Intracellular pH in Cnidarian-Dinoflagellate Symbiosis

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This thesis is dedicated to the loving memory of Ozzie
(12/ 2000 – 02/2014)

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“It is good to have an end to journey toward; but it is the journey that matters, in the end.” – Ernest Hemingway

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Abstract

Accumulation of anthropogenic CO₂ is fuelling the decline of coral reef ecosystems. Increasing sea surface temperatures disrupt the endosymbiotic relationship between cnidarians and their single-celled dinoflagellate partners (genus *Symbiodinium*), while ocean acidification is known to impede calcification. At the cellular level, however, ocean acidification also has the potential to cause acidosis, with negative impacts on cell structure and function. Yet, despite the importance of intracellular pH (pH_i), the mechanisms involved in pH regulation and the buffering capacity within coral cells are not well understood. Combining pH-sensitive fluorescent dyes with either confocal microscopy or flow cytometry enables the measurement of pH_i within live cells. Here, I employed these techniques to determine the relationship between symbiont photosynthesis and host- and symbiont pH_i under ocean acidification and thermal stress. The specific aims of the study were: (1) to design a protocol for measuring the pH_i of the *Symbiodinium* cell and to quantify the effect of the diel light cycle on the pH_i of both members of the endosymbiosis; (2) to determine the role of the symbiont in modifying host cellular responses to short-term CO₂-induced acidification; (3) to quantify how exposure to elevated temperature changes the responses of the host and the symbiont pH_i to short-term CO₂-induced acidification; and (4) to establish the relationship between photo-physiology and pH_i after longer-term exposure to CO₂-induced acidification.

In Chapter 2, I used flow cytometry in conjunction with the ratiometric fluorescent dye BCECF to quantify pH_i in *Symbiodinium* cells and to monitor the effect of the diel light/dark cycle on pH_i. The pH_i of ITS2 type B1 cells (freshly isolated from the sea anemone *Aiptasia pulchella*) was 7.25 ± 0.01 (mean $\pm$ S.E.M) in the light and 7.10 ± 0.02 in the dark. A comparable effect of irradiance was seen across a variety of cultured *Symbiodinium* genotypes (types A1, B1, E1, E2, F1, and F5) which varied between pH_i 7.21–7.39 in the light and 7.06–7.14 in the dark. Of note, there was a significant genotypic difference in pH_i, irrespective of irradiance, with this parameter being lowest in types E2 and F1. The pH_i of *A. pulchella* host cells was then measured using the SNARF-4F probe and confocal microscopy. Light-induced alkalinisation

observed in the dinoflagellate cells was reflected in the pH_i of the host cells, with pH_i increasing from 6.86 ± 0.04 in the dark, to 7.02 ± 0.06 in the light.

The inter-dependence of host cell pH_i on its symbiont is described in Chapter 3 using cells isolated from the coral *Pocillopora damicornis*. BCECF was used in conjunction with confocal microscopy to determine how host- and symbiont pH_i responds to pCO_2 -driven seawater acidification under saturating irradiance, in symbiotic and non-symbiotic states, with and without the photosynthetic inhibitor DCMU. Each treatment was run under control (pH 7.8) and CO_2 -acidified seawater conditions (decreasing pH from 7.8 - 6.8). After 105 min of CO_2 addition, by which time the external pH (pH_e) had declined to 6.8, the dinoflagellate symbionts had increased their pH_i 0.5 pH units above control levels. In contrast, in both symbiotic and non-symbiotic host cells, 15 min of CO_2 addition (0.2 pH unit drop in pH_e) in the presence and absence of DCMU led to cytoplasmic acidosis equivalent to 0.4 pH units. Despite further seawater acidification over the duration of the experiment, the pH_i of non-symbiotic coral cells did not change, though in host cells containing a symbiont cell the pH_i recovered to control levels. This recovery was negated when cells were incubated with DCMU, revealing that the photosynthetic activity of the endosymbiont is tightly coupled with the ability of the host cell to recover from cellular acidosis after exposure to high CO_2 / low pH .

This raised the interesting possibility that bleached corals may be more sensitive to cellular acidosis than are their non-bleached counterparts, a hypothesis that was tested in Chapter 4. I exposed *P. damicornis* (a thermally sensitive coral) and *Montipora capitata* (a thermally resilient coral) fragments to four temperature treatments: 23.8 (ambient), 25.5, 28 and 31°C. Host coral cells containing their symbionts were then isolated and subjected to CO_2 -addition, designed to mimic predicted the VI CO_2 stabilisation scenario (pH_e 7.6) provided by the Intergovernmental Panel on Climate Change (IPCC, 2014). Host cells in *P. damicornis* were much more susceptible to cellular acidosis under the highest temperature treatment (7.40 ± 0.07 at 23°C to 6.56 ± 0.03 at 31°C) than their counterparts in *M. capitata* (7.35 ± 0.07 at 23°C to 6.95 ± 0.05 at 31°C). A similar decrease was observed in the *Symbiodinium* cell, with pH_i dropping from 7.45 ± 0.02 to 6.86 ± 0.01 in *P. damicornis*, and from 7.43 ± 0.04 to

7.14 ± 0.03 in *M. capitata* after CO₂-addition, suggesting that thermally sensitive corals may be at the highest risk of cellular acidosis.

Finally in Chapter 5, I describe the relationship between CO₂-driven acidification, photo-physiological performance and pH_i in *A. pulchella*. I exposed anemones to ambient (289.94 ± 12.54 μ atm), intermediate (687.40 ± 25.10 μ atm) or high (1459.92 ± 65.51 μ atm) CO₂ conditions for two months, conditions that represent the IPCC IV and VI stabilisation scenarios (IPCC, 2014). At regular intervals I measured the maximum dark-adapted fluorescent yield of PSII (F_v/F_m), the gross photosynthetic rate, respiration rate, symbiont population density, and the light-adapted pH_i of both the symbiont and the host cell. I observed increases in all but one photo-physiological parameter (P_{gross} : R ratio). Increases in light-adapted symbiont pH_i were observed under both intermediate and high CO₂ treatments, relative to control conditions (pH_i 7.35 ± 0.03 and 7.46 ± 0.06 versus pH_i 7.25 ± 0.05 , respectively). The response of light-adapted host pH_i was more complex, with no change observed under the intermediate CO₂ treatment, but a 0.3 pH-unit increase under the highest CO₂ treatment (pH_i 7.19 ± 0.01 and 7.48 ± 0.02 , respectively). This suggests that, rather than causing cellular acidosis, the addition of CO₂ will enhance the organism's photosynthetic performance, enabling the host-symbiont association to withstand the predicted ocean acidification scenarios.

Overall, the results from this study provide new insight into the cellular interactions that underpin cnidarian-dinoflagellate symbiosis. Moreover, they highlight the dynamic and species-specific nature of the cellular responses, reinforcing the need to incorporate species-specific acidification/warming interactions into models that are designed to predict the response of marine organisms to global climate change.

List of abbreviations

$^1\text{O}_2$	singlet oxygen
β	a cell's innate buffering capacity
μ	cell-specific growth rate
ANOVA	analysis of variance
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ASH	aragonite saturation horizon
BCECF-AM	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CA	carbonic anhydrase
CaCO_3	calcium carbonate
CaFSW	calcium free seawater
CCM	carbon-concentrating mechanism
CE	calicoblastic epithelium
Chl-<i>a</i>	chlorophyll- <i>a</i>
CMC	carboxymethyl cellulose
CO_2	carbon dioxide
CO_3^-	carbonate ion
CSD	cell specific density
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DIC	dissolved inorganic carbon
DIN	dissolved inorganic nitrogen
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOM	dissolved organic matter
EDTA	ethylenediaminetetraacetic acid
ENSO	el niño southern oscillation
FCM	flow cytometry
FDA	fluorescein diacetate

FSC	forward scatter
FSW	0.22 μm filtered seawater
F_v/F_m	maximum dark-adapted yield of photosystem II
G0	gap phase zero of interphase
G1	gap phase one of interphase
G2	gap phase two of interphase
H^+	proton
H_2CO_3	carbonic acid
H_2O_2	hydrogen peroxide
H_2PO_4^-	monobasic inorganic phosphate
HCl	hydrochloric acid
HCO_3^-	bicarbonate
I	interphase stage of the cell cycle
IPCC	intergovernmental panel on climate change
ITS	internal transcribed spacer
LED	light emitting diode
MAA	mycosporine-like amino acid
MAMP	microbe-associated molecular pattern
MES	2-[N-morpholino] ethanesulfonic acid
MI	mitotic index
NaOH	sodium hydroxide
NBS	national bureau of standards
NHE	Na^+ / H^+ exchangers
NO	nitric oxide
O_2^-	superoxide
OH^-	hydroxide ion
PAM	pulse amplitude modulation
PAR	photosynthetically-active radiation
PCR	polymerase chain reaction
P_{gross}	gross photosynthetic oxygen evolution
pH_e	external pH
pH_i	intracellular pH
PI	propidium iodide
P_{net}	net photosynthetic oxygen evolution

POM	particulate organic matter
PRR	pattern recognition receptors
PSII	photosystem II
R	respiration
R123	rhodamine-123
rmANOVA	repeated measures analysis of variance
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROI	region of interest
ROS	reactive oxygen species
SCM	subcalicoblastic epithelium
S.E.M	standard error mean
SNARF	seminaphtharhodafluor
TA	total alkalinity
TDI	time-delayed integration
TRIS	tris(hydroxymethyl)aminomethane

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Chapter 1: General Introduction

1.1 Symbiosis

Symbioses are ubiquitous across terrestrial, aquatic and marine ecosystems, and have played a fundamental role in shaping biological life on Earth. Indeed, a transitory symbiosis between bacteria and eukaryotic cells is thought to have facilitated the development of both mitochondria and chloroplasts (Sagan, 1967), and so led to the oxygenation of our planet's atmosphere (Embley and Martin, 2006).

The term symbiosis was not formally coined until 1879, when it was described by the German mycologist Heinrich Anton de Bary as “the living together of unlike organisms” (de Bary, 1879). Today, that definition has been refined and is broadly accepted as being the co-evolution of two genetically distinct organisms, which interact and form an ecologically significant association that is persistent over time (Trench, 1979). However, the definition does not specify what kind of association this has to be. As such, symbiosis spans a broad continuum from parasitism (where one partner benefits at the other's expense) through to mutualism (where both partners benefit). In the middle somewhere sits commensalism, where one partner gains from the association, while there is no benefit or loss to the other (Douglas, 2003).

Symbioses can be further classified according to the level of dependency that each organism exhibits towards the association. Obligate relationships describe those in which neither organism can survive outside the partnership (e.g. the tube worm *Riftia pachyptila*'s reliance on sulphur-oxidising bacteria at hydrothermal vents (Minic and Herve, 2004). Facultative relationships describe those in which both partners can survive independently (e.g. the symbiosis between the bivalve *Thyasira flexuosa* and the chemolithotroph *Thiobacillus thyasiris* (Wood and Kelly, 1989). The association can be either intra- (endosymbiotic) or extracellular (ectosymbiotic). These terms describe the position of the symbiont (the smaller partner) relative to its host organism. For example, the gamma-proteobacterium *Teredinibacter turnerae* is found within host cells in the gills of its shipworm host rendering it endosymbiotic (Altamia et al., 2014), while the light organ harbouring the bioluminescent bacterium *Vibrio*

fischeri in the bobtail squid *Euprymna scolopes* is ectosymbiotic (Overmann, 2006). Ascribing specificity to these partnerships is rather more complex. Monospecific symbiosis refers to associations where one host only interacts with one symbiont type and *vice versa*. This type of symbiosis is rare, and provides evidence of co-evolution between the host and symbiont (McFall-Ngai, 2001). Far more frequent is monospecificity exhibited by either the host or the symbiont (Baker, 2003; see section 1.2.4 for further discussion).

1.2 Cnidarian-dinoflagellate symbiosis

Photosynthetic symbioses are particularly widespread in the marine environment [Fig. 1.1] and are prevalent across the Chordata, Mollusca, Porifera, and Platyhelminthes phyla (Trench, 1979; Pawlowski et al., 2001; Venn et al., 2008). However, it is the relationship between members of the Cnidaria (hard and soft corals, sea anemones, jellyfish, and hydrocorals) and unicellular dinoflagellates belonging to the genus *Symbiodinium* (Freudenthal, 1962) that provides the best-known example. This well-studied relationship is thought to have evolved some 200 million years ago in the Triassic, a period that coincides with the first paleolithic evidence of coral reef structures (Wood, 2001; Stanley, 2003; Muscatine et al., 2005).

Fig. 1.1 Photosynthetic symbioses in the marine environment: [A] the tropical sponge *Carteriospongia foliacens*, photograph by M.C. Pineda; [B] the giant clam *Tridacna gigas*; [C] the tropical sea anemone *Aiptasia pulchella* [D] the leather coral *Sarcophyton* sp.; and [E] reef-building corals.

1.2.1 Ecological significance of coral reefs

Coral reefs exist within a narrow band extending out from the Equator, to latitudes of approximately 30° north to 30° south (Muller-Parker and Davy, 2001), forming ecosystems of considerable environmental and socio-economic importance. The biogenic framework of a coral reef is formed from the calcium carbonate (CaCO_3) skeletons of scleractinian corals, which are fused together by coralline crustose algae. Despite covering less than 0.1% of the Earth's surface (Smith, 1978), reefs provide a habitat that is unparalleled in productivity, and support an extraordinarily high biodiversity of flora and fauna (Bellwood et al., 2001; Knowlton et al., 2010). These ecosystems are a vital food source for the burgeoning populations in the developing world (Hughes et al., 2013a; Teh et al., 2013) and also support a thriving tourism trade that is critical for the economic stability of many tropical countries (Barbier et al., 2012; Sarkis et al., 2013). Coupled with this anthropogenic dependence, reefs also play a critical role in protecting coastal shorelines (Villanoy et al., 2012), and in particular, defend ecologically significant mangrove and seagrass habitats that act as vital nursery grounds for many species (McMahon et al., 2012).

1.2.2 Structure of cnidarian-dinoflagellate symbiosis

Cnidarians are anatomically simple organisms. They are diploblastic, being composed of two epithelial layers - the outer epidermis and the inner endodermis, which are separated by a collagenous layer called the mesogloea. In Anthozoans, where the polyp life history phase dominates, the two layers are arranged to form a tubular shape called the body column. At the top of the column, the oral disc contains a mouth that acts as both the entrance and exit to the gastrovascular cavity – a digestive cavity spanning the length of the organism. The endodermal layer facing the gastrovascular cavity contains the phagocytic cells, which host the photosynthetic dinoflagellates. Here, the symbionts are bound within the symbiosome membrane complex (Roth et al., 1988) a series of phagocytically-derived algal membranes (Schwarz et al., 1999; 2002) encompassed by an outermost host-derived membrane (Wakefield and Kempf, 2001). The cells themselves are coccoid in shape, and between 5 and 15 μm in size (Fig. 1.2B; Trench, 1979). Closer inspection reveals that

the cells contain a ‘lobulate’ chloroplast(s) (LaJeunesse et al., 2010a), holding the photosynthetic pigments diadinoxanthin and peridinin (Hackett et al., 2004). The dinoflagellate life-history typically contains both a vegetative and a motile (dinomastigote) phase but, while free-living *Symbiodinium* cells are able to switch between the two, the flagella are lost *in hospite* and a vegetative cyst is dominant (Santos and Coffroth, 2003). In this phase, *Symbiodinium* cells propagate asexually through mitotic division (Fitt and Trench, 1983), though the remarkable diversity in this lineage, only now becoming apparent through the advancement of higher resolution molecular techniques (see section 1.2.4 for further details), provides growing evidence for sexual reproduction too (Porto et al., 2008; Chi et al., 2014).

Fig. 1.2 Cnidarian-dinoflagellate endosymbiosis. [A] Confocal image of the tropical anemone *Aiptasia pulchella*; red fluorescence indicates the position of *Symbiodinium* cells (ex: 635, em: 655-755 nm). [B] Bright field micrograph of cultured *Symbiodinium* cells originally isolated from *Acropora* sp. in Okinawa, Japan; photograph by T.D. Hawkins). [C] Confocal image of an endodermal cell containing two algal cells. Host cell is stained yellow with SNARF-4F dye (ex: 559 nm, em: 585 $\pm$ 10 nm and 635 nm $\pm$ 10 nm). [D] Time-delayed integration (TDI) light micrograph of the same cell. Scale bar represents 10 μ m.

Symbiodinium are closely related to parasitic apicomplexans and ciliates (Stat et al., 2006), so there has been a great deal of interest in determining what brings about the onset of symbiosis in this genus (Weis et al., 2001; Rodriguez-Lannetty et al., 2004; Wood-Charlson et al., 2006; Harii et al., 2009). We now know that more than 85% of

corals acquire their symbionts from the environment, a process known as horizontal transmission (Babcock and Heyward, 1986), mainly through phagocytosis (Schwarz et al., 1999; Schwarz et al., 2002). In contrast, brooding corals such as *Seriatopora hystrix* generally employ a vertical transmission strategy, transferring their symbiont cohort directly to their young (Baird et al., 2009; Bongaerts et al., 2010). There are benefits and disadvantages to both approaches. Horizontal transmission affords the host access to environmental ‘pools’ of symbionts, which can be better acclimated to their local environment (Buddemeier and Fautin, 1993; van Oppen et al., 2004), but also presents the risk of not gaining any symbionts at all (Genkai-Kato and Yamamura, 1999). In comparison, vertical transmission is energetically costly to the host and may result in a less-than-optimal cohort of symbionts (Weis et al., 2001).

Horizontally transmitting species ‘sort’ the symbionts post-infection, in a complex process known as ‘winnowing’ (Nyholm and McFall-Ngai, 2004). Winnowing involves a cascade of molecular signaling between the symbiont and the host. Little is known about this process, but lectin/glycan interactions are likely to play an important role (Wood-Charlson et al., 2006; Logan et al., 2010), as are pattern recognition receptors (PRR’s) and microbe-associated molecular patterns (MAMP’s; see Davy et al., 2012). Once inside the organism, live, functional cells are able to interfere with their host’s immune defense response and present as “self” to avoid destruction (Fitt and Trench, 1983). In contrast, heat-stressed or DCMU-treated *Symbiodinium* cells are destroyed (Chen et al., 2005). In part, these interactions are thought to depend on the symbiont’s ability to express the correct Rab protein. Healthy, intact symbionts are able to express the early endosomal marker Rab 5 (Chen et al., 2004). In contrast, the late endosomal Rab 7 marker is present around dead, and photosynthetically compromised symbionts (Chen et al., 2005; Fransolet et al., 2012).

1.2.3 Function of cnidarian-dinoflagellate symbiosis

The success of the symbiosis is largely founded on the recycling and conservation of nutrients, and the transfer of photosynthetic products between the two partners (Wang and Douglas, 1998). A truly mutualistic symbiosis, the coral affords its symbiont’s protection from predators and access to the inorganic nutrients (primarily CO₂, NH₃, and PO₄) present in the coral’s waste (Muscatine and Porter, 1977; Muller-Parker and

D'Elia, 1997). The *Symbiodinium* cells use these nutrients in photosynthesis, to synthesize a range of complex organic compounds, which are then returned to the host as sugars, alcohols, amino acids, fatty acids and lipids (Gordon and Leggat, 2010; Burriesci et al., 2012). In the nutrient-deplete waters of the tropics, this source of energy is invaluable to the coral, potentially meeting more than 100% of its energetic requirements (Falkowski et al., 1984; Muscatine, 1990).

Early studies were quick to recognise the nutritional value of photosynthetic products to the cnidarian host (Muscatine and Hand, 1958) and noted that the productivity of the symbiont appeared to be enhanced *in hospite* relative to free-living cultures (Muscatine et al., 1967). R.K. Trench published a seminal set of papers to this effect in the early 1970's, identifying and tracking (with ^{14}C) the compounds released by the symbiont (Trench 1971a-c). He analysed *Symbiodinium* cells freshly isolated from the temperate anemone *Anthopleura elegantissima* and concluded that 20-50% of all carbon was made available to the host. The majority of this is released in the form of glycerol and/or glucose (Lewis and Smith, 1971; Trench, 1974; Sutton and Hoegh-Guldberg, 1990; Whitehead and Douglas, 2003; Burriesci et al., 2012), although alanine, fumaric acid, succinic acid and glycolic acid derivatives are all present (Trench, 1971b). Despite decades of research, we still know little about the identity of the photosynthate released (Davy et al., 2012), although there is some evidence that lipids may also be translocated between the two (Kellog and Patton, 1983; Patton and Burris, 1983; Luo et al., 2009). We do however have a much greater appreciation for the role of these compounds in supporting growth, respiration, reproduction, and calcification (Falkowski et al., 1984; Muscatine et al., 1990; Gattuso et al., 1999; Al-Horani et al., 2003; Allemand et al., 2004).

Photosynthetic activity is dependent on an adequate concentration of CO_2 at the site of photosynthesis, and the provision of essential inorganic nutrients. *Symbiodinium* cells contain type II Rubisco, which has a much higher affinity for O_2 than CO_2 (Rowan et al., 1996). This, in addition to the intracellular location of the symbionts, and the low concentration of CO_2 in seawater ($\sim 30 \mu\text{M}$, $< 1\%$ of all dissolved inorganic carbon (DIC)) requires considerable adaptations for the symbiosis. These adaptations come in the form of carbon-concentrating mechanisms (CCM's) and H^+ -ATPases. CCM's facilitate the inter-conversion of CO_2 and bicarbonate (HCO_3^-), and

are found widely across algae (Giordano et al., 2005). Carbonic anhydrases (CA's) are often a crucial component of CCM's (Badger, 2003). There is now considerable evidence for the importance of CA's in symbiont photosynthesis: (1) CA activity is light-dependent (Leggat et al., 1999) (2) there is a much higher concentration of CA in the tissues of symbiotic relative to aposymbiotic sea anemones (29-fold; Weis et al., 1989) and (3) the infection of previously symbiont-free organisms induced a 2.5-fold increase in CA activity (Weis, 1991). Locating the CA activity, however, remains elusive (Davy et al., 2012).

Allemand and co-workers (1998) produced the first model to explain the mechanism of DIC uptake [Fig 1.3]. They hypothesised that a membrane-bound H^+ -ATPase pump exists on the ectodermal host cell, facilitating the dehydration of HCO_3^- into carbonic acid (H_2CO_3), and subsequently into CO_2 , which is then able to enter the cell via passive diffusion (Furla et al., 2000). Once inside the cell a cytosolic CA hydrates the CO_2 back into HCO_3^- , essentially trapping the HCO_3^- in the host's tissues (Bertucci et al., 2011). The exact mechanism by which carbon is transferred to the symbiont is presently unknown. However, it is clear that in order for the symbiont to be able to use the trapped HCO_3^- for photosynthesis, there must be a further dehydration step to produce CO_2 . An H^+ -ATPase pump that is localised to the symbiont's plasma membrane, and is only expressed *in hospite* (Bertucci et al., 2009), is likely to play a critical role in the delivery of CO_2 to the site of photosynthesis. This model enables symbiotic organisms to exploit the HCO_3^- reservoir in seawater (~2.2 mM), and explains the disproportionately high CO_2 concentration observed at the site of photosynthesis (up to 330 μ M) compared to the surrounding seawater (Aizawa and Miyachi, 1986). Yet, despite these adaptations, photosynthesis in many marine algae remains DIC-limited (Reibesell et al., 1993; Nimer et al., 1999); a trait that also appears to be true for *Symbiodinium in hospite* (Weis, 1993; Goiran et al., 1996; Davy and Cook, 2001; Suggett et al., 2012).

Similarly, the conservation and exchange of nutrients such as nitrogen and phosphate is an important factor in determining coral reef productivity (Wang and Douglas, 1998). Dissolved inorganic nitrogen (DIN) in particular is essential for the synthesis of amino acids, proteins, nucleic acids, chlorophyll and Rubisco (Markell and Trench, 1993; Jiang et al., 2014). Yet, despite attaining higher concentrations of DIN (5-50

μM) than the surrounding seawater (Crossland and Barnes, 1977; Wilkerson and Muscatine, 1984), levels are still considered limiting in *Symbiodinium* cells *in hospite* (Cook and D'Elia, 1987; Jackson et al., 1989; Cook et al., 1992; Koop et al., 2001). Nutrient limitation is thought to play a fundamental role in controlling the symbiont population density, and importantly, in stimulating the translocation of fixed carbon to the host (Cook et al., 1988; Falkowski, 1993; Smith and Muscatine, 1999), so it is in the host's interest to regulate the amount delivery of carbon, nitrogen, and phosphorus available to the symbiont [Fig. 1.3].

Fig. 1.3 Schematic depicting inorganic carbon acquisition and nutritional translocation between the three tissue layers (the ectodermis, mesogloea and the endodermis) of the cnidarian-dinoflagellate symbiosis. Red dashed arrows denote the passive diffusion of carbon dioxide (CO_2) and red solid arrows the transfer of bicarbonate (HCO_3^-). Blue arrows show the transfer of dissolved organic matter/particulate organic matter (DOM/POM), dissolved inorganic nitrogen (DIN) and phosphate (P). The carbon and nutrient cycles have been offset for clarity. Membrane-bound H^+ -ATP-ases are in blue, transmembrane exchanger pumps are in red and the starburst sign represents photosynthesis. A full explanation of the mechanism is provided in the text above. Figure adapted from Allemand et al., 1998.

1.2.4 Host and symbiont diversity, and symbiosis specificity

Originally, all symbiotic dinoflagellates were thought to belong to a single pandemic species, classified as *Symbiodinium microadriaticum* (Freudenthal, 1962). However advances in molecular genetics (Stat et al., 2006) have redefined the phylogeny of this lineage and revealed an astonishing diversity. Analysis of nuclear genes encoding for ribosomal DNA brought about a complete re-classification of the genus (Baker, 2003), with 9 clades of *Symbiodinium* (A-I) now formally recognised based on their internal transcribed tracer (ITS2) sequences (Pochon and Gates, 2010). Of these, just under half (A, B, C and D) frequently form associations with scleractinian corals (Baker, 2003), although there have been isolated reports of clades F and G in corals (Rodriguez-Lanetty et al., 2004; LaJeunesse et al., 2010b; Lien et al., 2012). Clade E has been isolated from the temperate anemone *Anthopleura elegantissima* (LaJeunesse and Trench, 2000) and from a water sample in Wellington Harbour, New Zealand (Baker, 2003), while, clades H and I are specific to Foraminifera (Pochon et al., 2006; Pochon and Gates, 2010). The evolution of cladal lineages is thought to have taken place some 50 million years ago in the Eocene (Pochon et al., 2006). Since then, numerous adaptive radiation events have transpired resulting in the high intra-subcladal diversification that exists today (LaJeunesse, 2005). Indeed, only now with the advances in genetic microsatellite markers (Pettay et al., 2009; LaJeunesse et al., 2012; Howells et al., 2013) are we beginning to comprehend the true diversity that exists within these sub-clades, with more than 100 ITS2 ‘types’ assigned to clade C alone (LaJeunesse et al., 2004). The genetic composition, physiology, ecology, and biogeography vary substantially between types (Coffroth and Santos, 2005; Stat et al., 2006; Thornhill et al., 2007; Brading et al., 2011). Specifically, pigment composition, chlorophyll-*a* content per cell, and the size and number of light-harvesting antennae differ between and within types (Chang and Trench, 1984; Iglesias-Prieto and Trench, 1994; Hennige et al., 2009), as does the ability to produce mycosporine-like amino acids (MAA’s) that are crucial for UV protection (Banaszak and Trench, 1995; Banaszak et al., 2000). These factors cause substantial variation in the bio-optical properties of the symbiont, specifically affecting photosynthetic performance and thus the algal growth rate (Tchernov et al., 2004; Robison and Warner, 2006; Brading et al., 2011). The importance of these physiological differences becomes apparent upon exposure to thermal stress and/or high light (Chang and Trench, 1984; Robison and

Warner, 2006; Suggett et al., 2008; Fisher et al., 2012; Howells et al., 2012). Some genotypes can modify their chlorophyll-protein complexes, reducing the impact of irradiance (Iglesias-Prieto and Trench, 1997), while others face decreases in their light-harvesting pigments, a reduction in the efficiency of photosystem II (PSII) and increased xanthophyll pool sizes (Krämer et al., 2012). As such, the type of symbiont that a host species associates with has generated considerable interest in the scientific community, because this effectively defines the environmental limits of the partnership in question (Buddemeier and Fautin, 1993; Iglesias-Prieto et al., 2004; Sampayo et al., 2008; Stat et al., 2009; Putnam et al., 2012).

Broadly speaking *Symbiodinium* genotypes can be classified into two distinct groups, ‘generalists’, which are able to form associations with many hosts (LaJeunesse et al., 2004) and ‘specialists’, which are endemic to specific locations and/or hosts (Fitt et al., 2009; Putnam et al., 2012). The former, comprised of the basal cladal members A1, C1, C3 and D1 (Putnam et al., 2012), are often fast growing and opportunistic (Stat et al., 2008; Stat and Gates, 2011). Conversely, the latter tend to be comprised of highly derived, hard-to-culture types such as C15 (Reimer and Todd, 2009; Krüeger and Gates, 2012). Symbiont specificity, however, is not synonymous with host specificity (Baker, 2003). A ‘specialist’ symbiont for example, may only be able to interact with one coral species, but the coral may be able to host more than one type of symbiont (Rowan and Knowlton, 1995; Goulet and Coffroth, 1997; Rowan et al., 1997; Santos et al., 2003; Silverstein et al., 2012). On the contrary (and perhaps more frequently), a coral may only be able to host one type of symbiont (Weis et al., 2001), whereas that type may be able to form associations across many host species (LaJeunesse et al., 2004). These patterns drive considerable temporal and biogeographical variation in the distribution of symbionts and their hosts (Rowan et al., 1996; Ulstrup and van Oppen, 2003; Schmidt et al., 2004; Stat et al., 2006). Broad inter-cladal patterns are now becoming apparent; corals in the tropics (e.g. the Indo-Pacific), predominantly harbour symbionts belonging to clade C, whereas those at higher latitudes often host clades A, B and/or F (Rodriguez-Lanetty et al., 2001; Savage et al., 2002; Thornhill et al., 2009). There is also considerable variation within these regions, not only in the depth distribution of symbionts (Rowan et al., 1997; Toller et al., 2001; Frade et al., 2008), but also within individual coral colonies (Stat

et al., 2011). The benefits of hosting more than one *Symbiodinium* type are still hotly debated (Putnam et al., 2012; Cunning et al., 2013). In theory, corals with a low-specificity for *Symbiodinium* types should be more flexible to respond to stress (Berkelmans and van Oppen, 2006), but in practice corals that fall into this category, such as pocilloporids and acroporids are often the most susceptible to extended periods of stress (Stat et al., 2011; Putnam et al., 2012). Resolving these interactions is of the upmost importance given the threats posed to reefs over the coming century.

1.3 Threats to the stability of cnidarian-dinoflagellate symbiosis

Coral reefs currently face a catalogue of threats, ranging in scale and severity. Land run-off, pollution and over-fishing are all examples of localised threats, which can dramatically alter the structure and abundance of coral species on reefs (Chin et al., 2011; Hoegh-Guldberg et al., 2011; Hughes et al., 2013b). However, it should be possible to implement management strategies on a small scale and mitigate the effect of these stressors. The emergence of global climate change over the past century, though, is a very different proposition.

1.3.1 Global warming

Atmospheric CO₂ levels have risen by 75% since 1970 and currently stand at 385 ppm (IPCC, 2014). Although Earth's early climate may have witnessed atmospheric CO₂ concentrations higher than this (Pearson and Palmer, 2000), the current rate of change is unprecedented in recent geological history, and shows no sign of slowing down (Hoegh-Guldberg et al., 2007). The most optimistic estimates place CO₂ levels for 2100 at 450 ppm (IPCC, 2014). Under this scenario there is a 66% chance of limiting warming (above temperatures from before the Industrial Revolution) to 2°C, although for this to eventuate, emissions must plateau and then decline. A more realistic 550 ppm trajectory reduces the chance to less than 50% (IPCC, 2014), while some models caution that levels could reach up to 1000 ppm by 2100 (Meehl et al., 2007; Vuuren et al., 2011). Considered as a global average, a 2°C rise may not appear significant, however if water temperatures exceed the summer maxima by 1-2°C (Fitt et al., 2001), corals visibly pale, a process termed 'coral bleaching'. Bleaching refers to the

characteristic whitening of tissues resulting from a decline in symbiont density and/or the degradation of photosynthetic pigments, resulting from the collapse of symbiosis (Douglas, 2003; Weis, 2008). The frequency, severity and duration of bleaching events have increased over the past 40 years and are expected to escalate further into the future (Hoegh-Guldberg et al., 2007; Pandolfi et al., 2011; Perry et al., 2013). Even under the most optimistic CO₂ scenario, 23% of coral reefs are projected to experience annual bleaching events by 2050 (Van Hooidonk et al., 2013). Indeed, some mesophotic reefs in the Red Sea already experience such episodes (Nir et al., 2014). Mass bleaching events can have catastrophic consequences for reefs. The most severe event in recent history was witnessed in 1997/1998, when high temperatures coincided with the El Niño Southern Oscillation (ENSO), resulting in the loss of over 16% of the world's coral (Hughes et al., 2003), and up to 85% of hard coral cover was lost from reefs in the Western Indian Ocean alone (McClanahan, 2000).

Coral bleaching is induced by a number of stressors, including (but not limited to) extended periods of thermal stress (Bruno and Selig, 2007; Eakin et al., 2010) high irradiance (Lesser et al., 2011; Hill et al., 2012), low salinity (Kerswell and Jones, 2003), microbial infection (Kushmaro et al., 1996; Mills et al., 2013) and heavy metal contamination (Douglas, 2003). These factors can initiate bleaching independently, but they can also interact, lowering the thermal threshold required for bleaching (Lesser, 2006). The effects of bleaching however – namely decreased growth rates (Cantin et al., 2010), a reduction in reproductive success (Sudek et al., 2012; Airi et al., 2014) and, in some cases, mortality (Glynn et al., 2001; McClanahan et al., 2007) – persist irrespective of the cause. Yet despite decades of research, the mechanism involved in bleaching remains contentious (Weis, 2008). It is widely accepted that heat stress disrupts photosynthesis, most likely by damaging PSII (Takahashi et al., 2004; Lesser, 2011; Bhagooli, 2013; Hill et al., 2012). Three sites appear particularly vulnerable to injury [Fig 1.4]: the D1 protein (Warner et al., 1999; Takahashi et al., 2004); the thylakoid membrane (Tchernov et al., 2004; Díaz-Almeyda et al., 2011); and the Calvin Benson cycle (Jones et al., 1998; Hill et al., 2004). However, damage to these three sites is unlikely to always be mutually exclusive due to the high degree of inter-relatedness between the processes (Weis, 2008). More specifically:

(1) The D1 protein is an integral part of the PSII water-splitting complex, but it is also sensitive to excessive irradiance and/or heat stress (Baroli and Melis, 1996; Warner et al., 1999). An active repair system ensures D1 continues to function, but under longer periods of elevated heat and/or light stress this mechanism becomes overwhelmed and can sustain direct damage itself (Takahashi and Murata, 2008; Takahashi et al., 2009).

(2) Lipids present in the thylakoid membranes can become destabilised under the two stressors, disrupting the transfer of electrons from PSII to PSI (Tchernov et al., 2004).

(3) The dark reaction of photosynthesis, particularly the fixation of CO₂ by Rubisco, is susceptible to damage, which is likely to further impede the flow of electrons between the two-photosystem complexes (Jones et al., 1998).

Fig. 1.4 Three sites of photosynthesis that are susceptible to damage upon exposure to elevated heat and/or light stress. [1] Photosystem II (PSII), more specifically the D1 protein; [2] destabilisation of the thylakoid membranes and [3] the Calvin cycle. All three lead to the formation of highly reactive molecules such as superoxide (O₂⁻), singlet oxygen (¹O₂) and/or hydrogen peroxide (H₂O₂), collectively known as reactive oxygen species (ROS). The antioxidant activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX) may reduce damage to the alga and/or the host, although the effectiveness of these measures depends on the rate of ROS production. Figure redrawn from Venn et al. (2008).

Ultimately, all three mechanisms result in the production of highly reactive molecules such as superoxide (O_2^-), singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2) and/or nitric oxide (NO), which are collectively termed reactive oxygen/nitrogen species (ROS/RNS; Lesser et al., 2006; Hawkins et al., 2012). Without adequate antioxidant mechanisms, ROS can denature proteins, mutate DNA, and oxidise lipids in cellular membranes (Richier et al., 2006). While failure to apprehend NO production can activate apoptotic and necrotic cell death pathways (Perez and Weis, 2006; Bouchard and Yamasaki, 2009; Hawkins et al., 2013). Yet, production is not uniform across the *Symbiodinium* genus. At elevated temperature, types B1 and C1, for example, produce far more H_2O_2 than types A1 and F2 (Suggett et al., 2008; McGinty et al., 2012), whereas types A1 and B1 produce more RNS than C1 (Hawkins et al., 2012). Considered together, these findings may underpin some, but not all (see Baird et al., 2009 for a full discussion) of the variation observed in the bleaching susceptibility of corals hosting different symbiont populations (Sampayo et al., 2008; van Oppen et al., 2009).

