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## The mechanism of calcification and its relation to photosynthesis and respiration in the scleractinian coral *Galaxea fascicularis*

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**Abstract** The mechanism of calcification and its relation to photosynthesis and respiration were studied with  $\text{Ca}^{2+}$ , pH and  $\text{O}_2$  microsensors using the scleractinian coral *Galaxea fascicularis*. Gross photosynthesis (Pg), net photosynthesis (Pn) and dark respiration (DR) were measured on the surface of the coral. Light respiration (LR) was calculated from the difference between Pg and Pn. Pg was about seven times higher than Pn; thus, respiration consumes most of the  $\text{O}_2$  produced by the algal symbiont's photosynthesis. The respiration rate in light was ca. 12 times higher than in the dark. The coupled Pg and LR caused an intense internal carbon and  $\text{O}_2$  cycling. The resultant product of this cycle is metabolic energy (ATP). The measured ATP content was about 35% higher in light-incubated colonies than in dark-incubated ones. Direct measurements of  $\text{Ca}^{2+}$  and pH were made on the outer surface of the polyp, inside its coelenteron and under the calicoblastic layer. The effects on  $\text{Ca}^{2+}$  and pH dynamics of switching on and off the light were followed in these three compartments.  $\text{Ca}^{2+}$  concentrations decreased in light on the surface of the polyp and in the coelenteron. They increased when the light was switched off. The opposite effect was observed under the calicoblastic layer. In light, the level of  $\text{Ca}^{2+}$  was lower on the polyp surface than in the surrounding seawater, and even lower inside the coelenteron. The concentration of calcium under the calicoblastic layer was about 0.6 mM higher than in the surrounding seawater. Thus  $\text{Ca}^{2+}$  can diffuse from seawater to the coelenteron, but

metabolic energy is needed for its transport across the calicoblastic layer to the skeleton. The pH under the calicoblastic layer was more alkaline compared with the polyp surface and inside the coelenteron. This rise in pH increased the supersaturation of aragonite from 3.2 in the dark to 25 in the light, and brought about more rapid precipitation of  $\text{CaCO}_3$ . When ruthenium red was added,  $\text{Ca}^{2+}$  and pH dynamics were inhibited under the calicoblastic layer. Ruthenium red is a specific inhibitor of Ca-ATPase. The results indicated that Ca-ATPase transports  $\text{Ca}^{2+}$  across the calicoblastic layer to the skeleton in exchange for  $\text{H}^+$ . Addition of dichlorophenyl dimethylurea completely inhibited photosynthesis. The calcium dynamics under the calicoblastic layer continued; however, the process was less regular. Initial rates were maintained. We conclude that light and not energy generation triggers calcium uptake; however, energy is also needed.

### Introduction

Scleractinian corals are abundant in shallow, tropical areas, which are oligotrophic environments. Their success in this environment is attributed to their association with symbiotic dinoflagellates called *Symbiodinium* sp. (commonly known as zooxanthellae). Corals precipitate aragonitic  $\text{CaCO}_3$  to produce supportive skeletons (Barnes 1970). It is well documented that light enhances calcification (e.g. Goreau 1959; Pearse and Muscatine 1971). The mechanism of this enhancement is still uncertain (Carlson 1996; Goreau et al. 1996; Marshall 1996). Two forms of Ca-ATPases were characterized that may play a role in calcification (Kingsley and Watabe 1985; Ip et al. 1991). However, the exact nature of this role has not yet been revealed. This lack of understanding limits predictions of future changes in the rate of calcification in response to global climate change (Gattuso et al. 1999). Furthermore, the interactions of calcification with

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endosymbiont photosynthesis are poorly understood (Gattuso et al. 2000), and many mechanisms have been proposed to explain this interaction, some of which are conflicting. The proposed mechanisms include that: (1) energy supply by zooxanthellae is responsible for light-enhanced calcification (Goreau 1959; Chalker and Taylor 1975), (2) calcification generates protons used to assimilate bicarbonate and nutrients (McConnaughey and Whelan 1997), (3) both processes are more efficient in a coexisting system than in isolated reactions (Suzuki et al. 1995) and (4) calcification does not enhance photosynthesis (Yamashiro 1995; Gattuso et al. 2000).

Corals possess a multi-compartment structure composed of two tissue layers (oral and aboral) separated by the coelenteron. The calcifying layer (calicoblastic layer) faces the skeleton. The symbionts (zooxanthellae) are present within a perisymbiotic membrane in the endodermal cells. Due to the close structural arrangement, the chemical dynamics of the ions involved have not been measured directly so far. Microsensors enabled us to measure biological processes occurring in different localities and compartments of the coral polyp, with high spatial and temporal resolution.

