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(Article begins on next page)

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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.

Keywords: Antarctic scallop, Multiple stressors, Climate Change, Cadmium, Oxidative stress, Antioxidant defences

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 (IPCC, 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 to 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 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.

Materials and methods:

Experimental design

Scallops, *A. colbeckii,* 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.

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).

Biomarker analyses

Sample preparation and analytical protocols have been fully detailed elsewhere (Regoli et al., 2000). Metallothioneins were analyzed 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 30,000g 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 110,000g 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, ε = 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 (λ = 340 nm, ε = 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 (ε = 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, 0.24 mM NADPH, and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates, respectively, for the selenium-dependent and for the sum of Se-dependent and Se-independent forms. The rate of the blank reaction was subtracted from the total rate. Glutathione S-transferases (GST) were determined at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH (ε = 9.6 mM^{-1} cm^{-1}).

Total glutathione was analyzed in samples of digestive gland and gill homogenized (1:5 and 1:3 w:v ratio respectively) in 5% sulfosalicylic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37,000g for 15 min. The resulting supernatants were enzymatically assayed (Benedetti et al., 2007).

The total oxyradical scavenging capacity (TOSC) assay measures the overall capability of cellular antioxidants to neutralize different forms of artificially generated oxyradicals, thus inhibiting the oxidation of 0.2 mM a-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Regoli et al., 2000). Peroxyl radicals (ROO·) were generated by the thermal homolysis of 20 mM 2–2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl radicals (·OH) were produced by the Fenton reaction of iron-EDTA (1.8 µM Fe3+, 3.6 µM EDTA) plus ascorbate (180 µM) in 100 mM K-phosphate buffer. Ethylene formation in control and sample reactions was analyzed at 10–12 min time intervals by gas-chromatographic analyses and the TOSC values quantified from the equation: TOSC=100-(∫SA/∫CA×100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay.

The content of malondialdehyde (MDA) was measured in homogenates of scallops digestive glands and gills derivatized with 1-metyl-2-phenylindole and spectrophotometrically determined after calibration against a malondialdehyde standard curve.

Acetylcholinesterase activity (AChE) was analyzed in hemolymph and gills: hemolymph was centrifuged at 3,000g for 5 min, while gills were homogenized in 100 mM Tris-HCl buffer (pH 7.2), 0.55 M sucrose and centrifuged at 10,000g for 10 min. Obtained supernatants were spectrophotometrically assayed by the Ellman's reaction at 18 ± 1 °C, λ = 412 nm, ε = 13.6 mM^{-1} cm^{-1}.

For the activity of Acyl CoA oxidase (AOX), samples were homogenized in 1 mM sodium bicarbonate buffer (pH 7.6) containing 1 mM EDTA, 0.1% ethanol, 0.01% Triton X-100 and centrifuged at 500g for 15 min at 4 °C. The H_2O_2 production was measured in a coupled assay by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 µM sodium azide, 0.01% Triton X-100, 1.2 U mL−1 HRP in a final volume of 1 mL. After a preincubation at 25 °C for 5 min in the dark with an appropriate volume of sample, reactions were started adding the substrates palmitoyl-CoA at final concentrations of 30 µM and 100 µM for Acyl-CoA oxidase (AOX) and readings were carried out against a blank without the substrates at 502 nm.

Protein concentrations were measured according to Lowry method (1951), using bovine serum albumin (BSA) as standard. All biochemical biomarkers are expressed as mean values ± standard deviations (n=5).

Lipofuscin content of tertiary lysosomes was determined on duplicate cryostat sections (8 µm thick) of digestive gland. Slides were fixed in Beker's fixative (+2.5% NaCl) and stained by Schmrol reaction before

mounting in glycerol gelatine. Five measurements were made on digestive tubules of each section (two sections for mussel, 10 scallops for each experimental condition). Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of digestive tubules.

The DNA integrity was evaluated at chromosomal level by measuring the micronucleus (MN) frequency in scallops haemocytes rapidly fixed in Carnoy's solution (3:1 methanol: acetic acid), dispersed on glass slides and stained with the fluorescent dye 4',6-diamidino- 2-phenylindole at 100 ng ml-1. For each experimental condition, 10 scallops were observed and for each specimen 2000 cells with preserved cytoplasm were scored to assess the presence of micronuclei, defined as round structures, smaller than 1/3 of the main nucleus diameter, on the same optical plan and clearly (Benedetti et al., 2014).

Statistical analyses

Analysis of variance (1-way ANOVA) was applied to test significance of effects on biochemical parameters caused by temperature, acidification, Cd exposure and their reciprocal interactions. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Cochram C and mathematical transformation applied if necessary; post-hoc comparison (Newman–Keuls) was used to discriminate between means of values. Multivariate statistical analysis (non-metric multidimensional scaling analysis) was applied to biomarkers data to discriminate various experimental treatments.