1.3.2 Ocean Acidification

Global warming is not the only consequence of having high atmospheric CO_2 levels. The world's oceans are estimated to have absorbed up to 30% of all of the carbon emitted during the Anthropocene (Sabine et al., 2004). While this process undeniably mitigates the effect on the atmosphere, it also causes the pH of the oceans to drop, a process termed ocean acidification. This process is a perfectly natural response to changes in CO_2 ; in the atmosphere CO_2 is a relatively un-reactive gas, however upon contact with the sea surface a series of dissolution reactions are initiated. CO_2 reacts with water to form H_2CO_3 . This weak acid quickly dissociates into HCO_3^- and H^+ , which are subsequently dehydrated to form carbonate (CO_3^{2-}) and two protons, according to the equilibrium: $\text{CO}_{2(\text{g})} \leftrightarrow \text{CO}_{2(\text{aq})} + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 + \text{H}^+ \leftrightarrow 2\text{H}^+ + \text{CO}_3^{2-}$. The relative proportions of these three forms of carbon (CO_2 , HCO_3^- and CO_3^{2-}) reflect the pH of the seawater. The speciation of carbon has been stable for the past 20 million years, with seawater comprised of approximately 90% HCO_3^- ($2000 \mu\text{mol kg}^{-1}$), 9% CO_3^{2-} ($240 \mu\text{mol kg}^{-1}$) and 1% CO_2 ($15 \mu\text{mol kg}^{-1}$), so oceanic pH has also been constant (~ 8.1 ; Millero et al., 2002). Increasing the concentration of CO_2 shifts the equilibrium to the left, resulting in a greater concentration of free H^+ . There are

two main effects of this: (1) the seawater becomes more acidic; and (2) the additional H^+ reacts with CO_3^{2-} , generating HCO_3^- and reducing the amount of CO_3^{2-} available. The pH of the world's oceans has already dropped 0.1 pH units from pre-industrial levels, and it is expected to fall another 0.3 - 0.4 pH units by 2100 leading to a bleak future for marine organisms (Silverman et al., 2009; Pandolfi et al., 2011). The future of calcifying organisms is strongly dependent on the CO_3^{2-} concentration in the ocean. Currently this is stable at $\sim 210 \mu\text{mol kg}^{-1}$, a level lower than at any point in the past 420,000 years (Hoegh-Guldberg et al., 2007). However, if this value falls below $200 \mu\text{mol kg}^{-1}$, aragonite (the structural polymorph of CaCO_3 responsible for forming the skeletons in scleractinian corals) becomes undersaturated and it becomes far more difficult for corals to calcify (Kleypas and Langdon, 2006). Calcite on the other hand, (the polymorph predominantly used by soft corals) is more robust, and is expected to lag aragonite undersaturation by 50 – 100 years (Orr et al., 2005).

Fig. 1.5 Schematic depicting the processes involved in ocean acidification: dashed red arrows represent the fluxes of carbon dioxide (CO_2) and solid red arrows denote the flux of protons $[\text{H}^+]$. [1] Upon contact with the sea surface, CO_2 dissolves in water and dissociates to form carbonic acid (H_2CO_3), which breaks down into bicarbonate (HCO_3^-) and an extra H^+ . [2 & 3] The excess H^+ ions react with carbonate ions (CO_3^{2-}), which are a principle component of the calcium carbonate skeletons (CaCO_3) of organisms such as corals. Changes in the concentration of these three carbon compounds influence the stability of seawater pH (Bering diagram, *inset*).

The effects of acidification are likely to be magnified in high latitude regions because colder waters have a higher capacity for CO_2 absorption (Fabry et al., 2009). Indeed,

areas of the Arctic Ocean are already experiencing seasonal aragonite-undersaturation events (Yamamoto-Kawai et al., 2009; Bates et al., 2013), a situation that is expected to become the norm in the Southern Ocean by 2050, and across the sub-Arctic Pacific Ocean by 2100 (Orr et al., 2005; McNeil and Matear, 2008). Likened to a ‘bellwether for global acidification’ by Fabry and co-workers (2009), these environments provide an opportunity to model the responses of calcifying organisms to ocean acidification. The effects observed so far have been stark and concerning; echinoderms, coralline red algae, gorgonians, and pteropods have all dissolved in undersaturated waters (Orr et al., 2005). The distribution of high latitude cold-water coral reefs, which occur in relatively deep waters, is limited by the aragonite saturation horizon (ASH; Roberts et al., 2006), the stratification between saturated and under-saturated waters. The depth of the ASH varies latitudinally, and between oceans; for example it is relatively deep in the South Pacific and the North Atlantic (> 800 m), but is much shallower in the North Pacific (< 600 m). Acidification is reducing the depth of the ASH (i.e. it is becoming shallower), a finding that has grave implications for deep-sea scleractinian corals (Turley et al., 2007) and commercial fishing grounds (Feely et al., 2004; Feely et al., 2008; Gruber et al., 2012). Indeed, by 2100, up to 70% of all deep-sea corals are expected to be at risk of dissolution (Guinotte et al., 2006; Turley et al., 2007). Furthermore, marginal reef communities already living at the limits of their thermal tolerance, such as those on the Lord Howe Rise, may be particularly susceptible to acidification (Beger et al., 2013), as may the highly productive reef systems in areas of significant upwelling, such as Palmyra Atoll in the Central Pacific (Knowlton and Jackson, 2008).

Several hundred publications have been published on the causes and consequences of ocean acidification in reef corals in the past decade alone, providing a thorough database of inter- and intraspecific calcification responses under a variety of CO₂-concentrations (Veron et al., 2011). It is beyond the scope of this introduction to describe the individual responses to CO₂ alone, but excellent reviews exist on this topic (Doney et al., 2009; Hofmann et al., 2010; Veron et al., 2011). Meta-analyses of these results concluded that the responses are usually, but not always (Ries et al., 2009), negative in calcifying organisms (Silverman et al., 2009; Hendriks et al., 2010; Chan and Connolly, 2013; Kroeker et al., 2013) and are likely to be compounded by the increases in temperature (Anthony et al., 2011; Edmunds et al., 2012; Evenhuis et

al., 2014). Exceptions to this rule are few and far between, but one such example found no change in the growth rate of the coral *Stylophora pistillata* after 5 weeks of exposure to $2 \times \text{CO}_2$ (Reynaud et al., 2003). Reductions in adult growth rate may be the tip of the iceberg considering the number of life-history stages that could be affected by acidification prior to this. Early life history phases are particularly susceptible to high CO_2 /low pH (Gibson et al., 2011); decreases in fertilisation and cleavage success (Kurihara et al., 2008), larval survival (Dupont et al., 2008) and larval settlement (Doropoulos et al., 2012) will all contribute to poor recruitment in an acidified ocean (Albright et al., 2010; Albright, 2011).

While laboratory experiments are undoubtedly important, many of the experiments are conducted under CO_2 levels that are many times greater than those predicted for 2100. Furthermore they do not take into consideration the effects of factors such as community metabolism and predator/prey interactions (Edmunds et al., 2012; Poore et al., 2013). Natural volcanic CO_2 seeps provide the perfect opportunity to examine these effects of ocean acidification *in situ*. Unsurprisingly, hard coral cover and species richness is negatively correlated with pH at sites across Papua New Guinea (Fabricius et al., 2011), Japan (Inoue et al., 2013), Mexico (Crook et al., 2012) and the Mediterranean (Hall-Spencer et al., 2008; Cigliano et al., 2010; Johnson et al., 2012), and no corals were found at sites of less than pH 7.7 in Papua New Guinea (Fabricius et al., 2011). However, these vents were by no means barren, and were instead dominated by resistant species such as poritids (Rodolfo-Metalpa et al., 2011; Crook et al., 2012), soft corals (Inoue et al., 2013) and non-calcifying organisms such as anemones (Suggett et al., 2012). These findings are supported by the results of several laboratory studies. Indeed, increases in primary productivity under CO_2 -addition were observed in both the Mediterranean coral *Stylophora pistillata* (Marubini et al., 2008) and the temperate anemone species *Anemonia viridis* (Suggett et al., 2012; Jarrold et al., 2013) and *Anthopleura elegantissima* (Towanda and Thuesen, 2012). The benefits could be a direct result of the increase in substrate (i.e. CO_2) for photosynthesis (Rodolfo-Metalpa et al., 2010; Gao et al., 2012), or indirect through a reduction in the energetic demands of HCO_3^- acquisition (Raven et al., 2012). Either way, they suggest that some species may be “winners” under ocean acidification (Fabricius et al., 2011) and that a shift in species dominance across ecosystems is likely (Norström et al., 2009; Bell et al., 2013).

1.4 pH and cellular acidosis

1.4.1 *The physiological role of pH*

An individual organism's response to ocean acidification is likely to depend on its ability to regulate its intracellular pH (pH_i ; Fabry, 2008; McCulloch et al., 2012). pH_i by definition is simply the intracellular ratio of H^+ ions to OH^- ions, yet its regulation is fundamental to all metabolic processes within a cell. Eukaryotic cells typically maintain their pH_i between 7 and 7.4 (Madshus, 1988), but values vary considerably between organelles. Plant thylakoid membranes, for example, can be as low as pH 4, while the stroma can reach pH 8 (Kurkdjian and Guern, 1989). The pH_i of microalgae normally falls within the typical range of values (Walker and Smith, 1975; Lane and Burris, 1981; Gehl and Colman, 1985) although variation does exist between species, with values reaching as low as pH 6.5 in the giant-celled alga *Chaetomorpha darwinii* (Raven and Smith, 1980) and as high as pH 7.6 in the blue-green alga *Coccochloris peniocyctis* (Coleman and Colman, 1981). These differences play an important role biochemically, affecting the structure and function of proteins, the activity of enzymes and the efficiency of contractile elements (especially important in DNA/RNA synthesis), all of which influence the conductivity of ion channels and therefore control what can enter a cell (Smith and Raven, 1979; Madshus, 1988; Taylor et al., 2011; Taylor et al., 2012).

1.4.2 *Regulation of pH and cellular acidosis*

Cellular acidosis occurs when a cell's buffering capacity is exceeded, leaving the cell unable to respond to an acid-base imbalance. Failure to mitigate a rise in H^+ can modify the structure and function of ion channels, lower the efficiency of CA's (Bozzo and Colman, 2000) and alter the permeability of plasma membranes (Sobrinho et al., 2005), all of which can seriously compromise an organism's fitness (Pörtner et al., 2005; Langenbuch et al., 2006; Rosa and Seibel, 2008). Exposure to a short-term reduction in external pH (pH_e) typically initiates a sub-lethal response in organisms. During this time, the cell reduces the most energetically expensive processes (usually protein synthesis; Hand, 1991). Over a longer period of time, metabolic dormancy can have severe repercussions on an organism's reproductive and therefore competitive

fitness (Pörtner et al., 2004; Havenhand et al., 2008; Wood et al., 2008). To combat these, eukaryotic cells have evolved multiple buffer mechanisms at different cellular levels to minimise changes in pH_i (Casey et al., 2010). These mechanisms can be divided into two categories: 1) the cell's innate buffering capacity (β), which regulates acute changes over a short period of time (e.g. hours); and 2) permanent structural adaptations, which regulate chronic changes over a longer period of time (e.g. weeks).

Fig. 1.6 Transmembrane protein pumps involved in the regulation of intracellular pH (pH_i). Acid extruders use energy to reduce the acidity of the pH_i , either by removing H^+ ions, or by increasing the concentration of HCO_3^- . Acid loaders reduce the pH_i by increasing the concentration of H^+ in the cytoplasm. Colours represent the type of pump: H^+ -ATPases are blue; exchangers are red; and cotransporters are green. Figure adapted from Boron (2004).

β is comprised of two mechanisms: a closed system ($\beta_{\text{intrinsic}}$) and an open system ($\beta_{\text{HCO}_3^-}$). $\beta_{\text{intrinsic}}$ represents the buffering capacity of the intracellular weak acids and weak bases that are already present in the cytoplasm, such as (but not limited to) NH_4^+ , H_2CO_3 , and monobasic inorganic phosphate ($H_2PO_4^-$). The nature of weak acids is to dissociate into equilibrium between a proton and its conjugate base, which together form a buffer pair. In general terms this can be explained as: $HB^{(n+1)} \leftrightarrow B^{(n)} + H^+$, where HB is a weak acid, B its conjugate weak base and n the valence. For each

of these buffers, the equilibrium constant (K) can be considered in general terms as: $K = [B^{(n)}].[H^+] / [HB^{(n+1)}]$; or in its logarithmic form related to pH as: $pH = pK + \log_{10} ([B^{(n)}] / [HB^{(n+1)}])$. When an acute change in pH occurs, the free H^+ in the cytoplasm buffers the change by binding to OH^- and forming water. Conversely, a weak base dissociates into its conjugate weak acid and releases OH^- , to counteract any protons that are added into the system (Boron, 2004).

The $\beta_{HCO_3^-}$ on the other hand, is an open system. This arises from the diffusive ability of the uncharged CO_2 molecule, which is readily transported across all biological membranes. Once inside the cell, hydration and subsequent deprotonation of H_2CO_3 means that CO_2 acts as a conjugate acid, splitting to form HCO_3^- and releasing a proton: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$. Considering this relationship, cells become increasingly resistant to changes in pH_i as pH_e increases (Boron, 2004). This is particularly important when cells are exposed to larger or longer lasting fluctuations in pH_i . Exposure to these chronic pH events requires structural adaptations in the form of a suite of acid-base membrane-bound transporters (Kaniewska et al., 2012). In general, these can be classified into two groups [Fig. 1.6]. First, acid extruders (e.g. V-type H^+ pumps, Na- H^+ exchangers, Na-driven Cl- HCO_3^- exchangers and Na- HCO_3^- co-transporters) use energy to reduce the acidity of the pH_i , either by removing H^+ ions, or by increasing the concentration of HCO_3^- . Second, under alkaline conditions, acid loaders (e.g. Cl- HCO_3^- exchangers and Na- HCO_2 co-transporters) regulate the pH_i by increasing the concentration of protons in the cytoplasm. Very little is known about the regulatory mechanisms involved in cnidarians; there is molecular evidence for the involvement of V-type H^+ pumps (Ganot et al., 2011; Kaniewska et al., 2012), but to date only one study has adopted a mechanistic approach to pH regulation. In this case Laurent et al. (2013b) used amiloride inhibitors, in combination with live cell pH_i measurements, to demonstrate a role for Na/ H^+ exchangers (NHE) in the sea anemone *Anemonia viridis*.

1.4.3 pH in the cnidarian-dinoflagellate symbiosis

In symbiotic cnidarians, pH_i plays a particularly important role in coral calcification (Venn et al., 2011; 2013). Briefly, the coral skeleton is lined by a thin epithelium, called the calicoblastic epithelium (CE), which is comprised of calicoblastic cells and

anchoring cells termed desmocytes (Muscatine et al., 1997). Above lies a fluid termed the subcalicoblastic medium (SCM). The pH_i of the SCM is vital in the calcification process, directly influencing the concentration of CO_3^{2-} in the CE. Values of this layer measured in the corals *Galaxea fascicularis* (pH 8.13 in the dark *versus* 9.29 in the light; Al-Horani et al., 2003) and *S. pistillata* (pH 8.36 *versus* 8.69, respectively; Venn et al., 2011) reveal the importance of irradiance (and therefore photosynthesis) in this process. The benefits of light-enhanced calcification rates have long been recognised (Kawaguti and Sakumoto, 1948), but the coupling between photosynthesis and calcification is less clear. The photosynthetic uptake of CO_2 may increase the alkalinity of the coral tissue, aiding the deposition of the $CaCO_3$ skeleton (Goreau, 1959). Alternatively, a Ca^{2+} -ATPase pump may remove H^+ from the site of deposition (Zoccola et al., 2004), fueling the dehydration of HCO_3^- into CO_2 in the gastrovascular cavity, and resulting in high calcification rates (Gattuso et al., 1999; Allemand et al., 2004; Holcomb et al., 2009). Either way, failure to regulate pH_i in the SCM is likely to explain the decrease in calcification rates described earlier. It is becoming clear that disentangling the effects of ocean acidification will require intimate knowledge of the relationship between carbon speciation, pH_i regulation, photosynthesis and calcification (Schneider and Erez, 2006; Brownlee, 2009; Venn et al., 2013).

The first measurements of pH_i in coral cells were only reported in 2009 (Venn et al., 2009). Prior to this study, investigators were largely restricted by the methods available. The development of fluorescent pH-sensitive intracellular dyes has been instrumental in furthering research in this field (Zhang et al., 2012). The use of fluorescent dyes in conjunction with confocal microscopy has significant advantages; in particular, membrane-permeable dyes facilitate real-time, high-resolution visualisation of pH changes in living cells. This is especially beneficial for the analysis of pH_i in spatially confined areas such as coral cells, where the region of interest (ROI) is reduced by the presence of the dinoflagellate symbionts. Of the fluorochromes, two in particular are favoured. The cell-permeant acetoxymethyl ester form of 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) has been widely used to study pH_i fluxes in marine organisms (Dixon et al., 1989; Davies et al., 1990; Hervé et al., 2012; Liu et al., 2012). While the recent development of the

seminaphthorhodafluor-1 (SNARF) probe facilitated the first measurements of pH_i in host cnidarian cells (from *S. pistillata* and *A. viridis*; Venn et al., 2009).

Venn and co-workers' initial study (Venn et al., 2009) had three aims: (1) to determine how symbiotic state influences the pH_i of the host coral cell; (2) to measure the effect of light/dark on pH_i ; and (3) to measure the pH_i of the symbiosome (i.e. the vacuolar space surrounding the alga). Unfortunately, the latter aim could not be resolved any further than $< \text{pH } 6$ (a value that is in agreement with other studies; Rands et al., 1993) because the SNARF probe only works reliably between $\text{pH } 6$ and 8 . Both cnidarians maintained a steep gradient between the pH_i and the external seawater ($\sim \text{pH } 8.1$), however this gradient was greater in dark-adapted cells, where *S. pistillata* reached 7.13 ± 0.24 and *A. viridis* reached 7.01 ± 0.27 . In contrast, the light-adapted pH_i was measured as 7.41 ± 0.22 in the former and 7.29 ± 0.15 in the latter. Because light-induced alkalinisation was only witnessed in symbiont-bearing host cells, the authors concluded that the photosynthetic activity of the symbionts is able to directly influence the host cell pH_i . In a further experiment by this same research group (Laurent et al., 2013a), fluctuations in pH_i were measured over a range of light levels, with and without a photosynthetic inhibitor, DCMU. Their results confirmed that the photosynthetic activity by the symbionts is responsible for the rise in alkalinity in their host cells' pH_i , and demonstrated that this change is proportional to the level of irradiance that the symbionts are exposed to. Starzak (2012) then investigated whether the pH_i of the host cell is affected by the symbiont genotype, by re-infecting aposymbiotic *A. pulchella* with different symbiont types and exposing the organism to saturating light before measuring the pH_i of the host cell. No statistical differences between genotypes were observed, although of note the pH_i of host cells containing type E2 (a free-living strain) were more acidic than those containing homologous or heterologous B1 strains both in the dark (6.6 ± 0.10 versus 6.9 ± 0.04 and 6.7 ± 0.14) and light (6.7 ± 0.08 versus 7.0 ± 0.04 and 6.9 ± 0.08) respectively.

These results raised several interesting questions: What is the pH_i of the symbiont? Does the pH_i of the symbiont vary between different *Symbiodinium* genotypes? How will both partners of the symbiosis be impacted by external seawater acidification? And finally, if the photosynthetic activity of the symbiont is compromised (e.g. by heat), how will this affect the pH_i of the host and its response to ocean acidification?

1.5 Aim and specific objectives

No study has yet determined the pH_i of the *Symbiodinium* cell. This will form a vital component of this thesis. I will then aim to determine the inter-relationship between symbiont photosynthesis, symbiont pH and host cell pH, and how this is impacted by ocean acidification and thermal stress. The specific objectives of the study were to:

1. Measure the pH_i of the dinoflagellate symbiont cell and establish (a) the effects of the diel light cycle on pH_i , (b) how this compares to the pH_i of the host cell and (c) whether pH_i differs between symbiont genotypes in culture.

Hypotheses: (a) Light will cause an alkalinisation of the *Symbiodinium* cell as CO_2 is removed during photosynthesis (b) this will cause a similar light-induced increase in pH_i to occur in the host cell and (c) the magnitude of light-adapted alkalinisation will differ between symbiont types according to the efficiency of their CO_2 removal (i.e. the culture with the highest light-adapted pH_i will have the greatest photosynthetic productivity).

2. Determine how the presence/absence of symbionts and photosynthetic activity affect the response of host cell pH_i to short-term CO_2 -driven seawater acidification in reef corals.

Hypotheses: (a) Endodermal coral cell's hosting *Symbiodinium* will be able to buffer short-term external CO_2 -addition by increasing their photosynthetic activity, (b) when photosynthesis (and hence CO_2 -removal) is inhibited, this additional buffering capacity will be lost, leading to acidosis of the host cell.

3. Measure and compare how elevated temperature impacts upon the cellular response to short-term CO_2 -driven seawater acidification in thermally sensitive *versus* thermally resilient reef corals.

Hypotheses: thermally sensitive corals will be more likely to suffer acidosis under high-temperature because the photosynthetic activity of the symbiont is compromised and CO_2 will not be removed as efficiently.

4. Establish the longer-term impacts of CO₂-driven seawater acidification on the photo-physiology, stability and pH_i of the intact cnidarian-dinoflagellate symbiosis.

Hypotheses: the addition of CO₂ will enhance photosynthetic performance in *A. pulchella* enabling both the symbiont and host cell to withstand cellular acidosis under predicted ocean acidification scenarios.

The thesis has been written as a series of individual manuscripts, which are either published or in preparation, so there may be a degree of overlap in the content of each chapter, and particularly in their introductions. The author completed all laboratory work, fieldwork, analyses and writing, with further assistance as described below:

Chapter 2: This chapter is formatted as a stand-alone manuscript based on a published study. Gibbin, E. M. and Davy, S. K. (2013) Intracellular pH of symbiotic dinoflagellates, *Coral Reefs* 32 (3): 859-863. doi: [10.1007/s00338-013-1048-7](https://doi.org/10.1007/s00338-013-1048-7). S. K. Davy assisted with experimental design, data analysis and writing.

Chapter 3: This chapter is formatted as a stand-alone manuscript based on a published study. Gibbin, E. M., Putnam, H. M., Davy, S. K., and Gates, R. D. (2014) Intracellular pH and its response to CO₂-driven seawater acidification in symbiotic versus non-symbiotic coral cells. *Journal of Experimental Biology* 217: 1963-1969. doi: [10.1242/jeb.099549](https://doi.org/10.1242/jeb.099549). H. M. Putnam assisted with experimental design, data analysis and writing, S. K. Davy and R. D. Gates assisted with experimental design and writing.

Chapter 4: This chapter is formatted as a stand-alone manuscript and is currently 'under review'. Gibbin, E. M., Putnam, H. M., Gates, R. D., Nitschke, M. R. and Davy, S. K. Species-specific susceptibility to ocean acidification and cellular acidosis in reef corals is influenced by thermal sensitivity. H. M Putnam assisted with experimental design, data analysis and writing, S. K. Davy and R. D. Gates assisted with experimental design and writing, and M. R. Nitschke assisted with data collection.

Chapter 5: This chapter is formatted as a stand-alone manuscript based on a published study. Gibbin, E. M. and Davy, S. K. (2014) The photo-physiological response of a model cnidarian-dinoflagellate symbiosis to CO₂-induced acidification at the cellular level. *Journal of Experimental Marine Biology and Ecology* 457: 1-7. doi: [10.1016/j.jembe.2014.03.015](https://doi.org/10.1016/j.jembe.2014.03.015). S. K. Davy assisted with experimental design, data analysis and writing.

All published papers are provided at the end of the thesis.

Chapter 2: Quantifying the effect of light on the intracellular pH of cultured *Symbiodinium* cells, freshly isolated symbionts and *Aiptasia pulchella* host cells

2.1 Introduction

Coral reefs are both ecologically and socio-economically important, and their success is founded on the mutualistic endosymbiosis between corals and dinoflagellate algae of the genus *Symbiodinium* (Hoegh-Guldberg, 1999). However, despite the importance of the cnidarian-dinoflagellate symbiosis, we know very little about its underlying cell biology (Weis et al., 2008; Davy et al., 2012). This hampers our understanding of how coral reefs function, both under ‘normal’ conditions and projected scenarios of environmental stress. The genus *Symbiodinium* is exceptionally diverse, with nine genetically distinct clades (A-I), which are further differentiated into sub-clades termed ‘types’ (Pochon and Gates, 2010). There is substantial variation in physiology between genotypes, particularly with respect to their photosynthetic response to light (Hennige et al., 2009) and thermal tolerance (Fisher et al., 2012). These physiological differences have the potential to confer significant advantages/disadvantages to a coral and can influence a host’s resistance to stress (Baker et al., 2004). Yet despite this, one of the most fundamental attributes of the symbiotic algal cell, its intracellular pH (pH_i) was, until now, unknown.

Virtually all metabolic activity is pH-dependent. pH also plays a key role in controlling progression through the cell cycle, protein synthesis, and the synthesis of both DNA and RNA (Madshus, 1988). Furthermore, the establishment of a proton gradient across a cell membrane creates an electrochemical potential, which is important for maintaining the flux of ions between cells, and in inter-cell signalling (Casey et al., 2010). Little, however, is known of the role of pH in the function of the cnidarian-dinoflagellate symbiosis, despite its potential significance in the regulation of inorganic carbon and nitrogen fluxes between the host and symbiont (Fitt et al., 1995; Brownlee, 2009; Venn et al., 2009), and the coordination of host and symbiont cell proliferation (Davy et al., 2012). Moreover, pH_i is also known to affect the formation of crystals during coral calcification (Venn et al., 2013). Indeed, only recently has the pH_i of the host cell been measured, with the photosynthetic activity of

the symbionts causing an alkalisation of the host cell (Venn et al., 2009), proportional to the irradiance intensity experienced by the symbiont (Laurent et al., 2013a). The aim of this study was to develop a method for measuring the pH_i of *Symbiodinium* cells, to measure pH_i over a daily light cycle in a range of *Symbiodinium* types, and to compare the pH_i of *Symbiodinium* with that of its host cell in the sea anemone *Aiptasia pulchella*, an important model for coral research.

2.2 Methods

2.2.1 Experimental organisms

Cultured *A. pulchella* anemones hosting *Symbiodinium* ITS2 type B1, were grown at $25 \pm 1^\circ\text{C}$ and an irradiance of $80 - 100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ on a 12 h light: 12 h dark cycle (Osram Dulux 36W/890 fluorescent bulbs). Anemones were kept in $0.22 \mu\text{m}$ filtered seawater (FSW) under these conditions for six months prior to experimentation. During this time, anemones were fed with *Artemia* sp. nauplii every 3 days. Six *Symbiodinium* types (A1, B1, E1, E2, F1 and F5) were also examined. One of the types, E2, was cultured from a free-living dinoflagellate whilst the others were originally isolated from a range of invertebrate hosts including corals, anemones and jellyfish [Table 2.1, page 52]. The cultures were grown in FSW enriched with Guillard's f/2 media (AlgaBoost, AusAqua Pty Ltd., Wallaroo, SA, Australia) and maintained under the same light regime and temperature as the anemones. The cells were kept in logarithmic growth phase (approximately $100000 \text{ cells mL}^{-1}$) by regular sub-culturing in new f/2 media. Prior to experimentation, the cultures were genetically identified according to the protocol described by Logan et al. (2010).

Briefly, a 1 mL sample of each culture was centrifuged for 6 min at $3000 \times g$, before being re-suspended in guanidinium solution (100 mL H_2O , 100 g guanidinium isothiocyanate, 10.6 mL tris(hydroxymethyl)aminomethane (Tris) 1 M pH 7.6, 4.25 mL ethylenediaminetetraacetic acid (EDTA) 0.5 M; 4.24 g sarkosyl and 2.1 mL β -mercaptoethanol) and left for five days at 4°C . The DNA that was extracted was then subjected to an optimised PCR protocol (10 min at 95°C followed by 39 cycles of 2 min at 94°C , 30 s at 55°C , 30 s 72°C , and then 10 min at 72°C with the final hold

temperature set to 14°C) in conjunction with *Symbiodinium*-specific ITS2 primers. Forward primer: 5'-GTG AAT TGC AGA ACT CCG TG-3'. Reverse primer: 5'-CCT CCG CTT ACT TAT ATG CTT-3' (Logan et al., 2010). The PCR products were refined using PCR purification kits (Invitrogen, Auckland, New Zealand) and the resulting product was sent for to Macrogen Inc. (Seoul, Korea) for sequencing. The ITS2 sequences [Appendix A.1] were analysed using the Basic Local Alignment Search Tool (BLAST). All chemicals were obtained from Life Technologies, Auckland, New Zealand, unless stated otherwise.

2.2.2 Developing a method for measuring the pH_i of *Symbiodinium* cells

BCECF (Molecular Probes, Invitrogen, Auckland, New Zealand) was used to measure the pH_i of *Symbiodinium* cells. The dye was dissolved in dimethyl sulfoxide (DMSO) at 1 mg mL⁻¹ and stored at -20°C until required. BCECF is non-fluorescent until it enters the cell, where it is cleaved by intracellular esterases. The fluorescence emission spectrum of BCECF then becomes pH-dependent [Fig. 2.1A]. With a pK_a of 7.0, BCECF is a particularly useful dye for measuring changes in eukaryotic cells (usually pH_i 6.8 – 7.4). As such, it has been widely used to measure pH_i (Dixon et al., 1989; Hervé et al., 2012; Ozkan and Mutharasan, 2002) and has been successfully used in conjunction with flow cytometry (FCM) in other model systems (Franck et al., 1996). It was selected here for its strong dual excitation properties that allow pH_i to be measured without interference from chlorophyll auto-fluorescence (>640 nm).

Fig. 2.1 Visualising the spectral emission of BCECF-AM ester at the acidic and the basic endpoints of the calibration (pH 6 and pH 8) and at the dyes pK_a (pH 7). (A) Typical lambda scans of BCECF inside *Symbiodinium* cells, imaged after excitation with a 473 nm laser with emission recorded every 10 nm. (B) Optimising the concentration of BCECF-AM ester to produce the greatest shift in the ratio of fluorescence at the limiting ends of the calibration.

Confocal microscopy (Fluoview 1000, Olympus, Pennsylvania, USA) was initially used to confirm that the BCECF was able to enter the cells [Fig. 2.2A], to check for dye homogeneity through the cell [Fig. 2.2B] and to check that neither centrifugation nor vortexing had damaged the cells. A 10 mL sample of cultured *Symbiodinium* cells (homologous type B1) was centrifuged for 5 min, at $1000 \times g$, at a temperature of 20°C . The algal pellet was then re-suspended in FSW at a density of 1×10^6 cells mL^{-1} . A 1 mL aliquot was then taken and incubated with $10 \mu\text{M}$ of BCECF ester in the presence of 0.01% pluronic F-127 (Molecular Probes, Invitrogen, Auckland); a non-ionic surfactant that reduces dye aggregation. Preliminary studies confirmed that this concentration of dye produced the greatest difference in fluorescence at both the acidic and basic endpoints of the calibration i.e. pH 6 and 8 [Fig. 2.1B]. The dye-loaded sample was then placed on an orbital shaker set to 250 RPM and incubated in the dark for 20 min. The sample was then thoroughly vortexed and $300 \mu\text{L}$ was pipetted into a poly-d-lysine coated glass-bottom culture dish (MatTek Corporation, Ashland, Massachusetts, USA). The cells were left to settle for 15 min before $50 \mu\text{L}$ of carboxymethyl cellulose (CMC) was added to immobilise the cells. Individual *Symbiodinium* cells were imaged using a 1.45 NA $\times 100$ oil immersion lens, with sequential excitation provided by 405 nm and 473 nm lasers.

Fig. 2.2 Quantifying the pH_i of *Symbiodinium*. (A) Confocal microscope image of *Symbiodinium* cells loaded with $10 \mu\text{M}$ of the pH-sensitive BCECF-AM ester, imaged after excitation with both 405 and 473 nm lasers, with fixed emission at $535 \pm 10 \text{ nm}$. Scale bar represents $20 \mu\text{m}$. (B) ‘Z-stack’ ($z = 5$) image of a single *Symbiodinium* cell imaged under the aforementioned conditions. Scale bar represents $10 \mu\text{m}$. While the dye consistently labelled cells, including their cytoplasm, note its uneven intracellular distribution. This was most likely caused by selective accumulation into certain organelles.

Each cell was imaged ten times in the z-plane with fluorescence detection fixed at 535 ± 10 nm as this produced the greatest relative shift in fluorescence [Fig. 2.1A], and therefore the highest resolution of changes in pH_i . Confocal settings were kept constant between images to ensure that measurements were comparable between treatments. Visual comparisons between dye-loaded cells and control cells confirmed that the dye was able to enter the cells but that the fluorescence was not homogenous through the cell. This could either be an artefact caused by selective accumulation in certain organelles (e.g. the nucleus and mitochondria; Weinlich et al., 1998) or could reflect regional pH differences between organelles (e.g. acidic endosomal vacuoles; Casey et al., 2010). Furthermore, *Symbiodinium* cells contain auto-fluorescent ‘inclusion bodies’ that, when excited by blue light, have the potential to interfere with fluorescence measurements (Kazandjian et al., 2008). In this respect, FCM is a very useful tool because it averages the fluorescence signal across each cell and measures large numbers of cells simultaneously, reducing the effect of inter-cellular variability.

2.2.2.1 *In vivo* calibration of pH_i with nigericin

In vivo calibration of BCECF was carried out using a high-potassium buffer series in combination with the K^+/H^+ ionophore nigericin. This is a widely used technique to calibrate pH-sensitive dyes (Weinlich et al., 1998; Buckler and Vaughan-Jones, 1990; Venn et al., 2009; Taylor et al., 2011; Suffrian et al., 2011). Nigericin eliminates the trans-membrane K^+ gradient making the plasma membrane permeable to the buffer surrounding the cells. The buffers were created by adding 60 mM Na^+ , 200 mM K^+ , and 190 mM Cl^- to distilled water in the presence of either 20 mM 2-[N-morpholino] ethanesulfonic acid (MES) for acidic buffers (pH 6 - 6.8), or 20 mM Tris for basic buffers (pH 7 - 8). Mannitol was added as appropriate to adjust the osmolality of each solution to 1100 mosm L^{-1} , to mimic the cnidarian intracellular environment of cnidarians (Herrera et al., 1989; Goiran et al., 1997; Venn et al., 2009). A series of pH solutions were created at 0.2 pH unit intervals between pH 6 and pH 8, by the addition of HCl or NaOH as appropriate. The calibration was then performed using homologous *Symbiodinium* cells (ITS2 type B1) in culture. The cells were sampled and dye-loaded as described previously, before being resuspended in the calibration buffers mixed with 5 μL of nigericin (Molecular Probes, Invitrogen, Auckland, New Zealand) and transferred to a flow cytometer (FACScan, Becton Dickinson, New

Jersey, USA). The dye was excited with a 488 nm argon laser and fluorescence was captured in linear collection mode using the 515-545 nm (FL1) and 564-606 nm (FL2) filter channels. Prior optimisation of the FCM settings, based on forward scatter (FSC) a proxy for particle size, and chlorophyll-*a* (Chl-*a*), ensured that only *Symbiodinium* cells were counted, excluding any remaining bacteria and/or cellular debris in the samples. 5,000 individual cells were recorded in each sample [Fig. 2.3].

Fig. 2.3 *In vivo* flow cytometer calibration of *Symbiodinium* cells loaded with BCECF and re-suspended in a range of calibration buffers in the presence of nigericin. Each dot represents a single *Symbiodinium* cell, and dots are coloured according to their density (5000 cells per plot; red for individual cells, yellow-orange for > 100 cells, green-purple for > 500 cells). The white gate is placed around the position of the population at pH 6 to highlight the shift in fluorescence (y-axis; FL1 channel; 515-545 nm) caused by increasing intracellular pH.

