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Coral calcifying fluid pH is modulated by seawater carbonate chemistry not solely seawater pH

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Reef coral calcification depends on regulation of pH in the internal calcifying fluid (CF) in which the coral skeleton forms. However, little is known about calcifying fluid pH (pH_{CF}) regulation, despite its importance in determining the response of corals to ocean acidification. Here, we investigate pH_{CF} in the coral *Stylophora pistillata* in seawater maintained at constant pH with manipulated carbonate chemistry to alter dissolved inorganic carbon (DIC) concentration, and therefore total alkalinity (A_T). We also investigate the intracellular pH of calcifying cells, photosynthesis, respiration and calcification rates under the same conditions. Our results show that despite constant pH in the surrounding seawater, pH_{CF} is sensitive to shifts in carbonate chemistry associated with changes in [DIC] and [A_T], revealing that seawater pH is not the sole driver of pH_{CF}. Notably, when we synthesize our results with published data, we identify linear relationships of pH_{CF} with the seawater [DIC]/[H^+] ratio, [A_T]/ [H^+] ratio and [CO_3^{2-}]. Our findings contribute new insights into the mechanisms determining the sensitivity of coral calcification to changes in seawater carbonate chemistry, which are needed for predicting effects of environmental change on coral reefs and for robust interpretations of isotopic palaeoenvironmental records in coral skeletons.

1. Introduction

The structural basis of one of the world's most biodiverse habitats, coral reef ecosystems, is composed of a $CaCO_3$ framework largely built by the calcification of scleractinian corals. Despite the importance of coral calcification in building and maintaining reef structures, relatively little is understood about calcification at a mechanistic, physiological level [1].

Critical to the calcification mechanism of reef corals is the ability to regulate pH in the extracellular calcifying fluid (CF) where the skeleton forms [2,3]. The CF is separated from the surrounding seawater by the overlying coral tissues, and is at the interface between the coral skeleton and the calcifying calicoblastic epithelium (figure 1*a*). Knowledge of the chemistry of the CF is limited, but it has been shown that corals increase pH_{CF} to exceed that of the surrounding seawater pH in order to increase the aragonite saturation state (Ω_{arag}) of the CF and promote precipitation of $CaCO_3$ [2,3,10–13].

Previous research has attempted to better characterize both biological and seawater environmental parameters that influence pH_{CF} in order to better understand the calcification process. At the cellular level, biological regulation

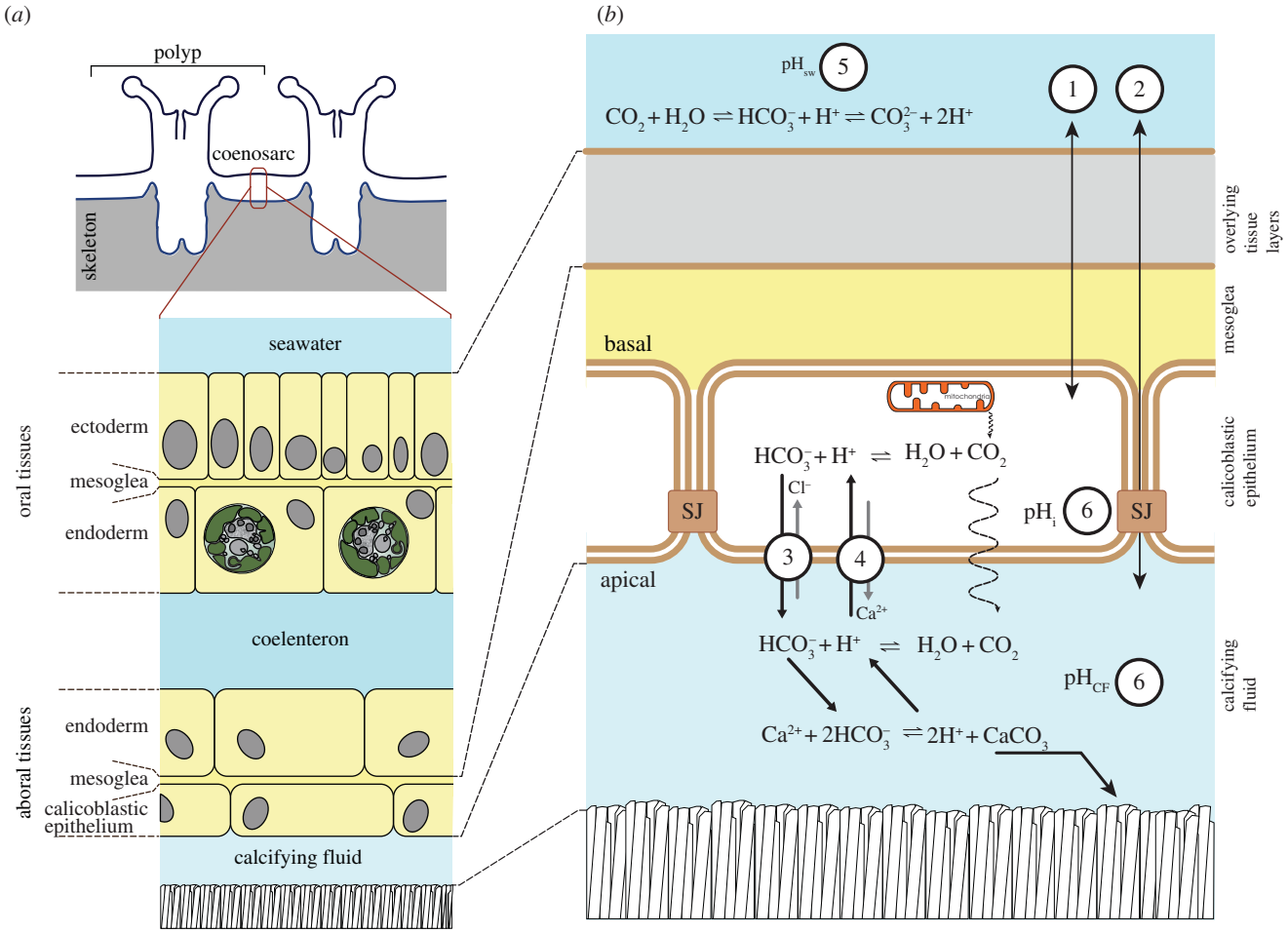

Figure 1. (*a*) Schematic of cross section through the coral tissues at the coenosarc. (*b*) Schematic of ion transport processes influencing calcifying fluid (CF) pH in *Stylophora pistillata*. (1–4) Ion exchange between seawater, the CF and calicoblastic cells occurs via (1) transcellular and (2) paracellular pathways [4–7]. (3 and 4) Transcellular pathways include HCO_3^- transport by a SLC4y transporter localized to the apical calicoblastic membrane [4] and the removal of protons from the CF via the Ca^{2+}ATPase [8]. (5 and 6) Changes in seawater carbonate chemistry associated with shifts in seawater [DIC] and A_T, and also seawater pH, affect pH_i in the calicoblastic cells and pH_{CF} (this study and [9]). SJ, septate junction.

of pH_{CF} is typically attributed to proton transporters situated in the calicoblastic epithelium, particularly Ca^{2+} ATPases, which have been proposed to remove protons from the CF, thus elevating pH [8] (figure 1*b*). Physiological studies and recent molecular work have also described cnidarian HCO_3^- transporters in the calicoblastic cell layer that may be involved in transporting dissolved inorganic carbon (DIC) for calcification, thus influencing CF chemistry [4,14] (figure 1*b*). At the organism scale, there is evidence that photosynthesis is also linked to pH_{CF} regulation, because pH_{CF} is elevated in light relative to dark conditions [2]. Respiration rate may also be a factor effecting pH_{CF}, because many studies suggest that respiratory CO_2 is a major source of DIC used in coral calcification [12,14–16].

