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REPORT

Dark calcification and the daily rhythm of calcification in the scleractinian coral, *Galaxea fascicularis*

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Abstract The rate of calcification in the scleractinian coral *Galaxea fascicularis* was followed during the daytime using ^{45}Ca tracer. The coral began the day with a low calcification rate, which increased over time to a maximum in the afternoon. Since the experiments were carried out under a fixed light intensity, these results suggest that an intrinsic rhythm exists in the coral such that the calcification rate is regulated during the daytime. When corals were incubated for an extended period in the dark, the calcification rate was constant for the first 4 h of incubation and then declined, until after one day of dark incubation, calcification ceased, possibly as a result of the depletion of coral energy reserves. The addition of glucose and *Artemia* reduced the dark calcification rate for the short duration of the experiment, indicating an expenditure of oxygen in respiration. Artificial hypoxia reduced the rate of dark calcification to about 25% compared to aerated coral samples. It is suggested that *G. fascicularis* obtains its oxygen needs from the surrounding seawater during the nighttime, whereas during the day time the coral exports oxygen to the seawater.

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Introduction

There have been extensive studies during the last two decades on coral calcification and its interactions at many levels. Calcification is important for reef building and plays a role in seawater carbonate chemistry, which is influenced by the atmospheric CO_2 levels (Barnes and Chalker 1990; Frankignoulle et al. 1994; Gattuso et al. 1998; Kleypas et al. 1999; Gattuso and Buddemeier 2000; Langdon et al. 2000; Marubini et al. 2001, 2002; Leclercq et al. 2002; Reynaud et al. 2003; Langdon and Atkinson 2005). Most of the information gained on coral calcification has focused on light calcification while little attention has been given to dark calcification. Calcification requires energy and involves various enzymes and carriers (Barnes and Taylor 1973; Chalker and Taylor 1975; Ip et al. 1991; Goiran et al. 1996; Al-Moghrabi et al. 1996; Allemand et al. 1998; Furla et al. 1998; Al-Horani et al. 2003a). It is accepted that corals calcify at a faster rate in the light compared with the dark, through a phenomenon usually termed light-enhanced calcification. In the light, photosynthetically produced reduced carbon compounds are transported to the growing parts of the coral to be used as energy resources and building blocks, while O_2 produced during photosynthesis supports metabolic respiration (Patton et al. 1977; Rinkevich and Loya 1983; Falkowski et al. 1984; Fang et al. 1989; Al-Horani et al. 2003b). Carbon dioxide fixation by photosynthesis removes this respiratory product and controls the pH in the coral tissues (Furla et al. 1998; Al-Horani et al. 2003a). This led to the belief that photosynthesis was the driving force behind the higher rate of calcification in the coral during the

daytime (Goreau 1959; Pearse and Muscatine 1971; Chalker and Taylor 1975). In the dark, photosynthesis ceases completely and the coral depends on a self-service mode of action to sustain growth and calcification (Muscatine 1990). To date, calcification in the dark has received little attention in coral research. Previous studies of dark calcification did not go beyond an estimation of the ratio between light and dark calcification rates (reviewed by Gattuso et al. 1999). As a result, there is currently a need for further information about the process of dark calcification in corals.

Various methods have been employed to study calcification, with the use of radioisotopes being one of the main techniques for work involving both corals and other marine-calcifying organisms. However, measuring ^{45}Ca exchange with ^{40}Ca produces errors because this exchange gives apparent calcification in the absence of real precipitation (Goreau 1959). The processing of samples also produces additional errors. In some cases, dark calcification has been confused with isotopic exchange (Chalker and Taylor 1975). In other cases, coral researchers have assumed that isotopic exchange is small compared to true calcification occurring in the light and have used dark calcification as a control for experiments involving light calcification (reviewed by Barnes and Chalker 1990). Using cultured microcolonies with no skeletons exposed to the radioisotope-labeled incubation medium, Tambutté et al. (1995, 1996) reduced the errors associated with isotopic exchange. Further improvements were made by including dead colonies in the incubation experiments to estimate the rate of isotopic exchange (Al-Horani et al. 2005). These results showed that dark calcification does occur in corals and that rates were always higher compared with those obtained using isotopic exchange methods. But the question of what drives calcification in the dark remains. The use of Ca^{2+} microsensors has proved to be a valuable tool for studying the dynamics and fluxes of Ca^{2+} at a microscale level in corals (De Beer et al. 2000; Al-Horani et al. 2003a). The application of Ca^{2+} and pH microsensors in different compartments of the coral, including the calcifying fluid between the skeleton and the tissue layer, has provided many details about the mechanism of light calcification (Al-Horani et al. 2003a).