Initial processing of the FACScan files was completed in CellQuest Pro (BD Biosciences), with detailed analysis performed in the program Weasel (Walter + Eliza Hall Institute of Medical Research, Melbourne, Australia). For each individual cell the fluorescence intensity ratio (R) was calculated as emission at FL1 / FL2 [Fig. 2.4A]. R was then related to pH by the following ratiometric equation: $\text{pH} = \text{pK}_a - \log \left[\frac{R - R_B}{R_A - R} \times \frac{F_{B(\text{FL1})}}{F_{A(\text{FL1})}} \right]$, where F represents the mean fluorescence per cell in channel FL1 at the acidic (A) and basic (B) limits of the calibration [Fig. 2.4B]. Using the logarithmic form of the equation minimises the impacts of non-uniform loading, leakage of dye out of the cell and photo-bleaching (Bright et al., 1989).

Fig. 2.4 *In vivo* calibration of intracellular pH in cultured B1 *Symbiodinium* cells. (A) The direct relationship between the fluorescence ratio (R; FL1 / FL2) of BCECF-AM and pH (mean values $\pm$ S.E.M, n = 3). (B) R was related to pH according to the following logarithmic equation: $\text{pH} = \text{pK}_A - \log [R - R_A / R_B - R * F_A / F_B]$, where F is fluorescence intensity measured at in FL1 (515-545 nm), and the subscripts A and B represent the limiting values at the acidic and basic endpoints of the titration (i.e. pH 6 and 8).

2.2.3 Obtaining viable *A. pulchella* cells

Viable endodermal cells (i.e. the host cell with its enclosed dinoflagellate symbiont) were isolated from *A. pulchella* [Fig. 2.5] using a protocol that was adapted from the method outlined in Gates et al. (1992).

Fig. 2.5 Viable intact endodermal *Aiptasia pulchella* host cells. (A) Endodermal cell containing a single algal cell. (B) Endodermal cell containing two algal cells. Host cells stained with Fluorescein Diacetate (FDA), Rhodamine 123 (R123) and Hoechst 33342. Colours represent the following: green = functional host mitochondria stained with FDA (ex: 473 nm, em: 510-550 nm), blue = host nuclear material (ex: 405 nm, em: 440-490 nm) and red = chl-*a* autofluorescence.

Anemones were initially bathed in calcium-free seawater (CaFSW), a relaxant, for an hour to relax the organism and ensure the tentacles were extended. The tentacles were then removed and cut into lengths of approximately 100 μm . These were transferred between a series of glass slides containing 10 μL CaFSW to remove excess mucus, before being placed in a watch glass, where the excess seawater removed, and 0.05% trypsin in CaFSW was added. The watch glass was then covered with parafilm to avoid evaporation, placed on an orbital mixer and agitated at 180 rpm for 40 min. The cells were transferred to a poly-d-lysine coated dish and left for 15 min for the cells to attach. After this time, the excess trypsin was removed and the cells were washed in the desired fluorescent dye, and incubated for 20 min before being transferred to the confocal microscope for imaging. Initially cells were loaded with a combination of dyes, designed to test the viability and structural conformity of cells following isolation. These were: 5 μM Fluorescein Diacetate (FDA), 5 μM Rhodamine 123 (R123) and 5 $\mu\text{g}/\text{mL}$ of Hoechst 33342 [Fig. 2.5].

2.2.3.1 Measuring the pH_i of *A. pulchella* cells

Cells were isolated using the protocol described above, however this time the cells were loaded with 10 μM SNARF-4F AM-ester in the presence of 0.01% pluronic F-127, and 5 μL of nigericin.

Fig. 2.6 *In vivo* calibration curve of SNARF-4F on the confocal microscope. (A) The direct relationship between R and pH (mean $\pm$ S.E.M). (B) R-value ($F_{(546\text{nm})}/F_{(635\text{nm})}$) log-transformed at each pH by the equation $\text{pH} = \text{pK}_a - \log [R-R_B/R_A-R * F_{B(635\text{nm})}/F_{A(635\text{nm})}]$, where R represents the ratio of fluorescence at $546 \pm 10 \text{ nm}$ / fluorescence at $635 \pm 10 \text{ nm}$, and F represents the mean fluorescence per cell at 635 nm at the acidic (A) and basic (B) endpoints of the titration (i.e. pH 6 and 8.5).

Calibration buffers were created at 0.5 pH unit intervals between pH 6 and pH 8.5 as described previously. The fluorescent stain was then washed off with the calibration buffer and 50 μL of CMC were added to immobilise the cells. The dish was transferred to the confocal microscope (Fluoview 1000, Olympus, Pennsylvania, USA) where the cells were excited with a 559 nm laser, and individual cells were imaged with the pinhole set at 1.51 units using a $\times 100$ oil immersion lens. Fluorescence emission was captured at 585 ± 10 nm and $635 \text{ nm} \pm 10$ nm and recorded within the region of interest (ROI). Each cell was imaged 10 times in the z-plane to produce the calibration curves [Fig. 2.6].

2.2.4 Experimental design

The effect of irradiance (12 h light adaptation at $80\text{-}100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and 12 h of dark adaptation) on pH_i was quantified in freshly isolated and cultured *Symbiodinium* cells, and in *A. pulchella* host cells. For freshly isolated algal cells, individual anemones ($n = 10$ per treatment) were exposed to light or dark before being homogenised in 500 μL of FSW in a hand-held glass tissue grinder. The resulting slurry was transferred to a 1.5-mL microcentrifuge tube and centrifuged for 5 min at $1000 \times g$, 25°C to separate the algal fraction from host tissue. The supernatant was discarded and the *Symbiodinium* pellet washed twice in FSW before the pellet was re-suspended in FSW at a final density of 1×10^6 cells mL^{-1} and incubated with 10 μM of BCECF-AM ester and 0.01% Pluronic F-127.

Cultured cells were grown in 250-mL flasks before being exposed to the same light or dark treatment as before ($n = 3$ per treatment). An aliquot (10 mL) was sampled from each flask, centrifuged for 5 min at $1000 \times g$, and re-suspended in FSW to give a final density of 1×10^6 cells mL^{-1} . The samples were then dye-loaded while maintaining them under their experimental conditions. Similarly, to quantify the pH_i of host cells, individual anemones ($n = 10$ per treatment) were exposed to light and dark as described above, after which viable endodermal cells were isolated following the protocol summarised above. In all cases, pH_i was then quantified using the methods stated previously.

2.3 Results

The pH_i of freshly isolated *Symbiodinium* cells and *A. pulchella* host cells was 7.10 ± 0.02 and 6.86 ± 0.04 (mean $\pm$ S.E.M) in the dark, increasing significantly (two-way ANOVA, $F_{1,51} = 12.219$, $p = 0.001$) to 7.25 ± 0.01 and 7.02 ± 0.06 in the light, respectively. Both the host cell and the symbiont responded in the same manner to irradiance ($F_{1,51} = 0.064$, $p > 0.05$), though the pH_i of the host cell was significantly lower than that of the symbiont at all times ($F_{1,51} = 26.325$, $p < 0.001$).

In the cultured *Symbiodinium* cells, genotypic differences in pH_i were apparent irrespective of the light/dark regime (two-way ANOVA, $F_{5,24} = 2.896$, $p = 0.035$). *Post hoc* analysis could not pinpoint where these genotypic differences lay (Tukey's HSD, $p > 0.05$ for all comparisons), though of note types E2 and F1 generally had the lowest values in both the light and dark [Table 2.1]. Light again significantly enhanced pH_i ($F_{1,24} = 73.784$, $p < 0.001$), with a similar response witnessed in all *Symbiodinium* types ($F_{5,24} = 1.192$, $p = 0.343$).

Table 2.1 Cultured *Symbiodinium* types categorised by the internal transcribed spacer 2 (ITS2) region of the nuclear rDNA, the host they were first isolated from, and the pH_i after 12 h light adaptation and 12 h dark adaptation (mean $\pm$ S.E.M; $n = 3$).

Culture name	Original host	Location	Clade (ITS2)	Light-adapted pH_i	Dark-adapted pH_i
CCMP 2467	<i>Stylophora pistillata</i>	Gulf of Aqaba, Jordan	A (A1)	7.33 ± 0.03	7.10 ± 0.03
Ap1	<i>Aiptasia pulchella</i>	Okinawa, Japan	B (B1)	7.32 ± 0.01	7.14 ± 0.01
FlCass	<i>Cassiopea xamachana</i>	Florida, USA	E (E1)	7.27 ± 0.04	7.14 ± 0.03
CCMP 421	Free-living dinoflagellate	Wellington Harbour, New Zealand	E (E2)	7.21 ± 0.04	7.08 ± 0.03
Mv	<i>Montipora verrucosa</i>	Hawai'i, USA	F (F1)	7.23 ± 0.08	7.06 ± 0.17
Pd	<i>Pocillopora damicornis</i>	Hawai'i, USA	F (F5)	7.39 ± 0.02	7.12 ± 0.27

2.4 Discussion

This study provides valuable information on pH_i in *A. pulchella*, an important model system for the study of the cnidarian-dinoflagellate symbiosis (Weis et al., 2008). However, more importantly, I describe a method for measuring pH_i in *Symbiodinium* and show genotypic diversity in this parameter. While this method provides resolution at the level of the whole cell rather than the specifics of the intracellular environment, it enables the rapid measurement of large numbers of cells simultaneously.

The pH_i values quantified in this study fall within those expected for eukaryotic cells, which typically maintain their cytosolic pH between 7 – 7.4 (Madshus, 1988), and are similar to light-adapted pH_i values measured in other marine algae (Lane and Burris, 1981). Furthermore, the more alkaline pH_i of *Symbiodinium* seen in the light than in the dark was consistent with the effect of light observed in higher plants (Yin et al., 1990). Light-induced alkalisation is likely a by-product of physiological changes in the *Symbiodinium* cell's cytoplasm during photosynthesis. In particular, CO_2 and H_2O exist in equilibrium with H_2CO_3 , so when CO_2 is removed for photosynthesis it causes an increase in the conversion of HCO_3^- to H_2CO_3 , a process that consumes protons H^+ (Allemand et al., 1998). Conversely, the subsequent reduction of pH_i in the dark is likely to be a consequence of respiration, as the products, CO_2 and H_2O , react to form HCO_3^- and H^+ . It is therefore perhaps unsurprising that the pH_i of *Symbiodinium* cells, the sites of photosynthesis in the symbiosis, is higher than that of the surrounding host cell in the light. Why this pattern is also present in the dark, however, is at present unclear. One possibility is that dark aerobic respiration may be greater in the host cell than the symbiont, resulting in a build up of CO_2 in the cell and thus reducing pH_i . Alternatively, the higher pH_i of the symbiont in the dark could be a consequence of residual dark or heterotrophic CO_2 fixation. Interestingly, the pH_i in the *A. pulchella* host cell mirrored the light-response seen in the symbiont. This has also been observed in host cells of the coral *S. pistillata* and the anemone *Anemonia viridis* (Venn et al., 2009), highlighting the inter-relationship between symbiont and host physiology. Indeed, recent research suggests that the pH of the host cell is directly dependent on the photosynthetic activity of its algal symbiont and, as such, any factor that either inhibits or enhances photosynthesis (e.g. irradiance) has the capability to influence the pH of the host cell (Laurent et al., 2013a).

The physiological and genetic diversity within the genus *Symbiodinium* is now well known, though most studies have focused on photophysiological responses to environmental stresses (Fisher et al., 2012). We can now extend this physiological diversity to the pH_i . The basis for this difference is unknown as, while it might be expected that differences in photosynthetic performance and/or efficiency (Hennige et al., 2009) could lead to a difference in pH_i in the light, genotypic differences were also maintained in the dark. Further research however, is needed to elucidate this matter, both in the *Symbiodinium* types tested here and across a range of types.

The cultures investigated here included two B1 strains, one that was freshly isolated from *A. pulchella* and the other that was grown *ex hospite* in culture. Interestingly, the light-adapted pH_i was maintained significantly higher in the cultured *Symbiodinium*, despite both being exposed to identical light and temperature regimes. This is perhaps not surprising, as *in hospite* *Symbiodinium* are often DIC-limited (Goiran et al., 1996; Al-Moghrabi et al., 1996) and light-adapted pH_i levels have been shown to correlate with the availability of carbon in the surrounding media (Nimer et al., 1997). It is also worth noting that the cultures in this study were grown in nutrient-enriched seawater, which would be much higher than those experienced *in hospite* (Koop et al., 2001), and so may not be entirely indicative of the implications of physiological diversity for symbiosis function. The novel techniques and findings presented in this chapter, do however build on the important work of Venn and coworkers (Venn et al., 2009), by providing a means of studying both partners in this ecologically important symbiosis, and addressing how the symbiosis may be impacted by the increasing global threat of ocean acidification.

Chapter 3: Intracellular pH and its response to CO₂-driven seawater acidification in symbiotic *versus* non-symbiotic coral cells

3.1 Introduction

Reef building corals depend on a symbiotic association with photosynthetic dinoflagellates of the genus *Symbiodinium* for survival. This intimate partnership evolved in the mid-Triassic period (Muscatine et al., 2005) and coral reefs have prospered in tropical oceans, particularly in areas characterised by high degrees of environmental stability (Hoegh-Guldberg, 1999). Corals have adapted to live within narrow physiological limits, and are highly sensitive to fluctuations in the surrounding environment (Jones et al., 1998). Rising sea surface temperatures (Hoegh-Guldberg et al., 2007) and the increasing acidity of the ocean (Orr et al., 2005) are threatening the stability of coral-dinoflagellate symbioses, leading to dire projections for the future of coral reefs (Pandolfi et al., 2011; Silverman et al., 2009). At present, our ability to accurately predict the response of corals to global climate change is severely hampered by our limited understanding of the cellular mechanisms that underpin coral-dinoflagellate symbiosis (Fabry et al., 2008; Weis et al., 2008; Davy et al., 2012), which ultimately frame how corals respond to environmental stress.

Intracellular pH (pH_i) is critical for virtually all elements of cellular homeostasis (Smith and Raven, 1979), directly influencing protein structure, enzymatic rates and membrane solubility (Madshus, 1988). The maintenance of pH within an optimal functional range therefore plays a critical role in determining the metabolic activity of the cell, and is specific to the metabolic pathway in question. Consequently, eukaryotic cells have evolved compartmentalised organelles to provide sites with specific pH conditions within the cell for different metabolic activities to occur (Casey et al., 2010). Disruptions to the pH_i have serious physiological consequences (Pörtner et al., 2004; Fabry et al., 2008; Hofmann et al., 2013). Indeed, a drop as small as 0.1 - 0.2 pH_i units can induce metabolic depression (Reipschläger and Pörtner, 1996), so it is not surprising that changes in pH_i are stringently avoided (Casey et al., 2010). In general, eukaryotic cells are protected from fluctuations in their pH_i at two levels. Acute, localised changes in pH_i , such as those arising from metabolic

reactions, are neutralised by manipulating the various weak acids and bases in the cytosol (Casey et al., 2010). Longer-term changes are buffered by more permanent mechanisms such as transmembrane exchangers (Boron, 2004). The mechanisms involved in pH regulation are not well understood in corals, and this is partly due to the intrinsic complexity associated with their endosymbiosis. By virtue of their intracellular location, the *Symbiodinium* cells can essentially be regarded as heavily fortified organelles belonging to the coral host cell. However, unlike other organelles, *Symbiodinium* cells, through their photosynthetic activity, are able to exert significant control over the pH of the surrounding host cell (Venn et al., 2009; Laurent et al., 2013a). Consequently, the response of corals to a change in ambient CO₂ / pH, is likely to be influenced by their own physiological capacity and that of their symbionts (McCulloch et al., 2012).

In this chapter, I investigate how the presence/absence of symbionts affects the response of pH_i to CO₂-driven seawater acidification in cells isolated from the Hawaiian reef coral *Pocillopora damicornis* (Linnaeus, 1758). Using the fluorescent dye BCECF, in conjunction with live cell imaging, I characterise the response of pH_i (National Bureau of Standards scale; NBS) in *Symbiodinium* cells freshly isolated from coral hosts, isolated non-symbiotic coral cells, and isolated coral host cells with their symbionts intact. The cells were exposed to control seawater (pH 7.8) and CO₂-acidified seawater, designed to expose the cells to a gradient of declining external pH (pH_e 7.8 - 6.8) over 105 min, to mimic diurnal changes in pH in reef water due to reef photosynthesis, respiration, and calcification (Hofmann et al., 2011; Price et al., 2012), which are particularly strong in Kaneohe Bay (Putnam, 2012). In both treatments, the cells were exposed to saturating white light in the presence and absence of the photosynthetic inhibitor DCMU, with measurements of both pH_i and pH_e taken every 15 min for 105 min. These findings demonstrate that CO₂ addition initiates very different responses in the pH_i of the symbiont compared to that of the coral host cell. Crucially, the results of this chapter suggest that the photosynthetic activity of the symbiont plays a key role in determining the intracellular buffering capacity of its coral host.

3.2 Methods

3.2.1 Coral collection and maintenance

One large normally pigmented adult *P. damicornis* colony was collected three weeks prior to experimentation (in February 2013) from a shallow fringing reef (< 3 m) in Kaneohe Bay, Hawaii. This single colony was cut into 50 genetically identical fragments (4 x 2 cm) that were secured to 3 x 3 cm plastic tiles with underwater epoxy (Z-spar, Splash Zone compound) and placed in a 50 L holding tank supplied with flowing seawater from Kaneohe Bay. Seawater chemistry was monitored frequently according to the recommended best practices for ocean acidification research and reporting (Riebesell et al., 2010), with daily measurements of salinity (psu) as well as pH (NBS scale) taken via the m-cresol dye method, stipulated in SOP 6B (Dickson et al., 2007); total alkalinity (TA) was measured on a weekly basis (Dickson et al., 2007). These characteristics were stable for the duration of the experiment, with an average salinity of 35.5 ± 0.1 ppt, pH of 7.8 ± 0.1 and a TA of 2166 ± 25 (mean $\pm$ S.E.M, $n = 5$). It is important to note that the $p\text{CO}_2$ of seawater in Kaneohe Bay is markedly higher than average oceanic conditions fluctuating between 450 and 650 ppm (Drupp et al., 2011; Putnam, 2012) and resulting in a lower ambient pH. An ambient seasonal temperature of $22.6 \pm 0.3^\circ\text{C}$ was maintained by a dual stage temperature controller (Aqualogic, TR115DN), while tanks were illuminated on a 12 h:12 h light/dark cycle by metal halide lights (Ice CapMetal Halide lights, 250 W DE 14K bulbs, 250 W double-ended pendants), which, mounted on motorised light rails, moved back and forth to provide irradiances ranging between 3.85 and 328.85 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. This set-up ensured an even light field corresponding to an average irradiance of $125 \pm 10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was maintained over each coral fragment.

3.2.2 Experimental Design

Four experimental treatments were designed to investigate how CO_2 -addition influenced pH_i (NBS scale) in cells isolated from the coral *P. damicornis*. Three symbiotic states were tested: (1) isolated *Symbiodinium* cells; (2) isolated non-symbiotic host coral cells and (3) isolated host coral cells containing their symbiotic algae. Visual inspection (by both light and confocal microscopy) failed to establish

conclusively whether isolated *Symbiodinium* cells were surrounded by an intact symbiosome membrane. Non-symbiotic host coral cells were classified as intact host cells not containing an algal symbiont. Typically 10 μm in diameter and spherical, it is not known whether these cells were ecto- or endodermal in origin (see discussion). Finally, only symbiotic cells containing two algal cells were used for calculating the pH_i change in symbiotic host coral cells. These were deemed the most suitable choice for two major reasons: (1) the host cell region of interest (ROI) is much larger in doublet cells than in host cells containing a single alga, making pH_i measurements much easier (Venn et al., 2009); and (2) this cell type is found in much greater abundance than triplet cells (Houlbrèque et al., 2004). Furthermore, standardisation of symbiont number allowed for the possibility that this parameter influences host pH_i (see discussion).

The cell and dye loading procedure was repeated five times for each treatment ($n = 5$) to achieve independent replicate cell preparations. Individual cells were then imaged every 15 min for 105 min, with pH_i calculated from the images taken. At each experimental time-point, 1.5 mL of seawater were carefully removed by pipette so as not to disrupt the cells and analysed for pH_e (NBS scale, $n = 5$) via the m-cresol dye method described in SOP 6B (Dickson et al., 2007). The treatments were carried out under an external white light source, provided by a variable-irradiance fibre optic cable (Halogen Reflector lamp, 150W GX5.3 21V 1CT bulb, Philips, Somerset, New Jersey, USA) that produced a saturating irradiance of approximately 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ photosynthetically active radiation. Previous studies have shown that photosynthetic activity in the symbiont modifies the pH_i of the host under normal CO_2 conditions (Venn et al., 2009), so a preliminary measurement was performed with no additional CO_2 added, in order to establish the control or baseline change in pH_i caused by photosynthesis. This treatment was then repeated with CO_2 addition (5.0% setting on the incubation unit of the LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany), resulting in a decreasing gradient of pH_e over time. The negative controls were run in the light, as before, under zero and high CO_2 conditions, but in the presence of 100 μM DCMU in 0.1% acetone, a photosynthetic inhibitor that blocks the plastoquinone binding site of PSII and thus prevents the transfer of electrons and formation of ATP. DCMU is an effective inhibitor of photosynthesis

that has been widely used in *Symbiodinium* research (Fitt et al., 1983; Iglesias-Prieto et al., 1992; Al-Horani et al., 2003; Takahashi et al., 2013).

3.2.3 *Isolation of cells and dye-loading procedure*

Coral fragments were selected randomly from the acclimation tank before the start of each experimental run. Cells were isolated immediately by gently brushing the tip of a partially submerged fragment in 50 mL of 0.22 μm filtered seawater (FSW) using a soft bristle toothbrush. The resulting slurry was centrifuged for 5 min at $1700 \times g$. The supernatant was discarded and the pellet re-suspended in a further 50 mL FSW. An additional centrifugation step was introduced to wash the pellet and remove any residual host-generated mucus. This time when the supernatant was discarded, the pellet was resuspended in 1 mL FSW containing 10 μM BCECF and 0.01% Pluronic F-127 with / without 100 μM DCMU and 0.1% acetone depending on the treatment in question, to give a final concentration of approximately 1×10^6 cells mL^{-1} . The dye-loaded cell suspension was then transferred to a 35 mm poly-d-lysine coated petri dish (MatTek Corporation, Ashland, Massachusetts, USA) and placed on the stage of a confocal microscope (LSM 710, Carl Zeiss, Oberkochen, Germany), where the cells were left to settle for 30 min in the dark at 22°C. After dye loading, the cells were carefully washed twice in 1 mL FSW to remove any residual dye without dislodging cells from the surface of the dish. Finally, 6 mL FSW were added and the dish was placed in a closed-exchange live-cell chamber (PeCon, Erbach, Germany) before experimental treatments were carried out. All dyes used for microscopy were purchased from Invitrogen (Grand Island, New York, USA).

3.2.4 *Measurement of pH_i by confocal microscopy*

The fluorescent dye BCECF, in conjunction with confocal microscopy, has been widely used to study pH_i in marine algae (Hervé et al., 2012) as its dual-excitation spectral properties allow fluorescence measurements to be taken that are not compounded by chlorophyll autofluorescence (>640 nm). In this study, confocal microscopy was conducted on a LSM 710 confocal microscope equipped with UV and visible laser lines. Cells loaded with BCECF were sequentially excited, first at 458 nm, then at 488 nm, both with laser strength set at 10% and pinhole set at 1.51

units using an $\times 63$ -fold oil immersion lens. Under both excitations, fluorescence emission was captured at 525 ± 10 nm by imaging the z-stack profile of each cell 10 times in the x/y plane. BCECF is able to enter both the host and the symbiont, and can therefore be used for imaging of both compartments of an intact symbiosis. However, the signal is much stronger in the host cell than in the symbiont, probably due to the thick cell wall that surrounds the symbiont. Therefore, prior optimisation of laser settings was essential to maximise the signal strength in the algae, without overexposing the host cell. *In vivo* calibration was carried out on each partner in the intact symbiosis to yield two separate calibration curves, one for the host [Fig 3.1A] and one for the symbiont [Fig. 3.1B]. The calibration series is dependent on the calculation of the fluorescence intensity ratio (R ; F_{488}/F_{458}) after cells are suspended in buffers of a known pH (pH 6 – 8.5) in the presence of 5 μ M nigericin (Venn et al., 2009). This R value can then be linked to pH_i by the logarithmic equation $pH = pK_a + \log [((R-R_A)/(R_B-R)) \cdot (F_{A(458)}/F_{B(458)})]$, where pK_a represents the acid dissociation constant and A and B represent the acidic and basic endpoints of the titration. To check the level of background fluorescence in the sample, cells were loaded separately with 10 μ M of BCECF-free acid (in the presence of 0.1% DMSO, and 0.01% Pluronic F-127), a membrane-impermeant form of the dye. Background fluorescence was negligible, with no accumulation in either the symbiont or host cells.

Fig. 3.1 Calibration of intracellular pH (pH_i) in (A) host coral cell and (B) *Symbiodinium* cells isolated from the coral *Pocillopora damicornis* using the fluorescent dye BCECF-AM ester. pH_i was calculated from the ratio of fluorescence emission, R (F_{488}/F_{458}). R was then linked to pH_i by the following logarithmic equation: $pH = pK_a + \log [((R-R_A)/(R_B-R)) \cdot (F_{A(458)}/F_{B(458)})]$, where pK_a represents the acid dissociation constant and A and B represent the acidic and basic endpoints of the titration (in this case, 6 and 8.5).

3.2.5 Manipulation of pH_e

pH_e was manipulated via in vitro addition of 99% CO_2 in a fully adjustable CO_2 / temperature controlled live-cell chamber attached to an Axiovert 200 microscope. The amount of CO_2 that was added was controlled using the Zen 2011 software (Carl Zeiss, Oberkochen, Germany), with the pre-defined volume mixed in a CO_2 module and directly injected into the chamber. The final pH_e and the time taken for the pH_e to stabilise were dependent on the amount of CO_2 added [Fig. 3.2]. The 5.0% CO_2 injection setting that produced a gradient of pH_e that spanned one pH unit, and stabilised below pH 7 was selected for this study. This provided an optimal timeframe for monitoring pH, as experiments lasting longer than two hours are often impacted by photo-bleaching or dye-leakage from the cells (Musgrove et al., 1986). The temperature of the microscope stage was maintained at a constant 22°C by a custom-made Plexiglas incubator encasing the entire system.

Fig. 3.2 Stability of the live-cell incubator. Values represent the pH of the surrounding seawater (mean $\pm$ S.E.M, $n = 5$) over time.

3.2.6 Statistics

Actual pH values are provided, and can be visualised in Fig. 3.3. However data were analysed as the relative change in pH_i [Fig. 3.5; the pH_i value after acidification minus the mean pH_i of the control at each time point]. The effect of the symbiotic state (between-subject factor), external pH, and external pH $\times$ symbiotic state (both within subject-factors) were analysed using a repeated measures analysis of variance

(rmANOVA). Initial analysis of the dataset confirmed that there was a significant interaction between external pH $\times$ symbiotic state $\times$ DCMU treatment (rmANOVA, $F_{(15.03, 160.34)} = 4.60$, $p < 0.001$), so subsequent analyses were carried out on the two separate treatments (no DCMU and DCMU added). Likewise *post hoc* analysis was carried out at the treatment level using Bonferroni corrected paired t-test comparisons ($\alpha = 0.001$). The assumptions of normality were confirmed using the Kolgomorov-Smirnov Test. The sphericity of the data was tested using Mauchly's Sphericity Test. Epsilon-adjusted univariate F-test (Greenhouse–Geisser; G–G) values are reported. All data were analysed using JMP 10.0.0 (SAS Institute Inc., USA).

3.3 Results

Initial analysis of the dataset confirmed that there was a significant interaction between external pH $\times$ symbiotic state $\times$ DCMU treatment (rmANOVA, $F_{(15.03, 160.34)} = 4.60$, $p < 0.001$), so subsequent analyses were carried out on the two separate treatments (no DCMU and DCMU added). The response of pH_i to pH_e was dependent on the symbiotic state of the cell in both the presence (rmANOVA, $F_{(14.23, 75.87)} = 7.51$, $p < 0.001$) and absence of DCMU (rmANOVA, $F_{(11.15, 59.44)} = 14.80$, $p < 0.001$). Subsequent *post hoc* analyses revealed where the differences in the response of pH_i to acidification lay [Table 3.1].

Fig. 3.3 Absolute change in the intracellular pH (pH_i) of four cell types isolated from the coral *Pocillopora damicornis* (*Symbiodinium* freshly isolated from host coral cell; isolated non-symbiotic host coral cell; isolated host coral cell and its symbiotic algae), under control (A-B) and CO₂ acidified conditions (C-D) under two treatments (top): in the light without DCMU and (bottom) under light in the presence of the photosynthetic inhibitor DCMU. At each time point, the pH_i (NBS scale) of each cell was measured using confocal microscopy ($n = 5$) and the pH (NBS scale) of the surrounding seawater media was measured via the spectrophotometric m-cresol dye method ($n = 5$). Values plotted represent mean $\pm$ S.E.M. The dashed line represents the pH_e of the surrounding seawater and is plotted on the secondary y-axis.

Fig. 3.4 Light and confocal microscopy images of the pH-sensitive dye BCECF-AM ester, showing the cells, and intracellular pH (pH_i) of cells isolated from *Pocillopora damicornis* after 105 min of exposure to either control pH conditions (left) or increasing CO_2 (right). (Rows A-C) Symbiotic state of the cell: (A) *Symbiodinium* freshly isolated from host coral cell; (B) isolated non-symbiotic host coral cell and (C) isolated host coral cell containing two symbiotic algae. (Column 1) TDI light micrograph; (Columns 2-5) Different treatments: (2) control conditions (pH 7.8) in the absence of DCMU [- DCMU]; (3) control conditions (pH 7.8) in the presence of DCMU [+ DCMU]; (4) increasing CO_2 (pH 6.8) in the absence of DCMU [- DCMU]; (5) increasing CO_2 (pH 6.8) in the presence of DCMU [+ DCMU]. All experiments were conducted under saturating ($400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) PAR. Scale bars represent $5 \mu\text{m}$.

The disparities in pH_i were driven primarily by the opposing reactions of the host coral cells and the algal cells to acidification. Within 15 min of CO_2 addition, the pH_i of both the isolated symbiotic and non-symbiotic host coral cells decreased by approximately 0.4 pH units, irrespective of whether DCMU was present [Fig. 3.5]. Following this initial drop, the response of pH_i to further acidification was dependent on the symbiotic state of the host cell, and the DCMU treatment it was exposed to. There was no change in the pH_i of the non-symbiotic host cell in either treatment [Fig. 3.5B]. Similarly, there was no change in the pH_i of the symbiotic host cell in the presence of DCMU [Fig. 3.5B]. In contrast, without DCMU, the pH_i of the host cell increased over time when in symbiosis with its dinoflagellate partner, returning to control levels within 75 min (when the pH_e reached pH 7). The response of the pH_i of the dinoflagellate symbiont to the addition of CO_2 differed between the DCMU

treatments [Fig. 3.4A; Fig. 3.5], irrespective of whether the alga was in isolation or in symbiosis [Table 3.1]. In the presence of DCMU there was no change in the pH_i of either the isolated or symbiotic algae [Fig. 3.5B], whereas without DCMU both the isolated and symbiotic algae were both able to increase their pH_i relative to control levels [Fig. 3.5A].

Fig. 3.5 The impact of CO_2 addition on the intracellular pH (pH_i) in cells isolated from *Pocillopora damicornis* (*Symbiodinium* freshly isolated from host coral cell; isolated non-symbiotic host coral cell; isolated host coral cell and its symbiotic algae). The figures show the relative change in pH_i after acidification (the pH_i value after acidification minus the mean pH_i of the control at each timepoint $\pm$ S.E.M; $n = 5$) under two treatments (A) saturating white $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR); (B) saturating white $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR in the presence of DCMU. The dashed line plotted on the secondary y-axis represents the pH of the surrounding medium at each time point ($n = 5$).

Table 3.1 *Post hoc* results of paired t-tests following rmANOVA, showing the effect of ‘symbiotic state x external pH’ on intracellular pH (pH_i) at each time-point. Asterisks indicate a significant interaction between the paired cell types (* p < 0.001). Pairs are as follows: 1 = non-symbiotic coral cell *versus* symbiotic coral cell; 2 = non-symbiotic coral cell *versus* isolated algal cell; 3 = non-symbiotic coral cell *versus* symbiotic algal cell; 4 = symbiotic coral cell *versus* isolated algal cell; 5 = symbiotic coral cell *versus* symbiotic algal cell; and 6 = symbiotic algal cell *versus* isolated algal cell. Only Bonferroni corrected significant interactions ($\alpha = 0.001$) are included.

Treatment	Pair	p-value							
		0 min	15 min	30 min	45 min	60 min	75 min	90 min	105 min
Light (- DCMU)	1	-	-	-	-	*	*	*	*
	2	-	*	*	*	*	*	*	*
	3	-	*	*	*	*	*	*	*
	4	-	*	*	*	-	*	*	*
	5	-	*	*	*	*	*	*	*
	6	-	-	-	*	*	-	-	-
Light (+DCMU)	1	-	-	-	-	-	-	-	-
	2	-	-	-	*	*	-	-	*
	3	-	-	-	*	*	-	-	*
	4	-	-	-	*	*	*	*	-
	5	-	-	-	*	*	*	*	*
	6	-	-	-	-	-	-	-	-

3.4 Discussion

Understanding the coral-dinoflagellate symbiosis is pivotal to accurately predicting the susceptibility of coral reefs to climate change and ocean acidification (Weis et al., 2008; Davy et al., 2012). Recent ecological research suggests that the symbiotic state may play a critical role in determining a coral's capacity to tolerate changes in seawater chemistry (Ohki et al., 2013). It is well established that photosynthesis in the dinoflagellate symbiont enhances host coral calcification rates in the light (Allemand et al., 2004). At the cellular level however, a more important role may be the ability of the symbiont to manipulate aspects of the host's physiology, such as cellular pH (Venn et al., 2009; Laurent et al., 2013a). As yet, no study has investigated how this relationship will be impacted by acidification. I addressed this knowledge gap by simultaneously quantifying pH_i in both partners of the symbiosis in response to CO₂

addition. The experimental design exposed isolated cells to a wide range of pH_e , at levels of CO_2 much greater than those predicted to arise from ocean acidification. These treatments were not designed to replicate climate change scenarios, but rather to provide a means of determining how the symbiotic state influences the recovery of its coral host cell under induced cellular acidosis. The results of this study reveal that the photosynthetic activity of the symbiont increases the ability of the host cell to recover from cellular acidosis after exposure to high CO_2 / low pH. The responses seen in the various symbiotic states are summarised in Figure 3.6 below.

Fig. 3.6 Model of intracellular pH (pH_i) fluctuations in cells isolated from *Pocillopora damicornis* after 105 min of exposure to either control pH conditions (left) or increasing CO_2 (right) according to the state of the equilibrium reaction that exists between $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$. (Rows A-C) Symbiotic state of the cell: (A) *Symbiodinium* freshly isolated from host coral cell; (B) isolated non-symbiotic host coral cell and (C) host coral cell containing symbiotic algae. (Columns 1-4) Different treatments: (1) control conditions (pH 7.8) in the absence of the DCMU [- DCMU]; (2) control conditions (pH 7.8) in the presence of DCMU [+ DCMU]; (3) increasing CO_2 (pH 6.8) in the absence of DCMU [- DCMU]; (4) increasing CO_2 (pH 6.8) in the presence of DCMU [+ DCMU]. Dashed arrows show passive CO_2 diffusion. Sunburst symbol represents photosynthetic processes and CA represents carbonic anhydrase. Colours indicate pH as identified in the colour gradient above. With increasing seawater CO_2 there is a shift in carbonate chemistry equilibrium, leading to an accumulation of internal H^+ in the absence of photosynthesis.

CO₂ and H₂O exist in equilibrium with H₂CO₃, so when CO₂ is removed for photosynthesis it causes an increase in the conversion of HCO₃⁻ to H₂CO₃, a process that consumes protons H⁺ (Allemand et al., 1998). Conversely, an increase in CO₂ will reverse the process, resulting in the production of H⁺ and leading to cellular acidosis. The pH_i of freshly isolated *Symbiodinium* cells is therefore strongly influenced by the availability of CO₂ in the surrounding seawater (Nimer et al., 1999). *Symbiodinium* cells are clearly able to increase their pH_i relative to a control, by up to 0.5 pH units [Fig. 3.6A]. This alkalisation demonstrates the capacity of *Symbiodinium* cells to strongly buffer the external environmental pH signal, probably due to a fertilising effect on photosynthesis in these normally CO₂-limited algae (Nimer et al., 1999). An increase in photosynthetic productivity after CO₂ addition has also been observed in other symbiotic associations, most notably in the temperate sea anemones *Anemonia viridis* (Suggett et al., 2012) and *Anthopleura elegantissima* (Towanda and Thuesen, 2012), and the benthic foraminiferan *Marginopora vertebralis* (Uthicke and Fabricius, 2012). The application of DCMU (a photosynthetic inhibitor) reversed the increase in pH_i, confirming that the change was a direct consequence of photosynthesis, as the inhibited photosynthetic machinery of the symbionts is not able to ameliorate the increasing H⁺ concentration [Fig 3.6A].