Previous research on the influence of seawater environmental parameters on pH_{CF} has almost exclusively focused on seawater pH. This includes palaeoenvironmental research that has sought to determine the relationship between seawater pH and pH_{CF} to better constrain the use of isotopic signatures in coral skeletons as indicators of seawater pH in earth's history [17–19]. It also includes a number of studies that have characterized how seawater pH affects pH_{CF}, in order to gain a better physiological understanding of how ocean acidification, which is considered to be one of the major environmental challenge to coral reefs, impacts coral calcification [3,9,11]. A decrease in seawater pH associated with ocean acidification can depress intracellular pH (pH_i) in calicoblastic cells and pH_{CF}, leading to a decrease in CF Ω_{arag} and declines in coral calcification rate [9]. Indeed, recent papers have linked the capacity of corals to regulate pH_{CF} with the low sensitivity of some scleractinians to ocean acidification [9,11].

While much attention has been paid to seawater pH, experiments have not yet been conducted on the influence of other seawater carbonate chemistry parameters on pH_{CF}. This is surprising given the potential role of several carbonate chemistry parameters in pH_{CF} regulation. For example, changes in seawater [DIC] and the concentrations of its components CO_2, HCO_3^- and CO_3^{2-} are likely to be of significance, because DIC species have integral roles in acid–base regulation of both cells and extracellular fluids in most organisms [20] (regardless of whether they engage in calcification). Furthermore, the total alkalinity [A_T] of seawater, which describes the buffering capacity of seawater to pH change, is also potentially important for the buffering of protons removed from the CF. At a wider physiological level, seawater carbonate chemistry can affect processes such as photosynthesis and calcification that may themselves have a bearing on pH_{CF}. Indeed, previous studies have demonstrated that calcification rates are affected by seawater [DIC] and [A_T]

[21–25], and it is also known that coral photosynthetic rates respond to seawater [DIC] [25,26]. All these reasons suggest that pH_{CF} may not simply be controlled by the pH gradient between the CF and the surrounding seawater.

Here, we test the hypothesis that variation in seawater [DIC] and [A_T] impacts pH regulation of the CF in the scleractinian *Stylophora pistillata*, which in turn affects calcification rates. We manipulated seawater carbonate chemistry to modify seawater [DIC] and [A_T] (i.e. our treatment levels) while maintaining seawater pH constant, thereby creating the potential to experimentally distinguish the effects of varying seawater [DIC] and [A_T] from varying seawater pH on the pH_{CF} in the corals. Under these conditions, we measured pH_{CF} *in vivo* by confocal microscopy, and assessed calcification in the light and dark. Additionally, we sought to gain insights into the physiological mechanisms underlying how pH_{CF} is regulated, through measurements of rates of photosynthesis, respiration and pH_i of the calicoblastic cells (measured by confocal microscopy).

2. Material and methods

(a) Experimental set-up

Coral samples were prepared from three colonies maintained in the coral culture facilities at the Centre Scientifique de Monaco. Samples were prepared as nubbins (22 per colony) suspended on monofilament threads or microcolonies grown laterally on glass coverslips (eight per colony) according to methods given in [20]. Four experimental treatments were maintained total scale pH (pH_T) at approximately 7.9, with manipulated [DIC] and [A_T] using combinations of CO_2-free air, pure CO_2, 1 M HCl and 1 M NaOH (electronic supplementary material table S1). Throughout the manuscript, these treatments are referred to as 'very low', 'low', 'ambient' and 'high' [DIC] and [A_T].

Eight nubbins mounted on threads and three microcolonies on coverslips were randomly selected and placed in each of eight 20 l tanks for DIC treatments and were maintained in the aquaria during 15 days. Details of the aquarium conditions, manipulations of DIC and monitoring of carbonate chemistry are provided in the electronic supplementary material.

(b) Calcification

Calcification of the coral nubbins was measured using two techniques: buoyant weight was used to evaluate calcification over the two weeks of incubation, and the alkalinity anomaly technique [27] was used to differentiate short-term (approx. 30 min) calcification in the light and dark at the end of the two week incubation period. Buoyant weight ($\pm$1 mg) was recorded before and after the 15 day incubation, using eight nubbins per DIC treatment, and the difference between the two was converted to dry weight, using an aragonite density of 2.93 g cm^{-3}.

Light and dark calcification were estimated on three coral pieces per treatment. After 10 days of incubation, nubbins were chosen randomly from the tanks and placed in separate 100 ml glass beakers containing seawater from the respective incubation tanks. Light incubations were performed in the morning after corals were exposed to a minimum of 1 h of light. Dark incubations were performed at the end of the night on corals that were acclimated to darkness for more than 8 h. Calcification rates determined by buoyant weighing and alkalinity anomaly were normalized to surface area, estimated using the wax dipping method [28].

(c) Experimental conditions for confocal microscopy

Measurements of pH_{CF} (=subcalicoblastic medium) and pH_i of the calcifying cells in the light and dark were made by inverted confocal microscopy (Leica SP5, Germany) as described previously [9]. Briefly, samples that had been grown laterally on glass coverslips were fitted in semi-closed perfusion chambers (4 cm internal diameter; PeCon, Germany), mounted on the confocal microscope and supplied with seawater drawn from the desired incubation treatment (renewal rate of 50% min^{-1} in 2.5 ml). Colony sizes were restricted to 1 cm^2 in surface area, irradiance was provided by fibre optic light source (Philips 21 V 150-W halogen bulb) and temperature was maintained at 25°C. Prior optimization of seawater flow rates and colony size has demonstrated that carbonate chemistry remains constant in the perfusion under these conditions [2,9,29] (see discussion of the experimental design in the electronic supplementary material).

(d) Analysis of pH_{CF} and pH_i of calicoblastic cells

Confocal pH measurements were made using ratiometric analysis of two forms of the dual emission pH-sensitive dye SNARF-1 (Invitrogen) according to methods published previously [2,9]. After being transferred from the treatment aquaria directly to the microscope, samples were first perfused with seawater from the desired experimental treatment for 10 min in either the light or dark. For pH_{CF}, samples were then perfused with seawater from the desired treatment containing 45 µM cell-impermeable SNARF-1 for a 5 min loading period, before making five measurements of pH_{CF} during a 10 min time window in light or dark. pH measurements were also taken in the seawater surrounding the corals in the perfusion chamber to confirm seawater pH remained stable during confocal analysis and that it remained the same between treatments.

For measurements of pH_i of calicoblastic cells, the procedure involved 10 min of dye loading by perfusion with seawater containing 10 µM cell-permeable SNARF-1 AM, followed by 10 min of seawater perfusion, during which pH_i measurements were taken to check pH_i was stable.

Calibration of intracellular SNARF-1 AM and extracellular (seawater and CF) SNARF-1 to pH was performed as described previously [16] to the National Bureau of Standards (NBS) pH scale and total scale pH, respectively.

pH_{CF} and calicoblastic cell pH_i measurements were carried out at 40× magnification by excitation at 543 nm at 30% laser intensity, and fluorescence captured at emission wavelengths of 585 $\pm$ 10 nm and 640 $\pm$ 10 nm. For each measurement, several optical sections were captured in a Z-stack, with an acquisition time of approximately 10 s. pH_{CF} was measured in light and dark conditions in six samples from each treatment. Calicoblastic cell pH_i was measured in three colonies from the very low, ambient and high [DIC] and [A_T] treatments.