The present study was carried out to develop our understanding of dark calcification in the coral *Galaxea fascicularis*, daytime calcification, and the driving forces of dark calcification in this species.

Materials and methods

Biological samples

Single polyps of *G. fascicularis* from colonies from the Gulf of Aqaba (Jordan), were suspended in seawater in the aquaria of the Centre Scientifique de Monaco using nylon threads as described by Al-Moghrabi et al. (1993). After about 3 months, the polyps developed into small microcolonies, which were used in the experiments described in this study. The aquarium was supplied with Mediterranean seawater with low nutrient and chlorophyll *a* concentrations (Ferrier-Pagès et al. 1998) and illuminated with metal halide lamps (HQI, 400 W) at a constant irradiance of 200-µmol photons m^{-2} s^{-1} (12 h:12 h light:dark cycles). Water temperature in the aquarium was 27°C while the seawater pH was between 8.1 and 8.2. The seawater had an aragonite saturation state of 3.1–3.7.

Efflux experiments

In order to test the efflux of ^{45}Ca from the coral coelenteron, three microcolonies were incubated in small plastic vials with 25 ml of seawater at 27°C for one hour. The specific activity of the water at the beginning was ca. 23,000 dpm. A 100-µl volume of the labeled seawater was used to count the activity (mixed with 4 ml scintillation fluid). Counting was carried out for 5 min in a scintillation counter. After the end of the incubation period, the microcolonies were dipped in nonlabeled seawater ten times to remove nonspecific bound ^{45}Ca. The microcolonies were then transferred to 100 ml nonlabeled seawater and incubated under the same conditions used in the experiment to determine the efflux rate. A 100-µl water sample was taken for counting at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 40, 50, 60, 70, 80, 100 and 120 min.

Processing of coral skeleton

At the end of the incubation period, the microcolonies were then blotted dry on tissue paper and boiled with ca. 10 ml 2 N NaOH solution for 30 min to hydrolyze the tissue layer. After the tissue hydrolysis was complete, the hydrolysate was decanted and the skeleton was washed with 10 ml distilled H_2O. The skeleton was then blotted dry on tissue paper and dried overnight at 70°C in oven. The next day, the dried skeletons were weighed and hydrolyzed completely by concentrated HCl solution and 250 µl of the hydrolysate was mixed with 4-ml scintillation fluid and counted for 5 min in the scintillation counter.

Daytime calcification rates

In order to measure changes in the rate of coral calcification during the day, the calcification rate was measured at 09:00, 12:00, 15:00, 18:00 and 20:00 h.

Dark incubations and measurement of calcification rates

Calcification in the dark was measured in triplicate for the coral microcolonies after incubating in the dark for 1, 2, 4,

12, 24, 48 and 72 h. After dark incubation, the corals were incubated with ^{45}Ca at 27°C in stirred seawater for 1 h each. The first four measurements were made during the night so that the normal light dark cycle of the coral was not disturbed.

Effects of addition of external energy sources

Replicates of *G. fascicularis* were pre-incubated for 3 h in the dark. One mM of glucose was added to one group; 2 mM of glucose was added to a second group while *Artemia salina* naupilii were added to a third group. A fourth control group was similarly incubated but without any additions. After this period, the colonies of each group were transferred to three incubating jars and ^{45}Ca was added and left one more hour in the dark.