Oxygen microsensors have been used to measure gross and net photosynthesis rates (Pg and Pn) and dark respiration (DR), as well as  $\text{Ca}^{2+}$  and pH dynamics on the surface of corals from the genera *Acropora* and *Favia* (Kühl et al. 1995; de Beer et al. 2000). In the present study, we placed microsensors within the coelenteron of the coral *Galaxea fascicularis* polyp and beneath the calicoblastic layer. Specific inhibitors for Ca-ATPase, photosystem II (PSII) and carbonic anhydrase (CA) were used.

## Materials and methods

### Biological samples

Colonies of *Galaxea fascicularis* were collected by SCUBA diving from 5 m depth south of the Marine Science Station at Aqaba, Jordan. Colonies were immediately transferred to a 2 m<sup>3</sup> aquarium illuminated with a HQI-lamp, with a light spectrum similar to natural light (12 h light:12 h dark cycle). The colonies were fragmented into smaller colonies and left for at least 2 weeks to acclimatize to the aquarium conditions before use.

### Inhibitors

The specific inhibitor of PSII, dichlorophenyldimethylurea (DCMU) was dissolved in ethanol and added to seawater to a final concentration of 1  $\mu\text{M}$ . Ruthenium red (RR), a specific inhibitor of Ca-ATPase (Watson et al. 1971; Ip et al. 1991), was dissolved in dimethylsulfoxide (DMSO) and used at a final concentration of 100  $\mu\text{M}$ . Acetazolamide (AZ), the specific inhibitor of carbonic anhydrase was dissolved in DMSO and used at a final concentration of 600  $\mu\text{M}$ . The final concentrations of DMSO and ethanol were 0.1%.

### Microsensors

$\text{Ca}^{2+}$  microsensors (de Beer et al. 2000), Clark-type  $\text{O}_2$  electrodes (Revsbech and Jørgensen 1983) and LIX pH electrodes (de Beer et al. 1997) were constructed and calibrated as described, and positioned on the polyp surface, inside the coelenteron and under

the calicoblastic layer. Pg was measured using the light–dark shift method (Kühl et al. 1995).  $\text{O}_2$  profiles in light and dark were used to calculate Pn and DR rates from the slope of the profile in the diffusive boundary layer using Fick's first law of diffusion, assuming 100  $\mu\text{m}$  thickness of photosynthetically active tissue. LR was calculated from the difference between Pg and Pn. For the dynamics of  $\text{Ca}^{2+}$  and pH, each cycle of illumination and darkening was repeated two to three times and a new coral colony was used for each experimental variable. Control measurements of the dynamics on the same coral colony preceded each experiment and are presented in the "Results" for comparison.

### Experimental set-up

Coral colonies were placed in a polycarbonate flow cell with a volume of 0.7 l for microsensor measurements. Filtered seawater was circulated between the flow cell and a 3 l reservoir at a constant rate (420 ml/min<sup>-1</sup>). The water had 40‰ salinity, pH 8.2–8.3 and an ambient temperature of 20–21°C. The reservoir was continuously aerated. Motorized micromanipulators fixed on a heavy stand were used to position the electrodes. A halogen light source (KL 1500 Schott Mainz, Germany) provided a light intensity of 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . An electronically controlled shutter (model Uniblitz, Vincent, Rochester, USA) was used to turn illumination of the coral on and off. Signals from the millivoltmeter (MasCom, Germany) and the picoamperometer (model PA2000, Unisense, Denmark) were plotted on a strip-chart recorder.

### Positioning of the microsensors

Positioning of the electrodes on the surface of *G. fascicularis* polyps was done as previously described (de Beer et al. 2000). The coelenteron was accessed through the mouth. The sensors were brought to the calcifying site by making an incision with a scalpel, lifting the tissue gently from the skeleton by a water jet and then positioning the sensors under the calicoblastic layer. Because this might disturb the chemical conditions of the microenvironment under the calicoblastic layer, the tissue was left for at least 1 h to relax and cover the microsensor, thus allowing enough time to re-establish normal conditions before measurements were taken.

### ATP determination

Small colonies of *G. fascicularis* were incubated in light (140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and dark in normal seawater (aerated and circulated at 26°C for 3 h incubation time). The incubated colonies were transferred to 1 M NaOH and 50 mM Na-ethylenediamine-tetraacetic acid solution and heated at 90°C for 15 min. The hydrolysate was centrifuged at 4500 rpm for 30 min. The pH of the supernatant was adjusted to ca. 7.8. The ATP content was determined following a luminometric method described in the Promega kit (Promega, USA). Protein content was analyzed by the Bradford method (Bradford 1976).

