Biogeosciences Discuss., 6, 7103–7131, 2009
www.biogeosciences-discuss.net/6/7103/2009/


Biogeosciences Discussions

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# Response of the temperate coral *Cladocora caespitosa* to mid- and long-term exposure to $pCO_2$ and temperature levels projected in 2100

**R. Rodolfo-Metalpa[1,2], S. Martin[3], C. Ferrier-Pagès[1], and J.-P. Gattuso[3]**

[1]Centre Scientifique de Monaco, Monaco (Principality), France
[2]International Atomic Energy Agency-Marine Environment Laboratories, 4 Quai Antoine Ier, 98000 Principality of Monaco, France
[3]CNRS, Laboratoire d'océanographie de Villefranche, Villefranche-sur-Mer Cedex, France; Université Pierre et Marie Curie–Paris 6, France

Received: 28 June 2009 – Accepted: 30 June 2009 – Published: 16 July 2009

Correspondence to: R. Rodolfo-Metalpa (riccardo@rodolfo-metalpa.com)

Published by Copernicus Publications on behalf of the European Geosciences Union.





## Abstract

Atmospheric $CO_2$ partial pressure ($pCO_2$) is expected to increase to 700 ppm or more by the end of the present century. Anthropogenic $CO_2$ is absorbed by the oceans leading to decreases in pH and the $CaCO_3$ saturation state ($\Omega$) of the seawater. While $pCO_2$ was shown to drastically decrease calcification rates in tropical, fast growing corals, here we show, using the Mediterranean symbiotic coral *Cladocora caespitosa*, that the conventional belief that an increase in $pCO_2$, in the range predicted to 2100, reduces calcification rates may not be widespread in temperate corals. We found that the seasonal change in temperature was the predominant factor controlling the physiology and growth of *C. caespitosa*, while an increase in $pCO_2$, alone or in combination with global warming, had no significant effect on photosynthesis, photosynthetic efficiency and calcification. This result differs from that obtained on reef-building corals, which exhibit lower rates of calcification at elevated $pCO_2$. The lack of sensitivity of temperate corals to high-$pCO_2$ levels might be due to its slow growth rates, which seem to be more dependent on temperature than on the saturation state of calcium carbonate in the range predicted for the end of the century.

## 1 Introduction

Atmospheric $CO_2$ partial pressure ($pCO_2$) increased by 32% between 1880 and 2005 (280 vs. 379 ppm; IPCC 2007) and it is expected to double present day concentration by the end of this century. Anthropogenic $CO_2$ emitted to the atmosphere is absorbed by the oceans leading to decreases in pH and the $CaCO_3$ saturation state ($\Omega$) of the seawater. Coral calcification is largely controlled by the degree of saturation of seawater with respect to aragonite ($\Omega_{aragonite}$; see review by Kleypas et al., 2006), which varies with latitude (Orr et al., 2005). As a result, coral calcification is expected to decline dramatically in the future, raising widespread concerns about the future of our oceans in a high-$CO_2$ world (Hoegh-Guldberg et al., 2007; Hall-Spencer et al., 2008; Wootton et



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al., 2008). Several studies on the effect of ocean acidification on fast-growing tropical corals show that calcification could decline by 0 to 56% under a doubling of $pCO_2$ alone (Kleypas et al., 2006) or in combination with a +3°C increase in temperature (Reynaud et al., 2003; Anthony et al., 2008). In contrast, rates of photosynthesis are either not affected (e.g. Langdon et al., 2003; Reynaud et al., 2003; Schneider and Erez, 2006; Marubini et al. 2008) or slightly increased (e.g. Langdon and Atkinson, 2005) at the level of $pCO_2$ expected in 2100. Similarly, Anthony et al. (2008) recently found that, in contrast to *Acropora intermedia*, *Porites lobata* decreased its net productivity under intermediate $CO_2$ concentrations (700 ppm), high temperature and irradiance regime. The only study that investigated the effect of high $pCO_2$ on a Mediterranean coral, *Oculina patagonica* (Fine and Tchernov, 2007) revealed a complete dissolution of the skeleton at pH 7.4, a value lower than that expected in 2100.

Climate change also includes the on-going increase in global sea temperature with an expected additional warming of ca. 3°C (Mc Neil and Matear, 2007). The effect of seawater warming has been extensively studied on corals because it is the primary cause of bleaching of tropical species (e.g. Brown, 1997) and of the mortality of warm-temperate Mediterranean species (Rodolfo-Metalpa et al., 2006, 2008c).

Ocean acidification and global warming will certainly cause a significant decrease in coral growth rates and, consequently, affect the stability of reef ecosystems (Hoegh-Guldberg et al., 2007). However, the response of corals to elevated $pCO_2$ has mostly been measured during short- and mid-term experiments (hours to weeks), therefore excluding any potential coral acclimatization/adaptation ability to the new conditions. In addition, the interactive effect of elevated $pCO_2$ and temperature has been poorly investigated (Reynaud et al., 2003; Langdon and Atkinson, 2005; Anthony et al., 2008). Therefore, multifactor long-term experiments are essential to investigate the response of corals to the synergistic or antagonistic effects of elevated $pCO_2$ and temperature, and to provide a more accurate prediction of their status in 2100. Consequently, we have investigated, for the first time, the effect of mid- and long-term exposure to normal and elevated temperatures ($T$ and $T$ +3°C) and $pCO_2$ (400 ppm and 700 ppm) on

calcification and photosynthesis of the Mediterranean zooxanthellate coral, *Cladocora caespitosa.* Colonies were acclimated to: (1) mid-term perturbations (1 month) in summer and winter conditions of irradiance and temperature and (2) a long-term perturbation (1 year), mimicking the natural variations in temperature and irradiance. Because shallow-water corals already live (during summer) near their upper thermal limits in the north Mediterranean Sea and suffer mass-mortality during abnormal long periods of elevated temperatures (Rodolfo-Metalpa et al., 2006, 2008c), we run the experiments on colonies collected at 20–30 m depth, where the seasonal thermocline generally limits the incidence of summer thermal anomalies.

## 2 Materials and methods

### 2.1 Coral collection

Three colonies of *C. caespitosa* were collected in the Bay of Villefranche (Ligurian Sea, France, 43°41′N, 7°18′E) at ca. 25 m depth in July 2006 and 3 others in February 2007 and transported in thermostated tanks to the laboratory. They were divided into fragments (10 to 20 polyps each), and single polyps which were carefully cleaned from epiphytes, associated fauna, and sediment, tagged on PVC supports and randomly assigned to one of the four treatments (see experimental set-up). Twenty-eight fragments (7 for each treatment) were randomly selected from the colonies collected in July 2006 for the long-term experiment; 20 fragments (5 for each treatment) and 24 single polyps (6 for each treatment) were selected for the mid-term experiments from colonies collected in July 2006 for the summer experiment, while other 20 fragments (5 for each treatment) and 32 single polyps (8 for each treatment) were selected from colonies collected in February 2007 for the winter experiments (see below). Corals were set in each aquarium and allowed to acclimate for one month prior to the beginning of the experiment. Corals were considered naturally fed because the seawater supplied to the aquaria was not filtered; therefore, no artificial food was supplied.



