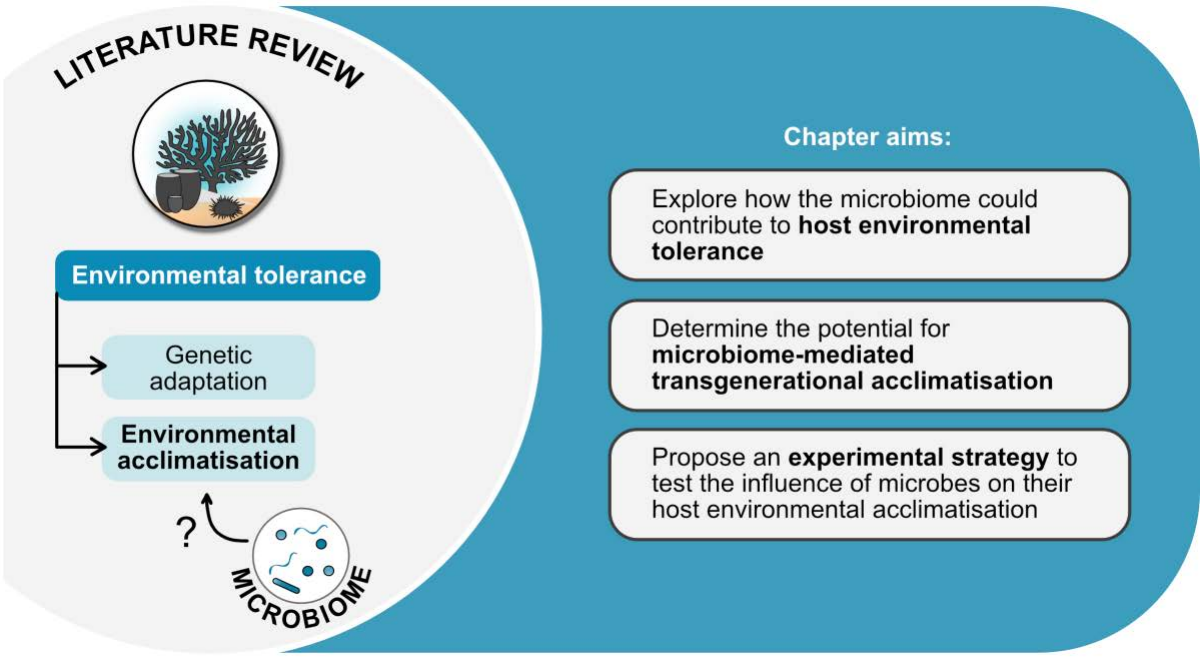


Fig. 1.1. Thesis outline.

CHAPTER 2 : MICROBIOME-MEDIATED MECHANISMS CONTRIBUTING TO THE ENVIRONMENTAL TOLERANCE OF REEF INVERTEBRATE SPECIES



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Microbiome-mediated mechanisms contributing to the environmental tolerance of reef invertebrate species

Abstract

Coral reefs globally are increasingly impacted by climate change. High temperature and pCO_2 levels disrupt multiple physiological and biochemical pathways in marine organisms, often leading to disease and mortality in sensitive reef species. Host-associated microbes contribute critical functions that underpin host health, and environmentally induced changes in microbial communities represent a potential source for new metabolic features within holobiont systems. However, whether the acquisition of new beneficial microbial functions contributes to environmental acclimatisation of reef species is currently unknown. Using extensively studied model systems, we identify potential direct and indirect microbiome-mediated mechanisms that may contribute to environmental acclimatisation in reef invertebrate species. These mechanisms include increasing energy metabolism in the host, reduction of oxidative stress, regulation of nutrients in host cells, and increased pathogen resistance. We also propose a robust experimental strategy to test how microbial metabolic pathways may facilitate environmental acclimatisation of reef taxa. Understanding the mechanisms of microbiome-mediated acclimatisation and the timescales over which it can occur will be critical for predicting reef ecosystem dynamics under future climate scenarios and applying effective reef conservation strategies.

Introduction

Coral reef ecosystems are declining around the world due to cumulative impacts from local and global pressures including overfishing, pollution and climate change (Carpenter et al., 2008; Hoegh-Guldberg et al., 2007; Hughes et al., 2018a; Zaneveld et al., 2016). For instance, increasing oceanic pCO_2 levels and sea surface temperatures affect marine organisms and ultimately drive shifts in ecosystem function (Doney et al., 2009; Hughes et al., 2017a; IPCC, 2014), leading to a variety of ecological, social and economic impacts (Cardinale et al., 2012; Hoegh-Guldberg et al., 2017; Moberg & Folke, 1999). Ocean acidification (OA) can reduce calcification rates of marine species including reef building corals (Doney et al., 2009), resulting in increased rates of reef erosion. Corals are also threatened by ocean warming (OW) which drives the expulsion of endosymbiotic dinoflagellates (Symbiodiniaceae) from tissues when seawater temperatures exceed the hosts thermal threshold (Hughes et al., 2018b, 2019b; Suggett & Smith, 2020). A global bleaching event between 2014-2017 resulted in the most extensive mortality of corals and damage to reefs ever recorded (Eakin et al., 2019). On the Great Barrier Reef (GBR), multiple bleaching events have occurred over the past three decades, with the extreme 2016 event resulting in coral bleaching on 91.1% of the surveyed reefs (Hughes et al., 2017b) and substantial coral mortality (Hughes et al., 2018b), which was exacerbated by further mass bleaching in 2017 (Hughes et

al., 2019b). The increasing severity and frequency of bleaching events is limiting the ability of many coral species to recover and altering the composition of coral reef assemblages (Hughes et al., 2017b, 2018b, 2019a). The persistence of coral reefs will depend on the ability of ecologically important reef species, in particular sensitive species, to rapidly increase their tolerance to OW and OA.

Genetic adaptation and environmental acclimatisation (see Box 2.1 for a definition of terms) can both increase the tolerance of an organism to environmental changes. Genetic adaptation increases organismal fitness via acquisition of beneficial genetic modifications between generations (Box 2.1). For example, a recent comparative genomic study of sensitive and tolerant Atlantic killifish populations revealed that changes in allele frequencies and deletion of genes involved in the aryl hydrocarbon receptor signalling pathway contributed to rapid adaptation of populations to high levels of toxic pollutants (Reid et al., 2016). While adaptation is based on genetic changes, environmental acclimatisation enhances organismal tolerance to environmental stress within a generation via phenotypic modifications (Box 2.1). Environmental acclimatisation can arise due to modifications in gene expression (Barshis et al., 2013; Dixon et al., 2018; Zhang et al., 2018), and/or changes in host-associated microbial communities (Bang et al., 2018; Berkelmans & Van Oppen, 2006; Nadeem et al., 2014; Webster & Reusch, 2017; Wilkins et al., 2019). Plants are well-established models for studying climate-induced acclimatisation. Changes in plant gene expression have been reported to facilitate environmental acclimatisation to extreme conditions, such as high salinity and low temperatures (Park et al., 2018; Zhang et al., 2018). In *Arabidopsis*, changes in chromatin status contributes to the regulation of cold response genes, which are responsible for cold tolerance (Park et al., 2018). Plant-associated microbial communities also enable their host to maintain normal growth rates under environmental stress via provision of essential nutrients and mediation of host-produced harmful compounds (Batool et al., 2020; Madhaiyan et al., 2006; Mayak et al., 2004; Sukweenadhi et al., 2018; Upadhyay & Singh, 2015). Environmental acclimatisation can persist between generations when these non-genetic beneficial traits are transmitted to offspring (i.e. transgenerational acclimatisation, Box 2.1), as described in several terrestrial model species (Cavalli & Paro, 1998; Klosin et al., 2017; Mirouze & Paszkowski, 2011; Wei et al., 2014). In the nematode *Caenorhabditis elegans*, for example, temperature-induced alterations in gene expression can be inherited via both oocytes and sperm across at least 14 generations (Klosin et al., 2017). In the reef environment, a growing body of literature is demonstrating that genetic adaptation and environmental acclimatisation can enhance thermal tolerance in some reef species, acting on both the host and its associated symbionts (Bay & Palumbi, 2014; Buerger et al., 2020; Dixon et al., 2018; Drury, 2020; Howells et al., 2016; Moran, 2007; Palumbi et al., 2014; Putnam et al., 2016; Putnam & Gates, 2015; Torda et al., 2017). Understanding whether genetic adaptation and/or environmental acclimatisation can play a role in increasing tolerance of sensitive reef species to OW and OA is critical for predicting coral reef persistence, as slight increases

in thermal tolerance by the end of the century may reduce the risk of extinction faced by many reef taxa (Drury, 2020; Hughes et al., 2019b; Logan et al., 2014).

Microorganisms are ubiquitously associated with coral reef invertebrates, where they play a crucial role in host health (Bourne et al., 2016; Webster & Thomas, 2016). Environmental perturbations can alter the composition of host-associated microbes, with negative or positive consequences for the host (Ainsworth et al., 2010; Ainsworth & Gates, 2016; Botté et al., 2019; Glasl et al., 2020; Pita et al., 2018). The onset of disease has been the primary focus of research investigating the effects of environmental stressors on the microbiome of coral reef species (Bourne et al., 2009; Egan & Gardiner, 2016; Thurber et al., 2009; Zaneveld et al., 2016). However, changes in host-associated microbiomes has recently been proposed as a mechanism that could contribute to environmental acclimatisation in reef taxa (Webster & Reusch, 2017). Given the role of bacteria and archaea in providing beneficial compounds to the host, and the ability of the microbiome to rapidly change under altered environmental conditions (McFall-Ngai et al., 2013; Reshef et al., 2006; Voolstra & Ziegler, 2020), a shift in the microbiome towards a species assemblage that supports host fitness could underpin rapid host acclimatisation. The potential for these microbial changes was initially defined as the Coral Probiotic Hypothesis, which postulated that variations in microbial communities under altered environmental conditions generates the most advantageous host-microorganism relationship (Reshef et al., 2006). The well-studied interactions between Symbiodiniaceae and corals exemplify the potential for genetically and functionally diverse eukaryotic microbes to enhance host tolerance to environmental stressors (Baker et al., 2004; Berkelmans & Van Oppen, 2006; Buerger et al., 2020; Howells et al., 2016). In a transplantation experiment using the coral *Acropora millepora* for example, Berkelmans and van Oppen (2006) demonstrated that bleaching thresholds of the host can increase up to 1-1.5˚C via community shifts towards the heat-tolerant clade *Durusdinium*. It is possible that, like Symbiodiniaceae, changes in the microbiome may contribute to environmental acclimatisation of some marine invertebrates, although few studies have explicitly tested this (Webster & Reusch, 2017). Only recently, experimental manipulation of the coral microbiome via inoculation of beneficial bacteria was shown to mitigate bleaching responses in the coral *Pocillopora damicornis* (Rosado et al., 2019), confirming the potential for microbes to enhance host thermal tolerance. Despite the possibility for microbiome-mediated acclimatisation, it is important to consider that changes in the microbiome could incur costs or trade-offs between host tolerance and fitness. For instance, within the coral-Symbiodiniaceae symbiosis, corals that associate with heat-tolerant symbiont clades had increased bleaching tolerance but also exhibited reduced fitness evidenced by decreased growth rates and eggs size (Jones & Berkelmans, 2011; Little et al., 2004; Stat & Gates, 2011). Thus, much more empirical data is needed to test whether changes in microbial community composition and function can contribute to increased environmental tolerance of reef taxa.

In this review we describe the functional roles of microbes in reef invertebrate species (hereafter 'reef species'), explore how the microbiome could contribute to host environmental tolerance and determine the potential for microbiome-mediated transgenerational acclimatisation (MMTA) in key reef taxa. Finally, we propose a robust experimental strategy to explicitly test microbial contributions to host environmental tolerance and highlight the importance of applying an integrated approach (-omics, advanced imaging and stable isotope analyses) to reveal the contribution of microbes to environmental acclimatisation in coral reef species.

Box 2.1. Glossary.

Genetic adaptation. Genetic adaptation acts at the population level through changes in allele frequencies between generations, resulting in modified phenotypes that increase population fitness. Organisms can adapt to environmental stress via two mechanisms: novel mutations and standing genetic variation. The former enables adaptation via the introduction of new beneficial genetic variants, while the latter operates via recombination and redistribution of pre-existing genetic variants. As adaptation via standing genetic variation can take place over short timescales, utilising existing genetic diversity within populations may enable species with long generation times, such as corals, to respond to rapid environmental changes.

Environmental acclimatisation. Environmental acclimatisation describes the ability of organisms to respond rapidly to abiotic stressors in the field via physiological adjustment (non-genetic changes; e.g. changes in gene expression). Evidence of rapid acclimatisation has been described in some reef taxa, suggesting that environmental acclimatisation may provide a survival buffer while genetic adaptation takes place. In host-microbe systems, it has been proposed that microbial community changes can facilitate host acclimatisation (i.e. **microbiome-mediated acclimatisation**).

Transgenerational acclimatisation. Transgenerational acclimatisation refers to a type of environmental acclimatisation, where non-genetically-determined acquired beneficial traits are transmitted to new generations. Mechanisms contributing to transgenerational acclimatisation include provision of nutrients via gametes or to embryos, transmission of somatic factors, hormones or proteins, and transmission of epigenetic markers (heritable changes in gene expression). In host-microbe systems, **microbiome-mediated transgenerational acclimatisation** refers to the acquisition of beneficial microbial community changes between generations. While some reef studies have revealed the potential for transgenerational acclimatisation, knowledge of the mechanisms underpinning this process in reef species is extremely limited.

The microbiome of key reef species: the functional role of bacteria and archaea

The association between microbes and host constitutes a system known as a holobiont (Bourne et al., 2009; McFall-Ngai et al., 2013). In reef holobionts, microbial associations are often host species-specific, and distinct from the microbes present in the surrounding environment (Botté et al., 2020; Bourne et al., 2016; Webster & Thomas, 2016). The majority of studies assessing microbial interactions in invertebrate reef taxa have focused on coral and sponge species, although more recently, microbial surveys have extended to other key reef organisms, including echinoderms, molluscs, foraminifera and ascidians (Botté et al., 2020; Dubé et al., 2019; Erwin et al., 2014; Hakim et al., 2016; Høj et al., 2018; Jackson et al., 2018; Morrow et al., 2018). Corals and sponges frequently harbour highly diverse microbial communities (Blackall et al., 2015; Thomas et al., 2016), with considerable variation in intra-species stability across taxa. Microbial associations in sponges tend to exhibit high intra-species stability and are largely conserved across temporal and geographic scales (Cárdenas et al., 2014; Erwin et al., 2012; Reveillaud et al., 2014; Thomas et al., 2016; Webster & Thomas, 2016), whereas they are more variable in corals (Bourne et al., 2016; Glasl et al., 2019; Li et al., 2014; Littman et al., 2009; Pantos et al., 2015). In corals, bacteria are also naturally associated with Symbiodiniaceae, where they may play a role in Symbiodiniaceae metabolism and, in turn, holobiont health (Bernasconi et al., 2019a; Camp et al., 2020; Maire et al., 2021; Matthews et al., 2020; Motone et al., 2020).

Microbes inhabit a wide range of anatomic compartments within their host and microbial dynamics can vary across these distinct micro-habitats (Bourne et al., 2016; Glasl et al., 2016; Pernice et al., 2020; Tianero et al., 2019). For example, microbial communities in coral tissue tend to be more specific and stable than in coral mucus, which harbours transient microbial communities influenced by both host exudates and the surrounding seawater (Glasl et al., 2016). Not only does community composition vary among microhabitats, microbial functions are strictly linked to the location of microbes within the host (Fraune et al., 2015; Hakim et al., 2016; Hentschel et al., 2001; Liu et al., 2018a; Oliphant & Allen-Vercoe, 2019; Raina et al., 2016; Ritchie, 2006; Tianero et al., 2019) (see Fig. 2.1). Coral skeleton-associated microbes, for example, play an important role in bioerosion and nutrient cycling in this microhabitat with unique physiochemical characteristics (Pernice et al., 2020).

The putative functional role of microbes in host metabolism has recently been characterized in select reef taxa via metagenomic, metatranscriptomic and single cell studies. These studies have shown that bacteria and archaea are involved in a diverse array of metabolic pathways in the holobiont (Moitinho-Silva et al., 2017; Neave et al., 2017b; Rix et al., 2020; Robbins et al., 2019, 2021). The genomic potential for several carbon fixation pathways has been described in bacteria and archaea associated with sponges and corals (Engelberts et al., 2020; Glasl et al., 2020; Hallam et al., 2006; Moitinho-Silva et al., 2017; Robbins et al., 2019). A recent metagenomic study on the reef sponge *Ircinia ramosa*

identified that microbes could contribute to their hosts energy demands via three separate autotrophic carbon fixation pathways (Engelberts et al., 2020). Similarly, microbes can contribute to nitrogen metabolism within the host, with nitrogen fixing bacteria enhancing nitrogen availability for some corals (Pogoreutz et al., 2017; Rädecker et al., 2015) and ammonia oxidising archaea detoxifying sponge-excreted ammonia (Bayer et al., 2008; Engelberts et al., 2020; Moeller et al., 2019; Robbins et al., 2021; Zhang et al., 2019). Phosphate sequestration has recently been proposed as another important role of sponge-associated microbes, as bacterial derived phosphate granules can represent up to 40% of the total phosphorous in sponge tissue (Zhang et al., 2015). Microbes can also contribute to sulphur cycling in both corals (Raina et al., 2010; Robbins et al., 2019) and sponges (Gauthier et al., 2016; Hoffmann et al., 2005; Jensen et al., 2017; Lavy et al., 2018; Tian et al., 2014). It has been proposed that a metabolic interplay between sulphate-reducing and sulphur-oxidizing microbes mediates sulphur cycling in sponges (Gauthier et al., 2016; Hoffmann et al., 2005), and coral-associated bacteria, together with Symbiodiniaceae, are responsible for the catabolism of dimethylsulfoniopropionate (DMSP) (Robbins et al., 2019). Microbes are therefore fundamental contributors to holobiont health in reef systems.

The critical role of microbes in host metabolism has led to co-dependence between some reef hosts and their microorganisms. Microbes are postulated to provide their host with essential compounds that cannot be synthesized by their eukaryotic partner, such as B-vitamins and essential amino acids (Fan et al., 2012; Robbins et al., 2019; Thomas et al., 2010). As an example, the genome of the coral *Acropora digitifera* lacks an essential enzyme for cysteine biosynthesis, suggesting that this amino acid may be provided by coral-associated microorganisms (Shinzato et al., 2011). A recent genomic study on the coral *Porites lutea* further reveals the integrated metabolism between bacteria, archaea, Symbiodiniaceae and their coral host, illustrating that the interplay between all components of the holobiont underpins coral health (Robbins et al., 2019). Additional evidence for metabolic complementation is that some reef host-associated bacterial genomes are reduced in size and lack essential metabolic pathways including amino acid biosynthesis, glycolysis and tricarboxylic acid cycle (He et al., 2018; Tianero et al., 2019), but are enriched in genes required to establish and maintain symbiotic interactions (Burgsdorf et al., 2015; Fan et al., 2012; Gao et al., 2014; Thomas et al., 2010). Eukaryotic-like proteins (ELPs) are enriched and expressed in sponge-associated microbes, likely interfering with eukaryotic processes to avoid digestion (Díez-Vives et al., 2017; Jahn et al., 2019; Liu et al., 2012; Nguyen et al., 2014; Robbins et al., 2021). Many coral associated microbes are also enriched in ELPs that may facilitate their tissue associated symbiotic lifestyle (Robbins et al., 2019). It has also been suggested that sponges may be capable of actively recognizing and selecting specific microbes via immune responses (Yuen, 2016). This process could occur via upregulation of immune receptors that permit recognition of specific microbial molecular patterns (Yuen, 2016). Overall, these

studies highlight the critical role of microbes in host fitness, and the potentially co-dependent nature of these associations.

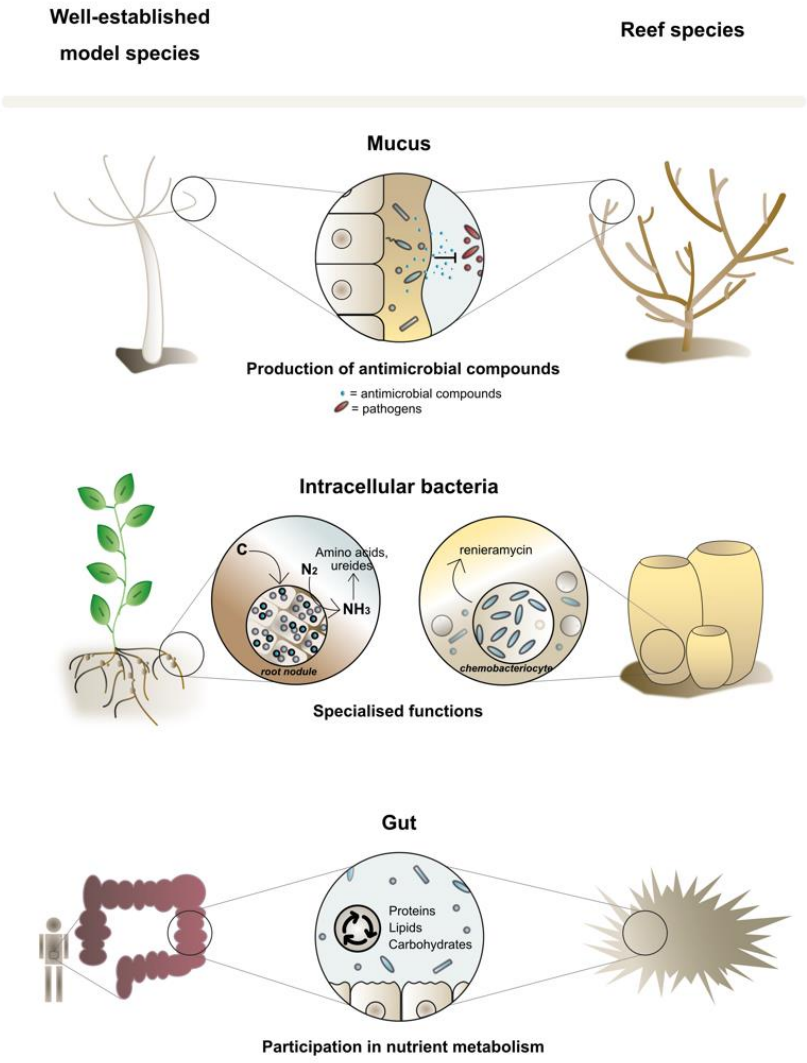


Fig. 2.1. Selected examples illustrating micro-habitat specificity: the location of microbes within the host (i.e. mucus, intracellular, gut) is linked to their functional role in the holobiont. In the mucus layer, antimicrobial compounds produced by microbial communities prevent the onset of diseases in *Hydra* and corals (Hentschel et al., 2001; Ritchie 2006; Fraune et al., 2015; Raina et al., 2016). Rhizobia located in root nodules of legumes can utilize atmospheric nitrogen to produce ammonium, which can be assimilated by their host (Liu et al., 2018a). Intracellular bacteria in *Haliclona* sponges are capable of synthesizing renieramycins, defence chemicals that may support to host health (Tianero et al., 2019). In the digestive systems of humans and echinoderms, microbes appear to participate in macronutrient metabolism (Hakim et al., 2016; Oliphant & Allen-Vercoe, 2019).

Contribution of microbes to the environmental tolerance of the host

Physiological tolerance to abiotic stressors varies widely among reef organisms. Many reef species live close to their thermal limits, with small increases in temperature above summer maxima triggering stress responses, and in some instances mortality (Hoegh-Guldberg, 1999; Hughes et al., 2017a; Webster et al., 2008). While several reef species are expected to experience reduced growth, reproduction and survival under conditions of OW predicted for 2100 (Hoegh-Guldberg et al., 2017), some taxa may be tolerant of these conditions. For example, in the 1998 bleaching event in Japan, branched coral species became locally extinct, while massive and encrusting corals survived (Loya et al., 2001). Similarly, responses to OA also vary between species. While most coral species are sensitive to high pCO_2, some species including the massive *Porites* spp. are abundant at volcanic CO_2 seeps where conditions reflect pCO_2 levels predicted for 2100 (Fabricius et al., 2011). Considering the abundance of reef species that are sensitive to projected future OW and OA, it is critical to determine whether their tolerance could be enhanced through processes of genetic adaptation and environmental acclimatisation (Box 2.1). While multiple host factors have been shown to contribute to these processes (reviewed in Drury, 2020; Torda et al., 2017), here we focus on microbial mechanisms facilitating tolerance of reef taxa to OW and OA. We hereafter use terms 'microbiome' and 'microbes' to specifically refer to the prokaryotic component (i.e. bacteria and archaea) of the host-associated microbial community.

Changes in the microbiome may facilitate acclimatisation of reef taxa to new environmental conditions. Microbial changes that benefit the host can come about through shifts in the abundance of specific microbial taxa (microbial shuffling), acquisition of new taxa from environmental pools (microbial switching), horizontal gene transfer (HGT) and/or changes in symbiont gene expression (Webster & Reusch, 2017). To date, most studies investigating microbiome-mediated acclimatisation in reef taxa have utilised 16S rRNA gene analysis, which only detects changes in the composition of the community (i.e. shuffling and switching). Under adverse conditions, microbes may enter an inactive state of dormancy (Lennon & Jones, 2011), which cannot be detected solely by 16S rRNA gene sequencing of DNA. Without direct empirical evidence of how microbial function changes with environmental conditions, the role of the microbiome in environmental acclimatisation of reef invertebrate species can only be inferred.

Changes in microbial community composition (shuffling and switching) have been documented under both OW and OA. Microbial shuffling was reported in the sponges *Coelocarteria singaporensis* and *Cinachyra* sp. inhabiting natural volcanic CO_2 seeps (Morrow et al., 2015). The pCO_2-tolerant sponges were 40-fold more abundant at the seep location than at a corresponding control site, and the higher sponge cover was correlated with an increased relative abundance of photosynthetic *Synechococcus*

(Morrow et al., 2015), which was assumed to provide additional nutritional benefits under high pCO_2. Another example is the coral *Fungia granulosa*, where reductions in photosynthesis and calcification were observed after short-term exposure to high salinity levels but the negative effects were reversed after long-term exposure, along with shifts in microbial communities (Röthig et al., 2016). Functional profiling based on bacterial taxonomy suggested that the microbial shift could facilitate increased potential for osmolytes, sulphur oxidation, and nitrogen fixation (Röthig et al., 2016). Microbial shuffling was also observed in corals reciprocally transplanted across thermally variable habitats, with variations in the microbiome linked to changes in host thermal tolerance (Ziegler et al., 2017). When coral colonies were exposed to short-term heat stress 17 months after transplantation, corals in the highly-variable warmer environment showed reduced bleaching responses and changes in taxonomy-based microbial functional potential that were independent of the habitat of origin, suggesting microbial changes as a potential acclimatisation mechanism (Ziegler et al., 2017). Finally, studies on echinoderm larvae exposed to changing environmental conditions suggest that shifts in microbial communities could also enhance host fitness (Carrier et al., 2018; Carrier & Reitzel, 2018). In terms of microbial switching, acquisition of new microbial taxa has been reported in several sponge species after exposure to environmental stressors such as high pCO_2, temperature, salinity and eutrophication (Luter et al., 2020; Ribes et al., 2016; Webster et al., 2011). The Mediterranean sponge *Dysidea avara*, for example, can maintain standard growth rates under high pCO_2 (Ribes et al., 2016). This higher tolerance to environmental stress was suggested to involve acquisition of novel microbes from the environment (Ribes et al., 2016), although a direct correlation was not demonstrated. The main limitation of community composition studies is that phylogenetically similar microbial taxa can undertake very distinct functions (Burke et al., 2011; Ranjan et al., 2016), hence investigating the microbiome from a functional genomic perspective will be a critical step for understanding the role played by microbes in host tolerance.

Microbial pathways that could contribute to microbiome mediated acclimatisation include the provision of beneficial compounds to the host (i.e. direct mechanisms) and/or disease prevention via inhibition of the onset of pathogens (i.e. indirect mechanisms) (Glick, 1995). These mechanisms have the capacity to reduce the negative effects of OW and OA on host physiology, enabling higher tolerance to climate stressors and, thus, contributing to environmental acclimatisation of reef taxa (Fig. 2.2). In organisms associated with Symbiodiniaceae, like corals, Symbiodiniaceae-bacteria interactions may also underpin host tolerance (Camp et al., 2020; Matthews et al., 2020).

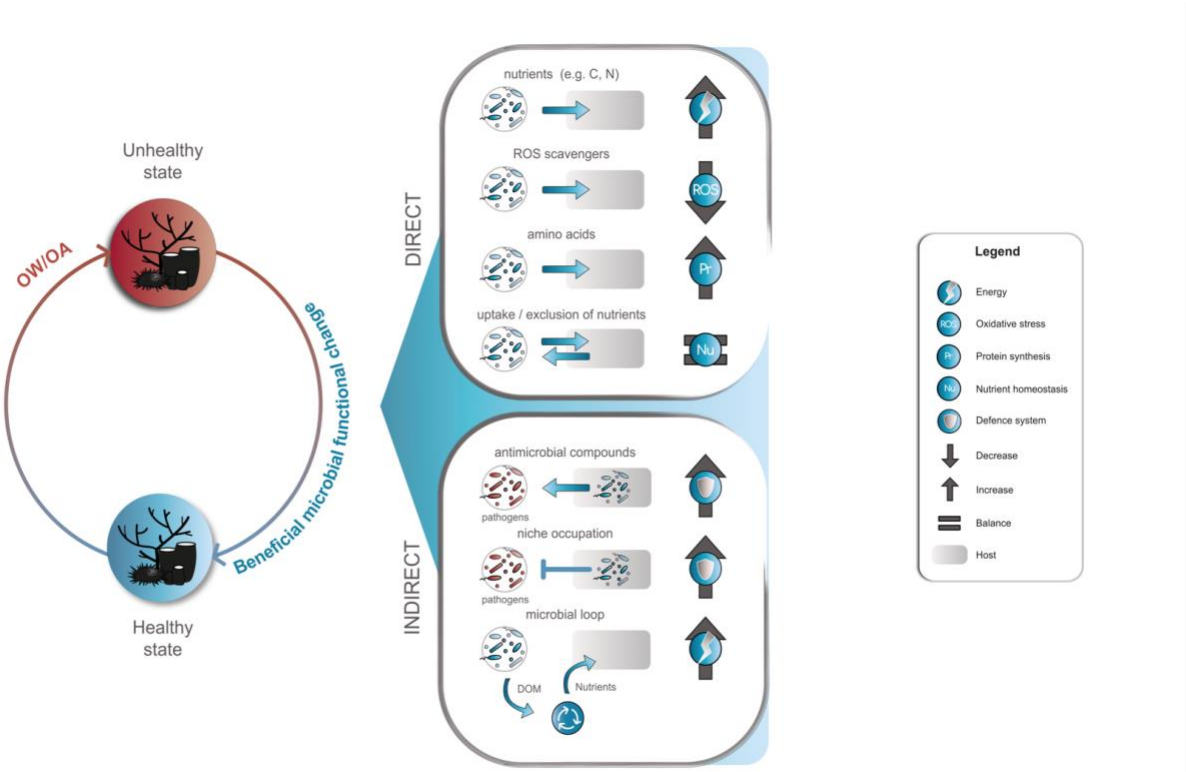


Fig. 2.2. Conceptual overview of the hypothetical contribution microbes could make to host health under altered environmental conditions. Direct and indirect microbiome-mediated mechanisms may alleviate OW/OA-driven detrimental changes in host physiological/biochemical pathways (detrimental changes based on Desalvo et al., 2008; Webster et al., 2013; Gullian Klanian & Terrats Preciat, 2017; Cornwall et al., 2018).

Direct mechanisms

In well studied model systems like plants, microbes can facilitate tolerance to various abiotic stressors including salinity, dryness and heavy metals (Etesami, 2018; Nadeem et al., 2014; Porter et al., 2020). Inoculation experiments have shown that in periods of high salinity, plant growth-promoting rhizobacteria (PGPR) improve host growth by fixing atmospheric nitrogen, facilitating uptake of phosphorous and potassium (Mayak et al., 2004; Upadhyay & Singh, 2015) and producing scavengers of reactive oxygen species (ROS), which reduces the harmful ROS produced by plants under drought and salinity stress (Batool et al., 2020; Sukweenadhi et al., 2018). Symbiotic PGPR also facilitate plant homeostasis by regulating the uptake/exclusion of nutrients in host cells exposed to environmental stress (Rojas-Tapias et al., 2012). Furthermore, plants often increase the production of ethylene under environmental stress, which inhibits plant growth (Kende, 1993). Notably, PGPR can neutralize the negative effects of ethylene by synthesising the enzyme ACC deaminase, which converts ethylene to ammonia and α-ketobutyrate (Madhaiyan et al., 2006). These microbiome-mediated mechanisms in

plants illustrate how microbes can interact with biochemical and physiological pathways of the host to have positive effects on host environmental tolerance.

Increased nutrient provision from symbiotic microbes represents a mechanism that could contribute to the environmental tolerance of reef species. Reef invertebrates require a high energetic supply under environmental stress to support physiological adjustments, such as changes in rates of calcification and respiration (Vidal-Dupiol et al., 2013). An increased energetic demand could foreseeably be met via a shift in the microbiome towards a community of bacteria that could utilize the higher inorganic carbon available under OA, assuming that the products of fixed carbon are transferred to the host. A higher relative abundance of photosynthetic bacteria observed in sponges inhabiting high pCO_2 conditions supports this hypothesis (Morrow et al., 2015). Furthermore, a recent metagenomic study revealed that the sponge *Coelocarteria singaporensis* has enriched potential for energy efficient archaeal carbon fixation at a CO_2 seep compared to an adjacent control reef (Botté et al., 2019). In corals, energy reserves play a crucial role in increasing resilience to bleaching events (Anthony et al., 2009; Rodrigues & Grottoli, 2007). For instance, alternative sources of energy can be utilized to meet energetic demand after the breakdown of coral-Symbiodiniaceae associations, and during the period when corals are re-acquiring Symbiodiniaceae post bleaching. For example, endolithic algae associated with the coral *Oculina patagonica* have been shown to translocate a higher quantity of photoassimilates to the host during bleaching (Fine & Loya, 2002), potentially contributing to the high resilience of some coral species (Sangsawang et al., 2017). Since metagenomic analyses have revealed the potential for bacteria and archaea to transfer fixed carbon to their coral host (Robbins et al., 2019), it has been hypothesized that changes in microbial metabolism could represent an alternative source of energy for corals under environmental stress.

Nitrogen is another important nutrient influencing coral and sponge health. For instance, alterations in the N:P ratio, and deficit / excess of nitrogen can have detrimental effects on coral health (Béraud et al., 2013; Ezzat et al., 2016; Morris et al., 2019; Wiedenmann et al., 2013). Nitrogen is a limiting nutrient in coral reef ecosystems and its uptake is expected to be reduced in corals exposed to both high temperature and pCO_2 (Godinot et al., 2011). Given that nitrogen-cycling microbes are key players in regulating nitrogen availability in corals (Ceh et al., 2013; Rädecker et al., 2015; Robbins et al., 2019), microbes may enable the coral holobiont to maintain N:P ratios under environmental stress conditions. In the resilient coral *Acropora hemprichii*, for example, an increase in di-nitrogen fixation under OW (Cardini et al., 2016) may facilitate a higher nitrogen uptake in the host (Bednarz et al., 2017). Metabolic shifts towards energy-efficient biochemical pathways may also contribute to environmental acclimatisation. In the OA-tolerant sponge *Coelocarteria singaporensis*, the capacity for microbial degradation of host-derived creatine is enriched at CO_2 seeps compared to adjacent control sites, where

creatinine metabolism is predominant (Botté et al., 2019), suggesting that energy-efficient nitrogen metabolism may contribute to host OA tolerance.

Regulation of oxidative stress via the microbiome could also contribute to counteracting the negative effects of OW and OA in reef holobionts (Peixoto et al., 2021). OW is known to alter multiple physiological pathways in reef species, leading to an increased production of ROS in sponges, corals, and echinoderms (Desalvo et al., 2008; Gullian Klanian & Terrats Preciat, 2017; Webster et al., 2013) that results in oxidative damage to host cells and bleaching in corals (Lesser, 1997). However, removal of excess ROS by antioxidant compounds may mitigate oxidative damage (Lesser, 1997). Heat stress experiments revealed that corals reared in highly-variable warmer environments harbour a microbiome with greater potential for production of ROS scavengers including possession of ferredoxin like proteins (Ziegler et al., 2017). Although this finding is based on preliminary analyses of microbial community composition, it raises the possibility that some microbes could remove excess ROS in corals exposed to OW. Carotenoids can also contribute to the mitigation of oxidative stress due to their antioxidant properties (Galasso et al., 2017). A link between these pigments and high thermal tolerance was proposed by Osman et al. (2020), after identifying *Erythrobacter* sp., rich in carotenoids, as the dominant indicator taxon in the mucus of corals from warm sites in the northern Red Sea (Osman et al., 2020). Further support comes from recent studies on cultured dinoflagellates where Symbiodiniaceae-associated bacteria were proposed to support the antioxidant systems of Symbiodiniaceae (Matthews et al., 2020; Motone et al., 2020) which would have positive effects for the health of the coral holobiont. Bacteria may also produce ROS scavengers via DMSP metabolism. In corals, DMSP is biosynthesised by Symbiodiniaceae as well as the host, with an increased production observed under thermal stress (Raina et al., 2013). Recent metagenomic studies revealed that Symbiodiniaceae and some coral-associated bacteria, such as *Endozoicomonas*, have the metabolic potential to metabolize DMSP into dimethyl sulphide (DMS) (Robbins et al., 2019; Tandon et al., 2020), which is well-known for its antioxidant activity (Sunda et al., 2002). Despite these studies revealing the potential for microbiome-mediated mitigation of thermal stress via production of ROS scavengers, further empirical validation is required to confirm this mechanism.

Finally, we hypothesise that biosynthesis of amino acids and uptake / exclusion of nutrients via the microbiome may be involved in host resilience. Alteration of protein synthesis in response to OW has been reported in both sponges and corals (Desalvo et al., 2008; Webster et al., 2013). Given that coral microbes are thought to provide essential amino acids to their host (Neave et al., 2017a; Robbins et al., 2019), microbes may be capable of reducing heat-stress induced biochemical deficiency through an increased synthesis of these compounds and their subsequent transfer to the host. One of the major triggers of physiological impairment in corals exposed to OW and OA is the disruption of intracellular Ca^{2+} homeostasis (Cornwall et al., 2018; Desalvo et al., 2008). This impairment appears to cause

cytoskeletal reorganization, decreased calcification, changes in cell adhesion and cell death (Desalvo et al., 2008). In the same way that microbes contribute to Na^+ homeostasis in plants exposed to high salinity (Rojas-Tapias et al., 2012), microbes associated with corals may be able to reduce physiological stress by modifying the presence of Ca^{2+} in host cells via changes in expression of Ca^{2+} transporters. Whilst all of these putative mechanisms have the potential to facilitate host tolerance to environmental stressors, empirical evidence is still required to validate them.

Indirect mechanisms

Indirect mechanisms may also play a role in environmental acclimatisation of reef holobionts. For instance, production of antimicrobial compounds can prevent the establishment of pathogens and opportunistic bacteria in the host and thereby control disease dynamics. In plants, PGPR provide their host with a variety of antimicrobial compounds that can reduce pathogen proliferation (Glick, 1995; Latha et al., 2009). Similarly, host-associated microbes in reef taxa are also capable of synthesizing a broad suite of antimicrobial compounds, and some of these have been implicated in preventing the onset of disease (Hentschel et al., 2001; Raina et al., 2016; Ritchie, 2006). Although antimicrobial production has been shown to decrease when cultures of coral-associated bacteria are grown under thermal stress (Raina et al., 2016), it is not clear whether this antimicrobial capacity would be altered in the coral holobiont exposed to OW. Exclusion of pathogens through niche occupation is another indirect mechanism contributing to host defense against opportunistic bacteria (Bourne et al., 2016; Glasl et al., 2016). Stable microbiomes, for example, may reduce pathogen colonization through long-term niche occupation, as these communities are not subject to vulnerable transition phases (Voolstra & Ziegler, 2020). Recently, inoculation of beneficial bacteria into the coral microbiome was shown to increase host resistance to the pathogen *Vibrio coralliilyticus*, even under thermal stress (Rosado et al., 2019). The mechanisms underlying the higher pathogen resistance were not established although pathogen exclusion through niche occupation was proposed as one potential process (Rosado et al., 2019). Finally, microbes colonizing and metabolizing coral mucus may indirectly increase their host tolerance to a changing environment through a microbial loop. Coral mucus is an important source of dissolved organic matter (DOM) and nutrient cycling in reef ecosystems (de Goeij et al., 2013; Rix et al., 2016; Wild et al., 2004), generating important trophic linkages. Microbial mediation of the recycling of coral mucus may promote increased nutrient availability for corals and sponges, and thereby support host health. Macro-organisms living in association with reef invertebrates may also underpin holobiont health, having been previously suggested to influence microbiome-coral interactions (Ainsworth et al., 2020), which, in turn, may contribute to the environmental tolerance of the host. For instance, the crab *Cymo melanodactylus* can consume tissue in *Acropora* corals affected by white syndromes, reducing the spread of these diseases (Pollock et al., 2013). This interaction may influence the coral microbiome,

which could potentially promote host resilience. Further studies are needed to assess how indirect microbiome mediated mechanisms contribute to enhancing host tolerance to environmental stress.

Microbial role in transgenerational acclimatisation

Whether microbiome-mediated acclimatisation can be transferred across generations (transgenerational acclimatisation, Box 2.1) is currently unknown in reef species (Webster & Reusch, 2017). For MMTA to occur, beneficial microbes would need to be acquired in each generation. In reef species, microorganisms can be transmitted between generations (i.e. vertical transmission), acquired from the environment (i.e. horizontal transmission), or obtained through a combination of these mechanisms (Bourne et al., 2016; Webster & Thomas, 2016). An example of high partner fidelity in marine invertebrates is well represented by the symbiosis between the squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri*, where *V. fischeri* is selected by the squid from the surrounding seawater through a winnowing process at each generation (Nyholm & McFall-Ngai, 2004). Transmission modes are still unknown for most reef species, but we here suggest that there appears to be a connection to the reproductive mode of each host species.

In brooder species, where developed larvae are released into the environment following internal fertilization, both vertical and horizontal symbiont transmission have been reported. Vertically transmitted microbes have been detected across early developmental stages in the brooder corals *Porites astreoides*, *Pocillopora acuta* and several brooding sponges, through a combination of 16S rRNA gene sequencing and microscopy techniques (Damjanovic et al., 2020a; Ereskovsky et al., 2005; Lee et al., 2009; Oren et al., 2005; Schmitt et al., 2007; Sharp et al., 2007, 2012; Wu et al., 2018). Vertical transmission has been also proposed for the brittle star *Amphipholis squamata* (Walker & Lesser, 1989), but a recent study has shown that *A. squamata* may also acquire microbes from the surrounding seawater and sediments, by selecting specific bacteria through a winnowing process (Morrow et al., 2018). This selection and acquisition of microbes from the surrounding environment has been widely described also in several brooding coral and sponge species (Webster et al., 2010; Wu et al., 2018), and some bacteria appear to have an active role in the establishment of this symbiosis by finding their sponge host through chemotaxis (Tout et al., 2017). Although offspring can acquire microbes from the surrounding environment, vertical acquisition seems to be widely adopted in brooding species.

Transmission of microbes in broadcast spawning reef species, which release gametes in the surrounding water for external fertilization, is less clear. Vertical transmission of microbes to eggs has been observed in some sponge species using transmission electron microscopy (TEM) and 16S rRNA gene sequencing (Gloeckner et al., 2013; Usher et al., 2001, 2005), and vertical transmission to sperm has been shown in the spawning sponge *Chondrilla australiensis* (Usher et al., 2005). It has also been suggested that

some host-associated bacteria may be released into the surrounding environment during spawning to facilitate the acquisition of specific microbes within planula larvae (Gloeckner et al., 2013). In addition to these examples of vertical transmission, microbes can also be acquired from the environment, as observed in the spawning sponge *Ectyoplasia ferox* (Schmitt et al., 2008). Overall, these studies suggest that a combination of vertical and horizontal transmission may be required to structure and preserve the diverse microbiome in sponges. In contrast to sponges, direct transmission to gametes has yet to be elucidated in spawning coral and echinoderm species. Despite 16S rRNA gene sequence analysis suggesting that several bacterial taxa are transferred to gametes and planula larvae in the corals *Mussismilia hispida* and *Acropora digitifera* (Bernasconi et al., 2019b; Leite et al., 2017), bacteria were not detected in gametes via microscopic analysis (Leite et al., 2017). Absence of bacteria in the tissue of early life stages in spawning corals has raised the hypothesis that bacteria may be located in the mucus layer (Damjanovic et al., 2020b). Although mucus-mediated transmission cannot be excluded, it appears more likely that microbes are not vertically transmitted to offspring in spawning corals, and microbial associations commence in developed coral planulae (Apprill et al., 2009), or after settlement (Sharp et al., 2010). Horizontal transmission is therefore crucial for maintaining a stable microbiome between generations in these spawning species. In echinoderms, despite 16S rRNA gene sequencing suggesting that microbes are associated with unfertilized eggs and larvae (Bosch, 1992; Cameron & Holland, 1983; Carrier et al., 2018; Carrier & Reitzel, 2018; Cerra et al., 1997; Galac et al., 2016; Ho et al., 2016), these associations have yet to be validated. However, vertical transmission of bacterial symbionts from adults to offspring is supported by similarity in the composition of the microbiome between sea star adults and larvae and the high relative abundance of bacteria known to be vertically transferred in other systems (Galac et al., 2016). In summary, horizontal transmission appears to be the dominant method for symbiont acquisition in spawning reef species studied to date.

MMTA has been proposed in reef species, but few studies have empirically tested whether altered microbiomes can be inherited across generations. Inheritance of temperature-induced alterations in Symbiodiniaceae communities was, however, confirmed in the coral *Montipora diigitata* (Quigley et al., 2019). Similarly, a recent study assessing microbial changes in the brooding sponge *Carteriospongia foliascens* across generations found that parental exposure to future climate scenarios altered a number of microbial taxa in the offspring they produced, thereby revealing the potential for MMTA through both symbiont shuffling and switching in a vertically acquired microbiome (Luter et al., 2020). Although these findings provide an early insight into MMTA in reef species, further studies are needed to better understand the potential for MMTA. Based on the dominant type of microbial transmission (vertical vs horizontal) across different reproductive modes, we suggest that MMTA would be more likely to occur in brooders compared to broadcast spawners, as transmission of altered beneficial microbiomes across generations may be less likely when microbes are acquired each generation from the surrounding environment.

Conclusions and future directions

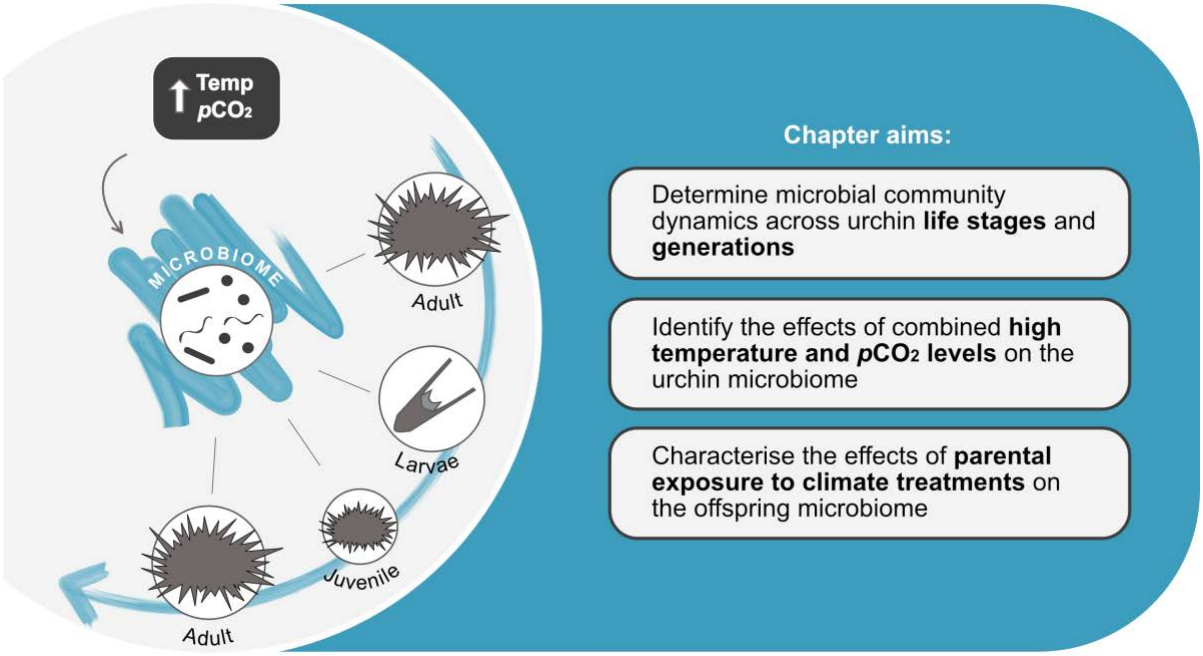
Recent commentaries have suggested a potential for microbiome-mediated acclimatisation to enhance tolerance of reef species to OW and OA. However, to date, analyses of environmentally induced microbial changes have primarily utilised community composition data, precluding analysis of the microbial pathways that would underpin acclimatisation. Throughout this review we have highlighted potential direct and indirect mechanisms that could contribute to microbiome-mediated acclimatisation of reef species to OW and OA, including provision of beneficial compounds, reduction of oxidative stress, maintenance of nutrient homeostasis, and increased pathogen resistance. Furthermore, we have suggested that the host reproductive mode may represent a critical factor determining the likelihood for MMTA. Considerable research effort is clearly required to experimentally validate the potential for microbiome-mediated environmental tolerance in reef species.

