© 2014. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2014) 217, 1963-1969 doi:10.1242/jeb.099549

RESEARCH ARTICLE

# Intracellular pH and its response to $CO_2$-driven seawater acidification in symbiotic *versus* non-symbiotic coral cells

**Emma M. Gibbin[1], Hollie M. Putnam[2], Simon K. Davy[1,*] and Ruth D. Gates[2]**

## 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 $P_{CO_2}$-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 $CO_2$-acidified seawater conditions (decreasing pH from 7.8 to 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 $pH_i$ 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 $CO_2$ 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 photsynthesis 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 $CO_2$/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 (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). 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 (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. 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 $CO_2$/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 $CO_2$-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 $CO_2$-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

[1]School of Biological Sciences, Victoria University of Wellington, Kelburn Parade, Wellington 6140, New Zealand. [2]Hawaii Institute of Marine Biology, University of Hawaii, PO Box 1346, Kaneohe, HI 96744, USA.

*Author for correspondence (simon.davy@vuw.ac.nz)

**Received 4 November 2013; Accepted 3 March 2014**

The Journal of Experimental Biology (2014) doi:10.1242/jeb.099549

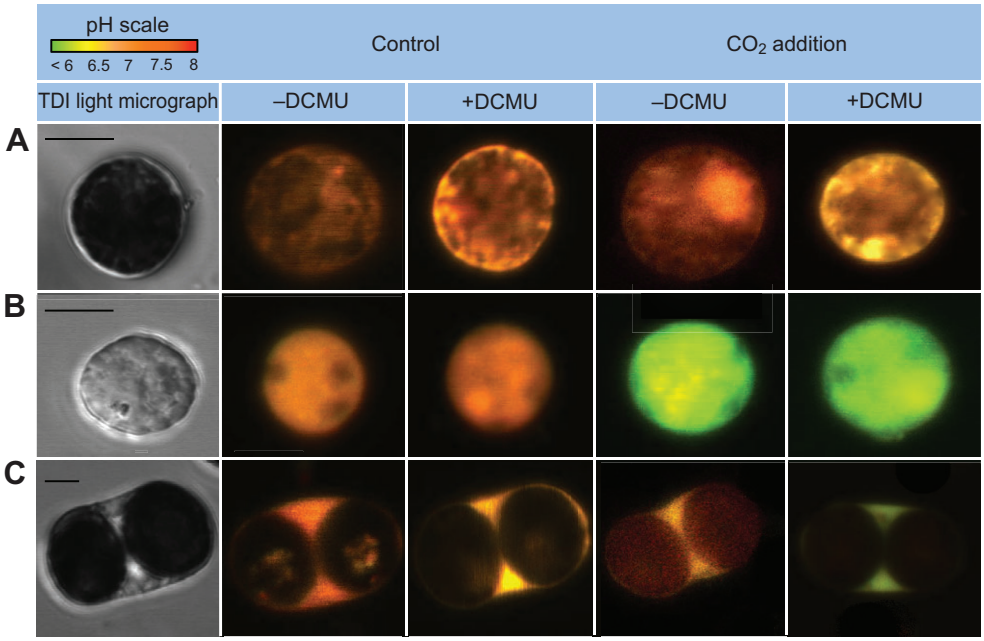


**Fig. 1. Light and confocal microscopy images of host *Pocillopora damicornis* cells and *Symbiodinium* cells.** The pH-sensitive dye BCECF-AM ester was used to monitor intracellular pH ($pH_i$) of cells isolated from *P. damicornis* after 105 min of exposure to either control pH conditions (left) or 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 µmol photons $m^{-2}$ $s^{-1}$) photosynthetically active radiation (PAR). Scale bars represent 5 µm.

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 pH × symbiotic state × 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

