Season specific influence of projected ocean changes on the response to cadmium of stress-related genes in Mytilus galloprovincialis

Seasonal and tissue specific interactions of temperature, pH and cadmium on transcriptional regulation of antioxidant and cellular stress genes in *Mytilus galloprovincialis*

Maria Elisa Giuliani[a], Giulia Filippini[b] and Alessandro Nardi[a*]

[a] Dipartimento di Scienze della Vita e dell'Ambiente (DiSVA), Università Politecnica delle Marche, Ancona, Italy

[b] Department of Environmental Sciences, Macquarie University, North Ryde, NSW 2109, Australia

*Corresponding author: Dr. Alessandro Nardi
Dipartimento di Scienze della Vita e dell'Ambiente (DiSVA),
Università Politecnica delle Marche,
Via Brecce Bianche 60131, Ancona, Italy
e-mail: a.nardi@univpm.it

Keywords: *Mytilus galloprovincialis*; ocean acidification; global warming; metal contamination; molecular biomarkers, oxidative stress

Abstract

Continuous anthropogenic inputs of carbon dioxide in the atmosphere are driving ocean warming and acidification. The potential threat represented by these changes for marine species could be amplified in coastal areas, characterized by higher levels of chemical pollutants. In addition, organisms living in temperate areas may exhibit a different seasonal tolerance to stressors influenced by fluctuations of environmental factors and physiological characteristics. In this study, mRNA levels of selected genes related to metal-induced stress response were investigated in the Mediterranean mussel *Mytilus galloprovincialis* collected both in summer and in winter and exposed to combinations of two temperatures (SST, seasonal surface temperature and SST+5 °C), two levels of pH (8.20 and 7.40) and two doses of cadmium (0 and 20 µg/L). Selected genes included metal detoxification pathway (metallothionein *mt-20*), cellular stress response (heat-shock protein *hsp70*), and antioxidants network (superoxide dismutase *Cu/Zn-sod*, catalase *cat*, glutathione peroxidase *gpx1* and glutathione S-transferase *gst-pi*). To further elucidate possible differences in responsiveness of various tissues related to metabolic function and physiological characteristics, such analyses were carried out in digestive gland and in gills of exposed mussels. Seasonal- and tissue-specific differences were observed for metallothioneins gene induction after Cd-exposure, while *hsp70* up-regulation was caused by acidification, both alone or in combination with other stressors. A complex array of interactions affected antioxidant genes, with strong differences as a function of analyzed tissue and experimental season and without a clear role of specific stressors and investigated pathways. The overall results highlighted the importance of considering seasonality and responsiveness of different tissues to predict the effects of sudden changes in environmental parameters on responsiveness and toxicity of chemicals to marine coastal organisms.

1.Introduction

As a consequence of increased anthropogenic carbon dioxide emissions, oceans are threatened by warming and acidification (IPCC, 2013). Since the beginning of the industrial era, ocean has warmed by almost 1 °C because of the greenhouse effect and captured about 30% of anthropogenic CO_2, resulting in a pH drop of 0.1 units (Hansen *et al.*, 2016). By the end of the century, temperature is projected to increase by additional 2°C, while ocean mean pH will further decrease by 0.3 – 0.5 units (IPCC, 2013). These changes could be even more pronounced in coastal areas, where large fluctuations and sudden peaks of temperature and pH naturally occur (Wallace *et al.*, 2014). In addition, these ecosystems are characterized by a higher anthropogenic footprint compared to open ocean and synergistic effects can be expected between a wide range of stressors and climate change (Hewitt *et al.*, 2016).

Ocean warming and acidification have been described as potential factors affecting trace metals speciation and bioavailability but also their biological effects and toxicity. Such interactions were demonstrated on impaired mitochondrial functioning, energy metabolism, oxidative unbalance, accumulation of lipid peroxidation products, damages to lysosomes, DNA and immune function, as well as to impair larval development (Regoli et al., 2002; Roberts *et al.*, 2013; Rodríguez-Romero *et al.*, 2014; Götze *et al.*, 2014; Izagirre *et al.*, 2014; Múgica *et al.*, 2015; Moreira *et al.*, 2018; Cao *et al.*, 2019).