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## 2.2 Experimental set-up

Two mid-term experiments (one month) were run in summer and winter in parallel to the long-term experiment and using the same set-up. Rates of calcification and photosynthesis, zooxanthellae density, as well as the content of chlorophyll (chl) and protein were measured. During the long-term experiment (one year) the rate of calcification and the effective quantum yields ($\Delta F/F'_m$) were measured during each season.

A 2×2 orthogonal, full cross factorial experiment was set-up using 4 independent aquaria. While this is, strictly speaking, a pseudo-replicate design (sensu Hurlbert 1984), it must be emphasized that in this experiment it was technically difficult to control $pCO_2$ and other parameters in several aquaria. However, to avoid undesirable "tank" effect, we carefully cleaned each aquarium every week and each tank reservoir every three weeks in order to prevent any growth of epiphytes and fouling communities or detritus accumulation; cleaning procedures and the high seawater renewal avoided any undesirable differences between aquarium in their seawater chemistry, except for the carbonate seawater. During the experiment irradiance, temperature, salinity (range: 37.9–38.3, $n$=194 for each tank), and the seawater carbonate chemistry were monitored in each aquarium every second days on average (see below).

Two aquaria were kept at a $pCO_2$ close to ambient values (ca. 400 ppm) while the remaining two were maintained at elevated $pCO_2$ (ca. 700 ppm). Within each $pCO_2$ treatment, one aquarium was maintained at normal temperature ($T$) while temperature was increased by 3°C in the other one ($T$+3°C). Aquaria were therefore labeled: 400 $T$ or 400 $T$+3°C for 400 ppm $pCO_2$ with normal ($T$) or elevated temperature ($T$+3°C) and 700 $T$ or 700 $T$+3° for 700 ppm $pCO_2$ and also normal or elevated temperature. Three experiments were concomitantly carried out: one long-term experiment from July 2006 to June 2007 on a set of corals collected in July 2006, and two mid-term experiments in August 2006 (summer) and January 2007 (winter) on two set of corals respectively collected in July 2006 and February 2007. Except $pCO_2$, which remained constant, temperature, irradiance and photoperiod were changed according to their seasonal



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values measured at ca. 20 m depth in the Bay of Villefranche. Temperature was gradually changed from 13.3°C in winter, to 17.7°C in autumn and spring and to 22°C in summer; these values were maintained for ca. 3 weeks in order to complete all the physiological measurements (Fig. 1a). Because irradiance data did not exist for the Bay of Villefranche when the experiment was planned, we chose four seasonal values that were measured for this species living at similar depths (Peirano et al., 1998, 2007), 15 $\mu$mol photon $m^{-2} s^{-1}$ in winter, 30 and 40 $\mu$mol photon $m^{-2} s^{-1}$ in autumn and spring, respectively and 60 $\mu$mol photon $m^{-2} s^{-1}$ in summer (Fig. 1a). These experimental irradiances were seasonally adjusted using neutral density filters and verified using a LI-COR spherical underwater quantum sensor (LI-193SA). In addition, their ecological relevance was verified by seasonal measurements at ca. 25 m depth in the Bay of Villefranche. In order to maintain a constant seawater temperature, aquaria were set-up in a thermostated room and each-one was equipped with a 150 W heater connected to a temperature electronic controller (Corema). The temperature control was ±0.1°C. Two submersible pumps (Micro-jet, Aquarium Systems) ensured water circulation. Irradiance was provided by fluorescent tubes (JBL Solar Ultra Marin Day 39W) at the required irradiance by using neutral density filters. Photoperiod was changed according to seasons (from 9:15 in winter to 15:9 in summer; light:dark, h).

### 2.3 *p*$CO_2$ aquaria set-up and carbonate chemistry calculations

*p*$CO_2$ was adjusted by bubbling $CO_2$-free air and $CO_2$-rich air in two large tanks reservoir (110 l), for either the normal and high *p*$CO_2$ treatment. Seawater was pumped from 10 m depth in the Bay of Villefranche to the two reservoir tanks. Each tank furnished two 26 l volume aquaria at a rate of ca. 13 l $h^{-1}$. Elevated *p*$CO_2$ in the aquaria was achieved at the desired level using a gas blender (series 850, Signal instrument) in combination with a gas divider (821, Signal Instrument). pH was measured every 1 or 2 days (183 times in total) using a Metrohm 826 pH meter and an 8103SC Orion electrode that was calibrated on the total scale ($pH_T$) and at the experimental temperatures as described by (DOE 1994). Seawater samples for total alkalinity (TA), were filtered



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on Whatman GF/F membranes, poisoned with 0.05 ml of 50% $HgCl_2$ to avoid biological alteration, and stored in the dark at 4°C pending analyses. TA was determined on 20 ml sub-samples using a custom-made titration system composed of a 20 ml open thermostated titration cell (kept at 25.0°C), an Orion 8103SC pH electrode calibrated on the National Bureau of Standards scale and a computer-driven Metrohm 665 Dosimat titrator. Parameters of the carbonate system ($pCO_2$, $CO_3^{2-}$, $HCO_3^-$, DIC concentrations, and $\Omega_{aragonite}$) were calculated from $pH_T$, TA, temperature and salinity using the R package *seacarb* (Proye and Gattuso, 2003). The mean seasonal parameters of the carbonate system are given in Table 1.

### 2.4 Calcification rates

Calcification rate was measured using the alkalinity anomaly (Smith and Kinsey, 1978) and the buoyant weight (Davies, 1989) techniques, respectively for the mid- and long-term experiments. The former measures calcification rate over hours (nmol $CaCO_3$ $cm^{-2}$ $h^{-1}$), while the latter technique integrates coral calcification rates over several weeks (mg $CaCO_3$ $cm^{-2}$ $day^{-1}$). In order to compare results from the two methods, calcification rates measured in summer and winter using the alkalinity anomaly technique were also recalculated as mg $CaCO_3$ $cm^{-2}$ $day^{-1}$ by multiplying light and dark calcification over the daily cycle and photoperiod (24-h integrate growth). It is important to note that both methods do not allow discriminating between the gross calcification ($CaCO_3$ deposition) and dissolution ($CaCO_3$ loss).

For the mid-term experiments (summer and winter), 20 fragments (5 for each treatment) were prepared in summer and 20 others in winter (see "coral collection"). They were maintained for one month to the summer and winter experimental conditions, respectively. The rate of calcification of each fragment was measured in the dark and light using the alkalinity anomaly technique. Fragments were incubated for 5 h in closed glass chambers filled with ca. 50 ml of seawater from the respective aquarium and continuously stirred with a stirring bar. Seawater was sampled for TA determination at the beginning and the end of the incubation, filtered onto 0.45 $\mu$m membranes (Whatman,

GF/C) and stored at 4°C pending analysis (within 7 days at most). At the end of the incubations, the fragments were frozen at −80°C for further analysis of protein content. The change in TA during the incubation was used to estimate the calcification rate (see Tentori and Allemand, 2006, for further details).

For the long-term experiment, 28 fragments (7 for each treatment) were repeatedly weighed using the buoyant weight technique at the end of each season, i.e. end of August 2006, November 2006, March 2007 and June 2007. They were weighed again 15 days after each initial measurement. Fragments were carefully cleaned from epiphytes before each measurement. The calcification rate was estimated as the difference between the two weights normalized per day (Davies, 1989) and unit surface area measured according to Rodolfo-Metalpa et al. (2006).