The Journal of Experimental Biology (2014) doi:10.1242/jeb.099549

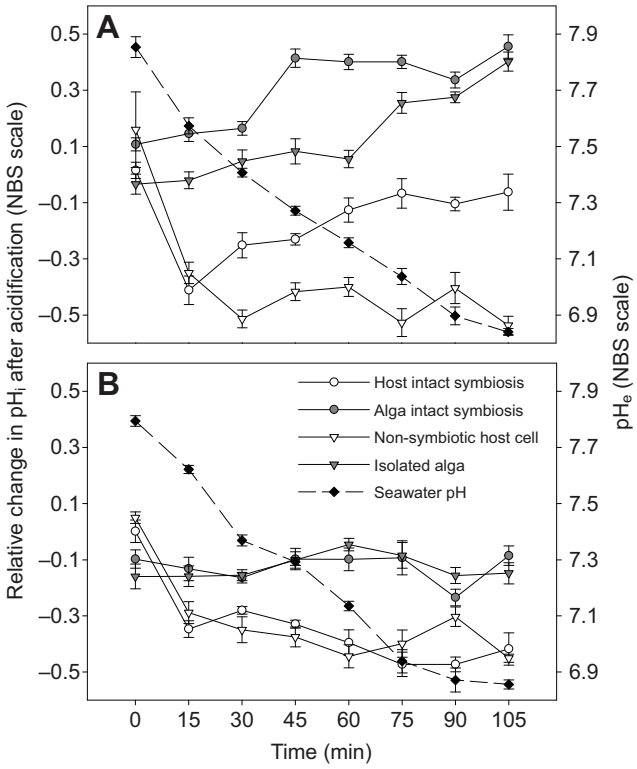


**Fig. 2. The impact of $CO_2$ addition on $pH_i$ in *P. damicornis* cells and *Symbiodinium* cells.** The figure shows the relative change in $pH_i$ after acidification (the $pH_i$ value after acidification minus the mean $pH_i$ of the control at each time point, means ± s.e.m., *N*=5) under two treatments: (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 $CO_2$ 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. 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 $CO_2$/low pH. The responses seen in the various symbiotic states are summarised in Fig. 3 and are discussed in more detail here.

$CO_2$ 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, an increase in $CO_2$ 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 $CO_2$ in the surrounding seawater (Nimer et al., 1999) (Fig. 3A). Our results clearly show that, upon $CO_2$ addition, *Symbiodinium* cells are able to increase their $pH_i$ relative to the control, by up to 0.5 pH units. This alkalinisation 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 $CO_2$-limited algae (Nimer et al., 1999). An increase in photosynthetic productivity after $CO_2$ 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).

$CO_2$ 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 $CO_2$ 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 $CO_2$, and active transport of $HCO_3^-$ 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**

| | | *P*-value | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | Pair | 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.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.099549