Effect of oxygenated and de-oxygenated seawater on coral calcification

Seawater was passed through a 0.2-µm filter and oxygenated with an air pump or de-oxygenated with N_2 gas for 30 min. During incubation, the microcolonies were incubated in sealed Erlenmeyer flasks in 50 ml of solution. The air and N_2 gas were continuously bubbled through syringe needles and after ca. 15 min of incubation at 27°C, ^{45}Ca was injected using a Hamilton syringe. Triplicates of microcolonies were incubated in the following conditions; O_2 bubbling in light: N_2 bubbling in light: O_2 bubbling in dark: N_2 bubbling in dark. These experiments were designed to test the presence of stored energy sources in the corals and the effect of ambient O_2 level on the rate of coral calcification.

Oxygen depletion and repletion in seawater

Four conditions were tested to determine rates of oxygen depletion and repletion from seawater. The first involved aeration, whilst the oxygen depletion rate from the water was measured in the absence of a coral microcolony. The second was similar to the first but with a microcolony suspended in the beaker containing the seawater. The third involved flushing for 15 min with N_2 gas and measuring the oxygen repletion rate into the seawater. The fourth was similar to the third but with a coral microcolony suspended in the seawater. Oxygen levels were measured in a respirometric glass chamber containing a Strathkelvin 928 electrode and Mediterranean seawater while the liquid was continuously stirred with a stirring bar.

All results were statistically analyzed using a one-way ANOVA.

Results

Efflux experiments showed that radioactive tracers were washed out after about 10 min of stirring in clean seawater; as a result, this time was used in treatment of samples after incubation in radioactive tracers.

The calcification rate measured in the light was about twice that in the dark (4.07 ± 0.15 µmol Ca^{2+}/g wt/h (mean ± SD) compared to 1.98 ± 0.31 µmol Ca^{2+}/gwt/h). In both treatments there was a significantly higher ($P < 0.05$) incorporation rate compared with the dead coral colonies (0.10 ± 0.1 µmol Ca^{2+}/gwt/h) (Fig. 1). The rates of calcification during the daytime under constant light intensity showed a clear temporal pattern (Fig. 2). In the morning (at 9:00 h), the rate of calcification was 2.84 ± 0.19 µmol Ca^{2+}/gwt/h, at noon the rate increased to 3.24 ± 0.46 µmol Ca^{2+}/gwt/h reaching its maximum at 15:00 h with a rate of 4.26 ± 0.60 µmol Ca^{2+}/gwt/h, and then decreasing to 3.58 ± 0.82 µmol Ca^{2+}/gwt/h by 18:00 h and finally decreasing to 1.84 ± 0.74 µmol Ca^{2+}/gwt/h by

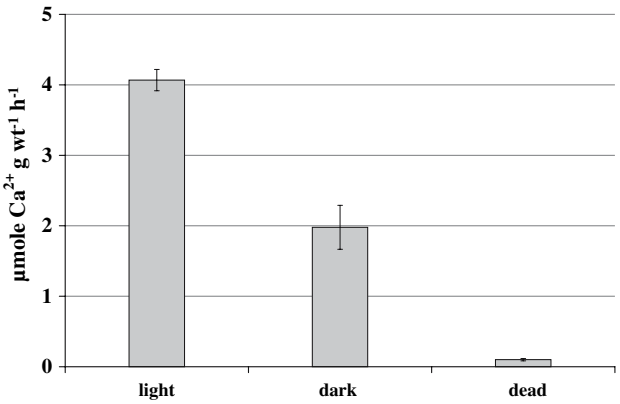

Fig. 1 Calcification rates in the light, the dark and for dead colonies of *Galaxea fascicularis*

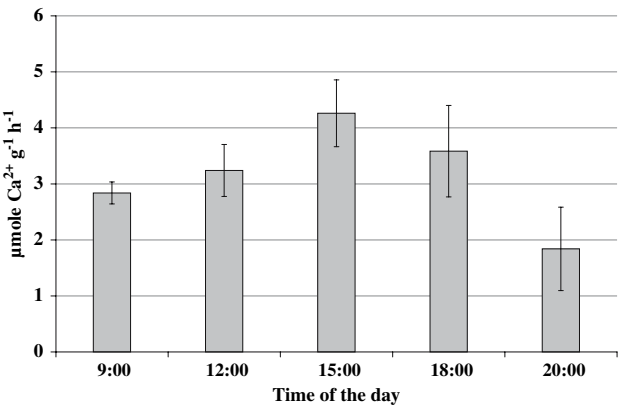

Fig. 2 Calcification rates of *Galaxea fascicularis* colonies over the course of a day under a constant irradiance of 200-µmol photons m^{-2} s^{-1}

20:00 h. This pattern was similar to that observed for dark calcification rates. There were significant differences between the calcification rates at 9:00 and 15:00, 12:00 and 20:00, 15:00 and 20:00, and 18:00 and 20:00 ($P < 0.05$).

In order to further characterize dark calcification in corals, the coral colonies were incubated in the dark for up to 72 h as detailed in the methods section. The rate of dark calcification remained constant for a few hours after incubation in the dark (at about 2 µmol Ca^{2+}/gwt/h) after which a drop occurred (i.e., after 12 h of incubation). After 24 h, there was a significant reduction in the rate of dark calcification (to about 0.17 µmol Ca^{2+}/gwt/h) (Fig. 3), which was about the same order as for the dead colonies. There were significant differences between all incubations of 12 h or less (i.e., 1, 2, 4 and 12 h) and all incubations more than 12 h (i.e., 24, 48 and 72 h) ($P < 0.05$).

For dark calcification, the addition of glucose or *Artemia* had a negative effect on the rate of coral calcification in both cases. The control rate of dark calcification was 1.98 ± 0.31 µmol Ca^{2+}/gwt/h (mean ± SD), while the rate decreased to 1.24 ± 0.26 µmol Ca^{2+}/gwt/h after 1 mM glucose was added, and 1.03 ± 0.16 µmol Ca^{2+}/gwt/h for 2 mM glucose. Addition of *Artemia* also decreased the rate to 1.02 ± 0.41 µmol Ca^{2+}/gwt/h (Fig. 4). There was a significant difference between the control rate and all the other treatments ($P < 0.05$), but not among treatments.

In order to study the effects of the ambient oxygen level on the rate of coral calcification, the coral colonies were suspended in oxygenated and de-oxygenated seawater and the rates of light and dark calcification were measured under the two conditions. There was no significant difference between the rates of light calcification in oxygenated (3.73 ± 0.15 µmol Ca^{2+}/gwt/h, (mean ± SD)) and de-oxygenated (3.14 ± 1.25 µmol Ca^{2+}/gwt/h) seawater, but a significant difference for dark calcification, where the rate in oxygenated seawater was more than three times higher than in de-oxygenated seawater (1.48 ± 0.16, 0.40 ± 0.6 µmol Ca^{2+}/gwt/h, respectively) (Fig. 5). There were significant differences between the rates of calcification in corals incubated in light and dark with N_2 bubbling ($P < 0.05$), and between the rates in light and dark incubations with O_2 bubbling ($P < 0.05$). Significant differences were also found between calcification rates in corals incubated in the dark with N_2 bubbling and corals incubated in the light with O_2 bubbling ($P < 0.05$), and between calcification rates in corals incubated in the light with N_2 bubbling and corals incubated in the dark with O_2 bubbling ($P < 0.05$). In the oxygen-depleted seawater, the oxygen level increased to saturation levels at a faster rate in the presence of a coral colony when compared with the same condition in the absence of a coral colony (Figs. 6, 7). In other cases where oxygen was bubbled before the start of measurements, the oxygen level increased steadily in the presence of a coral colony (i.e., over-saturation was observed), while the oxygen level decreased to reach a saturation level and was maintained at a steady state in the absence of the coral colony (Figs. 6, 7).

