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Oxidative responsiveness to multiple stressors in the key Antarctic species, *Adamussium colbecki*: Interactions between temperature, acidification and cadmium exposure

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ABSTRACT

High-latitude marine ecosystems are ranked to be among the most sensitive regions to climate change since highly stenothermal and specially adapted organisms might be seriously affected by global warming and ocean acidification. The present investigation was aimed to provide new insights on the sensitivity to such environmental stressors in the key Antarctic species, *Adamussium colbecki*, focussing also on their synergistic effects with cadmium exposure, naturally abundant in this area for upwelling phenomena. Scallops were exposed for 2 weeks to various combinations of Cd (0 and 40 μgL-1), pH (8.05 and 7.60) and temperature (−1 and +1 °C). Beside Cd bioaccumulation, a wide panel of early warning biomarkers were analysed in digestive glands and gills including levels of metallothioneins, individual antioxidants and total oxyradical scavenging capacity, onset of oxidative cell damage like lipid peroxidation, lysosomal stability, DNA integrity and peroxisomal proliferation. Results indicated reciprocal interactions between multiple stressors and their elaboration by a quantitative hazard model based on the relevance and magnitude of effects, highlighted a different sensitivity of analysed tissues. Due to cellular adaptations to high basal Cd content, digestive gland appeared more tolerant toward other prooxidant stressors, but sensitive to variations of the metal. On the other hand, gills were more affected by various combinations of stressors occurring at higher temperature.

1. Introduction

The concentration of CO_2 in the atmosphere has continuously increased from 278 ppm in pre-industrial time up to 400 ppm nowadays, representing one of the most important drivers of global climate change (http://www.sciencedirect.com/science/article/pii/S0022098114000077XIPCC, 2013). Approximately 30% of total CO_2 emissions has been absorbed by seawater, causing the well known process of ocean acidification, OA (Raven et al., 2005). Progressive changes in carbonate chemistry determined a decrease of 0.1 pH units compared to the pre-industrial levels, with a further expected reduction of 0.3–0.5 units by the end of the 21st century (Raven et al., 2005).

The Southern Ocean accounts for about 4% of the global uptake of CO_2 by the world oceans due to the high solubility of CO_2 at low temperature and mixing patterns from upwellings and deep water formation (Fabry et al., 2009). Similar characteristics lead to an increased rate of acidification and a more rapid shoaling of the saturation horizons (Fabry et al., 2009). In this respect, polar organisms, which have evolved in environmentally stable conditions, might be more vulnerable to climate change, in particular regarding calcification processes and variations of fundamental pathways like energy metabolism, growth, reproduction, larval development and oxidative stress. Compared to temperate models, however, only a few studies have investigated the possible effects of climate change on Antarctic marine species (Cubillos et al., 2007; McClintock et al., 2009; Moy et al., 2009; Seibel et al., 2012; Walker et al., 2013; Constable et al., 2014; Collard et al., 2015; Flynn et al., 2015).

In addition, while future scenarios of temperature and ocean acidification can be simulated from CO_2 emission models (IPCC, 2013), at this moment it is virtually impossible to predict the biological impact and synergistic effects of multiple stressors, which

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can be indirectly modulated by variations of environmental factors due to climate change. In this respect, it has been widely postulated that increased temperature and ocean acidification could influence chemical speciation and bioavailability of environmental pollutants, bioaccumulation processes, responsiveness of detoxification mechanisms, sensitivity and consequences on organisms health condition, but clear evidences of similar modulations are still lacking.

In the Ross Sea, local upwelling phenomena are responsible for a natural enrichment and elevated bioavailability of cadmium (Cd) which is accumulated in tissues of Antarctic invertebrates and fish at values typically 10–50 folds higher than those of similar temperate species (Nigro et al., 1997). Although this element does not apparently cause adverse effects on the organisms, in Antarctic fish it was shown to influence the responsiveness of fundamental metabolic pathways to other stressors, i.e. the cytochrome P450 biotransformation mechanisms, male vitellogenin expression and the antioxidant network (Regoli et al., 2005; Canapa et al., 2007; Benedetti et al., 2007, 2009). In the scallop *Adamussium colbecki* (Smith, 1902), another key sentinel species of the Antarctic environment, the enhanced accumulation of Cd during algal bloom is paralleled by a general increase of antioxidant defences (Regoli et al., 2000, 2002). Beside the role in counteracting a natural increase of prooxidant pressure during phytoplankton blooms, oxyradical metabolism and antioxidant defences have a fundamental role for polar organisms in adaptation mechanisms to high solubility of oxygen in cold seawater, elevated content of oxidizable poly-unsatured fatty acids (PUFAs) in membranes, high cellular mitochondrial densities, and the need of long-term protection of proteins and RNAs due to their low turnover rate (Abele and Puntarulo, 2004).

