

Annual response of two Mediterranean azooxanthellate temperate corals to low-pH and high-temperature conditions

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Abstract Ocean acidification (OA) and warming related to the anthropogenic increase in atmospheric CO₂ have been shown to have detrimental effects on several marine organisms, especially those with calcium carbonate structures such as corals. In this study, we evaluate the response of two Mediterranean shallow-water azooxanthellate corals to the projected pH and seawater temperature (ST) scenarios for the end of this century. The colonial coral *Astroides calycularis* and the solitary *Leptopsammia pruvoti* were grown in aquaria over a year under two fixed pH conditions, control (8.05 pH_T units) and low (7.72 pH_T units), and simulating two annual ST cycles, natural and high (+3 °C). The organic matter (OM), lipid and protein content of the tissue and the skeletal microdensity of *A. calycularis* were not affected by the stress conditions (low pH, high ST), but the species exhibited a mean 25 % decrease in calcification rate at high-ST conditions at the end of the warm period and a mean 10 % increase in skeletal porosity under the acidified treatment after a full year cycle. Conversely, an absence of effects on calcification and skeletal microdensity of *L. pruvoti* exposed to low-pH and high-ST treatments contrasted with a significant decrease in the OM, lipid and

protein content of the tissue at high-ST conditions and a 13 % mean increase in the skeletal porosity under low-pH conditions following a full year of exposure. This species-specific response suggests that different internal self-regulation strategies for energy reallocation may allow certain shallow-water azooxanthellate corals to cope more successfully than others with global environmental changes.

Introduction

The response of marine ecosystems to global environmental changes is one of the major challenges facing modern society. It is well known that increases in atmospheric CO₂ concentration are leading to changes in the carbonate chemistry and temperature of the ocean (Gattuso and Hansson 2011; Khatiwala et al. 2013). The impact of these processes on marine ecosystems will depend on the ability of organisms to cope with such changes (Ries et al. 2009; Byrne 2011; Wicks and Roberts 2012; Kroeker et al. 2013; Parker et al. 2013). Hence, numerous experiments have investigated the potential effects of ocean acidification (OA) and warming on different marine species (e.g., Hoegh-Guldberg et al. 2007; Guinotte and Fabry 2008; Doney et al. 2009, 2012; Ries et al. 2009; Wicks and Roberts 2012). Among them, coral reef communities have attracted special attention because their calcifying organisms may be severely affected by these two global stressors in the near future, particularly under the more pessimistic range of scenarios projected by the Intergovernmental Panel on Climate Change (IPCC 2013). Skeletal records from the Great Barrier Reef show, for instance, that coral calcification has dropped over the last few decades (Cooper et al. 2008; De'ath et al. 2009) and, although the causes of this decline remain unknown, increasing seawater temperature

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(ST) and declining pH are among the most likely drivers (e.g., Brown 1997; Hoegh-Guldberg 1999; Anthony et al. 2007; Fabricius et al. 2011). Since both stressors act simultaneously, a growing number of studies have evaluated the combined effect of increased ST and reduced pH on reef zooxanthellate corals. However, outcomes have revealed diverse and often contrasting responses as a result of the interaction between ST and pH, with warming counteracting the adverse effects of acidification in some instances (e.g., Muehllehner and Edmunds 2008; McCulloch et al. 2012) or leading to a synergy of the two stressors in others (e.g., Reynaud et al. 2003; Anthony et al. 2008).

In contrast to the large number of studies including shallow-water tropical species, only a handful of experiments have assessed the OA response of corals inhabiting temperate regions (e.g., Ries et al. 2010; Holcomb et al. 2010, 2012) and, among them, only five have been conducted with Mediterranean species so far. These five Mediterranean studies have also revealed high variability between and within species in their response to a range of acidified conditions. While some of this variability appears to be due to different methodological approaches, reported results vary from a complete dissolution of the skeleton in *Oculina patagonica* reared in aquaria at pH 7.4 (Fine and Tchernov 2007), to a decrease in calcification rate up to ~35 % in *Cladocora caespitosa* maintained in aquaria at pH 7.8 (Movilla et al. 2012) and ~18 % in *Balanophyllia europaea* transplanted along a natural CO₂ gradient at in situ pH 7.7 (Fantazzini et al. 2015). When the response to low pH was further assessed in combination with warming, no detectable effects on the calcification of *C. caespitosa* (Rodolfo-Metalpa et al. 2010) or *B. europaea* (Rodolfo-Metalpa et al. 2011) were found at in situ pH levels similar to those expected by year 2100 (7.8 pH units). All these previous studies with temperate species have targeted zooxanthellate specimens (i.e., most tropical species), in which the complexity of the combined effects of pH and ST on coral calcification can result, in part, from the tight relationship between photosynthesis and calcification (Gattuso et al. 1999). In this context, an advantage of working on asymbiotic corals, as we do in the present study, is that the direct impacts of OA and ST on the calcification process should be better discerned, with no confounding effects of photosynthesis and symbiotic processes. Furthermore, it is important to point out that all these previous studies assessed the responses of the organisms in terms of calcification rate, photosynthesis and respiration, yet did not assess how the two stressors may affect the biochemical composition of corals. It is still uncertain what the effects may be of the increase in energy consumption apparently required by these organisms to calcify under adverse conditions (Cohen and Holcomb 2009; Allemand et al. 2011) and whether ST and pH stress may affect other physiological

and biological processes (Hoegh-Guldberg et al. 2007; Brewer and Peltzer 2009; Pelejero et al. 2010). Therefore, studies assessing the effect of decreasing pH and increasing ST in both calcification and metabolic balance are critical to fully understand the potential response of temperate corals in a changing environment.