Results on biological parameters in scallops were further elaborated within a previously developed quantitative and software-assisted model (Sediqualsoft, Piva et al., 2011). Whole calculations, detailed flow-charts, rationale for weights and thresholds have been fully given elsewhere and successfully validated in filed conditions, during the characterization and classification of risk from industrial and harbour sediments, natural hydrocarbon seepage in coastal areas, the recent Costa Concordia wreck at Giglio Island and the ecotoxicological effects of microplastics (Piva et al., 2011, Benedetti et al., 2012; 2014; Regoli et al., 2014; Avio et al., 2015; Bebianno et al., 2015). Briefly, depending on species and tissue, the model assigns to each biomarker a "weight" based on the relevance of biological endpoint, and a "threshold" for changes of biological significance which consider both inductions and/or inhibitions of various responses. For every analysed biomarker, the measured variation is compared to the threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. Depending on the magnitude of the calculated effect, each biomarker response is assigned by the model to 1 of 5 classes of effect (from Absent to Severe); the calculation of the Hazard Quotient for biomarkers (HQ_{BM}) does not consider the contribution of responses with an effect lower or equal to threshold (Absent or Slight), calculates the average for those with an effect up to two-fold compared to the threshold (Moderate), and adds the summation (Σ) for the responses more than 2 fold greater than the respective threshold, i.e. Major or Severe (Piva et al., 2011):

$$HQ_{BM} = \left(\frac{\sum_{j=1}^{N} Effect_W(j)_{1<Effect(j)\leq 2}}{num\ biomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{M} Effect_W(k)_{Effect(j)>2} \right)$$

According to variations measured for various biomarkers, the model summarizes the level of cumulative HQ_{BM} in one of five classes of hazard for biomarkers, from Absent to Severe (Piva et al., 2011).

Results:

Cd concentrations significantly increased in both digestive glands and in gills of Cd exposed scallops with values 3 and 5 folds higher than those measured in respective control groups (Fig. 1A, 1B). Co-occurring variations of other factors had negligible effects on Cd accumulation with slightly lower values of this element measured only in digestive glands of organisms co-exposed in acidic conditions (Ac+Cd): higher temperature did not cause additional effects on the uptake of this element in both the tissues (Fig. 1A, 1B).

Levels of metallothioneins did not vary in digestive gland (Fig. 1C), while a significant increase was observed in gills after exposure to Cd, without any difference when the element was dosed alone or in combination with higher temperature and/or lower pH (Fig. 1D).

The antioxidant status was assessed by integrating analyses of individual antioxidants with the total capability to neutralize different forms of oxyradicals. In digestive gland, catalase showed a trend toward lowered enzymatic activities with significant effects after co-exposures between Cd and higher temperature, with or without acidified conditions (Fig. 2A). Increased temperature affected also the responses of GST, while no variations were observed for GR and GSH (Fig. 2B-D). Glutathione peroxidases, particularly Se-dependent forms, were slightly inhibited by co-exposures to higher temperature with Cd and/or acidification (Fig. 2E-F). Variations of individual antioxidants were not paralleled by any significant change in the TOSC values toward either ROO· or ·OH (Fig. 2G-H).

Antioxidant responses in gills exhibited lower values of catalase after exposure to Cd with increased temperature and acidification, while various combinations between Cd, acidification and/or temperature were often more effective than individual stressors in affecting responses of GST, GSH, Se-dependent GPx (Fig. 3A-F). Antioxidant variations in gills were reflected by a slight increase of TOSC toward ROO· and HO· in scallops exposed to higher temperature with or without Cd (Fig. 3G-H).

Among cellular biomarkers, malondialdehyde showed a general increase in digestive gland after treatment to multiple stressors, but no variations in gills (Fig. 4A-B). On the other hand, lipofuscin was increased by Cd, low pH and their combination, while it decreased after exposure to higher temperature

with or without the other stressors (Fig. 4C). Frequency of micronuclei significantly increased in scallops exposed to all treatments with both individual and multiple stressors (Fig. 4D). Fluctuating changes of AOX were observed in digestive gland and gills with a few effects observed after exposure to treatments involving higher temperature and other stressors (Fig. 4E-F). Acetylcholinesterase was increased in digestive gland by Cd with or without acidification, while similar effects were induced in gills by higher temperature with or without Cd and acidification (Fig. 4G-H); the combination of the 3 stressors tended to reduce this enzymatic activity in both tissues.

The nMDS ordination of biomarker responses in a multidimensional space (d = 2) analysis is given in Fig. 5. In digestive tissues results indicated a separation between treatments involving co-exposures at higher temperature and those with Cd and/or acidification at -1°C (environmental temperature) (Fig. 5A); in gills, a more evident difference was observed between organisms exposed to individual stressors compared to their multiple combinations (Fig. 5B). The elaboration of the overall biomarkers results through weighted criteria summarized in a single hazard index the biological significance of cellular responses observed in tissues of *A. colbeckii* exposed to different experimental treatments (Fig. 6). In digestive gland, the elaborated Hazard Quotient was Moderate for scallops exposed to Cd alone and in combination with low pH and higher temperature, Slight for all the other treatments. On the other hand, gills appeared more sensitive to co-exposures between higher temperature and the other stressors (HQ Moderate), compared to individual stressors or their combination at -1°C (HQ Slight).

Discussion:

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, *A. colbecki*, focussing also on their synergistic effects with Cd exposure. Cd accumulation is of special interest for marine organisms in the area of Terra Nova Bay due to a naturally elevated bioavailability of this metal enriched in water column by up-welling currents: during phytoplanktonic bloom, when the algae represent an important trophic source, Cd is transferred to both benthic species and pelagic food webs. As a consequence of this local peculiarity, tissue concentrations of Cd in organisms from Terra Nova Bay are up to 20 fold higher than those normally measured in similar temperate species (Nigro et al., 1997). The elevated Cd content in tissues, despite not directly toxic, was shown to influence metabolism of organic xenobiotics (Regoli et al., 2005; Benedetti et al., 2007), and probably interfere with endocrine receptor (ER) and expression of vitellogenin (VTG) in males of fish *T. bernacchii* (Canapa et al., 2007). In this respect, it was of interest to explore whether a similar peculiarity of Antarctic organisms might have reciprocal interactions with their sensitivity to the effects of climate change.