(e) Photosynthesis and respiration

Rates of net photosynthesis (P_n) and respiration (R) were assessed at 0 and 250 µmol quanta m^{-2} s^{-1} on five nubbins per treatment in 50 ml glass chambers. Chambers were equipped with a Unisense optode (oxygen-sensitive minisensor) connected to the Oxy-4 software (Chanel fibreoptic oxygen meter, Presens, Regensburg, Germany). The optodes were calibrated before each experiment against air-saturated (by bubbling) and nitrogen-saturated seawater (for the 100% and 0% oxygen, respectively). Stir bars were used to continuously create turbulent conditions in the chambers. P_n and R were calculated by regressing oxygen flux against time, and were corrected by control incubation performed with empty chambers. Gross photosynthesis was calculated by adding the absolute value of R to P_n. At the end of each incubation, nubbins were frozen (−20°C) before determining *Symbiodinium* densities, and total chlorophyll concentrations [30].

All the data presented in the manuscript were deposited in the PANGAEA database [31].

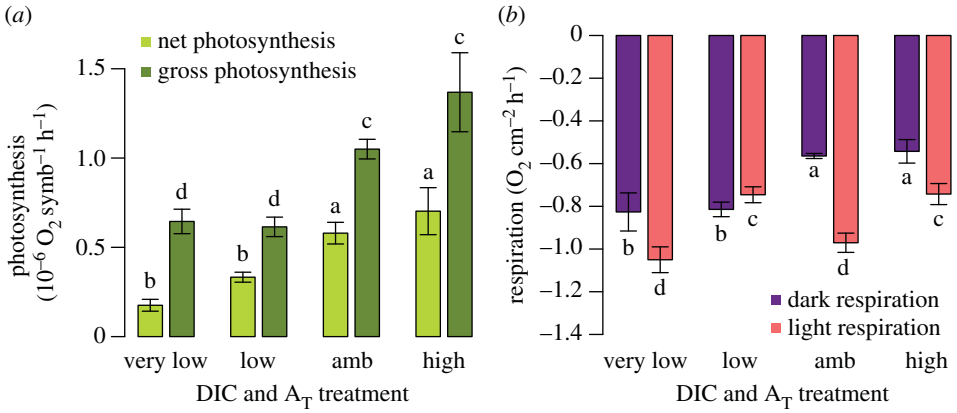

Figure 2. Photosynthesis and respiration (mean ± s.e.) of *S. pistillata* incubated at constant pH_T (approx. 7.9) and varying dissolved inorganic carbon concentration [DIC] and total alkalinity [A_T]. ([DIC] was approximately 800, 1500, 2200, 2900 µmol kg^{-1} in the very low, low, amb and high treatments, respectively). (*a*) Net photosynthesis and gross photosynthesis normalized by the *Symbiodinium* density measured at the end of the two week incubation. (*b*) Light and dark respiration normalized by the surface area of organisms. Columns with the same letters were not significantly different (*post hoc* analyses). (Online version in colour.)

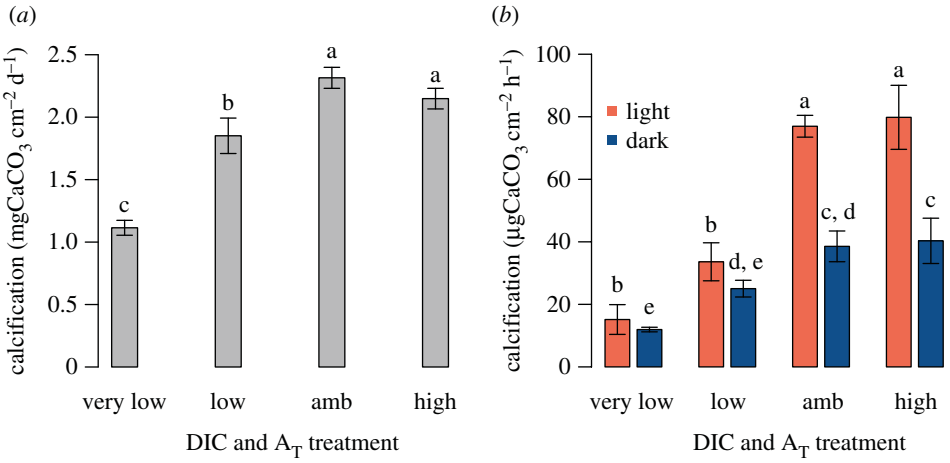

Figure 3. Calcification rates (mean ± s.e.) of *S. pistillata* at constant pH_T (approx. 7.9) and varying [DIC] and [A_T]. ([DIC] was approximately 800, 1500, 2200, 2900 µmol kg^{-1}, in the very low, low, amb and high treatments, respectively). (*a*) Net calcification measured by the buoyant weight technique over the two week incubation. (*b*) Net calcification measured in the light and the dark at the end of the incubations using the alkalinity anomaly technique. Columns with the same letters were not significantly different (post hoc analyses). (Online version in colour.)

3. Results

(a) Carbonate chemistry

Carbonate chemistry in the tanks was precisely regulated across all treatments, and tanks were accurately duplicated within treatments (electronic supplementary material, table S1). Seawater pH_T was maintained close to the pH in the ambient treatment (7.89 ± 0.1), with mean values of 7.92 ± 0.1, 7.94 ± 0.2 and 7.91 ± 0.3 in the very low, low and high [DIC] and [A_T] treatments, respectively (±s.e., $n = 15$, electronic supplementary material, table S1). [DIC] was maintained at 847 ± 57, 1554 ± 47, 2219 ± 10 and 2968 ± 39 µmol kg^{-1} in the very low, low, ambient and high [DIC] treatments (respectively), which corresponded to [A_T] values of 1001 ± 30, 1775 ± 31, 2461 ± 5 and 3278 ± 19 µmol kg^{-1} (mean ± s.e., $n = 15$, electronic supplementary material, table S1).

(b) Photosynthesis—respiration

In the following section, photosynthesis was normalized to *Symbiodinium* cells to assess the functioning of the symbionts under the different [DIC] and [A_T] treatments. Respiration was normalized to surface area to assess both the host's and the symbionts' responses to the treatments. *Symbiodinium*-normalized net photosynthesis (P_n) was maximal at ambient and high [DIC] and [A_T] (figure 2*a*, light green). There was an effect of treatment on *Symbiodinium*-normalized P_n ($p < 0.001$), with P_n increasing with [DIC] and [A_T] (figure 2*a*). Gross photosynthesis (P_g) followed similar trends, with a significant effect of treatment ($p < 0.001$) and greatest values for *Symbiodinium*-normalized rates at ambient and high [DIC] and [A_T] treatments (figure 2*a*).

Surface area-normalized dark respiration (R_d) was affected by treatment ($p = 0.004$) and slightly decreased with increasing [DIC] and [A_T] (figure 2*b*). Surface area-normalized respiration in the light (R_l) was affected by treatment when normalized to area ($p < 0.001$; figure 2*b*) although there was no overall decreasing or increasing trend across [DIC] and [A_T] treatments.

