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Physiological response of the symbiotic gorgonian *Eunicella singularis* to a long-term temperature increase

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SUMMARY

Increase in seawater temperature is one of the major effects of global climate change that affects marine organisms, including Cnidaria. Among them, gorgonians from the NW Mediterranean Sea, such as the species *Eunicella singularis*, have suffered spectacular and extensive damage. We thus investigated in a controlled laboratory experiment the response of *E. singularis* to a long-term increase in temperature and we took a special interest in its photosynthetic and calcification response to the stress. Two populations collected at 15 and 35 m depths were studied in order to determine whether there was a difference in sensitivity to thermal stress between living depths. Our results show: (a) that calcification and photosynthesis were impacted only when gorgonians were maintained for more than two weeks at 26°C, and (b) that colonies of *E. singularis* living in shallow waters were less tolerant than those living in deep waters. Because *E. singularis* is a symbiotic species, we have also discussed the potential role of symbiosis in the thermotolerance response.

Key words: gorgonians, symbiosis, calcification, photosynthesis, thermal stress, Mediterranean, climate change.

INTRODUCTION

Increase in seawater temperature is one of the major effects of global climate change. Considering the predictions of the Intergovernmental Panel on Climate Change, scientists have given alarmist predictions for the next decades on the future of several ecosystems, including the marine ones. Among them are coral reefs in tropical seawaters, which are known for many years to suffer from temperature increase with the well-described phenomenon of bleaching (Hoegh-Guldberg, 1999). Despite the growing interest of the scientific community to model how corals will respond to climate change, 'there is still much to learn about the stress response of corals and their inherent variability in those responses so we can make better predictions about the future effects of global climate change on coral reefs' (Lesser, 2007). If this observation is totally true for tropical corals, it can also be widened to other marine organisms and especially gorgonians from the NW Mediterranean Sea. Indeed major mortality events of marine benthic invertebrates, including Cnidaria, have occurred in the years 1999 and 2003 in the NW Mediterranean Sea, and have been related to positive temperature anomalies (Cerrano et al., 2000; Garrabou et al., 2001; Garrabou et al., 2009; Linares et al., 2005; Perez et al., 2000). Thermal anomalies during these mortality events were either characterised by moderate (+1 or 2°C above mean values) increases of sea surface temperature on a long-term period [one month such as in 1999 (Cerrano et al., 2000; Romano et al., 2000)] or by larger increases (up to 7.5°C) during a few hours such as in 2003 (Harmelin, 2004).

Among cnidarians, gorgonians have suffered spectacular and extensive damage, the species most affected depending on the location. *Eunicella singularis*, for example, suffered extensive damage both in 1999 and 2003 with mortality rates up to 90% (Garrabou et al., 2009; Perez et al., 2000). Despite these major mortality events, studies aimed at understanding the physiological basis of the response of these marine organisms towards an increase in temperature are still scarce or even lacking. Indeed, while three studies have investigated the response of the symbiotic Mediterranean coral *Cladocora caespitosa* to temperature increase both in laboratory and *in situ* conditions (Rodolfo-Metalpa et al., 2006a; Rodolfo-Metalpa et al., 2006b; Rodolfo-Metalpa et al., 2008), showing that five weeks at temperatures above 24°C were damaging the corals, only one experimental laboratory study has been performed on the gorgonian species *Corallium rubrum*, showing that the thermotolerance response was different depending on the depth at which colonies lived (Torrents et al., 2008).

The aim of our work was therefore to investigate the physiological response of the Mediterranean gorgonian *E. singularis* to a long-term increase in temperature. For this purpose, we chose to perform an experimental approach because it is the best way to assess the effect of temperature on the different physiological parameters investigated when no other preliminary studies have been performed on such gorgonian species. Two populations of *E. singularis*, one collected at 15 m depth and the other at 35 m depth, were studied, in order to determine whether there was a difference in their sensitivity to thermal stress. We maintained each population at the closest irradiance level as that measured in the field, to get the best answer of the gorgonian to the thermal stress. Because *E. singularis* is symbiotic, containing photosynthetic dinoflagellates (commonly called zooxanthellae), we studied both the photosynthetic and calcification parameters. Temperature increase is indeed known to severely damage the photosynthetic apparatus of the zooxanthellae, in symbiosis with both tropical (Jones et al., 2000; Warner et al.,

1996; Warner et al., 1999) and Mediterranean anthozoans (Rodolfo-Metalpa et al., 2006a; Rodolfo-Metalpa et al., 2006b), inducing a decrease in the maximal photochemical efficiency of the photosystem II (PSII) (Warner et al., 1996; Warner et al., 1999) and affecting several parameters (Venn et al., 2008). Temperature is also known to change calcification rates, both in tropical (Clausen, 1971; Clausen and Roth, 1975) and temperate corals (Howe and Marshall, 2002; Rodolfo-Metalpa et al., 2006a) as well as in gorgonians (Allemand and Grillo, 1992; Torrents et al., 2008; Velimirov and King, 1979). Indeed in gorgonians, calcification can be studied through the formation of spicules. These spicules are individual minute inclusions of calcium carbonate, which occur over an extended region of the animal's body and are secreted by scleroblasts in the extracellular mesoglea (Goldberg and Benayahu, 1987; Kingsley, 1984; Kingsley and Watabe, 1982). Moreover, in soft and scleractinian tropical corals, it has been shown that there is a link between photosynthesis and calcification, a process that is called light-enhanced calcification (LEC) and involves an enhancement of calcification by zooxanthellae photosynthesis (for a review, see Gattuso et al., 1999). As no study has ever been performed in Mediterranean gorgonians we also looked at this parameter and the effect of temperature on LEC in *E. singularis*.

