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Detrimental effects of Ocean Acidification on the economically important Mediterranean red coral (*Corallium rubrum*)

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Abstract

The mean predicted decrease of 0.3 to 0.4 pH units in the global surface ocean by the end of the century has prompted urgent research to assess the potential effects of ocean acidification on the marine environment, with strong emphasis on calcifying organisms. Among them, the Mediterranean red coral (*Corallium rubrum*) is expected to be particularly susceptible to acidification effects, due to the elevated solubility of its Mg-calcite skeleton. This, together with the large overexploitation of this species, depicts a bleak future for this organism over the next decades. In this study, we evaluated the effects of low pH on this species from aquaria experiments. Several colonies of *C. rubrum* were long-term maintained for 314 days in aquaria at two different pH levels (8.10 and 7.81, pH_T). Calcification rate, spicule morphology, major biochemical constituents (protein, carbohydrates and lipids) and fatty acids composition were measured periodically. Exposure to lower pH conditions caused a significant decrease in the skeletal growth rate in comparison to the control treatment. Similarly, the spicule morphology clearly differed between both treatments at the end of the experiment, with aberrant shapes being observed only under the acidified conditions. On the other hand, while total organic matter was significantly higher under low pH conditions, no significant differences were detected between treatments regarding total carbohydrate, lipid, protein and fatty acid composition. However, the lower variability found among samples maintained in acidified conditions relative to controls, suggests a possible effect of pH decrease on the metabolism of the colonies. Our results show, for the first time, evidence of detrimental ocean acidification effects on this valuable and endangered coral species.

1. Introduction

Ocean acidification (OA) is considered a major threat to the marine environment in the coming years (Doney et al. 2009). An average reduction of 0.1 pH units has already affected the surface waters of the world's oceans since the pre-industrial era (Orr et al. 2005) and future projections predict a decrease by 0.3 to 0.4 pH units by the end of the century, depending on the considered CO_2 emission scenarios (e.g. Joos et al. 2011). Although OA acts at a global scale, its impact varies locally. In the case of the Mediterranean Sea, the fast turnover time of its waters (50-100 years; Bethoux et al. 2005) and the very high concentration and fast penetration of anthropogenic CO_2 (Schneider et al. 2007; Schneider et al. 2010; Touratier & Goyet 2009) makes it one of the world's most sensitive regions to increasing atmospheric CO_2 (Ylmaz et al. 2008; Calvo et al. 2011, Ziveri 2012). A recent study estimated a pH decrease of up to 0.14 units since the pre-industrial era (Touratier & Goyet 2011), larger than the global averaged surface ocean pH decrease of ~0.1 pH units

recognised normally (e.g. Orr et al. 2005). The Mediterranean Sea is also characterized by other important environmental stressors (e.g. Calvo et al. 2011; Durrieu de Madron et al. 2011) which can impact marine organisms and ecosystems in different ways, in many instances synergistically (Crain et al. 2008; Lejeusne et al, 2010; Coll et al, 2010).

Several studies examining the potential impact of the projected pH levels on marine organisms have shown a wide variety of responses (e.g. Doney et al. 2009; Guinotte & Fabry, 2008; Ries et al. 2009; Wicks & Roberts 2012; Suggett et al. 2012). In the case of coral reef communities, most experiments indicate that the decreased concentration of carbonate ion associated to the reduction in pH has a detrimental effect, hindering the calcification process and the skeleton formation (e.g., Kroeker et al. 2010; Chan & Connolly 2012 and references therein). However, the calcification process in corals is highly complex and the impact of OA on critical physiological/biochemical mechanisms controlling the pH at the site of calcification is still poorly understood (Allemand et al. 2011; Cohen & Holcomb 2009). There is evidence that some species have the capacity to raise pH and [CO_3^{2-}] at the calcifying site (Al-Horani et al. 2003; Cohen & McConnaughey 2003; Ries 2011; Trotter et al, 2011), yet this active physiological process requires a significant amount of energy and a high metabolic cost for the organism (Wood et al. 2008; Cohen & Holcomb 2009; Edmunds et al. 2012; Li & Gao 2012; McCulloch et al, 2012). It is likely that the rate of change and the magnitude of seawater pH decrease are key factors in determining the energy demand for this process, reducing its availability to undertake other activities such as locomotion, reproduction, tissue growth or to counteract other environmental stresses (Brewer & Peltzer 2009; Hoegh-Guldberg et al. 2007). Therefore, studies aiming to identify which metabolic pathways can be more influenced by OA may play a helpful role to understand such an effect.