(c) Calcification

Buoyant weight analysis of *S. pistillata* was conducted to provide an integrated measurement of net calcification (dry weight) in the light and dark over the 15 days period (figure 3*a*). Treatment affected calcification (ANOVA, $F_{3,36} =$

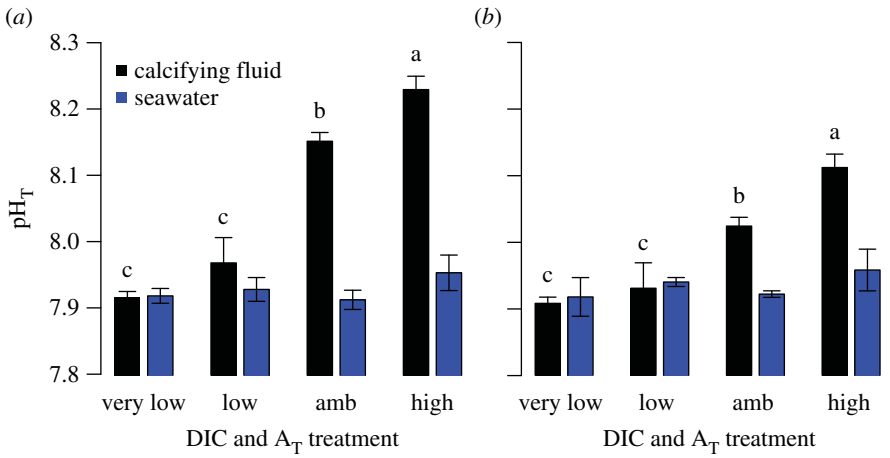

Figure 4. Confocal microscopy pH measurements (mean ± s.e.) made on organisms maintained under constant pH_T (approx. 7.9) and varying dissolved inorganic carbon concentration [DIC] and [AT]. ([DIC] was approximately 800, 1500, 2200, 2900 μmol kg^{-1}, in the very low, low, amb and high treatments, respectively). Measurements were done in the light (*a*) and dark (*b*) in the calcifying fluid and the seawater. Columns with the same letters were not significantly different (post hoc analyses). (Online version in colour.)

30.04, $p < 0.001$), with calcification increasing with [DIC] and [A_T] from the very low to the ambient treatment (figure 3*a*). *Post hoc* analyses showed that calcification in the very low and low [DIC] and [A_T] differed significantly from all the other treatments ($p < 0.01$).

Net light calcification of *S. pistillata* was impacted by treatment, with a maximum of 79 ± 10 μg $CaCO_3$ cm^{-2} h^{-1} in the high [DIC] and [A_T] treatment, and a minimum of 15 ± 5 μg $CaCO_3$ cm^{-2} h^{-1} in the very low [DIC] and [A_T] treatment (figure 3*b*). Treatment affected net calcification in the light (ANOVA, $p < 0.001$), and *post hoc* analyses revealed that calcification differed between treatments ($p < 0.01$), except between the very low and low [DIC] and [A_T] treatments ($p = 0.326$) and between the ambient and high [DIC] and [A_T] treatments ($p = 0.994$).

Similarly, maximal net calcification rates in the dark were recorded in the high and ambient [DIC] and [A_T] treatment, whereas minimal calcification (11 ± 1 μg $CaCO_3$ cm^{-2} h^{-1}) was measured in the very low [DIC] and [A_T] treatment (figure 3*b*). Treatment affected calcification in the dark ($p < 0.001$), with increasing [DIC] and [A_T] stimulating dark calcification. *Post hoc* analyses demonstrated that the very low treatment differed from the ambient and high [DIC] and [A_T], and the low [DIC] and [A_T] differed from the high [DIC] and [A_T] treatment ($p < 0.002$).

(d) Calcifying fluid pH

Coral samples were mounted in a perfusion chamber and analysed by confocal microscopy and the fluorescent pH dye SNARF-1 to determine pH_{CF}. The experimental set-up also allowed confocal measurements of pH to be taken in the seawater flowing through the perfusion chamber to confirm that seawater pH was consistent among treatments (figure 4).

pH_{CF} varied across the treatments during the light and dark ($p < 0.001$), and was more elevated at higher [DIC] and [A_T] (figure 4*a*,*b*). In the light, pH_{CF} varied from 7.92 ± 0.01 in the very low [DIC] and [A_T] treatment to 8.23 ± 0.02 in the high [DIC] and [A_T] treatment. *Post hoc* analyses revealed that pH_{CF} differed between treatments, except between the very low and low [DIC] and [A_T] ($p = 0.395$). In the dark, pH_{CF} varied from 7.91 ± 0.02 at very low [DIC], to 8.11 ± 0.01 at high [DIC] and [A_T]. Similar to pH_{CF} in the light, *post hoc* analyses showed that pH_{CF} in the dark differed between treatments, except between the very low and low [DIC] and [A_T] ($p = 0.904$).

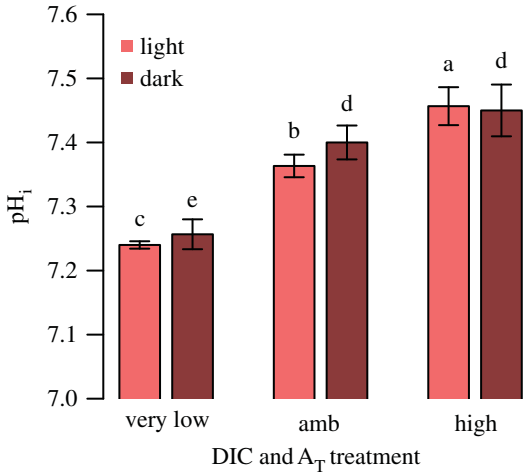

Figure 5. Confocal microscopy measurements of the pH in the calicoblastic cells (pH_i) (mean ± s.e.) of *S. pistillata* maintained under constant pH_T (approx. 7.9) and very low [DIC] and [A_T] (very low, DIC approx. 800 μmol kg^{-1}; [A_T] approx. 1000 μmol kg^{-1}), ambient DIC and [A_T] (amb; DIC approx. 2200 μmol kg^{-1}; [A_T] approx. 2500 μmol kg^{-1}), and high DIC (high, DIC approx. 2900 μmol kg^{-1}; [A_T] approx. 3300 μmol kg^{-1}). Measurements were done in the light and the dark. Columns with the same letters were not significantly different (post hoc analyses). (Online version in colour.)

(e) Calicoblastic cell pH_i

pH_i was measured in the high, ambient and very low DIC and [A_T] treatments (figure 5). pH_i varied according to [DIC] and [A_T] in both light and darkness ($p < 0.001$ and $p = 0.011$ in light and darkness, respectively) with pH_i decreasing with decreasing [DIC] and [A_T].

4. Discussion

Determining the factors that control pH regulation of the CF is a critical step towards a better mechanistic understanding of

coral calcification physiology, and identifying why corals are sensitive to changes in seawater carbonate chemistry. Previous investigations into pH_{CF} have focused on the influence of seawater pH on pH_{CF}, and no attention has been paid to other seawater carbonate parameters. Here we maintained seawater pH constant, while modifying seawater carbonate chemistry through a manipulation of $[A_T]$ and [DIC], which resulted in variation in the concentration of the components of seawater DIC, namely CO_2, HCO_3^- and CO_3^{2-} (electronic supplementary material, table S1). pH_{CF} varied across the treatments, displaying a positive relationship with increasing [DIC] and $[A_T]$ despite external seawater pH remaining constant. Notably, pH_{CF} was not regulated above the surrounding seawater pH by corals in the low and very low [DIC] and $[A_T]$ treatments. Our findings demonstrate that seawater pH is not the only driver of pH_{CF}.