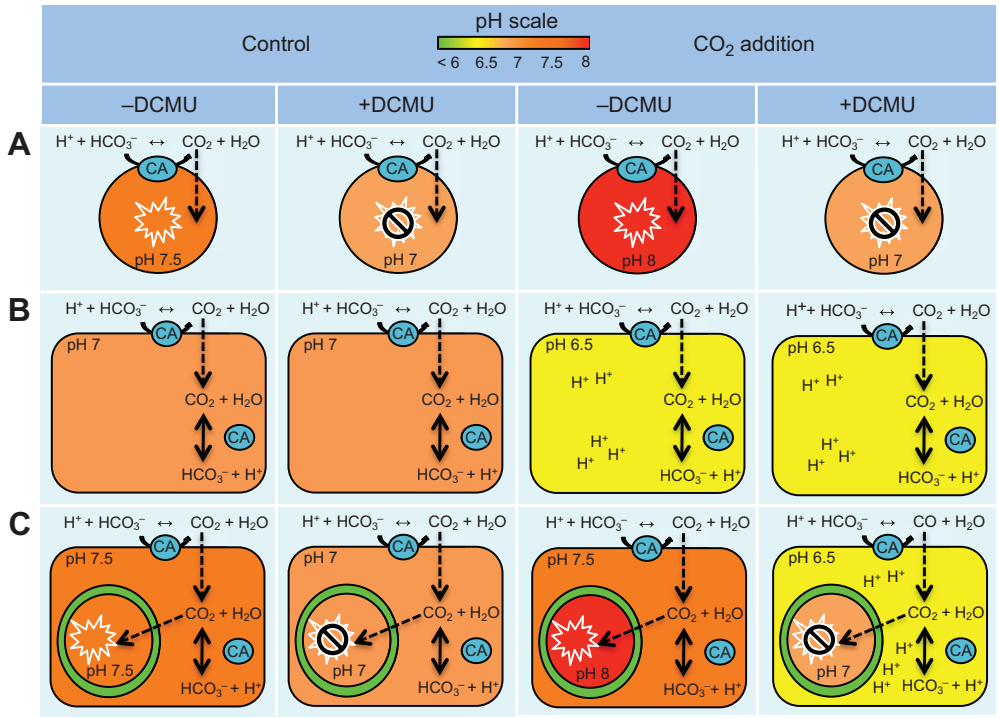


**Fig. 3. Model of $pH_i$ fluctuations in cells isolated from *P. damicornis*.** Cells were exposed for 105 min to either control pH conditions (left) or increasing $CO_2$ (right) according to the state of the equilibrium reaction that exists between $CO_2+H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$. (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 $CO_2$ (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 µmol photons $m^{-2}$ $s^{-1}$) PAR. 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. 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 $CO_2$-addition (Fig. 3C). 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., 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 $CO_2$-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 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 $H^+/Ca^{2+}$ 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 expoxy (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 ± 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±0.3°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 µmol photons $m^{-2}$ $s^{-1}$, corresponding to a mean irradiance of 125±10 µmol photons $m^{-2}$ $s^{-1}$ 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 µm 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 ~400 µmol photons $m^{-2}$ $s^{-1}$ 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 µmol $l^{-1}$ 3-(3-4-dichlorophenyl)-1,1-dimethylurea (DCMU) in 0.1% acetone, a photosynthetic inhibitor that blocks the plastiquinone 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 µm 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 µmol $l^{-1}$ BCECF-AM ester and 0.01% Pluronic F-127 with/without 100 µmol $l^{-1}$ DCMU and 0.1% acetone depending on the treatment in question, to give a final concentration of ~$1\times10^6$ 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 $pH=pK_a+\log\{[(R-R_A)/(R_B-R)]\times(F_{A,458}/F_{B,458})\}$, 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% $CO_2$ 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 Zen 2011 software (Carl Zeiss), 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 (supplementary material Fig. S3). We selected the $CO_2$ 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, 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 Kolgomorov–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).

#### Acknowledgements

We thank Shaun Wilkinson and Nyssa Silbiger for statistical advice.

#### 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.

#### Funding

This research was funded by a Commonwealth PhD Scholarship and a Kathleen Stewart Postgraduate Scholarship, both awarded to E.M.G. This manuscript is Hawaii Institute of Marine Biology (HIMB) contribution number 1587.

#### Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.099549/-/DC1

#### References

**Allemand, D., Furla, P. and Tambutté, S. B.** (1998). Mechanisms of carbon acquisition for endosymbiont photosynthesis in Anthozoa. *Botany* **76**, 925-941.

**Allemand, D., Ferrier-Pagès, C., Furla, P., Houlbrèque, F., Puverel, S., Reynaud, S., Tambutte, E., Tambutte, S. and Zoccola, D.** (2004). Biomineralisation in reef-building corals: from molecular mechanisms to environmental control. *Comptes Rendus Palevol* **3**, 453-467.

**Boron, W. F.** (2004). Regulation of intracellular pH. *Adv. Physiol. Educ.* **28**, 160-179.

**Brading, P., Warner, M. E., Davey, P., Smith, D. J., Achterberg, E. P. and Suggett, D. J.** (2011). Differential effects of ocean acidification on growth and photosynthesis among phylotypes of Symbiodinium (Dinophyceae). *Limnol. Oceanogr.* **56**, 927-938.

**Casey, J. R., Grinstein, S. and Orlowski, J.** (2010). Sensors and regulators of intracellular pH. *Nat. Rev. Mol. Cell Biol.* **11**, 50-61.

**Comeau, S., Carpenter, R. C. and Edmunds, P. J.** (2013). Coral reef calcifiers buffer their response to ocean acidification using both bicarbonate and carbonate. *Proc. Biol. Sci.* **280**, 20122374-20122374.

**Davy, S. K., Allemand, D. and Weis, V. M.** (2012). Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiol. Mol. Biol. Rev.* **76**, 229-261.

**Dickson, A. G., Sabine, C. L. and Christian, J. R.** (2007). Guide to best practices for ocean $CO_2$ measurements. *PICES Special Publication* **3**.

**Drupp, P., De Carlo, E. H., Mackenzie, F. T., Bienfang, P. and Sabine, C. L.** (2011). Nutrient inputs, phytoplankton response, and $CO_2$ variations in a semi-enclosed subtropical embayment, Kaneohe Bay, Hawaii. *Aquat. Geochem.* **17**, 473-498.

**Fabry, V. J., Seibel, B. A., Feely, R. A. and Orr, J. C.** (2008). Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES J. Mar. Sci.* **65**, 414-432.

**Furla, P., Allemand, D. and Orsenigo, M. N.** (2000). Involvement of $H^+$-ATPase and carbonic anhydrase in inorganic carbon uptake for endosymbiont photosynthesis. *Am. J. Physiol.* **278**, R870-R881.

