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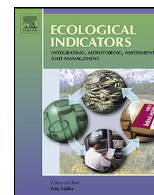

Role of thermal niche in the cellular response to thermal stress: Lipid peroxidation and HSP70 expression in coastal crabs

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ABSTRACT

The aim of this study was to investigate the effect of temperature on lipid peroxidation and HSP70 expression, in three species of crabs, which occupy three different thermal niches in the intertidal–subtidal gradient: *Pachygrapsus marmoratus* (supratidal), *Carcinus maenas* (intertidal) and *Liocarcinus marmoreus* (subtidal). Individuals were collected in the wild and subjected to a temperature increase of $1\,^{\circ}C\,h^{-1}$, until reaching their Critical Thermal Maximum. Haemolymph samples were taken every 2 h. Lipid peroxidation was quantified using the TBARS protocol (thiobarbituric acid reactive substances) and HSP70 was quantified using an ELISA (Enzyme Linked Immunosorbent Assay). Results showed that lipid peroxidation is highly sensitive to temperature in the 3 species studied, showing a significant increase with increasing temperature. HSP70 expression was also altered by temperature except in *C. maenas*. Different patterns of response were detected in lipid peroxidation and HSP70 expression, which appear to be related to the different thermal niches occupied by each species. Species that occupy colder and more stable thermal niches present peaks of cellular stress biomarkers at lower temperatures than species that occupy warmer and more variable thermal niches. The occurrence of cellular repair processes at lower temperatures is indicative of cellular stress at lower temperatures for species of colder and more stable environments.

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1. Introduction

Among marine habitats, coastal areas are the most variable ones. Environmental conditions vary in time and space. Temperature and salinity are the most variable abiotic factors in these areas. Coastal organisms have to deal with these changes, experiencing alterations in their physiological state in order to maintain homeostasis. When these alterations are beyond the organism's capacity to cope with them, the organism is under stress. Brett (1958) defined stress as "a state produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that in either case, the chances of survival are significantly reduced". More recently, Koolhaas et al. (2011) suggested that the term 'stress' should be restricted to conditions where an environmental demand exceeds the natural regulatory capacity of an organism, in particular situations that include unpredictability and uncontrollability.

The production of Reactive Oxygen Species (ROS) is very common in aquatic environments (Lesser, 2006, 2011). ROS can be naturally produced by cellular processes (e.g. respiration) and are important in maintaining cellular activity and signaling (Halliwell and Gutteridge, 1999). However, when their production is increased and there is an imbalance between pro-oxidants and antioxidant defenses (e.g. catalase, superoxide dismutase, glutathione, vitamins) it is said that the organism is under oxidative stress. Environmental conditions such as chemical pollution, thermal stress and ultraviolet radiation lead to oxidative stress, eliciting a stress response characterized by alterations in oxidative stress biomarkers (Lesser, 2006; Heise et al., 2006). Oxidative stress leads to tissue and cellular damage (Halliwell, 1994; Imlay, 2003; Ahmed, 2005) in several components such as proteins, free amino acids, lipids, DNA and carbohydrates (Toyokuni, 1999; Abele and Puntarulo, 2004). Lipid peroxidation is one of the most prevalent mechanisms of cellular injury (Halliwell and Gutteridge, 1999).

Many marine invertebrates are used as sentinel organisms in pollution and contamination studies (e.g., Regoli and Orlando,

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1994; Walsh et al., 1995; Viarengo et al., 1997; Dugan et al., 2005; Solé et al., 2009). In these studies oxidative stress parameters are used as biomarkers: increases in oxidation products or antioxidants are viewed as warning signals of environmental contamination (Haux and Förlin, 1988). Thus, oxidative stress biomarkers are useful in environmental monitoring. However, amongst marine ectotherms, studies of ROS related processes are still sparse and poorly understood and the basal dynamics of oxidative stress in marine invertebrates is not clear (Abele and Puntarulo, 2004). Furthermore, marine invertebrates such as crustaceans have been one of the less studied groups in terms of temperature effects on oxidative stress parameters (Liberato et al., 2003). Therefore, there seems to be a lack of information concerning oxidative stress in invertebrates.

Environmental conditions such as hyperthermia influence oxidative stress parameters (Vinagre et al., 2012a). Hyperthermia increases ROS production not only due to a higher respiratory rate (Abele et al., 2002; Lushchak, 2011; Freire et al., 2011) but also due to a hypoxic situation followed by tissue reoxygenation during recovery (Halliwell and Gutteridge, 1999).

One of the most widely analyzed thermal stress biomarkers is HSP70 (Heat Shock Protein 70 kDa). This protein is not only increased by thermal stress alone, but also by other factors. One of these factors is the production of ROS, which increase not only transcription factors of hsp-genes (Heat Shock Factor 1-HSF1) (Lesser, 2011) but also HSP70 itself (Snoeckx et al., 2001; Kregel, 2002; Heise et al., 2006). It is known that HSP70 activates ROS scavengers (Currie et al., 1988; Mocanu et al., 1993) and inhibits the depolarization of the mitochondrial membrane, maintaining its integrity (Snoeckx et al., 2001). Additionally, HSPs play a role in sensing oxidative stress (Jakob et al., 1999; Ruddock and Klappa, 1999; Calabrese et al., 2004) and they inhibit cell death pathways (Kalmar and Greensmith, 2009).