One of the main pathway of trace metals toxicity is exerted through oxidative insult: these elements enhance intracellular production of reactive oxygen species (ROS) affecting electron transport chains and catalyzing Fenton-like and Haber-Weiss reactions, but they can also reduce the amount or efficiency of antioxidant defenses (Regoli et al., 1997, 1998; Regoli and Giuliani, 2014). Oxidative unbalance has been demonstrated to be promoted also by thermal stress and reduced pH-hypercapnic condition in several species, both vertebrates and invertebrates (Tomanek *et al.*, 2011; Wang *et al.*, 2016; Freitas *et al.*, 2017; Andrade *et al.*, 2019; Liao *et al.*, 2019).

To reduce metal toxicity, the accumulation of these elements is associated with the induction of metallothioneins (MTs), low-molecular weight metal-binding proteins the transcription of which is up-regulated, through the metal transcription factor I (MTF-I) (Kimura *et al.*, 2009). The induction of MT can be affected also by other factors, such as ROS, temperature, nutritional status, salinity, and hypoxia (Le *et al.*, 2016). Similarly, environmental stressors including heat stress, metals, hypoxia and hypercapnia activate the heat shock factor (HSF) transcription factor leading to enhanced formation of heat shock protein (Hsp) families (Wang *et al.*, 2013). Among these, Hsp70s represents one of the most conserved families with chaperone activity, involved in folding and unfolding of damaged proteins and stress response (Wang *et al.*, 2013).

Previously published researches of our group (Nardi *et al.*, 2017, 2018b), demonstrated oxidative effects of cadmium in association with temperature and pH stress at biochemical and cellular level in *M. galloprovincialis*. Since the use of molecular biomarkers might represent a sensitive tool to identify early biological responsiveness to environmental stressors, this study aimed to characterize the key transcriptional effects in long-term studies to previously tested multiple stressors such as cadmium (Cd), temperature and acidification. The Mediterranean mussel *Mytilus galloprovincialis*, was chosen as a widely used bioindicator organism (Fattorini *et al.*, 2008; Regoli *et al.*, 2014), while selected target genes reflect pathways of metal-detoxification (the Cd-inducible isoform of metallothioneins, *mt-20*), cellular stress (the heat shock protein 70, *hsp70*) and antioxidants network (cytosolic superoxide dismutase, catalase, Se-dependent glutathione peroxidase and the pi isoform of glutathione S-transferase, *Cu/Zn-sod*, *cat*, *gpx1*, *gst-pi*). Gene expression was analyzed both in digestive gland and gills of exposed mussels due to their different ability to concentrate pollutants and tolerate environmental stressors; further, experiments were performed in summer and in winter to elucidate the potential tissue-dependent and seasonal modulation of observed effects of the investigated parameters. The overall results were expected to provide novel insights on mechanisms underlying the onset of biological and interactions of climate change with other environmental stressors.

2.Materials and Methods

2.1 Animal collection and experimental design

Mussels, *M. galloprovincialis* (6.0 ± 0.5 cm shell length), were obtained from a shellfish farm in an unpolluted area of Central Adriatic Sea (Regoli *et al.*, 2014) in summer (June 2014) and winter (January 2015). For each season, mussels were acclimatized in aerated artificial seawater (ASW; Instant Ocean®) for 7 days at pH 8.20 and salinity 37 (practical salinity unit). Different acclimation temperatures were used for summer (20 °C) and winter (10°C), representative of the mean seasonal surface temperature (SST) at the sampling site and time of collection. Water was changed every other day and mussels fed 12 hours prior the water change with a commercial mixture of zooplankton (50-300 µm) for filter-feeding organisms.