This study confirmed elevated basal concentrations of Cd in tissues of *A. colbecki,* but also the capability of this scallop to further accumulate the metal in response to enhanced bioavailability. Surprisingly, the Cd accumulation in both digestive gland and in gills was not affected by either temperature increase or acidification which were expected to influence metabolism and chemical availability respectively. In this respect, previous studies revealed that temperature increased metal uptake in some temperate and Arctic organisms (Baines et al., 2006; Sokolova and Lannig, 2008), while other investigations on temperate mussels (*Mytilus galloprovincialis*) did not support a similar modulation (Izagirre et al., 2014). Regarding acidification, PCO_2 was shown to enhance Cd and Cu accumulation in oysters and clams, but the rather subtle and tissue-specific variations suggested that CO_2-dependent effects on the metal uptake of marine bivalves are complex and not predictable from the chemical models of the metal speciation in seawater (Rodriguez-Romero et al., 2014; Ivanina et al., 2015).

The increase of Cd concentration in tissues of *A. colbecki* was not reflected by variations of metallothioneins in digestive gland of exposed scallops, while levels of these proteins increased in gills. The lack of response in digestive tissues can be related to the elevated basal levels of metallothioneins, reflecting an adaptation mechanism to high natural concentrations of Cd in these Antarctic organisms. Despite we did not analyse subcellular distribution of Cd in exposed scallops, previous studies and metallothioneins characteristics suggest this element as mostly present in a soluble form (Viarengo et al., 1997; Regoli et al., 1997, 2002): this hypothesis is also supported by the typical amino acid composition of these proteins in *A. colbecki* that confer a preferential binding capacity for Cd (Ponzano et al., 2001). High levels of metallothioneins have also been shown to provide protection against oxidative stress through reaction of sulfhydryl groups with reactive oxygen species, thus further representing an important defence in polar marine species toward the high environmental prooxidant pressure.

Oxyradical metabolism is of great importance for Antarctic organisms and in our study, a wide panel of antioxidant defences was integrated with measurement of Total Oxyradical Scavenging Capacity (TOSC) towards hydroxyl and peroxyl radicals. Despite the limited effects on Cd bioaccumulation, exposure to multiple stressors appeared to modulate oxidative responsiveness to this element. In digestive gland, co-exposures to Cd and higher temperature caused a significant decrease of catalase and glutathione peroxidases, confirming the sensitivity of these enzymes in revealing a prooxidant pressure, and the importance of H_2O_2 metabolism as a possible driver (Regoli and Giuliani, 2014). Similar effects, not compensated by a varied capability to neutralize bot ROO· and HO·, resulted in a slight increase of peroxidative processes and consequent enhancement of malondialdehyde content: in this respect, the lowered values of lipofuscin after treatments involving higher temperature might be the consequence of enhanced excretion processes of oxidized membranes through tertiary lysosomes. Acidification caused

generally slighter oxidative effects in digestive gland, and only when acting as co-stressor with higher temperature and/or Cd exposure.

Sensitivity to oxidative stress was highlighted also in gills where Se-dependent glutathione peroxidases confirmed decreased values in response to multiple combinations of Cd, temperature and acidification. Glutathione S-transferases and levels of GSH were upregulated in these tissues, modulating the responsiveness to Cd in combination with temperature and/or acidification. GSH can act as a direct scavenger toward H_2O_2 and, as cofactor of GST, contributes to removal of peroxidative products on damaged membranes: in this respect, no malondialdehyde accumulation was observed in gills which also exhibited a certain enhancement of the overall capability to neutralize both peroxyl and hydroxyl radicals, particularly in response to higher temperature and Cd. The different responsiveness of digestive gland and gills to investigated multiple stressors confirm that variations of antioxidants to environmental stressors can not be generalized, responding to specific signals, interactions, and pathways that differ in various tissues or exposure conditions (Regoli et al., 2011). Also for antioxidants, the more limited fluctuations observed in digestive gland compared to gills are probably related to the higher basal levels of such defences. The elevated protection in digestive tissues was shown as an important strategy of this scallop to cope with fluctuations of prooxidant pressure in the Antarctic marine environment characterized by low temperature and high levels of dissolved oxygen, marked seasonality in photochemical activation of dissolved organic matter and food availability (Viarengo et al., 1995; Regoli et al., 1997; 2000; 2002).

Overall, our results confirm previous evidences that elevated temperature and acidification may represent prooxidant stressors, as a common consequence of the metabolic and acid–base disturbance in animals (Matoo et al., 2013). Elevated temperature was shown to cause oxidative stress in marine molluscs also through a mismatch between generation and detoxification of reactive oxygen species (ROS) (Abele et al., 2001). On the other side, acidification was responsible for significant changes in gene expression and activity of antioxidant defences in the Arctic spider crab *Hyas araneus* and in the Eastern oyster, *Crassostrea virginica* (Tomanek et al., 2011; Harms et al., 2014). Different mechanisms have been suggested for prooxidant effects of acidification, including the reaction of CO_2 with peroxinitrite and formation of reactive carbonate and nitrogen species with elevated oxidizing potential (Dean, 2010). Elevated CO_2 and/or low environmental pH can also have indirect effects since molluscs have a limited capability for pH regulation, and sea water acidification would thus determine intracellular acidosis (Tomanek et al., 2011). Such intracellular conditions may negatively affect the efficiency of mitochondria by increasing the electron slip in ROS-generating mitochondrial complex I and II, and/or by partially inhibiting the flow through the downstream electron transport chain complex: in either case, these disturbances of the electron transport chain would result in elevated rates of ROS generation. Further, intracellular acidosis can cause the release of chelated transition metals such as Fe^{2+} from intracellular store, thus favouring the

onset of oxidative stress through Fenton reactions and generation of hydroxyl radicals (Tomanek et al., 2011).