A robust experimental approach coupled with appropriate microbial analyses is needed to confirm the potential for microbiome-mediated acclimatisation in reef taxa. Such an experimental design would comprise long-term, multifactorial (OW and OA) transgenerational exposures. Since microbial responses to climate stressors vary among reef taxa (Egan & Gardiner, 2016; Pita et al., 2018; Voolstra & Ziegler, 2020), species with different ranges of environmental tolerance need to be assessed. Furthermore, any experimental design should allow the disentanglement of critical factors influencing microbiome structure, including host genotype (Glasl et al., 2019; Hernandez-Agreda et al., 2018) and host micro-habitat (Bourne et al., 2016; Høj et al., 2018; Li et al., 2014; Sweet et al., 2011). To link microbiome changes with shifts in host health, host fitness also needs to be monitored throughout the experiments using a relevant suite of physiological parameters. This experimental approach should be combined with analyses that investigate microbiome changes at both the compositional and functional levels. DNA, RNA and protein sequencing approaches provide a way to characterize microbiome changes from a functional perspective (Glasl et al., 2020; Liu et al., 2012; Maron et al., 2007; Moitinho-Silva et al., 2017; Robbins et al., 2021), permitting the identification of direct and indirect microbial mechanisms that may be involved in environmental and transgenerational acclimatisation to OW and OA. These methods include metagenomics (Handelsman, 2004), metatranscriptomics (Bashiardes et al., 2016) and metaproteomics (Maron et al., 2007), for detecting changes in gene content, gene expression and protein expression linked to exposure to OW and OA. Complementary methodologies will also be required to validate any genomic-based hypotheses. Advanced imaging analyses, including nanoscale secondary ion mass spectrometry (NanoSIMS) and Raman spectroscopy (Berry & Loy, 2018; Volland et al., 2018; Wang et al., 2016), combined with stable isotope incubations (Berry & Loy, 2018; Rix et al., 2016, 2020), enable tracking of exchange of metabolites between microbes and their host at the single-cell level. In combination with techniques that permit visualisation and quantification of host-associated microbes, such as fluorescence *in situ* hybridization (FISH) (Amann & Fuchs, 2008), this

integrated methodological approach will help quantify the contribution microbes make to the environmental tolerance of their reef hosts.

Understanding if and how microbes contribute to environmental acclimatisation of reef species is one approach that may facilitate successful reef restoration measures. For example, active manipulation of the microbiome could be undertaken to enhance host resistance and resilience to environmental stressors in laboratory reared corals prior to deployment onto degraded reefs (Peixoto et al., 2017, 2021; Rosado et al., 2019; van Oppen et al., 2015; van Oppen & Blackall, 2019). Based on well-studied model systems, this review highlights several microbiome-mediated mechanisms that could contribute to host acclimatisation to OW and OA. The next step for the coral reef research community is to provide empirical evidence to validate these mechanistic hypotheses.

CHAPTER 3 : LIFE-STAGE SPECIFICITY AND CROSS-GENERATIONAL CLIMATE EFFECTS ON THE MICROBIOME OF A TROPICAL SEA URCHIN (ECHINODERMATA: ECHINOIDEA)



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Life-stage specificity and cross-generational climate effects on the microbiome of a tropical sea urchin (Echinodermata: Echinoidea)

Abstract

Microbes play a critical role in the development and health of marine invertebrates, though microbial dynamics across life stages and host generations remain poorly understood in most reef species, especially in the context of climate change. Here, we use a four-year multigenerational experiment to explore microbe-host interactions under the Intergovernmental Panel on Climate Change (IPCC)-forecast climate scenarios in the rock-boring tropical urchin *Echinometra* sp. *A*. Adult urchins (F_0) were exposed for 18 months to increased temperature and pCO_2 levels predicted for years 2050 and 2100 under RCP 8.5, a period which encompassed spawning. After rearing F_1 offspring for a further two years, spawning was induced and F_2 larvae were raised under current day and 2100 conditions. Cross-generational climate effects were also explored in the microbiome of F_1 offspring through a transplant experiment. Using 16S rRNA gene sequence analysis, we determined that each life stage and generation was associated with a distinct microbiome, with higher microbial diversity observed in juveniles compared to larval stages. Although life-stage specificity was conserved under climate conditions projected for 2050 and 2100, we observed changes in the urchin microbial community structure within life stages. Furthermore, we detected a climate-mediated parental effect when juveniles were transplanted amongst climate treatments, with the parental climate treatment influencing the offspring microbiome. Our findings reveal a potential for cross-generational impacts of climate change on the microbiome of a tropical invertebrate species.

Introduction

Microbial symbionts play a critical role in host health, contributing to host metabolism, immunity and development (Cavalcanti et al., 2020; McFall-Ngai, 2014; Robbins et al., 2021; Schuh et al., 2020; Weiland-Bräuer et al., 2020). In many animals, development and major life cycle transitions are influenced by the host-associated microbiome through interactions with regulatory networks at the base of these developmental processes (Russell & Castillo, 2020). In marine invertebrates with a biphasic life cycle, bacteria can provide the host with critical compounds necessary for regulating morphogenetic changes and completing the animal's life cycle (Cavalcanti et al., 2020; Song et al., 2021; Weiland-Bräuer et al., 2020). To maintain these crucial host-microbe relationships, key microbial taxa need to be faithfully acquired at each generation through vertical or horizontal transmission. In the sponge *Amphimedon queenslandica,* for example, vertical transmission from adult to embryos ensures the acquisition of three *Proteobacteria* taxa that are essential for settlement and metamorphosis (Fieth et

al., 2016; Song et al., 2021). Instead, relationships like the squid-*Vibrio* symbiosis relies on the acquisition of mutualistic microbial symbionts from the surrounding seawater for the formation of functional organs, through complex host-symbiont interactions resulting in the monospecific colonization of *Vibrio fischeri* (Koropatnick et al., 2004; McFall-Ngai, 2014).

Following the establishment of host-microbe associations, restructuring of the microbiome may occur throughout ontogeny (Bernasconi et al., 2019b; Carrier & Reitzel, 2019; Damjanovic et al., 2020b; Fieth et al., 2016). These changes are species-specific; some coral larvae harbour less-diverse microbial communities than their early juvenile stages (Bernasconi et al., 2019b), while some echinoid species are characterized by a decrease in microbial diversity from early development to juvenile stages (Carrier & Reitzel, 2019). Characterizing host-microbe relationships across life history stages is essential to better understand the role of microbes in host development and fitness, and identifying the impact of climate change on these functionally important microbial partnerships is critical.

In the marine environment, climate change not only compromises host fitness through physiological and behavioural impairments (Marangon et al., 2020; Pörtner & Farrell, 2008), but may also affect animal-microbe interactions. Ocean warming (OW) and acidification (OA) have been shown to drive compositional and functional shifts in the microbiome of marine invertebrates (Botté et al., 2019, 2020; Webster et al., 2016), with both positive and negative consequences for animal health depending on species, environmental stressors and environmental gradients (Egan & Gardiner, 2016; Marangon et al., 2021; Pita et al., 2018; Posadas et al., 2022; Ziegler et al., 2017). For example, a metagenomic study comparing the microbiome of the sponge *Coelocarteria singaporensis* between a CO_2 seep and an adjacent control reef, found an enriched potential for energy-efficient archaeal carbon fixation at the seep, suggesting microbes may contribute to host OA tolerance in this species (Botté et al., 2019). In contrast, detrimental changes in the microbial community associated with the reef sponge *Stylissa flabelliformis* were observed at both the compositional and functional levels under heat stress, including a reduced capacity for ammonia detoxification (Botté et al., 2023). Despite climate-induced changes on the microbiome structure being widely observed in marine organisms, other host associated microbial communities are stable upon environmental disturbances (Grottoli et al., 2018; Luter et al., 2020; Webster et al., 2016). This stability may reduce the occurrence of diseases through pathogen exclusion, though may also preclude beneficial microbial changes from occurring (Voolstra & Ziegler, 2020). Given the importance of host-associated microbes for the stability of ecological community structure and broader ecological processes that underpin ecosystem function and services (Bourne et al., 2009; Pita et al., 2018), it is crucial to understand microbiome changes under OW-OA.

Host-microbe responses to altered environmental conditions may vary when the stressors are experienced over multiple generations (Donelson et al., 2012; Karelitz et al., 2020; Uthicke et al., 2021).

Emerging evidence suggests that heritability of beneficial alterations to the microbiome may facilitate host acclimatisation over generations (Baldassarre et al., 2022). For example, it was recently shown that sea anemone juveniles from temperature-acclimated F_0 females were characterized by high thermal tolerance and were associated with specific members from the parental acclimated microbiota (Baldassarre et al., 2022). Similarly, Luter and colleagues (2020) confirmed that transgenerational effects of climate treatments can alter the microbiome of the sponge *Carteriospongia foliascens*, with specific microbial taxa exclusively identified in recruits whose parents were pre-exposed to climate stressors. Hence, characterizing the contribution of microbes to transgenerational plasticity is required for generating realistic predictions of organisms' responses to future climate, and inform reef conservation strategies.

To explore cross-generational effects of climate change on reef host-microbe dynamics, we employed a tropical sea urchin species as study system (*Echinometra* sp. *A*). Sea urchins are an emerging model for investigating animal-microbe symbiosis (Carrier et al., 2021), showing a re-shaping of the bacterial communities during major developmental transitions, from pelagic larvae to benthic juveniles and adults, with microbes inferred to contribute to larval immunity and nutrition in some species (Carrier et al., 2021; Carrier & Reitzel, 2019, 2020; Schuh et al., 2020). Urchins are susceptible to changing climate, in particular during larval life stages (Byrne & Przeslawski, 2013), though little is known on the effects of OW and OA on microbial dynamics in adult urchins and early developmental stages (Brothers et al., 2018; Carrier & Reitzel, 2020; Ketchum et al., 2021; Webster et al., 2016). Along with an important role in reef resilience through its grazing activity, *Echinometra* sp. *A* is widely distributed in reef ecosystems (McClanahan & Muthiga, 2013) and characterized by relatively short-generation times, making it an ideal model for our multigenerational experiment. In this study, we employed 16S rRNA gene sequencing analyses to 1. Characterize urchin ontogenetic microbial changes (*aim 1*); 2. Determine the combined effect of OW and OA on these host-microbe relationships (*aim 2*); and 3. Identify transgenerational effects of climate treatments on the urchin microbiome through a transplant experiment (*aim 3*). Our 4-year multigenerational study demonstrated that urchin microbial communities differ across life stages, and although combined OW / OA can alter these associations, life stage specificity was maintained under climate stress. Furthermore, transgenerational effects of climate treatments appeared to occur in *Echinometra* sp. *A.* microbiome, with potential implications for host health.

Material and methods

Urchin collection and experimental set up

Echinometra sp. *A* adults (F_0) were collected from the central Great Barrier Reef (GBR; Trunk Reef; 18.3188° S, 146.8662° E; Permit G12/35236.1) in February 2016 and transported to the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS). The experimental set up is described in detail in Uthicke et al. (2020). Briefly, urchins were randomly distributed across nine outdoor mesocosm tanks of 1,260 L each (n= 22-24 per tank), at ambient conditions (28 ˚C). Treatment conditions were gradually reached over a four-week period, with target temperature and pCO_2 reflecting present-day conditions, predictions for year 2050 (+1 ˚C; ~685 ppm) and year 2100 (+2 ˚C; ~940 ppm) according to an RCP 8.5 scenario (Collins et al., 2013; Meinshausen et al., 2011), resulting in 3 mesocosms under each climate treatment (i.e. ambient, year 2050, year 2100). Ambient temperature mimicked natural fluctuations on the central GBR based on the daily average sea surface temperature at Davies Reef (temperature from 1991 to 2012; Australian Institute of Marine Science, 2020), and ambient pCO_2 reflected present-day conditions (~400 ppm; Uthicke et al., 2014a). pCO_2 levels of all treatments simulated natural pCO_2 variations observed on the GBR (daily ±60 ppm fluctuations; Karelitz et al., 2020). Temperatures and pCO_2 levels were finely managed by three Programmable Logic Controllers (PLC), which were connected to temperature sensors and gas equilibration systems with Telaire CO_2 sensors. Urchins fed *ad libitum* on crustose coralline algae (CCA) and biofilms throughout the experimental period.

F_0 Urchin spawning and F_1 Urchin larvae

F_0 adults were spawned after 18-month exposure to treatment conditions (15 November 2017) by injecting 0.5 ml of 0.5 M KCl (Uthicke et al., 2020). Sperm from multiple adults of the same mesocosm was pooled (1 µL of sperm per individual into 20 ml Filtered Seawater [FSW]) and 1 ml of diluted sperm was used to fertilize eggs from females of the respective mesocosm (spawned eggs pooled in 1L FSW). Fertilization was accomplished in each mesocosm except for mesocosm #7 (2050 conditions) due to low abundance of individuals in this system. Fertilization success was high across treatments, as indicated by the presence of a fertilization envelope on 90-100% of eggs (Uthicke et al., 2020). Cultures were reared in Schott bottles under parental treatment conditions (1L of 0.5 µm FSW; n=1 per treatment) at a density of ~ 5 embryos ml^{-1}. pCO_2 levels were stable over the incubation period (Karelitz et al., 2020), and target temperatures were maintained by placing Schott bottles on rollers in temperature-controlled water baths. Seawater of the larval cultures was changed every second day with FSW at the same treatment conditions, and feeding was carried out twice per day (*Chaetoceros muelleri*;

5000 cells ml^{-1} from 2 to 10 days, 8000 cells ml^{-1} thereafter). Larvae competent to settlement were transferred to 0.5 L aquaria under ambient conditions 19 and 26 days after fertilisation, where settlement was induced by placing pre-conditioned aragonite plugs with CCA into the aquaria. Metamorphized juveniles were transferred to the mesocosm systems between 4 to 6 days post-settlement.

F_1 Urchin growth

In the mesocosms, juveniles were raised in 50 ml polypropylene centrifuge tubes covered with 150 µm mesh (40 juveniles per tube, n=2 tubes per mesocosm), and fed on CCA-encrusted aragonite plugs. In addition to rearing juveniles in the mesocosms under the respective parent treatments, juveniles from adults held at ambient conditions were also reared under 2050 and 2100 conditions, and juveniles from adults at 2050 conditions were also raised under 2100 conditions generating a transplant experiment (Fig. 3.1). For logistic reasons, 6-month juveniles (F_1) were transferred to indoor 50 L flow-through aquaria under the respective treatment conditions (n=3 tanks under ambient conditions; n=2 tanks for the other climate treatments), where they fed *ad libitum* on CCA, and reared until maturity (~25 months). In these aquaria, temperature and pCO_2 levels were finely controlled by a SeaSim computer system and temperature was further stabilized by temperature-controlled water baths. Light simulated the natural 12h light/dark cycle (6:00 am – 6:00 pm) with 4-h ramping at sunrise and sunset (AquaIllumination SOL LED lights; light 50 µE). At the end of October 2018 (~10.5-month juveniles) a pathogen-induced disease outbreak affected urchin survival across all treatments, with ambient-ambient juveniles showing a significant higher survival rate than the other treatment groups (see Uthicke et al., 2021 for detailed information on the outbreak).

F_1 Urchin spawning and F_2 Urchin growth

F_1 urchins reached maturity after > 2 years exposure to future climate scenarios. Urchins under ambient-ambient and 2100-2100 were spawned on 15 January 2020, following the same methodology as described above for F_0 adults. F_2 larvae were maintained under parental conditions in 1L Schott bottles (n=2 per treatment) on rollers in temperature-controlled water baths.

Urchin sampling

To assess microbial community dynamics across urchin life stages, generations and climate treatments, samples were collected from F_0 adult gonad tissue, most major life stages in F_1 (1-day larvae, 5-day larvae, 5-month juveniles, adult gonad) and F_2 larvae (1-day and 7-day). Following F_0 spawning, 6 female adults were dissected per treatment (ambient, 2050, 2100) and gonads were sampled using sterile

tweezers and scissors. Samples were rinsed with 0.2 µm filtered-sterilized seawater, snap frozen in liquid nitrogen and stored at – 80 ˚C until DNA extraction. F_1 larvae were sampled 1 day and 5 days after fertilization under ambient, 2050 and 2100 conditions. First, 500 mL of larval culture per each treatment was filtered through 50 µm membrane and rinsed in 0.2 µm filtered-sterilized seawater. Larvae within each treatment were then distributed into 3 aliquots, concentrated into a pellet by centrifugation to remove excess seawater, snap frozen and stored at -80˚C until DNA extractions. 5-month juveniles (F_1) were sampled under all treatment conditions (n= 6 treatments, Table S3.1), snap frozen in liquid nitrogen and stored at – 80 ˚C until processing. For F_1 adults, gonads were sampled as described per F_0 gonads. F_2 larvae were sampled 1 and 7 days after fertilization under ambient and 2100 conditions following the same sampling procedure as per F_1 larvae. Seawater samples were collected as environmental control in parallel with sampling of F_0 gonad adults, F_1 larvae, F_1 juveniles and F_1 gonad adults. For each seawater sampling time, 1L seawater was collected from each tank or larval culture and filtered through 0.2 µm sterivex filters (Millipore) and stored at – 80 ˚C until DNA extraction.

DNA extraction and sequencing

Genomic DNA was extracted from major life stages across the two generations and seawater samples (see Figure 3.1; n = 145, details in Table S3.1). DNA extractions were undertaken using the DNeasy PowerSoil Pro kit (QIAGEN) following the manufacture's protocol, with samples homogenised using the FastPrep-24 5G instrument (MP Biomedicals). DNA purity was examined using the Nanodrop 2000 spectrophotometer (Thermo Scientific) and DNA concentration was quantified using the Qubit 3.0 Fluorometer (Life Technologies) and then standardized within each sample type. The V4 variable region of the 16S rRNA gene was amplified with the primers 515F (Parada et al., 2016) and 806rB (Apprill et al., 2015), for targeting bacteria and archaea. PCRs were performed using the AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific), 0.2 µM primers (Sigma-Aldrich) and 3.1 µM $MgCl_2$ (QIAGEN). PCR conditions were the following: initial denaturation at 95˚C for 10 min, 30 cycles at 95˚C for 30 sec, 55˚C for 1 min, 72˚C for 30 sec, and final extension at 72˚C for 7 min. PCR amplification products were analysed by agarose gel electrophoresis, and then stored at -20 ˚C before submission to the Ramaciotti Centre for Genomics (UNSW, Australia) for completion of the 16S library preparation and sequencing on the Illumina MiSeq platform (2x250 bp paired-end reads). In addition to urchin and seawater samples, 3 blank DNA extractions were sequenced to detect any potential reagent contamination. Sequence data are deposited in NCBI (BioProject PRJNA1013433).

16S rRNA gene data processing and statistical analyses

Demultiplexed paired-end reads were analysed in QIIME2 (v 2020.8; Bolyen et al., 2019). First, poor quality reads and chimeras were removed using DADA2 (Callahan et al., 2016). High quality sequences were then grouped into amplicon sequence variants (ASVs) based on 100% sequence similarity in DADA2 (Callahan et al., 2016). Taxonomic assignment was performed using a naïve Bayes classifier trained with the feature-classifier plugin using the primer set 515F/806rB on the SILVA 132 database (Quast et al., 2012). FastTree method was used to build phylogenetic relationships. ASVs table, taxonomic table, phylogenetic tree and metadata were imported into R (v 4.0.3; R Core Team, 2020) for data analyses. Contaminants were identified and removed using the R package decontam (Davis et al., 2018) using stringent thresholds (*P* threshold = 0.5; n=32 contaminants identified, Table S3.2). Reads assigned to Chloroplasts, Eukaryotes and Mitochondria, as well as singletons and low-quality samples (< 8,400 reads; n=2, F_1 juvenile from 2050 treatment and F_0 gonads from ambient conditions) were removed prior to the analyses.

To test for differences in microbial community structure between host life stages, generations, and climate treatments (see below), we calculated beta diversity on non-rarefied data, with Bray-Curtis dissimilarities applied on square-root transformed data normalized using proportions (McKnight et al., 2019). For beta-diversity analyses, rare ASVs with an overall relative abundance < 0.001% were removed (34,412 ASVs). Analyses include Non-metric Multi-dimensional Scaling (NMDS, 'phyloseq package', McMurdie & Holmes, 2013), Permutation Multivariate Analysis of Variance using 10,000 permutations (Adonis, 'vegan package', Oksanen et al., 2020), Multivariate Homogeneity of Group Dispersions ('vegan package', Oksanen et al., 2020), pairwise comparisons using Benjamini-Hochberg correction ('RVAideMemoire package', Hervé, 2021). Differential abundance analyses ('DESeq2 package', Love et al., 2014) were run on filtered data using p-value of 0.01, and significant ASVs that were present across at least 50% of the samples in one of the compared groups were identified as differentially abundant between groups. Furthermore, a 1% mean relative abundance cutoff was included when determining differentially abundant ASVs across life stages (ambient conditions). Principal Component Analysis (PCA) and multilevel sparse Partial Least Squares – Discriminant Analysis (multilevel sPLS-DA) were performed in the mixOmics package (Rohart et al., 2017) on the same pre-processed data as above but applying a centered log-ratio transformation (offset by +1) (Chua et al., 2017). To determine which ASVs were shared between life stages / generations / climate conditions, ASVs present in at least 50% of samples within each group were analysed using the function 'ps_venn' in 'MicEco' (https://github.com/Russel88/MicEco) on rarefied data (8,400 sequences).

Analyses of alpha diversity were based on Shannon diversity index calculated on a dataset rarefied to 8,400 sequences ('phyloseq package', McMurdie & Holmes, 2013), with the effect of host life stages,

generations, and climate treatments tested through linear mixed effect models ('glmmTMB package', Brooks et al., 2017) and pairwise comparisons with Benjamini-Hochberg correction ('emmeans package', Lenth, 2022). The assumption of normality and homogeneity of variances were tested both visually and through DHARMa residual diagnostics ('DHARMa package', Hartig, 2021). Additional alpha diversity measures (i.e. Pielou's evenness, Simpson's diversity, richness, Faith's phylogenetic diversity) were calculated using the phyloseq (McMurdie & Holmes, 2013) and btools (https://github.com/twbattaglia/btools/) packages. Data manipulation was performed using the dpylr, forcats and tidyr packages (Wickham et al., 2019), graphs were generated using ggplot2 and RcolorBrewer (https://github.com/cran/RColorBrewer) packages, and illustration were further stylized in Affinity Designer. All analyses were performed in R (version 4.0.3, R Core Team, 2020).

Aim 1: microbial community across urchin life stages and generations under ambient conditions

To examine microbial community dynamics across life stages and generations, we first investigated urchins under ambient conditions (see Fig. 3.1, aim 1). The interaction between life stage (fixed factor with four levels: "adult gonad", "larvae [1-day and older larvae were pooled]", "juvenile", "seawater") and generation (fixed factor with three levels: "F_0", "F_1", "F_2") was explored using Permutation Multivariate Analysis of Variance (Adonis, 'vegan package', Oksanen et al., 2020) including tank as fixed factor fitted first in the model to account for tank-to-tank variation. The effect of age on the larval microbial community was assessed across generations using Permutation Multivariate Analysis of Variance (Adonis, 'vegan package', Oksanen et al., 2020) , with larval age (two levels: "1 day", "> 1 day") and tank as fixed factors. To test for differences in Shannon diversity among life stages, linear mixed effect models ('glmmTMB package', Brooks et al., 2017) were performed with life stage and generation tested as fixed effect (seven levels: "F_0 adult gonad", "F_1 larvae", "F_1 juvenile", "F_1 adult gonad", "F_2 larvae", "F_0 seawater", "F_1 seawater") and tank as random, nested effect.

Aim 2: effects of combined OW and OA on the urchin microbiome

Effects of future climate scenarios predicted for year 2050 and 2100 on urchin microbial community structure (see Fig. 3.1, aim 2) were investigated using Permutation Multivariate Analysis of Variance (Adonis, 'vegan package', Oksanen et al., 2020), including climate scenario (three levels: "ambient", "2050", "2100"), life stage (three levels: "F_0 adult gonad", "F_1 larvae [1- and 5- day larvae were pooled]", "F_1 juvenile") and tank as fixed factors, as well as climate scenario * life stage. As F_1 adults were not reared under 2050 conditions, the effect of climate scenario (two levels: "ambient", "2100"; fixed factor) on the microbiome associated with adult gonads (F_1) was tested separately. Climate effects on each F_1 larval stage (1- and 5-day) were also explored, with climate scenario (three levels: "ambient",

"2050", "2100") included as fixed factor. Finally, the effect of climate treatments on the seawater microbiome was tested, including climate scenario (three levels: "ambient", "2050", "2100"), time point ("F_0 adult gonad", "F_1 larvae", "F_1 juvenile", "F_1 adult gonad") and tank as fixed factors, as well as climate scenario * time point. Climate effects on F_2 larvae were not statistically investigated due to insufficient replication. Liner mixed effect models ('glmmTMB package', Brooks et al., 2017) were used to test any climate treatment, life stage and generation effects on the Shannon diversity index.

Aim 3: effects of parental exposure to climate treatments on the offspring microbiome

Finally, transgenerational effects of climate change on the urchin microbiome were tested through a transplant experiment (see Fig. 3.1, aim 3). Permutation Multivariate Analysis of Variance (Adonis, 'vegan package', Oksanen et al., 2020) were performed to test any effect of the interaction between parental (F_0) climate treatment (three levels: "ambient", "2050", "2100") and offspring climate treatment (three levels: "ambient", "2050", "2100") on the 5-month juvenile microbiome, with tank included as fixed effect. Differences in alpha diversity (Shannon index) among juveniles exposed to the 6 reciprocal climate treatments were tested using linear mixed effect models ('glmmTMB package', Brooks et al., 2017) with climate treatment as fixed factor and tank as random, nested effect. Multilevel sPLS-DA was run following the example Case Study Multilevel sPLS-DA: Vac18 available on the mixOmics website (http://mixomics.org/case-studies/multilevel-vac18-case-study/). For this analysis, Ambient-Ambient juveniles were excluded. Multilevel sPLS-DA is a supervised approach for data classification of repeated measures ('tank' in our study). Briefly, a sPLS-DA was run with 10 components and then tuned using the 'perf' and 'tune.splsda' (n=5 folds, n=50 repeat, Maximum Distance, Balanced Error Rate measure) functions to select the optimal number of components and variables.

Results

We performed a 4-year multigenerational experiment to characterize the combined effect of high temperature and pCO_2 levels on the microbial community dynamics in the tropical sea urchin *Echinometra* sp. *A* across major life stages (experimental design illustrated in Fig. 3.1; replicates per life stage / treatment conditions listed in Table S3.1). A total of 4,767,687 high-quality 16S rRNA amplicon reads was obtained, with an average of 35,580 reads per sample ranging between 8,495 and 104,419 reads. Following quality trimming, chimera removal and data filtration, a total of 40,599 amplicon sequence variants (ASVs) were identified. Rarefaction curves based on sample diversity reached an asymptote, confirming sequencing depth for this study was adequate (Fig. S3.1).

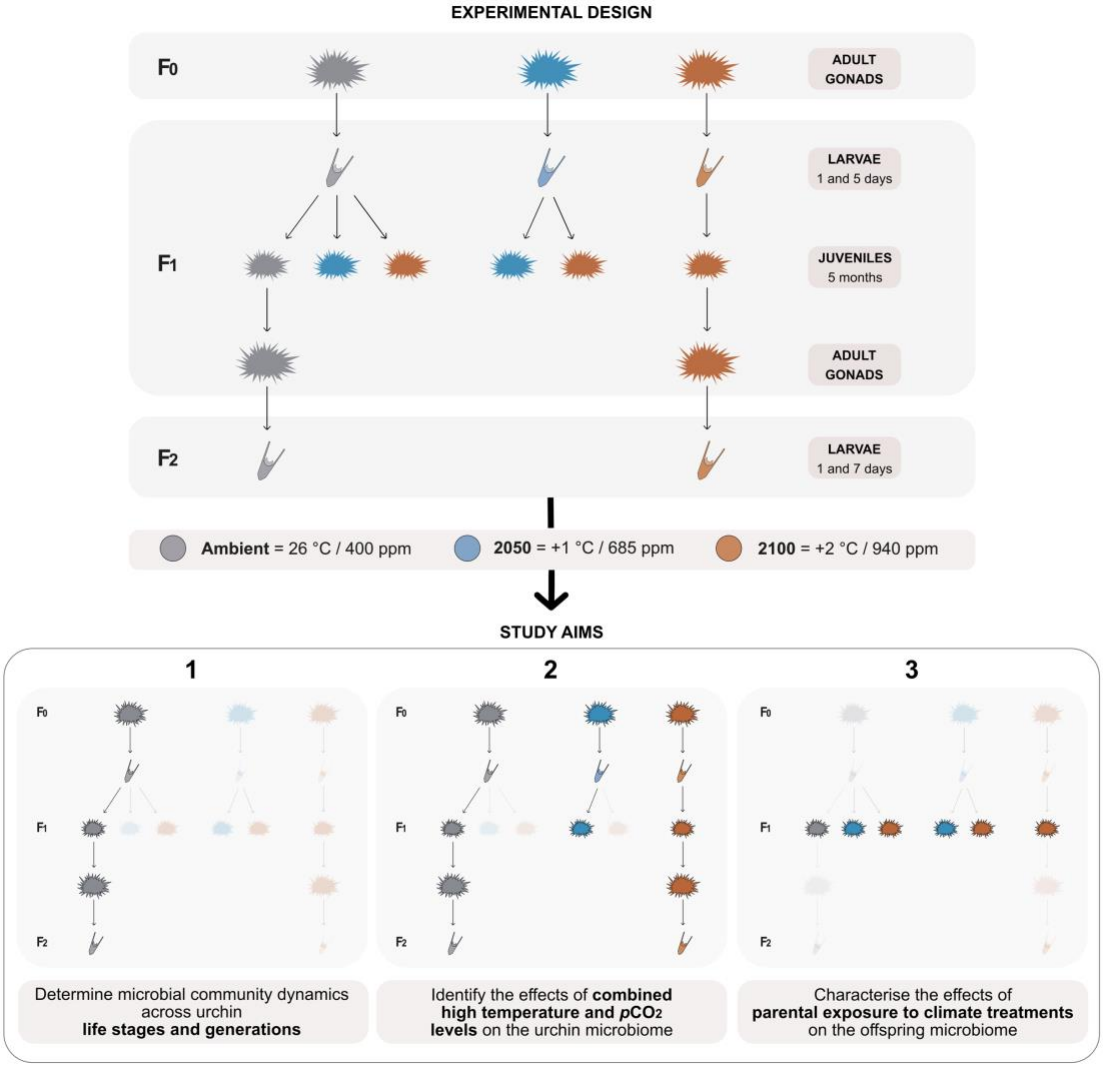

Fig. 3.1. Experimental design and research aims of the transgenerational urchin experiment. Ambient temperature mimicked natural fluctuations of the central GBR, with an average of 26 ˚C. Samples used for addressing each study aim are highlighted in the illustration. Sample replication: F_0 adult gonads $n \geq 5$; F_1 larvae $n = 3$; F_1 juveniles $n \geq 8$; F_1 adult gonads $n \geq 3$; F_2 larvae $n = 2$. Details in Table S3.1.

Microbial dynamics across urchin life stages and generations under ambient conditions (aim 1)

The microbial community across life history stages and generations held under ambient conditions, including adult gonads (F_0 and F_1), larvae (F_1: 1- and 5-day; F_2: 1- and 7-day) and 5-month juveniles (F_1) were characterized (Fig. 3.1, aim 1). Although there were significant differences in dispersion (dispersion in adult gonads was lower than larvae and juvenile), non-metric Multi-dimensional Scaling (NMDS) and permutational multivariate analysis of variance revealed that microbial communities shifted significantly during developmental stages and between generations, with changes in the presence / absence of taxa as well as in their relative abundance (PERMANOVA, life stage x

generation: pseudo-F = 4.1, $p < 0.001$; Fig. 3.2 and 3.3, Table S3.3). Each life stage (i.e. adult [gonad], larvae and juvenile) was significantly different to one another ($p < 0.001$), as well as to the seawater-associated microbial community ($p < 0.001$; Fig. 3.2, Table S3.3). Age also affected the microbial community associated with urchin larvae, with 1-day larvae significantly different to older larvae (PERMANOVA, pseudo-F =8.1, $p < 0.001$; Fig. 3.2, Table S3.3B). In addition to these microbial changes during host development, microbial community structure shifted across generations within adult gonads (PERMANOVA, $p = 0.002$; Fig. 3.2, Table S3.3) and larvae (larval ages were pooled; PERMANOVA, $p = 0.03$; Fig. 3.2, Table S3.3).

Overall, 23 microbial classes represented at least 1% mean relative abundance across life stages and generations, of which *Alphaproteobacteria* (*Proteobacteria*), *Deltaproteobacteria* (*Proteobacteria*), *Gammaproteobacteria* (*Proteobacteria*), and *Bacteroidia* (*Bacteroidetes*) were the only classes with >1% abundance in all life stages (Fig. 3.3A). Some microbial classes were characteristic of specific life stages, for example *Fusobacteriia* affiliated sequences were highly abundant only in adult gonads (F_0: 14.3%, F_1: 13.5%), *Nitrososphaeria* in F_1 1d-larvae (8.2%) and *Oxyphotobacteria* in juveniles (7.5%). To better characterize the potential drivers of the differences among life history stages, we explored microbial dynamics at the family level (Fig. 3.3B and S3.2). The microbiome of adult gonads (F_0 and F_1) was primarily comprised of Vibrionaceae (*Gammaproteobacteria*), Desulfobulbaceae (*Deltaproteobacteria*), Fusobacteriaceae (*Fusobacteriia*), Cryomorphaceae (*Bacteroidia*) and Prolixibacteraceae (*Bacteroidia*); and F_1 gonads showed a high relative abundance also in Kiritimatiellaceae (*Kiritimatiellae*) affiliated sequences (Fig. S3.2). In contrast, all larval stages were dominated by Rhodobacteraceae (*Alphaproteobacteria*) and Alteromonadaceae (*Gammaproteobacteria*) affiliated sequences, constituting up to 27% and 32% respectively in mean relative abundances (Fig. 3.3B). Other bacterial families were abundant at this life stage, such as Halomonadaceae (*Gammaproteobacteria*) in F_1 larvae, and Oleiphilaceae (*Gammaproteobacteria*) and Cellvibrionaceae (*Gammaproteobacteria*) in F_2 larvae (Fig. S3.2). In juveniles, Rhodobacteraceae relative abundance was similar to the larvae life stage (11.9%), while Alteromonadaceae and Vibrionaceae represented 1.5% and 16.3% of the community respectively (Fig. 3.3B). The most abundant families in the urchin-associated tissue across life stages were a minor component of the seawater samples, indicating that urchin microbial communities are host-specific (Fig. 3.3B and S3.2), which was also confirmed by the small proportion of ASVs shared between life stages and seawater (Fig. S3.3).

When exploring microbial community structure at the ASV level, DESeq analyses identified ASVs that were differentially abundant between life stages. In F_1 larvae, 7 ASVs were present exclusively at 1- or 5-day, and sequences affiliated to the archaea *Nitrosopumilus* were significantly reduced from 1-day to older larvae (F_1; from 7.3% to 2.2%) (Fig. 3.3D). Some ASVs were also differentially abundant between

F_1 larvae and juveniles (Fig. 3.3E). For example, one ASV assigned to *Ruegeria* constituted on average 2.1% of the microbial community in juveniles, though was absent in F_1 larvae; and *Alteromonas* affiliated sequences showed about a 10-fold reduction in juveniles compared to F_1 larvae. This clear distinction between life history stages was also reflected in the low number of ASVs observed across all life stages, with few ASVs present in at least 50% of the samples and shared between developmental stages (Fig. 3.3C; Table S3.4). Only four microbial taxa were shared between F_0 adult gonads and F_1 1-day larvae, which were not retained in older larvae (Fig 3.3C; Table S3.4). While a high number of ASVs (n = 31) were retained from 1-day to older larvae, only three of them remained in association with the juveniles (Fig. 3.3C; Table S3.4). These three ASVs were also present in F_2 larvae at both sampling times, and were assigned to *Alcanivorax*, *Hyphomonas* and *Oleiphilus* strains. When comparing the microbial composition between generations, 44% of ASVs associated with F_0 adult gonads was also present in F_1 gonads, and 29% of the microbiome in F_1 1-day larvae was retained in the next generation (Fig. 3.3C). Some ASVs associated with the urchin tissue were also present in the seawater, with the proportion of shared microbial taxa between urchin and seawater varying among life stages (Fig. S3.3).

Alpha diversity based on Shannon index varied significantly across life stages but not generations under ambient conditions (Fig. 3.4A, Table S3.5). Specifically, post-hoc comparisons revealed that juveniles (4.97 ± 0.77 SE) had significantly higher alpha diversity than F_1 larvae (3.83 ± 0.40 SE; $p=0.002$), F_2 larvae (3.47 ± 0.32 SE; $p=0.0005$), F_0 adult gonads (3.42 ± 0.38 SE; $p<0.0002$) and F_1 adult gonads (3.30 ± 0.50 SE; $p<0.0001$; Fig. 3.4A, Table S3.5). Similar patterns were observed for Pielou's evenness, Simpson's diversity, richness (total number of ASVs) and Faith's phylogenetic diversity (Fig. S3.4).

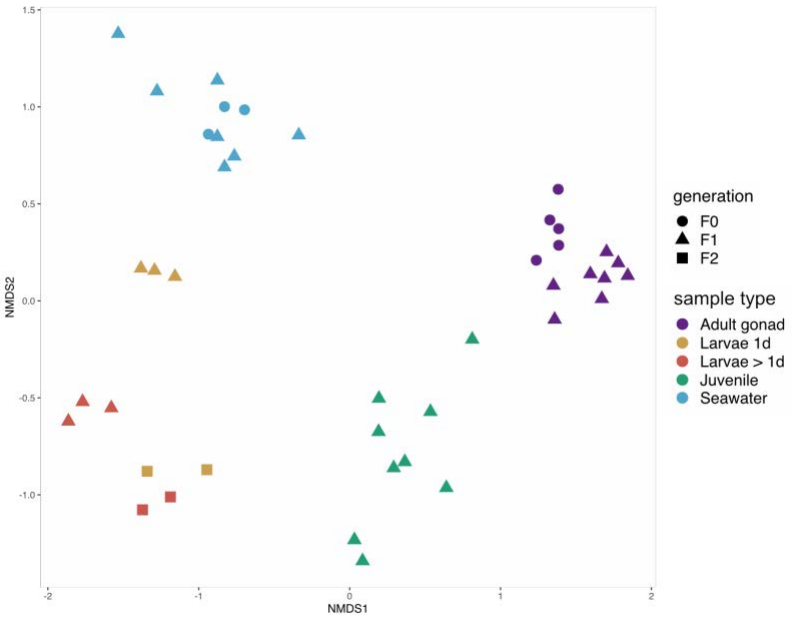

Fig. 3.2. Non-metric Multi-dimensional Scaling (NMDS; stress = 0.13) based on Bray-Curtis dissimilarities on the microbial communities (ASVs level) associated with urchin adult gonad, juvenile, larvae (1 day; >1 day) and seawater across generations (F_0, F_1, F_2) under ambient conditions. Relative abundances were sqrt-root transformed.

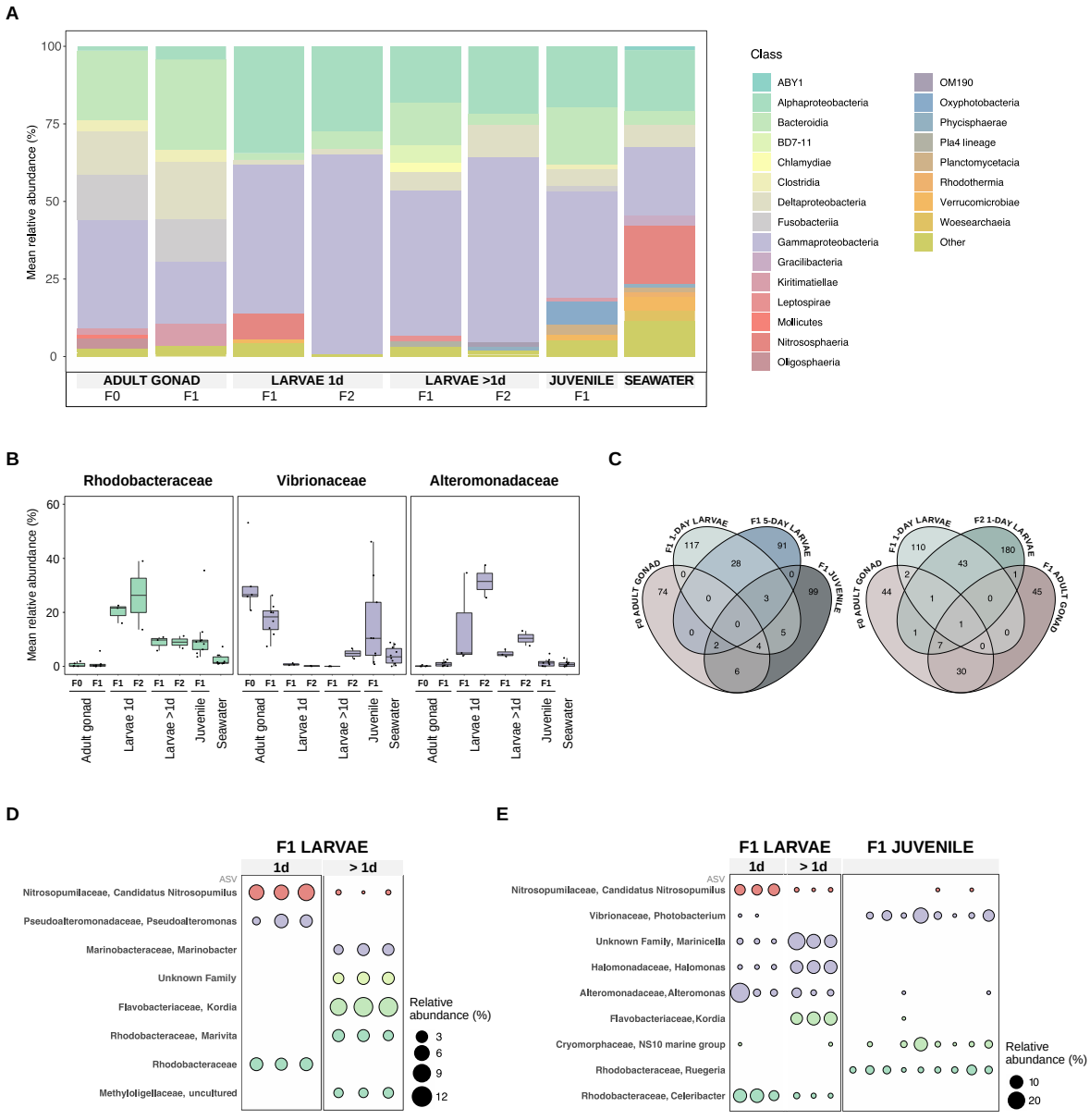

Fig. 3.3. Urchin microbial community structure under ambient conditions. (A) Mean relative abundance of prevalent microbial classes (>1% relative abundance; relative abundance mean in larvae F_2 is based on 2 replicates / larval age) and (B) boxplot of dominant 3 families across sample types and generations (colours based on taxonomic classes, see A). Box = inter-quartile range (IQR), line in box = median, whiskers = minimum and maximum values not outliers (i.e. -/+1.5*IQR). Seawater represents an environmental control, and we here show the seawater-associated microbial community pooled across time and generations. (C) Venn diagrams illustrating the number of shared ASVs between life stages and generations. Only ASVs present in at least 50% of the samples within each life stage / generation were considered; data were rarefied. (D) ASVs with significantly different relative abundances between 1-day and 5-day F_1 larvae, and I larvae F_1 (pooled 1-day and 5-day) and juvenile F_1 identified using DESeq analyses (1% mean relative abundance cutoff). Taxonomy is shown as Family and Genus, and colours are based on taxonomic classes, see A.

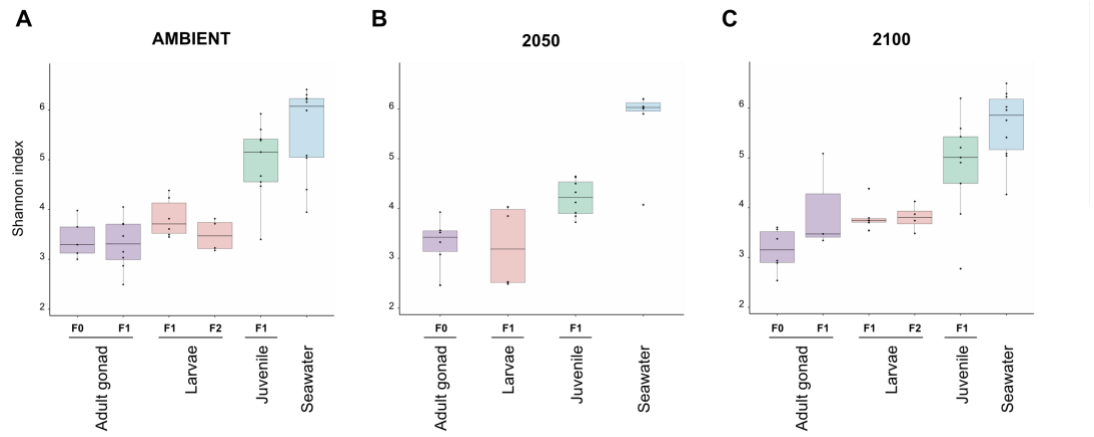

Fig. 3.4. Boxplots of Shannon diversity index representing alpha diversity of the microbiome in adult gonads, larvae (1d and >1d larvae were pooled), juveniles and seawater under (A) ambient, (B) 2050 and (C) 2100 treatments.

Effects of combined OA and OW predicted for years 2050 and 2100 on the urchin microbiome (aim 2)

Urchins were reared under future RCP 8.5 climate scenarios predicted for year 2050 and 2100 (see Fig. 3.1, aim 2; experimental design described in Uthicke et al., 2021) and the microbial community changes to OW / OA across host generations were assessed. NMDS visualization showed that the greatest difference in microbial communities was driven by life stage rather than combined temperature and pCO_2 levels (Fig. 3.5A). However, Principal Component Analysis (PCA) revealed that climate treatment had an effect on the microbial structure within life stages (Fig. 3.5B) and permutational multivariate analysis of variance confirmed this climate effect on F_0 adult gonads and F_1 juveniles (PERMANOVA, life stage*climate scenario: pseudo-F = 1.86, p = 0.01), 1-day F_1 larvae (PERMANOVA, climate scenario: pseudo-F = 3.38, p = 0.004), 5-day F_1 larvae (PERMANOVA, climate scenario: pseudo-F = 6.69, p = 0.003) and F_1 adult gonads (PERMANOVA, climate scenario: pseudo-F = 1.84, p = 0.028, Table S3.6). Microbial community dispersion was significantly different across life stages. Post-hoc comparisons showed that the microbial community of adult gonads (F_0) reared under ambient conditions was significantly different from adult gonads under the 2050 treatment (p =0.01; Fig. 3.5B, Table S3.6), and juveniles (F_1) under ambient were different to juveniles under 2050 ($p < 0.001$) and 2100 (p=0.04, Table S3.6) treatments.

Changes in relative abundances of Fusobacteriaceae, Prolixibacteraceae and Desulfobulbaceae were observed in gonads of adults exposed to 2050 treatments (Fig. S3.5), and for example, an ASV assigned to Desulfobulbaceae was significantly enriched in both 2050 and 2100 treatments compared to ambient conditions (Fig. S3.6). In juveniles (F_1), *Haliangium* strains (Haliangiaceae) were differentially

abundant among ambient and 2100 juveniles (F_1), while two ASVs assigned to Flavobacteriaceae were differentially abundant between ambient and 2050 F_1 juveniles (Fig. 3.5C). Climate treatment appeared to have an effect on the larval microbial structure at each age / generation (Fig. 3.5), but low replicate numbers precluded statistical confirmation of this pattern in F_2 larvae. An increase in Pseudomonadaceae and decrease in Rhodobacteraceae affiliated sequences was observed in 1-day F_1 larvae reared under climate treatments, while a decrease in Flavobacteriaceae and increase in Alteromonadaceae and Vibrionaceae occurred in 5-day F_1 larvae under 2100 conditions (Fig. S3.5). DESeq analyses revealed that distinct ASVs were differentially abundant among the ambient and two climate treatments (Fig. 3.5C and S3.6). Importantly, 48% of the microbial community in 1d-larvae (F_1) under 2050 conditions was comprised of one ASV identified as a *Pseudomonas* strain, and ASVs identified as *Psychrobacter*, *Acinetobacter*, *Aestuariibacter* and *Vibrio* were lost in 1-d larvae reared under 2050 and 2100 climate scenarios (Fig. 3.5C). Despite the microbial variation observed in urchins exposed to future climate scenarios, linear mixed effects models revealed that Shannon diversity was not affected by climate treatment, with life stage being the major driver of differences in microbial diversity among samples (Fig. 3.4, Table S3.7, Fig. S3.7).

We also explored the number of ASVs retained across life stages and generations under climate treatments. No ASVs were shared between adult gonads (F_0) and 1-day larvae (F_1) under 2050 conditions, while one ASV was shared under 2100 conditions (Fig. S3.8). In contrast, 31% and 38% of ASVs associated with 1-day larvae were also present in 5-day larvae (F_1) under 2050 and 2100 conditions respectively. These findings indicate that a higher proportion of microbial taxa was retained during larval development under these treatments compared to ambient conditions (20%, Fig. 3.3C). Finally, the proportion of ASVs retained from 5-day larvae to 5-month juveniles was minor (2% under 2050; 4% under 2100; Fig. S3.8). The three ASVs that were consistently associated with larvae and juveniles under ambient conditions, were not present at each life stage under climate treatments (Table S3.8 and S3.9). Under 2050 and 2100 conditions, the ASV affiliated to *Alcanivorax* was absent in juveniles; and 1-day larvae (F_1) were not associated with *Hyphomonas* under 2050 conditions, and *Oleiphilus* under 2100 conditions (Table S3.8 and S3.9). When exploring differences in microbial composition between generations of urchins reared under 2100 treatment, we found that 42% of ASVs associated with F_0 adult gonads was also present in F_1 gonads, and 35% of ASVs in F_1 1-day larvae was present in F_2 1-day larvae (Fig. S3.8), similar to the pattern observed under ambient conditions (Fig. 3.3C).

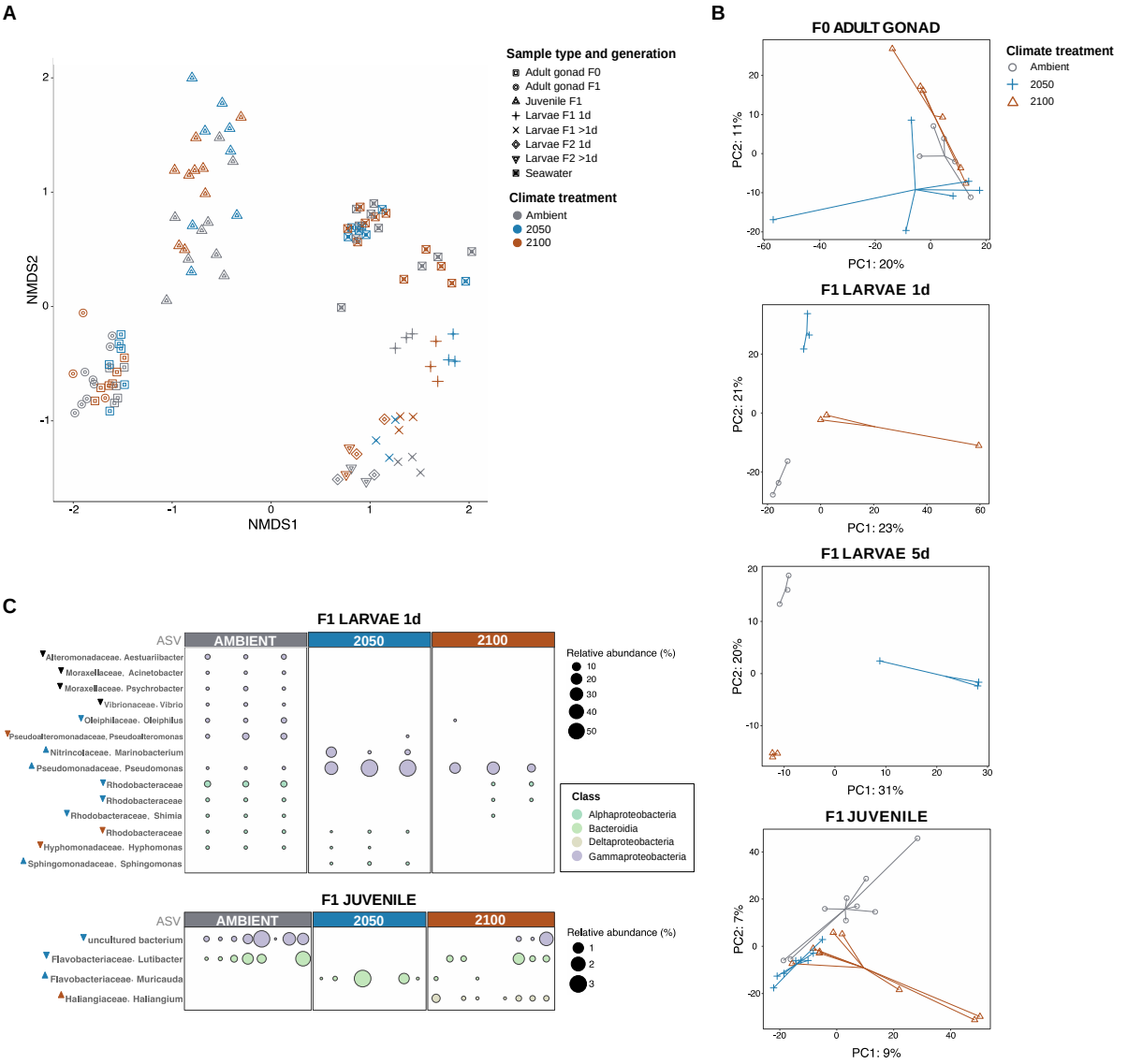

Fig. 3.5. Urchin microbial responses to combined OW and OA predicted for year 2050 and 2100 (RCP 8.5). (A) Non-metric Multi-dimensional Scaling (NMDS, sqrt-rooted data; stress = 0.14) based on Bray-Curtis dissimilarities calculated on relative abundance of ASVs present in adult gonad, juvenile, larvae and seawater across generations (F_0, F_1, F_2) and climate treatments (ambient, 2050, 2100). (B) Principal Component Analysis (PCA, centered log-ratio transformation) on relative abundance of ASVs in F_0 adult gonads, F_1 larvae at 1 and 5 days, and F_1 juveniles. (C) ASVs with significantly different relative abundances between climate treatments within F_1 juveniles and 1-day F_1 larvae identified using DESeq analyses. Only ASVs present across at least 50% of the samples in one of the compared groups are illustrated. Taxonomy is shown as Family and Genus, and a significant increase (▲) or decrease (▼) in relative abundance between Ambient and 2050 treatment (blue), Ambient and 2100 (red), and Ambient and both climate treatments (black) is illustrated for each ASV.

Effects of parental exposure to climate treatments on the offspring microbiome (aim 3)

Parental effect on the urchin microbiome was investigated through a transplant experiment in which F_1 juveniles from ambient parents were reared under ambient, 2050 and 2100 conditions, and juveniles from 2050 parents were reared under 2050 and 2100 conditions (Fig. 3.1, aim 3). Parental climate treatment, as well as offspring climate treatment, were sufficient for discrimination of the F_1 juvenile samples by multilevel sparse Partial Least Squares – Discriminant Analysis (sPLS-DA, six optimal components; Fig. 3.6A). Permutational multivariate analysis of variance confirmed this pattern (PERMANOVA, parental climate treatment*offspring climate treatment: pseudo-F = 1.78 $p < 0.001$; Table S3.10), with the microbiome of juveniles whose parents were exposed to 2050 or 2100 conditions different to juveniles from parents under ambient conditions (Fig 3.6A). However, the dominant microbial families (>2%) appeared to have similar relative abundances across parental climate treatments (Fig. S3.9). At the ASV level, DESeq analyses identified a wide range of ASVs differently abundant across parental and offspring treatments (Fig. 3.6B). However, parental treatment did not have a significant effect on the Shannon diversity index of F_1 juveniles, although highest diversity was observed in parents exposed to ambient conditions and lowest under 2050 treatment (Fig. S3.10, Table S3.11).

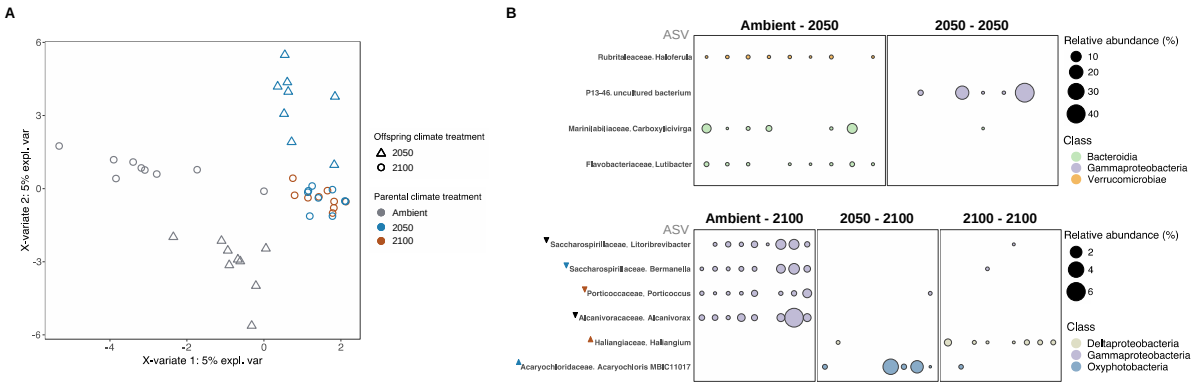

Fig. 3.6. F_1 5-month juvenile urchin microbiome in the transplant experiment. (A) Multilevel sPLS-DA on the relative abundance of ASVs in F_1 juveniles following centered log-ratio transformation. (B) Top ASVs with significantly different relative abundances across parental climate treatments (DESeq analysis). Only ASVs present across at least 50% of the samples in one of the compared groups are illustrated. Treatments are reported as 'parental (F0) climate treatment – juvenile (F1) climate treatment', and taxonomy is shown as Family and Genus. A significant increase (▲) or decrease (▼) in relative abundance between Ambient-2100 and 2050-2100 (blue), Ambient-2100 and 2100-2100 (red), and Ambient-2100 and both parental climate treatments (black) is illustrated for each ASV.

Discussion

Marine organisms are frequently associated with microbial communities that underpin their health and survival (McFall-Ngai, 2014; Robbins et al., 2021). Characterizing microbial dynamics throughout the development of marine invertebrate species and determining the effect of climate stressors on microbe-host relationships is critical for making predictions on ecological community structure in the context of environmental change. Using 16S rRNA gene sequencing, we determined that within the tropical sea urchin *Echinometra* sp. *A*, microbial life-stage specificity was maintained across generational exposure to future OW / OA conditions. However, results also indicated that shifts in microbial community composition occurred for specific urchin life stages under climate treatments, with a transplant experiment demonstrating that exposure of parents to climate treatments influenced the microbiota in subsequent urchin generations, thereby indicating potential for transgenerational effects of climate change on the microbiome.

Major life-history stages harbour a distinct microbiome under ambient conditions (aim 1)

We first investigated microbial community dynamics throughout the urchin life cycle (Fig. 3.1, aim 1), and found that adult gonads, larvae and juveniles harboured a distinct microbiota under ambient conditions (Fig. 3.2 and 3.3). Microbiome tissue-specificity has been widely reported for many reef marine invertebrates, including other urchin species (Bernasconi et al., 2019b; Carrier & Reitzel, 2019). In urchins, Carrier and Reitzel (2019) suggested that the establishment of a microbial community commences prior to fertilization since bacteria were detected in unfertilized eggs of three confamilial echinoids using 16S rRNA gene sequencing. However, ASVs retrieved from adult female gonads were not similar to larvae in our experiment; while only four ASVs were shared between F_0 gonads and 1-day F_1 larvae (Fig. 3.3C), a higher number of ASVs was shared between 1-day F_1 larvae and seawater (Fig. S3.3), indicating that microbial vertical transmission from parents to offspring may not be the primary mode of acquisition of these communities in *Echinometra* sp. *A*. Importantly, gonad sampling was performed following spawning, hence the sampling methodology could also have precluded the detection of some microbial taxa associated with the pre-spawning gonads. In regard to microbial diversity, an increase was observed from larvae to juveniles (Fig. 3.4A), suggesting that more bacterial niches become available for colonization throughout development. Although these results contrast with previous findings in other urchin species (Carrier & Reitzel, 2019), they are consistent with studies on other reef species with biphasic life cycles, where microbial alpha diversity increases during development, such as in the coral *Acropora digitifera* (Bernasconi et al., 2019b). Although urchin microbial diversity was conserved within each life stage between generations (Fig. 3.4A), transgenerational changes in microbial community composition were observed. For example, a higher

abundance of Alteromonadaceae, Oleiphilaceae and Cellvibrionaceae affiliated taxa was present in F_2 larvae compared to F_1 larvae (Fig. S3.2). And in the adult gonads, less than 50% of ASVs were shared between generations (Fig. 3.3C). Given that *Echinometra* sp. *A* appears to acquire microbes primarily from the surrounding environment, we hypothesize that microbial composition may be influenced by the available environmental microbial pool at each generation. We do not exclude that these microbial changes may be driven by stochastic mechanisms, as previously observed in the microbiota associated with echinoids eggs over multiple years (Carrier et al., 2020).

The microbiome of urchin larvae was dominated by Alteromonadaceae and Rhodobacteraceae families at 1-day in both F_1 and F_2 generations (Fig. 3.3B). These bacterial taxa have been observed at high relative abundance in larvae of other reef species, including the corals *Acropora digitifera* and *Acropora tenuis* (Bernasconi et al., 2019b; Damjanovic et al., 2020b). The larval microbial community structure was shaped by age (Fig. 3.3D), with the observed microbial changes likely linked to the emergence of new niches available for colonization during development. However, these changes may also occur due to the commencement of feeding in > 1-day larvae, as food has been shown to be the main source of bacterial uptake within larvae across multiple urchin species (Carrier et al., 2021; Schuh et al., 2020).

In urchin juveniles, members of the class *Oxyphotobacteria* were at higher abundances compared to all other life stages (Fig. 3.3A), and ASVs affiliated to Rhodobacteraceae and Vibrionaceae were also dominant at this stage (Fig. 3.3B and S3.2), in line with previous studies on cnidarian species (Bernasconi et al., 2019b; Mortzfeld et al., 2016; Quigley et al., 2020). In contrast, Alteromonadaceae affiliated sequences were present in low relative abundances (Fig. 3.3B), mirroring the observed reduction of Alteromonadaceae reported in the coral *Acropora digitifera* during development into juveniles (Bernasconi et al., 2019b). While members of the Rhodobacteraceae family were abundant in both juveniles and larvae, ASV level abundance within this family was widely variable across these life stages (Fig. 3.3E).

Urchin adult gonads (female) were dominated by *Gammaproteobacteria*, *Bacteroidia*, *Deltaproteobacteria* and *Fusobacteriia* classes (Fig. 3.3A), as well as several families, including Vibrionaceae, Desulfobulbaceae, Cryomorphaceae and Fusobacteriaceae (Fig. S3.2). In contrast to juvenile and larvae, adult gonads were characterized by low abundance in Rhodobacteraceae (Fig. 3.3B). However, it is worth noting that our sampling design was unable to separate tissue type effects (gonad) from life stage effects in adults. Hence, the observed differences between adult gonads and larvae / juveniles may be due to gonad microbiome specificity. Although the echinoderm gonad microbiome remains considerably underexplored, the microbiome associated with female gonads in the crown-of-thorns sea star *Acanthaster* cf. *solaris* appears to be highly variable (Høj et al., 2018), and a recent study reports high relative abundance of *Tenericutes* and *Spirochaetae* in multiple sea star taxa

(Jackson et al., 2018), which has not been observed in the *Echinometra* gonads collected within our study.

Microbial changes under exposure to 2050 and 2100 climate scenarios (aim 2)

To determine the multigenerational effects of OW and OA on the urchin microbiome, we assessed microbial community composition in urchin adult gonads, larvae and juveniles reared under future climate scenarios across three generations (Fig. 3.1, aim 2). Despite microbial life-stage specificity being maintained under OW / OA (Fig. 3.5A), treatment-attributed microbial changes were observed within each life stage (Fig. 3.5B). In adult gonads (F_0), microbial shifts were observed in urchins raised under 2050 and 2100 for ~ 2 years, with an ASV belonging to Desulfobulbaceae being present only at both high OW / OA conditions (Fig. S3.6). Although these results contrast to previous experimental studies on adult urchins (spines, gut) showing that the urchin microbial community is stable under exposure to OW / OA (Brothers et al., 2018; Webster et al., 2016), the discrepancy in microbial responses may be explained by the different tissue type as well as the extended exposure period investigated within this study. Indeed, Ketchum et al. (2021) recently confirmed that temperature-driven microbial shifts occur in the gut of the urchin *Echinometra* sp. *EZ* in natural conditions, suggesting that temperature can be a predictor of community variation.

Similar to adult gonads, the microbial community associated with larvae and juveniles was also altered under climate treatments. In 1-day larvae (F_1), for example, an ASV identified as *Pseudomonas* significantly increased in larvae exposed to 2050 conditions, representing the dominant ASV under this climate treatment (Fig. 3.5C). While in juveniles, *Muricauda* strains, which have been suggested to mitigate light and temperature stress through the provision of the antioxidant carotenoid zeaxanthin in cultured Symbiodiniaceae (Motone et al., 2020), were absent under ambient conditions but present at both climate treatments (Fig. 3.5C). Furthermore, the three ASVs consistently associated with each developmental stage (1-day larvae, older larvae and juvenile) under ambient conditions, were not present at each life stage under 2050 and 2100 conditions, indicating potential disruption of critical host-microbe interactions under climate treatments.

In parallel to characterizing the urchin microbial community, host health was also assessed under exposure to future climate conditions (see Uthicke et al., 2020, 2021). In contrast to previous short-term experiments (Uthicke et al., 2014b), F_0 adult health (growth, respiration rates and gonad development) was not impacted by climate conditions in our long-term experiment (analysed in Uthicke et al., 2020), suggesting adult urchins may have acclimated to the treatment conditions. Despite the maintenance of standard physiological performances in F_0 adults, a negative carryover effect was observed between F_0 and F_1 generations (Karelitz et al., 2020; Uthicke et al., 2021). Indeed, Karelitz and colleagues (2020)

assessed F_1 larval survival rates under ambient and 2100 conditions in the same experiment, and found that larvae from 2100-acclimated parents were characterized by a reduced survival. Furthermore, juveniles from 2050 and 2100 parents showed a decline in physiological performances at 9 months of age, until a pathogenic mortality event occurred in 10-month juveniles, which may have selected for phenotypes adapted to climate conditions (Uthicke et al., 2021).

Host physiological responses may be associated to the restructuring of the microbial community, as observed in other marine invertebrates (Botté et al., 2019). While changes in the gonad microbiome could be related to positive acclimatory mechanisms in our urchin species, the microbial alteration observed in larvae reared under climate treatments is likely a sign of dysbiosis occurring in parallel to a decrease in survival. The dominance of specific ASVs under climate conditions, which were absent or a minor component under ambient, further supports the hypothesis that larval microbial changes were detrimental. Similarly, microbial shifts in 5-month juveniles may represent an early sign of decline in urchin health due to negative parental carryover effects, although juvenile's size was not impacted by climate treatments at this sampling time (Uthicke et al., 2020). Finally, microbial alterations observed in F_2 adult gonads may be linked to the host phenotypic adaptation under treatment conditions following the mortality event. Stochastic processes, however, may also underlie the observed microbial changes (Sieber et al., 2019). It should also be noted that the urchin genetic diversity was reduced over generations, with potential implications for host acclimatory responses. To conclude, although microbial restructuring occurred under climate treatments, the influence of the microbiome in mitigating host stress and maintaining host physiology cannot be determined and additional studies investigating the link between microbial metabolic activities and host performance under environmental stress would be required.

Parental cross-generational effects observed on the offspring microbiome (aim 3)

Cross-generational effects on the microbiome have been observed in some marine species, but few studies have confirmed this mechanism in coral reef invertebrates. In our transplant experiment, parental (F_0) exposure to climate treatment affected the offspring microbiome (F_1 5-month juvenile; Fig. 3.1 aim 3, and Fig. 3.6), with potential implications for host health. For example, some ASVs belonging to Gammaproteobacteria were present in juveniles raised under 2100 conditions whose parents were under ambient treatment, but not in juveniles whose parents were exposed to 2050 or 2100 conditions (Fig. 3.6B). Given the likelihood that most *Echinometra* sp. *A* ASVs are acquired from the environment, experimental treatment conditions combined with host physiology and niche colonisation likely drive these microbiome changes, rather than cross-generational transmission. Despite these differences at the ASV level, major microbial changes were not detectable at the family level (Fig. S3.9). However, larvae were kept at the same treatment conditions as their parents, and thus, our

experimental design may have impacted our ability to fully identify cross-generational effects. Given older juveniles (9 months old) whose parents were reared under climate treatments showed signs of carryover effects on their physiology and behavioural responses (Uthicke et al., 2021), we hypothesize the microbial shifts observed in 5-month juveniles may be linked to early signs of decline in host health. Functional studies are needed to validate this hypothesis and characterize any metabolic change associated with the observed microbiome re-structuring.

Conclusions

Understanding how microbe-host interactions vary across host generations in a warming and acidifying ocean is critical for predicting realistic responses of reef species to future climate. Our 4-year experiment provides the first insights into microbial dynamics across life stages in a tropical urchin species exposed to OW / OA over multiple generations. Microbial communities were distinct across life stages, and this life-stage specificity was maintained under temperature and pCO_2 levels predicted for years 2050 and 2100 (RCP 8.5). However, microbial dynamics were affected by exposure to OW / OA within life stages, and the offspring microbiome was also influenced by parental exposure to climate treatments. As *Echinometra* sp. *A* appears to acquire microbes primarily from the surrounding environment, we exclude that inheritance of shuffled microbes is the basis of the observed parental effect, but postulate that urchin hosts may modify microbiome recruitment in response to parental exposure to OW / OA.

Acknowledgements

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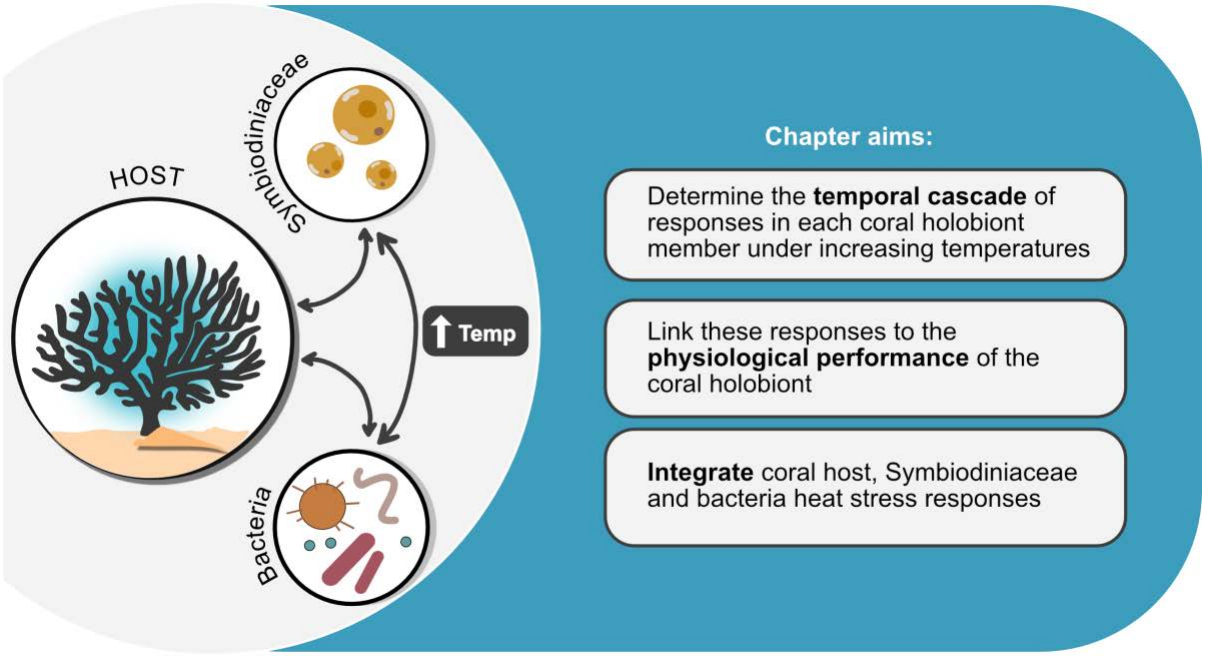
CHAPTER 4 : DESTABILIZATION OF MUTUALISTIC INTERACTIONS SHAPES THE EARLY HEAT STRESS RESPONSE OF THE CORAL HOLOBIONT



The associated publication is in preparation:

Marangon E, Rädecker N, Li JYQ, Terzin M, Buerger P, Bourne DG, Webster NS, Laffy PW (2023) Destabilization of mutualistic interactions shapes the early heat stress response of the coral holobiont, *in preparation*.

Destabilization of mutualistic interactions shapes the early heat stress response of the coral holobiont

Abstract

The stability of the symbiotic relationship between coral and their dinoflagellate algae (Symbiodiniaceae) is disrupted under the increasing rates of ocean warming. Although coral thermal response depends on the interactions between host, Symbiodiniaceae and prokaryotes, the mechanisms underlying the initial destabilisation of these symbioses are poorly understood. In a 2-month manipulative experiment, we exposed the coral *Porites lutea* to gradually increasing temperatures corresponding to 0 – 8 Degree Heating Weeks (DHW) and assessed the response of the coral holobiont through coral and Symbiodiniaceae transcriptomics, microbial 16S rRNA gene sequencing and physiological measurements. From early stages of heat stress (< 1 DHW), the increase in metabolic turnover shifted the holobiont to a net heterotrophic state in which algal-derived nutrients were insufficient to meet host energy demands, resulting in reduced holobiont performance at 1 DHW. The altered nutrient cycling also affected the coral-associated microbial community, with the relative abundance of *Endozoicomonas* bacteria showing a decline under increasing heat stress. Integration of holobiont stress responses linked this decline to an increase in the expression of a host ADP-ribosylation factor suggesting that Symbiodiniaceae and *Endozoicomonas* may underlie similar endosymbiotic regulatory processes. The thermotolerance of coral holobionts therefore integrates the nutritional status of its members and their interactions.

Introduction

Climate change and other anthropogenic stressors are driving a decline of coral reefs worldwide (Souter et al., 2021). Increasing sea surface temperatures trigger coral bleaching (loss of endosymbiotic dinoflagellates of the family Symbiodiniaceae), often resulting in widespread coral mortality (Hughes et al., 2018a). Given the increased frequency and severity of bleaching events in the last few decades (Hughes et al., 2021), a greater focus is required to characterize the mechanisms underlying bleaching susceptibility to apply effective reef conservation strategies. Coral responses to heat stress are complex and influenced by multiple factors, including the interplay between the coral host and its associated symbionts (Avila-Magaña et al., 2021; Cziesielski et al., 2019; Rädecker et al., 2021). The exchange and recycling of limiting nutrients between Symbiodiniaceae and their host are critical for the ecological success of corals in oligotrophic environments (Weis, 2008). Altered nutrient cycling (especially carbon and nitrogen) may thus be a major driver of the initial destabilization of the coral symbiosis under high temperatures (Baker et al., 2013; Cui et al., 2019; Rädecker et al., 2021). In addition, bacteria and

archaea (i.e. 'microbes' hereafter) also contribute to holobiont functioning, for example in the form of nutrient transformation and antimicrobial activity, and may therefore influence coral thermotolerance (Avila-Magaña et al., 2021; Baldassarre et al., 2022; Marangon et al., 2021; Robbins et al., 2019). However, our understanding of the ecological and metabolic processes initiating the destabilization of these symbiotic relationships under heat stress is still incomplete. Elucidating and integrating the response of each specific holobiont member (i.e. host, Symbiodiniaceae, microbes) to increasing temperatures is therefore critical for deciphering the mechanisms underlying coral holobiont breakdown.

Within coral symbiosis, stress intensity strongly influences the holobiont response (Dixon et al., 2020; Savary et al., 2021). Transcriptomic analyses of heat-stressed corals revealed that immunity responses, cytoskeleton organization, antioxidant expression, heat-shock-proteins and pre-apoptotic responses are among the most commonly enriched pathways under high temperatures, and have been linked to severe stress responses (Cziesielski et al., 2019). However, mechanisms underlying the initial destabilization of the symbiosis are highly debated. The leading hypothesis stating that the production of reactive oxygen species (ROS) by the Symbiodiniaceae triggers host immune response, and in turn, symbiosis breakdown (Weis, 2008), has been recently challenged (Dungan et al., 2022; Krueger et al., 2015; Nielsen et al., 2018; Tolleter et al., 2013). Growing evidence suggests that nitrogen availability may play a central role in the regulation of coral symbiosis (Cui et al., 2019; Morris et al., 2019; Rädecker et al., 2021). In a healthy state, nitrogen limitation within the holobiont induces Symbiodiniaceae to release the excess photosynthates (e.g. glucose) to the host fuelling host respiration and efficient carbon recycling (Cui et al., 2023; Pernice et al., 2012; Xiang et al., 2020). Heat stress, however, disrupts this fine equilibrium by enhancing energy demand and catabolic ammonium production in the host metabolism (Rädecker et al., 2021). Increased nitrogen availability within the holobiont may thus stimulate Symbiodiniaceae to proliferate and retain photosynthates thereby destabilizing the ecological basis of the mutualism (Rädecker et al., 2021; Xiang et al., 2020).

Adding to this complexity, microbial communities may affect the thermotolerance of their cnidarian hosts (Baldassarre et al., 2022). Indeed, bacterial metabolic capabilities may represent a key advantage under heat stress providing potential alternative metabolic pathways to maintain coral holobiont functioning (Avila-Magaña et al., 2021). Coral-associated microbes may also affect the stability of the coral-Symbiodiniaceae symbiosis, altering antioxidant capacity (e.g. through DMSP cycling) or nutrient levels (e.g. nitrogen transformation) (Ceh et al., 2013; Motone et al., 2020; Rädecker et al., 2015). For example, elevated nitrogen availability within the coral *Stylophora pistillata* under heat stress alleviates the host and symbionts' metabolic dependency on nitrogen-fixing bacteria, driving compositional and functional changes in the diazotrophic microbial community (Rädecker et al., 2021,

2022). Integrating microbial community dynamics within the holobiont is thus required when exploring the fundamental mechanisms of coral stress responses.

Here, we combined physiological and molecular tools (mRNA and 16S rRNA gene sequencing) to characterize the relationship among the thermotolerant coral *Porites lutea* host, Symbiodiniaceae and associated microbes under gradually increasing temperatures (28 – 32˚C; equivalent to 0 - 8 Degree Heating Weeks) over a 56-day experiment (Fig. 4.1). This manipulative heat stress experiment, i) characterizes the temporal cascade of responses in host and Symbiodiniaceae gene expression as well as microbial community structure with increasing stress intensity, ii) links the individual changes in the response of holobiont members to the physiological performance of the holobiont, and iii) integrates holobiont member responses to early heat stress by identifying the main correlations between changes in host and Symbiodiniaceae gene expression and microbial community structure.

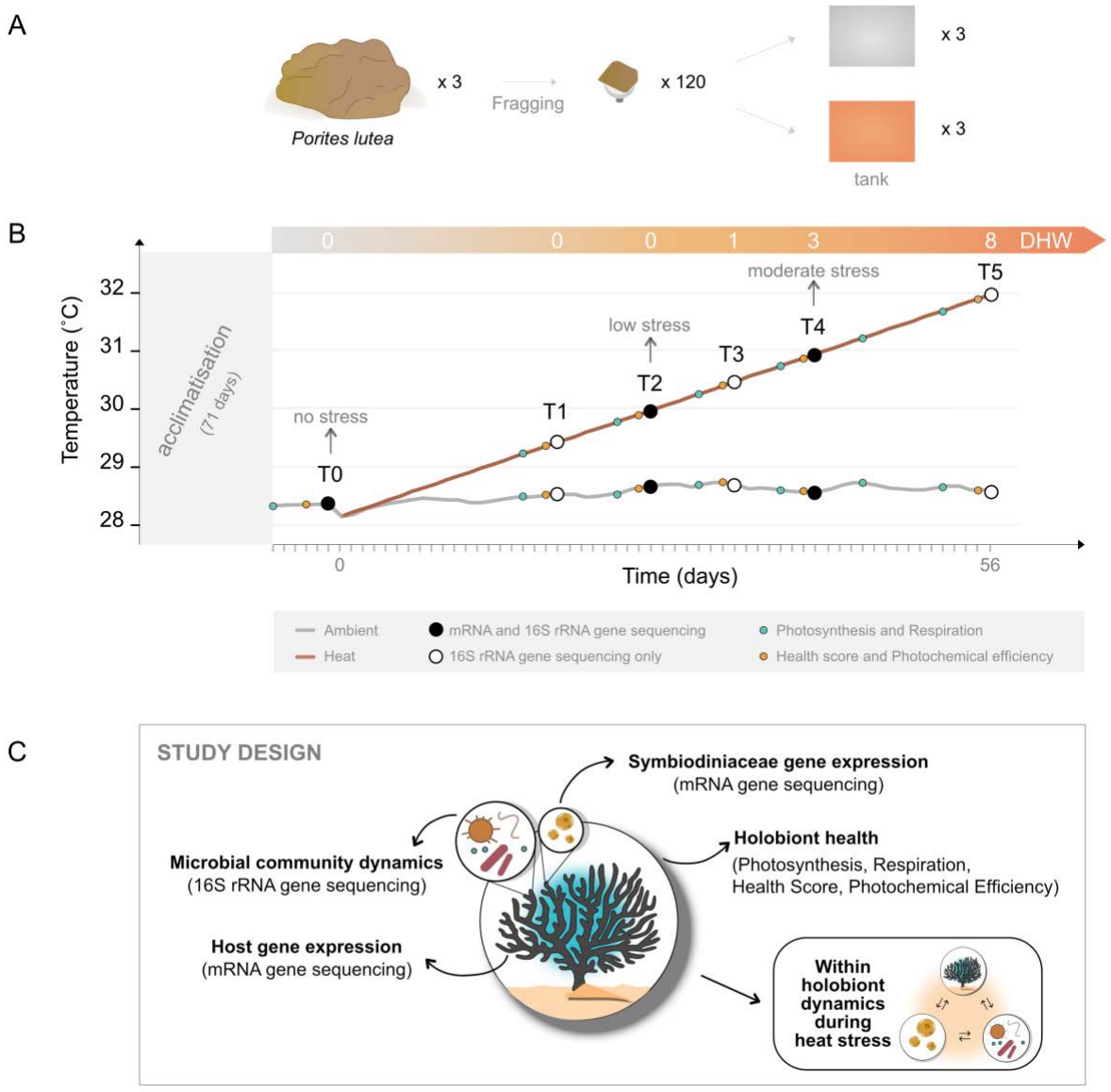


Fig. 4.1. Experimental design of the two-month incremental heat stress experiment on the thermal tolerant coral Porites lutea. A) Three coral colonies were collected from Orpheus Island (central GBR), fragged into smaller pieces, and randomly distributed across six tanks (ambient and heat treatment). B) For determining Symbiodiniaceae, host and microbial responses to increasing temperatures, coral fragments were sampled at T0-T5 for mRNA and / or 16S rRNA gene sequence analyses. Degree Heating Weeks (DHW), representing the accumulated heat stress, are shown for each sampling time. Health score, net photosynthesis, respiration and photochemical effective efficiency were assessed over time as proxy for host health. C) Overview of the sampling design.

Material and methods

Coral collection and experimental design

Three colonies of the reef-building coral *Porites lutea* were collected (~ 5 m depth) from Orpheus Island (central GBR, Australia) in September 2019 (Permit G12/35236.1). Colonies were transferred to the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (Townsville, Australia), where they were maintained in independent flow-through tanks at ambient temperature in the outdoor SeaSim area for 3 weeks. Following the acclimation period, each colony was sampled for 16S rRNA gene sequencing ("baseline" samples; n=4), and fragmented into at least 38 pieces (approximately 4x4 cm) using an underwater drill, ring saw and band saw. Fragments were glued onto aragonite plugs and transferred to indoor flow-through tanks (n= 6; 50L; 0.8 L min^{-1}), distributing 5 - 7 fragments from each colony of origin per tank. Coral fragments were maintained at ambient temperature for 10 weeks, with temperatures reflecting the average daily temperature of the Central GBR (Davies Reef, 1991 – 2012; 27 ±0.8 ˚C; Australian Institute of Marine Science, 2020). During this period, corals were fed daily with microalgae (*Nannochloropsis oceania*, *Isochrysis* sp., *Chaetoceros muelleri*, *Dunaliella* sp., *Proteomonas sulcate*; 2000 cells mL^{-1} in tank) and rotifers (480 rotifers L^{-1} in tank).

Following the acclimation period, coral fragments under the heat treatment were experimentally exposed to incremental temperatures for 56 days (0.07 ˚C daily increment, from 28.16 ˚C to 32.01˚C corresponding to 0-8 Degree Heating Week; n=3 tanks), while ambient conditions mimicked daily natural fluctuations in summer temperatures in the Central GBR (Australian Institute of Marine Science, 2020; 28.5±0.12 ˚C; n=3 tanks). Seawater temperatures in both ambient and heat tanks were regulated by a computer-controlled system which provides fine scale control of environmental parameters, and further stabilized through water jackets. A natural 12:12h light:dark cycle was set up using SOL LED lights with 4-h ramping at sunrise and sunset (max light intensity 130 µmol photons m^{-2} s^{-1}), and corals were fed with microalgae and rotifers three times per week.

During the experiment, several physiological parameters were regularly measured to assess holobiont health: photosynthesis and respiration rates, photochemical efficiency and bleaching score (Fig. 4.1). In parallel, sampling was performed at six time points (weeks 0, 3, 4, 5, 6, 8 hereafter referred to as T0 – T5) by collecting one fragment of each colony of origin per tank for mRNA and 16S rRNA gene sequencing (Fig. 4.1). Each fragment was cut into smaller pieces using a sterile band saw, rinsed with filtered seawater (0.22 µL), snap frozen in liquid nitrogen and stored at -80˚C until processing. Seawater was also sampled at T0, T3 and T5 as environmental control for 16S rRNA gene analyses; 1 L of seawater per each tank was filtered through 0.22 µL Sterivex filters (Millipore), which were subsequently stored at -80˚C until DNA extraction. Additionally, microalgae and rotifers used to feed

the corals were sampled for 16S rRNA gene analyses to control for diet-introduced microbes in the coral microbiome.

Holobiont physiological health metrics

Coral health was regularly assessed using the CoralWatch Coral Health Chart (Siebeck et al., 2006) to detect visible signs of bleaching (reduction in pigmentation). Coral pictures were taken under constant settings, and analysed in ImageJ (Schneider et al., 2012), where the mean grey value per each fragment (based on n=5 measurements) was standardized to the CoralWatch D1-D6 references using linear regressions to determine a comparable Health score (Quigley et al., 2019). Health score was measured on each coral fragment over time (T0: n = 60 per treatment; T1: n = 49 – 52 per treatment; T2: n = 42; T3: n = 32 – 33 per treatment; T4: 23 – 24 per treatment; T5: 13 – 15 per treatment).

Net photosynthesis (oxygen production, P) and respiration (oxygen consumption, R) rates were measured to estimate the carbon flux between host and Symbiodiniaceae through the photosynthesis-to-respiration ratio (P / R), and were performed using a modified process previously described in Schoepf et al. (2019). Briefly, dissolved oxygen was measured using an Optical probe after placing coral fragments (T0 - T4: n = 18 per treatment; T5: n = 13 - 15 per treatment) in 600 mL plastic chambers for 50-min incubation under constant light intensity (130 µmol photons m^{-2} s^{-1}) and 75-min incubation in dark conditions, respectively. Corals were acclimated for at least 1h to the respective light or dark conditions prior to incubation. During the incubation period, chambers were placed in water baths with temperature reflecting treatment conditions, and water motion within each chamber was generated by a magnetic stirrer. To account for the background variation in oxygen flux, chambers containing only seawater were incubated alongside the coral fragments (i.e. blanks; n=2 per tank/time point). P and R rates were adjusted by the oxygen concentration in the blanks and standardized by the volume of water in each chamber and the surface area of the fragment. The water volume was calculated using the displacement method, while each fragment's surface area was estimated via the aluminium foil method (Marsh, 1970). P / R was calculated as ratio of 12 h of gross P (= net P + R) and 24 h of R (Schoepf et al., 2019). Notably, this approach likely overestimates daily gross photosynthesis as the ramping of light intensities at sunrise and sunset is not accounted for in the analyses. While these P / R ratios may not provide an accurate assessment of daily carbon budgets, they do provide a robust estimate of relative changes between treatments and facilitate comparison with other studies.

To assess the photosynthetic efficiency of coral-associated Symbiodiniaceae under increasing temperatures, photochemical effective efficiency (ΔF/Fm') was analysed (Warner et al., 1999). Briefly, ΔF/Fm' was measured using a mini-PAM fluorometer (Walz, Germany; settings: MI = 4; SI = 8; SW

= 0.8; G=2; D=2) on corals acclimated to peak light conditions for at least 1 h. Measurements (n=3 per fragment at each time point; all fragments were assessed) were taken at a constant distance of 0.9 cm from the coral tissue, and the mean photochemical effective efficiency was calculated per fragment at each time point.

The effect of incremental temperatures on physiological host health metrics was tested using generalized linear mixed models ('glmmTMB package', Brooks et al., 2017) in R (version 4.0.3, R Core Team, 2020). Specifically, the effect of treatment and time on Health Score and Photosynthesis rates (square-root transformed) were analysed with a gaussian model, Respiration rates (absolute value) as well as P / R ratio with a gamma distribution, and Photochemical Effective Efficiency (square-root transformed) with a beta distribution. For testing net P rates, an offset was applied to the data to remove negative values prior to the square-root transformation. In the models, colony of origin was also included as fixed effect, while tank and fragment were included to account for repeated measures. Model assumptions were checked with 'DHARMa' (Hartig, 2021), and model selection was completed based on the corrected Akaike information criterion (AICc) when more than one model distribution was appropriate for the analyses. Pairwise comparisons were run using 'emmeans' (Lenth, 2022) with Šidàk correction.

Coral host and Symbiodiniaceae transcriptomics

After crushing the fragments at low temperatures using a French-press, RNA was isolated using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific), including a Trizol/Chloroform step. First, coral samples (tissue and skeleton) were added to bead tubes (MP; n=2 tubes per sample) containing 1 mL Trizol, and then homogenised using the FastPrep-24 5G instrument (MP Biomedicals). After spinning down the homogenised tissue, the supernatant was mixed with 200 µL chloroform and centrifugated, the upper phase containing RNA was then purified following the manufacturer protocol, which included also an on-column DNase treatment (Thermo Fisher Scientific). Purified RNA was diluted in 34 µL of nuclease-free water, and RNA quality and concentration were assessed using Nanodrop 2000 spectrophotometer (Thermo Scientific) and Agilent 2100 Bioanalizer with the RNA 6000 Pico assay. High-quality RNA was stored at -80˚C until submission to the Ramaciotti Centre for Genomics (UNSW, Australia) for Illumina stranded mRNA library preparation (including a polyA step selecting eukaryotic mRNA) and sequencing on NovaSeq 6000 S2 2 x 100 bp. Sequencing generated an average of ~ 75 million reads per sample.

To determine the gene expression patterns of coral host and associated Symbiodiniaceae to incremental temperatures, reads were processed for reference-based transcriptomics as previously described in (Buerger et al., 2020). First, read quality was assessed using FastQC (Andrews, 2010), and results were summarised with MultiQC (Ewels et al., 2016). For identifying and separating the holobiont members (i.e. host and Symbiodiniaceae), reads were then mapped to the *Porites lutea* reference genome (Robbins et al., 2019) using the RNA-seq aligner STAR with the --outFilterScoreMinOverLread and --outFilterMatchNminOverLread parameters set to 0.55 (version 2.7.9a, Dobin et al., 2013). The unmapped reads were then aligned to the *Cladocopium goreaui* genome (https://espace.library.uq.edu.au/view/UQ:fba3259; Liu et al., 2018b). The gene counts generated by STAR (reverse strandness) were processed and imported in R (R Core Team, 2020) for gene expression analyses. Overall, two samples were excluded from subsequent analyses based on quality control, and one (Symbiodiniaceae) and two (host) samples were further identified as outliers using a standardized connectivity test on network analyses and removed from the analysis (Wright et al., 2015; Fig. S4.1). Genes were assigned to euKaryotic Orthologous Groups (KOG), Gene Ontology (GO) terms, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways through eggNOG-mapper (version 2.1.6, Huerta-Cepas et al., 2017) on the Galaxy Australia platform (Afgan et al., 2018), with query sequences searched against the eggNOG database 5.0.2 (Huerta-Cepas et al., 2016) using Diamond.

Data normalization and transformation (log-CPM), removal of genes with low counts ('filterByExpr' function), as well as construction of mixed effect models for identifying Differentially Expressed Genes (DEGs; false-discovery rate, Benjamini–Hochberg adjusted p-value ≤ 0.05) were run on the genome-mapped reads using the limma-voom pipeline (version 3.46.0, (Law et al., 2018; Ritchie et al., 2015)). For the identification of DEGs under incremental temperatures, samples under low heat stress (Heat T2) were compared to samples under ambient conditions (Heat T0, Ambient T0, Ambient T2), and samples under moderate heat stress (Heat T4) were compared to Heat T0, Ambient T0, Ambient T2 and Ambient T4 samples; colony of origin was included in all mixed models as random effect.

To identify KOG classes that were significantly enriched with up- or down-regulated genes between treatments, KOG enrichment analyses were run using the Mann–Whitney U test (R package 'KOGMWU'; Dixon et al., 2015), and similarities among up- and down-regulated KOG classes across heat treatments using Pearson correlations. Through the KOGMWU function, delta ranks for 23 KOG gene classes were calculated based on log fold-changes (logFC) generated from the pairwise comparisons between treatments (limma-voom analyses described above). Similarly, GO enrichment analyses were run following the GO_MWU pipeline (https://github.com/z0on/GO_MWU) on logFC to determine which biological processes were differentially regulated under heat stress.

Multilevel sparse partial least-squares discriminant analysis (sPLS-DA) was performed separately on host and Symbiodiniaceae transcriptomic data using the R mixOmics package (v6.14.1, Rohart et al., 2017), following variance stabilizing transformation ('DESeq2', Love et al., 2014). The effect of colony of origin was accounted in the model through a multilevel approach. Tuning of sPLS-DA was performed to select the most discriminative genes between treatment conditions at the lowest error rate, using repeated cross validation (5 folds, 100 repeats) and mahalanobis distance; it resulted in the selection of one component for both datasets, with an error rate of 0.42 and 0.23 for host and Symbiodiniaceae, respectively. Among the most discriminative genes selected from the sPLS-DA models, loading weights were used to identify the most influential genes within the Symbiodiniaceae dataset.

16S rRNA gene amplicon analyses

DNA derived from coral and seawater was extracted using the DNeasy PowerSoil Pro kit (QIAGEN), according to the manufacturer's protocol, with the cell lysis step performed using the FastPrep-24 5G bead beater (MP Biomedicals). DNA was also extracted from feeds (rotifers and microalgae) added to the tanks, to act a control and ensure recovered bacterial 16S rRNA gene reads were derived from coral samples. The V4 region of the 16S rRNA gene was amplified using the forward primer 515F (Parada et al., 2016) and the reverse primer 806rB (Apprill et al., 2015), for targeting both bacteria and archaea taxa, in a 30 cycle PCR using the AmpliTaq Gold 360 Master Mix (ThermoFisher) under the following conditions: 95 ˚C for 10 min, 30 cycles of 95˚C for 30 sec, 56 ˚C for 1 min and 72˚C for 30 sec, followed by a final elongation at 72 ˚C for 7 min. PCR products were sent to the Ramaciotti Centre for Genomics (University of New South Wales, Australia) for standard Illumina library preparation and sequencing on the Illumina MiSeq 2x250 bp platform.

Sequence data underwent analyses in QIIME2 (v 2020.8, Bolyen et al., 2019). Following removal of poor quality reads and chimeras, amplicon sequence variants (ASVs) were identified based on 100% sequence similarity using DADA2 (Callahan et al., 2016). Taxonomy was assigned through a naïve Bayes classifier trained with the feature-classifier plugin using the primers 515F/806rB on the SILVA 132 database (Quast et al., 2012). Phylogenetic relationships were built using FastTree. Microbial community analyses were run in R (R Core Team, 2020), where reads assigned to Chloroplasts, Eukaryotes and Mitochondria were removed as well as contaminants identified using stringent thresholds in the R package 'decontam' (Davis et al., 2018; *P* threshold = 0.5; n=1 contaminant identified, Table S4.1). Singletons and samples with < 5,400 reads (n=4) were also excluded from the analyses.

Shannon diversity index was measured on the dataset rarefied to 5,400 sequences ('phyloseq package', McMurdie & Holmes, 2013). Differences in alpha diversity among treatment and time were tested using a linear mixed model ('glmmTMB package', Brooks et al., 2017) with colony of origin as fixed effect and tank as random effect; model assumptions were checked through DHARMa residual diagnostics (Hartig, 2021). For beta diversity analyses, Bray-Curtis dissimilarities were applied to non-rarefied data normalized using proportions and square-root transformation (McKnight et al., 2019), excluding ASVs with an overall relative abundance < 0.001% (9,800 ASVs). To determine differences in microbial composition across time (baseline, T0-T5), treatment (ambient, heat), and samples types (coral, seawater, feed), non-metric Multi-dimensional Scaling (NMDS) were generated using 'phyloseq' (McMurdie & Holmes, 2013) and Permutation Multivariate Analysis of Variance (adonis) was run in 'vegan' (10,000 permutations) (Oksanen et al. 2020). Dispersion was checked using Multivariate Homogeneity of Group Dispersions ('vegan package', Oksanen et al., 2020), and pairwise comparisons were calculated using Benjamini-Hochberg correction in 'RVAideMemoire' (Hervé, 2021). Differential abundance analyses ('DESeq2 package', Love et al., 2014) were run on filtered data using p-value of 0.01, and only significant ASVs present across at least 50% of the samples in one of the compared groups were identified as differentially abundant between groups.

Multilevel integration of omics-data analysis

Microbial community composition, Symbiodiniaceae gene expression and host gene expression data were integrated using DIABLO (Data Integration Analysis for Biomarker discovery using a Latent component method for Omics studies) in mixOmics (Rohart et al., 2017), following data normalization (microbiome: centered log-ratio transformation; transcriptomics: normalized variance stabilized counts). DIABLO is a supervised approach that maximises the common or correlated features across omics-datasets retrieved from the same biological samples, to identify key molecular signatures (Singh et al., 2019). A multilevel approach was applied to account for the effect of colony of origin, and annotated genes (KEGG Orthology) were used as input for these analyses, which corresponded to 7,627 genes for the Symbiodiniaceae dataset and 12,435 for the host. A design matrix of 0.6 was applied using 50 × 5-fold cross-validation.

Results

Holobiont performance declines from 1 DHW

Corals exposed to increasing temperatures showed signs of reduced physiological performance at 1 DHW. Photosynthesis rates gradually declined with increasing temperatures and significant differences between treatments were observed from 3 DHW (Fig. 4.2, Table S4.2). In contrast, R rates showed a

(albeit insignificant) tendency to increase with elevated temperatures (Fig. S4.2, Table S4.2). Consequently, P / R ratio significantly declined with increasing temperature from 1 DHW onwards with ratios dropping below the autotrophic compensation point of 1. This coincided with a significant reduction in the effective quantum yield of coral holobionts (Fig. 4.2, Table S4.2). Ultimately, the reduced photophysiological performance of coral holobionts was also reflected in their health scores (pigmentation). A significant decline was observed from 3 DHW, with corals under 3 and 8 DHW showing an average decrease of 2.6 and 4.1 units respectively (Fig. 4.2 and Table S4.2). Despite the significant loss in pigmentation compared to ambient conditions, corals under 3 DHW were characterized by an average health score above 6 (Health score 6.8 ± 1.1; Siebeck et al., 2006), indicating corals were still healthy, whereas exposure to 8 DHW resulted in coral bleaching (Health score 3.8 ± 0.7).

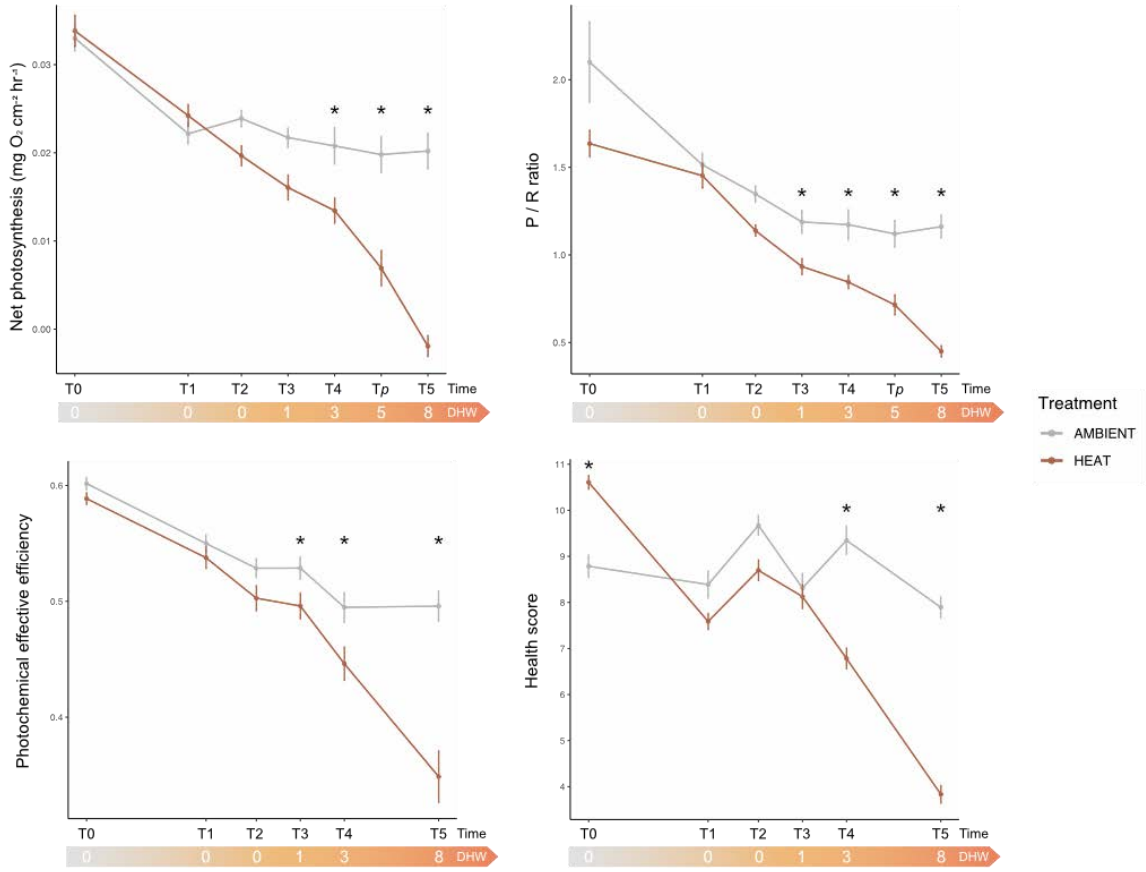


Fig. 4.2. Holobiont physiological health metrics under increasing heat stress. Net photosynthesis, photosynthesis / respiration ratio, photochemical effective efficiency (ΔF /Fm') and health score (health score ≥ 6 indicates healthy corals) are shown over time (T0 – T5) under increasing temperatures; DHW are reported for the heat treatment only (DHW = 0 under ambient conditions). Net photosynthesis and P / R ratio were measured at an additional time point (T*p*) corresponding to 5 DHW. Mean ± SE is shown over time, with asterisks indicating significantly different physiological responses between ambient and heat treatment at each time point ($p < 0.05$; adjusted post hoc tests).

Host transcriptional responses to incremental heat stress

An average of 47% of host derived transcriptional reads mapped to the reference (*Porites lutea* genome; Table S4.3), representing 35.1 million reads per sample. Despite a strong genotype effect, with colony of origin explaining the majority of the total variation in gene expression patterns (Fig. S4.3), expression profiles were also affected by heat stress. Compared to ambient conditions, 15 and 12 KOGs were enriched under low (< 1 DHW; T2) and under moderate (3 DHW; T4) stress, respectively, with overall significant correlation between low and moderate stress enrichment patterns ($r = 0.6$, $p < 0.01$; Fig. 4.3). Among these patterns, the expression of genes related to 'energy production' were upregulated under heat stress while processes related to 'inorganic ion and amino acid transport and metabolism' were downregulated. Furthermore, genes related to 'replication, recombination and repair' were significantly downregulated under low stress while upregulated under moderate stress; while genes associated with 'translation, ribosomal structure and biogenesis' were significantly upregulated under low stress only (Fig. 4.3A).

These overall expression patters were corroborated by the GO enrichment analysis. Heat stress caused a significant upregulation of genes involved in processes related to the tricarboxylic acid (TCA) cycle, as well as adenosine triphosphate (ATP), lipid, and glucose metabolism, and protein catabolism (Table S4.4 and S4.5). At the same time, apoptotic pathways were positively regulated under both low and moderate stress (Table S4.4 and S4.5). In addition to these universal heat stress responses, there were also differences in the GO enrichment patterns between low and moderate heat stress. While anion transport was positively regulated under low stress conditions, it underwent downregulation under moderate stress conditions, together with bicarbonate transport (Table S4.4 and S4.5).

Differential expression analyses identified 151 genes differentially expressed under moderate stress compared to ambient conditions (total of 24,207 genes; Fig. 4.4 and Table S4.6), while no DEGs were detected at low stress. Among the identified DEGs under moderate stress, genes encoding for glutamate synthase (GOGAT) and phosphoserine aminotransferase (PSAT), which are key genes in amino acids biosynthetic processes, were significantly downregulated. Additional genes associated with the *de novo* serine biosynthesis pathway (Cui et al., 2019) were also downregulated (albeit not significantly) under low and moderate stress, while multiple genes involved in the glycine/serine biosynthesis pathway dependent on food-derived choline (Cui et al., 2019) showed an increase in expression under these conditions (i.e. dimethylglycine dehydrogenase [DMGDH], sarcosine dehydrogenase [SARDH]).

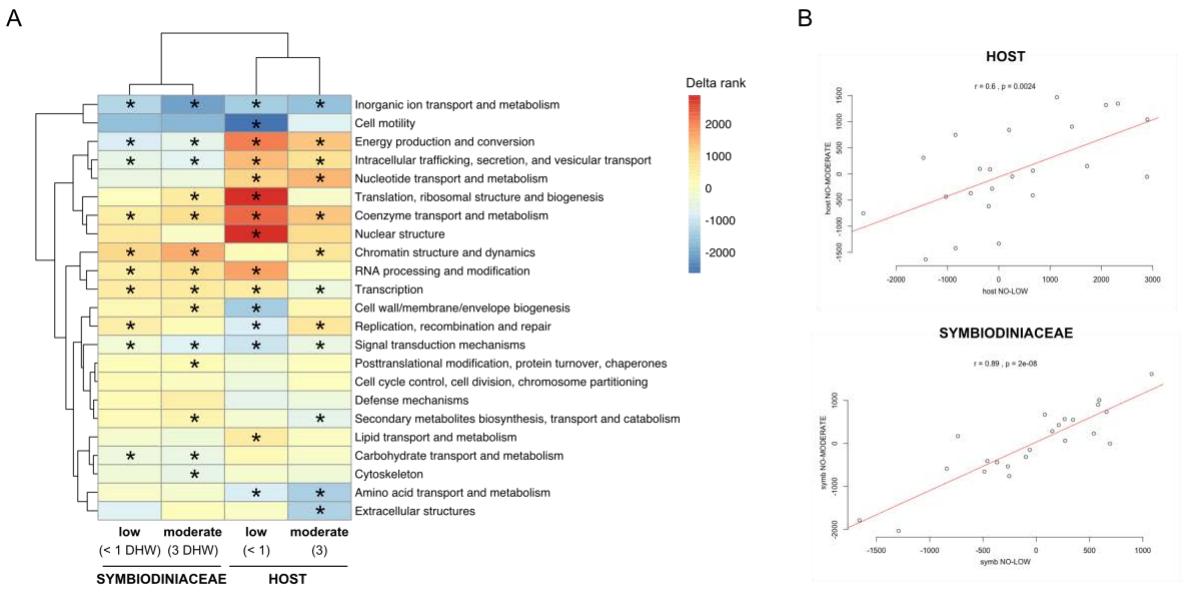


Fig. 4.3. EuKaryotic Orthologous Group (KOG) enrichment analysis in host and Symbiodiniaceae during early heat stress. A) Heat map illustrating KOG categories up (red) and down (blue) regulated in low (< 1 DHW; T2) and moderate (3 DHW; T4) heat stress compared to ambient conditions in Symbiodiniaceae and host. Asterisks indicate KOG categories significantly enriched with up or down regulated genes. B) Correlation between KOG delta ranks between low stress vs ambient, and moderate stress vs ambient in host and Symbiodiniaceae.

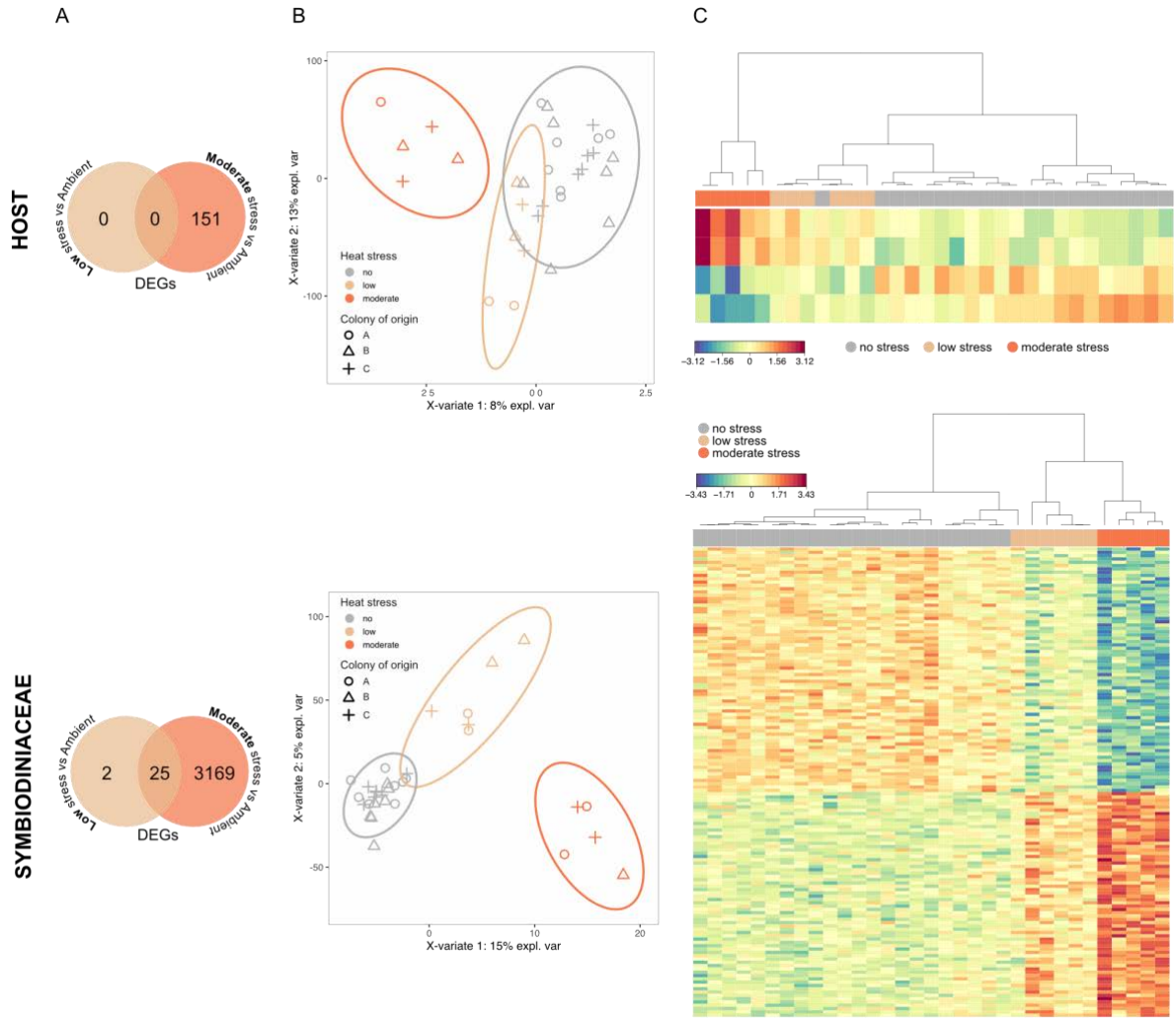


Fig. 4.4. Gene expression profiles of coral host and Symbiodiniaceae during early heat stress. A) Venn diagram of the number of Differentially Expressed Genes (DEGs) in corals exposed to low (< 1 DHW; T2) or moderate (3 DHW; T4) stress compared to ambient conditions determined by voom-limma analyses. B) Multilevel sPLS-DA for host and Symbiodiniaceae gene profiles; samples between treatment conditions are discriminated along component 1. C) Heat map illustrating the top discriminative genes between ambient, low and moderate stress, identified along with component 1 of multilevel sPLS-DA analysis for host (n = 4 genes) and Symbiodiniaceae (n = 142 genes).

Symbiodiniaceae transcriptional responses to incremental heat stress

On average, 11.2 million reads per sample uniquely mapped to the *Cladocopium* reference genome (Table S4.3), representing therefore the gene expression of host-associated Symbiodiniaceae. Similar to host gene expression analyses, colony of origin had a strong effect on Symbiodiniaceae transcriptional responses, representing the largest source of variation (Fig. S4.3). However, transcriptional changes were also detected under heat stress conditions. At a broad functional level,

KOG enrichment analyses revealed that genes related to 10 functional classes were differentially regulated under low stress, and 14 classes under moderate stress (Fig. 4.3A). Genes related to 'replication, recombination and repair' were the most upregulated under low stress and 'RNA processing and modification' under moderate stress (adjusted p values), while genes related to 'inorganic ion and carbohydrate transport and metabolism', and 'energy production and conservation', were consistently downregulated under both low and moderate stress. Importantly, enrichment patterns observed under low stress were similar to moderate stress, with some additional KOG classes significantly enriched under moderate stress only, such as 'posttranslational modification, protein turnover, chaperones' and 'cytoskeleton' (Fig. 4.3A). This incremental response to stress intensity was confirmed by a high correlation of KOG delta ranks for low stress vs ambient to moderate stress vs ambient ($r = 0.89$, $p < 0.01$; Fig. 4.3B); this relationship was stronger than in the host.

GO enrichment analyses further characterized the differences in expression among functional classes. Genes encoding for inorganic ion transmembrane transport, in particular for ammonium transmembrane transport, were significantly downregulated under low stress in Symbiodiniaceae (Table S4.7). This expression profile was intensified under moderate stress, in conjunction with the upregulation of more general heat stress responses such as transcription regulation, histone modification, protein folding and lipid membrane responses (Table S4.8).

When comparing Symbiodiniaceae transcription profiles under heat stress to ambient conditions at the gene level, differential expression analyses identified a total of 27 genes differentially expressed under low stress, and 3,194 genes under moderate stress (total of 23,345 genes; Fig. 4.4A, Table S4.9 and S4.10). Among the latter, genes related to ammonium transport, glutamine synthetase and glutamate synthase were consistently downregulated while genes related to positive TORC1 signalling were upregulated (Table S4.10).

Microbial community shifts from 1 DHW

A total of 2,723,455 high-quality 16S rRNA amplicon reads, with an average of 19,315 reads per sample (5,411 min, 84,479 max; blanks excluded) were retrieved with 12,558 amplicon sequence variants (ASVs) identified after quality trimming, chimera removal and data filtration. Rarefaction curves plateaued indicating that sequencing depth was sufficient to assess diversity of the coral associated microbes (Fig. S4.4). Although homogeneity of dispersion was not met, microbial communities associated with the coral tissue were clearly distinct from the microbiomes of the seawater and feeds (rotifers and microalgae; Fig. S4.5 and S4.6, Table S4.11). When exposed to incremental temperatures, the coral microbial community remained stable until cumulative thermal stress reached 1 DHW, after which significant differences in community structure were observed for the 1, 3 and 8 DHW samples

when directly compared to the respective time point under ambient conditions (Fig. 4.5 and Table S4.12). Specifically, Rhodobacteraceae (*Alphaproteobacteria*) affiliated sequences increased from 6% (ambient) to 21% under 8 DHW (Fig. 4.5B and S4.7). In contrast, the dominant microbial family under ambient conditions, Endozoicomonadaceae (*Gammaproteobacteria*), represented on average 57% of the community at the ambient T0 time point, but accounted for only 0.2% of retrieved sequences when thermal stress exposure reached 8 DHW (Fig. 4.5B and S4.7).

When exploring microbial changes at the ASV level, we identified one, three and 15 differentially abundant ASVs between ambient and 1, 3 and 8 DHW respectively (present in at least 50% of the samples within each category; Fig. 4.5C and S4.8). Among these ASVs, two members of the family Endozoicomonadaceae were consistently reduced in relative abundance at 3 and 8 DHW (Fig. 4.5C), while ASVs affiliated to Rhodobacteraceae and Flavobacteriaceae, for example, were significantly increased under heat stress (Fig. 4.5 and S4.8). Importantly, the coral microbial community was influenced by time in addition to heat stress, with a distinct community structure at T5 compared to T0 under ambient conditions (Fig. 4.5B and Table S4.12). An effect of the colony of origin was also detected, with microbial structure being significantly different among the three colonies (Table S4.12). Despite these differences, samples within each colony clustered at ≥ 3 DHW (Fig. S4.9), suggesting similar responses among colonies to the increasing thermal stress. Alpha diversity (Shannon diversity index) differed between colonies, but did not vary significantly across time and treatment with the exception of an overall higher diversity at T5 compared to the other time points (Fig. S4.10, Table S4.13).

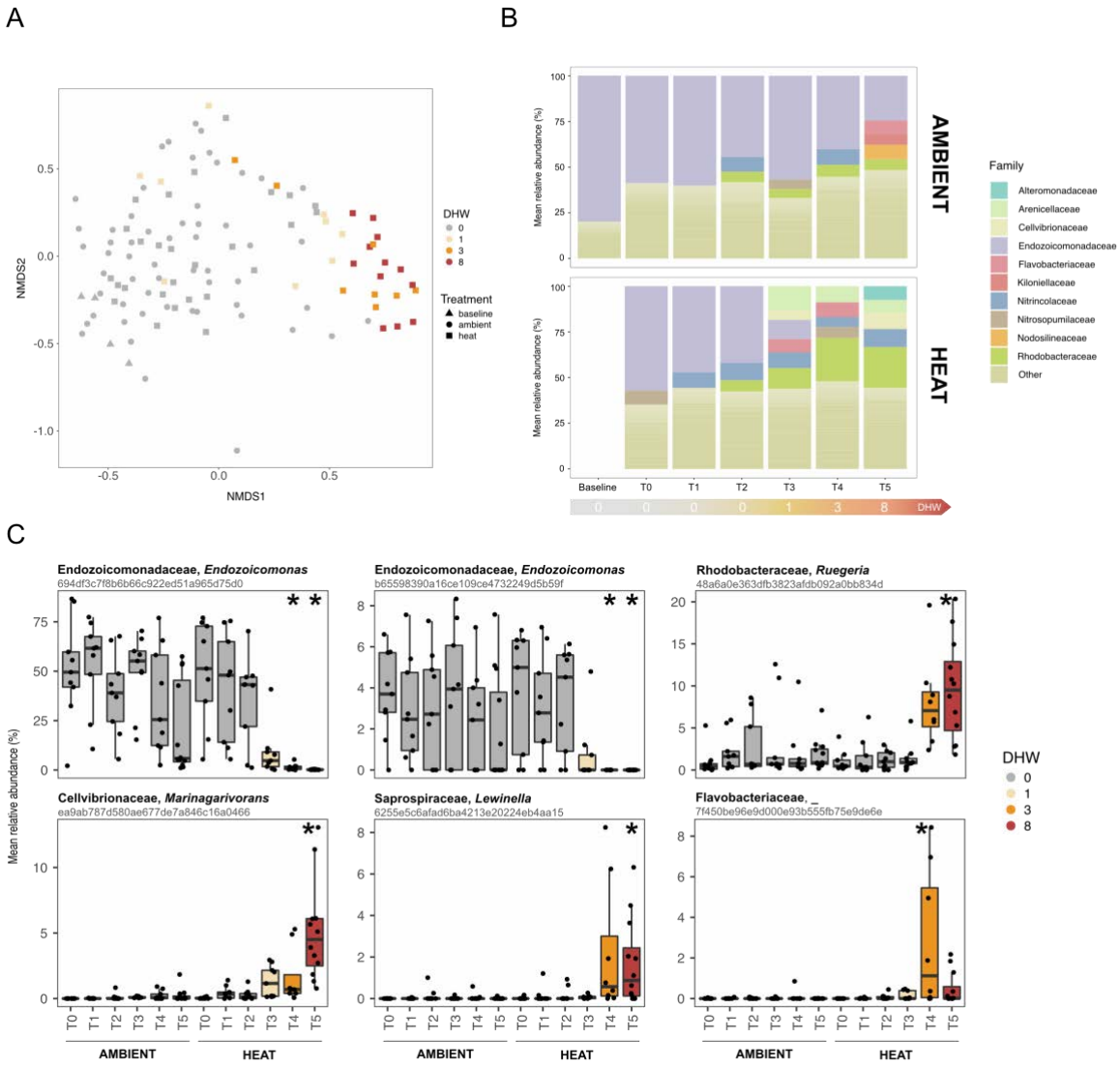


Fig. 4.5. Coral microbial responses to increasing heat stress determined by 16S rRNA gene sequencing. A) Non-metric Multi-dimensional Scaling (NMDS, sqrt-rooted data; stress = 0.21) based on Bray-Curtis dissimilarities calculated on relative abundance of ASVs present in the coral samples preceding fragging (baseline) and during the heat stress experiment (ambient and heat treatments). B) Mean relative abundance of prevalent microbial families (> 5% relative abundance) in the coral samples under ambient and heat conditions across time (baseline, T0-T5). DHW are reported for the heat treatment only (DHW = 0 under ambient conditions). C) Top differentially abundant ASVs between ambient and heat treatment identified using DESeq analyses ($p < 0.01$; adjusted post hoc tests). The relative abundance of the ASVs is shown over time in each treatment. Significantly different relative abundance of a specific ASV under heat treatment compared to ambient conditions (for the respective time point) are indicated with an asterisk; the taxonomic assignment for each ASV is shown as Family, Genus. Box = inter-quartile range (IQR), line in box = median, whiskers = minimum and maximum values not outliers (i.e. -/+1.5*IQR).

Integrated responses to heat stress

To link the heat-stress responses among holobiont members, we integrated the three omics-datasets (i.e. microbiome, gene expression of Symbiodiniaceae and host) through DIABLO. These analyses confirmed that samples under moderate stress were distinct from ambient conditions across datasets (Fig 4.6A). However, there was similarity between samples under low stress and ambient conditions for host gene expression and particularly for microbial structure (Fig 4.6A). When exploring associations between features from the three datasets, we identified a total of 88 highly correlated variables (i.e. 47 host genes, 26 Symbiodiniaceae genes, and 15 ASVs) along the first two principal components (Fig. 4.6B, Table S4.14). Importantly, a host ADP-ribosylation factor (plut2.m8.16024) that was most expressed under moderate stress correlated with 11 Symbiodiniaceae genes (including a negative correlation with symbiont glutamine synthetase) and two ASVs (including a negative correlation with an *Endozoicomonas* ASVs). Among the Symbiodiaceae genes, a nitrite transporter (Cgor.gene11242) that was most expressed under moderate stress correlated with 28 host genes (largely dominated by negative correlations with genes involved in transcriptional and translational regulation) and one Arenicellaceae ASV. Lastly, the relative abundance of four ASVs (assigned to Cellvibrionaceae, Rhodobacteraceae, Sandaracinaceae and Arenicellaceae families) was positively correlated with a wide range of host genes, but no strong correlations were detected with any Symbiodiniaceae genes (except for one Arenicellaceae ASV negatively correlated to one Symbiodiniaceae nitrite transporter).

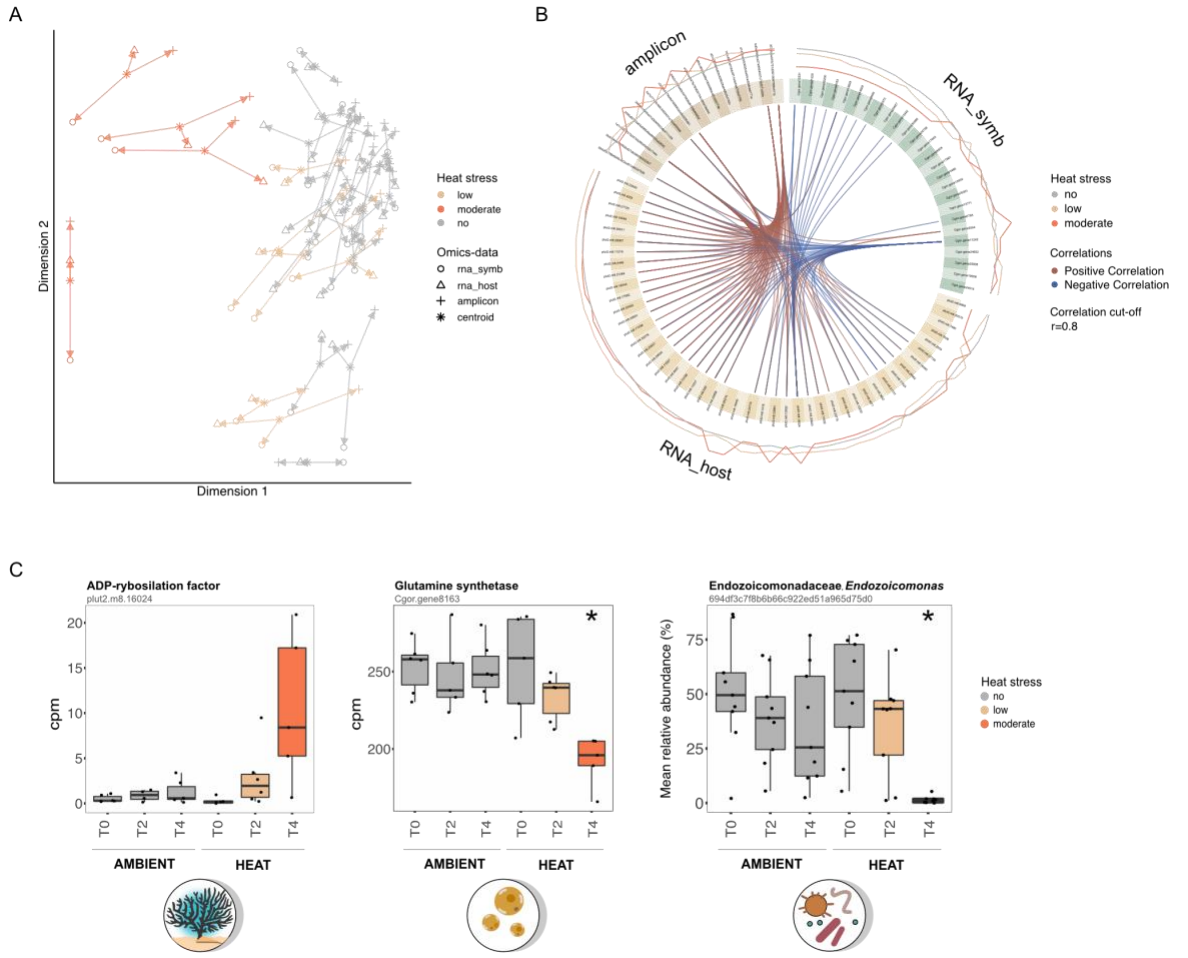


Fig. 4.6. Integrative analysis of host (transcriptomics), Symbiodiniaceae (transcriptomics) and microbial (amplicon) profiles in coral samples under ambient ('no stress'), low (< 1 DHW) and moderate (3 DHW) stress conditions using DIABLO (annotated genes only). A) Arrow plot showing the agreement between the different omic-datasets at the sample level, with * indicating the centroid between the datasets for each sample under treatment conditions shown by colour. Short arrows indicate strong agreement between datasets. B) Circos plot visualizing positive and negative correlations (r > 0.8; represented as red and blue lines) between the most discriminative features of each omics-dataset along component 1 and 2. Each quadrant represents an omics-dataset: microbial taxa ('amplicon', light brown), host genes ('rna_host'; yellow) and Symbiodiniaceae genes ('rna-symb'; green). The most outer lines represent the abundance level of each variable under ambient, low stress and moderate stress. C) Based on DIABLO analyses, the abundance of the host gene encoding for ADP-rybosilation factor was negatively correlated to the Symbiodiniaceae gene encoding for glutamine synthetase, as well as the relative abundance of Endozociomonas bacteria; abundance levels for each feature are shown here in counts per million (cpm; genes) and mean relative abundance (ASV).

Discussion

The stability of the coral holobiont under heat stress depends on the interactions between all its members (host, Symbiodiniaceae and microbes) (Avila-Magaña et al., 2021). In a stable state, mutualistic nutrient recycling within the holobiont underpins its ecological success in oligotrophic waters. Here, our integrative analysis within a thermotolerant coral reveals that even low levels of heat stress (i.e. < 1 DHW) may undermine this ecological foundation. Reduced photosynthesis and enhanced host metabolic energy demand drive the coral holobiont to a net heterotrophic state (1 DHW) in which algal-derived photosynthates are insufficient to cover its metabolic requirements. The destabilization of this association is linked to a shift of gene expression profiles towards an aposymbiotic-resembling state, and a strong restructuring of the microbial community.

The host gene expression profiles revealed that this metabolic switch occurred well before any visual signs of symbiosis breakdown (i.e. coral bleaching). Even at low heat stress (< 1 DHW), genes related to cellular energy production, ATP, lipid, and carbohydrate metabolism were significantly enriched, indicating elevated carbon turnover and consumption in the host cells. This aligns with previous studies suggesting that heat stress can shift the host metabolism from a nitrogen to a carbon-limited state (Cunning et al., 2017; Rädecker et al., 2021). The reduced availability of algal-derived carbon may, in turn, alter host amino acid metabolism, promoting a net release of ammonium (Rädecker et al., 2021). The increased protein catabolism observed in our study suggests that even early stages of heat stress may reduce the host ability of maintaining a nitrogen-limited state, which is prerequisite of a stable symbiosis (Pernice et al., 2012; Xiang et al., 2020).

The transcriptional changes observed in the host were strictly linked to the Symbiodiniaceae gene expression profile. A significant downregulation of genes involved in ammonium transport and fixation was observed from low heat stress levels (Table S4.7 and S4.8), reflecting the potential increased nitrogen availability for the algal symbionts under these conditions, which occurred prior to the observed thermal-induced decline in holobiont health. Our findings suggest that this decoupling of carbon recycling and the loss of a nitrogen-limited state in the symbiosis gradually accelerates with increasing temperatures. In the host, the significant downregulation of key genes in the *de novo* serine biosynthesis pathway (GOGAT, PSAT) indicates that a reduced availability of algal-derived carbon alters the amino acid metabolism, forcing the host to rely on fixed nitrogen sources (e.g. choline; Cui et al., 2019). Indeed, multiple host genes involved in the glycine/serine biosynthesis via food-derived choline were upregulated (albeit not significantly) under moderate stress. In this context, the downregulation of ammonium and anion transporters coupled with the upregulation of Symbiodiniaceae genes involved in positive TORC1 signalling support the idea that enhanced inorganic nutrient availability may stimulate anabolic activity and growth of algal symbionts at early stages of

heat stress. However, host genes related to immune responses and the apoptotic clearance of cells were also upregulated under these conditions. Similarly, Symbiodiniaceae showed an upregulation of multiple biological processes commonly related to heat stress, including transcription regulation, histone modification, protein folding and lipid membrane responses, suggesting host and Symbiodiniaceae cell homeostasis was impaired at 3 DHW. Consistent with this, holobiont health metrics showed a significant decline in photosynthetic performance and effective photochemical efficiency. Hence, our results indicate that the destabilization of nutrient cycling in the coral-Symbiodiniaceae precedes the onset of the processes ultimately leading to the complete breakdown of the symbiosis (i.e. bleaching), which in our study was observed at 8 DHW.

Based on the number of DEGs between heat stress and ambient conditions, Symbiodiniaceae showed a stronger response than the host, potentially suggesting Symbiodiniaceae may be the first responding to environmental changes. These results contrast previous studies identifying the host being more transcriptionally responsive to high temperatures (Avila-Magaña et al., 2021; Barshis et al., 2013, 2014; Davies et al., 2018; Leggat et al., 2011; Li et al., 2021; Savary et al., 2021). However, it is possible that these opposite patterns are linked to distinct mechanisms underpinning stress responses between thermotolerant (this study) and environmentally sensitive coral species (Barshis et al., 2013, 2014; Leggat et al., 2011; Li et al., 2021). Another factor likely contributing to these contrasting results is stress severity; while our study focusses on early stages of stress, the majority of heat stress studies performed to date have explored transcriptomic profiles under higher stress intensity (Avila-Magaña et al., 2021; Davies et al., 2018; Dixon et al., 2020; Savary et al., 2021).

The effects of heat stress within the coral holobiont were not restricted to the destabilization of the coral-Symbiodiniaceae symbiosis. Coral exposure to incremental temperatures resulted in a significant shift of the microbial community structure from as early as 1 DHW (i.e. coinciding with a metabolic shift within the coral holobiont towards a net heterotrophic state), with shifts becoming increasingly pronounced through to 8 DHW. The significant reduction of two *Endozoicomonas* ASVs under heat stress indicates the restructuring of the microbial community was likely related to dysbiosis. Indeed, these bacteria commonly dominate the microbiome of healthy corals and show high degrees of host specificity and co-phylogeny (Neave et al., 2017b; O'Brien et al., 2021; Pollock et al., 2018). While the specific contribution of *Endozoicomonas* to coral holobiont functioning is yet not fully understood, these bacteria are considered beneficial for coral health due to their putative role in carbon, phosphate and sulphur cycles, as well as amino acids and vitamin biosynthesis (Pogoreutz et al., 2022; Robbins et al., 2019; Tandon et al., 2020; Wada et al., 2022). Concurrent with a decrease in *Endozoicomonas* affiliated sequences, we observed an increase in the relative abundance of opportunistic bacteria, such as Rhodobacteraceae. Similar microbial shifts have been previously reported across a wide range of coral taxa exposed to environmental stress (Li et al., 2021; Pootakham et al., 2019; Savary et al., 2021),

indicating these microbial responses are commonly associated with general coral stress responses. Hence, the shifts in microbial composition observed in this study are unlikely an adaptive response, instead they may exacerbate the negative impacts of heat stress on other holobiont members.

We identified strong correlations between the responses of all coral holobiont members. Among these correlations, the upregulation of one host gene (an ADP-ribosylation factor) during heat stress coincided with the downregulation of critical genes involved in algal nitrogen metabolism (glutamine synthetase) and the decline in *Endozoicomonas*-affiliated sequences. ADP-ribosylation factors regulate host intracellular vesicle transport and have been proposed to be key negative regulators in cnidarian-Symbioniaceae symbioses (Chen et al., 2004). In support of this hypothesis, our results suggest that the upregulation may not only negatively affect host-associated Symbiodiniaceae populations but may also influence the regulation of the dominant bacterial symbiont, *Endozoicomonas*. Previous studies showed that *Endozoicomonas* occupy an endosymbiotic niche, forming aggregates termed coral-associated microbial aggregates (CAMAs) within the coral holobiont tissues (Neave et al., 2017b; Wada et al., 2022) and could thus be subjected to the same host regulatory machinery. Furthermore, the regulation of genes involved in symbiosis networks have been suggested to be critical in the stability of cnidarian symbiotic relationships under heat stress, with symbiosis-related genes responding prior to any visible signs of symbiosis breakdown (Cleves et al., 2020a).

Taken together, our results suggest that the breakdown of the symbiosis under heat stress is not driven by one coral holobiont member in isolation. Physiological and transcriptome data suggest that changes in nutrient cycling and availability begins under early stages of heat stress. Increased metabolic turnover and catabolic production of inorganic nutrients gradually shift the holobiont from a nitrogen- to a carbon-limited state. Under these conditions, Symbiodiniaceae may initially benefit from the enhanced nutrient availability but, in turn, may reduce the translocation of fixed carbon to the host, destabilizing their mutualistic interaction. In addition, our results reveal that the altered symbiotic and nutritional state within the holobiont may also drive changes in the associated microbial community, with mutualistic symbionts being gradually replaced by more opportunistic microbes. Hence, a gradual destabilization of mutualistic interactions within the coral holobiont begins already at low heat stress levels (< 1 DHW). This destabilization ultimately leads to coral physiology and gene expression profiles increasingly resembling a cnidarian aposymbiotic-like state, with a reduced contribution of symbionts to host metabolism and functioning.

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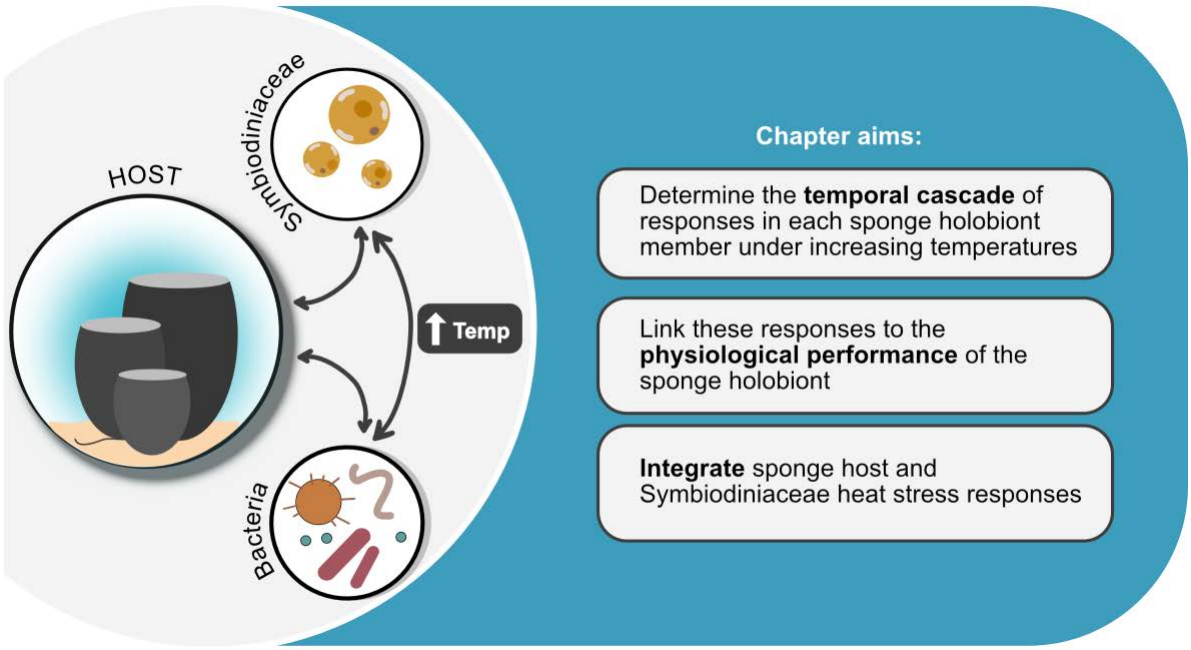
CHAPTER 5 : INTEGRATING HOST, SYMBIODINIACEAE AND MICROBIAL RESPONSES TO HEAT STRESS IN A TROPICAL SPONGE SYMBIOSIS



The associated publication is in preparation:

Marangon E, Bourne DG, Webster NS, Laffy PW (2023) Integrating host, Symbiodiniaceae and microbial responses to heat stress in a tropical sponge symbiosis, *in preparation*.

Integrating host, Symbiodiniaceae and microbial responses to heat stress in a tropical sponge symbiosis

Abstract

Some tropical sponges are associated with intracellular dinoflagellates (family Symbiodiniaceae), which facilitate nutrient assimilation and recycling, underpinning host health and ecological success. These sponge-Symbiodiniaceae relationships appear more resistant to prolonged heat stress than tropical coral species, though the molecular pathways contributing to this response in sponges are unknown. Here, we experimentally characterized host, Symbiodiniaceae and bacterial responses to increasing temperatures (0 – 9 Degree Heating Weeks, DHW) in the tropical sponge *Cliona orientalis* through combining transcriptomics, microbial 16S rRNA gene sequencing and physiological measurements. A decline in Symbiodiniaceae photochemical efficiency from 3 DHW was identified, coupled with an impairment of the energetic balance within the sponge holobiont and a strong upregulation of host immune responses. Integrated gene expression analyses revealed a negative correlation between host and Symbiodiniaceae genes involved in cytoskeleton proteins, suggesting structural change in cell organization may occur from early stages of heat stress. A complete breakdown of the symbiosis was observed from 6 DHW, and was characterized by signs of bleaching as well as a shift towards a dominant heterotrophic state. Whilst a strong transcriptional and physiological response was observed in both host and Symbiodiniaceae, the microbial community structure appeared stable under increasing temperatures. Overall, our integrative study reveals key processes underlying heat stress response within sponge-Symbiodiniaceae holobionts.

Introduction

The evolutionary and ecological success of many marine invertebrates lies in their symbiosis with photosynthetic dinoflagellates of the family Symbiodiniaceae. The mutualistic interactions between Symbiodiniaceae and cnidarians has received considerable scientific attention as it enables corals to thrive in nutrient-poor tropical oceans, providing the ecological foundation of coral reef ecosystems (Cui et al., 2019; Muscatine & Porter, 1977; Xiang et al., 2020). However, these photosynthetic symbionts are also integral to other ecologically relevant organisms, including some clionaid sponges (Hill et al., 2011; Schönberg & Loh, 2005). Bioeroding sponges within the '*Cliona viridis* species complex' harbour intracellular *Gerakladium* symbionts, belonging to one of the more ancestral Symbiodiniaceae lineages (LaJeunesse et al., 2018), which have been inferred to confer higher thermotolerance to their host compared to Symbiodiniaceae species commonly associated with

scleractinian corals (e.g. *Cladocopium*, *Durusdinium*) (Chakravarti & van Oppen, 2018; Matsuda et al., 2022). Rapid warming of the oceans and associated marine heat waves drive severe bleaching events (i.e. loss of Symbiodiniaceae), posing a major threat to the ongoing persistence of coral ecosystems and the species that build reefs (Hughes et al., 2018a). However bleaching has rarely been reported for Symbiodiniaceae-associated sponges (Ramsby et al., 2018a; Vicente, 1990), raising the key question of which mechanisms may underlie the high thermotolerance of sponge-Symbiodiniaceae relationships.

Multiple processes contribute to the destabilization of coral symbiosis under high temperatures, including increased host energetic demand, alteration of nutrient availability (i.e. C, N) and symbiont production of reactive oxygen species (Morris et al., 2019; Rädecker et al., 2021; Weis, 2008). It is currently unclear whether similar mechanisms may influence heat-stress responses within sponge symbioses, as very limited work has been conducted on these unique symbiotic relationships. The transcriptional response of sponge hosts experimentally exposed to high temperatures aligns with general responses to heat stress, with the consistent enrichment of apoptosis-like genes, heat shock proteins (HSP), and genes related to signal transduction and innate immunity pathways (Fan et al., 2013; Guzman & Conaco, 2016; Koutsouveli et al., 2020). In Symbiodiniaceae-associated sponges, the increased host energy demand under future climate conditions was shown to alter the carbon energy balance within the sponge holobiont (Fang et al., 2014; Ramsby et al., 2018a), leading to a shift from a prevalent autotrophic to an heterotrophic state. However, moderately high temperatures and pCO_2 levels (RCP 4.5) potentially benefitted sponge holobiont functioning, with an increased provision of photosynthates from symbiont to host, and increased host growth (Fang et al., 2014). While it is clear that heat stress can affect both the sponge host and its associated symbionts, the cellular and metabolic pathways involved in this integrated response remain poorly understood.

Sponge resilience to environmental stressors may also be influenced by a complex and diverse host-associated prokaryotic microbial community (hereafter 'microbes') (Botté et al., 2019; Webster & Reusch, 2017). In sponges, microbes extensively contribute to host nutrient recycling as well as the removal of host-produced waste compounds (Hudspith et al., 2021, 2022; Rix et al., 2020; Robbins et al., 2021; Webster & Thomas, 2016), thereby demonstrating a high metabolic co-dependency. The presence of Symbiodiniaceae, however, adds complexity to this integrated host-microbe metabolism. In clionaid sponges, Symbiodiniaceae appears to play a primary role in the direct assimilation of inorganic carbon and nitrogen from the surrounding seawater, and in the recycling of host nitrogenous waste (Achlatis et al., 2018, 2019b, 2019a), with a limited microbial contribution to these processes. The presence of putative diazotrophs and ammonia oxidizers in the microbiome of the bioeroding sponge *Cliona orientalis* may however indicate a prokaryote link in nitrogen metabolism within this species (Ramsby et al., 2018b). Hence, understanding the interplay between microbes, Symbiodiniaceae

and the host will identify key drivers of symbiosis destabilization within sponge holobionts under heat stress.

Here, we characterized host, Symbiodiniaceae and microbial responses to increasing temperatures in the sponge *Cliona orientalis* over a 2-month period (28 – 32 ˚C, corresponding to a maximum of 9 Degree Heating Weeks, DHW). Specifically, we determined gene expression changes in host and Symbiodiniaceae at 3 DHW using reference-based transcriptomics, and assessed microbial community structure (16S rRNA gene sequencing) as well as physiological responses to incremental temperatures. We also identified the correlations in gene expression profiles between host and Symbiodiniaceae under ambient and heat stress conditions, facilitating an understanding of how heat stress responses within the sponge host and its symbionts are integrated.

Material and methods

Sponge collection and experimental set up

Three individuals of the bioeroding sponge *Cliona orientalis* were collected at 5 m from Little Pioneer Bay at Orpheus Island in the central Great Barrier Reef (Australia) in September 2019 (Permit G12/35236.1). Sponges were transported to the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (Townsville, Australia), and maintained in outdoor independent flow-through tanks at ambient temperatures. After four weeks of acclimation, sponges were fragmented in clones using an underwater drill, ring saw and band saw, and then glued onto aragonite plugs. Sponge clones were subsequently transferred to six indoor flow-through tanks (50L; 0.8 L min^{-1}) that were maintained at ambient temperatures (27.3 ±0.6 ˚C; Central Great Barrier Reef [GBR] daily average temperature 1991 – 2012; http://data.aims.gov.au) over eight weeks (i.e. acclimation period). To account for any genotype effect, at least six clones from each parental colony were randomly distributed to each tank.

Following the acclimation period, sponge clones were exposed to two experimental treatments (n=3 tanks each). The ambient treatment mirrored the daily average sea surface temperatures in the central GBR (28.5 ± 0.12 ˚C; 1991 – 2012; Australian Institute of Marine Science, 2020), while the heat treatment consisted of a daily temperature increment of 0.07 ˚C over 55 days, from 28.16 ˚C to 32 ˚C, followed by four days of constant exposure to 32 ˚C (corresponding to 0 – 9 Degree Heating Week). SeaSim computer-control systems finely managed seawater temperatures within the experimental tanks, with temperatures further stabilized through water jackets. During the experimental period, SOL LED lights mimicked a natural 12:12h light:dark cycle with 4-h ramping at sunrise and sunset (max

light intensity 130 µmol photons m^{-2} s^{-1}); and sponges were fed twice per week with microalgae (2000 cells mL^{-1} in tank).

Sponge holobiont responses to incremental temperatures were characterized through gene expression analyses (host, Symbiodiniaceae) and 16S rRNA gene amplicon sequencing (microbes), coupled with physiological health metrics reflecting holobiont health (i.e. photosynthesis and respiration rates, photochemical efficiency and health score) (Fig. 5.1). For generating microbial profiles, sponge clones were sampled at six time points (weeks 0, 3, 4, 5, 6, 9, hereafter referred to as T0 – T5; n=3 per tank) while samples for generating transcriptomic data were taken at two time points (T0, T4; n=2 per tank) (Fig. 5.1). When sampling, clones were first cut into smaller pieces using a sterile band saw, rinsed with sterile seawater (0.22 µL), immediately frozen in liquid nitrogen and stored at -80˚C until processing for DNA and / or RNA extractions. Additional physiological measures were taken at T*p* (week 7; Fig. 5.1). Furthermore, seawater (1 L per tank) was sampled at T0, T1 and T5 as an environmental control for 16S rRNA gene analyses. Seawater samples were filtered through 0.22 µL Sterivex filters (Millipore), and then stored at -80˚C until DNA extraction. Microalgae used to feed the sponges were also sampled for 16S rRNA gene analyses as control for diet-introduced microbes within the system.

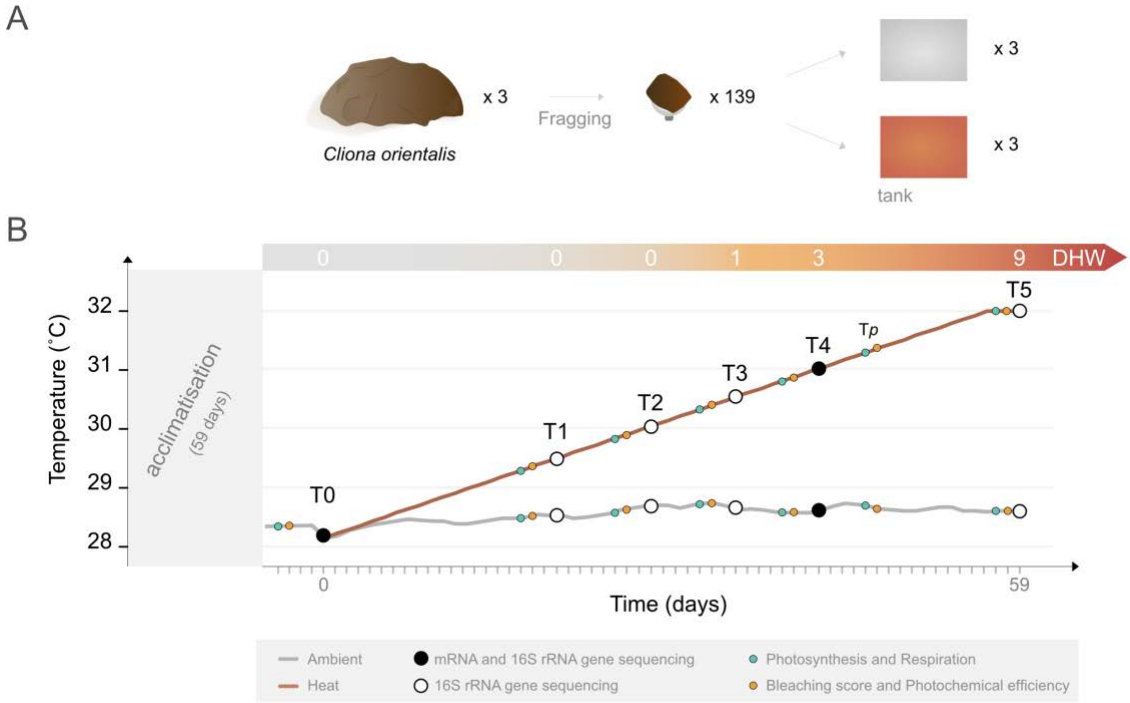


Fig. 5.1. Experimental design of the two-month incremental heat stress experiment on the photosymbiotic bioeroding sponge *Cliona orientalis*. A) Three sponge colonies were collected from Orpheus island (central GBR), fragged into clones and distributed across ambient and heat treatments (n=3 tank per treatment). B) Sponge holobiont responses to incremental heat stress were characterized over time using mRNA (host, Symbiodiniaceae) and / or 16S rRNA gene sequence (microbes). Accumulated heat stress (Degree Heating Weeks, DHW) is shown for each sampling time (T0-T5). Net photosynthesis rates, respiration rates, health score and photochemical effective efficiency were regularly assessed throughout the experiment to assess sponge holobiont health (T*p* represents an additional point at which all physiological parameters were assessed, but transcriptomic and amplicon sampling was not performed; photosynthesis, respiration and photochemical efficiency were measured at 4 DHW, while bleaching score at 6 DHW).

Holobiont physiological health metrics

To determine any heat-induced variation in carbon flux between host and Symbiodiniaceae, photosynthesis (oxygen production, P) and respiration (oxygen consumption, R) rates were assessed under increasing temperatures following a modified process from Schoepf et al. (2019). Briefly, sponge clones were first acclimated to light conditions for at least 60 min and then placed in sealed 600 mL plastic chambers within water baths with temperatures reflecting treatment conditions. After incubating the chambers (n = 15 - 20 per time point / treatment) for 40-min under constant light intensity (130 µmol photons m^{-2} s^{-1}), dissolved oxygen was measured (i.e. net P rates) using an Optical Dissolved Oxygen probe (Hach Intellical LDO101). On the same day, R rates were assessed using the same

procedure under dark conditions (60 min dark acclimation, 60-min incubation). P and R rates were then adjusted for the background oxygen concentration, which was estimated by incubating empty chambers containing only seawater (n=2 per tank/time point), and standardized by the volume of water in each chamber (displacement method) and by the surface area (top) of the sponge clone (aluminium foil method; Marsh, 1970). P / R ratio was based on 12 h of gross P (= net P + R) divided by 24 h of R (Schoepf et al., 2019).

Photochemical effective efficiency (ΔF/Fm') of sponge-associated Symbiodiniaceae was measured using a mini-PAM fluorometer (Walz, Germany; settings: MI = 5; SI = 8; SW = 0.8; G = 2; D = 2; 0.9 cm from sponge tissue) to assess the symbionts' photochemical capacity under increasing heat stress. Measurements (n=3 per sponge clones at each time point) were undertaken following at least 60 min of acclimation to light conditions and mean photochemical effective efficiency of each sponge clone was calculated per time point. In addition, sponge colouration was regularly assessed throughout the experiment to detect signs of bleaching (bleaching score; Fig. 5.1). Specifically, the colouration of 17 representative sponge clones was tracked over time and categorized into 'unbleached' (brown), 'moderately bleached' (pale brown) and 'bleached' (white).

Generalized linear mixed models ('glmmTMB package', Brooks et al., 2017) were applied to test the fixed effect of treatment and time on the sponge health metrics. Specifically, net P was analysed using a tweedy distribution (log link) on positive-transformed values, R analysed using a gamma distribution on square-root transformed (absolute) data, P / R ratio analysed through a gaussian model on log-transformed data and photochemical effective efficiency analysed using a beta distribution with a logit link. Colony of origin was included as a fixed effect in the models, while experimental tank and sponge clone were included to account for repeated measures. Model assumptions were verified through DHARMa (Hartig, 2021), and model selection was achieved by comparing relevant models using the corrected Akaike information criterion (AICc). Pairwise comparisons were then run using 'emmeans' (Lenth, 2022) with 'BH' correction; all analyses were run in R (version 4.0.3, R Core Team, 2020).

Sponge and Symbiodiniaceae gene expression analyses

Sponge clones were broken into smaller pieces using a French-press at low temperature. RNA was then extracted using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific), including a Trizol/Chloroform homogenization step (as described in Chapter 4). Briefly, sponge samples were homogenised in bead tubes (MP; n=1 tube per sample) containing 1 mL Trizol using the FastPrep-24 5G instrument (MP Biomedicals). The homogenized tissue was centrifuged, and the supernatant mixed with 200 µL chloroform. After centrifugation of the resulting solution at 12,000 x g for 15 minutes, the upper phase containing RNA was purified in accordance with the manufacturer protocol, which

included an on-column DNase treatment (Thermo Fisher Scientific). Purified RNA was diluted in 40 µL of nuclease-free water, and RNA quality and concentration were measured using the Nanodrop 2000 spectrophotometer (Thermo Scientific), Agilent 2100 Bioanalizer (RNA 6000 Pico assay; Agilent Technologies) and Qubit 3.0 Fluorometer (Life Technologies). High-quality RNA was standardized and stored at -80˚C until submission to the Ramaciotti Centre for Genomics (UNSW, Australia) for Illumina stranded mRNA library preparation (including a polyA step selective for eukaryotic mRNA) and sequencing on NovaSeq 6000 S2 2 x 100 bp. On average, sequencing yielded ~ 82 million reads per sample.

To characterise the transcriptional responses of sponge host and associated Symbiodiniaceae to heat stress, reference-based gene expression analyses were performed as previously described (Buerger et al., 2020). Following quality checking (FastQC and MultiQC; Andrews, 2010; Ewels et al., 2016), sequence reads were mapped to the *Cliona orientalis* and *Gerakladium endoclionum* transcriptome references (Strehlow et al., 2021) using Bowtie 2 (Langmead & Salzberg, 2012). Gene counts were generated using RSEM (version 1.3.0, Li & Dewey, 2011), pre-processed and imported into R (R Core Team, 2020) for subsequent analyses. One sample was excluded from the analyses as not passing quality control. Host and Symbiodiniaceae gene functional annotations (euKaryotic Orthologous Groups [KOG], Gene Ontology [GO] terms, and Kyoto Encyclopedia of Genes and Genomes [KEGG] pathways) were generated using eggNOG-mapper (version 2.1.6, Huerta-Cepas et al., 2017) on the Galaxy Australia platform (Afgan et al., 2018), based on sequence similarity comparisons to the eggNOG database 5.0.2 (Huerta-Cepas et al., 2016) using Diamond (Buchfink et al., 2021). One representative annotation was selected at the isoform level for each gene.

Gene expression analyses were performed following data normalization, transformation (log-CPM) and removal of genes with low counts ('filterByExpr' function, including treatment, time and parental colony in the model matrix). Changes in broad gene functional classes were tested using KOG enrichment analyses, which identifies KOG gene classes with up- or down-regulated genes at high temperatures (Mann–Whitney U test, R package 'KOGMWU'; Dixon et al., 2015). The input data for these analyses were the log fold-changes (logFC) generated from the pairwise comparisons between ambient and heat treatments (limma-voom pipeline described below). Delta ranks (i.e. difference between the mean rank of genes within a KOG class and the mean rank of the other genes) were calculated through the KOGMWU function for each of the 23 KOG classes. In addition, GO enrichment analyses were performed on logFC to identify which biological processes were differentially regulated under heat stress (GO_MWU pipeline; https://github.com/z0on/GO_MWU).

Differentially Expressed Genes (DEGs; false-discovery rate, Benjamini–Hochberg adjusted p-value ≤ 0.05) were identified between ambient and heat conditions using mixed effect models through the

limma-voom pipeline (version 3.46.0, (Law et al., 2018; Ritchie et al., 2015)). Specifically, samples at 3 DHW (Heat T4) were compared to samples under ambient conditions (Heat T0, Ambient T0, Ambient T4), with colony of origin included as a random effect. Heat maps were generated using Z-scores on logarithm of counts per million reads.

16S rRNA gene amplicon analyses

DNA was extracted from sponge, seawater and feed (i.e. microalgae) samples using the DNeasy PowerSoil Pro kit (QIAGEN) following the manufacturer's protocol, and using the FastPrep-24 5G bead beater (MP Biomedicals) for the cell lysis step. The V4 region of the 16S rRNA gene was targeted for amplification using the forward primer 515F (Parada et al., 2016) and the reverse primer 806rB (Apprill et al., 2015) in a 25 cycle PCR using the AmpliTaq Gold 360 Master Mix (ThermoFisher) under the following amplification cycles: initial denaturation at 95˚C for 10 min, 25 cycles of 95˚C for 30 sec, 55˚C for 1 min, 72˚C for 30 sec, and final elongation at 72˚C for 7 min. Amplified, samples were sent to the Ramaciotti Centre for Genomics (University of New South Wales, Australia) for standard Illumina library preparation and sequencing on the Illumina MiSeq 2x250 bp platform.

Amplicon sequence reads were processed using QIIME2 (v 2020.8, Bolyen et al., 2019). Briefly, poor quality reads (i.e. low quality score) and chimeras were removed, and amplicon sequence variants (ASVs) were identified based on 100% sequence similarity using DADA2 (Callahan et al., 2016). Taxonomic classification was assigned through a naïve Bayes classifier trained with the feature-classifier plugin using the primers 515F/806rB on the SILVA 132 database (Quast et al., 2012), and phylogenetic relationships were constructed using FastTree. The resulting ASV count table was imported into R (R Core Team, 2020) and reads assigned to Chloroplasts, Eukaryotes and Mitochondria were removed using the R packages 'phyloseq' (McMurdie & Holmes, 2013), as well as ASVs identified as contaminants through the R package 'decontam' (threshold = 0.5; n=3 contaminants identified, Table S5.1; Davis et al., 2018). Singletons and samples with < 3,200 reads (n=5) were also excluded from the analyses.

Beta diversity analyses were performed on Bray-Curtis dissimilarities calculated on square-root normalized (proportions) data (McKnight et al., 2019). ASVs with an overall relative abundance < 0.001% (8,427 ASVs) were excluded from the analyses. Non-metric Multi-dimensional Scaling (NMDS; 'phyloseq', McMurdie & Holmes, 2013) and Permutation Multivariate Analysis of Variance (adonis, 10,000 permutations; 'vegan', Oksanen et al., 2020) were performed to detect differences in the sponge-associated microbial community across time (baseline, T0-T5), treatment (ambient, heat) and sample types (sponge, seawater and feed). Dispersion was assessed using Multivariate

Homogeneity of Group Dispersions ('vegan package', Oksanen et al., 2020), and pairwise comparisons were performed in 'RVAideMemoire' (Hervé, 2021) using Benjamini-Hochberg correction. Differential abundance analyses were run on filtered data (p-value < 0.05; 'DESeq2 package', Love et al., 2014) with only ASVs present across at least 50% of the samples, in one of the compared groups (e.g. at least 50% of samples under T5 ambient conditions when compared to T5 heat), identified as differentially abundant.

Alpha diversity analyses were performed on data rarefied to 3,200 sequences ('phyloseq package', McMurdie & Holmes, 2013). Specifically, the effect of treatment and time was tested on the Shannon diversity index using linear mixed models ('glmmTMB package', Brooks et al., 2017), including colony of origin as fixed effect and tank as random effect. Model assumptions were assessed using DHARMa residual diagnostics (Hartig, 2021).

Multilevel integration of gene expression profiles

Symbiodiniaceae and host transcriptomic data (normalized variance stabilized counts) were integrated through DIABLO (Data Integration Analysis for Biomarker discovery using a Latent component method for Omics studies; 'mixOmics', Rohart et al., 2017), a supervised approach identifying key molecular signatures across omics-datasets generated from the same biological samples (Singh et al., 2019). Given the stability of the microbial community structure under increasing temperatures, this data was excluded from the analyses. Only annotated genes were used for performing DIABLO (KEGG Orthology, i.e. 5,761 Symbiodiniaceae genes, 31,675 host genes) and the effect of colony of origin was included in the analyses through a multilevel approach. A design matrix of 0.6 was applied, with 50 × 4-fold cross-validation; and the circosPlot function was used to visualize correlations (r >0.8) between host and Symbiodiniaceae genes (González et al., 2012).

Results

Holobiont health is stable until 3 DHW

A reduction in sponge holobiont performance was observed from 3 DHW, as effective quantum yield showed a significant decline from this time point onwards between the two treatments (Fig. 5.2A and Table S5.2). In contrast, holobiont P / R rates significantly decreased compared to ambient conditions at higher heat stress (i.e. 9 DHW; Fig. 5.2B and Table S5.2), reaching levels far below 1, indicating

autotrophy does not fully support holobiont energetic requirements at 9 DHW. Importantly, the lower P / R rates under heat stress compared to ambient conditions are a result of the alterations in both net photosynthesis and respiration rates observed at 9 DHW (Fig. S5.1 and Table S5.2). This strong impairment of both photochemical effective efficiency (3 DHW) and P / R rates (9 DHW) coincided with the onset of visual signs of bleaching on sponge clones from 6 DHW (Fig. 5.2C). Specifically, 35% of sponge clones were fully bleached at 6 DHW, with the remaining sponges showing moderate bleaching. Under higher heat stress (i.e. 9 DHW) the percentage of bleached sponges increased from 35% to 65% (Fig. 5.2C).

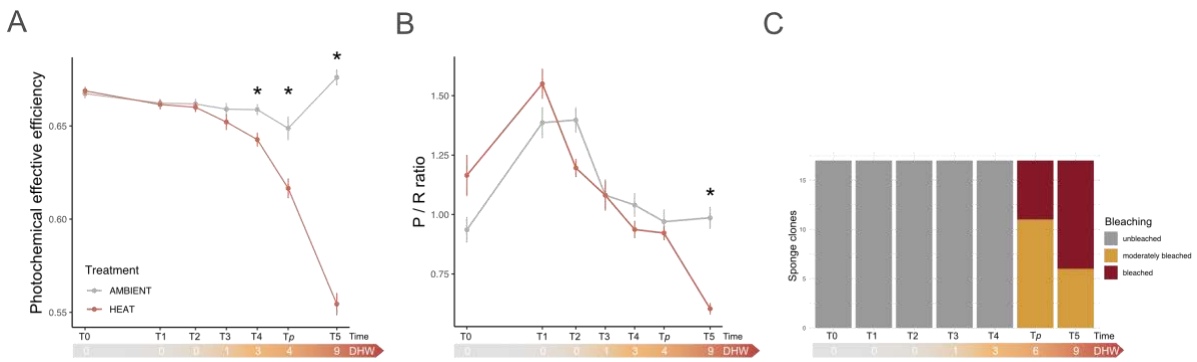


Fig. 5.2. Sponge holobiont physiological health metrics under incremental heat stress. A) Photochemical effective efficiency, B) photosynthesis / respiration ratio, and C) bleaching score are shown over time (T0 – T5) under ambient and heat treatments; DHW are illustrated for the heat treatment only, as DHW corresponds to 0 under ambient conditions. T*p* (i.e. time point at which physiological measures were performed but sponge clones were not sampled) corresponds to 4 DHW for photochemical effective efficiency and P / R ratio, and 6 DHW for the bleaching score. For P / R ratio and photochemical effective efficiency, mean ± SE is shown over time for each physiological response; asterisks indicate significantly different responses between ambient and heat treatment at each time point ($p < 0.05$; adjusted post hoc tests).

Changes in sponge host gene expression under 3 DHW

On average, 48% of reads mapped to the host reference transcriptome (i.e. *Cliona orientalis*; ~ 58.8 million reads per sample; Table S5.3). Significant changes in host gene expression were observed between heat stress and ambient conditions, though a strong effect of colony of origin on gene expression patterns was also observed in the principal component analysis (PCA) (Fig. S5.2). A total of five KOG functional classes were significantly enriched with up- or down- regulated genes between treatment conditions, including a downregulation of genes involved in 'energy production and conservation' and an upregulation of genes related to 'replication, recombination and repair' processes (Fig. 5.3). Similarly, GO enrichment analyses identified strong host functional responses to heat stress,

with the majority of genes within significant GO classes being downregulated under 3 DHW (Table S5.4). For example, GO categories related to glucose metabolism (e.g. 'cellular glucose homeostasis', 'glycolysis'), energy reserve metabolic process, acquisition and transport of nutrients (e.g. 'regulation of feeding behaviour', 'pinocytosis', 'regulation of transporter activity'), and processes involved in cell growth and division were downregulated under heat stress. These changes in host energy balance were coupled with a significant upregulation of multiple GO terms associated with host general and adaptive immunity responses, and a negative regulation of processes involved in innate immune responses, cellular structure and movement (i.e. 'cytoskeleton organization' and 'cytoplasmic actin-based contraction involved in cell motility').

Differential expression analyses identified a total of 395 up- or down-regulated genes under heat stress compared to ambient conditions (Fig. 5.4, Table S5.5). Among these DEGs, genes related to chaperone proteins, transcription factors (i.e. bZIP), scavenger receptor activities and NF-kappaB-inducing kinase activity (cellular signalling) were upregulated under heat stress conditions; while genes related to signal transduction (i.e. G protein-coupled receptors) and transient receptor potential channels (TRPML) were downregulated. While the expression patterns of DEGs clustered according to treatment conditions, time also appeared to have an effect on host transcriptional responses (Fig. 5.4).

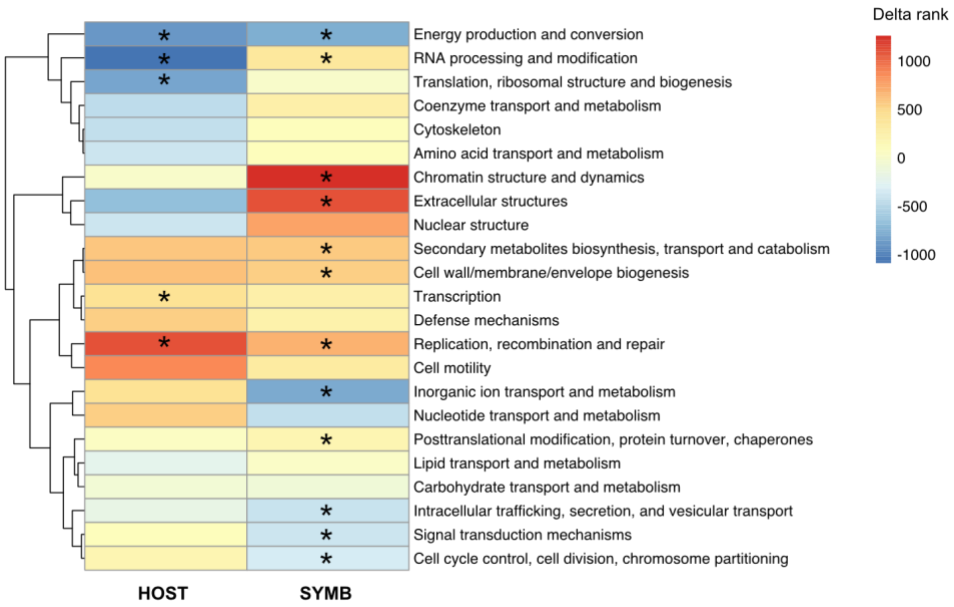


Fig. 5.3. EuKaryotic Orthologous Group (KOG) enrichment analysis in host and Symbiodiniaceae under heat stress. Heat map illustrating KOG categories up (red) and down (blue) regulated under heat stress (3 DHW) compared to ambient conditions (0 DHW) in both holobiont members; KOG categories significantly enriched (adj-p < 0.05) with up or down regulated genes are indicated with asterisks.

Fig. 5.4. Host and Symbiodiniaceae differentially expressed genes (DEGs) between 0 and 3 DHW. Heat maps representing gene expression levels (Z-scores) of DEGs under heat stress (3 DHW; T4 heat) compared to ambient conditions (0 DHW; T0 ambient, T0 heat, T4 ambient) in host (left) and Symbiodiniaceae (right); DEGs were identified using the voom-limma pipeline. Colony of origin (C1, C2, C3) of each sample is also shown on the top of the heat maps. The total number of up (↑) and down (↓) regulated genes for both holobiont members is illustrated in the centre of the figure (orange circles), as well as the number of genes not showing significant differences in the expression levels between experimental treatments (white circles).

Changes in Symbiodiniaceae gene expression under 3 DHW

An average of 18% of reads mapped to the Symbiodiniaceae reference transcriptome (i.e. *Gerakladium endoclionum*; Table S5.3), corresponding to ~ 21.6 million reads per sample. While colony of origin had a strong effect on host gene expression profile, it did not explain the majority of the variation observed in Symbiodiniaceae transcriptional responses (Fig. S5.2). Symbiodiniaceae functional profiles were affected by heat stress, with 12 KOG functional classes enriched under 3 DHW (Fig. 5.3). In particular, genes involved in 'energy production and conservation' and 'inorganic ion and carbohydrate transport and metabolism' were downregulated under heat stress, while genes related to 'posttranslational modification, protein turnover, chaperones' and 'RNA processing and modification' were upregulated under these conditions. GO enrichment analyses confirmed these functional changes (Table S5.6), identifying a strong downregulation of genes related to photosynthetic processes, energy metabolism and ion transport, and an upregulation of genes involved in protein production (e.g. rRNA and tRNA modification). In addition, genes related to the biosynthesis of nitrogen compounds were also negatively regulated under heat stress conditions.

Accumulated heat stress induced significant changes in the expression of 2,470 genes (Fig. 5.4, Table S5.7). Interestingly, a gene related to the Photosystem II was strongly downregulated under heat stress; and multiple genes encoding for transporters (i.e. phosphate, zinc, amino acids) were consistently negatively regulated under these conditions. Genes related to chaperone proteins showed contrasting patterns, with both up- and down-regulation under high temperatures. Symbiodiniaceae DEGs clustered based on heat treatment only, and not time (Fig. 5.4), in contrast to host differential expression analyses.

Stability in the microbial community under incremental temperatures

In total, 1,960,673 high-quality 16S rRNA amplicon reads were generated (ranging from 3,250 to 50,542 reads per sample, with an average of 15,199; blanks excluded). Following quality trimming, chimera removal and data filtration, a total of 10,900 amplicon sequence variants (ASVs) were identified and rarefaction curves of sponge microbial diversity reached a plateau, confirming an appropriate sequencing depth was achieved for effective community analysis (Fig. S5.3). Permutation Multivariate Analysis of Variance revealed sponge-associated microbial community was host-specific, significantly different to seawater and feed samples, despite homogeneity of dispersion not being met (Fig. S5.4, Table S5.8). Under increasing temperatures, the sponge microbial community structure was affected by both treatment and time, but a significant interaction between these two factors was not identified (Fig. 5.5 and Table S5.9). The microbial community composition was dominated by *Alphaproteobacteria* at each treatment / time point, with the exception of *Dadabacteriia* dominating samples collected at ambient T5 and heat T0. At the family level, Rhodobacteraceae (*Alphaproteobacteria*), P3OB-42 (*Deltaproteobacteria*) and an ambiguous taxon belonging to *Dadabacteriia* were the dominant taxa (Fig. 5.5). DESeq analyses revealed only six differentially abundant ASVs between 0 and 9 DHW, including an enrichment of an ASV assigned to *Marinagarivorans* (Fig. S5.5). At the family level, P30B-42 (*Deltaproteobacteria*) and Rhodobacteraceae-associated taxa (*Alphaproteobacteria*) were consistently prevalent across treatments and time, as well as one ambiguous taxon (*Dadabacteriia*) (Fig. 5.5). Importantly, colony of origin had an effect on the microbial community composition (Table S5.9), but sponge samples showed similar patterns in response to heat stress across colonies of origin (Fig. S5.6). Assessment of the microbial alpha diversity metrics using a Permutation Multivariate Analysis of Variance, revealed that treatment and time did not affect the Shannon diversity index (Fig. S5.7 and Table S5.10).

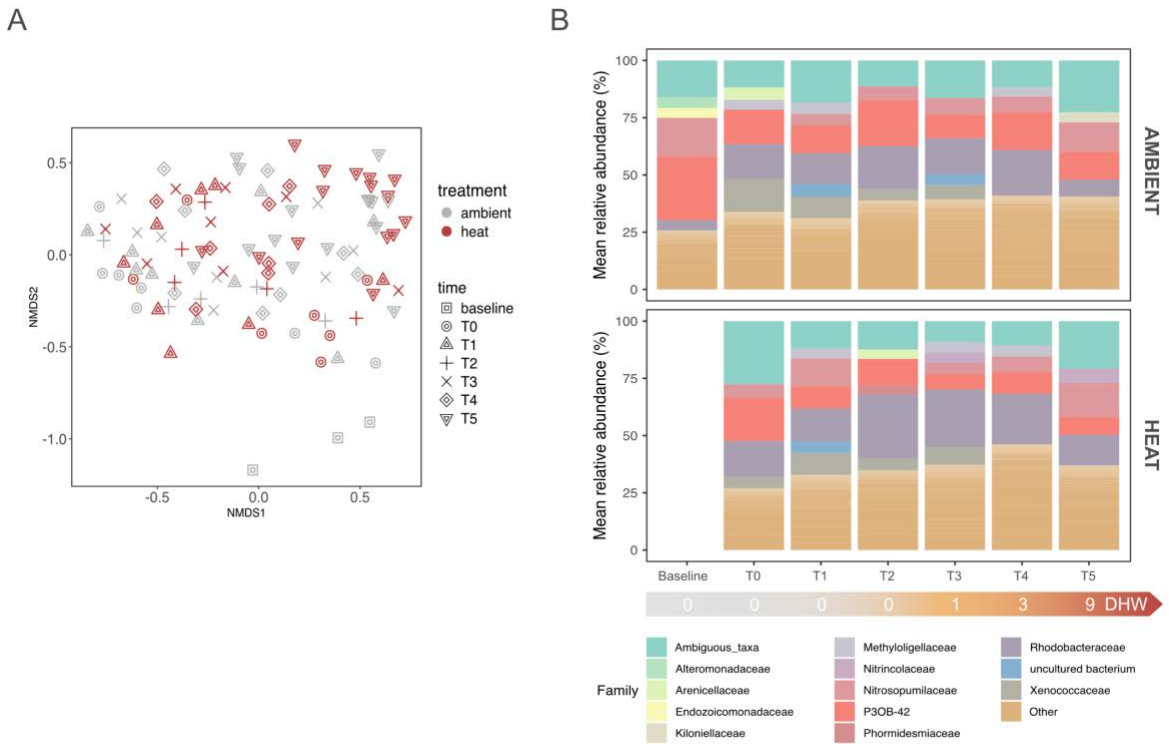


Fig. 5.5. Sponge microbial community structure under increasing heat stress characterized through 16S rRNA gene sequencing. A) Non-metric Multi-dimensional Scaling (NMDS, sqrt-rooted; stress = 0.19) based on Bray-Curtis dissimilarities calculated on ASVs relative abundance in the sponges preceding fragging (baseline), and in the sponge samples under ambient and heat treatments over time (T0 – T5). B) Mean relative abundance of dominant microbial families (> 4% relative abundance) under ambient and incremental heat stress.

Integrating host and Symbiodiniaceae transcriptional responses to heat stress

The integration of host and Symbiodiniaceae transcriptomic datasets through DIABLO revealed 81 highly correlated genes (i.e. 51 host and 30 Symbiodiniaceae genes) along the first four components, and a strong correlation (r = 0.94) between the two transcriptional profiles (Fig. 5.6, Table S5.11). Among these correlated genes, a host gene encoding an actin-related protein (most expressed under heat stress) was negatively correlated to many Symbiodiniaceae genes, including genes related to cytoskeleton proteins and G-protein coupled receptors (most expressed under ambient). In addition, one host and one Symbiodiniaceae gene encoding for G-protein coupled receptors were positively correlated with each other; while host and Symbiodiniaceae genes encoding for chaperone proteins were negatively correlated to one another.

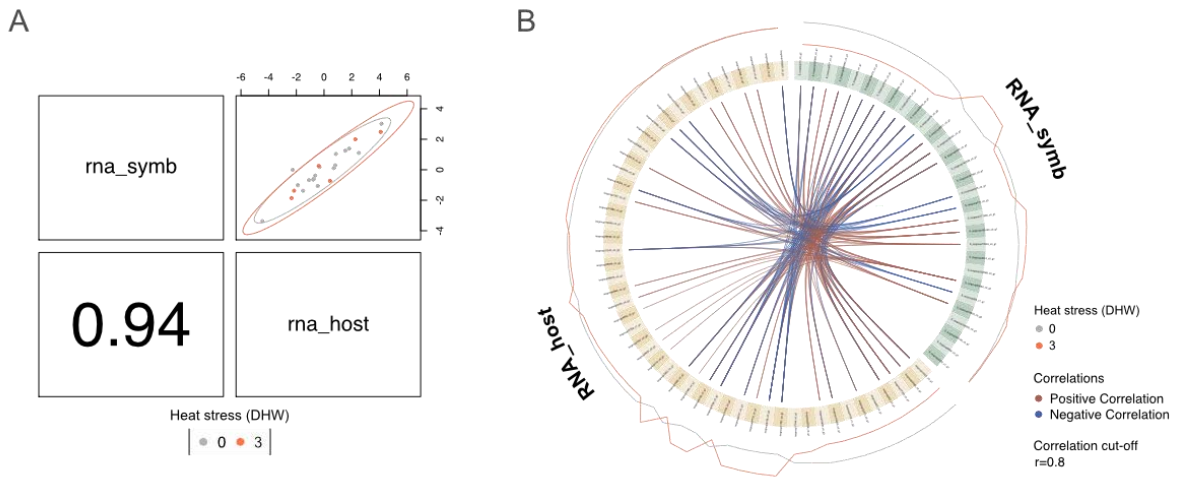


Fig. 5.6. Integrative analysis of host and Symbiodiniaceae gene expression profiles (annotated genes only) under ambient (0 DHW) and heat stress (3 DHW) conditions using DIABLO. A) Correlation between host (rna_host) and Symbiodiniaceae (rna_symb) transcriptional profiles. B) Circos plot illustrating positive and negative correlations (r > 0.8; blue and red lines) between the most discriminative host and Symbiodiniaceae genes under ambient and heat stress treatments along component 1 - 4. The yellow and green quadrants indicate host and Symbiodiniaceae genes, respectively. The most outer lines represent gene expression levels under ambient (grey) and heat stress (orange).

Discussion

The mutualistic association between Symbiodiniaceae and sponges within the '*Cliona viridis* species complex' not only enhances sponge growth and fuels its bioerosion activity, but is also essential for meeting the basic host metabolic requirements (Achlatis et al., 2017, 2018, 2019b, 2019a; Fang et al., 2014; Weisz et al., 2010). As sponge-Symbiodiniaceae associations appear to be more thermotolerant than the counterpart coral symbioses (Ramsby et al., 2018a), understanding how the sponge holobiont responds to increasing temperatures can reveal important insights into the mechanisms underpinning this relationship. In our manipulative heat stress experiment, we show that an initial destabilization of the symbiosis occurred at moderate heat stress levels (i.e. 3 DHW), with a decrease in Symbiodiniaceae photochemical effective efficiency and a downregulation of symbiont genes involved in photosynthetic processes. This reduction in Symbiodiniaceae photosynthetic performance was coupled with a downregulation of host genes involved in energy metabolism, as well as an upregulation of host immune responses. Our integrative analyses revealed a correlation between host and Symbiodiniaceae genes involved in cellular organization and structure, suggesting a key role of cytoskeleton proteins in the sponge heat stress response. With increasing heat stress, signs of bleaching were observed at > 6 DHW, as well as a shift towards a heterotrophic state (P / R ratio <1), indicating a breakdown of the symbiosis.

At a broad functional scale (i.e. KOG classes), host gene expression profiles showed limited alterations under heat stress (i.e. 3 DHW), while multiple GO terms were up- or down- regulated under these conditions. The significant downregulation of host genes within the KOG class identified as host energy production and conservation was confirmed by the downregulation of multiple GO terms including glucose metabolism. These gene expression changes indicate an overall decrease in host energy availability under heat stress, likely as a consequence of an altered metabolic exchange between host and Symbiodiniaceae. Indeed, the observed downregulation in Symbiodiniaceae genes involved in photosynthetic activity, potentially linked to symbiont photodamage (i.e. reduced photochemical effective efficiency), may have resulted in a reduction in energy production by the algal symbionts. Despite the potential alteration in nutrient exchange, further supported by a downregulation of host genes encoding for multiple ion transporters, physiological measures did not reveal significant changes in either net photosynthesis or P / R ratio between treatments (i.e. ambient vs 3 DHW), indicating the sponge host was still supported by its symbionts' autotrophy under these conditions. This finding aligns with Fang and colleagues (2014), who showed that provision of fixed carbon from symbionts to the sponge host was not reduced (i.e. increased carbon availability for the sponge host) under moderate heat stress conditions (+2 ˚C), but contrasts markedly to what is reported in Symbiodiniaceae-cnidarian symbioses where high temperatures induces the retention of carbon by the algal symbionts (Rädecker et al., 2021; Xiang et al., 2020).

We postulate that reduced energy production within the sponge holobiont was not compensated by an increase in heterotrophic feeding, as indicated by a significant downregulation of multiple genes involved in the host feeding behaviour. This hypothesis was further supported by the downregulation of the host TRPML gene (transient receptor potential channels), as genes within this channel superfamily have been linked to cilia flow sensory perception in sponges (Ludeman et al., 2014). Reduced feeding was also observed in the sponges *Rhopaloeides odorabile* exposed to high temperature due to a decrease in flow rate, filtration efficiency and choanocyte chamber density and size (Massaro et al., 2012). While in *C. orientalis*, the net uptake of dissolved organic carbon (DOC) and particulate organic carbon (POC) was previously shown not to increase under high temperatures, with the limited pumping capacities of this species likely being the driver of the observed response (Fang et al., 2014). The reduction in heterotrophic feeding contrasts with general coral heat responses, where the heterotrophic acquisition of carbon has been suggested as a potential strategy to increase coral resilience to ocean warming (Grottoli et al., 2006), indicating distinct mechanisms may underpin heat stress responses between disparate Symbiodiniaceae-associated marine taxa.

In addition to alterations within carbon metabolism, early responses to heat stress were linked to changes in gene expression associated with several host immune-related pathways. Immune responses have been shown to play a key role in symbiosis regulation, for example activating bacteria and

Symbiodiniaceae-recognition pathways (Hu et al., 2023; Matthews et al., 2017; Posadas et al., 2022; Weis, 2019). In our study, the upregulation of genes encoding for scavenger receptors and G-protein coupled receptors under heat stress, could thereby help maintain holobiont function through the recognition of symbionts from pathogens. Indeed, the regulation of these critical environmental sensors showed opposite patterns in the thermal sensitive sponge *Leucetta chagosensis* exposed to high temperatures (Posadas et al., 2022). The thermotolerance of *C. orientalis* may therefore lie in the activation of specific immune-related functions. While heat-induced immune responses commonly trigger autophagy and apoptotic cell death in sponges (Fan et al., 2013; Guzman & Conaco, 2016; Koutsouveli et al., 2020; Webster et al., 2013), apoptotic-related genes were not significantly upregulated in our study. Similarly, the downregulation of a gene encoding for thioredoxin, which promotes post-apoptosis cell regeneration in sponges (Guzman & Conaco, 2016), as well as a GO term assigned to 'tumor necrosis factor superfamily cytokine production', suggest *C. orientalis* cellular pathways were not impaired at 3 DHW, which was also confirmed by the maintenance of host physiological performances under these conditions. This suppression of the apoptotic pathway may have been induced by the upregulation of Heat Shock Proteins genes, as these molecular chaperones are well known for increasing host stress resistance (Sørensen et al., 2003). Together, our findings show that *C. orientalis* initiates strong molecular responses under moderate heat stress, which are potentially linked to its high thermotolerance, and precede any decline in host physiological metrics.

Integrative gene expression analyses between host and Symbiodiniaceae genes indicated strong correlation ($r = 0.94$) between the two transcriptomic profiles across treatments. Among the 81 highly correlated genes, the upregulation of a host gene encoding for an actin-related protein was linked to the downregulation of Symbiodiniaceae cytoskeleton proteins, suggesting cellular reorganization is connected between the two holobiont members, and show opposite responses under heat stress. In coral-Symbiodiniaceae associations, actin cytoskeletal rearrangements have been reported in both host and symbionts under high temperatures (Chakravarti et al., 2020; Desalvo et al., 2008), but actin can also be involved in host phagocytic processes leading to the establishment of the symbiosis (Hu et al., 2023). In addition, algal inoculation experiments on freshwater sponges revealed that actin-related proteins were upregulated in aposymbiotic compared to symbiotic hosts (Hall et al., 2021), indicating a potential role in the regulation of sponge-algal symbiosis. Hence, alteration in gene expression patterns in both host and algal cytoskeleton may contribute to the destabilization of sponge-Symbiodiniaceae association under heat stress.

In contrast to the strong transcriptional response observed in host and Symbiodiniaceae under heat stress, the sponge microbiome showed no significant structural changes under incremental temperatures, with *Alphaproteobacteria* representing the dominant microbial class as previously observed in Ramsby et al. (2018a). This microbial stability contrasts previous findings within the same

sponge species, describing a shift in the microbial community from 29˚C (Ramsby et al., 2018b). However, the distinct temperature range at which sponges were experimentally exposed may underlie the contrasting microbial responses, as this study focussed on summertime maxima only (28 ˚C - 32˚C), while Ramsby and colleagues (2018a) exposed sponges to a broad temperature shift, from 23˚C (i.e. winter temperatures) to 32˚C. The sponge microbiome constitutes a dynamic equilibrium (Pita et al., 2018), with previous studies linking high microbial stability with sponge resistance to environmental stressors (Posadas et al., 2022; Simister et al., 2012). Our findings suggest that the microbiome associated with *C. orientalis* may not be actively involved in the destabilization of the sponge holobiont under heat stress, as microbial communities did not significantly shift following host transcriptional changes nor the onset of bleaching. Similar results were found in the coral *Montipora aequituberculata* exposed to elevated temperatures and challenged by coral bacteria pathogens, with changes in host and Symbiodiniaceae gene expression profiles preceding significant microbial compositional shifts (Water et al., 2018). Further investigation is required to understand whether the microbial community maintains its stability at both the compositional and the functional level.

The high thermotolerance of *C. orientalis* identified through our manipulative experiment is consistent with other studies performed on the same species across the GBR (Achlatis et al., 2017; Fang et al., 2013; Ramsby et al., 2018a). Multiple factors may contribute to this trait, including the capacity of *Gerakladium* spp. to migrate into deeper layers of the sponge tissue (Fang et al., 2016), enhancing therefore its protection against light stress. However, the high thermotolerance observed of *Gerakladium* spp. when isolated in cultures as well as associated with coral larvae (Chakravarti & van Oppen, 2018; Matsuda et al., 2022), suggests intrinsic features may confer its resistance to high temperatures. Understanding heat stress responses within this genus in isolation and symbiosis is critical for predicting reef ecosystems dynamics under future climate. Our study reveals host immune-related pathways as well as nutrient imbalance between host and Symbiodiniaceae may underlie heat stress responses within sponge-Symbiodiniaceae symbiosis, with microbial communities not exacerbating the destabilisation of this mutualistic relationship.

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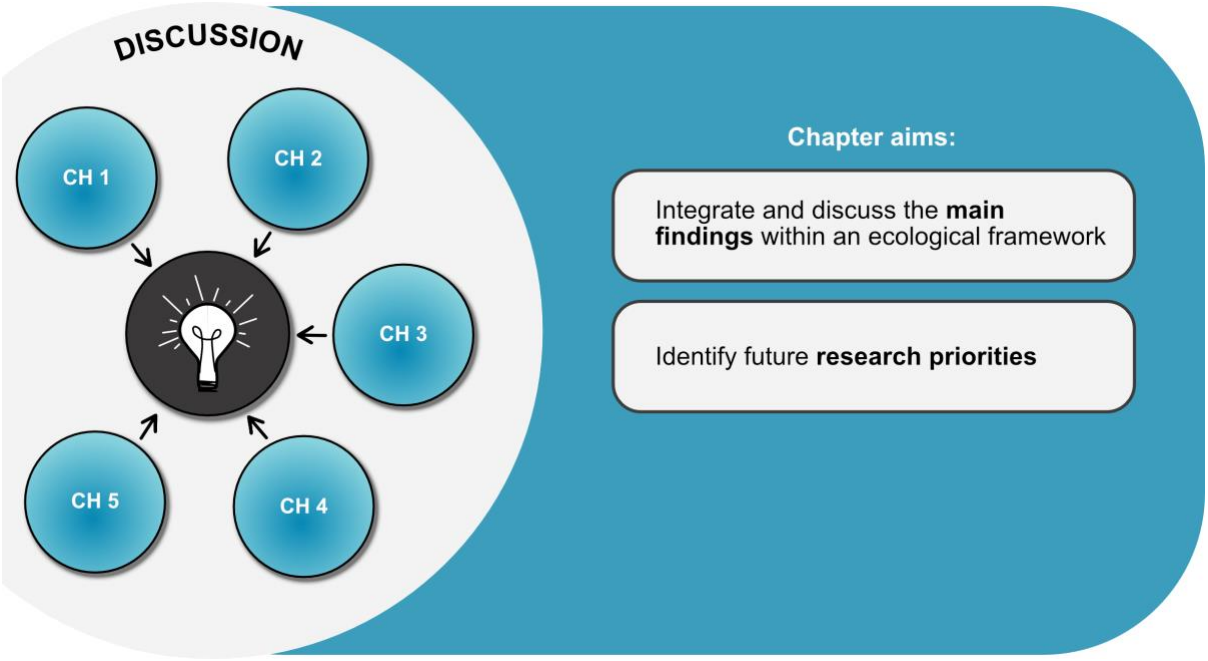
CHAPTER 6 : GENERAL DISCUSSION



General discussion

Summary of thesis findings

The research studies presented within this thesis provide evidence of the effects of environmental stresses related to predicted climate changes on host-symbiont interactions within important coral reef organisms. Reef ecosystems are marine diversity hotspots and one of the most sensitive ecosystems to climate change (Hughes et al., 2017b), with coral reef loss expected to raise in the near future even under optimistic warming scenarios (Frieler et al., 2013; McWhorter et al., 2022). As ocean warming and acidification intensify (Hoegh-Guldberg et al., 2017; McWhorter et al., 2022), understanding how these climate stressors will affect coral reef symbioses, which underpin reef ecosystem functioning, is critical for applying effective reef conservation and restoration strategies. Our understanding of the role symbionts (e.g. Symbiodiniaceae, bacteria, archaea) play in reef systems has improved dramatically in the past decade due to major advances in sequencing technologies, stable isotope labelling and imaging approaches (Achlatis et al., 2018; Nielsen et al., 2018; Robbins et al., 2021). Yet, many questions remain to be resolved on how climate-driven changes will affect host-symbiont dynamics, and their consequences for reef holobiont health. In this thesis, the effect of environmental stresses related to climate change on host-symbiont interactions was extensively explored, through the integration of a wide range of molecular and physiological approaches on ecologically important coral reef species.

First, I explored the effects of high temperature and pCO_2 levels on host-microbe dynamics, with the overall aim of linking microbial responses to host performance under future climate scenarios (Chapters 2 and 3). In doing so, I identified direct and indirect microbial mechanisms with the potential of enhancing host environmental tolerance based on a thorough review of studies performed on established terrestrial model systems as well as coral reef invertebrates (Chapter 2, published as Marangon et al., 2021). The microbial potential for coral reef intervention measures has gained increasing attention in recent years, as the provision of beneficial microorganisms in the form of probiotics has been proposed as an effective strategy to enhance coral resilience to heat stress (Peixoto et al., 2021; Rosado et al., 2019; Santoro et al., 2021). Although emerging microbial approaches may provide options for mitigating the impact of environmental stressors on coral health, the functional mechanisms underlying the increased resilience are poorly understood. My literature review highlights important host-microbe mechanisms that may contribute to the environmental tolerance of reef species (Chapter 2, published as Marangon et al., 2021), which could be further investigated for reef intervention measures.

While climate change may influence host-microbe dynamics within one host generation, climate-driven transgenerational effects may also occur, with beneficial or detrimental consequences for host health (Webster & Reusch, 2017). Within reef invertebrates, our current understanding of climate-driven

microbial dynamics across multiple host generations is limited (Baldassarre et al., 2022; Luter et al., 2020; Webster & Reusch, 2017), due to challenges in maintaining multi-generational experiments over extended time periods. In the research presented in my thesis, these technical challenges were overcome by testing climate transgenerational effects on the microbiome of the tropical reef urchin *Echinometra* sp. *A* (Chapter 3, published as Marangon et al., 2023), a common coral reef species characterized by relative short generation times. Through a four-year experiment, parental exposure to climate treatments was found to influence the offspring microbiome, with microbial changes potentially linked to early signs of decline in urchin health, suggesting future climate may alter microbiome recruitment and / or maintenance in the offspring of this species. To generate realistic predictions on reef ecosystem functioning, there is an urgent need of understanding climate-driven cross-generational effects on microbial community structure. Taken together, these findings stress the importance of host-microbe relationship in reef holobiont health, and their influence on host responses to a changing climate.

In addition to prokaryotic microbial communities, some coral reef species are also associated with algal dinoflagellates (Symbiodiniaceae), which provide their host with phototrophic compounds critical for the support of host energetic demand (Cui et al., 2023; Fang et al., 2014; Muscatine & Porter, 1977). However, the equilibrium sustaining these mutualistic associations may be disrupted under heat stress (i.e. bleaching), impacting host health and often resulting in mortality events (Hughes et al., 2017b; Ramsby et al., 2018a). Identifying the mechanisms that underlie the destabilization of these symbiotic interactions remains the focus of many research projects (Cleves et al., 2020a; Nielsen et al., 2018; Rädecker et al., 2021; Weis, 2008), with few studies assessing heat stress responses at the holobiont level (Avila-Magaña et al., 2021; Savary et al., 2021). Integrative analyses on the responses of multiple holobiont members can enhance our understanding of the mechanisms underpinning the initial destabilization of coral reef symbioses, which may inform and guide future reef intervention measures. By combining transcriptomics (host, Symbiodiniaceae), 16S gene sequencing analysis (microbes) and physiological assessments, I explored heat stress responses in two ecologically important coral reef taxa. In the thermotolerant coral *Porites lutea*, a decline in host physiological performances was preceded by an alteration in carbon and nitrogen recycling within the holobiont, which was coupled with microbial community changes with an increased relative abundance of opportunistic bacteria. These findings indicate the coral holobiont heat stress response integrates the nutritional status of its members, as well as their interactions, highlighting the importance of applying an integrative approach in coral heat stress studies. When exploring the destabilization of the symbiosis within the sponge *Cliona orientalis*, I found that the sponge heat stress response was also linked to changes in carbon availability (i.e. reduction in Symbiodiniaceae photosynthetic-associated pathways, and decrease in host glucose metabolism), although nitrogen cycling did not seem to be impaired under heat stress in this species. In addition, the sponge microbial community remained stable under increasing temperatures, even when the sponge physiological performance declined and bleaching occurred.

Hence, species-specific mechanisms appear to underlie reef holobiont responses to environmental changes, highlighting the importance of applying integrative approaches across reef taxa for better predicting coral reef functioning under future climate.

Overall, the research presented in this thesis stresses the importance of incorporating host-microbe relationships into the predictions of reef ecosystem health, as the host species, as well as their microbial symbionts, can contribute to the stability and resilience of reef holobionts. Furthermore, this thesis shows that integrating holobiont member responses through a multi-omics approach represents a comprehensive tool to help identify mechanisms that underpin symbiosis stability under heat stress, providing valuable insights for reef restoration efforts.

Duality in microbial community responses to climate change within reef holobionts

Host-associated prokaryotes (hereafter 'microbes') are key players in reef holobiont health (Bourne et al., 2016; Carrier et al., 2021; Chapter 1; Webster & Thomas, 2016). Under the increasing pressures of climate change, microbial communities may either undergo compositional and functional shifts, or maintain a high degree of stability (Pita et al., 2018; Voolstra & Ziegler, 2020). When microbial community structure is flexible, microbial changes have been postulated to influence host resilience to environmental stressors through a wide range of direct and indirect mechanisms (Chapter 2 published as Marangon et al., 2021; Reshef et al., 2006; Webster & Reusch, 2017). An objective of this thesis was exploring microbial responses to predicted future seawater conditions within (Chapter 2, 3, 4 and 5) and between host generations (Chapter 2 and 3), as well throughout host developmental stages (Chapter 3), to compare microbial responses across coral reef taxa and link them to host environmental tolerance.

The thesis findings highlight host- and developmental stage- specificity in microbial responses to future climate. While a strong microbial compositional shift was observed under climate stressors in urchin larvae (Chapter 3, published as Marangon et al., 2023) and in the coral *Porites lutea* (Chapter 4), microbial stability characterized the sponge-associated microbial community in the same conditions (Chapter 5). Microbial shifts occurred in parallel with a decline in host physiological performance, indicating the observed microbial changes were indicative of dysbiosis rather than beneficial acclimatory responses in these coral reef species. In contrast, the high stability of the sponge microbiome under increasing temperatures constitutes further evidence of the resistance of some sponge-associated microbial communities to environmental stressors (Gantt et al., 2017; Glasl et al., 2018). When comparing urchin microbial community composition across host generations and life stages under climate treatments, environmental conditions at which parents were exposed influenced the offspring microbial community composition (Chapter 3, published as Marangon et al., 2023),

highlighting the importance of multi-generational studies in predicting future host-microbe dynamics. Taken together, my thesis findings show that microbial detrimental changes will likely take place in many coral reef taxa under future climate, although microbial responses may vary across host species (Chapters 3, 4, 5). Given intensity and duration of environmental stressors can also influence microbial community dynamics (Chapters 3 and 4), performing experiments under biologically relevant environmental conditions is critical for predicting host-microbe interactions in coral reef systems. In addition to taxonomic analyses, determining changes in microbial metabolic pathways would further elucidate the relationship between microbial community dynamics and host fitness under a changing climate.

Altered nutrient recycling underpins the initial destabilization of host-Symbiodiniaceae symbiosis

In reef ecosystems, Symbiodiniaceae-associated taxa including coral, foraminifera, bivalve molluscs (giant clams) and some sponge species, rely on dinoflagellate algae to meet their energy requirements. In comparison to coral-Symbiodiniaceae associations, sponge symbiosis shows a higher thermotolerance (Ramsby et al., 2018a), which may be due to specific physiological properties of sponge-associated Symbiodiniaceae (Chakravarti & van Oppen, 2018; Matsuda et al., 2022), as well as distinct processing governing these symbioses. It has been identified that the carbon available for growth of the host increases under early stages of combined heat stress and ocean acidification in the marine sponge *C. orientalis* (Fang et al., 2014), while it is well known that carbon availability is reduced in coral holobionts (Rädecker et al., 2021), raising the question as to which metabolic processes contribute to Symbiodiniaceae stability within sponge symbiosis. To better understand the molecular mechanisms underlying the distinct heat stress responses in these two reef taxa, here I compare coral (i.e. *Porites lutea*) and sponge (i.e. *Cliona orientalis*) transcriptional responses to heat stress (Chapter 4 and 5), and propose that differences within metabolic co-dependency between host and Symbiodiniaceae in the two species may explain the distinct holobiont resistance to environmental stressors (Rädecker, 2019).

In the coral holobiont, gene expression analyses have revealed a decoupling of carbon recycling as well as an increase in nitrogen availability within the coral holobiont under early stages of heat stress (Chapter 4), supporting previous findings that suggested host maintenance of a nitrogen-limited state may underpin the stability of coral symbiosis (Cui et al., 2019; Cunning et al., 2017; Rädecker et al., 2021; Xiang et al., 2020). Indeed, nitrogen limitation induces a release of the excess algal photosynthates to the host, which supports host energy demand (Cui et al., 2023; Pernice et al., 2012; Xiang et al., 2020). However, high temperatures drive an increase in host energy requirements, resulting

in a higher production of host catabolic ammonium (Chapter 4, Rädecker et al., 2021). The higher nitrogen availability within the holobiont may therefore induce Symbiodiniaceae to grow and retain photosynthates, depriving the coral host of its main carbon source (Chapter 4, Rädecker et al., 2021; Xiang et al., 2020).

When investigating sponge transcriptional responses, abundance levels of genes involved in carbon recycling were reduced under high temperatures, while nitrogen cycling did not show strong alterations (Chapter 5). If nitrogen availability within the sponge holobiont does not increase under early stages of heat stress, Symbiodiniaceae may still translocate the surplus carbon to the host, until reaching a point where their photochemical capacities are impaired by high temperatures. When compared to each other, these combined heat stress studies (Chapter 4 and 5) suggest that distinct mechanisms may regulate host-Symbiodiniaceae metabolic interdependency between the two reef taxa. Future research should confirm whether a nitrogen-limited state is maintained in sponge symbiosis in a healthy state, and explore the effects of high temperatures on this carbon / nitrogen equilibrium. In addition, these two reef taxa are dominated by distinct Symbiodiniaceae species (Ramsby et al., 2018a; Robbins et al., 2019), and an incomplete understanding of their symbiont metabolic repertoire limit our ability of characterizing host-symbiont metabolic relationship. Future studies should better define the metabolic differences between coral- and sponge-associated Symbiodiniaceae, to elucidate the regulatory mechanisms underlying these symbioses.

Although host-Symbiodiniaceae molecular processes being distinct between coral and sponge symbioses, similar functional classes (euKaryotic Orthologous Groups, KOG) were enriched in the Symbiodiniaceae transcriptomic profiles under heat stress (i.e. 3 DHW; Chapter 4 and 5). For example, a downregulation of genes involved in ‘inorganic ion transport and metabolism’ and ‘energy production and conservation’ was observed in the Symbiodiniaceae associated with both species, as well as an upregulation of genes encoding for ‘posttranslational modification, protein turnover, chaperones’ and ‘RNA processing and modification’. These findings suggest general heat stress responses may be conserved in Symbiodiniaceae despite the evolutionary distance between symbionts hosted within distinct coral reef hosts.

Towards an integrative symbiotic framework

Assessment of reef holobiont responses under heat stress mainly focusses on single holobiont members, or interactions between two holobiont partners (for example (Chakravarti & van Oppen, 2018; Cleves et al., 2020a; Rädecker et al., 2021)). While these studies provide critical insights into the mechanisms involved in single holobiont heat stress responses, growing evidence indicate all members

simultaneously contribute to host physiological responses to increasing temperatures (Chapter 4 and 5, Avila-Magaña et al., 2021; Rädecker et al., 2021, 2022; Savary et al., 2021). Hence, applying an integrative multi-omics approach between multiple holobiont partners could enhance our understanding on the underlying drivers of heat-induced symbiosis breakdown. In my thesis, I integrated host (gene expression profile), Symbiodiniaceae (gene expression profile) and microbial (16S rRNA gene analysis) responses to heat stress within the coral *P. lutea* and the sponge *C. orientalis*. My findings demonstrate that changes in relative abundance of microbial taxa under heat stress were strongly correlated with coral host gene responses, but not with Symbiodiniaceae genes, suggesting microbial community dynamics may be regulated by host control mechanisms in this species (Chapter 4). In addition, a decline of *Endozoicomonas* bacteria, which are putative beneficial bacteria within coral holobionts (Pogoreutz et al., 2022; Robbins et al., 2019; Wada et al., 2022), was correlated to an increase in expression of a host ADP-ribosylation factor, which is involved in host intracellular vesicle transport and was identified as negative regulator in cnidarian-Symbiodiaceae symbioses (Chen et al., 2004). Taken together, these results suggest both *Endozoicomonas* and Symbiodiniaceae population may be regulated by similar host regulatory processes in this coral species (Chapter 4). In contrast, sponge microbial community remained stable under increasing temperatures, and integrative analyses revealed a negative correlation between sponge host and Symbiodiniaceae genes encoding for cytoskeleton proteins, suggesting structural change in cell organization may play a critical role in sponge early heat stress response (Chapter 5).

In addition to identifying important correlations between holobiont member responses, multi-omics approaches can also reveal the temporal scale at which these changes occur, which may be of particular importance in the context of future reef intervention strategies. In both the coral *P. lutea* and the sponge *C. orientalis*, differential gene expression analyses revealed a higher number of Symbiodiniaceae genes differentially expressed between heat and ambient treatments compared to the host, indicating Symbiodiniaceae may first respond to heat stress (Chapter 4 and 5). This result contrasts with previous findings showing that coral host genes respond earlier than Symbiodiniaceae genes (Barshis et al., 2013, 2014; Leggat et al., 2011; Li et al., 2021), however these studies were performed on heat-sensitive coral species, while I focused my studies on thermotolerant taxa. Although we cannot exclude methodological biases (e.g. lower variability between samples) may have influenced the transcriptomic outputs, the observed transcriptional response may suggest that Symbiodiniaceae are more sensitive to increasing temperatures than their hosts in thermotolerant species. Overall, my thesis demonstrates that multi-omics approaches facilitate linkages in environmental responses between holobiont members, providing an integrative overview on the key processing constituting the basis of symbiotic interactions.

Research priorities

Given the increasing pressure and threat of climate change, one of the major challenges within reef conservation efforts is identifying the mechanisms that underpin thermotolerance in coral reef species, and host-symbiont relationships complicate our ability to achieve this task. Technical methodological challenges and reef ecosystem complexities limit our ability to achieve these goals. Research within this thesis shows both host and symbionts contribute to the stability of reef symbioses under future climate, and cross-generational climate effects may further influence host-microbe interactions. To better understand the mechanisms underpinning the environmental tolerance of reef symbioses, future research would greatly benefit from targeted experimental validation, with the overall aim of moving from correlation to causation within the field of coral reef microbial ecology. Cutting-edge techniques such as CRISPR technology (Cleves et al., 2020b), spatial ecology (Geier et al., 2020) and single cell functional studies (Nielsen et al., 2018) would enable refined characterisation of host-symbiont and symbiont-symbiont functional relationships under a changing climate. To better apply these techniques, it would be beneficial to first improve our understanding on microbial functional potential within reef species, particularly in coral species showing taxonomic microbial changes under heat stress, as metagenomics and metatranscriptomics are currently highly challenging in this reef taxa due to difficulties in achieving high-quality microbial enrichments.

Comparative functional studies between sensitive and thermotolerant reef holobionts across multiple species could further assist in identifying critical mechanisms underpinning symbiosis stability, which could be used for developing reef intervention strategies. As the findings of this thesis suggest that nitrogen availability may play a key role in host thermotolerance, experimental validation should confirm the importance of this nutritional state for the stability of the symbioses. Inoculating Symbiodiniaceae with reduced nitrogen utilization into coral hosts, for example, could experimentally validate my thesis findings. In addition, combining stable isotope labelling and nanoscale secondary ion mass spectrometry (NanoSIMS) analyses for identifying host and symbiont metabolic activity under heat stress within the sponge holobiont, would enable a fine-scale comparison of sponge and coral responses to heat stress, as these analyses have been performed in some coral species (Rädecker et al., 2021).

Future research should also focus on the mechanisms driving the taxonomic microbial shifts observed in many coral reef species under environmental changes (Chapter 2, 3 and 4), as multiple processes may be involved in microbial community changes such as disruption in host-symbiont recognition mechanisms, modification of microbiome recruitment, competition with other symbionts, alteration in nutrient availability within the holobiont, and stochastic mechanisms. Overall, this thesis highlights the

importance of providing an ecological context to microbial data; linking holobiont physiological responses to host and symbionts functional changes opens up new avenues for understanding how the environment shapes host-symbiont interactions.

Concluding remarks

Predicting host-microbe dynamics in reef holobionts and identifying the molecular processes that underpin symbiosis destabilization is essential for targeting intervention strategies within coral reef conservation avenues. Here, I demonstrated how transgenerational effects, species specificity and host-symbiotic interactions are all important drivers of reef holobiont responses to a changing climate. In addition, nutrient availability was identified through integrative analyses as a critical factor underpinning the stability of reef symbioses, stressing the need for using integrative approaches to generate a comprehensive understanding of holobiont functioning. The research presented in this thesis (summarised in Fig. 6.1) reinforces the importance of creating an ecological framework for interpreting host-microbe responses to a changing climate, and applying multi-omics approaches for integrating reef holobiont member responses.

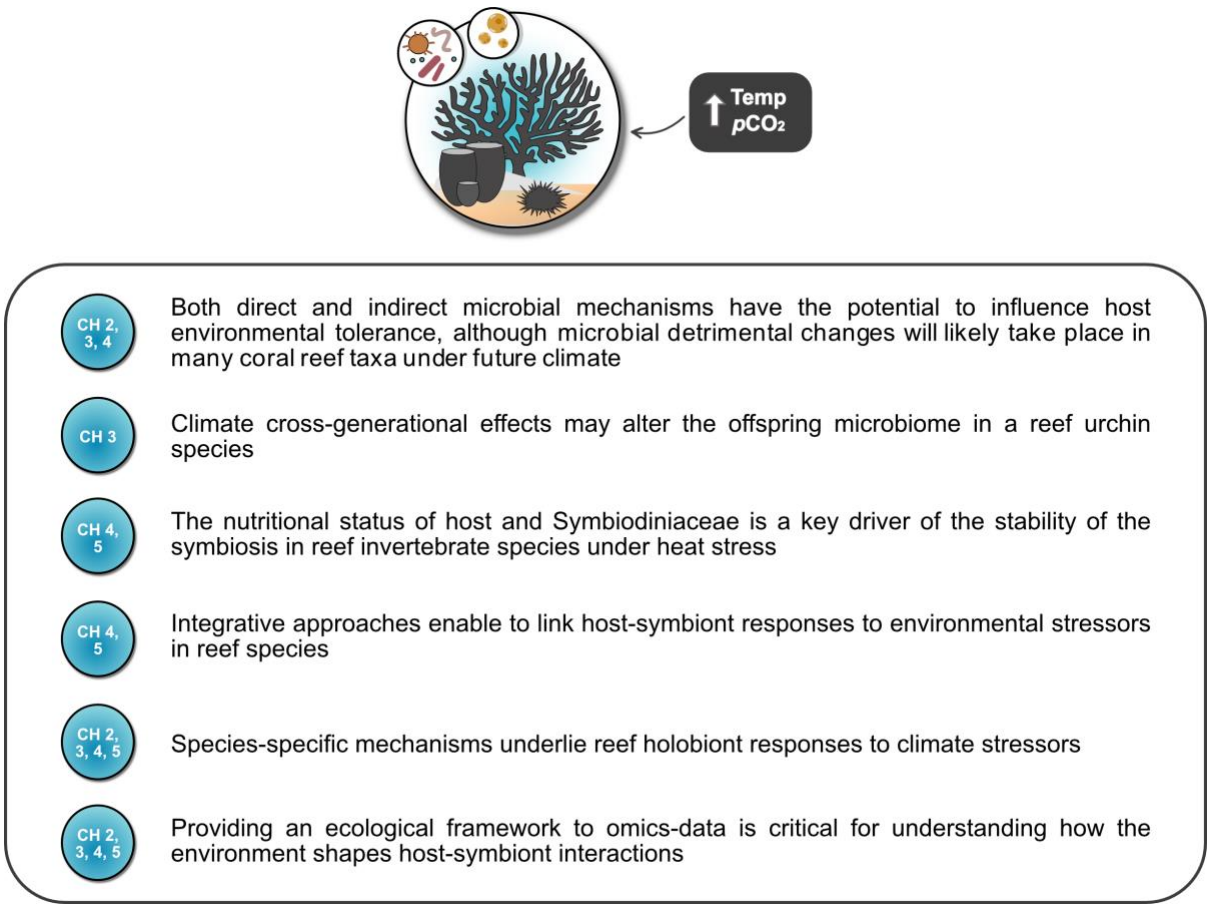


Fig. 6.1. Thesis main findings

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APPENDICES

Appendix A – Supplementary Material for Chapter 3

Table S3.1. (**A**) Number of urchin replicates per life stage and climate treatment after data filtration. Replicates for the transplant experiment are shown in the format 'parental climate treatment – offspring climate treatment' (e.g. Ambient-2050: parents exposed to ambient and offspring exposed to 2050 conditions). (**B**) Seawater replicates across climate treatments (Ambient, 2050, 2100) and life stages (F_0 Adult gonad, F_1 Larvae, F_1 Juvenile, F_1 Adult gonad).

A)

Urchin samples

	Ambient	**2050**	**2100**
F_0 Adult gonad	5	6	6
F_1 1-d larvae	3	3	3
F_1 5-d larvae	3	3	3

	Ambient - Ambient	**Ambient - 2050**	**Ambient - 2100**	**2050 - 2050**	**2050 - 2100**	**2100 - 2100**
F_1 Juvenile	9	9	9	8	9	9
F_1 Adult gonad	8	-	-	-		3
F_2 1-d larvae	2	-	-	-		2
F_2 7-d larvae	2	-	-	-		2

B)

Seawater samples

	Ambient	**2050**	**2100**
F_0 Adult gonad	**3**	**3**	**3**
F_1 Larvae	**1**	**1**	**1**
F_1 Juvenile	**3**	**3**	**3**
F_1 Adult gonad	**3**	**-**	**3**

Table S3.2. List of the 32 ASVs that were identified as contaminants using the R package decontam (https://github.com/benjjneb/decontam). Class, Family and Genus are reported for each ASV identifier; and unidentified family and genera are reported as "-".

ASV identifier	Class	Family	Genus
420e7973dcc614c06e823acd64399046	Oligosphaeria	uncultured bacterium	-
e88e9cbc6d53354d2de24dffc7a5e93c	Woesearchaeia	-	-
47528e332c77ba4cef8d067390f8b3b1	Woesearchaeia	-	-
bbe968da0a6da2ce9f66c7ab5ea15425	Gracilibacteria	uncultured bacterium	-
8424ac265142fe201c78bc93949a1ed8	BD7-11	-	-
baf7e5d44d4a61f89c1bdc6ab7a446c4	Bacteroidia	Flavobacteriaceae	Mesoflavibacter
b47dcf513fb6be7c020be3216ec4a639	Bacteroidia	Flavobacteriaceae	Aquimarina
de8144e92fbe0d32a5a90b8c1133f23b	Lineage IIb	-	-
7a71f57deebcdbc6796cbab3a587bd5a	Dehalococcoidia	hydrothermal vent metagenome	-
fd98346394a5c79e554003012cb33826	Actinobacteria	Micrococcaceae	Renibacterium
4668ae4998e1edc9996f75e7c09f7bac	Planctomycetacia	Gimesiaceae	uncultured
71cef5b1174477c4ffc4ad09a21f4c63	Planctomycetacia	Pirellulaceae	Pir4 lineage
ebd0562fa73b889281c0ded93518b7fa	Omnitrophia	Omnitrophaceae	Candidatus Omnitrophus
0b80a2392f611b736d856d177d560bf8	Alphaproteobacteria	Hyphomonadaceae	-
118735e135c14c9c69b787423d51ac40	Alphaproteobacteria	Hyphomicrobiaceae	uncultured
0c0044e3d1acdbb71c7cf4bc581bd27e	Alphaproteobacteria	Rhizobiaceae	-
afb1de4000e2688b434dacab00c9ae4c	Clostridia	Family XIII	-
5f56ed10a6d42d666669971daba052da	Fusobacteriia	Fusobacteriaceae	Cetobacterium
c8b083c2a3aef63ed75421f998aaa72f	Deltaproteobacteria	uncultured bacterium	-
529ff5c6b52a153757f4437c5722d155	Deltaproteobacteria	Bacteriovoracaceae	-
06a6e5477a6e3b608494ee0adccb4a94	Deltaproteobacteria	Bacteriovoracaceae	Peredibacter
75c904abd5443e40e28c688641317b7c	Deltaproteobacteria	Bacteriovoracaceae	-
ecb39484d5cfb68cd99bd33debef4fd4	Alphaproteobacteria	Magnetospiraceae	Magnetospira
550a0f69768245c3debe5262c3c6d668	Gammaproteobacteria	Cycloclasticaceae	Cycloclasticus
511a1dc820a661caeb441b3e5a55fb2f	Gammaproteobacteria	Coxiellaceae	Coxiella
39385ee32866666be718160857c5bcb6	Gammaproteobacteria	Spongiibacteraceae	BD1-7 clade
a166af5a482fb08f9ffbdcc3cc588e83	Gammaproteobacteria	Unknown Family	Marinicella
b99be30a570f9fb32fff706db4ae4609	Gammaproteobacteria	Alteromonadaceae	uncultured
8c44c6e0e7f7629878fe6666c139ef3d	Gammaproteobacteria	Cellvibrionaceae	uncultured
a93e5ddc25eca21fbfbdb99d40634ca7	Gammaproteobacteria	Shewanellaceae	Ferrimonas
be066e42c37b89db6680195009e0f55b	Gammaproteobacteria	Burkholderiaceae	-
7d217addfd80cb5393d81dae5f160367	Gammaproteobacteria	Burkholderiaceae	Schlegelella

Table S3.3. Permutational multivariate analysis of variance (adonis function in R vegan package) based on Bray-Curtis dissimilarities applied on square-root-transformed relative abundances under ambient conditions to examine the effects of (**A**) Life stage (fixed, four levels: "adult gonad", "larvae [1-day and older larvae were pooled]", "juvenile", "seawater"), generation (fixed, orthogonal, three levels: "F_0", "F_1", "F_2") and (**B**) larval age (fixed, two levels: "1 day", "> 1 day"). Tank was fitted first in the models to account for tank-to-tank variation before testing for effects of the other factors and their interactions. P-values were calculated using 10,000 permutations and statistical significance ($p < 0.05$) is indicated in bold.

A)

Source	*df*	*SS*	*pseudo-F*	*P-value*
Tank	10	7.01	4.18	**9.999e-05**
Life Stage	2	4.00	11.90	**9.999e-05**
Generation	1	0.30	1.80	**0.0386**
Life Stage x Generation	1	0.69	4.09	**9.999e-05**
Residual	27	4.53		

Pairwise tests:

Life Stage:
Adult gonad ≠ Larvae ≠ Juvenile ≠ Seawater

Generation:
$F_0 \neq F_1 \neq F_2$

Life Stage x Generation:

For life stage:
F_0: adult gonad ≠ seawater
F_1: adult gonad ≠ larvae ≠ juvenile ≠ seawater

For generation:
Adult gonad: $F_0 \neq F_1$
Larvae: $F_1 \neq F_2$

B)

Source	*df*	*SS*	*F*	*P-value*
Tank	3	1.75	10.96	**9.999e-05**
Larval Age	1	0.43	8.05	**9.999e-05**
Residual	5	0.27		

Table S3.4. Taxonomic classification of the ASV shared between life stages (F_0 adult gonad, 1-day F_1 larvae, 5-day F_1 larvae, F_1 juvenile) and present in at least 50% of the samples within each life stage under ambient conditions. Unassigned taxa are indicated with "-".

Present in F_0 adult gonad, F_1 1-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
68b9ce06fc98f29759c01b03263a5d67	Gammaproteobacteria	Vibrionaceae	*Vibrio*
0429a8a999c3238e12bbfaa1714d385e	Gammaproteobacteria	Vibrionaceae	*Vibrio*
4ee16f924a58f3d50a57e9b5c692f1c8	Gammaproteobacteria	Vibrionaceae	*Photobacterium*
689f1824bb6bdee2c08f619da1b57eb4	Gammaproteobacteria	Burkholderiaceae	-

Present in F_0 adult gonad, F_1 5-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
1824b94db3ba4ad470b4ef5580939b63	Fusobacteriia	Fusobacteriaceae	*Propionigenium*
1a23272bda7dbd3bd12860278e853693	Deltaproteobacteria	Desulfobulbaceae	unculutured

Present in F_1 1-day larvae, F_1 5-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
f420b5340d48941cc0e22ffef62e3df1	Alphaproteobacteria	Hyphomonadaceae	*Hyphomonas*
8787dc295950c53873b7ade8df61d312	Gammaproteobacteria	Alcanivoracaceae	*Alcanivorax*
3b237ba949f7dc62b497ba0049fbc0b8	Gammaproteobacteria	Oleiphilaceae	*Oleiphilus*

Present in F_0 adult gonad and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
48a6a0e363dfb3823afdb092a0bb834d	Alphaproteobacteria	Rhodobacteraceae	*Ruegeria*
0677d9f732493f832161376bda44672f	Bacteroidia	Cryomorphaceae	NS10 marine group
e4463785d08597b064903c399e26dd13	Gammaproteobacteria	Vibrionaceae	*Vibrio*
d3381ed3365e985eda8cf88bc4b1a016	Gammaproteobacteria	Vibrionaceae	*Vibrio*
346dd1e1f1d4684b1eb49c6187e51e5b	Gammaproteobacteria	Vibrionaceae	*Vibrio*
47a6c3d4b77e22e2363b6f567f6b6ff5	Gammaproteobacteria	Vibrionaceae	*Photobacterium*

Present in F_1 1-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
c3c32c0479bf4d183832a2e3cf235481	Alphaproteobacteria	Rhodobacteraceae	*Shimia*
7188ce140c148b15b672115f6b03944b	Gammaproteobacteria	Alteromonadaceae	*Aestuariibacter*
094ec68c611b5150657d2ca5d66df532	Gammaproteobacteria	Cellvibrionaceae	*Aestuariicella*
f9318f7ceb256618865b714eaf2bb57a	Gammaproteobacteria	Saccharospirillaceae	*Oleibacter*
8af3387cd4c1ab76183b20cf1b132d4b	Gammaproteobacteria	Oleiphilaceae	*Oleiphilus*

Present in F_1 1-day larvae and F_1 5-day larvae			
ASV identifier	**Class**	**Family**	**Genus**
7f0ce4f17ba4f5a4ecccf1816c6f599f	Nitrososphaeria	Nitrosopumilaceae	Candidatus *Nitrosopumilus*
3d685ccfb0ba01d3b90f5034bd711c59	Bacteroidia	Cyclobacteriaceae	*Reichenbachiella*
e308344afbdee0e94dbbbce16f4036dd	Bacteroidia	Saprospiraceae	*Rubidimonas*
c1682f506dbfad005be574be98cb06ca	BD7-11	-	-
e68fb7b3415cf827b2ed9cf8f93378b2	Verrucomicrobiae	uncultured bacterium	-
5eeba95b30322d684e95baa2e5149ebb	Alphaproteobacteria	Rhodobacteraceae	-
901bdd384be1d151e57df47fc0f66110	Alphaproteobacteria	Rhodobacteraceae	*Celeribacter*
09f7734468cb4192425e5d2808d54473	Alphaproteobacteria	Rhodobacteraceae	*Thalassobius*
7c96d8954560aee0155b635945a904ca	Alphaproteobacteria	Rhodobacteraceae	-
0da5e652e22483b15cea40a707af0bc4	Alphaproteobacteria	Rhodobacteraceae	-
e4304c9496921fcf8a88c1ef30dfd0eb	Alphaproteobacteria	Sphingomonadaceae	-
4366b9d73f1cae9a6e25bbfbaf772c56	Rhodothermia	Rhodothermaceae	uncultured
c771652b378108351c189ee8aa9e964d	Alphaproteobacteria	Hyphomonadaceae	*Maricaulis*
4a4899fc2175607ea55fe5a974f0aa0f	Alphaproteobacteria	Nisaeaceae	OM75 clade
3f2600c0719c081f1bbc976fb2d6576f	Gammaproteobacteria	uncultured bacterium	-
1b9abf85b0ab956755a3c0682ee4932d	Gammaproteobacteria	Diplorickettsiaceae	uncultured
e3ce2b2e4389c869b545c5eeb68577be	Gammaproteobacteria	SS1-B-06-26	uncultured
8ddcce2d1b11b48779fe048944173dc0	Gammaproteobacteria	Alcanivoracaceae	*Alcanivorax*
cd3c68e64285e960b6c46d8bf4cd75e6	Gammaproteobacteria	Unknown Family	*Marinicella*
3b5e5025d3eb814d0abf098474ba41c7	Gammaproteobacteria	Alteromonadaceae	*Alteromonas*

27f8b975f616fc83f4918afb2f83a40f	Gammaproteobacteria	Alteromonadaceae	*Aestuariibacter*
74ea9c538434b649ee8de4f42942b554	Gammaproteobacteria	Cellvibrionaceae	*Marinagarivorans*
78db7fa6b16b73b101fbb8b282b5330e	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*
6f3f68e5c8e2a11b388ddbbea9fa182d	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*
12c4cf4a9b6a4541ef111f02191bae96	Gammaproteobacteria	Halomonadaceae	*Halomonas*
d84d6fae24b0e5e82c7f93bef6bf70c3	Gammaproteobacteria	Saccharospirillaceae	*Oleibacter*
beed64a8017b2e1fdfe99d2fe8bca629	Gammaproteobacteria	Methylophilaceae	OM43 clade
3cf3c2c41dc63c196b8add9d3dd99b28	Gammaproteobacteria	Woeseiaceae	*Woeseia*

Table S3.5. Linear mixed effect models testing the fixed effect of life stage and generation (seven levels: "F_0 adult gonad", "F_1 larvae", "F_1 juvenile", "F_1 adult gonad", "F_2 larvae", "F_0 seawater", "F_1 seawater") on the Shannon diversity index on rarefied data under ambient conditions. Tank was included in the models as random effect. Contrasts are shown below, with statistical significance (Benjamini–Hochberg adjusted $p < 0.05$) indicated in bold.

Contrast	*Estimate*	*SE*	*df*	*P-value*
gonad F0 - gonad F1	0.12	0.36	40	0.7804
gonad F0 - juvenile F1	-1.55	0.35	40	**0.0002**
gonad F0 - larvae F1	-0.41	0.38	40	0.3509
gonad F0 - larvae F2	-0.05	0.42	40	0.9035
gonad F0 - seawater F0	-2.72	0.46	40	**<.0001**
gonad F0 - seawater F1	-1.91	0.37	40	**<.0001**
gonad F1 - juvenile F1	-1.66	0.30	40	**<.0001**
gonad F1 - larvae F1	-0.53	0.34	40	0.1754
gonad F1 - larvae F2	-0.17	0.38	40	0.7315
gonad F1 - seawater F0	-2.83	0.42	40	**<.0001**
gonad F1 - seawater F1	-2.03	0.32	40	**<.0001**
juvenile F1 - larvae F1	1.14	0.33	40	**0.0023**
juvenile F1 - larvae F2	1.50	0.38	40	**0.0005**
juvenile F1 - seawater F0	-1.17	0.42	40	**0.0124**
juvenile F1 - seawater F1	-0.36	0.32	40	0.3347
larvae F1 - larvae F2	0.36	0.40	40	0.4408
larvae F1 - seawater F0	-2.31	0.44	40	**<.0001**
larvae F1 - seawater F1	-1.50	0.35	40	**0.0002**
larvae F2 - seawater F0	-2.67	0.48	40	**<.0001**
larvae F2 - seawater F1	-1.86	0.40	40	**0.0001**
seawater F0 - seawater F1	0.80	0.43	40	0.1032

Table S3.6. Permutational multivariate analysis of variance (adonis function in R vegan package) based on Bray-Curtis dissimilarities applied on sqrt-root-transformed relative abundances to examine the effects of **A)** climate scenario (fixed, three levels: "Ambient", "2050", "2100") and life stage (fixed, three levels: "F_0 adult gonad", "F_1 larvae", "F_1 juvenile") on the urchin microbiome; **B)** climate scenario (fixed, two levels: "Ambient", "2100") on the F_1 adult gonad microbiome; **C)** climate scenario (fixed, three levels: "Ambient", "2050", "2100") on the F_1 1-day larvae microbiome; **D)** climate scenario (fixed, three levels: "Ambient", "2050", "2100") on the F_1 5-day larvae microbiome; and **E)** climate scenario (fixed, three levels: "Ambient", "2050", "2100") on the seawater samples collected at multiple time points ("F_0 adult gonad", "F_1 larvae", "F_1 juvenile", "F_1 adult gonad") along with the urchin samples. Tank was also fitted in the model (except for analyses reported in C) and D) as there was no tank replication), and statistical significance ($p < 0.05$) is indicated in bold.

A) F_0 adult gonad, F_1 larvae (1- and 5-day pooled), F_1 juvenile

Source	*df*	*SS*	*pseudo-F*	*P-value*
Life Stage	2	8.94	23.37	**9.999e-05**
Climate Scenario	2	0.76	1.98	**0.0081**
Tank	11	5.32	2.53	**9.999e-05**
Life Stage * Climate Scenario	2	0.71	1.86	**0.010399**
Residual	43	8.22		

Pairwise tests:

Life Stage * Climate Scenario:

For climate scenario:
F_0 adult gonad: Ambient ≠ 2050; Ambient ≠ 2100
F_1 larvae (1- and 5-day larvae pooled): ns
F_1 juvenile: Ambient ≠ 2050 ≠ 2100

For life stage:
Ambient: F_0 adult gonad ≠ F_1 larvae ≠ F_1juvenile
2050: F_0 adult gonad ≠ F_1 larvae ≠ F_1 juvenile
2100: F_0 adult gonad ≠ F_1 larvae ≠ F_1 juvenile

B) F_1 adult gonad

Source	*df*	*SS*	*pseudo-F*	*P-value*
Climate Scenario	1	0.28	1.84	**0.0281**
Tank	3	0.52	1.15	0.2240
Residual	6	0.90		

C) F_1 1-day larvae

Source	*df*	*SS*	*pseudo-F*	*P-value*
Climate Scenario	2	0.84	3.38	**0.004**
Residual	6	0.74		

D) F_1 5-day larvae

Source	*df*	*SS*	*pseudo-F*	*P-value*
Climate Scenario	2	0.52	6.69	**0.0033**
Residual	6	0.23		

E) Seawater

Source	*df*	*SS*	*pseudo-F*	*P-value*
Time Point	3	3.40	10.96	**9.999e-05**
Climate Scenario	2	0.41	1.96	**0.03770**
Tank	13	1.88	1.40	0.06869
Time Point * Climate Scenario	2	0.21	1.01	0.42106
Residual	6	0.62		

Table S3.7. Linear mixed effect models testing the fixed effects of Climate Scenario on urchin microbiome across life stages and generations (19 levels: "F_0 adult gonad ambient [A]", "F_0 adult gonad 2050", "F_0 adult gonad 2100", "F_1 larvae ambient [A]", "F_1 larvae 2050", "F_1 larvae 2100", "F_1 juvenile ambient [A]", "F_1 juvenile 2050", "F_1 juvenile 2100", "F_1 adult gonad ambient [A]", "F_1 adult gonad 2100", "F_2 larvae ambient [A]", "F_2 larvae 2100", "F_0 seawater ambient [A]", "F_0 seawater 2050", "F_0 seawater 2100", "F_1 seawater ambient [A]", "F_1 seawater 2050", "F_1 seawater 2100") on the Shannon diversity index on rarefied data. Tank was included in the models as random effect. Statistical significance (Benjamini–Hochberg adjusted $p < 0.05$) is indicated in bold. Only ecological relevant pair comparisons are reported here.

Contrast	*Estimate*	*SE*	*df*	*P-value*
gonad F0 A - gonad F0 2100	0.19	0.46	105	0.7629
gonad F0 A - gonad F0 2050	0.023	0.48	105	0.9704
gonad F0 2100 - gonad F0 2050	-0.17	0.45	105	0.7859
gonad F0 2100 - gonad F1 2100	-1.02	0.59	105	0.1612
gonad F0 2100 - juvenile F1 2100	-1.68	0.32	105	**<.0001**
gonad F0 2100 - larvae F1 2100	-0.82	0.51	105	0.1902
gonad F0 2100 - seawater F0 2100	-3.02	0.42	105	**<.0001**
gonad F0 2050- juvenile F1 2050	-0.92	0.34	105	**0.0172**
gonad F0 2050 - larvae F1 2050	-0.08	0.51	105	0.9193
gonad F0 2050 - seawater F0 2050	-2.69	0.43	105	**<.0001**
gonad F1 A - gonad F1 2100	-0.63	0.51	105	0.3213
gonad F1 2100 - juvenile F1 2100	-0.66	0.57	105	0.3563
gonad F1 2100 - larvae F2 2100	0.22	0.58	105	0.7859
gonad F1 2100 - seawater F1 2100	-1.44	0.46	105	**0.0062**
juvenile F1 A - juvenile F1 2100	0.05	0.40	105	0.9382
juvenile F1 A - juvenile F1 2050	0.64	0.43	105	0.2331
juvenile F1 2100 - juvenile F1 2050	0.59	0.40	105	0.2364
juvenile F1 2100 - larvae F1 2100	0.86	0.49	105	0.1527
juvenile F1 2100 - larvae F2 2100	0.88	0.52	105	0.1712
juvenile F1 2100 - seawater F1 2100	-0.78	0.39	105	0.0973
juvenile F1 2050 - larvae F1 2050	0.85	0.48	105	0.1520
juvenile F1 2050 - seawater F1 2050	-1.55	0.41	105	**0.0010**
Larvae F1 A - larvae F1 2100	0.02	0.49	105	0.9704

larvae F1 A - larvae F1 2050	0.59	0.49	105	0.3391
larvae F1 2100 - larvae F1 2050	0.57	0.49	105	0.3527
larvae F1 2100 - larvae F2 2100	0.02	0.52	105	0.9759
larvae F1 2100 - seawater F1 2100	-1.64	0.43	105	**0.0010**
larvae F1 2050 - seawater F1 2050	-2.39	0.49	105	**<.0001**
larvae F2 A - larvae F2 2100	-0.32	0.55	105	0.6779
seawater F0 A - seawater F0 2100	-0.11	0.56	105	0.8961
seawater F0 A - seawater F0 2050	0.06	0.57	105	0.9430
seawater F1 A - seawater F1 2100	-0.12	0.37	105	0.8176
seawater F1 A - seawater F1 2050	-0.30	0.43	105	0.6134

Table S3.8. Taxonomic classification of the ASV shared between life stages (F_0 adult gonad, 1-day F_1 larvae, 5-day F_1 larvae, F_1 juvenile) and present in at least 50% of the samples within each life stage under 2050 conditions. Unassigned taxa are indicated with "-".

Present in F_0 adult gonad, F_1 5-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
68b9ce06fc98f29759c01b03263a5d67	Gammaproteobacteria	Vibrionaceae	*Vibrio*

Present in F_1 1-day larvae, F_1 5-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
f420b5340d48941cc0e22ffef62e3df1	Alphaproteobacteria	Hyphomonadaceae	*Hyphomonas*

Present in F_0 adult gonad and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
516743b16d4dd61231b836db630eb141	Erysipelotrichia	Erysipelotrichaceae	*Turicibacter*
48a6a0e363dfb3823afdb092a0bb834d	Alphaproteobacteria	Rhodobacteraceae	*Ruegeria*
e2c3de7db50582cdafccb3b166b68b4a	Alphaproteobacteria	Rhizobiaceae	-
e9590b36781b69df7b2f7958c8003724	Deltaproteobacteria	Desulfovibrionaceae	*Halodesulfovibrio*
66e4cd4e9f92f88b55017bcc5a571164	Alphaproteobacteria	Kiloniellaceae	*Pelagibius*
0677d9f732493f832161376bda44672f	Bacteroidia	Cryomorphaceae	NS10 marine group
0429a8a999c3238e12bbfaa1714d385e	Gammaproteobacteria	Vibrionaceae	*Vibrio*
47a6c3d4b77e22e2363b6f567f6b6ff5	Gammaproteobacteria	Vibrionaceae	*Photobacterium*
4ee16f924a58f3d50a57e9b5c692f1c8	Gammaproteobacteria	Vibrionaceae	*Photobacterium*

Present in F_0 adult gonad and F_1 5-day larvae			
ASV identifier	**Class**	**Family**	**Genus**
1824b94db3ba4ad470b4ef5580939b63	Fusobacteriia	Fusobacteriaceae	*Propionigenium*

Present in F_1 1-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
65d43491988bfe557da4d86a5ba25dae	Bacilli	Staphylococcaceae	*Staphylococcus*

Present in F_1 5-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
c3c32c0479bf4d183832a2e3cf235481	Alphaproteobacteria	Rhodobacteraceae	*Shimia*
3b237ba949f7dc62b497ba0049fbc0b8	Gammaproteobacteria	Oleiphilaceae	*Oleiphilus*

Present in F_1 1-day larvae and F_1 5-day larvae			
ASV identifier	**Class**	**Family**	**Genus**
7f0ce4f17ba4f5a4ecccf1816c6f599f	Nitrososphaeria	Nitrosopumilaceae	*Candidatus Nitrosopumilus*
e68fb7b3415cf827b2ed9cf8f93378b2	Verrucomicrobiae	uncultured bacterium	-
93d599229f1b55f21eec84519bd4807f	Alphaproteobacteria	AEGEAN-169 marine group	-
c595de1845ebd5bb497bed4ad0a56431	Alphaproteobacteria	Rhodobacteraceae	-

09f7734468cb4192425e5d2808d54473	Alphaproteobacteria	Rhodobacteraceae	*Thalassobius*
0da5e652e22483b15cea40a707af0bc4	Alphaproteobacteria	Rhodobacteraceae	-
573091d7ddd85f9c5f14a7e53d2ba0bf	Alphaproteobacteria	Caulobacteraceae	*Caulobacter*
0ed014312927af3e1c7ea99794894747	Alphaproteobacteria	Caulobacteraceae	*Phenylobacterium*
4366b9d73f1cae9a6e25bbfbaf772c56	Rhodothermia	Rhodothermaceae	-
c771652b378108351c189ee8aa9e964d	Alphaproteobacteria	Hyphomonadaceae	*Maricaulis*
6bda65cf1d3307845f45b2c904f78e50	Alphaproteobacteria	Magnetospiraceae	-
4a4899fc2175607ea55fe5a974f0aa0f	Alphaproteobacteria	Nisaeaceae	OM75 clade
3d02e1bee03ef38ee83307443759fd37	Bacteroidia	Saprospiraceae	*Lewinella*
a589eca3c1f2bd55df83077542adc727	Gammaproteobacteria	Pseudohongiellaceae	*Pseudohongiella*
5cdf326d1392b9053408d8b7e8135342	Gammaproteobacteria	Alteromonadaceae	*Rheinheimera*
e3ce2b2e4389c869b545c5eeb68577be	Gammaproteobacteria	SS1-B-06-26	-
8ddcce2d1b11b48779fe048944173dc0	Gammaproteobacteria	Alcanivoracaceae	*Alcanivorax*
8787dc295950c53873b7ade8df61d312	Gammaproteobacteria	Alcanivoracaceae	*Alcanivorax*
cd3c68e64285e960b6c46d8bf4cd75e6	Gammaproteobacteria	-	*Marinicella*
3b5e5025d3eb814d0abf098474ba41c7	Gammaproteobacteria	Alteromonadaceae	*Alteromonas*
7188ce140c148b15b672115f6b03944b	Gammaproteobacteria	Alteromonadaceae	*Aestuariibacter*
58e29ce3fbf1effd0ab41fdb554fc0a1	Gammaproteobacteria	Saccharospirillaceae	-
9155702ba18adf5965ba430ef315d30f	Gammaproteobacteria	Saccharospirillaceae	*Thalassolituus*
78db7fa6b16b73b101fbb8b282b5330e	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*
6f3f68e5c8e2a11b388ddbbea9fa182d	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*
e59c5cdfbaf34e9c42adb42ab05ee84a	Gammaproteobacteria	Saccharospirillaceae	*Oleibacter*
beed64a8017b2e1fdfe99d2fe8bca629	Gammaproteobacteria	Methylophilaceae	OM43 clade
3cf3c2c41dc63c196b8add9d3dd99b28	Gammaproteobacteria	Woeseiaceae	*Woeseia*

Table S3.9. Taxonomic classification of the ASV shared between life stages (F_0 adult gonad, 1-day F_1 larvae, 5-day F_1 larvae, F_1 juvenile) and present in at least 50% of the samples within each life stage under 2100 conditions. Unassigned taxa are indicated with "-".

Present in F_0 adult gonad, F_1 1-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
68b9ce06fc98f29759c01b03263a5d67	Gammaproteobacteria	Vibrionaceae	*Vibrio*

Present in F_0 adult gonad, F_1 5-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
47a6c3d4b77e22e2363b6f567f6b6ff5	Gammaproteobacteria	Vibrionaceae	*Photobacterium*

Present in F_1 1-day larvae, F_1 5-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
3b237ba949f7dc62b497ba0049fbc0b8	Gammaproteobacteria	Oleiphilaceae	*Oleiphilus*

Present in F_0 adult gonad and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
516743b16d4dd61231b836db630eb141	Erysipelotrichia	Erysipelotrichaceae	*Turicibacter*
48a6a0e363dfb3823afdb092a0bb834d	Alphaproteobacteria	Rhodobacteraceae	*Ruegeria*
66e4cd4e9f92f88b55017bcc5a571164	Alphaproteobacteria	Kiloniellaceae	*Pelagibius*
0677d9f732493f832161376bda44672f	Bacteroidia	Cryomorphaceae	NS10 marine group
e4463785d08597b064903c399e26dd13	Gammaproteobacteria	Vibrionaceae	*Vibrio*
0429a8a999c3238e12bbfaa1714d385e	Gammaproteobacteria	Vibrionaceae	*Vibrio*
6e0f21beba4fceae378d9efbabf9083c	Gammaproteobacteria	Vibrionaceae	*Vibrio*
4ee16f924a58f3d50a57e9b5c692f1c8	Gammaproteobacteria	Vibrionaceae	*Photobacterium*

Present in F_1 1-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
a3595435c3205f8e0630d087bd0f0f1f	Alphaproteobacteria	Rhodobacteraceae	-
689f1824bb6bdee2c08f619da1b57eb4	Gammaproteobacteria	Burkholderiaceae	-

Present in F_1 5-day larvae and F_1 juvenile			
ASV identifier	**Class**	**Family**	**Genus**
c3c32c0479bf4d183832a2e3cf235481	Alphaproteobacteria	Rhodobacteraceae	*Shimia*
f420b5340d48941cc0e22ffef62e3df1	Alphaproteobacteria	Hyphomonadaceae	*Hyphomonas*
7188ce140c148b15b672115f6b03944b	Gammaproteobacteria	Alteromonadaceae	*Aestuariibacter*

Present in F_1 1-day larvae and F_1 5-day larvae			
ASV identifier	**Class**	**Family**	**Genus**
7f0ce4f17ba4f5a4ecccf1816c6f599f	Nitrososphaeria	Nitrosopumilaceae	*Candidatus Nitrosopumilus*
c41aa9c9ce9467cf47962a420beaaa94	Bacteroidia	Cryomorphaceae	-
bed4df5dd89852e1e36acc676cc57bd6	Bacteroidia	Flavobacteriaceae	-
651083dd15565eead4fdf9cd1344a6e5	Bacteroidia	Flavobacteriaceae	-
504a6b184c4a2127a0eb7511c76c9cca	Pla4 lineage	-	-
e68fb7b3415cf827b2ed9cf8f93378b2	Verrucomicrobiae	-	-
147e525a5a7535a5e15bfd8ee3c8e497	Alphaproteobacteria	-	-
84b738cddad75358f87ba65365842a87	Alphaproteobacteria	AEGEAN-169 marine group	-
93d599229f1b55f21eec84519bd4807f	Alphaproteobacteria	AEGEAN-169 marine group	-
b73f98faae7971641f34649a9e11857a	Alphaproteobacteria	Rhodobacteraceae	*Ascidiaceihabitans*
5610ad9b17dac6248943d00b55bf0df7	Alphaproteobacteria	Rhodobacteraceae	-
5eeba95b30322d684e95baa2e5149ebb	Alphaproteobacteria	Rhodobacteraceae	-
901bdd384be1d151e57df47fc0f66110	Alphaproteobacteria	Rhodobacteraceae	*Celeribacter*
09f7734468cb4192425e5d2808d54473	Alphaproteobacteria	Rhodobacteraceae	*Thalassobius*
0da5e652e22483b15cea40a707af0bc4	Alphaproteobacteria	Rhodobacteraceae	-
e4304c9496921fcf8a88c1ef30dfd0eb	Alphaproteobacteria	Sphingomonadaceae	-
e4c42127aac0dbe32c10ff6f7e4832d5	Alphaproteobacteria	Sneathiellaceae	*AT-s3-44*
4366b9d73f1cae9a6e25bbfbaf772c56	Rhodothermia	Rhodothermaceae	-
c771652b378108351c189ee8aa9e964d	Alphaproteobacteria	Hyphomonadaceae	*Maricaulis*
e37077e9ac44d90bdd436d86bb28df80	Alphaproteobacteria	-	-
cffd0fbfc405bd03bcb298c70494c1e8	Alphaproteobacteria	Magnetospiraceae	-
c70565b6fa4c8ab447fd3d843dd98072	Alphaproteobacteria	Magnetospiraceae	-
4a4899fc2175607ea55fe5a974f0aa0f	Alphaproteobacteria	Nisaeaceae	OM75 clade
a589eca3c1f2bd55df83077542adc727	Gammaproteobacteria	Pseudohongiellaceae	*Pseudohongiella*
bf493dd27b858bfbba917beb6fa28af4	Gammaproteobacteria	Legionellaceae	-
e3ce2b2e4389c869b545c5eeb68577be	Gammaproteobacteria	SS1-B-06-26	-
8ddcce2d1b11b48779fe048944173dc0	Gammaproteobacteria	Alcanivoracaceae	*Alcanivorax*
8787dc295950c53873b7ade8df61d312	Gammaproteobacteria	Alcanivoracaceae	*Alcanivorax*
86ae6ec32d3ba2b072a7c1803cfa40d9	Gammaproteobacteria	Nitrincolaceae	*Neptuniibacter*
cd3c68e64285e960b6c46d8bf4cd75e6	Gammaproteobacteria	-	*Marinicella*
3b5e5025d3eb814d0abf098474ba41c7	Gammaproteobacteria	Alteromonadaceae	*Alteromonas*
6f3f68e5c8e2a11b388ddbbea9fa182d	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*
12c4cf4a9b6a4541ef111f02191bae96	Gammaproteobacteria	Halomonadaceae	*Halomonas*
dae22455cb0e8aa1fab3d09eba54fc5f	Gammaproteobacteria	-	-
50e2c2f2162356c0e3c76edf634ad8ca	Gammaproteobacteria	Nitrosomonadaceae	-
beed64a8017b2e1fdfe99d2fe8bca629	Gammaproteobacteria	Methylophilaceae	OM43 clade
3cf3c2c41dc63c196b8add9d3dd99b28	Gammaproteobacteria	Woeseiaceae	*Woeseia*

Table S3.10. Permutational multivariate analysis of variance (adonis function in R vegan package) based on Bray-Curtis dissimilarities applied on sqrt-root-transformed relative abundances to examine the effect of: parental climate treatment (fixed, three levels: "Ambient", "2050", "2100") and offspring climate treatment (fixed, three levels: "Ambient", "2050", "2100") on the urchin microbiome of 5-month juveniles. Tank was also fitted in the model and statistical significance ($p < 0.05$) is indicated in bold.

Source	*df*	*SS*	*pseudo-F*	*P-value*
Parental Climate Treatment	2	1.02	1.78	**9.999e-05**
Offspring Climate Treatment	2	0.91	1.58	**9.999e-05**
Tank	6	2.52	1.47	**9.999e-05**
Parental Climate Treatment * Offspring Climate Treatment	1	0.51	1.78	**9.999e-05**
Residual	41	11.78		

Pairwise tests:

Parental Climate Treatment * Offspring Climate Treatment:

For Parental Climate Treatment:
Ambient: offspring Ambient ≠ offspring 2100; offspring 2050 ≠ offspring 2100
2050: offspring 2050 ≠ offspring 2100

For Offspring Climate Treatment:
2050: parent Ambient ≠ parent 2050
2100: parent Ambient ≠ parent 2050 ≠ parent 2100

Table S3.11. Linear mixed effect models testing the fixed effect of the reciprocal climate treatments (six levels: "A-A", "A-2050", "A-2100", "2050-2050", "2050-2100", "2100-2100"; treatments are reported as 'parental (F_0) climate treatment – juvenile (F_1) climate treatment') on the Shannon diversity index on rarefied data; "A" = ambient. Tank was included in the models as random effect. Contrasts are reported below, with statistical significance (Benjamini–Hochberg adjusted $p < 0.05$) indicated in bold.

Contrast	*Estimate*	*SE*	*df*	*P-value*
A -A – A-2100	-0.39	0.37	51	0.5585
A -A – A-2050	-0.13	0.37	51	0.8014
A -A – 2100-2100	0.14	0.37	51	0.8014
A -A – 2050-2100	0.12	0.37	51	0.8014
A -A – 2050-2050	0.77	0.38	51	0.2535
A-2100 – A-2050	0.27	0.37	51	0.7003
A-2100 – 2100-2100	0.53	0.37	51	0.3754
A-2100 – 2050-2100	0.51	0.37	51	0.3754
A-2100 – 2050-2050	1.16	0.38	51	0.0589
A-2050 – 2100-2100	0.26	0.37	51	0.7003
A-2050 – 2050-2100	0.25	0.37	51	0.7003
A-2050 – 2050-2050	0.89	0.38	51	0.1802
2100-2100 – 2050-2100	-0.02	0.37	51	0.9692
2100-2100 – 2050-2050	0.63	0.38	51	0.3151
2050-2100 – 2050-2050	0.65	0.38	51	0.3151

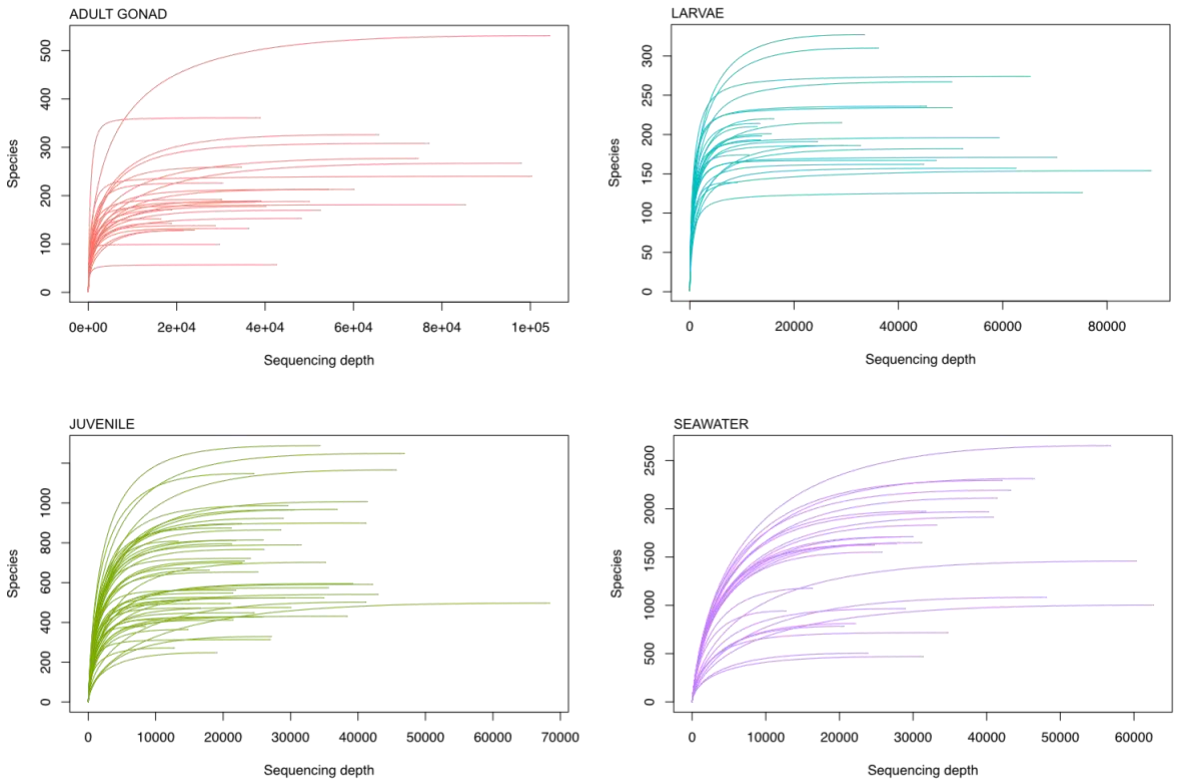

Fig. S3.1. ASV rarefaction curves of 16S rRNA gene sequences showing diversity in urchin (adult gonad, larvae, juvenile) and seawater samples.

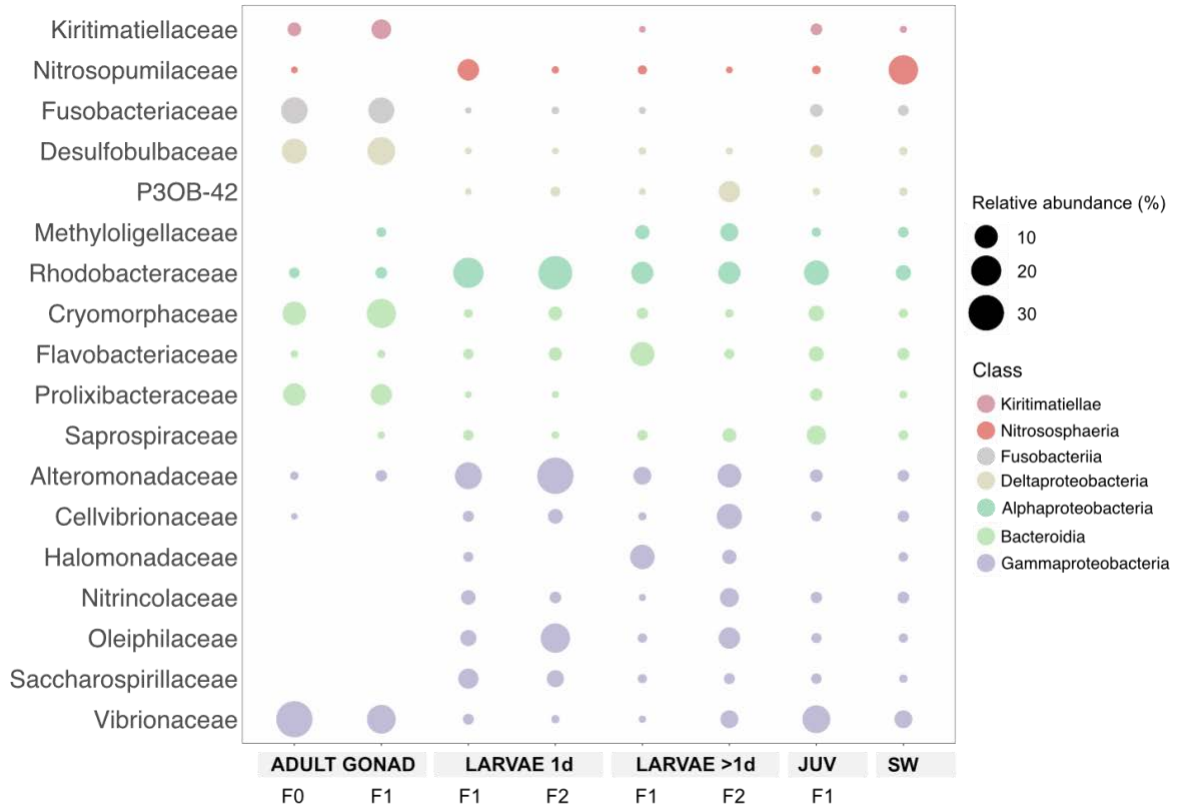

Fig. S3.2. Dominant families (>5% relative abundance) across life stages (adult gonad, larvae 1d, larvae >1d, juvenile [JUV]) and generations (F_0, F_1, F_2) under ambient conditions. Seawater-associated microbial community (SW) was pooled across time and generations.

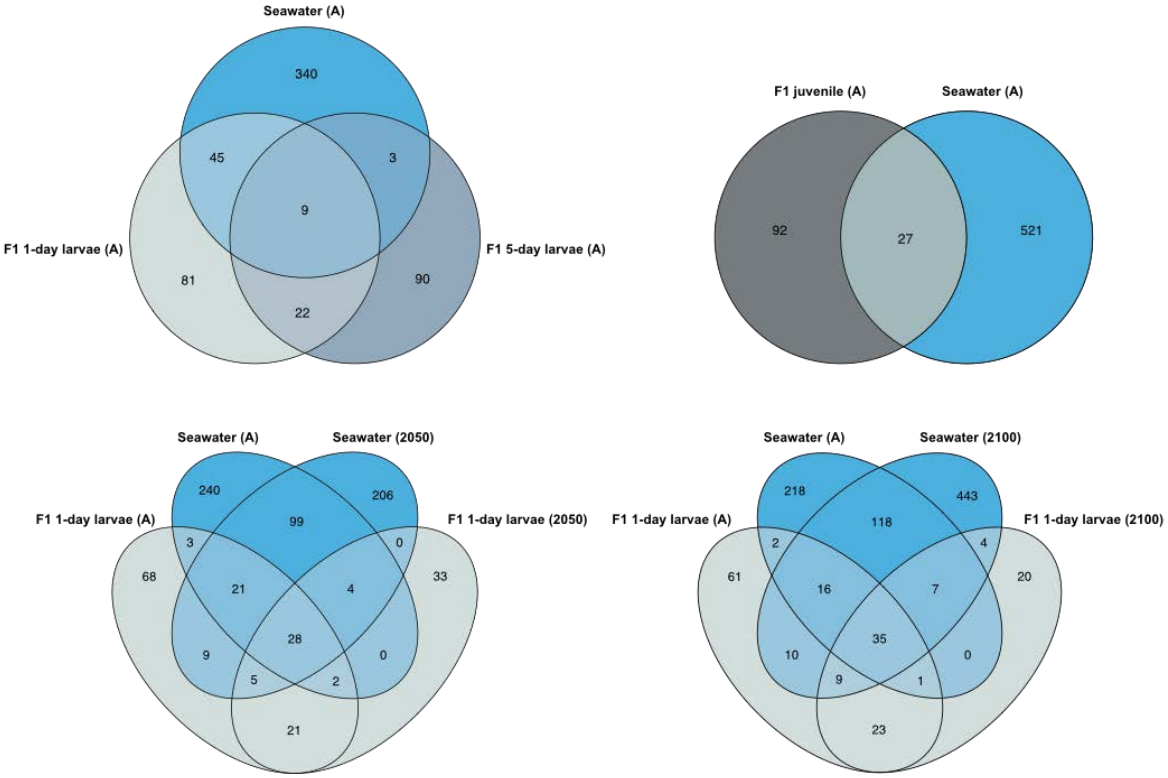

Fig. S3.3. Venn diagrams illustrating the number of shared ASVs between F1 larvae, F1 juveniles and surrounding seawater under treatment conditions (ambient [A], 2050 and 2100). Only ASVs present in at least 50% of the samples at each life stage / generation were analysed; data were rarefied.

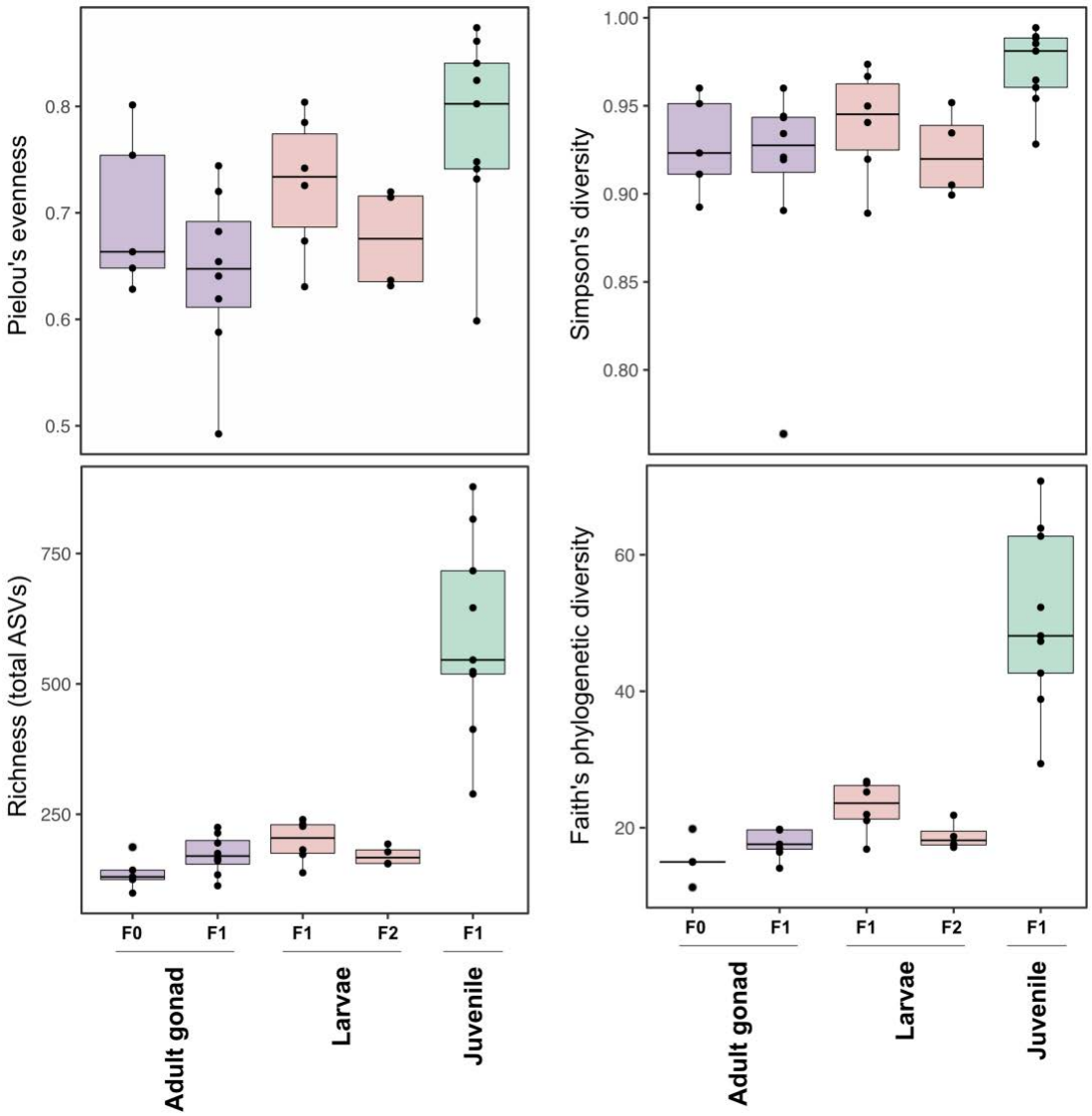

Fig. S3.4. Alpha diversity measures (Pielou's evenness, Simpson's diversity, richness measured as total number of ASVs, Faith's phylogenetic diversity) of the microbiome in adult gonads, larvae (1- and older larvae were pooled) and juveniles across generations (F_0, F_1, F_2) under ambient conditions.

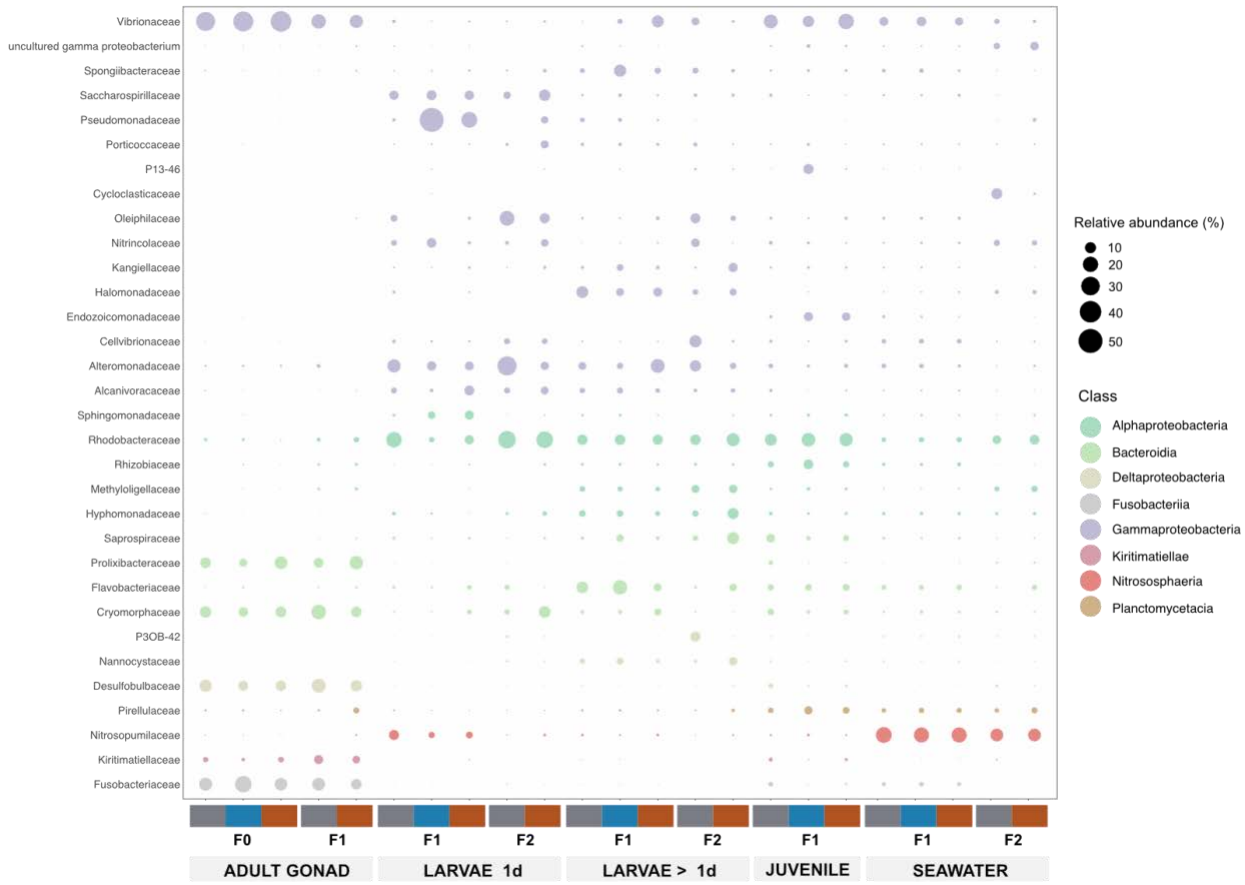

Fig. S3.5. Bubble plot illustrating mean relative abundance of dominant microbial families (> 5%) across sample types, host generations and climate treatments (ambient = grey; 2050 = blue; 2100 = red).

Fig. S3.6. ASVs with significantly different relative abundances between climate treatments within F_0 adult gonads and F_1 5-day larvae identified using DESeq analyses. Only ASVs present across at least 50% of the samples in one of the compared groups are illustrated. Taxonomy is shown as Family and Genus, and a significant increase (▲) or decrease (▼) in relative abundance between Ambient and 2050 treatment (blue), Ambient and 2100 (red), and Ambient and both climate treatments (black) is illustrated for each ASV.

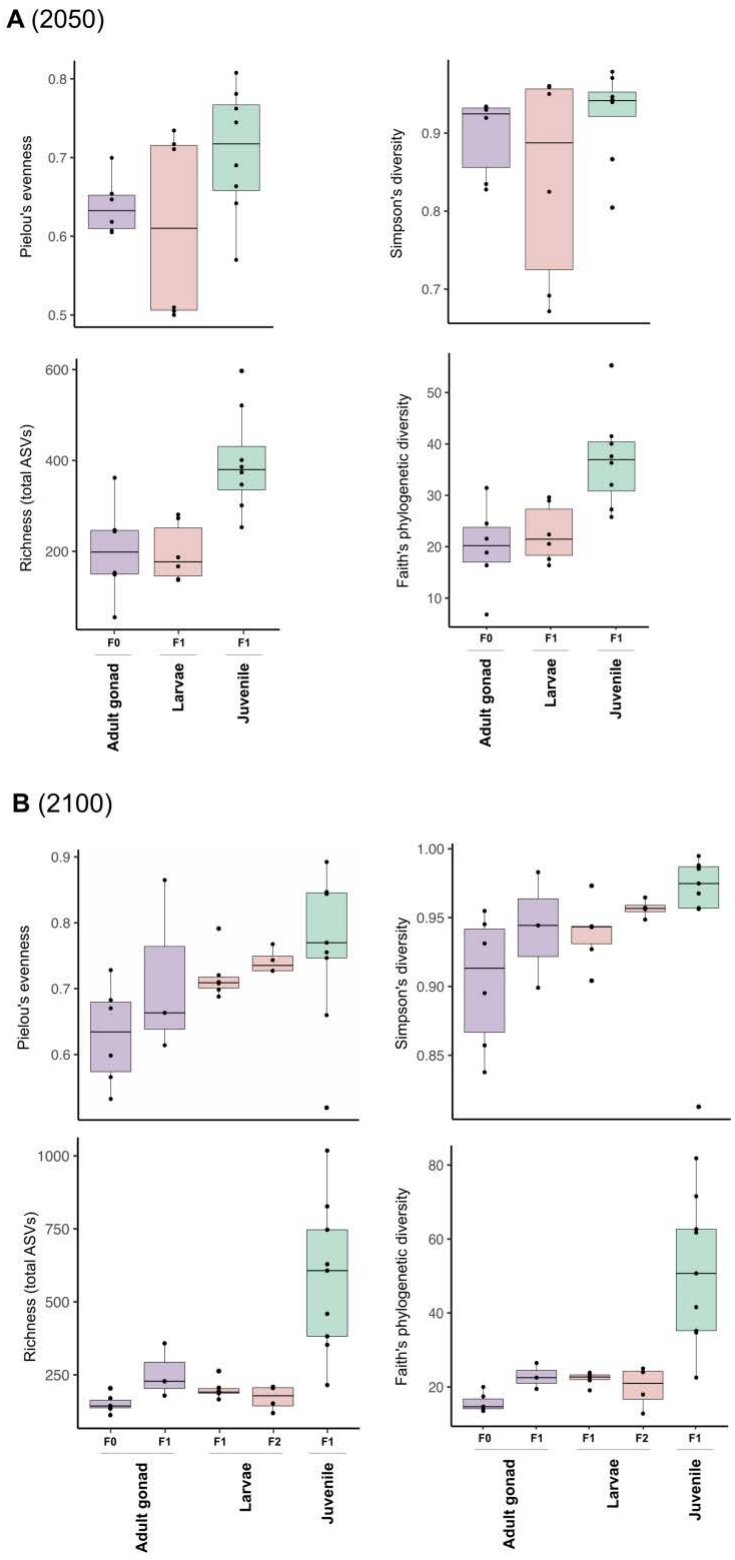

Fig. S3.7. Alpha diversity measures (Pielou's evenness, Simpson's diversity, richness measured as total number of ASVs, Faith's phylogenetic diversity) of the microbiome in adult gonads, larvae (1- and older larvae were pooled) and juveniles across generations (F_0, F_1, F_2) under (A) 2050 and (B) 2100 conditions.

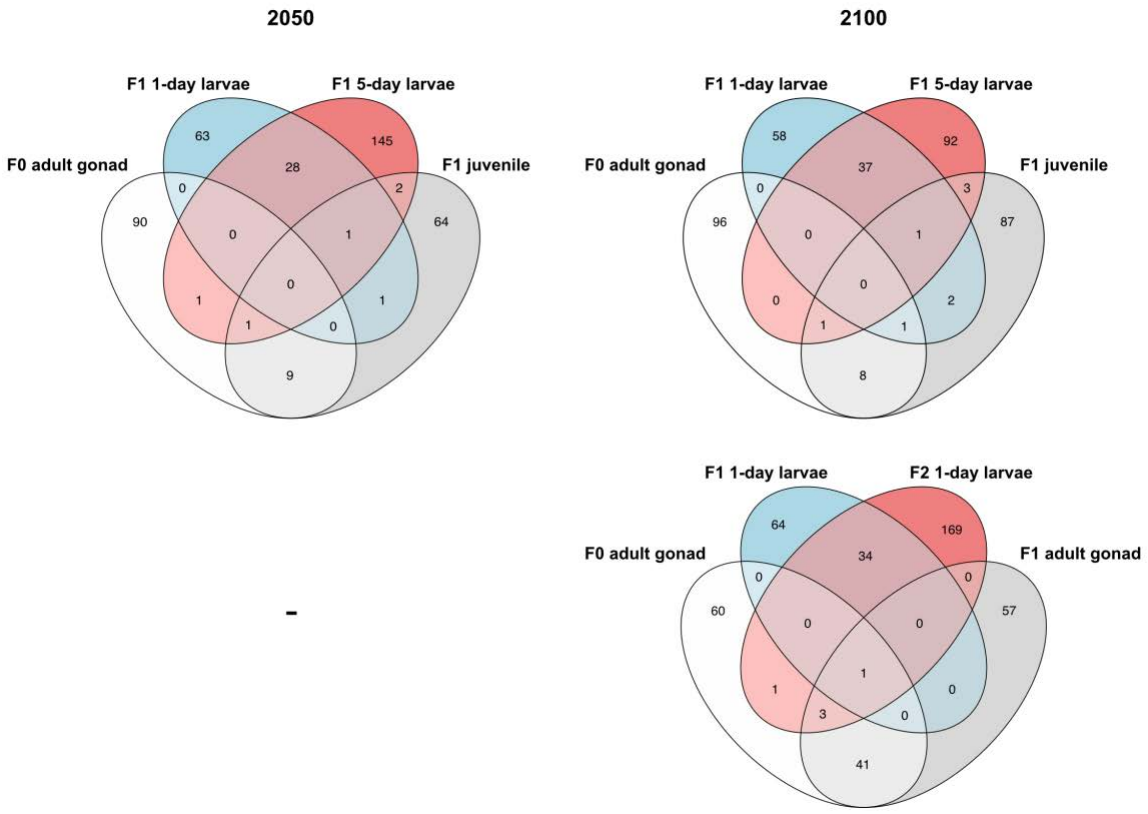

Fig. S3.8. Venn diagrams illustrating the number of shared ASVs between life stages (top) and generations (bottom) under 2050 (left) and 2100 (right) conditions. Only ASVs present in at least 50% of the samples at each life stage / generation were analysed; data were rarefied. Number of ASVs shared between generations under 2050 conditions are not shown as F_1 adult gonads and F_2 larvae were not reared under this treatment (see Fig. 3.1).

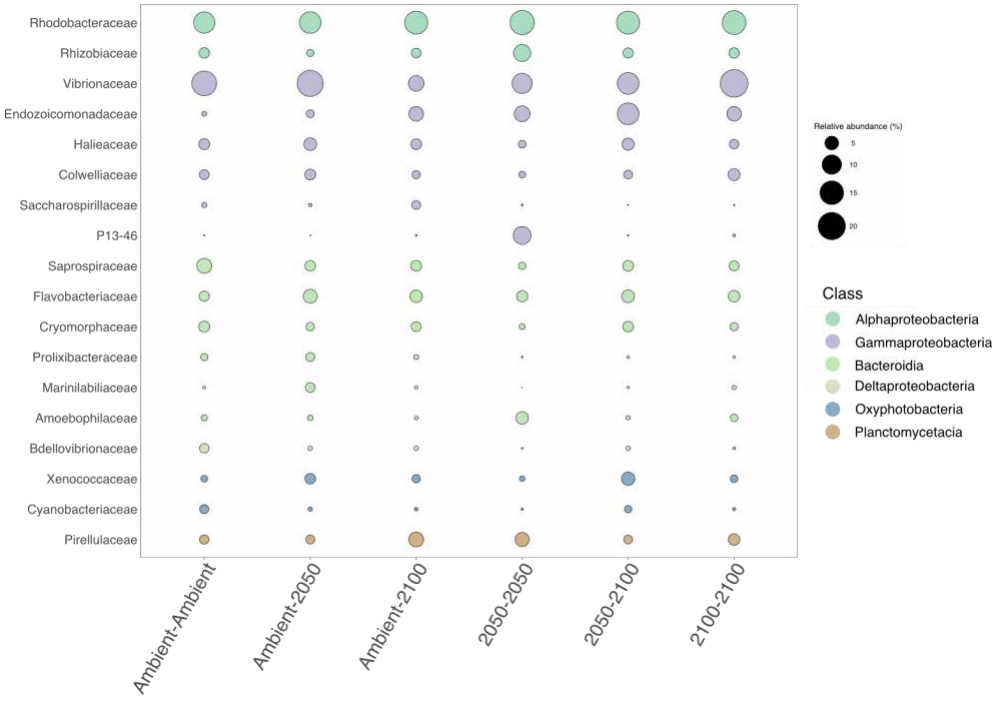

Fig. S3.9. Bubble plot illustrating mean relative abundance of dominant (>2%) microbial families in the urchin juveniles. Treatments are reported as 'parental (F_0) climate treatment – offspring (F_1 juvenile) climate treatment' (e.g. Ambient-2050: parents exposed to ambient and offspring exposed to 2050 conditions).

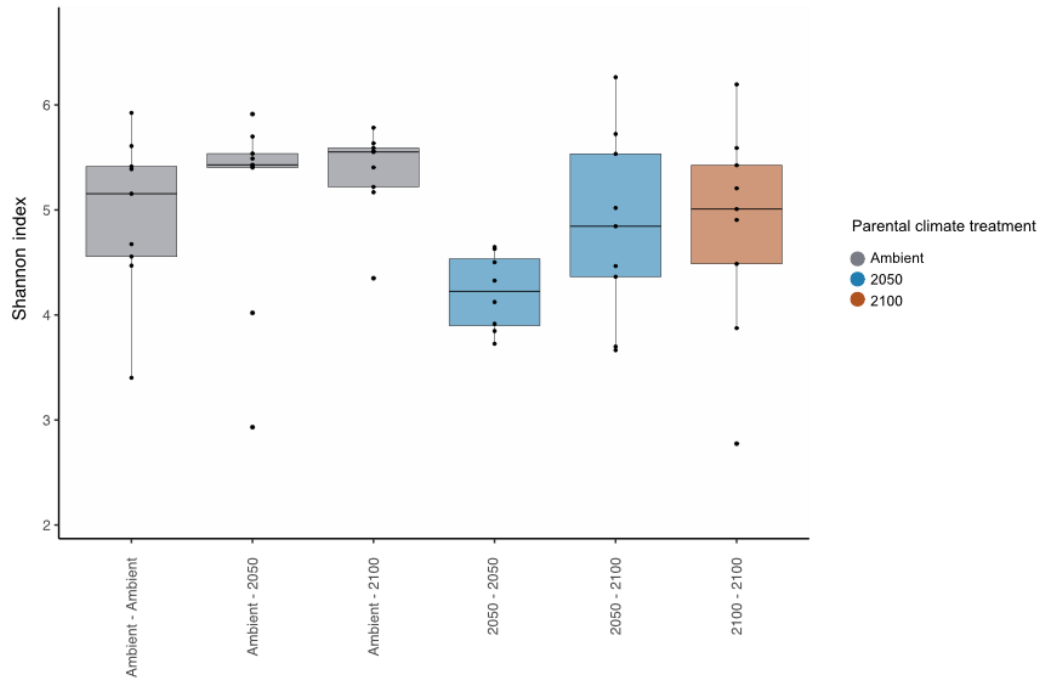

Fig. S3.10. Boxplot of Shannon diversity index of 5-month juveniles (F_1) across climate treatments in the transplant experiment. Treatments are reported as 'parental (F_0) climate treatment – juvenile (F_1) climate treatment'. Box = inter-quartile range (IQR), line in box = median, whiskers = minimum and maximum values not outliers (i.e. -/+1.5*IQR).

Appendix B – Supplementary Material for Chapter 4

Table S4.2. Generalized linear mixed models tested the fixed effect of Treatment (Ambient, Heat), Time (T0, T1, T2, T3, T4, T5A, T5B), Colony of origin (A, B, C) and the interaction between Treatment and Time on: health score, photosynthesis (P; sqrt-transformed), respiration (R; absolute), Gross P:R and photochemical effective efficiency (sqrt-transformed) in the coral *Porites lutea.* Tank and Fragment were included in the model to account for repeated measures. The pairwise comparisons (Šidàk correction) for significant interactions are reported here. Estimates are not back-transformed. Significant factors ($p<0.05$) are indicated in bold.

Var.	*Time Point*	*Contrast*	*Estimate*	*SE*	*df*	*P-value*
Health score	T0	Ambient-Heat	-1.822	0.482	442	**0.001**
	T1	Ambient-Heat	0.765	0.495	442	0.544
	T2	Ambient-Heat	0.969	0.510	442	0.303
	T3	Ambient-Heat	0.188	0.537	442	0.100
	T4	Ambient-Heat	2.556	0.578	442	**<0.001**
	T5	Ambient-Heat	4.142	0.670	442	**<0.001**
P (sqrt)	T0	Ambient-Heat	-0.002	0.006	233	1.000
	T1	Ambient-Heat	-0.006	0.006	233	0.934
	T2	Ambient-Heat	0.011	0.006	233	0.466
	T3	Ambient-Heat	0.017	0.006	233	0.0521
	T4	Ambient-Heat	0.021	0.006	233	**0.012**
	T4.5	Ambient-Heat	0.046	0.007	233	**<0.001**
	T5	Ambient-Heat	0.090	0.007	233	**<0.001**
R (absolute)	T0	Ambient-Heat	18.41	5.51	233	**0.007**
	T1	Ambient-Heat	14.56	5.72	233	0.079
	T2	Ambient-Heat	3.46	5.07	233	0.992
	T3	Ambient-Heat	6.97	4.57	233	0.618
	T4	Ambient-Heat	9.43	4.57	233	0.251
	T4.5	Ambient-Heat	4.19	5.05	233	0.974
	T5	Ambient-Heat	10.19	5.10	233	0.285
P/R ratio	T0	Ambient-Heat	-0.118	0.045	233	0.062
	T1	Ambient-Heat	-0.014	0.052	233	1.000
	T2	Ambient-Heat	-0.117	0.061	233	0.330
	T3	Ambient-Heat	-0.216	0.071	233	**0.017**
	T4	Ambient-Heat	-0.310	0.075	233	**<0.001**
	T4.5	Ambient-Heat	-0.500	0.097	233	**<0.001**

	T5	Ambient-Heat	-1.358	0.138	233	**<0.001**
Phot. Efficiency (sqrt)	T0	Ambient-Heat	0.039	0.038	356	0.889
	T1	Ambient-Heat	0.055	0.040	356	0.681
	T2	Ambient-Heat	0.090	0.040	356	0.153
	T3	Ambient-Heat	0.130	0.045	356	**0.025**
	T4	Ambient-Heat	0.202	0.049	356	**<0.001**
	T5	Ambient-Heat	0.528	0.057	356	**<0.001**

Table S4.3 (separate Dataset file https://tinyurl.com/389pr32p). Summary of sequencing statistics of the transcriptomic dataset, and percentage of reads mapping to the reference genomes.

Table S4.4 (separate Dataset file https://tinyurl.com/389pr32p). Outcome of the Gene Ontology (GO) enrichment analyses (Biological Processes) for the coral host between low stress (< 1 DHW) and ambient conditions; only significant genes (adjusted $p < 0.05$) are shown.

Table S4.5 (separate Dataset file https://tinyurl.com/389pr32p). Outcome of the Gene Ontology (GO) enrichment analyses (Biological Processes) for the coral host between moderate stress (3 DHW) and ambient conditions; only significant genes (adjusted $p < 0.05$) are shown.

Table S4.6 (separate Dataset file https://tinyurl.com/389pr32p). Overview of the coral host Differentially Expressed Genes (DEGs) detected between moderate stress (3 DHW) and ambient conditions.

Table S4.7 (separate Dataset file https://tinyurl.com/389pr32p). Outcome of the Gene Ontology (GO) enrichment analyses (Biological Processes) for the coral-associated Symbiodiniaceae between low stress (< 1 DHW) and ambient conditions; only significant genes (adjusted $p < 0.05$) are shown.

Table S4.8 (separate Dataset file https://tinyurl.com/389pr32p). Outcome of the Gene Ontology (GO) enrichment analyses (Biological Processes) for the coral-associated Symbiodiniaceae between moderate stress (3 DHW) and ambient conditions; only significant genes (adjusted $p < 0.05$) are shown.

Table S4.9 (separate Dataset file https://tinyurl.com/389pr32p). Overview of Symbiodiniaceae Differentially Expressed Genes (DEGs) detected between low stress (< 1 DHW) and ambient conditions.

Table S4.10 (separate Dataset file https://tinyurl.com/389pr32p). Overview of Symbiodiniaceae Differentially Expressed Genes (DEGs) detected between moderate stress (3 DHW) and ambient conditions.

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Table S4.11. Permutation multivariate analysis of variance (adonis function in R vegan package) based on Bray-Curtis dissimilarities applied on square-root-transformed relative abundances to examine the effects of Sample type (fixed, three levels: "coral", "seawater", "feed") on the microbiome. P-values were calculated using 10,000 permutations and statistical significance ($p < 0.05$) is shown in bold.

Source	*df*	*SS*	*F*	*P-value*
Sample type	2	7.99	13.77	**<0.001**
Residual	138	40.04		
Pairwise tests: Sample type: Coral ≠ Seawater Coral ≠ Feed Seawater ≠ Feed				

Table S4.12. Permutation multivariate analysis of variance (adonis function in R vegan package) based on Bray-Curtis dissimilarities applied on square-root-transformed relative abundances to test the effect of Time (fixed, six levels: "T0", "T1", "T2", "T3", "T4", "T5"), Treatment (fixed, two levels: "ambient", "heat") and Colony of origin (fixed, three levels: "A", "B", "C") on the coral microbiome. Tank was also fitted in the model. P-values were calculated using 10,000 permutations and statistical significance ($p < 0.05$) is shown in bold.

Source	*df*	*SS*	*F*	*P-value*
Time	5	3.30	2.69	**<0.001**
Treatment	1	1.30	5.28	**<0.001**
Colony	2	3.87	7.87	**<0.001**
Tank	4	1.63	1.66	**<0.001**
Time x Treatment	5	1.98	1.61	**<0.001**
Residual	95	23.33		
Pairwise tests: Colony of origin: A ≠ B ≠ C Time x Treatment: *For time:* Ambient: T0 ≠ T5 Heat: T0 ≠ T3, T4, T5; T1 ≠ T4, T5; T2 ≠ T4, T5; T3 ≠ T5, T4 ≠ T5				

For treatment:
T0: ns
T1: ns
T2: ns
T3: Ambient ≠ Heat
T4: Ambient ≠ Heat
T5: Ambient ≠ Heat

Table S4.13. A linear mixed model (gaussian) testing the effect of Treatment (fixed, two levels: "Ambient", "Heat") Time (fixed, six levels: "T0", "T1", "T2", "T3", "T4", "T5") and Colony of origin (fixed, 3 levels) on the Shannon diversity index of the coral microbiome (rarefied data). Tank was included in the model as random effect. Statistical significance ($p < 0.05$) is shown in bold.

Source	*Estimate*	*SE*	*P-value*
T1	0.30	0.38	0.433
T2	0.63	0.38	0.095
T3	0.41	0.38	0.281
T4	0.72	0.38	0.059
T5	1.12	0.36	**0.002**
Heat	0.57	0.40	0.155
Colony B	-0.35	0.19	0.059
Colony C	-0.69	0.18	**<0.001**
T1:heat	-0.27	0.54	0.611
T2:heat	-0.53	0.54	0.321
T3:heat	0.47	0.54	0.381
T4:heat	0.43	0.55	0.428
T5:heat	-0.02	0.50	0.970

Table S4.14 (separate Dataset file https://tinyurl.com/389pr32p). Overview of the 88 highly correlated variables across datasets identified through DIABLO analyses.

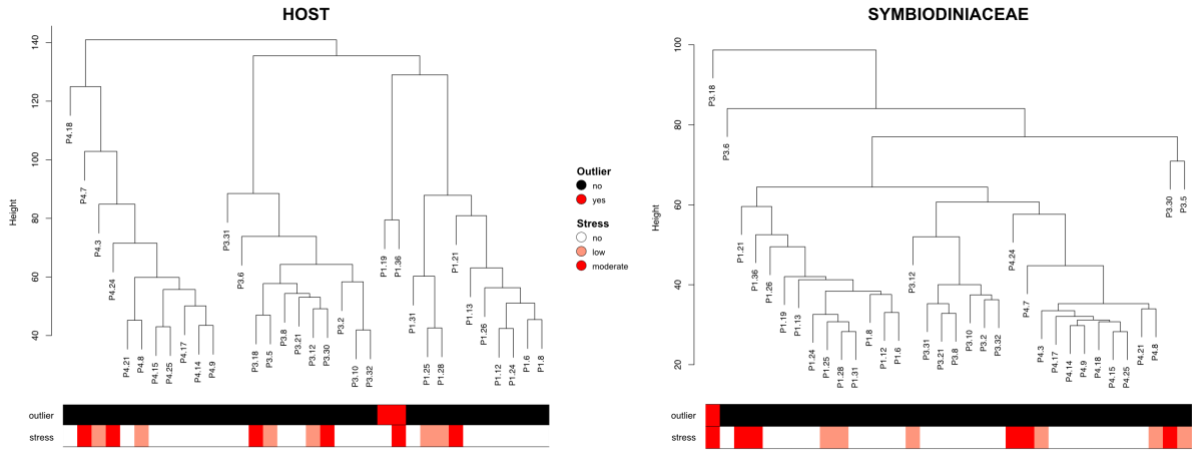

Fig. S4.1. Sample clustering for outlier detection in host and Symbiodiniaceae. Only samples passing QC are included.

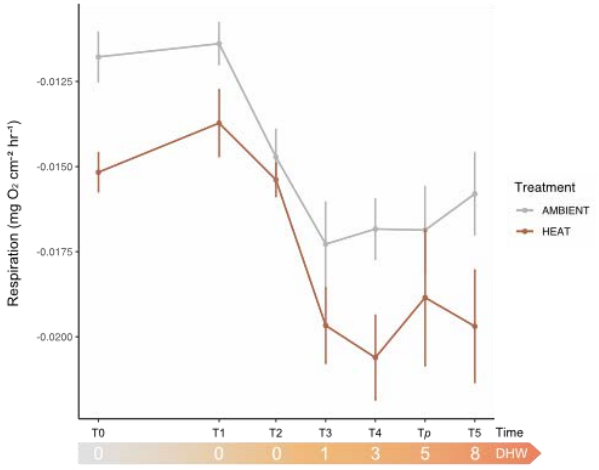

Fig. S4.2. Coral respiration rates throughout the heat stress experiment. Mean ± SE is shown over time, and DHW are reported for the heat treatment only (DHW = 0 under ambient conditions).