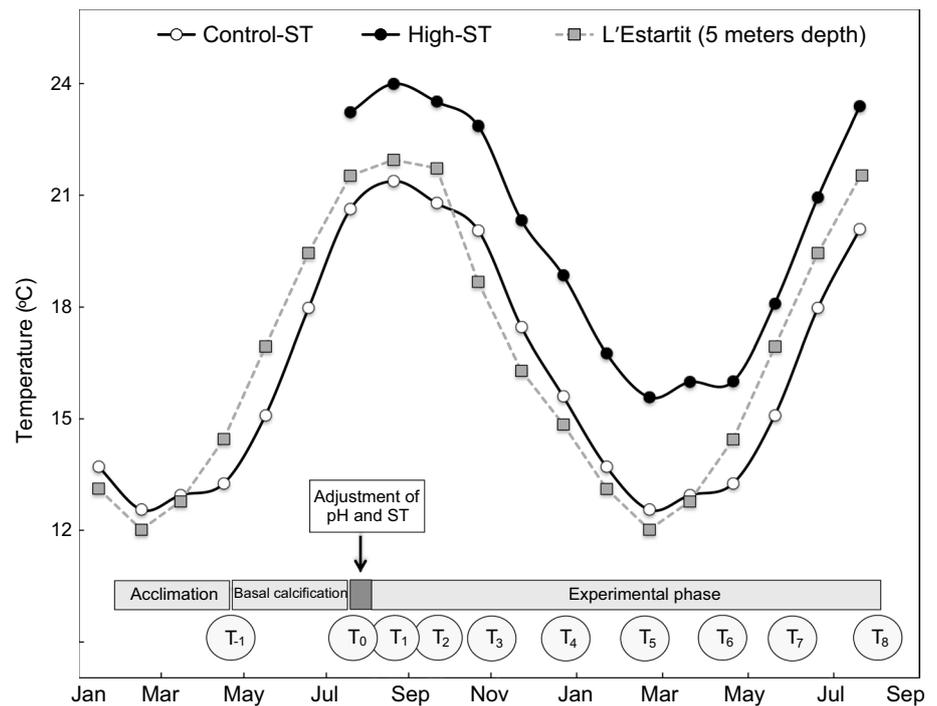
The present study investigates the cumulative effects of acidification and warming on the calcification rate, skeleton properties (microdensity and porosity) and biochemical composition of the tissue [organic matter (OM), lipid and protein content] in two temperate azooxanthellate corals from the Mediterranean, *Astroides calycularis* and *Leptopsammia pruvoti*, by simulating an annual temperature cycle in an aquarium experiment. The bright orange scleractinian *A. calycularis* is a colonial coral with a limited geographic distribution, being present along the Atlantic coasts of Morocco and Spain, in the Mediterranean Iberian Peninsula from the Strait of Gibraltar to Cape Palos, in the south Alboran Sea to the Algeria and Tunisian waters and in the south-central part of the Western Mediterranean Sea (Zibrowius 1980, 1995; Bianchi 2007), with some recent recordings in northern areas of the Tyrrhenian and Adriatic Seas (Bianchi and Morri 1994; Kružić et al. 2002; Grubelić et al. 2004; Casellato et al. 2007). The sunset cup coral *L. pruvoti* is a solitary species distributed in the Mediterranean basin and along the eastern Atlantic coast from Portugal to southern England (Zibrowius 1980). These two non-symbiotic scleractinian corals typically inhabit low irradiance habitats (i.e., vertical walls, overhangs and caves) at depths from 0 to 50 m (Rossi 1971; Zibrowius 1980; Cebrian and Ballesteros 2004; Goffredo et al. 2006). To our knowledge, this is the first multi-seasonal assessment using these two coral species, where the potential response to future acidification and to the regional variation in ST across the Western Mediterranean Sea is investigated.

Materials and methods

Specimen collection

In January 2011, 15 colonies of *A. calycularis* and 60 individuals of *L. pruvoti* were collected by scuba diving between 6 and 12 m depth in Cartagena (SE Spain, 37°33'N, 0°58'W) and in L'Estartit (NE Spain, 42°03'N, 3°13'E), respectively. Coral specimens were immediately transported to the Experimental Aquarium Zone at the Institute of Marine Sciences (ICM-CSIC) in Barcelona and placed in a 225-L tank with 50 µm filtered running natural seawater. Shortly after arrival, four fragments (~3 cm in diameter) harvested from each of the 15 collected colonies of *A. calycularis* and the 60 individuals of *L. pruvoti* were carefully cleaned of epiphytes and sediment, and glued

Fig. 1 Mean monthly seawater temperature (ST) variation over the study period (January 2011–August 2012) in the experimental control-ST and high-ST conditions (empty and solid circles, respectively). Gray squares indicate the mean monthly ST logged in situ at 5 m depth in the reference collection site (L'Estartit). The different buoyant weight sampling times (T_{-1} to T_8) and the corresponding experimental phases (acclimation, basal calcification and exposure to pH and ST treatments) are indicated



onto labeled methacrylate plates with an inert mastic compound. The specimens of *L. pruvoti* were randomly distributed in 12 experimental aquaria (five specimens per aquarium), whereas specimens of *A. calycularis* were distributed in these aquaria such that there was at least one representative from each colony in each treatment. Seawater renewal rate in the 11 L experimental aquaria was 20 times per day. Light intensity was adjusted to $\sim 9 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (measured with a Li-cor Li-1935B; Lincoln, NE; USA) in a 12:12 light:dark cycle, thus simulating the low irradiance habitats commonly inhabited by these azooxanthellate coral species (Weinberg 1979). A mixed diet including freeze-dried *Tetraselmis* sp. and *Mysidacea* (Ocean Nutrition™) and fresh *Artemia salina* nauplii was supplied three times per week.

Experimental setup and treatment conditions

In situ ST was recorded hourly using an Onset HOBO Pendant Temperature Data Logger (UA-002–64, Onset Computer Corporation) placed at the two collection locations (Cartagena and L'Estartit) at 5 m depth over a 2-year period (July 2010 to June 2012). For each location, mean monthly STs were determined on the basis of averaged daily values. The ST regime at L'Estartit was selected as a reference for setting the experimental ST conditions.

An acclimation phase simulating mean winter-ST conditions at the reference location ($13.0 \pm 0.5 \text{ }^\circ\text{C}$) was conducted in the aquaria from January to March 2011, thereby ensuring a proper healing of the tissue following the

transfer and mounting operations. Over the next 3 months (April to June 2011), coral specimens were exposed to the natural springtime ST rise from 13 to 20 °C, adjusted at $0.5 \text{ }^\circ\text{C week}^{-1}$ (Fig. 1). At this point (July 2011), pH and ST were gradually adjusted to the experimental conditions (at $0.03 \text{ pH units day}^{-1}$ and $0.3 \text{ }^\circ\text{C day}^{-1}$, respectively) and maintained over an annual cycle (July 2011 to August 2012). The four experimental treatments crossed two fixed pH levels (control pH and low pH) with two annual cycle STs (control ST and high ST), using triplicate aquaria in each treatment. The mean pH_T (total scale at the in situ ST) used in the control-pH conditions was 8.05 units, similar to the mean annual pH_T measured at the reference location of L'Estartit (unpublished data). The decreased pH_T used in the low-pH conditions was pH 7.72, following the 'business-as-usual' RCP8.5 (Representative Concentration Pathway, 8.5 W m^{-2}) scenario by the year 2100 (IPCC 2013). The annual cycle of ST used in the control-ST conditions was adjusted weekly according to the mean monthly ST measured in situ at the reference location (L'Estartit), whereas the ST in the high-ST conditions was adjusted $\sim 3 \text{ }^\circ\text{C}$ above the control ST, simulating a rise in ST as expected in the Mediterranean by the end of this century (Solomon et al. 2007; Sokolov et al. 2009).