MATERIALS AND METHODS

Characterisation of *in situ* thermal regime

Temperature (*T*) data recorded since June 2003 at 15 and 35 m depths at Riou Island (<7 nautical miles from Planier Island) were examined to document the vertical temperature gradients and past thermal history of the shallow and deep populations sampled. Temperature was recorded hourly by *in situ* autonomous sensors (StowAway Tidbit, Bourne, MA, USA; –5 to +35°C; ±0.2°C). Annual temperature cycle was examined from May 2006 to May 2007 (*N*=9267). In addition, mean vertical gradients, maximal temperature (T_{max}) and total duration above 24°C were calculated for the warm period (July–September) from 2003 to 2006.

Biological material

Experiments were carried out on two populations of the gorgonian *Eunicella singularis* Esper 1794 inhabiting the rocky coast of Marseille at the 'Planier Island' (43°11′54,27″N 05°13′48,71″E, NW Mediterranean, France). These populations were located in shallow (15 m) and deep (35 m) waters. About 160 colony tips (between 5 and 7 cm in length) of healthy gorgonian colonies from both populations were randomly sampled by SCUBA diving in May 2007 from *ca*. 100 mother colonies. Samples were then maintained in an aerated cooled water box and immediately transported to the laboratories. Gorgonian tips were then placed in experimental tanks (see below).

Experimental design

The experimental set up consisted in two 142 l buffer tanks continuously supplied with oligotrophic Mediterranean seawater pumped from 50 m depth at a flow rate of 1–3 l min^{-1}. Each buffer tank supplied seawater to four experimental 27 l tanks, containing for two of the tanks the 15 m gorgonian population and for the other two tanks the 35 m population. Acclimation to laboratory conditions lasted for two weeks with a constant temperature of 18±0.5°C. Then the first system (one buffer + four small tanks) was maintained under a 'control' temperature of 18°C during the whole experiment while temperature was regularly increased in the second system (Fig. 1). Temperature increase mimicked a thermal stress event, such as monitored in the Mediterranean Sea (Rodolfo-Metalpa et al., 2006a).

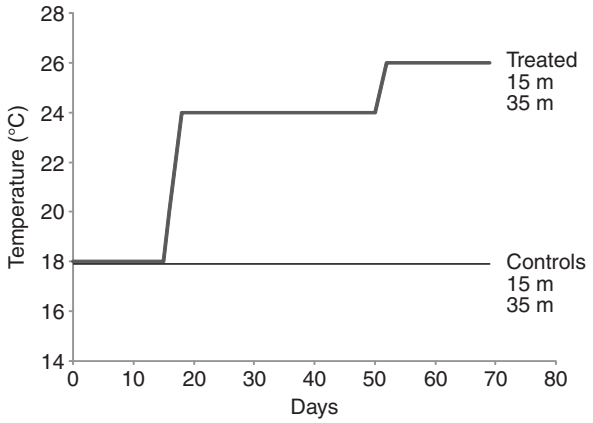

Fig. 1. Experimental design showing the temperature increase as a function of the incubation period for the control and treated samples.

The temperature was raised from 18°C to 24°C in one day and kept constant at 24°C for 33 days. After this first period, temperature was raised again from 24°C to 26°C in one day and was kept constant at 26°C for 19 days. Irradiance (12 h:12 h light:dark cycle) was set up to match the light intensity that the populations received in the field as closely as possible, and was therefore equal to 100 and 50 µmol photons m^{-2} s^{-1} for the population of 15 and 35 m, respectively. Seawater temperature was maintained constant using temperature controllers (Toshniwal N6100, Toshcon®, West Instruments, Brighton, East Sussex, UK; ±0.1°C) and submersible resistance heaters (Visi-Therm® Deluxe, Aquarium Systems, Sarrebourg, France). Salinity values were constant at 38 PSU. Colonies were fed twice a week with a combination of an equal quantity of krill, nauplii of *Artemia salina*, grinded frozen shrimps and mussels (total quantity per tank: 4 g of food). All tanks were regularly cleaned in order to avoid algal growth on the walls. Samples were collected once a week for measurements of calcification rates and photosynthetic parameters.

Measurements

Calcification rates

Calcification rates were measured by incubating colony tips (five replicates) with ^{45}Calcium (^{45}Ca) and measuring the radioisotope uptake in the spicules according to the protocol adapted from Grillo et al. (Grillo et al., 1993). Briefly, colony tips were removed from the experimental tanks and incubated for 24 h in 25 ml beakers of filtered seawater (FSW, filtration on 0.45 µm Millipore membranes, Molsheim, Alsace, France) containing 400 kBq of ^{45}Ca (as $^{45}CaCl_2$, Perkin-Elmer®, Courtaboeuf, Essonne, France). Salinity, light and temperature in the beakers were the same as in the experimental set up. Samples (100 µl) of seawater were counted during each incubation period for the determination of specific radioactivity. Water movement was maintained with a magnetic stirring bar. At the end of the incubation, the samples were rapidly rinsed in 1 l of unlabelled FSW (<1 min). Because there was no non-specific adsorption of radioactivity on the spicules (data not shown), an efflux period was not necessary. Tissues were then completely dissolved in NaOCl 9.6% at room temperature for 10 min under stirring. The spicules were separated from the tissues by centrifuging at 4000 ***g*** for 1 min. The supernatant was discarded and the pellet containing the spicules was rinsed in 4 ml of distilled water. Rinsing was performed three times and each time the solution was centrifuged at 4000 ***g*** for 1 min and the supernatant was discarded. Finally the

pellet of spicules was dried overnight at 90°C and weighed. Spicules were then dissolved in HCl 6 mol l^{-1}. Aliquots of the spicules fraction were counted with 4 ml of Ultima Gold AB (Perkin Elmer 6013309). Beta-emission was measured using a liquid scintillation counter (2100 TR Packard, Tricarb, Courtaboeuf, Essonne, France). Results are expressed as nmol Ca. mg spicules^{-1} h^{-1} .