Experimental conditions are those already described in Nardi *et al*., 2017 and 2018b. After the acclimation, mussels (36 individuals in 20 liters) were randomly assigned to one of eight combinations of a multifactorial experimental design with two temperatures (SST and SST+5°C), two pH/pCO_2 (8.20/~400 µatm and 7.40/~3000 µatm) and two doses of added cadmium (0 and 20 µg/L). The resulting experimental treatments were: 1) control condition (CTRL), with seasonal mean surface temperature (SST) and normocapnia (pH=8.20/ pCO_2=~400 µatm); 2) cadmium exposure (Cd), SST, normocapnia and 20 µg/L Cd; 3) acidification (A), SST, hypercapnia (pH=7.40/ pCO_2=~3000 µatm); 4) warming (W), 5°C temperature increase in respect to the SST (SST+5°C) and normocapnia; 5) acidification + Cd (A-Cd), SST, hypercapnia and 20 µg/L Cd; 6) warming + Cd (W-Cd), SST+5°C, normocapnia and 20 µg/L Cd; 7) acidification + warming (A-W), SST+5°C and hypercapnia; 8) acidification + warming + Cd (A-W-Cd), SST+5°C, hypercapnia and 20 µg/L Cd. Exposure cadmium concentration is representative of a polluted but environmentally realistic scenario in Mediterranean coastal waters (Neff, 2002), while selected pH and temperature were adapted from scenario RCP 8.5 and IPCC WGII AR5 (IPCC, 2013), predicting more pronounced variations in coastal areas than in open ocean. The hypercapnic condition was obtained mixing ASW (pH=8.2) with small amounts of CO_2-saturated ASW, resulting of bubbling pure CO_2 in ASW for at

120 least 24h (Nardi *et al*., 2017). For each experimental condition temperature, pH and salinity were
121 measured daily, while total alkalinity (A_T) was measured twice per week. Seawater carbonate
122 parameters (pCO_2, and saturation state (Ω) for calcite and aragonite) were calculated in CO2SYS
123 (Pierrot *et al.*, 2006), using barometric pressure values, as well as A_T, pH, temperature and salinity
124 values for the respective samples (see Nardi *et al.*, 2017, 2018b for details on calculation). Full
125 seawater chemistry, along with Cd bioaccumulation in digestive gland and gills of exposed mussels
126 (previously published in Nardi *et al*., 2017 and 2018b) are provided in Table 1. During the
127 experimental phase, water renewal and feeding regime were the same as in the acclimation phase,
128 and Cd dosed after every water change.
129 After four weeks, animals were sampled from each tank, digestive gland and gills rapidly excised,
130 pooled in 12 samples each constituted by tissues of 3 individuals, frozen in liquid nitrogen and
131 maintained at -80°C until molecular analyses.
132
133 *2.2 RNA isolation and cDNA synthesis*
134 Total RNA was purified from digestive glands and gills, using the Hybrid-RTM kit (GeneAll
135 Biotechnology) according to the manufacturer's protocol. Total RNA concentrations and purity were
136 measured using Nano-Drop ND-1000 UV-Visible Spectrophotometer (NanoDrop Technologies,
137 Wilmington, DE, USA). RNA quality was verified on an agarose-formaldehyde gel. Total cDNA was
138 generated by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) from 1 µg of total RNA
139 for each sample using combined oligo(dT) and random hexamer primers (iScript cDNA Synthesis
140 Kit, Bio-Rad).
141
142 *2.3 Quantitative real-time PCR*
143 Absolute quantitative real-time PCR with gene-specific primer pairs (Table 2) was performed for
144 evaluating the mRNA levels of individual target genes, using SYBR green method in StepOnePlus®
145 Real-Time PCR System (Applied Biosystems). Each 15 µL DNA amplification reaction contained

