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Response of the temperate coral *Cladocora caespitosa* to mid- and long-term exposure to pCO_2 and temperature levels projected for the year 2100 AD

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Abstract. Atmospheric CO_2 partial pressure (pCO_2) is expected to increase to 700 µatm 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 (Ω) of the seawater. Elevated pCO_2 was shown to drastically decrease calcification rates in tropical zooxanthellate corals. Here we show, using the Mediterranean zooxanthellate coral *Cladocora caespitosa*, that an increase in pCO_2, in the range predicted for 2100, does not reduce its calcification rate. Therefore, the conventional belief that calcification rates will be affected by ocean acidification may not be widespread in temperate corals. Seasonal change in temperature is the predominant factor controlling photosynthesis, respiration, calcification and symbiont density. An increase in pCO_2, alone or in combination with elevated temperature, had no significant effect on photosynthesis, photosynthetic efficiency and calcification. The lack of sensitivity *C. caespitosa* to elevated pCO_2 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 projected for the end of the century.

1 Introduction

Atmospheric CO_2 partial pressure (pCO_2) has increased by 32% between 1880 and 2005 (280 vs. 379 µatm) and a further doubling is expected by the end of this century (IPCC, 2007). Anthropogenic CO_2 emitted to the atmosphere is absorbed by the oceans leading to decreases in pH, CO_3^{2-} concentration [CO_3^{2-}] and the related $CaCO_3$ saturation state (Ω) of seawater. A number of studies have shown that coral calcification rate 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 (e.g. Hoegh-Guldberg et al., 2007). 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) found that, in contrast to *Acropora intermedia*, *Porites lobata* exhibited a lower net productivity under intermediate CO_2 concentrations (700 µatm), 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 the one expected in 2100.

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Rising pCO_2 is also causing an increase in global sea temperature with an expected additional average 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 acclimation to the new conditions. In addition, the interactive effects of elevated pCO_2 and temperature have 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 synergistic or antagonistic responses of corals to elevated pCO_2 and temperature, and to provide a more accurate prediction of their future status. 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 µatm and 700 µatm) on calcification and photosynthesis of the Mediterranean zooxanthellate coral *Cladocora caespitosa.* Colonies were subject 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 seasonal changes 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), the experiments were carried out on colonies collected at 20 to 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 carefully cleaned from epiphytes, associated fauna and sediment. They were attached to PVC plates 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 additional food was supplied.

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‘ effects, we carefully cleaned each aquarium every week and each header tank every three weeks to prevent the growth of epiphytes and fouling communities or the accumulation of detritus. This maintenance and the high seawater renewal (see below) ensured that similar conditions prevailed in each aquarium, except for the carbonate chemistry. Irradiance, temperature, salinity (range: 37.9 to 38.3, $n = 194$ for each tank), and seawater carbonate chemistry were monitored in each aquarium every second day on average (see below).

Two aquaria were kept at a pCO_2 close to ambient values (ca. 400 µatm) while the remaining two were maintained at elevated pCO_2 (ca. 700 µatm). Within each pCO_2 treatment, one aquarium was maintained at normal temperature (T, i.e. the in situ temperature that the corals experience at 25 m depth) while the temperature was increased by 3 °C in the other one (T+3 °C). Aquaria were labeled: 400 T or 400 T+3 °C for 400 µatm pCO_2 with normal (T) or elevated temperature (T+3 °C) and 700 T or 700 T+3 °C for 700 µatm pCO_2 and also normal or elevated temperature. Three experiments were carried out concomitantly: 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 sets of corals collected in July 2006 and February 2007, respectively. With the exception of pCO_2, which was held constant, temperature, irradiance as well as the photoperiod was changed according to their seasonal 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 (Fig. 1a). Since in situ irradiance data were not available for the Bay of Villefranche during the experiment, we chose four seasonal values that were measured for this species living at similar depths (Peirano et al., 1998, 2007), 15 µmol photons m^{-2} s^{-1} in winter, 30 and 40 µmol photons m^{-2} s^{-1} in autumn and spring, respectively and 60 µmol photons m^{-2} s^{-1} in summer (Fig. 1a). These experimental irradiances were seasonally adjusted using neutral density filters and checked using a LI-COR spherical underwater quantum sensor (LI-193SA). The aquaria were setup in a thermostated room and the temperature control was performed at ±0.1 °C using 150 W heaters connected to electronic temperature controllers (Corema). 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. The photoperiod was changed according to seasons (from 9:15 in winter to 15:9 in summer; light:dark, *h*).