Chlorophyll *a* fluorescence of PSII

Maximal photosynthetic efficiency of PSII was measured on dark-adapted gorgonian tips (six replicates per condition) using a PAM fluorometer [DIVING-PAM, Walz, Germany (Schreiber et al., 1986)]. During measurements, a black jacket of neoprene was mounted on the free-end of the 8 mm optical fibre in order to keep a fixed distance of 5 mm from the gorgonian surface. The minimal (F_0) and maximal (F_m) fluorescence yields were measured by applying a weak pulsed red light (max. intensity <1 mol photon m^{-2} s^{-1}, width 3 µs, frequency 0.6 kHz) and a saturating pulse of actinic light (max. intensity 5000 µmol photon m^{-2} s^{-1}, width 800 ms), respectively. The maximum photosynthetic efficiency was calculated as follows: $F_v/F_m=(F_m–F_0)/F_m$, where F_v is the variable fluorescence.

The relative electron transport rate (rETR) was also assessed using the rapid light curve (RLC) function of the PAM fluorometer, followed by 5 min relaxation in the dark for five tips maintained in each condition. The effective quantum yield ($\Delta F/F'_m$) and the rETR were therefore measured after exposure during 10 s to eight light intensities (from 0 to 1155 µmol photons m^{-2} s^{-1}). At the end of the last light level of the RLC, tips remained in the dark and $\Delta F/F'_m$ was determined after 30 s, 1, 2 and 5 min. The non-photochemical quenching (NPQ), a measure of the heat-dissipation process of excessive absorbed energy from PSII, was also calculated during the RLC and the relaxation period. The kinetics of relaxation of NPQ is a method that allows the discrimination of the processes that led to the dissipation of excess absorbed light. Two main components of NPQ can be distinguished: (i) the energy-dependent quenching (qE), which is a protective mechanism, which relaxes quickly (<10 min); and (ii) the photoinhibitory quenching (qI), which results from damage to photosystems and relaxes much slowly [>10 min to hours (Ralph and Gademann, 2005)]. In non-stressful culture conditions, NPQ would dissipate quickly following the exposition to a saturating light pulse. By contrast, if the PSII experiences photodamage, NPQ relaxes much more slowly.

Photosynthesis and respiration rates

Oxygen changes were assessed at 0 (respiration, R) and at the culture irradiance [net photosynthesis (Pn) at 100 and 50 µmol photons m^{-2} s^{-1} for the 15 and 35 m depth population, respectively]. Photosynthesis and respiration rates were measured in all conditions, at the end of each temperature step, on six gorgonian tips for each condition. Tips were incubated in small glass chambers, thermostated to the given temperature (from 18°C to 26°C according to the treatment). Each chamber was equipped with a Strathkelvin 928 oxygen electrode (North Lanarkshire, Scotland, UK), filled with 0.45 µm FSW and continuously stirred with a stirring bar. The electrodes were calibrated before each experiment against air-saturated and nitrogen-saturated seawater for the 100% and 0% oxygen, respectively. Changes in dissolved oxygen concentrations were monitored on a computer during 15 min, at each light level for each gorgonian population. Light was provided by a metal halide lamp (Philips, HPIT 400 W, Distrilamp, Bossee, Indre et Loire, France). Rates of Pn and R were estimated by regressing oxygen data against time, taking into account the seawater volume in the chamber. Gross photosynthetic rates (Pn+R) were normalised to chlorophyll *a*.

Chlorophyll concentration and surface determination

At the end of each temperature step, six samples were frozen at –80°C for chlorophyll measurements (chl *a* and c_2). Pigments from each tip were extracted at 4°C during 24 h in 95% acetone. The extraction was repeated twice to get all pigments. The extracts were centrifuged at 10,000 ***g*** for 15 min, and chl *a* and c_2 were measured according to the method of Jeffrey and Humphrey (Jeffrey and Humphrey, 1975). Data were normalised to the surface area of the tips. As tips were regular, the diameter and the length of each tip were measured using a calliper and the surface determined.

Statistical treatment of the data

Statistics were performed using SAS 9.1 (Cary, NC, USA). Because all parameters remained constant during each step of temperature tested [repeated-measures analysis of variance (ANOVA), $P>0.05$], only the last measurements for each temperature step were tested and presented in the following results. Two-way ANOVAs, taking into account the 'population depth' and the 'temperature' factors, were performed on all parameters except F_v/F_m. Temperature was the main factor that we wanted to test in this study. Population depth was however tested along with the temperature, even if light levels were different for each population. Results obtained were used to discuss the differences that might have occurred *in situ* for the two populations. When ANOVAs showed significant differences, Tukey honest test (HSD) was used to attribute differences between specific factors or their interaction only. All data were expressed as means ± standard deviation (s.d.). All data were also tested for assumptions of normality and homoscedasticity using a Shapiro–Wilk test and were transformed when required (as for chl c_2). However, for the F_v/F_m, no transformation could be used. Therefore, the effects of depth and temperature were tested using the two-way rank approach of Conover, followed by a Dunn test when significant. Normality of the ranks was tested using the Shapiro–Wilk test.

RESULTS

Characterisation of *in situ* thermal regime

Temperature records at 15 and 35 m depths from May 2006 to May 2007 are shown in Fig. 2. On an annual basis, mean *T* differences between the two depth levels was only 1°C (17.1±2.9°C *vs* 16.1±2.4°C) but it could punctually reach 10°C during the seasonal stratification. Annual *T* range varied from 12.7°C to 9.7°C at 15 and 35 m depth, respectively. Large and rapid *T* fluctuations were observed at both depth levels. Indeed, the seasonal thermal stratification was frequently disrupted from August to October, with a sudden *T* drop below 14°C, consecutive to coastal upwelling of deep and cold waters triggered by northern winds.

Examination of Table 1 shows vertical gradients of about 3°C for mean *T* during the warm period (July–September) and contrasted T_{max} (22–23.8°C *vs* 25–25.7°C).