CO₂ supplementation initiated a markedly different response in the host coral cells compared to the symbiont. In the host cells, acidosis of the cytoplasm was observed within 15 min of CO₂ addition, with a decline in pH_e of 0.2 pH units causing the pH_i to fall 0.4 pH units below the usual pH of the cell, irrespective of the presence or absence of DCMU and the symbiotic state of the cell [Fig 3.6B]. This strongly suggests that the host's intrinsic buffering capacity is, initially at least, overwhelmed by the accumulation of protons resulting from the passive diffusion of CO₂, and active transport of bicarbonate (HCO₃⁻) into the cell (Furla et al., 2000), which drives the aforementioned equilibrium reaction. The decline in pH_i however, was halted after 15 min in both the symbiotic and non-symbiotic host cells. It is likely that this represents a time lag between the onset of acidosis and the activation of the regulatory membrane transporters. Indeed, such activity could explain the subsequent stability (~pH 6.6) of the pH_i in non-symbiotic host cells, which was achieved irrespective of the surrounding seawater being subject to further acidification. Nevertheless, the pH_i of these non-symbiotic host cells never recovered to pre-acidosis levels. In contrast,

the pH_i of symbiotic host cells showed a full recovery to control pH levels within 105 min of CO_2 -addition [Fig 3.6C]. Again, this recovery was negated in the presence of DCMU, confirming that the photosynthetic removal of CO_2 by the symbiont (and hence the consumption of protons) was responsible for the increase in the host's pH_i .

These results confirm that the symbiont is able to exert a significant level of control over its host's cellular pH, corroborating the findings of previous research on reef corals (Laurent et al., 2013a). Furthermore, they demonstrate that the photosynthetic activity of the symbionts plays a key role, at least in the short-term, in regulating the cellular response of their host to external CO_2 -driven acidification. Perhaps more importantly, however, the inability of non-symbiotic or photosynthetically-compromised symbiotic host cells to recover from cellular acidosis in the short-term (at least under the experimental conditions here) suggests that they may be more susceptible than are host cells that contain fully functional *Symbiodinium* cells. However, whether the cells have the potential for recovery in the longer term, especially under constant pH, warrants further investigation. While some caution needs to be exercised when interpreting the results for the non-symbiotic coral cells, as we cannot be sure of their precise origin (i.e. ectodermal or endodermal), it is notable that the symbiotic endodermal host cells responded in the same manner when photosynthesis was inhibited. This limited capacity to withstand acidosis in the absence of a functional symbiont could therefore be a general response. If this is the case, it raises the possibility that the number of *Symbiodinium* cells that a coral host cell contains may also influence the cellular response to acidification. In *Stylophora pistillata*, for example, the majority of host cells (~60%) typically contain one algal cell, while fewer contain two (~35%) or more (<5%) (Houlbrèque et al., 2004). Given that we focused on host cells that harboured two algal symbionts only, there is a need for future studies that clarify the influence of symbiont number on the pH_i , and the overall response at the organismal level.

The regulation of pH_i in corals is an important area for future research as cellular acidosis has serious physiological repercussions for the fitness of the individual. At a biochemical level, acidosis disrupts ion transport, nutrient trafficking and carbon acquisition (Fabry, 2008), causing metabolic suppression (Pörtner et al., 2004) and inevitably leading to shortfalls in the energy available for other cellular processes

(Reipschläger and Pörtner, 1996). In addition to these fundamental cellular attributes, regulating pH_i is particularly important in reef-building corals for calcification. In the recently proposed ‘proton flux hypothesis’, Jokiel (2011) presented the first organism-scale model of acid–base balance in corals (Jokiel, 2011). The hydroxide ions (OH^-) produced as an indirect by-product of CO_2 removal (Furla et al., 2000) are proposed to play a critical role in calcification, neutralising protons released by $\text{H}^+/\text{Ca}^{2+}$ ion exchangers in the gastrovascular cavity, and thus facilitating a pH gradient high enough for the precipitation of calcium carbonate (CaCO_3) (Jokiel, 2011; Comeau et al., 2013). Acidosis of the host coral cells could therefore place further chemical and energetic constraints on corals by affecting their ability to calcify (Jokiel, 2011; Venn et al., 2013). It remains to be seen whether a non-symbiotic host cell can reverse the changes in pH_i over a longer timeframe, though this might depend, in part, on its ability to up-regulate the expression of membrane transporters (Kaniewska et al., 2012), as this will afford greater control over pH_i . Indeed, several organisms are able to reverse initial decreases in pH_i (Michaelidis et al., 2005; Stumpp et al., 2012).

The ability of corals to respond to pH_e change is likely to depend on the response of both the symbionts and coral host (McCulloch et al., 2012). There is considerable diversity within the genus *Symbiodinium* (Pochon and Gates, 2010), which translates into substantial genotypic differences in physiology (Tchernov et al., 2004; Hennige et al., 2009; Brading et al., 2011). This experiment used genetically identical fragments from a single colony, which had two advantages: firstly, it minimised the baseline variation in host cellular activity; and secondly, it reduced the chances of sampling corals that contained very different *Symbiodinium* clades. However, future research should aim to determine whether the host and symbiont responses, and their relative abilities to buffer the effects of CO_2 -driven acidification, vary according to host and symbiont genotype. In addition, it will be important to determine whether the patterns observed in isolated coral cells are replicated in intact coral tissues, and whether longer-term incubations at more moderate levels of acidification (i.e. predicted climate change scenarios) induce similar responses to those reported here. Moreover, the findings here highlight the possibility that bleached corals may be more sensitive to cellular acidosis than are their non-bleached counterparts; this is an especially interesting topic for future research, and so shall form the foundation for the experiments in Chapter 4.

Chapter 4: Species-specific susceptibility to ocean acidification and cellular acidosis in reef corals is influenced by thermal sensitivity

4.1 Introduction

Atmospheric CO₂ levels have been increasing since the Industrial Revolution, but only now is the scale of the threat to marine ecosystems becoming apparent (Pandolfi et al., 2011; Silverman et al., 2012). Rising sea surface temperatures and the declining pH of the world's oceans are driving the global collapse of coral reefs, potentially leading to phase shifts away from calcifying species towards ecosystems dominated by algal- or soft-bodied organisms (Norström et al., 2009; Suggett et al., 2012; Bell et al., 2013; Inoue et al., 2013). Individually, the effects of increasing temperature and declining pH on reef-building corals are becoming well established. Chronic exposure to high temperatures often results in the photosynthetic dysfunction, and subsequent loss of single-celled symbiotic dinoflagellates (genus *Symbiodinium*) and/or their photosynthetic pigments (Fitt et al., 2000). This breakdown in symbiosis, a process termed “coral bleaching” (Weis et al., 2008), has a major impact on the coral's fitness, reducing photosynthetic activity and efficiency (Iglesias-Prieto et al., 1992; Fitt and Warner, 1995; Warner et al., 1996; Jones et al., 2000; Rodolfo-Metalpa et al., 2006), colony growth, reproductive output (Ward et al., 2000; Michalek-Wagner and Willis, 2001; Mendes and Woodley, 2002), and resilience to disease (Selig et al., 2006; Bruno and Selig, 2007; Mydlarz et al., 2010), all of which exacerbate mortality rates (Baird and Marshall, 2002).

There is substantial variation in bleaching susceptibility between (Loya et al., 2001; Marshall and Baird, 2000; McClanahan et al., 2004) and within coral species (Edmunds, 1994; Glynn et al., 2001; Yee et al., 2008). Traditionally these differences in thermal sensitivity have been attributed to the composition of the *Symbiodinium* population (Rowan et al., 1997; Rowan, 2004; Tchernov et al., 2004; Berkelmans and van Oppen, 2006; Ulstrup et al., 2006; Sampayo et al., 2008; van Oppen et al., 2009; Weis, 2010). At a cladal level, corals hosting *Symbiodinium* ITS2 clade D tend to be more resistant to thermal stress than corals of the same species harbouring clade C symbionts (Toller et al., 2001; Rowan, 2004; Tchernov et al., 2004; Berkelmans and

van Oppen, 2006), and so there are disproportionately higher survival rates in clade D-containing corals following bleaching events (Glynn et al., 2001; Fabricius et al., 2004; van Oppen et al., 2005). There is also a great deal in variation in resilience at the sub-cladal level; corals containing types C78, C8/a and C15 for example, are often more tolerant of thermal stress than those hosting C1, C8a, C79, C35/a and C3 (Sampayo et al., 2008; Fitt et al., 2009; Fisher et al., 2012). But, this is not always the case; *Acropora tenuis* juveniles containing ITS2 type D for example, are more susceptible to thermal stress compared with those hosting type C1 (Abrego et al., 2008). So, while it is true that the response of corals to elevated temperature can be influenced by the variation in the thermo-tolerance of different *Symbiodinium* genotypes (Berkelmans and van Oppen, 2006; Robison and Warner, 2006; Suggett et al., 2008; Ragni et al., 2010; Fisher et al., 2012; Howells et al., 2012), it is becoming clear that the host also plays an important part in determining the holobiont's resilience to thermal stress (Baird et al., 2009). At the simplest, anatomical level, a species' skeletal morphology (Kühl et al., 1995; Fabricius et al., 2006; Jimenez et al., 2011; Yost et al., 2013), and tissue pigmentation (Dove et al., 2001; Salih et al., 2006) can heavily influence its sensitivity to elevated temperatures. In addition, the ability to synthesise/acquire mycosporine-like amino acids and the capacity produce antioxidants vary substantially between species (Dunlap and Shick, 1998; Yakovleva et al., 2004; Richier et al., 2005). These differences in thermal sensitivity could have implications for the sensitivity to ocean acidification and cellular acidosis. The photosynthetic activity of *Symbiodinium* plays a key role in regulating the cellular response of its host cell to external CO₂-driven acidification through the removal of CO₂, and hence H⁺. When DCMU was used to block photosynthesis the host cell's ability to recover from cellular acidosis was compromised (Chapter 3). This raises the possibility that thermally sensitive corals (be it through the type of *Symbiodinium* cells that they contain, or through differences in the resilience of the host) may be more prone to cellular acidosis. Short-term acidosis reduces protein synthesis (Hand, 1991) and induces a period of metabolic suppression (Reipschläger and Pörtner, 1996; Langenbuch et al., 2006; Fabry, 2008; Rosa and Seibel, 2008). But, if this state is not reversed, the associated reductions in reproductive output and growth (Pörtner et al., 2004; Havenhand et al., 2008; Wood et al., 2008), will severely impact a corals competitive fitness over longer time-scales.

Here, I take advantage of the differential thermal sensitivities of two coral species - *Pocillopora damicornis*, a branching coral that is highly sensitive to heat stress, and the more robust perforate coral *Montipora capitata* (Jokiel and Brown, 2004; Yost et al., 2013) - to investigate whether a coral's thermal sensitivity can influence its sensitivity to cellular acidosis during exposure to high CO₂/low pH. Specifically, I hypothesised that: (1) bleached corals would be more sensitive to cellular acidosis than their non-bleached counterparts; and (2) the threshold for this would differ between species according to their temperature tolerance.

4.2 Methods

4.2.1 Collection and manipulation of coral fragments

M. capitata and *P. damicornis* colonies were collected from a shallow reef in Kaneohe Bay, Hawaii (21°25'24.60" N, 157°47'40.13" W) in January 2014. Symbiont cells were not genotyped in these corals, but *M. capitata* is typically a flexible generalist, hosting ITS2 types C3, C17, C21, C31, and D1. In contrast, *P. damicornis*, normally hosts C1 and C3 (LaJeunesse et al., 2004; Padilla-Gamiño et al., 2012; Putnam et al., 2012). Pronounced annual and diurnal fluctuations in temperature exist in the bay [Fig. 4.1], so corals directly adjacent to each other were selected to ensure that both species had experienced similar thermal and irradiance histories. Individual coral fragments (~2 × 3 cm, n = 50 per species) were cut, mounted onto 3 × 3 cm plastic tiles using underwater epoxy (Z-spar, Splash Zone compound and placed in a 50-L tank and supplied with ambient flowing seawater for two weeks, a sufficient period of time for tissue to regrow over any skeleton that may have been exposed in the collection process. Four experimental tanks were set up, with each tank set to one of the following temperatures: 23.8 ± 0.3°C (ambient), 25.5 ± 0.4°C, 28 ± 0.8°C and 31 ± 0.3°C. These values were selected to span the annual range of temperatures observed in Kaneohe Bay [Fig. 4.1A] and, in the case of 31°C, provide a treatment high enough to initiate bleaching in *P. damicornis* (Jokiel and Brown, 2004).

Fig. 4.1 Fluctuations in seawater temperature and irradiance from the collection site in Kaneohe Bay, Hawaii. [A] Daily temperature changes in Kaneohe Bay, Hawaii across the period of one year (January - December 2013) and [B] Daily temperature (black line, primary y-axis) and irradiance (grey line, secondary y-axis) collected four days prior to coral collection. Data obtained from NOAA: <http://tidesandcurrents.noaa.gov/>.

Treatment tanks above ambient seawater temperature were heated using aquarium heaters (WON pro-heat IC heaters, Fredericksburg, Virginia, USA) and temperatures were monitored for the duration of the experiment using HOBO data loggers (Onset Corporation, Bourne, MA, USA). Seawater chemistry was uniform across the experimental tanks, with an average weekly salinity of 34.4 ± 0.29 ppt, a pH of 7.88 ± 0.02 (total scale, SOP 6a Dickson et al., 2007) and a TA of 2166.7 ± 10.62 ($\mu\text{mol kg}^{-1}$ sw; SOP 3b Dickson et al., 2007); values represent mean $\pm$ S.E.M ($n = 4$). A period of 12 h light was provided by an actinic LED system (AI Sol, 72 W 100 to 240 VAC / 50-60Hz, C2 Development Inc., Ames, Iowa). The irradiance profile [Fig. 4.2] was designed to mimic that experienced by corals on the reef flat [Fig. 4.1B]. Lights were switched on at sunrise (06:15 h) and ramped for 4 h until an irradiance of $600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was reached. Maximum irradiance was then maintained for 4 h, before being slowly ramped back down to zero by sunset (18:15 h). Four fragments of each species were haphazardly selected and placed in the ambient-temperature experimental tank. After 24 h, three of the four fragments were transferred to the 25.5°C treatment tank, a process that was repeated sequentially over the following two days until one fragment was present in each thermal treatment tank. This ramping technique reduced the likelihood of acute temperature shock. The fragments were left in their final treatment tank for a further four days, so the whole experimental treatment took a week. This whole procedure was repeated five times for each species, to achieve independent replicate fragments (i.e. $n = 5$).

Fig. 4.2 Daily (24 h) irradiance curve. Lights were switched on at sunrise (06:15 h) and ramped for 4 h until an irradiance of $600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was reached. Maximum irradiance was then maintained for 4 h, before being slowly ramped back down to zero by sunset (18:15 h).

4.2.2 *Quantifying the effects of temperature stress on coral fragments*

After imaging the cells (described below), coral fragments were frozen at -20°C ($n = 5$ per species). These samples were subsequently analysed for chlorophyll-*a* (chl-*a*) content and symbiont density, enabling comparisons to be made between the cellular and the physiological response to heat stress. The samples were defrosted, placed on ice and processed in the dark to prevent pigment degradation. Tissue was removed from the coral skeleton by air brushing into 30 mL of 0.22- μM filtered seawater (FSW) and the resulting homogenate was centrifuged for 5 min at $4000 \times g$. Once pelleted, the supernatant was discarded and the residue resuspended in either 10 mL or 3 mL FSW, depending on the apparent density of symbionts present. The slurry was vortexed before 1 mL was removed and frozen at -20°C for subsequent *Symbiodinium* cell counts. The remaining homogenate was re-centrifuged at $4000 \times g$ for 5 min, the supernatant discarded and the pellet dissolved in 3 mL 90% acetone. Samples were transferred to a 4°C fridge and left to extract for 24 h. The samples were centrifuged at $10,000 \times g$ for 3 min after 24 h, to remove any cellular debris. Triplicate aliquots of 300 μL were removed from each sample and transferred to a 96-well plate in preparation for analysis on the spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, California, USA). Absorbance was read at 663 and 630 nm, with chl-*a* concentrations subsequently calculated from the equations of Jeffrey and Humphrey (1975). These values were then normalised to symbiont density and expressed as pg cell^{-1} . Cell counts ($n = 10$ per sample) were conducted using an Improved Neubauer haemocytometer (Boeco, Hamburg, Germany), with values subsequently normalised to coral surface area following the paraffin wax dipping method outlined in Holmes (2008).

4.2.3 *Isolation of cells and quantification of pH_i*

Cells were isolated following the protocol described in Chapter 3. Preliminary experiments determined that an injection of 1.3, 1.2, 1.1 and 1% of CO_2 (99%) was required at 23.8, 25.5, 28 and 31°C , respectively, to produce a uniform 0.3 pH unit decline within 30 min of CO_2 addition to the external seawater (i.e. a pH_e of 7.6). Each sample was maintained on the stage for 30 min, with images collected at the end of the exposure time. This technique was highly reproducible (pH_e 7.59 ± 0.02 , $n =$

40), though for verification, 900 μL seawater were removed at the end of each experiment and pH_e was analysed in triplicate (total scale, $n = 3$) using the m-cresol dye method described in SOP 6B (Dickson et al., 2007). All experiments were conducted at a photosynthesis-saturating irradiance of $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Appendix, Fig. A.2), with white light provided by a variable-irradiance fibre optic cable (Halogen Reflector lamp, 150 W GX5.3 21V 1CT bulb, Philips, Somerset, New Jersey, USA). Both host and symbiont pH_i were determined from the ratio of fluorescence emitted at $525 \pm 10 \text{ nm}$ after sequential excitation using the 458 nm and then the 488 nm laser (R ; F_{458}/F_{488}). BCECF fluorescence itself is influenced by temperature, so calibration curves were produced at each temperature, and the appropriate curve was applied during the image analysis [Fig. 4.3]. All isolation and quantification steps were carried out at the same temperature as the fragment had been previously exposed to, by using the control unit on the confocal microscope stage.

Fig. 4.3 Temperature-sensitive calibration of intracellular pH in (A) host coral cell and (B) symbiont cells isolated from the coral *P. damicornis* using the fluorescent dye BCECF-AM ester. Preliminary experiments determined no difference in the response of the dye between *P. damicornis* and *M. capitata* cells, but did detect a small reduction in the ratio of fluorescence emission, R (F_{488}/F_{458}) at high temperature. As such, we calculated and applied individual calibration curves for each temperature (equations plotted on the graphs above).

4.2.4 Statistical analysis

Symbiodinium cell density and the amount of chl-*a* per cell were analysed by a two-way analysis of variance (ANOVA) with species (two levels) and temperature (four levels) as fixed factors. A three-way ANOVA with species (two levels), cell type (host or symbiont) and temperature (four levels) was used to examine the response of pH_i . All data met the assumptions of normality stated by the Kolgomorov-Smirnov Test and for each dataset, the homogeneity of variance was verified by Levene's test.

4.3 Results

Symbiont density differed between the two coral species, regardless of the temperature treatment ($F_{(1,40)} = 65.310$, $P < 0.001$). Indeed, the mean density of symbionts per cm^2 in *M. capitata* was almost double that of *P. damicornis* in ambient seawater temperatures [Fig. 4.4A]. *P. damicornis* fragments paled markedly after exposure to the highest temperature [Appendix; A.3], losing an average of 70% of their symbionts relative to controls. In contrast, *M. capitata* lost an average of just 7% of their symbionts. Despite this difference, there was no significant effect of temperature or species-temperature interaction on symbiont density [Table 4.1]. High temperatures did, however, cause a significant decline in the chl-*a* content per symbiont cell ($F_{(3,40)} = 4.023$, $P = 0.015$), consistent with pigment degradation as a result of damage to the photosystem II complex, and specifically the oxygen-evolving complex, the thylakoid membranes, and the D1 protein/D1 protein repair mechanism. More precisely, the highest temperature caused a 74% and 15.4% reduction in the cell-specific chl-*a* content in *P. damicornis* and *M. capitata*, respectively (Tukey's HSD, $P = 0.011$), with the response to temperature being significantly different between species ($F_{(1,40)} = 5.804$, $P = 0.022$); again the species-temperature interaction was not significant however [Table 4.1].

The importance of thermal resilience in determining pH_i became apparent under CO_2 -addition. Symbiont and host cells in *P. damicornis* were much more susceptible to cellular acidosis than their counterparts in *M. capitata* [Fig. 4.4C; Table 4.1]. In fact, the magnitude of acidosis was almost double in cells isolated from *P. damicornis*. On average, when incubated at pH_e 7.6, the pH_i of isolated *P. damicornis* host cells was 7.403 ± 0.072 at 23.8°C compared to 6.555 ± 0.025 at 31°C , a decline of 0.85 pH_i units. A similar pattern was observed in the symbiont cell, where pH_i was 7.448 ± 0.023 at 23.8°C versus 6.744 ± 0.012 at 31°C . In contrast, this $\sim 7^\circ\text{C}$ increase in temperature caused a 0.4 pH_i unit decline in *M. capitata* host cells (from pH_i 7.348 ± 0.068 to 6.946 ± 0.045), and had even less of an effect on the pH_i of the symbiont, which dropped 0.3 pH_i units from 7.436 ± 0.043 at 23.8°C to 7.138 ± 0.034 at 31°C .

Fig. 4.4 Effect of temperature on [A] symbiont density (10^6 cells cm^{-2}), [B] chlorophyll-*a* content per symbiont (pg cell^{-1}) and [C] light-adapted intracellular pH (NBS scale) of host endodermal cells (circles) and *Symbiodinium* cells within host cells (triangles) following exposure to CO_2 -addition for half an hour (resulting in a 0.3 pH unit drop in the external pH from 7.9 to 7.6) in two coral species, *Montipora capitata* (black) and *Pocillopora damicornis* (grey). Values represent mean $\pm$ S.E.M for $n = 5$ independent replicates.

Table 4.1 Two-way ANOVA results comparing the density of *Symbiodinium* cells per cm² of coral surface area and the chlorophyll-*a* content per symbiont cell in two species (*Pocillopora damicornis* and *Montipora capitata*) that were exposed to four temperatures (23.8, 25.5, 28, and 31°C), and the results of a three-way ANOVA examining how cell type (host or symbiont), temperature and host species influence intracellular pH. Where appropriate, significant differences were explored further by a Tukey's *post hoc* test. Asterisks denote significant interactions (* $p < 0.05$, ** $p < 0.001$).

Variable	Analysis	Interaction term	F-value	P-value	Tukey's test
<i>Symbiodinium</i> cells cm ⁻²	Two-way ANOVA	Species	$F_{1,32} = 65.310$	$< 0.001^{**}$	
		Temperature	$F_{3,32} = 1.849$	0.158	
		Species × Temperature	$F_{3,32} = 1.634$	0.201	
Chlorophyll- <i>a</i> pg cell ⁻¹	Two-way ANOVA	Species	$F_{1,32} = 5.804$	0.022*	
		Temperature	$F_{3,32} = 4.023$	0.015*	23.8 = 25.5 = 28 > 31.5 °C
		Species × Temperature	$F_{3,32} = 1.832$	0.161	
Intracellular pH	Three-way ANOVA	Species	$F_{1,64} = 21.507$	$< 0.001^{**}$	
		Cell type	$F_{1,64} = 15.662$	$< 0.001^{**}$	
		Temperature	$F_{3,64} = 105.569$	$< 0.001^{**}$	23.8 > 25.5 > 28 > 31.5 °C
		Species × Cell type	$F_{1,64} = 0.171$	0.680	
		Species × Temperature	$F_{3,64} = 18.311$	$< 0.001^{**}$	
		Cell type × Temperature	$F_{3,64} = 1.910$	0.137	
		Species × Cell type × Temperature	$F_{3,64} = 0.372$	0.773	

4.4 Discussion

The goal of this study was to induce thermal stress in corals with differing bleaching susceptibilities, to measure how reduced photosynthetic performance impacts the pH_i of both symbiont and host cells. The results of this study suggest that there is a clear link between temperature and sensitivity to cellular acidosis under acidification. Here, I argue that the pH_i response observed under the high temperature treatment results from photosynthetic dysfunction in the symbiont, primarily by photo-inhibition and/or the degradation of chlorophyll-*a*.

The mechanism involved in regulating pH_i under CO_2 -addition is likely to be similar to that described in Chapter 3, regardless of the temperature that the coral fragment was exposed to. Briefly, acidosis is likely to arise from the passive diffusion of CO_2 into the cell. Once in the cytoplasm, CO_2 dissociates into the intermediate compound carbonic acid (H_2CO_3), and then further into bicarbonate (HCO_3^-) and H^+ , so creating a more acidic environment. Under ‘normal’ temperature conditions, photosynthesis removes some of the CO_2 , keeping the pH_i constant or at least mitigating the influx of H^+ (Furla et al., 2000). Reductions in cell-specific chl-*a* content and/or damage to PSII, however, reduce the activity and efficiency of photosynthesis (Iglesias-Prieto et al., 1992; Fitt and Warner, 1995; Warner et al., 1996; Jones et al., 2000; Rodolfo-Metalpa et al., 2006), thus preventing the removal of CO_2 and hence H^+ , increasing the potential for cellular acidosis. The physiological responses of the two corals were markedly different, with a significant reduction in the symbiont cell-specific chl-*a* content observed in *P. damicornis* but not in *M. capitata*, which could explain why cells isolated from the thermally sensitive *P. damicornis* showed less capacity to buffer against acidification than their counterparts in *M. capitata*. The degradation of algal pigments reflects damage to the photosystem II complex (Takahashi et al., 2004; Lesser 2011; Hill et al., 2012; Bhagooli, 2013), specifically affecting the oxygen-evolving complex (Becker et al., 1990), the thylakoid membranes (Tchernov et al., 2004), and the D1 protein/D1 protein repair mechanism (Warner et al., 1999; Takahashi et al., 2004). Pigmentation loss and the expulsion of algal cells are not mutually exclusive in the bleaching response; a reduction in chlorophyll content can occur with or without a reduction in algal density (Hoegh-Guldberg and Smith, 1989;

Kleppel et al., 1989; Porter et al., 1989; Lesser et al., 1990), but the outcome for pH_i is the same; anything that disrupts the removal of CO_2 will reduce a cell's ability to buffer changes in external pH (pH_e).

The impact of thermal stress on pH_i may not only be related to the removal of CO_2 and H^+ through photosynthesis, however, as a cell's capacity to regulate its pH_i may be directly impacted by elevated temperature. Cellular acidosis is a common response to thermal stress in non-photosynthetic organisms, and is strongly linked to both oxidative stress and metabolic activity (Pörtner et al., 1999; Sartoris et al., 2003; Strobel et al., 2013). For example, exposure to temperatures 4°C above control levels caused a decline in pH_i of 0.2 pH units in the mantle tissue of the bivalve *Limopsis marionensis* (Pörtner et al., 1999), while pH_i in the gill arches of the Atlantic cod *Gadus morhua* decreased by 0.025 pH units per $^\circ\text{C}$ increase (Sartoris et al., 2003). There may be parallels between the responses observed in these studies and the acidosis observed here in coral cells, as oxidative stress and the increased formation of ROS are key components of coral bleaching (Lesser, 2006), causing significant damage to homeostatic mechanisms such as acid-base regulation (Strobel et al., 2013), non-photochemical quenching (Gorbunov et al., 2001) and antioxidant defense (Császár et al., 2009). Almost all cellular processes are up-regulated under thermal stress in accordance with Michaelis-Menten kinetics, leading to a shift in metabolic activity and an increase in ATP-demand (Lesser et al., 1997; DeSalvo et al., 2008; Seneca et al., 2010). Given this, the energy gained from photosynthesis in corals could play an important role in enabling both the host and symbiont to regulate their pH_i and counter changes in the pH_e . Indeed, under 'normal' conditions, the energy required to regulate pH_i relative to the energy gained from photosynthesis is minor; buffering a 0.4 pH_e unit decrease for example, is estimated to cost between $1\text{--}2 \text{ kJ mol}^{-1} \text{ C}$, just a fraction of the $\sim 475 \text{ kJ mol}^{-1} \text{ C}$ produced during photosynthesis (McCulloch et al., 2012). Amid the photosynthetic dysfunction and physiological disorder caused by thermal stress in corals, however, meeting the additional energy demand is likely to be far more problematic.

The mechanism(s) described here could partly explain why interactions between the two stressors are antagonistic – i.e. elevated temperatures compound the effect of

ocean acidification (Anthony et al., 2008; Martin and Gattuso, 2009; Anlauf et al., 2011; Rodolfo-Metalpa et al., 2011; Gao et al., 2012; Byrne and Przeslawski, 2013). Such an inter-relationship has been reported previously for corals and other phototrophic organisms (Hendriks et al., 2010; Kroeker et al., 2010; Kroeker et al., 2013). For example, necrosis in the crustose coralline alga *Lithophyllum cabiochae* was initiated by exposure to high temperature but not CO₂-addition; the cumulative effect of both, however, was a two- to threefold increase in mortality (Martin and Gattuso, 2009). A similar relationship has been observed in both adult and juvenile corals (Manzello, 2010; Anlauf et al., 2011; Rodolfo-Metalpa et al., 2011), though not in all species (e.g. the coral *Cladocora caespitosa*) (Rodolfo-Metalpa et al., 2010). At a community level, the two stressors are likely to reduce the resilience of the reef, leading to a reduction in coral cover, structural complexity and biodiversity (Jokiel et al., 2008; Anthony et al., 2011; Hale et al., 2011). Future work should incorporate corals from across the thermal-tolerance spectrum, to clarify the role of thermal resilience in determining the response to CO₂. Ideally, this research would encompass a range of coral morphologies and *Symbiodinium* genotypes, both of which can influence thermal sensitivity (Rowan et al., 1997; Rowan, 2004; Jimenez et al., 2008; van Oppen et al., 2009; Fabricius et al., 2006; Sampayo et al., 2008; Jimenez et al., 2011). Furthermore, longer-term experiments are needed to test for recovery from and adaptation to the environmental changes applied in this study. Together, these approaches will better enable the modeling and prediction of how coral reefs will respond to future, combined warming and acidification scenarios.

Chapter 5: The photo-physiological response of a model cnidarian-dinoflagellate symbiosis to CO₂-induced acidification at the cellular level

5.1 Introduction

At 380 ppm, present-day atmospheric CO₂ concentrations are higher than at any point in the past 740,000 years (Hoegh-Guldberg et al., 2007), and are predicted to reach 500–1,000 ppm by 2100 (Vuuren et al., 2011). It is estimated that approximately one third of all anthropogenic CO₂ is sequestered by the oceans (Sabine et al., 2004). This continuous dissolution of CO₂ lowers the pH of seawater and alters the speciation of dissolved inorganic carbon (DIC), in a process known as ocean acidification. Such changes are of great concern for marine ecosystems, but are particularly pertinent for those in which calcifying organisms play a major role (Fabry, 2008). Coral reefs, therefore, which are recognised for their high diversity and productivity (Bellwood and Hughes, 2001), have received considerable attention (Hall-Spencer et al., 2008; Fabricius et al., 2011; Crook et al., 2012). Predicting the response of reef-building corals to ocean acidification is complicated by the presence of the single-celled photosynthetic dinoflagellates (genus *Symbiodinium*) that they harbour within their gastrodermal tissue layer. The photosynthetic products that are supplied by these dinoflagellate symbionts are of great metabolic importance to their host, facilitating growth, reproduction and, ultimately, survival (Muscatine, 1990). Therefore, the adaptive capacity of corals is not only determined by the responses of the host but also those of the symbionts it contains (McCulloch et al., 2012). Understanding the cellular mechanisms that underpin the complex inter-relationship between the symbiont and host is a vital step towards understanding why the physiological response to CO₂-addition differs so substantially between species (Anthony et al., 2008; Ries et al., 2009; Kroeker et al., 2010; Pandolfi et al., 2011). Of specific interest is characterising the response of intracellular (pH_i) to ocean acidification (Schneider and Erez, 2006; Brownlee, 2009).

Acid-base regulation is an essential facet of cellular metabolism in all organisms (Busa and Nuccitelli, 1984), but is particularly important for symbiotic anthozoan cells that are subjected to significant fluctuations in pH_i through the photosynthetic

and respiratory activity of their symbionts (Venn et al., 2009; Laurent et al., 2013a). Deviation from the optimal pH_i , a process known as acidosis, often leads to cellular dysfunction (Pörtner et al., 2004), and while short-term acidosis can typically be recovered from (Fabry, 2008), longer periods of exposure can result in metabolic depression (Reipschläger and Pörtner, 1996) and reduced rates of protein synthesis (Hand, 1991), which are detrimental to fitness. Little is currently known about how the cnidarian-dinoflagellate symbiosis might respond to CO_2 -induced acidification at the cellular level. Indeed, there has been just one study to focus on this, in which short-term experimental perfusions (< 2 h) of isolated, symbiotic gastrodermal cells from the sea anemone *A. viridis* in HCl-acidified seawater (Laurent et al., 2013b). The results of this study revealed no detrimental effects on the pH_i of the host cell. On the contrary, there is growing evidence that increased CO_2 availability associated with ocean acidification could enhance primary productivity in *Symbiodinium* (Marubini et al., 2008; Brading et al., 2011; Suggett et al., 2012; Towanda and Thuesen, 2012; Jarrold et al., 2013), which could limit the potential for host cell acidosis (Chapter 3). This photosynthetically driven up-regulation of pH_i under high CO_2 has been postulated as one potential mechanism for countering the negative impacts of acidification on coral calcification (Ries et al., 2009). Yet, to date, no study has examined the longer-term impacts of CO_2 -addition on both host and symbiont pH_i in the cnidarian-dinoflagellate symbiosis. This is an essential step if we are to fully appreciate the impacts that ocean acidification might have on this ecologically important association.

In this chapter, I investigate the relationship between photo-physiology and pH_i under long-term CO_2 addition. To achieve this, the sea anemone *Aiptasia pulchella* a model system for the study of cnidarian-dinoflagellate symbiosis (Weis et al., 2008), was exposed to conditions representative of the intermediate (520–700 ppm) and high-end (1000–1300 ppm) CO_2 stabilisation scenarios (IPCC, 2014), over a two-month period. A suite of physiological responses were quantified at regular intervals: maximum dark-adapted fluorescent yield of PSII (F_v/F_m), gross photosynthetic rate, respiration rate, symbiont population density, and pH_i of both the dinoflagellate symbiont and host anemone. This study tested the prediction that, rather than causing cellular acidosis, the addition of CO_2 would enhance photosynthetic performance, enabling both the host-symbiont association to withstand future ocean acidification scenarios.

5.2 Methods

5.2.1 Experimental organisms

A long-term aquarium stock of the sea anemone *A. pulchella*, originating from an unknown location in the Pacific, was grown at 25 °C under a 12 h light:12 h dark cycle, with 80–100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided by cool white fluorescent lamps (Osram Dulux 36W/890 fluorescent bulbs), and fed freshly-hatched *Artemia* sp. nauplii every 3 days. These anemones contain *Symbiodinium* ITS2 type B1 (Chapter 2). One month prior to experimentation, 300 anemones (oral disc ~1 cm diameter) were randomly selected and transferred to 2-L glass beakers (20 individuals per beaker) containing 1 μm filtered seawater. The beakers were split evenly between three water baths (i.e. $n = 5$ independent beakers per water bath), under the same light and temperature conditions described above. During this acclimation period, water was changed daily to ensure that pH (8.1 ± 0.1) and salinity (35.5 ± 0.1 ppt) were kept constant. Nutrient concentrations were not monitored during the experiment, but levels are typically low (dissolved inorganic nitrogen (DIN) levels are approximately $\sim 2 \mu\text{M}$, NH_3 and $\text{NO}_3^- \sim 1 \mu\text{M}$, NO_2^- and $\text{PO}_4^{3-} < 1 \mu\text{M}$), and relatively stable in the coastal waters around Wellington, New Zealand, from the seawater collection point (Dudley and Shima, 2010). Tanks were cleaned weekly and water temperatures were monitored using HOBO data loggers (Onset Corporation, Bourne, MA, USA).