The responsiveness of *A. colbecki* to combinations of various stressors was confirmed by the enhanced levels of micronuclei observed after all treatments to individual and multiple stressors: considering the effects observed on antioxidants, the higher frequency of MN might confirm a certain unbalance of oxyradical metabolism but also suggest a modulation of cellular turnover: in this respect, beside an oxidative damage on DNA, micronuclei formation would be, at least partially, favoured by a higher mitotic rate. More limited variations occurred for Acyl CoA oxidase, indicating that higher temperature, in combination with Cd and/or acidification, may represent the primary stressor affecting β-oxidation of fatty acids and lipid metabolism. Finally, acetylcholinesterase was particularly modulated by Cd exposures with or without other stressors, confirming the neurotoxic potential of this element (Del Pino et al., 2014). Reported effects of Cd include block of cholinergic transmission by decrease of acetylcholine synthesis and release, inhibition of AChE activity and of postsynaptic transmission, blockage of cholinergic receptors (Del Pino et al., 2014).

When all the complex variations on biomarkers effects were elaborated through multivariate analysis or the quantitative hazard model (Sediqualsoft) interesting trends could be summarized. The multivariate scaling analysis (nMDS) revealed that temperature influence the pattern of biological responses in digestive gland where the analysis tended to separate cold from warm exposed organisms. On the other hand, considering both the biological relevance and the magnitude of observed responses, the overall quantitative effects were always higher for organisms exposed to Cd, alone and in combination with other factors: after these treatments, the elaborated HQ was assigned as Moderate, compared to Slight in organisms exposed to higher temperature, acidification or their combination. This result would further confirm that the adaptation to the high basal content of Cd, which involves specific cellular strategies like elevated antioxidant defences, makes these tissues more tolerant toward other potentially prooxidant stressors like temperature or acidification: at the same time, however, such delicate homeostatic equilibrium, appears overwhelmed when cellular concentrations of the metal increase, not being further exacerbated by contemporary variations of temperature and pH. A different trend of responsiveness was observed in gills which, according to both nMDS and hazard model, generally appeared more sensitive to various combinations of stressors occurring at higher temperature.

In conclusion, this study provided new insights on the reciprocal, synergistic and modulatory effects of ocean warming, acidification and metals bioaccumulation in a key Antarctic invertebrate. Despite the elevated basal levels of antioxidants as adaptive mechanism to the high environmental prooxidant pressure, *A. colbecki* was sensitive to interactions between climate change and Cd bioavailability, with different sensitivity among analysed tissues toward different factors. Further studies are needed to better

understand long term effects, as the onset of earlier biological responses at transcriptional levels or the link with physiological status of the organisms, as well as the different sensitivity of polar organisms in comparison with similar temperate species.

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LEGENDS OF FIGURES

Figure 1. Cadmium concentrations (A-B) and levels of metallothioneins (C-D) in digestive glands and gills of *A. colbecki* exposed to different experimental conditions. Letters indicate significant differences between groups of means. Data are given as mean values ± standard deviations, *n* = 5.

Figure 2. Activities of individual antioxidant enzymes and Total Oxyradical Scavenging Capacity toward peroxyl and hydroxyl radicals in digestive gland of *A. colbecki* exposed to different experimental conditions. Letters indicate significant differences between groups of means. Data are given as mean values ± standard deviations, *n* = 5.

Figure 3. Activities of individual antioxidant enzymes and Total Oxyradical Scavenging Capacity toward peroxyl and hydroxyl radicals in gills of *A. colbecki* exposed to different experimental conditions. Letters indicate significant differences between groups of means. Data are given as mean values ± standard deviations, *n* = 5.

Figure 4. Levels of malondialdehyde, lipofuscin, micronuclei, acyl CoA oxidase and acetylcholinesterase in tissues of *A. colbecki* exposed to different experimental conditions. Panels A, C, E: digestive gland; B, F: gills; D,G: haemolymph. Letters indicate significant differences between groups of means. Data are given as mean values ± standard deviations, *n* = 5.

Figure 5. Non-metric multidimensional scaling analysis (MDS) ordination plots of biological responses in digestive glands (A) and in gills (B) of *A. colbecki* exposed to different experimental conditions. White points indicate samples exposed to environmental temperature (-1°C) and dark points indicate samples exposed to warm conditions (+1°C).

Figure 6. Weight of Evidence (WOE) classification of biomarkers data, in scallops exposed to different laboratory conditions. The quantitative Hazard Quotients (HQ) and the assigned classes of hazard are given.