### Calculation of the saturation state

The aragonite saturation state was calculated using the Solmineq.88 software, assuming a dissolved inorganic carbon concentration of 2.4 mM, temperature of 21°C and 40‰ salinity.

## Results

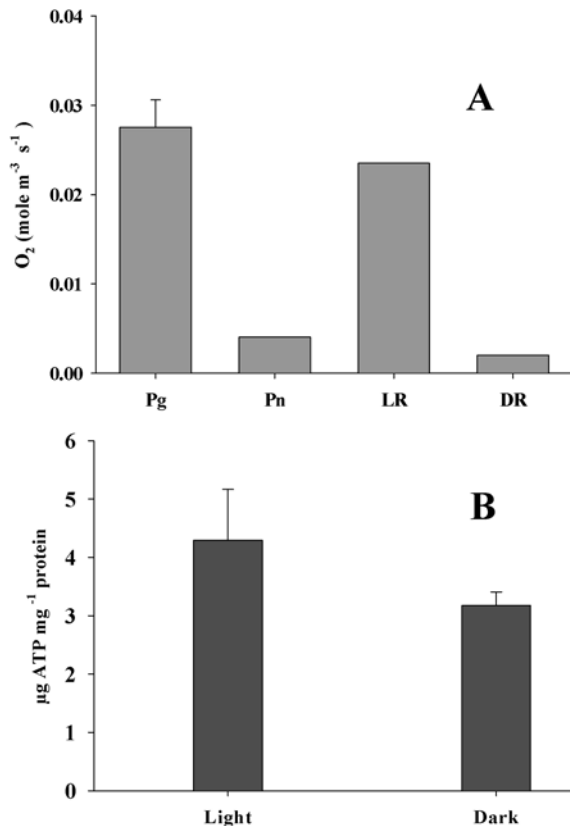
### Photosynthesis, respiration and energy budget

In the light, Pg was ca. seven times higher than the Pn rate. The LR was ca. 80–90% of Pg. LR was ca. 12 times

higher than DR (Fig. 1A). The measured ATP concentration in colonies incubated in light was ca. 35% higher than those incubated in dark (illuminated samples:  $4.30 \pm 0.87 \mu\text{g ATP mg}^{-1}$  protein,  $n = 9$ ; dark incubated samples:  $3.17 \pm 0.23 \mu\text{g ATP mg}^{-1}$  protein,  $n = 9$  (Fig. 1B).

### Calcium and pH dynamics

The effects of switching the light on and off upon  $\text{Ca}^{2+}$  and pH dynamics were measured on the polyp surface, inside the coelenteron and under the calciblastic layer. Switching the light on decreased  $\text{Ca}^{2+}$  concentration on the polyp surface and inside the coelenteron and increased it under the calciblastic layer, while switching the light off reversed the situation. Switching the light on increased the pH and switching it off decreased pH in all three compartments. The  $\text{Ca}^{2+}$  dynamics under the calciblastic layer were the opposite of the dynamics on the polyp surface and inside the coelenteron (Fig. 2). In light, the concentration of  $\text{Ca}^{2+}$  on the surface ( $9.82 \pm 0.06 \text{ mM}$ ) was higher than the concentration inside the coelenteron ( $9.76 \pm 0.5 \text{ mM}$ ) and lower than the concentration in seawater ( $10.0 \text{ mM}$ ). Under the calciblastic layer, the level of calcium was  $10.58 \pm 0.29 \text{ mM}$



**Fig. 1A, B** *Galaxea fascicularis*. **A** Rates of gross (*Pg*) and net (*Pn*) photosynthesis, and light (*LR*) and dark (*DR*) respiration. **B** ATP concentration in light- and dark-incubated colonies

in light and the pH was  $9.28 \pm 0.03$  (see Fig. 7). The calculated aragonite saturation state was ca. 25 in light compared with ca. 3.2 in dark and ca. 4 in seawater.

### Effect of inhibitors

Addition of Ca-ATPase inhibitor (RR) inhibited  $\text{Ca}^{2+}$  and pH dynamics under the calciblastic layer in light to dark level (Fig. 3). Inhibition of CA by AZ increased the concentration dynamics of calcium under the calciblastic layer (Fig. 4). DCMU completely inhibited Pg (Fig. 5) and decreased the amplitude of the  $\text{Ca}^{2+}$  concentration dynamics under the calciblastic layer by ca. 50% (Fig. 6). The dynamics after inhibition were less regular. The initial  $\text{Ca}^{2+}$  concentration increase under the calciblastic layer observed upon switching on the light was not affected by DCMU.