OA could also have clear regional socio-economic ramifications such as those related with the reduction in the harvest of high commercial interest species (Cooley et al. 2009). One of these key species is the red coral, *Corallium rubrum* (Linnaeus 1758), the so-called "Mediterranean Red Gold". It is a long-lived, slow-growing gorgonian endemic to the Mediterranean Sea and its neighbouring Atlantic rocky shores, where it can be found between 10 and 600 m depth (Costantini et al. 2010; Rossi et al. 2008). It is considered as one of the most valuable precious corals due to its bright red durable skeleton used as raw material in the jewellery industry (Tsounis et al. 2010). For these reasons, *C. rubrum* has been harvested since ancient times and it is now considered overexploited (Santangelo et al. 2004). Moreover, due to its rarity, cultural importance and landscape aesthetic value, red coral can be considered as a patrimonial or flagship species of the Mediterranean Sea (Bramanti et al. 2011). Red coral is composed of an axial skeleton and sclerites coated with living tissue (coenenchyma). The external surface of the coenenchyma is formed by the ectoderm, under which the mesoglea lies, a thick acellular layer of collagen containing small sclerites (30-50 μm). The axial skeleton has the same mineralogical composition than the free sclerites (Mg-rich calcite, Vielzeuf et al. 2008) but it is not the product of their fusion (Grillo et al. 1993). The main function of the sclerites is to provide mechanical protection against abrasion (Allemand 1993), although it has been suggested that they may act as temporary stocks of $CaCO_3$, readily available for the formation of the axial skeleton (Vielzeuf et al., 2008). The solubility of Mg-rich calcite minerals is greater than that of aragonite or calcite (Plummer & Mckenzie, 1974), and the seawater saturation state with respect to carbonate minerals decreases with increasing latitude (Andersson et al. 2008; Orr et al. 2005). Thus, it is conceivable that Mg-rich calcite, temperate calcifying organisms such as *C. rubrum* should be more susceptible to the decrease of pH conditions and among the first to be affected by these new environments.

Owing to the demand for management and conservation plans for a sustainable harvesting of this precious octocoral (Brukner & Roberts 2009; Bussoletti et al. 2010; GFCM 2010, 2011), dynamic models have been developed, projecting demographic trends over time (Santangelo et al. 2007; Bramanti et al. 2009). However, there is a lack of information regarding future OA effects in these colonies with potential to be harvested. To shed light on this topic, we investigated the effect of OA on *C. rubrum* by simulating in aquaria the future pH conditions projected by the end of the century. The potential changes in calcification rate, sclerite morphology and biochemical composition of the colonies reared under different pH conditions were evaluated. The results provide valuable information on the response of *C. rubrum* to low pH conditions that will increase our understanding and capacity to forecast the economic and ecological consequences of OA impacts on this precious species.

In this study, we aimed at answering the following questions: (1) Does a lowering in pH affect the $CaCO_3$ deposition in *C. rubrum*?; (2) What is the effect of OA on the morphology of sclerites?; (3) How does OA affect the metabolic balance of colonies in terms of total organic matter, carbohydrates, proteins, lipids and fatty acids?; (4) Could OA lead to different physiological responses related with the organism's capability to store energy?; (5) How will OA affect the harvestable stocks of red coral?

2. Materials and methods

2.1 Specimen collection and experimental setup

Colonies of *C. rubrum* were carefully removed from rocky substrates at 35-40 m depth from the Marine Protected Area of Cap de Creus (Spain, NW Mediterranean Sea, 42°19'N; 003°19'E) in November 2010 when in situ temperature was 13 ºC. The collected specimens were transported in large seawater containers at constant temperature (12.5 ± 0.5ºC) to the Institut de Ciències del Mar (ICM CSIC) in Barcelona (Spain). Colonies were