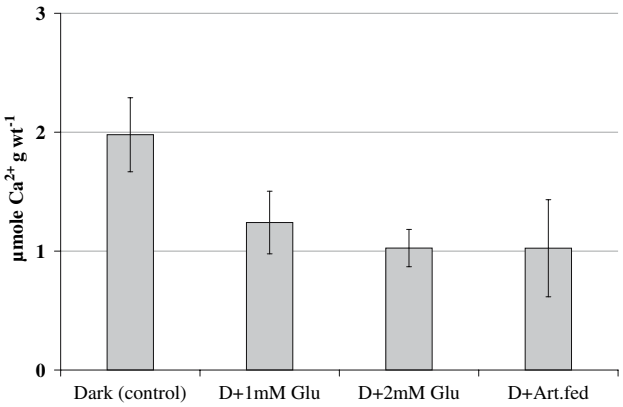

Fig. 4 Rates of dark calcification in *Galaxea fascicularis* upon addition of 1 mM (D + 1 mM Glu) and 2 mM of glucose (D + 2 mM Glu), and *Artemia* (D + Art. fed)

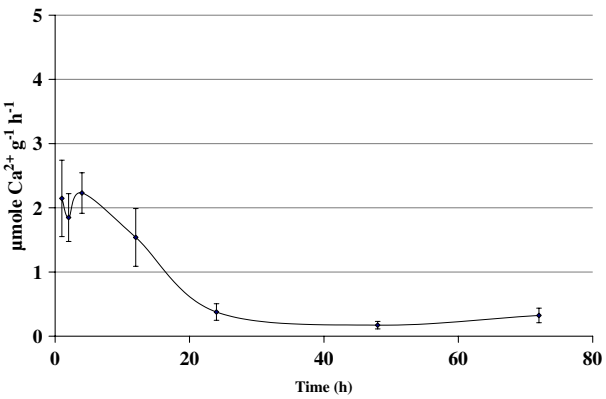

Fig. 3 Dark calcification in *Galaxea fascicularis* colonies incubated for up to 72 h

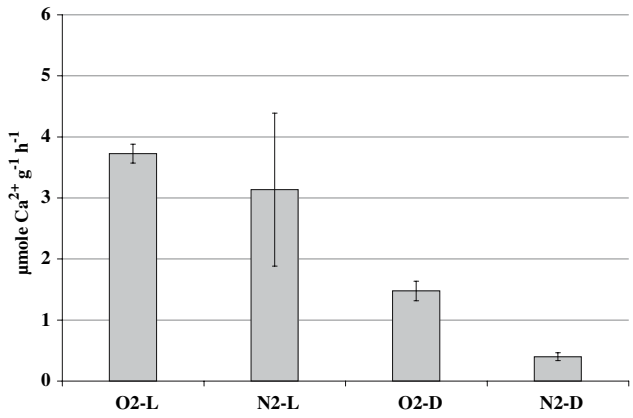

Fig. 5 Effects of oxygenation (O_2) and de-oxygenation (N_2) on the rates of light (L) and dark (D) calcification in *Galaxea fascicularis* colonies

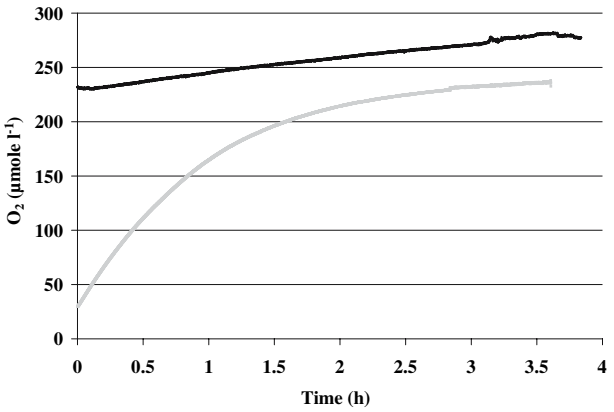

Fig. 6 Change in oxygen level in seawater with coral colony after oxygen depletion (*grey line*) and oxygen bubbling (*black line*)