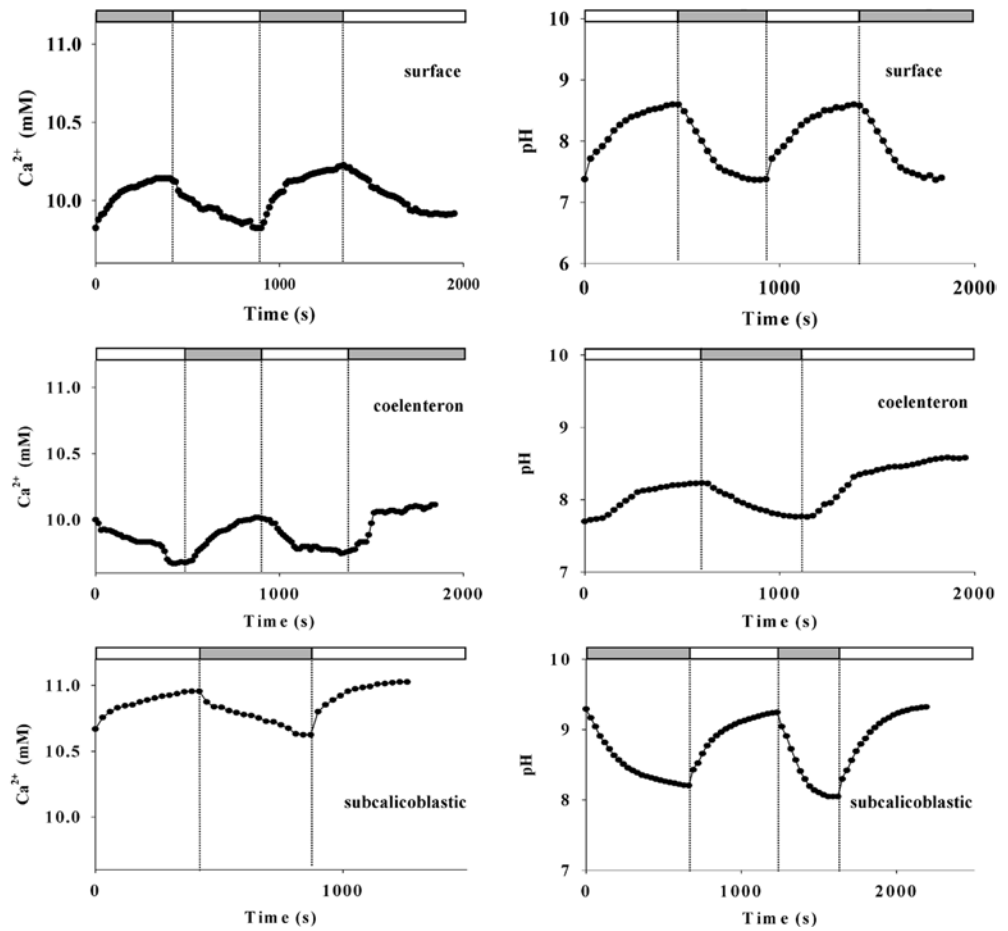
## Discussion and conclusions

### Energy budget

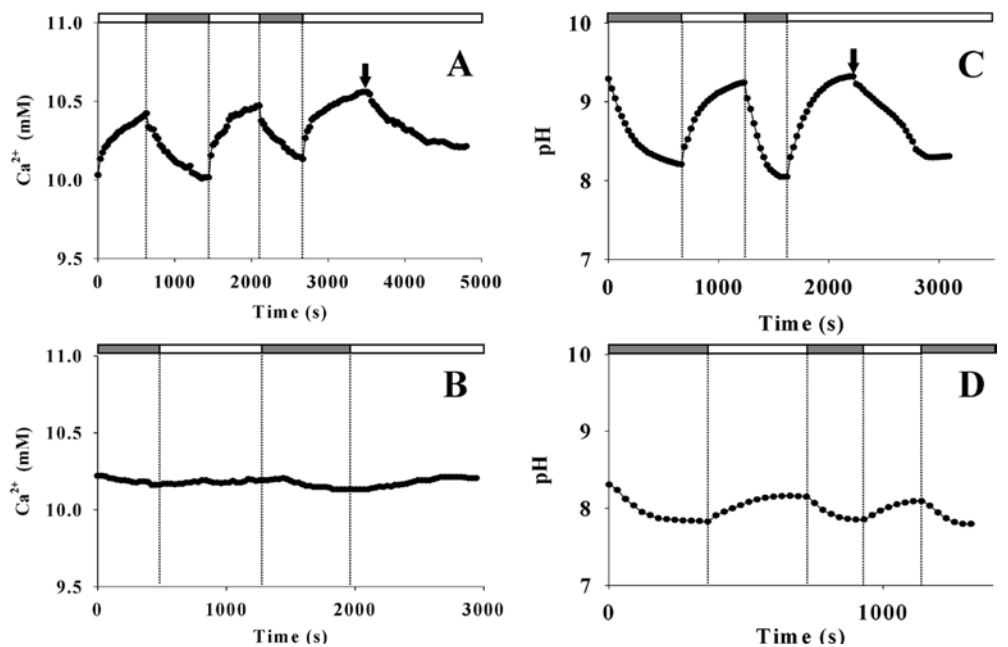
In the untreated experiments (regular conditions), the rate of oxygen production (gross photosynthesis) by the symbiont exceeded the rate of its consumption (respiration); thus,  $\text{O}_2$  is released to the environment (net photosynthesis is positive). In light, oxygen is consumed by photorespiration (Marx 1973), the Mehler reaction by the reduced donors associated with PSI (Walker 1992) and oxidative phosphorylation by mitochondria. Of these, only oxidative phosphorylation generates ATP for the coral. Metabolic respiration by the animal and the symbiont and photosynthesis by the symbiont form a highly active internal carbon cycle in the coral (Al-Horani et al., unpublished data). The main function of this carbon cycle is the production of ATP to support energy-requiring processes in the animal. The respiration rate in light is much higher than its rate in dark; thus, more ATP is generated by the polyp in light than in dark. This is supported by the finding that the amount of ATP extracted from colonies incubated in light was ca. 35% higher than dark-incubated colonies. Such increase in the ATP concentration in the light leads to an increase in the