maintained in a 100 L acclimation tank with 50 μm filtered running natural seawater at *in situ* temperature and salinity conditions (12ºC and 37.6, respectively). As the red coral commercially harvested populations dwells between 80 and 100 meters depth (Tsounis et al, 2013), where the seasonal temperature variation are very small, colonies in the tanks were maintained at constant temperature and in complete darkness. For feeding, de-frozen *Cyclops* (Ocean Nutrition ™) were supplied daily (~55 mg of dry weight per aquarium). After 1 month of acclimation, 48 colonies of *C. rubrum* (Fig. S1) were selected and randomly distributed among 6 aquaria (30 litres each). Aquaria were further subdivided into 2 treatments (3 replicates per treatment), control pH and low pH (see below). The experimental set up is shown in Fig. S2. Seawater pH was gradually adjusted (0.03 units per day) in two large tanks of 150 L up to ~8.10 and ~7.81 pH units (total scale) simulating, respectively, the Mediterranean seawater in equilibrium with an atmosphere of ~380 ppm CO_2 (current levels) and ~800 ppm CO_2 (future levels predicted for year 2100 following A2 IPCC SRES; Plattner et al. 2008). In order to achieve the desired pH levels, seawater was bubbled with CO_2 (99.9% purity) or CO_2-free air (using a filter filled with soda lime absorber, Sigma Aldrich). Seawater pH was continuously monitored by glass electrodes (LL Ecotrode plus - Metrohm) connected to a pH controller (Consort R316, Topac Inc., USA). The glass electrodes were calibrated on a daily basis with a Tris buffer, following standard procedures (SOP6a of Dickson et al. 2007). In addition, small volumes of water were taken periodically (once a month during the first 3 months and bimonthly for the rest of the experiment) to analyse total alkalinity (TA) by potentiometric titration (Perez & Fraga, 1987; Perez et al. 2000) and pH using spectrophotometry (Clayton & Byrne 1993), which provides better precision than electrodes. TA and pH were used to calculate the rest of parameters of the carbonate system in seawater in both treatments, using the CO_2calc software (Robbins et al. 2010) (Table S1). Water from the large tanks flowed continuously (12 L per hour) to the experimental aquaria where colonies were maintained. Water in each aquarium was mixed with a pump (HYDOR

Koralia; 4.5W, 1500 l h^{-1}) and a plastic wrap was used to reduce evaporation and surface-air gas exchange. The pH-manipulative experimental set-up was installed inside a temperature-controlled room, ensuring constant values (~12°C) during the whole experiment.

C. rubrum colonies were sampled quarterly, from December 2010 (Time 0) to November 2011 (Time 3). During each sampling event, the buoyant weight of all the colonies was measured and 6 random colonies (1 from each experimental aquarium) were removed and kept at -80ºC, subsequently freeze-dried and then stored frozen at -20ºC until morphological and biochemical analyses were undertaken. Furthermore, at the end of the experiment, one extra colony was randomly selected from each aquarium to estimate specific microdensity and porosity.

2.2 Skeletal measurements

Changes in growth of the *C. rubrum* colonies (n = 30) were assessed from measurements of buoyant weight (Jokiel et al. 1978; Davies 1989), using a 0.1 mg resolution balance (Mettler Toledo AB204 SFACT). During the measurements, temperature and salinity of seawater were constantly monitored using an YSI-30M probe. The net buoyant weight of the corals (total coral weight minus the coral holder and glue) was transformed to dry weight using the specific density value of 2.66 g cm^{-3} (see below). The increase in weight was normalized to their initial mass and the growth rate (G) is expressed as mg of $CaCO_3$ increase per gram of initial weight per day. This normalization was used for statistical analyses.

Microdensity and porosity of the skeleton of *C. rubrum* were evaluated following the Bucher et al.'s (1998) technique. For this, one colony from each aquarium (3 per treatment) was dipped in sodium hypochlorite during two days to remove the organic matter and washed with distilled water. Buoyant weight and dry weight of each sample were recorded before and

after the inclusion in molten paraffin wax (105–110ºC) to form a water-tight barrier. In both cases, the buoyant weight was measured in distilled water at 20ºC with specific density 1.00 g cm^{-3}. Total enclosed volume, skeleton matrix volume and bulk density were also calculated for each sample using the equations described in Bucher et al. (1998).

2.3 Sclerite morphology

Following the methodology described in Lewis & von Wallis (1991) and Gori et al. (2012a), a sample of about 5 mm length of each colony, taken at approximately mid height of the branches, was dipped in sodium hypochlorite solution until organic matter was dissolved and sclerites were disaggregated. After sedimentation, the samples were rinsed with distilled water and subsequently with absolute ethanol for faster drying during mounting. A small portion of the sample was deposited on a glass cover attached to an aluminium stub with colloidal silver and covered with a thin layer of gold palladium (< 200 Å). Sclerite morphological observations were performed with a Scanning Electron Microscope (SEM) HITACHI S-3500 N working at 5.0 KV. A total of 12 colonies (3 replicates per treatment for time 0 and time 3) were examined and categorized in two main morphological types (Fig S3; Carpine & Grasshoff 1975). For each colony, 35 sclerites (Type I = 20, Type II = 15) were measured using a magnification of 1300 X. Sclerites with an anomalous morphology were labelled as aberrant (Fig. S3). For each sclerite the following measures were recorded by means of the graphic software Image J (Abramoff et al. 2004): Area, perimeter, maximum width and maximum height. In order to describe the overall shape of each sclerite, the following ratios were calculated: area/perimeter, width/height and circularity. The latter, defined as the ratio between the area of the sclerite and the area of a circle with the same perimeter, represents a measure of compactness (Turon & Becerro, 1992).