Based on previous issues, the main aim of this study was to investigate whether variations of temperature and pH may singularly or synergistically affect the sensitivity of *A. colbecki* to Cd, highlighting a potentially reciprocal modulation of key cellular responses by multiple stressors. Scallops were exposed to various combinations of treatments including two different levels of temperature, pH and Cd concentrations, opportunely chosen as reflecting environmentally realistic or future scenarios for Antarctic marine environment.

Analyses of Cd bioaccumulation were integrated with a wide panel of early warning biomarkers and results were elaborated within a quantitative model (Sediqualsoft) which, based on biological relevance and magnitude of observed variations, summarize an hazard index for biomarkers results (Piva et al., 2011; Benedetti et al., 2012).

Overall, this study was expected to provide new insights on mechanisms underlying the responsiveness of a model Antarctic species to variations of temperature and acidification, interactions occurring between multiple stressors, and potential consequences of climate change in areas characterized by elevated environmental pollution or geochemical anomalies.

2. Materials and methods

2.1. Experimental design

Scallops, *A. colbecki*, were sampled during the XXIX Italian Antarctic Expedition (2013–2014) from Terra Nova Bay (Ross Sea) and acclimatized to laboratory conditions for 10 days with running, unfiltered seawater at the controlled temperature of −1 °C and pH 8.05. A total of 240 organisms were randomly distributed in eight tanks (150 L each) and exposed to one of the following experimental conditions: 1) control (CTRL), at environmental temperature (−1 °C) and environmental pH (8.05); 2) Cd exposure (Cd), at 40 μg/L of Cd, −1 °C, pH 8.05; 3) acidified water condition (Ac), at pH 7.60 and −1 °C; 4) warm exposure (Warm), at +1 °C and pH 8.05; 5) acidified and Cd exposure (Ac + Cd), at pH 7.60, 40 μg/L of Cd and −1 °C; 6) warm and Cd exposure (W + Cd), at +1 °C, 40 μg/L of Cd and pH 8.05; 7) warm and acidified condition (W + Ac), at +1 °C and pH 7,6; 8) warm, acidified and Cd exposure (W + Ac + Cd), at +1 °C, pH 7.6 and 40 μg/L of Cd. After 14 days, organisms were sacrificed, haemolymph, gills and digestive glands were rapidly dissected, frozen in liquid nitrogen and stored at-80 °C until analyses. A portion of haemolymph and gills was maintained in Carnoy's solution (3:1 methanol:acetic acid) for micronuclei frequency analyses. No mortality was observed during the experiments. For both chemical and biochemical analyses, 5 pools, each constituted by tissue of 6 specimens, were prepared for digestive glands, gills and haemolymph.

2.2. Chemical analyses

Cd concentration in scallops tissues was analysed according to previously described methods (Regoli et al., 2005). For every treatment, digestive glands and gills were dried at 70 °C until constant weight and digested under pressure with nitric acid in microwave digestor systems (CEM, Mars Systems). Quality assurance and quality control was assessed by processing blank samples and reference standard material (Mussel Tissue Standard Reference Material SRM 2977, National Institute of Standards and Technology). Cd was analysed by atomic absorption spectrophotometry with electrothermal atomization. The concentrations obtained for the standard reference material were always within the 95% confidence interval of certified values. Data are expressed as μg/g dry weight (mean values ± standard deviations, n = 5).

2.3. Biomarker analyses

Sample preparation and analytical protocols have been fully detailed elsewhere (Regoli et al., 2000). Metallothioneins were analysed in digestive glands and gills homogenized in 20 mM Tris-HCl buffer (pH 8.6), 0.5 M sucrose, 0.006 mM phenylmethylsulfonyl fluoride (PMSF), and 0.01% β-mercaptoethanol and centrifuged at 30,000 g for 45 min. After acidic ethanol/chloroform fractionation of tissue supernatants, metallothioneins were quantified by a spectrophotometric assay using reduced glutathione (GSH) as standard.

For measurement of enzymatic antioxidants, tissues (digestive gland and gills) were homogenized (1:5 and 1:3 w:v ratio respectively) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg mL^{-1} bacitracin, 0.008 TIU mL−1 aprotinin, 1 mg mL^{-1} leupeptin, 0.5 mg/mL pepstatin, NaCl 2.5%, and centrifuged at 110,000 g for 1 h at 4 °C. Measurements were made with a Varian (model Cary 3) spectrophotometer at a constant temperature of 18 °C. Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04$ mM^{-1} cm^{-1}) due to the consumption of hydrogen peroxide, H_2O_2 (12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0). Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of oxidized glutathione, GSSG ($\lambda = 340$ nm, $\varepsilon = 6.22$ mM^{-1} cm^{-1}). The final assay condition were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 60 mM NADPH. Glutathione peroxidases (GPx) activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide (NaN_3) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase,