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ORIGINAL PAPER

Physiological resilience of a temperate soft coral to ocean warming and acidification

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Abstract

Atmospheric concentration of carbon dioxide (CO_2) is increasing at an unprecedented rate and subsequently leading to ocean acidification. Concomitantly, ocean warming is intensifying, leading to serious and predictable biological impairments over marine biota. Reef-building corals have proven to be very vulnerable to climate change, but little is known about the resilience of non-reef-building species. In this study, we investigated the effects of ocean warming and acidification on the antioxidant enzyme activity (CAT—catalase, and GST—glutathione S-transferase), lipid peroxidation (using malondialdehyde, MDA—levels as a biomarker) and heat shock response (HSP70/HSC70 content) of the octocoral *Veretillum cynomorium*. After 60 days of acclimation, no mortalities were registered in all treatments. Moreover, CAT and GST activities, as well as MDA levels, did not change significantly under warming and/or acidification. Heat shock response was significantly enhanced under warming, but high CO_2 did not have a significant effect. Contrasting to many of their tropical coral-reef relatives, our findings suggest that temperate shallow-living octocorals may be able to physiologically withstand future conditions of increased temperature and acidification.

Keywords Climate change · Heat shock proteins · Antioxidant enzymes · Lipid peroxidation · *Veretillum cynomorium*

Introduction

Since the start of the industrial revolution, atmospheric concentrations of carbon dioxide (CO_2) have been rising, reaching for the first time levels above 400 ppm (Lüthi et al. 2008) and being expected to reach 730–1020 ppm by the end of the century (Pörtner et al. 2014). Nearly 30% of atmospheric CO_2 is absorbed by the oceans (Hoegh-Guldberg et al. 2014), resulting in a change in seawater chemistry that is expected to prompt a 0.13–0.42 pH unit drop in seawater pH by the year 2100 (Pörtner et al. 2014). Increasing atmospheric CO_2 concentration is also increasing global temperature, with future projections pointing out to a rise up to 4 °C in sea surface temperature until the end of the century (Collins et al. 2013). Additionally, the frequency and extent of extreme weather events, such as heat waves, are expected to increase at a global scale (Pörtner et al. 2014). These changes are expected to cause major shifts in species distribution, phenology, and physiology (e.g., Edwards and Richardson 2004; Harvey et al. 2013; Kroeker et al. 2013; Parmesan and Yohe 2003), which can ultimately lead to worldwide extinction events (Thomas et al. 2004).

Important constituents of coral reef ecosystems in tropical regions and abundant components of shallow-water habitats in temperate environments, corals harbor an overwhelming complexity of species and play an essential ecological role within benthic communities (Carpenter et al. 2008; Freiwald et al. 2004). Considering their ecological relevance, large efforts have been undertaken in order to study the effects of climate change on corals worldwide (e.g., Baker et al. 2008; Hofmann et al. 2008). In fact, temperature and CO_2 levels

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have been proven to affect several life-history processes of reef-building corals (e.g., development and reproduction), besides damaging several key physiological functions, such as calcification, photosynthesis, and respiration (Ateweberhan et al. 2013; Pandolfi et al. 2011). Moreover, increased seawater temperature is also threatening the symbiosis between corals and zooxanthellae, in a phenomenon known as coral bleaching (Glynn 1996). In contrast to reef-building corals, studies targeted to non-reef-building corals are still scarce. In fact, investigations addressing the effects of climate change on soft corals are mainly focused on community spatial distribution rather than in understanding the effects upon species ecophysiology (e.g., Gómez et al. 2015; Inoue et al. 2013; Ruzicka et al. 2013).