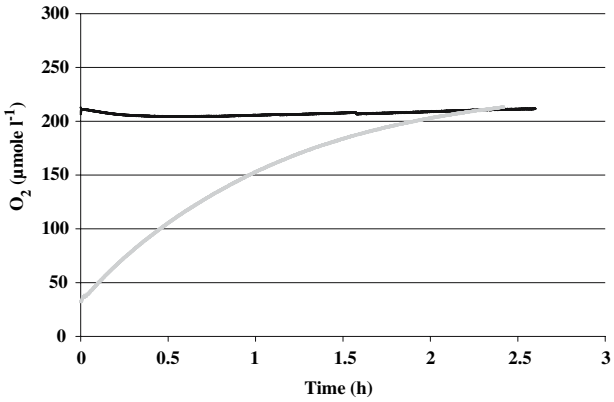

Fig. 7 Change in oxygen level in seawater without coral colony after oxygen depletion (*grey line*) and oxygen bubbling (*black line*)

Discussion

The present study had two objectives. The first was to test whether corals have a fixed rate of calcification when held under a uniform light intensity, and the second to investigate the driving force behind coral calcification in the absence of photosynthesis (i.e., dark calcification).

The ratio between light and dark calcification in *G. fascicularis* was two to one. This difference could not be attributed to isotopic exchange as this has previously been shown, by including dead corals in the incubation experiments (Al-Horani et al. 2005), to be much lower than the rates obtained for light and dark calcification (Fig. 1). This value also lies within the ratios obtained for corals in general (reviewed by Gattuso et al. 1999). The rate difference between light and dark calcification has always been attributed to photosynthesis enhancement of calcification during the light (e.g., Goreau 1959; Pearse and Muscatine 1971). In the dark, the calcification rate is reduced to about one half of its rate in the light, but calcification still takes place, although at lower rate.

Pätzold (1984) found that the growth of corals followed seasonal rhythms between summer and winter as a result of sea temperature variations. Aside from his work, few studies have been conducted to investigate daily changes in the rate of coral calcification. Most have been conducted under natural daylight (Clausen and Roth 1975; Chalker 1977; Barnes and Crossland 1978; Hidaka 1991). Moya et al. (2006) alone investigated coral calcification rates during a diel cycle using *Stylophora pistillata* under controlled conditions. They found no evidence of a daily cycle in the calcification rate under constant irradiance. In the present study, calcification rates for *G. fascicularis* were followed during the time course of a day. In the morning, the coral started with a relatively low rate of calcification, which increased to a maximum at 15:00 h. Thereafter the rate decreased until 20:00 h when it mirrored the dark rate of calcification (Fig. 2). These rate changes were not related to changes in light intensity since all experiments were conducted under a fixed light intensity in a darkened fume hood space, thus suggesting that *G. fascicularis* has an intrinsic rhythm for the regulation of calcification rate during the day. Field studies of O_2 flux and photosynthesis have demonstrated a similar rhythm in three other coral species, *Goniopora lobata*, *Favia favus* and *Plerogyra sinuosa* (Levy et al. 2004). Another study found a daily photosynthetic rhythm with a maximum value at sunrise and sunset and a minimum value occurring at noon for *Acropora cervicornis* (Chalker and Taylor 1978). The mechanism behind this rhythm is not known.

Calcification in the dark remained nearly constant for the first 4 h of incubation, then reduced by about 25% (i.e., after incubation in the dark for 12 h), and finally dropped to about 10% of the original rate, close to the value recorded for the dead colonies attributable to isotopic exchange (Fig. 3). This suggests that the coral had a reservoir of energy that was used to support calcification for the first few hours in the dark, with the calcification rate then declining after this energy reservoir was exhausted. Under normal conditions, corals are able to restore their energy reserves during the next daylight period through photosynthesis, but because in this experiment they were kept in dark after the 12-h incubation, the calcification rate decreased to zero. The addition of energy resources such as glucose and *Artemia salina* naupilii did not increase dark calcification over the short duration of the experiment, rather, a decrease was observed (Fig. 4). The addition of 1 mM glucose decreased the rate of dark calcification by ca. 37% relative to the dark control. This reduction could be due to diversion of energy into glucose consumption and cleavage. Also in support of this assumption was the finding that the rate further decreased to about 50% of the control when the concentration of glucose was increased to 2 mM. Additionally, adding *Artemia* also decreased the rate