2.3 Control of pCO_2 and carbonate chemistry calculations

The partial pressure of CO_2 was adjusted and controlled to the desired level by bubbling CO_2-free air and CO_2-enriched air in two header tanks (110 l), for either the normal and high pCO_2 treatment. Seawater was pumped from a 10 m depth in the Bay of Villefranche into the two header tanks. Each tank was connected to two 26 l aquaria and supplied seawater at a rate of ca. 13 l h^{-1}. Elevated pCO_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 (90 ml) for total alkalinity (TA), were filtered 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 until analysis. TA was determined on 20 ml sub-samples using a custom-made titration system comprising of a 20 ml open titration cell thermostated 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 accuracy of the pH-meter and the titrator were 0.003 pH units and ±3 µmol kg^{-1} (see: Martin and Gattuso, 2009 for more details). The mean seasonal parameters of the carbonate system are given in Table 1.

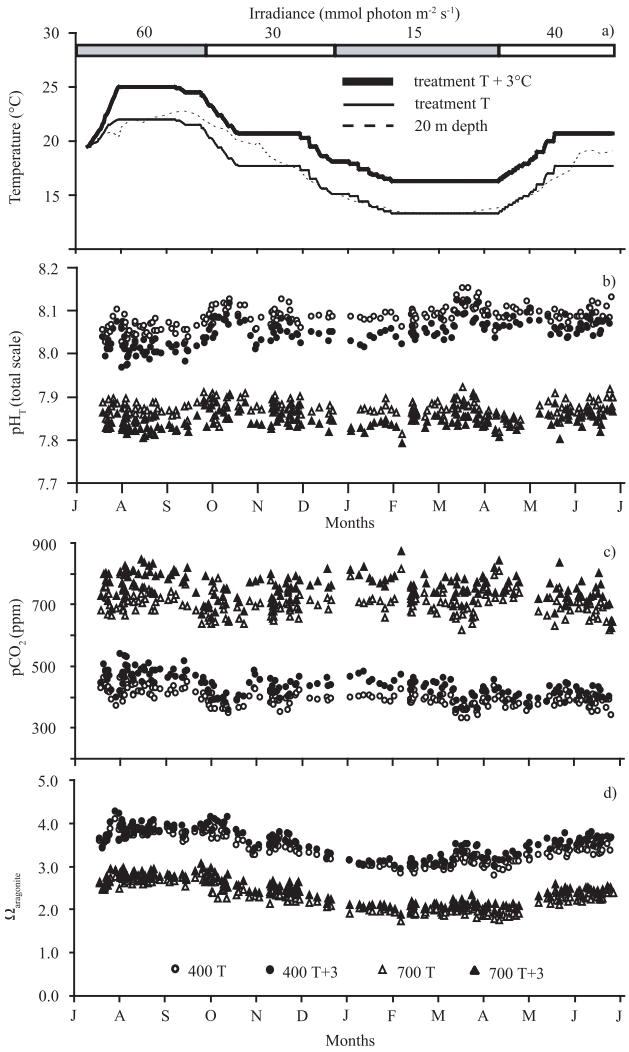

Fig. 1. **(a)** seasonal variation of temperature and irradiance in the Bay of Villefranche (mean between 1995 and 2006 at 20 m depth; data from SO-Rade, *Service d'Observation Rade de Villefranche* of the *Observatoire Océanologique* and SOMLIT, *Service d'Observation en Milieu Littoral*, CNRS, INSU) 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).

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 integrated 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. It is important to note that both methods do not discriminate 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 at 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 end of the incubation, filtered onto 0.45 µm membranes (Whatman, GF/C), preserved with mercuric chloride, 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 (Smith, 1978).