Therefore, both HSPs and antioxidants seem to be important in the survival after a heat shock (Heise et al., 2006). There is also evidence that the thermal niche occupied by coastal organisms may result in different strategies of cellular defense against thermal stress (Tomanek and Somero, 1999; Somero, 2002; Madeira et al., 2012a).

This work aimed to test (1) the effect of temperature on lipid peroxidation; (2) the effect of temperature on HSP70 expression and (3) if there is any correlation between lipid peroxidation (oxidative stress biomarker) and HSP70 expression (thermal stress biomarker). The tests were performed in three species of crabs that occupy different thermal niches in the intertidal–subtidal gradient: *Pachygrapsus marmoratus* (supratidal), *Carcinus maenas* (intertidal), and *Liocarcinus marmoreus* (subtidal).

2. Materials and methods

2.1. Species collection and acclimation conditions

Crabs were captured during July 2010 in the central Portuguese coast (approximate latitude 38° N, Northeast Altlantic) using hand nets, and beam trawling. Individuals belonging to three abundant species from the supratidal, *P. marmoratus* ($N = 22$), intertidal, *C. maenas* ($N = 24$), and subtidal, *L. marmoreus* ($N = 26$), were collected. After capture, organisms were transported to the laboratory and placed in aquaria of 70 L with aerated sea water, and constant levels of temperature (24 °C) and salinity (35‰), conditions that mimic what was present in the field. The water dissolved O_2 level varied between 95% and 100%. Organisms were left there to acclimate for two weeks, being fed twice a day. They were starved for 24 h before the experiments.

2.2. Experimental setup

The temperature was increased as described by Mora and Ospina (2001), until the individuals reached their Critical Thermal Maximum (CTMax), which is defined as the “arithmetic mean of the collective thermal points at which the end-point is reached” (Mora and Ospina, 2001). This end-point is loss of equilibrium. Crabs need to be stimulated with a lab tweezer to force them upside down, allowing the identification of equilibrium loss; they are unable to get back upright if they have reached the end-point (Vinagre et al., 2012b). CTMax for these species was published in Madeira et al. (2012b). The CTMax value for *P. marmoratus* is 35.7 °C, for *C. maenas* is 35.1 °C and for *L. marmoreus* it is 32.2 °C. During the trial, a thermostatized bath was used to increase water temperature at a constant rate of 1 °C h^{-1}, stopping when the organisms reached their end-point. This required continuous observation. All experiments were carried out in shaded day light (15L; 09D). Haemolymph samples from 3 individuals were taken every 2 °C. All samples taken were immediately frozen in liquid nitrogen and then stored in a regular freezer at −20 °C. Individuals used for haemolymph collection were removed from the trial.

2.3. HSP70 extraction and quantification

Haemolymph samples were centrifuged for 5 min at 16,000 × g and then diluted 1:100 in 0.05 M carbonate–bicarbonate buffer, with a pH was 9.6 at 25 °C (Sigma–Aldrich, USA). Heat Shock Protein 70 (hsc70/hsp70) was quantified through Enzyme Linked Immunosorbent Assay (ELISA) (Njemini et al., 2005) using 96-well microplates (Nunc-Roskilde, Denmark, reference Cat.-No.: 655061). Three replicates of 50 μL of the supernatant were taken from each diluted sample, transferred to the microplate wells and incubated overnight at 4 °C. The microplate was washed (3×) in PBS 0.05% Tween-20 and then blocked by adding 200 μL of 1% BSA (Bovine Serum Albumin, Sigma–Aldrich, USA). The microplate was incubated at 37 °C for 90 min. After microplate washing, the primary antibody (anti-Hsp70/Hsc70, Acris USA), diluted to 0.5 μg mL^{-1} in 1% BSA, was loaded to microplate wells (50 μL each). Then the microplate was incubated for 90 min at 37 °C. After a new washing stage, the secondary antibody (anti-mouse IgC, fab specific, alkaline phosphatase conjugate, Sigma–Aldrich, USA) was diluted (1 μg mL^{-1} in 1% BSA) and added (50 μL) to each well, followed by incubation at 37 °C for 90 min. The microplate was washed again; 100 μL of substrate (SIGMA *FAST*™ p-Nitrophenyl Phosphate Tablets, Sigma–Aldrich, USA) was added to each well and incubated for 30 min at room temperature. Fifty μL of stop solution (3 M NaOH) were added to each well and absorbance was read in a 96-well microplate reader at 405 nm (BIO-RAD, Benchmark, USA). For quantification purposes, an 8-point calibration curve was constructed using serial dilutions of purified HSP70 active protein (Acris, USA) to give a range from 0 to 2000 ng mL^{-1}.

For normalization purposes, the Bradford Assay (Bradford, 1976) was used to quantify the total amount of protein in each sample. The analysis was carried out in 96-well microplates (Nunc-Roskilde, Denmark) by adding 200 μL of Bradford reagent in each well and 10 μL of each sample or standards. After 10 min of reaction, the absorbance was read at 595 nm in a microplate reader (BIO-RAD, Benchmark, USA). A calibration curve was calculated using BSA standards.

The primary antibody used cross-reacts and detects 72 and 73 kDa proteins corresponding to the molecular mass of inducible and constitutive heat shock proteins. In cells, the constitutive form hsc70 remains unchanged (e.g. LeBlanc et al., 2011) or is only slightly upregulated (up to 2-fold) in certain tissues (e.g. Liu et al., 2004; Rendell et al., 2006) whereas the inducible form hsp70, is highly up-regulated from low basal levels (Deane and Woo, 2005).

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