5.2.2 Experimental design

Anemones were exposed to three pH treatments over a two-month period: 8.14 ± 0.01 , 7.83 ± 0.01 and 7.54 ± 0.02 [Table 5.1]. These values correspond to $p\text{CO}_2$ concentrations of $289.94 \pm 12.54 \mu\text{atm}$, $687.40 \pm 25.10 \mu\text{atm}$ and $1459.92 \pm 65.51 \mu\text{atm}$, respectively. Photosynthesis caused the seawater pH to fluctuate by ~ 0.1 pH unit over 24 hours, so water changes were performed daily. Addition of gaseous CO_2 to seawater was performed in a 50-L black plastic reservoir, via a CO_2 solenoid valve connected to a PINPOINT pH controller (American Marine Inc., Ridgefield, CT, USA). The final pH of the seawater was checked with an independent pH probe, adjusted accordingly if needed, and recorded before being added to the beakers. In addition to this, pH readings were taken daily before the water change, to provide a

full record of the pH experienced in each beaker [Fig. 5.1]. Anemones were haphazardly selected for sampling at 0, 1, 2, 4, 6 and 8 weeks. Not all of the anemones placed in each beaker were sampled during the experiment. At each time-point, two anemones were removed from each beaker. One was used to quantify symbiont pH_i , in addition to a suite of photo-physiological parameters (described below), and the other was used to determine the host cell pH_i .

Table 5.1 Summary of the carbonate chemistry of the control and experimental pH treatments. pH and temperature were measured directly, with total alkalinity (TA) measurements provided by the National Institute of Water and Atmospheric Research (NIWA), NZ. The remaining carbon parameters were estimated using the program CO2SYS (Lewis and Wallace, 1998). Values represent daily mean $\pm$ S.E.M ($n = 60$).

pH (NBS-Scale)	Temperature (°C)	TA ($\mu\text{mol kg}^{-1}$)	pCO_2 (μatm)	HCO_3 ($\mu\text{mol kg}^{-1}$)
8.14 ± 0.02	25.46 ± 0.07	2258.81 ± 4.06	289.94 ± 1.77	1648.40 ± 2.52
7.84 ± 0.02	25.78 ± 0.06	2258.81 ± 4.06	687.40 ± 3.55	1909.89 ± 1.58
7.57 ± 0.02	25.84 ± 0.06	2258.81 ± 4.06	1459.92 ± 9.27	2066.22 ± 1.18

Fig. 5.1 Record of the pH changes in each beaker for the duration of the two-month experiment. These values represent the mean seawater $\text{pH} \pm$ S.E.M ($n = 5$ per treatment, $n = 3$ treatments), upon addition to and 24-h after addition to the experimental beakers.

5.2.3 Photo-physiological responses

Maximum dark-adapted yield of PSII (F_v/F_m) of each anemone was measured 30 min before the lights were switched on, using pulse amplitude modulated fluorometry (Diving-PAM, Walz, Effeltrich, Germany; settings: measuring light = 8, saturation intensity = 8, saturation width = 0.8 s, gain = 5 and damping = 2). Triplicate measurements were performed for each anemone, with readings taken 1 cm immediately above the oral disc, and 45 degrees either side of this point. Anemones were then placed singly with 0.22 μm filtered seawater (FSW) into a 10-mL glass chamber, and left to settle for an hour; this was sufficient time for attachment to a thin nylon mesh grid that overlaid a magnetic spin-bar. A glass lid, held in place by a rubber O-ring, sealed the chamber, into which a temperature probe and an oxygen electrode (FIBOX 3 – fiber-optic oxygen meter, PreSens, GmbH, Germany) were inserted such that both probes were positioned ~ 1 cm above the anemone's oral disc. The chamber was then wrapped in aluminium foil to ensure that no light could enter and placed on top of a submersible magnetic stirrer, itself placed in a transparent water bath set to 25°C. Respiratory oxygen consumption (R ; $\text{mL O}_2 \text{ h}^{-1}$) was measured every 10 sec in darkness until a constant rate was observed (normally after ~ 30 min). The foil was then removed and the chamber illuminated at 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ by a 150 W sealed beam PAR38 lamp (Thorn Lighting Ltd., Durham, UK). This light level was chosen because it is well above the photosynthesis-saturating irradiance for *A. pulchella*, which is typically between 200 and 250 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Muller-Parker, 1984) but is not high enough to cause photoinhibition, which usually begins at irradiances greater than 600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in this anemone (Lesser and Shick, 1989). Net photosynthetic oxygen evolution (P_{net}) was then measured until a constant rate was observed. Gross photosynthesis (P_{gross}) was calculated by adding P_{net} to R , and the resulting values were used to determine the $P_{\text{gross}}:R$ ratio for each anemone on an idealized day (assuming 12 h light and 12 h dark), a proxy for autotrophic potential. It was assumed that R in the light was the same as in the dark (i.e. total daily respiration = $R \times 24$ h). After the O_2 -flux measurements were complete, each anemone was then placed in a 1.5-mL microcentrifuge tube and homogenised in 1.1 mL FSW using a plastic tissue grinder. From the resulting slurry, a 100 μL aliquot was removed, and frozen at -20°C. This

was later used to determine symbiont density ($n = 6$ counts per sample) using an Improved Neubauer haemocytometer (Boeco, Hamburg, Germany). The remaining 1 mL was centrifuged for 5 min at $1,000 \times g$ to separate the algal fraction from host tissue. Once isolated, the host supernatant was decanted and stored at -20°C prior to analysis of the host protein content, using the Bradford assay (Bradford, 1976) and bovine serum albumin (BSA) as the standard. These values were subsequently used to normalise P_{gross} , R and symbiont density values. The remaining algal pellet was re-suspended in FSW at a final density of 1×10^6 cells mL^{-1} , for analysis of symbiont pH_i (see below). Light-adapted symbiont pH_i and light-adapted host cell pH_i were quantified according to the protocol described in Chapter 2.

5.2.4 Statistics

Repeated measures analysis of variance (rmANOVA) was used to test the response of each variable to pH over time. *Post hoc* analyses represent Bonferroni-corrected pairwise comparisons of estimated marginal means between each of the two enhanced- CO_2 treatments and the ambient CO_2 control at a particular time-point. Epsilon-adjusted Greenhouse-Geisser (G-G) values are reported whenever Mauchly's Sphericity Test returned a significant result. Linear regression analyses were performed to evaluate the relationship between symbiont and host pH_i . Data were examined for normality prior to parametric analysis using the Shapiro-Wilk Test, and were transformed where necessary to satisfy these requirements. All data were analysed using the PASW Statistics 20.0 package (IBM, Armonk, NY, USA).

5.3 Results

5.3.1 Photo-physiological responses to CO_2 addition

In general, the addition of CO_2 caused a significant increase in all but one of the photo-physiological parameters measured (P_{gross} : R ratio) [Table 5.2]. Typically these increases were detected within 2-3 weeks of CO_2 addition, and in most cases the increased response appeared to be proportional to the amount of CO_2 added [Fig. 5.2]. However, in some cases, the full treatment effect was not fully apparent until week 8.

Table 5.2 Statistical analysis of the response variables in *Aiptasia pulchella* when exposed to different pH treatments for two months. The F-statistic represents the overall rmANOVA interaction (time $\times$ pH treatment) for each parameter and asterisks denote statistical significance at * $p < 0.05$ or ** $p < 0.001$.

Response variable	F-statistic	P-value
Symbiont density ($\times 10^6$ cells mg^{-1} protein)	$F_{10, 60} = 5.006$	$<0.001^{**}$
Max. Quantum yield (F_v/F_m)	$F_{10, 60} = 2.088$	0.039^*
P_{gross} ($\mu\text{g O}_2 \text{ mg}^{-1}$ protein h^{-1})	$F_{3.90, 23.403} = 11.181$	$<0.001^{**}$
P_{gross} ($\text{pg O}_2 \text{ cell}^{-1} \text{ h}^{-1}$)	$F_{10, 60} = 2.204$	0.030^*
R ($\mu\text{g O}_2 \text{ mg}^{-1}$ protein h^{-1})	$F_{10, 60} = 2.191$	0.031^*
$P_{\text{gross}}: R$	$F_{4.957, 29.745} = 0.597$	0.701
Symbiont pH_i	$F_{10, 60} = 3.403$	0.001^*
Host cell pH_i	$F_{10, 60} = 3.436$	0.001^*

Moderate increases were observed from week 2 onwards in the intermediate treatment and densities were 32% higher than in the control by the end of the experiment [Fig. 5.2A]. High CO_2 addition induced a greater response, with a 54% increase evident by week 2, increasing to 81% by the end of the experiment. Maximum dark-adapted yield (F_v/F_m) showed no response to the intermediate CO_2 treatment but under high CO_2 there was a significant increase in F_v/F_m relative to both the control and the intermediate treatment at weeks 1, 6 and 8 [Fig. 5.2B].

The response of total maximum gross photosynthetic rate (P_{gross}) to CO_2 addition was pronounced [Fig. 5.2C; Table 5.2]. Under intermediate CO_2 addition, a two-fold increase in P_{gross} was evident by week 2, which remained more or less constant thereafter. In comparison, exposure to high CO_2 induced a 2.5-fold increase in P_{gross} after two weeks, and an almost 4-fold increase from week 6 onwards. These patterns were similar when P_{gross} was expressed on a cell-specific basis [Fig. 5.2D]. Over the duration of the experiment, cell-specific photosynthetic rate increased by 81.6% and 114% in the intermediate and high CO_2 treatments, respectively. Total respiration (R) also responded to the addition of CO_2 [Fig. 5.2E; Table 5.2]. R tended to increase in the intermediate CO_2 treatment from at least week 2 onwards, but this increase was not significant. In contrast, there was a significant, two-fold increase in R from week 2 onwards in the high CO_2 treatment. Given the more marked influence of CO_2

addition on total photosynthesis than respiration, the ratio of P_{gross} to R increased, though variability in this parameter masked any statistical significance [Fig. 5.2F; Table 5.2].

Fig. 5.2 Photo-physiological responses observed in *Aiptasia pulchella* after exposure to pH 8.1, pH 7.8 and pH 7.5 over two months. Response variables measured are as follows: [A] symbiont cell density ($\times 10^6$ cells mg^{-1} protein); [B] maximum quantum yield of PSII (F_v/F_m); [C] maximum rate of gross photosynthesis (P_{gross} , $\mu\text{g O}_2 \text{ mg}^{-1} \text{ protein h}^{-1}$); [D] maximum rate of gross photosynthesis per *Symbiodinium* cell ($\text{pg O}_2 \text{ cell}^{-1} \text{ h}^{-1}$); [E] maximum respiration rate ($\mu\text{g O}_2 \text{ mg}^{-1} \text{ protein h}^{-1}$); [F] ratio of the maximum rate of gross photosynthesis to respiration ($P_{\text{gross}}:R$) for an idealized day (12 h light and 12 h dark); values above the line denote a potentially autotrophic symbiotic association where $P_{\text{gross}}:R > 1$. Data represent means $\pm$ S.E.M for $n = 5$ individuals per time-point, and asterisks indicate significant differences between elevated CO₂ treatments relative to the control at each time point (rmANOVA, pairwise post hoc with Bonferroni correction, * $p < 0.05$, ** $p < 0.001$).

5.3.2 Cellular pH_i response to CO_2 addition

The light-adapted pH_i of both the symbiont [Fig. 5.3A] and host [Fig. 5.3B] was significantly modified *via* the addition of CO_2 [Table 5.2]. Symbiont cells isolated from anemones exposed to the highest CO_2 treatment showed the most rapid response, with pH_i increasing ~ 0.2 pH units, from 7.25 ± 0.05 to 7.46 ± 0.06 , within a week, and stabilising at this level for the remainder of the experiment. The pH_i of symbiont cells exposed to intermediate CO_2 levels showed the largest increase between weeks 1 and 4, when it reached 7.37 ± 0.09 ; the pH_i remained at a similar level for the remainder of the experiment.

There were discernible differences between the response of host pH_i and symbiont pH_i under acidification [Fig. 5.3]. Interestingly, exposure to intermediate CO_2 had no effect on host cell pH_i [Fig. 5.3B], with mean values comparable to those observed in the control anemones (7.19 ± 0.01 *versus* 7.17 ± 0.01) throughout the experiment. In stark contrast, the pH_i of host cells exposed to high CO_2 increased by almost 0.3 pH units within a week, rising from 7.19 ± 0.03 to 7.48 ± 0.02 , and remained relatively constant thereafter. Linear regression showed a strong predictive relationship between symbiont and host pH_i ($p < 0.001$), and suggests that a 0.1 pH unit increase in symbiont pH_i is associated with a 0.63 pH unit increase in host pH_i [Fig. 5.3C].

Fig. 5.3 The response of intracellular pH (pH_i) to CO_2 -driven acidification. [A] *Symbiodinium* and [B] symbiotic *Aiptasia pulchella* cells at normal CO_2 levels (pH 8.1), and IPCC CO_2 stabilisation scenarios IV (pH 7.8) and VI (pH 7.5) over a two-month time-frame. [C] The linear relationship between host and symbiont pH_i . [D] Schematic to explain host cell pH_i fluctuations as a function of gross photosynthesis (P_{gross}) and total symbiosis respiration (R). Host cell is represented by the rectangle, and symbiont is represented by the dotted oval. From clockwise: if $P_{gross} < R$ then host pH_i decreases; if $P_{gross} > R$ then host pH_i increases; and if both P_{gross} and R either both increase or decrease in proportion to one another then there is no change in pH_i . Data represent means $\pm$ S.E.M for $n = 5$ individuals per time-point and asterisks indicate significant differences between elevated CO_2 treatments relative to the control at each time point (rmANOVA, pairwise *post hoc* with Bonferroni correction, * $p < 0.05$, ** $p < 0.001$).

Fig. 5.4 Confocal microscope images of symbiotic endodermal cells isolated from *Aiptasia pulchella* and loaded with the pH-sensitive SNARF-4F fluorescent dye. (Columns A-C) CO₂ treatment: (A) pH 8.1, (B) pH 7.8 and (C) pH 7.5. (Rows 1-2) Time of exposure to CO₂ treatment: (1) before exposure to treatment and (2) after two months of exposure to each treatment. Colours indicate light-adapted (400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR) pH_i in the host cell as identified in the colour gradient above. White triangle on image 1A indicates the region of interest in the host cytoplasm used for pH measurements and scale bar represents 10 μm . Red represents chlorophyll autofluorescence in the *Symbiodinium* cells and is not related to pH_i. Similarly yellow fluorescence in the algal cell is not pH-related and is likely to denote the position of the pyrenoid. Note the increase in alkalinity after two months of exposure to the highest CO₂ treatment, represented by the shift from yellow to orange in the host cell.

5.4 Discussion

Ocean acidification - once referred to as “the other CO₂ problem” (Doney et al., 2009) - is now recognised as a major selective force in determining ecosystem structure and function (Hall-Spencer et al., 2008; Christen et al., 2013; Godbold and Calosi, 2013). Marine organisms exhibit high variability in their physiological and cellular tolerance to CO₂ addition (Fabry, 2008). While considered detrimental on the whole for calcifying organisms (Hoegh-Guldberg et al., 2007; Marubini et al., 2008; Kroeker et al., 2013), there is growing evidence that enhanced CO₂ levels may benefit soft-bodied photosynthetic anthozoans (Suggett et al., 2012; Towanda and Thuesen, 2012; Inoue et al., 2013) through the alleviation of carbon limitation, that is thought typical of the symbiont population (Weis, 1993; Davy and Cook, 2001). The increased photosynthetic production that arises as a consequence of this likely has two major

advantages for the host: (1) increased translocation of photosynthate for growth and reproduction (Brownlee, 2009); and (2) the prevention of cellular acidosis that arises from increased concentrations of CO₂ and hence protons. The results of this chapter lend support to the potential success of non-calcifying symbiotic cnidarians in a CO₂-enriched world, and confirm the positive relationship between CO₂ supply, photosynthesis and the prevention of cellular acidosis in the model symbiotic cnidarian, *A. pulchella*.

Increases in symbiont density, photosynthetic health (F_v/F_m), overall and cell-specific gross photosynthetic rate (P_{gross}), and respiration rate (R) were commensurate with the CO₂ concentration, signifying enhanced primary productivity and hence symbiont growth in response to the increased availability of dissolved inorganic carbon (DIC). Similar findings were reported for two temperate sea anemone species exposed to elevated CO₂. *Anemonia viridis* sampled from high CO₂ sites (corresponding to 1428 μ atm) along a naturally occurring CO₂ vent, contained up to four-times more *Symbiodinium* cells than when at control sites (300 μ atm), resulting in enhanced rates of both P_{gross} and R ; though, as in the current study, P_{gross} increased more than R (Suggett et al., 2012). These patterns were mirrored in *Anthopleura elegantissima* after six weeks of incubation at pH 7.3, although there was no accompanying change in symbiont density (Towanda and Thuesen, 2012). The increased primary productivity seen here in *A. pulchella* is unsurprising given that seawater (pH 8.2) contains less than half of the DIC (2.2 mM HCO₃⁻, 30 μ M CO₂) required to saturate photosynthesis in this species under normal circumstances (5 mM DIC; Weis, 1993). Nevertheless, *A. pulchella* can still achieve its autotrophic potential (i.e. $P_{gross}: R > 1$) with respect to carbon (Starzak et al., 2014), in part because of the activity of carbon-concentrating mechanisms (CCM's) that concentrate CO₂ at the site of photosynthesis (Weis et al., 1989; Leggat et al., 1999). CO₂ enrichment may therefore provide a further benefit through reducing the metabolic costs of maintaining CCM activity (Raven et al., 2012).

However, not all studies report increases in anemone productivity after CO₂ addition. Six weeks of exposure to pH 7.6 had no effect on symbiont density or physiological performance in the temperate anemone *Anthopleura aureoradiata* (Doherty, 2009). Similarly, two weeks of exposure to pH 7.4 caused declines in symbiont density, but

did not affect either P_{gross} or R in laboratory-maintained *A. viridis* (Jarrold et al., 2013); though our results suggest that a two-week time period may not always be long enough to observe significant changes in physiology. Differences in the organismal response to CO_2 addition are potentially attributable to the type of *Symbiodinium* present and/or the amount light available for photosynthesis (Suggett et al., 2013; Comeau et al., 2014). There is certainly growing evidence for the former, with research suggesting that the response to CO_2 -addition may be type-specific (Buxton et al., 2009; Brading et al., 2011). For example, when a range of cultured *Symbiodinium* types were exposed to a two-fold increase in CO_2 concentration (corresponding to pH 7.79 – the intermediate treatment in our study), there was an increase of ~60% in the growth rate and photosynthetic capacity of types A13 and A2, respectively, while types A1 and B1 did not respond at all (Brading et al., 2011). Of note, this lack of response by *Symbiodinium* B1 in culture is different to our observations here, when this same type was *in hospite*, so future studies should consider how CO_2 concentration may influence different host-symbiont combinations.

One of the key objectives of this study was to establish the response of pH_i to CO_2 -addition, simultaneously in the host and symbiont. To achieve this, I measured the pH_i of both partners at photosynthesis-saturating light levels ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The light level chosen is important because irradiance can modify the response of cnidarians to CO_2 -driven acidification (Suggett et al., 2013; Comeau et al., 2014). The values measured in the control anemones were similar to those measured in Chapter 2 (*Symbiodinium* types ranged from pH 7.21–7.39, while the host and symbiont cells isolated from *A. pulchella* were $\text{pH } 7.02 \pm 0.06$ and 7.25 ± 0.01 , respectively). Light-adapted host pH_i in this anemone species was, however, slightly lower than that measured in the cnidarians *A. viridis* ($\text{pH } 7.41 \pm 0.22$) and *Stylophora pistillata* (7.29 ± 0.15) (Venn et al., 2009). Under normal conditions, photosynthesis in the symbiont results in an alkalinisation of the host cell (Venn et al., 2009), the magnitude of which is proportional to the irradiance (Laurent et al., 2013a). As such, we predicted that CO_2 addition would increase the photosynthetic rate and hence symbiont pH_i , and trigger a similar increase in the host cell pH_i . The results of the high CO_2 treatment (pH 7.5) were consistent with this prediction, with both symbiont pH_i and host pH_i rising by ~0.3 pH units in proportion to the external CO_2 concentration. However, the same cannot be said of the host cell pH_i under the intermediate CO_2 treatment (pH

7.8), despite the *Symbiodinium* cell pH_i increasing by ~ 0.12 pH units. While the high buffering capacity of the cnidarian cell likely plays a role (Laurent et al., 2013b), the results presented here suggest a strong link between the relative rates of photosynthesis and respiration in dictating the pH_i of the host in response to external, CO_2 -induced pH change. In particular, the increase in alkalinity reported for the high CO_2 treatment is potentially attributable to the elevated $\text{P}_{\text{gross}} : \text{R}$ ratio seen under these conditions (in contrast to the intermediate CO_2 treatment). This could indicate a partial decoupling of the rates of photosynthesis and respiration, which typically facilitate one another through the evolution of O_2 and CO_2 respectively (Harland and Davies, 1995), and a concomitant decrease in the intracellular CO_2 concentration. The mechanism by which the pH_i of the host cell may be increased by CO_2 enrichment is summarised in Figure 5.4D.

In summary, these findings demonstrate that the photo-physiological response to predicted ocean acidification scenarios has the potential to alter the pH_i of both host and symbiont simultaneously, and therefore affect the functioning of the cnidarian-dinoflagellate symbiosis. Of particular importance, these results suggest improved photosynthetic performance and an absence of cellular acidosis in the light in response to CO_2 -driven acidification. pH_i in the dark was not measured, and it would be interesting to know what impact these different conditions have on night-time pH_i , though the results of Chapter 3 suggest that isolated host cells of the reef coral *P. damicornis* are more susceptible to acidification when the photosynthetic activity of their symbionts is inhibited by DCMU. The enhanced performance in the light, however, raises important questions about the future of non-calcareous symbiotic anthozoans in a high- CO_2 ocean. In particular, several studies have reported that ocean acidification initiates a shift in ecosystem structure from a calcareous to a non-calcareous, algal-dominated community (Hall-Spencer et al., 2008; Suggett et al., 2012; Inoue et al., 2013) that has the potential to act as a significant carbon sink (Anthony et al., 2011; Russell et al., 2013). Similarly, these results, along with those of Suggett et al. (2012), suggest that symbiotic sea anemones could not only survive ocean acidification, but thrive under future conditions. However, it is important to bear in mind that ocean acidification is only once aspect of climate change; with the effects of global warming likely to influence the physiological response to elevated CO_2 levels (Chapter 4).

Chapter 6: General Discussion

6.1 Summary

This thesis aimed to determine the inter-relationship between photosynthesis, symbiont pH and host cell pH in the cnidarian-dinoflagellate symbiosis, and quantify how this is impacted by ocean acidification and thermal stress. Live confocal imaging was used to monitor fluxes in pH_i of the symbiont and the host simultaneously, providing new insights into the cell biology of this ecologically significant association. My findings demonstrate that pH_i in the host cell is tightly linked to the photosynthetic performance of the symbiont, and show that both host and symbiont display synchronicity with the diel light cycle (Chapters 2, 3). This corroborates the findings of studies with the anemone *Anemonia viridis* and coral *Stylophora pistillata* (Venn et al., 2009; Laurent et al., 2013a; Laurent et al., 2013b). Of most significance, the results presented here suggest that if CO_2 -driven seawater acidification enhances photosynthetic productivity in the symbiont, then the host cells is better equipped to response to changes in external pH (pH_e). Host cell's that contain *Symbiodinium* that are able to use the additional CO_2 in photosynthesis, are therefore less susceptible to cellular acidosis, after both short- and longer-term exposure to CO_2 (Chapters 3-5). Conversely, host cells containing less productive and/or thermally sensitive symbionts may not be at greater risk of cellular acidosis (Chapter 4). These findings raise several important questions: what are the potential costs/benefits of CO_2 supplementation for marine phototrophs, and what cellular mechanisms are likely to be affected? How will global warming influence the cellular response to CO_2 ? And, what are the ecological implications of these findings for the survival of coral reefs?

6.2 The cellular response to CO_2 enrichment

6.2.1 *What are the costs/benefits of CO_2 supplementation for marine phototrophs?*

Photosynthetic organisms are dependent on a plentiful supply of CO_2 to the enzyme Rubisco (the site of CO_2 -fixation), to maximise photosynthetic productivity. Yet the

concentration of CO₂ in seawater is low, being < 1% of all dissolved inorganic carbon (DIC) present in oceanic surface waters (Millero et al., 2002). The evolution of carbon concentrating mechanisms (CCM's) has enabled organisms to exploit the bicarbonate (HCO₃⁻) reservoir, but these are energetically costly to maintain (Raven et al., 2012), so the majority of marine phototrophs remain DIC-limited (Levavassur et al., 1991; Riebesell et al., 1993; Nimer et al., 1997; Carpenter and Williams, 2007; Herfort et al., 2008). This is particularly the case in dinoflagellates, phototrophic proteobacteria and cyanobacteria, because they contain a type II form of Rubisco that is inefficient at discriminating between O₂/CO₂ (Tabita, 1995; Rowan et al., 1996; Sderberg and Hansen, 2007). CO₂ accumulation resulting from fossil fuel burning could thereby alleviate DIC-limitation through the provision of an additional source of carbon for photosynthesis (Schippers et al., 2004; Rodolfo-Metalpa et al., 2010). Alternatively, it could promote an organism's fitness indirectly, by reducing the energetic costs of HCO₃⁻ acquisition (Beardall and Giordano, 2002; Raven et al., 2012). There have been mixed responses to CO₂-addition in marine organisms (Ries et al., 2009; Kroeker et al., 2010; Kroeker et al., 2013). Macroalgae such as *Dictyota dichotoma* and *Hildenbrandia rubra* can survive in seawater as low as pH 6.7 (Porzio et al., 2011). And while seagrasses (Orth et al., 2008; Jiang et al., 2010), sea anemones (Suggett et al., 2012; Towanda and Thuesen, 2012) sponges (Bell et al., 2013; Goodwin et al., 2013) and some members of the phytoplankton (Hutchins et al., 2007; Fu et al., 2008) appear to thrive under intermediate and/or high CO₂ conditions (pH_e 7.6 - 7.8), these same conditions typically have negative impacts on calcifying organisms such as hard corals (Schneider and Erez, 2006; Anthony et al., 2008; Sweatman et al., 2011), molluscs (Comeau et al., 2009; Rodolfo-Metalpa et al., 2011; Kroeker et al., 2013), foraminiferans (De Moel et al., 2009; Moy et al., 2009); coccolithophores (Beaufort et al., 2011) and crustose coralline algae (Jokiel et al., 2008; Kuffner et al., 2008; Martin and Gattuso, 2009). The increases in cell-specific productivity and symbiont density seen in the ITS2 type B1 *Symbiodinium* cells isolated from *Aiptasia pulchella* (Chapter 5) follow these general trends, and suggest that increased carbon fixation, in response to the available CO₂ is responsible for the enhanced growth rate. These patterns are consistent with the response observed in type A19 *Symbiodinium* that were isolated from *A. viridis* (Suggett et al., 2012) and in cells from *Anthopleura elegantissima* (Towanda and Thuesen, 2012). They contrast however, with those in cultured *Symbiodinium* (types A1, A13, A2 and B1), which

showed either no change in growth or photosynthesis (types A1 and B1); no change in growth but an increase in photosynthesis (type A2); or accelerated growth without a change in photosynthetic output (type A13) (Brading et al., 2011). The disparity in the responses between the cultured B1 and freshly isolated B1 cells used in this study probably arises from the different mechanisms of DIC acquisition that they employ. *Ex hospite Symbiodinium* mediate their DIC supply one of two ways; (1) through the uptake of HCO_3^- via a Na^+ -dependent HCO_3^- mechanism (Al-Moghrabi et al., 1996; Goiran et al., 1996; Leggat et al., 1999); or (2) via the passive diffusion of CO_2 (Brading et al., 2013). Symbionts that employ the former mechanism may not be affected by CO_2 -driven acidification to the same degree as those that use the latter, because they can typically achieve (or come close to achieving) photosynthetic saturation from the HCO_3^- reservoir in ambient seawater. The situation is rather different for *in hospite Symbiodinium* because they are reliant firstly, on their host cell to acquire the initial DIC, and secondly, on having enough energy to fuel the H^+ -ATPase proteins, which are thought to be responsible for the final transfer of DIC from host to symbiont (Bertucci et al., 2009). These additional stages mean that *Symbiodinium* are almost always DIC-limited *in hospite* in ambient seawater (Weis, 1993; Bénazet-Tambutté et al., 1996; Davy and Cook, 2001; Suggett et al., 2012; Towanda and Thuesen, 2012). The results presented in Chapter 5 support this assertion, and suggest that in type B1 at least, the enhanced availability of CO_2 may alleviate these limiting conditions.

Variation in the response of different *Symbiodinium* types to CO_2 is hardly surprising given the immense physiological diversity that exists within the genus (Baker, 2001; Baker, 2003; Berkelmans and van Oppen, 2006; Suggett et al., 2008; Ragni et al., 2010; Howells et al., 2012). Indeed, like all marine phototrophs (Riebesell, 2004), the degree of DIC required for photosynthetic saturation varies considerably between symbiont types. *Symbiodinium* type A20 for example, is saturated at ambient levels of DIC whereas type A13 is not (Brading et al., 2013). The reasons for these differences are unclear, but one suggestion is that it could be related to the efficiency of a cells' CCM (Brading et al., 2011). If the CCM's are highly efficient, then it is possible to achieve photosynthetic saturation at present day CO_2 levels. In this case, adding CO_2 will not increase the photosynthetic yield, but it could reduce the amount of energy required by the CCM's, resulting in the reallocation of energy into population growth

(Rost et al., 2008). Conversely, if cells possess inefficient CCM's, then they will be more reliant on the passive diffusion of CO₂ at ambient conditions, and CO₂-addition is likely to result in an increase in photosynthetic productivity. The half-saturation constant of photosynthesis (widely used as an index of CCM function) is particularly low in type B1 *Symbiodinium* (Oakley et al., 2014). It is therefore plausible that the cells isolated from *A. pulchella* in this study possess inefficient CCM's, and so are under-saturated at present day, and even intermediate ocean acidification trajectories (Chapter 5). If this is the case, then organisms such as anemones could become an important sink for CO₂ (Anthony et al., 2011) in the future, the implications of which are discussed further in section 6.3.

The stimulation of photosynthesis could further benefit organisms by reducing the potential for cellular acidosis under ocean acidification. The magnitude of light-adapted intracellular alkalinisation increased under both short (< two hours) and longer-term (two months) CO₂-addition (Chapters 3, 5), providing further evidence that *Symbiodinium* cells are DIC-limited under normal *in hospite* conditions. Similar rises were observed in the free-living bloom-forming dinoflagellate *Prorocentrum micans*, where cytosolic and chloroplast pH_i increased from 6.9 ± 0.10 and 7.14 ± 0.12, respectively, under DIC-limiting conditions (1.0 mM DIC) to 8.3 ± 0.13 and 7.7 ± 0.11 under DIC-replete conditions (2.0 mM DIC) (Nimer et al., 1999). In both cases, incubating the cells with DCMU (a photosynthetic inhibitor) before exposing them to either HCO₃⁻ addition (Nimer et al., 1999) or CO₂ addition (Chapter 3) suppressed the degree of light-induced pH_i alkalinisation, indicating that photosynthesis plays a fundamental role in protecting the intracellular environment from cellular acidosis. Moreover, it suggests that DIC-limited cells that can 'soak up' excess CO₂ may be better placed to counter acidosis, and thus prevent metabolic suppression, providing that the removal of CO₂ is greater than the influx of CO₂ (Pörtner et al., 2004; Melzner et al., 2009; see Figure 6.1). Future studies should investigate these phenomena further (see section 6.4 for details), while it would also be interesting to clarify the interactions between pH_i and CO₂-driven acidification in the dark, because, despite the obvious importance of photosynthesis, it is by no means the only mechanism involved in pH_i regulation. While no studies have assessed the mechanisms of pH_i regulation in *Symbiodinium* cells, Cl⁻/H⁺, Ca²⁺/H⁺ and Na⁺/H⁺

exchangers (NHE's) have all been documented in marine algae (Katz et al., 1992; Thaler et al., 1992; Okazaki et al., 1996; Balnokin et al., 1997; Plieth et al., 1997). In contrast, only one study to date has adopted a mechanistic approach for understanding pH regulation in cnidarian host cells. Laurent and co-workers (2013b) used amiloride and 5-(N-ethyl-N-isopropyl) amiloride (EIPA) inhibitors with confocal imaging to assess whether NHE's were involved in pH_i regulation in *A. viridis*. Both symbiotic cells and non-symbiotic cells were bathed in seawater that had been adjusted to pH 7.4. Without the inhibitors, the pH_i decreased to 6.70 ± 0.07 pH and 6.64 ± 0.06 pH units within 15 min, respectively, but recovered to $\text{pH } 7.02 \pm 0.02$ after 20 min in both cases. No recovery was observed in the presence of inhibitors, providing evidence that NHE's play an important role in determining the response to acidification. Interestingly, the authors of this study reported no difference in the buffering capacity between symbiotic and non-symbiotic cells in the dark (Laurent et al., 2013b). This contrasts with the results presented in Chapter 3, where symbiotic cells recovered from an initial 0.4 pH unit drop, but non-symbiotic host cells did not. One potential reason for these differences is the pH_e employed in the two studies: the former used a pH_e of 7.4 whereas the final pH_e in my study was 6.8, creating a steeper proton gradient ($\text{pH}_e - \text{pH}_i = 0.4$ versus 0.8 pH units).

Proton fluxes, and the effect of ocean acidification on photosynthesis, take on renewed importance when they are considered in the context of calcification (Brownlee, 2009). Light-enhanced calcification is a well-established phenomenon, although the mechanism(s) involved in this process remain contentious (Gattuso et al., 1999; Al-Horani et al., 2003; Mass et al., 2007; Moya et al., 2008), not least because photosynthesis and skeletogenesis occur in separate cell layers (the oral endodermal cell layer and aboral endodermal cell layer, respectively). Mechanisms proposed to link the two are hotly debated and often contradict one another (Al-Horani et al., 2003). These include: (1) photosynthesis reducing the partial pressure of CO_2 at the site of calcium carbonate (CaCO_3) deposition (Goreau, 1959); (2) calcification producing H^+ that the symbionts absorb, thus fuelling the assimilation of HCO_3^- and nutrients (McConnaughey and Whelan, 1991); (3) the increasing concentration of OH^- arising from photosynthesis, that enables the neutralisation of H^+ produced during CaCO_3 deposition (Furla et al., 1998); (4) the phosphorylation of photosynthate

produced during photosynthesis which fuels the ATP-demand of Ca-ATPases pumping calcium (Ca^{2+}) to the site of calcification (Al-Horani et al., 2003); and (5) the ‘Proton Flux Hypothesis’ (Jokiel, 2011), the theory that net calcification rates are dependent on the gradient in pH that exists between the external seawater and a 200 - 300 μm thick, oxygen-saturated ‘boundary layer’ that overlies the photosynthetic coral tissue (Kühl et al., 1995). This layer is maintained well above the pH of the surrounding seawater, reaching up to pH 8.6 in *Acropora* sp. and *Favia* sp. (Kühl et al., 1995). Calcification is the result of an equilibrium reaction that exists between HCO_3^- and Ca^{2+} , which combine to form CaCO_3 and result in the release of H^+ . The rate of CaCO_3 is therefore limited by the extrusion, and subsequent neutralisation of H^+ . Under ‘normal’ conditions the gradient between the boundary layer and the surrounding seawater is approximately 0.5 pH units (Kühl et al., 1995). If CO_2 is added to the surrounding seawater, however, the gradient increases between the two, causing a shift to the left of the equilibrium, rendering it more difficult for the coral to extrude H^+ and hence to calcify (Jokiel, 2011). If, as my results suggest, photosynthesis is fertilised by the addition of CO_2 (Chapters 3, 5), then the increased removal of H^+ could mitigate some of the energetic costs associated with H^+ extrusion (Schneider and Erez, 2006; Ries et al., 2009; McCulloch et al., 2012). In addition, the energy derived from the increased rate of photosynthesis could contribute to the energetic demands of converting HCO_3^- to CO_3^{2-} , potentially offsetting the reduction in the aragonite saturation state (Ries et al., 2009). This could explain why not all not all calcifying species exhibit negative calcification rates after CO_2 addition (Hendriks et al., 2010; Kroeker et al., 2013). The temperate coral *Oculina arbuscula*, for example, showed no response to CO_2 at 606 and 903 ppm, while the coralline red alga *Neogoniolithon* sp. and the calcareous green alga *Halimeda incrassata* achieved net growth at the same levels (Ries et al., 2009). In summary, it appears that future oceanic CO_2 levels are likely to benefit non-calcifying marine phototrophs, but that reduced calcification may negate the benefits to calcifying marine phototrophs. However, neither of these outcomes considers the influence of global warming, the potential impacts of which are considered in the next section.

