Responses of corals and coral reef ecosystems to ocean acidification under variable temperature and light

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Statement of the contribution of others

All data chapters of this thesis have been published. Details of the authors' contributions are as follows:

Chapter	Publication details	Author contribution
2	Noonan SHC, Fabricius KE and Humphrey C (2013) *Symbiodinium* community composition in scleractinian corals is not affected by life-long exposure to elevated carbon dioxide. PLoS ONE 8(5): e63985	The authors co-developed the manuscript idea and collected field samples. SN conducted all lab work and statistical analyses, wrote first draft of paper, developed the figures and tables, and addressed co-author and reviewers' comments during publication. CH and KF contributed to paper preparation providing comments on the draft and during review.
3	Noonan SHC and Fabricius KE (2016) Ocean acidification affects productivity but not the severity of thermal bleaching in some tropical corals. ICES Journal of Marine Science 73 (3): 715-726	The authors co-developed the manuscript idea and conducted field bleaching surveys. SN ran laboratory experiment and completed all lab work and statistical analyses, wrote first draft of paper, developed the figures and tables, and addressed co-author and reviewers' comments during publication. KF contributed to paper preparation comments on the draft and during review.
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5	Noonan SHC, Kluibenschedl A and Fabricius KE (2018) Ocean acidification alters early successional coral reef communities and their rates of community metabolism. PLoS ONE 13(5): e0197130	The authors co-developed the manuscript idea and conducted field experiment. AK completed photo analyses of community composition. SN completed all lab work, conducted all statistical analyses, wrote first draft of paper, developed the figures and tables, and addressed co-author and reviewers' comments during publication. KF and AK contributed to paper preparation comments on the draft and during review.

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Abstract

Coral reefs are under increasing pressure from ocean acidification. However, much of our understanding is based on single-species aquarium experiments made in isolation from realistic environmental parameters (e.g. light, water flow, food supply) and other co-occurring stressors (e.g. increasing sea surface temperatures, reduced water clarity due to terrestrial runoff). In my PhD project I aimed to understand how ocean acidification affects the ecophysiology of reef corals and reef communities in natural settings, and how effects may differ with concurrent exposure to variable temperature and light. I used a combination of experimental and observational studies at unique field sites with naturally high levels of CO_2 (CO_2 seep sites), and multi-factor experiments in the aquarium facilities of The Australian Institute of Marine Science's National Sea Simulator to address these questions.

In chapter 2, I investigated if corals can acclimate to ocean acidification by switching their photo-symbionts to types that may be able to utilise the more abundant CO_2 in photosynthesis. I used molecular techniques to investigate the dominant photosymbiont types in six species of coral from the field and found them to be highly conserved within species between CO_2 seep and control sites. In chapter 3, I used a combination of field surveys and a multifactor laboratory experiment to investigate if elevated CO_2 increased the severity of coral thermal bleaching. Field surveys during a bleaching event at the CO_2 seeps, as well as the experimental study, both showed that corals were not significantly more susceptible to thermal stress under high CO_2. In chapter 4, I used a multifactor laboratory experiment to investigate if reduced or variable daily light availability affected the responses of corals to high CO_2. Here I found that reductions in light levels, regardless of the variability in daily light integrals, can reduce coral growth rates more than high CO_2. In chapter 5, I followed the development of early successional coral reef benthic communities on settlement tiles along a gradient of CO_2 exposure at the seep sites, and further measured rates of community metabolism. Here high CO_2 strongly influenced the development of communities, shifting them away from a dominance of calcifying taxa under present day conditions to a range of non-calcifying algae as CO_2 levels increased. These high CO_2 communities progressively recorded lower rates of calcification and higher rates of photosynthesis at high CO_2.

Results from this thesis show that the considerable changes to the CO_2 seep benthic communities are likely due to secondary ecological effects, rather than the physiological effects on corals alone. Moreover, the negative effects of cooccurring stressors on corals and coral reefs will also be substantial.

Hence there is an immediate need to reduce atmospheric CO_2 emissions and improve the management of local stressors to prevent further declines to the health and functioning of coral reef ecosystems.

Contents

Chapter 1: General introduction

1.1 Ocean acidification

Human activities, such as the burning of fossil fuels in transportation and industry, and the clearing of land for agriculture, are raising atmospheric carbon dioxide (CO_2) concentrations (Friedlingstein *et al.* 2020; IPCC 2021). We currently emit around 40 gigatons $year^{-1}$, resulting in a yearly increase in atmospheric CO_2 concentrations of ~2.5 ppm (Feely *et al.* 2009; Friedlingstein *et al.* 2020), the fastest rate of increase in the last 55 million years (Gingerich 2019). Monthly averages of atmospheric CO_2 are currently ~415 ppm, which is the highest they have been in at least 800 000 years (Lüthi *et al.* 2008; Hönisch *et al.* 2012; Doney *et al.* 2020). Following Henry's gas law, approximately 25% of the CO_2 we emit is absorbed by the surface waters of the world's oceans (Friedlingstein *et al.* 2020). On the one hand this is beneficial, as it is slowing the effects of global climate change (Friedlingstein *et al.* 2020). However, absorption of CO_2 by the ocean is also causing a suite of seawater chemical changes in a process known as ocean acidification (Freely 2004). When CO_2 dissolves in seawater it forms carbonic acid (H_2CO_3), but this readily disassociates into free hydrogen ions (H^+) and bicarbonate ions (HCO_3^-). The increased concentration of H^+ lowers seawater pH, and this is where ocean acidification derives its name. However, ocean acidification also increases the partial pressure of carbon dioxide (pCO_2), as well as the concentration of total dissolved inorganic carbon (C_T). While C_T increases, the ratio of its constituent chemical species changes. Here free H^+ binds to CO_3^{2-} forming HCO_3^-, resulting in more dissolved CO_2 ($CO_{2\,aq}$) and less CO_3^{2-}. Ocean acidification is steadily progressing (Fabricius *et al.* 2020), and without considerable changes to anthropogenic activity, we will likely more than double CO_2 levels in the atmosphere and ocean by the year 2100 (IPCC 2021). Ocean acidification should be considered an environmental change which is increasingly affecting the metabolism, physiological performance, and subsequent distribution of species (Kroeker *et al.* 2013; Smith *et al.* 2020).

1.2 Biological effects of ocean acidification on coral reef corals

Coral reefs are predicted to be amongst the ecosystems most affected by ocean acidification (Doney *et al.* 2020; IPCC 2021). This is because they are characterised by an abundance of organisms with calcium carbonate ($CaCO_3$) skeletons, the most conspicuous of which are the scleractinian corals. Reduced CO_3^{2-}

concentrations under ocean acidification lowers the thermodynamic potential for $CaCO_3$ to precipitate, a value numerically represented by the saturation state of $CaCO_3$ (Ω). Lowered Ω and pH make it more difficult for calcifying organisms to form their $CaCO_3$ skeletons (Feely *et al.* 2004; Orr *et al.* 2005). Of the three main $CaCO_3$ mineral phases, calcite is the most stable, followed by aragonite, with high-Mg-calcite being the most soluble. Thus, the form of $CaCO_3$ used by an organism can also influence their susceptibility to ocean acidification. For example, the high-Mg-calcite skeletons of many crustose coralline algae (CCA) have been shown to be particularly sensitive (Fabricius *et al.* 2015). Scleractinian corals utilise aragonite, and we are on track to reduce the aragonite saturation state (Ω_{Ar}) from present day values in the tropics of ~3.5 - 4 to ~2 by the year 2100 (IPCC 2021). Some studies argue that reefs as we know them cannot persist once Ω_{Ar} is less than three (Ricke *et al.* 2013; Eyre *et al.* 2018) although it is clear that the effects of ocean acidification on corals and other reef organisms can be species specific.

The negative effects of ocean acidification on calcification has long been identified as an issue for corals (Gattuso *et al.* 1998; Marubini & Atkinson 1999; Langdon *et al.* 2000). Meta-analyses have further indicated that every unit decrease of Ω_{Ar} can reduce coral calcification by an average of 15% (range 0 – 30%) (Chan & Connolly 2013). For calcification to occur the coral must raise the pH of the fluid at the site of calcification (McCulloch *et al.* 2012). But this becomes increasingly energetically expensive in seawater with lower pH, and the ability to do so appears to be species specific (Venn *et al.* 2019). Increased food supply can mitigate some of the negative effects of ocean acidification on coral calcification, however results differ among species and locations (Edmunds 2011; Comeau *et al.* 2013a; Houlbréque *et al.* 2015; Kornder *et al.* 2018). Other environmental factors, including light availability and water flow, also influence coral calcification (Gattuso *et al.* 1999a; Al-Horani *et al.* 2003; Comeau *et al.* 2014b), complicating predictions of the effects of ocean acidification.

Reduced calcification is not the only experimentally observed biological effect of ocean acidification. Photosynthesis may also be stimulated in a range of coral reef primary producers, including coral photosymbionts (Semesi *et al.* 2009; Suggett *et al.* 2012; Uthicke & Fabricius 2012; Connell *et al.* 2013; Kroeker *et al.* 2013; Anderson *et al.* 2019). Marine primary producers often rely on energetically expensive carbon concentrating mechanisms (CCMs) to convert abundant forms of C_T (mainly HCO_3^-) to CO_2 for fixation (Allemand *et al.* 1998; Leggat *et al.* 1999; Furla *et al.* 2005). Many can also utilise $CO_{2\,aq}$, but under present day conditions, $CO_{2\,aq}$ makes up <1% of the C_T pool and can be limiting (Tansik *et al.* 2017). Increases in $CO_{2\,aq}$ under ocean acidification may therefore stimulate photosynthesis. Certain coral

photosymbiont types can grow and photosynthesise more under elevated CO_2, presenting a potential mechanism of acclimation for corals which contain these symbiont types (Brading *et al.* 2011). A suite of non-calcifying photosynthetic taxa, including algae, anemones and seagrasses, have also been shown to benefit from elevated CO_2 by increasing growth and photosynthetic rates (Suggett *et al.* 2012; Connell *et al.* 2013; Kroeker *et al.* 2013; Takahashi *et al.* 2015). However, this effect is not universal (Kroeker *et al.* 2013; Comeau *et al.* 2017a) and likely depends on a variety other factors (e.g. organism CCM efficiency, limitation in other photosynthesis substrates). The physiological changes to corals and other calcifying taxa under ocean acidification may result in altered species distributions and ultimately change the productivity and functioning of coral reef benthic communities.

1.3 Multiple stressors on coral reefs

Ocean acidification is not occurring in isolation, and often multiple stressors impact coral reefs simultaneously (Crain *et al.* 2008; Ban *et al.* 2014). These can be from a range of other global (e.g. climate change and cyclone intensities) and local sources (e.g. terrestrial runoff, sedimentation and eutrophication, over-fishing, pollution etc.). When stressors cooccur, the combined effects may simply be the sum of individual stressors (i.e. additive effects), or effects may be enhanced or diminished (i.e. synergistic vs antagonistic effects) (Dunne 2010). Unfortunately there is little consistency in the response of multiple stressors for organisms across all marine systems (Crain *et al.* 2008; Ban *et al.* 2014; Uthicke *et al.* 2016). For example, meta-analyses conducted by Crain *et al.* (2008), which summarised the results of 171 multiple stressor studies across all marine systems, found approximately one third of studies reported either additive (26%), synergistic (36%) or antagonistic (38%) results. For coral reef organisms, results are also mixed. Ban *et al.* (2014) summarised results from 111 multiple stressor studies primarily focussing on coral reef corals, and found 54% reported synergistic effects, with additivity (30%) and antagonism (15%) being less common. Furthermore, there have been very few studies conducted on the majority of stressor combinations for most coral reef organisms. Uthicke *et al.* (2016) summarised 102 multiple stressor studies on different groups of coral reef taxa (e.g. coral, algae, seagrass, etc.), and found that 62% of studies have been conducted on corals, 18% on algae, and less than 10% of all studies were conducted on a combined group of other reef taxa including crustaceans, molluscs and porifera. Hence, we know very little about

how the majority of stressor combinations will affect the majority of coral reef taxa and have a limited ability to predict effects given the variety of responses.

1.4 Field sites at CO_2 seeps

Much of our understanding of the effects of ocean acidification and other stressors on coral reefs stems from small-scale perturbation experiments, on few or single species, with short incubations and often abrupt changes in treatment conditions. Such experiments are often isolated from realistic environmental parameters, such as light, water flow, and food supply. While this large number of small-scale studies has given us great insight into the predicted effects of ocean acidification on the physiology of individual coral reef organisms, a knowledge gap exists in scaling up these results to coral reef communities. Direct physiological effects on certain organisms can result in considerable ecological effects for other organisms with whom they interact (Fabricius *et al.* 2014a). These downstream ecological effects are hard to predict without examining communities directly. Short-term experiments also fail to account for any mechanisms of acclimation that may occur over longer periods of time in realistic environmental settings. These shortcomings are of concern as an organism's response to multiple stressors, and the response of communities, can be different from the sum of individual responses (Gunderson *et al.* 2016; Uthicke *et al.* 2016).

To overcome some of these limitations and improve predictions for the future of coral reefs, a field of study has emerged centring around reefs with naturally high levels of CO_2. These natural analogues have provided much information on the community and ecological effects on coral reef communities (reviewed by González-Delgado & Hernández 2018; Hill & Hoogenboom 2022). Most of these sites occur around volcanic CO_2 seeps, where emerging gas has a high percentage of CO_2 and locally alters seawater carbon chemistry. All field components of this thesis are conducted at such volcanic CO_2 seep sites in Milne Bay, PNG. At three reefs in the region (Fig 1.1), near pure, unheated CO_2 emerges from the seafloor in streams of gas bubbles, locally producing ocean acidification conditions predicted for the future (Fabricius *et al.* 2011).

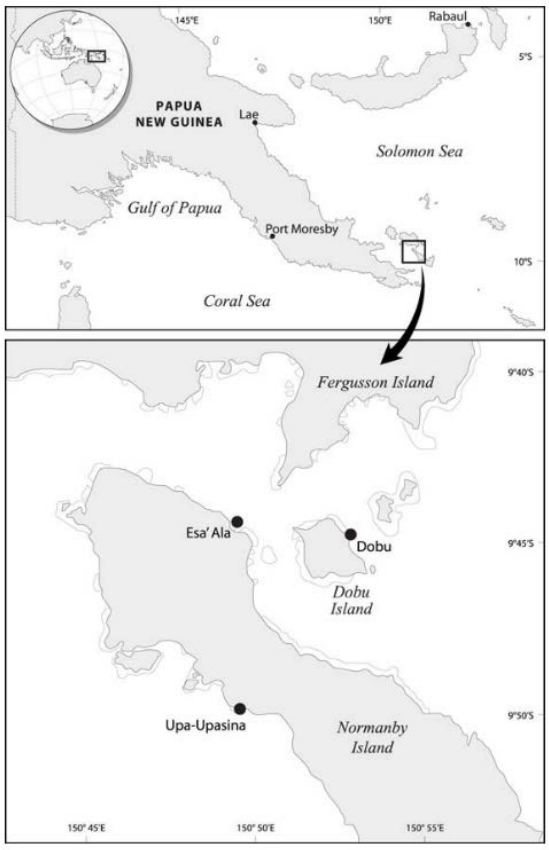

Fig 1.1: Study site map indicating the location of the three CO_2 seep sites used throughout this thesis, Milne Bay, PNG. Map taken from Fabricius *et al.* (2011).

Initial investigations at the CO_2 seeps revealed benthic communities considerably altered from adjacent control sites, with many calcifying taxa being negatively affected (Fabricius *et al.* 2011). Here the total percent cover of hard cover remained unchanged between sites, due to a two-fold increase in massive *Porites* spp. cover within the seeps (Fig 1.2). At the same time, the cover of structurally complex hard corals (i.e. the branching, tabular and foliose morphologies) reduced three-fold, and hard coral taxonomic richness reduced ~40%. The cover of CCA also reduced seven-fold. Positive effects were documented in a suite of non-calcifying taxa: seagrass and non-calcifying algal cover increasing eight- and two-fold, respectively. Similar patterns have also been documented at a range of other seep sites in both tropical, and temperate locations (Hall-Spencer *et al.* 2008; Kroeker *et al.* 2012; Enochs *et al.* 2015;

González-Delgado & Hernández 2018). However, the mechanisms responsible for these drastic changes in communities remain largely uninvestigated.

Fig 1.2: Contrasting reef-scape images taken at a control reef (a) and the CO_2 seep site at Upa Upasina (b), Milne Bay, PNG (photo credit: Sam Noonan).

1.5 Thesis aims

The overarching objective of my PhD project is to understand how ocean acidification affects reef corals and coral communities in natural settings, and how effects may differ with concurrent exposure to variable temperature and light. I also aim to further predictions of how coral reefs will be shaped under progressive ocean acidification by contrasting the physiological response of corals from experiments with the ecological patterns seen at the Milne Bay CO_2 seep sites.

Specifically, I aim to determine:

Chapter 2: Are corals that settled and had life-long exposure to ocean acidification conditions able to acclimate to ocean acidification by switching the types of algal photosymbiont they associate with? Certain laboratory in-vitro studies have shown contrasting photosynthesis and growth responses to ocean acidification by different coral dinoflagellate photosymbionts (Brading *et al.* 2011). It is thus hypothesised that some algal types may be favoured and become more prevalent in symbiosis with corals growing under elevated CO_2.

Chapter 3: Are corals more susceptible to thermal stress under ocean acidification conditions? Corals are particularly thermally sensitive, and steadily increasing global temperatures have seen an increase in the frequency and severity of coral bleaching and mortality events (Hughes *et al.* 2017). Early lab-based experimental work suggested that coral thermal tolerance may be reduced when exposed to high CO_2 (Anthony *et al.* 2008), however results are not universal. Moreover, the relationship between coral thermal bleaching susceptibility and ocean acidification has not been explored under realistic environmental settings.

Chapter 4: Are the negative physiological effects of ocean acidification on corals exacerbated under low or variable light? A reduction in light reduces photosynthetically derived energy supplies in corals, leading to lower rates of calcification. While some studies have shown the combined effects of ocean acidification and low light additively reduce coral calcification (Marubini *et al.* 2001; Ohde & Hossain 2004), this is not universal. The light environment on coral reefs is naturally variable, and the cumulative amount of light reaching the seafloor and maximum daily irradiance values can vary four-fold day to day (Anthony *et al.* 2004). It remains unknown how environmentally relevant variability in light levels interacts with ocean acidification to affect coral calcification and photosynthesis.

Chapter 5: How does ocean acidification affect the development of benthic coral reef communities, and what are the consequences for community metabolism? Many calcifying coral reef benthic taxa are expected to be negatively affected by ocean acidification, while non-calcifiers may benefit. Changes to water chemistry can also directly reduce community metabolism (e.g. rates of photosynthesis and

calcification), as can changing the composition of communities (e.g. the abundance of calcifying and non-calcifying taxa) (Langdon *et al.* 2003; Doo *et al.* 2019). Examining community development and metabolism rates along gradients of CO_2 exposure will give further insights into the expected outcomes for reefs of the future.

By using a combination of field studies at unique CO_2 seep sites and a series of multi-factor laboratory experiments, I will contrast the physiological response of corals and reef communities to high CO_2. In doing so I aim to determine how reef communities will be shaped as ocean acidification progresses, and if the expected changes are due to direct physiological effects to corals themselves, or to secondary ecological effects and altered species interactions.

Chapter 2: *Symbiodinium* community composition in scleractinian corals is not affected by life-long exposure to elevated carbon dioxide

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Chapter addendum: the taxonomy of the coral symbionts examined in this Chapter has been revised since publication. They are now designated to the family Symbiodiniaceae, and the different Clades are distinct genera (LaJeunesse *et al.* 2018). This does not affect the results or conclusions of this Chapter.

2.1 Abstract

Ocean acidification (OA) is expected to negatively affect coral reefs, however little is known about how OA will change the coral-algal symbiosis on which reefs ultimately depend. This study investigated whether there would be differences in coral *Symbiodinium* types in response to OA, potentially improving coral performance. We used denaturing gradient gel electrophoresis (DGGE) of the internal transcribed spacer 2 (ITS2) region of ribosomal DNA to investigate the dominant types of *Symbiodinium* associating with six species of scleractinian coral that were exposed to elevated partial pressures of carbon dioxide (pCO_2) *in situ* from settlement and throughout their lives. The study was conducted at three naturally occurring volcanic CO_2 seeps (pCO_2 ~500 to 900 ppm, pH_{Total} 7.8 – 7.9) and adjacent control areas (pCO_2 ~390 ppm, pH_{Total} ~8.0 – 8.05) in Papua New Guinea. The *Symbiodinium* associated with corals living in an extreme seep site (pCO_2 >1000 ppm) were also examined. Ten clade C types and three clade D types dominated the 443 coral samples. *Symbiodinium* types strongly contrasted between coral species, however, no differences were observed due to CO_2 exposure. Within five species, 85 – 95% of samples exhibited the same *Symbiodinium* type across all sites, with remaining rare types having no patterns attributable to CO_2 exposure. The sixth species of coral displayed site specific differences in *Symbiodinium*

types, unrelated to CO_2 exposure. Symbiodinium types from the coral inhabiting the extreme CO_2 seep site were found commonly throughout the moderate seeps and control areas. Our finding that symbiotic associations did not change in response to CO_2 exposure suggest that, within the six coral hosts, none of the investigated 13 clade C and D *Symbiodinium* types had a selective advantage at high pCO_2. Acclimatisation through changing symbiotic association therefore does not seem to be an option for Indo-Pacific corals to deal with future OA.

2.2 Introduction

Present atmospheric carbon dioxide (CO_2) levels have surpassed 390 ppm, the highest they have been in at least two million years (Honisch *et al.* 2009). Since the beginning of the industrial revolution, anthropogenic CO_2 emissions, from the burning of fossil fuels and land clearing, have dramatically increased and continue to do so on a trajectory to reach or exceed 500ppm by the year 2100 (IPCC 2021).These increases are causing a planetary warming (Oreskes 2004) and ocean acidification (OA). Following Henry's gas law, as the partial pressure of atmospheric CO_2 (pCO_2) increases, more is dissolved into the surface waters of the world's oceans, raising levels of dissolved inorganic carbon (DIC) and lowering carbonate saturation states and pH (Langdon & Atkinson 2005). Declining carbonate saturation states are predicted to have negative consequences for calcifying organisms (Hofmann *et al.* 2010), however the increased levels of DIC may actually benefit some primary producers, enhancing the photosynthetic capacity of those limited by DIC (Schippers *et al.* 2004; Hall-Spencer *et al.* 2008; Rost *et al.* 2008; Crawley *et al.* 2010; Brading *et al.* 2011; Uthicke & Fabricius 2012).

Coral reefs are the most diverse marine ecosystems on our planet, primarily owing to the physical framework constructed by scleractinian corals as they secrete their calcium carbonate skeleton (Connell 1978). This process is made possible through a symbiotic relationship formed between the coral cnidarian host and single-celled photosynthetic dinoflagellates of the genus *Symbiodinium* (Muscatine *et al.* 1981). Corals meet much of their energy requirements through translocation of photosynthetically fixed carbon from their symbionts (Muscatine *et al.* 1981). While the coral host provides their *Symbiodinium* many of the substrates for photosynthesis, a significant proportion of the inorganic carbon required for fixation is still derived from the surrounding seawater (Al-Moghrabi *et al.* 1996). Dinoflagellates, including *Symbiodinium,* are unique amongst eukaryotes in that they utilise type II ribulose biphosphate carboxylase/oxygenase (RuBisCO) during the onset of carbon fixation (Whitney *et al.* 1995). This enzyme

has a much lower affinity with inorganic carbon than RuBisCo I (Rowan *et al.* 1996; Tortell 2000), leaving it under-saturated with CO_2 under present-day pCO_2 levels despite the apparent ability to also use bicarbonate (HCO_3^-) and the existence of a carbon concentrating mechanism (CCM) (Leggat *et al.* 1999). As pCO_2 increases under OA, both CO_2 and HCO_3^- substrates for photosynthesis will become more abundant.

The genus *Symbiodinium* is presently delineated phylogenetically into nine lineages (clades A-I) using nuclear (18S, 28S, ITS1 and ITS2 regions) and chloroplast (23S) ribosomal DNA (LaJeunesse 2001; Pochon & Gates 2010; Pochon *et al.* 2012). These clades are further divided into types which are usually identified by a single haploytype of the highly variable nuclear internal transcribed spacer (ITS1 and ITS2) regions of the rDNA operon (LaJeunesse *et al.* 2004; Sampayo *et al.* 2009; Bongaerts *et al.* 2011). While nuclear ribosomal DNA cistrons are multicopy regions, where there may be considerable intra-genomic variation among copies, they are frequently used to distinguish *Symbiodinium* types at ecologically relevant levels (Baker 2001; LaJeunesse *et al.* 2004, 2010; Berkelmans & van Oppen 2006; Thornhill *et al.* 2007; Bongaerts *et al.* 2011).

Different types of *Symbiodinium* are physiologically adapted to certain environments (Baker 2001; Fabricius *et al.* 2004; Jones *et al.* 2008). Indeed, *Symbiodinium* types may vary among geographic locations and along natural environmental gradients of temperature, depth and water quality (Fabricius *et al.* 2004; LaJeunesse *et al.* 2004, 2010; Garren *et al.* 2006; Bongaerts *et al.* 2010), suggesting physiological differences (Little *et al.* 2004; Berkelmans & van Oppen 2006). For example, both observational and experimental evidence indicates that *Symbiodinium* D types are generally more thermally tolerant than clade C types in the same coral host, and that a switch in dominance, from C to D, can occur in some hosts following heat stress (Baker 2001; Fabricius *et al.* 2004; Berkelmans & van Oppen 2006; Abrego *et al.* 2008). Recent work with *Symbiodinium in vitro* indicates that the physiological response to increased pCO_2 may also be type specific; Brading *et al.* (2011) showed that *in vitro* the growth and photosynthetic capacity of two different clade A *Symbiodinium* types increased with elevated pCO_2, while that of another type A and a type B *Symbiodinium* remained unaffected. Types of *Symbiodinium* that are capable of utilising the more abundant pCO_2 may therefore be expected to become dominant within a coral host and out-compete types that cannot (Brading *et al.* 2011; van Oppen *et al.* 2011b). However, to date it remains unknown if corals are able to respond to rising CO_2 concentrations by changing to better adapted dominant *Symbiodinium* types after long-term exposure to elevated pCO_2 in the field.

Other studies that have investigated the response of *Symbiodinium* to OA were based on algal cultures (Buxton *et al.* 2009; Brading *et al.* 2011), relatively short-term exposure experiments of *in hospite Symbiodinium* communities in corals (Reynaud *et al.* 2003; Anthony *et al.* 2008; Herfort *et al.* 2008; Marubini *et al.* 2008; Crawley *et al.* 2010; Suwa *et al.* 2010; Iguchi *et al.* 2012) or *Symbiodinium* in host taxa other than corals (Towanda & Thuesen 2012; Uthicke & Fabricius 2012). While these works have been informative, they do not have the capacity to predict the long-term effects of OA on potentially dynamic coral-algal symbioses.

Corals acquire their *Symbiodinium* either maternally, from already infected eggs or as brooded planula larvae (vertical transmission), or from the environment during the juvenile phase (horizontal transmission). Vertically transmitting species have been shown to have higher fidelity for *Symbiodinium* type than horizontal transmitters (Fabricius *et al.* 2004; LaJeunesse *et al.* 2004). Multiple *Symbiodinium* types can infect juveniles in horizontally transmitting coral species (Weis *et al.* 2001; Ulstrup & Van Oppen 2003; Little *et al.* 2004; Abrego *et al.* 2009b, a) and recent work has identified multiple background symbiont types occurring within a single coral (Mieog *et al.* 2007; Correa *et al.* 2009; Silverstein *et al.* 2012). These features present avenues for symbiont differences to arise between con-specific colonies growing in different environments (Rowan *et al.* 1997; Cooper *et al.* 2011).

The recent discovery of three volcanic CO_2 seeps in Milne Bay, Papua New Guinea (PNG) (Fabricius *et al.* 2011), provides a unique opportunity to investigate the long term effects of increased pCO_2 on the adjacent coral reef communities *in situ*. In this study we compare the dominant *Symbiodinium* types harboured by six species of scleractinian coral that have settled and grown within three CO_2 seep sites to those of three adjacent control areas. The *Symbiodinium* type associating with a coral species from an extreme seep environment is also examined. Because the productivity of *Symbiodinium* may be limited by available inorganic carbon (Rowan *et al.* 1996; Leggat *et al.* 1999; Buxton *et al.* 2009) and certain *Symbiodinium* types may be able to out compete others under OA scenarios (Brading *et al.* 2011; Towanda & Thuesen 2012), we hypothesised that the frequency of certain *Symbiodinium* types within hosts may change at the seep sites in response to life-long exposure to high CO_2.

2.3 Materials and Methods

Study Site and Species

Samples were collected from three shallow water (3 - 5m), CO_2 seeps in Milne Bay, PNG, named Upa-Upasina, Esa' Ala and Dobu. Three control areas with ambient pH conditions, one adjacent to each seep site, were also sampled. Samples were collected under research permit by the Department of Environment and Conservation of Papua New Guinea to the Australian Institute of Marine Science (AIMS). Seep and control sites are described in detail by Fabricius *et al.* (Fabricius *et al.* 2011). Notably, seep areas are dominated by massive *Porites* spp. and scleractinian coral diversity declines sharply within the areas influenced by the seeps (Fabricius *et al.* 2011). Sample collection at seep sites was restricted to areas with pH values of 7.8 - 7.9 (pCO_2 ~500 to 900 ppm) as this is what is predicted for the world's oceans by the end of the century and thus considered ecologically relevant (IPCC 2021). The extreme seep samples were collected from the seep at Upa-Upasina from the most intense bubbling areas where individual faviid and *Porites* coral colonies still occurred. Here pH values were observed to decline to a pH of 6.9 during the day (unpublished data), far beyond those predicted for the end of the century. Samples were collected on SCUBA over the course of three field trips from August 2010 to December 2011. During a sample collection dive, <2 cm coral fragments were removed from adult colonies of each species that were at least 5m apart, placed into separate bags, and preserved in 100% ethanol upon surfacing.

For the main CO_2 comparison study, a total of 433 colonies were sampled from the six species of scleractinian coral across the six sites (three seep and three controls). The species sampled were *Acropora millepora, Pocillopora damicornis, Seriatopora hystrix, Porites cylindrica,* massive *Porites sp. and Galaxea fascicularis.* The massive *Porites sp.* designation potentially consisted of a number of *Porites* species with massive growth forms, and was left with the *sp.* label as accurate species identification was not obtained. A summary of the sample numbers for each species at each site is given in Table 2.1. The species of coral sampled included one horizontally transmitting species (*A. millepora*) and five vertically transmitting species. While it would have been preferable to sample more horizontally transmitting species it was not possible as there is an under-representation of these corals within the seep sites (Fabricius *et al.* 2011) and sufficient sample sizes were not attainable for other coral species at all sites. Two species are predominately brooders (*P. damicornis* and *S. hystrix*; both vertical transmitters) while the other species are broadcast spawners. Due to the calm conditions and absence of cyclones at Latitude 9°S and the <150

m length of the seep sites, there is little potential even for the branching colonies to have entered the seep sites via fragmentation rather than during settlement.

Favites pentagona was the only species that occurred in moderate abundance at the extreme seep site and 10 colonies were sampled from the Upa-Upasina seep, bringing the total sample number to 443 (Table 2.1). While this species was not used to compare *Symbiodinium* types between CO_2 exposures and sites, it was investigated to examine whether extreme pCO_2 environments would result in the occurrence of unusual *Symbiodinium* types.

Table 2.1: The number of samples and the *Symbiodinium* ITS2 DGGE profiles for each coral species from each site used in this study.

Coral species[a]	**Symb. Acqu.**[b]	**Dispersal**[c]	**Symb. Profile**[d]	**Upa-U Seep**[e]	**Upa-U Ctr**	**Dobu Seep**	**Dobu Crt**	**Esa' A Seep**	**Esa' A Ctr**
A. millepora	Horizontal	Broadcast	Am1	15	15	10	13	13	12
			Am2	0	0	2	2	0	0
			Am3	0	0	2	0	0	3
P. damicornis	Vertical	Brooding	Pd1	15	15	15	14	14	13
			Pd2	0	0	0	1	1	1
S. hystrix	Vertical	Brooding	Sh1	15	14	0	8	0	0
			Sh2	0	1	0	0	0	0
			Sh3	0	0	0	2	9	14
			Sh4	0	0	0	3	0	1
			Sh5	0	0	15	2	6	0
P. cylindrica	Vertical	Broadcast	Pc1	7	10	0	9	10	10
			Pc2	1	0	1	0	0	0
			Pc3	1	0	0	0	0	0
Porites sp.	Vertical	Brooding	Pm1	10	10	10	9	8	10
			Pm2	0	0	0	1	1	0
			Pm3	0	0	0	0	1	0
G. fascicularis	Vertical	Broadcast	Gf1	2	3	1	1	2	0
			Gf2	8	8	9	5	9	10

F. pentagona	Horizontal	Broadcast	Fp1	2	0	0	0	0	0
			Fp2	8	0	0	0	0	0

[a]The coral species *Acropora millepora, Pocillopora damicornis, Seriatopora hystrix, Porites cylindrica,* massive *Porites* sp., *Galaxea fascicularis* and *Favites pentagona* used in this study.

[b] The modes of *Symbiodinium* acquisition (*Symb.* Acqu.) employed by each coral species. Horizontal being from the environment (post-settlement) and vertical from maternal sources.

[c]The reproductive strategy of each coral species with either broadcast spawning gametes, larvae brooded in the parental colony or a combination of the two.