In order to avoid deleterious effects caused by environmental disturbances, marine organisms display a diverse set of physiological regulatory defense mechanisms, which include heat shock and antioxidant responses (Feder and Hofmann 1999; Lesser 2006). Heat shock response involves the synthesis of heat shock proteins (HSP), which possess an important role by promoting stabilization and refolding of denatured proteins (Dong et al. 2008; Tomanek 2010). On the other hand, antioxidant response is characterized by a powerful set of enzymatic antioxidants [e.g., catalase (CAT) and glutathione S-transferase (GST)], which act against toxic effects of reactive oxygen species [ROS, i.e., superoxide radicals (O_2^-), hydrogen peroxides (H_2O_2), singlet oxygens ($^1O_2$), and hydroxyl radicals ($HO^•$)] (Apel and Hirt 2004). Under environmental stress, high levels of ROS production may cause cellular damage through lipid peroxidation, one of the most frequent cellular injury processes where ROS react with membrane-associated lipids (Lesser 2011).

Over the last few decades, the effects of increasing temperature and hypercapnia on the physiological defense mechanisms of corals have been extensively studied (see Supplementary Table SI). However, the bulk of these studies are focused on hexacorals species (e.g., Agostini et al. 2016; Downs et al. 2013; Downs et al. 2000; Griffin and Bhagooli 2004; Griffin et al. 2006), while studies on octocorals remain scarce (Madeira et al. 2015; Mydlarz and Jacobs 2006; Wiens et al. 2000). Moreover, most of the above-mentioned studies focus on the effects of temperature alone, without considering the effect of ocean acidification or the interaction between stressors.

The finger-shaped sea pen, *Veretillum cynomorium* (Pallas 1766), is a colonial octocoral belonging to the order Pennatulacea. This species is widely distributed in the eastern Atlantic Ocean (Vander Land 2008), and it is found on coastal shallow waters, inhabiting the soft sediment of beaches and sand plains (Cornelius et al. 1995), with a bathymetric distribution between 0 and circa 200 m (López-González et al. 2001). Previous studies have already addressed the great tolerance of this species to deal with the extreme abiotic conditions of the intertidal environment, as they are fitted with an anticipatory response to cope with oxidative stress during air exposure at low tide conditions (Teixeira et al. 2013). Additionally, it was also seen that they tolerate well the seasonal variation in temperature regimes, being able to withstand low tide conditions during summer without undergoing cellular damage (Madeira et al. 2015). Nevertheless, this species resilience toward climate change-related conditions remains unknown. To that end, the aim of the present study was to investigate the physiological mechanisms that may enable *V. cynomorium* to withstand future ocean warming and acidification conditions. More specifically, we analyzed (i) antioxidant enzyme activities (GST and CAT), (ii) lipid peroxidation (malondialdehyde—MDA), and (iii) heat shock response (HSP70/HSC70).

Materials and methods

Coral collection and laboratory acclimation

Twenty-four *V. cynomorium* colonies were hand-collected near the mouth of the Sado estuary (38° 29′ 11″ N, 8° 53′ 13″ W, Setúbal, Portugal) in March 2014. After field collection, organisms were immediately transported to the aquatic facilities of Laboratório Marítimo da Guia (Cascais, Portugal).

Octocorals were maintained in twelve 50-L holding tanks coupled to recirculation aquaculture systems filled with 0.35 μm-filtered (Harmsco, USA) and UV-irradiated (V^2ecton 600, TMC Iberia, Portugal) natural seawater, directly pumped from the sea. Each system was fitted with mechanical (Glass wool, Fernando Ribeiro Lda, Portugal), biological (Ouriço®, Fernando Ribeiro Lda, Portugal), and physicochemical filtration (V^2Skim Pro 450, TMC Iberia, Portugal), with additional UV-irradiation (V^2ecton 300, TMC Iberia, Portugal). A 10% seawater renewal was daily performed. Ammonia, nitrite, and nitrate levels were daily monitored by means of colorimetric tests (Profi Test, Salifert, Holland) and kept below detectable levels. During the experimental exposure, a photoperiod of 14 h:10 h (light:dark cycle) was performed. All colonies were fed (twice a day) with a mixture of frozen *Artemia* spp. and *Mysis* spp. (TMC Iberia, Portugal).

