RESEARCH ARTICLE

The influence of photosynthesis on host intracellular pH in scleractinian corals

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SUMMARY

The regulation of intracellular pH (pHi) is a fundamental aspect of cell physiology that has received little attention in studies of reef-building corals and symbiotic cnidarians. Here, we investigated the hypothesis that dynamic changes in the pHi of coral host cells are controlled by the photosynthetic activity of the coral's dinoflagellate symbionts. Using live cell imaging and the pH-sensitive dye SNARF-1, we tracked pH in symbiont-containing and symbiont-free cells isolated from the reef coral *Stylophora pistillata*. We characterised the response of coral pHi in the presence of a photosynthetic inhibitor, the dynamics of coral pHi during light exposure and how pHi values vary on exposure to a range of irradiance levels lying within the coral's photosynthesis–irradiance response curve. Our results demonstrate that increases in coral pHi are dependent on photosynthetic activity of intracellular symbionts and that pHi recovers under darkness to values that match those of symbiont-free cells. Furthermore, we show that the timing of the pHi response is governed by irradiance level and that pHi increases to irradiance-specific steady-state values. Minimum steady-state pHi values of 7.05±0.05 were obtained under darkness and maximum values of 7.46±0.07 were obtained under saturating irradiance. As changes in pHi often affect organism homeostasis, there is a need for continued research into acid/base regulation in symbiotic corals. More generally, these results represent the first characterization of photosynthesis-driven pHi changes in animal cells.

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INTRODUCTION

Photosynthetic symbioses in Cnidaria are major contributors to the structural and trophic foundation of shallow water coral reef ecosystems at tropical and subtropical latitudes. Reef-building corals form a symbiosis with photosynthetic dinoflagellates belonging to the genus *Symbiodinium*, which reside intracellularly in host endodermal cells (Muscatine, 1990; Trench, 1993). This endosymbiosis provides corals with access to a valuable source of photosynthetically fixed carbon that is used for host respiration and other essential processes (Muscatine, 1990; Venn et al., 2008; Yellowlees et al., 2008).

The relationship between corals and their symbiotic dinoflagellates is sensitive to changes in the marine environment associated with climate change (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007). Elevations in seawater temperature and ocean acidification can exert physiological stress on both coral and dinoflagellate partners (Lesser, 2007; Anthony et al., 2008; Crawley et al., 2010). Much research on coral biology is currently directed towards improving our understanding of how and why corals are sensitive to environmental change, but the field is currently impeded by a limited knowledge of the basics of the cell physiology underpinning the coral–dinoflagellate symbiosis (Weis et al., 2008; Weis and Allemand, 2009).

Intracellular cytosolic pH (pHi) is a fundamental parameter of cell physiology and is potentially very important to the cell biology of the coral–dinoflagellate symbiosis. pHi affects most aspects of cell biology, including protein synthesis, enzyme activity and cell signalling. As such, it has a strong influence over the physiology of all organisms and most organisms seek to minimise variations in pHi by a system of intracellular buffers and membrane transporters in order to maintain steady-state metabolism (Busa and Nuccitelli, 1984; Casey et al., 2010).

When changes in pHi do occur, they are frequently linked with transitions such as changes in rates of cell metabolism and events such as cell activation and division (Roos and Boron, 1981; Busa, 1986; Casey et al., 2010). In algae, pHi also significantly increases on exposure to light because of the activity of photosynthesis (Smith and Raven, 1979; Kurkdjian and Guern, 1989). For example, differences of 0.4–0.5 pH units have been observed between photosynthesising and non-photosynthesising cells of the giant single-celled algae *Chaetomorpha darwinii* (Raven and Smith, 1980) and the dinoflagellate *Prorocentrum micans*. The single previous study performed on cnidarian pHi suggests that coral pHi may also be responsive to light (Venn et al., 2009). It was observed that pHi in coral cells containing dinoflagellate symbionts exposed to light have a higher pHi than those kept in dark conditions. However, for a more complete understanding of the interactions between host pHi and photosynthesis, further research into pHi dynamics is required as this previous study was built on a single time point measurement with a fixed light intensity.

Important advances in the understanding of intracellular pH regulation in many organisms including corals have been facilitated by the use of pH-sensitive intracellular dyes (Dubbin et al., 1993; Lemasters et al., 1999). Certain dyes [such as carboxyseminaphthorhodafluor-1 (SNARF-1)] emit fluorescence at two wavelengths, which can be calibrated to the concentration of

ions in the cell (e.g. H^+). Used together with confocal microscopy, this approach allows the monitoring of dynamic pH changes in living cells. In the case of coral cells, the use of confocal microscopy has proved advantageous for cells containing algal symbionts, where high spatial resolution is required to analyse the coral cell cytoplasm, which is stretched tightly around intracellular symbionts (Venn et al., 2009).

The present study investigated the hypothesis that changes in the pHi of corals are shaped by the photosynthetic activity of coral symbionts. This involved: (1) testing whether changes in coral pHi could be blocked in the presence of a photosynthetic inhibitor; (2) characterising the dynamics of coral pHi during light exposure; (3) determining whether pHi values vary on exposure to a range of irradiance levels lying within the corals' photosynthesis–irradiance (PI) response curve; and (4) examining the rate of pHi recovery under darkness. To achieve this we analysed pHi in cells isolated from the reef coral *Stylophora pistillata* (Esper 1797) using confocal microscopy and the pH-sensitive intracellular probe SNARF-1. We monitored pHi in symbiont-containing and symbiont-free cells under controlled conditions of constant seawater pH and oxygen levels in flowing seawater to focus on the effect of intracellular mechanisms.