[d]The assigned *Symbiodinium* ITS2 DGGE profiles (Figs 2.1, 2.3)

[e]The Seep and control (Ctr) sites at Upa-Upasina (Upa-U), Dobu and Esa' Ala (Esa' A)

DNA extraction and Denaturing Gradient Gel Electrophoresis Profiling of the Internal Transcribed Spacer 2 region

DNA was extracted using a modified Chelex extraction protocol (Walsh *et al.* 1991) which allows the inexpensive and rapid extraction of a high volume of samples. A small fragment of coral tissue of approximately 2 mm^2 was removed from the coral branch with a fine pair of forceps and placed into a well of a 300 µL 96 well plate (Axygen). To each well 100 µL of extraction buffer was added. This buffer contained 10 µL of 20 g/L Proteinase K solution and 100 µL of 5% Chelex buffer (Chelex-100 BioRad) in 0.01 M Tris (pH 8.3). The plate was incubated at 55°C for 3 hours, with a vortex every hour, and then heat shocked at 95°C for 20 min to denature the Proteinase K enzyme. The plate was then centrifuged at 335.4 g for 5 min and stored at -20°C before polymerase chain reactions.

The use of denaturing gel gradient electrophoresis (DGGE) profiling of the internal transcribed spacer region 2 (ITS2) is a widely used method for identifying distinct *Symbiodinium* lineages (Sampayo *et al.* 2009; LaJeunesse *et al.* 2010; Thornhill *et al.* 2010; Bongaerts *et al.* 2011; Silverstein *et al.* 2011) and was the method employed in this study. DGGE produces a profile for each sample that consists of one or more bands of the most numerically abundant ITS2 variants within the ribosomal array (Thornhill *et al.* 2007), that can differ from one another by a single base pair (Sampayo *et al.* 2009; LaJeunesse *et al.* 2010; Thornhill *et al.* 2010). This results in multiple bands being evident in DGGE profiles. One µL of the supernatant from the Chelex extraction was taken as DNA template from each sample and amplified under standard conditions using the Multiplex Kit (Qiagen). The primers "ITS2 clamp" and "ITSintfor 2" were used in 12 µL reactions following a touchdown thermal cycle, including a 30 min final extension at 72°C,

following LaJeunesse (2002). PCR products were visually checked on 1% agarose gels stained with ethidium bromide prior to DGGE. Amplified ITS2 PCR products were separated using 8% poly-acrylamide gels with a 35-55% denaturant gradient (formamide and urea) in an INGENY PhorU DGGE unit for 15 hrs at 75V. Gels were stained with SYBR Gold (Invitrogen) prior to examination on a transilluminator.

Sequencing and Statistical analyses

Each sample was assigned a profile based on common banding patterns following Sampayo *et al*. (2007). Profiles are defined as the dominant subset of the *Symbiodinium* ITS2 community present within each sample. Profiles were assigned a *Symbiodinium* community by sequencing the dominant bands from at least two representative samples of each profile. In each case, identical sequences were obtained for the analogous bands in the same profile from different samples, confirming profile and band designations. These profiles were given an alphanumeric designation which comprised of a species code and then a profile number. For example Am1 and Am2 were two different profiles seen in *A. millepora*. Where samples from the same species were run on different gels the most common profiles from earlier gels were used as references on latter ones. A representative of each profile from each different gel was then run next to one another on a single gel to confirm category designations between gels. To determine symbiont type, a representative of each dominant band from the lowest relative position on the DGGE gel from each profile was cut from the gel, left to elute overnight in 40 µL of UV sterilized, ultrapure H_2O, and re-amplified without the GC-rich reverse primer for direct sequencing in the forward direction (Macrogen Ltd., Korea). Following LaJeunesse *et al.* (2010), bands that were relatively high on the DGGE gel were excluded from the study to minimise the sequencing of heteroduplexes that run higher on the gel as they denature more readily. Many of the minor and higher bands were also cut and sequenced to check for background types and/or heteroduplexes. However, these were excluded from later analyses as no patterns were evident between CO_2 exposures and all minor bands clustered around the dominant band from the same profile. Each sequence was aligned with ClustalW and visually checked (BioEdit Sequence Alignment Editor) before being compared with sequences in the public library of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). An unrooted haplotype network was constructed from the sequence alignments using the program TCS (version 1.21). Networks were constructed by treating gaps as a fifth character state and with a 90% connection limit between haplotypes. Band II was included in the clade D network even though it had a 26 base pair indel as it matched the same Genbank sequences as

the other clade D bands. Published sequences in Genbank that matched the newly obtained ITS2 sequences most closely were included in the haplotype networks for type identification.

A representative of each of the profiles, from all species, was then run on a single DGGE gel and, in conjunction with sequence data (to check for co-migration of dissimilar ITS2 types), the presence/absence of each of the dominant bands was scored to allow for between species comparisons of *Symbiodinium* profiles (Table 2.S1) (Sampayo *et al.* 2007). This presence/absence matrix was used to conduct a sequential permutational multivariate analysis of variance based on redundancy analysis to compare the distribution of *Symbiodinium* types between the six species, the three sites, and the two CO_2 exposures nested within each of the three sites (Legendre *et al.* 2011). All statistics were completed using the vegan package in the statistical program R (version 2.15.1) (R Development Core Team 2021).

2.4 Results

A total of 20 *Symbiodinium* profiles, characterised by 13 distinct dominant bands in DGGE profiles, were identified across the seven species of coral (Fig 2.1). This includes both the six species of coral investigated in the main CO_2 comparison study and *F. pentagona* from the extreme seep site. Twelve out of the 20 DGGE profiles contained more than one dominant band (Table 2.S1), and often bands occurred in more than one profile within species. For example all but one of the profiles found in *P. cylindrica* and massive *Porites* sp. contained band IV, while band IX was common amongst three of the five *S. hystrix* profiles. Bands VI and VII displayed very similar migration across the DGGE gel (Fig 2.1), however sequence data indicated they differed by six base pairs (Fig 2.2). Four profiles were identical across coral species (Am1 and Pc3, Pm1 and Pc1, Pm3 and Pc2 as well as Gf2, Sh3 and Fp2) and the identity of these *Symbiodinium* communities was confirmed with the sequence data. All other profiles differed from one another by at least one dominant band. Therefore, the 443 samples contained a total of 15 distinct *Symbiodinium* communities.

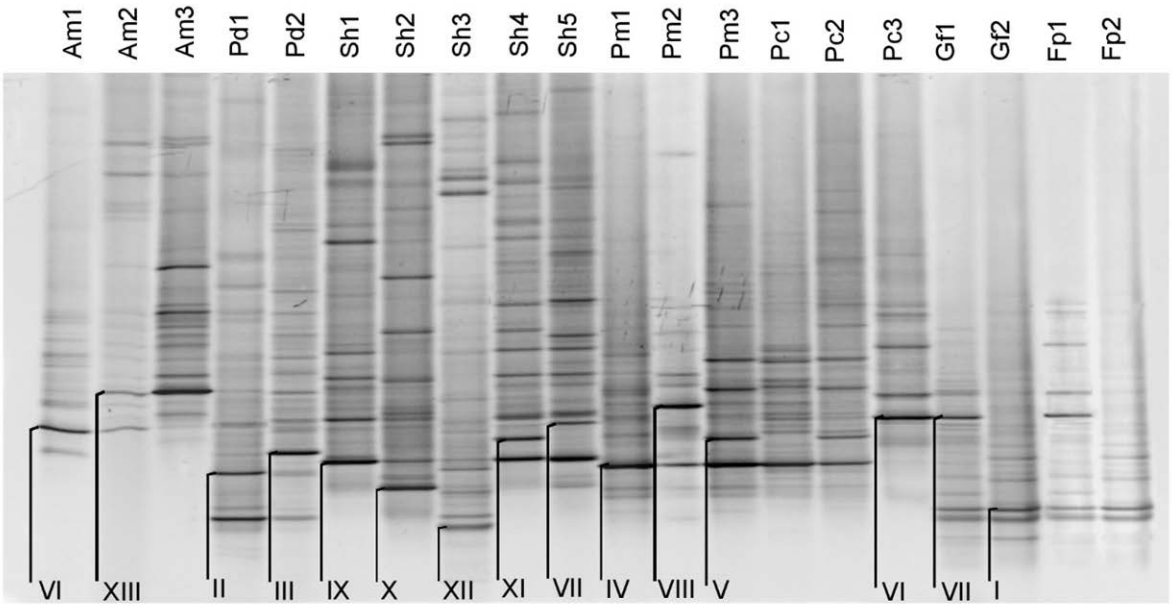

Fig 2.1: A representative of each *Symbiodinium* ITS2 DGGE profile from the seven coral species investigated. The *Symbiodinium* profiles from the species *Acropora millepora* (Am)*, Pocillopora damicornis* (Pd)*, Seriatopora hystrix* (Sh)*, Porites cylindrica* (Pc)*,* massive *Porites* sp. (Pm), *Galaxea fascicularis* (Gf) and *Favites pentagona* (Fp) are shown at the top of each DGGE column. Each of the 13 dominant bands (I-XIII), which characterise the profiles, are also indicated. Bands VI and VII are labelled twice as they appear to co-migrate, however sequence data differentiates them.

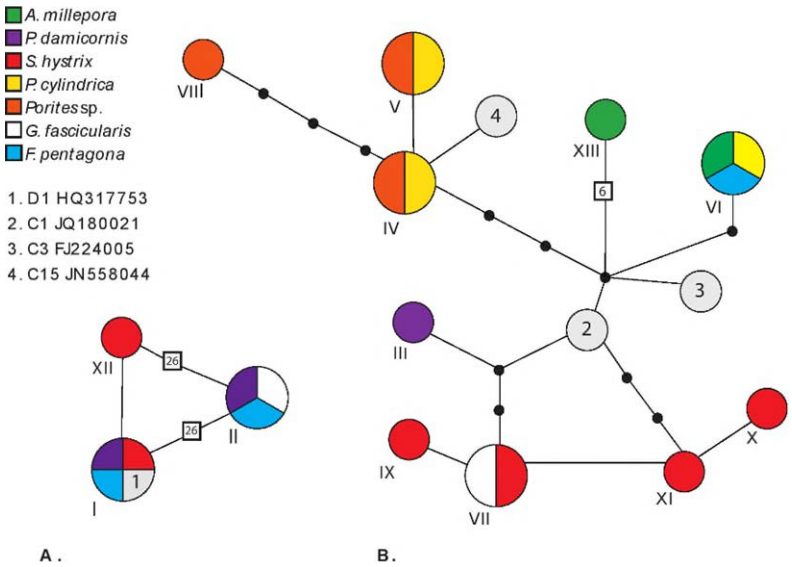

Fig 2.2: *Symbiodinium* ITS2 haplotype networks of the 13 dominant bands identified in this study. Parsimony networks of Clade D (A) and Clade C (B) *Symbiodinium* ITS2 haplotypes from dominant bands identified in this study. Coral species are shown in different colours and Roman numerals indicate dominant band numbers. Previously published sequences are also indicated (1 - 4) along with their Genbank accession number. Each node represents a base pair change and indel lengths are shown by the boxed numbers along branches. Bands III to XI and XIII represent clade C types, while bands I, II and XII represent clade D type *Symbiodinium.* Pies indicate the presence of *Symbiodinium* types in multiple coral species and are not indicative of frequency.

All 13 dominant ITS2 bands belonged to either clade C (61% of samples, 10 haplotypes) or D (39% of samples, 3 haplotypes). Twelve of these bands were novel types (Genbank accession numbers KC631398-KC631409), not previously recorded in the Genbank database. Some of these types differed from one another by a single base pair substitution or by a single insertion or deletion (Fig 2.2). While it was not the purpose of this study to name these new types, they did cluster most closely to D1 and C1, C3 and C15 (Genbank Accession numbers HQ317753, JQ180021, FJ224005 and JN558044, respectively) (Fig 2.2a and b). The ITS2 diversity in clade D was comparably low with band I matching the D1 sequence exactly, band XII being only one base pair different and band II being 26 base pairs different due to a large indel (Fig 2.2a). The clade C network was considerably more complex (Fig 2.2b). While none of the C type bands had a 100% match with the C1, C3 and C15 sequences, many clustered within a few base pair substitutions.

In the coral species *A. millepora,* all sequenced bands were closely related to C1 and C3 (Fig 2.2b). In *P. damicornis,* the abundant profile Pd1 contained ITS2 variants that either matched or clustered most closely to D1 while the less frequent profile, Pd2, was most closely related to C1. The *Symbiodinium* profiles of *S. hystrix* were the most diverse of the species investigated in the present study. Of these, the most common variants Sh1 as well as Sh5 and Sh3 clustered with both C1 and D1, respectively (Fig 2.2a and b). The majority of both the *P. cylindrica* and massive *Porites sp.* samples clustered with the C15 type, however one *P. cylindrica* sample was more closely related to C1 and C3. The vast majority of *G. fascicularis* samples contained profiles with bands that clustered with the D1 sequence only. The remaining *G. fascicularis* samples displayed the same banding pattern but also included an extra band that clustered closely with C1 (Fig 2.2a and b) indicating both *Symbiodinium* clades C and D were present within the same coral host.

In the main CO_2 comparison study differences between locations were minor in all of the six investigated species except *S. hystrix*, regardless of CO_2 exposure (Fig 2.3). Approximately 85 - 95% of the samples exhibited the same symbiont profiles at all locations (Table 2.1). The remaining percentage comprised of rare types that only occurred in one or two samples, and for which no correlations were evident with CO_2 exposures (Fig 2.3, Table 2.1). There were strong differences between species and weak differences between sites and CO_2 exposures in *Symbiodinium* types when all coral species were combined in the one analysis (sequential permutation test for RDA, species: $F_{(5, 422)} = 62.7$, $p = 0.01$; site: $F_{(2, 422)} = 4.5$, $p = 0.01$; CO_2 exposure nested within site: $F_{(3, 422)} = 2.3$, $p = 0.01$). This pattern was primarily driven by site-specific differences in *S. hystrix* profiles (sequential permutation test for RDA, site: $F_{(2, 84)} = 27.6$, $p = 0.01$; CO_2 exposure nested within site: $F_{(3, 84)} = 5.9$, $p = 0.01$). For *S. hystrix,* the Dobu seep site was comprised entirely of Sh5, while Sh1 dominated both Upa-Upasina sites. The Esa' Ala sites were dominated by Sh3, with one third of samples at the Esa' Ala seep site characterised as Sh5 (Fig 2.3, Table 2.1). Profiles Sh1 and Sh5 were characterised by two dominant bands for which band IX was common between the two profiles (Fig 2.1). Band VII was also present in the Sh5 profile, differentiating it from Sh1, however it only deviated from band IX by a single base pair substitution (Fig 2.2). All other species were non-significant, however there was a marginally insignificant effect of CO_2 exposure on *P. cylindrica* (sequential permutation test for RDA, site: $F_{(2, 43)} = 1.0$, $p = 0.97$; CO_2 exposure nested within site: $F_{(3, 43)} = 3.9$, $p = 0.06$). which was not considered to be ecologically relevant (sampling was unbalanced as only one sample was found at Dobu High CO_2, and only three of 49 samples yielded different types in the collection). As such, CO_2 exposures did not lead to environmentally significant changes in symbiont types, regardless of the mode of symbiont acquisition or reproductive strategy, for all six coral species investigated in this study.

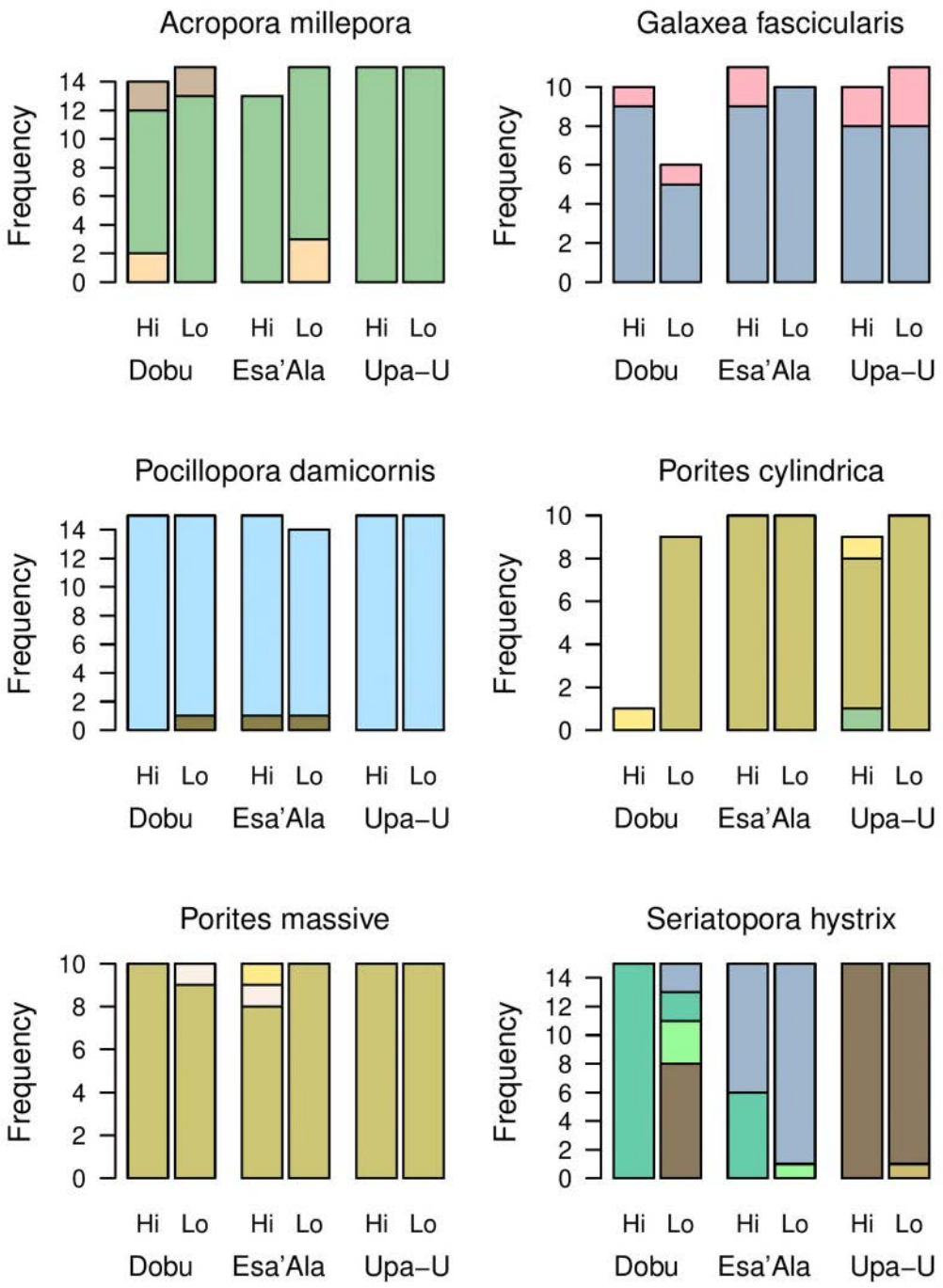

Fig 2.3: The frequency of different *Symbiodinium* ITS2 profiles between sites and CO_2 exposures. The different *Symbiodinium* ITS2 profiles from the corals *Acropora millepora, Pocillopora damicornis, Seriatopora hystrix*, *Porites cylindrica*, massive *Porites sp.* and *Galaxea fascicularis* found at the three sites, each with High (Hi) and control (Lo) CO_2 exposures. Each colour corresponds to one of the 14 unique

Symbiodinium ITS2 profiles found in these coral species. Colours common between sites or coral species indicate synonymous *Symbiodinium* profiles (See also Table 2.S1). The different colour schemes represent clade C (shades of green, yellow and brown), D (shade of grey and blue) and mixed (pink) *Symbiodinium.*

The groupings in the RDA plot based on the presence/absence of DGGE bands between profiles further indicated that some *Symbiodinium* types were common between coral species but that some coral species contained *Symbiodinium* types that differed from one another (Fig 2.S1). This constrained analysis and the associated permutation analysis showed that differences between species were very strong compared to the differences between Sites and CO_2 levels.

In the *F. pentagona* samples collected from the extreme seep site, all dominant *Symbiodinium* types were also observed in other coral species examined in this study. Eighty per cent of the *F. pentagona* samples had the Fp2 profile, consisting of bands closely related to D1, while the remaining samples (Fp1) also contained a clade C type (Band VI) (Fig 2.2). The profiles shared between *F. pentagona, G. fascicularis* and *S. hystrix* (Fp2, Gf2 and Sh4) as well as the joint occurrence of dominant bands between Fp1 and ten other profiles (Table 2.S1) indicated that the *Symbiodinium* types that occur in the extreme seep area were also commonly found at both the less extreme seep and the control sites. As such, there was no evidence to suggest that the type of *Symbiodinium* associating with the seven species of coral investigated in this study was influenced by the exposure to the CO_2 seeps.

2.5 Discussion

This study shows that the dominant *Symbiodinium* community in scleractinian corals did not change despite a life-time (and for brooding species possibly even trans-generational) exposure to elevated concentrations of CO_2 around volcanic CO_2 seeps. The seep sites represent oceanic pCO_2 conditions in line with IPCC scenarios predicted towards the year 2100, albeit without the predicted rise in temperature (Fabricius *et al.* 2011; IPCC 2021). While it was hypothesised that a change in *Symbiodinium* types would occur, no such change was observed. Instead, the *Symbiodinium* of five of the six coral species investigated between sites was dominated by a single ITS2 profile consisting of clade C or D types. The majority of symbiont types were consistent between sites within species, and some of the types were also observed in several coral species. Furthermore, the *Symbiodinium* types found in a seventh species of coral

from the extreme seep area (dominated by types similar to D1) were also found commonly at the moderate seep and control areas. (Fabricius *et al.* 2011; IPCC 2021).

The *Symbiodinium* types identified in the present study clustered closely to C1, C3, C15 and D1 sequences from Genbank, however the vast majority were novel types whose ITS2 haplotype had not previously been recorded. *Symbiodinium* types C1, C3, C15 and D1 are common throughout the Indo-Pacific and may form symbiosis with a variety of taxa (LaJeunesse *et al.* 2010). To date there have been hundreds of unique *Symbiodinium* ITS2 haplotypes reported (LaJeunesse 2002) and, as per the present study, new sites often reveal further diversity (Silverstein *et al.* 2011). The ITS2 diversity of clade C *Symbiodinium* types is greater than that of clade D types (Correa & Baker 2009; Bongaerts *et al.* 2010; Silverstein *et al.* 2011; van Oppen *et al.* 2011a). The high representation of D type *Symbiodinium* in the present study (found in 4 of the seven species, at a total of 39% of all samples) may reflect the low latitude and subsequent warm waters of the study sites (approximately 9° South), as the frequency of certain D1 types have been observed to increase in warm waters (Baker 2001; Fabricius *et al.* 2004; Jones *et al.* 2008).

The few studies that have investigated the physiological response of *Symbiodinium* to OA, either *in hospite* or in culture, have found conflicting results. Increased DIC and pCO_2 has been reported to increase net production in some studies (Herfort *et al.* 2008; Marubini *et al.* 2008; Brading *et al.* 2011; Towanda & Thuesen 2012; Uthicke & Fabricius 2012), while others have found negligible or negative effects (Reynaud *et al.* 2003; Langdon & Atkinson 2005; Anthony *et al.* 2008; Buxton *et al.* 2009). These studies not only utilised different experimental methodologies and host species, but few have identified the sub-cladal type of *Symbiodinium* under experimentation, further limiting comparisons. Work by Brading *et al.* (Brading *et al.* 2011) indicated that in culture, two A type *Symbiodinium* were better able to take advantage of elevated levels of inorganic carbon than another type A and type B through increased growth and photosynthesis. Clade C and D types, which are dominant in corals of the Indo-Pacific (van Oppen *et al.* 2001; Jones *et al.* 2008; LaJeunesse *et al.* 2010), have not been subject to similar physiological studies, and such work on common Indo-Pacific Clade C and D type *Symbiodinium* is warranted.

In the present study there was no indication that the coral investigated had acclimatised to high pCO_2 at the seeps by changing their dominant type of *Symbiodinium*. If indeed certain *Symbiodinium* types outperform others in response to OA (van Oppen *et al.* 2011b), those types were not found at the study sites due to environmental or geographic constraints (Fabricius *et al.* 2004; LaJeunesse *et al.* 2010; Bongaerts *et al.* 2011), or in the host species investigated due to host-symbiont specificity (Baker 2003; Little *et al.* 2004; Goulet 2006; Baird *et al.* 2007). While more work at CO_2 seep sites is needed to determine

if the increased DIC and pCO_2 increases production in coral holobionts *in situ*, we have found no evidence to suggest that any difference is sufficient enough for one *Symbiodinium* type to outcompete another. Moreover, recent work by Howells *et al.* (Howells *et al.* 2012) indicates that there may be substantial adaptation within the same sub-cladal types of *Symbiodinium* to local environmental conditions. This indicates that there is potential for the seep *Symbiodinium* to have undergone local adaptation to the OA conditions that is sufficient to prevent selection of certain types over others. Physiological studies that monitor the response of both the coral and the algal partners, as well as fine scale population genetic studies, are needed to identify any potential local acclimatisation or even adaptations.

The diversity of coral communities is sharply reduced at the three seep sites compared with the control sites, although coral cover remains similar (Fabricius *et al.* 2011). Seep communities are dominated by massive *Porites* spp.*,* while adjacent control reefs are comparatively rich in *Acropora* spp. (Fabricius *et al.* 2011). Our study has shown that the massive *Porites* sp. at the seeps house the same C15-like *Symbiodinium* as at the control sites. It is possible that C15-like types can take advantage of the additional CO_2, buffering the host from the negative effects of OA. However, it is unlikely that the association with C15-like *Symbiodinium* types alone accounts for the dominance of massive *Porites* spp. at seep sites, as *P. cylindrica* contained the same C15-like *Symbiodinium* but is uncommon at the seeps (Fabricius *et al.* 2011). It is hypothesised that the observed difference in community structure may therefore be related to differences in the inherent stress tolerances of the coral hosts themselves (Abrego *et al.* 2008), resulting in shifts in competitive advantages from sensitive to persistent and long-lived taxa. Massive *Porites* are comparatively tolerant to a variety of stressors (Colgan 1987; Done & Potts 1992; Marshall & Baird 2000; Fabricius *et al.* 2005) and may be less affected by the negative effects of OA compared to branching *Acropora* spp*.* (Albright *et al.* 2008, 2010; Anthony *et al.* 2008).

Reduced recruitment success at high pCO_2 may also contribute to the observed shift in coral community structure. Of the coral species examined in the present study, *A. millepora* was the sole horizontally transmitting species that occurred at sufficient numbers to be sampled at seep sites. Such under-representation of horizontally transmitting species at the seeps may be due to constraints intrinsic to their mode of symbiont acquisition, potentially suggesting a high sensitivity of free-living *Symbiodinium* to high CO_2. This theory appears possible as about 75% of Pacific coral species are horizontally transmitting (Baird *et al.* 2009), yet very few are found at the seep sites. Although juvenile *A. millepora* are obviously able to take up symbionts at the seep sites, even moderate declines in algal infection rates under OA may

reduce recruitment success of horizontal transmitters (Suwa *et al.* 2010), potentially contributing to their under-representation in the coral community.

This study has shown that the observed differences in scleractinian coral communities at the Milne Bay CO_2 seep sites is unlikely to be due to differences in the dominant type of *Symbiodinium* harboured by the particularly successful corals. The data suggest that the inherent stress tolerance and resilience of the coral holobiont, rather than a change in symbiotic association with more tolerant *Symbiodinium* types, determined the ability of massive *Porites* to live under high CO_2 conditions. No evidence was detected to suggest that any of the other coral species may be able to adapt or acclimatise to OA conditions by switching or shuffling the dominant type of *Symbiodinium* they harbour to types that are better able to utilise the more abundant DIC. This was reiterated by the overlap in types found commonly throughout control sites and the extreme seep site. However, the relative contribution of reduced recruitment success of horizontally transmitting corals, and the physiological performance of sensitive host corals associated with different *Symbiodinium* types requires further investigation to better understand the underlying mechanisms responsible for structuring these coral reef communities, and to predict how coral reefs will be shaped by ongoing and rapidly progressing acidification of the world's oceans.

2.6 Supplementary material

Supporting supplementary material can be found at:
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0063985#s5

Chapter 3: Ocean acidification affects productivity but not the severity of thermal bleaching in some tropical corals

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3.1 Abstract

Increasing carbon dioxide emissions are raising sea surface temperature (SST) and causing ocean acidification (OA). While higher SST increases the frequency of mass coral bleaching events, it is unclear how OA will interact to affect this process. In this study, we combine *in situ* bleaching surveys around three tropical CO_2 seeps with a two-month two-factor (CO_2 and temperature) tank experiment to investigate how OA and SST in combination will affect the bleaching susceptibility of tropical reef corals. Surveys at CO_2 seep and control sites during a minor regional bleaching event gave little indication that elevated pCO_2 influenced the bleaching susceptibility of the wider coral community, the four most common coral families (Acroporidae, Faviidae, Pocilloporidae or Poritidae), or the thermally sensitive coral species *Seriatopora hystrix*. In the tank experiment, sub-lethal bleaching was observed at 31°C after 5 days in *S. hystrix* and 12 days in *Acropora millepora*, while controls (28°C) did not bleach. None of the measured proxies for coral bleaching were negatively affected by elevated pCO_2 at pH_T 7.79 (vs 7.95 pH_T in controls), equivalent to ~780 µatm pCO_2 and an aragonite saturation state of 2.5. On the contrary, high pCO_2 benefitted some photophysiological measures (although temperature effects were much stronger than CO_2 effects): maximum PSII quantum yields and light-limited electron transport rates increased in both species at high pCO_2, while gross photosynthesis and pigment concentrations increased in *S. hystrix* at high pCO_2. The field and laboratory data in combination suggest that levels of OA up to a pH_T of 7.8 will have little effect on the

sensitivity of tropical reef corals to thermal bleaching. Indeed, some coral species appear able to utilise the more abundant dissolved inorganic carbon to increase productivity, however, these gains offset only a small proportion of the massive bleaching-related energy losses during thermal stress.

3.2 Introduction

The continual anthropogenic pollution of Earth's atmosphere with carbon dioxide (CO_2) is causing planetary warming and ocean acidification (OA). These global changes are ongoing and projected to be exacerbated into the future as atmospheric CO_2 levels continue to rise (IPCC 2021). In the marine environment, climate change is raising sea surface temperature (SST), while the oceanic uptake of CO_2 is causing a suite of chemical changes, increasing dissolved inorganic carbon (C_T) and reducing carbonate saturation states and pH (Langdon & Atkinson 2005). The warming and OA stressors are occurring simultaneously and are considered amongst the greatest threats to marine biodiversity (Kleypas 1999; Hoegh-Guldberg *et al.* 2007). It is thus necessary to consider the effects of these global stressors in conjunction with one another as their effects may differ in combination and isolation (Harvey *et al.* 2013).

Coral reefs are amongst the most vulnerable ecosystems to the changes associated with CO_2 emissions (Hoegh-Guldberg 1999; Anthony *et al.* 2011). The primary concern under increasing SST is the breakdown of the symbiosis between scleractinian corals and dinoflagellate algae of the genus *Symbiodinium* (zooxanthellae) in a process known as bleaching (e.g. Brown 1997; Dubinsky *et al.* 2011). During thermal bleaching, damage to the photosynthetic machinery in *Symbiodinium*, including photosystem II (PSII), reduces their photosynthetic capacity and may eventually lead to their expulsion from the coral host (Dubinsky *et al.* 2011). Loss of autotrophy can starve the coral, and large-scale coral mortality has been observed when conditions that induce bleaching persist for some time (Brown 1997; e.g. Berkelmans *et al.* 2004). Periodic heat stress events, where temperatures exceed the long-term summer maximum for weeks at a time, are superimposed upon the gradual warming trend and often prompt bleaching (Brown 1997; Berkelmans *et al.* 2004). The frequency of extreme weather conditions and subsequent heat stress events are projected to increase with rising atmospheric CO_2 (IPCC 2021). However, it remains unclear how the thermal bleaching susceptibility of corals will be influenced by OA.

The issue of OA has received less attention than global warming; however recent years have seen a surge in research effort. To date, coral reef research has primarily focused on the effects of OA on rates of calcification (e.g. Anthony *et al.* 2008; Uthicke & Fabricius 2012) and the metabolic demands that potentially increase in order to maintain high calcification rates (Cohen & Holcomb 2009; Cyronak *et al.* 2015). However, other effects of increasing pCO_2 remain less clear, especially on the corals' *Symbiodinium* partners, and many results are contradictory. Some authors report that pCO_2 increases can enhance primary production in *Symbiodinium* in a range of host taxa and suggest carbon is the limiting substrate for photosynthesis (Crawley *et al.* 2010; Brading *et al.* 2011; Uthicke & Fabricius 2012), while other studies have seen negligible (Wall *et al.* 2013) and even negative effects of increased pCO_2 on photophysiology (Anthony *et al.* 2008).

Studies examining the interactive effects of elevated SST and DIC on the bleaching susceptibility and photobiology of corals are few, and often have contradictory results. Some investigators have found increases in pigment content and the number of *Symbiodinium* per coral cell under elevated temperatures and CO_2 (Reynaud *et al.* 2003), while others have found null (Schoepf *et al.* 2013; Wall *et al.* 2013) or opposite results. Anthony *et al.* (2008) observed declining pigmentation and oxygen production in two species of coral exposed to OA and temperature, suggesting that elevated CO_2 may increase thermal bleaching severity in corals. On the other hand, pCO_2 increases may boost primary production, and could reduce the severity of thermally induced bleaching (Hoogenboom *et al.* 2012).