ST was measured continuously in the two controlled-temperature baths in which the experimental aquaria were placed and temperature was controlled with two electronic Pt100 regulators (Delta Ohm HD9022) connected to a water cooler and 300 W heaters. Seawater pH was adjusted by bubbling CO_2 into the overhead feed tanks and the pH

levels were continuously monitored by glass electrodes (LL Ecotrode plus—Metrohm) connected to two pH controllers (Consort R362; Topac Inc., USA). For further details on the experimental setup, see Movilla et al. (2012) and Bramanti et al. (2013). Samples of seawater from each treatment were taken bimonthly for total alkalinity (TA) analyses by potentiometric titration (Perez and Fraga 1987; Perez et al. 2000) and pH by spectrophotometry (Clayton and Byrne 1993). At the same time, temperature and salinity of each treatment aquarium were also monitored using a YSI-30 M probe and used to calculate the rest of the parameters of the carbonate system in seawater using the CO2sys.xls software (version 2.1; Pelletier et al. 2007) with the carbonic acid dissociation constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987).

Calcification rates

Skeletal calcification rate of coral specimens was assessed by means of the buoyant weight (BW) technique (Jokiel et al. 1978, Davies 1989), using a 0.1 mg resolution balance (Mettler Toledo AB204 SFACT). An initial measurement of the BW was taken at the end of the acclimation phase (April 2011, T_{-1}), a second right before setting the experimental pH and ST conditions (July 2011, T_0), and subsequent weighing was conducted throughout the annual experiment (July 2011 to August 2012, at 20, 49, 91, 143, 200, 258, 307 and 369 days; T_1 to T_8 , respectively; Fig. 1). The BW of the corals was transformed to dry weight (DW) using the seawater density (δ_{SW}) and the specific value of the aragonite skeleton microdensity (δ_{ar}) determined for each species (see the next section), using the function:

$$DW = BW \left[1 - \left(\frac{\delta_{SW}}{\delta_{ar}} \right) \right]$$

Calcification rate was normalized to the initial skeletal DW of coral specimens and expressed as $\text{mg CaCO}_3 \text{ g}^{-1} \text{ d}^{-1}$ using the exponential growth function:

$$G = 1000 * \left[\left[\left(\frac{DW_n}{DW_0} \right)^{\left(\frac{1}{n} \right)} \right] - 1 \right]$$

where G is the net calcification rate, DW_0 is the initial dry weight, DW_n is the dry weight after n days, and n is the time interval (in days) since the beginning of the exposure (e.g., Reynaud et al. 2007; Maier et al. 2013). The basal coral calcification rate in the aquaria was calculated during the spring season (April–July 2011; T_{-1} to T_0) right before setting the experimental treatments. During the experimental stage, coral calcification rate was calculated both for the summer period (T_0 to T_3) and over the whole annual cycle (T_0 to T_8).

Skeletal microdensity and porosity

For each coral species, three specimens of each treatment (one specimen per individual replicate aquarium) were randomly selected at the end of the experiment to estimate skeleton microdensity and porosity following the Bucher et al. (1998) technique. Coral specimens were submerged in sodium hypochlorite for 48 h to remove the OM and washed with distilled water. BW and DW of each specimen were recorded before and after their coating in molten paraffin wax (105–110 °C) to form a watertight barrier. The BW was measured in distilled water at 20 °C with a specific gravity of $\sim 1.00 \text{ g cm}^{-3}$. Total enclosed volume, skeleton matrix volume and bulk density were also calculated for each specimen using the equations described in Bucher et al. (1998).

Biochemical composition of the tissue

For each coral species, six specimens of each treatment (two specimens per individual replicate aquarium) were randomly selected at the end of the experiment to estimate potential changes on the biochemical composition of the coral tissue (OM, protein and lipid content). Samples were frozen at -80 °C, subsequently freeze-dried, crushed with a mortar and pestle (including tissue and skeleton) and then stored frozen at -20 °C until biochemical analyses were conducted. Approximately 250 mg from each sample were heated at 80 °C for 48 h and weighed to obtain the DW, then combusted at 450 °C for 5 h and weighed again to obtain the ash weight (AW). The OM content was calculated as the difference between DW and AW and expressed as a percentage with respect to the DW (Slattery et al. 1995). For protein extraction, ~ 75 mg from each sample were homogenized in NaOH (1 N) and heated to 90 °C for 30 min. The protein content was quantified colorimetrically using the BCA Assay Kit (Interchim) with bovine serum albumin as standard and measured using a multimode microplate reader Tecan Infinite M200 spectrophotometer. For lipid extraction, ~ 75 mg from each sample were homogenized in 3 mL of chloroform–methanol (2:1). The lipid content was quantified colorimetrically according to the method of Barnes and Blackstock (1973), using cholesterol as standard, and measured with a Varian Cary 100 UV–Vis spectrophotometer. Protein and lipid contents were standardized by the OM content and expressed as $\mu\text{g protein or lipid (mg OM)}^{-1}$.

Statistical analyses

Data were tested for assumptions of normality and homoscedasticity using the Kolmogorov–Smirnov and Levene tests, respectively. A root square or arctan transformation

Table 1 Parameters of the seawater carbonate system in the aquaria for each treatment. All parameters are expressed as mean \pm SE; $N = 7$

Treatment		Measured parameters			Calculated parameters							
ST	pH	pH _T	TA	Sal	pCO ₂	χ CO ₂	DIC	[CO ₂] _{aq}	[HCO ₃ ⁻]	[CO ₃ ²⁻]	Ω_c	Ω_A
Control (12.4–22.5)	Control	8.045 \pm 0.004	2527 \pm 6	37.9 \pm 0.1	432 \pm 5	441 \pm 5	2249 \pm 14	14.7 \pm 0.6	2033 \pm 20	201 \pm 7	4.7 \pm 0.2	3.1 \pm 0.1
	Low	7.717 \pm 0.006	2532 \pm 6	37.9 \pm 0.2	1028 \pm 14	1049 \pm 14	2414 \pm 13	35.0 \pm 1.6	2273 \pm 16	106 \pm 5	2.5 \pm 0.1	1.6 \pm 0.1
High (15.2–24.9)	Control	8.017 \pm 0.004	2526 \pm 6	37.9 \pm 0.1	466 \pm 5	477 \pm 5	2240 \pm 14	14.6 \pm 0.6	2017 \pm 20	208 \pm 7	4.9 \pm 0.2	3.2 \pm 0.1
	Low	7.706 \pm 0.004	2531 \pm 6	37.8 \pm 0.1	1056 \pm 5	1081 \pm 5	2401 \pm 14	33.4 \pm 0.6	2255 \pm 20	113 \pm 7	2.6 \pm 0.2	1.7 \pm 0.1