MATERIALS AND METHODS
Coral culture and preparation of cells
Stylophora pistillata colonies were maintained at the Centre Scientifique de Monaco in aquaria supplied with flowing Mediterranean seawater (salinity 38.2) with a 2% h^{-1} exchange rate, at 25±0.5°C. Irradiance levels were provided at 275 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) on a 12 h:12 h light:dark cycle. Corals were fed four times a week with frozen shrimp, krill and live *Artemia salina* nauplii. Cells were isolated from branches of *S. pistillata* colonies immediately before each experiment by gentle brushing of the tissue with a soft bristle-tooth brush into 50 ml filtered seawater (FSW). The resulting cell suspension was centrifuged once (350 g, 4 min) and the pellet of cells was resuspended in FSW as described previously (Venn et al., 2009). In the case of experiments on the photosynthetic response of coral cells to irradiance (PI response), preparations of isolated cells were adjusted to a density of 2.5×10^6 cells ml^{-1} and a 3 ml aliquot was taken for oxygen electrode analysis. A second aliquot (5 ml) was stored frozen (–20°C) for quantification of chlorophyll (chl). For pHi experiments, cell preparations were adjusted to a density of 2.5×10^5 cells ml^{-1} with FSW. Viability staining using AnnexinV-conjugate (Invitrogen, Grand Island, NY, USA) and Sytox-green (Invitrogen) confirmed that cells remained viable during experiments, as in a previous study (Venn et al., 2009).

Analysis of the photosynthetic response of cell preparations to irradiance by oxygen electrode
Cell preparations were transferred to a closed combined plate chamber (Hydro-Bios, Halifax, NS, Canada) illuminated by a fibre optic variable-irradiance light source (Bioblock, Fisher Scientific, Illkirch, France, with a Philips 21 V 150 W halogen bulb). The cell suspension in the cuvette was agitated using a magnetic stirrer and was maintained at 25±0.5°C by a recirculating water bath. An oxygen optode sensor system (oxy-4 mini, PreSens, Regensburg, Germany) was used to quantify oxygen flux. Samples were exposed to increasing irradiances (0, 20, 40, 60, 100, 150, 200, 300, 500, 800 and 1000 μmol photons m^{-2} s^{-1}) after a period of steady dark respiration rate. Irradiance levels were measured by a 2π quantum light meter (LI-Cor LI-250A, Lincoln, NB, USA) and were controlled by varying the light source settings and the distance

between light source and the sample. For each value of light intensity the rate of oxygen production/uptake was quantified for 4 min once stable values were reached. Data were recorded with OXY4v2_11FB software (PreSens).

Chlorophyll measurements
Aliquots stored for chlorophyll analysis were thawed, centrifuged (8000 g for 10 min at 4°C) and resuspended in 5 ml fresh acetone (100%). Samples were vortexed, and then incubated overnight at 4°C and centrifuged (11,000 g for 15 min at 15°C) to remove cell debris. A 2.5 ml aliquot of the supernatant was used to measure absorbance at 750, 663 and 630 nm using a spectrophotometer (SAFAS UV mc2, Monte Carlo, Monaco). Concentrations of chl a and c_2 were calculated using the equations of Jeffrey and Humphrey (Jeffrey and Humphrey, 1975).

Calculating the PI response curve
Oxygen flux was expressed per total chl concentration. The PI curve (Fig. 1) was fitted iteratively to the data to create nonlinear regression values using the following exponential equation as in previous studies (Romaine et al., 1997; Ferrier-Pagès et al., 2000):

$$P_{net} = P_{gmax} \left[1 - \exp(-I/I_k) \right] + R, \qquad (1)$$

where P_{net} is the net photosynthetic rate (nmol O_2 μg^{-1} chl min^{-1}), P_{gmax} is the gross maximum photosynthetic rate (nmol O_2 μg^{-1} chl min^{-1}), I is the irradiance (μmol photons m^{-2} s^{-1}), I_k is the irradiance at which the initial slope (α) intersects P_{gmax} (μmol photons m^{-2} s^{-1}) and R is the respiration rate in the dark (nmol O_2 μg^{-1} chl min^{-1}).

Analysis of pHi in coral cells by confocal microscopy
One millilitre aliquots of cell preparations (described above) were transferred to an open perfusion chamber (POC cell cultivation system, PECON, Erbach, Germany) and mixed with 2 ml of the cell permeant acetoxymethyl ester acetate of SNARF-1 (SNARF-1 AM) (Invitrogen) in FSW (10 μmol l^{-1} SNARF-1 AM, 0.01% pluronic F-127 and 0.1% DMSO 0.01%). Cell preparations were then dark-incubated for 30 min at 25°C to load cells with SNARF-1 AM and washed by 5 min perfusion with FSW in the dark to remove residual traces of the dye.
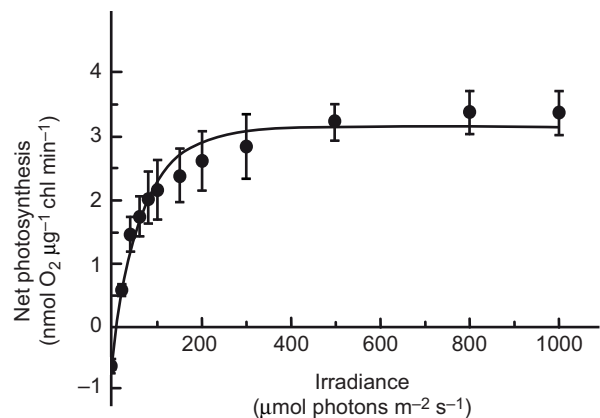