2.4 Major biochemical constituents

Organic matter (OM) of primary branches was determined in 10-12 mg of dry coenenchyma from each colony (n = 24). To obtain dry weight, samples were heated at 80ºC

for 48 h. The same samples were then ashed for 5 hours at 450ºC and weighted again. OM was then calculated as the difference between dry and ash weight (Slattery & McClintock 1995). Results are expressed as percentage with respect to the initial dry weight of the sample. Approximately 8 mg of dry coenenchyma from each sample were homogenized in 3ml of double distilled water, and total carbohydrates content was quantified colorimetrically according to the method of Dubois et al. (1956), with glucose as standard. Another 8 mg of coenenchyma dry weight from each sample were homogenized in 2 ml of 1N NaOH and total protein content was quantified colorimetrically using the method of Lowry et al. (1951), with albumin as standard. Finally, approximately 12 mg of coenenchyma dry weight from each sample were homogenized in 3 ml of chloroform-methanol (2:1) and total lipid content was quantified colorimetrically according to the method of Barnes & Blackstock (1973), using cholesterol as standard. All the results are presented in µg of constituent per mg of OM. These methodologies have already been applied with *C. rubrum* (Rossi & Tsounis, 2007).

For fatty acid proportions estimation, approximately 10 mg of dry coenenchyma from each sample were homogenized in 3:1 DCM:MeOH (dichloromethane-methanol), spiked with an internal standard mixture (2-octyldodecanoic acid and 5β-cholanic acid) and extracted using microwave assisted extraction for 5 min at 70ºC. After centrifugation, the extract was taken to near dryness in a centrifugal vacuum concentrator at a constant temperature and fractionated by solid phase extraction according to Ruiz et al. (2004). The sample was redissolved in 0.5 ml of chloroform and eluted through a 500 mg aminopropyl glass column (Russell & Werne 2007) that was previously activated with 4 ml of *n*-hexane. The first fraction was eluted with 3 ml of chloroform:2-propanol (2:1) and the fatty acids recovered with 8.5 ml of diethyl ether:acetic acid (98:2). The fatty acids fraction was methylated using a solution of 20% MeOH/BF3 and heated at 90ºC for 1 h. The reaction was quenched with 4 ml of NaCl saturated water. The methyl esters of fatty acids were recovered

by extracting twice with 3 ml of *n*-hexane. The combined extracts were taken to near dryness, redissolved in 1.5 ml of chloroform and eluted through a glass column filled with Na_2SO_4 to remove residual water. After removal of the chloroform under nitrogen gas, the extracted sample was stored at -20ºC until analysis by gas chromatography. Samples were redissolved in 80 µl of isooctane and gas chromatography analysis was performed with an Agilent Technologies 7820A GC equipped with a flame ionization detector, a splitless injector and a DB5MS Agilent column (60 m length, 0.25 mm internal diameter and 0.25 µm phase thickness). Hydrogen was used as a carrier gas at 30 ml min^{-1}. The oven temperature was programmed to increase from 50ºC to 160ºC at 20ºC min^{-1}, from 160ºC to 188ºC at 0.5ºC min^{-1}, from 188ºC to 299ºC at 20ºC min^{-1}, from 299ºC to 235ºC at 2ºC min^{-1} and from 235ºC to 300ºC at 4ºC min^{-1}. Injector and detector temperatures were set at 300ºC and 320ºC, respectively. Methyl esters of fatty acids were identified by comparing their retention times with those of standard fatty acids (29 FAME compounds, Supelco® Mix C4-C24). Fatty acids were quantified by integrating the peak areas using the Chromquest 4.1 software, and converting them into concentrations from the area *vs* concentration of the internal standards.

For fatty acid grouping into classes (total Saturated Fatty Acids, SAFA; Mono Unsaturated Fatty Acids, MUFA and Poly Unsaturated Fatty Acids, PUFA), only those with concentrations higher than 1% of the total fatty acids were considered (Daalsgaard et al. 2003). This methodology has already been applied in gorgonians (Gori et al. 2012b).