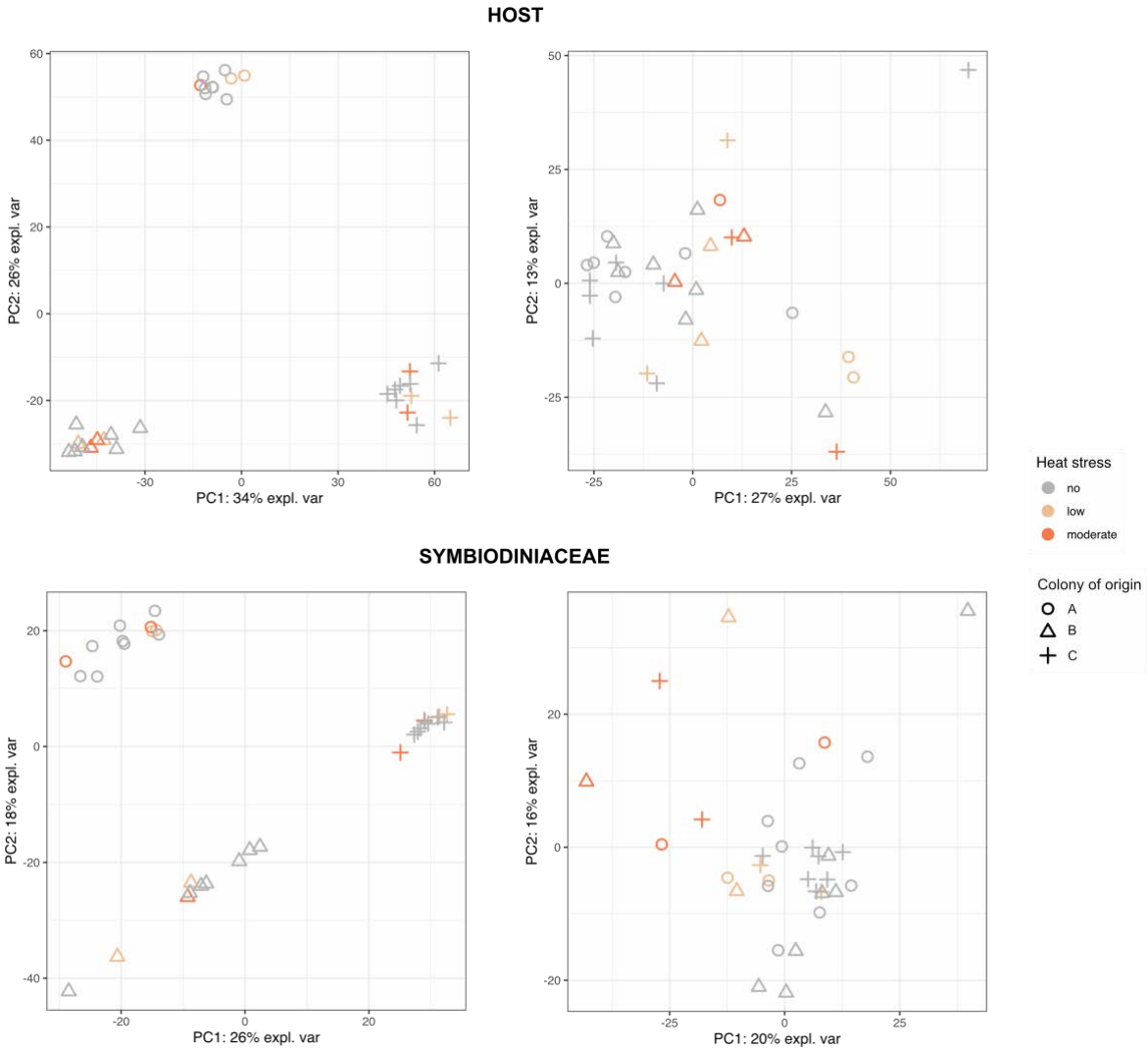

Fig. S4.3. Principal Component Analysis (PCA) of gene expression profiles in host and Symbiodiniaceae on transformed (variance stabilizing transformation) and centred data (left) and multilevel PCA on the same dataset (right) to account for the effect of colony of origin.

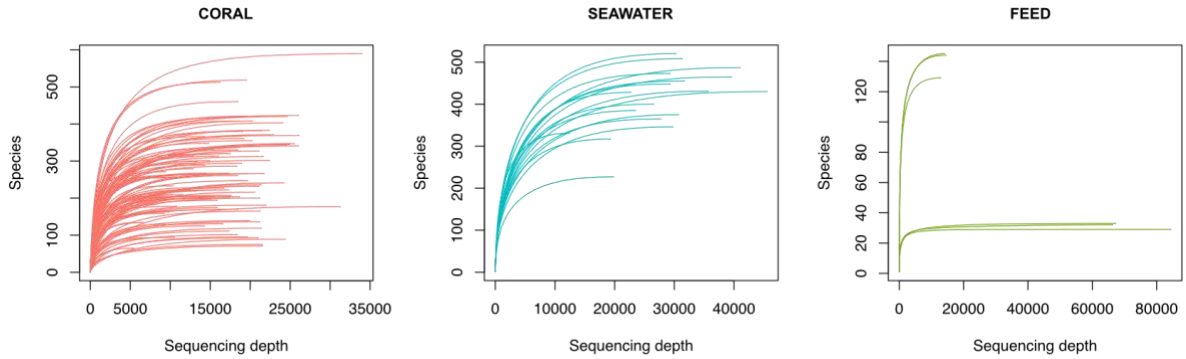

Fig. S4.4. ASV rarefaction curves of 16S rRNA gene sequences for coral (Baseline, T0-T5), seawater and feed (rotifers and microalgae) samples.

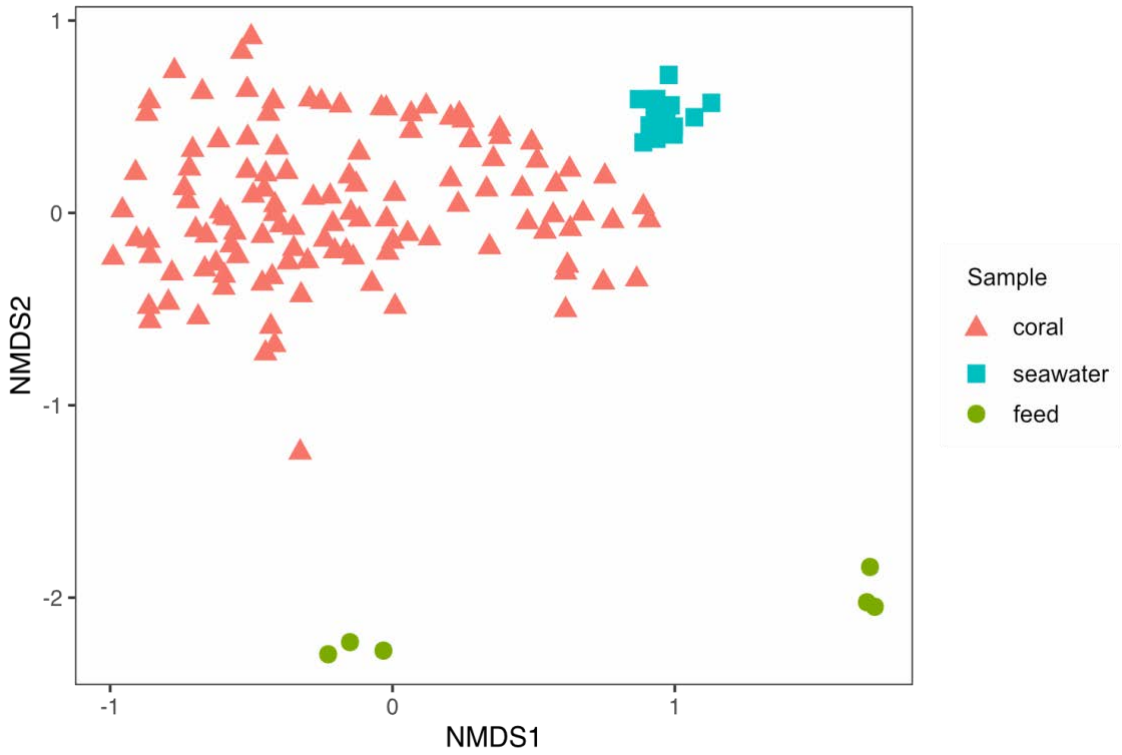

Fig. S4.5. Non-metric Multi-dimensional Scaling (NMDS, sqrt-rooted data; stress = 0.17) based on Bray-Curtis dissimilarities calculated on relative abundance of ASVs in coral (*Porites lutea*), seawater and feed (rotifers and microalgae) samples.

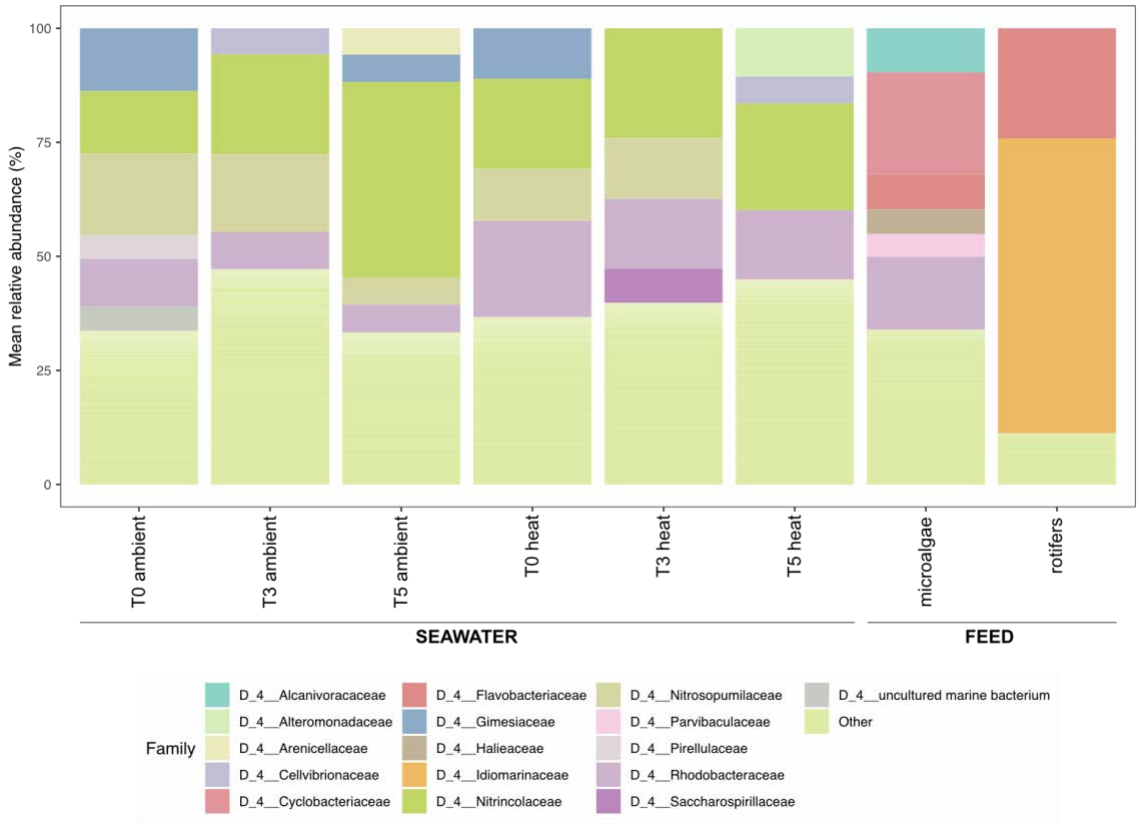

Fig. S4.6. Mean relative abundance of dominant microbial families in seawater and feed (rotifers and microalgae) samples. For seawater, microbial community structure is shown under ambient and heat treatment over time (T0, T3, T5).

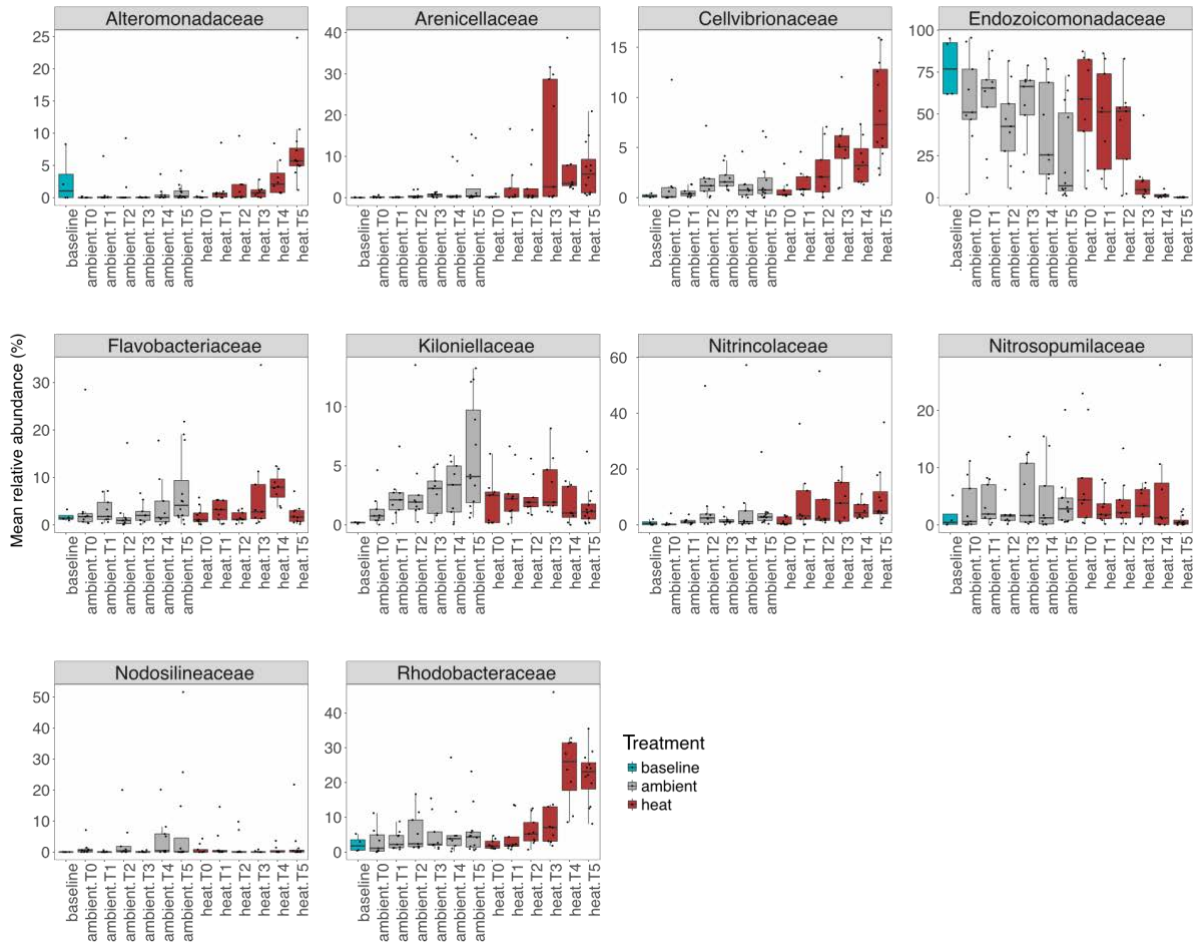

Fig. S4.7. Changes in relative abundance of the dominant 10 microbial families (> 5 % mean relative abundance) across treatments (ambient, heat) and time (baseline, T0 - T5) in the coral samples throughout the heat stress experiment. Box = inter-quartile range (IQR), line in box = median, whiskers = minimum and maximum values not outliers (i.e. -/+1.5*IQR).

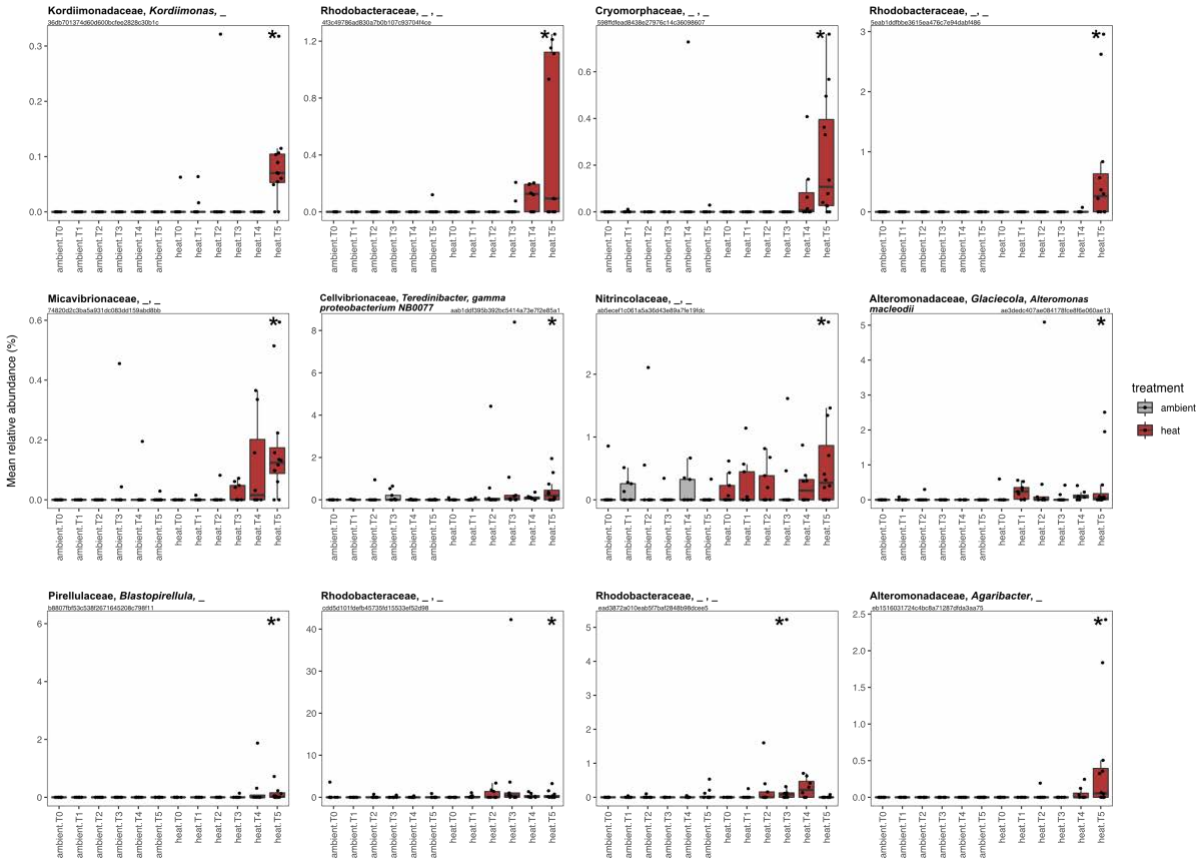

Fig. S4.8. Changes in mean relative abundance of differentially abundant ASVs between ambient and heat treatment over time (T0-T5). Differentially abundant ASVs were identified using DESeq ($p < 0.01$; adjusted post hoc tests), and significant differences are indicated with asterisks (heat vs ambient for the respective time point). Taxonomic assignment for each ASV is shown as Family, Genus, Species. Box = inter-quartile range (IQR), line in box = median, whiskers = minimum and maximum values not outliers (i.e. -/+1.5*IQR).

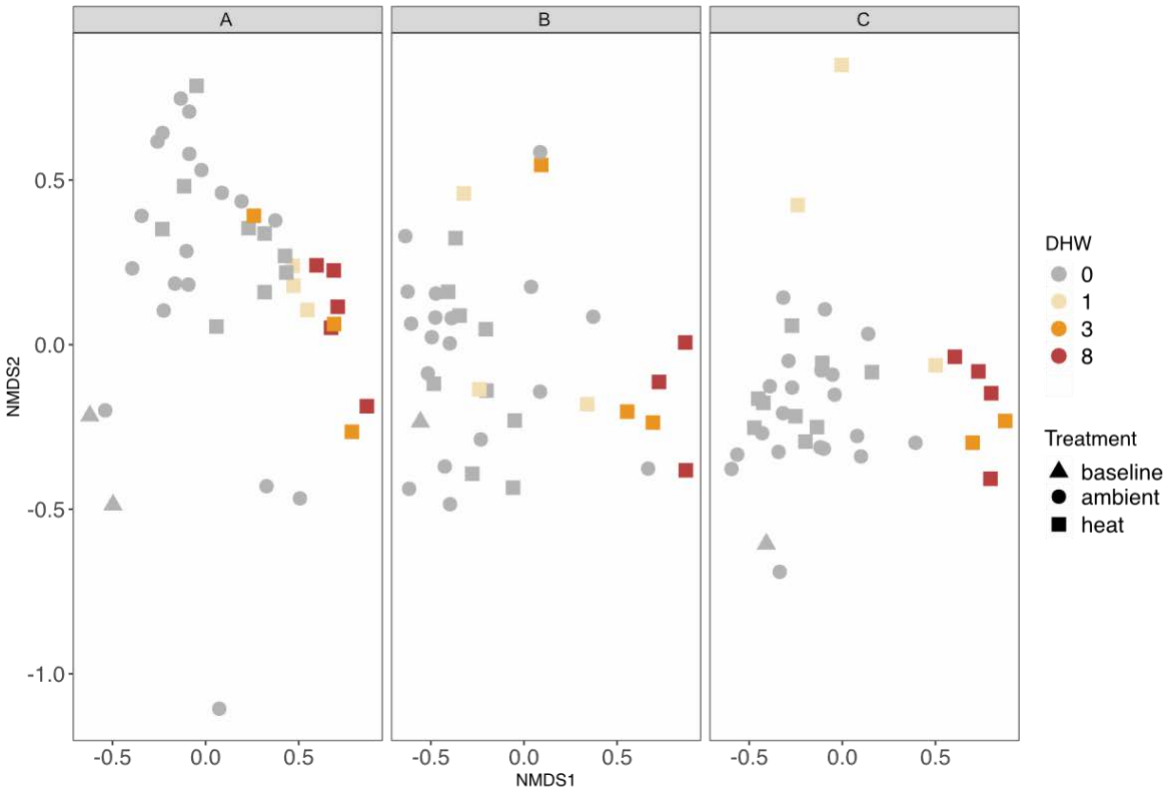

Fig. S4.9. NMDS (sqrt-rooted data; stress = 0.21) based on Bray-Curtis dissimilarities calculated on relative abundance of ASVs in coral samples during the heat stress experiment (baseline, ambient, heat) in the three colonies of origin (A, B, C).

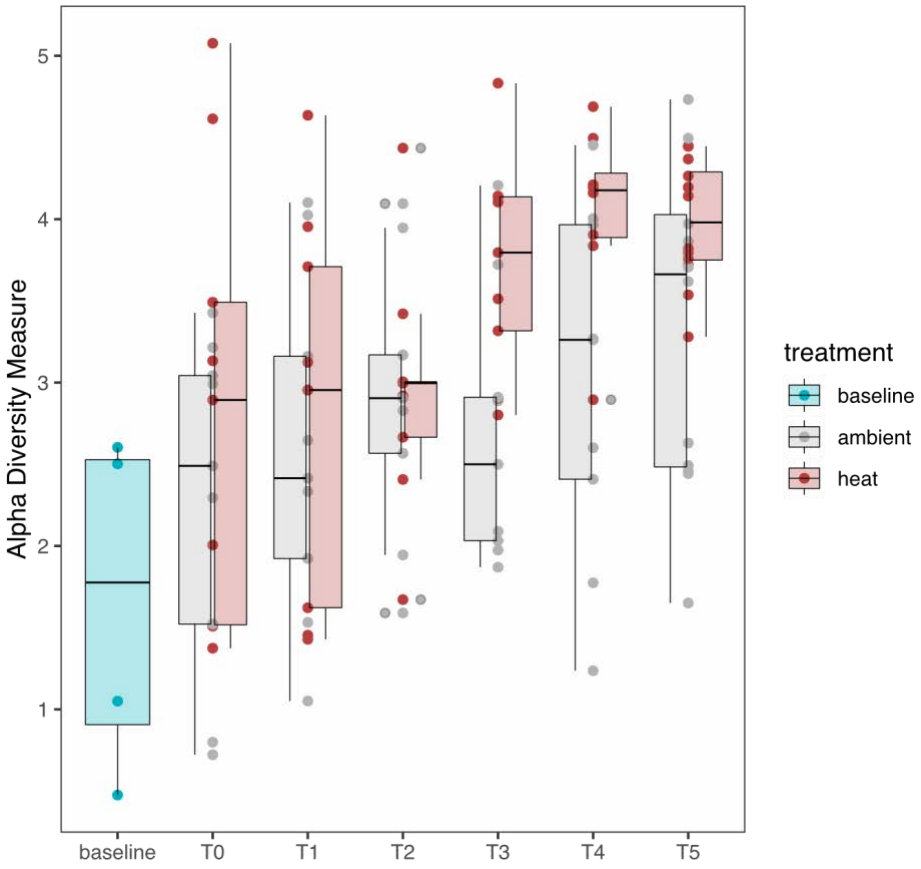

Fig. S4.10. Shannon diversity index representing alpha diversity of the microbiome in the coral samples over time (baseline, T0-T5) and across treatments (ambient and heat). Box = inter-quartile range (IQR), line in box = median, whiskers = minimum and maximum values not outliers (i.e. -/+1.5*IQR).

Appendix C – Supplementary Material for Chapter 5

Table S5.1. Three ASVs were identified as contaminant using the R package decontam (https://github.com/benjjneb/decontam). Class, Family and Genus are reported for each ASV identifier; and unidentified genera are reported as "-".

ASV identifier	Class	Family	Genus
ed8957a6eedb2183fb8bb92a6d8b53c4	Bacteroidia	Amoebophilaceae	uncultured
1ecc6c44aebeb49bee553dc886d99edb	Alphaproteobacteria	Rhodobacteraceae	-
cbe14950a2b13c46ac72c998469b8af0	Gammaproteobacteria	Saccharospirillaceae	Oleibacter

Table S5.2. Generalized linear mixed models tested the fixed effect of treatment (ambient, heat), time (T0, T1, T2, T3, T4, T4.5, T5), colony of origin (C1, C2, C3) and the interaction between treatment and time on net photosynthesis, respiration (sqrt-transformed), gross photosynthesis / respiration ratio (log-transformed) and photochemical effective efficiency in the sponge *Cliona orientalis*. Tank and fragment were included in the model to account for repeated measures. The pairwise comparisons (BH correction) for significant interactions are reported here. Estimates are not back-transformed. Significant factors ($p<0.05$) are indicated in bold.

Var.	*Time Point*	*Contrast*	*Estimate*	*SE*	*df*	*P-value*
Net photosynthesis	T0	Ambient-Heat	-0.2812	0.133	236	0.1235
	T1	Ambient-Heat	-0.2112	0.127	236	0.2289
	T2	Ambient-Heat	0.0175	0.133	236	0.8952
	T3	Ambient-Heat	-0.0590	0.126	236	0.8016
	T4	Ambient-Heat	-0.0964	0.132	236	0.8016
	T4.5	Ambient-Heat	-0.0510	0.126	236	0.8016
	T5	Ambient-Heat	1.2041	0.147	236	**<.0001**
Respiration (sqrt)	T0	Ambient-Heat	-0.02892	0.0150	237	0.1916
	T1	Ambient-Heat	-0.02295	0.0147	237	0.2801
	T2	Ambient-Heat	0.00237	0.0154	237	0.8779
	T3	Ambient-Heat	-0.00530	0.0146	237	0.8427
	T4	Ambient-Heat	-0.01115	0.0150	237	0.7998
	T4.5	Ambient-Heat	-0.00545	0.0153	237	0.8427
	T5	Ambient-Heat	0.12713	0.0153	237	**<.0001**
P/R ratio (log)	T0	Ambient-Heat	-4.74e-02	0.0211	237	0.0897
	T1	Ambient-Heat	-2.83e-02	0.0209	237	0.3080
	T2	Ambient-Heat	3.90e-02	0.0216	237	0.1702
	T3	Ambient-Heat	-5.43e-05	0.0208	237	0.9979

	T4	Ambient-Heat	2.27e-02	0.0211	237	0.3987
	T4.5	Ambient-Heat	1.68e-02	0.0215	237	0.5081
	T5	Ambient-Heat	9.93e-02	0.0215	237	**<.0001**
Photochemical effective efficiency	T0	Ambient-Heat	-0.00400	0.0165	505	0.8089
	T1	Ambient-Heat	0.00596	0.0166	505	0.8089
	T2	Ambient-Heat	0.01046	0.0181	505	0.7905
	T3	Ambient-Heat	0.03470	0.0228	505	0.2254
	T4	Ambient-Heat	0.07373	0.0201	505	**0.0006**
	T4.5	Ambient-Heat	0.14579	0.0356	505	**0.0002**
	T5	Ambient-Heat	0.50055	0.0290	505	**<.0001**

Table S5.3 (separate Dataset file https://tinyurl.com/2yuzmtxr). Summary of sequencing statistics of the mRNA dataset, and percentage of reads mapping to the reference transcriptomics.

Table S5.4 (separate Dataset file https://tinyurl.com/2yuzmtxr). Outcome of the Gene Ontology (GO) enrichment analyses (Biological Processes) for the sponge host between heat stress (3 DHW) and ambient conditions (0 DHW); only significant genes (adjusted $p < 0.05$) are shown.

Table S5.5 (separate Dataset file https://tinyurl.com/2yuzmtxr). Overview of the sponge host Differentially Expressed Genes (DEGs) detected between heat stress (3 DHW) and ambient conditions (0 DHW).

Table S5.6 (separate Dataset file https://tinyurl.com/2yuzmtxr). Outcome of the Gene Ontology (GO) enrichment analyses (Biological Processes) for the sponge-associated Symbiodiniaceae between heat stress (3 DHW) and ambient conditions (0 DHW); only significant genes (adjusted $p < 0.05$) are shown.

Table S5.7 (separate Dataset file https://tinyurl.com/2yuzmtxr). Overview of Symbiodiniaceae Differentially Expressed Genes (DEGs) detected between heat stress (3 DHW) and ambient conditions (0 DHW).

Table S5.8. Permutation multivariate analysis of variance (adonis function in R vegan package; 10,000 permutations) based on Bray-Curtis dissimilarities applied on square-root-transformed relative abundances (ASV level) to determine the effects of sample type (fixed, three levels: "sponge", "seawater", "feed") on the sponge-associated microbial community. Statistical significance ($p < 0.05$) is shown in bold.

Source	*df*	*SS*	*F*	*P-value*
Sample type	2	5.538	11.28	**<0.001**
Residual	123	30.194		
Pairwise tests: Sample type: Sponge ≠ Seawater Sponge ≠ Feed Seawater ≠ Feed				

Table S5.9. Permutation multivariate analysis of variance (adonis function in R vegan package; 10,000 permutations) based on Bray-Curtis dissimilarities applied on square-root-transformed ASV relative abundances to test the effect of time (fixed, six levels: "T0", "T1", "T2", "T3", "T4", "T5"), treatment (fixed, two levels: "ambient", "heat") and colony of origin (fixed, three levels: "C1", "C2", "C3") on the sponge-associated microbial community. Tank was also fitted in the model. Statistical significance ($p < 0.05$) is shown in bold.

Source	*df*	*SS*	*F*	*P-value*
Time	5	4.4172	4.4978	**<0.001**
Treatment	1	0.6328	3.2215	**0.0003**
Colony	2	2.0023	5.0970	**<0.001**
Tank	4	1.9525	2.4851	**<0.001**
Time x Treatment	5	1.1380	1.1588	0.1390
Residual	84	16.4992		
Pairwise tests: Colony of origin: C1 ≠ C2 ≠ C3 Time: T0 ≠ T3, T4, T5 T1 ≠ T4, T5				

T2 ≠ T4, T5 T3 ≠ T5 T4 ≠ T5

Table S5.10. The effect of treatment (fixed, two levels: "ambient", "heat"), time (fixed, six levels: "T0", "T1", "T2", "T3", "T4", "T5") and colony of origin (fixed, 3 levels) were tested on the Shannon diversity index of the sponge microbiome (rarefied data) using a linear mixed model (gaussian). Tank was included in the model as random effect. Statistical significance ($p < 0.05$) is shown in bold.

Source	*Estimate*	*SE*	*P-value*
T1	-0.22386	0.38015	0.5559
T2	0.01011	0.44070	0.9817
T3	0.01862	0.39099	0.9620
T4	0.10430	0.39537	0.7919
T5	-0.16329	0.34724	0.6382
Heat	-0.70068	0.42758	0.1013
Colony C2	-0.88726	0.20367	**<0.001**
Colony C3	-0.29861	0.20893	0.1529
T1:heat	0.81492	0.54394	0.1341
T2:heat	0.64388	0.62175	0.3004
T3:heat	0.84029	0.55071	0.1271
T4:heat	0.94283	0.56308	0.0941
T5:heat	0.58284	0.48786	0.2322

Table S5.11 (separate Dataset file https://tinyurl.com/2yuzmtxr). Similarity matrix between host and Symbiodiniaceae genes selected through DIABLO.

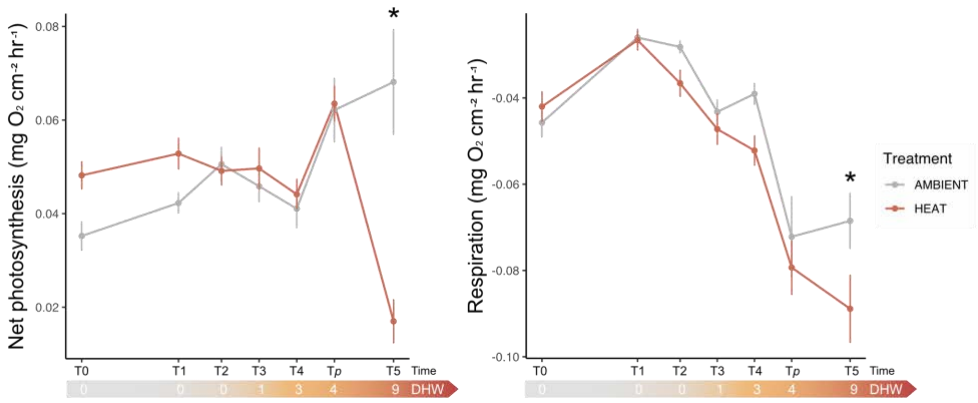

Fig. S5.1. Net photosynthesis and respiration rates in the sponge clones over time (T0 – T5) under ambient and heat treatments. DHW are shown for the heat treatment only (0 DHW under ambient conditions). Mean ± SE is shown over time for each physiological response. Significant different ($p < 0.05$; adjusted post hoc tests) responses between ambient and heat treatments are indicated with asterisks.

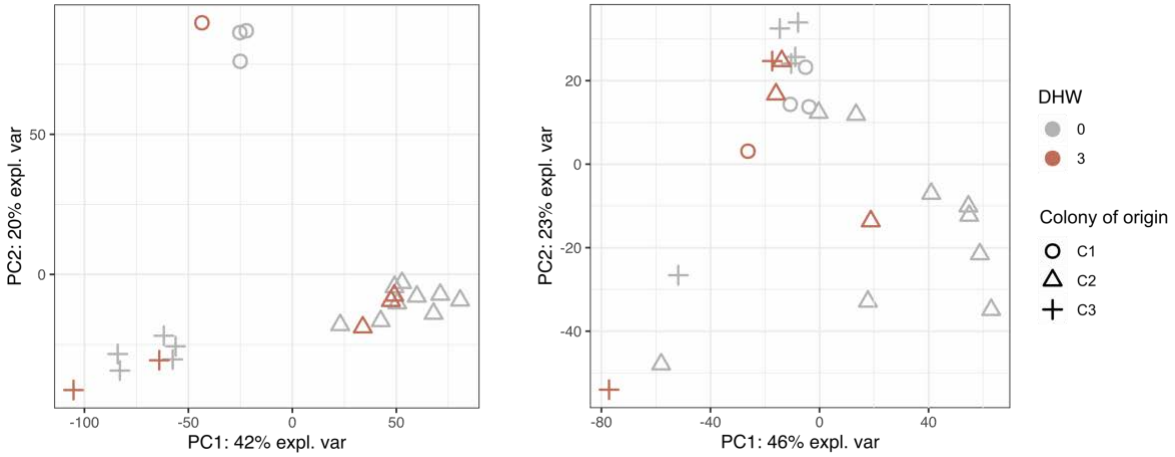

Fig. S5.2. Principal Component Analysis (PCA) of host (left) and Symbiodiniaceae (right) gene expression profiles on transformed (variance stabilizing transformation) and centered data.

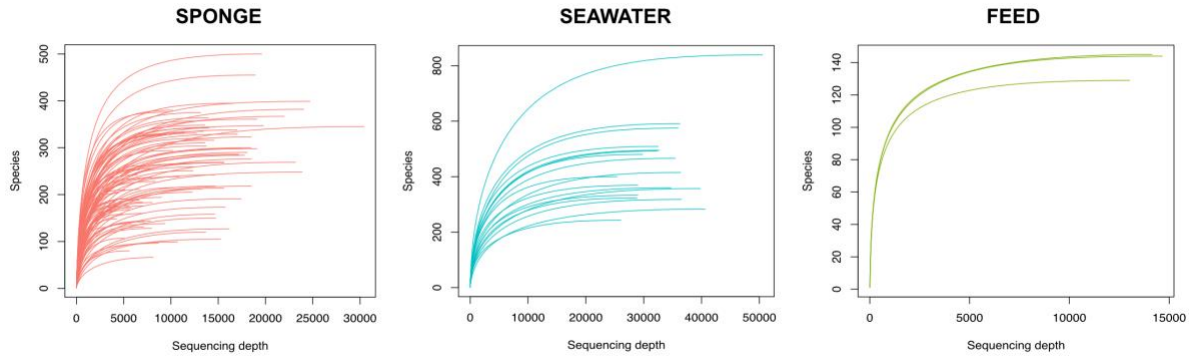

Fig. S5.3. ASV rarefaction curves of 16S rRNA gene sequences for sponge (Baseline, T0-T5), seawater and feed (microalgae) samples.

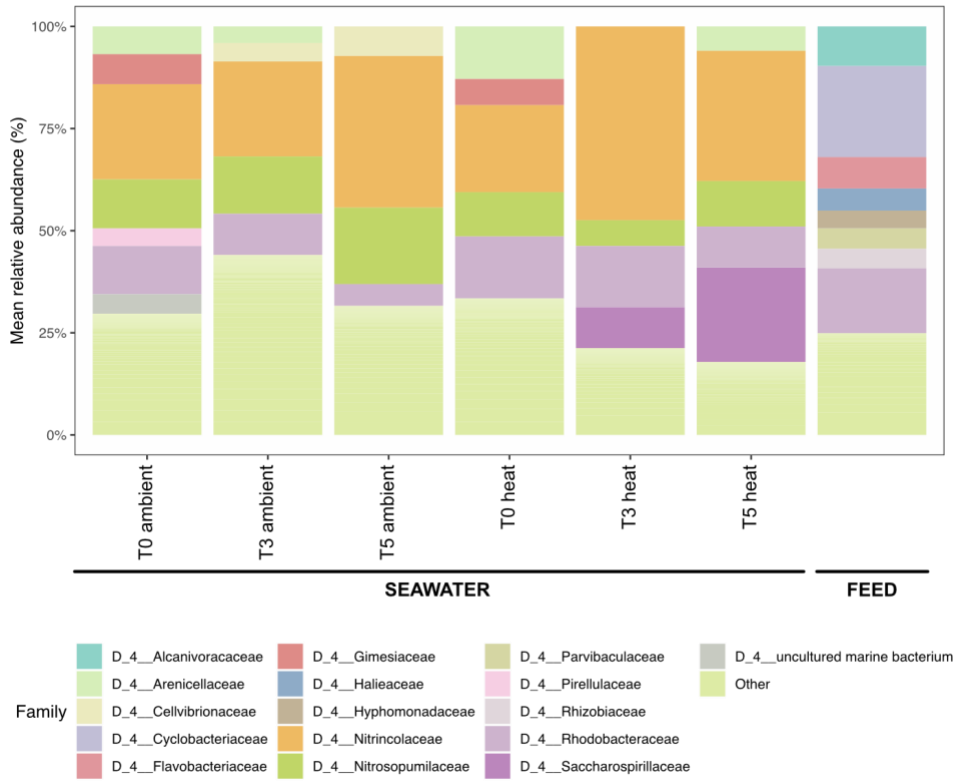

Fig. S5.4. Mean relative abundance of dominant microbial families (> 4% relative abundance) in seawater and feed (microalgae) samples. Seawater microbial community composition is shown over time (T0, T3, T5) under ambient and heat treatments.

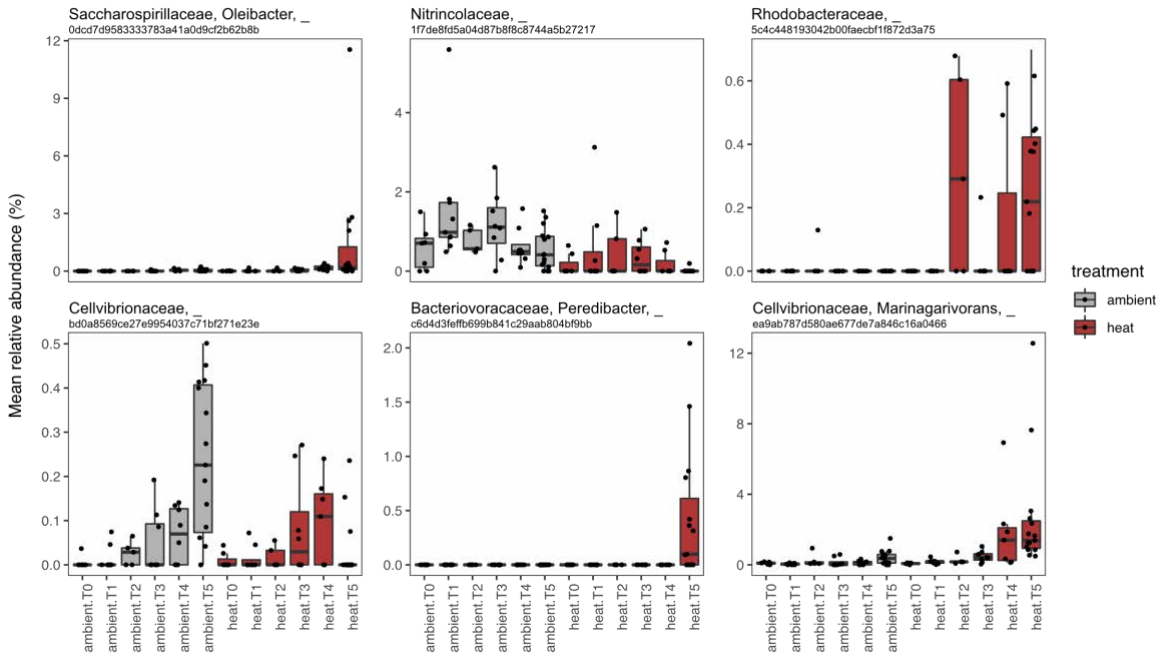

Fig. S5.5. Changes in mean relative abundance over time (T0 – T5) of ASVs differentially abundant between T5 ambient and T5 heat (9 DHW). ASVs were identified through DESeq ($p < 0.05$; adjusted post hoc tests). Taxonomic assignment for each ASV is shown at the Family and Genus levels. Box = inter-quartile range (IQR), line in box = median, whiskers = minimum and maximum values not outliers (i.e. -/+1.5*IQR).

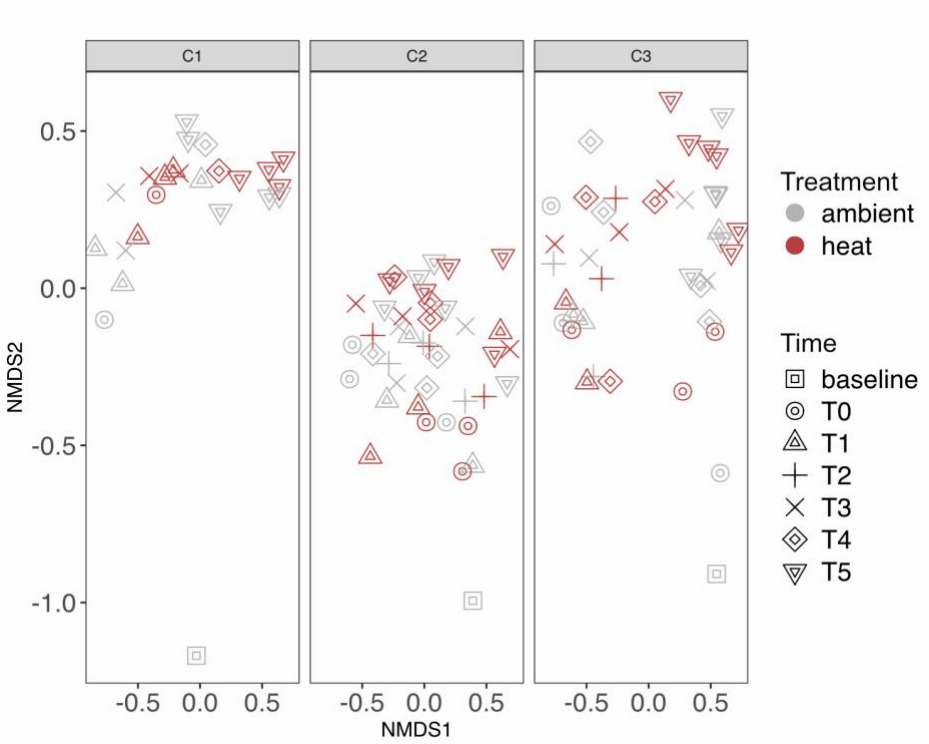

Fig. S5.6. NMDS (sqrt-rooted data; stress = 0.19) showing sponge-associated microbial community structure based on Bray-Curtis dissimilarities calculated on relative abundance of ASVs across time (baseline, T0-T5) and treatment (ambient, heat) in the three colonies of origin (C1, C2, C3).

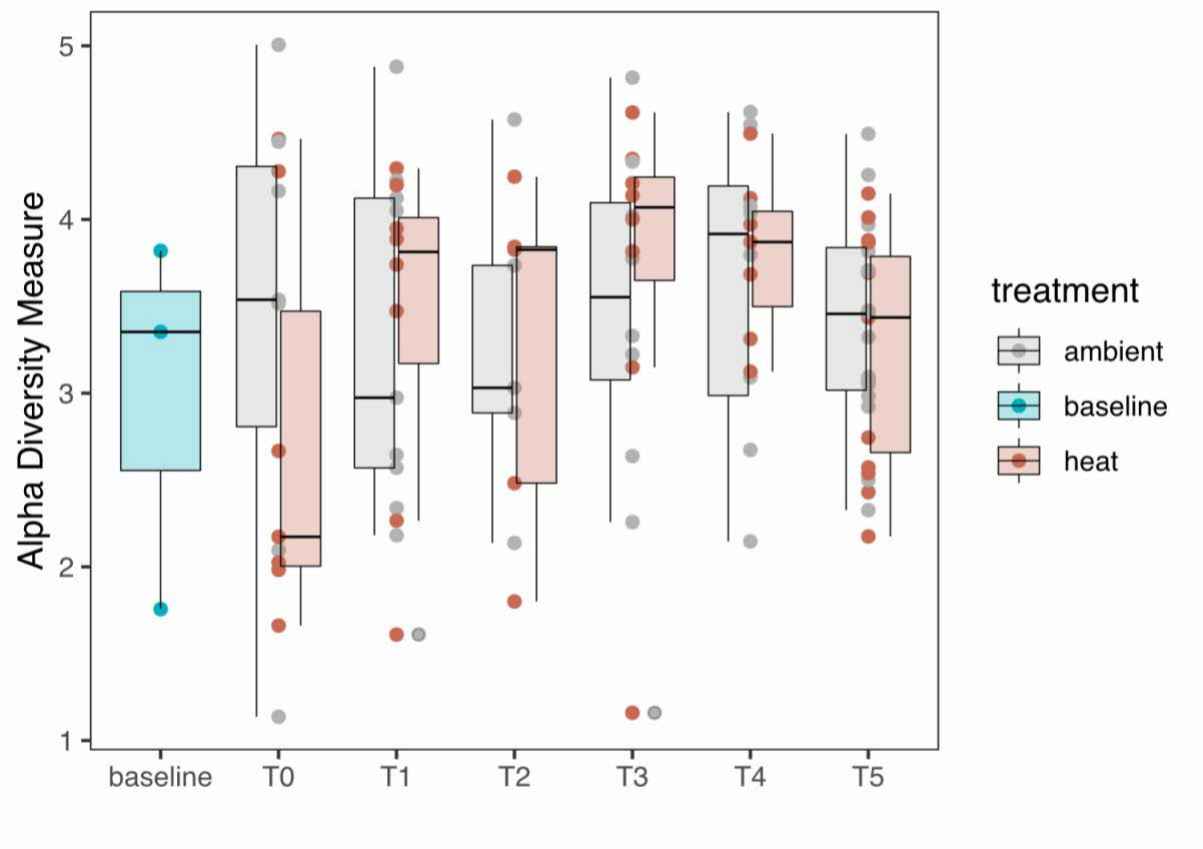

Fig. S5.7. Shannon diversity index estimating the alpha diversity in the sponge microbiome across time (baseline, T0 – T5) and treatments (ambient, heat). Box = inter-quartile range (IQR), line in box = median, whiskers = minimum and maximum values not outliers (i.e. -/+1.5*IQR).

Appendix D – List of co-authorships during the PhD

During my PhD, I have collaborated on additional projects which have resulted in the below publications:

1. Terrell, A. P., **Marangon**, **E.,** Webster, N. S., Cooke, I., & Quigley, K. M. (2023). The promotion of stress tolerant Symbiodiniaceae dominance in juveniles of two coral species under simulated future conditions of ocean warming and acidification. *Front Ecol Evol*, 11, 1113357. https://doi.org/10.3389/fevo.2023.1113357
2. Engelberts, J. P., Abdul Wahab, M. A., Maldonado, M., Rix, L., **Marangon, E**., Robbins, S. J., Wagner, M., & Webster, N. S. (2022). Microbes from Mum: symbiont transmission in the tropical reef sponge *Ianthella basta*. *ISME Communications*, 2(1), 90. https://doi.org/10.1038/s43705-022-00173-w
3. Robbins, S. J., Song, W., Engelberts, J. P., Glasl, B., Slaby, B. M., Boyd, J., **Marangon, E.**, Botté, E. S., Laffy, P., Thomas, T., & Webster, N. S. (2021). A genomic view of the microbiome of coral reef demosponges. *The ISME Journal*, *15*(6), 1641–1654. https://doi.org/10.1038/s41396-020-00876-9
4. Botté, E. S., Luter, H. M., **Marangon, E.**, Patel, F., Uthicke, S., & Webster, N. S. (2020). Simulated future conditions of ocean warming and acidification disrupt the microbiome of the calcifying foraminifera *Marginopora vertebralis* across life stages. *Environmental Microbiology Reports*, *12*(6), 693–701. https://doi.org/10.1111/1758-2229.12900
5. Glasl, B., Robbins, S., Frade, P. R., **Marangon, E**., Laffy, P. W., Bourne, D. G., & Webster, N. S. (2020). Comparative genome-centric analysis reveals seasonal variation in the function of coral reef microbiomes. *The ISME Journal*, 14(6), 1435–1450. https://doi.org/10.1038/s41396-020-0622-6

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