by about 50%. Edmunds and Davies (1988) found that the respiration rate in cnidarians and other animals increased after feeding on particulate food, which they attributed to energy expenditure in growth. These results can be interpreted as a reduction in the calcification rate and a reallocation of energy into respiration and uptake of *Artemia* at least for the short period after addition of glucose and *Artemia*. This link between respiration and calcification in corals has previously been demonstrated through the inhibition of calcification by inhibiting oxidative phosphorylation (Chalker and Taylor 1975). Furthermore, in experiments which were conducted for longer time periods (weeks or months), feeding the coral *S. pistillata* with *Artemia* and freshly collected zooplankton resulted in enhanced coral growth both in the light and the dark (Houlbreque et al. 2003; Ferrier-Pages et al. 2003). The finding that reductions in dark calcification rates with depth was not proportional to the reduction in photosynthesis and light energy (Drew 1973), also supports the idea that feeding on particulate matter has a role in dark calcification.

In order to elucidate the role of ambient oxygen in coral calcification, *G. fascicularis* colonies were incubated in the light and dark in aerated and oxygen-depleted seawater. In the light, there were no significant difference in calcification rate between N_2 bubbling and aerated treatments, while in the dark the rate was about 75% less in the case of N_2 treatment compared with the aerated samples (Fig. 5). Although aeration increased dark calcification, this enhancement was not to the same degree as seen in the light. This suggests that O_2 is only one factor amongst several that are responsible for the higher rate of calcification in the light. Other factors may include the supply of reduced organic carbon compounds, the removal of byproducts, and the control of pH at the calcification site. Calcification rates for *S. pistillata* in aerated dark experiments were also found to be significantly higher than calcification rates of non-aerated dark controls (Rinkevich and Loya 1984). Similarly, it was found that in unstirred conditions, the rates of light and dark calcification were reduced by 25 and 60%, respectively (Dennison and Barnes 1988) suggesting that photosynthesis can possibly compensate the coral with oxygen in cases where the oxygen level is reduced, while in the dark the ambient oxygen is crucial for the coral to calcify. Corals may experience low ambient oxygen levels (i.e., hypoxia) during calm sea conditions when water movement around the coral colony is minimal (Ulstrup et al. 2005). This could lead to an increased boundary layer effect with low gas exchange and thereby increased oxidative stress, which in turn may lead to coral death at elevated water temperatures (Lesser et al. 1994; Nakamura and Woesik 2001; Ulstrup et al. 2005). In the present study, when changes in ambient oxygen level change were followed over time in oxygenated and de-oxygenated seawater with and without a coral present, the oxygen-depleted seawater was able to replace oxygen and reach a steady state in both cases, although the process was faster when a colony was present. This implies that seawater can exchange gases with the atmosphere, a factor, which might be important when seawater oxygen levels are low. The aerated seawater reached a steady state in the absence of coral colony, while it became supersaturated when a colony was present, a result which indicates that corals play an important role in supplying oxygen to the surrounding seawater. In previous experiments, it has been shown that the O_2 concentration measured at the coral surface was higher than in the ambient seawater (Kuehl et al. 1995; De Beer et al. 2000; Marshall and Clode 2003). This additional O_2 diffuses into the surrounding seawater and could support the respiratory needs of other reef organisms as well as being a reservoir of O_2 for night respiration by corals themselves (Al-Horani et al. 2003b).

From the results of this study it is concluded that in *G. fascicularis* the daily rate of calcification is regulated by an intrinsic rhythm whose mechanism is yet unknown. In contrast, the dark calcification rate is mainly controlled by the level of energy reserves in the coral and the oxygen level in the surrounding seawater.

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