7.5 µL of SYBR Select Master Mix (Life Technologies), 5 µL of total cDNA (synthesized as described above and diluted 1:5) and 200 nM of each forward and reverse primers. The real-time PCR program included an enzyme activation step at 95 °C (2 min) and 40 cycles composed by 15 s at 95 °C and 1 min at the annealing temperature (Table 2). The specificity of target cDNA amplification was checked by including controls lacking cDNA template and by a melting analysis (95 °C for 1 min, 65 °C for 10 s and fluorescence detection at increasing temperature between 65 and 95 °C). For quantification, serial dilutions of known amounts of plasmid containing the amplicon of interest were used as standards to build a standard plot of Ct versus log copy number (for each target gene). Samples and standards were run in duplicate in the same run. Cycle threshold (Ct) values of unknown samples were converted into mRNA copy number interpolating the standard plot. The values were expressed as $\log_2$ of fold change (FC: exposed samples relative to control samples).

2.4 Statistical analyses

Non-parametric one-way analysis of variance (Kruskal-Wallis test) was used to evaluate the effect of the different treatments. Level of significance was set to $p < 0.05$; *post-hoc* Dunn's test, allowed to compare differences between groups of means. Non-metric multidimensional scaling (NMDS) was applied to each tissue dataset. Statistical analyses were performed using RStudio (version 0.99.491).

3. Results

3.1 Digestive gland

A significant increase of *mt-20* mRNA was observed in the digestive gland of organisms exposed to all experimental combinations containing Cd (Cd, A-Cd, W-Cd and A-W-Cd), with a lower level of induction in summer compared to winter (Fig. 1a and 2a). Acidification increased significantly *hsp70* mRNA levels independently of additional stressors in summer, while in winter no significant variation occurred despite a slight up-regulation due to acidification alone (Fig. 1b, 2b). Changes in

172 *Cu/Zn-sod* expression were not statistically significant in mussels exposed to various treatments in
173 summer (Fig. 1c), while in winter a significant up-regulation was caused by Cd and acidification,
174 alone or in combination with higher temperature (Fig. 2c). Similarly to *hsp70*, *cat* mRNA was higher
175 in organisms exposed to A, A-Cd and A-W during the summer (Fig.1d), while in winter acidification
176 alone caused a significant induction (Fig. 2d). Levels of *gpx1* mRNA were significantly down-
177 regulated in summer by the exposure to Cd, W, A-Cd and A-W (Fig. 1e), while in winter this effect
178 was observed in organisms exposed to higher temperature with Cd and/or reduced pH (Fig. 2e). The
179 *gst-pi* mRNA was significantly increased in summer by acidification, alone or when organisms were
180 co-exposed to Cd and warming (A and A-W-Cd, Fig.1f), while in winter *gsti-pi* induction occurred
181 in organisms exposed to Cd, acidification and acidification at higher temperature (Cd, A and A-W,
182 Fig. 2e).
183
184 *3.2 Gills*
185 Expression of *mt-20* in summer was upregulated in gills of the organisms treated with Cd, but this
186 increase was statistically significant only in those exposed at control temperature with or without
187 acidification (Cd and A-Cd, Fig. 3a); in winter mussels exhibited the induction of *mt-20* when
188 exposed to Cd at control temperature, while increased mRNA levels observed after W-Cd and A-W-
189 Cd treatments did not reach the statistical significance (Fig. 4a) and a statistically significant
190 downregulation of *mt-20* mRNA occurred in organisms exposed to acidification or higher
191 temperature alone (A and W, Fig. 4a). Expression of *hsp70* was downregulated in organisms co-
192 exposed in summer to Cd and reduced pH (A-Cd, Fig. 3b), while in winter a significant upregulation
193 of this gene occurred in organisms exposed to acidification at control and higher temperature (A and
194 A-W, Fig. 4b). No significant variations of *Cu/Zn-sod* and *cat* expression (Fig.3c and 3d) were found
195 in summer treatments, while in winter both genes were affected: *Cu/Zn-sod* was upregulated in gills
196 of mussels co-exposed to Cd, acidification and warming (A-W-Cd, Fig. 4c), while *cat* was
197 significantly downregulated in organisms exposed to Cd and acidification, both alone or in