rate of the ATP-consuming processes (Raven 1976), such as calcification. Although, the LR/DR ratio is ca. 12, the amount of ATP extracted from colonies incubated in light is only 35% higher than those incubated in dark. Most likely, ATP is consumed at a faster rate in light than in the dark; thus, the relative size of the two ATP pools does not reflect ATP production rate in the light versus the dark.

The use of metabolic inhibitors, such as sodium cyanide and iodoacetic acid, showed that energy is required for calcification (e.g. Chalker and Taylor 1975; Tambutte et al. 1996; Lucas and Knapp 1997). Energy is needed in calcification for the transport of ions and synthesis of organic matrix (Chalker and Taylor 1975).

**Fig. 2** *Galaxea fascicularis*. Representatives of  $\text{Ca}^{2+}$  and pH dynamics on the polyp surface, inside the coelenteron and under the calicoblastic layer (dark bars dark periods; light bars periods of illumination)

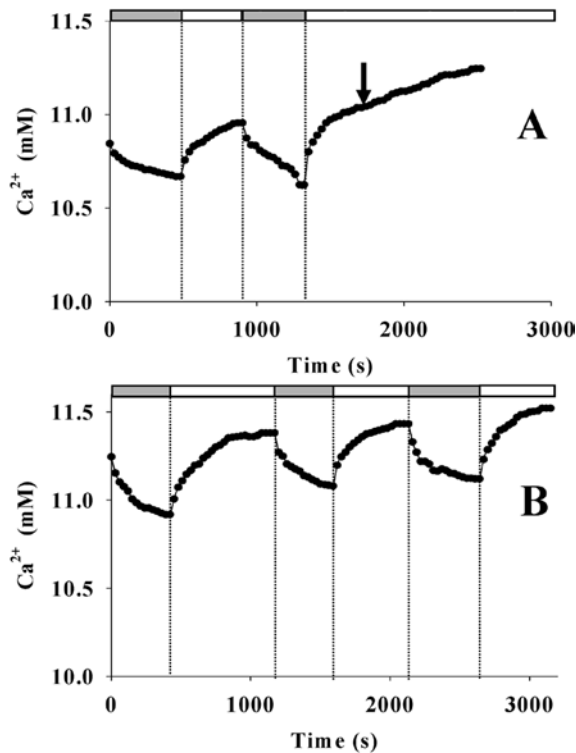


**Fig. 3A–D** *Galaxea fascicularis*. Effects of illumination on  $\text{Ca}^{2+}$  (A, B) and pH (C, D) dynamics under the calicoblastic layer before (A, C) and after (B, D) addition of the inhibitor ruthenium red (RR) (arrows addition of RR; dark bars dark periods; light bars periods of illumination)