### 2.5 Rates of photosynthesis and respiration

Twenty-four (6 polyps for each treatment) and 32 single polyps (8 polyps for each treatment) were used at the end of summer and winter, respectively, to measure their rates of net photosynthesis ($P_n$) and dark respiration ($R$). Each polyp was placed in a closed thermostated Perspex chamber filled with ca. 50 ml of seawater at the treatment $pCO_2$ and temperature and continuously stirred with a stirring bar. Changes in the concentration of dissolved oxygen were measured using a Strathkelvin oxygen electrode system (Clark-type electrodes connected to a Strathkelvin 928 oxygen meter and a computer). The electrode was calibrated against $O_2$-free (using sodium dithionite) and air-saturated (100% $O_2$) seawater. The 100% $O_2$ concentration was calculated according to the experimental temperature, salinity values at the ambient barometric pressure (http://www.unisense.com/Default.aspx?ID=117). Polyps were allowed to acclimate for at least 15 min, and $P_n$ was measured at the experimental winter and summer temperatures and irradiance conditions followed by $R$ in the dark. Changes in dissolved $O_2$ were also measured in chambers without polyps ($n$=6 for each treatment) and served as controls to account for bacterial and phytoplankton activities. Gross photosynthesis ($P_g$) at the culture irradiance was calculated by summing the rates of $R$ (absolute value)



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and $P_n$ corrected from the blank activities and assuming that $R$ in the light equaled $R$ in the dark. summer and winter values were separately compared between treatments. Molar unit were normalized to the surface area of polyps (nmol $O_2$ $cm^{-2}$ $h^{-1}$). The polyps were frozen at −80°C immediately after measurements for subsequent determination of zooxanthella density and chl concentrations.

### 2.6 Chlorophyll fluorescence yields

Photosynthetic efficiency ($\Delta F/F'_m$) were measured at the end of each season using an underwater PAM fluorometer (Diving-PAM, Walz). The 8 mm fiber was placed at a fixed distance from the coral tissue using a black jacket. After 5–10 s of darkness the effective quantum yield was measured by exposing the same 28 fragments (7 for each treatment) used during the long-term calcification measurements to a 0.8 s period of saturating light (ca. 8000 $\mu$mol photon $m^{-2}$ $s^{-1}$).

### 2.7 Zooxanthellae, chlorophyll and protein content

Twenty polyps used during $P$ and $R$ measurements in summer (6 polyps for each treatment) and 32 others in winter (8 polyps for each treatment) were processed for their zooxanthellae and chl content. Tissues were separated from the skeleton using an air-pick and homogenised using a hand-held Potter in 7 ml of filtered seawater (Whatman GF/C). One ml sub-sample was used to measure the density of zooxanthellae while the remaining homogenate was used to measure chl concentration. At least 300 zooxanthellae were counted in 10 sedimentation chambers of known volume, using an inverse microscope (Leica, Wetzlar, Germany) and the Histolab 5.2.3 image analysis software (Microvision, Every, France). Chl $a$ and $c_2$ were determined according to the equations of Jeffrey and Humphrey (1975) using a spectrophotometer. Details of both methods can be found in Rodolfo-Metalpa et al. (2006).

Protein content were measured on the 20 fragments used for the alkalinity anomaly technique in summer (6 polyps for each treatment) and in winter (6 polyps for each

treatment). Polyps were treated in 1 N NaOH at 90°C for 30 min and the protein content was measured using the BCA assay Kit (Interchim). The standard curve was established with bovine serum albumin and the absorbance was measured with a multiscan bichromatic spectrophotometer (Labsystem®). Data were normalized to the surface area of polyps as described above.

### 2.8 Statistical analysis

Data were tested for homogeneity of variances using Cochran's test and were log-transformed if necessary; plots of residuals were also used to verify conformity to ANOVA assumptions. Results of the mid-term experiments (summer and winter) were analyzed separately by two-way ANOVAs with $pCO_2$ (400 and 700 ppm) and temperature treatment ($T$ and $T$+3°C) as fixed factors. When ANOVA showed significant differences, Tukey HSD multiple-comparisons were used to attribute differences between specific treatments. STATISTICA® software (StatSoft) was used to perform these analyses. Calcification rates and the photosynthetic efficiency throughout the one-year experiment were analysed using repeated measures ANOVA in SPSS 16.0, with seasons (summer, autumn, winter and spring) as the within-subjects factor and $pCO_2$ (400 and 700 ppm) and temperature treatment ($T$ and $T$+3°C) as fixed main effects. Data for repeated measures analyses were first tested for equality of covariances using Box's M test, then Mauchly's test for sphericity was used to test for deviation from sphericity in the variance-covariance matrix of the orthonormalized dependent variable. Both sets of data passed Box's M test, but calcification rate data deviated from assumptions of sphericity ($W_5$=0.434, P=0.03); we therefore adjusted degrees of freedom in all tests involving within-subjects effects using the Greenhouse-Geisser method (based on e values; e=0.666) to produce a more conservative F-ratio, and calculated polynomial contrasts where appropriate for all these effects. Photosynthetic efficiency data passed all tests and more liberal F ratios were used in repeated measures design accordingly. Subsequent to a lack of significant interactions between the within-subjects



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and fixed factors, we used Levene's tests to confirm homogeneity of variances in the data within each season and type III SS ANOVA to test between-subjects main effects using aggregate data. Finally, relationships between calcification rates (long-term exposure, pooled data), $\Omega_{aragonite}$, and temperature were assessed by multiple regression analysis. Results are expressed as mean ± standard error of the mean (s.e.m.).

## 3 Results

### 3.1 Effect of a mid-term exposure

A 3 ˚ C rise in temperature caused significant changes in $P_n$ and $R$ in winter (from 13 to 16˚C), resulting in a 72% increase in $P_g$ at elevated temperatures (Table2; Fig.2a). The enhancement of photosynthesis occurred despite a 40% decrease in the zooxanthellae density but with no change in the chl content (Table 2; Fig. 3a, b). A 3˚C rise in temperature in winter also caused a significant increase in the light calcification rate (Table 2; Fig. 2b). Dark and light calcification rates (Fig. 2b, c) were significantly higher in summer (97.1±14.4 and 246.8±10.3 nmol $CaCO_3$ $cm^{-2}$ $h^{-1}$, respectively) than in winter (39.7± 2.7 and 90.4±5.6 nmol $CaCO_3$ $cm^{-2}$ $h^{-1}$, respectively). Calcification was always ca. 2.3 times higher in the light than in the dark, both in winter and summer. The protein content did not change significantly between treatments (Table 2), in winter (averaged data with $n$=20: 1.01±0.04 mg $cm^{-2}$) and summer (averaged data with $n$=20: 1.32±0.10 mg $cm^{-2}$).

$P_n$, $P_g$ and $R$ were not affected significantly by elevated $pCO_2$, neither in summer or winter (Table 2, Fig. 2a). The same result was obtained with light and dark calcification rates, both in winter and summer (Table 2, Fig. 2b, c). However, elevated $pCO_2$ increased significantly the zooxanthellae density and chl content in winter (Table 1; Fig. 3b), without any interacting effect with temperature treatment.