198 combination (Cd, A, A-Cd, A-W-Cd, Fig. 4d). A lower expression of *gpx1* was caused in summer
199 warming and warming with Cd (W and W-Cd, Fig. 3e), while in winter downregulation of *gpx1*
200 expression was caused by A, W and A-W-Cd (Fig, 4e). No significant variations were observed for
201 *gst-pi* mRNA in gills for mussels from both the seasons (Fig. 3f and Fig. 4f).
202
203 *3.3 Non-metric multidimensional scaling (NMDS)*
204 Non-metric multidimensional scaling provided clear separation between summer and winter
205 experiments in both tissues (Fig. 5a,b), mostly due to *mt20*, *Cu/Zn-sod* and *gpx* in digestive gland,
206 and to *mt20*, *gpx1*, *Cu/Zn-sod* and *gst-pi* in gills. In each tissue, separation between Cd-exposed and
207 non-exposed mussels was also explicitly driven by *mt20*.
208
209 **4. Discussion**
210 The present study showed that both acidification and warming can synergistically affect the
211 transcription of genes associated to metal exposure, with the sensitivity of these diverging in different
212 seasons and tissues.
213 ***4.1 Digestive gland***
214 Different isoforms of metallothioneins genes have been described in *M. galloprovincialis*, among
215 which *mt-20* has been shown to be induced by cadmium and oxidative stress (Dondero *et al.*, 2005).
216 Here, *mt-20* gene in the digestive gland showed to be responsive to Cd, independently of temperature
217 and pH, in both summer and winter experiments. However, *mt-20* upregulation was not always
218 proportional to Cd uptake (previously published in Nardi *et al.*, 2017; 2018b): in fact, while exposure
219 at higher temperature always increased Cd accumulation, a parallel modulation of *mt20* induction
220 was observed only in summer for the digestive gland. From these data, a lower responsiveness of
221 metallothionein gene may be hypothesized in a potential scenario of increased temperature in winter.
222 Scientific literature on the role of temperature and pH on *mt-20* transcription in mussels is scarce, and
223 the few available data described an up-regulating effect of temperature on *mt-20* gene expression in

224 embryos and larvae of *M. galloprovincialis* exposed to copper (Boukadida *et al.*, 2017; Mlouka *et al.*,
225 2019).

226 Hsp70s are usually associated to folding or degradation of damaged and unrepairable proteins
227 (Mayer and Bukau, 2005) and their induction is a marker of thermal or cellular stress (Wang *et al.*,
228 2013). In our study, the absence of *hsp70* upregulation after 28 days of exposure to increased
229 temperature alone, confirms that the response to heat-shock is typical of acute stress (Franzellitti and
230 Fabbri, 2005), supporting a physiological adaptation of mussels to long-term warming. Acidification
231 was instead the main driver of *hsp70* induction in mussels digestive gland after 28 days exposure in
232 summer, particularly evident when acidification was combined with other stressors. Similar results
233 would indicate that mechanisms of protein damage are enhanced by changes in the intracellular
234 mileau due to hypercapnic condition (Wang *et al.*, 2016). Acute effects of lowered pH on hsp70s
235 were already demonstrated in several marine species after hours to days of exposure (Hernroth *et al.*,
236 2011; Moya *et al.*, 2015; Feidantsis *et al.*, 2015), but the induction after longer periods of reduced pH
237 exposure has been documented only in the Antarctic bivalve *Laternula elliptica* (21 days, Cummings
238 *et al.*, 2011) and the cold-water coral *Desmophyllum dianthus* (8 months exposure, Carreiro-Silva *et*
239 *al.*, 2014). On the other hand, in mussels exposed during the winter the trend toward an induction of
240 *hsp70* expression by lower pH was abolished when also warming and/or Cd were combined as
241 multiple stressors: the different physiological state of mussels in winter period would then reflect a
242 lower sensitivity to cellular impairment compared to summer organisms.