Fig. 1. Photosynthesis–irradiance (PI) curve. Net photosynthesis (nmol O_2 μg^{-1} chl min^{-1}; mean ± s.d.) of isolated cells from *Stylophora pistillata* (N=5) exposed to different irradiance levels (from 0 to 1000 nmol photons m^{-2} s^{-1}). Maximum rate of photosynthesis (P_{max})=3.33 nmol O_2 μg^{-1} chl min^{-1}, saturating irradiance (I_k)=74.47 μmol m^{-2} s^{-1}, photosynthetic efficiency (α)=0.052 and respiration (R)=–0.56 nmol O_2 μg^{-1} chl min^{-1}. Goodness of fit r^2=0.6789.

SNARF-1 fluorescence was measured by confocal microscope (Leica SP5, Buffalo Grove, IL, USA) and calibrated to pHi (NBS scale) using methods published previously (Venn et al., 2009). Briefly, cells were excited at 543 nm and SNARF-1 fluorescence emission was captured in two channels at 585 and 640±10 nm whilst simultaneously monitoring in transmission. In cells containing symbiont, the use of 543 nm as the excitation wavelength minimised chlorophyll autofluorescence, as 543 nm lies outside of absorption spectra of chl a and in the low region of absorption of the peridinin–chlorophyll–protein complex (Frank and Cogdell, 1996). pHi image analysis was performed using LAF-AS software (Leica) using digital regions of interest (ROI) to confine fluorescence analysis to the coral cell cytoplasm, avoiding dinoflagellate symbionts and their autofluorescent inclusion bodies, which appear with extended exposure time (Kazandjian et al., 2008) (Fig. 2). The 585/640 nm fluorescence intensity ratio (r) was calculated after subtracting background fluorescence recorded in a second ROI in the surrounding cell medium. r was related to pHi by the following equation:

$$pH = pK_a - \log [r - r_B / r_A - r \times F_{B(\lambda2)} / F_{A(\lambda2)}], \qquad (2)$$

where pK_a is the logarithmic acid dissociation constant, F is fluorescence intensity measured at 640 nm ($\lambda2$) and the subscripts A and B represent the values at the acidic and basic end points of the calibration, respectively. Intracellular calibration of pHi with SNARF-1 was performed for each experiment *in vivo* by ratiometric analysis of SNARF-1 fluorescence in cells exposed to buffers ranging from pH 6 to pH 8.5 containing the ionophore nigericin (Venn et al., 2009).

The fluorescence ratio of SNARF-1 was sensitive to differences in illumination caused by the halogen lamp used for photosynthesis and pHi experiments in the light. These changes were not related to photodegradation, but rather to the weak excitation of the dye by the wider spectral range of illumination added by the lamp. As such, it was necessary to perform *in vivo* calibrations for each light level used in the investigation.

Experiments to determine the influence of photosynthesis on pHi

In all pHi experiments, cell preparations were perfused at a rate of 50 ml h^{-1} and temperature maintained at 25°C by a thermostatic microscope stage insert (PECON). Illumination was provided by the same irradiance halogen lamp used for the PI response experiments.

Three sets of experiments were performed to investigate the influence of photosynthesis on pHi. The first set of experiments compared the response of coral cell pHi in the presence and absence of the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Sigma-Aldrich, St Louis, MO, USA). Experiments were carried out over 30 min, during which cell preparations were perfused with a seawater solution containing 100 μmol l^{-1} DCMU and 0.1% acetone (DCMU stock solutions were prepared in acetone) or a control seawater solution containing 0.1% acetone. pHi measurements were made on symbiont-containing and symbiont-free cells in the dark or under 300 μmol photons m^{-2} s^{-1} irradiance in the presence and absence of DCMU. Measurements were taken at 1, 5, 10, 15, 20, 25 and 30 min. Separate experiments conducted by oxygen electrode analysis (as described above) confirmed that 100 μmol l^{-1} DCMU was sufficient to block photosynthetic evolution of oxygen by coral cell preparations (data not shown).

The second set of experiments examined the influence of light intensity on pHi dynamics of coral cells. We examined the pHi dynamics of coral cells containing symbionts on exposure to various light levels; experiments were conducted over 30 min at each light level (dark, 50, 100, 200, 250, 300, 400, 500 and 800 μmol photons m^{-2} s^{-1}). Measurements were taken at 1, 5, 10, 15, 20, 25 and 30 min. Values at 0 min were obtained using a separate calibration, with cells analysed immediately after SNARF-1 loading and washing with no light exposure, and are presented with the time course data.

In the third set of experiments, we investigated pHi dynamics on return to darkness after exposure to light. Cells were exposed to 400 μmol photons m^{-2} s^{-1} for 20 min before measurements were taken for 35 min at 0 μmol photons m^{-2} s^{-1}.