Figure

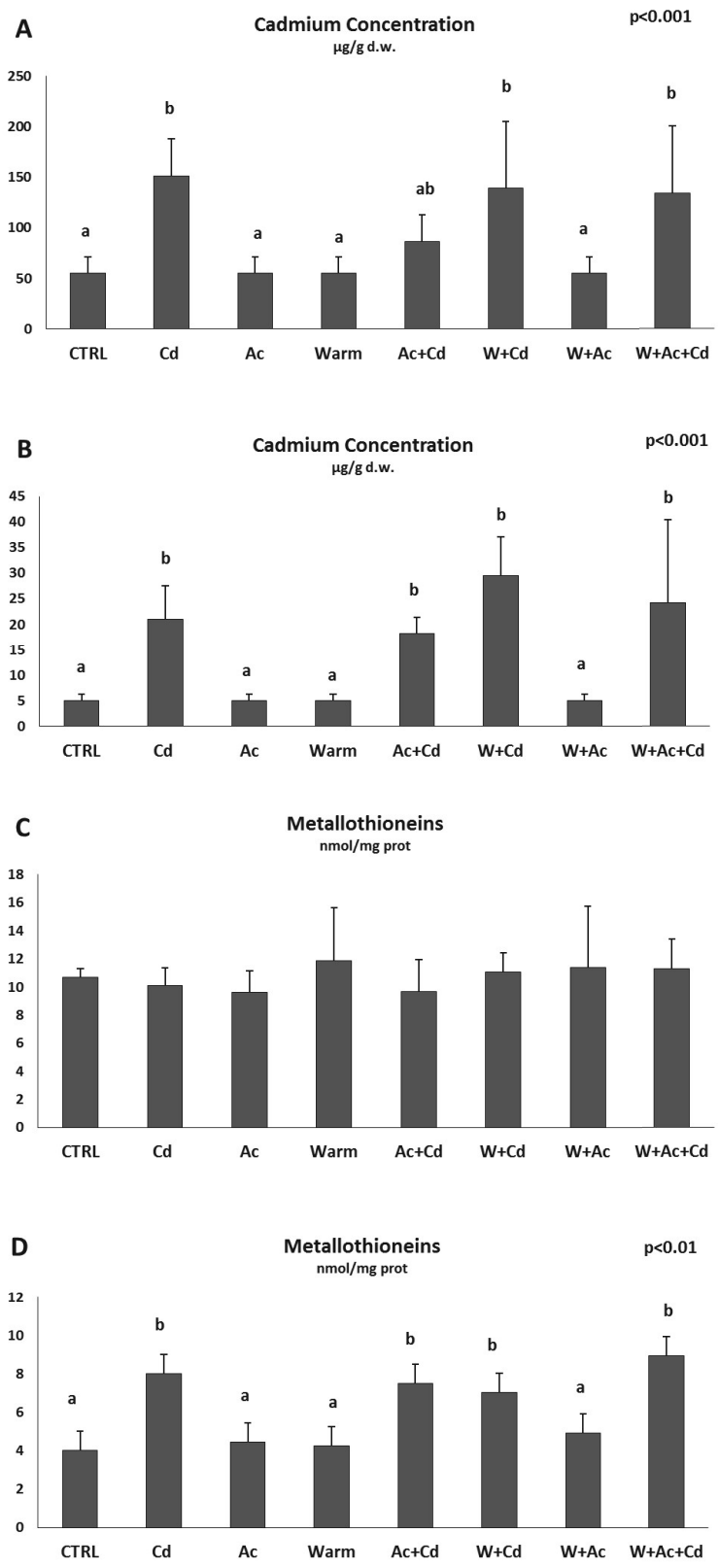
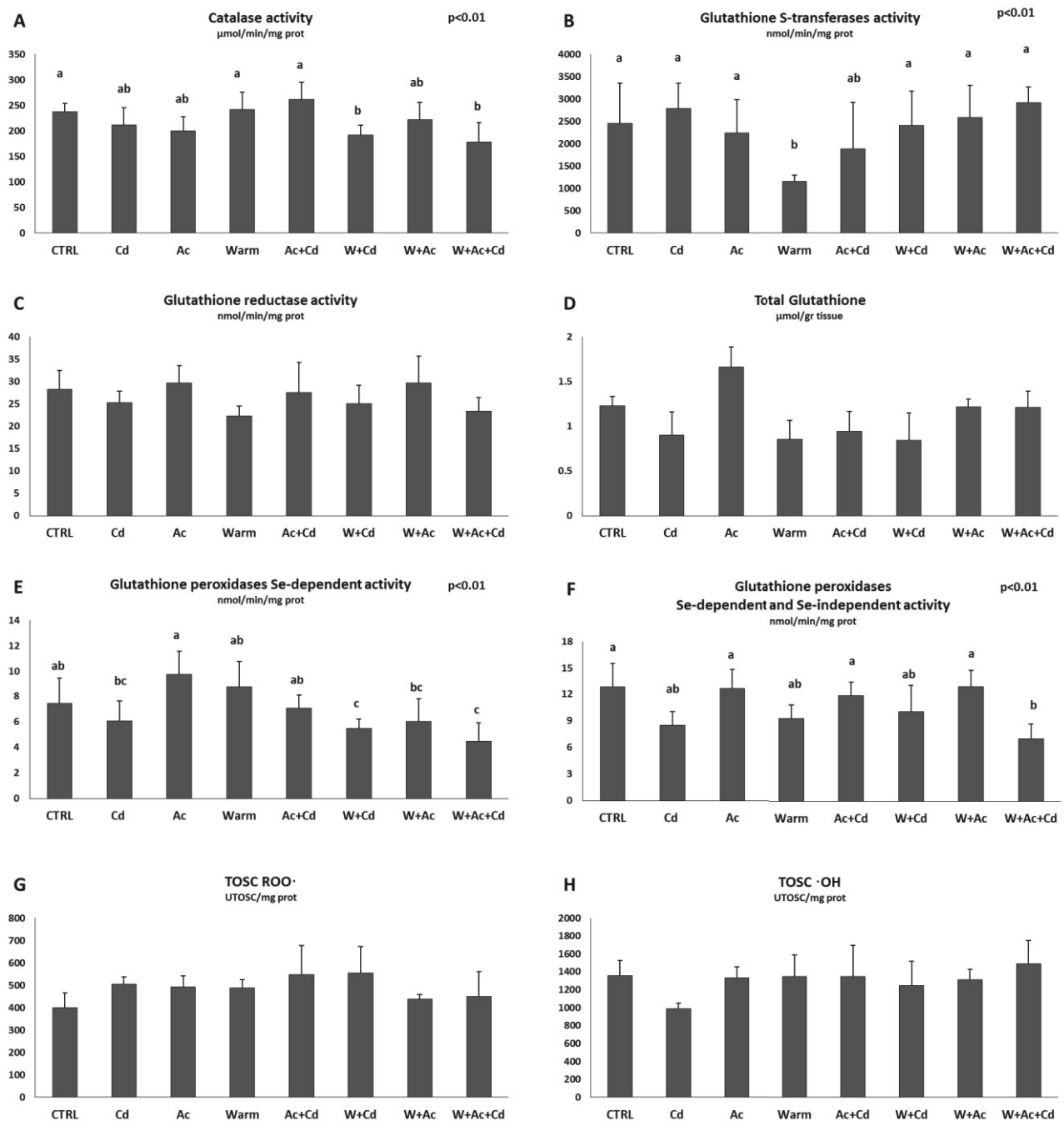