Colony tips and spicules

Colony tips and spicules used in this study are shown in Fig. 3. Colony tips had a diameter varying from 2 to 3 mm. Polyps were about 3 mm long when fully expanded (Fig. 3A,B). The brown colour of expanded polyps in bright light (Fig. 3A,B) was due to the presence of zooxanthellae, which appeared in red when illuminated with fluorescent blue light (Fig. 3C). When polyps were fully retracted, the colony tips appeared white due to the spicules present

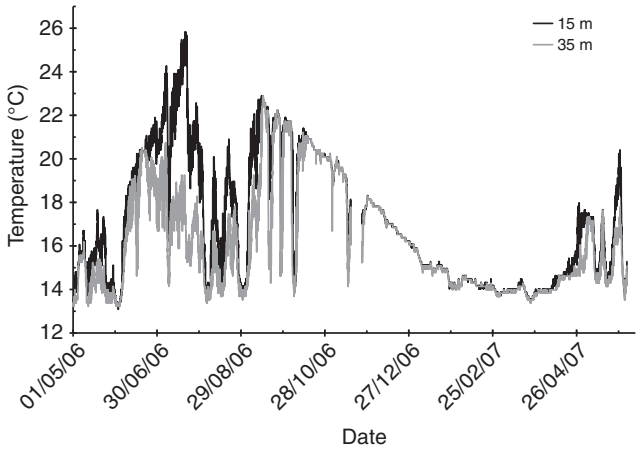

Fig. 2. Hourly temperature records at 15 and 35 m depths (black and grey lines, respectively) from May 2006 to May 2007 at Riou Island (Marseille, France).

in the tissues (Fig. 3D). Physiological experiments were only performed with colony tips having polyps fully expanded. Spicules used to measure calcification after incubation in ^{45}Ca are shown in Fig. 3E,F.

Light-enhanced calcification

Fig. 4 shows the calcification rates of the spicules of the population from 15 m under light (24 h at 100 µmol photons $m^{-2} s^{-1}$) and dark conditions (24 h at 0 µmol photons $m^{-2} s^{-1}$) at 18°C. The rate was about 1.7 higher in the light than in the dark. This result shows that the spiculogenesis in *E. singularis* was dependent on light and could be described under the term of 'light-enhanced calcification' as for scleractinian corals.

Effect of thermal stress on calcification

Values of the calcification rates of the spicules from the two populations of *E. singularis* during the thermal stress are shown in Fig. 5. At 18°C (control conditions), calcification rates of the 15 m population were higher than those of the 35 m population (respectively, 0.91±0.06 nmol Ca. mg spicules^{-1} h^{-1} *vs* 0.78±0.05 nmol Ca. mg spicules^{-1} h^{-1}), even if the difference was not significant (Table 2). There was also no significant difference in the calcification rates of the heated (24°C) and control samples (18°C) independently of the population considered (0.89±0.12 nmol Ca. mg spicules^{-1} h^{-1} at 24°C *vs* 0.91±0.06 nmol Ca. mg spicules^{-1} h^{-1} at 18°C for the 15 m depth population and 0.68±0.09 nmol Ca. mg spicules^{-1} h^{-1} at 24°C *vs* 0.78±0.05 nmol Ca. mg spicules^{-1} h^{-1} at 18°C for the 35 m depth population) (Fig. 5; Table 2). Calcification rates were however significantly lower for the 15 m depth population maintained at 26°C than at 24°C (0.46±0.02 nmol Ca. mg spicules^{-1} h^{-1} at 26°C v*s* 0.89±0.12 nmol Ca. mg spicules^{-1} h^{-1} at 24°C) (Fig. 5; Table 2). No significant temperature effect was observed for the population of 35 m depth maintained at 26°C (0.73±0.08 nmol Ca. mg spicules^{-1} h^{-1} at 26°C *vs* 0.68±0.09 nmol Ca. mg spicules^{-1} h^{-1} at 24°C) (Fig. 5; Table 2). The different responses of the two populations to temperature increase indicate that there was a combined effect of temperature and depth on the calcification rates (Table 2).

Chlorophyll concentrations

Chl *a* and c_2 concentrations were significantly different between gorgonian populations with a higher content in the 15 than in the 35 m population maintained under control conditions (Table 2). Temperature had a strong effect on the chlorophyll contents of the gorgonians, because concentrations of both chlorophylls significantly decreased at 26°C (Fig. 6; Table 2), this decrease being comparable in both populations.

Photosynthetic efficiency of PS II, rates of photosynthesis and respiration

The maximal photosynthetic efficiency, F_v/F_m, was not significantly different between the two gorgonian populations, for all conditions (Table 2). At the control temperature of 18°C, values were maximal (F_v/F_m=0.6). Temperature increase had a significant effect on the F_v/F_m, mostly between 24 and 26°C (Table 2); however, no change was monitored between 18 and 24°C. The maximal values of 0.64–0.65 at 18 and 24°C therefore decreased to 0.60–0.61 at 26°C for the 15 and 35 m populations, respectively (Fig. 7). The amplitude of the shift in F_v/F_m was not significantly different between the two populations.