In all three sets of experiments, five cells were measured at each time point and experiments were conducted three times for each cell type, light level and treatment. Preliminary tests were performed to see whether oxygen levels or pH values shifted in the seawater
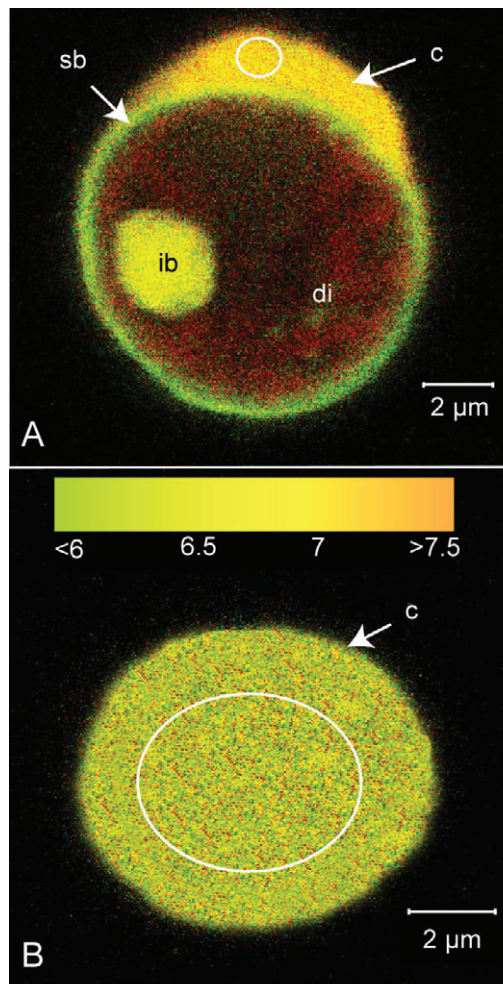

Fig. 2. Ratiometric image of SNARF-1 AM emission (585 and 640 nm) in isolated cells of *S. pistillata* exposed for 30 min to 300 μmol photons m^{-2} s^{-1} irradiance. (A) Cell containing a dinoflagellate symbiont. (B) Symbiont-free cell. c, animal cytoplasm; di, dinoflagellate; sb, symbiosome membrane complex; ib, inclusion body. White circle indicates the region of interest (ROI) in host cytoplasm used for pH measurements. Red indicates chlorophyll autofluorescence of intracellular algae. Colour scale represents fluorescence of SNARF-1 AM, indicating pHi (except autofluorescence of inclusion body). Note the difference in pHi between symbiont-containing and symbiont-free cells.

in the perfusion chamber during the course of the experiments under the range of light levels used. Oxygen levels were monitored by placing a needle type oxygen microsensor (PreSens) in the seawater within the perfusion chamber and values were recorded with TX3v602 software (PreSens). Seawater pH was monitored by adding SNARF-1 to the perfused seawater and measuring the fluorescence ratiometrically as described previously (Venn et al., 2011). pHi data were analysed using SPSS statistical software by repeated-measures ANOVA or one-way ANOVA after establishing that data conformed to a normal distribution and homogeneity of variance.

RESULTS
PI response of isolated cell preparations

Prior to performing analysis of pHi in coral endoderm cells, we characterised the PI relationship of isolated cell preparations (Fig. 1). Generation of a PI curve established that the maximum rate of photosynthesis was reached at approximately $300\,\mu mol\,photons\,m^{-2}\,s^{-1}$ PAR and remained stable up to $1000\,\mu mol\,photons\,m^{-2}\,s^{-1}$.

Establishing the link between coral cell pHi and symbiont photosynthesis

Coral cell pHi was analysed using ratiometric analysis of the pH-sensitive dye SNARF-1 AM in cells with and without symbionts (Fig. 2). In symbiont-containing cells, the low pH symbiosome was visible around the dinoflagellate as described previously (Venn et al., 2009). When symbiont-containing cells were exposed to light in the absence and presence of DCMU, pHi significantly increased in control (non DCMU treated) cells from pH 7.03 ± 0.03 to pH 7.37 ± 0.03 over 20 min (repeated-measures ANOVA, $F_{6,84}=17.372$, $P<0.001$), while pHi in DCMU-treated cells remained unchanged (pH 7.04 ± 0.03; repeated-measures ANOVA, $F_{6,84}=0.557$, $P>0.05$; Fig. 3A). pHi also remained unchanged in symbiont-containing cells exposed to dark conditions (with and without DCMU) (Fig. 3A) and in symbiont-free cells in the light and dark (with and without DCMU) (Fig. 3B). Separate experiments that
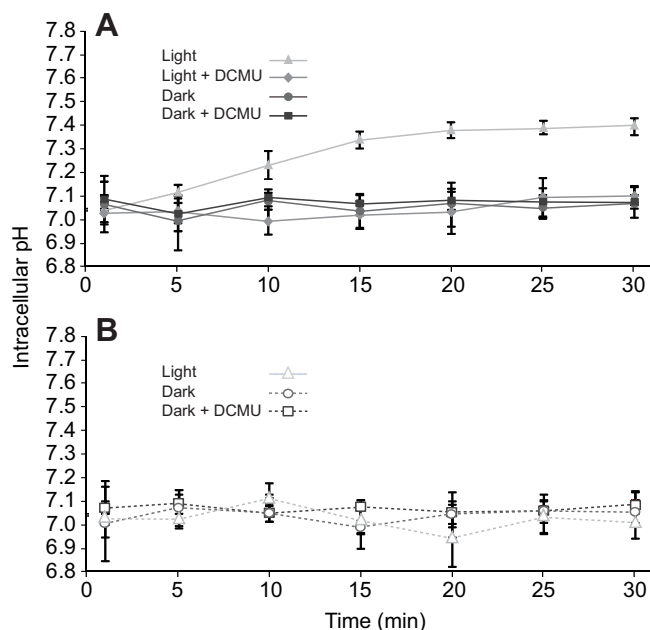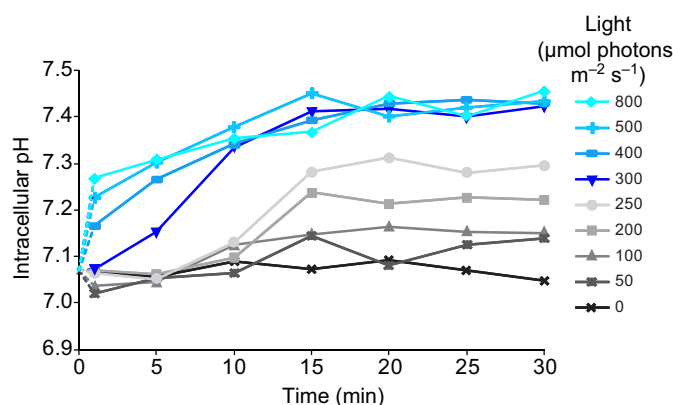

