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Subcellular evidences of redox imbalance in well-established populations of an endangered limpet. Reasons for alarm?

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ABSTRACT

Intertidal species are more vulnerable to anthropogenic disturbances than others inhabiting subtidal and offshore habitats. Coastal development frequently results in trace-metal pollution. For endangered species such as *Patella ferruginea* it can be a high risk that leads local populations to extinction. Three localities were surveyed, one within a natural and unpolluted area and the other two within the harbor of Ceuta (Strait of Gibraltar), on breakwaters outside and inside.

The specimens collected inside the harbor reached 3-fold higher Hg content than for those incoming from the natural area. PERMANOVA test indicated that metal composition of the specimens from inside the harbor was different from the rest. In addition, evidence of cell damage was detected in the specimens from the harbor area. This highlights the urgency of undertaking a physiological evaluation of some of the most vulnerable populations, establishing eco-physiological protocols for monitoring and managing populations settled on artificial substrata.

1. Introduction

Intertidal species are usually more vulnerable to anthropogenic disturbances than others inhabiting subtidal and offshore habitats (Thompson et al., 2002). Among the many direct reasons, we can point to the frequent interaction between humans and intertidal organisms (e.g. through collection) since intertidal ecosystems are generally easily accessible. In addition, 60% of human population is settled in coastal areas (Cinzano et al., 2001), resulting not only on physical alterations of the habitat (e.g. construction of coastal structures) but also in chemical modifications through waste discharges from industrial, agriculture and/or domestic source which have a significant and direct impact on intertidal organisms.

Technological development in coastal areas frequently results in trace-metal pollution (Rivera-Ingraham et al., 2013a), a type of impact considered as one of the main threats to Mediterranean marine environments (Cubadda et al., 2001). An increasing number of studies have reported raised levels of several trace metals (Zatta et al., 1992; Malea et al., 1994), which is specially worrying given the status of the Mediterranean basin as a biodiversity 'hot spot' (Coll et al., 2010), and the large number of endangered and endemic species it hosts. It thus becomes imperative to monitor the ecological status of populations of such species and evaluate their vulnerability towards trace-metal pollution events which would allow taking decisions of conservational and management concern in order to avoid massive mortalities of threatened organisms.

For the specific case of mollusks, many are the factors determining metal bioavailability and accumulation in soft tissues. Among those of biotic nature, body size and sex have been proposed to be highly significant (Boening, 1999; Cravo and Bebianno, 2005) to the extent that the comparison of populations differing in size of its individuals can potentially lead to unreliable results and misleading interpretations. Limpets in particular satisfy many of the requirements to be considered biomonitoring organisms (Brown et al., 2004), due to their high capacity to accumulate metals in their tissues (Navrot et al., 1974; Cravo and Bebianno, 2005), which are taken up and accumulated by contact with polluted medium through permeable areas such as gills and/or through consumption of other polluted organisms (Depledge and Rainbow, 1990; Rainbow, 1993). Exposure to such compounds often produces alterations in the organisms' fitness (Harada et al., 2007). Even though the physiological stress of accumulating pollutants may not be immediately lethal, it can prevent the growth and reproduction of the affected individuals (Fukuyama et al., 2000).

Because of this, biomarkers of oxidative stress, such as antioxidant activity and/or quantifications of redox imbalance-induced by-products, are routinely used because they frequently suffer significant modifications upon pollutant (e.g. trace metal) exposure (Regoli and Principato, 1995; Abele et al., 2002): such events are known to enhance reactive oxygen species (ROS) formation, creating an imbalance between prooxidants and antioxidants (favoring the former), generating the widely-characterized effect of oxidative stress as defined by Sies

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(1991). Thus, an increasing number of studies attempt to monitor the status and vulnerability of intertidal organisms by characterizing their response in terms of variations in the levels of their enzymatic (e.g. catalase, CAT and superoxide dismutase, SOD) but also non-enzymatic antioxidants (e.g. glutathione pool, α-tocopherol or β-carotene contents). Limpets are not an exception, as they have frequently been subject of such approaches (Malanga et al., 2004; Rivera-Ingraham et al., 2013a).

The giant limpet *Patella ferruginea* is declared as the most endangered marine macroinvertebrate in the western Mediterranean (Ramos, 1998), and the sole study approaching its tolerance and vulnerability to deteriorated environmental quality had a community approach (Espinosa et al., 2007). Indeed, up to date, no physiological studies have been conducted to determine their response to pollutants such as the heavy metals that, as abovementioned, are so largely present across the Mediterranean Sea. Due to its critical situation, this species is included in several directives at the European level (MMAMRM, 2008). Furthermore, and since many of the most important remaining populations of this species are present on Spanish coasts (Casu et al., 2011; Espinosa et al., 2013), the government of Spain has developed a national strategy of conservation for this species where the monitoring of populations has been recognized as a relevant tool for conservational purposes, in order to avoid pollution-derived mortality events (MMAMRM, 2008).