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. The fragments were carefully cleaned of 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, for measurements 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 seawater. The O_2 concentration at saturation was calculated according to the experimental temperature, salinity values at the ambient barometric pressure (http://www.unisense.com/Default.aspx?ID=117). The 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 measurement of 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 blank metabolism. Gross photosynthesis (P_g) at the culture irradiance was calculated by summing the rates of R (absolute value) and P_n corrected from the blank activities and assuming that R is identical in the light and dark. Data were normalized by the surface area of polyps and expressed in nmol O_2 cm^{-2} h^{-1}. The summer and winter values were compared between treatments. 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 ($\Delta F/F'_m$) was measured at the end of each season using an underwater Pulse Amplitude Modulated fluorometer (Diving-PAM, Walz). The 8 mm fibre 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 28 fragments (7 for each treatment), used during the long-term calcification measurements, to a 0.8 s period of saturating light (ca. 8000 µmol photons m^{-2} s^{-1}).

2.7 Zooxanthellae, chlorophyll and protein content

Twenty polyps used for the determination of P and R in summer (6 polyps for each treatment) and 32 others in winter (8 polyps for each treatment) were processed to measure their zooxanthellae and chl content. Tissues were separated from the skeleton using an air-pick and homogenised using a hand-held tissue grinder 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 Ulthermöhl sedimentation chambers of known volume, using an inverted 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. The details of both methods can be found in Rodolfo-Metalpa et al. (2006).

The protein content was 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). Tissues were extracted 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

The data were tested for homogeneity of variances using Cochran's test and were log-transformed if necessary; plots

Table 1. Mean values of parameters of the carbonate chemistry for each treatment of pCO_2 (400 and 700 µatm) and temperature treatment (T, $T+3\,°C$) and season. Data are mean ± s.e.m. (in bracket), n=9–12 for TA and n=35–55 for the other measurements. Salinity ranged from 37.9 to 38.3. Total alkalinity in the Bay of Villefranche measured from January 2007 to December 2008 was 2545±15 µmol kg^{-1} at the surface and 2546±10 µmol kg^{-1} at 50 m depth (J.-P. Gattuso, unpubl. data).

400 T	Temperature (°C)	Total alkalinity (µmol kg^{-1})	pCO_2 (µatm)	pH_T (total scale)	CO_2 (µmol kg^{-1})	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	DIC (µmol kg^{-1})	$\Omega_{aragonite}$
Summer	21.7 (0.1)	2538 (4)	423 (3)	8.06 (0.002)	12.83 (0.1)	1941 (3)	247 (1)	2201 (2)	3.77 (0.02)
Autumn	18.0 (1.2)	2526 (5)	387 (3)	8.09 (0.003)	13.00 (0.1)	1959 (5)	233 (2)	2205 (3)	3.53 (0.03)
Winter	13.4 (0.1)	2540 (2)	386 (3)	8.10 (0.003)	14.85 (0.1)	2043 (3)	203 (1)	2262 (2)	3.04 (0.02)
Spring	17.2 (0.2)	2486 (4)	381 (3)	8.09 (0.003)	13.43 (0.1)	1948 (4)	221 (1)	2182 (2)	3.33 (0.02)
400 $T+3$									
Summer	24.5 (0.1)	2541 (3)	475 (4)	8.01 (0.003)	13.40 (0.1)	2203 (3)	249 (1)	2203 (2)	3.86 (0.02)
Autumn	21.0 (0.2)	2533 (6)	424 (4)	8.06 (0.003)	13.10 (0.2)	2202 (5)	242 (2)	2203 (3)	3.69 (0.04)
Winter	16.4 (0.1)	2540 (2)	425 (4)	8.06 (0.004)	14.94 (0.1)	2053 (3)	211 (1)	2254 (2)	3.17 (0.02)
Spring	20.1 (0.2)	2487 (4)	407 (4)	8.07 (0.003)	13.01 (0.1)	2169 (3)	231 (1)	2181 (2)	3.52 (0.02)
700 T									
Summer	21.7 (0.1)	2543 (2)	713 (4)	7.87 (0.002)	21.64 (0.1)	2317 (2)	175 (1)	2317 (1)	2.68 (0.01)
Autumn	18.0 (0.2)	2521 (4)	695 (6)	7.88 (0.003)	23.31 (0.2)	2322 (3)	156 (1)	2322 (2)	2.35 (0.02)
Winter	13.4 (0.1)	2538 (1)	709 (5)	7.87 (0.003)	27.27 (0.2)	2378 (2)	131 (1)	2378 (1)	1.96 (0.01)
Spring	17.2 (0.2)	2486 (4)	693 (5)	7.87 (0.003)	24.03 (0.3)	2300 (4)	146 (2)	2300 (2)	2.21 (0.02)
700 $T+3$									
Summer	24.5 (0.2)	2546 (3)	779 (6)	7.84 (0.003)	22.00 (0.2)	2114 (2)	180 (1)	2315 (2)	2.78 (0.02)
Autumn	21.0 (0.2)	2530 (3)	733 (6)	7.86 (0.003)	22.65 (0.3)	2127 (4)	167 (2)	2317 (3)	2.55 (0.03)
Winter	16.4 (0.1)	2545 (2)	763 (5)	7.85 (0.003)	26.78 (0.2)	2208 (2)	139 (1)	2374 (1)	2.10 (0.01)
Spring	20.1 (0.2)	2491 (4)	733 (6)	7.85 (0.003)	23.43 (0.5)	2126 (6)	150 (3)	2301 (4)	2.29 (0.02)