ST Seawater temperature range ($^{\circ}$ C), pH_T pH in total scale, TA total alkalinity (μ mol/kg-SW), Sal salinity, pCO₂ partial pressure of CO₂ (μ atm), χ CO₂ mol fraction of CO₂ in dry air (ppm), DIC dissolved inorganic carbon (μ mol/kg-SW), [CO₂]_{aq} CO₂ concentration in seawater (μ mol/kg-SW), [HCO₃⁻] bicarbonate ion concentration (μ mol/kg-SW), [CO₃²⁻] carbonate ion concentration (μ mol/kg-SW), Ω_c saturation state of seawater with respect to calcite, Ω_A saturation state of seawater with respect to aragonite

was applied when normality was not fulfilled. For each coral species, the variation in calcification rate at two sampling points (summer period from T₀ to T₃, and annual cycle from T₀ to T₈), in skeletal properties (microdensity and porosity) and biochemical composition of the tissue (OM, protein and lipid contents) at the end of the experiment (T₈), was compared among treatments using two-way ANOVAs with pH and ST as fixed factors. All results are expressed as mean \pm standard error of the mean (SE). Statistical analyses were performed using the JMP 9.0.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Carbonate system and temperature of seawater in the aquaria

Our experimental setup allowed a precise regulation of the selected pH conditions, which were maintained at 8.045 \pm 0.004 and 7.717 \pm 0.006 pH_T units for control and low-pH conditions, respectively (Table 1). The mean calculated partial pressure of CO₂ (pCO₂) and Ω_A for the control-pH conditions were \sim 430 μ atm and 3.1, respectively. In the low-pH conditions, these values changed to \sim 1030 μ atm and 1.6, respectively. TA and salinity values were similar in all treatments and remained constant throughout the whole experiment, with average values of \sim 2530 μ mol kg⁻¹ seawater (SW) and 38, respectively. Average values of other calculated parameters of the carbonate system are summarized in Table 1.

Over the whole experiment (January 2011 to August 2012), the mean monthly ST in the control-ST conditions was similar to that logged at the reference location of L'Estartit over a 2-year period (July 2010 to June 2012; Fig. 1). Mean monthly ST in the control-ST conditions ranged from 12.4 $^{\circ}$ C in winter to 22.5 $^{\circ}$ C in summer, whereas the high-ST conditions were, on average,

2.9 \pm 0.3 $^{\circ}$ C above the control (ranging from 15.2 to 24.9 $^{\circ}$ C; Fig. 1). During the summer period (July–September 2011; T₀ to T₃), mean monthly ST was 21.2 \pm 0.1 and 23.7 \pm 0.1 $^{\circ}$ C for the control-ST and the high-ST conditions, respectively. Over the same period, mean monthly ST logged at the sampling location for *A. calycularis* (Cartagena) was evenly higher than ST in the high-ST experimental conditions, with a mean value during the summer period of 26.4 $^{\circ}$ C and reaching a peak of 27.1 $^{\circ}$ C.

Calcification rates

During the previous phase to the adjustment of the experimental conditions (T₋₁ to T₀), the two species showed similar mean basal calcification rates (0.75 \pm 0.04 and 0.88 \pm 0.08 mg CaCO₃ g⁻¹ day⁻¹ for *A. calycularis* and *L. pruvoti*, respectively), coinciding with the 13–20 $^{\circ}$ C natural ST rise associated with the spring season. During this preliminary phase (T₋₁ to T₀), no significant differences were detected between treatments in specimens of each species (Table 2). Over the experimental stage, the specimens from both species reared under control-pH and control-ST conditions displayed similar values of calcification rate throughout the summer period (T₀ to T₃; Fig. 2a). However, when the whole annual cycle (T₀ to T₈) was considered, the mean calcification rate in control-pH and control-ST conditions decreased by 36–42 % (0.47 \pm 0.06 and 0.52 \pm 0.10 mg CaCO₃ g⁻¹ day⁻¹ for *A. calycularis* and *L. pruvoti*, respectively; $N = 15$ for each; Fig. 2b). This indicates that about two-third of the annual skeletal growth occurred during the late spring–early fall period.

Regarding the response of *A. calycularis* under different experimental conditions, the effect of high ST caused a \sim 25 % significant reduction in its calcification rate when compared to that in control ST at the end of the summer period (T₀–T₃), while no effect was observed under low pH or the combination of low-pH and high-ST conditions (Table 2; Fig. 2a). Conversely, calcification rate of *L.*

Table 2 Summary of two-way ANOVAs assessing the effect of pH and temperature on the calcification rate of *Astroides calycularis* and *Leptopsammia pruvoti* previous to the beginning of the experiment (T_0), at the end of the summer season (T_3) and at the end of the whole annual cycle (T_8)

Source	ANOVA two-way			
	DF	SS	F value	p value
Calcification <i>A. calycularis</i> T_0				
Temperature	1	0.006	0.075	0.786
pH	1	0.2 E-4	0.3 E-3	0.987
Temp*pH	1	0.035	0.423	0.519
Residual	50	4.084		
Calcification <i>L. pruvoti</i> T_0				
Temperature	1	0.1 E-3	0.005	0.942
pH	1	0.2 E-3	0.009	0.925
Temp*pH	1	0.3 E-2	0.133	0.717
Residual	53	1.444		
Calcification <i>A. calycularis</i> T_3				
Temperature	1	0.238	4.448	0.040
pH	1	0.054	1.010	0.320
Temp*pH	1	0.049	0.918	0.343
Residual error	53	2.833		
Calcification <i>L. pruvoti</i> T_3				
Temperature	1	0.001	0.010	0.921
pH	1	0.437	3.188	0.080
Temp*pH	1	0.071	0.517	0.475
Residual	51	6.992		
Calcification <i>A. calycularis</i> T_8				
Temperature	1	0.031	1.089	0.302
pH	1	0.014	0.513	0.477
Temp*pH	1	0.003	0.101	0.752
Residual	50	1.399		
Calcification <i>L. pruvoti</i> T_8				
Temperature	1	0.037	0.741	0.394
pH	1	0.084	1.678	0.201
Temp*pH	1	0.007	0.137	0.713
Residual	50	2.495		

Boldface numbers indicate $p < 0.05$

pruvoti during the summer period did not differ between ST or pH conditions (Table 2; Fig. 2a). When the whole annual cycle was considered (T_0 – T_8), the negative effect of high-ST conditions on *A. calycularis* calcification rate observed during the summer period was attenuated (Fig. 2b). Thus, no significant effect of ST, pH or the combination of both factors was observed in *A. calycularis* or in *L. pruvoti* by integrating a whole annual growth cycle, (Table 2). None of the coral specimens of either species showed any signs of mortality (denuded skeleton) during the experiment and no tank effect was detected between triplicate aquaria of each treatment in any of the analyses.