2.6 Statistical analyses

A two-way nested ANOVA was used to examine whether calcification rate varied between treatments (i.e., exposure to low pH conditions and exposure to control pH conditions) and aquaria. Aquarium was considered as a random factor nested within treatment. A total of 30 colonies (5 per aquarium, 15 per treatment) were weighted. Calcification rate data are expressed as mean ± standard error (SE). A one-way ANOVA was used to examine differences between both treatments (Control

and low pH) in microdensity and porosity of the Mg-rich calcite skeleton. Sclerites morphology results were standardized with respect to their median absolute deviation (MAD), and a Euclidean distance matrix was built based on the standardized data. A distance-based permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) was employed to test the null hypothesis of no significant differences between treatments. Factors were time (initial and final time), treatment (control and low pH) and aquaria (random factor nested within treatment). Finally, a univariate two-way ANOVA was used to analyse biochemical results. Factors in this case were treatment (control and low pH) and time (4 sampling points). When ANOVA showed significant differences, Tukey's honest significant difference test (HSD) was used to attribute differences between specific factors or their interaction only. All biochemical data are expressed as mean ± standard deviation (SD). In order to have a measure of the variability associated to the response to low pH, the Coefficient of Variation (Standard Deviation / Mean) was calculated. Multivariate analysis was performed using the PERMANOVA software (Anderson 2005), while all the other statistical analyses were performed using the R software platform (R development Team 2012).

3. Results

3.1 Skeletal measurements

Microdensity and porosity of *C. rubrum* were 2.66 ± 0.06 g cm^{-3} and 25.7 ± 5.3 % (mean ± SD; n = 6), respectively. No significant differences between treatments were observed, neither in microdensity (ANOVA, $F_{1, 5} = 5.25$, $p = 0.08$) nor on porosity (ANOVA, $F_{1, 5} = 0.42$, $p = 0.55$), indicating that the structural material composition of this gorgonian was not affected by the level of acidification to which it was subjected. Regarding the effect of the lower pH treatment on the skeletal growth of *C. rubrum*, a significant decrease was observed at the end of the experiment in their averaged calcification rates, which were found to be 59% lower compared to control conditions (Table 1 and Fig. 1). The survivorship in each treatment was 100% and no tank effect was detected between aquaria replicates of the same treatment in any analysis.

3.2 Sclerites morphology

PERMANOVA results showed a significant interaction between the factor Time and the factor Treatment for both types of sclerites (type I and II, Fig. S3, Table 2). These results confirm the hypothesis of an effect of low pH on the overall sclerite shape. The post hoc test showed that at Time 0 (at the beginning of the experiment) there were no significant differences between both treatments while at Time 3 (after 314 days) the overall shape of sclerites of the colonies grown under low pH conditions was significantly different compared with the control treatment. Furthermore, the presence of anomalous shapes classified as aberrant type (Fig. S3) was detected only in colonies reared in the acidified treatment.

3.3 Major biochemical constituents

The analysis of the OM content showed a significant interaction between the factor "Treatment" and the factor "Time" (Table 3 and Fig. 2). Post hoc comparisons (Table 3) indicated no differences between treatments in T0 and T1, while in T2 and T3, the mean OM content of samples in the low pH treatment remained higher than that in control conditions. While the OM of *C. rubrum* grown in the control treatment significantly changed from T0 to T3 (Table 3 and Fig. 2), no significant differences were found in the lower pH conditions throughout the experiment.

Regarding the metabolic balance, no differences between treatments were found in total protein, carbohydrate or lipid content during the whole experiment (Two-way ANOVA, $F_{3,16} = 0.093$, $P > 0.05$; $F_{3,16} = 1.22$, $p > 0.05$; $F_{3,16} = 0.009$, $p > 0.05$, respectively). Furthermore, the Coefficient of Variation (CV) was higher in the control samples (Fig 3).

Regarding fatty acids, no significant differences were found between treatments, neither in the total fatty acid levels among the different times (Fig 4; Two-way ANOVA, $F_{3,16} = 0.49$, $P > 0.05$), nor in the fatty acid proportions (SAFA, MUFA and PUFA; Fig S4). As in the previous case, samples reared under control conditions showed higher coefficient of variability values in times 2 and 3 (Fig 4).