of residuals were also used to ascertain that the ANOVA requirements were met. The results of the mid-term experiments (summer and winter) were analyzed separately by two-way ANOVAs with pCO_2 (400 and 700 µatm) and temperature treatment (T and T+3 °C) as fixed factors. When significant differences were found, 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 µatm) 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 the 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 the calcification data deviated from assumptions of sphericity ($W_5 = 0.434$, $P = 0.03$); the degrees of freedom were, thus, adjusted 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 designed accordingly. Subsequent to a lack of significant interactions between the within-subjects 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 linear 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 ($P < 0.05$) changes in P_n and R in winter (from 13 to 16 °C), resulting in a 72% increase in P_g at elevated temperatures (Table 2; 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). The rates of calcification in the dark and light (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), both 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 significantly affected by elevated pCO_2, neither in summer nor 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 significantly increased the zooxanthellae density and chl content in winter (Table 1; Fig. 3b), without any interacting effect with temperature.

3.2 Effect of a long-term exposure

Elevated pCO_2, alone or in combination with elevated temperature, did not significantly affect calcification and $\Delta F/F'_m$ of zooxanthellae over one year (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 aggregated 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; $R^2 = 0.15$, $F_{(1,14)} = 2.59$, $P = 0.13$).

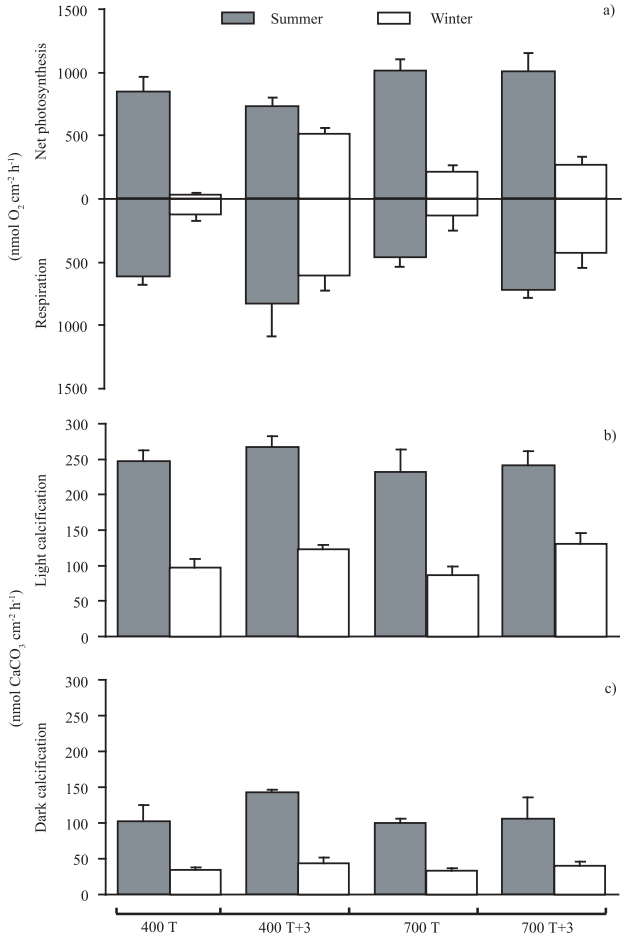

Fig. 2. Net photosynthesis and respiration **(a)**, and calcification rates measured in the dark and at the culture irradiance level **(b)** during the mid-term experiments (summer and winter) on *Cladocora caespitosa* exposed to the combined effect of normal (400 µatm) and elevated (700 µatm) pCO_2,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

4 Discussion

While temperature is a critical environmental parameter controlling the physiology and calcification of *C. caespitosa*, an increase in CO_2 partial pressure, within the values expected by the end of 2100, did not significantly affect either their photosynthetic performance or the calcification rates.

Although treatments in this experiment were not replicated, it is very unlikely that the observed lack of 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 °C, respectively); their values changed concomitantly during the experiment in each

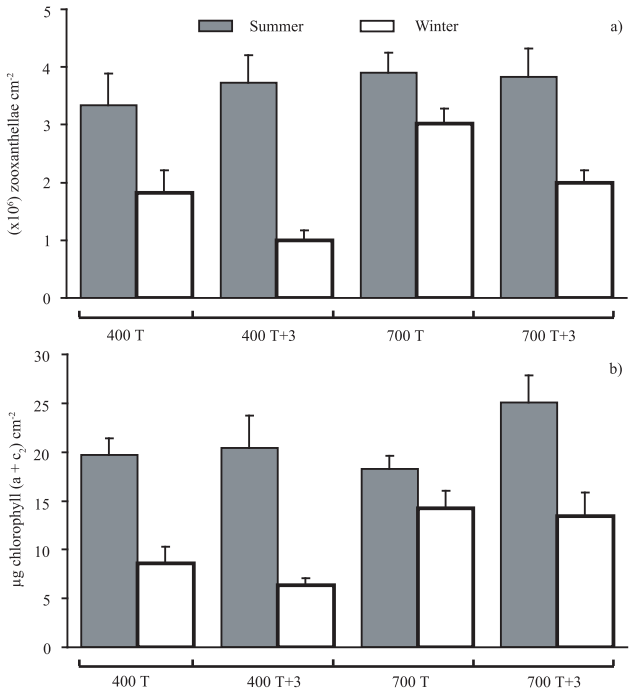

Fig. 3. Zooxanthellae density **(a)** and chlorophyll content **(b)** measured after one-month acclimation during the mid-term experiments (summer and winter) on *Cladocora caespitosa* exposed to the combined effect of normal (400 µatm) and elevated (700 µatm) pCO_2, and normal (T) and elevated (T+3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