### 3.2 Effect of a long-term exposure

Elevated $pCO_2$, alone or in combination with elevated temperature, did not affect significantly the calcification and $\Delta F/F'_m$ of zooxanthellae over one-year experiment (Fig. 4a, b). Repeated measures ANOVA indicated that calcification rates were significantly different across seasons ($F_{1.9,45.9}$=23.03, $P$<0.01); however interactions of the within-subjects effect (season) with fixed between-subjects main effects were all non-significant ($P$>0.05). A similar effect of seasons was also apparent for photosynthetic efficiency ($F_{3,66}$=11.39, $P$<0.001), a significant interaction with temperature regime was also indicated ($F_{3,66}$=8.68, $P$<0.001). Polynomial contrasts for calcification rates revealed significant linear ($F$=28.74, $p$<0.001), quadratic ($F$=21.84, $p$<0.001) and cubic ($F$=5.31, $p$<0.031) trends in seasonal data; no contrasts values for interaction terms involving the within-subjects term indicated any significant trends. For photosynthetic efficiencies, contrasts indicated significant linear and quadratic components for Season and interactions between Season and both $pCO_2$ and Temperature. Subsequently, ANOVA on aggregate data confirmed a lack of significant differences in calcification rates between temperature regimes or $pCO_2$ levels ($P$>0.05); no such ANOVA was conducted on photosynthetic efficiency data due to the significant interactions described above.

A significant and positive relationship was found between temperature (from 13.3–25°C) and calcification rates Fig. 5a; $R^2$=0.62, $F_{(1,14)}$=23.41, $p$=0.0002)) but not between calcification rates and $\Omega_{aragonite}$ (Fig. 5b; $R^2$=0.15, $F_{(1,14)}$=2.59, $p$=0.13).

## 4 Discussion

While temperature was found as one of the most critical environmental parameter controlling the physiology and calcification of *C. caespitosa*, an increase in $CO_2$ concentration, within the values expected to the end of 2100, did not affect significantly either their photosynthetic performance or the calcification rates.

Although treatments in this experiment were not replicated, it is very unlikely that the observed treatment effects result from differences between the aquaria other than those imposed by the treatment regimes. All environmental parameters that could affect the comparison between treatments were monitored. Salinity and irradiance were identical in each treatment. Seawater temperature did not differ within treatments ($T$ and $T+3^{\circ}C$, respectively); their values changed concomitantly during the experiment in each aquarium accordingly to measurements made in the Bay of Villefranche (Fig. 1a). Its effect on calcification and photosynthetic performances of *C. caespitosa*, is consistent with previous findings in aquaria as well as in situ (Rodolfo-Metalpa et al., 2008b, c), thus confirming the validity of the experimental approach, and suggesting the lack of a "tank" effect during the experiment. Only $pCO_2$ (ca. 400 and 700 ppm), temperature ($T$ and $T+3^{\circ}C$), and season (4 seasons) changed according to the treatment regime. The seasonal evolution of $pCO_2$, $pH_T$ and $\Omega_{aragonite}$ are presented in Fig. 1b, c, d, respectively). Because the seawater $pCO_2$ was manipulated by bubbling gasses, the TA did not change between treatments at all seasons (Table 1). These values were within the range measured in the Bay of Villefranche from January 2007 to December 2008 both at the surface and at 50 m depth (Table 1). In contrast, $pH_T$, $pCO_2$ and $\Omega_{aragonite}$ values differed between treatments (400 ppm vs. 700 ppm) but not within treatments ($T$ (400 and 700 ppm) vs. $T+3^{\circ}C$ (400 and 700 ppm)) (Table 1; Fig. 1b, c, d). This demonstrates that aquarium cleaning, and the high seawater renewal rate we used, successfully prevented any "tank" effect related to the above-mentioned factors. We are therefore confident that the corals responses were only caused by the treatment ($pCO_2$, temperature treatment, and season).

Our study confirms previous findings on the effect of temperature on the calcification rates of *C. caespitosa* (Rodolfo-Metalpa et al., 2008b, c) and other temperate corals (e.g. Jacques et al., 1993, Howe and Marshall, 2002). Rates of photosynthesis and respiration were much lower in winter (13.3°C and irradiance of 15 $\mu$mol photon $m^{-2}\,s^{-1}$), compared to summer values. This reduction of photosynthetic activity could explain the difference in calcification rates between the two seasons (Fig. 2b, c). Low



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metabolism is a typical feature of temperate benthic species in winter when both irradiance and temperature are also at their lowest levels. Previous studies have demonstrated that *C. caespitosa* relies greatly on the heterotrophic nutrition, when available, to increase its zooxanthellae density and chl content, thereby increasing photosynthate production (Rodolfo-Metalpa et al., 2008a, b). In contrast, in the present study, because corals were not fed, zooxanthellae density and chl concentrations remained low during winter. A 3°C increase in temperature during this winter period (passing from 13 to 16°C) was sufficient to activate the coral metabolism, enhancing both its photosynthesis and respiration rates. This acclimation ability was also more important during summer, following an increase in both temperature and irradiance (22°C and 60 $\mu$mol photon $m^{-2} s^{-1}$); these conditions caused a significant parallel increase in the calcification rates (Fig. 2b, c) in agreement with the stimulating effect of temperature reported for both tropical (e.g. Clausen and Roth, 1975) and temperate corals (Jacques et al., 1983; Howe and Marshall, 2002). At 22°C, a 3°C increase in temperature did not affect *C. caespitosa* photosynthesis, symbiont biomass and calcification rates (Fig. 2a, b; 0.49 mg $CaCO_3$ $cm^{-2}$ $h^{-1}$, and 0.74 mg $CaCO_3$ $cm^{-2}$ $day^{-1}$, for the mid- and the long-term experiments, respectively), suggesting that its metabolism was at its upper temperature limits. The highest temperature only increased, but not significantly, the respiration rates (Fig. 2a).

A very sensitive marker of thermal stress in symbiotic corals is the decrease in photosynthetic efficiency ($\Delta F/F'_m$) after an exposition to elevated temperatures (Warner et al., 1996). In this experiment, and in agreement with Rodolfo-Metalpa et al. (2006), $\Delta F/F'_m$ decreased after four weeks at 25°C. If the elevated temperature was maintained for longer (>6 weeks), this decrease would be the upper step of a cascading effect leading to the death of the corals (Rodolfo-Metalpa et al., 2006, 2008c). Coral mortality has become frequent on colonies living in the first 10–20 m because they are exposed during summer to prolonged periods of high temperatures (mean 25–26°C). There is no doubt that subjecting shallower colonies to a 3°C increase of temperature as expected for the end of the century would have led, as described before, to major



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response and mortality, potentially confounding any evaluation on the effect of high $pCO_2$.

In order to compare the two methods used to measure the calcification rates, data from the alkalinity anomaly technique were recalculated on a 24-h growth cycle, therefore considering the light and dark growth rates to be constant during the seasonal photoperiod. Using this technique, corals calcified 0.15 and 0.45 mg $CaCO_3$ $cm^{-2}$ $day^{-1}$ in winter and summer respectively. However, using the buoyant weight technique, these rates increased to 0.27 mg $CaCO_3$ $cm^{-2}$ $day^{-1}$ in winter and 0.74 mg $CaCO_3$ $cm^{-2}$ $day^{-1}$ in summer. This difference is not surprising because the two methods measure calcification rates over different time-scales (hours vs. weeks). To our best of knowledge only Steller et al. (2007) compared the two methods on the growth rates of the coralline algae *Lithophyllum margaritae*, and found a 2-fold enhancement of the growth rates using the buoyant weight technique. A possible underestimation of calcification rates by the alkalinity method could also be due to the fact that both light and dark calcification rates can slightly change over time, due to different feeding inputs, use of lipid reserves, or acclimation. In addition, during this study the dark calcification rates were measured during the day in darkness, and can be different from night calcification rates. Therefore, the buoyant weight technique integrates on a long-term several factors and seems more appropriate to investigate integrate physiological response of the whole coral to environmental conditions.

