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Depth-dependant thermotolerance of the symbiotic Mediterranean gorgonian *Eunicella singularis*: Evidence from cellular stress markers

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ABSTRACT

Over the last decades, increases in Mediterranean seawater temperature have caused several mass mortality episodes in gorgonian populations. Along the Northwestern Mediterranean coast, *Eunicella singularis*, the only symbiotic Mediterranean gorgonian, was particularly affected. The mortalities were observed to be "patchy", however, suggesting differential resistance within this species. Following the same experimental design used in Ferrier-Pagès et al. (2009), *E. singularis* tips were collected at −15 and −35 m depth (Marseilles, France) and then subjected to two steady-step temperature increases under aquarium conditions: the first from 18 °C to 24 °C over 33 days and the second up to 26 °C over 19 days. Consequences of hyperthermia were evaluated by studying three cellular stress markers: the total oxyradical scavenging capacity (TOSC), ubiquitinated proteins and heat-shock protein (Hsp70) levels. This is the first time that these markers have been studied in such a temperate gorgonian. The first temperature increase to 24 °C did not produce any response in stress marker levels. In contrast, the 26 °C step generated a transient stimulation, 24 h after the increase, of antioxidant defenses for both populations. After 2 weeks of exposure to 26 °C, no Hsp70 induction was detected, but an increase in protein damages indicated a lower resistance in shallow populations corroborating the previous observation of differential resistance capacity between deep and shallow populations of *E. singularis*, with a higher thermotolerance in deep populations. The intensification of mass mortality episodes highlights the urgency to improve our knowledge of thermal resistance mechanisms. Such knowledge will help administrators to establish conservation plans to protect these emblematic gorgonians.

1. Introduction

During the past few decades, the increase in mass mortality episodes among marine invertebrates in coastal ecosystems has been linked to modifications of environmental conditions caused by global climate change (Harley et al., 2006), mostly with increased temperature conditions (Harvell et al., 1999; Lesser, 2007). The intensification of mass mortalities in marine organisms is a worldwide phenomenon that affects many regions, including temperate seas such as the Mediterranean (Turley, 1999). In the Northwestern Mediterranean basin, at the end of summers of 1999 and 2003, two extensive and spectacular damage episodes affected marine rocky benthic communities (Cerrano et al., 2000; Fava et al., 2009; Garrabou et al., 2001, 2009; Perez et al., 2000). The damage had dramatic consequences not only for the species concerned but also for the conservation of their entire ecosystems. A wide array of Cnidarian species and more particularly of Anthozoans, such as *Corallium rubrum*, *Paramuricea clavata*, *Cladocora caespitosa* and *Eunicella* spp, were severely affected. These Anthozoans all share a number of common characteristics: they are long-lived animals, have a low resilience, are sessile, and form adult colonies that have a high natural heritage value. Notably, because they are fixed in place, they are unable to migrate when faced with unfavorable conditions.

Among these Anthozoans, the white gorgonian *Eunicella singularis* (Esper, 1791) is characteristic of coralligenous biocenosis and is among the most representative gorgonian species in Western Mediterranean sublittoral communities (Weinberg and Weinberg, 1979). During the past mass mortality events, particularly the abnormal summer heat waves in 1999 and 2003, *E. singularis* was reported to be one of the most impacted Anthozoans (Cerrano et al., 2000; Fava et al., 2009; Garrabou et al., 2009). Furthermore, even several years after these events, some populations of *E. singularis* were still observed to be injured (Linares et al., 2008). Interestingly, despite an overall anomalous warming of the seawater in the whole Northwestern Mediterranean Sea, differential impacts were observed at various spatial scales within sites, and also between individuals living in shallow and deeper waters (Garrabou et al., 2009; Perez et al., 2000). These discrete mortalities evoke that there are differences in resistance capacity to thermal stress, but the biological causes of such resistance *vs* sensitivity to this stress remain unknown.

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E. singularis is the only symbiotic gorgonian of the Mediterranean Sea (Carpine and Grasshof, 1975). Around the world, a common symptom of stress in symbiotic Anthozoans is mass bleaching, which mainly consists of a loss of the symbiotic dinoflagellates living inside the host tissue (Douglas, 2003). Bleaching can subsequently lead to mortality, depending on the amplitude and duration of the stress, and also on species or population resistance to stress (Prada et al., 2010). For *E. singularis*, the bleaching phenomenon has never been recorded in the field, but has been recently measured during thermal stress in controlled aquarium conditions (Ferrier-Pagès et al., 2009). In this previous study, both photosynthetic capacity of the symbiont and calcification rates of *E. singularis* colonies were significantly more affected by thermal stress in shallow (−15 m) than in deep (−35 m) populations. However, reasons for the better thermal stress resistance in populations from deep waters are still needed to be investigated at the cellular and the physiology levels.

In tropical ecosystems, studies on the molecular basis of thermal stress resistance capacity in corals have been multiplied since the 1990s. Some of these studies looked at the effects of heat stress alone or in combination with other environmental parameters, mainly UV and irradiance (Lesser, 2004; Lesser and Farell, 2004; Shick et al., 1995). Very briefly, the main cellular pathways that have been suggested to play a role in coral thermal sensitivity are antioxidant capacity (Richier et al., 2008), zooxantellae photosynthetic activity (for review, see Smith et al., 2005), heat-shock protein levels and ubiquitination (Downs et al., 2000), and apoptosis or autophagy process (Dunn et al., 2007; Richier et al., 2006). In contrast, only a little knowledge has been gathered about the thermotolerance of Mediterranean Anthozoans (Fava et al., 2009; Previati et al., 2010; Rodolfo-Metalpa et al., 2006a; Torrents et al., 2008; Weinberg and Weinberg, 1979), and molecular mechanisms of such thermal tolerance remain unknown.