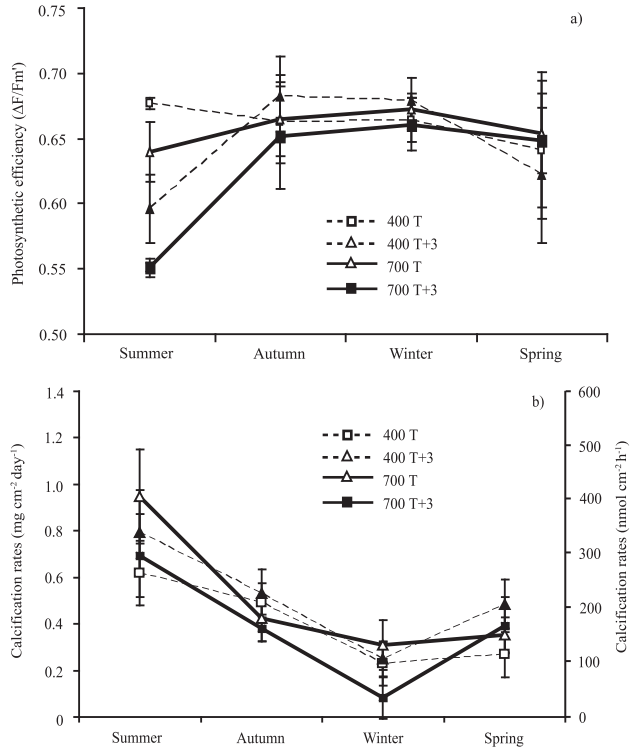

Fig. 4. Effective photosynthetic efficiency **(a)** and seasonal calcification rates **(b)** measured during the long-term experiment (one year) on *Cladocora caespitosa* exposed to the combined effect of normal (400 µatm) and elevated (700 µatm) pCO_2, normal (T) and elevated (T+3 °C) temperatures.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

aquarium according to measurements made in situ (Fig. 1a). Its effect on calcification and photosynthetic performances of *C. caespitosa*, is consistent with previous findings obtained in aquaria as well as in situ (Rodolfo-Metalpa et al., 2008b, c), thus, supporting the validity of the experimental approach, and suggesting the lack of a "tank" effect during the experiment. Only pCO_2 (ca. 400 and 700 µatm and the related parameters of the carbonate chemistry), temperature (T and T+3 °C), and season changed according to the treatment. The changes in pCO_2, pH_T and $\Omega_{aragonite}$ during the experiment are shown 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, $\Omega_{aragonite}$, carbonate and bicarbonate concentrations differed between treatments (400 µatm vs. 700 µatm) but not within treatments T (400 and 700 µatm) vs. T+3 °C (400 and 700 µatm) (Table 1; Fig. 1b, c, d). This demonstrates that aquarium cleaning, and the high seawater renewal rate used, successfully prevented any "tank" effect. We are, therefore, confident that the corals responses were only caused by the treatment (pCO_2 and seawater carbonate chemistry, temperature treatment, and season).

The present 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 than in summer. Such reduction of photosynthetic activity could explain the difference in calcification rates between the two seasons (Fig. 2b, c). Low metabolism is a typical feature of temperate benthic species in winter when both irradiance and temperature are at their lowest levels. Previous studies have demonstrated that *C. caespitosa* relies greatly on the heterotrophic nutrition, when available, to increase the zooxanthellae density and chl content, thereby increasing photosynthesis (Rodolfo-Metalpa et al., 2008a, b). In contrast, in the present study, because corals were not artificially fed, zooxanthellae density and chl concentrations remained low in winter. A 3 °C increase in temperature during this winter period stimulated the coral metabolism, enhancing both photosynthesis and respiration. This stimulation was higher in summer and caused a significant 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;

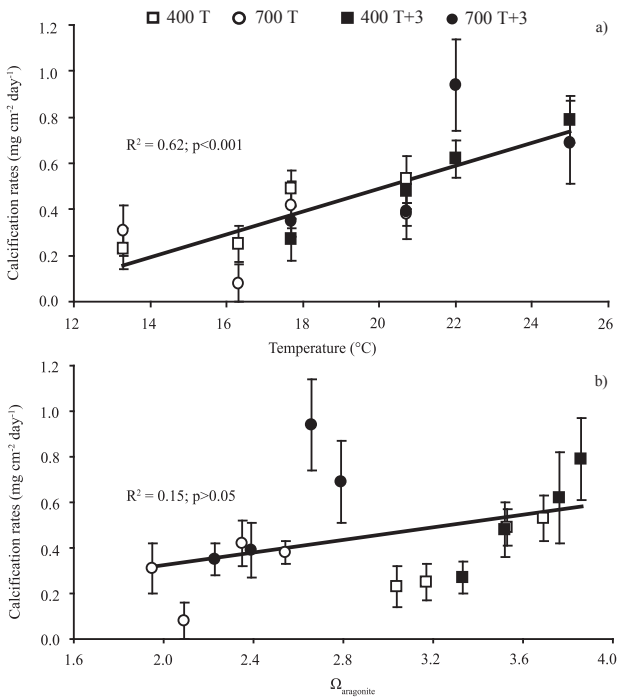

Fig. 5. Relationships between the mean calcification rates of *Cladocora caespitosa* measured during the long-term experiment and: **(a)** temperature regimes (range 13.3–25 °C); **(b)** $\Omega_{\text{aragonite}}$. Open sqare 400$T$; closed square 400$T$+3; open circle 700$T$; closed circle 700$T$+3. The coefficient of the regression is reported as R^2. Data are mean±s.e.m. ($n = 7$).