(a) Influence of photosynthesis and respiration on pH_{CF}

We analysed photosynthesis and respiration, because previous research suggests that both physiological parameters are likely to have major roles in influencing pH_{CF} levels [2,14]. Studies have shown that photosynthesis increases pH_{CF} in the light versus dark [2,10]. The data in this study are consistent with these previous studies, because under ambient [DIC] and $[A_T]$, pH_{CF} was higher in the light than in the dark, and when net photosynthetic rates decreased in low and very low [DIC] and $[A_T]$ treatments, there was a concomitant drop in pH_{CF}. It is possible that the drop in photosynthetic rate occurred owing to DIC-limitation, because corals use external seawater DIC as the principal source of carbon for photosynthesis [14]. Overall, this could point to a mechanism by which seawater [DIC] modulates pH_{CF} by affecting photosynthetic rate. Although this mechanism may provide a partial explanation for the response of pH_{CF} to seawater [DIC] and $[A_T]$ treatments in the light, it does not, however, explain the dark pattern and why pH_{CF} increased in high [DIC] and $[A_T]$ treatment relative to ambient [DIC] and $[A_T]$, despite net photosynthetic rates remaining unchanged.

In the case of respiration, previous studies indicate that DIC derived from coral respiration accounts for a large portion of the DIC used in coral calcification [14,16]. It follows, therefore, that variation in production of respiratory CO_2 may have an effect on pH_{CF}. Reductions in pH_{CF} at low and very low [DIC] and $[A_T]$ observed in darkness are consistent with this possibility, because respiration rates were significantly elevated in these treatments relative to ambient and high DIC and $[A_T]$ levels. This could suggest a mechanism where at lower seawater [DIC] and $[A_T]$, increased respiration led to higher rates of CO_2 diffusion from calicoblastic cells into the CF with an acidifying effect. Under such a scenario, cellular mechanisms of pH_{CF} regulation (e.g. previously proposed Ca^{2+} ATPase activity) appeared not to have prevented CF acidosis. However, changes in respiration rates do not explain increases in pH_{CF} that occurred at high [DIC] and $[A_T]$ in darkness, as respiration rates were the same in the ambient and high [DIC] and $[A_T]$ treatments.

(b) Influence of pH_i of calcifying cells on pH_{CF}

Variation in rates of photosynthesis and respiration may have contributed to the observed response of pH_{CF} to changes in seawater carbonate chemistry associated with shifts in [DIC] and $[A_T]$, but these parameters do not explain certain aspects of our data. As noted in §4a, pH_{CF} was higher in high [DIC] and $[A_T]$ treatments relative to ambient [DIC] and $[A_T]$, with no concomitant change in photosynthesis or respiration. Furthermore, in low and very low [DIC] and $[A_T]$ treatments, pH_{CF} was no longer regulated above seawater pH in either light or dark conditions. These data suggest that seawater carbonate chemistry parameters may affect pH_{CF} by an additional mechanism not directly related to photosynthesis or respiration.

It is unlikely that this mechanism involves seawater leakage (e.g. by paracellular transport) into the CF exerting direct control of pH_{CF}, because maintaining elevated pH under elevated [DIC] and $[A_T]$, with higher buffering capacity, is chemically more challenging than at low [DIC] and $[A_T]$ [32]. Thus, if the mechanism by which corals regulate pH_{CF} remains the same between treatments, we would not expect to see decreases in pH_{CF} in the low and very low [DIC] and $[A_T]$ treatments relative to high [DIC] and $[A_T]$.

Instead, our calicoblastic cell pH_i data point to another candidate mechanism by which pH_{CF} is affected, involving an effect of changes in seawater carbonate chemistry on calicoblastic pH_i. Calicoblastic pH_i decreased in parallel with changes in seawater carbonate chemistry associated with decreases in seawater [DIC] and $[A_T]$ at constant pH. These data indicate that the concentration of one or more DIC species (e.g. CO_2, HCO_3^- or CO_3^{2-}) or seawater A_T itself has a direct bearing on pH_i regulation in these cells. Although the available data in our paper and in the literature provide little insight into identifying which carbonate chemistry parameter is responsible for the changes in calicoblastic cell pH_i, disruption to pH_i impairs cellular processes including the functioning of membrane transporters [33]. Therefore, it follows that a decrease in calicoblastic cell pH_i may directly impinge on the ability of this tissue layer to regulate pH of the CF, contributing to the observed declines in pH_{CF} in the lower [DIC] and $[A_T]$ treatments. Indeed, previous research involving seawater acidification has also argued that decreases in pH_i of calicoblastic cells may lead to decreases in pH_{CF} [9].

Determining the effects of carbonate chemistry on both calcifying cell pH_i and pH_{CF} will depend crucially on a better understanding of ion transport from seawater across the coral tissues. Currently, the literature on this subject is not clear, because certain previous studies have pointed to a largely unrestricted exchange of ions between the CF and the surrounding seawater [5], whereas other studies have pointed to a more restricted exchange of ions mediated by transcellular transport mechanisms and septate junctions between cells [6,7].

(c) Calcification and calcifying fluid Ω

Several studies manipulating seawater carbonate chemistry have found that varying [DIC] and $[A_T]$ affects coral calcification rates [21–23]. These studies include the work of Jury *et al*. [21] and Schneider & Erez [25], who recorded a strong decline in calcification rates at constant ambient seawater pH over a decrease in seawater [DIC] and $[A_T]$ similar to that of this study. Schneider & Erez [25] also demonstrated a decrease in calcification with [DIC] when pH was held constant. These results have been interpreted to mean that decreases in seawater [DIC] restrict the supply of inorganic carbon as a reactant in the calcification reaction [21–25].