6.2.2 How will global warming influence the cellular response to CO₂?

The data presented in Chapter 4 suggest that exposure to elevated temperatures can significantly reduce a cell's ability to regulate its pH_i under CO₂-addition. The drop in pH_i following an 8°C increase in seawater temperature was substantial, particularly in *Pocillopora damicornis* cells where pH_i declined from 7.40 ± 0.07 at 23°C to 6.56 ± 0.03 at 31°C in host cells, and from 7.45 ± 0.02 to 6.86 ± 0.01 in the symbionts. The reduction in pH_i was much less in *Montipora capitata*, but still present, with pH_i declining from 7.35 ± 0.07 to 6.95 ± 0.05 in host cells, and from 7.43 ± 0.04 to 7.14 ± 0.03 in the symbionts. Quite how and why thermal stress induces acidosis, and whether or not this effect is reversible is unclear, but given that these patterns mirror those observed in host cells that are incubated with DCMU (Chapter 3), it is likely that photosynthesis plays a critical role in governing a cell's susceptibility to changes in pH_e. Under high temperatures, the photosynthetic machinery of the *Symbiodinium* cell is impaired, reducing the amount of carbon that is fixed, and triggering the production of damaging reactive oxygen and nitrogen species (ROS/RNS). This has two effects on the pH_i: firstly, CO₂ is not removed at a quick enough rate to mitigate the influx of H⁺, leading to cellular acidosis; and secondly, the energy remaining in the cell is directed towards the up-regulation of antioxidant production (e.g. superoxide dismutase, catalase, peroxidases) to counter the build-up of ROS, rather than to pH_i regulation (Richier et al., 2005; DeSalvo et al., 2008; Császár et al., 2009; Seneca et al., 2010). Failure to suppress the production of ROS/RNS leads to metabolic suppression (Muller et al., 2007; DeSalvo et al., 2008) and, under prolonged circumstances, to apoptosis (Buttke and Sandstrom, 1994; Richier et al., 2006). It is conceivable that thermal stress could have similarly detrimental effects on the capacity for pH regulation, with negative consequences for holobiont fitness (Kaniewska et al., 2012). Resolving the interrelationship between the physiological impacts of thermal stress and the potentially beneficial role of CO₂ addition should therefore be considered a high research priority for future studies, especially given the increased frequency and/or severity of bleaching events expected over the 21st century (Perry et al., 2013; Van Hooidonk et al., 2013). Furthermore, if a coral's thermal sensitivity does indeed influence its survivorship under ocean acidification, then understanding how and why bleaching thresholds differ between coral species (Loya et al., 2001; van Woesik et al., 2011) takes on renewed importance.

A coral's thermal vulnerability is determined by the genetic constitution of its symbiont community (Rowan et al., 1997; Baker, 2001; Toller et al., 2001), in addition to many numerous host-derived factors including: coral colony morphology (Fabricius et al., 2006; Jimenez et al., 2011), tissue thickness (Hoegh-Guldberg 1999; Loya et al., 2001; Yost et al., 2013), and green fluorescent protein concentration (Bou-Abdallah et al., 2006). While I did not test the genetic diversity of the symbionts of the corals used in Chapter 4, it is likely that they contained different *Symbiodinium* types. *M. capitata* is a flexible generalist that typically contains a combination of types C3, C17, C21, C31 and/or D1, whereas *P. damicornis* is only able to host C1 and C3 (LaJeunesse et al., 2004; Padilla-Gamiño et al., 2012; Putnam et al., 2012). There is a high degree of variation that exists in thermal tolerance between *Symbiodinium* ITS2 types (Robison and Warner, 2006; Sampayo et al., 2008; Fitt et al., 2009; Fisher et al., 2012). Types A1 and F2, for example, are much more susceptible to thermal stress than are A1.1 and B1 (Robison and Warner, 2006), while both C1 and C3 have particularly low thermal stress thresholds (Fitt et al., 2009; Fisher et al., 2012). Corals containing C3, for example, exhibited declines in the maximum dark adapted yield of photosystem II (F_v/F_m) of 63.8, 67.5, 76.6 and 91.1% in *Platygyra daedalea*, *Acropora millepora*, *Acropora formosa* and *Acropora aspera*, respectively, after being subjected to an 8°C increase in temperature over a 4 day period (Fisher et al., 2012). The results presented in Chapter 4 were derived from a single colony of each species, so caution needs to be exercised when making generalisations, but there were clear differences in the two species' abilities to regulate pH_i after thermal stress. Considering whether the type of symbiont that they contain influences the host cell's susceptibility to acidosis certainly offers an exciting avenue for future research, not least because corals may be able to alter the types of *Symbiodinium* that they host to increase their resilience to thermal stress (Buddemeier and Fautin, 1993; Berkelmans and van Oppen, 2006; Howells et al., 2012; Putnam et al., 2012). This could occur *via* the uptake of a new symbiont type from the external environment ("switching") following coral bleaching or the production of symbiont-free larvae, or through repopulation by a type that was previously kept at low background levels within the coral ("shuffling"). Convincing empirical evidence for switching is lacking in reef corals, but there is evidence of shuffling (Berkelmans and van Oppen, 2006; Mieog et al., 2007; van Oppen et al., 2009; Gilbert et al., 2010; Stat

and Gates, 2011; Silverstein et al., 2012). My results suggest that, in warm, acidified oceans, a switch in symbiont dominance (either by switching or shuffling; Fig. 6.1A) will only benefit the coral if that symbiont type is thermally resilient and is able to increase its photosynthetic productivity in response to CO₂-addition [Fig. 6.1B]. If the new symbiont is susceptible to heat stress [Fig. 6.1C] or has a lower photosynthetic rate [Fig. 6.1D] then it will provide less protection against ocean acidification. The worst-case scenario would eventuate if a coral acquired a symbiont that is thermally susceptible and has a low photosynthetic output [Fig. 6.1E]. Under this outcome, the host cell succumbing to acidosis appears to be inevitable.

Fig. 6.1 Schematic diagram illustrating how intracellular pH (pH_i) fluctuations in the coral host cell are influenced by the thermal resilience and productivity of the *Symbiodinium* cells that they host. [A] Following a bleaching event corals can acquire *Symbiodinium* cells via [1] switching or [2] shuffling. If the symbiont has: [B] high thermal resilience and high productivity under CO₂ addition, the pH_i of the host cell can be elevated above normal levels (~ 8); if the symbiont has either [C] low thermal resilience and high productivity under CO₂ addition; or [D] high thermal resilience and low productivity under CO₂ addition, the pH_i is maintained within normal levels (~ 7-7.4); but if the symbiont has [E] low thermal resilience and low productivity under CO₂ addition, the host cell will experience cellular acidosis (~ 6.5). Note that the colour of the corals indicates the pH_i of the host cell as identified in the colour gradient above, and *Symbiodinium* cells are coloured black/white according to their thermal tolerance (black = thermally sensitive; white = thermally resilient)

One such example of this is clade D *Symbiodinium*. Once heralded as a “nugget of hope” because of the additional 1-1.5°C thermal tolerance it affords *A. millepora*

(Berkelmans and van Oppen, 2006), it has since been shown to reduce growth rates by 38%, lipid stores by 26% and egg sizes by up to 28% (Jones and Berkelmans, 2010; 2011), due to its relatively low photosynthetic rate (Abrego et al., 2008), and is now deemed an “opportunistic” (Stat and Gates, 2011). Under elevated temperatures alone, associating with clade D for a short period can be beneficial in that it can prevent mortality before a coral reverts back to its optimal symbiont cohort (Thornhill et al., 2006). However, under the combined stressors of both elevated temperature and ocean acidification, this advantage may be lost, leading to acidosis of the host cell. In conclusion, it appears that global warming, and symbiont productivity will profoundly influence the cellular response of coral cells to CO₂. Given that the nature of the response will be both species- and symbiont specific, these findings have important ecological implications for the future of coral reefs in warm, acidified oceans.

6.3 Implications for the future of coral reefs: a shift in dominance?

The effects of ocean acidification and global warming will not be homogeneous in space or time (Hoegh-Guldberg et al., 2007; Pandolfi et al., 2011). High latitude reefs, for example, are likely to experience acidification earlier than reefs close to the Equator, because the aragonite saturation horizon (ASH) is shallower in these regions (Fabry et al., 2009). Conversely, equatorial reefs will be subjected to earlier, and more severe coral bleaching episodes (Van Hooidonk et al., 2013). Between these areas lie the tropics, home to the majority of the world’s coral reefs, and the habitats that are at the greatest risk from the combined effects of sea surface temperature rises and ocean acidification. Within these areas, variation in local and regional ecosystem drivers such as eutrophication (Szmant, 2002; Fabricius et al., 2005; Wiedenmann et al., 2012; Bell et al., 2014; Vega Thurber et al., 2014), over-fishing (Jackson et al., 2001; Hughes et al., 2007), pollution (Hughes et al., 2010) and disease (Richardson, 1998) all shape the community structure of reefs. Coral reefs that are already degraded are beginning to show signs of “phase shifts” (Norström et al., 2009) to “alternate stable states” [Fig. 6.2] such as those dominated by macroalgae (Hughes, 1994; Aronson and Precht, 2006); sponges (Goodwin et al., 2011; Bell et al., 2013); sea anemones (Chen and Dai, 2004, Tkachenko et al., 2007) and corallimorpharians (Chadwick-Furman and Spiegel, 2000; Rajasuriya and Karunaratna, 2000; Work et al., 2008).

Fig. 6.2 Alternative stable states. [A] A scleractinian-dominated reef (Great Barrier Reef, Australia) may shift to either: [B] an algal dominated ecosystem (Western Rocky Island, Myanmar); [C] a sponge-dominated reef (Richelieu Rock, Thailand); [D] an anemone-dominated reef (Hin Muang, Thailand); or [E] a corallimorpharian reef (Hienghene, New Caledonia). Photographs B, C and D provided by T.D. Hawkins.

The prospect of anemone-dominated reefs [Fig 6.2D] is particularly interesting in light of the positive and rapid response ($< \text{two weeks}$) of *A. pulchella* to CO_2 -addition (Chapter 5). The concurrent, and sustained increase in gross (P_{gross}) and cell-specific photosynthesis across the two-month experimental period suggests that anemones are not only well equipped to colonise any space that becomes available, but also to retain it. This has important implications for protecting the biodiversity of reefs; maintaining a degree of structural complexity and providing refuge from predators (Holbrook and Schmitt, 2005) by reducing algal cover (Taylor and Littler, 1982). Under specific high-nutrient conditions, some anemones are not only able to colonise space rapidly,

but can actively outcompete live coral. *Mesactinia genesis*, for example, increases its rate of reproduction and the size of its acrorhagi tentacles (Williams, 1991), and actively attacks nearby *Acropora muricata* colonies (Liu et al., 2009). Surprisingly then, anemone reefs are relatively rare in the tropics. Those that do persist are dominated by the giant anemone *Condylactis* sp. (Tkachenko et al., 2007), and tend to form in areas characterised by high nutrient and sedimentation rates (Chen and Dai, 2004; Liu et al., 2009) such as around Taiwan, where they can reach percentage covers as high as 40% (Tkachenko et al., 2007). Rising sea surface temperatures, however, could inadvertently provide an opportunity for anemone-dominated reefs to increase in prevalence, including in higher latitude areas as they warm (Guinotte et al., 2003; Veron, 2011). A poleward migration of tropical coral reef species has largely been dismissed, because the shallowing of the ASH would prevent corals from calcifying at higher latitudes (Kleypas et al., 1999), but there has been little consideration of the implications that this may hold for non-calcifying phototrophs. If, as my results suggest, phototrophic organisms are able to benefit from CO₂ addition, and the increase in primary productivity is enough to prevent the negative aspects of acidification, namely acidosis (Chapter 3, 5), then it is possible that soft-bodied phototrophic cnidarians could dominate at marginal latitudes that are undersaturated with aragonite in the future. There is direct support for this hypothesis. Firstly, in natural Mediterranean CO₂ vent communities, *A. viridis* abundances increased two-fold at sites with *p*CO₂ values of 500 - 1250 ppm relative to control sites, and then doubled again at the site of the highest *p*CO₂ of 1450 ppm (Suggett et al., 2012). Secondly, in three temperate anemones, *Anthopleura aureoradiata* (Doherty, 2009), *A. elegantissima* (Towanda and Thuesen, 2012) and *A. viridis* (Jarrold et al., 2013), experimental CO₂-addition either had no effect (Doherty, 2009), or increased photosynthetic output (Towanda and Thuesen, 2012; Jarrold et al., 2013). A further key piece of evidence can be derived from the geological records spanning the two major mass extinctions of coral reefs – at the end of the Triassic and Cretaceous periods (Stanley, 2003; Veron, 2008). Both of these mass extinction events were characterised by extremely high CO₂ levels - up to eight times higher than those experienced today (Hautmann, 2004). The persistence of corals despite a 6-8 million year absence from the fossil record (Veron, 2008) led to the ‘naked coral hypothesis’, the theory that corals are able to survive the increase in acidity, just not in the calcified form we recognise today (Stanley, 2003; Medina et al., 2006; Kitahara et al.,

2014). Fine and Tchernov (2007) tested this theory using the Mediterranean corals *Oculina patagonica* and *Madracis pharencis*. Both corals were able to survive CO₂ enrichment scenarios much higher than those predicted for 2100 (pH_e 7.3 - 7.6) by shifting to a soft-bodied polyp form, functioning in manner similar to the anemones in this study. Selecting this life-history strategy would render the polyps at greater risk of predation in the short-term, but could increase survivorship of corals in the longer-term, where the deterioration in reef health (and habitat complexity) would reduce the abundance and diversity of predators (Guinotte and Fabry, 2008). In this respect, it will be important for future studies to consider the effects of global climate change and ocean acidification on predator-prey dynamics (Suggett et al., 2012).

6.4 Limitations of the present study, and priorities for future work

This thesis provides a number of methodological advances that can be used in future studies to determine the cellular implications of ocean acidification and global climate change. In Chapter 2, I provide a simple, quick and cheap method for analysing pH_i at the level of the whole *Symbiodinium* cell. But, while this technique is adequate for measuring average pH_i, it is not able to provide information on areas of differential pH_i within the cell. Given that pH_i varies substantially between cellular organelles in other organisms (Roos and Boron, 1981; Kurkdjian and Guern, 1989; Casey et al., 2010), increasing the resolution of this approach should be a priority for future research. Furthermore, attention should be focused on accurately measuring the vacuolar space of the symbiosome (i.e. the perialgal vacuole) in the intact symbiosis, because this region serves as the functional interface between the host and symbiont, and thus plays a critical role in the translocation of metabolites. Previous studies have noted the presence of an acidic ring, which is thought to be less than pH 5.7 (Rands et al., 1993; Venn et al., 2009; Laurent et al., 2013a), but this should be resolved. The technique developed here was, however, sensitive enough to measure the intrinsic differences between light- and dark-adapted pH_i in six *Symbiodinium* genotypes (Chapter 2). A great deal of physiological variation exists between and within *Symbiodinium* types (Warner et al., 1996; Bhagooli and Hidaka, 2003; Fabricius et al., 2004; Takahashi et al., 2009; Ragni et al., 2010), so it would be pertinent for future studies to assess a wider range of types in order to fully encompass both the genetic

and physiological diversity in this genus. Given the importance of the symbiont in determining its host cell's sensitivity to acidification (Chapters 3-5), it will be especially important to quantify how a symbiont's genotype affects the buffering capacity of its host cell. In this regard, it would be particularly interesting to consider whether thermally tolerant types, such as C15 (Fitt et al., 2009; Fisher et al., 2012), may be able to offer greater resistance to their host under ocean acidification (Chapter 4; Fig. 6.1). Moreover, given that the measurements in this study were conducted on doublet cells (host cells containing two algal symbionts) only, it will be important to investigate whether the number of *Symbiodinium* cells that a coral host cell contains can influence the host's cellular response to acidification. For example, does a triplet host cell (i.e. a host cell containing three algal symbionts) have a greater capacity to withstand acidification than a host cell containing a single alga? And, if indeed they do, how does the average carrying capacity of the host cells across the holobiont influence its susceptibility to acidification? The average number of symbiont cells per anthozoan host cell (the cell-specific density; CSD) varies from 1.11 in *Condylactis gigantea*, *Montastrea cavernosa* and *Agaricia* sp. to 2.19 in *A. viridis*, with the mean of 33 different species being 1.54 ± 0.30 (Muscattine et al., 1998). Could a high CSD in *A. pulchella* contribute to its success under CO₂-addition? And can corals exert control over their symbiont population density as a means of regulating their pH_i? These are both interesting questions for the future.

The majority of the work in this thesis used isolated cells, yet bathing cells in seawater does not necessarily replicate the conditions that a cell would experience in intact coral tissues. There is a great deal of variation in pH within the coral biome; cells facing the gastrovascular cavity, for example, can be exposed to seawater of up to pH 9 (Al-Horani et al., 2003). In this respect, it would be interesting to extend this work to different tissue layers. Quantifying the pH_i of ectodermal cells, for example, will improve our understanding of carbon speciation and DIC-transport, while measuring the pH_i of cells in the aboral tissue layer may shed light on the fluxes of DIC that connect photosynthesis and calcification (Brownlee, 2009).

6.5 Conclusions

This thesis provides the first simultaneous measurements of pH_i in *Symbiodinium* and their host coral cells, using a novel live-cell imaging technique, which will facilitate future investigations into the role of pH_i in symbiotic partnerships. My findings further the pioneering work of Venn and co-authors (2009), by considering the impact of CO_2 -addition and thermal stress on pH_i dynamics. I demonstrate inter- and intra-taxonomic differences in the susceptibility of organisms to cellular acidosis; specifically I confirm previous observations that CO_2 -addition can have benefits for soft-bodied non-calcifying organisms such as anemones, in contrast to the negative impacts for corals. Furthermore, I show that thermal stress enhances an individual's sensitivity to acidosis, and that the magnitude of this sensitivity is increased/reduced according to its thermal resilience. The interactions between photosynthesis, symbiont pH and host cell pH drive the cellular mechanisms that underpin symbiosis. Understanding the biology behind these interactions is essential if we are to understand how coral and/or *Symbiodinium* physiology contributes to symbiosis collapse (Weis et al., 2008; Venn et al., 2008; Davy et al., 2012), and indeed, if we are to make accurate predictions about the future status of coral reefs.

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A: Appendices

A.1 ITS2 sequences for the cultured *Symbiodinium*

Culture name: CCMP 2467 (A1)

GCGCCGAACTGCATTCAAATGACTTCATGCTAGGAAGTGTTAAGAACCACATGAGCTTTT
GTTTCGCCAACTGAAACGACATGAGATGTTGATCAGTCACAACAGCAAGCAAACTTGAT
ATTTAGCATGCCAGTGCCACTTGCAGAAGCATGCAGCAACACTGCTCCTGATAACAAGAG
CAGCAGAAAATGAAAGTAGAAGCACTGAAGCAGACATGCTCTCAGGCATATCCCAAGAG
CGCAATGCACGTTCAAGAGGCCATTGGTTCACGGAGTTCTGCAATTCACAAAAGT

Culture name: Ap1 (B1)

GGACGATCGTATTACTAGCTGTACACATGCCACGGGAGTTCTGCAATTCACAGGACAGCT
GCGCGCTTGAGCTGCACGATGCAAGCAAAGCTTGGAACAACAGTACGCTCAAGCTTGCGC
CATGGGCCAGGCAGCAAACAAGCCATTTACGAACACAAAAGCGATCGATGACTTGTTGGA
AAGCGAAGCAGCAATGCAGACATACATGCTGCTTGCAAGCACAGGTAGATAATGCTAAGC
ACTGAAGCAGACCTGCTCTCAGGAAATCCCGAGAGCGCAATGCGCGTTCAGGAGGCCATC
GGTTCACGGAGTTCGGCAATTCACAAAAATTTT

Culture name: FlCass (E1)

TCCCTTGATCTGACTTCATGCTAAGATTGGTATTTGAGCACCAATTGCTTGGCAGGCTGGC
ATGATTGGGCCAAAACGGACACACGTGCCCAGTGATGCGCGACAAATCAGTCCAGCTTAC
CTCCCGGTTTGTGTTACAGCGCGCCGCTGCAAATTCTGATGCAGCAGCGCACAAAGAGGCT
CAAGGCTTCTCCAGG

Culture name: CCMP 421 (E2)

CCGGTTTGCTGCTGACTCTGCTAAGATTGGTTATTTGAGCACCAATTGCTTGGCAGGCTGG
CATGATTGGGCCAAAACGGACACACGTGCCCAGTGATGCGCGACAAATCAGTCCAGCTTA
CCTCCCGGTTTGTGTTACAGCGCGCCGCTGCAAATTCTGATGCAGCAGCGCGCAAAGAGGC
TCAAGGCTTCTCCAGGAAGCCCGCACTGCGAAGATATGAAAAGCACTGAAGCAGACATAC
TCTCAGGCGTGTCCTCAAGAGTGCAATGCGAGTTACGGGTGCTATTGGTTCACGGGCTTCCA
CATATCCACCAAAAAGGAAAAAAAAAAGGGAAAAAGGGGGGGGAAAA

Culture name: Mv (F1)

CGGGGGGATCGTTTGATCTGACTTCCTGCCTAGAAAGGCATGCGCCTAACATCCCCATCACT
TTGCAAGCATTGGCAGCGTTACTCAAGAAAATACACATTTTCAGGCGGAGAACTCGAAAC
GCATTGCGCAAAGCTCTTTGCAAGTCTCATACTCATCAATCAACCCCATTCGACTATC
TGATCTCAATCGGCTGCACAGAGGGCGCCACGAGCAC

Culture name: Pd (F5)

GCGGGCAAATCGAATTCACAAGATCATCTTGCCACGAGAGTTCTGCAATTCACACAGCAG
CTGCAAGCATAAACAGCGTCACTCAAGCAATACCACTTTAAGGTGGAATCTTGAATAGCA
GCGCGCAAAGCTTTTGCAAGACCAAAATCATTCAATAAGCCACTGAGTAGACATTCAATGG
CTCACAGGGGGCGCAAGACAGAAACACAATCTGTCCGCAAGATTGGGCAAGCTAAGCACT
GAAGCAGACATACTCTCAGGAAATCCCAAGAGTGCAACGTACGTTACAGGAGGCCATTGGT
TCACGGAGTTCTGCAATTCACAAAACA

A.2 Photosynthesis-irradiance curves

Photosynthesis-irradiance curves were conducted prior to experimentation to calculate the saturating irradiance for photosynthesis in both species. This value was subsequently used during intracellular pH (pH_i) measurements. Briefly, fragments were randomly selected and placed inside a 250 mL plastic chamber. A glass lid, held in place by a rubber O-ring, sealed the chamber, into which a temperature probe and a fibre-optic oxygen sensor were inserted such that both probes were positioned ~ 2 cm above the coral surface (Ocean Optics, Dunedin, Florida, USA). A constant water temperature of 25°C was maintained by recirculating water around the chamber, and water motion was generated by a submersible magnetic stirrer. O_2 -evolution ($\text{mL O}_2 \text{ h}^{-1}$) was measured over five light levels (100, 150, 250, 400 and 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). The desired light level was achieved by manipulating the power and position of a variable-irradiance fibre optic light (Halogen Reflector lamp, 150 W GX5.3 21V 1CT bulb, Philips, Somerset, New Jersey, USA), and the final irradiance was checked using a 4π quantum light meter connected to a data logger (Li-Cor Biosciences, Lincoln, Nebraska, USA). Oxygen readings were taken every 10 s for a minimum of 20 min, or until a clear slope was observed, and the data were recorded using the Neofox Viewer software (Ocean Optics, Dunedin, Florida, USA). Coral fragments were then frozen at -20°C for subsequent chl-*a* analysis (protocol described in section 4.2.2), which was used to normalise oxygen evolution ($\mu\text{g O}_2 \text{ mg}^{-1} \text{ chl h}^{-1}$).

Fig. A.2 Photosynthesis-irradiance curves for [A] *P. damicornis* and [B] *M. capitata*. Values represent net photosynthesis (mean $\pm$ S.E.M, $n = 3$ replicates per species).

A.3 Visual bleaching in *Pocillopora damicornis* and *Montipora capitata*

Fig. A.3 Visual effect of temperature on [A-D] *Montipora capitata* and [E-H] *Pocillopora damicornis* fragments. Images are ordered (top-bottom) via increasing temperature treatment. Note the severe loss of pigmentation in [H] – the 31°C treatment.

B: Additional Work

B.1 Determining the effect of pH on the cell cycle in *Symbiodinium*

The original proposal for Chapter 4 was to investigate how the *Symbiodinium* cell cycle is affected by acidification. Regulation of pH_i plays an important role in cellular differentiation, specifically in the interphase (I) stage of the cell cycle (Aerts et al., 1985). A rise in alkalinity is responsible for the transition between the resting phase of I (G_0), the gap phase of I (G_1) and subsequently into the synthesis (S) phase, during which DNA replication occurs (Madhus, 1988). The role of pH_i was first detected in quiescent fibroblast cells, where it was determined that a pH_i of greater than 7.2 was to initiate S (Pouysségur et al., 1985). Similarly, a 0.25 pH_i unit rise between S stage and final mitosis, was demonstrated for the both the slime mold *Dictyostelium discoideum* (Aerts et al., 1985), and the yeast species *Saccharomyces cerevisiae* (Orij et al., 2012). However this was not the case in *Schizosaccharomyces pombe*, which kept a constant pH_i of 7.3 throughout the cell cycle (Karagiannis, 2001). *Symbiodinium* cells exhibit phased division rates, peaking at dawn upon illumination (Fitt and Trench, 1983). However, little is known about control of the cell cycle, other than that it does not appear to be linked to nutrient limitation by the host (Smith and Muscatine, 1999), and that light is likely to play an important role in the S phase of the cycle (Wang et al., 2008). These preliminary experiments were carried out at an pH_e of 7. These conditions were not intended to replicate ocean acidification predictions (IPCC, 2014) but rather to investigate the dynamics of the *Symbiodinium* cell cycle.

Stock cultures of two *Symbiodinium* types, CCMP 2467 and Ap1 (identified in Chapter 2) were grown in f/2-enriched FSW, in a water bath set to 25°C and an irradiance of 80-100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Osram Dulux 36W/890 fluorescent bulbs). When logarithmic growth was achieved, 1 mL aliquots were removed and added to six 250 mL beakers, each containing 200 mL of FSW. The beakers were then sealed with rubber stoppers ($n = 3$, per species, per treatment). At a density of $\sim 100,000$ cells mL^{-1} two holes were cut in the stoppers and two hand-blown glass tubes, of differing lengths (5 cm and 10 cm) were inserted. CO_2 -insulated tubing connected the shorter glass tube to one of two treatment reservoir tanks, while the longer glass tube led to a

waste tank. Together, the two glass rods created a vacuum within the flask, enabling water to be dripped into the cultures at a constant flow rate. A pH of 7.05 ± 0.1 was maintained in the reservoir tank by a CO₂ solenoid valve, which was connected to a PINPOINT pH controller (American Marine Inc., Ridgefield, CT, USA). Cultures were exposed to the new conditions for 48 h. Samples were then taken every 3 h over a 72 h period. At each time point 10 mL were removed from each flask. A 1 mL aliquot was stored at -20°C for subsequent cell counts using an Improved Neubauer haemocytometer (Boeco, Hamburg, Germany) and a light microscope ($\times 400$ magnification). The resulting values were used to determine the cell-specific growth rate (μ) and the % of cells dividing (mitotic index; MI). The remaining 9 mL were centrifuged for 5 min at $800 \times g$ until an algal pellet was formed. The supernatant was discarded and the pellet was resuspended in 1 mL of 70% ethanol. Samples were stored on ice under high light in order to bleach the sample of chlorophyll. They were then stored in a 4°C fridge for later analysis.

Fig. B.1 Confocal visualisation of Propidium Iodide (PI) staining solution in *Symbiodinium* cells. Images depict different cell cycle phases [A] Synthesis (S) phase, during which DNA replication occurs and [B] Mitotic division (M) phase before cytokinesis cleaves the two cells. Where fluorescence is enhanced PI is binding to genetic material (ex: 473, em: 600-700 nm).

The number of cells at each stage of the cell cycle (G1, S and G2) was calculated using a protocol adapted from Pozarowski and Darzynkiewicz (2004). Samples were centrifuged for 5 min at $800 \times g$ and all ethanol was thoroughly decanted before the cells were re-suspended in 1 mL of PI staining solution (0.1% Triton X-100, 10 µg/mL PI and 100 µg/mL DNase-free RNase A in PBS) and left in the dark for 10

min. The samples were then transferred to a FACSAN flow cytometer (FACScan, Becton-Dickinson, Franklin Lakes, NJ, USA) where they were excited with a 488 nm argon laser. Fluorescence was collected in the FL3 filter channel (red; 610–700 nm). The technique relies on the relative fluorescence of the PI stain upon binding to DNA (Fig. B.1); i.e. the strength of the signal received from single cells in interphase (one nucleus; not dividing) should be exactly half of that when the cell is in G2 (when two nuclei are present). Data were analysed using the FlowJo software package (Tree Star Inc., Ashland, OR, USA) with Watson's Pragmatic algorithm selected to separate the cyclical stages (Watson et al., 1987). The final dataset was analysed as the relative change in the % of cells (calculated as the mean % of cells after acidification minus the mean % of cells in the control) in three phases of the cell cycle – G1, S and G2 using one-way ANOVA. Additional analysis was carried out on the incremental cell population $(t + 1) - t$ in order to establish the number of new cells entering a particular stage at each time point. The assumptions of normality were confirmed using the Kolgomorov-Smirnov Test and sphericity of the data was tested using Mauchly's Sphericity Test.

In general, division rates were higher in the B1 (Ap1) than the A1 strain (CCMP 2467; $F_{(1,43.97)} = 8.17$, $p = 0.007$). Both cultures exhibited phased division rates, with the % of cells in mitosis significantly higher in the light than the dark ($F_{(1,43.65)} = 5.35$, $p = 0.026$). The addition of CO₂ had a significant impact on cell cycle dynamics (Fig. B.2). The % of cells in G1 was significantly higher in the control treatment for both Ap1 ($F_{(1,14)} = 6.72$, $p = 0.021$) and CCMP 2467 ($F_{(1,22)} = 11.13$, $p < 0.003$). In contrast, there was a significantly lower % of cells in the S phase in the control treatment. Again, this pattern was observed in both the Ap1 ($F_{(1,14)} = 11.373$, $p = 0.005$) and CCMP 2467 ($F_{(1,22)} = 18.96$, $p < 0.001$) cultures. Acidification however, had no effect on the % of cells in G2 in either Ap1 ($F_{(1,14)} = 2.72$, $p = 0.122$) or CCMP 2467 ($F_{(1,23)} = 0.019$, $p = 0.891$). Examination of the incremental increases in cells at each phase revealed that transitions between %S and %G2 are tightly linked (Fig. B.2), but that both stages are negatively correlated with the % of cells in G1, regardless of the pH treatment and symbiont genotype. In addition, the amplitude of the hourly increments was smaller towards end of experiment.

Fig. B.2 The effect of acidification on the cell cycle dynamics of two *Symbiodinium* cultures, CCMP2467 (A1; black) and Ap1 (B1; grey). Data represent the relative change in the % of cells in three phases of the cell cycle – [A] the gap phase of interphase (G1), [B] the synthesis stage (S) when DNA replication occurs and [C] gap phase two (G2) preceding cytokinesis, and mitotic division, and the incremental increase in cells entering [D] G1, [E] S and [F] G2.

Intracellular pH of symbiotic dinoflagellates

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Abstract Intracellular pH (pH_i) is likely to play a key role in maintaining the functional success of cnidarian–dinoflagellate symbiosis, yet until now the pH_i of the symbiotic dinoflagellates (genus *Symbiodinium*) has never been quantified. Flow cytometry was used in conjunction with the ratiometric fluorescent dye BCECF to monitor changes in pH_i over a daily light/dark cycle. The pH_i of *Symbiodinium* type B1 freshly isolated from the model sea anemone *Aiptasia pulchella* was 7.25 ± 0.01 (mean $\pm$ SE) in the light and 7.10 ± 0.02 in the dark. A comparable effect of irradiance was seen across a variety of cultured *Symbiodinium* genotypes (types A1, B1, E1, E2, F1, and F5) which varied between pH_i 7.21–7.39 in the light and 7.06–7.14 in the dark. Of note, there was a significant genotypic difference in pH_i , irrespective of irradiance.

Keywords pH_i · *Symbiodinium* · Cnidarian–dinoflagellate symbiosis · Zooxanthellae

Introduction

Coral reefs are both ecologically and socio-economically important, and their success is founded on the mutualistic endosymbiosis between corals and dinoflagellate algae of

the genus *Symbiodinium* (Hoegh-Guldberg 1999). However, despite the importance of the cnidarian–dinoflagellate symbiosis, we know very little about its underlying cell biology (Weis et al. 2008; Davy et al. 2012). This hampers our understanding of how coral reefs function, both under ‘normal’ conditions and projected scenarios of environmental stress. The genus *Symbiodinium* is exceptionally diverse, with nine genetically distinct clades (A–I), which are further differentiated into sub-clades termed ‘types’ (Pochon and Gates 2010). There is substantial variation in physiology between genotypes, particularly with respect to their photosynthetic response to light (Hennige et al. 2009) and thermal tolerance (Fisher et al. 2012). These physiological differences have the potential to confer significant advantages/disadvantages to a coral and can influence a host’s resistance to stress (Baker et al. 2004). Yet despite this, one of the most fundamental attributes of the symbiotic algal cell, its internal pH, was, until now, unknown.

Virtually, all metabolic activity is pH-dependent. pH also plays a key role in controlling progression through the cell cycle, protein synthesis, and the synthesis of both DNA and RNA (Madhus 1988). Furthermore, the establishment of a proton gradient across a cell membrane creates an electrochemical potential, which is important for maintaining the flux of ions between cells, and in inter-cell signalling (Casey et al. 2009). Little, however, is known of the role of pH in the function of the cnidarian–dinoflagellate symbiosis, despite its potential significance in the regulation of inorganic carbon and nitrogen fluxes between the host and symbiont (Fitt et al. 1995; Brownlee 2009; Venn et al. 2009), and the coordination of host and symbiont cell proliferation (Davy et al. 2012). Moreover, pH_i is also known to affect the formation of crystals during coral calcification (Venn et al. 2012). Indeed, only recently has the pH_i of the host cell been measured, with the

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photosynthetic activity of the symbionts causing an alkalisation of the host cell (Venn et al. 2009), proportional to the irradiance intensity experienced by the symbiont (Laurent et al. 2012). The aim of this study was to develop a method for measuring the pH_i of *Symbiodinium* cells, to measure pH_i over a daily light cycle in a range of *Symbiodinium* types, and to compare the pH_i of *Symbiodinium* with that of its host cell in the sea anemone *Aiptasia pulchella*, an important model for coral research.

Methods

Cultured *A. pulchella* anemones, hosting *Symbiodinium* ITS2 type B1, were grown at 25 °C under 80–100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light (Osram Dulux 36W/890 fluorescent bulbs) on a 12 h light:12 h dark cycle and fed *Artemia* sp. nauplii every 3 days. The anemones were not fed for the week prior to sampling. Six *Symbiodinium* types (A1, B1, E1, E2, F1, and F5) were also examined in culture; these types were identified via the method of Stat

et al. (2008). The cultures, originally isolated from a range of invertebrate hosts (Table 1), were grown to densities of $2 \times 10^5 \text{ cells mL}^{-1}$ in 0.22 μm filtered seawater (FSW) enriched with f/2 medium and sampled while the cells were in logarithmic growth phase.