An important factor determining responses to rising SST and OA is acclimatisation through prolonged or repeated prior exposure to the stressor. While short-term experimental studies are certainly informative, in isolation they are unable to account for potential acclimatisation. Longer-term tank experiments provide more robust results as study organisms become acclimatised with the experimental environment (Krief *et al.* 2010). Some evidence is emerging which suggests corals from reefs with a history of heat stress tend to bleach less severely during subsequent heat stress events (Berkelmans *et al.* 2004; Maynard *et al.* 2008). Tropical CO_2 seeps also allow studies to be conducted on organisms that have been acclimatised to higher levels of pCO_2 throughout their lifetime (Fabricius *et al.* 2011). More longer-term experiments and *in situ* studies around CO_2 seeps are needed to more confidently predict how chronic OA will interact with warming SST to shape coral reefs into the future.

This two-part study combines field observations and an experiment to investigate the effects of increased pCO_2 on coral thermal bleaching susceptibility. Part 1 reports on field data collected during a mild bleaching event in Milne Bay Province, Papua New Guinea in April 2011, including at coral reefs surrounding three CO_2 seeps. To determine if zooxanthellate corals are more or less susceptible to thermal bleaching if they had been exposed to elevated levels of pCO_2 since settlement, surveys were used to quantify coral pigmentation near and away from the seeps. This represents the first set of direct measurements of *in situ* coral thermal bleaching susceptibility in the face of OA (but see Manzello 2010). The field data were complemented by a two month crossed two-factor laboratory experiment to investigate the interactive effects of elevated pCO_2 and temperature on the bleaching susceptibility, photobiology, photosynthetic production and *Symbiodinium* pigment dynamics in two common coral species, namely *Seriatopora hystrix and Acropora millepora*.

3.3 Materials and methods

Bleaching surveys of reefs with elevated and ambient pCO_2

Bleaching surveys were conducted at three locations with volcanic CO_2 seeps and adjacent control sites (namely Upa Upasina, Esa'Ala and Dobu, in Milne Bay Province, Papua New Guinea) over a one week period in April 2011. Seep and control sites are described in detail by Fabricius *et al.* (2011). The emerging gas is >98% CO_2 and the seep and control sites are very similar in their geomorphology, flow, light, wave exposure and nutrients (Tables S1 and S2 of Fabricius *et al.* 2011). Three years of continuous benthic temperature logging since April 2011 (Reef net, Census Ultra, Canada) has shown that temperatures are similar between seep and control sites (Fig 2.S2, Table 2.S1). Spatial maps of mean seawater carbonate chemistry (Fabricius *et al.* 2011) were used to confine surveys to seep areas with mean seawater pH_T 7.8 – 7.9 ± ~0.2SD. We observed mild bleaching (i.e. a proportion of colonies were pale and a very small number were almost white) at all visited reefs (Fig 2.S1).

The first set of surveys was a series of 20 * 0.5 m belt transects at the six seep and control sites, at 3 – 4 m depth (n = 4 each at the seep and control sites of Upa Upasina and Dobu, and n = 2 per site at the smaller Esa'Ala reef). A single observer recorded the taxonomic identity (mainly genus but family for less common taxa) and pigmentation of any zooxanthellate hard coral and octocoral colonies within the

belts (n = 874 colonies). Pigmentation was estimated on the upper surface of the colony to the nearest 0.25 colour chart units of the Coral Watch Coral Health Chart (http://www.coralwatch.org) on a scale of 1 (severely bleached) to 6 (dark pigmentation), which correlates well with symbiont density and chlorophyll *a* content (Siebeck *et al.* 2006) (Fig 3.S1d). Of the 874 colonies surveyed, 780 came from the families Acroporidae, Faviidae, Pocilloporidae or Poritidae.

During the surveys the coral *Seriatopora hystrix* was noted to be particularly bleached. As the belt transects failed to capture enough of these colonies for statistical analyses, a second set of surveys was conducted at the Upa Upasina between 2 and 6 m depth, recording the depth and pigmentation on the upper surface of the first 14 - 15 colonies of *S. hystrix* encountered each at the control and seep site.

Laboratory experiment

A two-month, two-factor aquarium experiment (two temperatures: 28° and 31°C, and two pH_T levels: 7.8 and 8.0, Table 2.1) was conducted at the Australian Institute of Marine Science in September to December 2011 to investigate coral bleaching susceptibility to pCO_2 and temperature exposures at levels projected to occur before the end of the century (atmospheric CO_2 ~750 ppm in RCP 8.5) (Moss *et al.* 2010; IPCC 2021). The elevated temperature treatment of 31°C is considered the ten-day summer bleaching threshold for corals from the study area (long-term summer mean ~28°C) (Berkelmans *et al.* 2004). The two common and widely distributed coral species used, *Seriatopora hystrix* and *Acropora millepora*, are both highly susceptible to thermal bleaching.

Table 3.1: Treatment conditions in the 54 day flow-through experiment: pH_T and temperature (measured daily), and seawater carbonate parameters (measured weekly). The four experimental treatments are a combination of low and high temperature (TL, TH) and low and high pCO_2 (CL, CH). Measured values of temperature, salinity (35 ppt), total alkalinity (A_T) and dissolved inorganic carbon (C_T) were used to calculate the seawater carbonate parameters (n = 9 per treatment) including the saturation state of aragonite (Ωar). Standard deviations are shown in brackets.

	Measured Parameters				Calculated Parameters		
Treatment	pH_T	Temperature (°C)	A_T (µmol kg^{-1} SW)	C_T (µmol kg^{-1} SW)	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1} SW)	Ωar
TL.CL	7.97 (0.05)	28.07 (0.17)	2318 (22)	2026 (13)	479 (38)	1819 (45)	3.27 (0.31)
TL.CH	7.79 (0.05)	27.85 (0.25)	2325 (24)	2116 (35)	738 (65)	1950 (47)	2.44 (0.21)
TH.CL	7.99 (0.05)	30.81 (0.33)	2328 (23)	2019 (9)	500 (32)	1789 (15)	3.57 (0.30)
TH.CH	7.79 (0.03)	30.83 (0.36)	2326 (23)	2119 (35)	835 (85)	1940 (41)	2.56 (0.26)

Eighteen partial colonies of *S. hystrix* and *A. millepora* were collected from two reefs in the central Great Barrier Reef (Orpheus Island and Davies Reef), Australia, between 3–5 m of depth. Each colony was divided into four ~5 cm nubbins (n = 72 nubbins per species) and one nubbin per colony was assigned to one of the four experimental treatments to remove any effects of parental colony identity. Initial measurements of net oxygen production and PAM fluorometer parameters, taken after the acclimation period (outlined below) did not differ between corals collected from the two sites (ANOVA, all $p > 0.05$).

Nubbins were allowed seven days of acclimation in partially shaded (maximum irradiance ~500 µmol photons m^{-2} s^{-1}), outdoor holding tanks with flow through seawater (~28°C) before being distributed across 12 glass aquaria (17 L volume) in a temperature controlled room (25 ± 1°C), supplied (400 mL min^{-1}) with filtered (5 µm) seawater at 28°C and ambient atmospheric pCO_2 for a further 17 days acclimation. There were three aquaria per treatment, each containing six nubbins per species. Illumination was delivered in 12 hours cycles by white fluorescent light (10 000 K), and was consistent between aquaria (180 µmol photons m^{-2} s^{-1}, 15.6 mol photons day^{-1}, meter LI205A, sensor LI201SA, LICOR, USA). Each aquarium had an individual power head for circulation.

After acclimation, the elevated pCO_2 treatment was applied directly for 21 days prior to the onset of heat stress by controlling pH in header tanks (n = 4; one per experimental treatment) through a diffused feedback control CO_2 gas injection system (Aquamedic, Germany). Daily pH_{NBS} measurements were conducted in each experimental aquarium (meter: Oakton pH 1100, USA; electrode: Eutech, USA), with measurements being compared to the Dickson seawater TRIS pH standard (Table 3.1) and converted to pH_T (Dickson 2007).

A heat stress event was then simulated by ramping the temperature in heated treatments at 0.5°C per12 hours with a separate feedback control system (Neptune Apex aqua controller, USA), and then maintained in the header tanks with a computer-controlled data logger (CR 1000, Campbell Scientific, Australia). Treatments were alternated to eliminate any potential environmental effects within the room. Aquarium water temperatures were monitored daily and remained constant (Table 3.1). Water samples were taken weekly throughout the experiment for C_T and A_T analyses (Marianda VINDTA 3C, Germany) which were used to calculate carbonate system parameters with the program CO2SYS (Lewis & Wallace 1998) (Table 3.1).

Coral nubbins were inspected for survivorship and visual signs of bleaching nearly daily. To ensure there would be living samples for final analyses, the experiment was terminated once ~60% of nubbins per species within the heated treatments showed visual signs of bleaching. Nubbins were immediately snap frozen in liquid nitrogen and stored at -80°C for later analyses. We used PAR absorptivity and Fv/Fm measurements (outlined below) as bleaching indices throughout the experiment and further examined the content of different pigments at the experiment's end.

Pulse amplitude modulated fluorometry

Pulse amplitude modulated (PAM) fluorometry measurements of dark adapted (> 30 min) maximum quantum yields (F_v/F_m) and PAR absorptivity were taken with an Imaging PAM fluorometer (IPAM, Walz, Germany, Unit IMAG-CM fitted with a Maxi head). Measurements of all 144 nubbins were recorded weekly from the start of the acclimation period, and then every four days once the heat treatment began. F_v/F_m provides a measure of the maximum proportion of available light that can be

photochemically quenched through PSII. PAR absorptivity is the fraction of incident red light that is absorbed by photosynthetically active pigments (Ralph *et al.* 2005).

Rapid light curve (RLC) measurements were taken with the IPAM (Ralph *et al.* 2005) on the day before each species was removed from the aquaria ($n \geq 15$ per species per treatment) applying 10 s exposures to increasing irradiances (38, 88, 160, 264, 309, 369, 504, 658, 861 and 995 µmol photons m^{-2} s^{-1}). Measured RLCs were fitted with an exponential function to derive light-limited electron transport rate (α) and the maximum electron transport rate (ETR_{max}) following standard procedures (Ralph & Gademann 2005).

Oxygen flux

Gross photosynthesis and respiration was measured after the acclimation period, after 21 days of CO_2 treatment, and in the closing days of the experiment ($n > 6$ per species per treatment per time point). Real-time changes in oxygen concentration were measured in stirred and temperature controlled 210 mL clear Perspex incubation chambers fitted with oxygen sensor spots (''optodes'', Ø 0.5 cm, Presens, Germany), and an Oxy-4 fibre-optic oxygen meter (Presens, Germany; for details see Uthicke *et al.* 2012). The same lights and intensities used in the aquaria were used in the gross photosynthesis runs. Treatment water was obtained from the header tanks of the experiment and further filtered to 0.5 µm. Measurements lasted ~30 min and each run included a blank chamber. Respiration and gross photosynthesis rates were normalised to nubbin surface area. Net production was calculated by subtracting hourly respiration from hourly gross photosynthesis.

Protein and pigment content

Three nubbins per species per tank were water-picked to remove coral tissue in 10 mL of ultra-filtered seawater (0.05 µm). This slurry was homogenised and a supernatant of coral tissue was prepared for spectrophotometric protein quantification following Dove *et al.* (2006) using the DC protein assay kit (Bio-Rad laboratories, Australia). Protein content was standardised to nubbin surface area, determined using the single wax dipping technique (Veal *et al.* 2010).

Pigments from the *Symbiodinium* pellet obtained after centrifuging the water-picked coral nubbins were sonicated and extracted on ice in the dark in two one-hour extractions in 1 mL of chilled (4°C) buffered methanol (98% MeOH/2% 0.5 M tetrabutylammonium acetate [TBAA] pH 6.5). Extracts were prepared for analysis with an ultra-performance liquid chromatography (UPLC) system (Waters Acquity UPLC) following Uthicke *et al.* (2012) and were standardised to nubbin surface area for analysis.

Statistical analyses

In the wider bleaching surveys, two-factor analysis of variance (ANOVA) was used to compare mean pigmentation between locations and CO_2 exposures for all taxa combined, and separately for the four common families. Tukey's HSD was used for *post hoc* examinations. ANOVA was used in the *S. hystrix* bleaching surveys to compare the mean pigmentation of colonies between the seep and control site, with colony depth as a covariate.

In the experiment, data were averaged across nubbins for each species within aquaria. Unless otherwise stated, all reported statistics satisfied the assumptions of homoscedasticity, Gaussian distributions and independence. Generalised additive mixed models (GAMMs) were fitted to assess trends in PAR absorptivity and quantum yields across treatments over time. One-way and two-factor ANOVA were used to compare quantum yields and PAR absorptivity between the treatments at specific dates, as well as RLC parameters, protein and pigment contents, pigment ratios and oxygen flux at three time points (after the acclimation period, after 21 days CO_2 treatment, and in the last days of the experiment). The statistical program R (version 3.0.2) was used including the packages mgcv and nlme (R Development Core Team 2021).

3.4 Results

Bleaching surveys

Mild bleaching was observed at all six sites. Coral pigmentation data from all 874 colonies combined displayed a significant interaction between location and CO_2 exposure (two-factor ANOVA, Location * CO_2: $F_{(2, 868)} = 5.616$, $p = 0.004$), as the mean pigmentation at the Dobu control site was ~0.2

colour chart units darker than that of the Upa-Upasina control and Esa'Ala and Dobu seep sites (Fig 3.1, Tukey's HSD: $p < 0.05$, Table 3.S2). No differences were detected between any of the other CO_2 and site combinations for mean pigmentation across all coral taxa (Fig 3.1).

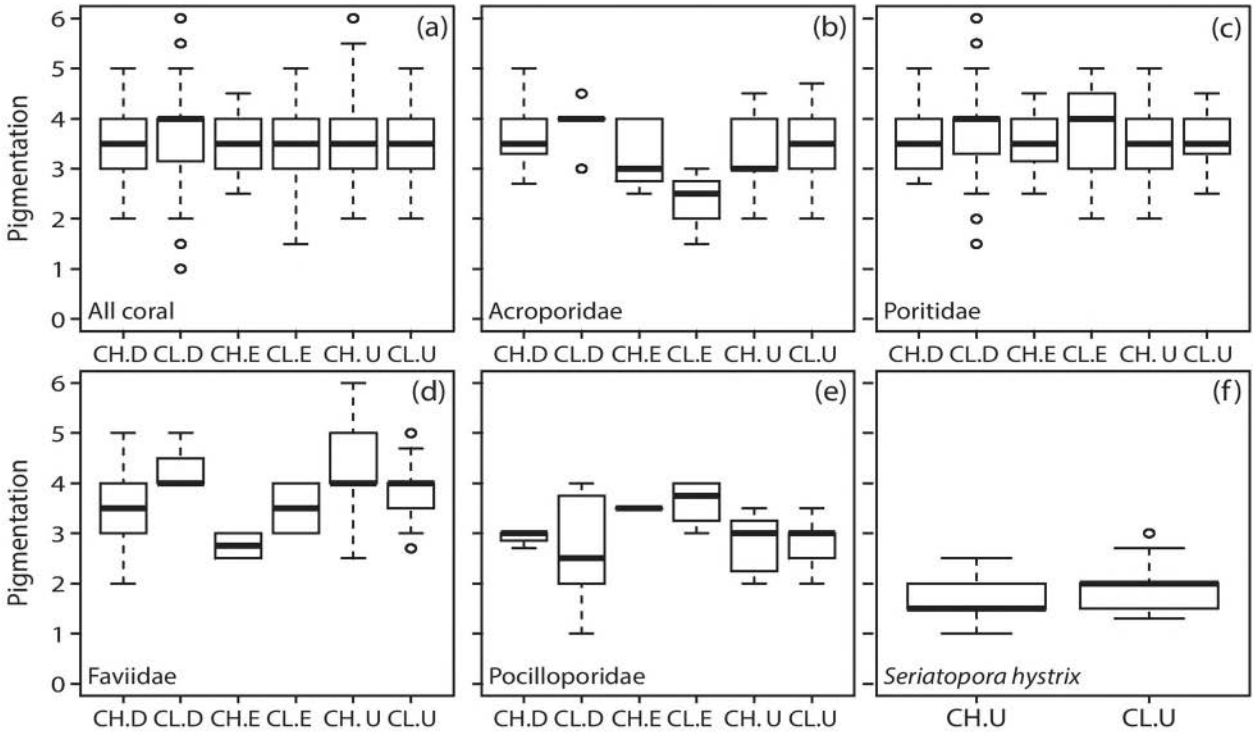

Fig 3.1: Field surveys of coral pigmentation during a mild bleaching event. Coral colour chart scores (6 = darkest and 1 = lightest) for all coral taxa combined (a: n = 874), the Acroporidae (b: n = 128), Poritidae (c: n = 466), Faviidae (d: n = 145), Pocilloporidae (e: n = 41) and *Seriatopora hystrix* (f: n = 29) at seep and control sites (pCO_2 high and low: CH and CL) at the three reefs Dobu (D), Esa'Ala (E) and Upa Upasina (U). Plots are standard boxplots, with the top and bottom of the box enclosing the first to third quartiles (horizontal bar: median, the whiskers: 1.5 inter-quartile ranges, and the circles are outliers.

The pigmentation values for the Acroporidae and Faviidae differed between some specific sites (Location * CO_2 interaction), but no differences were attributable to CO_2 exposure as a main effect (Fig 3.1, Table 3.S2). Pigmentation values in the Poritidae (n = 466 colonies) displayed a significant main effect of CO_2 exposure, with colonies at the seep sites being ~4% paler (3.59 ± 0.55 SD colour chart units) than the control sites (3.76 ± 0.74). No differences in pigmentation were detected in the Pocilloporidae.

In *S. hystrix,* most colonies were obviously pale (mean 1.81 ± 0.47), indicating moderate bleaching in this species, and 57% and 33% of colonies had a pigmentation value of ≤ 1.5 at the high CO_2 and control site, respectively (i.e. were almost completely white, Fig 3.1f). There was no difference in the mean pigmentation of the upper surfaces of *S. hystrix* between the seep and control site ($F_{(1,26)} = 2.073$, $p = 0.16$), nor was depth a significant covariate ($F_{(1,26)} = 0.753$, $p = 0.39$).

Laboratory experiment

Mortality and visual signs of bleaching

In the laboratory experiment, none of the nubbins displayed visual loss in pigmentation at control temperatures (28°C). In *S. hystrix*, the first visual signs of bleaching were recorded at 31°C five days after the temperature ramp finished, 60% of *S. hystrix* appeared visually bleached four days later, and the final measurements were taken before removing the surviving nubbins from the experiment. All bleached and dead nubbins (n = 3 total) were confined to, and evenly distributed across, the two heated treatments. In *A. millepora,* visual signs of bleaching were first observed 12 days after the temperature ramp finished, and ~60% of nubbins in the heated treatments appeared visually bleached and the experiment ended on the 16th day after the temperature ramp. No mortality was recorded in *A. millepora*.

The PAR absorptivity initially increased in both species during the acclimation period, and all but plateaued by the time the pCO_2 treatments began and remained relatively constant throughout the pCO_2 exposure period (Figs 3.S3 and 3.S4). Absorptivity values did not differ in either species at the end of the acclimation period between tanks that would later become treatments, or after three weeks of CO_2 treatment (ANOVA: all $p > 0.05$). However, absorptivity values declined once temperatures were ramped (Fig 3.S3 and 2.S4), and final values were significantly lower in the heated treatments compared to the control temperature (Table 3.2). Absorptivity values from both species changed significantly over the course of the experiment (GAMM smooth term: *S. hystrix*: $F_{(5.984)} = 14.05$, $p < 0.001$; *A. millepora*: $F_{(5,27)} = 8.75$, $p < 0.001$), however pCO_2 did not affect absorptivity ($p > 0.1$), while the heated treatments had significant reductions in both species (GAMM: *S. hystrix*: $T = 14.05$, $p < 0.05$; *A. millepora*: $T = 2.97$, $p < 0.01$). The models explained 42% and 35% of the variation in PAR absorptivity for *S. hystrix* and *A. millepora,* respectively (Fig 3.S3 and 2.S4).

Table 3.2**:** ANOVA results comparing photophysiological parameters for *Seriatopora hystrix* and *Acropora millepora* between the experimental treatments of CO_2 (C), temperature (T) and their interaction (C:T): mean absorptivity and maximum quantum yield (Fv/Fm, both averaged over the final two measurements); alpha and ETR_{max} values derived from rapid light curves, and final net oxygen flux and chlorophyll *a* concentration at the end of the 54 day experiment.

S. hystrix				*A. millepora*	
	Df	F	p	F	p
Absorptivity					
C	1	0.34	0.54	0.93	0.35
T	1	15.17	<0.01	11.18	<0.01
C:T	1	0.50	0.49	0.92	0.35
Res	20				
Fv/Fm					
C	1	64.14	<0.01	5.78	0.03
T	1	11.68	<0.01	11.27	<0.01
C:T	1	0.72	0.41	1.19	0.29
Res	20				
Alpha (α)					
C	1	17.28	<0.01	17.44	<0.01
T	1	32.98	<0.01	11.72	<0.01

C:T	1	0.13	0.73	0.10	0.76
Res	8				
ETR_{max}					
C	1	2.92	0.13	2.31	0.17
T	1	24.72	<0.01	0.81	0.39
C:T	1	0.36	0.57	0.80	0.40
Res	8				
Respiration					
C	1	1.00	0.33	0.60	0.45
T	1	1.55	0.23	0.22	0.64
C:T	1	0.56	0.46	0.97	0.34
Res	20				
Production					
C	1	4.99	0.04	0.01	0.93
T	1	31.36	<0.01	38.40	<0.01
C:T	1	0.7	0.41	0.81	0.38
Res	20				
Net O_2 production					
C	1	6.52	0.02	0.01	0.93
T	1	28.51	<0.01	38.40	<0.01

C:T	1	1.13	0.30	0.81	0.38
Res	20				
Chlorophyll *a*					
C	1	12.62	<0.01	0.34	0.58
T	1	51.80	<0.01	67.28	<0.01
C:T	1	1.72	0.23	1.57	0.24
Res	8				

Quantum yields and rapid light curves

After acclimation, the F_v/F_m for both species did not differ between tanks that would later become treatments (Two-factor ANOVA, all p >0.05). F_v/F_m was 0.65 ± 0.03 (SD) and 0.67 ± 0.03 for *S. hystrix* and *A. millepora*, respectively. After three weeks of pCO_2 exposure, F_v/F_m in *S. hystrix* was significantly higher at elevated compared to ambient pCO_2 (0.53 ± 0.03 vs 0.63 ± 0.04, one-way ANOVA: $F_{(1,10)} = 22.43$, p <0.001). In contrast, F_v/F_m in *A. millepora* remained similar between pCO_2 treatments (0.61 ± 0.05 vs 0. 64 ± 0.04, one-way ANOVA, p >0.05). While declines in Fv/Fm were observed in all treatments, values in the final days after heat stress, for both species, were influenced significantly by both pCO_2 and temperature in an additive fashion (Fig 3.2c and d, Table 3.2). In both species, the highest mean F_v/F_m values were recorded in the high pCO_2 + low temperature treatment, while the lowest values were observed in the low pCO_2 + high temperature treatment. This decline was ~20% in both species (*S. hystrix*: 0.64 ± 0.03 vs 0.50 ± 0.03; *A. millepora* 0.62 ± 0.02 vs 0.53 ± 0.09). The changes over time in F_v/F_m were significant in both species (GAMM smooth term: *S. hystrix*: $F_{(6.18)} = 14.43$, p <0.001; *A. millepora*: $F_{(4.58)} = 16.94$, p <0.001), with CO_2 addition significantly increasing F_v/F_m in *S. hystrix* (GAMM: T = 2.66, p <0.01) and elevated temperature significantly reducing it in *A. millepora* (GAMM: T = 2.67, p <0.01). The models explained 62% and 52% of the variation in F_v/F_m for *S. hystrix* and *A. millepora*, respectively (Fig 3.S5 and 2.S6).

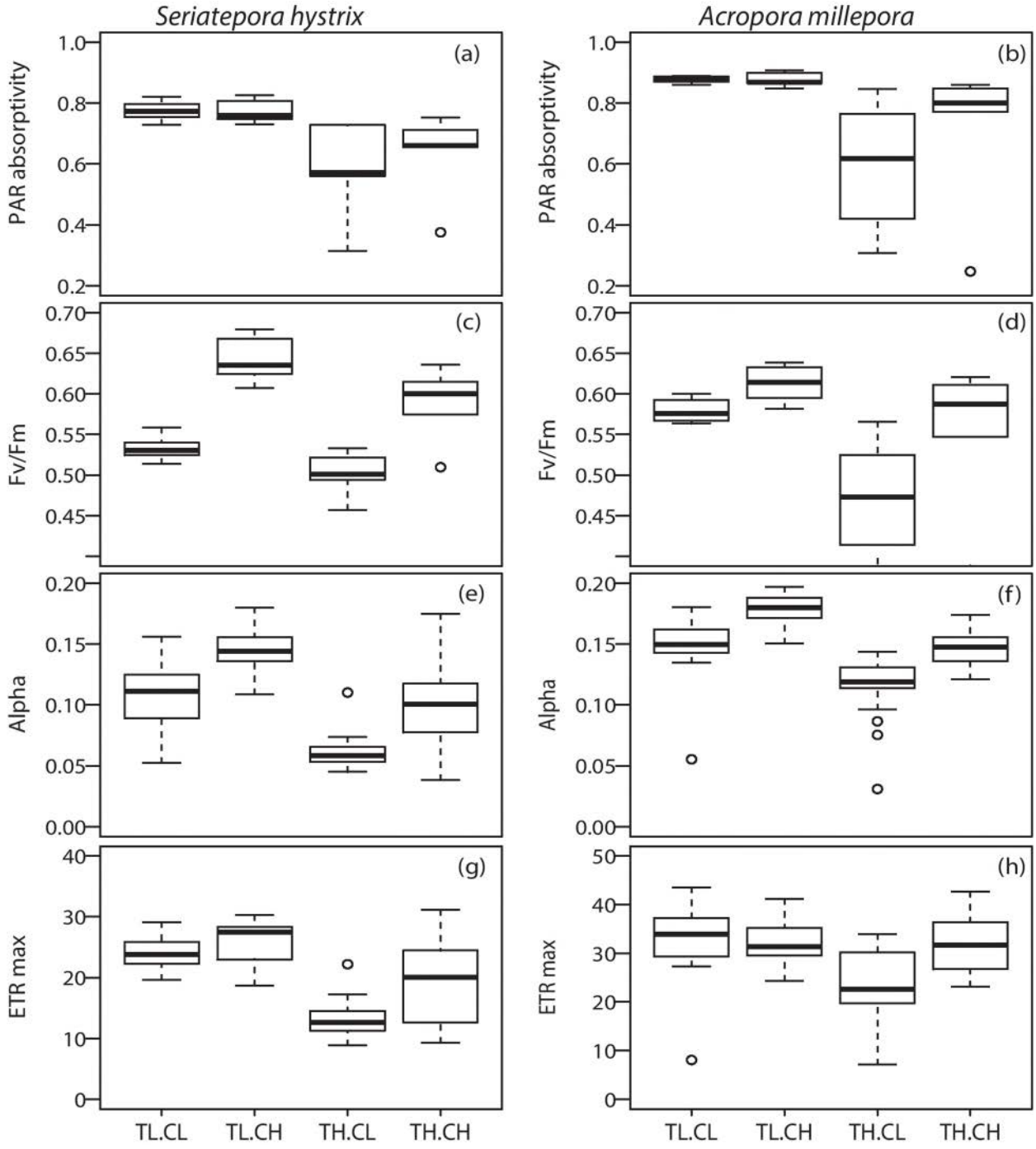

Fig 3.2**:** Photophysiological responses in corals in the laboratory to the treatments of low and high temperature (TL, TH) and low and high pCO_2 (CL, CH). Shown are PAR absorptivity (a and b) and maximum PSII quantum yield (Fv/Fm, c and d) in the last 5 days of heat stress, and light-limited electron transport rate (photosynthetic efficiency, α) (e and f) and the maximum electron transport rate ETR_{max} (g and h) at

the end of the experiment. n = 3 tanks per treatment (averaging 6 colonies per species) for Fv/Fm and absorptivity, n ≥15 per treatment for α and ETR_{max}. See Fig 3.1 legend for boxplot description.

At the end of the experiment, RLCs in *S. hystrix* indicated that light-limited electron transport rates (α) were influenced by both pCO_2 and temperature, and that their effects were additive (Fig 3.2e and f, Table 3.2). Elevated pCO_2 increased α, while increasing temperature lowered them. Mean values of α were lowest in the low pCO_2 + high temperature treatment (0.07 ± 0.01), being 43% of those observed in the high pCO_2 + low temperature treatment (0.16 ± 0.01, Table 3.2). Maximum electron transport rate (ETR_{max}) in *S. hystrix* was influenced by temperature alone (Fig 3.2g and h, Table 3.2). ETR_{max} values in the higher temperature treatment were 63% of those observed in the lower temperature treatment (Fig 3.2). The α values in *A. millepora* were also significantly affected by both CO_2 and temperature in an additive fashion (Table 3.2). As per *S. hystrix*, they were lowest in the low CO_2 + high temperature treatment (0.11 ± 0.03), being 64% of the values observed in the high CO_2 + low temperature treatment (0.18 ± 0.01). No significant differences in ETR_{max} values were detected between the experimental treatments in *A. millepora* (Table 3.2).

Oxygen flux

Rates of respiration and photosynthesis did not differ after three weeks of differential pCO_2 exposure (prior to heat treatment) in either species (one-way ANOVA: p >0.05), and final rates in the control treatments closely matched initial rates. Respiration rates after three weeks of pCO_2 treatment were -9.65 ± 0.90 and -9.90 ± 2.61 μg O_2 cm^{-2} h^{-1} for *S. hystrix* and *A. millepora*, respectively, while gross photosynthesis rates were 29.64 ± 5.35 and 36.80 ± 10.79 μg O_2 cm^{-2} h^{-1}. In *S. hystrix*, the final net oxygen production rates were influenced by both pCO_2 and temperature in an additive fashion (Table 3.2), with CO_2 addition increasing and increased temperature reducing net production (Fig 3.3e). This was driven by changes in gross photosynthesis, as respiration remained unchanged between treatments (Fig 3.3a, c). Net production was highest in the high pCO_2 + low temperature treatment (43.47 ± 7.75 μg O_2 cm^{-2} h^{-1}) and lowest in the low pCO_2 + high temperature treatment (15.42 ± 9.29), i.e. a 2.5-fold difference (Fig 3.3e). In *A. millepora,* net O_2 production measurements at the end of the experiment were influenced by

temperature alone (Table 3.2). Corals in the two heated treatments produced approximately 2.5 times less O_2 than in low temperature treatments (15.88 ± 12.10, vs 37.30 ± 7.19 µg O_2 cm^{-2} h^{-1}; Fig 3.3f). This difference was driven by changes in gross photosynthesis, as respiration rates remained unaffected (Table 3.2). Analyses of O_2 flux and pigment contents (see below), standardised by units of protein, gave no further insights compared to the values standardised by surface area.

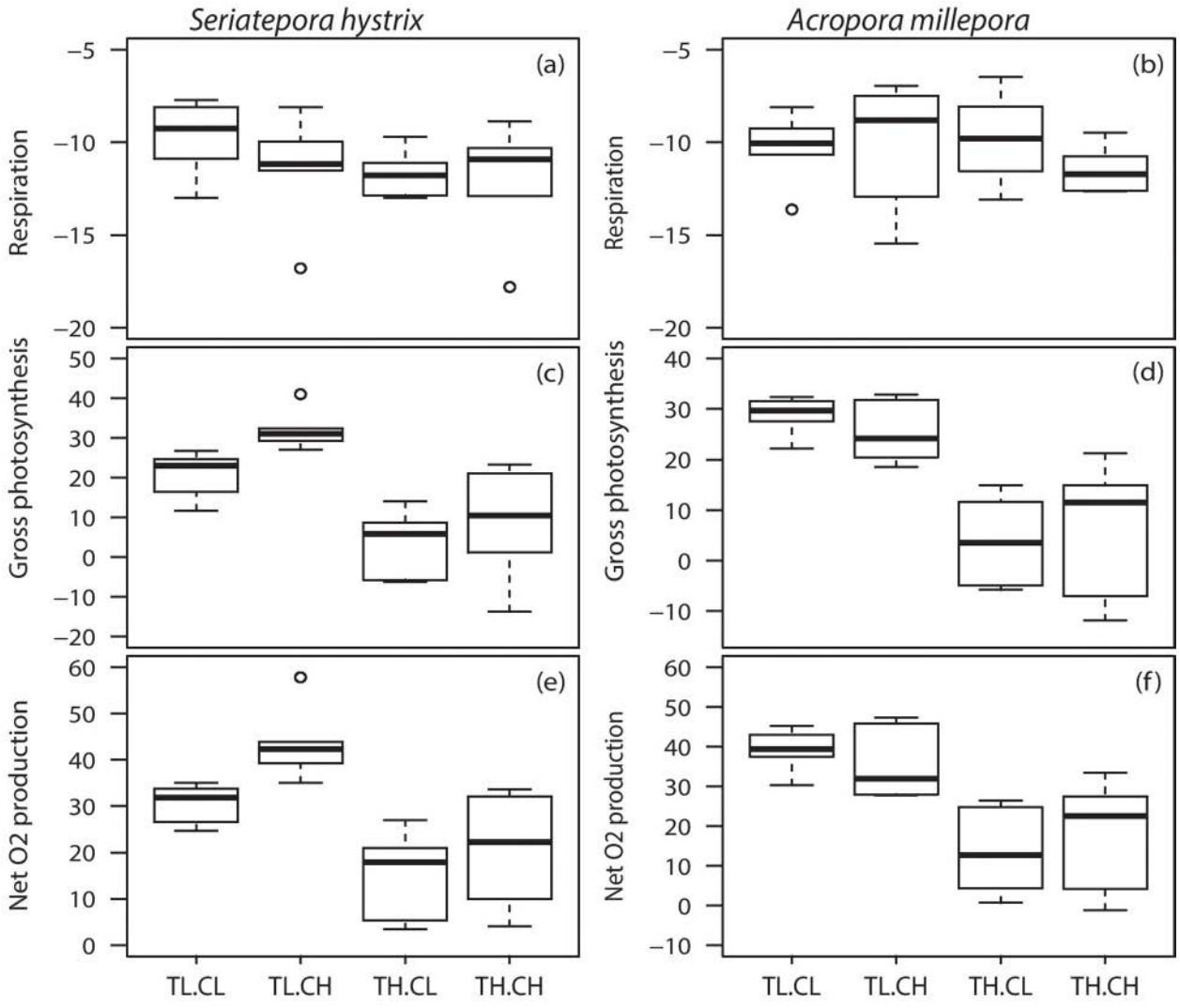

Fig 3.3**:** Respiration (a and b), gross photosynthesis (c and d) and average hourly net production (e and f) (all in µg O_2 cm^{-2} h^{-1}) in *Seriatopora hystrix* and *Acropora millepora* at the end of the experiment. Values are standardised per unit surface area of the coral nubbins (n = 6 per treatment). Legends as in Fig 3.2.