The responses of *C. caespitosa* to elevated $pCO_2$ were in part not consistent with previous studies on tropical corals as it did not exhibit strong changes in physiological parameters such as photosynthesis, photosynthetic efficiency and calcification. While photosynthesis on symbiotic corals seems relatively less affected by ocean acidification (e.g. Langdon et al., 2003; Reynaud et al., 2003; Schneider and Erez, 2006), likely because corals do not rely on dissolved $CO_2$ for photosynthesis (Gattuso et al., 1999), laboratory and mesocosm experiments have shown a common trend of decreased calcification rates with increased acidification (e.g. Gattuso et al., 1999; Langdon et al., 2005; Kleypas et al., 2006). For the tropics, it has been suggested that below a



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value of $\Omega_{aragonite}$=3.3, corresponding to a $pCO_2$ level of around 480 ppm, calcification rates will approach zero (Hoegh-Guldberg et al., 2007; Silverman et al., 2009). In contrast, the temperate coral *C. caespitosa* maintained similar calcification rates for $\Omega_{aragonite}$ ranging from 1.95 to 3.86 ($pCO_2$ values of 709–475 ppm), suggesting that this species may be resistant to an increase in $pCO_2$ in the range predicted for the end of the century. Similarly, Ries et al. (2008) recently reported that the calcification rate of the temperate coral *Oculina arbuscula* is unaffected by an increase of $pCO_2$ up to 840 ppm ($\Omega_{aragonite}$=1.8). A drastic decrease in calcification was only found at a $pCO_2$ of 2240 ppm, corresponding to an $\Omega_{aragonite}$ of 0.8. The low sensitivity of these two temperate corals to an increase in $pCO_2$ is at odds with the general consensus on the negative relationship between $pCO_2$ and calcification of tropical corals (14–30% less by 2100, Gattuso et al., 1999; Kleypas et al., 1999).

There are several possible reasons why temperate corals may be relatively resistant to the changes in carbonate chemistry expected in the present century. The first explanation lies in the very different methods used to manipulate $\Omega_{aragonite}$ in experimental studies. For example, methodological differences were held responsible for variable effects of acidification

experiments on coccolithophores (Iglesias-Rodriguez et al., 2008). However, this seems unlikely in corals because coral communities have in general shown decreased calcification whether $\Omega_{aragonite}$ was manipulated by acid addition (e.g. Marubini and Thake, 1999, Jokiel et al., 2008), gas bubbling (e.g. Reynaud et al., 2003), or manipulation of the calcium concentration (e.g. Gattuso et al., 1998). Second, except for Ries et al. (2008) and the present study, all experiments carried out on corals used tropical, fast-growing species which grow up to 5 times faster (Rodolfo-Metalpa et al., 2006). It is likely that fast-growing corals need higher $\Omega_{aragonite}$ concentrations than the slow-growing *C. caespitosa* or other temperate or cold species. It is conceivable that the carbonate requirement of slow growing corals is low and that the concentration of carbonate ions therefore does not become limiting, even under high $pCO_2$ concentrations (ca. 700 ppm). This would explain why Reynaud et al. (2003) did not find a significant





increase in growth rates of *Stylophora pistillata* maintained at 734 ppm and 25.1˚C but only when the corals were exposed to the 28.3˚C, i.e. when calcification rates were faster under normal $pCO_2$ conditions. Similarly, preliminary observations using the reef coral *Montipora capitata* showed that the impact of acidification on growth was more pronounced at summer temperatures, when corals were growing faster (Jokiel, personal communication). Anthony et al. (2008) recently found differences in calcification rates (% weight increase per month) on the fast-growing coral *A. intermedia* exposed to high $pCO_2$ (700 ppm) but not on the massive coral *P. lobata*. It is therefore possible that the effect of low $\Omega_{aragonite}$ concentrations on coral growth rates is more noticeable, on fast-growing corals in which the calcification rate is also the highest.

Alternatively, some marine invertebrates may be able to calcify in the face of ocean acidification or, contrary to what is generally expected, may increase their calcification rates as recently reported on the ophiourid brittlestar *Amphiura filiformis* (Wood et al., 2008), the seastar *Pisaster ochraceus* (Gooding et al., 2009) exposed to lowered pH (7.8–7.3), and suggested for other calcifying species (Ries et al., 2008). In contrast, the calcification rate of *C. caespitosa* did not increase at a pH of ca. 7.85. However, it is known that exposing calcifying species to low pH levels could both change their calcification rates and, concomitantly, increase dissolution of the existing $CaCO_3$ skeleton. To date, no conclusion can be drawn on the gross calcification ability of *C. caespitosa* because both methods used in this study measure net calcification and do not allow discriminating the gross calcification and dissolution. However, at a pH of ca. 7.85, the dissolution rate should be negligible because the seawater is still oversaturated with respect to $CaCO_3$ (Fig. 1d). In the light of these evidences, more experiments are necessary at lower pH levels, in order to discriminate between the two processes by using methods such as the $^{45}Ca$ uptake (Tambutté et al., 1995).

Wood et al. (2008) showed that even though *A. filiformis* increased its calcification rate in order to regenerate arms, it failed to form efficient muscle apparatus, therefore decreasing some physiological functions such as feeding, burrow aeration and predator avoidance. This highlights how important it is to consider the whole organisms

responses (e.g. respiration, growth, mobilisation of energy stores, feeding rates, reproduction, etc.) when the effect of acidification is investigated. The response of *C. caespitosa* to high $pCO_2$ conditions was investigated at the symbiont level (chlorophyll fluorescence yields, density and chl content), animal level (protein content and respiration rates), as well at their symbiosis level (oxygen metabolism and calcification). For instance, as clearly shown here as well as in several other marine organisms (Reynaud et al., 2003; Pörtner, 2009), seawater temperature can greatly affect their physiological performances and in particular to marine invertebrates which, in turn, may reduce or alter their performances facing to ocean acidification. Therefore, in order to predict correctly the fate of corals, future research on ocean acidification should take into consideration the whole organism response, over its whole temperature range, including an increase in temperature by global warming (Pörtner and Knust, 2007).

Although the negative relationship between calcification and $pCO_2$ is widely accepted for corals and other calcifying organisms, it is likely that the response of the two temperate corals *C. caespitosa* (this study) and *O. arbuscula* (Ries et al., 2008) and potentially other slow-growing corals, to an increase in $pCO_2$ concentrations (700 ppm) might be frequent also in tropical corals. However, experimental artifacts (e.g. temperature, irradiance and methods used to acidify seawater), species-specific responses and short incubations might sometimes have confounded the interpretation of results. Temperature at which corals are maintained is by far the main causative agent affecting their calcification under $\Omega_{aragonite}$ saturated conditions, as predicted for the end of the century in most seawater surface areas.