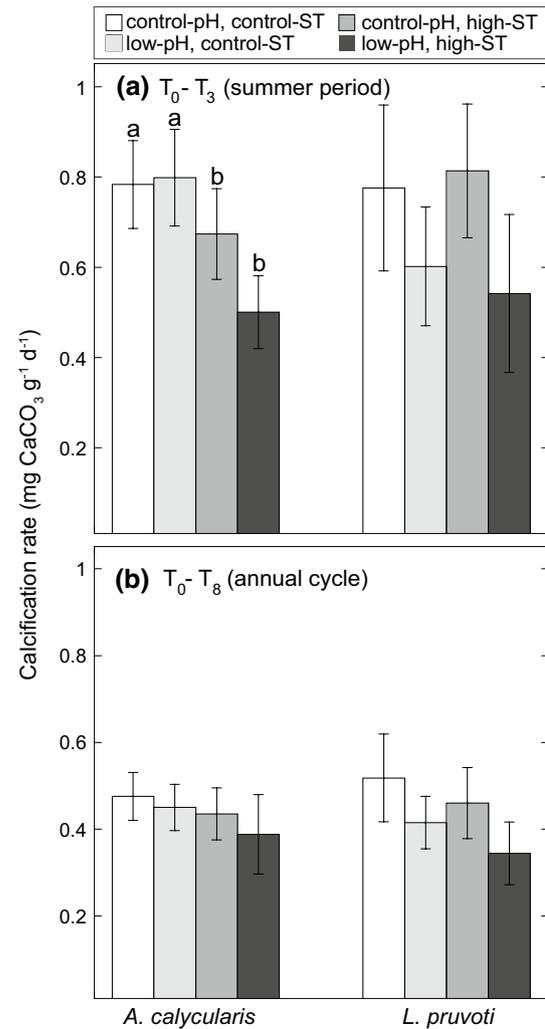


Fig. 2 Calcification rate of *Astroides calycularis* and *Leptopsammia pruvoti* under the four experimental pH and ST treatments **a** over the summer period (T_0 – T_3) and **b** over the whole annual cycle (T_0 to T_8). Different letters indicate significant differences between treatments ($p < 0.05$)

Skeletal microdensity and porosity

Skeletal microdensity was not significantly affected by low pH, high ST or the combination of both factors, for either *A. calycularis* or *L. pruvoti* at the end of the experiment (Table 3). The mean skeletal microdensity was $2.3 \pm 0.1 \text{ g cm}^{-3}$ for *A. calycularis* and $2.5 \pm 0.1 \text{ g cm}^{-3}$ for *L. pruvoti* ($N = 12$ for each species; Fig. 3a). For both tested species, skeletal porosity in control-pH and control-ST conditions was affected significantly by low pH, but not by high ST or the combination of the two factors at the end of the experiment (Table 3). Thus, the mean skeletal porosity of *A. calycularis* in low-pH conditions ($63 \pm 16 \%$; $N = 6$) was found to be 10 % higher when compared to that in control-pH conditions ($58 \pm 12 \%$, $N = 6$; Fig. 3b).

Table 3 Summary of two-way ANOVAs on microdensity, porosity, organic matter amount, protein and lipid content of *Astroides calycularis* and *Leptopsammia pruvoti* at the end of the experiment (T₈)

Source	ANOVA two-way			
	DF	SS	F value	p value
Microdensity <i>A. calycularis</i>				
Temperature	1	0.067	1.924	0.208
pH	1	0.086	2.457	0.161
Temp*pH	1	0.058	1.672	0.237
Residual	7	0.244		
Microdensity <i>L. pruvoti</i>				
Temperature	1	0.012	0.293	0.603
pH	1	0.016	0.394	0.548
Temp*pH	1	0.003	0.078	0.788
Residual	8	0.316		
Porosity <i>A. calycularis</i>				
Temperature	1	60.363	3.693	0.096
pH	1	102.622	6.278	0.041
Temp*pH	1	5.609	0.343	0.576
Residual	7	114.427		
Porosity <i>L. pruvoti</i>				
Temperature	1	37.163	2.148	0.181
pH	1	146.776	8.484	0.020
Temp*pH	1	26.360	1.524	0.252
Residual	8	138.405		
Organic matter <i>A. calycularis</i>				
Temperature	1	19.861	1.343	0.285
pH	1	15.979	1.081	0.333
Temp*pH	1	0.004	0.2 E-3	0.988
Residual	17	103.522		
Organic matter <i>L. pruvoti</i>				
Temperature	1	0.2 E-2	6.217	0.037
pH	1	0.2 E-3	0.848	0.384
Temp*pH	1	0.3 E-3	1.146	0.316
Residual	18	0.002		
Proteins <i>A. calycularis</i>				
Temperature	1	7.5 E+4	1.765	0.201
pH	1	2.6 E+4	0.609	0.445
Temp*pH	1	1.0 E+4	0.245	0.627
Residual	18	0.8 E+4		
Proteins <i>L. pruvoti</i>				
Temperature	1	1.1 E-6	8.114	0.010
pH	1	7.5 E-8	0.541	0.471
Temp*pH	1	2.2 E-8	0.163	0.691
Residual	19	2.6 E-6		
Lipids <i>A. calycularis</i>				
Temperature	1	0.1 E-4	3.589	0.075
pH	1	0.5 E-5	1.036	0.323
Temp*pH	1	0.7 E-6	0.143	0.710
Residual	17	0.8 E-4		
Lipids <i>L. pruvoti</i>				

Table 3 continued

Source	ANOVA two-way			
	DF	SS	F value	p value
Temperature	1	2.1 E+5	11.806	0.003
pH	1	56.790	0.003	0.955
Temp*pH	1	1.0 E+4	0.573	0.458
Residual	19	0.3 E+6		

Bold faces numbers indicate *p* values < 0.05

Likewise, a 13 % increase in the mean skeletal porosity of *L. pruvoti* was detected in the low-pH conditions with respect to control-pH conditions (56 ± 2 and 50 ± 1 %, $N = 6$, respectively; Fig. 3b).