Coral protein content

Protein content per unit surface area at the end of the experiment, showed no difference between treatments in either species (Two-factor ANOVA: all p >0.1). *S. hystrix* nubbins had 3.43 ± 0.65 mg cm^{-2} protein (mean of all treatments), while in *A. millepora* this value was 5.10 ± 0.92 mg cm^{-2}, and was significantly higher than in *S. hystrix* (One-way ANOVA: $F_{(1,22)} = 26.36$, p <0.001).

***Symbiodinium* pigment content**

At the end of the experiment, many of the *Symbiodinium* pigment concentrations in *S. hystrix* were influenced by both pCO_2 exposure and temperature, without major interactions between these treatments (Table 3.S3). Temperature and pCO_2 changes had an additive effect on concentrations of chlorophyll *a* and *c2* and peridinin, which increased with elevated pCO_2 and declined at high temperature (Fig 3.4a – d, f). Pigment concentrations in the high pCO_2 + low temperature treatment were ~5-fold higher than at low pCO_2 + high temperature. B-carotene and the combination of diadinoxanthin (Ddx) and dinoxanthin (Dnx) were significantly reduced at high temperatures (Table 3.S3). Conversely, the concentration of diatoxanthin (Dtx), the relative proportion of Dtx to the total xanthophyll pool and the ratio of photo-protective (PP: Ddx, Dnx, Dtx and β-carotene) to light harvesting (LH: chlorophyll a, chlorophyll c2 and peridinin) pigments showed the opposite patterns, increasing with temperature and declining with elevated pCO_2.

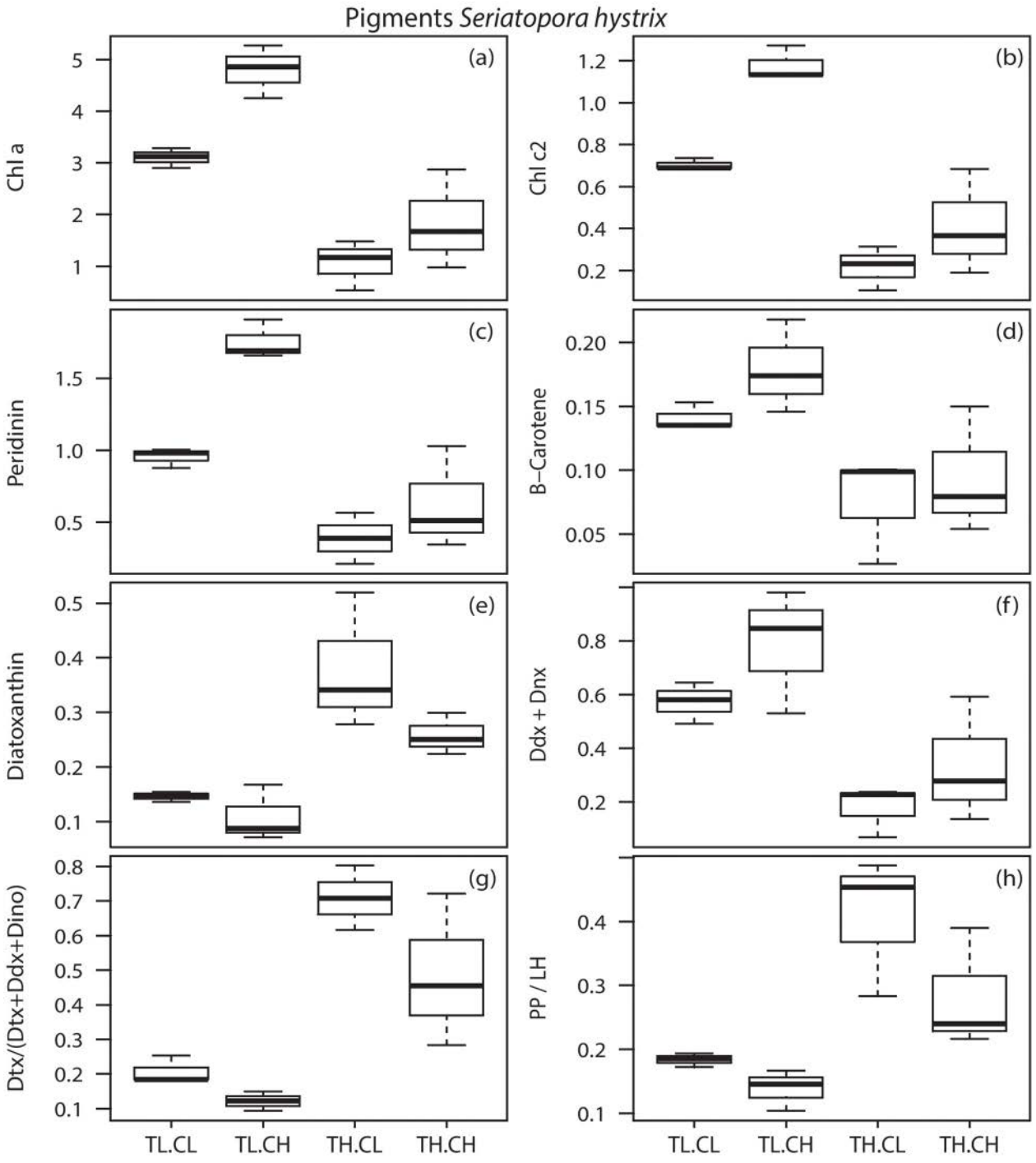

Fig 3.4: Molar concentrations of pigments (nmol cm^{-2} coral surface area) and pigment ratios from the *Symbiodinium* of *Seriatopora hystrix* at the end of the experiment (n = 9 per treatment). Legend as in Fig 3.2.

In *A. millepora,* the *Symbiodinium* pigment concentrations and ratios were influenced by temperature only (Fig 3.5, Table 3.S3). Concentrations of chlorophyll *a* and *c2*, peridinin, B-carotene and the combination of diadinoxanthin (Ddx) and dinoxanthin (Dnx) all declined in the heated treatments (Fig 3.5a – d, f), while Dtx, the relative proportion of Dtx to the total xanthophyll pool and the ratio of photo-protective (PP) to light harvesting (LH) pigments increased (Fig 3.5e, g, h). Chlorophyll *a* and *c2*, peridinin and B-carotene showed a 5-fold reduction in heated compared to control temperatures, while Ddx + Dnx was reduced ~3-fold. Concentrations of Dtx were approximately twice as high in the heated compared to the control treatments, while xanthophyll cycling and PP:LH increased 5 to 6-fold (Fig 3.5).

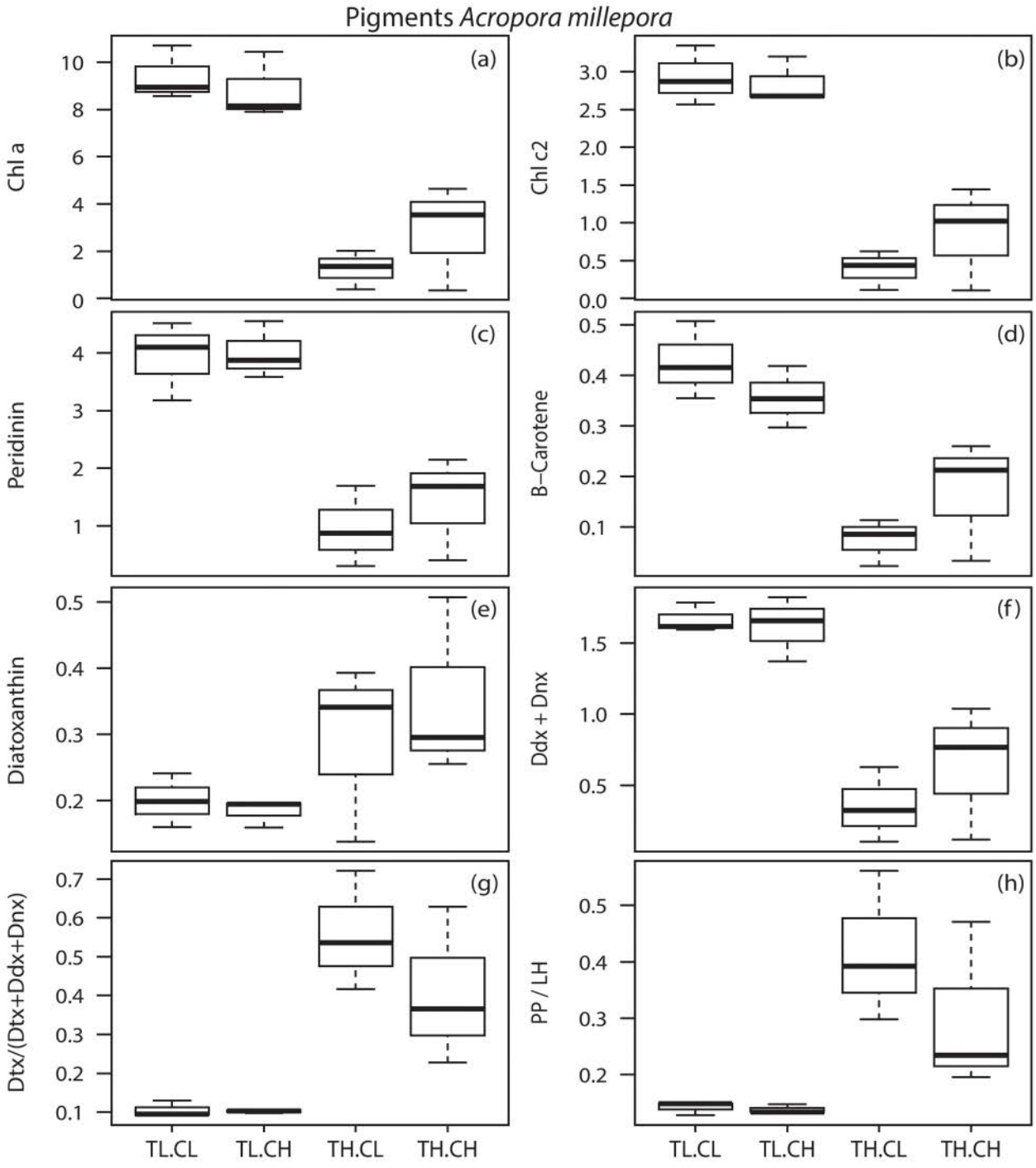

Fig 3.5: Molar concentrations of pigments (nmol cm^{-2} coral surface area) and pigment ratios from the *Symbiodinium* of *Acropora millepora* at the end of the experiment (n = 9 per treatment). Legend as in Fig 3.2.

3.5 Discussion

Despite rapidly progressing global climate change and OA, which is irreversible on a time scale of thousands of years, science is still largely uncertain about the interactive effects of these changes on marine organisms. The present study investigated whether the thermal bleaching tolerance of tropical reef corals would be influenced by elevated pCO_2 at levels predicted for later this century under OA. Contrary to some earlier observations and predictions (e.g. Anthony *et al.* 2008), our field and experimental data showed minimal negative effects of CO_2 exposure on the bleaching susceptibility of symbiotic corals. Furthermore, we found that pCO_2 increases can provide some benefits to the symbionts' photosystem, enhancing maximum PSII quantum yields and light-limited electron transport rates in both species, and additionally promoting photosynthetic productivity and pigment concentrations in *S. hystrix*. This study shows that although OA may provide an avenue to improve photosynthetic carbon gains for some corals, even during heat stress, the detrimental effects of warming temperatures remained disproportionately stronger, and did not fully offset bleaching-related losses in productivity.

The bleaching surveys at the three CO_2 seep and control sites represent the first *in situ* data that specifically investigates coral thermal bleaching susceptibility under elevated pCO_2. They gave little indication that the thermal bleaching susceptibility of the major components of the coral community was influenced by elevated pCO_2. Slight CO_2 effects were detected in the Poritidae at all sites, and in the Faviidae at Dobu, however differences were very minor (~4% change in pigmentation in the Poritidae). Pervious work in the same study area also detected reduced colour chart scores in massive *Porites* at the seep compared to the control sites during winter when bleaching was not observed (Fabricius *et al.* 2011). We therefore conclude that the observed minor reductions in *Porites* pigmentation at the seeps cannot be unequivocally attributed to a significant increase in bleaching sensitivity.

Thermal bleaching in corals can be co-determined by natural dynamics in food supply, water flow and light regimes (Fabricius 2006; Anthony *et al.* 2009; Hoogenboom *et al.* 2012), the combined effects of which cannot be effectively replicated in the laboratory. Furthermore experiments are relatively short-term by nature and hence unable to fully account for potential acclimatisation. Seep sites are not perfect representations of future reefs, as temperatures remain at ambient levels and CO_2 regimes are more temporarily variable. However, organism acclimatisation and ecological interactions between taxa can be

examined. The use of seep sites as natural laboratories, in conjunction with controlled experiments, provide the best available information to predict how OA will impact marine ecosystems.

In the laboratory experiment, heat stress induced a bleaching response in both coral species. *S. hystrix* was more sensitive to thermal stress than *A. millepora*, but heat stress was not exacerbated by increased CO_2 in either species as shown in the field. While CO_2 addition reduced the severity of temperature effects on some photophysiological parameters, thermal bleaching (as quantified by commonly used photophysiological measures) was still observed in both coral species in both pCO_2 treatments, and the temperature effects were much stronger than the pCO_2 effects. It remains to be seen whether this finding will hold for other species and other experimental conditions. For example, corals in the present study included sensitive species, were not fed and were kept under moderate light levels, and previous studies have shown that all these factors co-determine thermal tolerance (Marshall & Baird 2000; Anthony *et al.* 2009; Hoogenboom *et al.* 2012). The fact that our field study resulted in similar findings for both highly sensitive and more thermally tolerant taxa (e.g., *S. hystrix* vs *Faviidae*), under natural levels of light, flow and food supply, confirm that the findings from the laboratory study are likely to apply to other species and other study conditions.

The extent of coral thermal bleaching is influenced by factors within the host coral and their *Symbiodinium*. While different *Symbiodinium* types have been shown to vary in their temperature tolerance (Berkelmans & van Oppen 2006), previous work at the same CO_2 seep locations did not detect any difference in *Symbiodinium* types due to CO_2 exposure in six common corals (Noonan *et al.* 2013). In our laboratory experiment, the parental corals were divided evenly across all experimental treatments to prevent differences in genotypes or symbiont identity from confounding our results.

The results of previous works that have examined the effects of elevated pCO_2 or the interactive effect of elevated pCO_2 and increased temperature on coral bleaching and photobiology have been highly inconsistent. Anlauf *et al.* (2011) showed that *Porites panamensis* bleached at increased temperature under ambient pH, but not under reduced pH (the number of *Symbiodinium* per coral polyp remained unaffected). Schoepf *et al.* (2013) documented a range of responses to OA and increased temperature across four species of corals, with no clear pattern emerging. Reynaud *et al.* (2003) showed an increase in chlorophyll *a* and the number of *Symbiodinium* per coral cell in *Stylophora pistillata* with increased temperature and CO_2, respectively, while Anthony *et al.* (2008) found pigmentation (measured by

luminance) decreased in two species of coral under similar treatments. Moreover, Wall *et al.* (2013) concluded that changes in CO_2 had no influence on the bleaching susceptibility of *Seriatopora caliendrum*. Anthony *et al.* (2008) attributed the differences between their results and that of Reynaud *et al.* (2003) to the higher light levels used in their study, however the null effects observed by Wall *et al.* (2013) were in corals exposed to saturating light levels. The use of different species, methodologies and metrics of bleaching may be contributing to the disparities seen between works to date.

The present study confirmed that increases in pCO_2 can stimulate photosynthesis in corals, suggesting carbon supply may limit their photosynthesis. A greater proportion of quanta were funnelled through PSII for use in photosynthesis under higher pCO_2, and light-limited electron transport rate and maximum quantum yields in both *S. hystrix* and *A. millepora* increased. In *S. hystrix*, this further manifested in greater oxygen production. Carbon limitation has been reported in *Symbiodinium* in numerous taxa including corals and in culture (Brading *et al.* 2011; Uthicke & Fabricius 2012), with photosynthesis being stimulated by the addition of CO_2 (Crawley *et al.* 2010) or bicarbonate (Herfort *et al.* 2008). These results are not universal however, with some authors reporting either null or negative effects of elevated pCO_2 on photosynthesis (Langdon & Atkinson 2005; Anthony *et al.* 2008; Edmunds 2012; Wall *et al.* 2013). Carbon may only become limiting in high light, as well as in relatively nutrient-rich waters (Chauvin *et al.* 2011) such as those of the inshore GBR lagoon used in the present experiment and that conducted by Crawley *et al.* (2010). In contrast, experiments conducted using relatively oligotrophic waters (Langdon & Atkinson 2005; Anthony *et al.* 2008; Edmunds 2012) may experience limitation in other substrates required for photosynthesis before carbon supply becomes limiting. Moreover, other carbonate system changes associated with OA, such as pH declines, may contribute to increased productivity and warrant further investigation.

In the present study, the effects of increased pCO_2 were more evident in *S. hystrix* than in *A. millepora*. In *S. hystrix*, pigment dynamics, including the xanthophyll cycle which non-photochemically quenches excess light energy, net photosynthetic oxygen production, maximum PSII quantum yields and electron transport rates were all up-regulated at high pCO_2 and reduced with temperature stress. In contrast, pigments and net photosynthetic oxygen production responded only to temperature stress in *A. millepora*. Such species-specific responses may help explain the disparities seen between different experimental works conducted to date and may be due to differences in the efficiency of their carbon concentrating mechanism (Comeau *et al.* 2012). Furthermore, many of the negative effects of elevated

pCO_2 on coral photophysiology and photosynthetic production documented in previous studies have occurred in treatments where CO_2 values were experimentally increased to very high levels (Krief *et al.* 2010). For example, Anthony *et al.* (2008) found net productivity in *Acropora intermedia* and *Porites lobata* did not change with moderately increased pCO_2 (similar to those in the present study), however productivity dramatically declined once pCO_2 was further increased. Similarly, Crawley *et al.* (2010) documented a 38% increase in photosynthetic capacity in *A. formosa* under conservative but not under high emission scenarios. Such non-linear (Gil 2013; Schoepf *et al.* 2013), species-specific (Marshall & Baird 2000; Schoepf *et al.* 2013) responses to environmental pressures are not uncommon. It may be that groups of closely related species have separate non-linear responses to CO_2 where minor increases have negligible effects or are beneficial for net photosynthesis, while additional increases in CO_2 may result in negative effects.

Maintaining rates of calcification in corals potentially becomes increasingly energy demanding with increasing seawater pCO_2 (Cohen & Holcomb 2009; Comeau *et al.* 2013b; Cyronak *et al.* 2015; Jokiel 2015). During times of thermal stress, energetic demands are also placed on corals to maintain the symbiosis with their *Symbiodinium* partners (Dubinsky *et al.* 2011). With OA progressing and SST anomaly frequencies increasing, the opportunity cost for corals to maintain the *status quo* may include declines in calcification, bleaching resistance, fecundity or other energetically demanding processes. As carbon emissions continue to increase, we are likely to see the gradual deterioration of coral species that are more susceptible to OA effects on calcification (Comeau *et al.* 2013b) and temperature stress (Marshall & Baird 2000) and declines in those species that are unable to utilise the more abundant CO_2 for photosynthesis in eutrophic waters (Crawley *et al.* 2010; Brading *et al.* 2011). Unfortunately for the majority of reef associated taxa, it appears the most competitive species of coral, in the face of OA and increasing SST, are massive varieties that support a low diversity of associates (Marshall & Baird 2000; Fabricius *et al.* 2011, 2014b). While it is difficult to be sanguine in the face of projected trajectories for coral reefs, the only feasible option to prevent the exacerbation of these effects is to reduce anthropogenic CO_2 emissions.

3.6 Supplementary material

Supporting supplementary material can be found at:
https://academic.oup.com/icesjms/article/73/3/715/2458717

Chapter 4: Effects of light variability and elevated CO_2 on the adult and juvenile performance of two *Acropora* corals

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4.1 Abstract

Reef building coral performance strongly depends on light availability and seawater carbon chemistry. We experimentally assessed whether the effects of ocean acidification on adult and early post-settlement *Acropora tenuis* and *A. hyacinthus* corals depend upon light availability (both light intensity and light variability). Four light treatments – one treatment with variable daily light integrals (DLI) that averaged 7.6 mol photons m^{-2} d^{-1} and three treatments with static DLIs (12.6, 7.6, 2.5 mol photons m^{-2} d^{-1}) were fully crossed with two levels of pCO_2 (400 and 900 ppm) in a 63-day aquarium experiment. Growth and protein content in the adult corals declined as average DLI declined, regardless of whether DLI was static or variable. The depressed growth showed that photoacclimation was insufficient to compensate for light reductions, although both effective (ϕ_{PSII}) and maximum (F_v/F_m) quantum yields of photosystem two (PSII) varied by <5% between all static light treatments. Under variable light, both species adjusted their ϕ_{PSII} on the day of change in DLI, whereas F_v/F_m remained relatively constant despite five-fold fluctuation in light intensity between days. Elevated CO_2 increased protein content in adult *A. tenuis* at all light levels, but otherwise had little effect on measured parameters. For juveniles, the survival of both species reduced at low light due to overgrowth by *Peyssonnelia* algae, whereas *A. tenuis* growth was

highest at low light. Our study shows that periods of DLI reductions accumulate over time for corals, negatively affecting *Acropora* juvenile survival and adult growth rates, and hence slowing reef recovery after disturbance.

4.2 Introduction

Benthic irradiance, or the amount of light reaching the seafloor, has profound effects on the distribution and demography of photo-symbiotic reef corals (Muir *et al.* 2015). This is because many coral species rely on the translocation of photosynthates from their dinoflagellate photosymbionts to meet their carbon requirements (Muscatine 1990). Benthic irradiance levels depend on the intensity of light reaching the sea surface, attenuation in the water column, and water depth. Light intensity at the sea surface varies spatially and temporally (e.g. due to differences in latitude, season, time of day, cloud cover and wind/waves), while light attenuation with depth varies with the concentration and type of suspended particulate matter and dissolved organic matter (Storlazzi *et al.* 2015). The cumulative amount of light reaching the seafloor per day (daily light integral: DLI) can vary five-fold from one day to the next (Anthony *et al.* 2004), presenting physiological challenges to corals in these environments.

Corals and their photosymbionts photoacclimate to their light environment to maximise photosynthetic rates and avoid photo-damage. Both partners employ a range of strategies, and typical responses include increased density of photosymbionts, increased content of symbiont photosynthetic pigments (i.e. chlorophyll *a* and c_2, and peridinin), as well as increased photosynthetic capacity (i.e. quantum yields of PSII) at lower irradiance (Titlyanov *et al.* 2001b; Mass *et al.* 2007; Roth 2014; Bessell-Browne *et al.* 2017a). However, previous studies of coral photoacclimation have typically used light regimes that are consistent over time (e.g. Hoogenboom *et al.* 2009), and it remains unclear how corals photoacclimate to environments where maximum light intensity and DLI vary frequently. If corals photoacclimate to mean DLI during periods of variable light, they might be exposed to damage due to light stress during periods of high irradiance (Baker 2001; Richier *et al.* 2008). Conversely, if corals photoacclimate to maximum irradiance, they are unlikely to be able to maximise photosynthetic rates during periods of low irradiance (Iglesias-Prieto & Trench 1994). Some plants photoacclimate to average DLI rather than maximum irradiance (Chabot *et al.* 1979), and this has also been suggested for corals (Falkowski *et al.* 1990; Anthony & Hoegh-Guldberg 2003a; DiPerna *et al.* 2018). Understanding how light

influences the depth distributions of different coral species requires knowledge of the mechanisms of photoacclimation, and identification of the light thresholds above which photoacclimation is no longer sufficient to prevent light stress. Here we use an experimental approach to assess photoacclimation of adult and juvenile corals to light regimes that have the same mean DLI but varying peak irradiance.

Growth via calcification is energetically expensive and is stimulated by light in photosymbiotic corals (Gattuso *et al.* 1999a; Al-Horani *et al.* 2003). Photosynthetically derived sugars are transferred from symbiont to host, which in turn are metabolised and provide much of the ATP necessary to import calcium and export hydrogen ions to and from the site of calcification (Al-Horani *et al.* 2003). The removal of intracellular CO_2 due to photosynthesis also raises internal pH, further facilitating the precipitation of calcium carbonate (Cohen & Holcomb 2009; Venn *et al.* 2019). High light availability thus increases coral photosynthesis and calcification (Chalker & Taylor 1975; Gattuso *et al.* 1999a). However, the relationship between light intensity and photosynthetic rate is affected by photoacclimation (Anthony & Hoegh-Guldberg 2003a; Hoogenboom *et al.* 2009) whereby corals acclimated to low light levels have higher photosynthesis rates at low light compared to corals acclimated to high light (Anthony & Hoegh-Guldberg 2003b; DiPerna *et al.* 2018). This means that under variable DLIs, irradiance will be higher or lower than what the corals are acclimatised to and, therefore, corals may experience suboptimal performance during photoacclimation periods. Consequently, photoacclimation status is likely to track the variability of the light regime that corals experience to influence colony growth rates, but the nature of this relationship is largely unknown.

Complicating the corals' response to light variability is ongoing ocean acidification (OA); the process whereby the carbonate chemistry of the world's oceans is being altered by anthropogenic carbon dioxide (CO_2) emissions (Orr *et al.* 2005). The ocean surface water pCO_2 is currently ~415 ppm, already ~130 ppm higher than in pre-industrial times, and depending on future emissions, could more than double by the end of the century (IPCC 2021). Although higher CO_2 may serve as a substrate for enhanced photosynthesis, coral calcification becomes increasingly energetically expensive with the concurrent declining seawater pH (Cohen & Holcomb 2009). Subsequently, high levels of CO_2 have often been shown to reduce rates of coral calcification, although there is substantial variation in responses among species and studies (Kroeker *et al.* 2010). Corals might be somewhat buffered against this negative effect under high irradiance through increased photosynthesis (Edmunds 2011), however this is unlikely to be the case at lower irradiance levels (Suggett *et al.* 2013; Comeau *et al.* 2014a; Enochs *et al.* 2014; Vogel *et al.* 2015).

It is therefore unclear whether corals exposed to low light (e.g. in deeper waters), or variable light (e.g. during cloudy periods or times of high turbidity from sediment resuspension) experience more detrimental effects of future OA than corals growing in high light environments.

To complicate matters further, preferred habitats and stress responses may vary across life stages. For example, many juvenile corals avoid horizontal high-light surfaces during settlement (Tomascik 1991; Babcock & Mundy 1996; Doropoulos *et al.* 2016), as these environments can lower post-settlement survival (Sato 1985; Mundy & Babcock 2000). However, the growth of juvenile and adult corals alike may increase in such high light habitats (Gattuso *et al.* 1999a; Doropoulos *et al.* 2016). Furthermore, elevated CO_2 can reduce juvenile growth (Albright *et al.* 2008; Albright & Langdon 2011; Foster *et al.* 2016), while promoting the photosynthesis and growth of competitive turf algae (Connell *et al.* 2013). Microhabitat differences in light and CO_2 levels, as well as increased competition with other benthic taxa, may expose juvenile corals to environmental conditions that differ from those to which the adults are exposed. However, the complementarity of responses of adult and juvenile corals to the same light and CO_2 environments remains largely unexplored.

In this study we examined how contrasting DLIs (both static and variable) in combination with elevated CO_2 would alter the growth, photophysiology and survival of adult and early post-settlement juvenile corals. By comparing the photophysiology of corals exposed to variable and static light treatments with the same DLI we investigate if corals can rapidly photoacclimate to changing light conditions to maintain calcification relative to DLI, or if they are negatively influenced by periods of high and low intensity.

4.3 Materials and methods

Experimental setup

All experiments were conducted at the National Sea Simulator (SeaSim), at the Australian Institute of Marine Science (AIMS), Townsville Australia in January to March 2017. Twenty-four 50 L aquaria were set up each with one of two CO_2 treatments, fully crossed with four light treatments, with three replicate tanks per treatment. Light treatments alternated between tanks around the aquaria room, while CO_2 treatments were blocked on each side due to constraints within the room design. Water temperature was

maintained at 27 ^{o}C ± 0.3 ^{o}C SE. Water movement was provided by individual pumps in each tank (Turbelle 6015, Tunze, Germany) and flow-through water exchange rates were set at 600 mL min^{-1} of ultrafiltered (0.4 µm) seawater plus 100 mL min^{-1} of unfiltered seawater. The unfiltered seawater was added as a food supplement including naturally occurring plankton and detrital material, benefitting the growth and survival of coral recruits in this system (Conlan *et al.* 2017).

The CO_2 treatment consisted of two levels, nominally set to 400 and 900 ppm pCO_2. Seawater was delivered to the tanks after in-line CO_2 injection with a mass flow controller (Aalborg GFC17s 0-10 std. mL min^{-1}) and membrane contactor (Liqui-Cell Extra-Flow 2.5, 3M Business Group, USA). The mole faction of CO_2 (pCO_2 in ppm) was continually monitored in one tank per CO_2 treatment by an equilibration setup following SOP5 in Dickson et al. (2007), which was providing feedback to control the CO_2 dosage system. The gas analyser used in the equilibration setup (Telaire T6613) was calibrated against 0.0 and 1500 ppm CO_2 gas standards, and then confirmed with a mid-range reading at 600 ppm. To ensure the ambient treatment was maintained at 400 ppm, seawater was first stripped of dissolved CO_2 with soda-lime and then dosed as per the elevated treatment method. Sixteen pH sensors (CPS471D isFET, Endress and Hauser, Switzerland) were distributed across the 24 aquaria (eight per CO_2 treatment) to ensure the pH in all aquaria did not deviate from the aquarium used in the CO_2 feedback control.

Each tank was illuminated with LED lights (Hydra HD, Aquaillumination USA) over a 12 hour light/dark cycle, with light intensity linearly ramping up and down for five hours, and including two noon hours at maximum intensity. All four light treatments ('low', 'medium', 'high' and 'variable') had the same 12 hr ramping light schedule, but at different intensities. The high light treatment (HL) had a noon maximum of 500 µmol photons m^{-2} s^{-1} and a DLI of 12.6 mol photons m^{-2} d^{-1}, the medium treatment (ML) had a noon maximum of 300 µmol photons m^{-2} s^{-1} and a DLI of 7.6 mol photons m^{-2} d^{-1}, while the low light treatment (LL) had a noon maximum of 100 µmol photons m^{-2} s^{-1} and a DLI of 2.5 mol photons m^{-2} d^{-1}. The variable light treatment (VL) oscillated on a five day cycle, with four days at the LL intensity, a ramp day at ML, four days at the HL intensity, another ramp day, and then repeating. Long-term mean DLI in VL was therefore the same as ML. Light intensities were measured weekly with a calibrated underwater PAR sensor (meter: LI 1400, sensor: LI 192, Licor, USA) and were maintained at set values. Both adult and juvenile corals (details below) were kept in the same experimental aquaria. All aquaria began at 400 ppm pCO_2 and the LL light levels and were gradually ramped to their set values (VL began in the high light phase) one day after juvenile corals were added. The CO_2 and light treatments were ramped evenly over four and

three days, respectively. The adult corals, which came from a field location where light levels were similar to the high light treatment (see below; Fig 4.S1), were added directly into the aquaria 15 days after the juveniles.

Duplicate samples were taken weekly from the unfiltered and filtered incoming seawater, prior to CO_2 manipulation, for salinity (HQ30d, Hach USA) and dissolved nutrients (ammonium (NH_4), nitrate (NO_3), nitrite (NO_2), phosphate (PO_4), and silicon dioxide (SiO_2)), assessed with an autoanalyser following Ryle (1981) after 0.04 µm filtration. The water in the experimental aquaria averaged 34.8 ± 0.36 SE PSU salinity, 0.39 ± 0.05 µmol L^{-1} NH_4, 1.55 ± 0.07 µmol L^{-1} NO_3, 0.24 ± 0.03 µmol L^{-1} NO_2, 0.15 ± 0.01 µmol L^{-1} PO_4, 7.82 ± 0.65 µmol L^{-1} SiO_2, and 2301.93 ± 4.33 µmol kg^{-1} A_T. These values are typical for of coastal waters where the experiment was conducted, but higher than Davies Reef where the adults were collected (Schaffelke *et al.* 2012).

Additional samples were taken throughout the experiment (n = 5) for total alkalinity (A_T) and dissolved inorganic carbon (C_T) analyses (Marianda VINDTA 3C, Germany) following Dickson (2007). The CO_2/pH treatments were successfully maintained for the duration of the experiment (Fig 4.S1), and pCO_2 and pH_{Total} ranged between 390 - 430 ppm and 7.97 - 8.16 in the control treatment, and 852 – 992 ppm and 7.64 - 7.88 in elevated CO_2 treatment. All samples were processed within the Analytical Services laboratory at AIMS.