Howe and Marshall, 2002). At 22 °C, a 3 °C increase in temperature did not affect 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). The highest temperature slightly increased, albeit not significantly, the respiration rate (Fig. 2a). These results suggest that its metabolism was at its upper temperature limit.

A very sensitive marker of thermal stress in symbiotic corals is the decrease in photosynthetic efficiency ($\Delta F/F'_m$) after exposure 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 could have triggered a cascading effect leading to the death of the corals (Rodolfo-Metalpa et al., 2006, 2008c). Coral mortality has become increasingly frequent in the first 10–20 m because colonies are exposed to prolonged periods of high summer 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 (Rodolfo-Metalpa et al., 2006, 2008c) to major response and mortality, potentially confounding any evaluation on the effect of elevated pCO_2.

In order to compare the two methods used to measure the calcification rates, data from the alkalinity anomaly technique were expressed per day. The rate of calcification was 0.15 and 0.45 mg $CaCO_3$ cm^{-2} day^{-1} in winter and summer, respectively. However, the rates measured with the buoyant weight technique were 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 the best of our knowledge, only Steller et al. (2007) compared the two methods in the coralline alga *Lithophyllum margaritae* and found a rate of calcification twice as high using the buoyant weight technique. A possible reason for lower calcification rates obtained using the alkalinity anomaly technique could be due to the fact that it assumes a constant rate of calcification in the light and dark throughout the season considered. However, the rates can change over time due to different food supply, use of lipid reserves, or acclimation. Also, in the present 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 seems better suited to investigate the response of corals to environmental conditions over extended periods of time.

The response of *C. caespitosa* to elevated pCO_2 partly contrasts with previous studies on tropical zooxanthellate corals as it does not exhibit large changes in physiological parameters such as photosynthesis, photosynthetic efficiency and calcification. Photosynthesis does not seem to be greatly affected by ocean acidification in symbiotic corals (e.g. Langdon et al., 2003; Reynaud et al., 2003; Schneider and Erez, 2006), probably because corals do not rely on dissolved CO_2 for photosynthesis (Gattuso et al., 1999). However, laboratory and mesocosm experiments have shown a common trend of decreased calcification rates with increased pCO_2 in reef-building corals (e.g. Gattuso et al., 1999; Langdon et al., 2005; Kleypas et al., 2006). It has been suggested that below a value of $\Omega_{\text{aragonite}} = 3.3$, corresponding to a pCO_2 level of around 480 µatm, 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 $\Omega_{\text{aragonite}}$ ranging from 1.95 to 3.86 (pCO_2 values of 709–475 µatm), 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. (2009) reported that the calcification rate of the temperate coral *Oculina arbuscula* is unaffected by an increase of pCO_2 up to 840 µatm ($\Omega_{\text{aragonite}} = 1.8$). A drastic decrease in calcification was only found at a pCO_2 of 2240 µatm, corresponding to an $\Omega_{\text{aragonite}}$ 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 to 30% decrease by 2100; Gattuso et al., 1999; Kleypas et al., 1999).