243 The battery of antioxidant genes indicated a certain disturbance of the oxidative balance toward
244 investigated stressors, confirming a generally higher responsiveness during the summer.
245 Acidification, alone or in combination with other stressors, was the main factor promoting the up-
246 regulation of *cat* and *gst-pi* both, suggesting a CO_2-mediated increase of oxidative challenge as
247 already hypothesized by other authors (Tomanek *et al.*, 2011). Interestingly, heat shock proteins were
248 previously suggested to act as redox sensors activating some antioxidant genes (Madeira *et al.*, 2017
249 and ref. therein): this hypothesis seems to be confirmed by our observations with similar responses

to acidification for *cat*, *gst-pi* and *hsp70*. The expression of *gpx1* exhibited a generalized downregulation, with major effects due to Cd-exposure in summer, and by various combinations of temperature with other stressors in winter. Inhibitory effects of Cd on *gpx1* expression have been reported in *Danio rerio* (Banni *et al.*, 2011) and in *Oncorhynchus kisutch* (Wang *et al.*, 2012), while increased temperature was shown to upregulate *gpx1* in the gastropod *Haliotis discus discus* (De Zoysa *et al.*, 2009). The downregulation of *gpx1* is mainly due to degradation through the so-called "nonsense mediated decay" mechanism, which may occur e.g. when intracellular Se concentration are limiting (Sun *et al*., 2000). Although few evidences are available for bivalves, both Cd exposure and changes in pH have been shown to modulate uptake and intracellular levels of Se (Dorey *et al.*, 2018).

4.2 Gills

In the gills, interactive effects of warming and acidification showed to interfere with Cd-induced *mt-20* transcription in both seasons. Although Cd was bioaccumulated in all treatments with this element (Tab. 1), responsiveness to *mt20* seems more variable in organisms co-exposed to higher temperature in summer and weakened by lowered pH in winter, corroborating the hypothesis of differential sensitivity of mussels to environmental factors in different seasons, and a compromised capacity of gills to counteract Cd contamination in case of prolonged temperature and/or pH stress.

Similarly to the digestive tissues, *hsp70* was not induced by warming alone in gills, and acidification was confirmed to provoke a certain up-regulation, although less intense and limited to winter mussels. On the other hand, a marked downregulation of *hsp70* was observed in organisms co-exposed to Cd and acidification during the summer: downregulation of *hsp70* mRNA was observed in the haemocytes of clam *Mercenaria mercenaria* exposed to Cd and hypercapnia (Ivanina *et al.*, 2014), in the haemocytes of oyster *Saccostrea glomerate,* and in the digestive gland of *M. galloprovincialis* due to Cd exposure (Thompson *et al.*, 2012; Izagirre *et al.*, 2014); lowered levels of heat shock proteins were also observed in the oysters *Crassostrea virginica*, *Pinctada fucata* and

276 *Crassostrea gigas* exposed to acidification (Liu *et al*. 2012; Ivanina *et al.*, 2014; Dineshram *et al.*,
277 2016). The downregulation of this cellular response has been described as a mechanism of energy
278 allocation trade-off (Goncalves *et al.*, 2017), allowing to hypothesize a decreased adaptability to new
279 environmental scenarios, since the capacity to activate gene expression influence the tolerance of
280 marine species to climate changes (Somero 2010, Logan and Somero, 2011).

281 Weak sensitivity of antioxidant responses was generally observed in gills of exposed mussels
282 exposed during both seasons. Most of the observed variations were not statistically significant; only
283 *gpx1* and *cat* were downregulated by single or combined stressors with unclear causative stressor-
284 effect relationships, confirming a lower involvement of gills compared to digestive tissues in
285 counteracting oxidative insult deriving from environmental stressors (Regoli, 1998). Also for the
286 gills, non-metric multidimensional scaling analysis (Fig. 5b) confirms seasonal differences between
287 summer and winter organisms.