Adult corals

Adult corals were obtained from four colonies each of *Acropora tenuis* and *Acropora hyacinthus*, collected from ~4 m depth at Davies Reef (18.83 S, 147.63 E) in January 2017. At this time of year daily light levels can be both high due to the high summer sun angles but are highly variable due to monsoonal clouds (Fig 4.1). Subsequently DLIs can be higher or lower than other times of the year, and daily maxima vary ~five-fold over periods of days. The median DLI from the depth of coral collection was approximately the same as our HL treatment (~12 mol photons m^{-2} d^{-1}), and the variation in DLI was such that corals naturally encounter conditions equivalent to our LL treatment (2.52 mol photons m^{-2} d^{-1}). The median DLI of the collection site at 5 m depth is 9.34 mol photons m^{-2} d^{-1}, with a range of 0.40 – 18.61 mol photons m^{-2} d^{-1} (Fig 4.1).

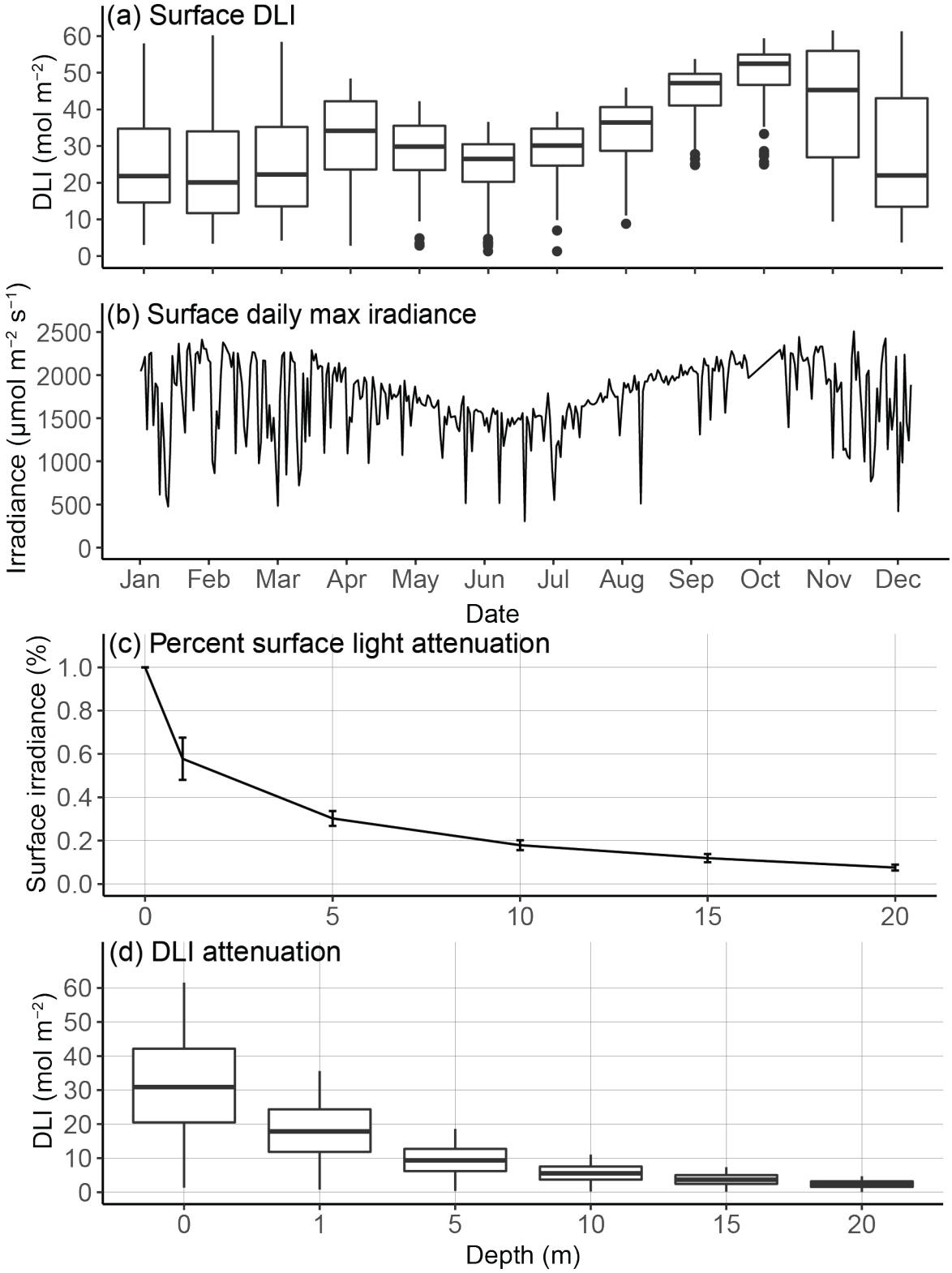
(a) Surface DLI
DLI (mol m⁻²)
0
10
20
30
40
50
60
(b) Surface daily max iradiance
Irradiance (µmol m⁻² s⁻¹)
0
500
1000
1500
2000
2500
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Date
(c) Percent surface light attenuation
Surface irradiance (%)
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
20
(d) DLI attenuation
DLI (mol m⁻²)
0
10
20
30
40
50
60
0
1
5
10
15
20
Depth (m)

Fig 4.1 The light environment of Davies Reef where the adult corals from the experiment were collected. The plot shows: daily light integrals (DLI: mol photons m^{-2} d^{-1}) at the water surface by month (combined 2013 – 2016, i.e., the four years prior to coral collection) (a), maximum instantaneous irradiance (µmol photons m^{-2} s^{-1}) per day in 2016 (b), the percent attenuation of surface irradiance by water depth ± SE (c), and the attenuation of DLI with depth (d). Surface light readings are recorded by the Australian Institute of Marine Science weather station at Davies Reef (http://data.aims.gov.au/aimsrtds/datatool.xhtml?from=1980-01-01&thru=2020-04-15&qc=LEVEL1&channels=72: accessed April 2020). Boxplots are standard (the horizontal line is the median, the box encloses the first to third quartiles, the whiskers are 1.5 the interquartile range and the circles are outliers). The percent depth attenuation relationship is based on a series of light profiles performed in July 2016. See DiPerna et al. (2018) Fig 4.S1 for information on attenuation profiles (N = 3). The DLIs at depth are calculated based on the average DLI in panel (a) multiplied by the percentage attenuation in panel (c)

Each colony was cut into 12 nubbins of ~5 cm, superglued to aragonite discs, and given two days to heal before recording their buoyant weights (Shimadzu AUW220D, Japan). Two nubbins per species were placed into each of the 24 aquaria so that one or two of the 12 nubbins per genotype were allocated to each of the eight treatments (i.e. the four light * two CO_2 treatments). The aragonite discs were embedded in PVC racks, minimising their exposure to seawater and potential dissolution under high CO_2. In addition to including unfiltered seawater in the experiment aquaria, freshly hatched *Artemia* nauplii were added to the aquaria daily at densities of 350 L^{-1} to feed the corals, as photoacclimation may not be maintained in starved corals (Titlyanov *et al.* 2001a). Feeding densities of *Artemia* followed Petersen et al. (2008), and were lower than zooplankton densities typically found on coral reefs (Roman *et al.* 1990). The adults remained under the experimental conditions for 51 days before the experiment ended. Mortality was limited to a single nubbin in a low light + 900 ppm pCO_2 tank.

After 41 days of acclimation to the experimental treatments, effective quantum yields of photosystem II (ϕ_{PSII}), as well as maximum quantum yields of photosystem II (F_v/F_m), were measured with a pulse amplitude modulated fluorometer (Diving PAM, Waltz, Germany). Quantum yield measurements were used to assess photoinhibition, where declines in ϕ_{PSII} alone would suggest dynamic photoinhibition,

whereas declines in F_v/F_m, coupled with an increase in dark adapted minimum fluorescence (F_o), would suggest chronic photoinhibition (Osmond & Grace 1995; Maxwell & Johnson 2000; Gorbunov *et al.* 2001). The ϕ_{PSII} measurements were taken at noon after at least one hour of maximum irradiance, then lights were turned off for at least one hour before measuring F_v/F_m. PAM measurements of the VL nubbins were made daily during the final ten days of the experiment to capture a complete light treatment cycle, with light levels as follows: day one: high light; day two: medium light; days three to six: low light; day seven: medium light; and days eight to ten: high light. The static light treatments were measured three times within this period (days two, six, nine). PAM settings were constant throughout: measuring intensity 2, saturating intensity 8, saturating width 0.8 s, gain 2 and damping 2. Three PAM measurements were taken per nubbin and averaged prior to analysis.

Buoyant weights of the adult nubbins were recorded again at the end of the experiment (49 days from initial measurements). The initial weight of each aragonite disc was subtracted from the combined nubbin/disc weight and growth was estimated following Jokiel et al. (1978). Growth was defined as percent weight increase, relative to the initial weight of each nubbin, as well as the increase in total dry weight of skeletal material (mg) per day standardised by nubbin surface area.

A small branch from each nubbin was snap-frozen in liquid nitrogen and stored at -80 ^{o}C for later analyses for soluble protein and pigment content (chlorophylls *a* and c_2, and total carotenoids) following standard procedures. Briefly, each nubbin was water-picked in 10 mL of 0.04 µm filtered seawater to remove coral tissue. This tissue slurry was then homogenised and centrifuged to separate coral and symbiont components following Dove et al. (2006). Coral soluble protein content was quantified from the coral tissue supernatant with the DC protein assay kit (Bio-Rad Laboratories, Australia), while algal pigments in the pellet were determined spectrophotometrically (Powerwave microplate reader, Bio Tek, USA), following Ritchie (2008) for chlorophyll *a* and c_2, and Lichtenthaler (1987) for total carotenoids. Soluble protein and pigment content were standardised to nubbin surface area, estimated with the single wax-dipping technique (Veal *et al.* 2010). Pigment content was also standardised by soluble protein content.

Juvenile corals

To obtain cohorts of coral juveniles for the experiment, 990 aragonite discs (20 mm diameter), preconditioned and covered in crustose coralline algae (CCA) and other biofilms, were randomly distributed across six separate 52 L aquaria for bulk larval settlement. Settlement aquaria were kept at 27 ^{o}C, had a water exchange rate of 200 mL min^{-1} of 0.04 µm ultra-filtered seawater, did not have their CO_2 manipulated, and had ramping 12 hr light/dark cycles at a maximum irradiance of 120 µmol photons m^{-2} s^{-1} (DLI of 2.59 mol photons m^{-2} d^{-1}). Each tank had ~1500 one-month old larvae added of either *A. tenuis* or *A. hyacinthus,* derived from the gametes of five and three adult colonies respectively. Larvae were allowed 24 hrs to settle before coral photosymbiotic dinoflagellates from the AIMS Symbiont Culture Facility were added at ~1000 algal cells mL^{-1} to facilitate algal inoculation. The algae provided comprised even parts of C1, D1a, A and A3c ITSII types (SCF IDs: 010.02, 022.01, 055-01.10 and 082). After three weeks the discs with the most juveniles (n = 226 discs for *A. tenuis* and 288 for *A. hyacinthus*), were evenly distributed across the 24 experimental aquaria.

Macro photographs of the aragonite discs were taken on the day they were placed in the experimental aquaria, and after 63 days (the experiment's end). The photographs were used to visually assess the juveniles' survivorship (alive vs dead) and growth (change in the number of corallites) by a single observer (Fig 4.S2). Discs were also assessed visually for any algal overgrowth and were categorised at each census point as either not being overgrown, or being overgrown with filamentous turf algae, *Peysonnelia* spp., or a combination of turf algae and *Peysonnelia* spp. (Fig 4.S3).

Statistical analyses

All statistics were conducted in the program R version 3.6.1 (R Development Core Team 2021). Generalised liner models (GLMs) were conducted on both the adult and juvenile data. Models for differences in adult photophysiological parameters between the static light treatments, as well as juvenile growth, were based on Gaussian distributions with identity link functions as data approximated normality. A series of GLMs with the same distributions and link functions were also used to compare the photophysiological parameters from the static light treatments to periods of the variable light treatment with the same light intensity (e.g. HL vs VL days 1, 8 - 10 and LL vs VL days 3 - 6). Models for percent adult

growth, adult soluble protein and pigment content per colony surface area, and the proportion of juvenile survival were fit to quasipoisson distributions as they were either proportion data, right skewed, or over-dispersed. Light and CO_2 treatments and adult genotype were included in the GLMs as fixed factors, while experimental aquaria identity was removed from final models due to statistical insignificance. Models investigating the proportion of juvenile survivors were weighted by the initial number of recruits per settlement disc. All GLMs were performed in R using base features.

Changes in photophysiology in adult corals from the variable light treatment over the final ten days of the experiment were analysed using generalised additive mixed models (GAMMs). Here, light, CO_2 and genotype were included as fixed factors, while date was included as the smooth term. Models were constructed in R with the gamm function of the mgcv package.

Multinomial log-linear models were used to examine patterns in the algal overgrowth of the juvenile settlement discs in relation to experimental light and pCO_2 treatments at the end of the experiment. These models compute the probability of the different algal overgrowth categories occurring under the experimental treatments (Fig 4.S3). Likelihood ratio tests were used to compare different models with and without each of the main effects (light and CO_2), their interaction, and the experimental aquaria identity, via analysis of variance (ANOVA) to determine which were influencing the probability of the algal overgrowth (Table 4.S3). Pairwise Tukey's HSD tests were then used to determine if there were statistical differences between treatment levels. Multinomial models were performed in R using the multinom function in the nnet package.

4.4 Results

Adult coral growth

Growth rates of both species were significantly affected by light, but not by CO_2 or the interaction between these two treatments (Fig 4.2, Table 4.1). For both species, adult growth rates decreased with DLI, from high to low light, and did not differ between the medium- and variable-light treatments with the same DLI (i.e. growth in HL > ML = VL > LL, Tukey's HSD, all $p < 0.02$). The 80% light reductions between the high and low light treatments resulted in growth rates of *A. tenuis* decreasing by 63% (0.35 ± 0.05% d^{-1} to 0.11 ± 0.01% d^{-1}), and in *A. hyacinthus* by 72% (from 0.19 ± 0.2% d^{-1} to 0.05 ± 0.01% d^{-1}) (Fig 4.2). Growth rates

also differed between genotypes in both species, however the effects of the light treatment were stronger (Table 4.1).

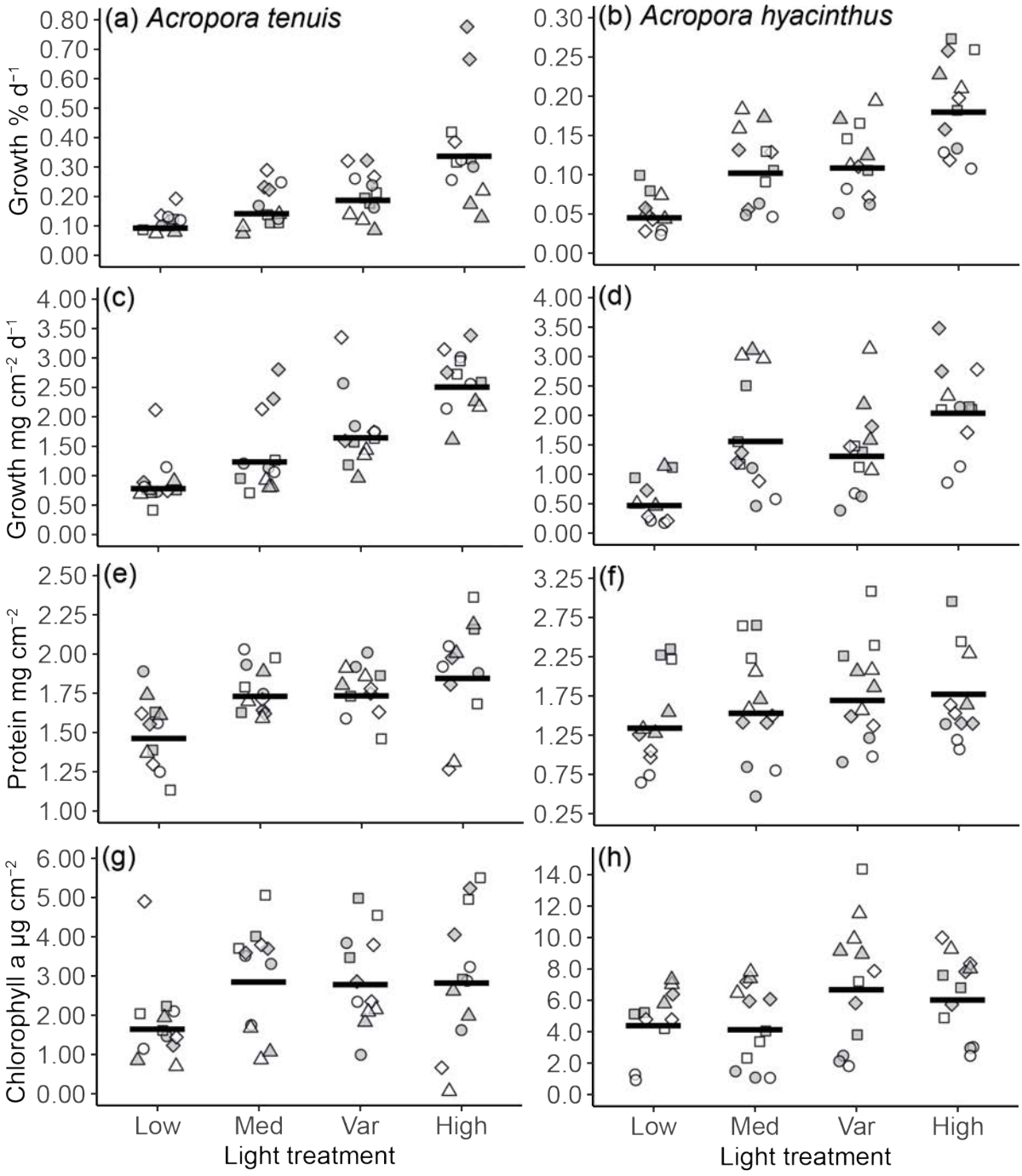

Fig 4.2 The effects of light intensity and CO_2 on percent daily growth (a and b), daily calcification standardised by surface area ($CaCO_3$ mg cm^{-2} d^{-1}) (c and d), and tissue protein (e and f) and chlorophyll *a*

(g and h) standardised by surface area, in *Acropora tenuis* (a, c, e and f) and *A. hyacinthus* (b, d, f and h). The experimental light treatments were low, medium (Med), variable (Var) and high intensity. Symbols represent the different coral genotypes (n = 4), and colours represent the 400 (white) or 900 (grey) ppm pCO_2 treatments. The horizontal bars show mean values per light treatment

Table 4.1 Effects of light and CO_2 treatments, and genotypes on adult *Acropora tenuis* and *A. hyacinthus* growth, and protein and symbiont pigment contents. Results of generalised linear models, bold values indicate $p < 0.05$. Pigment responses standardised by protein content are summarised in Table S1

		A. tenuis		*A. hyacinthus*	
	Df	F	p	F	p
Growth (% d^{-1})					
Light	3	53.850	**<0.001**	37.157	**<0.001**
CO_2	1	0.088	0.768	0.035	0.853
Genotype	3	37.103	**<0.001**	19.662	**<0.001**
Light * CO_2	3	2.152	0.110	0.631	0.600
Growth cm^{-2} ($CaCO_3$ mg cm^{-2} d^{-1})					
Light	3	32.91	**<0.001**	17.35	**<0.001**
CO_2	1	0.024	0.877	3.996	0.053
Genotype	3	11.83	**<0.001**	14.06	**<0.001**
Light * CO_2	3	0.389	0.649	1.537	0.222
Protein (mg cm^{-2})					
Light	3	7.423	**<0.001**	7.606	**<0.001**
CO_2	1	5.918	**0.020**	0.433	0.515
Genotype	3	1.483	0.235	103.8	**<0.001**
Light * CO_2	3	1.746	0.174	0.470	0.705
Chlorophyll *a* (µg cm^{-2})					

Light	3	3.700	**0.020**	8.988	**<0.001**
CO_2	1	0.097	0.757	1.038	0.315
Genotype	3	10.05	**<0.001**	50.15	**<0.001**
Light * CO_2	3	0.476	0.701	2.690	0.061
Chlorophyll c_2 (μg cm^{-2})					
Light	3	1.210	0.319	0.965	0.420
CO_2	1	1.020	0.319	3.522	0.069
Genotype	3	2.900	**0.048**	9.360	**<0.001**
Light * CO_2	3	0.350	0.789	0.276	0.844
Carotenoids (μg cm^{-2})					
Light	3	4.566	**0.008**	18.11	**<0.001**
CO_2	1	0.160	0.692	0.026	0.874
Genotype	3	5.410	**0.004**	63.61	**<0.001**
Light * CO_2	3	0.375	0.771	4.927	**0.006**

Adult coral soluble protein content

Tissue soluble protein content per unit surface area in both species was also negatively affected by declining DLI (Fig 4.2, Table 4.1), and there was no difference between the ML and VL treatments (Tukey's HSD, $p > 0.05$) (Table 4.1). *Acropora tenuis* soluble protein content was reduced ~10% in the LL treatment compared to the other light treatments (i.e. soluble protein in LL < ML = VL = HL, Tukey's HSD, all $p < 0.02$), and it was also 10% lower in the 400 ppm pCO_2 treatment compared to 900 ppm. In combination, *A. tenuis* soluble protein content in the LL + 400 ppm treatment was 68% lower than the HL + 900 ppm treatment (1.37 ± 0.08 vs 2.01 ± 0.06 mg cm^{-1}). *Acropora hyacinthus* soluble protein content was reduced by 12 - 24% in the LL treatment compared to the other light treatments (i.e. soluble protein in LL < ML = VL = HL, Tukey's HSD, all $p < 0.04$), and significant differences were also seen between genotypes (Table 4.1).

Adult coral symbiont pigments

The pigment responses differed between coral taxa and standardisation method. In *A. tenuis*, mean chlorophyll *a* content standardised by coral surface area was 39% lower in the low vs. high light treatments (Fig 4.2), while total carotenoid content was 57% lower. However, variability was high and genotype effects strong, and pigments did not differ in response to the light treatments in *post-hoc* examination (Tukey's HSD, all $p > 0.05$). *A. tenuis* chlorophyll c_2 cm^{-2}, and chlorophyll *a* and c_2 and total carotenoids standardised by soluble protein were unaffected by the light and CO_2 treatments, and only differed between genotypes (Table 4.S1).

For *A. hyacinthus*, chlorophyll *a* cm^{-2} was affected by light but not by CO_2 treatments (Fig 4.2, Table 4.1). Concentrations were equally high in the high and variable light treatments, while the medium and low light levels were equal and ~50% reduced (i.e. chlorophyll *a* cm^{-2} in (HL = VL) > (ML = LL), Tukey's HSD, all $p < 0.02$). No consistent patterns were detected in response to the light or CO_2 treatments for any of the other pigments, regardless of standardisation method, which only differed between genotypes (Table 4.S1).

Adult coral photophysiology: static light treatments

In each of the three static light treatments, both ϕ_{PSII} and F_v/F_m did not differ between measurement days (GLMs, all $p > 0.05$), and days were thus averaged prior to analyses. Differences in ϕ_{PSII} were greatest between genotypes in both species (Table 4.2), but in *A. tenuis*, values also decreased with DLI and elevated CO_2 (Table 4.2, Fig 4.3). These effects were additive, and ϕ_{PSII} in the LL + 400 ppm treatment was ~10% higher than in the HL + 900 ppm treatment (0.64 ± 0.01 vs. 0.58 ± 0.01) (Fig 4.3). In *A. hyacinthus,* ϕ_{PSII} was affected by light but not by CO_2, and LL values were ~2% lower than HL values (0.59 ± 0.01 vs 0.58 ± 0.01).

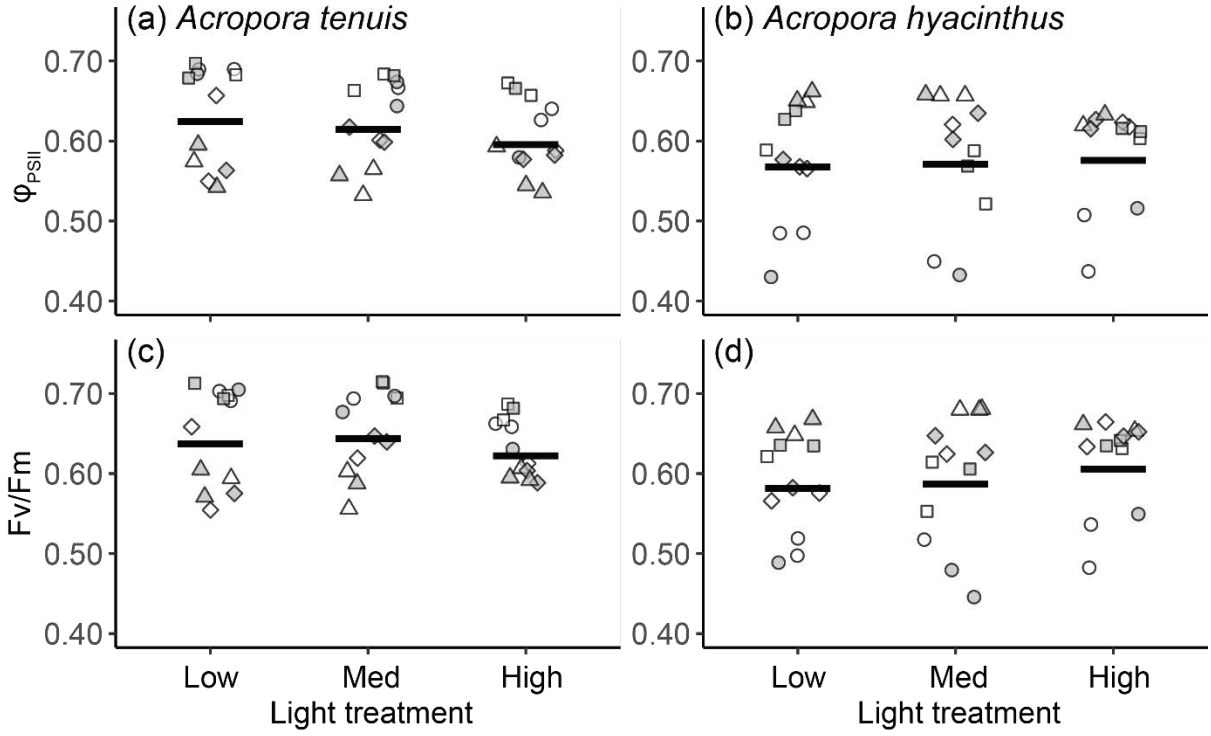

Fig 4.3 Photophysiological responses in the static light treatments, measured as effective (ϕ_{PSII}: a and b) and maximum (F_v/F_m: c and d) quantum yield of photosystem II, of *Acropora tenuis* (a and c) and *A. hyacinthus* (b and d). Colours represent the 400 (white) or 900 (grey) ppm pCO_2 treatments, and symbols represent the different coral genotypes (n = 4). Horizontal bars are means per light treatment. Values are averages for each genotype across the three time points in the final ten days of the experiment

Table 4.2 Photophysiological response of adult *Acropora tenuis* and *A. hyacinthus* corals from the static light treatments. Generalised linear models examined the effects of experimental light, CO_2 and genotype on the effective (ϕ_{PSII}) and maximum (F_v/F_m) quantum yield of photosystem II. Measurements from the three time-points in the final ten days of the experiment combined. Bold values indicate $p < 0.05$

		A. tenuis		*A. hyacinthus*	
	df	F	p	F	p
ϕ_{PSII}					
Light	2	10.17	**<0.001**	4.351	**0.015**

CO_2	1	11.06	**0.001**	3.735	0.056
Genotype	3	102.9	**<0.001**	98.55	**<0.001**
Light * CO_2	2	2.731	0.070	1.251	0.291
F_v/F_m					
Light	2	6.740	**0.002**	5.530	**0.005**
CO_2	1	3.149	0.079	10.90	**0.001**
Genotype	3	111.4	**<0.001**	156.9	**<0.001**
Light * CO_2	2	1.650	0.197	1.289	0.280
F_o					
Light	2	20.86	**<0.001**	6.001	**0.003**
CO_2	1	6.953	**0.009**	4.148	**0.044**
Genotype	3	117.0	**<0.001**	19.27	**<0.001**
Light * CO_2	2	1.780	0.174	1.713	0.186

Differences in F_v/F_m values in the static light treatments were greatest between genotypes in both species (Table 4.2, Fig 4.3). The light treatment also significantly affected F_v/F_m in both species, however differences between high and low light were inconsistent between species and only varied by ~3% (Fig 4.3). In *A. hyacinthus*, F_v/F_m also increased by ~3% in the elevated CO_2 treatment compared to the control.

Values of F_o in both species from the static light treatments were significantly affected by light, CO_2, and genotype, with genotype explaining the most amount of variation (Table 4.2, Fig 4.S5). Patterns in F_0 values between the light treatments were inconsistent between species. In *A. tenuis*, F_o values were ~10% lower, while in *A. hyacinthus*, they were ~10% higher under LL than under ML or HL.

Adult coral photophysiology: variable light treatments

Values of ϕ_{PSII} and F_v/F_m in VL corals during the final 10 days of the experiment strongly differed between genotype, and were largely unaffected by CO_2, except for a minor increase in *A. tenuis* ϕ_{PSII} at

elevated CO_2 (Table 4.3; Fig 4.4). For both parameters, the changing light intensity explained more variability than date, CO_2, or the interaction between light and CO_2 (GAMMs, Table 4.3). ϕ_{PSII} values increased in both species by ~10% as light transitioned from high to low intensity (day 1 to 3) and decreased by the same amount as light transitioned from low to high intensities (day 6 to 8), while remaining stable during the periods of static light (2 - 3% change during days 3 - 6 and 1, 8 - 10) (Fig 4.4). F_v/F_m values remained relatively stable through the light cycle, and like ϕ_{PSII} did not differ significantly within either the periods of high or low light (GLM, all $p > 0.05$). In both species, F_v/F_m increased by 2 - 5% when light transitioned from high to low intensities (day 1 to 3), and similarly decreased by the same amount from low to high light (day 6 to 8), while only varying by 1 – 4 % within the high and low light periods (days 3 to 6 and 8 to 1; Fig 4.4). Values of F_o from both species were unaffected by changes in light intensity through the variable light cycle (Table 4.3, Fig 4.S5). Instead F_o differed predominantly between coral genotypes and increased throughout the whole cycle regardless of the changing light intensity (Table 4.3, Fig 4.S6). Hence, there was little evidence of chronic photoinhibition or photodamage in corals from the variable light treatment.