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 µatm), 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			

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, mostly by bubbling with CO_2-enriched air and the addition of acid. These two approaches result in different outcomes (Gattuso and Lavigne, 2009). The former approach increases the concentration of dissolved inorganic carbon (DIC) at constant total alkalinity and, therefore, mimics well the changes generated by ocean acidification. The latter approach decreases total alkalinity at constant DIC, which does not mimic future changes well in the carbonate chemistry. Methodological differences were suggested in explaining the various responses to ocean acidification reported in coccolithophores (Iglesias-Rodriguez et al., 2008) but this suggestion was subsequently discounted (Shi et al., 2009). It is also unlikely that a methodological difference exists between previous studies on isolated

corals and coral communities because almost all experiments conducted so far have reported a decreased in calcification, whether the carbonate chemistry was manipulated by adding acid (e.g. Marubini and Thake, 1999; Jokiel et al., 2008), bubbling with CO_2-enriched air (e.g. Reynaud et al., 2003) or by manipulating the calcium concentration (e.g. Gattuso et al., 1998). Second, except for Ries et al. (2009) and the present study, all experiments carried out on corals used tropical, fast-growing species which grow up to 5 times faster than their tropical counterparts (Rodolfo-Metalpa et al., 2006). It is likely that fast-growing corals need higher $\Omega_{aragonite}$ than the slow-growing *C. caespitosa* or other temperate or cold species. It is conceivable that the requirement for carbonate ions of slow growing corals is low and that the concentration of carbonate ions does not become limiting, even under high pCO_2 concentrations (ca. 700 µatm). This could partly explain why Reynaud et al. (2003) did not find a significant decrease in the rate of calcification of colonies of *Stylophora pistillata* in response to elevated pCO_2 concentrations at 25.1 °C, but did find a decrease at 28.3 °C. The calcification rate was stimulated by the higher temperature but, in turn, it was affected by the decrease in saturation state. Similarly, preliminary observations on the reef-building coral *Montipora capitata* showed that the impact of acidification on the growth rate was more pronounced at summer temperatures when the colonies grow faster (P. L. Jokiel, personlal communication, RRM 2008). Anthony et al. (2008) found differences in calcification rates (% weight increase per month) on the fast-growing coral *A. intermedia* exposed to high pCO_2 (700 µatm) but not on the massive coral *P. lobata*. It is, therefore, possible that the effect of low $\Omega_{aragonite}$ on calcification rates is more pronounced in fast-growing zooxanthellate corals.

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 reported on the ophiourid brittlestar *Amphiura filiformis* (Wood et al., 2008), the seastar *Pisaster ochraceus* (Gooding et al., 2009) exposed to lowered pH_{NBS} (7.8–7.3), the Caribbean coral *Madracis mirabilis* at pH_T 7.6 (Jury et al., 2010), and shown for coralline red algae, calcareous green algae, temperate urchins, limpets, crabs, lobsters and shrimp (Ries et al., 2009). 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 the two methods used in this study measure net calcification and cannot discriminate between 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 aragonite (Fig. 1d).

Many studies have shown a negative relationship between $[CO_3^{2-}]$ in the external seawater and calcification rates (e.g. Langdon and Atkinson, 2005), making the carbonate ion the principal driver of coral calcification. Although ocean acidification also increases the concentration of bicarbonate, its role on calcification has generally not been considered. Jury et al. (2010) recently tested the separate effect of various parameters of the carbonate on the calcification of the Caribbean coral *Madracis mirabilis*. They suggest that the concentration of bicarbonate explains the changes better in the calcification rate than the concentration of carbonate.

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 whole organism responses (e.g. photosynthesis, 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 (chlorophyll fluorescence yield, zooxanthellae density and chl content) and whole organism levels (protein content and respiration rates, oxygen metabolism and calcification). For instance, as shown here and in several other marine organisms (Reynaud et al., 2003; Pörtner, 2009), seawater temperature can greatly affect the physiological performance, particularly in marine invertebrates and may reduce their fitness in the face of ocean acidification. Therefore, future research on ocean acidification should take into consideration the whole organism response, over its entire temperature range, including an increase in temperature due to global warming (Pörtner and Knust, 2007).

Although the negative relationship between calcification and pCO_2 is widely accepted for tropical corals and other calcifying organisms, temperate species may be less sensitive to an increase in seawater pCO_2. For the Mediterranean zooxanthellate coral *C. caespitosa*, temperature is by far the main factor controlling the rate of calcification, even at the lower $\Omega_{aragonite}$ values predicted for the end of the century for surface seawater in most regions.

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