Fig. 4. Effect of irradiance level and duration on pHi in symbiont-containing cells isolated from *S. pistillata*. *In situ* calibrations were carried out for each light intensity. Dotted lines link $t=0$ values (obtained with a separate calibration in the dark) with values at 1 min. Black to grey lines represent light below saturating irradiance; blue lines are used for light above saturating irradiance. Mean values are shown and s.e.m. are given in Table 1.

monitored external seawater $[O_2]$ and pH by oxygen and pH electrode showed the renewal seawater in the perfusion chamber was sufficient to keep seawater $[O_2]$ and pH stable throughout the 30 min exposure to $300\,\mu mol\,photons\,m^{-2}\,s^{-1}$ (supplementary material Fig. S1) and the full range of irradiances used in subsequent experiments (not shown).

Impact of irradiance level and duration on pHi in coral cells containing symbionts

pHi was measured in coral cells containing dinoflagellate symbionts exposed to a range of light levels (0 to $800\,\mu mol$) for 30 min (Fig. 4). pHi increased over the course of the experiment at all light levels, but remained stable in cells kept in the dark. pHi did not increase immediately on exposure to lower irradiances. At irradiances of 50 to $250\,\mu mol\,photons\,m^{-2}\,s^{-1}$, a lag phase of 10 min occurred before significant increases in pHi were measured (Fig. 4, Table 1). At $300\,\mu mol\,photons\,m^{-2}\,s^{-1}$, this lag phase shortened to 5 min. For irradiances of $400\,\mu mol\,photons\,m^{-2}\,s^{-1}$ and above, values of pHi measured were higher than dark values at 1 min, suggesting that pHi increases occurred with the first minute of light exposure. Following 20 min of light exposure, pHi values reached a plateau at all irradiances, with successively higher values of pHi associated with higher irradiances. The highest pHi values (ranging from pH 7.4 to 7.46) were measured at $300\,\mu mol\,photons\,m^{-2}\,s^{-1}$ and above (supersaturating irradiances) (Fig. 4, Table 1).

Recovery of pHi in the dark

Having established that host pHi reaches a plateau at pH 7.4–7.46 after 20 min of supersaturating irradiance, we investigated whether pHi declined if cells were returned to dark conditions. After increasing to values of pH 7.36 on exposure to $400\,\mu mol\,photons\,m^{-2}\,s^{-1}$, pHi significantly declined in the dark to 7.08 ± 0.04 over 35 min (repeated-measures ANOVA, $F_{8,104}=2.850$, $P<0.05$; Fig. 5). This value was similar to initial values (prior to light exposure) and pHi values in symbiont-free cells.

Fig. 3. pHi (mean ± s.e.m) in symbiont-containing (A) and symbiont-free cells (B) from *S. pistillata* exposed to combinations of light ($300\,\mu mol\,photons\,m^{-2}\,s^{-1}$), dark and DCMU.

DISCUSSION

The results of the present study demonstrate that the photosynthetic activity of coral symbionts drives changes in coral host pHi in an irradiance intensity-dependent manner. To our knowledge this is

Table 1. pHi (mean ± s.e.m.) in symbiont-containing cells isolated from *Stylophora pistillata* exposed to different light intensities for 30 min