**Gibbin, E. M. and Davy, S. K.** (2013). Intracellular pH of symbiotic dinoflagellates. *Coral Reefs* **32**, 859-863.

**Hennige, S., Suggett, D., Warner, M., McDougall, K. and Smith, D.** (2009). Photobiology of Symbiodinium revisited: bio-physical and bio-optical signatures. *Coral Reefs* **28**, 179-195.

**Hervé, V., Derr, J., Douady, S., Quinet, M., Moisan, L. and Lopez, P. J.** (2012). Multiparametric analyses reveal the pH-dependence of silicon biomineralization in diatoms. *PLoS ONE* **7**, e46722.

**Hoegh-Guldberg, O.** (1999). Climate change, coral bleaching and the future of the world's coral reefs. *Mar. Freshw. Res.* **50**, 839-866.

**Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Harvell, C. D., Sale, P. F., Edwards, A. J., Caldeira, K. et al.** (2007). Coral reefs under rapid climate change and ocean acidification. *Science* **318**, 1737-1742.

**Hofmann, G. E., Smith, J. E., Johnson, K. S., Send, U., Levin, L. A., Micheli, F., Paytan, A., Price, N. N., Peterson, B., Takeshita, Y. et al.** (2011). High-frequency dynamics of ocean pH: a multi-ecosystem comparison. *PLoS ONE* **6**, e28983.

**Hofmann, L. C., Straub, S. and Bischof, K.** (2013). Elevated $CO_2$ levels affect the activity of nitrate reductase and carbonic anhydrase in the calcifying rhodophyte *Corallina officinalis*. *J. Exp. Bot.* **64**, 899-908.

**Houlbrèque, F., Tambutté, E., Allemand, D. and Ferrier-Pagès, C.** (2004). Interactions between zooplankton feeding, photosynthesis and skeletal growth in the scleractinian coral *Stylophora pistillata*. *J. Exp. Biol.* **207**, 1461-1469.

**Iglesias-Prieto, R., Matta, J. L., Robins, W. A. and Trench, R. K.** (1992). Photosynthetic response to elevated temperature in the symbiotic dinoflagellate *Symbiodinium microadriaticum* in culture. *Proc. Natl. Acad. Sci. USA* **89**, 10302-10305.