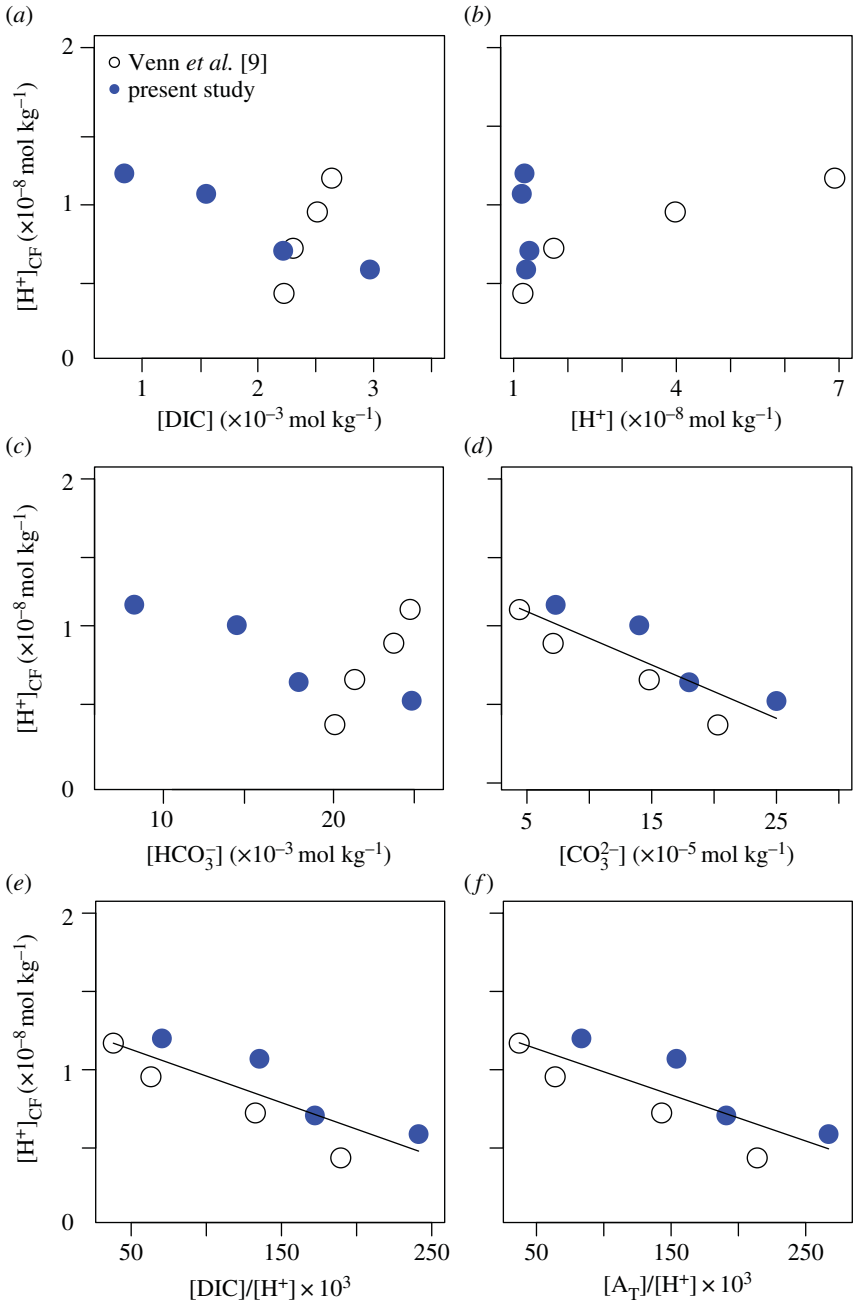

Figure 6. Relationship between the proton concentration in the calcifying fluid ($[H^+]_{CF}$) and various parameters of the carbonate chemistry. Data from this study (filled circles) were combined with data from Venn *et al.* [9] (open circles) to compare CO_2-driven decreases in seawater pH with relatively small increases in seawater [DIC] and no change in $[A_T]$ with large change of [DIC] and $[A_T]$ at constant pH. $[H^+]_{CF}$ was plotted against (*a*) seawater [DIC], (*b*) seawater $[H^+]$, (*c*) seawater $[HCO_3^-]$, (*d*) seawater $[CO_3^{2-}]$, (*e*) the ratio $[DIC]/[H^+]$ and (*f*) the ratio $[A_T]/[H^+]$. There were strong linear correlations of $[H^+]_{CF}$ with $[CO_3^{2-}]$, the ratio $[DIC]/[H^+]$ and the ratio $[A_T]/[H^+]$ ($R^2 = 0.71$, $p = 0.010$; $R^2 = 0.75$, $p = 0.006$, and $R^2 = 0.68$, $p = 0.012$, respectively). (Online version in colour.)

Our study provides a new, complementary perspective where changes in carbonate chemistry associated with shift in seawater [DIC] and $[A_T]$ also modulate pH_i and pH_{CF}. As pH_{CF} affects aragonite saturation state (Ω_{arag}) of the CF, changes in pH_{CF} are expected to correspond to changes in calcification rate. Our observations of decreased calcification rates of *S. pistillata* measured by buoyant weight and alkalinity anomaly methods are consistent with this expectation, as calcification rates follow a similar trend to pH_{CF} in very low, low and ambient [DIC] and $[A_T]$ treatments under both light and dark conditions.

We note that there are differences between the patterns of pH_{CF} and calcification, which suggest that pH_{CF} is not the only factor influencing the calcification response to seawater [DIC] and $[A_T]$. For example, while calcification rates saturate at ambient and high [DIC] and $[A_T]$, pH_{CF} increased between ambient and high [DIC] and $[A_T]$. Similarly, a comparison between similar light and dark pH_{CF} values (e.g. pH_{CF} 8.15 in the light at ambient [DIC] and $[A_T]$ versus pH_{CF} 8.11 in the dark in high [DIC] and $[A_T]$) does not yield similar calcification rates (light ambient [DIC] and $[A_T]$ calcification rates are two times higher than dark calcification rates at high [DIC] and $[A_T]$). This indicates that although regulation of pH_{CF} is essential to provide a favourable physiochemical environment for $CaCO_3$ precipitation, the physiology and biochemistry involved in the calcification process is complex [6] and involves additional factors that are likely to influence calcification rates. These include synthesis of the skeletal organic

matrix by the calicoblastic cells partly from precursors produced by the coral's symbiotic algae [34]. The organic matrix serves to catalyse nucleation of aragonite crystals and potentially act as template for skeleton formation [6,35].

In addition to pH_{CF}, Ω_{arag} of the CF and calcification rates may also be determined by CF [DIC], but we are not able to determine this parameter from our data. Currently, the literature is divided over whether or not [DIC] is elevated in the CF with respect to seawater. While geochemical analysis of coral skeletons suggests that DIC is concentrated at the site of calcification [12], [DIC] derived from microelectrode measurements of carbonate in the CF indicate that [DIC] is similar to that of the surrounding seawater [13]. Further research is required to definitively resolve this issue.

(d) Correlation of carbonate chemistry parameters with pH_{CF}

The role of seawater carbonate chemistry in controlling coral calcification has been described previously [35–37] in terms of the ratio of seawater concentrations $[DIC]_{SW}:[H^+]_{SW}$. The authors of these previous pieces of work proposed that calcification is influenced by the $[DIC]_{SW}:[H^+]_{SW}$ ratio, as rates of calcification are limited by the supply of DIC and the accumulation of H^+ (i.e. a product of calcification) [36–38]. Reanalysis of data from several previous ocean acidification experiments supports the proposed relationship between $[DIC]_{SW}/[H^+]_{SW}$ and calcification rates [36–38].

To investigate whether there also was a relationship between $[DIC]_{SW}/[H^+]_{SW}$ ratio and pH_{CF}, we combined the data of this study with a previous confocal investigation on pH_{CF} in *S. pistillata* [9]. Seawater treatments in this previous study involved CO_2-driven decreases in seawater pH with relatively small increases in seawater [DIC] and no change in $[A_T]$ (as occurring with ocean acidification). First, net calcification was plotted (as values relative to controls) against $[DIC]_{SW}/[H^+]_{SW}$ (electronic supplementary material, figure S1) to reveal a linear relationship similar to those described previously [36–38]. pH_{CF} (expressed as CF proton concentrations ($[H^+]_{CF}$)) was then plotted against various carbonate chemistry parameters and the $[DIC]_{SW}/[H^+]_{SW}$ and $[A_T]_{SW}/[H^+]_{SW}$ ratios (figure 6). These plots reveal that the compiled $[H^+]_{CF}$ of both studies exhibits a strong linear correlation with the $[DIC]_{SW}/[H^+]_{SW}$ ratio, the $[A_T]/[H^+]_{SW}$ ratio and $[CO_3^{2-}]_{SW}$ ($R^2 = 0.75$, $p = 0.006$; $R^2 = 0.68$, $p = 0.012$ and $R^2 = 0.71$, $p = 0.010$, respectively). By contrast, compiled $[H^+]_{CF}$ values do not correlate with $[H^+]_{SW}$, $[DIC]_{SW}$ or $[HCO_3^-]_{SW}$ ($p > 0.20$).