Biochemical composition of the tissue

A different pattern in the biochemical composition of the tissue (OM, protein and lipid content) was observed in the two species investigated. In *A. calycularis*, biochemical results were not significantly affected by low pH, high ST or the combination of the two factors at the end of the experiment (Table 3). Thus, the mean proportion of OM of the tissue was 12.9 ± 1.1 %, the mean protein content was 503 ± 43 μg protein (mg OM)⁻¹ and the mean lipid content was 190 ± 17 μg lipid (mg OM)⁻¹ ($N = 24$ for each parameter; Fig. 4). Conversely, specimens of *L. pruvoti* reared under high-ST conditions displayed a significant reduction in all the biochemical components of the tissue at the end of the experiment (Table 3). The mean percentage of OM of *L. pruvoti* in high-ST conditions (9.9 ± 0.4 %; $N = 12$) was found to be 28 % lower when compared to that in control-ST conditions (13.7 ± 1.5 %; $N = 12$; Fig. 4a). Similarly, the mean protein and lipid content in high-ST conditions [580 ± 43 μg protein (mg OM)⁻¹ and 278 ± 28 μg protein (mg OM)⁻¹; $N = 12$ for each parameter] were reduced by 24 and 40 %, respectively, when compared to that in control-ST conditions [762 ± 54 μg protein (mg OM)⁻¹ and 467 ± 44 μg lipid (mg OM)⁻¹; $N = 12$ for each parameter; Fig. 4b, c].

Discussion

Effects of acidification and warming on calcification rates

Our results show that the calcification rate of the azooxanthellate coral species *Astroides calycularis* and *Leptopsammia pruvoti* is unaffected by the low-pH conditions expected by the year 2100. These results match the lack of response to acidified conditions observed in a previous

study on another temperate coral, *Cladocora caespitosa*, although the simulated pH was slightly higher in that study (7.9 pH units; Rodolfo-Metalpa et al. 2010). However, further work has shown that the response in terms of calcification of temperate corals reared under low-pH seawater is quite variable. For example, the zooxanthellate *C. caespitosa* and *Oculina patagonica* reared in aquaria at pH of 7.8 and 20 °C exhibited a ~35 and 32 % decrease in calcification rate, respectively (Movilla et al. 2012). Colonies of *C. caespitosa* transplanted for three months along a natural CO₂ gradient exhibited a ~80 % decrease in calcification rate at pH 7.8 and ~26 °C, and net calcification of this species became negative at pH 7.5 (Rodolfo-Metalpa et al. 2011). Along a similar pH gradient, *Balanophyllia europaea* was more resistant to low-pH conditions

than *C. caespitosa* exhibiting a slight decrease in calcification rate at pH 7.8–7.7 (Rodolfo-Metalpa et al. 2011; Fantazzini et al. 2015) and up to ~50 % decrease at pH 7.5

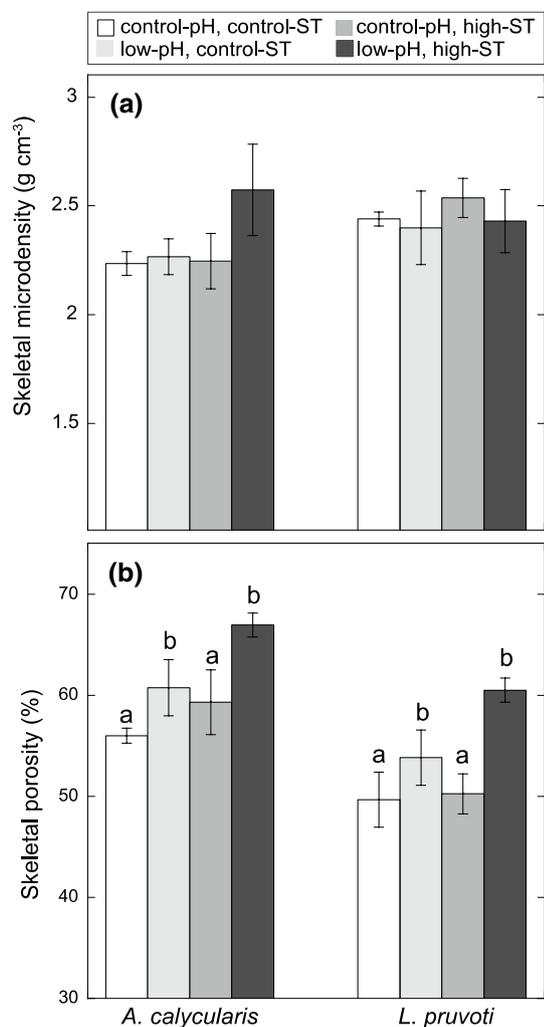


Fig. 3 Skeletal properties of *Astroides calycularis* and *Leptopsammia pruvoti* under the four experimental pH and ST treatments at the end of the annual cycle experiment. **a** Skeletal microdensity and **b** porosity. Different letters indicate significant differences between treatments ($p < 0.05$)

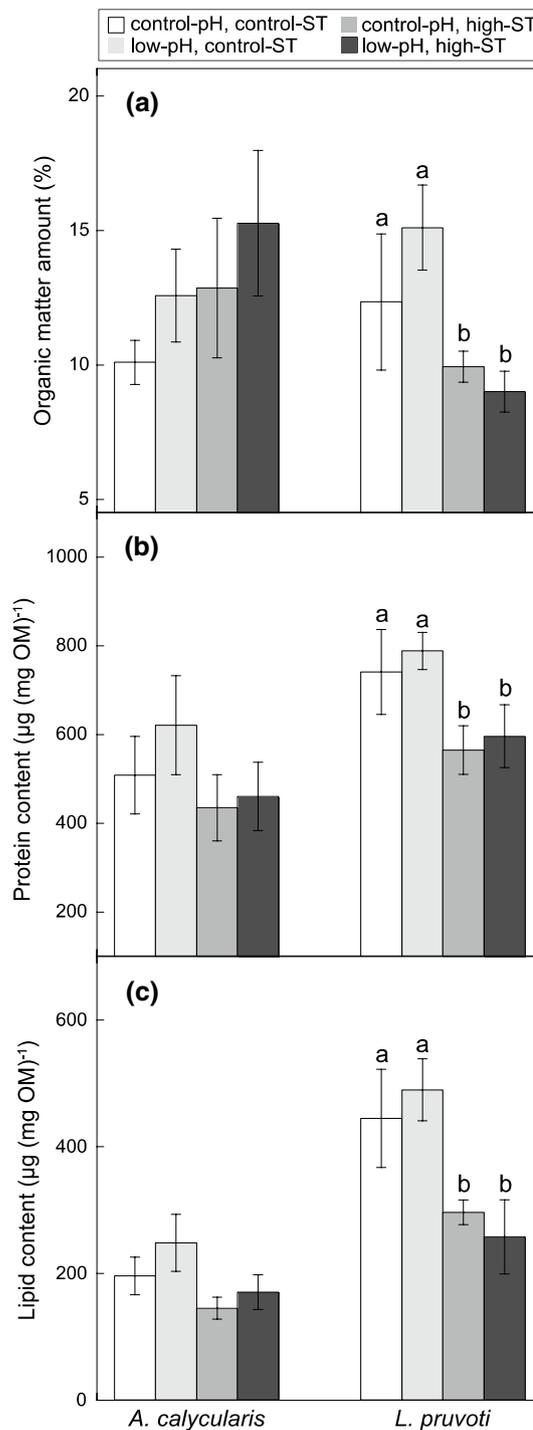


Fig. 4 Biochemical composition of the tissue of *Astroides calycularis* and *Leptopsammia pruvoti* under the four experimental pH and ST treatments at the end of the annual cycle experiment. **a** Organic matter, **b** protein and **c** lipid content. Different letters indicate significant differences between treatments ($p < 0.05$)

(Rodolfo-Metalpa et al. 2011). Considering that all these previous studies with Mediterranean corals have included only zooxanthellate temperate species, our results therefore suggest that calcification in azooxanthellate shallow-water corals, similar to the response observed in most cold-water corals (e.g., Movilla et al. 2014 and references therein), presents greater resistance to acidified conditions than symbiotic species.