**Jokiel, P. L.** (2011). Ocean acidification and control of reef coral calcification by boundary layer limitation of proton flux. *Bulletin of Marine Science* **87**, 639-657.
**Jones, R. J., Hoegh-Guldberg, O., Larkum, A. W. D. and Schreiber, U.** (1998). Temperature-induced bleaching of corals begins with impairment of the $CO_2$ fixation mechanism in zooxanthellae. *Plant Cell Environ.* **21**, 1219-1230.
**Kaniewska, P., Campbell, P. R., Kline, D. I., Rodriguez-Lanetty, M., Miller, D. J., Dove, S. and Hoegh-Guldberg, O.** (2012). Major cellular and physiological impacts of ocean acidification on a reef building coral. *PLoS ONE* **7**, e34659.
**Laurent, J., Tambutté, S., Tambutté, É., Allemand, D. and Venn, A.** (2013). The influence of photosynthesis on host intracellular pH in scleractinian corals. *J. Exp. Biol.* **216**, 1398-1404.
**Madshus, I. H.** (1988). Regulation of intracellular pH in eukaryotic cells. *Biochem. J.* **250**, 1-8.
**McCulloch, M., Falter, J., Trotter, J. and Montagna, P.** (2012). Coral resilience to ocean acidification and global warming through pH up-regulation. *Nature Climate Change* **2**, 623-627.
**Michaelidis, B., Ouzounis, C., Paleras, A. and Pörtner, H. O.** (2005). Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Mar. Ecol. Prog. Ser.* **293**, 109-118.
**Muscatine, L., Goiran, C., Land, L., Jaubert, J., Cuif, J. P. and Allemand, D.** (2005). Stable isotopes (δ13C and δ15N) of organic matrix from coral skeleton. *Proc. Natl. Acad. Sci. USA* **102**, 1525-1530.
**Musgrove, E., Rugg, C. and Hedley, D.** (1986). Flow cytometric measurement of cytoplasmic pH: a critical evaluation of available fluorochromes. *Cytometry* **7**, 347-355.
**Nimer, N. A., Brownlee, C. and Merrett, M. J.** (1999). Extracellular carbonic anhydrase facilitates carbon dioxide availability for photosynthesis in the marine dinoflagellate prorocentrum micans. *Plant Physiol.* **120**, 105-112.
**Ohki, S., Irie, T., Inoue, M., Shinmen, K., Kawahata, H., Nakamura, T., Kato, A., Nojiri, Y., Suzuki, A. and Sakai, K.** (2013). Symbiosis increases coral tolerance to ocean acidification. *Biogeosciences Discuss.* **10**, 7013-7030.
**Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F. et al.** (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681-686.
**Pandolfi, J. M., Connolly, S. R., Marshall, D. J. and Cohen, A. L.** (2011). Projecting coral reef futures under global warming and ocean acidification. *Science* **333**, 418-422.
**Pochon, X. and Gates, R. D.** (2010). A new Symbiodinium clade (Dinophyceae) from soritid foraminifera in Hawai'i. *Mol. Phylogenet. Evol.* **56**, 492-497.
**Pörtner, H. O., Langenbuch, M. and Reipschläger, A.** (2004). Biological impact of elevated ocean $CO_2$ concentrations: lessons from animal physiology and earth history. *J. Oceanography* **60**, 705-718.
**Price, N. N., Martz, T. R., Brainard, R. E. and Smith, J. E.** (2012). Diel variability in seawater pH relates to calcification and benthic community structure on coral reefs. *PLoS ONE* **7**, e43843.
**Putnam, H. M.** (2012). *Resilience and Acclimatization Potential of Reef Corals Under Predicted Climate Change Stressors.* University of Hawai'i at Manoa, HI: ProQuest, UMI Dissertations Publishing, 3569106.
**Reipschläger, A. and Pörtner, H. O.** (1996). Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. *J. Exp. Biol.* **199**, 1801-1807.
**Riebesell, U., Fabry, V. J., Hansson, L. and Gattuso, J.-P.** (2010). *Guide to Best Practices for Ocean Acidification Research and Data Reporting*. Luxembourg: Publications Office of the European Union.
**Silverman, J., Lazar, B., Cao, L., Caldeira, K. and Erez, J.** (2009). Coral reefs may start dissolving when atmospheric $CO_2$ doubles. *Geophys. Res. Lett.* **5**, L05606.
**Smith, F. A. and Raven, J. A.** (1979). Intracellular pH and its regulation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **30**, 289-311.
**Stumpp, M., Hu, M. Y., Melzner, F., Gutowska, M. A., Dorey, N., Himmerkus, N., Holtmann, W. C., Dupont, S. T., Thorndyke, M. C. and Bleich, M.** (2012). Acidified seawater impacts sea urchin larvae pH regulatory systems relevant for calcification. *Proc. Natl. Acad. Sci. USA* **109**, 18192-18197.
**Suggett, D. J., Hall-Spencer, J. M., Rodolfo-Metalpa, R., Boatman, T. G., Payton, R., Tye Pettay, D., Johnson, V. R., Warner, M. E. and Lawson, T.** (2012). Sea anemones may thrive in a high $CO_2$ world. *Glob. Change Biol.* **18**, 3015-3025.
**Tchernov, D., Gorbunov, M. Y., de Vargas, C., Narayan Yadav, S., Milligan, A. J., Häggblom, M. and Falkowski, P. G.** (2004). Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. *Proc. Natl. Acad. Sci. USA* **101**, 13531-13535.
**Towanda, T. and Thuesen, E. V.** (2012). Prolonged exposure to elevated $CO_2$ promotes growth of the algal symbiont Symbiodinium muscatinei in the intertidal sea anemone *Anthopleura elegantissima*. *Biol. Open* **1**, 615-621.
**Uthicke, S. and Fabricius, K. E.** (2012). Productivity gains do not compensate for reduced calcification under near-future ocean acidification in the photosynthetic benthic foraminifer species *Marginopora vertebralis*. *Glob. Change Biol.* **18**, 2781-2791.
**Venn, A. A., Tambutté, E., Lotto, S., Zoccola, D., Allemand, D. and Tambutté, S.** (2009). Imaging intracellular pH in a reef coral and symbiotic anemone. *Proc. Natl. Acad. Sci. USA* **106**, 16574-16579.
**Venn, A. A., Tambutté, E., Holcomb, M., Laurent, J., Allemand, D. and Tambutté, S.** (2013). Impact of seawater acidification on pH at the tissue-skeleton interface and calcification in reef corals. *Proc. Natl. Acad. Sci. USA* **110**, 1634-1639.
**Weis, V. M., Davy, S. K., Hoegh-Guldberg, O., Rodriguez-Lanetty, M. and Pringle, J. R.** (2008). Cell biology in model systems as the key to understanding corals. *Trends Ecol. Evol.* **23**, 369-376.