To compare RLC, we used the values of the maximal electron transport rate (ETR_{max}). RLC were significantly different between populations and temperature conditions (Table 2). At the control condition of 18°C, ETR_{max} was significantly higher for the shallow population than for deep one (Fig. 8, 65 *vs* 50). Increasing temperature from 18 to 24°C had no significant effect on the ETR_{max}. However, an increase to 26°C significantly decreased the ETR_{max} of both populations after one week incubation and further decreased it after two additional weeks (Fig. 8; Table 2). The decrease in ETR_{max} was larger for the shallow population than for the deep population. Gorgonians from both populations maintained at 18 and 24°C showed a small increase in the minimum fluorescence (F_0) during the RLC, corresponding to a decrease in maximal fluorescence (F'_m) (data not shown) and an increase in NPQ (Fig. 9). After relaxation of the PSII in the dark for 10 min, the NPQ rapidly decreased to a value <0.1, showing that most of the quenching was energy dependent (qE). Conversely, gorgonians from both populations maintained at 26°C presented the lowest ETR_{max} values, a high increase in the minimum fluorescence yield (F_0), reflecting the closure of the photosystems (photochemical quenching). During the whole

Table 1. Comparative analysis of warm period (July–September) temperature conditions at 15 and 35 m depth in Marseille area from 2003 to 2006

Year	15–35 m gradient (°C)	T_{max} 15 m (°C)	Duration 24°C (days)	T_{max} 35 m (°C)
2003	2.9	26.7	2.2	22
2004	2.9	25.3	4	23.1
2005	2.8	25	1.7	23.8
2006	2.7	25.8	4.7	22.9

T_{max} is maximum temperature.

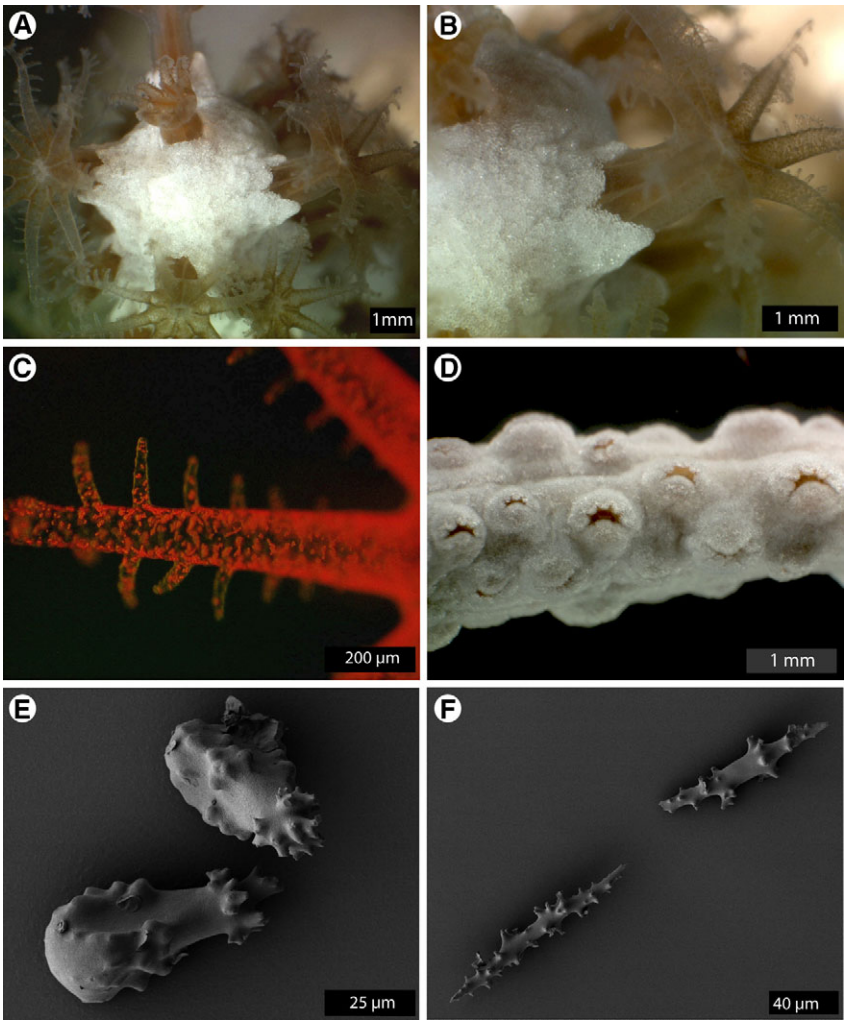

Fig. 3. Photographs of colony tips and spicules of *Eunicella singularis*. (A) Expanded polyps (in brown) and spicules (in white) of a tip. (B) Magnification of (A) showing an expanded polyp with tentacles. (C) Tentacle of a polyp showing red zooxanthellae when illuminated with fluorescent blue light. (D) Tip with retracted polyps appear white due to spicules. (E,F) Spicules with different shapes observed in scanning electron microscopy.

relaxation period in the dark, NPQ did not decrease, suggesting that a significant fraction of the quenching was due to qI and not to qE.

Rates of gross photosynthesis were very low (10- to 100-fold) compared with values usually obtained with tropical or temperate corals. They ranged from 2.5±2.0 to 4.0±2.5 nmol O_2 µg^{-1} chl *a* h^{-1} for the 35 and 15 m depth populations, respectively. These low values, with large variations within treatments, were due to the limitations of our technique, using small tips of gorgonians. Therefore, these data are just indicative of a level of photosynthesis.

DISCUSSION

This study provides the first physiological data on the thermotolerance of the Mediterranean gorgonian species, *E.*

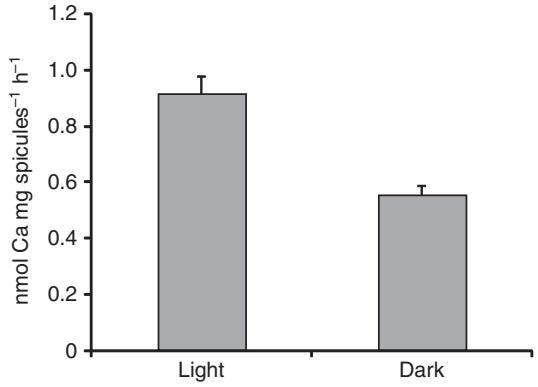

Fig. 4. Calcification rates of the spicules for the 15 m depth population under light (100 µmol photons m^{-2} s^{-1}) or dark conditions (0 µmol photons m^{-2} s^{-1}) at 18°C.