4. Discussion

4.1 Changes in the structural features of C. rubrum

After 314 days of exposure, colonies of *C. rubrum* reared under lower pH conditions (pH 7.81) suffered a decrease in skeletal growth rate of 59% compared with those maintained in the control treatment (pH 8.10). This reduction confirms the expected detrimental effects on skeletal formation and it is consistent with previous experimental studies examining the response to OA of other benthic calcifying species. In the case of tropical scleractinian corals, the decline in the calcification rate ranges from 10% to 60% at double elevated pCO_2 conditions (Guinotte and Fabry, 2008; Kleypas et al. 2006), reaching up to 70% of reduction in the case of *Porites rus* at pH 7.80 (Muehllehner & Edmunds, 2008). Some species, however, are able to calcify even under low saturation state conditions (Jury et al. 2010; Krief et al. 2010; Ries et al. 2010; Comeau et al, 2013), pointing to some complexity in the mechanisms controlling the intracellular calcification process (Ries, 2011 and references therein). Similarly, a wide range of responses has been observed in Mediterranean coral species. Fine & Tchernov (2007) reported total skeleton dissolution for *Oculina patagonica* at a pH of 7.4, while Movilla et al (2012) found a 35% reduction in calcification rates for O. patagonica and *Cladocora caespitosa* at a pH of 7.83, and Rodolfo-Metalpa et al (2010a) showed no effects of decreased seawater pH (7.88) on the calcification rates of C. caespitosa. In addition, the only experiment to date in which temperate corals were transplanted to a natural pH gradient influenced by volcanic CO_2 vents, showed evidences of dissolution on the exposed skeleton of *C. caespitosa* at pH 7.5 environments, while no effects were observed in *Balanophyllia europaea*, which skeleton was completely covered by tissue, at pH levels as low as 7.3 (Rodolfo-Metalpa et al. 2011). Regarding Mediterranean bryozoans, *Schizoporella errata* transplanted to the same naturally acidified site showed skeletal corrosion and disruption in calcification at pH 7.76 (Lombardi et al. 2011a). However, the skeletal growth rate and appearance of *Myriapora truncata* were not affected until a pH of

7.43 (Lombardi et al. 2011b; Rodolfo-Metalpa et al, 2010b), suggesting that the presence of organic tissue enveloping the skeleton could play a key role in these organisms (Ries et al, 2009; Rodolfo-Metalpa et al, 2011), providing protection against the corrosiveness of lower pH seawater. It should be noted that, unlike scleractinian corals or bryozoans as *S. errata*, whose skeletons are made of calcite or aragonite (Cohen & McConnaughey, 2003; Smith et al. 2006), the red coral skeletal structure consists entirely of Mg-rich calcite (Vielzeuf et al. 2008). As previously mentioned, the solubility of this latter form is much higher than the first two and, therefore, this species could be considered as one of the most susceptible organisms to OA in the Mediterranean.

Our results also showed an effect of lower pH on the morphology of microscopic sclerites, with an overall shape different between treatments and anomalous forms observed only in acidified conditions. Sclerites abnormal skeletogenesis has been observed previously in sea urchin larvae exposed to high pCO_2 conditions (Kurihara & Shirayama, 2004). According to Allemand (1993), the main function of these sclerites in *C. rubrum* is to ensure the mechanical protection against abrasion, although it has been suggested that they may act as $CaCO_3$ temporary stocks readily available for the axial skeleton formation through mechanisms of dissolution, transport, and recrystallization (Vielzeuf et al. 2008). Furthermore, in the order Alcyonacea, the skeleton is typically proteinaceous (Grillo et al. 1993) with elastic properties that allow the colonies to bend forward and back to an upright position (Jeyasuria & Lewis, 1987). The morphology and abundance of sclerites in the tissue limit the extent of these movements, being a determinant factor defining their overall structure (Lewis & Von Wallis 1991). Therefore, our results suggest that, in the long term, OA will affect *C. rubrum* and other gorgonians in compromising the $CaCO_3$ stocking capacity as well as the skeleton biomechanical properties.

This study focused on evaluating the effect of OA on the growth of a Mediterranean anthozoan with high commercial value such as the red coral. However, in addition to OA, other environmental pressures may affect future red coral populations, acting independently or in synergy with OA. While increased atmospheric pCO2 is responsible for OA, it is also causing an increase in global seawater temperature. In the Mediterranean Sea, increased seawater temperature has been shown to translate into longer stratification periods associated with mass mortality events (Coma et al. 2009; Garrabou et al. 2009). Although the synergistic effects of warming and ocean acidification could well affect shallow water red coral populations (Santangelo et al. 2012a), the present study has a particular focus on populations with harvest potential (i.e. below 60 meters depth, Rossi et al. 2008), where temperature changes are not as marked and tend to remain relatively constant throughout the year. Nevertheless, high temperatures induce increased metabolic and respiration rates and depress the polyp activity in red coral and other gorgonians (Previati et al. 2010). In addition, long-term exposure to high temperature can result in partial or total mortality of the colonies and recruits (Bramanti et al. 2005; Garrabou et al. 2001, 2009; Torrents et al, 2008). Thus, it is essential to perform future studies manipulating OA and temperature to assess the existence of possible interactions between multiple stressors and establish to which extent coral colonies inhabiting shallower ranges will be threatened.