The ratiometric fluorescent dye BCECF-AM ester was used to measure pH_i of *Symbiodinium* via flow cytometry, with the dispersant Pluronic F-127 being employed during the loading procedure to reduce dye aggregation (see below for loading protocol). BCECF-AM has been widely used to measure pH_i (Ozkan and Mutharasan 2002) and has been successfully used in conjunction with flow cytometry in other model systems (Franck et al. 1996). It was selected here for its strong dual excitation properties that allow pH_i to be measured without interference from any chlorophyll autofluorescence ($>640 \text{ nm}$). Confocal microscopy was initially used to confirm that the dye was able to enter the cells (Fig. 1a) and to check for dye homogeneity through the cell (Fig. 1b). It was apparent that, despite the use of a dispersant, BCECF-AM-derived fluorescence was not evenly distributed within each cell. This could either be an

Table 1 Cultured *Symbiodinium* types categorised by the internal transcribed spacer 2 (ITS2) region of the nuclear rDNA, the host they were first isolated from, and the pH_i after 12 h light adaptation and 12 h dark adaptation (mean $\pm$ SE; $n = 3$)

Culture name	Original host	Location	Clade (ITS2 type)	Light-adapted pH_i	Dark-adapted pH_i
CCMP 2467	<i>Stylophora pistillata</i>	Gulf of Aqaba, Jordan	A (A1)	7.33 ± 0.03	7.10 ± 0.03
Ap1	<i>Aiptasia pulchella</i>	Okinawa, Japan	B (B1)	7.32 ± 0.01	7.14 ± 0.01
FlCass	<i>Cassiopea xamachana</i>	Florida, USA	E (E1)	7.27 ± 0.04	7.14 ± 0.03
CCMP 421	Free-living dinoflagellate	Wellington Harbour, New Zealand	E (E2)	7.21 ± 0.04	7.08 ± 0.03
Mv	<i>Montipora verrucosa</i>	Hawai'i, USA	F (F1)	7.23 ± 0.08	7.06 ± 0.17
Pd	<i>Pocillopora damicornis</i>	Hawai'i, USA	F (F5)	7.39 ± 0.02	7.12 ± 0.27

Fig. 1 Quantifying the pH_i of *Symbiodinium*. **a** Confocal microscope image of *Symbiodinium* cells loaded with 10 μM of the pH-sensitive BCECF-AM ester, imaged after excitation with both 405 and 473 nm lasers, with fixed emission at $535 \pm 10 \text{ nm}$. Scale bar represents 20 μm . **b** Single *Symbiodinium* cell imaged under the aforementioned

conditions. Scale bar represents 10 μm . While the dye consistently labelled cells, including their cytoplasm, note its uneven intra-cellular distribution. This was most likely caused by selective accumulation into certain organelles

artefact caused by selective accumulation in certain organelles (e.g. the nucleus and mitochondria; Weinlich et al. 1998) or could reflect regional pH differences between organelles (e.g. acidic endosomal vacuoles; Casey et al. 2009). Furthermore, *Symbiodinium* cells contain autofluorescent ‘inclusion bodies’ that, when excited by blue light, have the potential to interfere with fluorescence measurements (Kazandjian et al. 2008). When calibrating the dye with flow cytometry (Electronic Supplementary Material, ESM S1), we therefore used the logarithmic form of the in vivo calibration equation, as this minimises the impacts of non-uniform loading, as well as leakage of dye out of the cell and photo-bleaching (Bright et al. 1989). It is also important to note that flow cytometry averages the fluorescence signal across each cell and measures large numbers of cells simultaneously, so reducing errors caused by intra- and inter-cellular variability.

The effect of irradiance (12 h light adaptation at $80\text{--}100\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$ and 12 h of dark adaptation) on pH_i was quantified in freshly isolated and cultured *Symbiodinium* cells, and in *A. pulchella* host cells. For freshly isolated algal cells, individual anemones ($n = 10$ per treatment) were exposed to light or dark before being homogenised in 500 μL of FSW in a hand-held glass tissue grinder. The resulting slurry was transferred to a 1.5-mL Eppendorf tube and centrifuged for 5 min at $1,000\times g$, $25\ ^\circ\text{C}$ to separate the algal fraction from host tissue. The supernatant was discarded, and the *Symbiodinium* pellet washed twice in FSW. The pellet was re-suspended in FSW at a final density of 1×10^6 cells mL^{-1} and incubated with 10 μM of BCECF-AM ester and 0.01 % Pluronic F-127 for 20 min on an orbital shaker set to 250 rpm. The analysis of pH_i was conducted on a FACScan flow cytometer. Samples were excited with a 488 nm argon laser, and *Symbiodinium* cells were gated on particle size (FSC) and chlorophyll fluorescence ($>670\text{ nm}$, FL3) to exclude cellular debris. BCECF-AM fluorescence was measured in linear mode in the 515–545 nm (FL1) and 564–606 nm (FL2) filter channels, with 5,000 individual cells recorded per sample. Cultured cells were grown in 250-mL flasks before being exposed to the same light or dark treatment as before ($n = 3$ per treatment). An aliquot (10 mL) was sampled from each flask, centrifuged for 5 min at $1,000\times g$, and re-suspended in FSW to give a final density of 1×10^6 cells mL^{-1} . The samples were then dye-loaded and analysed as before, while maintaining them under their experimental conditions.

To quantify the pH_i of host cells, individual anemones ($n = 10$ per treatment) were exposed to light and dark as described above, before intact endodermal cells were isolated using the enzymatic maceration procedure described by Gates and Muscatine (1992). The cells were loaded with 10 μM of the cell-permeant acetoxymethyl ester acetate of

SNARF-4F, in the presence of 0.01 % Pluronic F-127 for 20 min, before being pipetted onto poly-D-lysine glass culture dishes and transferred to the confocal microscope (Fluoview 1000, Olympus, Pennsylvania, USA), where they were analysed via the method of Venn et al. (2009). SNARF has previously been used in coral endodermal cells (Venn et al. 2009) and provides a higher resolution than does BCECF-AM; it could be used on this occasion due to the lack of interference from chlorophyll autofluorescence in animal cells lacking symbionts. Detailed information of the in vivo calibration is provided in ESM S2.

Results and discussion

The pH_i of freshly isolated *Symbiodinium* cells and *A. pulchella* host cells was 7.10 ± 0.02 and 6.86 ± 0.04 (mean $\pm$ SE) in the dark, increasing significantly (two-way ANOVA, $F_{1,51} = 12.219$, $p = 0.001$) to 7.25 ± 0.01 and 7.02 ± 0.06 in the light, respectively. Both the host cell and the symbiont responded in the same manner to irradiance ($F_{1,51} = 0.064$, $p > 0.05$), though the pH_i of the host cell was significantly lower than that of the symbiont at all times ($F_{1,51} = 26.325$, $p < 0.0001$).

In the cultured *Symbiodinium* cells, genotypic differences in pH_i were apparent irrespective of the light/dark regime (two-way ANOVA, $F_{5,24} = 2.896$, $p = 0.035$). *Post hoc* analysis could not pinpoint where these genotypic differences lay (Tukey’s HSD, $p > 0.05$ for all comparisons), though, of note, types E2 and F1 generally had the lowest values in both the light and dark (Table 1). Light again significantly enhanced pH_i ($F_{1,24} = 73.784$, $p < 0.0001$), with a similar response witnessed in all *Symbiodinium* types ($F_{5,24} = 1.192$, $p = 0.343$).

Our study provides valuable information on pH_i in *A. pulchella*, an important model system for the study of the cnidarian–dinoflagellate symbiosis (Weis et al. 2008). However, more importantly, we describe a method for measuring pH_i in *Symbiodinium* and show genotypic diversity in this parameter. While this method provides resolution at the level of the whole cell rather than the specifics of the intra-cellular environment, it enables the rapid measurement of large numbers of cells simultaneously.

The pH_i values quantified in this study fall within those expected for eukaryotic cells, which typically maintain their cytosolic pH between 7 and 7.4 (Madhus 1988), and are similar to light-adapted pH_i values measured in other marine algae (Lane and Burris 1981). Furthermore, the more alkaline pH_i of *Symbiodinium* seen in the light than in the dark was consistent with the effect of light observed in higher plants (Yin et al. 1990). Light-induced alkalinisation is likely a by-product of physiological changes in the *Symbiodinium* cell’s cytoplasm during photosynthesis. In

particular, CO_2 and H_2O exist in equilibrium with carbonic acid (H_2CO_3), so when CO_2 is removed for photosynthesis, it causes an increase in the conversion of bicarbonate (HCO_3^-) to H_2CO_3 , a process that consumes protons (H^+) (Allemand et al. 1998). Conversely, the subsequent reduction of pH_i in the dark is likely to be a consequence of respiration, as the products, CO_2 and H_2O , react to form HCO_3^- and H^+ . It is therefore perhaps unsurprising that the pH_i of *Symbiodinium* cells, the sites of photosynthesis in the symbiosis, is higher than that of the surrounding host cell in the light. Why this pattern is also present in the dark, however, is at present unclear. One possibility is that dark aerobic respiration may be greater in the host cell than the symbiont, resulting in a build-up of CO_2 in the cell and thus reducing pH_i . Alternatively, the higher pH_i of the symbiont in the dark could be a consequence of residual dark or heterotrophic CO_2 fixation. Interestingly, the pH_i in the *A. pulchella* host cell mirrored the light-response seen in the symbiont. This has also been observed in host cells of the coral *Stylophora pistillata* and the anemone *Anemonia viridis* (Venn et al. 2009), highlighting the inter-relationship between symbiont and host physiology. Indeed, recent research suggests that the pH of the host cell is directly dependent on the photosynthetic activity of its algal symbiont, and, as such, any factor that either inhibits or enhances photosynthesis (e.g. irradiance) has the capability to influence the pH of the host cell (Laurent et al. 2012).

The physiological and genetic diversity within the genus *Symbiodinium* is now well known, though most studies have focused on photophysiological responses to environmental stresses (Fisher et al. 2012). We can now extend this physiological diversity to the pH_i . The basis for this difference is unknown as, while it might be expected that differences in photosynthetic performance and/or efficiency (Hennige et al. 2009) could lead to a difference in pH_i in the light, we found that genotypic differences were also maintained in the dark. Further research is needed to elucidate this matter, however, both in the *Symbiodinium* types tested here and across a wider range of types. Moreover, the implications of this physiological diversity for symbiosis function warrant investigation.

The methods and findings in our study build on the important work of Venn and co-workers (Venn et al. 2009), by providing a means of studying both partners in this ecologically important symbiosis, and addressing the many questions surrounding carbon transport (Brownlee 2009) and how the symbiosis may be impacted by the increasing global threat of ocean acidification.

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RESEARCH ARTICLE

Intracellular pH and its response to CO₂-driven seawater acidification in symbiotic *versus* non-symbiotic coral cells

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ABSTRACT

Regulating intracellular pH (pH_i) is critical for optimising the metabolic activity of corals, yet the mechanisms involved in pH regulation and the buffering capacity within coral cells are not well understood. Our study investigated how the presence of symbiotic dinoflagellates affects the response of pH_i to P_{CO₂}-driven seawater acidification in cells isolated from *Pocillopora damicornis*. Using the fluorescent dye BCECF-AM, in conjunction with confocal microscopy, we simultaneously characterised the pH_i response in host coral cells and their dinoflagellate symbionts, in symbiotic and non-symbiotic states under saturating light, with and without the photosynthetic inhibitor DCMU. Each treatment was run under control (pH 7.8) and CO₂-acidified seawater conditions (decreasing pH from 7.8 to 6.8). After 105 min of CO₂ addition, by which time the external pH (pH_e) had declined to 6.8, the dinoflagellate symbionts had increased their pH_i by 0.5 pH units above control levels when in the absence of DCMU. In contrast, in both symbiotic and non-symbiotic host coral cells, 15 min of CO₂ addition (0.2 pH unit drop in pH_e) led to cytoplasmic acidosis equivalent to 0.3–0.4 pH units irrespective of whether DCMU was present. Despite further seawater acidification over the duration of the experiment, the pH_i of non-symbiotic coral cells did not change, though in host cells containing a symbiont cell the pH_i recovered to control levels when photosynthesis was not inhibited. This recovery was negated when cells were incubated with DCMU. Our results reveal that photosynthetic activity of the endosymbiont is tightly coupled with the ability of the host cell to recover from cellular acidosis after exposure to high CO₂/low pH.

KEY WORDS: *Symbiodinium*, Acidosis, Climate change

INTRODUCTION

Reef-building corals depend on a symbiotic association with photosynthetic dinoflagellates of the genus *Symbiodinium* for survival. This intimate partnership evolved in the mid-Triassic period (Muscattine et al., 2005) and coral reefs have prospered in tropical oceans, particularly in areas characterised by high degrees of environmental stability (Hoegh-Guldberg, 1999). Consequently, corals have adapted to live within narrow physiological limits, and are highly sensitive to fluctuations in the surrounding environment (Jones et al., 1998). Rising sea surface temperatures (Hoegh-Guldberg et al., 2007) and the increasing acidity of the ocean (Orr et al., 2005) are threatening the stability of coral–dinoflagellate symbioses, leading to dire projections for the future of coral reefs (Pandolfi et al., 2011; Silverman et al., 2009). At present, our ability

to accurately predict the response of corals to global climate change is severely hampered by our limited understanding of the cellular mechanisms that underpin coral–dinoflagellate symbiosis (Fabry et al., 2008; Weis et al., 2008; Davy et al., 2012), which ultimately frame how corals respond to environmental stress.

Intracellular pH (pH_i) is crucial for virtually all elements of cellular homeostasis (Smith and Raven, 1979), directly influencing protein structure, enzymatic rates and membrane solubility (Madhus, 1988). The maintenance of pH within an optimal functional range therefore plays a critical role in determining the metabolic activity of the cell, and is specific to the metabolic pathway in question. Thus, eukaryotic cells have evolved compartmentalised organelles to provide sites with specific pH conditions within the cell for different metabolic activities to occur (Casey et al., 2010). Disruption of pH_i has serious physiological consequences (Pörtner et al., 2004; Fabry et al., 2008; Hofmann et al., 2013). Indeed, a drop as small as 0.1–0.2 pH_i units can induce metabolic depression (Reipschläger and Pörtner, 1996), so it is not surprising that changes in pH_i are stringently avoided (Casey et al., 2010). In general, eukaryotic cells are protected from fluctuations in their pH_i at two levels. Acute, localised changes in pH_i, such as those arising from metabolic reactions, are neutralised by manipulating the various weak acids and bases in the cytosol (Casey et al., 2010). Longer-term changes are buffered by more permanent mechanisms such as transmembrane exchangers (Boron, 2004). The mechanisms involved in pH regulation are not well understood in corals, and this is partly due to the intrinsic complexity associated with their endosymbiosis. By virtue of their intracellular location, the *Symbiodinium* cells can essentially be regarded as heavily fortified organelles belonging to the coral host cell. However, unlike other organelles, *Symbiodinium* cells, through their photosynthetic activity, are able to exert significant control over the pH of the host cell (Venn et al., 2009; Laurent et al., 2013). Consequently, the response of corals to a change in ambient CO₂/pH is likely to be influenced by their own physiological capacity and that of their symbionts (McCulloch et al., 2012).

Here, we investigated how the presence/absence of symbionts affects the response of pH_i to CO₂-driven seawater acidification in cells isolated from the Hawaiian reef coral *Pocillopora damicornis* (Linnaeus 1758). Using the fluorescent dye BCECF-AM, in conjunction with live cell imaging, we characterised the response of pH_i (NBS scale) in *Symbiodinium* cells freshly isolated from coral hosts, isolated non-symbiotic coral cells, and isolated coral host cells with their symbionts enclosed. The cells were exposed to control seawater (pH 7.8) and CO₂-acidified seawater, designed to expose the cells to a gradient of declining external pH (pH_e 7.8–6.8) over a 105 min period, to mimic diurnal changes in pH in reef water due to reef photosynthesis, respiration and calcification (Hofmann et al., 2011; Price et al., 2012), which are particularly strong in Kaneohe Bay, Hawaii (Putnam, 2012). In both treatments, the cells were exposed to saturating white light in the presence and absence of the

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Fig. 1. Light and confocal microscopy images of host *Pocillopora damicornis* cells and *Symbiodinium* cells. The pH-sensitive dye BCECF-AM ester was used to monitor intracellular pH (pH_i) of cells isolated from *P. damicornis* after 105 min of exposure to either control pH conditions (left) or increasing CO_2 (right). (A–C) Symbiotic state of the cell: (A) *Symbiodinium* freshly isolated from host coral cell; (B) isolated non-symbiotic host coral cell; and (C) isolated host coral cell containing two symbiotic algae. The columns show the time-delayed integration (TDI) light micrograph and images from the different treatments (from left to right): control conditions (pH 7.8) in the absence of the photosynthetic inhibitor DCMU (–DCMU); control conditions (pH 7.8) in the presence of DCMU (+DCMU); increasing CO_2 (pH 6.8) in the absence of DCMU (–DCMU); and increasing CO_2 (pH 6.8) in the presence of DCMU (+DCMU). All experiments were conducted under saturating ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) photosynthetically active radiation (PAR). Scale bars represent $5 \mu\text{m}$.

photosynthetic inhibitor DCMU, with measurements of both pH_i and pH_e taken every 15 min. Our findings demonstrate that CO_2 addition initiates a very different response in the pH_i of the symbiont compared with that of the coral host cell. Crucially, we show that the photosynthetic activity of the symbiont plays a key role in determining the intracellular buffering capacity of its coral host cell to changes in pH_e .

RESULTS

We analysed the relative change in pH_i (calculated as the pH_i after acidification minus the mean control pH_i) in three symbiotic states – *Symbiodinium* cells freshly isolated from coral hosts, isolated non-symbiotic coral cells, and isolated coral host cells with their symbionts enclosed (Fig. 1) – across a gradient of pH_e under light, in the presence and absence of the photosynthetic inhibitor DCMU (Fig. 2). Actual changes in pH are provided in supplementary material Fig. S1. Initial analysis of the dataset confirmed that there was a significant interaction between external $\text{pH} \times$ symbiotic state $\times$ DCMU treatment [repeated measures (rm)ANOVA, $F_{15,03,160,34}=4.60$, $P<0.001$], so subsequent analyses were carried out on the two separate treatments (no DCMU and DCMU added). The response of pH_i to pH_e was dependent on the symbiotic state of the cell in both the presence (rmANOVA, $F_{14,23,75,87}=7.51$, $P<0.001$) and absence of DCMU (rmANOVA, $F_{11,15,59,4}=14.80$, $P<0.001$). Subsequent *post hoc* analyses revealed where the differences in the response of pH_i to acidification lay (Table 1).

The disparities in pH_i were driven primarily by the opposing reactions of the host coral cells and the algal cells to acidification. Within 15 min of CO_2 addition, the pH_i of both the isolated symbiotic and non-symbiotic host coral cells decreased by 0.3–0.4 pH units, irrespective of whether DCMU was present (Fig. 2). Following this initial drop, the response of pH_i to further

acidification was dependent on the symbiotic state of the host cell, and the DCMU treatment it was exposed to. There was no change in the pH_i of the non-symbiotic host cell in either treatment (Fig. 2). Similarly, there was no change in the pH_i of the symbiotic host cell in the presence of DCMU (Fig. 2B). In contrast, without DCMU, the pH_i of the host cell increased over time when in symbiosis with its dinoflagellate partner, returning to control levels within 75 min (when the pH_e reached pH 7). The response of the pH_i of the dinoflagellate symbiont to the addition of CO_2 differed between the DCMU treatments (Fig. 2), irrespective of whether the alga was in isolation or in symbiosis (Table 1). In the presence of DCMU there was no change in the pH_i of either the isolated or symbiotic algae (Fig. 2B), whereas without DCMU both the isolated and symbiotic algae were able to increase their pH_i relative to control levels (Fig. 2A).

DISCUSSION

Understanding the coral–dinoflagellate symbiosis is pivotal to accurately predicting the susceptibility of coral reefs to climate change and ocean acidification (Weis et al., 2008; Davy et al., 2012). Recent ecological research suggests that the symbiotic state may play a critical role in determining a coral's capacity to tolerate changes in seawater chemistry (Ohki et al., 2013). It is well established that photosynthesis in the dinoflagellate symbiont enhances host coral calcification rates in the light (Allemand et al., 2004). At the cellular level, however, a more important role may be the ability of the symbiont to manipulate aspects of the host's physiology, such as cellular pH (Venn et al., 2009; Laurent et al., 2013). As yet, no study has investigated how this relationship will be impacted by acidification. We addressed this knowledge gap by simultaneously quantifying pH_i in both partners of the symbiosis in response to CO_2 addition. Our experimental design exposed isolated

Fig. 2. The impact of CO₂ addition on pH_i in *P. damicornis* cells and *Symbiodinium* cells. The figure shows the relative change in pH_i after acidification (the pH_i value after acidification minus the mean pH_i of the control at each time point, means ± s.e.m., *N*=5) under two treatments: (A) saturating white 400 μmol photons m⁻² s⁻¹ PAR; (B) saturating white 400 μmol photons m⁻² s⁻¹ PAR in the presence of the photosynthetic inhibitor DCMU. Data are shown for *Symbiodinium* freshly isolated from the host coral cell; the isolated non-symbiotic host coral cell; and the isolated host coral cell and its symbiotic algae. The dashed line plotted on the secondary y-axis represents the pH of the surrounding medium (external pH, pH_e) at each time point (*N*=5).

cells to a wide range of pH_e, at levels of CO₂ much greater than those predicted to arise from ocean acidification. These treatments were not designed to replicate climate change scenarios but rather

to provide a means of determining how the symbiotic state influences the recovery of its coral host cell under induced cellular acidosis. Our results reveal that the photosynthetic activity of the symbiont increases the ability of the host cell to recover from cellular acidosis after exposure to high CO₂/low pH. The responses seen in the various symbiotic states are summarised in Fig. 3 and are discussed in more detail here.

CO₂ and H₂O exist in equilibrium with carbonic acid (H₂CO₃), so when CO₂ is removed for photosynthesis it causes an increase in the conversion of bicarbonate (HCO₃⁻) to H₂CO₃, a process that consumes protons (H⁺) (Allemand et al., 1998). Conversely, an increase in CO₂ will reverse the process, resulting in the production of H⁺ and leading to cellular acidosis. The pH_i of freshly isolated *Symbiodinium* cells is therefore strongly influenced by the availability of CO₂ in the surrounding seawater (Nimer et al., 1999) (Fig. 3A). Our results clearly show that, upon CO₂ addition, *Symbiodinium* cells are able to increase their pH_i relative to the control, by up to 0.5 pH units. This alkalisation demonstrates the capacity of *Symbiodinium* cells to strongly buffer the external environmental pH signal, probably due to a fertilising effect on photosynthesis in these normally CO₂-limited algae (Nimer et al., 1999). An increase in photosynthetic productivity after CO₂ addition has also been observed in other symbiotic associations, most notably in the temperate sea anemones *Anemonia viridis* (Suggett et al., 2012) and *Anthopleura elegantissima* (Towanda and Thuesen, 2012), and the benthic foraminiferan *Marginopora vertebralis* (Uthicke and Fabricius, 2012). The application of DCMU (a photosynthetic inhibitor) reversed the increase in pH_i, confirming that the change was a direct consequence of photosynthesis, as the inhibited photosynthetic machinery of the symbionts is not able to ameliorate the increasing H⁺ concentration (Fig. 3A).

CO₂ supplementation initiated a markedly different response in the host coral cells compared with the symbiont. In the host cells, acidosis of the cytoplasm was observed within 15 min of CO₂ addition, with a decline in pH_e of 0.2 pH units causing the pH_i to fall 0.3–0.4 pH units below the usual pH of the cell, irrespective of the presence or absence of DCMU and the symbiotic state of the cell (Fig. 3B). This strongly suggests that the host’s intrinsic buffering capacity is, initially at least, overwhelmed by the accumulation of protons resulting from the passive diffusion of CO₂, and active transport of HCO₃⁻ into the cell (Furla et al., 2000), which drives the

Table 1. Post hoc results of paired *t*-tests following rmANOVA, showing the effect of symbiotic state × external pH on pH_i at each time point

Treatment	Pair	P-value							
		0 min	15 min	30 min	45 min	60 min	75 min	90 min	105 min
Light (–DCMU)	1	–	–	–	–	*	*	*	*
	2	–	*	*	*	*	*	*	*
	3	–	*	*	*	*	*	*	*
	4	–	*	*	*	–	*	*	*
	5	–	*	*	*	*	*	*	*
	6	–	–	–	*	*	–	–	–
Light (+DCMU)	1	–	–	–	–	–	–	–	–
	2	–	–	–	*	*	–	–	*
	3	–	–	–	*	*	–	–	*
	4	–	–	–	*	*	*	*	–
	5	–	–	–	*	*	*	*	*
	6	–	–	–	–	–	–	–	–

Asterisks indicate a significant interaction between the paired cell types (*P*<0.001). Pairs are as follows: 1, non-symbiotic coral cell versus symbiotic coral cell; 2, non-symbiotic coral cell versus isolated algal cell; 3, non-symbiotic coral cell versus symbiotic algal cell; 4, symbiotic coral cell versus isolated algal cell; 5, symbiotic coral cell versus symbiotic algal cell; and 6, symbiotic algal cell versus isolated algal cell. Only Bonferroni-corrected significant interactions (α=0.001) are included.

Fig. 3. Model of pH_i fluctuations in cells isolated from *P. damicornis*. Cells were exposed for 105 min to either control pH conditions (left) or increasing CO₂ (right) according to the state of the equilibrium reaction that exists between $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+$. (A–C) Symbiotic state of the cell: (A) *Symbiodinium* freshly isolated from host coral cell; (B) isolated non-symbiotic host coral cell; and (C) host coral cell containing symbiotic algae. The columns show the different treatments (from left to right): control conditions (pH 7.8) in the absence of the photosynthetic inhibitor DCMU (–DCMU); control conditions (pH 7.8) in the presence of DCMU (+DCMU); increasing CO₂ (pH 6.8) in the absence of DCMU (–DCMU); and increasing CO₂ (pH 6.8) in the presence of DCMU (+DCMU). All experiments were conducted under saturating (400 μmol photons m⁻² s⁻¹) PAR. Dashed arrows show passive CO₂ diffusion. Sunburst symbol represents photosynthetic processes and CA represents carbonic anhydrase. Colours indicate pH as identified in the colour gradient above. With increasing seawater CO₂ there is a shift in carbonate chemistry equilibrium, leading to an accumulation of internal H⁺ in the absence of photosynthesis. Note that for simplicity not all active inorganic carbon uptake pathways are shown.

aforementioned equilibrium reaction. The decline in pH_i, however, was halted after 15 min in both the symbiotic and non-symbiotic host cells. It is likely that this represents a time lag between the onset of acidosis and the activation of the regulatory membrane transporters. Indeed, such activity could explain the subsequent stability (~pH 6.6) of pH_i in non-symbiotic host cells, which was achieved irrespective of the surrounding seawater being subject to further acidification. Nevertheless, the pH_i of these non-symbiotic host cells never recovered to pre-acidosis levels. In contrast, the pH_i of symbiotic host cells showed a full recovery to control pH levels within 105 min of CO₂-addition (Fig. 3C). Again, this recovery was negated in the presence of DCMU, confirming that the photosynthetic removal of CO₂ by the symbiont (and hence the consumption of protons) was responsible for the increase in the host's pH_i.

These results confirm that the symbiont is able to exert a significant level of control over its host's cellular pH, corroborating the findings of previous research on reef corals (Laurent et al., 2013). Furthermore, they demonstrate that the photosynthetic activity of the symbionts plays a key role, at least in the short term, in regulating the cellular response of their host to external CO₂-driven acidification. Perhaps more importantly, however, the inability of non-symbiotic or photosynthetically compromised symbiotic host cells to recover from cellular acidosis in the short term (at least under the experimental conditions used here) suggests that they may be more susceptible than are host cells that contain fully functional *Symbiodinium* cells. However, whether the cells

have the potential for recovery in the longer term, especially under constant pH, warrants further investigation. While some caution needs to be exercised when interpreting the results for the non-symbiotic coral cells, as we cannot be sure of their precise origin (i.e. ectodermal or endodermal), it is notable that the symbiotic endodermal host cells responded in the same manner when photosynthesis was inhibited. This limited capacity to withstand acidosis in the absence of a functional symbiont could therefore be a general response. If this is the case, it raises the possibility that the number of *Symbiodinium* cells that a coral host cell contains may also influence the cellular response to acidification. In *Stylophora pistillata*, for example, the majority of host cells (~60%) typically contain one algal cell, while fewer contain two (~35%) or more (<5%) (Houlbrèque et al., 2004). Given that we focused on host cells that harboured two algal symbionts only, there is a need for future studies that clarify the influence of symbiont number on pH_i, and the overall response at the organismal level.

The regulation of pH_i in corals is an important area for future research as cellular acidosis has serious physiological repercussions for the fitness of the individual. At a biochemical level, acidosis disrupts ion transport, nutrient trafficking and carbon acquisition (Fabry et al., 2008), causing metabolic suppression (Pörtner et al., 2004) and inevitably leading to shortfalls in the energy available for other cellular processes (Reipschläger and Pörtner, 1996). In addition to these fundamental cellular attributes, regulating pH_i is particularly important in reef-building corals for calcification. In the recently proposed 'proton flux hypothesis', Jokiel presented the first

organism-scale model of acid–base balance in corals (Jokiel, 2011). The hydroxide ions (OH^-) produced as an indirect by-product of CO_2 removal (Furla et al., 2000) are proposed to play a critical role in calcification, neutralising protons released by $\text{H}^+/\text{Ca}^{2+}$ ion exchangers in the gastrovascular cavity, and thus facilitating a pH gradient high enough for the precipitation of CaCO_3 (Jokiel, 2011; Comeau et al., 2013). Acidosis of the host coral cells could therefore place further chemical and energetic constraints on corals by affecting their ability to calcify (Jokiel, 2011; Venn et al., 2013). It remains to be seen whether a non-symbiotic host cell can reverse the changes in pH_i over a longer time frame, though this might depend, in part, on its ability to upregulate the expression of membrane transporters (Kaniewska et al., 2012), as this will afford greater control over pH_i . Indeed, several organisms are able to reverse initial decreases in pH_i (Michaelidis et al., 2005; Stumpp et al., 2012).

The ability of corals to respond to external pH change is likely to depend on the response of both the symbionts and coral host (McCulloch et al., 2012). There is considerable diversity within the genus *Symbiodinium* (Pochon and Gates, 2010), which translates into substantial genotypic differences in physiology (Tchernov et al., 2004; Hennige et al., 2009; Brading et al., 2011; Gibbin and Davy, 2013). Our experiment used genetically identical fragments from a single colony. This had two advantages: firstly, it minimised the baseline variation in host cellular activity; and secondly, it reduced the chances of sampling corals that contained very different *Symbiodinium* clades. However, future research should aim to determine whether the host and symbiont responses, and their relative abilities to buffer the effects of CO_2 -driven acidification, vary according to host and symbiont genotype. In addition, it will be important to determine whether the patterns we observed in isolated coral cells are replicated in intact coral tissues, and whether longer-term incubations at more moderate levels of acidification (i.e. predicted climate change scenarios) induce similar responses to those reported here. Nevertheless, our model system elucidates the interrelationship between symbiont photosynthesis and the capacity of host coral cells to withstand CO_2 -driven acidification. Moreover, our findings highlight the possibility that bleached corals may be more sensitive to cellular acidosis than are their non-bleached counterparts; this is an especially interesting topic for future research.

MATERIALS AND METHODS

Coral collection and maintenance

One large normally pigmented adult *P. damicornis* colony was collected 3 weeks prior to experimentation (in February 2013) from a shallow fringing reef (<3 m) in Kaneohe Bay, Hawaii. This single colony was cut into 50 genetically identical fragments (4×2 cm) that were secured to 3×3 cm plastic tiles with underwater epoxy (Z-spar, Splash Zone compound) and placed in a 50 l holding tank supplied with flowing seawater from Kaneohe Bay. Seawater chemistry was monitored frequently according to the recommended best practices for ocean acidification research and reporting (Riebesell et al., 2010), with daily measurements of salinity (psu) as well as pH (NBS scale), taken via the *m*-Cresol dye method stipulated in SOP 6B (Dickson et al., 2007); total alkalinity (TA) was measured on a weekly basis (Dickson et al., 2007). These characteristics were stable for the duration of the experiment, with an average salinity of 35.5 ± 0.1 ppt, pH of 7.8 ± 0.1 and a TA of 2166 ± 25 (means $\pm$ s.e.m., $N=5$). It is important to note that the P_{CO_2} of seawater in Kaneohe Bay is markedly higher than average oceanic conditions (Drupp et al., 2011), resulting in a lower ambient pH. An ambient seasonal temperature of $22.6 \pm 0.3^\circ\text{C}$ was maintained by a dual-stage temperature controller (Aqualogic, TR115DN), while tanks were illuminated on a 12 h:12 h light/dark cycle by metal halide lights (Ice CapMetal Halide lights, 250 W DE 14K bulbs, 250 W double-ended pendants), which were

mounted on motorised light rails and provided irradiances ranging between 3.85 and $328.85 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, corresponding to a mean irradiance of $125 \pm 10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ over each coral fragment.

Experimental design

Four experimental treatments were designed to investigate how CO_2 addition influenced pH_i (NBS scale) in cells isolated from the coral *P. damicornis*. Three symbiotic states were tested: (1) isolated *Symbiodinium* cells; (2) isolated non-symbiotic host coral cells; and (3) isolated host coral cells containing their symbiotic algae (Fig. 1). Visual inspection (by both light and confocal microscopy) failed to establish conclusively whether isolated *Symbiodinium* cells were surrounded by an intact symbiosome membrane. Non-symbiotic host coral cells were classified as intact host cells not containing an algal symbiont. Typically $10 \mu\text{m}$ in diameter and spherical, it is not known whether these cells were ectodermal or endodermal in origin (see Discussion). Finally, only symbiotic cells containing two algal cells were used for calculating the pH_i change in symbiotic host coral cells. These were deemed the most suitable choice for two major reasons: (1) the host cell region of interest (ROI) is much larger in doublet cells than in host cells containing a single alga, making pH_i measurements much easier (Venn et al., 2009); and (2) this cell type is found in much greater abundance than triplet cells (Houlbrèque et al., 2004). Furthermore, standardisation of symbiont number allowed for the possibility that this parameter influences host pH_i (see Discussion).

The cell and dye loading procedure was repeated five times for each treatment ($N=5$) to achieve independent replicate cell preparations. Individual cells were then imaged every 15 min for 105 min, with pH_i calculated from the images taken. At each experimental time point, 1.5 ml of seawater was carefully removed by pipette so as not to disrupt the cells and analysed for pH_e (NBS scale, $N=5$) via the *m*-Cresol dye method described in SOP 6B (Dickson et al., 2007). The treatments were carried out under an external white light source, provided by a variable-irradiance fibre optic cable (Halogen Reflector lamp, 150 W GX5.3 21V 1CT bulb, Philips, Somerset, NJ, USA) that produced a saturating irradiance of $\sim 400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ photosynthetically active radiation. Previous studies have shown that photosynthetic activity in the symbiont modifies the pH_i of the host under normal CO_2 conditions (Venn et al., 2009), so a preliminary measurement was performed with no additional CO_2 added, in order to establish the control or baseline change in pH_i caused by photosynthesis. This experimental run was then repeated with CO_2 addition (5.0% setting on the incubation unit of the LSM 710 confocal microscope, Carl Zeiss, Oberkochen, Germany), resulting in a decreasing gradient of pH_e over time. The negative controls were run in the light, as before, under zero and high CO_2 conditions, but in the presence of $100 \mu\text{mol l}^{-1}$ 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in 0.1% acetone, a photosynthetic inhibitor that blocks the plastoquinone binding site of photosystem II and thus prevents the transfer of electrons and formation of ATP. DCMU has been used extensively to study photosynthesis in *Symbiodinium*, as it effectively blocks photosynthesis without impairing cellular functioning (Iglesias-Prieto et al., 1992).

Isolation of cells and dye-loading procedure

Coral fragments were selected randomly from the acclimation tank before the start of each experimental run. Cells were isolated immediately by gently brushing the tip of a partially submerged fragment in 50 ml of $0.22 \mu\text{m}$ filtered seawater (FSW) using a soft bristle toothbrush. The resulting slurry was centrifuged for 5 min at 1700 g . The supernatant was discarded and the pellet re-suspended in a further 50 ml FSW. An additional centrifugation step was introduced to wash the pellet and remove any residual host-generated mucus. This time, when the supernatant was discarded, the pellet was resuspended in 1 ml FSW containing $10 \mu\text{mol l}^{-1}$ BCECF-AM ester and 0.01% Pluronic F-127 with/without $100 \mu\text{mol l}^{-1}$ DCMU and 0.1% acetone depending on the treatment in question, to give a final concentration of $\sim 1 \times 10^6$ cells ml^{-1} . The dye-loaded cell suspension was then transferred to a 35 mm poly-D-lysine-coated Petri dish (MatTek Corporation, Ashland, MA, USA) and placed on the stage of a confocal microscope (LSM 710), where the cells were left to settle for 30 min in the dark at 22°C . After dye loading,

the cells were carefully washed twice in 1 ml FSW to remove any residual dye without dislodging cells from the surface of the dish. Finally, 6 ml FSW was added and the dish was placed in a closed-exchange live-cell chamber (PeCon, Erbach, Germany) before experimental treatments were carried out. All dyes used for microscopy were purchased from Invitrogen (Grand Island, NY, USA).