Light	pHi							ANOVA
(μmol photons m^{-2} s^{-1})	1 min	5 min	10 min	15 min	20 min	25 min	30 min	
800	7.26±0.08	7.31±0.05	7.35±0.04	7.37±0.06	7.44±0.04[1,5,10]	7.4±0.04[1,5]	7.46±0.07[1,5,10]	$F_{6,72}$=4.618; P=0.001
500	7.22±0.02	7.3±0.03[1]	7.38±0.06[1]	7.45±0.03[1,5]	7.4±0.05[1,5]	7.42±0.05[1,5]	7.44±0.04[1,5]	$F_{6,54}$=3.776; P=0.003
400	7.17±0.05	7.27±0.08[1]	7.34±0.07[1]	7.39±0.08[1]	7.43±0.08[1,5]	7.44±0.14[1,5]	7.44±0.08[1,5]	$F_{6,54}$=3.140; P=0.010
300	7.07±0.1	7.15±0.11	7.33±0.07[1]	7.41±0.03[1,5]	7.42±0.03[1,5]	7.4±0.03[1,5]	7.42±0.05[1,5]	$F_{6,48}$=5.500; P<0.001
250	7.06±0.05	7.05±0.06	7.13±0.04	7.28±0.09[1,5]	7.31±0.09[1,5]	7.28±0.08[1,5]	7.30±0.08[1,5]	$F_{6,54}$=4.812; P=0.001
200	7.07±0.06	7.06±0.08	7.1±0.08	7.24±0.06[1,5]	7.21±0.04[1,5]	7.23±0.06[5]	7.22±0.02[1,5]	$F_{6,54}$=2.532; P=0.042
100	7.04±0.06	7.05±0.03	7.13±0.08	7.15±0.07[5]	7.16±0.01[1,5]	7.15±0.11[1,5]	7.15±0.12[1,5]	$F_{6,72}$=2.868; P=0.015
50	7.02±0.08	7.05±0.12	7.07±0.09	7.15±0.08[1]	7.08±0.07	7.13±0.08[1]	7.14±0.1[1]	$F_{6,48}$=2.595; P=0.034
0	7.07±0.02	7.06±0.03	7.09±0.03	7.07±0.03	7.09±0.03	7.07±0.05	7.05±0.05	$F_{6,114}$=0.237; P>0.05

Post hoc analysis was performed using paired-sample *t*-tests, following repeated-measures ANOVA.
Superscripted numbers (corresponding to time points) indicate mean pHi values that are significantly different to pHi at other time points.

the first characterisation of the role of algal photosynthesis in altering pH in the cytoplasm of an animal host. In the first part of the investigation we tested whether changes in coral pHi could be blocked in the presence of a photosynthetic inhibitor. After determining the value of irradiance for maximal rate of photosynthesis, we conducted experiments with the photosynthetic inhibitor DCMU. This inhibitor has been widely used in experiments on coral symbionts (Jones, 2004), free-living algae and plants (Garrigue et al., 1992) as it blocks photosystem II by binding to plastoquinone, inhibiting the light reactions of photosynthesis. Our experiments showed that while control symbiont-containing cells exposed to a saturating irradiance of 300 μmol photons m^{-2} s^{-1} displayed a significant increase of 0.3 pH units in pHi, symbiont-containing cells treated with DCMU exhibited no changes in pHi over a 30 min period. As the pHi of DCMU-treated cells was not distinguishable from the pHi of cells kept in the dark or cells that did not contain symbionts in the light or dark, it is likely that DCMU did not alter coral cell pHi regulation by mechanisms other than inhibiting photosynthesis.

One possible way that photosynthesis may have induced an increase in pHi of host cells is through modifications of the surrounding seawater in the perfusion chamber during the time course of each experiment, such as an increase in pH resulting from CO_2 removal from seawater by the cells. Indeed, the acid/base balance of most organisms may be affected by changes in the pH of the surrounding environment (Boron, 2004). We ruled this possibility out by perfusing cell preparations with FSW at a rate that kept pH and O_2 levels stable in the perfusion chamber in all

our treatments. Thus our data point to a role of symbiont photosynthesis in modifying the pH of host cytoplasm by intracellular mechanisms rather than changes in the pH of the surrounding medium. One likely candidate mechanism is the photosynthetic consumption of intracellular CO_2. Depletion of CO_2 from the cytoplasm will cause the conversion of HCO_3^- (the dominant form of dissolved inorganic carbon in the cell) to CO_2 + H_2O, consuming protons, which would contribute to alkalisation of the cell (Allemand et al., 1998). Conversely, the observed recovery in pHi occurring in darkness after pre-exposure to irradiance may be due to net production of CO_2. CO_2 is produced by both host and symbiont respiration in the dark, and combined with H_2O is converted back to HCO_3^- and H^+, which acidifies the cell.

In the following stages of the study we investigated the dynamics of pHi on exposure to a range of light levels. Several observations taken from these experiments provide insight into the mechanisms underlying how photosynthesis may drive pHi changes in corals. Concerning the dynamics of pHi, the important result is that the presence of a lag phase is dependent on the light intensity. Indeed, there is a 10 min time lag between the beginning of the exposure to light and when increases in pHi occur for irradiances that ranged from 0 to 250 μmol photons m^{-2} s^{-1}. This time lag is absent for higher irradiances (300–800 μmol photons m^{-2} s^{-1}) as increases in pHi occur earlier in the time course. The existence of the time lag could be interpreted as evidence for the gradual depletion of a CO_2 pool that built up within dinoflagellate cells and the symbiosome membrane complex during dark loading with SNARF-1 AM, delaying the eventual consumption of CO_2 from host cells. In this scenario, the depletion of the dinoflagellate CO_2 pool occurs more rapidly at higher irradiances (when rates of photosynthesis are higher) and thus removal of CO_2 from the host cell and the resulting increases in host pHi occur at an earlier point in the time course.