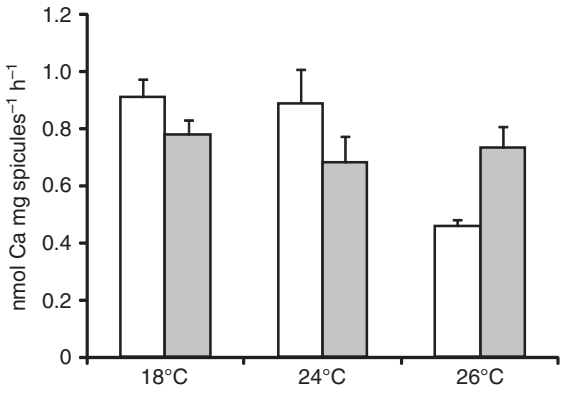

Fig. 5. Calcification rates of the spicules *vs* incubation temperature. White bars for the population from 15 m depth and grey bars for the population from 35 m depth.

Table 2. Statistical results on the effects of population depth (15 or 35 m) and seawater temperature (18, 24 and 26°C) on the main physiological parameters

	d.f.	*F*	*P*	*Post-hoc* test
Chlorophyll *a*				
Population depth	1	9.83	0.003*	15 m≠35 m
Temperature	2	37.29	<0.0001*	18°C≠26°C; 24°C≠26°C
Depth × temperature	2	2.28	0.1133	
Chlorophyll c_2				
Population depth	1	4.61	0.039*	15 m≠35 m
Temperature	2	21.21	<0.0001*	18°C≠26°C; 24°C≠26°C
Depth × temperature	2	1.35	0.2700	
F_v/F_m				
Population depth	1	0.70	0.4107	
Temperature	2	7.50	0.0031*	18°C≠26°C; 24°C≠26°C
Depth × temperature	2	0.17	0.8485	
ETR_{max}				
Population depth	1	14.78	0.0007*	15 m≠35 m
Temperature	2	6.03	0.0071*	18°C≠26°C; 24°C≠26°C
Depth × temperature	2	0.52	0.5992	
Calcification rates				
Population depth	1	0.09	0.7583	
Temperature	2	5.59	0.005*	24°C≠26°C
Depth × temperature	2	5.92	0.003*	

Significant *P* values are labelled with *. ≠ means significantly different. F_v is the variable fluorescence, F_m is maximal dark-adapted fluorescence, ETR_{max} is maximal electron transport rate.

singularis. To mimic conditions experienced *in situ* by the gorgonians, the difference in irradiance levels between the two sampling depths was conserved. Results demonstrated the extreme thermal resistance of this gorgonian compared with the other temperate cnidarians tested to date (Rodolfo-Metalpa et al., 2006a; Torrents et al., 2008), because temperature affected *E. singularis* only when it rose up to 26°C.

A long-term exposure of 33 days to 24°C, as might happen *in situ*, did not affect the rates of calcification and the photosynthetic parameters measured for both the shallow (15 m) and deep (35 m) populations of *E. singularis*. For calcification, this result is in contrast to those obtained for the two other Mediterranean cnidarian species previously investigated, the symbiotic coral *C. caespitosa* and the asymbiotic gorgonian *C. rubrum*, for which the same 6°C increase in temperature, from 18°C to 24°C, induced a significant decrease in calcification after 34 and 21 days, respectively (Rodolfo-Metalpa et al., 2006a; Torrents et al., 2008). The same comparison can be made with the photosynthetic parameters, which remained constant and unaffected in the gorgonian *E. singularis*, while the F_v/F_m, ETR_{max} as well as the rates of photosynthesis decreased in *C. caespitosa* after the same incubation length.

This result has several implications for the understanding of the *in situ* response of the different anthozoan species. Indeed, by maintaining a temperature of 24°C during 33 days, this experiment aimed at reproducing a thermal stress event as those having occurred in the NW Mediterranean Sea these last 10 years. Elevated temperatures were indeed suggested to be the primary cause of benthos mortality in 1999 (Cerrano et al., 2000; Garrabou et al., 2001), because they remained at 24°C for more than one month in surface waters [from mid August 1999 to the end of September 1999 (Perez et al., 2000; Rodolfo-Metalpa et al., 2006a)]. Even if similar studies have shown an impact of such temperatures on the physiology of *C. caespitosa* and *Oculina patagonica* (Rodolfo-Metalpa 2006b), results obtained in the present study show that a temperature of 24°C maintained during several weeks is not the primary cause of *E. singularis* death. Genetic differences in the populations cannot be excluded to explain the different sensitivity to high temperatures but mortality can also be a combined effect of punctual high temperatures and past history of long-term elevated temperatures. *In situ* mortalities all occurred in September, at the

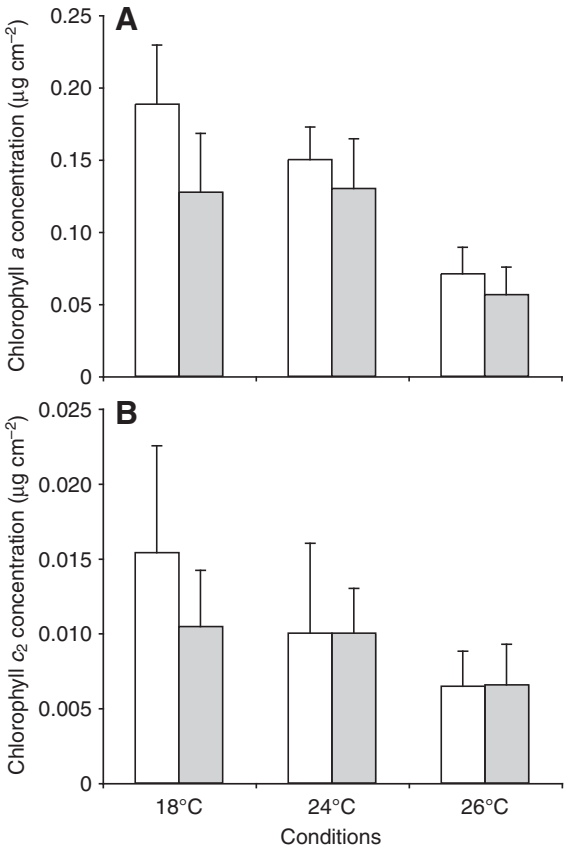

Fig. 6. Chlorophyll *a* (A) and c_2 (B) concentrations (µg cm^{-2}) measured at the end of the different treatments, for the 15 m (white bars) and 35 m (grey bars) populations, respectively.