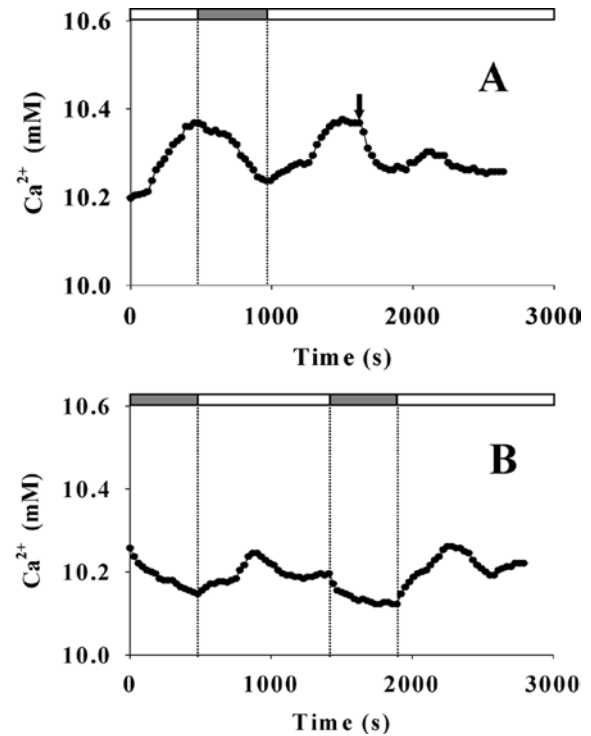


The organic matrix might include glycoproteins, proteins and phospholipids, and it may function in nucleation and control crystal growth (Young et al. 1971; Mitterer 1978;

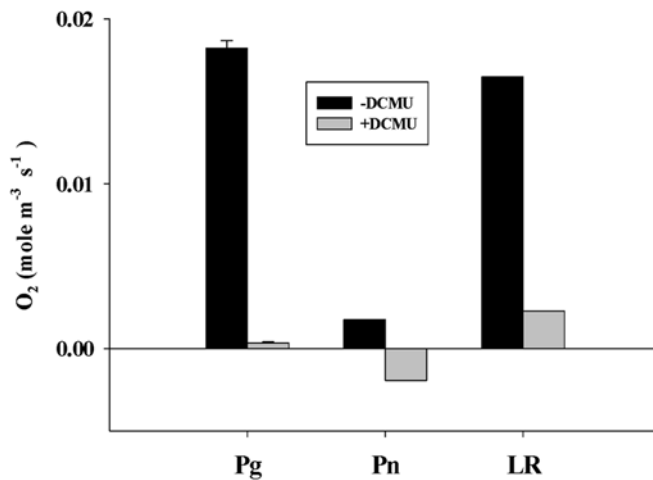
Isa and Okazaki 1987; Constantz and Weiner 1988; Allemand et al. 1998). The synthesis and translocation of such organic matrix was suggested to be a prerequisite for



**Fig. 4A, B** *Galaxea fascicularis*. Effect of carbonic anhydrase inhibition on the dynamics of  $\text{Ca}^{2+}$  under the calciblastic layer. **A** Dynamics before and upon addition of the inhibitor acetazolamide (AZ). **B** Dynamics after addition of AZ (arrow addition of AZ; dark bars dark periods; light bars periods of illumination)



**Fig. 6A, B** *Galaxea fascicularis*. Effect of illumination and DCMU addition on  $\text{Ca}^{2+}$  dynamics under the calciblastic layer. **A** Dynamics before and upon addition of DCMU. **B** Dynamics after addition of DCMU (arrow addition of DCMU; dark bars dark periods; light bars periods of illumination)



**Fig. 5** *Galaxea fascicularis*. Effect of dichlorophenyl dimethylurea (DCMU) addition on the rates of gross photosynthesis (Pg), net photosynthesis (Pn) and light respiration (LR)

calcification (Allemand et al. 1998). The main source of organic carbon respired comes from photosynthesis by the zooxanthellae (McCloskey and Muscatine 1984; Porter et al. 1984). Various forms of carbohydrates are synthesized by the symbionts (Streamer et al. 1993) that are transported to growing parts of the polyp to generate

ATP (Pearse and Muscatine 1971; Fang et al. 1989). Cyclic photophosphorylation, another light-dependent process, supplies the ATP needed for the synthesis of proteins and lipids (Furbank and Horton 1987; Herzig and Dubinsky 1993) that might be used by calcification. The ATP molecules per se, produced by cyclic photophosphorylation, cannot be used in animal calcification, as they are produced in the zooxanthellae and the membranes are not permeable for ATP.