On the other hand, the calcification rate of *L. pruvoti* was unaffected by high-ST conditions, which is in agreement with the high thermal tolerance previously described for this species (Goffredo et al. 2007; Caroselli et al. 2011, 2012). In contrast, the calcification rate of *A. calycularis* at high-ST experimental conditions during the summer period was 25 % lower than that observed at control-ST conditions. The growth of scleractinian corals in temperate seas has been described as temperature-dependent, being enhanced up to a sublethal ST threshold and declining thereafter (e.g., Rodolfo-Metalpa et al. 2008; Dimond and Carrington 2007). Previous studies with other Mediterranean coral species indicate that the exposure to positive ST anomalies may lead to physiological stresses such as decreased calcification (Rodolfo-Metalpa et al. 2006, 2008, 2014), increased respiration (Coma et al. 2002; Rodolfo-Metalpa et al. 2006), higher susceptibility to pathogens (Bally and Garrabou 2007), bleaching (Kushmaro et al. 1998) or tissue necrosis (Coma et al. 2006; Rodolfo-Metalpa et al. 2006). When these ST anomalies are maintained for an extended period, global warming has been linked to significant changes in marine coastal ecosystems, resulting in mass mortality outbreaks and changes in the geographic distribution of species (Garrabou et al. 2009; Coma et al. 2009; Coll et al. 2010; Doney et al. 2012; Trenberth 2012). In fact, a mass mortality event of *A. calycularis* has already been reported during summer 2009 at Ischia (Tyrrhenian Sea), when the shallow-water coral populations (up to 15 m) were affected by a thermal anomaly elevating surface ST to 28–29 °C (Gambi et al. 2010).

Interestingly, the high-ST conditions during the summer period in our experiment (23.7 °C) were still ~3 °C lower than those registered at the collection site of *A. calycularis* in Cartagena (26.4 °C). Thus, the negative effects of high-ST conditions reported in our study may be magnified for coral populations living in this area, located ~30 km southwestward from the northernmost limit of their distribution along the Spanish coast (Cape Palos, Balearic Sea, 37°38'N; Zibrowius 1995). The particular local conditions found in the eastern Iberian Peninsula, northward from Cape Palos and up to Ebro river delta (40°45'N), led to an unexpected increase in summer ST with increasing latitude (Marbà et al. 2015). We hypothesize that high ST during the summer period found north of Cape Palos may be constraining the upper limit of *A. calycularis* distribution in the

Balearic Sea. This species has recently expanded its distribution northward in both the Tyrrhenian and Adriatic Seas (Bianchi and Morri 1994; Kružić et al. 2002; Grubelić et al. 2004). Several studies based on *A. calycularis* fossil records and biogeography have hypothesized that the recent expansion of its previously limited distribution, confined to the southwestern Mediterranean Sea between the 14 and 15 °C isotherms for February, could be related to the increasing winter ST due to surface water warming (Zibrowius 1995; Bianchi 2007; Casado-Amezúa et al. 2012). An alternative hypothesis suggested by Bianchi and collaborators (2007) is that these recent coral expansions could be related to an unusual inversion of surface currents in the Ionian Sea. Additional field and mesocosm experiments are needed to gain insight into the ST mortality thresholds that may constrain the *A. calycularis* geographic distribution under the distinct ST regimes found in the western Mediterranean Sea.

When the whole annual cycle was considered, the calcification rates of both tested species were about half of that during the warm period and the effect of high ST observed during the fast growing period in *A. calycularis* was clearly attenuated (Fig. 2b). As discussed earlier, this is most probably related to the positive effect that ST has on growth within the optimum temperature thresholds, as observed in other Mediterranean temperate coral species (e.g., Rodolfo-Metalpa et al. 2008). Thus, detection of an observable effect of the examined environmental variables on coral calcification rate could be better assessed during their maximum growth period in summer. However, it should be taken into account that consideration of summer growth only could overestimate the predicted annual effects of the examined factors and would neglect the potential role that phenotypic plasticity may play in enhancing the resistance of the species to stressful conditions (Chevin and Lande 2010). This would be consistent with the interpretation that the response of corals to OA and global warming may not be as rapid and geographically consistent as expected (Pandolfi et al. 2011), highlighting the necessary but challenging task of long-term experiments to detect with more reliability the likely effects in the field.

Potential to compromise the carbonate skeletal framework

Skeletal porosity and microdensity are factors influencing the ability of corals to withstand breakage caused by natural or anthropogenic disturbance (Brown et al. 1985). However, studies evaluating the potential effect of OA and global warming on the coral structural framework are scarce in the literature. A study of different Mediterranean corals along a latitudinal temperature gradient documented that the skeletal porosity of the zooxanthellate *B. europaea*

correlated positively with ST increase (Caroselli et al. 2011). According to the authors, the attenuation of calcification due to the inhibition of the photosynthetic activity by high ST could have caused the observed increase in the porosity of this coral. The hypothesis was supported by the fact that this effect was not observed in the non-symbiotic species *L. pruvoti*, where, conversely, high ST correlated with an increase in the microdensity of the skeleton. In contrast, our results did not exhibit any significant difference in skeletal microdensity in *A. calycularis* or *L. pruvoti* reared under acidified and warmer conditions with respect to the control treatment (Fig. 3a). However, slight differences between the two studies (including ST regime, exposure time, natural vs. aquarium conditions and technique applied) may explain the divergent results for ST-dependent microdensity effects.