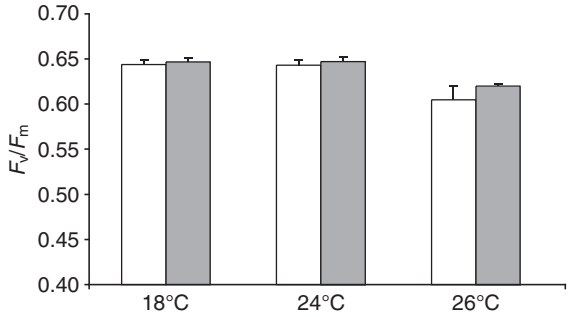

Fig. 7. Maximal photosynthetic efficiency measured at the end of the different treatments, for the 15 m (white bars) and 35 m (grey bars) populations, respectively.

end of the warm period. Conversely, the populations studied in this experiment were collected in May 2007, and did not experience increases in temperature during the whole summer period. They were also not engaged in their reproductive effort [which generally occurs in late May–June (Ribes et al., 2007)], which can weaken organisms by pumping a large part of their energy supply. *In situ* mortalities can also be the combined effect of high temperatures and lack of food. Indeed, in the field, it has been suggested that some anthozoan populations are suffering from starvation during the whole summer (Coma et al., 2006; Coma et al., 2009) because thermocline prevents the upwelling of nutrients and the populations are thus more sensitive to stress. Conversely, in our experiments, populations were fed, because we didn't want to induce an additional stress. Feeding might have had a positive effect on the gorgonian physiology, as already observed in tropical corals (Borell et al., 2008) and Mediterranean gorgonians (Coma et al., 2009). This different supply in nutrients can also explain the different impacts of elevated temperatures between the east and west Mediterranean (Perez et al., 2000).

Results obtained also showed that both gorgonian populations could not afford the second step of the thermal stress event, consisting in an additional temperature increase of 2°C (from 24 to 26°C). The photosynthetic parameters of the shallow and deep samples of *E. singularis* were particularly affected. Indeed, there was a rapid decrease in the maximal photosynthetic efficiency (F_v/F_m) and in the maximal rETR. Moreover, the dark relaxation kinetics (NPQ curves) showed that, contrary to control gorgonians, the photosystem did not recover well from the stress, because it did not show a great ability to dissipate energy in the dark (Ralph and Gademann, 2005). Indeed, in control gorgonians the NPQ first increased in the light but rapidly relaxed in the dark within 5 min (recovered its initial value), suggesting that most of the quenching was energetic (qE). Only 20% was associated to the qI. Conversely, NPQ in gorgonians maintained at 26°C did not recover its initial value, suggesting that the quenching was mostly due to photoinhibition (qI).

For the shallow population, calcification rates were also impacted by the additional increase in temperature and decreased by 2-fold in parallel to the decrease in the photosynthetic capacity. Since we have shown in the Results section the existence of a LEC process in the spicules of *E. singularis*, the decrease in calcification during the thermal stress should certainly be linked to a decrease in photosynthesis. This decrease was also in the same range (50%) than the decrease observed between light and dark conditions (40%). The diminution of the photosynthetic parameters and/or the chlorophyll concentration during the thermal stress, could explain the inhibition of the LEC process for the population of 15 m since it has been shown that this process is the first parameter of calcification specifically affected during an environmental stress (Moya et al., 2008).

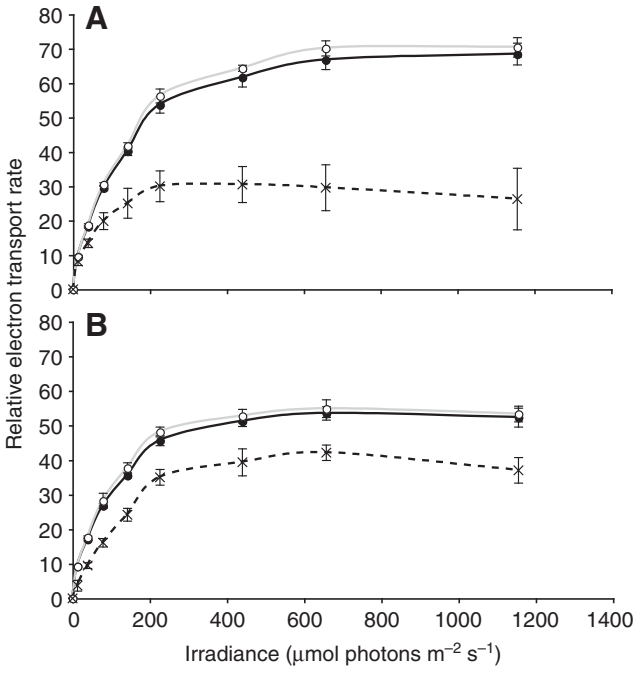

Fig. 8. Rapid light curves (RLC) obtained for the 15 m population (A) and the 35 m population (B) at the end of the incubation at 18°C (black circles), 24°C (white circles) and 26°C (crosses).