#### Calcification mechanism

The concentration dynamics of calcium in light showed that the animal transports  $\text{Ca}^{2+}$  from seawater to the skeleton site by passive and active transport mechanisms (Fig. 2). Comparison of  $\text{Ca}^{2+}$  concentrations in seawater, on the polyp surface and inside the coelenteron shows a downward gradient of  $\text{Ca}^{2+}$  between seawater and the coelenteron. This concentration gradient drives  $\text{Ca}^{2+}$  diffusion from seawater to the coelenteron (Benzet-Tambutte et al. 1996). In light, the level of  $\text{Ca}^{2+}$  under the calciblastic layer is ca. 0.8 mM above the level inside the coelenteron. Thus, active transport of  $\text{Ca}^{2+}$  must take place to transport the ion against its concentration gradient to the skeleton. Ruthenium red (a specific inhibitor of Ca-ATPase) stopped the dynamics of  $\text{Ca}^{2+}$  and pH under the calciblastic layer (Fig. 3). Thus, Ca-ATPase transports  $\text{Ca}^{2+}$  to the

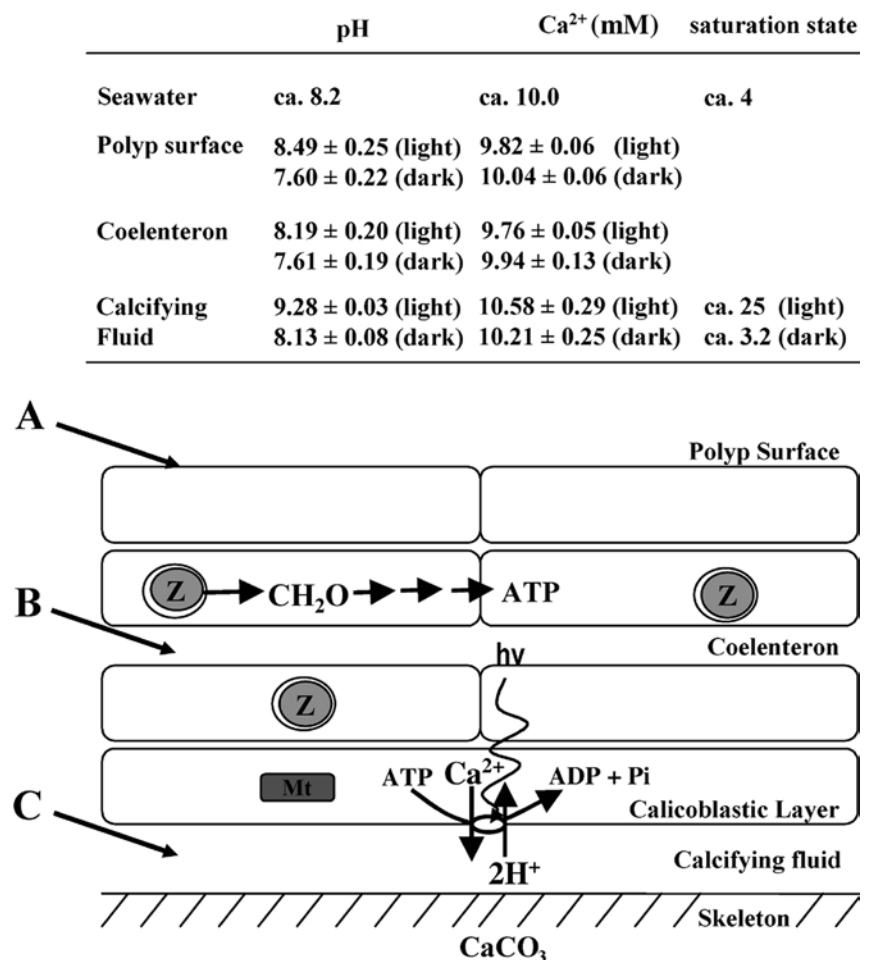
skeleton site in exchange for  $H^+$ , thereby increasing the saturation states of  $Ca^{2+}$  and  $CO_3^{2-}$ . The calcification reaction is chemically represented by:  $Ca^{2+} + CO_2 + H_2O \rightleftharpoons CaCO_3 + 2H^+$ . The only way in which the equilibrium is shifted towards calcification is by removal of the protons; therefore, the enzyme Ca-ATPase has a dual function. The enzyme transports  $Ca^{2+}$  to the site of calcification at the same time it removes protons away from it, thereby driving the reaction of calcification towards  $CaCO_3$  formation. The aragonite saturation state under the calcicoblastic layer increases from ca. 3.2 in dark to ca. 25 in light compared with ca. 4 in seawater. This change in the aragonite saturation state drives calcification at the skeleton. When such environment was mimicked by increasing the pH of filtered seawater to a value of 9.3,  $CaCO_3$  immediately precipitated (results not shown). The changes in  $Ca^{2+}$  and pH were fast, indicating high activity of the enzyme Ca-ATPase. Corals, like other marine invertebrates, maintain a very low intracellular  $Ca^{2+}$  level compared with the surrounding seawater (Barnes and Chalker 1990); therefore, the high activity of Ca-ATPase is important in keeping such a low intracellular  $Ca^{2+}$  level. The ATP needed for the functioning of Ca-ATPase could be supplied from respiration as indicated from the higher rate of LR compared with DR.