Recently, a new figure of protection has been promoted under the name of Artificial Marine Micro Reserves (AMMRs) (García-Gómez et al., 2011, 2015), considering using coastal artificial structures such as breakwaters, docks, ripraps, or moles as conservation tools, given that many endangered species naturally settle on these man-made structures, as it is the case of *P ferruginea*. A good example of this is provided by the harbor of the north-African city of Ceuta, hosting thousands of specimens of *P. ferruginea* on its artificial dikes and other structures (Rivera-Ingraham et al., 2011). However, as García-Gómez et al. (2011) pointed out, one of the potentially negative side effects of AMMRs was that such artificial environments are always at risk of pollution. In this sense, there is a complete lack of information about the pollution levels of *P. ferruginea* populations and their eco-physiological status.

Therefore, the aim of the present study was to characterize for the first time the trace metal profile of the highly endangered species *P. ferruginea* throughout a pollution gradient. Since pollutants first interact at the subcellular levels we conducted a physiological evaluation (based on a characterization of cellular redox balance) on selected populations that have been previously described as being well-established and healthy, but exposed to different degrees of pollution to explore the correlation between populational and physiological biomarkers of vulnerability.

2. Material and methods

2.1. Study site

Taking into account the highly endangered status of the species and the legal restrictions, a limited number of specimens were taken under permission of competent authorities (Consejería de Medio Ambiente, Ciudad Autónoma de Ceuta). Specimens of *P. ferruginea* were collected from three different localities in Ceuta (Strait of Gibraltar): a natural rocky shore at Desnarigado Beach, located within the protected area of "Acantilados del Monte Hacho" (Natural: N), an artificial breakwater outside of the harbor of Ceuta (Dique de Levante: DL) and another one inside of the harbor (Parque del Mediterráneo: PM) (see Fig. 1). Within each locality, three different sites were established separated tens of meters from each other, and six limpets were collected at each site (6 limpets × 3 sites × 3 localities = 54 specimens).

Limpets were detached from the substrata, weighted and measured in height and length with a Vernier caliper to the nearest mm. Since size and sex are factors significant determining trace-metal bioaccumulation levels in organisms, as it was briefly reviewed above, care was taken to use animals of similar size. Sex was also determined following the method originally described by Wright and Lindberg (1979) and consisting in conducting a biopsy through the use of a syringe. Gill samples were collected by dissection. Specimens and gills were flash-frozen by immersion in liquid nitrogen and stored at −80 °C until further analyses.

2.2. Chemical analysis

Whole soft tissues were digested by wet oxidation with concentrated HNO_3 under pressure in a microwave digester (Microwave Laboratory Station Milestone START D with built-in ATC-400-CE automatic temperature control). Dry weight determination was performed by oven drying at 105 °C until constant weight. Analysis of trace metals (As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn) in the digests of soft tissues were performed by inductively coupled plasma spectrophotometry (ICP-OES) using a simultaneous spectrometer Varian ICP 720-ES with axially viewed plasma. Results were expressed as μg/g of dry weight.

For the analysis of Hg in soft tissue, 5 mL of the digested samples diluted to 50 mL, was treated with 0.5 mL of potassium bromate/bromide reagent, and then after 1 h with 0.05 mL of a solution of hydroxylamine chloride (13% *w*/*v*). These extracts were then analyzed by an atomic fluorescence spectrometer (Mercury plus Analytikjena model; Germany).

The accuracy and precision of the analytical methods was assessed by routine analyses of the reference material mussel tissue ERM-CE278. Recovery rates for reference samples were between 90 and 100%.

2.3. Assessments on the oxidative balance

All analyses were carried out using gills, as representative of one of the most active tissues in molluscs but also due to the fact that they constitute the first barrier and thus the first line of the defense against environmental stressors.

Antioxidant defense in *P. ferruginea* was assessed as catalase (CAT) and superoxide dismutase (SOD) activities. These were measured spectrophotometrically in gill homogenates diluted at a ratio 1:5 and 1:30 (*w*:*v*) for CAT and SOD measurements, respectively in a 50 mM KPi buffer 120 mM KCl pH 7.4 supplemented with 0.1% Triton X-100 when meant for CAT measurements. Gills were homogenized using four stainless steel milling balls (Retsch, no. FR0120) in a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany). Two cycles of 1 min at 30 beats/s were required for complete tissue homogenization, and in-between cycles samples were cooled in ice for 5 min to reduce protein degradation. CAT activity was measured in supernatants as the decomposition of a 0.3 M H_2O_2 solution in a 50 mM KPi buffer after Aebi (1984) while SOD activities were determined using the cytochrome oxidase essay following the protocol described by Livingstone et al. (1992). All measurements were carried out in triplicate in a microplate reader (Tecan Infinite M200, TECAN, Männedorf, Switzerland) and results were expressed as UCAT and USOD per mg protein content.

Cell damage was assessed as the activity of Caspases 3 and 7, involved in apoptotic cell death. Frozen gill samples were homogenized at 4 °C as previously described at a ratio 1:50 (*w*:*v*) in a lysis buffer composed of 25 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA and 1 μL mL^{-1} of each of the protease inhibitors leupeptin, pepstatin and aprotinin (Rivera-Ingraham et al., 2013b; Strahl and Abele, 2010). Apoptosis intensities in supernatants were measured using the Caspase-Glo® 3/7 kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions at 20 °C. Luminosity values were recorded in a Tecan microplate reader and expressed as relative light units (RLU) per mg of protein content.