Upon arrival, colonies were acclimated for 15 days to the prevailing natural conditions of the collection site (temperature 19 ± 1 °C, pH 8.0 ± 0.1, pCO_2~500 μatm, salinity 35 ± 1). Subsequently, organisms were exposed for 60 days to different experimental conditions (three tanks per treatment): (i) control temperature and normocapnia (19 °C, pH 8.0, pCO_2~460 μatm); (ii) control temperature and hypercapnia (19 °C, pH 7.7, pCO_2~1020 μatm); (iii) warming and normocapnia (26 °C, pH 8.0, pCO_2~430 μatm); and (iv) warming and hypercapnia (26 °C, pH 7.7, pCO_2~1060 μatm). The warming condition was chosen based

on the temperature values observed during heat events in the collection site (see Supplementary Fig. S1). Regarding the pCO_2 scenarios, one should keep in mind that this species inhabits the Western Iberian Upwelling Ecosystem, part of the Canary Current Upwelling System. In these regions, actual pCO_2 levels may reach up to 500 μatm (Álvarez-Salgado et al. 1997; Perez et al. 1999) and are thus expected to exceed the level of 420–940 μatm projected for 2100 (Pörtner et al. 2014).

Water temperature and pH were continuously controlled and adjusted by means of an automatic monitoring device (Profilux 3.1, GHL, Germany), connected to temperature and pH probes (PL-0094 and PL-0071, respectively, GHL, Germany). Upon demand, seawater temperature was upregulated using submerged digital heaters (V^2Therm 200 W, TMC Iberia, Portugal) or downregulated using seawater chillers (HC-250A, Hailea, China). Adjustment of pH levels was performed automatically via solenoid valves connected to the Profilux system. Reduction of pH values was accomplished by the injection of a certified CO_2 gas (Airliquide, Portugal), while upregulation was achieved by the injection of CO_2-filtered atmospheric air (using soda lime, Sigma-Aldrich, Portugal). Additionally, a daily monitoring of seawater temperature (thermometer TFX 430, WTW GmbH, Germany), salinity (V^2 Refractometer, TMC Iberia, Portugal), and pH (pH/ion meter SG8, Mettler-Toledo, Switzerland) was performed using handheld equipment. Seawater carbonate system speciation (see Supplementary Table SII) was calculated weekly from total alkalinity (determined according to Sarazin et al. 1999) and pH measurements, using the CO_2SYS software, with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

At the end of the experimental exposure, colonies were collected, immediately placed in liquid nitrogen, and stored at − 80 °C for subsequent biochemical analyses.

Biochemical analyses

Preparation of tissue extracts

Tissue samples from different colonies ($n = 2$ per tank, $n = 6$ per treatment) were individually homogenized in phosphate buffer saline (PBS), according to Lopes et al. (2013). Homogenates were centrifuged at 14,000x*g* for 20 min at 4 °C. Antioxidant enzyme activity, lipid peroxidation, and the heat shock response were quantified in the supernatant fraction. Each sample was run in triplicate (technical replicates) and results were normalized to total protein content, as described by Bradford (1976).

Antioxidant enzymes

CAT activity was determined following Aebi (1984). A total of 100 μL of each sample was added to 2.9 mL of substrate solution [50 mM potassium phosphate buffer (pH 7.0) and 12.1 mM H_2O_2], into quartz cuvettes. Absorbance was measured at 240 nm (Helios spectrophotometer, Unicam, UK), during 15-s intervals across a 180-s incubation period. Bovine CAT solution was used as a positive control to validate the assay. CAT activity was calculated based on the absorbance increase per minute, using the H_2O_2 extinction coefficient (0.04 mM).

GST activity was determined according to Habig et al. (1974). A total of 180 μL of substrate solution [200 mM L-glutathione reduced, Dulbecco phosphate-buffered saline and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution] was added to 96-well microplates, alongside with 20 μL of GST standard or sample. Equine liver GST was used as a positive control to validate the assay. Enzyme activity was determined spectrophotometrically at 340 nm (Bio-Rad, Benchmark, USA), every minute during a 6-min time frame. GST activity was calculated based on the absorbance increase per minute, using the CDNB extinction coefficient (5.3 mM).