To examine these mechanisms in more detail in a Mediterranean gorgonian *E. singularis*, a parallel trial was conducted during the experiment carried out by Ferrier-Pagès et al. (2009). Additional colony tips were placed in similar stress conditions in order to record, in *E. singularis* cell extracts, the consequences of hyperthermia for several molecular markers. In the present study we report the first results obtained in this species by studying three biomarkers of both cellular defenses and cellular damages: total oxyradical scavenging capacity (TOSC), proteins ubiquitination and heat-shock protein (Hsp70) levels.

2. Materials and methods

2.1. Biological materials and experimental design

All the present experiments were carried out at the same time and under the same conditions as those previously described in Ferrier-Pagès et al. (2009). Briefly, the experimental set up was designed to minimize the number and quantity of gorgonians collected. Experiments were carried out on two populations of the white gorgonian *E. singularis* from the rocky coast of Marseille, at the south side of the Planier Island (43° 11 54,27 N 05° 13 48,71 E, Marseille, France). These populations were collected in May 2007 at either the shallow (−15 m) or deep (−35 m) limits of *E. singularis* distribution. Each apical colony tip (6 cm long) was collected at random on 80 different healthy adult of *E. singularis* (body size>30 cm) in each population by scuba divers (with authorization for sampling wild marine species). Gorgonian tips were then placed in two sets of aquaria, each composed of four 30 l tanks (20 gorgonian tips per tank) supplied with oligotrophic Mediterranean seawater from a 140 l buffer tank. A first set (one buffer tank + 4 small tanks containing gorgonians) was maintained under a "control" temperature of 18 °C for the whole experiment. In the second set, temperature was regularly and simultaneously increased in the four tanks over the experiment

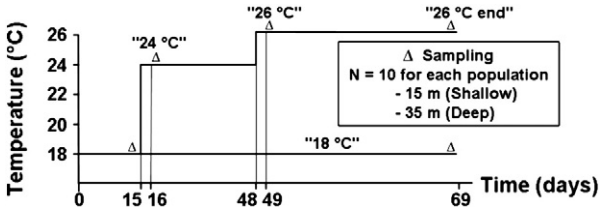

Fig. 1. Experimental design showing the temperature increase applied to specimens from shallow and deep populations of *Eunicella singularis* (after a 15 day acclimation time). Δ represents samplings made at 18 °C, 24 °C, 26 °C and after 19 days in seawater at 26 °C (26 °C end). (Modified from Ferrier-Pagès et al., 2009)

(Fig. 1). Temperature increase mimicked a thermal stress event, such as those recorded in the Mediterranean Sea (Rodolfo-Metalpa et al., 2006a). Previous experiments demonstrated that none of the molecular markers investigated in this study were significantly different between analyzes made on extracts of colony tips frozen immediately after harvesting and those made after tips had been fixed in aquaria and further left in resting condition for 50 days (data not shown). However, a 2 week acclimation time at 18 °C was respected before the rising temperature to 24 °C (over 8 h) and kept constant at 24 °C for 33 days (Fig. 1). The temperature was then raised from 24 °C to 26 °C (over 8 h) and kept constant at 26 °C for 19 days. Seawater was heated using submersible resistance heaters (Visi-Therm® Deluxe) in the buffer tank. Temperature was recorded using Testo T175-T3 data loggers. Salinity values were constants (about 38‰) during all experiments. Irradiance, with 12 h/12 h light/dark cycles, was set up to match the light intensity received in the field as closely as possible, with levels equal to 100 and 50 μmol photons m^{-2} s^{-1} for depth populations from −15 to −35 m, respectively (Ferrier-Pagès et al., 2009). Specimens were fed twice a week, with an equal combination of *Artemia salina* nauplii and a mixture of frozen shrimps and mussels (total quantity per tank: 4 g of food). At each sampling point (indicated as Δ in the Fig. 1), 10 samples were randomly collected in each tank and conserved at −80 °C until extraction.

2.2. Tissue and protein extraction

Frozen gorgonians were weighed and soluble cytoplasmic proteins extracted by grinding in liquid nitrogen and powdering in a mortar. Soluble cytoplasmic proteins were then resuspended by putting 0.2 g ml^{-1} of gorgonian powder in ice-cold extraction buffer (0.05 M phosphate buffer, pH 7.8; 0.4 M Sorbitol; 10 μg ml^{-1} protease inhibitor cocktail). All further extraction steps were performed at 4 °C. Samples were then filtered through a Nylon mesh (100 μm) in order to eliminate skeleton residues as much as possible. Extracts (containing animal debris and zooxanthellae) were sonicated (6 times 10 s, with a resting time of at least 30 s) and centrifuged at 12 000 g for 10 min. Supernatants corresponding to total animal and zooxanthellae soluble protein fractions were then used in the subsequent assays. Complementary experiments, carried out on the same crude extract with or without tissue fractionation by centrifugation, revealed that around 90% of the extracted proteins come from the host tissues, while 10% are from the symbiotic dinoflagellates.

2.3. Total oxyradical scavenging capacity (TOSC) assay

The TOSC assay evaluates the global biological resistance to oxyradicals, thus providing useful indications to predict adverse pro-oxidant effects on the physiological condition of organisms (Regoli, 2000). The oxygen radical scavenging activities of *E. singularis* samples were determined using a fluorometric assay according to Naguib (2000), with modifications to allow measurements in 96-well microplates. Briefly, this TOSC assay is based on the oxidation of 6-carboxyfluoroscein by peroxyl radical generator AAPH (2,2 -azobis (2-