Concerning the influence of irradiance levels on pHi values, the primary observation is that pHi values plateau after 20 min at all light intensities and the value of pHi at the plateau increases with increasing light intensity. Maximum pHi values (pHi 7.4–7.46) were obtained at the maximum rate of photosynthesis and supersaturating light levels. As pHi increases do not surpass values of 7.46, it is likely that after a 20 min delay, regulatory mechanisms intervene to prevent pHi increasing indefinitely in the cell. Membrane-bound transporters involved in the regulation of pHi, well known in many organisms but yet to be characterised in corals, are likely to be involved in this process (Casey et al., 2010). These may include extruders of OH$^-$ and bicarbonate-linked transport mechanisms, which are highly important for pHi regulation (Russell and Boron, 1976; Furla et al., 2000; Bonar and Casey, 2008) and potentially
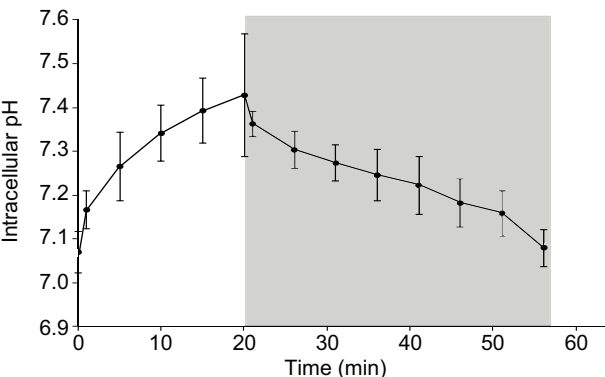

Fig. 5. pHi (mean ± s.e.m.) in symbiont-containing cells isolated from *S. pistillata* exposed to 20 min of 400 μmol photons m^{-2} s^{-1} irradiance (white) followed by 35 min in the dark (grey).

also important for dissolved inorganic carbon transport and CO_2-concentrating mechanisms in corals (Al-Moghrabi et al., 1996; Allemand et al., 1998; Brownlee, 2009). Similarly, we presume that regulation by membrane-bound transporters, particularly acid extruders, contributes to the stable values of pH obtained in darkness (pH 7.05). Additionally, as CO_2 regularly traverses cell membranes and if the partial pressure of CO_2 of cnidarians cells is estimated to be higher than that of the surrounding seawater (Venn et al., 2009), respiratory CO_2 may also leak out of the cell.

In our study, external variation in seawater pH was deliberately controlled in order to identify intracellularly driven pHi variations. However, one important avenue of future research will be to characterise the interaction of photosynthesis-driven changes in coral pHi with changes in extracellular pH in both the coelenteron lumen and the external seawater pH. Previous studies on pH at the coral's surface have characterised pH variation in the diffusive boundary layer (Kühl et al., 1995; De Beer et al., 2000; Al-Horani et al., 2003). For example, working with microsensors and the coral *Galaxea fascicularis*, Al Horani and coworkers observed variations of diffusive boundary layer (DBL) pH ranging from pH 7.6 in the dark to 8.5 in the light (Al-Horani et al., 2003). In the coelenteron, variations of 0.6 pH units have been measured between light and dark conditions (Al-Horani et al., 2003). These data from the coelenteron may be of particular relevance when considering our results in the context of the intact organism, because coral symbionts reside in the endoderm cell layer lining the coelenteron, rather than directly facing the surrounding seawater. The mechanistic basis underlying these changes in coelenteron pH may be linked to regulation of pHi at the maximum and minimum pHi values observed under constant conditions in the present study. Under irradiance, membrane-bound transporters that prevent intracellular pH rising above pH 7.46, such as extrusion of OH^- or uptake of H^+, may drive alkalinisation of the coelenteron (Furla et al., 2000). Equally, in the dark, regulatory mechanisms that prevent decreases in pHi below 7.05 may involve extrusion of H^+, which reduces pH in the coelenteron.

Together with knowledge on pH in the DBL and the coelenteron, understanding changes in pHi will eventually lead towards a better understanding of ion gradients and ion transport (particularly H^+ and dissolved inorganic carbon) across coral tissues. Such an understanding is imperative for a better grasp of the physiological response of corals to environmental changes such as ocean acidification (reduced seawater pH driven by ocean uptake of anthropogenic CO_2). A recently published hypothesis by Jokiel illustrates this point. Jokiel argues that reduced rates of calcification in corals exposed to ocean acidification arise through a decrease in the proton gradient across the DBL, impeding the efflux of protons generated by the calcification reaction (Jokiel, 2011). It is proposed that photosynthesis may alleviate this unfavourable gradient by increasing pH in the DBL during daylight hours. Allemand et al. have argued that increases in pH of the coelenteron may also provide a more favourable gradient for the movement of protons from away the site of calcification (Allemand et al., 2004). However, much future research is still required to address gaps in our understanding of how protons and other ions are transported across coral tissues. Indeed, the relative contributions of paracellular and transcellular flux of ions in corals are still a matter of debate (Tambutté et al., 2011).