These compiled data suggest that neither $[H^+]_{SW}$ (i.e. seawater pH), $[DIC]_{SW}$ nor $[HCO_3^-]_{SW}$ are controlling parameters of pH_{CF}. Instead the mechanism of pH_{CF} depends on either the $[DIC]_{SW}/[H^+]_{SW}$ ratio, the $[A_T]/[H^+]_{SW}$ ratio or $[CO_3^{2-}]_{SW}$. Distinguishing between the $[DIC]_{SW}/[H^+]_{SW}$ ratio, the $[A_T]/[H^+]_{SW}$ ratio or $[CO_3^{2-}]_{SW}$ is not possible in the current experimental scenario. Deciphering the parameters modulating pH_{CF} will rely on improving our current understanding of ion transport mechanisms in corals, particularly in terms of the exchange of H^+, HCO_3^- and CO_3^{2-} between the CF and the surrounding seawater.

Beyond gaining insights into biomineralization mechanisms in scleractinians, the correlations of $[H^+]_{CF}$ with carbonate chemistry parameters are of relevance to research into geochemical proxies, which have the goal of using isotopic signatures in coral skeletons as indicators of previous environmental conditions. For example, boron isotope systematics are used widely to determine pH_{CF} [29,39], which used to be assumed to reflect seawater pH with an offset owing to the effects of pH_{CF} regulation by the coral (sometimes referred to as a 'vital effect') [40,41]. However, the observed dependence of pH_{CF} upregulation on $[DIC]_{SW}$ and the lack of correlation with seawater $[H^+]$ and $[H^+]_{CF}$ (figure 6) in this study serve to illustrate that a robust calibration of the vital effect cannot be uniquely conducted using an assumed offset of pH_{CF} from seawater pH.

5. Conclusion

This study determined that pH_{CF} is influenced by shifts in seawater chemistry associated with changes in seawater [DIC] and $[A_T]$ at constant seawater pH. This complements previous studies that have shown that similar carbonate chemistry treatments also modulate coral calcification rates [25]. In the light, availability of seawater [DIC] may affect photosynthetic rates, which is likely to influence pH_{CF} and CF Ω, and therefore calcification rates. In darkness, the observed increases in respiration rates may have contributed to lower pH_{CF} in the lower seawater [DIC] and $[A_T]$ treatments. Our calicoblastic cell pH_i data suggest that despite constant seawater pH, changes in seawater carbonate chemistry may disturb calicoblastic cell pH_i regulation, which may impair the ability of this cell layer to regulate pH_{CF}. Future cellular investigations into ion transport in corals and a better understanding of calicoblastic cell physiology are needed to explore this possibility further. Most notably, we demonstrate that pH_{CF} expressed as $[H^+]_{CF}$ shows a strong linear correlation with seawater $[CO_3^{2-}]_{SW}$, the $[DIC]_{SW}/[H^+]_{SW}$ ratio and the $[A_T]/[H^+]_{SW}$ ratio.

Overall, the observed effects of manipulated seawater carbonate chemistry on pH_{CF} regulation in *S. pistillata* highlight that there is still much to learn about the physiological mechanisms controlling CF chemistry in scleractinians. Characterizing these mechanisms in scleractinians (as well as other calcifiers) is important to diverse lines of research including palaeoenvironmental reconstructions and predicting the response of calcifying organisms to the impending oceanic changes.

Data accessibility. See [31].

Authors' contributions. S.C., E.T., P.J.E., R.C.C., S.T. and A.V. conceived and designed research. S.C., E.T., N.R.E., C.F.-P. and A.V. performed the laboratory experiments. S.C., E.T., S.T., P.J.E., R.C.C., C.F.-P., D.A. and A.V. analysed data. All co-authors contributed to the manuscript and participated in scientific discussions of the findings.

Competing interests. We have no competing interests.