Figure 1.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Catalase activity
µmol/min/mg prot
p<0.01
B
Glutathione S-transferases activity
nmol/min/mg prot
p<0.01
C
Glutathione reductase activity
nmol/min/mg prot
D
Total Glutathione
µmol/gr tissue
E
Glutathione peroxidases Se-dependent activity
nmol/min/mg prot
p<0.01
F
Glutathione peroxidases
Se-dependent and Se-independent activity
nmol/min/mg prot
p<0.01
G
TOSC ROO·
UTOSC/mg prot
H
TOSC ·OH
UTOSC/mg prot
CTRL
Cd
Ac
Warm
Ac+Cd
W+Cd
W+Ac
W+Ac+Cd

Figure 2.

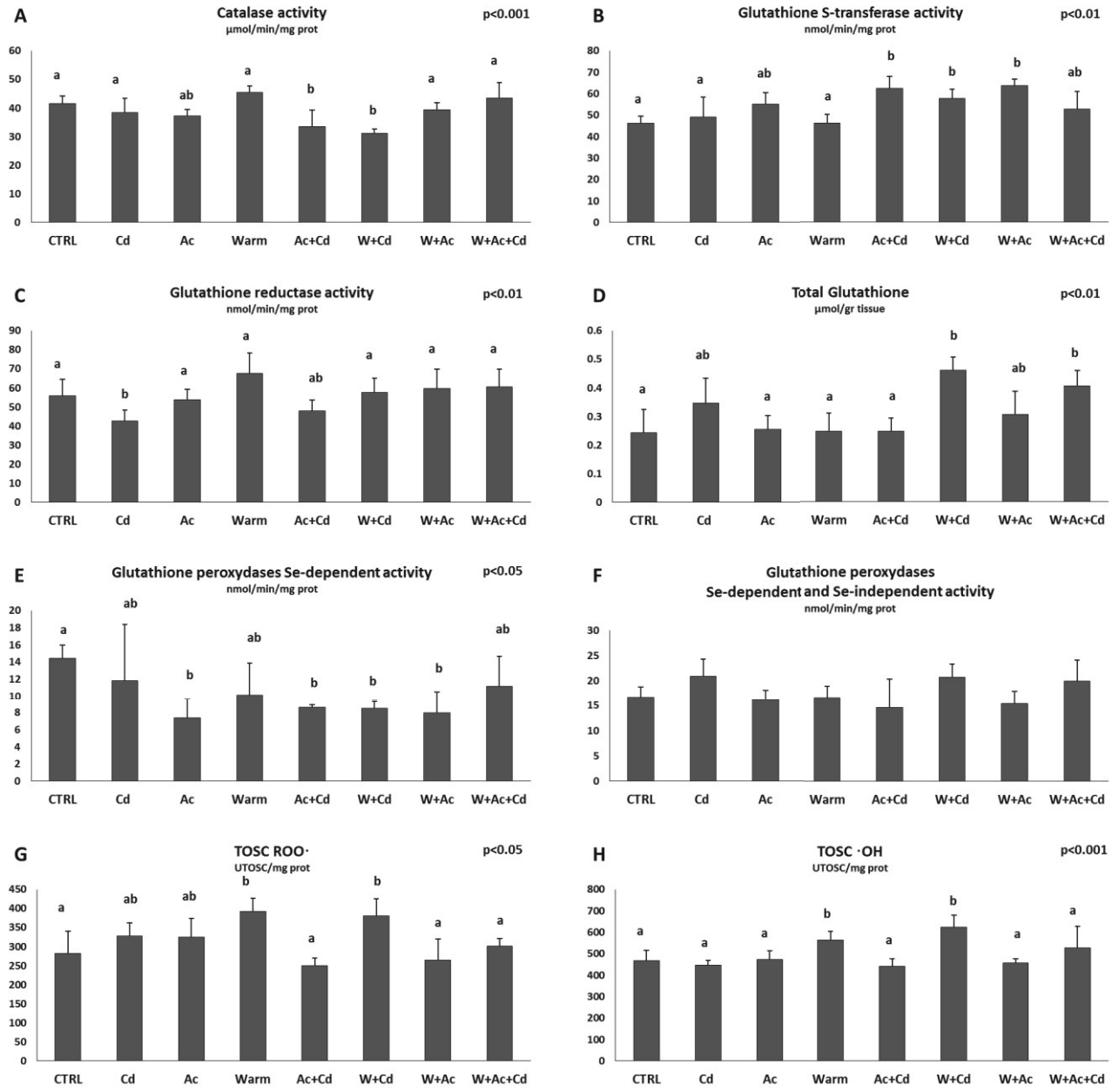

A