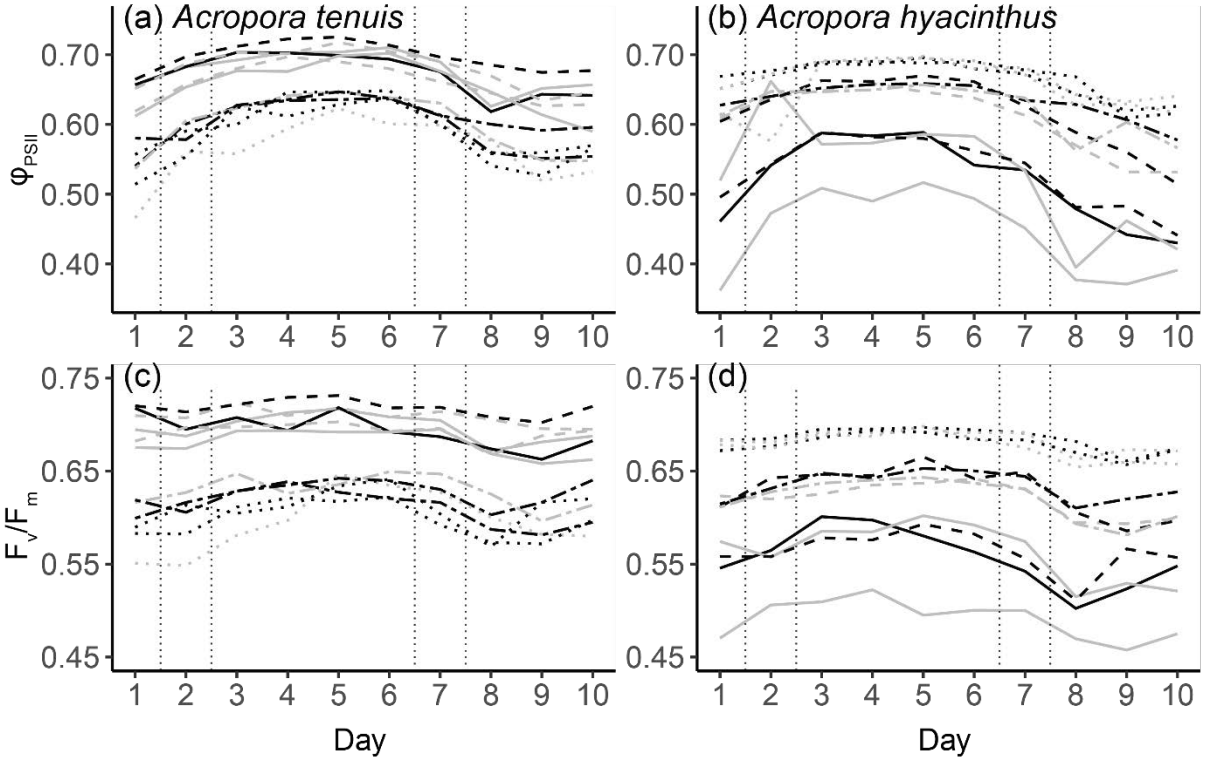

Fig 4.4 Daily changes in the photophysiology of *Acropora tenuis* (a and c) and *A. hyacinthus* (b and d) in the variable light treatment over the final ten days of the experiment. Line type represent the 400 (black) and 900 (grey) ppm pCO_2 treatments. Line types represent the different coral genotypes, with each line following an individual coral nubbin through the variable light cycle. The vertical lines indicate the light transition days. The light schedule during these ten days was - day one and days eight to ten: high light; days two and seven: medium light; days three to six: low light. The photophysiological response included the effective (ϕ_{PSII}: a and b) and maximum (F_v/F_m: c and d) quantum yield of photosystem II

Table 4.3 Generalised additive mixed model (GAMM) results of the effective (ϕ_{PSII}) and maximum (F_v/F_m) quantum yield of photosystem II, and the dark adapted minimum fluorescence (F_o) in *Acropora* tenuis and *A. hyacinthus* photosymbionts in the variable light treatment during the final ten days of the experiment. The noon light intensity on the day of measurement was included in the model as a factor (VLight- three values). The measurement date was included in models as the smooth term. Bold values indicate $p < 0.05$

		A. tenuis		*A. hyacinthus*	
	df	F	p	F	p
ϕ_{PSII}					
CO_2	1	12.35	**<0.001**	0.005	0.941
VLight	2	35.49	**<0.001**	20.64	**<0.001**
Genotype	3	282.0	**<0.001**	141.9	**<0.001**
VLight * CO_2	2	1.333	0.268	0.363	0.697
Date	1	9.156	**<0.001**	7.592	**0.007**
F_v/F_m					
CO_2	1	2.126	0.148	0.187	0.666
VLight	2	15.26	**<0.001**	11.60	**<0.001**
Genotype	3	313.9	**<0.001**	386.2	**<0.001**
VLight * CO_2	2	0.366	0.694	0.029	0.972
Date	1	0.641	0.425	4.065	**0.046**
F_o					

CO_2	1	6.062	**0.015**	1.688	0.197
VLight	2	0.953	0.389	0.486	0.616
Genotype	3	213.7	**<0.001**	59.03	**<0.001**
VLight * CO_2	2	2.463	0.090	0.180	0.836
Date	1	53.58	**<0.001**	14.38	**<0.001**

In both species there were differences in ϕ_{PSII} between the static low light treatments and the equivalent period in the variable light cycle (i.e., LL vs. VL average low-light days 3 – 6), additionally to differences between genotypes (Table 4.S3). ϕ_{PSII} was 5% lower in *A. tenuis* and 10% lower in *A. hyacinthus* in LL compared to the VL treatment during the low light phase (in contrast to the 10% increase in ϕ_{PSII} when transferred into low light). Furthermore, there was no or minor differences in ϕ_{PSII} between the static high light treatments and the equivalent period in the variable light cycle (i.e. HL vs. average VL high-light days 1, 8 – 10) (Table 4.S3). The difference in F_v/F_m between static and variable light treatments in the respective high light phases were <3% in both species, but for the low light phase they were <3% in *A. tenuis* and 8% in *A. hyacinthus.*

Juvenile coral growth and survival

The number and size of coral juveniles occupying the discs did not differ between treatments at the beginning of the experiment (GLM, all $p > 0.05$). Initially *A. tenuis* averaged 3.93 ± 0.23 juveniles per disc, with 1.45 ± 0.04 corallites per juvenile, and *A. hyacinthus* juveniles averaged 3.11 ± 0.12 per disc, with 1.74 ± 0.05 corallites per juvenile (Table 4.S5). The growth (change in corallite number) of *A. tenuis* juveniles over the course of the experiment was affected by the light treatment alone (Table 4.4), declining with increasing DLI. At the end of the experiment, the mean number of corallites per *A. tenuis* juvenile had increased by 1.47 ± 0.31 in the high-, 1.93 ± 0.50 in the medium-, 2.68 ± 0.83 in the variable-, and 2.81 ± 0.58 in the low-light treatment. However, this light effect was weak (Table 4.4), and differences between light treatment levels were insignificant in *post-hoc* examination (Tukey's HSD, all $p > 0.05$). Growth in *A. hyacinthus* juveniles was faster than in *A. tenuis* but was unaffected by the experimental treatments (Table 4.4). By the end of the experiment the mean number of *A. hyacinthus* corallites had increased to 6.04 ±

0.36 across all treatments. Neither species' growth rate was affected by pCO_2 or algal overgrowth (Table 4.4).

Table 4.4 Results of generalised linear models examining growth and survival of juvenile *Acropora tenuis* and *A. hyacinthus* over the duration of the experiment in response to experimental light, pCO_2 and algal overgrowth. Bold values indicate $p < 0.05$.

		A. tenuis		*A. hyacinthus*	
	df	F	p	F	p
Juvenile growth					
Light	3	3.963	**0.010**	1.742	0.160
pCO_2	1	0.658	0.419	0.187	0.666
Overgrowth	3	1.510	0.216	1.875	0.135
Light * pCO_2	3	1.600	0.194	0.552	0.647
Juvenile survival					
Light	3	2.118	0.099	1.425	0.236
pCO_2	1	2.236	0.136	2.364	0.125
Overgrowth	3	11.66	**<0.001**	15.19	**<0.001**
Light * pCO_2	3	0.199	0.897	0.401	0.752

The survival of *A. tenuis* juveniles averaged 36.85 ± 0.03 %, and of *A. hyacinthus* 45.27 ± 0.02 after 63 days at the end of the experiment. The algal communities on the aragonite discs with the coral juveniles changed throughout the experiment (Fig 4.S4), and overgrowth affected coral survival more than the direct effects of the experimental treatments (Fig 4.S4, Table 4.4). In *A. tenuis*, juvenile survival was significantly lower on discs overgrown with *Peyssonnelia* spp. (17.4 ± 2.98%) compared to discs overgrown with turf (37.59 ± 5.47%) and discs not overgrown (46.54 ± 5.04%) (Tukey's HSD, all $p < 0.05$). Discs overgrown with a combination of turf and *Peyssonnelia* spp. recorded 20.18 ± 5.73% *A. tenuis* survival. Juvenile *A. hyacinthus* survival was also significantly lower on discs overgrown with *Peyssonnelia* (21.68 ± 3.58%) compared to those overgrown with *Peyssonnelia* spp. and turf (51.70 ± 5.53%), those overgrown

with turf (51.43 ± 3.06%), as well as discs not overgrown (55.90 ± 5.46%) (Tukey's HSD, all $p < 0.05$) (Fig 4.S4).

The algal overgrowth of discs containing the juveniles was affected by light, pCO_2 and their interaction, as well as by experimental aquaria identity (Table 4.S3, all $p < 0.05$), however the light treatment was most influential (Table 4.S3, χ^2 values). Initially 96% of discs were not being overgrown, but by the end of the experiment *Peyssonnelia* spp. dominated low light discs, and turf algae dominated high light discs. Here 58% of discs in the low light treatment were overgrown with *Peyssonnelia* spp., and 60% of discs in the high light treatment were overgrown by turf, both of which were significantly higher than all other overgrowth categories at these light levels (Tukey's HSD, all $p < 0.05$) (Fig 4.S3). Algal overgrowth did not differ between the medium and variable light intensity treatments except for the category of combined *Peyssonnelia* spp. and turf algae, which more frequently occurred in the variable treatment (Tukey's HSD, $p < 0.05$) (Fig 4.S3). Furthermore, elevated pCO_2 resulted in significantly higher turf algal overgrowth, as well as fewer discs without any algal overgrowth, compared to the control treatment (Tukey's HSD, both $p < 0.05$) (Fig 4.S3). Consequently, juvenile survival rates appeared more affected by the increased out-competition by *Peysonnellia* spp. at low light rather than by direct light effects on their physiology.

4.5 Discussion

In this study we investigated the joint effects of contrasting and variable light and ocean acidification regimes on two reef corals. We found that variation in daily light integrals (DLIs), at environmentally relevant levels, can act additively over time, with fluctuations between low and high light periods accumulating to reduce coral growth. Overall, adult corals performed best at high light (fastest growth, most soluble protein per unit surface area), while juvenile survival was lowest at low light. The light treatments affected the measured parameters in both species and both life stages more than the 900 ppm pCO_2. Elevated CO_2 increased adult *A. tenuis* soluble protein content at all light levels, but otherwise had little effect at any light level.

The effect sizes of low DLI were substantial. Growth declined by 60 - 70%, and soluble protein by 10 - 20% per unit surface area, in both species with the 80% light reductions between the static-high and

-low treatments. Importantly, corals in the variable and static-medium light treatments, with the same cumulative DLI, displayed intermediate growth rates. This shows that within the light intensities used, it is the sum of DLI accumulating over time which determines these parameters, regardless of the level of DLI variability. This is perhaps not surprising given that the time taken for most corals to photoacclimate is generally longer than the variable light cycle used in this study (Roth 2014). We found similar results in a previous study, where growth rates of *A. millepora* declined with the accumulated sum of DLIs over time, across both static and variable light treatments (DiPerna *et al.* 2018). Sexual maturity and colony fecundity are largely size dependent in corals (Babcock 1991), and are hence affected by growth rates. These results indicate that even temporary reductions in benthic light could have negative consequences for coral populations, especially in the context of recovery following disturbance.

Adult coral chlorophyll *a* and total carotenoids per coral surface area were found to be reduced in the low light treatments. Coral low light photoacclimation typically results in an increase in such parameters (Roth 2014). However, similar declines have been documented in a range of species when DLI < 1 (Mass *et al.* 2007; Bessell-Browne *et al.* 2017a, b; Jones *et al.* 2020). Jones et al. (2020) found symbiont densities and the amount of chlorophyll *a* and c_2 per surface area of coral skeleton increased in *A. millepora* at light levels similar to our low light treatment (2.3 vs. 2.52 mol photons m^{-2} d^{-1}), however these values rapidly declined as light reduced further. While the cause of this discrepancy is unknown, clearly the light levels in the low light treatment of the present study were insufficient for these highly autotrophic species that were high-light acclimated prior to collection. It is unknown if the reductions in chlorophyll *a* and total carotenoids per coral surface area constituted declines in photosymbiont numbers or the amount of pigments per symbiont, but either likely result in a reduction of photosystem reaction centres and the capacity for photosynthesis (Mass *et al.* 2007).

Noontime ϕ_{PSII} differed by ~3% between the static light treatments, while it changed by 10% in the variable light treatment between periods of low and high light. This suggests that the corals in the static treatment had partially acclimated to their light treatments, but that photoacclimation takes longer than the 4-day duration of the variable light cycle to stabilise. Other studies documenting coral photoacclimation through light transition events report the majority of acclimation metrics similarly taking longer than four days (Anthony & Hoegh-Guldberg 2003a; Roth *et al.* 2010; Langlois & Hoogenboom 2014; Roth 2014). It is uncertain how ϕ_{PSII} equates to photosynthesis and downstream carbon fixation here, primarily because ϕ_{PSII} values are complicated by non-photochemical quenching, which can be four times

higher than photochemical quenching during periods of high irradiance (Gorbunov *et al.* 2001). However, reduced growth rates with lower DLI indicate that any acclimation to lower light in the static treatment was not enough for corals to maintain rates of calcification.

Corals in the variable light treatment showed clear patterns of dynamic photoinhibition through the light cycle. Noontime ϕ_{PSII} in both species changed by ~10% when the light levels transitioned between low and high days and remained relatively stable through the periods of static light. There was little evidence of chronic photoinhibition, as F_o did not respond to the variable light cycle and F_v/F_m changed by <5%. Dynamic photoinhibition under increased irradiance is a normal photo-protective mechanism in corals, and chronic photoinhibition is indicated by larger and sustained decreases in F_v/F_m coupled with an increase in F_o (Brown *et al.* 1999; Gorbunov *et al.* 2001; Iglesias-Prieto *et al.* 2004; Hoogenboom *et al.* 2006). If the high light periods had induced chronic photoinhibition, growth in the variable light treatment might have been lower than in the medium treatment with the same DLI. The adult corals were collected from a variable light environment with high DLIs, which may account for their better performance under higher light and the ability to handle such light fluctuations.

The *Acropora* species used in these experiments are highly autotrophic, and are largely restricted to shallow waters (Done 1982; Muir *et al.* 2015). Light reductions have been repeatedly shown to lower the growth of *Acropora* spp. (Enochs *et al.* 2014; Vogel *et al.* 2015; Bessell-Browne *et al.* 2017a). Moreover, Jones et al. (2020) reported declining growth of *A. millepora* with declining DLI from sedimentation, while other coral species were less negatively affected. *Acropora* corals require above 5.2 mol photons m^{-2} d^{-1} in winter to meet their basal metabolic needs (Muir *et al.* 2015), and light levels on inshore GBR reefs below 5 m depth are frequently below this value (Kleypas *et al.* 1999; Anthony *et al.* 2004). In the present study, declines in pigmentation in the static low light treatment (DLI = 2.52 mol photons m^{-2} d^{-1}), as well as the lower ϕ_{PSII} in static low light compared to the low light phase of variable light treatment, suggest the low light DLI was chronically too low, and periods of high light were likely beneficial for the variable light treatment corals. Hence factors such as optimal light levels, extent of heterotrophy, or trophic plasticity between autotrophic and heterotrophic energy supplies (Anthony & Fabricius 2000) may well determine the corals' responses to light intensity and light variability.

Surprisingly, increased CO_2 had few detectable effects on any of the measured responses of *A. hyacinthus* adults and on the juveniles of both species in our study. While increased CO_2 did raise the

soluble protein content of adult *A. tenuis* and their ϕ_{PSII} in the variable light treatment, their effect sizes were weak (~5%), and they did not translate into measurable effects on colony growth or survival. Many studies have found increased CO_2 to reduce coral calcification, which may be further exacerbated under low irradiance (Marubini *et al.* 2001; Ohde & Hossain 2004; Suggett *et al.* 2013; Comeau *et al.* 2014a; Vogel *et al.* 2015). However, many of these studies did not feed corals during experimentation, and the provision or deprivation of heterotrophic nutrients can effect coral calcification under OA (Edmunds 2011). Corals in the present experiment may have maintained calcification and growth at elevated CO_2 because they were fed *Artemia* nauplii. Even though *Acropora* spp. are highly light dependent, they still depend on food to obtain essential nutrients for tissue growth (Houlbreque & Ferrier-Pages 2009).

For the coral juveniles of *A. tenuis*, growth rates and colony size at the end of the experiment were highest in the constant low light treatment and declined with increasing DLI. This is contrary to the adults of this study, as well as previous studies that have seen juvenile coral growth increase with irradiance (Babcock & Mundy 1996; Box & Mumby 2007; Doropoulos *et al.* 2016). It is unknown why our results are contrary to previous work, however these previous studies were conducted over longer timescales (~1 yr) and did not specifically investigate early post-settlement juveniles. Furthermore, the microenvironment the juveniles were growing in in the present experiment responded to the light and CO_2 treatments. The thallus lobes of *Peyssonnelia* spp. can smother and kill even adult corals (Tanner 1995), and their proliferation at low light increased juvenile mortality. However, juveniles in the low light treatment that were not overgrown with *Peyssonnelia* spp. were otherwise left to grow uninhibited. The high light intensities induced turf algal coverage, which, although not as lethal as *Peysonellia* may have lowered *A. tenuis* growth by shading or physically abrading the juveniles, or through the production of allelochemicals (McCook *et al.* 2001; Box & Mumby 2007; Venera-Ponton *et al.* 2011). Furthermore, our juvenile corals had been reared under low light levels in the settlement tanks, while the adult corals came from the field with higher light. It remains unknown whether this difference in prior photoacclimation negated potential growth advantages for the juveniles at high light in a 63 day long experiment.

This study has shown that any temporary or chronic reductions in light reaching the seafloor can accumulate to limit the growth of two common coral reef corals, despite acclimation. The effects of reduced light on coral growth were stronger than those of ocean acidification at levels predicted for the end of the century. There are always limitations in translating aquarium experiments to the reef, as the acclimation process could differ between corals in these two environments, and light, water flow and other

interacting processes vary. Nevertheless, our study adds further evidence for the importance of DLIs, and for preserving water clarity around coral reefs, to maintain rates of coral growth and accelerating reef recovery following disturbance.

4.6 Supplementary material

Supporting supplementary material can be found at: https://doi.org/10.1007/s00227-021-03992-y

Chapter 5: Ocean acidification alters early successional coral reef communities and their rates of community metabolism

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5.1 Abstract

Ocean acidification is expected to alter community composition on coral reefs, but its effects on reef community metabolism are poorly understood. Here we document how early successional benthic coral reef communities change *in situ* along gradients of carbon dioxide (CO_2), and the consequences of these changes on rates of community photosynthesis, respiration, and light and dark calcification. Ninety standardised benthic communities were grown on PVC tiles deployed at two shallow-water volcanic CO_2 seeps and two adjacent control sites in Papua New Guinea. Along the CO_2 gradient, both the upward facing phototrophic and the downward facing cryptic communities changed in their composition. Under ambient CO_2, both communities were dominated by calcifying algae, but with increasing CO_2 they were gradually replaced by non-calcifying algae (predominantly green filamentous algae, cyanobacteria and macroalgae, which increased from ~30% to ~80% cover). Responses were weaker in the invertebrate communities, however ascidians and tube-forming polychaetes declined with increasing CO_2. Differences in the carbonate chemistry explained a far greater amount of change in communities than differences between the two reefs and successional changes from five to 13 months, suggesting community successions are established early and are under strong chemical control. As pH declined from 8.0 to 7.8, rates of gross photosynthesis and dark respiration of the 13-month old reef communities (upper and cryptic surfaces

combined) significantly increased by 10% and 20%, respectively, in response to altered community composition. As a consequence, net production remained constant. Light and dark calcification rates both gradually declined by 20%, and low or negative daily net calcification rates were observed at an aragonite saturation state of <2.3. The study demonstrates that ocean acidification as predicted for the end of this century will strongly alter reef communities, and will significantly change rates of community metabolism.

5.2 Introduction

The oceanic uptake of anthropogenic carbon dioxide (CO_2) emissions is causing ocean acidification (OA) (Doney *et al.* 2009). OA not only lowers seawater pH, but also reduces the saturation state (Ω) of calcium carbonate ($CaCO_3$) minerals, and increases CO_2 and bicarbonate ion concentration. Predicting how marine communities will respond to OA is complicated, as many of these chemical alterations can act as drivers of change (Doney *et al.* 2009; Connell *et al.* 2013). For example, the inhibition of calcification from declining pH and Ω (Hofmann *et al.* 2010), or the stimulus of photosynthesis from the increases in dissolved inorganic carbon (C_T) (Connell *et al.* 2013), may affect species performances. Such physiological responses may also cause disruptions of ecological interactions, further altering communities (Kroeker *et al.* 2012; Fabricius *et al.* 2014b). As OA is occurring progressively, the response of species and communities is likely to occur along a continuum as well. Individual species have displayed both linear responses (McCulloch *et al.* 2012; Comeau *et al.* 2013b), as well as non-linear thresholds or tipping points (Fabricius *et al.* 2015) along gradients of CO_2, while the response of communities remains largely uninvestigated. To better predict how communities will be shaped under OA, there is thus a need for studies which investigate the response curves of communities to increasing CO_2.

Coral reefs are likely to be among the ecosystems most affected by OA (Hoegh-Guldberg *et al.* 2007). Predictions are based on a multitude of single-species physiological studies (Kroeker *et al.* 2013), and several that have investigated changes at the community level. Community scale studies have centred around naturally occurring high-CO_2 analogues, such as volcanic CO_2 seep sites (Fabricius *et al.* 2011; Inoue *et al.* 2013; Enochs *et al.* 2015) or other oceanographic features affecting their carbonate chemistry (Manzello 2010; Crook *et al.* 2012; Barkley *et al.* 2015), as well as larger-scale multi-species tank

experiments (Langdon *et al.* 2000; Dove *et al.* 2013; Comeau *et al.* 2015). While there is substantial variation in the responses between taxa, the general consensus predicts declines in biodiversity, a retraction of many calcifying species (e.g. scleractinian corals, coralline algae and foraminifera), an expansion of non-calcifying phototrophs (e.g. algae and seagrasses), and increased bioerosion. (Enochs *et al.* 2016).

Coupled with the predicted changes in community composition under OA will likely be changes in community metabolism. However, scaling up OA effects on metabolic processes from individuals and species to the community level has proven difficult, and our current understanding is poor (Edmunds *et al.* 2016). To date the best inferences have been based on naturally occurring seasonal carbonate chemistry changes (Bates *et al.* 2010; Albright & Langdon 2011; Andersson *et al.* 2014), or the manipulation of seawater carbonate chemistry on coral reefs *in situ* (Albright *et al.* 2016, 2018) and in experimentation (Langdon *et al.* 2000, 2003), as well as larger-scale mesocosm experiments (Leclercq *et al.* 2002; Dove *et al.* 2013; Comeau *et al.* 2015, 2016). These studies generally predict that rates of community photosynthesis and respiration will remain relatively unchanged from the reefs of today, while calcification and net $CaCO_3$ accumulation will decline. However, these investigations have mainly examined effects due to changes in seawater carbonate chemistry, without fully accounting for changes due to the longer-term shifts in benthic community composition that may occur under OA. For example, Ω declines may directly reduce calcification rates in numerous taxa (Kroeker *et al.* 2013), but if these taxa are then outcompeted by non-calcifers, community calcification rates may further decline. Similarly, OA can increase rates of community production by directly stimulating photosynthesis in some species (Suggett *et al.* 2012; Noonan & Fabricius 2016), or indirectly by increasing the benthic cover of certain phototrophs (Connell *et al.* 2013). To gain further insight into the community metabolic dynamics of coral reefs under OA, measurements must be made on communities that have developed in their entirety under altered seawater carbonate chemistries.

The frequency and severity of disturbances affecting coral reefs is increasing (Hughes *et al.* 2017), and scleractinian coral cover is now often well below 30% (De'ath *et al.* 2012). Scleractinian corals eventually re-enter communities, however it is early-successional non-scleractinian taxa (e.g. algae, sponges, and other sessile invertebrates) that increasingly dominate light exposed benthic reef communities (Hughes *et al.* 2003). Furthermore, shade exposed cryptic taxa within crevices of the reef matrix can account for the largest fraction of biomass in reef systems (Richter *et al.* 2001). Both of these

communities - the early successional benthic taxa on illuminated and shaded surfaces - are often overlooked in reef community metabolism studies, although their metabolism co-determines the carbonate chemistry conditions for newly settling corals within the benthic boundary layer.

In this study we investigate how OA will shape the composition of early-successional benthic communities that live on the carbonate substrata of coral reefs, and the metabolic rates of the non-scleractinian components of reef communities that have developed under altered carbonate chemistries. To do so, non-carbonate settlement tiles were deployed *in situ*, under natural levels of light and shade, temperature and water flow, along CO_2 gradients at two volcanic CO_2 seep and two control sites in Papua New Guinea. Benthic communities developing on the upper light exposed tile sides, as well as the shaded crevice-dwelling taxa on the lower sides were investigated after five and 13 months, and their successional changes, and taxa-specific responses along the CO_2 gradients were explored. Response curves in community photosynthesis, respiration and light and dark calcification were then determined after 13 months.

5.3 Materials and Methods

Study site and carbonate chemistry

This study was conducted at two tropical shallow water (<5 m depth) CO_2 seeps and adjacent control sites in Milne Bay Province, Papua New Guinea. Seep and control sites at Upa-Upasina and Dobu are located adjacent to Normanby and Dobu Islands, respectively, and are described in detail by Fabricius *et al.* (2011). At both seep sites there is an area where near-pure (~99%) CO_2 gas emerges from the seafloor, locally altering the carbonate chemistry of the seawater without altering its temperature (Noonan & Fabricius 2016). The seeping has resulted in an altered benthic community, with hard and soft coral diversity, calcifying algal cover and the abundance and diversity of numerous mobile invertebrate taxa declining, while non-calcifying algae and seagrass cover is increased (Fabricius *et al.* 2011, 2014b). This research was conducted under permits issued to Dr Katharina Fabricius from Papua New Guinea's Department of Environment and Conservation, and the National Research Institute.

Data on the seawater carbonate chemistry for the sites has been published by Fabricius *et al.* (2011, 2014b), as have seawater measurements above settlement tiles used in this study (Fabricius *et al.*

2015, 2017; Enochs *et al.* 2016). Specifically, during four ~2-week expeditions between 2011 and 2013, 911 pH measurements were recorded across the 90 settlement tiles (median n = 8 per tile), and a further 625 samples (median n = 5 per tile) were taken for total alkalinity (A_T) (Fabricius *et al.* 2015). Given seeping intensity varies spatially and temporally within the mosaic of gas streams that comprises the seep sites, these samples were used to form long-term medians of the seawater carbonate chemistry at the exact location of each tile. Unless otherwise stated, all pH values are presented in the total scale (pH_T).

Benthic community composition

In December 2011, 90 labelled settlement tiles were evenly distributed across seep and control sites at both Dobu and Upa-Upasina reefs (n = 15 control and 30 seep site tiles per reef). Tiles were 11.5 * 11.5 cm, made of 3 mm thick polyvinyl chloride (PVC) and roughened on both sides with sandpaper. Kennedy *et al.* (2017) have shown that the percent coverage of benthic taxa settling on PVC tiles more closely matched adjacent reef substrata than other tile materials. Tiles were deployed horizontally on numbered baseplates, at ~3 m depth, ~2 cm from the reef substratum, >2m apart. The gap between the tile and the reef substrate allowed communities to not only develop on the upper light exposed tile side, but also for cryptic taxa to recruit to the lower shaded side. The tiles were first collected after five months (May 2012), photographed on both sides while being continuously submerged, and redeployed to their original location. In January 2013 (after 13 months deployment) the tiles were again collected, physiological measurements were made (details below), and they were rephotographed, before being dried and transported to the laboratories at the Australian Institute of Marine Science. Eighty-eight of the 90 settlement tiles were recovered during both census periods, with one missing from each seep site at Dobu and Upa-Upasina.

The benthic community composition of the upper and lower sides of the tiles, from the two census periods, was assessed on the tile photographs using the evenly spaced point analysis method (Meese & Tomich 1992). To do so, photographs were imported into image editing software (Photoshop CS6, Adobe Systems, USA), and digitally overlaid with a grid consisting of 7 * 7 evenly spaced lines. The identity of the benthos occurring under the resultant 49 cross points was recorded and classified into 15 operational taxonomic units (OTUs, Table 5.S1), including seven groups of algae and cyanobacteria, six phyla of benthic invertebrates, bare tile space, and a category for any taxa that could not be identified. The point counts

were converted into percent coverage data and some OTUs were further grouped into functional categories (non-calcifying algae, calcifying and non-calcifying invertebrates) for later statistical analysis (Table 5.S1). Photographs from 15 of the 30 seep tiles at Upa-Upasina at the 13 month census were lost (the camera memory card was accidently formatted prior to backup) and hence not included in analyses. The abundances of sessile tube-forming polychaetes and of coral recruits were counted directly on each tile once dried in the laboratory using a dissection microscope.

Benthic community metabolism

To determine rates of community gross photosynthesis, dark respiration, and light and dark calcification, all settlement tiles were incubated in the light and dark on the day of their collection in January 2013. For the incubations, tiles were transferred from their holding containers in running seawater into clear rectangular-prism chambers (920 mL volume), atop spacers which left ~1.5 cm gap between the tile and the walls, bottom and lid of the chamber. Chambers were placed in black flow-through bins that served as water baths and were maintained at ambient *in situ* temperatures from a 2m deep intake (30 °C). To minimise boundary layers, a 35 mm magnetic stirrer bar was placed into each chamber, activated with a custom made system of rotating magnets and pullies placed under the water baths. Communities from control sites were incubated in seawater at pH_T 8.08, while those originating from the seep sites were placed in seawater with pH_T 7.70. Four chambers per seep, and two per control site, were incubated with a blank settlement tile as a control. Water obtained from the seep sites was mixed with control seawater immediately prior to incubations to make a large batch at the target pH level, which was then used to fill the chambers. pH was determined with a portable pH meter (SG23, Mettler Toledo, USA) calibrated on the NBS scale. Measurements of pH_{NBS} and A_T from the incubation water were used to calculate carbonate chemistry parameters using the macro CO2SYS with the constraints set by Dickson and Millero (Dickson & Millero 1987), following Lewis *et al.* (1998) (Table 5.S2).

Light incubations were conducted in black bins under four white fluorescent tubes (10 000K) at their maximum output of 180 µmol photons m^{-2} s^{-1}. After ~90 minutes, the O_2 concentration in each chamber was measured (meter: HQ30d; probe: LDO101 IntelliCAL, Hach, USA) and a sample of water was retained and fixed with saturated $HgCl_2$ (>7 g L^{-1}) for calcification assays. Tile communities were allowed >30 min dark adaptation in the water baths under black lids, before the chambers were closed again and

the process was repeated in the dark. Light and dark calcification rates were determined with the alkalinity anomaly technique (Chisholm & Gattuso 1991), using open cell titration (Metrohm 855 robotic titrosampler, Switzerland) fitted with a gran function following Vogel *et al.* (2014). The alkalinity anomaly technique assumes incubation water A_T is only affected by the removal (calcification) or addition (dissolution) of $CaCO_3$, however some organisms in the tile communities (e.g. molluscs) would have altered A_T through the release or uptake of nutrients (Gazeau *et al.* 2015). This would have added some unaccounted measurement error to calcification rate estimates, however the technique has been used successfully to estimate rates of coral reef community calcification (Langdon *et al.* 2003; Albright *et al.* 2013), and is considered robust in coral reef environments when compared to other techniques (Gattuso *et al.* 1999b). The incubations of tile communities resulted in a change in A_T from blank-tile control chamber values that ranged from -219 to -6 µmol kg^{-1} SW in the light, and -76 to +92 µmol kg^{-1} SW in the dark. Primary A_T standards (CRMs; A. Dickson Laboratory, Scripps Institution of Oceanography) were titrated to calculate titrant concentration, and replicate secondary seawater standards (n = 15) were interspaced and titrated throughout incubation samples and were highly consistent (SE = 2.19 µmol kg^{-1} SW). Rates of gross photosynthesis and dark respiration (µg O_2 cm^{-2} min^{-1}), and light and dark calcification (µM $CaCO_3$ cm^{-2} min^{-1}), were calculated by subtracting the values of blank tile O_2 or A_T values, at the end of the incubation runs, from values of chambers with tile communities, and then standardised to incubation time and planar surface area of each tile. Changes in the surface area of the tiles due to differences in settling benthos were not accounted for. Estimates of daily net photosynthesis and calcification were calculated by combining light and dark rates, assuming a square profile of 11.5 hrs light and 12.5 hrs dark. This daily estimate equated to a cumulative daily light integral of 7.45 mol photon m^{-2} d^{-1}, which is similar to those at the study site and depth (Vogel *et al.* 2014).

Statistical analyses

Generalised linear models (GLM) were used to examine how percent cover of the different benthos OTUs on the tiles differed with median pH_T, and across Reef (Dobu and Upa-Upasina) and Time (five vs 13 month censuses). Median pH_T was used in models as it was directly measured many times at each tile (rather than being calculated), and correlated better with the other carbonate chemistry parameters than A_T (which is not directly affected by CO_2 addition). Percent cover data were fit using a

quasibinomial distribution accounting for the larger proportion of values at the upper and lower bounds of the distribution. Non-significant ($p > 0.05$) main effects and interaction terms were removed in the final models following a backward stepwise approach to minimise collinearity issues. For comparison to control values (seawater pH_T 8.05, or Ω_{Ar} = 3.84), percent coverages of taxa were also predicted for seawater pH_T 7.80 or Ω_{Ar} = 2.50, using the predict function in R, as this value may be expected by the year 2100 in tropical oceans (IPCC 2021).

Redundancy analysis (RDA) was also conducted to visually examine how the community data separated out in two-dimensional space, and to further test the significance of each explanatory variable via permutation. The explanatory variables in the analysis included reef and census period as categorical factors, as well as the median pH_T at each tile as a continuous quantitative variable.

GLMs were fitted to the metabolic measurements from all tiles, using Reef and median pH_T of the tile origin as predictors for measurements of gross photosynthesis and respiration, and Reef and Ω_{Ar} for calcification. For the calcification assay, two chambers opened, and five outliers (with values >8-fold from the confidence intervals) were removed from final models (n = 81). A second series of GLMs was conducted on the metabolism measurements which incorporated the carbonate chemistry of the tiles, as well as the taxonomic benthic cover data (n = 66 due to the loss of 15 photos). An identity link function was used when response parameters approximated a Gaussian distribution. Quasipoisson distributions and log link functions were used for over-dispersed data. The appropriate model distribution was selected by comparing dispersion factors, preferring those which approached a value of 1. All statistical procedures were conducted with the statistical software R version 3.2.5 (R Development Core Team 2021) using the packages vegan and gmodels.

5.4 Results

Carbonate chemistry

The settlement tile communities were developed along clear CO_2 gradients at both the Dobu and Upa-Upasina seeps (Fig 5.1). Both control sites had similar seawater chemistry, however the pH gradient at the Dobu seep reached lower levels than at the Upa-Upasina seep. The median seawater pH_T over the tiles ranged from 8.0 ± 0.001 (SE) at the control sites of both reefs, to 7.7 ± 0.10 as lowest values at the

Upa-Upasina seep, and to 7.4 ± 0.10 at Dobu seep (Fig 5.1). Median aragonite saturation state (Ω_{Ar}) was 3.84 ± 0.05 at the control sites, declining to 2.10 ± 0.25 and 1.16 ± 0.43 at the Upa-Upasina and Dobu seeps, respectively (Fig 5.1). Similarly, calcite saturation state (Ω_{Ca}) values decreased from 5.75 ± 0.10 at the control sites along the carbonate chemistry gradient to 3.14 ± 0.38 and 1.73 ± 0.64 at the Upa-Upasina and Dobu seep, respectively. While none of the communities were exposed to median Ω_{Ar} values <1.0, 55% had median Ω_{Ar} <3.0, a value which has been suggested to be the limit for reef development (Silverman *et al.* 2009).