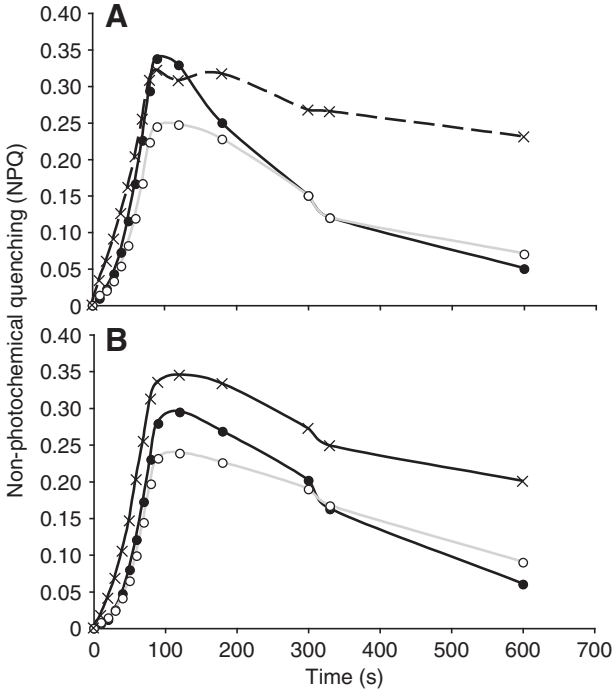

Fig. 9. Non-photochemical quenching (NPQ) curves, obtained for the 15 m population (A) and the 35 m population (B) at the end of the incubation at 18°C (black circles), 24°C (white circles) and 26°C (crosses).

Even if this point remains to be further investigated, the effect of the 26°C increase in temperature seemed to be different between the shallow and deep populations, and higher for the shallow population, for which photosynthetic activity but also calcification rates were impacted. This result is again different to what was obtained in the laboratory for the calcification of *C. rubrum*, where the shallow population was consistently less sensitive to thermal stress than the deeper one (Torrents et al., 2008). In the field, shallow populations are usually exposed to higher temperatures than the deep ones. If we refer to what is known in scleractinian corals, the upper thermal limits are correlated with their previous history, with a lower sensitivity for thermal pre-stressed symbiotic species (Middlebrook et al., 2008). This theory seems to be applicable to the non-symbiotic *C. rubrum*, for which populations exposed to large temperature variations in the field are less sensitive to thermal stress in the laboratory. However, the response is more complex with symbiotic species, when light is interacting with temperature. Again, it was demonstrated in tropical corals that the effect of temperature was exacerbated under high light conditions (Lesser and Farrell, 2004; Torregiani and Lesser, 2007). In the present study, but also *in situ*, shallow gorgonian populations are experiencing higher levels of irradiance than deep populations. They therefore also showed a higher photosynthetic activity than the deep population, which might have weakened the organisms by producing more free radicals with which they have to cope. Animal cnidarian cells have set up anti-oxidative defence systems but when these defences are insufficient (especially during an environmental stress event) the active oxygen species accumulate and damage the cells (Richier et al., 2005; Merle et al., 2007). In tropical corals such a phenomenon leads to bleaching, which was also observed in the symbiotic Mediterranean coral *C. caespitosa* (Rodolfo-Metalpa et al., 2006a), as well as in *E. singularis*, in this study (Fig. 6). Several other hypotheses can also be proposed to explain the different response between shallow and deep gorgonian populations, such as a weakening of the shallow population by elevated surface temperatures in summer 2006 (up to 26°C) (Fig. 2; Table 1) or genetic variations among gorgonians.

Conclusion

Understanding how Mediterranean marine ecosystems will respond to climate change is a question of fundamental interest for sciences, politics and management. The present study has demonstrated that for the gorgonian *E. singularis* calcification and photosynthesis are two good tools to evaluate organism health state. Calcification was already a good tool for another Mediterranean anthozoan *C. rubrum* (Torrents et al., 2008), and the maximal photosynthetic efficiency was also proved to be an excellent tool for all photosynthetic tropical and temperate anthozoans (Fitt and Warner, 1995; Hill et al., 2004; Jones et al., 2000; Warner et al., 1996; Rodolfo-Metalpa et al., 2006). The present study also highlighted the difficulties in assessing a clear response of these Mediterranean anthozoans to a thermal stress, due to the low number of investigations performed until now. Indeed, whereas *E. singularis* showed susceptibility to thermal anomalies during the natural thermal stress events of 1999 and 2003, it was more resistant under laboratory conditions. Moreover, shallow populations seemed to be less tolerant than those living in deep waters, and the role of the symbiosis in this different tolerance pattern has been discussed.

Complementary experiments are need to answer to the hypotheses proposed in this manuscript, such as: (1) the comparison with a non-symbiotic *Eunicella* (such as *E. cavolinii*) and with other gorgonians such as *Paramuricea clavata*; (2) the genetic determination of the populations which are sampled for thermotolerance studies; and (3) the environmental history of the site in which the samples are collected and especially the light profile and the nutritional status. Despite the need of these necessary data for a better understanding of the thermotolerance response of gorgonians, one very interesting result of this study is that the physiological parameters that we have measured in laboratory are good indicators of the thermotolerance features of gorgonians and can thus be used to help discriminate populations which in the field are more resistant than others and thus help managers to protect marine areas.

LIST OF ABBREVIATIONS

Chl	chlorophyll
ETR_{max}	maximum electron transport rate
F	fluorescence
F_0	minimum dark-adapted fluorescence
F_m	maximal dark-adapted fluorescence
F'_m	maximal light-adapted fluorescence
FSW	filtered seawater
F_v	variable fluorescence
$\Delta F/F'_m$	effective quantum yield
LEC	light-enhanced calcification
NPQ	non-photochemical quenching
Pn	net photosynthesis
PSII	photosystem II
qE	energy-dependent quenching
qI	photoinhibitory quenching
R	respiration
rETR	relative electron transport rate
RLC	rapid light curve
T	temperature
T_{max}	maximum temperature

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