Measurement of pH_i by confocal microscopy

The fluorescent dye BCECF-AM, in conjunction with confocal microscopy, has been widely used to study pH_i in marine algae (Hervé et al., 2012) as its dual-excitation spectral properties allow fluorescence measurements to be taken that are not compounded by chlorophyll autofluorescence (>640 nm). In this study, confocal microscopy was conducted on a LSM 710 confocal microscope equipped with UV and visible laser lines. Cells loaded with BCECF-AM were sequentially excited, first at 458 nm then at 488 nm, both with laser strength set at 10% and pinhole set at 1.51 units using an X63-fold oil-immersion lens. Under both excitations, fluorescence emission was captured at 525±10 nm by imaging the z-stack profile of each cell 10 times in the x/y plane. BCECF-AM is able to enter both the host and the symbiont, and can therefore be used for imaging of both compartments of an intact symbiosis. However, the signal is much stronger in the host cell than in the symbiont, probably due to the thick cell wall that surrounds the symbiont. Therefore, prior optimisation of laser settings was essential to maximise the signal strength in the algae, without overexposing the host cell. *In vivo* calibration was carried out on each partner in the intact symbiosis to yield two separate calibration curves, one for the symbiont and one for the host (supplementary material Fig. S2). The calibration series is dependent on the calculation of the fluorescence intensity ratio (R ; F_{488}/F_{458}) after cells are suspended in buffers of a known pH (pH 6–8.5) in the presence of 5 μmol l⁻¹ nigericin (Venn et al., 2009). This R value can then be linked to pH_i by the logarithmic equation $\text{pH} = \text{pK}_a + \log\{[(R - R_A)/(R_B - R)] \times (F_{A,458}/F_{B,458})\}$, where pK_a represents the acid dissociation constant and A and B represent the acidic and basic end points of the titration. To check the level of background fluorescence in the sample, cells were loaded separately with 10 μmol l⁻¹ BCECF-free acid (in the presence of 0.1% DMSO and 0.01% Pluronic F-127), a membrane-impermeant form of the dye. Background fluorescence was negligible, with no accumulation in either the symbiont or host cells.

Manipulation of pH_e

pH_e was manipulated via *in vitro* addition of 99% CO₂ in a fully adjustable CO₂/temperature-controlled live-cell chamber attached to an Axiovert 200 microscope. The amount of CO₂ that was added was controlled using Zen 2011 software (Carl Zeiss), with the pre-defined volume mixed in a CO₂ module and directly injected into the chamber. The final pH_e and the time taken for the pH_e to stabilise were dependent on the amount of CO₂ added (supplementary material Fig. S3). We selected the CO₂ injection setting that produced a gradient of pH_e that spanned one pH unit and stabilised below pH 7. This also provided an optimal time frame for monitoring pH_i, as experiments lasting longer than 2 h are often impacted by photo-bleaching or dye-leakage from the cells (Musgrove et al., 1986). The temperature of the microscope stage was maintained at a constant 22°C by a custom-made Plexiglas incubator encasing the entire system.

Statistics

Data were analysed as the relative change in pH_i (the pH_i value after acidification minus the mean pH_i of the control at each time point). Actual pH values are provided in supplementary material Fig. S1. The effect of the symbiotic state (between-subject factor), external pH and external pH × symbiotic state (both within-subject factors) were analysed using rmANOVA. Initial analysis of the dataset confirmed that there was a significant interaction between external pH × symbiotic state × DCMU treatment (rmANOVA, $F_{15.03,160.34}=4.60$, $P<0.001$), so subsequent analyses were carried out on the two separate treatments (no DCMU and DCMU added). Likewise, *post hoc* analysis was carried out at the treatment level using Bonferroni-corrected paired *t*-test comparisons ($\alpha=0.001$). The assumptions of normality were confirmed using the Kolmogorov–Smirnov test. The sphericity of the data was tested using Mauchly's sphericity test.

Epsilon-adjusted univariate *F*-test (Greenhouse–Geisser) values are reported. All data were analysed using JMP 10.0.0 (SAS Institute Inc., USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

E.M.G., H.M.P., S.K.D. and R.D.G. designed the research; E.M.G. performed the research; E.M.G. and H.M.P. analysed the data; and E.M.G., H.M.P., S.K.D. and R.D.G. wrote the paper.

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Supplementary material

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The photo-physiological response of a model cnidarian–dinoflagellate symbiosis to CO₂-induced acidification at the cellular level

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ABSTRACT

We measured the relationship between CO₂-induced seawater acidification, photo-physiological performance and intracellular pH (pH_i) in a model cnidarian–dinoflagellate symbiosis – the sea anemone *Aiptasia* sp. – under ambient (289.94 ± 12.54 μatm), intermediate (687.40 ± 25.10 μatm) and high (1459.92 ± 65.51 μatm) CO₂ conditions. These treatments represented current CO₂ levels, in addition to CO₂ stabilisation scenarios IV and VI provided by the Intergovernmental Panel on Climate Change (IPCC). Anemones were exposed to each treatment for two months and sampled at regular intervals. At each time-point we measured a series of physiological responses: maximum dark-adapted fluorescent yield of PSII (F_v/F_m), gross photosynthetic rate, respiration rate, symbiont population density, and light-adapted pH_i of both the dinoflagellate symbiont and isolated host anemone cell. We observed increases in all but one photo-physiological parameter (P_{gross}:R ratio). At the cellular level, increases in light-adapted symbiont pH_i were observed under both intermediate and high CO₂ treatments, relative to control conditions (pH_i 7.35 and 7.46 versus pH_i 7.25, respectively). The response of light-adapted host pH_i was more complex, however, with no change observed under the intermediate CO₂ treatment, but a 0.3 pH-unit increase under the high CO₂ treatment (pH_i 7.19 and 7.48, respectively). This difference is likely a result of a disproportionate increase in photosynthesis relative to respiration at the higher CO₂ concentration. Our results suggest that, rather than causing cellular acidosis, the addition of CO₂ will enhance photosynthetic performance, enabling both the symbiont and host cell to withstand predicted ocean acidification scenarios.

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1. Introduction

At 380 ppm, present-day atmospheric CO₂ concentrations are higher than at any point in the past 740,000 years (Hoegh-Guldberg et al., 2007), and are predicted to reach 500–1000 ppm by 2100 (Vuuren et al., 2011). It is estimated that approximately one third of all anthropogenic CO₂ is sequestered by the oceans (Sabine et al., 2004). This continuous dissolution of CO₂ lowers the pH of seawater and alters the speciation of dissolved inorganic carbon (DIC), in a process known as ocean acidification. Such changes are of great concern for marine ecosystems, but are particularly pertinent for those in which calcifying

organisms play a major role (Fabry et al., 2008). Coral reefs, therefore, which are recognised for their high diversity and productivity (Bellwood and Hughes, 2001), have received considerable attention (Crook et al., 2012; Fabricius et al., 2011; Hall-Spencer et al., 2008).

Predicting the response of reef-building corals to ocean acidification is complicated by the presence of the single-celled photosynthetic dinoflagellates (*Symbiodinium* spp.) that they harbour within their gastrodermal tissue layer. The photosynthetic products that are supplied by these dinoflagellate symbionts are of great metabolic importance to their host, facilitating growth, reproduction and, ultimately, survival (Muscatine, 1990). Therefore, the adaptive capacity of corals is not only determined by the responses of the host but also those of the symbionts it contains (McCulloch et al., 2012). Understanding the cellular mechanisms that underpin the complex interrelationship between the symbiont and host is a vital step towards understanding why the physiological response to CO₂-addition differs so substantially between species (Anthony et al., 2008; Kroeker et al., 2010; Pandolfi et al., 2011; Ries et al., 2009). Of specific interest in this context is characterising intracellular pH (pH_i) under normal conditions and determining its response to ocean acidification (Brownlee, 2009; Schneider and Erez, 2006).

Acid–base regulation is an essential facet of cellular metabolism in all organisms (Busa and Nuccitelli, 1984), but is particularly important

Abbreviations: BCECF-AM ester, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; BSA, bovine serum albumin; CaFSW, calcium-free seawater; CCM, carbon-concentrating mechanism; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DIC, dissolved inorganic carbon; DMSO, dimethyl sulfoxide; F_v/F_m, maximum dark-adapted yield of photosystem II; IPCC, intergovernmental panel on climate change; PAR, photosynthetically active radiation; P_{gross}, gross photosynthesis rate; pH_i, intracellular pH; P_{net}, net photosynthesis rate; PSII, photosystem II; R, respiration rate; ROI, region of interest; SNARF-4F, 5-(and-6)-carboxylic acid, acetoxymethyl ester acetate.

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for symbiotic anthozoan cells that are subjected to significant fluctuations in pH_i through the photosynthetic and respiratory activity of their symbionts (Gibbin and Davy, 2013; Laurent et al., 2013a; Venn et al., 2009). Deviation from the optimal pH_i, a process known as acidosis, often leads to cellular dysfunction (Pörtner et al., 2004), and while short-term acidosis can typically be recovered from (Fabry et al., 2008), longer periods of exposure can result in metabolic depression (Reipschläger and Pörtner, 1996) and reduced rates of protein synthesis (Hand, 1991), which are detrimental to fitness. We currently know little about how the cnidarian–dinoflagellate symbiosis might respond to CO₂-induced acidification at the cellular level. Indeed, there have been just two studies to focus on this, in which short-term experimental perfusions (<2 h) of isolated, symbiotic gastrodermal cells from the sea anemone *Anemonia viridis* in HCl-acidified seawater (Laurent et al., 2013b) and the coral *Pocillopora damicornis* in CO₂-acidified seawater (Gibbin et al., 2014) revealed no detrimental effects on the pH_i of the host cell, and host and symbiont cells, respectively. On the contrary, there is growing evidence that increased CO₂ availability associated with ocean acidification could enhance primary productivity in *Symbiodinium* cells (Brading et al., 2011; Jarrold et al., 2013; Marubini et al., 2008; Suggett et al., 2012; Towanda and Thuesen, 2012), and thus may limit the potential for host cell acidosis (Gibbin et al., 2014). This photosynthetically driven up-regulation of pH_i under high CO₂ has been postulated as one potential mechanism for countering the negative impacts of acidification on coral calcification (Ries et al., 2009). Yet, to date, no study has examined the longer-term impacts of CO₂-addition on both host and symbiont pH_i in the cnidarian–dinoflagellate symbiosis. This is an essential step if we are to fully appreciate the impacts that ocean acidification might have on this ecologically important association.

Here, we investigated the relationship between photo-physiology and pH_i under long-term CO₂ addition. To achieve this, we exposed the sea anemone *Aiptasia* sp., a model system for the study of cnidarian–dinoflagellate symbiosis (Weis et al., 2008), to conditions representative of the intermediate (IV; 520–700 ppm) and high-end (VI; 1000–1300 ppm) CO₂ stabilisation scenarios provided by the Intergovernmental Panel on Climate Change (IPCC, 2007). Over a two-month period we measured a suite of physiological responses: maximum dark-adapted fluorescent yield of PSII (F_v/F_m), gross photosynthetic rate, respiration rate, symbiont population density, and pH_i of both the dinoflagellate symbiont and host anemone. We tested the prediction that, rather than causing cellular acidosis, the addition of CO₂ would enhance photosynthetic performance, enabling both the symbiont and host cell to withstand the predicted ocean acidification scenarios.

2. Materials and methods

2.1. Experimental organisms

A long-term aquarium stock of the sea anemone *Aiptasia* sp., originating from an unknown location in the Pacific, was grown at 25 °C under a 12 h light:12 h dark cycle, with 80–100 μmol photons m⁻² s⁻¹ provided by cool white fluorescent lamps (Osram Dulux 36W/890 fluorescent bulbs), and fed freshly-hatched *Artemia* sp. nauplii every 3 days. These anemones contain *Symbiodinium* ITS2 type B1 (Gibbin and Davy, 2013). One month prior to experimentation, 300 anemones (oral disc ~1 cm diameter) were randomly selected and transferred to 2-L glass beakers (20 individuals per beaker) containing 1 μm-filtered seawater (FSW). The beakers were then split evenly between three water baths (i.e. n = 5 independent beakers per water bath), under the same light and temperature conditions described above. During this acclimation period, water was changed daily to ensure that pH (8.1 ± 0.1) and salinity (35.5 ± 0.1 ppt) were kept constant. Nutrient concentrations were not monitored during the experiment, but levels are typically low (DIN ~2 μM, NH₃ and NO₃⁻

~1 μM, NO₂⁻ and PO₄³⁻ <1 μM), and relatively stable in the coastal waters around Wellington, New Zealand, from where the seawater was collected (Dudley and Shima, 2010). Tanks were cleaned weekly and water temperature was monitored using HOBO data loggers (Onset Corporation, Bourne, MA, USA).

2.2. Experimental design

Anemones were exposed to three pH treatments over a two-month period: 8.14 ± 0.01, 7.83 ± 0.01 and 7.54 ± 0.02 [Table 1]. These values correspond to pCO₂ concentrations of 289.94 ± 12.54 μatm, 687.40 ± 25.10 μatm and 1459.92 ± 65.51 μatm, respectively. The treatments were chosen to represent current CO₂ levels, plus CO₂ stabilisation scenarios IV and VI provided by the IPCC. Photosynthesis caused the seawater pH to fluctuate by ~0.1 pH unit over 24 h, so water changes were performed daily. Addition of gaseous CO₂ to seawater was performed in a 50-L black plastic reservoir, via a CO₂ solenoid valve connected to a PINPOINT pH controller (American Marine Inc., Ridgefield, CT, USA). The final pH of the seawater was checked with an independent pH probe, adjusted accordingly if needed, and recorded before being added to the beakers. In addition to this, pH readings were taken daily before the water change, to provide a full record of the pH experienced in each beaker [Supplementary information, Fig. S1]. Anemones were haphazardly selected for sampling at 0, 1, 2, 4, 6 and 8 weeks. Not all of the anemones placed in each beaker were sampled during the experiment. At each time-point, two anemones were removed from each beaker. One was used to quantify symbiont pH_i, in addition to a suite of photo-physiological parameters (described below), and the other was used to determine the host cell pH_i.

2.3. Photo-physiological responses

Maximum dark-adapted yield of PSII (F_v/F_m) of each anemone was measured 30 min before the lights were switched on, using pulse amplitude modulated fluorometry (Diving-PAM, Walz, Effeltrich, Germany; settings: measuring light = 8, saturation intensity = 8, saturation width = 0.8 s, gain = 5 and damping = 2). Triplicate measurements were performed for each anemone, with readings taken 1 cm immediately above the oral disc, and 45° either side of this point. Anemones were then placed singly with FSW into a 10-mL glass chamber, and left to settle for an hour; this was sufficient time for attachment to a thin nylon mesh grid that overlaid a magnetic spin-bar. A glass lid, held in place by a rubber O-ring, sealed the chamber, into which a temperature probe and an oxygen electrode (FIBOX 3 – fibre-optic oxygen meter, PreSens, GmbH, Germany) were inserted such that both probes were positioned ~1 cm above the anemone's oral disc. The chamber was then wrapped in aluminium foil to ensure that no light could enter and placed on top of a submersible magnetic stirrer, itself placed in a transparent water bath set to 25 °C. Respiratory oxygen consumption (R ; mL O₂ h⁻¹) was measured every 10 s in darkness until a constant rate was observed (normally after ~30 min). The foil was then removed and the chamber illuminated at 400 μmol photons m⁻² s⁻¹ by a 150 W sealed beam PAR38 lamp (Thorn Lighting Ltd., Durham, UK). This light level was chosen because it is well above the photosynthesis-saturating irradiance for *Aiptasia* sp., which is typically between 200 and 250 μmol photons m⁻² s⁻¹ (Muller-Parker, 1984) but is not high enough to cause photoinhibition, which usually begins at irradiances greater than 600 μmol photons m⁻² s⁻¹ in this anemone (Lesser and Shick, 1989). Net photosynthetic oxygen evolution (P_{net}) was then measured until a constant rate was observed. Gross photosynthesis (P_{gross}) was calculated by adding P_{net} to R , and the resulting values were used to determine the $P_{gross}:R$ ratio for each anemone on an idealized day (assuming 12 h light and 12 h dark), a proxy for autotrophic potential. It was assumed that R in the light was the same as in the dark (i.e. total respiration per day = $R \times 24$ h).

Table 1

Summary of the carbonate chemistry of the control and experimental pH treatments. pH and temperature were measured directly, with total alkalinity (TA) measurements provided by the National Institute of Water and Atmospheric Research (NIWA), New Zealand. The remaining carbon parameters were estimated using the programme CO2SYS (Lewis et al., 1998). Values represent daily mean $\pm$ SE ($n = 60$).

pH (NBS-scale)	Temperature (°C)	TA ($\mu\text{mol kg}^{-1}$)	pCO ₂ (μatm)	HCO ₃ ⁻ ($\mu\text{mol kg}^{-1}$)	CO ₃ ²⁻ ($\mu\text{mol kg}^{-1}$)
8.143 $\pm$ 0.015	25.461 $\pm$ 0.070	2258.810 $\pm$ 4.056	289.939 $\pm$ 1.773	1648.396 $\pm$ 2.521	247.661 $\pm$ 1.017
7.843 $\pm$ 0.019	25.783 $\pm$ 0.062	2258.810 $\pm$ 4.056	687.404 $\pm$ 3.550	1909.885 $\pm$ 1.578	141.979 $\pm$ 0.642
7.570 $\pm$ 0.023	25.843 $\pm$ 0.056	2258.810 $\pm$ 4.056	1459.916 $\pm$ 9.265	2066.220 $\pm$ 1.181	78.485 $\pm$ 0.481

After the O₂-flux measurements were complete, each anemone was then placed in a 1.5-mL microcentrifuge tube and homogenised in 1.1 mL FSW using a plastic tissue grinder. From the resulting slurry, a 100 μL aliquot was removed, and frozen at -20°C . This was later used to determine symbiont density ($n = 6$ counts per sample) using an Improved Neubauer haemocytometer (Boeco, Hamburg, Germany). The remaining 1 mL was centrifuged for 5 min at 1000 $\times g$ to separate the algal fraction from host tissue. Once isolated, the host supernatant was decanted and stored at -20°C prior to analysis of the host protein content, using the Bradford assay (Bradford, 1976) and bovine serum albumin (BSA) as the standard. These values were subsequently used to normalise P_{gross} , R and symbiont density values. The remaining algal pellet was re-suspended in FSW at a final density of 1×10^6 cells mL^{-1} , for analysis of symbiont pH_i (see below).

2.4. Measuring pH_i of the symbiont and host

Light-adapted symbiont pH_i was measured according to the protocol described in Gibbin and Davy (2013). Briefly, cells were incubated with FSW containing 10 μM BCECF-AM ester, 0.1% DMSO and 0.01% Pluronic F-127 for 20 min on an orbital shaker set to 250 rpm. The samples were then transferred to a flow cytometer (FACScan, Becton-Dickinson, Franklin Lakes, NJ, USA), where they were illuminated by 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light, provided by an external variable-irradiance fibre optic cable (Halogen Reflector lamp, 150W GX5.3 21V 1CT bulb, Philips, Somerset, New Jersey, USA), for 20 min. Samples were excited with a 488 nm argon laser, and BCECF-AM fluorescence was measured in linear mode in the 515–545 nm (FL1) and 564–606 nm (FL2) filter channels, with 5000 individual cells recorded per sample. The average ratio of fluorescence, R (FL1/FL2), was calculated per sample and converted to pH_i using a calibration curve [Supplementary information, Fig. 2A].

The light-adapted pH_i of host cells was calculated from viable symbiotic endodermal cells (i.e. the host cell with its enclosed dinoflagellate symbiont) isolated using a protocol adapted from Gates and Muscatine (1992). Briefly, tentacles were excised and sliced into 1–2 mm lengths. These were transferred between a series of glass slides containing 10 μL of calcium-free filtered seawater (CaFSW) to remove mucus. The tentacles were then placed in a watch glass and the excess seawater removed, before being subjected to tryptic maceration (0.1% w/v in CaFSW). The watch glass was covered with parafilm to avoid evaporation, placed on an orbital mixer and agitated at 180 rpm for 30 min. The cells were transferred to a poly-L-lysine coated dish (MatTek Corporation, Ashland, MA, USA) and left to settle for 15 min. After this time, any excess trypsin-CaFSW solution was carefully pipetted off, and 1 mL FSW containing 10 μM SNARF-4F AM-ester, 0.1% DMSO and 0.01% Pluronic F-127 was added. The samples were left for a further 20 min before the stain was washed off with FSW. The cells were then transferred to a confocal microscope (Fluoview 1000, Olympus, Pennsylvania, USA), excited with a 559 nm laser, and imaged with the pin-hole set at 1.51 Airy units, using a $\times 63$ oil immersion lens. Only doublet cells (i.e. host cells containing two symbiont cells) were selected for analysis, since they were more numerous than triplet cells, and have a larger region of interest (ROI) than singlet symbiotic cells (Venn et al., 2009). This should be kept in mind when extrapolating results to the organismal level, because symbiont number could influence

host cell pH_i. Individual cells were imaged 10 times in the z-plane, with fluorescence emission captured at 585 ± 10 nm and 635 ± 10 nm. Three cells were imaged per replicate anemone to account for any variability, and subsequent image analysis and estimation of pH_i were carried out as described by Venn et al. (2009), using a calibration curve [Supplementary information, Fig. 2B].

2.5. Data analysis

Repeated measures analysis of variance (rmANOVA) was used to test the response of each variable to pH over time. *Post hoc* analyses represent Bonferroni-corrected pair-wise comparisons of estimated marginal means between each of the two enhanced-CO₂ treatments and the ambient CO₂ control at a particular time-point. Epsilon-adjusted Greenhouse–Geisser (G–G) values are reported whenever Mauchly's Sphericity Test returned a significant result. Linear regression analyses were performed to evaluate the relationship between symbiont and host pH_i. Data were examined for normality prior to parametric analysis using the Shapiro–Wilk Test, and were transformed where necessary to satisfy these requirements. All data were analysed using the PASW Statistics 20.0 package (IBM, Armonk, NY, USA).

3. Results

In general, the addition of CO₂ caused a significant increase in all but one of the photo-physiological parameters measured (P_{gross} :R ratio) [Table 2]. Typically these increases were detected within 2–3 weeks of CO₂ addition, and in most cases the increased response appeared to be proportional to the amount of CO₂ added [Fig. 1]. However, in some cases, the full treatment effect was not fully apparent until the end of the experiment.

3.1. Photo-physiological responses to CO₂ addition

Over the course of the experiment, anemones exposed to high and intermediate levels of CO₂ exhibited increased symbiont densities relative to the control group [Fig. 1A; Table 2]. Moderate increases were observed from week 2 onwards in the intermediate treatment and densities were 32% higher than in the control by the end of the experiment [Fig. 1A]. High CO₂ addition induced a greater response, with a 54% increase evident by week 2, increasing to 81% by the end of the experiment.

Table 2

Statistical analysis of the response variables in *Aiptasia* sp. when exposed to different pH treatments. The F-statistic represents the overall rmANOVA interaction (time $\times$ pH treatment) for each parameter and asterisks denote statistical significance at * $p < 0.05$ or ** $p < 0.001$.

Response variable	F-statistic	p-Value
Symbiont density ($\times 10^6$ cells mg^{-1} protein)	$F_{10, 60} = 5.006$	$<0.001^{**}$
Max. quantum yield (F_v/F_m)	$F_{10, 60} = 2.088$	0.039*
P_{gross} ($\mu\text{g O}_2 \text{ mg}^{-1} \text{ protein h}^{-1}$)	$F_{3, 90, 23, 403} = 11.181$	$<0.001^{**}$
P_{gross} ($\text{pg O}_2 \text{ cell}^{-1} \text{ h}^{-1}$)	$F_{10, 60} = 2.204$	0.030*
R ($\mu\text{g O}_2 \text{ mg}^{-1} \text{ protein h}^{-1}$)	$F_{10, 60} = 2.191$	0.031*
P_{gross} :R	$F_{4, 957, 29, 745} = 0.597$	0.701
Symbiont pH _i	$F_{10, 60} = 3.403$	0.001*
Host cell pH _i	$F_{10, 60} = 3.436$	0.001*

Fig. 1. Photo-physiological responses observed in *Aiptasia* sp. after exposure to control CO₂ levels (pH 8.1), and IPCC CO₂ stabilisation scenarios IV (pH 7.8) and VI (pH 7.5) over two months. Response variables measured are as follows: [A] symbiont cell density ($\times 10^6$ cells mg^{-1} protein); [B] maximum quantum yield of PSII (F_v/F_m); [C] maximum rate of gross photosynthesis (P_{gross} , $\mu\text{g O}_2 \text{ mg}^{-1}$ protein h^{-1}); [D] maximum rate of gross photosynthesis per *Symbiodinium* cell ($\text{pg O}_2 \text{ cell}^{-1} \text{ h}^{-1}$); [E] maximum respiration rate ($\mu\text{g O}_2 \text{ mg}^{-1}$ protein h^{-1}); [F] ratio of the maximum rate of gross photosynthesis to respiration ($P_{\text{gross}}:R$) for an idealized day (12 h light and 12 h dark); values above the line denote a potentially autotrophic symbiotic association where $P_{\text{gross}}:R > 1$. Data represent means $\pm$ SE for $n = 5$ individuals per time-point, and asterisks indicate significant differences between elevated CO₂ treatments relative to the control at each time point (rmANOVA, pairwise post hoc with Bonferroni correction, * $p < 0.05$, ** $p < 0.001$).

Maximum dark-adapted yield (F_v/F_m) showed no response to the intermediate CO₂ treatment but under high CO₂ there was a significant increase in F_v/F_m relative to both the control and the intermediate treatment at weeks 1, 6 and 8 [Fig. 1B].

The response of total maximum gross photosynthetic rate (P_{gross}) to CO₂ addition was pronounced [Fig. 1C; Table 2]. Under intermediate CO₂ addition, a two-fold increase in P_{gross} was evident by week 2, which remained more or less constant thereafter. In comparison, exposure to high CO₂ induced a 2.5-fold increase in P_{gross} after two weeks, and an almost 4-fold increase from week 6 onwards. These patterns were similar when P_{gross} was expressed on a cell-specific basis [Fig. 1D]. Over the duration of the experiment, cell-specific photosynthetic rate increased by 81.6% and 114% in the intermediate and high CO₂ treatments, respectively.

Total respiration (R) also responded to the addition of CO₂ [Fig. 1E; Table 2]. R tended to increase in the intermediate CO₂ treatment from at least week 2 onwards, but this increase was not significant. In contrast, there was a significant, two-fold increase in R from week 2 onwards in the high CO₂ treatment. Given the more marked influence of CO₂ addition on total photosynthesis than respiration, the ratio of

P_{gross} to R increased, though variability in this parameter masked any statistical significance [Fig. 1F; Table 2].

3.2. Cellular pH_i response to CO₂ addition

The light-adapted intracellular pH (pH_i) of both the symbiont [Fig. 2A] and host [Fig. 2B] was significantly modified via the addition of CO₂ [Table 2]. Symbiont cells isolated from anemones exposed to the highest CO₂ treatment showed the most rapid response, with pH_i increasing ~ 0.2 pH units, from 7.25 ± 0.05 to 7.46 ± 0.06 , within a week, and stabilising at this level for the remainder of the experiment. The pH_i of symbiont cells exposed to intermediate CO₂ levels showed the largest increase between weeks 1 and 4, when it reached 7.37 ± 0.09 ; the pH_i remained at a similar level for the remainder of the experiment.

There were discernible differences between the response of host pH_i and symbiont pH_i under acidification [Fig. 2]. Interestingly, exposure to intermediate CO₂ had no effect on host cell pH_i [Fig. 2B], with mean values comparable to those observed in the control anemones (7.19 ± 0.01 versus 7.17 ± 0.01) throughout the experiment. In stark

Fig. 2. The response of light-adapted intracellular pH (pH_i) to CO_2 -driven acidification. [A] *Symbiodinium* and [B] symbiotic *Aiptasia* sp. cells at normal CO_2 levels (pH 8.1), and IPCC CO_2 stabilisation scenarios IV (pH 7.8) and VI (pH 7.5) over a two-month time-frame. [C] The linear relationship between host and symbiont pH_i . [D] Schematic to explain host cell pH_i fluctuations as a function of gross photosynthesis (P_{gross}) and total symbiosis respiration (R). Host cell is represented by the rectangle, and symbiont is represented by the dotted oval. From clockwise: if $P_{gross} < R$ then host pH_i decreases; if $P_{gross} > R$ then host pH_i increases; and if both P_{gross} and R either both increase or decrease in proportion to one another then there is no change in pH_i . Data represent means $\pm$ SE for $n = 5$ individuals per time-point and asterisks indicate significant differences between elevated CO_2 treatments relative to the control at each time point (RMANOVA, pairwise *post hoc* with Bonferroni correction, * $p < 0.05$, ** $p < 0.001$).

contrast, the pH_i of host cells exposed to high CO_2 increased by almost 0.3 pH units within a week, rising from 7.19 ± 0.03 to 7.48 ± 0.02 , and remained relatively constant thereafter. Linear regression showed a strong predictive relationship between symbiont and host pH_i ($p < 0.001$), and suggests that a 0.1 pH unit increase in symbiont pH_i is associated with a 0.63 pH unit increase in host pH_i [Fig. 2C].

4. Discussion

Ocean acidification (OA) – once referred to as “the other CO_2 problem” (Doney et al., 2009) – is now recognised as a major selective force in determining ecosystem structure and function (Christen et al., 2013; Godbold and Calosi, 2013; Hall-Spencer et al., 2008). Marine organisms exhibit high variability in their physiological and cellular tolerance to CO_2 addition (Fabry et al., 2008). While considered detrimental on the whole for calcifying organisms (Hoegh-Guldberg et al., 2007; Kroeker et al., 2010; Marubini et al., 2008), there is growing evidence that enhanced CO_2 levels may benefit soft-bodied photosynthetic anthozoans (Inoue et al., 2013; Suggitt et al., 2012; Towanda and Thuesen, 2012) through the alleviation of carbon limitation, that is thought typical of the symbiont population (Davy and Cook, 2001; Weis, 1993). The increased photosynthetic production that arises as a consequence of this likely has two major advantages for the host: (1) increased translocation of photosynthate for growth and reproduction (Brownlee, 2009); and (2) the prevention of cellular acidosis that arises from increased concentrations of CO_2 and hence protons (Gibbin et al., 2014). Our study lends support to the potential success of non-calcifying symbiotic cnidarians in a CO_2 -enriched world, and confirms the positive relationship between CO_2 supply, photosynthesis and the

prevention of cellular acidosis in the model symbiotic cnidarian, *Aiptasia* sp.

Increases in symbiont density, photosynthetic health (F_v/F_m), overall and cell-specific gross photosynthetic rate (P_{gross}), and respiration rate (R) were commensurate with the CO_2 concentration, signifying enhanced primary productivity and hence symbiont growth in response to the increased availability of dissolved inorganic carbon (DIC). Similar findings were reported for two temperate sea anemone species exposed to elevated CO_2 . *A. viridis* sampled from high CO_2 sites (corresponding to 1428 μatm) along a naturally occurring CO_2 vent, contained up to four-times more *Symbiodinium* cells than when at control sites (300 μatm), resulting in enhanced rates of both P_{gross} and R ; though, as in the current study, P_{gross} increased more than R (Suggitt et al., 2012). These patterns were mirrored in *Anthopleura elegantissima* after six weeks of incubation at pH 7.3, although there was no accompanying change in symbiont density (Towanda and Thuesen, 2012). The increased primary productivity seen here in *Aiptasia* sp. is unsurprising given that seawater (pH 8.2) contains less than half of the DIC (2.2 mM HCO_3^- , 30 μM CO_2) required to saturate photosynthesis in this species under normal circumstances (5 mM DIC; Weis, 1993). Nevertheless, *Aiptasia* sp. can still achieve its autotrophic potential (*i.e.* $P_{gross} : R > 1$) with respect to carbon (Starzak et al., 2014), in part because of the activity of carbon-concentrating mechanisms (CCM's) that concentrate CO_2 at the site of photosynthesis (Leggat et al., 1999; Weis et al., 1989). CO_2 enrichment may therefore provide a further benefit through reducing the metabolic costs of maintaining CCM activity (Raven et al., 2012).

However, not all studies report increases in anemone productivity after CO_2 addition. Six weeks of exposure to pH 7.6 had no effect on symbiont density or physiological performance in the temperate

anemone *Anthopleura aureoradiata* (Doherty, 2009). Similarly, two weeks of exposure to pH 7.4 caused declines in symbiont density, but did not affect either P_{gross} or R in laboratory-maintained *A. viridis* (Jarrold et al., 2013); though our results suggest that a two-week time period may not always be long enough to observe significant changes in physiology. Differences in the organismal response to CO_2 addition are potentially attributable to the type of *Symbiodinium* present. Indeed, there is growing evidence that the response to CO_2 -addition may be type-specific (Brading et al., 2011; Buxton et al., 2009). For example, when a range of cultured *Symbiodinium* types were exposed to a two-fold increase in CO_2 concentration (corresponding to pH 7.79 – the intermediate treatment in our study), there was an increase of ~60% in the growth rate and photosynthetic capacity of types A13 and A2, respectively, while types A1 and B1 did not respond at all (Brading et al., 2011). Of note, this lack of response by *Symbiodinium* B1 in culture is different to our observations here, when this same type was *in hospite*. Future studies should consider how CO_2 concentration may influence different host–symbiont combinations.

One of the key objectives of this study was to establish the response of intracellular pH (pH_i) to CO_2 -addition, simultaneously in the host and symbiont. To achieve this, we measured the light-adapted pH_i of both partners. The values measured in the control anemones were similar to those measured previously in a range of cultured *Symbiodinium* types (pH 7.21–7.39), and both the host and symbiont cells of *Aiptasia* sp. (pH 7.02 ± 0.06 and 7.25 ± 0.01 , respectively; Gibbin and Davy, 2013). Light-adapted host pH_i in this anemone species was, however, slightly lower than that measured in the cnidarians *A. viridis* (pH 7.41 ± 0.22) and *Stylophora pistillata* (7.29 ± 0.15) (Venn et al., 2009). Under normal conditions, photosynthesis in the symbiont results in an alkalinisation of the host cell (Venn et al., 2009), the magnitude of which is proportional to the irradiance (Laurent et al., 2013a). As such, we predicted that CO_2 addition would increase the photosynthetic rate and hence symbiont pH_i , and trigger a similar increase in the host cell pH_i . Our results were consistent with this prediction, with symbiont pH_i increasing in proportion to the CO_2 concentration. The response in the host cell was not quite so marked, however, with there being no change in the pH_i of the host cell under the intermediate CO_2 treatment (pH 7.8), but a substantial increase in alkalinity (~0.3 pH units) under the high CO_2 treatment (pH 7.5). While the high buffering capacity of the cnidarian cell likely plays a role (Laurent et al., 2013b), our results suggest a strong link between the relative rates of photosynthesis and respiration in dictating the pH_i of the host in response to external, CO_2 -induced pH change. In particular, the increase in alkalinity reported for the high CO_2 treatment is potentially attributable to the elevated $P_{\text{gross}}:R$ ratio seen under these conditions (in contrast to the intermediate CO_2 treatment). This could indicate a partial decoupling of the rates of photosynthesis and respiration, which typically facilitate one another through the evolution of O_2 and CO_2 respectively (Harland and Davies, 1995), and a concomitant decrease in the intracellular CO_2 concentration. The mechanism by which the pH_i of the host cell may be pushed upwards by CO_2 enrichment is summarized in Fig. 2D.

In summary, these findings demonstrate that the photo-physiological response to predicted ocean acidification scenarios has the potential to alter the pH_i of both host and symbiont simultaneously, and therefore affect the functioning of the cnidarian–dinoflagellate symbiosis. Of particular importance, our data suggest improved photosynthetic performance and an absence of cellular acidosis in the light in response to CO_2 -driven acidification. We did not measure pH_i in the dark and it would be interesting to know what impact these different conditions have on night-time pH_i , though we have previously shown that isolated host cells of the reef coral *P. damicornis* are more susceptible to acidification when the photosynthetic activity of their symbionts is inhibited by DCMU (Gibbin et al., 2014). The enhanced performance in the light, however, raises important questions about the future of non-calcareous symbiotic anthozoans in a high- CO_2 ocean. In particular, several studies have reported that ocean acidification initiates a shift in ecosystem

structure from a calcareous to a non-calcareous, algal-dominated community (Hall-Spencer et al., 2008; Inoue et al., 2013; Suggett et al., 2012) that has the potential to act as a significant carbon sink (Anthony et al., 2011; Russell et al., 2013). Similarly, our results, along with those of Suggett et al. (2012), suggest that symbiotic sea anemones could not only survive ocean acidification, but also thrive under future conditions. However, it is important to bear in mind that ocean acidification is only one aspect of climate change, with the effects of global warming likely to influence the physiological response to elevated CO_2 levels. In particular, thermal stress and the resulting photosynthetic dysfunction may increase the likelihood for cellular acidosis in CO_2 -enriched waters. The model system we describe here provides a basis for future study of this topic in the intact cnidarian–dinoflagellate symbiosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jembe.2014.03.015>.

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