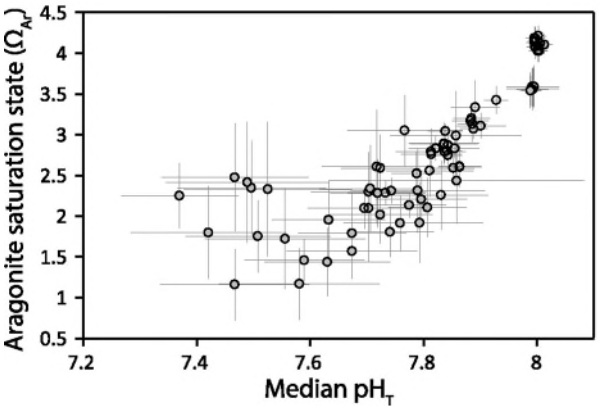

Fig 5.1. Median pH_T and saturation state of aragonite (Ω_{Ar}) from the 88 settlement tiles. Median n = 8 pH and 5 Ω_{Ar} measures per tile. White points are from Upa-Upasina, grey are from Dobu. Error bars are standard errors.

Values of pCO_2, C_T and A_T were also spread along the carbonate chemistry gradient. The daytime pCO_2 at the control sites averaged 392.15 ± 9.32 µatm, increasing to 1008 ± 291.99 and 2253 ± 759.63 µatm at Upa-Upasina and Dobu seeps respectively. Control C_T values averaged 1926 ± 0.70 µmol kg^{-1}, and increased to >2100 at the seeps. Median A_T values were slightly elevated within the seep sites (perhaps due to $CaCO_3$ dissolution), and differed from control values by no more than 6%. A_T values increased from an average of 2252.68 ± 3.33 µmol equivalents kg^{-1} at the controls to 2339.60 ± 16.29 and 2329.68 ± 18.89 µmol equivalents kg^{-1} within the Upa-Upasina and Dobu seeps, respectively (see also Fig S2 in Enochs *et al.* (2016) as their experimental units were deployed alongside the settlement tiles of the present study).

Carbonate chemistry parameters were more variable within the seeps compared to the control sites (Fig 5.1).

Benthic community composition

Both tile surfaces were covered by a significant amount of macrobenthos after five months, and after 13 months, the tiles were all but indistinguishable from the adjacent substrata (Fig 5.2). Communities on the upper tile sides, being exposed to higher light intensities and grazing, only included several algal OTUs and bare space, and no invertebrates. This contrasted with the lower sides of the tiles, where light and grazing intensities are low; these communities consisted of algal OTUs, as well as many invertebrate taxa and bare space (Fig 5.2). Coral recruits were observed on the lower tile sides (Fabricius *et al.* 2017), however, due to their small size (typically <2mm diameter), they failed to contribute to community cover estimates.

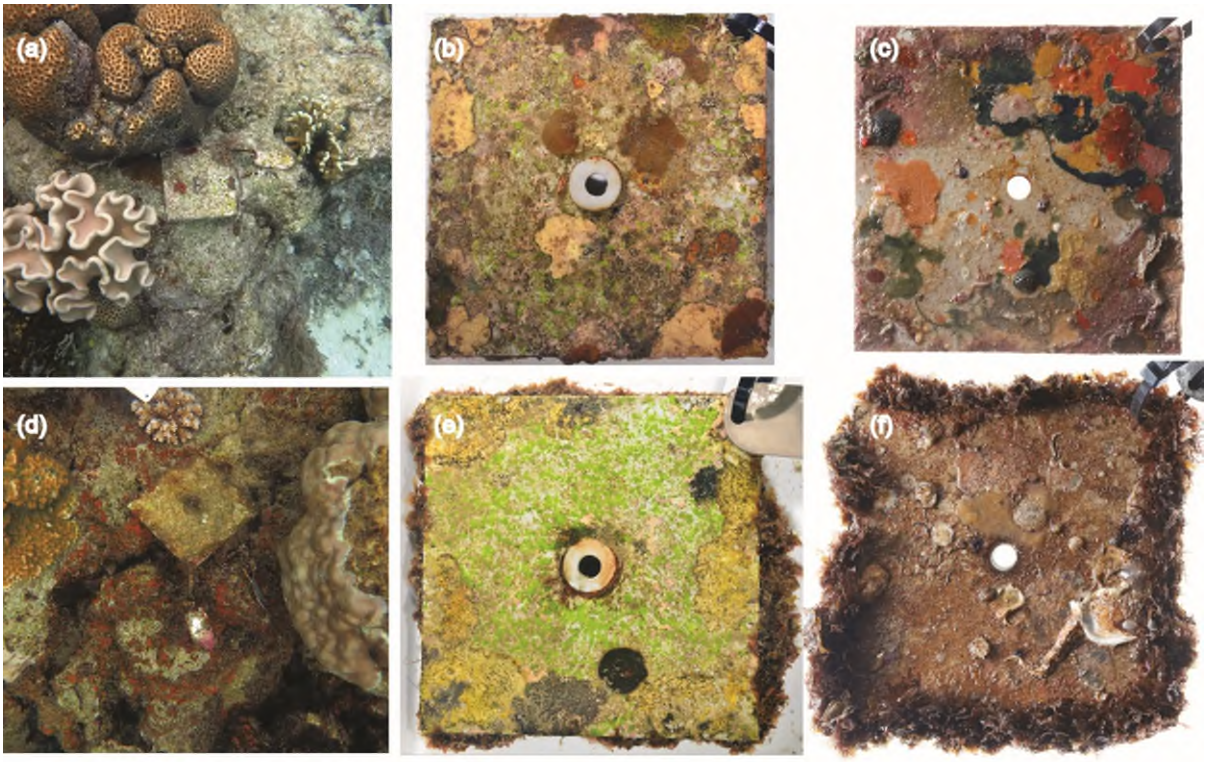

Fig 5.2. Settlement tiles from control sites (a, b and c) and volcanic CO_2 seep sites (d, e and f) in Papua New Guinea after 13 months deployment. Tiles *in situ* (a and d), upper sides (b and e), and lower sides (c and f).

The communities underwent some successional changes between the two census periods. Pioneering algal taxa, including green filamentous algae on the upper tile side and turf algae and cyanobacteria on the lower tile side, all recorded significantly lower percent cover after 13 months compared to the five month census (GLM significant main effect of Time, all $p < 0.05$; Table 5.1). Turf algae on the upper side displayed the opposite pattern, increasing in cover between the five and 13 month censuses (Table 5.S3). At 13 months, several of the slower growing taxa had increased in abundance: *Peyssonnelia* spp. on the upper sides and macroalgae and sponges on the lower sides had all increased in cover, as did the combined cover of both the calcifying and the non-calcifying invertebrate groups (Table 5.1). The amount of unoccupied space on both the upper and lower tile sides similarly declined between the census periods as available space was progressively occupied (Table 5.1).

Table 5.1: Changes in percent cover of the main benthic taxonomic and functional categories, in response to pH_T (median per tile), Reef (contrasting Dobu against Upa Upasina) and Time (contrasting 13 against five months of deployment). Responses are for the upper (up) and lower (low) sides of the settlement tiles. Parameter estimates from the best fitting generalised linear models, with quasibinomial distributions. Non-significant terms removed from final models. * indicates taxa only found on one tile side.

	Estimate	SE	t	p
Non-calcifying algae up				
Intercept	18.88	3.13	6.04	<0.001
pH	-2.35	0.39	-5.94	<0.001
Reef	-0.45	0.13	-3.49	<0.001
Non-calcifying algae low				
Intercept	40.36	4.42	9.13	<0.001
pH	-5.20	0.56	-9.20	<0.001

Time	16.61	6.79	2.45	0.016
pH:Time	-2.17	0.87	-2.50	0.014
Green filaments up*				
Intercept	10.91	2.59	4.21	<0.001
pH	-1.60	0.33	-4.88	<0.001
Reef	0.79	0.13	6.15	<0.001
Time	-0.45	0.11	-4.02	<0.001
Turf up				
Intercept	-0.68	0.13	-5.15	<0.001
Reef	-0.93	0.17	-5.48	<0.001
Time	0.51	0.17	3.01	0.003
Turf low				
Intercept	-1.38	0.07	-18.43	<0.001
Time	-1.15	0.14	-7.82	<0.001
Macroalgae low*				
Intercept	26.84	4.85	5.54	<0.001
pH	-4.18	0.61	-6.83	<0.001
Reef	2.57	0.59	4.31	<0.001
Time	1.55	0.25	6.17	<0.001
Cyanobacteria low*				
Intercept	67.31	12.79	5.26	<0.001
pH	-8.79	1.63	-5.40	<0.001
Reef	-31.41	16.50	-2.33	0.021
Time	-0.32	0.17	-1.91	0.057
pH: Reef	3.99	1.72	2.32	0.022
Peyssonnelia up				
Intercept	14.50	4.27	3.40	<0.001
pH	-2.38	0-55	-4.33	<0.001
Time	1.74	0.26	6.65	<0.001
Peyssonnelia low				

Intercept	26.73	11.26	2.37	0.019
pH	-3.64	1.43	-2.54	0.012
Reef	-33.07	12.82	-2.58	0.011
Time	17.11	16.46	1.04	0.300
pH: Reef	4.15	1.63	2.54	0.012
pH: Time	-2.02	2.08	-0.97	0.334
Reef: Time	-72.00	19.88	-3.62	<0.001
pH: Reef: Time	9.04	2.52	3.59	<0.001
CCA up				
Intercept	-64.69	6.86	-9.43	<0.001
pH	7.89	0.86	9.13	<0.001
Reef	1.65	0.21	7.75	<0.001
Time	27.24	8.96	3.04	0.003
pH: Time	-3.37	1.13	-2.99	0.003
Reef: Time	-0.95	0.30	-3.17	0.002
CCA low				
Intercept	-53.013	8.45	-6.28	<0.001
pH	6.47	1.06	6.08	<0.001
Reef	-93.28	23.06	-4.04	<0.001
Time	-0.26	0.17	-1.56	0.122
pH: Reef	11.64	2.89	4.03	<0.001
Reef: Time	1.08	0.27	4.03	<0.001
Non-calcifying invertebrates low*				
Intercept	-20.90	5.71	-3.66	<0.001
pH	2.16	0.72	3.00	0.003
Reef	1.29	0.37	3.48	<0.001
Time	1.34	0.37	3.59	<0.001
Reef: Time	-1.36	0.46	-2.95	0.004
Calcifying invertebrates low*				
Intercept	-50.90	15.48	-3.29	0.001

pH	6.00	1.95	3.08	0.002
Reef	42.69	16.64	2.57	0.011
Time	1.02	0.29	3.47	<0.001
pH: Reef	-5.35	2.10	-2.55	0.012
Reef: Time	-1.03	0.41	-2.50	0.013
Ascidians low*				
Intercept	-98.37	33.45	-2.94	0.004
pH	12.00	4.20	2.86	0.005
Reef	77.74	34.19	2.27	0.024
pH: Reef	-9.65	4.29	-2.25	0.026
Polychaetes low (counts per tile)*				
Intercept	-1395.59	436.92	-3.19	0.002
pH	188.78	55.35	3.41	0.001
Reef	-39.26	17.84	-2.20	0.031
Unoccupied space up				
Intercept	-0.66	0.09	-7.41	<0.001
Reef	-0.62	0.14	-4.51	<0.001
Time	-1.49	0.20	-7.53	<0.001
Reef: Time	1.11	0.25	4.43	<0.001
Unoccupied space low				
Intercept	-13.83	4.42	-3.13	0.002
pH	1.61	0.56	2.88	0.004
Reef	-0.78	0.19	-4.04	<0.001
Time	-1.76	0.27	-6.47	<0.001
Reef: Time	0.98	0.37	2.65	0.009

Changes in pH explained two and 50 times more variation in the cover of non-calcifying algae on the upper and lower tile sides, respectively, compared to the explanatory variables Reef and Time (GLM F ratios, Table 5.S3), indicating many patterns in the tile communities were established within five months and were largely consistent between reefs. Non-calcifying algal cover increased as pH declined from

relatively low control values to ~80% at the lower end of pH gradients, without a clear threshold at which point non-calcifying algae came to dominate communities (Fig 5.3a, b). On the upper tile side the cover of non-calcifying algae increased from control values of 40.3 ± 1.7 (SE) to 56.3 ± 2.0% at pH 7.8 after five months, and 49.4 ± 2.07 to 59.2 ± 2.3% at pH_T 7.8 after 13 months (Fig 5.3), predominantly due to green filamentous algae (Fig 5.S1). Similarly, on the lower sides, non-calcifying algae increased from control values of 19.3 ± 1.7% to 45.1 ± 2.1% by pH 7.8 at five months, and from 8.6 ± 0.6 to 37.4 ± 2.5% by pH_T 7.8 at 13 months (Fig 5.3). Lower side communities were dominated by cyanobacteria at both seeps, and also by macroalgae at Dobu (Fig 5.S1).

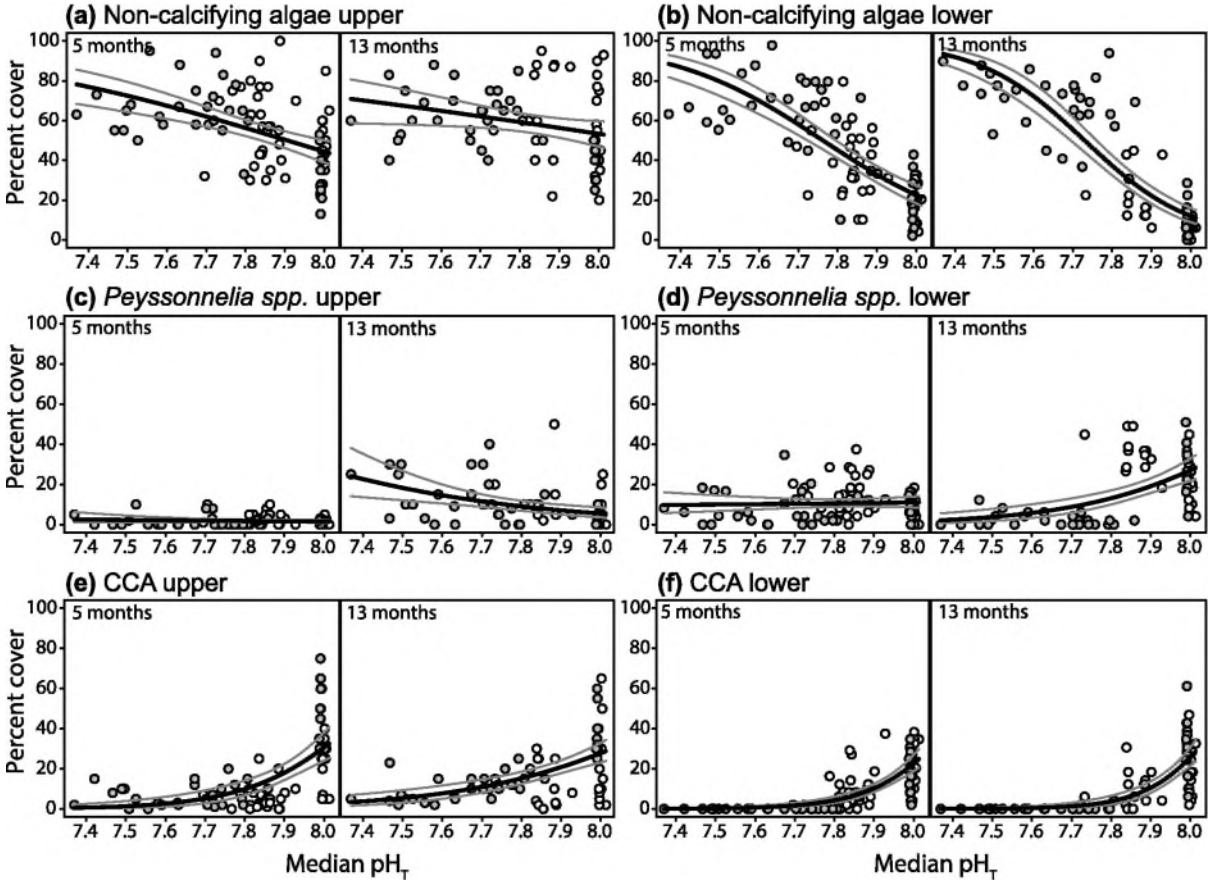

Fig 5.3. Shifts in settlement tile community composition along the pH gradients. Percent cover of non-calcifying algae on the upper (a) and lower (b) settlement tile sides, *Peyssonnelia* spp. from the upper (c) and lower (d) sides, and crustose coralline algae (CCA) from the upper (e) and lower (f) sides in relation to pH_T. Left and right panels per plot represent the five and 13 month old communities, respectively. White

points are tiles from Upa-Upasina and grey are from Dobu. The black lines represent the modelled means, while the grey lines are confidence intervals.

The different taxa of calcifying algae contrasted in response to changes in pH. The very mildly calcifying red alga *Peyssonnelia* spp. on the upper surface increased in cover between census periods. In 13 month old communities, upper side *Peyssonnelia* spp. cover also increased with declining pH from control values of 4.1 ± 0.3 to 9.1 ± 1.2% at pH_T 7.8 (Table 5.1, Fig 5.3c, d). On the lower sides, *Peyssonnelia* spp. cover showed no clear patterns, indicating light limitation or competition with other benthos was co-limiting their distribution (Fig 5.3). The cover of the heavily calcified crustose coralline algae (CCA) declined steeply along the pH gradient on both the upper and lower tile sides (Fig 5.3e, f), and the effects of pH changes were again far stronger than Reef or Time (GLM F ratios, Table 5.S3). On the upper sides, CCA cover declined from control values of 35.2 ± 2.4 to 9.9 ± 1.8% by pH_T 7.8 in five month old communities, and from 29.6 ± 1.8 to 15.2 ± 1.5% by pH_T 7.8 in 13 month old communities. An apparent threshold in CCA cover was observed on the lower tile sides at pH_T 7.8, where CCA was virtually absent below pH_T 7.8, while they continued to persist with low cover on the upper sides below this pH level (Fig 5.3).

Patterns in the invertebrate communities were less clear compared to those seen in the algae. The cover of both calcifying and non-calcifying groups declined with pH at both reefs after five months, and at Upa-Upasina but not Dobu after 13 months (Fig 5.S2). Ascidian cover and the number of polychaetes significantly declined with pH at both reefs and both times, but cover differed between the reefs (Table 5.1). No distinct patterns were observed in the cover of bivalves, foraminifera or bryozoa (Table 5.S3).

Results of the RDA agreed with the GLM analyses: all explanatory variables accounted for significant variation in benthic communities (ANOVA, all $p = 0.001$), and tile pH explained more of this variation than differences between census periods or reef (F ratios of 22.37, 19.29 and 14.67 for pH, census period and reef, respectively). In the ordination, the first RDA axis separated communities between seep and control sites. This was primarily driven by high cover of cyanobacteria, macroalgae, and green and brown filamentous algae in the high CO_2 communities, and high CCA cover in control sites (Fig 5.4). Communities from Upa-Upasina clustered closer together between high CO_2 and control tiles in comparison to Dobu, perhaps reflecting the greater intensity of CO_2 exposure at Dobu seep. The second RDA axis divided communities between census periods: the five month old communities on the lower sides

were associated with more empty space and turf algae, and on the upper sides with green filamentous algae (Fig 5.4). At 13 months, the communities were associated with an increased cover of *Peyssonnelia* spp., sponges, foraminifera (on the lower sides of the control tiles), and macroalgae (on the lower sides of high CO_2 tiles).

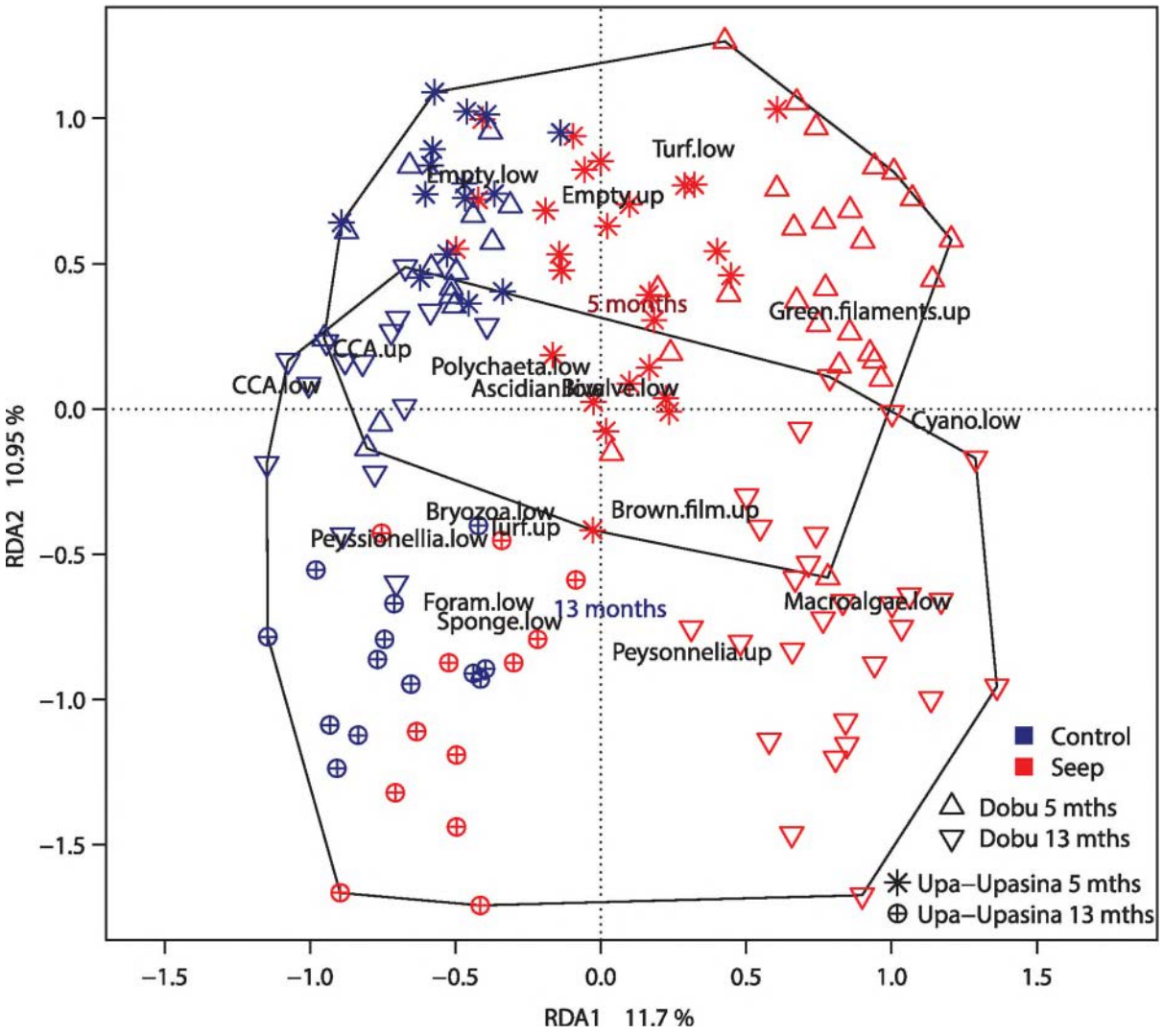

Fig 5.4. Redundancy analysis ordination of the settlement tile benthic communities at the seep and control sites. Red points represent tiles from the seeps, and blue from the controls, of Dobu and Upa-Upasina Reefs. Points in the upper polygon are five month (mths) old communities, while those in the lower polygon are at 13 months.

Benthic community metabolism

Rates of community metabolism were related to both changes in the seawater carbonate chemistry and changes in benthic communities. All metabolic measurements gradually changed along the carbonate chemistry gradient, and no abrupt changes or threshold responses were detected (Fig 5.5). Gross photosynthetic rates increased linearly as pH declined along the gradient (Fig 5.5a, Table 5.2). On average, control tiles produced 10.37 ± 0.4 µg O_2 cm^{-2} hr^{-1}, increasing by 10% to 11.54 ± 0.3 µg O_2 cm^{-2} hr^{-1} at pH_T 7.8. Increased gross photosynthetic rates were also positively related to the increasing cover of non-calcifying algae in the upper communities, but declined with the increasing cover of non-calcifying invertebrates along the pH gradient (Table 5.2).

Table 5.2: Changes in community metabolism in response to median pH (gross photosynthesis; respiration) or the saturation state of aragonite (Ω_{Ar}: calcification) and Reef (contrasting Dobu against Upa Upasina). Models were then run again but the cover of the main community members was additionally included as co-variates (benthos). Parameter estimates from the best fitting generalised linear models (^{G}: Gaussian; ^{Q}: quasipoisson distributions), with estimates for the quasipoisson models back-transformed to aid interpretation. Non-significant terms were removed from final models.

	Estimate	SE	t	p
Gross photosysthesis^{Q}				
Intercept	6.39	1.25	1.48	0.142
pH	-1.57	0.16	-2.81	0.006
Gross photosysthesis benthos^{Q}				
Intercept	-6.64	0.09	-20.21	<0.001
Non-calc invert low	-1.01	0.002	-3.78	<0.001
Non-calc algae up	1.00	0.001	3.84	<0.001
Respiration$^{\wedge 0.25G}$				
Intercept	0.16	0.48	0.34	0.735
pH	0.05	0.06	0.80	0.427

Reef	1.75	0.53	3.29	0.001
pH: Reef	-0.22	0.07	-3.28	0.002
Respiration benthos$^{\wedge 0.25G}$				
Intercept	1.77	0.18	9.61	<0.001
pH	-0.16	0.02	-6.69	<0.001
Bivalve	0.01	0.001	3.43	0.001
Non-calc invert low	0.001	<0.001	4.32	<0.001
Net daily production benthos^{G}				
Intercept	78.10	6.48	12.04	<0.001
Bivalve	-4.97	1.70	-2.92	0.005
Non-calc invert low	-1.35	0.31	-4.37	<0.001
Light calcification^{G}				
Intercept	0.06	0.02	2.76	0.007
Ω_{Ar}	0.02	<0.01	2.89	0.005
Dark calcification^{G}				
Intercept	-0.06	0.01	-4.27	<0.001
Ω_{Ar}	0.01	<0.01	3.15	0.002
Reef	0.02	0.01	2.33	0.023
Net daily calcification^{G}				
Intercept	-0.37	19.47	-0.02	0.985
Ω_{Ar}	22.34	6.34	3.52	<0.001

Respiration rates increased 20% from control site values (5.18 ± 0.6 μg O_2 cm^{-2} hr^{-1} consumption) to those at pH_T 7.8 (6.21 ± 0.2), and continued to increase along the pH gradient (Fig 5.5b). The increase in respiration with pH was stronger at Dobu, however the pH main effect explained three times the variation in respiration compared to the interaction between pH and Reef (Table 5.S4, F = 33 and 10 for pH and the interaction, respectively). Respiration rates also increased with the cover of bivalves and non-calcifying invertebrates on the lower sides of the tiles (Table 5.2). Since declining pH elevated rates of both gross photosynthesis and respiration, no difference was detected in daily net production, which averaged 55 ± 13.2 μg O_2 cm^{-2} day^{-1} across all tiles (Fig 5.5c). Daily net production was instead reduced by an increase

in the cover of bivalves and non-calcifying invertebrates on the lower sides of the tiles (Table 5.2). Mean gross photosynthetic rates were on average twice that of respiration, and 90% of tiles recorded positive daily net production values.

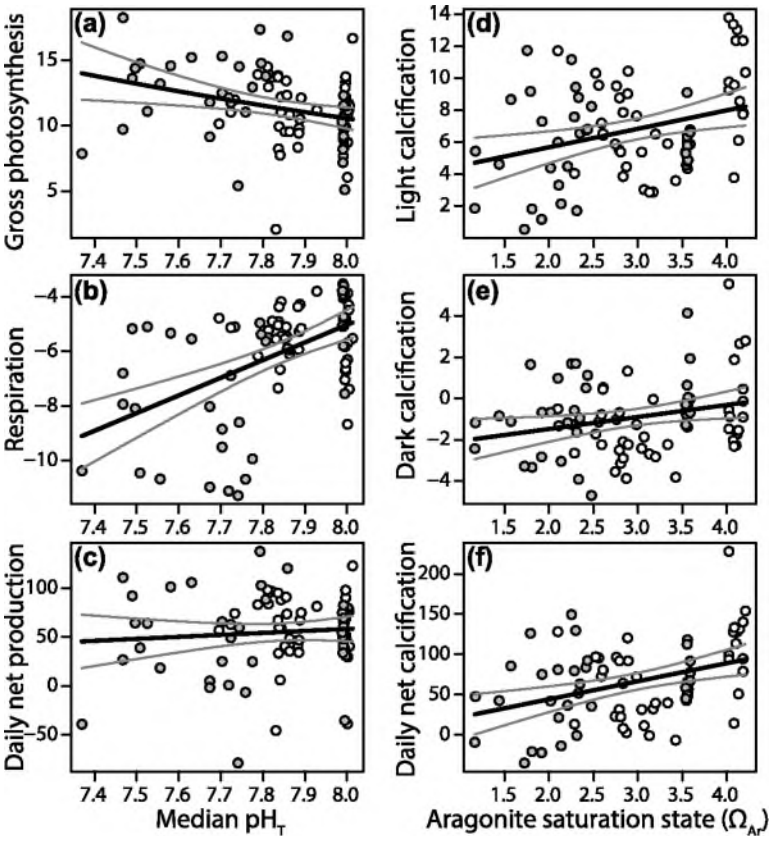

Fig 5.5. Metabolic rates from benthic communities on settlement tiles across the seawater carbon chemistry gradients. Rates of gross photosynthesis (a), respiration (b) and net daily production (c) in 13 month old communities are plotted against the median pH_T from the tiles. Gross photosynthesis and respiration are displayed in µg O_2 cm^{-2} hr^{-1}, and daily net production in µg O_2 cm^{-2} day^{-1}. Rates of light (d), dark (e) and net daily calcification (f) are plotted against tile median aragonite saturation state (Ω_{Ar}) from the tiles. Light and dark calcification rates are displayed in µg $CaCO_3$ cm^{-2} hr^{-1}, and daily net calcification in µg $CaCO_3$ cm^{-2} day^{-1}. White points are from Upa-Upasina and grey are from Dobu. The black lines represent the modelled mean, while the grey lines are confidence intervals.

Rates of community calcification in the light and dark, and net 24-h community calcification, all declined along the Ω_{Ar} gradient (Table 5.2). Mean light calcification rates averaged 7.73 ± 1.3 μg $CaCO_3$ cm^{-2} h^{-1} at the control sites and decreased by 20% to 6.25 ± 0.4 μg $CaCO_3$ cm^{-2} h^{-1} by Ω_{Ar} 2.5. None of the communities recorded net decalcification in the light, despite median Ω_{Ar} reaching as low as 1.16, while 70% of control and 80% of seeps communities showed net decalcification in the dark. Dark calcification declined from -0.01 ± 0.3 μg $CaCO_3$ cm^{-2} h^{-1} at the control sites to -1.19 ± 0.2 μg $CaCO_3$ cm^{-2} h^{-1} by 2.5 Ω_{Ar}, and continued to decrease along the gradient (Fig 5.5e, Table 5.2). Dark calcification rates were also lower at Upa-Upasina compared to Dobu (Table 5.2). Daily net calcification rates of 89.14 ± 19.3 μg $CaCO_3$ cm^{-2} day^{-1} at the control sites declined by 38% to 55.49 ± 5.9 at Ω_{Ar} 2.5 (equalling annual $CaCO_2$ deposition of 325 vs 204 g m^{-2} yr^{-1}). Given rates of light calcification were greater than those in the dark, 90% of the tiles recorded positive daily net calcification, with negative rates all observed at <2.3 Ω_{Ar} (Fig 5.5f). Interestingly, the declines in calcification were not significant when calcification was modelled against tile pH. Tile pH_T and Ω_{Ar} were highly correlated until the lower end of the pH gradient (Fig 2), where Ω_{Ar} increased relative to pH. This decoupling is likely driven by $CaCO_3$ dissolution in the seeps, raising A_T and subsequently Ω_{Ar}, and may explain the differences between model results. The inclusion of the benthic OTUs did not improve the GLM fits to the carbonate chemistry parameters for rates of light, dark or daily net calcification. No relationships were detected between gross photosynthesis and light calcification rates, or between respiration and dark calcification rates (linear regressions, all $p > 0.05$).

5.5 Discussion

Ocean acidification is predicted to fundamentally alter benthic marine communities. Here we report a drastic shift in the composition and metabolism of early successional benthic coral reef communities along seawater carbonate chemistry gradients. The carbonate chemistry explained a far greater amount of change in communities than successional changes from five to 13 months, and differences between the two reefs. Shifts were more pronounced in the algae compared to the invertebrate communities, where a suite of non-calcifying algal groups largely replaced CCA on seep site tiles. Changes in CO_2 and community composition also affected community metabolism; rates of gross photosynthesis and respiration increased with increasing CO_2, and with the cover of certain taxonomic groups, while 24-h net calcification decreased to low or even negative values.

The present study adds to the mounting body of evidence predicting ecosystem-wide changes in benthic communities under OA. Here we found rapid increases in non-calcifying algal cover as pH declined along the carbonate chemistry gradients, and little evidence of threshold responses for these taxa. This pattern was largely consistent between light exposed and cryptic communities, with green filaments establishing dominance on the upper sides, and cyanobacteria and macroalgae on the lower sides. While results are not universal (Barkley *et al.* 2015), patterns in benthic communities at CO_2 seeps in the temperate Mediterranean (Hall-Spencer *et al.* 2008; Porzio *et al.* 2011; Kroeker *et al.* 2012; Linares *et al.* 2015), as well as multiple tropical sites in the Indo-Pacific (Fabricius *et al.* 2011; Enochs *et al.* 2015), concur with the present study. These studies similarly predict an increase in non-calcifying algae under OA, and there are suggestions that other non-calcifying phototrophs, such as seagrasses (Fabricius *et al.* 2011) and anemones (Suggett *et al.* 2012), may also thrive. Interestingly, non-calcifying algae have increased abundances in the wider community at the seep sites in Milne Bay (Fabricius *et al.* 2011), however they do not dominate the benthos like on the settlement tiles of the present study, or at another tropical seep (Enochs *et al.* 2015). Grazers are diverse and abundant at the Milne Bay seeps (Munday *et al.* 2014), which may prevent the proliferation of algae on upper surfaces in the wider community. Similarly, longer-term competition with benthos that had not developed fully on the tiles (e.g. the scleractinian corals) may also constrain algal growth.