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References

1. Weis VM, Allemand D. 2009 What determines coral health? *Science* **324**, 1153–1155. (doi:10.1126/science.1172540)
2. Venn A, Tambutté E, Holcomb M, Allemand D, Tambutté S. 2011 Live tissue imaging shows reef corals elevate pH under their calcifying tissue relative to seawater. *PLoS ONE* **6**, e20013. (doi:10.1371/journal.pone.0020013)
3. Ries JB. 2011 A physicochemical framework for interpreting the biological calcification response to CO_2-induced ocean acidification. *Geochim. Cosm. Acta* **75**, 4053–4064. (doi:10.1016/j.gca.2011.04.025)
4. Zoccola D *et al.* 2015 Bicarbonate transporters in corals point towards a key step in the evolution of cnidarian calcification. *Sci. Rep.* **5**, 09983. (doi:10.1038/srep09983)
5. Gagnon AC, Adkins JF, Erez J. 2012 Seawater transport during coral biomineralization. *Earth Planet. Sci. Lett.* **329–330**, 150–161. (doi:10.1016/j.epsl.2012.03.005)
6. Tambutté S, Holcomb M, Ferrier-Pagès C, Reynaud S, Tambutté É, Zoccola D, Allemand D. 2011 Coral biomineralization: from the gene to the environment. *J. Exp. Mar. Biol. Ecol.* **408**, 58–78. (doi:10.1016/j.jembe.2011.07.026)
7. Ganot P, Zoccola D, Tambutté E, Voolstra CR, Aranda M, Allemand D, Tambutté S. 2015 Structural molecular components of septate junctions in cnidarians point to the origin of epithelial junctions in eukaryotes. *Mol. Biol. Evol.* **32**, 44–62. (doi:10.1093/molbev/msu265)
8. Zoccola D, Tambutté E, Kulhanek E, Puverel S, Scimeca JC, Allemand D, Tambutté S. 2004 Molecular cloning and localization of a PMCA P-type calcium ATPase from the coral *Stylophora pistillata*. *Biochim. Biophys. Acta* (*BBA*) - *Biomembranes* **1663**, 117–126. (doi:10.1016/j.bbamem.2004.02.010)
9. Venn A, Tambutté E, Holcomb M, Laurent J, Allemand D, Tambutté S. 2015 Impact of seawater acidification on pH at the tissue–skeleton interface and calcification in reef corals. *Proc. Natl Acad. Sci. USA* **110**, 1634–1639. (doi:10.1073/pnas.1216153110)
10. Al-Horani FA, Al-Moghrabi SM, De Beer D. 2003 The mechanism of calcification and its relation to photosynthesis and respiration in the scleractinian coral *Galaxea fascicularis*. *Mar. Biol.* **142**, 419–426. (doi:10.1007/s00227-002-0981-8)
11. McCulloch M, Falter J, Trotter J, Montagna P. 2012 Coral resilience to ocean acidification and global warming through pH up-regulation. *Nat. Clim. Change* **2**, 623–627. (doi:10.1038/nclimate1473)
12. Allison N, Cohen I, Finch AA, Erez J, Tudhope AW. 2014 Corals concentrate dissolved inorganic carbon to facilitate calcification. *Nat. Commun.* **5**, 5741. (doi:10.1038/ncomms6741)
13. Cai W-J *et al.* 2016 Microelectrode characterization of coral daytime interior pH and carbonate chemistry. *Nat. Commun.* **7**, 11144. (doi:10.1038/ncomms11144)
14. Furla P, Galgani I, Durand I, Allemand D. 2000 Sources and mechanisms of inorganic carbon transport for coral calcification and photosynthesis. *J. Exp. Biol.* **203**, 3445–3457.
15. Erez J. 1978 Vital effect on stable-isotope composition seen in foraminifera and coral skeletons. *Nature* **273**, 199–202. (doi:10.1038/273199a0)
16. Pearse VB. 1970 Incorporation of metabolic CO_2 into coral skeleton. *Nature* **228**, 383. (doi:10.1038/228383a0)
17. Gaillardet J, Allègre CJ. 1995 Boron isotopic compositions of corals: seawater or diagenesis record? *Earth Planet. Sci. Lett.* **136**, 665–676. (doi:10.1016/0012-821X(95)00180-K)
18. Pelejero C, Calvo E, McCulloch MT, Marshall JF, Gagan MK, Lough JM, Opdyke BN. 2005 Preindustrial to modern interdecadal variability in coral reef pH. *Science* **309**, 2204–2207. (doi:10.1126/science.1113692)
19. Hönisch B *et al.* 2012 The geological record of ocean acidification. *Science* **335**, 1058–1063. (doi:10.1126/science.1208277)
20. Muscatine L, Tambutté E, Allemand D. 1997 Morphology of coral desmocytes, cells that anchor the calicoblastic epithelium to the skeleton. *Coral Reefs* **16**, 205–213. (doi:10.1007/s003380050075)
21. Jury CP, Whitehead RF, Szmant AM. 2010 Effects of variations in carbonate chemistry on the calcification rates of *Madracis auretenra* (= *Madracis mirabilis* sensu Wells, 1973): bicarbonate concentrations best predict calcification rates. *Glob. Change Biol.* **16**, 1632–1644. (doi:10.1111/j.1365-2486.2009.02057.x)
22. Comeau S, Carpenter RC, Edmunds PJ. 2013 Coral reef calcifiers buffer their response to ocean acidification using both bicarbonate and carbonate. *Proc. R. Soc. B* **280**, 20122374. (doi:10.1098/rspb.2012.2374)
23. Marubini F, Ferrier-Pagès C, Furla P, Allemand D. 2008 Coral calcification responds to seawater acidification: a working hypothesis towards a physiological mechanism. *Coral Reefs* **27**, 491–499. (doi:10.1007/s00338-008-0375-6)
24. de Putron SJ, McCorkle DC, Cohen AL, Dillon AB. 2010 The impact of seawater saturation state and bicarbonate ion concentration on calcification by new recruits of two Atlantic corals. *Coral Reefs* **30**, 321–328. (doi:10.1007/s00338-010-0697-z)
25. Schneider K, Erez J. 2006 The effect of carbonate chemistry on calcification and photosynthesis in the hermatypic coral *Acropora eurystoma*. *Limnol. Oceanogr.* **51**, 1284–1293. (doi:10.4319/lo.2006.51.3.1284)
26. Goiran C, Al-Moghrabi S, Allemand D, Jaubert J. 1996 Inorganic carbon uptake for photosynthesis by the symbiotic coral/dinoflagellate association I. Photosynthetic performances of symbionts and dependence on sea water bicarbonate. *J. Exp. Mar. Biol. Ecol.* **199**, 207–225. (doi:10.1016/0022-0981(95)00201-4)
27. Chisholm JRM, Gattuso J-P. 1991 Validation of the alkalinity anomaly technique for investigating calcification and photosynthesis in coral reef communities. *Limnol. Oceanogr.* **36**, 1232–1239. (doi:10.4319/lo.1991.36.6.1232)
28. Veal CJ, Carmi M, Fine M, Hoegh-Guldberg O. 2010 Increasing the accuracy of surface area estimation using single wax dipping of coral fragments. *Coral Reefs* **29**, 893–897. (doi:10.1007/s00338-010-0647-9)
29. Holcomb M, Venn AA, Tambutté E, Tambutté S, Allemand D, Trotter J, McCulloch M. 2014 Coral calcifying fluid pH dictates response to ocean acidification. *Sci. Rep.* **4**, 5207. (doi:10.1038/srep05207)
30. Hoogenboom M, Béraud E, Ferrier-Pagès C. 2010 Relationship between symbiont density and photosynthetic carbon acquisition in the temperate coral *Cladocora caespitosa*. *Coral Reefs* **29**, 21–29. (doi:10.1007/s00338-009-0558-9)
31. Comeau S, Tambutté E, Carpenter RC, Edmunds PJ, Evensen NR, Allemand D, Ferrier-Pagès C, Tambutté S, Venn A. 2016 Coral calcifying fluid pH is modulated by seawater carbonate chemistry not solely seawater pH. Dataset no. 868353. (doi:pangaea.de/10.1594/PANGAEA.868353)
32. Wolf-Gladrow DA, Zeebe RE, Klaas C, Körtzinger A, Dickson AG. 2007 Total alkalinity: the explicit conservative expression and its application to biogeochemical processes. *Mar. Chem.* **106**, 287–300. (doi:10.1016/j.marchem.2007.01.006)
33. Busa WB, Nuccitelli R. 1984 Metabolic regulation via intracellular pH. *Am. J. Physiol.* **246**, R409–R438.
34. Muscatine L, Goiran C, Land L, Jaubert J, Cuif J-P, Allemand D. 2005 Stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) of organic matrix from coral skeleton. *Proc. Natl Acad. Sci. USA* **102**, 1525–1530. (doi:10.1073/pnas.0408921102)
35. Mass T, Drake JL, Haramaty L, Dongun Kim J, Zelzion E, Bhattacharya D, Falkowski PG. 2013 Cloning and characterization of four novel coral acid-rich proteins that precipitate carbonates *in vitro*. *Curr. Biol.* **23**, 1126–1131. (doi:10.1016/j.cub.2013.05.007)
36. Jokiel PL. 2011 The reef coral two compartment proton flux model: a new approach relating tissue-level physiological processes to gross corallum morphology. *J. Exp. Mar. Biol. Ecol.* **409**, 1–12. (doi:10.1016/j.jembe.2011.10.008)
37. Jokiel PL. 2013 Coral reef calcification: carbonate, bicarbonate and proton flux under conditions of increasing ocean acidification. *Proc. R. Soc. B* **280**, 20130031. (doi:10.1098/rspb.2013.0031)
38. Bach LT. 2015 Reconsidering the role of carbonate ion concentration in calcification by marine

organisms. *Biogeosciences* **12**, 4939–4951. (doi:10.5194/bg-12-4939-2015)

39. Georgiou L, Falter J, Trotter J, Kline DI, Holcomb M, Dove SG, Hoegh-Guldberg O, McCulloch M. 2015 pH homeostasis during coral calcification in a free ocean CO_2 enrichment (FOCE) experiment, Heron Island reef flat, Great Barrier Reef. *Proc. Natl Acad. Sci. USA* **112**, 13 219–13 224. (doi:10.1073/pnas.1505586112)
40. Trotter J *et al.* 2011 Quantifying the pH 'vital effect' in the temperate zooxanthellate coral *Cladocora caespitosa*: validation of the boron seawater pH proxy. *Earth Planet. Sci. Lett.* **303**, 163–173. (doi:10.1016/j.epsl.2011.01.030)
41. Hönisch B, Hemmings NG, Grottoli AG, Amat A, Hanson GN, Bijma J. 2004 Assessing scleractinian corals as recorders for paleo-pH: empirical calibration and vital effects. *Geochim. Cosmochim. Acta* **68**, 3675–3685. (idoi:10.1016/j.gca.2004.03.002)