4.2 Metabolic response of red coral to a lowering in pH

Gorgonians skeleton consists of internal formations (central axis and sclerites) protected by a thick layer of mesoglea gel (Alderslade & Fabricius 2008), a composite material of collagen-like fibrils randomly oriented in a hydrated polymer matrix (Lewis & von Wallis 1991). The presence of a tissue layer prevents the direct exposure of the skeleton to seawater and may modulate the effects of OA in coral and other organisms (Hoffman et al. 2010; Ries et al. 2009; Rodolfo-Metalpa et al. 2011). However, in our experiment, although

the tissue of colonies remained intact, a clear effect of high pCO_2 in coral growth was observed. In addition to minimizing growth, the colonies of *C. rubrum* reared under acidified conditions may have activated physiological processes to offset the new conditions, with the consequent increase in energy consumption (Al-Horani et al. 2003; Cohen & Holcomb 2009; Tsounis et al. 2012). Our results on Total Organic Matter tend to point in the direction of this hypothesis. For example, throughout the experiment, higher content of organic matter was observed in the coral colonies reared under lowered pH conditions in comparison to those in the control, which displayed a progressive lowering in organic matter content with time (Fig. 2). Similar responses were found in more drastic pH lowering (pH 7.4) in which the temperate corals *O. Patagonica* and *Madracis pharencis* were exposed (Fine & Tchernov, 2007). In this case, polyps of these species suffered a complete dissolution of the skeleton, but their biomass was three times higher than those maintained in control conditions (Fine & Tchernov, 2007). As it has been described in the case of gastropods, the energy allocation for the shell construction *vs* that needed to build the organic matrix is very different and changes under stress conditions (Palmer 1983, 1992). Under lowered pH conditions the metabolism is affected (Edmunds 2012; Edmunds et al. 2012) and the energetic cost of calcification is expected to be higher (e.g. Cohen & Holcomb, 2009). Therefore we hypothesize that *C. rubrum* may respond to decreased seawater pH be increasing the formation of organic matter, consequently decreasing calcification rates. This response is mediated by an alteration of *C. rubrum* metabolism caused by an increase in the cost of calcification in a low pH environment.

Total carbohydrate, protein and lipid content in colonies grown under control conditions in the present work were similar to those observed by Rossi & Tsounis (2007) in natural conditions (Fig. 3), indicating that the biochemical composition of *C. rubrum* tissue in aquaria is comparable to that observed *in situ*. Protein and lipid content was also comparable

to other gorgonians such as *Paramuricea clavata* and *Leptogorgia sarmentosa* (Rossi 2002; Rossi et al. 2006). Data on fatty acid concentration of *C. rubrum* are presented in this work for the first time. Regarding composition, a wide range of fatty acid profiles were observed (Fig. S4) but most of the fatty acid esters were quite similar to those described in the case of zooxanthellate anthozoans (Meyers & Quinn 1974; Latyshev et al. 1991; Gori et al. 2012b), although azooxanthellate organisms may have other physiological and metabolic mechanisms to counter the effect of external stress.

As mentioned, a larger variability in the total concentration of carbohydrate, protein and lipid content was observed throughout the experiment in the control colonies, whereas the variability was much more reduced in the colonies under the acidified conditions (Fig 3). This difference in response could perhaps be an indication of a more dimmed seasonal trend in the colonies under low pH pressure than those in the control treatment or the observed under natural conditions (Rossi and Tsounis 2007). This could be due to endogenous processes, dormancy or some kind of life cycle disruption experimented under certain environmental pressures, with the aim of saving energy by reducing or shutting down some physiological processes (e.g. respiration; Previati et al. 2010) and thus, survive. This conforms with the findings on sea urchins *Hemicentrotus pulcherrimus* and *Echinodetra mathei* where the fertilization success, developmental rates, larval size, and sclerite skeletogenesis also decreased in those specimens reared under low pH (Kurihara & Shirayama 2004) probably because a considerable part of the energy is invested in respiration and in protein synthesis related with basic metabolic paths as found in barnacle and copepods (Wong et al. 2011, Li & Gao 2012). In coral species, metabolism reduction has also been observed as a defensive mechanism to survive under adverse conditions (Rossi 2002; Previati et al. 2010). Nonetheless, this could translate into less available energy for processes such as growth or reproduction (Brewer & Peltzer 2009; Hoegh-Guldberg et al. 2007). Our study was

focused on assessing the effect of lower pH on skeletal growth rates of *C. rubrum*. However, further research on the effects on reproduction and on the response in a natural seasonal environment should be undertaken to gain deeper insights on the response of red coral populations to OA.