Our results show, however, that the low-pH treatment was conducive to an increase in porosity in the azooxanthellate *A. calycularis* and *L. pruvoti* with respect to control conditions (Fig. 3b). Similar results, which in our case contrast with the lack of OA effect in the calcification rate of both tested species at the end of our experiment, have been reported in recent publications. Increasing acidity along a natural pH gradient in a CO₂ vent was associated with an increasing skeletal porosity in the symbiotic coral *B. europaea* (Fantazzini et al. 2015). However, a significant reduction in the calcification rate was detected in this case, leading the authors to suggest that the response could be related to the need to maintain constant linear extension rates. It is therefore possible that the decrease in the skeletal porosity observed in *A. calycularis* and *L. pruvoti* under the low-pH treatment, along with the ability to keep constant their calcification rates, may lead to higher linear extension rates in these species under reduced pH. Unfortunately, we could not assess properly this biometric parameter in our studied species. According to Fantazzini et al. (2015), coral efforts to maintain extension rates could be related to a need to achieve a critical size at sexual maturity, at the expense of increasing their susceptibility to mechanical stress under acidified conditions. In the same way, the higher porosity values observed in the tropical coral *Stylophora pistillata* reared under acidified conditions (pH 7.4 and 7.2) were related to changes in its skeleton phenotype, such as the enlargement of the corallite calyx and thinning of septae and thecae (Tambutté et al. 2015). A similar phenotypic response was detected in the zooxanthellate Mediterranean coral *C. caespitosa* reared under more similar pH values than the present work (pH 7.8), although porosity of the skeleton was not measured in that study (Movilla et al. 2012). Therefore, the similar response of skeleton porosity between zooxanthellate and azooxanthellate corals suggest that factors other than the inhibition of symbionts by ST must be affecting the calcium carbonate structure of some

coral species. Further research on testing the fragility of the coral supporting framework to the long-term exposure to unfavorable conditions, such as those caused by the combination of an acidified and warmed environment, should promote better understanding of the calcification physiology in coral species.

Biochemical balance in the tissue and ecological implications

Elevated ST may stimulate enzyme activity and metabolic rate of organisms within their window of thermal tolerance (Ip et al. 1991), although it could also cause a rapid deterioration of cellular processes when certain ST threshold is exceeded (Pörtner and Farrell 2008; Kroeker et al. 2013). The observed lower values of OM, protein and lipid content of the tissue in *L. pruvoti* reared under high-ST conditions (Fig. 4) could be related to increased metabolic activity. However, this effect manifested as a species-specific response that was not observed in *A. calycularis*. In a previous work assessing two Mediterranean cold-water coral species (Movilla et al. 2014), and similar to our results with *A. calycularis*, no significant effect was detected in the OM amount or the lipid content in the tissue of *Dendrophyllia cornigera* and *Desmophyllum dianthus* after more than 300 days of exposure to a 7.8 pH treatment. Similarly, a previous study with the octocoral *Corallium rubrum* (Bramanti et al. 2013) reported no effect of similar pH levels on the lipid, protein or carbohydrate content of the tissue. However, a significant increase in the OM amount was observed in those specimens of *C. rubrum* reared under low-pH conditions, at the expense of a reduction in the calcification rate.

The calcification process is still poorly understood and predicting how corals will respond to an increase in CO₂ requires a better understanding of the mechanisms involved (Cohen and Holcomb 2009; Cohen et al. 2009; Allemand et al. 2011; Venn et al. 2011; Mass et al. 2012; Holcomb et al. 2014). It has been proposed recently that the physiological mechanisms that drive calcification itself could be crucial in how some coral species are able to resist increasing acidification (Ries et al. 2010; Tambutté et al. 2011; McCulloch et al. 2012). Venn et al. (2013) proposed two models to explain different responses to acidification in reef corals: (1) the energy pool for maintaining internal pH is constant and, therefore, a reduction in the environmental pH would result in a significant reduction in the calcification rate; and (2) the energy input is compensated as the external pH is reduced to maintain constant calcification rate, to the detriment of other vital activities such as locomotion, reproduction, tissue regeneration or to counteract other environmental stresses (Hoegh-Guldberg et al. 2007; Brewer and Peltzer 2009). In this context, our results on

the temperate azooxanthellate corals *A. calycularis* and *L. pruvoti* conform, respectively, to the energetic scenarios 1 and 2 proposed by Venn et al. (2013). The OM and the lipid and protein content of the tissue of *A. calycularis* did not become affected by the stress conditions of low pH and high ST, but the species exhibited a decrease in calcification rate of about 25 % under the high-ST conditions during the summer period. Conversely, the absence of effect of low pH and high ST on calcification of *L. pruvoti* is concomitant with a significant decrease in OM and on the lipid and protein content of the tissue under the high-ST conditions. This could be indicative of the activation of physiological processes to offset the new conditions at the expense of an increase in the energy consumption that leads to a decrease on the global energy resources of the species. Accordingly, the lipid and protein reserves in *L. pruvoti* under control conditions were twice as high as those in *A. calycularis* (Fig. 4c), which would allow the former to have a greater energy availability to maintain the energetically costly calcification rate. The species-specific observed response suggests therefore that different internal self-regulation mechanisms may be in place, allowing certain species to cope more successfully with environmental changes.

The Mediterranean is a biodiversity hotspot (Coll et al. 2010; Templado 2014) that is highly vulnerable to global change (e.g., Calvo et al. 2011; Herrmann et al. 2014; Marbà et al. 2015) and where OA and global warming seem to be occurring more rapidly than in the global oceans (Vargas-Yáñez et al. 2010; Touratier and Goyet 2011; Touratier et al. 2012; but see Palmiéri et al. 2014). In this semi-enclosed sea, global warming has been shown to be responsible of a significant lengthening of summer conditions (Coma et al. 2009), being an energetically unfavorable season with high respiratory demand and low food availability due to thermal stratification of the water column. The combination of both factors forces marine benthic invertebrates to a decreased activity pattern described as ‘summer dormancy’ (Coma et al. 2000; Coma and Ribes 2003), and may restrict the accumulation of energy resources that could be devoted to calcification, tissue growth and/or reproduction (Brewer and Peltzer 2009; Hoegh-Guldberg et al. 2007). Nonetheless, although it has been suggested that reduced pH can cause metabolic depression in some marine invertebrates (Edmunds 2012), little information is available about the potential effect of OA, alone or in combination with high ST, on the metabolism and biochemical balance. The decrease in the biochemical variables observed in *L. pruvoti* in our experiment suggests that, in the near future, the continuing reduced-pH conditions, along with increased ST, may push the metabolism of some of these organisms close to their limits. The potential synergistic effect of the two factors suggests that, in some temperate species, OA could enhance the severity of mass die-off events of benthic

organisms associated with long-term warm periods, which are increasing in frequency and intensity in the Mediterranean Sea (Coma et al. 2009). Understanding their biochemical strategies for the reallocation of metabolic energy can contribute to assessing the potential effects and relative resilience under future scenarios of anthropogenic global change and bring new insights into eventual shifts under large-scale stresses. This understanding will be useful for the management and preservation of the Mediterranean infralittoral (Ballesteros 2006), one of the richest and most diverse marine ecosystems on earth.

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