Catalase activity
µmol/min/mg prot
p<0.001
B
Glutathione S-transferase activity
nmol/min/mg prot
p<0.01
C
Glutathione reductase activity
nmol/min/mg prot
p<0.01
D
Total Glutathione
µmol/gr tissue
p<0.01
E
Glutathione peroxydases Se-dependent activity
nmol/min/mg prot
p<0.05
F
Glutathione peroxydases
Se-dependent and Se-independent activity
nmol/min/mg prot
G
TOSC ROO·
UTOSC/mg prot
p<0.05
H
TOSC ·OH
UTOSC/mg prot
p<0.001
CTRL
Cd
Ac
Warm
Ac+Cd
W+Cd
W+Ac
W+Ac+Cd

Figure 3.

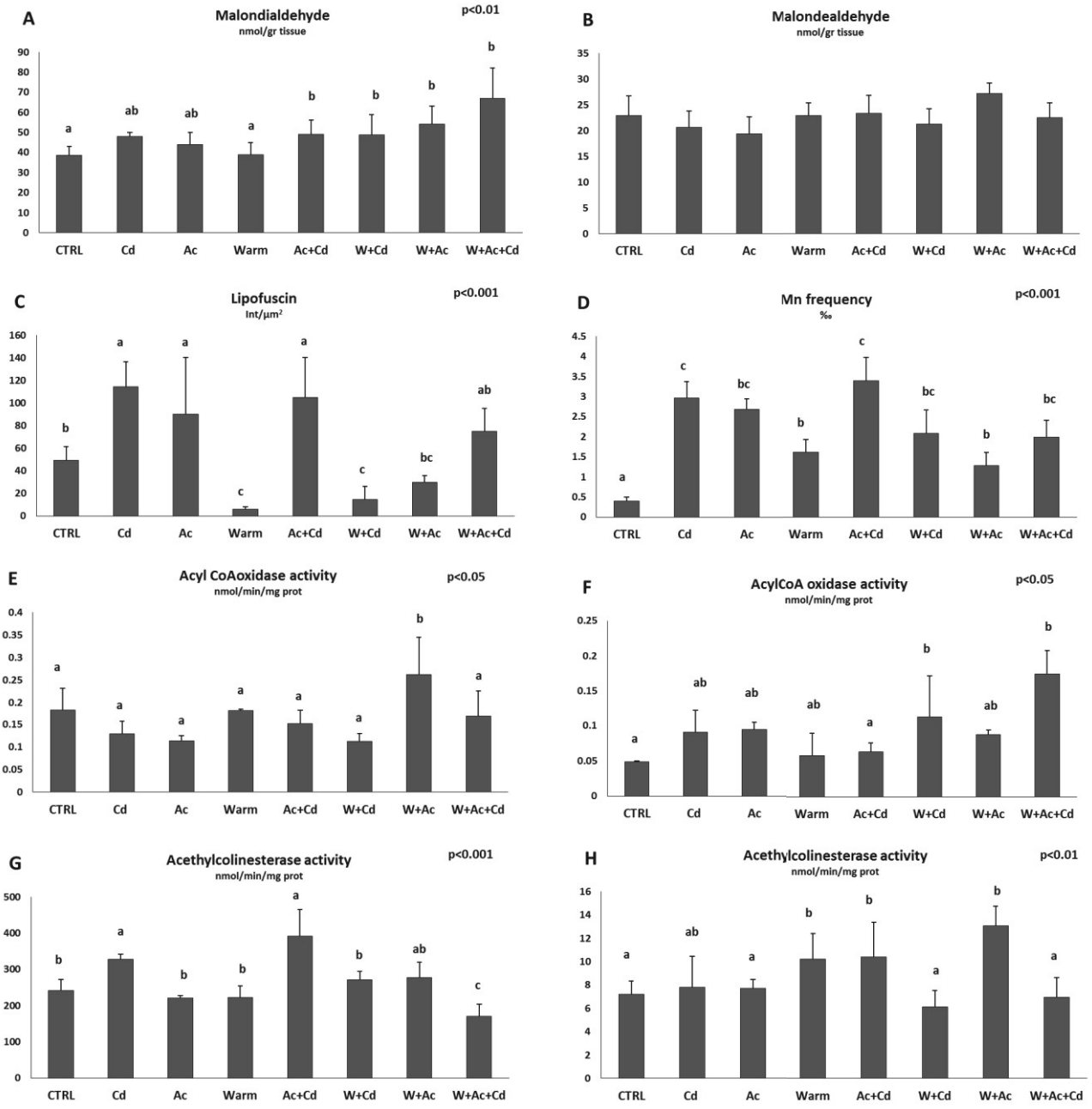
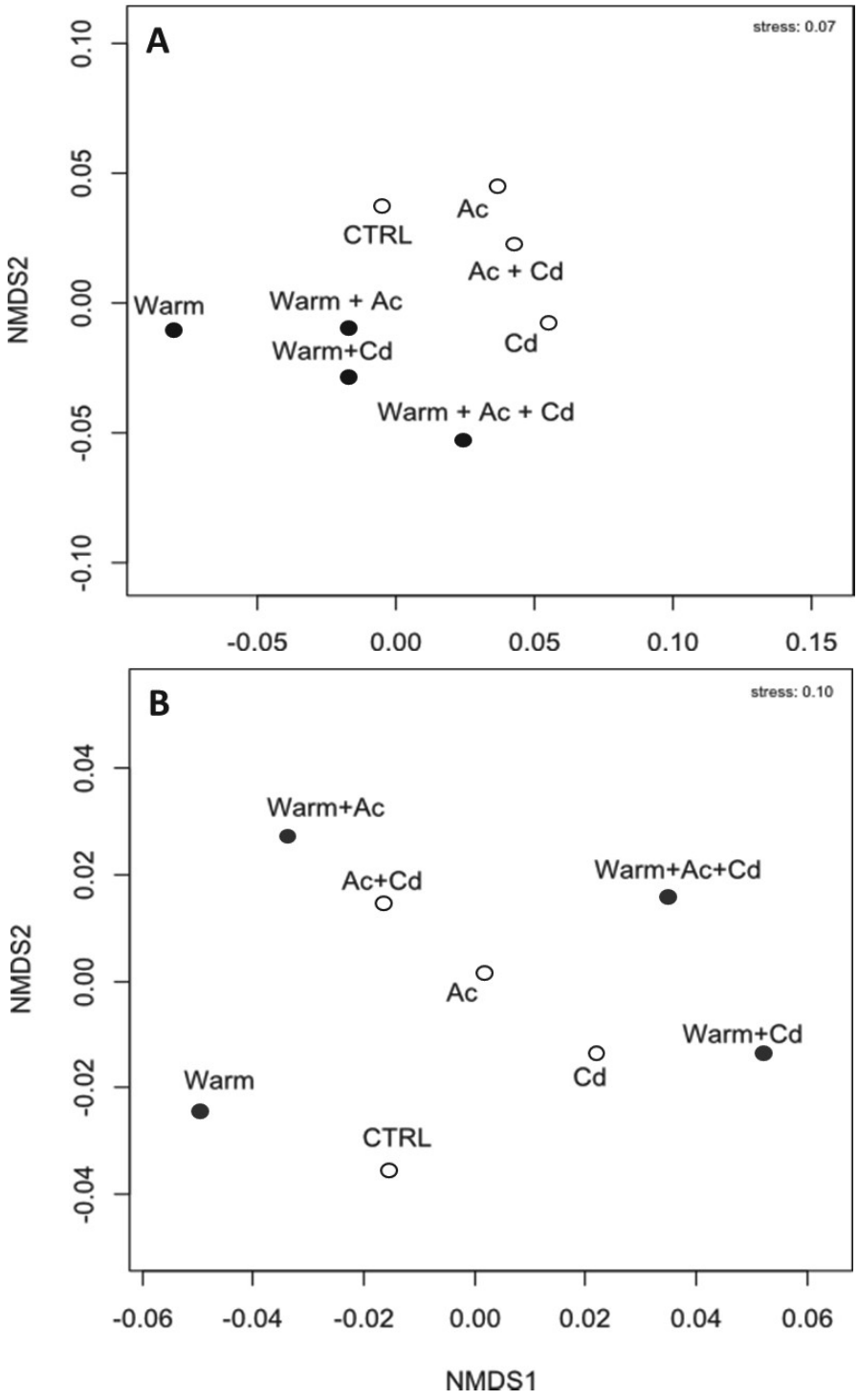