Lipid peroxidation

Lipid peroxidation was determined by MDA quantification, according to the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara 1978). Briefly, 10 μL of each sample was added to 50 mM of monobasic sodium phosphate buffer (45 μL), followed by the addition of 8.1% of sodium dodecyl sulphate (12.5 μL), 20% of trichloroacetic acid (93.5 μL), and 1% of thiobarbituric acid (93.5 μL). A volume of 50.5 μL of Milli-Q ultrapure water was added to this mixture, being subsequently mixed for 30 s and incubated in boiling water for 10 min. The resulting mixture was placed on ice for 3 min to lower temperature. Afterwards, 62.5 μL of Milli-Q ultrapure water and 312.5 μL of *n*-butanol pyridine (15:1 *v*/*v*) were added and microtubes centrifuged at 2000x*g* for 5 min. The supernatant fraction (150 μL) was added to 96-well microplates and absorbance was read at 532 nm (Bio-Rad, Benchmark, USA). MDA concentrations were calculated based on a calibration curve (0–0.3 μM) using MDA bis (dimethyl acetal) standards.

Heat shock response

The HSP70/HSC70 content was assessed through enzyme-linked immunosorbent assay (ELISA), according to an adaptation of the method described by Njemini et al. (2005). Each sample (10 μL) was diluted in 990 μL of PBS. Afterwards, 50 μL of each diluted sample was added to 96-well microplates (Microloan 600, Greiner, Germany) and incubated

overnight at 4 °C. After 24 h, microplates were washed (four times) using PBS containing 0.05% Tween-20. Then, 100 μL of blocking solution (1% bovine serum albumin) was added to each well and microplates were incubated in darkness for 2 h at room temperature. Consequently, 50 μL of primary antibody HSP70/HSC70 (5 μg mL^{-1}, Acris, USA) was added to each well. Microplates were incubated overnight at 4 °C and washed 24 h afterwards to remove non-linked antibodies. The alkaline phosphatase-conjugated anti-mouse IgG (Fab specific, Sigma-Aldrich, USA) was used as a secondary antibody, by adding 50 μL (1 μg mL^{-1}) to each well, and microplates were incubated for 90 min at 37 °C. After an additional washing procedure, 100 μL of substrate p-nitrophenyl phosphate tablets were added to each well and incubated for 30 min at room temperature. Lastly, 50 μL of stop solution (3 M NaOH) were added to each well, with absorbance being read at 405 nm in a microplate reader (BIO-RAD, Benchmark, USA). Heat shock protein content was calculated from the calibration curve, based on serial dilutions (0–2000 μg mL^{-1}) of purified HSP70 active protein (Acris, USA).

Statistical analyses

Generalized linear models (GLM) analysis was used to ascertain significant differences between temperature and pH treatments. For each dependent variable (CAT, GST, MDA, and HSP70/HSC70), temperature (2 levels: 19 and 26 °C) and pH (2 levels: 8.0 and 7.7) were used as explanatory variables, as well as their interaction. Mixed models were used to infer significant differences between replicate tanks within each treatment. Since there were no significant differences, the random effect of the tank was removed from the models. Our data was fitted using Gaussian family models. Model residuals were checked for departures from the assumed distributions and no significant deviations were found. Homogeneity and normality assumptions were checked through Levene and Shapiro tests, respectively. The most parsimonious models were selected based on the Akaike Information Criterion (Quinn and Keough 2002). Independence and leverage of the residuals were used to perform model validation. All statistical analyses were performed on R Studio (R Development Core Team 2016), using the lme4 and nlme packages.

Results

After 60 days of exposure to upcoming ocean warming and acidification conditions, no colony mortality was observed in all experimental treatments.

The activity of the antioxidant enzymes CAT and GST (Fig. 1) did not change significantly under warming and/or acidification ($p > 0.05$, GLM analysis in Supplementary Table SIII).

MDA levels (Fig. 2), a specific end-product of lipid peroxidation, also did not change significantly after exposure to ocean warming and/or acidification conditions ($p > 0.05$, GLM analysis in Supplementary Table SIII).