In conclusion, the present study provides fundamental information about how symbiont photosynthesis changes coral host pHi. Such information provides a basis for future studies into acid–base regulation, which are essential for a better understanding of coral biology in general. In eukaryotes, shifts in pHi of 0.1 pH units and greater are usually associated with changes in primary metabolic processes such as respiration rate, protein synthesis, cell division and cell cycle progression (Busa and Nuccitelli, 1984; Madshus, 1988; Kurkdjian and Guern, 1989; Boussouf and Gaillard, 2000; Denker et al., 2000; Putney and Barber, 2003). It follows therefore that the relatively large light-dependent shifts in pHi observed in the present study have the potential to contribute to many physiological processes, including diel patterns of coral metabolism and growth. Physiological tuning of coral metabolism to light has been studied for several years. The expression of genes and proteins linked to cellular redox states, division rates of both endoderm cells and symbionts, and rates of calcification all show diel periodicity (Wilkerson et al., 1983; Fitt, 2000; Levy et al., 2006; Levy et al., 2011; Tambutté et al., 2011). The potential role of light-driven pHi variation in contributing to regulation of coral cell physiology is worthy of future research.

LIST OF SYMBOLS AND ABBREVIATIONS

chl	chlorophyll
DBL	diffusive boundary layer
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
F	fluorescence intensity
FSW	filtered seawater
I	irradiance
I_k	irradiance at which the initial slope (α) intersects P_{gmax}
PAR	photosynthetically active radiation
P_{gmax}	gross maximum photosynthetic rate
pHi	intracellular pH
PI	photosynthesis–irradiance
P_{net}	net photosynthetic rate
r	fluorescence intensity ratio
R	respiration rate in the dark
ROI	regions of interest
SNARF-1	carboxyseminaphthorhodafluor-1
SNARF-1 AM	cell permeant acetoxymethyl ester acetate of SNARF-1
$\lambda 2$	640 nm

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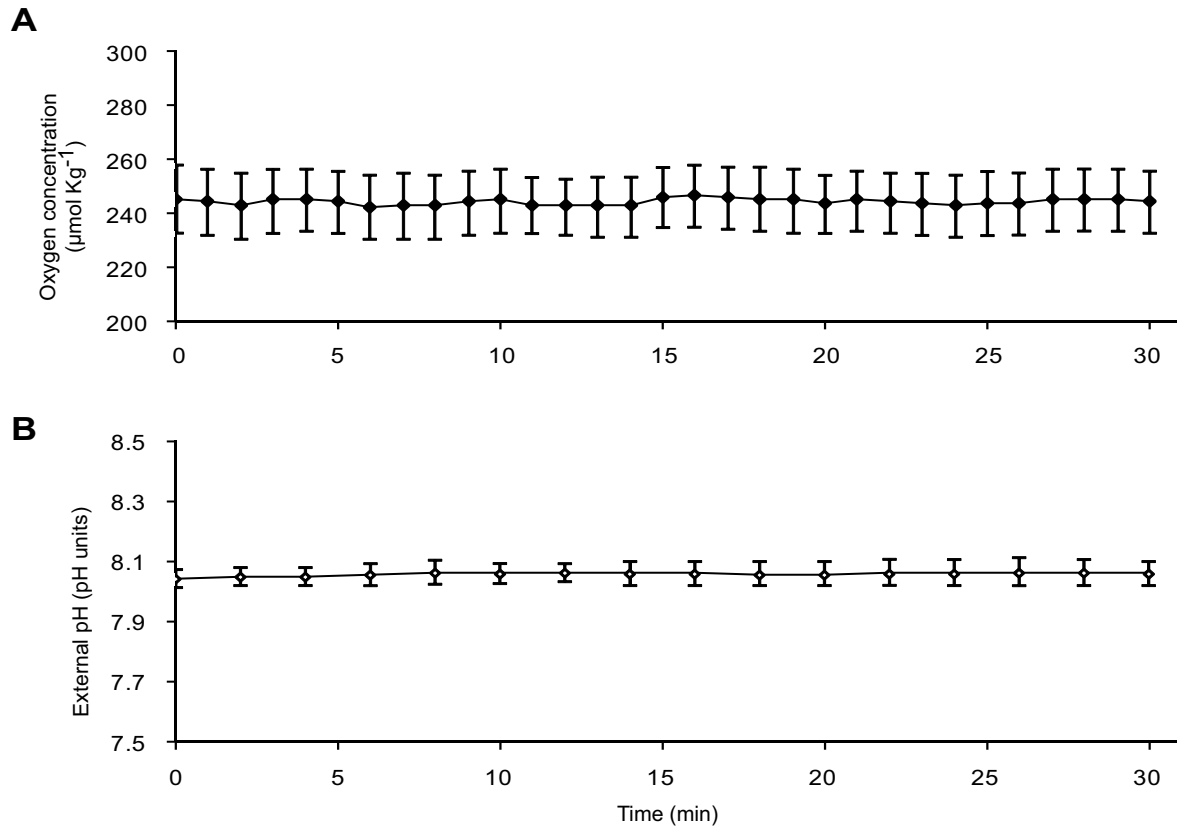
Fig. S1. Mean (±s.e.m.) (A) O_2 concentration (µmol kg^{-1}) and (B) pH in seawater of the perfusion chamber containing isolated cells with and without symbionts from *S. pistillata*. Data shown represent an example at 300 µmol photons m^{-2} s^{-1} irradiance. Data are not shown for other treatments (e.g. dark, DCMU, etc.).