A
Malondialdehyde
nmol/gr tissue
p<0.01
B
Malondealdehyde
nmol/gr tissue
C
Lipofuscin
Int/µm²
p<0.001
D
Mn frequency
‰
p<0.001
E
Acyl CoAoxidase activity
nmol/min/mg prot
p<0.05
F
AcylCoA oxidase activity
nmol/min/mg prot
p<0.05
G
Acethylcolinesterase activity
nmol/min/mg prot
p<0.001
H
Acethylcolinesterase activity
nmol/min/mg prot
p<0.01
CTRL
Cd
Ac
Warm
Ac+Cd
W+Cd
W+Ac
W+Ac+Cd

Figure 4.



21

22 Figure 5.

23

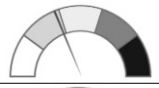
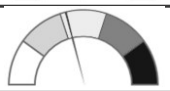
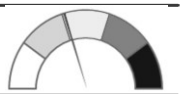
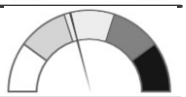
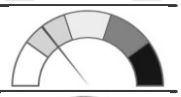
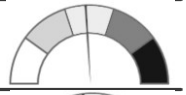
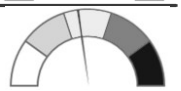
Tissues	Experimental groups	Hazard Quotients (HQ)	Class of Hazard	Level
Digestive glands	Cd	8.9	Moderate	
Digestive glands	Ac	7.99	Slight	
Digestive glands	W	5.92	Slight	
Digestive glands	Ac+Cd	8.62	Moderate	
Digestive glands	W+Cd	6.13	Moderate	
Digestive glands	W+Ac	1.9	Slight	
Digestive glands	W+Ac+Cd	5.5	Moderate	
Gills	Cd	3.53	Slight	
Gills	Ac	5.75	Slight	
Gills	w	2.35	Slight	
Gills	Ac+Cd	1.61	Slight	
Gills	W+Cd	10.58	Moderate	
Gills	W+Ac	9.03	Moderate	
Gills	W+Ac+Cd	13.15	Moderate	

Figure 6.

Highlights:

- Effects of multiple stressors were analysed in the Antarctic scallop *A. colbecki*
- Synergistic effects occurred between temperature, acidification and cadmium
- Oxidative responsiveness was evident in digestive gland and gills
- Cadmium was the primary stressor for digestive gland
- Gills were more sensitive to treatments at higher temperature