*Acknowledgements.* Thanks are due to D. Allemand for the loan of the gas blender and divider. S. M. and J.-P. G. were supported by the CarboOcean IP of the European Commission (grant 511176-2). S. Reynaud, C. Richard, and F. Houlbrèque helped during the experiment. Thanks to J. Hall-Spencer, and M. Hoogenboom for a critical revision, A. Foggo for statistical assistance. We acknowledge O. Passafiume, L. Mousseau, S. Comeau, the *Service d'Observation Rade de Villefranche,* SO-Rade, of the *Observatoire Océanologique* and the *Service d'Observation en Milieu Littoral*, SOMLIT/CNRS-INSU, for their kind permission to use in situ data.





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**Table 1.** Mean values of parameters of the carbonate chemistry for each treatment of $pCO_2$ (400 and 700 ppm) and temperature treatment ($T$, $T$+3°C) and season. Data are mean ±S. D. (in bracket), $n$=35–55. Salinity ranged 37.9 to 38.3. Temperature ($T$) were 22°C (summer), 17.7°C (autumn and spring), and 13.3°C (winter). Total alkalinity in the Bay of Villefranche measured from January 2007 to December 2008 was 2545.3±15.3 mmol kg$^{-1}$ at the surface and 2546.5±9.3 mmol kg$^{-1}$ at 50 m depth (J.-P. Gattuso, unpubl. data).

| | Temperature (°C) | Total alkalinity (mmol kg$^{-1}$) | $pCO_2$ (ppm) | $pH_T$ (total scale) | $CO_2$ (mmol kg$^{-1}$) | $HCO_3^-$ (mmol kg$^{-1}$) | $CO_3^{2-}$ (mmol kg$^{-1}$) | DIC (mmol kg$^{-1}$) | $\Omega_{aragonite}$ |
|---|---|---|---|---|---|---|---|---|---|
| *400 T* | | | | | | | | | |
| Summer | 22 (0.5) | 2538 (11) | 423 (20) | 8.06 (0.02) | 12.83 (0.64) | 1941 (20) | 247 (8) | 2201 (12) | 3.77 (0.13) |
| Autumn | 18 (1) | 2526 (15) | 387 (20) | 8.09 (0.02) | 13.00 (0.90) | 1959 (31) | 233 (13) | 2205 (19) | 3.53 (0.20) |
| Winter | 13 (8) | 2540 (7) | 386 (22) | 8.10 (0.02) | 14.85 (0.81) | 2043 (19) | 203 (8) | 2262 (12) | 3.04 (0.12) |
| Spring | 16 (1) | 2486 (15) | 381 (17) | 8.09 (0.02) | 13.43 (0.86) | 1948 (25) | 221 (11) | 2182 (15) | 3.33 (0.17) |
| *400 T+3* | | | | | | | | | |
| Summer | 24 (1) | 2541 (11) | 475 (27) | 8.01 (0.02) | 13.40 (0.81) | 2203 (15) | 249 (10) | 2203 (14) | 3.86 (0.15) |
| Autumn | 21 (1) | 2533 (17) | 424 (28) | 8.06 (0.02) | 13.10 (1.09) | 2202 (22) | 242 (15) | 2203 (22) | 3.69 (0.24) |
| Winter | 16 (10) | 2540 (7) | 425 (32) | 8.06 (0.03) | 14.94 (1.07) | 2053 (16) | 211 (10) | 2254 (16) | 3.17 (0.15) |
| Spring | 19 (1) | 2487 (19) | 407 (19) | 8.07 (0.03) | 13.01 (0.65) | 2169 (14) | 231 (10) | 2181 (15) | 3.52 (0.16) |
| *700 T* | | | | | | | | | |
| Summer | 22 (0.5) | 2543 (7) | 713 (31) | 7.87 (0.02) | 21.64 (0.95) | 2317 (9) | 175 (6) | 2317 (9) | 2.68 (0.09) |
| Autumn | 18 (1) | 2521 (14) | 695 (39) | 7.88 (0.02) | 23.31 (1.55) | 2322 (15) | 156 (9) | 2322 (15) | 2.35 (0.15) |
| Winter | 13 (6) | 2538 (4) | 709 (41) | 7.87 (0.02) | 27.27 (1.55) | 2378 (10) | 131 (6) | 2378 (10) | 1.96 (0.09) |
| Spring | 16 (1) | 2486 (18) | 693 (37) | 7.87 (0.02) | 24.03 (2.05) | 2300 (25) | 146 (11) | 2300 (18) | 2.21 (0.17) |
| *700 T+3* | | | | | | | | | |
| Summer | 24 (1) | 2546 (9) | 779 (40) | 7.84 (0.02) | 22.00 (1.12) | 2114 (18) | 180 (7) | 2315 (11) | 2.78 (0.12) |
| Autumn | 21 (1) | 2530 (11) | 733 (41) | 7.86 (0.02) | 22.65 (1.70) | 2127 (27) | 167 (11) | 2317 (17) | 2.55 (0.18) |
| Winter | 16 (5) | 2545 (8) | 763 (38) | 7.85 (0.02) | 26.78 (1.27) | 2208 (12) | 139 (5) | 2374 (8) | 2.10 (0.08) |
| Spring | 19 (1) | 2491 (17) | 733 (42) | 7.85 (0.02) | 23.43 (3.94) | 2126 (49) | 150 (21) | 2301 (32) | 2.29 (0.33) |



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**Table 2.** Summary of two-way ANOVAs followed by Tukey honest significant difference post-hoc tests testing the effect of short-term incubation at two levels of $pCO_2$ (400 and 700 ppm), and 2 temperature treatments ($T$, $T$+3°C) on *Cladocora caespitosa* main physiological parameters during summer and winter. Bold face numbers indicates $P<0.05$.

| | | Summer | | | | Winter | | |
|---|---|---|---|---|---|---|---|---|
| Source of variation | df | MS | F-ratio | P-value | df | MS | F-ratio | P-value | Post-hoc |
| Net photosynthesis | | | | | | | | | |
| $pCO_2$ | 1 | 572.05 | 6.64 | 0.02 | 1 | 3.95 | 0.13 | 0.72 | |
| Temperature (T) | 1 | 51.82 | 0.60 | 0.45 | 1 | 422.13 | 14.48 | **0.001** | 13<16 |
| $pCO_2 \times T$ | 1 | 76.14 | 0.88 | 0.36 | 1 | 524.51 | 17.97 | <**0.000** | |
| Error | 20 | 86.13 | | | 28 | 29.18 | | | |
| Gross photosynthesis | | | | | | | | | |
| $pCO_2$ | 1 | 5.62 | 0.06 | 0.81 | 1 | 247.15 | 0.51 | 0.49 | |
| Temperature | 1 | 98.71 | 1.03 | 0.32 | 1 | 5301.4 | 10.93 | **0.003** | 13<16 |
| $pCO_2 \times T$ | 1 | 46.28 | 0.48 | 0.49 | 1 | 86.90 | 0.18 | 0.67 | |
| Error | 20 | 96.13 | | | 28 | 485.11 | | | |
| Respiration | | | | | | | | | |
| $pCO_2$ | 1 | 135.38 | 1.74 | 0.20 | 1 | 57.08 | 0.81 | 0.38 | |
| Temperature | 1 | 313.67 | 4.02 | 0.06 | 1 | 1180.9 | 16.70 | <**0.000** | 13<16 |
| $pCO_2 \times T$ | 1 | 4.17 | 0.05 | 0.82 | 1 | 110.65 | 1.56 | 0.22 | |
| Error | 20 | 77.93 | | | 28 | 70.75 | | | |
| Zooxanthellae $cm^{-2}$ | | | | | | | | | |
| $pCO_2$ | 1 | 6.72 | 0.54 | 0.47 | 1 | 4.43 | 7.68 | **0.010** | 400<700 |
| Temperature | 1 | 1.50 | 0.12 | 0.73 | 1 | 7.64 | 13.24 | **0.001** | 13>16 |
| $pCO_2 \times T$ | 1 | 3.21 | 0.26 | 0.61 | 1 | 4.46 | 0.77 | 0.39 | |
| Error | 20 | 1.23 | | | 28 | 5.77 | | | |
| $Chl_a+c_2$ $cm^{-2}$ | | | | | | | | | |
| $pCO_2$ | 1 | 15.10 | 0.51 | 0.48 | 1 | 321.7 | 16.66 | <**0.000** | 400<700 |
| Temperature | 1 | 85.12 | 2.86 | 0.11 | 1 | 19.02 | 0.98 | 0.33 | |
| $pCO_2 \times T$ | 1 | 55.62 | 1.87 | 0.19 | 1 | 4.27 | 0.22 | 0.64 | |
| Error | 20 | 29.75 | | | 28 | 19.31 | | | |
| Protein $cm^{-2}$ | | | | | | | | | |
| $pCO_2$ | 1 | 0.22 | 0.92 | 0.35 | 1 | 0.025 | 0.85 | 0.37 | |
| Temperature | 1 | 0.10 | 0.42 | 0.53 | 1 | 0.001 | 0.03 | 0.85 | |
| $pCO_2 \times T$ | 1 | 0.40 | 0.16 | 0.69 | 1 | 0.037 | 1.24 | 0.28 | |
| Error | 16 | 0.24 | | | 16 | 00.30 | | | |
| Light calcification | | | | | | | | | |
| $pCO_2$ | 1 | 2147.0 | 0.91 | 0.35 | 1 | 1.41 | 0.00 | 0.95 | |
| Temperature | 1 | 956.9 | 0.41 | 0.53 | 1 | 4172.3 | 8.98 | **0.009** | 13<16 |
| $pCO_2 \times T$ | 1 | 127.5 | 0.05 | 0.82 | 1 | 290.8 | 0.63 | 0.44 | |
| Error | 16 | 2345.2 | | | 16 | 464.36 | | | |
| Dark calcification | | | | | | | | | |
| $pCO_2$ | 1 | 1203.1 | 0.66 | 0.44 | 1 | 21.03 | 0.13 | 0.72 | |
| Temperature | 1 | 1654.3 | 0.91 | 0.37 | 1 | 325.7 | 2.04 | 0.17 | |
| $pCO_2 \times T$ | 1 | 881.6 | 0.49 | 0.50 | 1 | 2.24 | 0.01 | 0.91 | |
| Error | 8 | 1810.3 | | | 16 | 159.6 | | | |

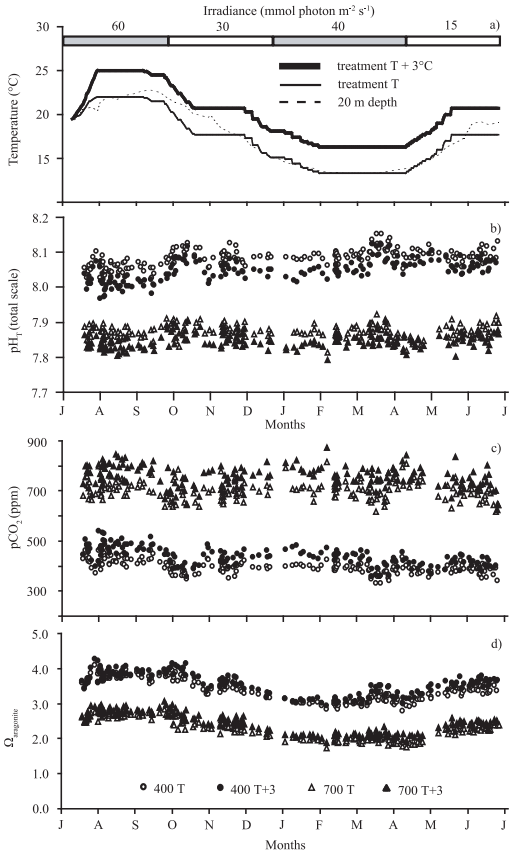


**Fig. 1.** **(a)** seasonal evolution of temperature and irradiance in the Bay of Villefranche (mean between 1995 and 2006 at 20 m depth; data from SO-Rade, *Service d'Observation Rade de Villefranche* of the *Observatoire Océanologique* and SOMLIT, *Service d'Observation en Milieu Littoral*, CNRS, INSU) and in the experimental aquaria; **(b)** $pH_T$, **(c)** $pCO_2$, and **(d)** $CaCO_3$ saturation state ($\Omega_{aragonite}$) of seawater during the one-year experiment for each treatment (400 *T*, 400 *T* +3, and 700, 700 *T* +3; $n$=191 to 194).



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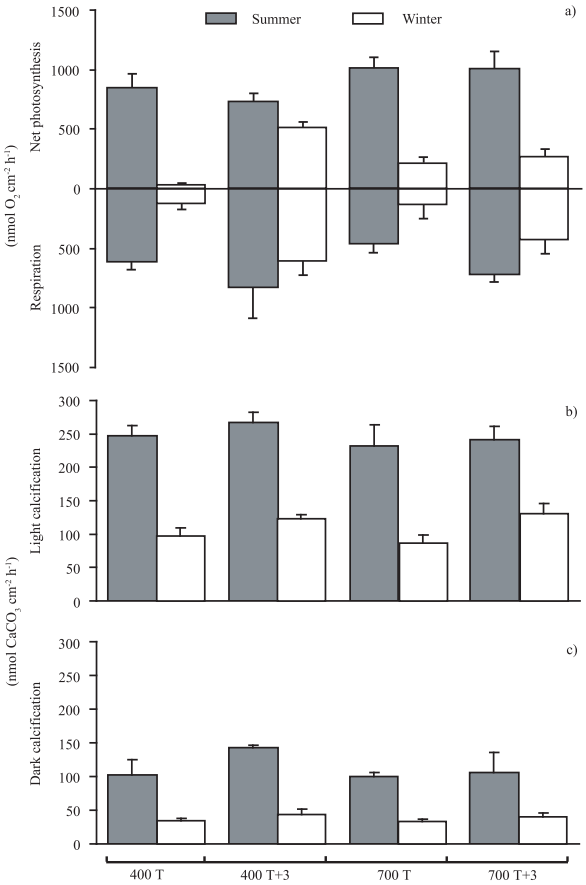


**Fig. 2.** Net photosynthesis and respiration **(a)**, and calcification rates measured in the dark and at the culture irradiance level **(b)** during the mid-term experiments (summer and winter) on *Cladocora caespitosa* exposed to the combined effect of normal (400 ppm) and elevated (700 ppm) $pCO_2$, and normal ($T$) and elevated ($T$ +3°C) temperatures. Data are mean ±s.e.m. (see Table 2 for degrees of freedom).



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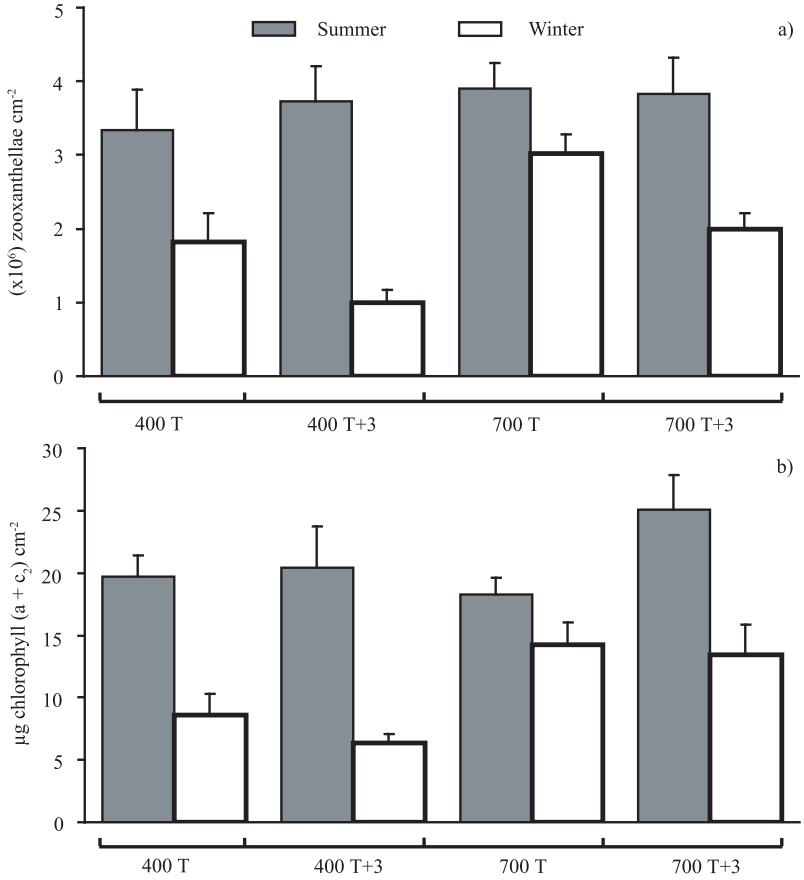


**Fig. 3.** Zooxanthellae density **(a)** and chlorophyll content **(b)** measured after one-month acclimation during the mid-term experiments (summer and winter) on *Cladocora caespitosa* exposed to the combined effect of normal (400 ppm) and elevated (700 ppm) $pCO_2$, and normal ($T$) and elevated ($T$ +3°C) temperatures. Data are mean ±s.e.m. (see Table 2 for degrees of freedom).



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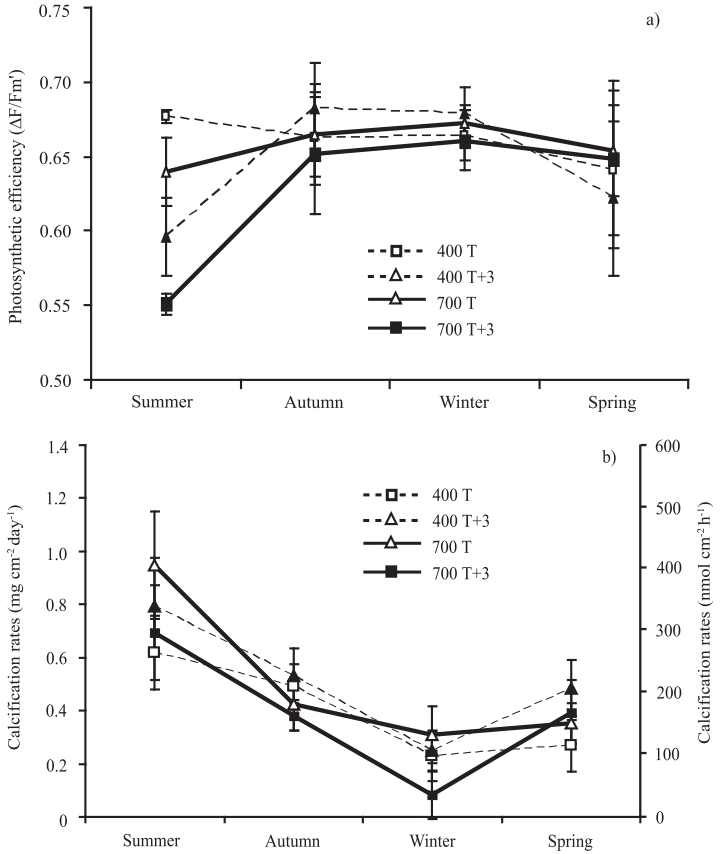


**Fig. 4.** Effective photosynthetic efficiency **(a)** and seasonal calcification rates **(b)** measured during the long-term experiment (one year) on *Cladocora caespitosa* exposed to the combined effect of normal (400 ppm) and elevated (700 ppm) $pCO_2$, normal ($T$) and elevated ($T$ +3°C) temperatures. Data are mean ±s.e.m. (see Table 2 for degrees of freedom).



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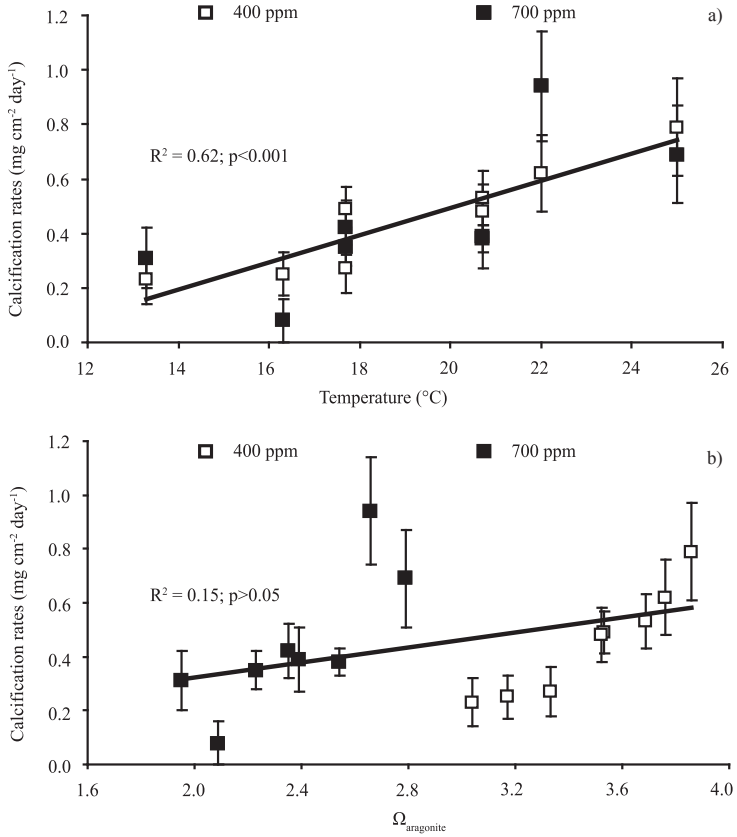


**Fig. 5.** Relationships between the mean calcification rates of *Cladocora caespitosa* measured during the long-term experiment and: (*a*) temperature regimes (range 13.3-25°C); (*b*)$\Omega_{aragonite}$. The coefficient of the regression is reported as $R^2$. Data are mean ±s.e.m. ($n$=7).



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