4.3 Economy and management

Understanding the population dynamics of the precious Mediterranean red coral is essential to implement best management and conservation strategies for this species under the ongoing rapid climate and environmental change. In summer 1999 and 2003, shallow populations of *C. rubrum*, among other benthic suspension feeders in the northwestern Mediterranean Sea, suffered a mass mortality event due to a temperature anomaly (Bramanti et al. 2005; Cerrano et al. 2000; Coma et al. 2006; Garrabou et al. 2001; 2009; Santangelo et al. 2007; Cupido et al. 2012). These mass mortality events, coupled with the uncontrolled harvesting, could bring some populations to local extinction (Santangelo et al. 2012a). In fact, the distribution and size of red coral have been altered over time (Rossi et al. 2008, Linares et al. 2010). In Cap de Creus (NE Spain), shallow and deep population structures have been affected by management rules (Santangelo et al. 2012b) turning the "coral forest" into "coral grass plain" (Rossi et al. 2008, 2012; Tsounis et al. 2007).

On the other hand, depending on the CO_2 emission scenarios, seawater pH and carbonate saturation states could drop significantly by the end of the century (Joos et al. 2011). The present study shows, for the first time, a negative response of *C. rubrum* deep-water populations to OA. The observed decrease in the calcification rate together with some evidence of a potential reduction in the metabolism of red coral colonies as possible survival mechanism against low pH conditions, could have negative rebounds on the economy of the jewellery industry linked to this species and lead to a price deflation for years. The market price of the red coral colonies is currently very high: thin

juvenile branches can be sold for 230-300 US$ kg^{-1} while Pacific Corallium sp. colonies with diameter >4cm can reach 50.000 US$ kg^{-1} (Tsounis et al. 2007, 2010). Under the increasing global environmental and economic pressures, causing limited raw material production and possible price deflation, the red coral industry may suffer a decline worldwide. In Europe, red coral industry has significantly declined over time and, today, only 30% of the coral processed in Torre del Greco (Italy) is *C. rubrum*, whereas 70-80% is represented by other species of precious corals imported from Japan and Taiwan (Nonaka & Muzik 2009; Tsounis et al. 2010; Chen 2012). These economic losses represent more than 230 million US$$yr^{-1}$ (Tsounis et al. 2010).

Furthermore, without proper protection and management plans, this slow-growing, endemic precious species of Mediterranean Sea may suffer local extinction in the near future. Young populations of *C. rubrum* may be unable to cope with combined pressures of heat waves, ocean acidification, overexploitation and other anthropogenic activities. Moreover, local extinction and changes in the population structure could affect the role that engineering species (Jones et al. 1994) as *C. rubrum* play in the benthic pelagic coupling and biogeochemical cycles (Rossi et al., 2012). Data from the present study provide experimental input that can be used in simulations of population trends overtime based on matrix models (Caswell 2001) that will allow making projections on population dynamics under the expected future acidification scenarios. Management and conservation actions should be then planned on the basis of the outcome of these simulations in order to preserve this precious species with the associated biodiversity and the economy linked to its exploitation.

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Figure captions

Fig S1. Colony of *Corallium rubrum* in the experimental aquarium

Fig. S2. Experimental setup used to control and modify seawater pH in each aquarium. A) and B) large 150 l tanks for seawater conditioning at pH_T 7.81 and 8.10, respectively; C) glass electrodes for pH and PT100 probes for temperature measurements; D) pH controller and data logger; E) solenoid valves; F) soda lime filter; G) 50 kg CO_2 bottle; H) and I) control and low pH experimental aquaria, respectively (three replicates per treatment); J) Microbubble diffusers.

Fig. S3. Examples of Scanning Electron Microscope (SEM) images of the three types of sclerites observed in *C. rubrum.*

Fig. S4. Fatty acids composition. SAFA: total Saturated Fatty Acids; MUFA: Mono Unsaturated Fatty Acids; PUFA: Poly Unsaturated fatty acids.

Fig. 1. Calcification rates of *C. rubrum* after 314 days under low pH_T (grey bars, pH_T 7.81 units) and control conditions (black bars, pH_T 8.10 units). Calcification rates are expressed as mg $CaCO_3$ per gram of initial weight per day. n = 15, error bars represent SE.

Fig. 2. Total organic matter % in the coenenchyma of *Corallium rubrum* colonies (n = 24) during the whole experiment. Black and grey bars represent control (8.10 pH_T units) and treatment (7.81 pH_T units), respectively (mean ± SD).

Fig. 3. Total protein (a), lipid (b) and carbohydrate (c) concentration (µg mg^{-1} OM) in the coenenchyma of *Corallium rubrum* colonies (n = 24) during all the periods of time when colonies were sampled. Black and grey bars represent control (pH_T 8.10 units) and treatment (7.81 pH_T units), respectively (mean ± SD). CV= coefficient of variation.

Fig. 4. Total fatty acid concentration (µg mg^{-1} OM) in the coenenchyma of *Corallium rubrum* colonies (n=24). Black and grey bars represent control (pH_T 8.10 units) and treatment (7.81 pH_T units), respectively (mean ± SD). CV= coefficient of variation.