In contrast, the heat shock response (HSP70/HSC70, Fig. 3) was significantly enhanced under warming ($p = 0.009$, GLM analysis in Supplementary Table SIII), increasing from 210.1 ± 131.6 to 588.6 ± 271.9 μg mg^{-1} protein under normocapnia, and from 158.6 ± 179.1 to 349.0 ± 190.2 μg mg^{-1} protein under hypercapnia. Neither the effect of acidification nor the interaction between warming and acidification were significant ($p > 0.05$, GLM analysis in Supplementary Table SIII).

Discussion

In the last decade, a significant body of research has been accumulated on the impact of climate change on coral ecophysiology, mainly on reef-building species (see Supplementary Table SI). Nonetheless, the impact of future ocean conditions on soft corals remains poorly known. To the best of our knowledge, only three studies were conducted so far in order to infer the impact of rising sea temperature on octocorals (Madeira et al. 2015; Mydlarz and Jacobs 2006; Wiens et al. 2000), and none of these studies have evaluated the effect of ocean acidification or the combination between increasing temperature and acidification.

Increased seawater temperature is known for enhancing ROS production and activating antioxidant enzymes, which are essential to eliminate ROS and prevent cellular damage such as lipid peroxidation (Lesser 2011). In most hard corals, the heat shock and antioxidant defense mechanisms are triggered under warming and/or acidified conditions (see Supplementary Table SI). However, in many cases, they are not able to avoid cellular damage in coral tissues (e.g., Downs et al. 2000; Flores-Ramírez and Liñán-Cabello 2007; Ritson-Williams et al. 2016; Soriano-Santiago et al. 2013; Yakovleva et al. 2009). Contrarily to reef-building corals, the present study showed that in the octocoral *V. cynomorium*, CAT and GST activities were not significantly affected by warming and/or acidification, and neither was cellular damage caused by lipid peroxidation. On the other hand, heat shock proteins, which play an important role in thermotolerance by helping denatured proteins to stabilize and refold (Tomanek 2010), were activated as a defense mechanism against high temperature, i.e., increasing significantly under warming conditions.

It is worth mentioning that the observed high tolerance of *V. cynomorium* to such abiotic conditions is not surprising, since this species can be found in coastal shallow habitats that are daily subject to extreme abiotic fluctuations (e.g., temperature,

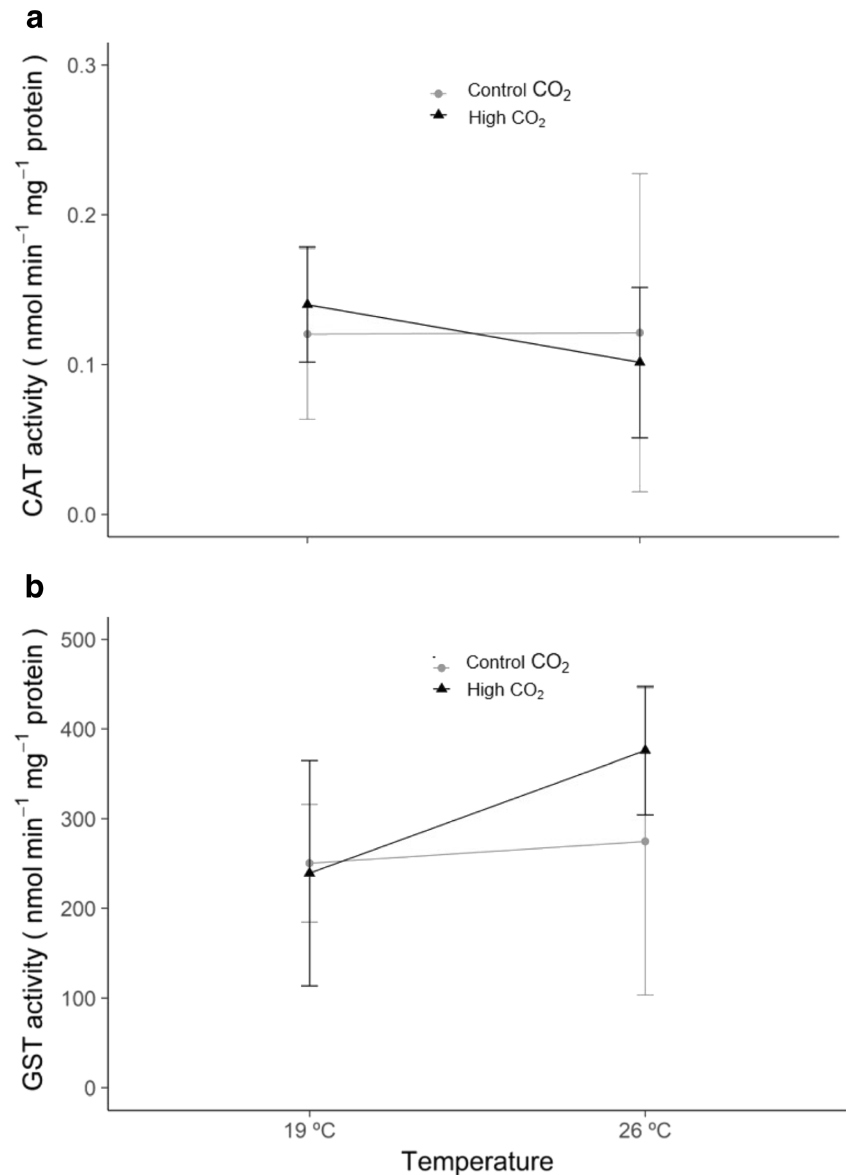

Fig. 1 Antioxidant enzyme activities in *Veretillum cynomorium* under ocean warming and acidification conditions: **a** Catalase (CAT) and **b** glutathione S-transferase (GST). Values represent mean ± standard deviation

salinity, oxygen). Indeed, this species proved to tolerate rapid cyclical fluctuations of the intertidal environment, by presenting integrated heat shock and antioxidant responses that allow them to cope with the underlying oxidative stress to which they are frequently exposed during the emersion stage (Teixeira et al. 2013) and across thermal gradients (Madeira et al. 2015). In both studies, *V. cynomorium* proved to be equipped with powerful defense mechanisms that enable them to avoid peroxidative damage under stressful conditions. Thus, in a time when climate change threatens coral reefs all over the world, the octocoral *V. cynomorium* stands out for its great resilience to warming and acidification.

Comparative studies between symbiotic and non-symbiotic corals clearly showed that symbiotic corals are more vulnerable to high temperatures and are at greater risk (Baker et al. 2008; Pandolfi et al. 2011). This may be due to the fact that increased seawater temperature leads to coral bleaching and mortality, which is primarily initiated with an overproduction of ROS in the symbionts (Downs et al. 2002; Lesser 2006; Mydlarz et al. 2009), impairing the association between corals and zooxanthellae (Glynn 1996). In contrast, octocorals such as *V. cynomorium*, instead of an endosymbiotic algae assemblage, harbor microbial communities within their tissues (Baptista et al. 2012), which might confer broader tolerance to heat and chemical stress. In fact, recent studies have brought to light that the resistance of a coral is not only determined by the coral itself, but rather to the association between all its parts, i.e., coral, zooxanthellae, and associated microorganisms (Grottoli et al. 2018; Roche et al. 2018), and that a shift in coral symbiosis elements to specific microorganisms

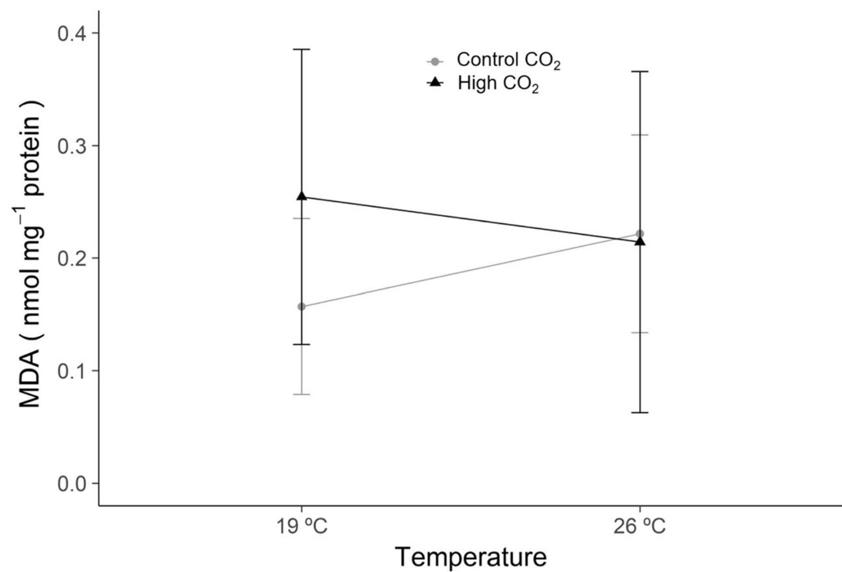

Fig. 2 Lipid peroxidation (MDA—malondialdehyde) levels in *Veretillum cynomorium* under ocean warming and acidification conditions. Values represent mean ± standard deviation

could eventually enhance their thermal resistance (Torda et al. 2017).

On the other hand, hard corals have also shown to be quite vulnerable to ocean acidification, since hypercapnia is reducing ocean carbonate ion availability and compromising the capacity of hard corals to build their skeletons (Carpenter et al. 2008). In contrast, *V. cynomorium* lacks an external calcium carbonate body. Instead, it presents a central axial skeleton composed by a fibrillar collagenous matrix calcified with calcite (Ledger and Franc 1978) and covered by an external tissue that may act as a barrier against decreased seawater pH, as previously observed in another octocoral species (Gabay et al. 2014).

In conclusion, the present study shows that exposure to ocean warming and acidification conditions did not have a negative impact on *V. cynomorium* physiology. Warmer conditions enhanced the heat shock response, a defense mechanism that allows them to tolerate higher temperatures, while the antioxidant response and cellular damage were not significantly affected. In contrast to reef-building corals that have shown to be particularly sensitive to climate-induced changes (Baker et al. 2008), the present findings show that *V. cynomorium* is a resilient species in the face of warming and acidified conditions and is expected to be able to withstand predicted future ocean conditions associated with climate change. Nevertheless, further studies are essential to evaluate the impact of future ocean scenarios on octocoral larvae, since the greater vulnerability of the early stages of development may become the bottleneck for species persistence in a changing ocean.

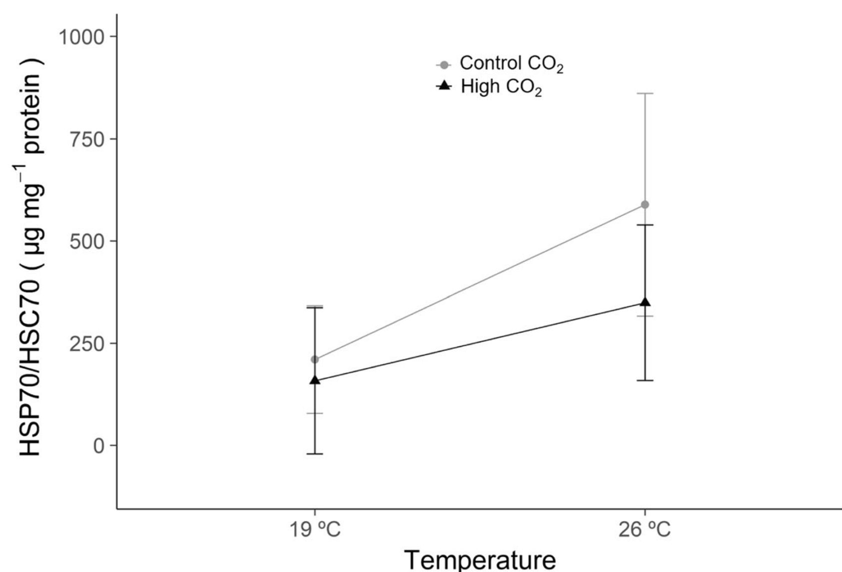

Fig. 3 Heat shock protein (HSP70/HSC70) concentrations in *Veretillum cynomorium* under ocean warming and acidification conditions. Values represent mean ± standard deviation

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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