Upon switching the light off, it was observed that  $Ca^{2+}$  leaks out for the short period of time of the experiment (Fig. 2). Such ion leakage might be due to a change in the pH at the skeleton site, which could release  $Ca^{2+}$  from the phospholipids in the organic matrix (Isa and Okazaki 1987). It is, however, unknown how  $Ca^{2+}$  leaks through the calcicoblastic layer, and further research is needed.

The pH in the three compartments increased in light and decreased in dark (Fig. 2). Under the calcicoblastic layer, the pH was more alkaline compared with the pH on the surface and inside the coelenteron in light. Here, the pH reached ca. 9.3 in light and decreased to ca. 8.1 in dark. At these pH values, the concentration of  $CO_3^{2-}$  is seven times higher in light than in dark and is the dominant form of inorganic carbon under the calcicoblastic layer in light (pKa=9.18 in seawater at 20°C; Stumm and Morgan 1996).

Pumping of protons from the subcalcicoblastic layer to the coelenteron by  $Ca^{2+}$ - $H^+$ -ATPase could decrease the pH inside the coelenteron in light, but this was not observed (Fig. 2). The decrease in pH by proton pumping as a result of calcification is opposed by the photosynthetic  $CO_2$  uptake by the symbiont in the adjacent endodermal cells. Thus, the expected decrease

**Fig. 7** *Top panel:* summarized table for calcium and pH levels in the three compartments illustrated in the drawing below (A-C) and the calculated aragonite saturation state of the calcifying fluid under the calcicoblastic layer in light and dark compared with seawater. *Bottom panel:* a conceptual model to explain the mechanism of light-enhanced calcification in *Galaxea fascicularis* illustrated on a simplified cross section of the coral. Ca-ATPase present at the calcicoblastic cells pumps  $Ca^{2+}$  against its concentration gradient in exchange for protons, thereby increasing the saturation states of  $Ca^{2+}$  and  $CO_3^{2-}$ , and thus  $CaCO_3$  precipitates. Light triggers enzyme function, while the ATP needed is supplied mainly from respiration of photosynthates (Z zooxanthellae; Mt mitochondria; arrows position of the electrode; hv light)



in the coelenteron pH cannot be seen, as it is influenced by more than one process.

The role of CA in calcification was tested through the use of AZ. This CA inhibition caused an increase in  $\text{Ca}^{2+}$  concentration under the calcicoblastic layer (Fig. 4). This is probably due to a reduced calcification rate caused by the decrease in supply of carbonate ions at the skeleton site. This result agrees with data obtained by Furla et al. (2000), whereby calcification decreased with CA inhibition. Thus, CA in the calcifying zone facilitates uptake of inorganic carbon for calcification, as it is  $\text{C}_i$  limited (Marubini and Thake 1999). The enzyme might also function in regulating the local pH by using protons generated by calcification in the conversion of bicarbonate to  $\text{CO}_2$  (Al-Horani et al., unpublished data).

Addition of 1  $\mu\text{M}$  of DCMU stopped photosynthesis instantly (Fig. 5). However, the light-dependant calcium dynamics continued, but at a lower amplitude (by ca. 50%) (Fig. 6). The dynamics were weaker and showed some irregularity, i.e. the instant increase after illumination was followed by a decrease. The data are consistent with the hypothesis that the calcium transport to the skeleton site is directly triggered by light and not by energy generation. However, energy is needed, and ATP is constantly re-supplied, mainly from photosynthesis-driven respiration.

### Working model

We have integrated our results with those from the literature in a model to explain the mechanism of calcification in corals (Fig. 7). In this model, Ca-ATPase generates two gradients of  $\text{Ca}^{2+}$  by continuously pumping  $\text{Ca}^{2+}$  to the calcifying site in light and, thus, decreasing the concentration of calcium in the calcicoblastic cells and the coelenteron. At the same time it increases the calcium concentration at the calcifying site. This leads to continuous diffusion of  $\text{Ca}^{2+}$  to the calcicoblastic cells along the concentration gradient from seawater. The aragonite saturation state is increased strongly, from ca. 3.2 in the dark to ca. 2.5 in the light at the calcifying site. The transport of  $\text{Ca}^{2+}$  to the calcifying site is coupled with the transport of protons away from it, and the pH is increased to ca. 9.3, thereby increasing the carbonate concentration at the calcification site. These conditions favor high rates of calcification in the light compared with the dark. Oxidative phosphorylation of photosynthates is a main process that supplies the ATP needed to power energy-requiring processes in light.

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