Calcifying algae on the settlement tiles displayed dissimilar results between taxa along the CO_2 gradient. The cover of the lightly calcifying algae *Peyssonnelia* spp. increased at lower pH, albeit only in high-light environments. Some *Peyssonnelia* species have increased in abundance at other seep sites (Porzio *et al.* 2011; Linares *et al.* 2015), suggesting that certain species of calcifying algae may be resilient to or even benefit from OA (Johnson *et al.* 2012; Vogel *et al.* 2014), perhaps due to the use of aragonite over high magnesium-calcite in their skeletons (Linares *et al.* 2015), and by using the additional C_T for photosynthesis. The steep decline in CCA cover is consistent with data from multiple seep sites (Enochs *et al.* 2015; Fabricius *et al.* 2015) and in experimentation (Kuffner *et al.* 2007) with potentially profound effects on coral reef communities (Fabricius *et al.* 2017). The steeper decline in CCA on the lower tile surfaces, as well as the increase in *Peyssonnelia* spp. on the upper tile surfaces, indicates light intensity is playing a role in the response of these taxa to OA.

Invertebrate responses to carbonate chemistry changes varied between taxa, and overall their cover and abundances did not respond as strongly as the algae did. It is important to note that the

invertebrate communities in the present study were in relatively early successional stages, and consisted of shade-adapted communities without scleractinian corals (no invertebrates were found on the upper tile surfaces). The number of tube-dwelling polychaetes per tile declined with pH, as did the cover of ascidians. Similar declines were seen in tube-dwelling polychaete species at a Mediterranean seep (Cigliano *et al.* 2010), possibly due to reduced calcification in the juvenile stage (Lane *et al.* 2013). Little is known about why ascidians appear to respond negatively to elevated CO_2, however a previous study found the abundances of ascidians on natural reef substrata also declined with CO_2 exposure at the Milne Bay seeps (Fabricius *et al.* 2014b). Our study found no apparent effect of carbonate chemistry on the cover of the diverse groups of bivalves, bryozoans or sponges. Both bivalves and bryozoans are calcifying and considered sensitive to carbonate chemistry changes, but their $CaCO_3$ skeletons are somewhat protected from the surrounding seawater through external tissue layers (Lombardi *et al.* 2011; Kroeker *et al.* 2013). Sponges have shown a mixed response to elevated CO_2, with some species negatively responding, while species with phototrophic symbionts or siliceous spicules may respond positively (Morrow *et al.* 2015).

Patterns in the 13 month old tile communities were largely established in the first five months, and successional changes between census periods were considerably weaker than the influence of the carbonate chemistry gradient for the majority of taxa. While the cover of some ephemeral (turf and green filamentous algae and cyanobacteria) and slower growing taxa (*Peyssonnelia spp*., other macroalgae and the invertebrate groups) changed between census periods, this did not significantly alter the patterns in the rest of the tile communities. For example, patterns along the CO_2 gradients in the cover of non-calcifying algae and CCA, which accounted for the majority of the tile communities, were largely consistent between census periods. This consistency between census periods contrasts a similar study at Mediterranean seeps, where Kroeker *et al.* (2012) found commonalities between early settlement seep and control communities progressively diverged as competitive hierarchies were disrupted. Fabricius *et al.* (2015), who closely examined patterns in CCA distributions on the tiles of the present study, concluded that it was recruitment limitation in the CCA at lower pH, rather than competition with other taxa, that established the patterns seen here.

It is unknown to what extent the successional tile communities reflect the surrounding mature benthic communities. After 13 months, the tile communities blended in and greatly resembled the surrounding benthos (personal observation). Previous work at the Milne Bay seeps has similarly shown higher turf and macroalgae cover, and lower CCA cover, on natural substrate within the seep reefs

compared to control reefs (Fabricius *et al.* 2011). Regardless of any disparities between tile and mature benthic communities, early successional communities may become increasingly prevalent on coral reefs as the frequency and severity of disturbances increases (Hughes *et al.* 2017), making larger contributions to overall reef composition and metabolic signals, with potentially important complications for the carbonate chemistry newly settling corals will experience within the benthic boundary layer.

The present study presents the first investigation of metabolic changes for combined surface and cryptic subsurface reef communities that have developed entirely *in situ* under high CO_2. Here we documented a 10% increase in gross photosynthesis and a 20% increase in respiration at pH_T 7.8 compared to control sites with a pH_T 8.0, but no change in net community production. Gross photosynthesis may increase under OA by directly stimulating photosynthesis (Suggett *et al.* 2012; Noonan & Fabricius 2016), and/or by increasing the benthic cover of phototrophs (Connell *et al.* 2013). Studies that have investigated metabolic changes under OA at the reef community scale are few and from quite different communities, however they have not observed the increases in gross photosynthesis reported here (Leclercq *et al.* 2002; Langdon *et al.* 2003; Dove *et al.* 2013; Enochs *et al.* 2016). In the present study, models which included benthic community cover indicated that increases in gross photosynthesis were predominantly due to increases in the cover of non-calcareous algae, rather than the changing seawater carbonate chemistry *per se*. Respiration may increase as a consequence of increasing biomass or increased metabolism. Biomass estimates are unavailable for the tiles, however our models indicated that declining seawater pH, and increasing invertebrate cover, both significantly contributed to the observed increase in respiration at lower pH.

OA is likely reducing reef calcification rates on coral reefs (Albright *et al.* 2016; Comeau *et al.* 2016). Light, dark and net calcification rates on the tiles all declined along the Ω_{Ar} gradient, and our models indicated that it was these changes in Ω_{Ar}, rather than shifts in tile communities, that were responsible. While results are not universal, OA is widely reported to reduce calcification in individual coral reef organisms (Comeau *et al.* 2013b; Kroeker *et al.* 2013) and at the community scale (Langdon *et al.* 2000; Dove *et al.* 2013; Comeau *et al.* 2015, 2016; Enochs *et al.* 2016). For example, Enochs *et al.* (Enochs *et al.* 2016) found net daily calcification rates of light exposed coral reef communities on $CaCO_3$ substrata decreased linearly along CO_2 gradients, and became negative by pH_T 7.8. The stronger response found by Enochs *et al.* (Enochs *et al.* 2016) compared to the present study is thought to be because their study used

$CaCO_3$ blocks as settlement substrata, and attracted many macro-boring organisms, yet did not include cryptofauna on the lower surfaces.

When predicting OA effects on coral reef calcification, one must also take the permeable carbonate matrix and sediments into consideration. These are the largest sources of reef $CaCO_3$ (Eyre *et al.* 2014), and they are more vulnerable to dissolution than calcifying organisms as they lack tissue layers to buffer them from the surrounding seawater (Bates *et al.* 2010; Rodolfo-Metalpa *et al.* 2011; Eyre *et al.* 2014; Comeau *et al.* 2015). For example, Comeau *et al.* (2012) documented a 60% decline in the calcification of experimental coral reef communities at 1300 µatm pCO_2, with half of this being attributed to sediment decalcification. Some estimates suggest that even if coral calcification rates are maintained under OA, the dissolution of carbonate sediments alone would result in reef loss (Cyronak *et al.* 2013; Eyre *et al.* 2014). Results from the calcification assays in the present study, lacking sediments and a $CaCO_3$ substrata, are thus likely to considerably under-estimate reef-wide dissolution rates expected under OA. Instead, they provide insight into how the calcification dynamics of a part of the biological components of coral reef communities may respond.

There are several factors that preclude CO_2 seep sites from perfectly representing the future of the world's oceans. Firstly, they are relatively small, and scaling up predictions to the world's coral reefs will undoubtedly introduce some uncertainty. Secondly, the altered carbonate chemistry at the seeps is occurring in isolation from the warming that is also predicted for a high CO_2 world (IPCC 2021), and the combined effect of these two stressors can be greater than either in isolation (Kroeker *et al.* 2013). Thirdly, the seep site A_T is slightly elevated (by ≤ 6% of control values), which may increase calcification rates at the seep sites (Albright *et al.* 2016). Increased dissolution of carbonate sediments under OA may locally increase A_T, however sediment dissolution- and dilution-rates from the surrounding seawater are largely unknown (Eyre *et al.* 2014). And finally, the seep seawater carbonate chemistry is characteristically variable over short (i.e. hourly) time-scales (Hofmann *et al.* 2011) with uncertain consequences for coral reef communities. Scleractinian corals have been shown to be largely robust, or to even benefit from increased pH variability (when compared to static lowered pH), while other coral reef organisms (e.g. CCA) can be negatively affected (Rivest *et al.* 2017). On the other hand, community scale studies, which allow for interactions between species and their environment, are not easily conducted in laboratory settings. While seep sites studies are not definitive, they do provide unique opportunities to overcome some issues with laboratory-based OA studies (i.e. organism acclimation and species/environment interactions) and

provide further contributions to scientific consensus about the severe effects ocean acidification is afflicting on marine communities.

Results from the two seep sites investigated here, as well as other naturally occurring high CO_2 analogues (Inoue *et al.* 2013; Barkley *et al.* 2015; Enochs *et al.* 2015), generally agree with a plethora of experimental work from the small- (Diaz-Pulido *et al.* 2011; Comeau *et al.* 2013b; Kroeker *et al.* 2013) to large-scale (Langdon *et al.* 2000; Leclercq *et al.* 2000; Dove *et al.* 2013; Comeau *et al.* 2015, 2016; Albright *et al.* 2018), *in situ* seasonal comparisons (Bates *et al.* 2010; Albright *et al.* 2013) and quantitative models (Orr *et al.* 2005; Anthony *et al.* 2011; Ricke *et al.* 2013). All these generally predict considerable changes for coral reefs under 'business as usual' carbon emissions scenarios and that we will likely see shifts in community composition, with the proliferation of non-calcifying taxa and a retraction of many calcifiers. Increase in non-calcifying algae may lead to increase in community gross production, however gross gains may be balanced by increased respiration. Ecosystem-wide calcification and $CaCO_3$ accumulation rates will likely decline, owing to the carbonate chemistry changes and the dissolution of carbonate sediments and increased bio-erosion (Barkley *et al.* 2015; Enochs *et al.* 2016). Unfortunately neither this study, meta-analyses (Doney *et al.* 2009; Kroeker *et al.* 2013), nor experimental comparisons of pre-industrial to present-day conditions (Dove *et al.* 2013; Albright *et al.* 2016) have shown signs that ecosystem acclimation will prevent the expected changes. This study further shows that many changes expected on coral reefs under increasing OA will occur along a continuum, indicating that the less CO_2 emitted into the atmosphere, the less deviation we will see from the reefs of today.

5.6 Supplementary material

Supporting supplementary material can be found at:
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0197130#sec012

Chapter 6: General Discussion

The overall objective of this Thesis was to enhance our ability to predict how ocean acidification will affect the ecophysiology of reef corals and of reef communities in natural settings, and how effects may differ with concurrent exposure to variable temperature and light. To do so, I have used field studies at unique CO_2 seep sites and a series of multi-factor laboratory experiments. The CO_2 seep sites have given insights into the ecological effects of ocean acidification that are not possible to measure in the laboratory alone. I have documented changes to seep benthic communities that are greater than predicted based on the documented changes to the physiology of individual organisms. This suggests that the observed changes in the coral communities at the CO_2 seep sites are likely due to secondary effects or altered ecological interactions, as well as direct physiological effects on corals. In addition to the data chapters of this Thesis, the conclusions presented here also draw from my other co-authored studies (Appendix 1).

Results from this thesis indicate that the physiological effects of elevated CO_2 on corals may be weaker than the changes expected for communities. Reductions in pH and Ω will make calcification more energetically expensive, and this will likely reduce the calcification rates of sensitive coral species (Strahl *et al.* 2015; Albright *et al.* 2018; Chapter 5). However, many coral species will be robust to these effects, and able to maintain calcification rates as ocean acidification progresses (Strahl *et al.* 2015; Chapter 4). Rates of coral reef calcification and $CaCO_3$ accumulation will likely decline, as the seawater chemical environment becomes less favourable (Eyre *et al.* 2018), as calcifying species become less abundant in communities (Fabricius *et al.* 2011; Connell *et al.* 2013; Chapter 5), and as bioerosion increases (Enochs *et al.* 2016). Coral photosynthesis may increase in situations where CO_2 levels limit photosynthesis. Reef-wide photosynthesis may also increase, due to the chemical stimulation of CO_2 addition, and if certain algae become more abundant. Many of these changes will likely occur gradually over time, rather than abruptly at a certain tipping point (Chapter 5).

Other co-occurring stressors will also increasingly impact coral reefs, and these effects may be stronger than those of high CO_2 alone. Elevated sea surface temperatures are of particular concern for coral reefs due to the negative effect on corals and their dinoflagellate symbionts. However, reductions in light intensities will also have negative effects on corals and coral reefs. These results suggest that managing local stressors as well as reducing CO_2 emissions is needed to promote coral reef resilience and prevent future reef degradation.

6.1 Coral reef community changes under ocean acidification

Ocean acidification is an environmental change, and as it progresses we will likely see shifts in species distributions and alterations to reef communities. Predicting the outcome of this change is difficult, as the response ultimately depends on direct physiological effects on individual species, and on secondary ecological effects (Hill & Hoogenboom 2022). Ecological effects can be substantial, and hard to predict from physiological studies alone (Falkenberg *et al.* 2013; Fabricius *et al.* 2014a; Gaylord *et al.* 2015). Changes in benthic communities seen at the CO_2 seeps in Milne Bay are more drastic than predicted based on the physiological response of individual organisms. For example, Fabricius *et al.* (2014a) show that changes to coral habitat and the loss of structural complexity within the seeps sites dramatically reduced the diversity and abundance of a range of mobile invertebrate groups which rely on them for shelter. This loss was greater than expected, especially for groups predicted to be relatively robust to the seawater chemical changes expected under ocean acidification (e.g. crustaceans).

Changes in the distribution and abundance of corals within the Milne Bay CO_2 seep sites are also greater than expected based on physiological effects alone. For example, the percent cover of branching, tabulate and foliose coral taxa, predominantly from the Acroporidae, are reduced three-fold within the seep sites compared to adjacent controls (Fabricius *et al.* 2011). This Thesis aimed to determine if these patterns were likely due to the direct physiological effects of OA on *Acropora* corals, and found weak or null effects of high CO_2 on the physiology of the adult corals investigated (Chapters 3 and 4). Certain *Acropora* spp. have been shown to be sensitive to elevated CO_2, including those within the CO_2 seeps. For example, Strahl *et al.* (2015) documented *A. millepora* net calcification to decline by 44% within the seep sites. Strahl *et al.* (2015) further attributed the concurrent proliferation of *Porites* spp. to be due to their ability to upregulate photosynthesis and maintain calcification rates within the seep sites. However, calcification responses to high CO_2 are mixed when looking at the Acroporidae and Poritidae as a whole (Fabricius *et al.* 2011; Kornder *et al.* 2018). Hence, the wholescale reduction in the abundance of the Acroporidae, and the proliferation of the Poritidae seen at the seeps, is unlikely due to physiological constraints alone.

There are several potential ecological mechanisms that may contribute to alterations in coral communities within the seep sites. In this thesis I have documented that a range of non-calcifying algal

groups can benefit from elevated CO_2 (Chapter 4 and 5), and this pattern has been widely observed elsewhere (Fabricius *et al.* 2011; Connell *et al.* 2013; Kroeker *et al.* 2013; Sunday *et al.* 2016; Doo *et al.* 2019). Using a lab experiment I further investigated if the reduced coral abundance at the CO_2 seep sites may have been due to altered recruitment. To do so, the changes to coral settlement substrate under high CO_2 were investigated, and I found that the discs containing the coral juveniles in Chapter 4 were more frequently overgrown with turf algae under elevated CO_2 compared to the ambient treatment, and a range of non-calcifying algal groups came to progressively dominate both the upper and lower sides of settlement tiles within the CO_2 seeps in Chapter 5. Algal overgrowth can impede coral settlement, reduce survivorship of coral juveniles (Chapter 4), and compete with adult corals for space (McCook *et al.* 2001; Arnold *et al.* 2010; Venera-Ponton *et al.* 2011), potentially altering seep site coral communities. Moreover, crustose coralline algae (CCA) cover was seen to decline on settlement tiles as CO_2 levels increased (Chapter 5), matching patterns seen within the seep sites themselves (Fabricius *et al.* 2011, 2015). Numerous studies have shown CCA to be particularly susceptible high CO_2 (Comeau *et al.* 2013b; Fabricius *et al.* 2015; Smith *et al.* 2020; Peña *et al.* 2021). CCA play several important functions on coral reefs, including consolidating the reef substrate, and providing settlement cues for numerous benthic invertebrate taxa (Harrington *et al.* 2010). They are particularly important for the settlement of *Acropora* spp., and hence, a decline in CCA abundance would likely have negative effects for *Acropora* recruitment. Here I have further shown that the negative effects of a reduction in CCA cover on *Acropora* spp. recruitment can be greater than the direct effects of high CO_2 on juvenile *Acropora* spp. survival (Chapter 3; Doropoulos *et al.* 2012; Webster *et al.* 2013; Fabricius *et al.* 2017). Furthermore, Smith *et al.* (2016a) documented a three-fold reduction in demersal zooplankton abundance within the seep sites, likely reducing coral food supply, and affecting the distribution and abundance of the more heterotrophic coral taxa. Coral reefs are incredibly complex ecosystems, where numerous species interact with one another and the environment. Ocean acidification will likely cause a range of other ecological changes yet to be determined, contributing to alterations of reef communities from those of today.

6.2 Physiological effects of ocean acidification on corals and coral reef communities

Ocean acidification is generally predicted to reduce coral calcification. However, the growth of corals in my laboratory experiments was largely unaffected by elevated CO_2. Here the growth of the adults (changes in buoyant weight) and juveniles (change in number of corallites) of two *Acropora* species were unaffected by pCO_2 levels of ~900 µatm (Ω_{Ar} = ~2.5, Chapter 4). While coral calcification rates under ocean acidification are generally expected to decline, results are highly variable, and inter-specific variation is substantial (Kroeker *et al.* 2010; Chan & Connolly 2013; Kornder *et al.* 2018; Bove *et al.* 2020; Klein *et al.* 2022). For example, meta-analyses by Chan and Connolly (2013) report a mean reduction of coral calcification of 15% per unit Ω_{Ar} decrease, however values ranged from 0 – 30%. Even Acroporidae corals, where the calcification of some species has been shown to be highly sensitive to high CO_2 (Comeau *et al.* 2013b, 2017b; Schoepf *et al.* 2013; Vogel *et al.* 2015), display mixed effects when combined at the family level (Kornder *et al.* 2018). A variety of mechanisms have been suggested to be responsible for this variation, including differences in coral colony morphology, tissue layer thickness, life-history stage, experimental heterotrophic food supply and PAR levels, energy reserves prior to exposure, within reef habitat differences, latitude of study site, and differences between experimental systems (reviewed by Kornder *et al.* 2018). Several of these factors may have influenced the null results observed in this thesis.

Elevated CO_2 may negatively affect reef-scale calcification rates more than the calcification rates of corals themselves. These effects are often greater in the dark, due to a lack of photosynthetic CO_2 removal in the system, making the chemical conditions of seawater less favourable for $CaCO_3$ precipitation (Gattuso *et al.* 1999a). Chapter 5 of this Thesis aimed to compare the coral calcification rates investigated in Chapters 3 and 4 to values seen in early successional reef communities, to see if community effects were greater compared to the corals themselves. My field studies demonstrated declines in both light and dark rates of calcification in early successional communities under elevated CO_2, such that net daily calcification declined ~40% at Ω_{Ar} 2.5 compared to control values of Ω_{Ar} ~4 (Chapter 5). Effects were pronounced in the dark: rates of calcification reduced 25% in the light, while carbonate dissolution rates increased 280% in the dark, per unit Ω_{Ar} decline. Similar community wide declines in calcification under elevated CO_2 have been demonstrated in several large-scale ocean acidification mesocosm experiments (Langdon *et al.* 2000; Leclercq *et al.* 2002; Comeau *et al.* 2016; Chou *et al.* 2020), as well as in studies which manipulated dissolved CO_2 in reef environments (Albright *et al.* 2018; Doo *et al.* 2019). For example,

Albright *et al.* (2018) documented a 34% reduction in reef wide calcification with a 0.79 reduction in Ω_{Ar} during daytime manipulations of reef-wide CO_2 levels. Chou *et al.* (2020) similarly documented a 54% decline in net daily community calcification per unit Ω_{Ar} in mesocosms with manipulated CO_2 levels, driven by increases in night time carbonate dissolution of 136% per unit Ω_{Ar}.

There are several potential mechanisms responsible for the stronger effects of ocean acidification on coral reef communities compared to the corals themselves. Firstly, corals utilise $CaCO_3$ in the form aragonite, and are hence thermodynamically more resilient to ocean acidification than reef taxa with high-Mg-calcite skeletons. If a reef environment is dominated by organisms with high-Mg-calcite skeletons (e.g. CCA), they will likely have lower calcification rates under ocean acidification. This was the conclusion drawn by Albright *et al.* (2018), as their study site contained a higher percent cover of CCA compared to scleractinian corals (26 vs 15%). Settlement tile communities in the present study similarly had a combined upper and lower side cover of CCA of ~30%, which likely contributed to the considerable calcification declines observed as CO_2 levels increased (Chapter 5). Secondly, many corals and other invertebrates with a tissue layer separating their skeleton from the surrounding seawater can upregulate calcifying fluid pH to maintain calcification rates against a background of lowered pH (Venn *et al.* 2011; Guillermic *et al.* 2021). However, CCA, foraminifera and other coral reef calcifiers that have cell membranes rather than tissue layers to protect them may not be able to so (De Beer & Larkum 2001; McCulloch *et al.* 2012). Hence communities that are comprised of a range of different species, including those with little or no tissue layer, may be more negatively affected by high CO_2 than corals themselves. Thirdly, calcareous sediments, completely lacking tissue layers to buffer them from the surrounding seawater, are particularly susceptible to ocean acidification conditions in the dark and can contribute largely to the dissolution signal in reef-scale studies (Cyronak *et al.* 2013; Cyronak & Eyre 2016; Eyre *et al.* 2018). The settlement tile communities in Chapter 5 completely lacked calcareous sediments and hence the estimates of calcification rates and potential $CaCO_3$ accumulation may be an overestimate of reef-wide values we see in the future. Additionally, bioerosion may also increase under high CO_2 (Enochs *et al.* 2016), which, coupled with declining calcification rates and the dissolution of sediments, may cause reef-wide net $CaCO_3$ loss before the end of the century (Eyre *et al.* 2018). Finally, ocean acidification will likely give some non-calcifying taxa, especially photosynthetic taxa such as fleshy macroalgae and seagrasses, a competitive advantage and calcifiers may become progressively less abundant in the community (Hall-Spencer *et al.* 2008; Fabricius *et al.* 2011; Kroeker *et al.* 2011; Enochs *et al.* 2015). This shift in community composition away

from a dominance of calcifying taxa will likely reduce community calcification rates further. In a recent review examining the indirect effects of ocean acidification on corals and coral communities, Hill and Hoogenboom (2022) similarly concluded that the effects of ocean acidification were larger at the population or community level, suggesting that this was due to an accumulation of sublethal physiological effects on individual organisms.

In this Thesis, elevated CO_2 promoted photosynthesis in adult corals in a tank experiment, and in settlement tile communities from the seep sites (Chapters 3 and 5). Previous studies investigating the effect of CO_2 addition on photosynthesis, at both the coral-colony and -reef scale, have seen mixed results (Langdon *et al.* 2003; Kroeker *et al.* 2013; Anderson *et al.* 2019; Doo *et al.* 2019). In Chapter 3, I concluded that studies that had seen corals increase photosynthesis under elevated CO_2 may have been influenced by the location the study was being conducted. For example, those conducted in coastal waters with higher nutrient loads more frequently found CO_2 addition stimulated photosynthesis, while those in more oligotrophic waters did not. However, increased photosynthesis with high CO_2 is not only due to location specific factors. Investigations of coral photosynthesis at a single Milne Bay CO_2 seep site have shown that two out of four target species significantly increased rates of photosynthesis at the seep site compared to the controls (Strahl *et al.* 2015). This suggests other species-specific physiological mechanisms also affect photosynthesis, rather than the chemical stimulation of photosynthesis alone. This is unlikely due to differences in photosymbiont types, as those associating with six species of coral, including the four investigated by Strahl *et al.* (2015), did not differ between seep and control sites (Chapter 2).

At the coral reef community scale, increases in photosynthesis under ocean acidification may similarly be due to a removal of carbon limitation for different photosynthetic organisms. Increased CO_2 has been shown to stimulate photosynthesis in a range of reef taxa (Suggett *et al.* 2012; Uthicke & Fabricius 2012; Kroeker *et al.* 2013), however results are certainly not universal (Hill & Hoogenboom 2022). Productivity increases at the community scale may also result from altered community composition. If coral settlement is disrupted and coral and CCA cover declines, these taxa may be replaced by a range of non-calcifying algae (Chapter 4 and 5), which can have a higher net oxygen production rates per unit area (Connell *et al.* 2013). In Chapter 5, rates of net community production were unaffected by high CO_2 as gains in gross photosynthesis were negated by increased respiration. This suggests that high CO_2 may have little effect on the net energy budgets of early successional coral reef communities if gains from

photosynthesis are offset by increased respiration. How this translates to fully developed coral reef communities requires further investigation.

6.3 Responses of corals to ocean acidification in combination with variable temperature and light

A range of multiple stressors are increasingly impacting coral reefs (Ban *et al.* 2014; Uthicke *et al.* 2016). Here I have further investigated if the effects of OA on coral reef corals is altered by the cooccurring stressors of increased temperature and reduced light (Chapters 3 and 4). I found few negative additive, synergistic or antagonistic effects of the different stressor combinations. Here both increased temperature and reduced light were seen to negatively affect adult corals more than elevated CO_2 (Chapters 3 and 4). For example, my field surveys during a bleaching event, and the aquarium based experiment, showed that increased temperatures induced coral bleaching, but this was not exacerbated by high CO_2 (Chapter 3). Similarly, an 80% reduction in cumulative daily light integrals lowered adult coral growth by 60 – 70%, however elevated CO_2 did not reduce growth further (Chapter 4). There are likely several reasons the corals in these studies were largely unaffected by high CO_2 and the different stressor combinations, as discussed in the respective chapters. In contrast to many previous studies, I used experimental conditions as environmentally relevant as possible, i.e., corals were fed in the aquarium experiments, and CO_2 treatments were not particularly extreme. Heterotrophic feeding is an important component of coral energy supply and nutrient acquisition, and the extent to which corals rely on heterotrophy varies between species (Anthony & Fabricius 2000; Houlbreque & Ferrier-Pages 2009). Feeding not only provides nutrients and trace elements necessary for skeletal and tissue growth (Houlbreque & Ferrier-Pages 2009), but it can also mitigate the negative effects of elevated CO_2 and other stressors in some coral species. This is the case even for some *Acropora* spp. that are considered highly autotrophic (Edmunds 2011; Towle *et al.* 2015). Moreover, we found that feeding rates in the highly heterotrophic coral *Galaxea fascicularis* were reduced at CO_2 seep sites compared to controls (Smith *et al.* 2016b) suggesting either a reduced ability to feed, or an increase in autotrophic energy supply. Thus both autotrophy and heterotrophy are likely important for corals species, regardless of if they rely on one mode of energy acquisition over the other.

While I detected few negative physiological effects of high CO_2 on corals, many previous studies have done so, and these responses can be influenced by co-occurring stressors (Ban *et al.* 2014; Uthicke *et al.* 2016). A recent meta-analysis by Klein et al. (2022), which summarised the results of 1788 experimental studies examining the combined effects of high CO_2 and temperature on corals similarly found that high temperatures had a stronger effect than CO_2 on a range of response parameters, but that the addition of CO_2 further reduced coral photosynthesis and survival. Thus a reduction in CO_2 emissions is needed to prevent the further degradation of coral reefs. Similarly, maximising the amount of light reaching the seafloor will likely have positive effects on coral growth and may assist reef recovery and resilience in the face of other stressors including elevated CO_2 (Gattuso *et al.* 2006; Suggett *et al.* 2013). One way to achieve this would be to reduce the amount of terrestrial runoff and suspended sediment loads reaching coastal reefs (Fabricius *et al.* 2013, 2014c; Storlazzi *et al.* 2015). Suspended sediments not only negatively impact corals by reducing light (Jones *et al.* 2020), but also by increasing sediment deposition rates and coral smothering (Jones *et al.* 2019). Hence, management strategies are needed to reduce the effects of these stressors at both local and global scales.

6.4 Conclusions

Coral reefs are under increasing pressure from a range of anthropogenic threats. A reduction in CO_2 emissions is needed to prevent worsening ocean acidification and climate change. Coral reefs are being increasingly affected by these two global stressors, and community changes will likely occur gradually over time rather than at a specific tipping point or threshold. Hence, the less CO_2 released into the atmosphere, the less deviation we will see from the reefs of today. A range of local stressors are also impacting reefs, and there is an increasing need for effective local management to promote reef resilience and recovery in the face of global stressors.

Ocean acidification will affect the physiology of many taxa. Changes to the distributions of species that are most susceptible to the physiological effects of high CO_2 (e.g. non-calcifying algae and CCA) will likely cause flow-on effects on the species they interact with. The effects of these secondary ecological changes may be greater than direct physiological effects for many corals and could result in considerable changes to communities. Such ecological effects are difficult to predict from laboratory studies alone. Hence, the

use of CO_2 seep sites as natural laboratories has given insights into the ecological effects of ocean acidification that would not be possible otherwise.

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Appendix 1: Published co-authored journal articles

1. Zitoun, R., Connell, S., Cornwall, C., Currie, K., Fabricius, K., Hoffmann, L., Lamare, M., Murdoch, J., Noonan, S., Sander, S., Sewell, M., Shears, N., van den Berg, C. & Smith, A. (2020). A Unique temperate rocky coastal hydrothermal vent system (Whakaari/White Island, Bay of Plenty, New Zealand): constraints for ocean acidification studies. *Mar. Freshw. Res*.,71, 321-324.
2. DiPerna, S., Hoogenboom, M., Noonan, S. & Fabricius, K. (2018). Effects of variability in daily light integrals on the photophysiology of the corals *Pachyseris speciose* and *Acropora millepora*. *PLoS One,* 13, e0203882.
3. Kandler, N.M., Abdul Wahab, M.A., Noonan, S.H.C., Bell, J.J., Davy, S.K., Webster, N.S. & Luter, H.M. (2018). *In situ* response of the sponge microbiome to ocean acidification. *FEMS Micro. Ecol.,* 94, fiy205.
4. Fabricius, K.E., Noonan, S.H.C., Abrego, D., Harrington, L. & De'ath, G. (2017). Low recruitment due to altered settlement substrata as primary constraint for coral communities under ocean acidification. *Proc. R. Soc. London. Ser. B Biol. Sci.,* 284: 20171536.
5. Enochs, I.C., Manzello, D.P., Kolodziej, G., Noonan, S.H.C., Valentino, L. & Fabricius, K.E. (2016). Enhanced macroboring and depressed calcification drive net dissolution at high-CO_2 coral reefs. *Proc. R. Soc. London. Ser. B Biol. Sci.,* 283, 20161742.
6. Smith, J.N., Strahl, J., Noonan, S.H.C., Schmidt, G.M., Richter, C. & Fabricius, K.E. (2016). Reduced heterotrophy in the stony coral *Galaxea fascicularis* after life-long exposure to elevated carbon dioxide. *Sci. Rep.,* 6, 27019.
7. Takahashi, M., Noonan, S.H.C., Fabricius, K.E. & Collier, C.J. (2016). The effects of long-term *in situ* CO_2 enrichment on tropical seagrass communities at volcanic vents. *ICES J. Mar. Sci.,* 73, 976-996.

8. Webster, N.S., Negri, A.P., Botté, E.S., Laffy, P.W., Flores, F., Noonan, S., Schmidt, C. & Uthicke, S. (2016). Host-associated coral reef microbes respond to the cumulative pressures of ocean warming and ocean acidification. *Sci. Rep.,* 6, 19324.

9. Rocker, M.M., Noonan, S.H.C., Humphrey, C., Moya, A., Willis, B. & Bay, L.K. (2015). Expression of calcification and metabolism-related genes in response to elevated pCO_2 and temperature in the reef-building coral *Acropora millepora*. *Mar. Gen.,* 24, 314-318.

10. Fabricius, K.E., Kluibenschedl, A., Harrington, L., Noonan, S. & De'ath, G. (2015). *In situ* changes of tropical crustose coralline algae along carbon dioxide gradients. *Sci. Rep.,* 5, 9537.

11. Strahl, J., Stolz, I., Uthicke, S., Vogel, N., Noonan, S.H.C. & Fabricius, K.E. (2015). Physiological and ecological performance differs in four coral taxa at a volcanic carbon dioxide seep. *Comp. Biochem. Phys. Part A,* 184, 179-186.

12. Vogel, N., Fabricius, K.E., Strahl, J., Noonan, S.H.C., Wild, C. & Uthicke, S. (2015). Calcareous green alga *Halimeda* tolerates ocean acidification conditions at tropical carbon dioxide seeps. *Limnol. Oceanogr.,* 60, 263-275.

13. Fabricius, K.E., De'ath, G., Noonan, S.H.C. & Uthicke, S. (2014). Ecological effects of ocean acidification and habitat complexity on reef-associated macroinvertebrate communities. *Proc. R. Soc. London. Ser. B Biol. Sci.,* 281, 20132479.

14. Fabricius, K.E., Langdon, C., Uthicke, S., Humphrey, C., Noonan, S., De'ath, G., Okazaki, R., Muehllehner, N., Glas, M.S. & Lough, J.M. (2011). Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations. *Nat. Clim. Chang.,* 1, 165-169.