288

289 ***4.3 Transcriptional vs. functional investigations***

290 Responses analyzed in this study at gene expression level were previously characterized in terms
291 of functional effects measuring protein levels of MTs and enzymatic activities of CAT, GST and Se-
292 GPX in mussels exposed to the same experimental conditions as those presented here (Nardi *et al.*,
293 2017, 2018b).

294 These overall results highlight some discrepancies between transcriptional responses and protein
295 or enzymatic activities, as already observed in the European eel *Anguilla anguilla* and in *M.*
296 *galloprovincialis* exposed to polluted sediments (Regoli *et al.*, 2011; Giuliani *et al.*, 2013), and in the
297 Pacific oyster *C. gigas* exposed to ibuprofen (Serrano *et al.*, 2015). In particular, the magnitude of
298 *mt20* mRNA induction was not always paralleled by a comparable increase of MTs protein levels in
299 Cd-exposed organisms, suggesting a potential steady state in protein synthesis capability,
300 independently on the rate of gene induction.

Differences between mRNA levels and enzymatic activities are also observed for antioxidants. The mRNA upregulations often observed in the digestive gland were not reflected in a similar increase of the corresponding enzymatic activities. Such evidence may imply that under stressful conditions a higher mRNA transcription is needed to maintain the physiological catalytic level, since mRNA, proteins and enzymatic activities could be target of post-transcriptional and/or post-translational toxicity (*e.g.* reduced mRNA stability, slower protein synthesis, incorrect folding, cofactor depletion). As a consequence, despite the attempt of the cell to counteract the stressors at transcriptional level, the reduced functional response might limit the capacity to adapt to environmental changes.

In conclusion, this study provided clear evidences that future ocean temperature and pH can interactively modulate transcriptional responses associated both directly and indirectly to metal-exposure; the observed effects are highly tissue- and season-specific, thus depending on tissue metabolic function and physiological characteristics influenced by seasonal life cycle. Nevertheless, our findings further contribute to the growing awareness on discrepancies between biological responses measured at transcriptional and catalytic level, suggesting a complementary use of these approaches, and confirming that mechanisms underlying the effects of future ocean changes are still to be fully elucidated.

5. References

Andrade M, De Marchi L, Soares AMVM, Rocha RJM, Figueira E, Freitas R (2019). Are the effects induced by increased temperature enhanced in *Mytilus galloprovincialis* submitted to air exposure? Science of the Total Environment, 647, pp. 431-440.

Banni M, Chouchene L, Said K, Kerkeni A, Messaoudi I (2011). Mechanisms underlying the protective effect of zinc and selenium against cadmium-induced oxidative stress in zebrafish *Danio rerio*. BioMetals, 24, pp. 981-992.

Bocchetti R, Regoli F (2006). Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. Chemosphere, 65, pp. 913-921.

Boukadida K, Cachot J, Clérandeaux C, Gourvesb P-Y, Banni M (2017). Early and efficient induction of antioxidant defense system in *Mytilus galloprovincialis* embryos exposed to metals and heat stress. Ecotoxicology and Environmental Safety, 138, pp. 105-112.

Cao C, Leng Y, Huang W, Liu X, Kufe D (2003). Glutathione peroxidase 1 is regulated by the c-Abl and Arg tyrosine kinases. Journal of Biological Chemistry 278(41), pp. 39609-39614.

Cao R, Zhang T, Li X, Zhao Y, Wang Q, Yang D, Qu Y, Liu H, Dong Z, Zhao J (2019). Seawater acidification increases copper toxicity: A multi-biomarker approach with a key marine invertebrate, the Pacific Oyster *Crassostrea gigas*. Aquatic Toxicology, 210, pp. 167-178.

Carreiro-Silva M, Cerqueira T, Godinho A, Caetano M, Santos RS, Bettencourt R (2014). Molecular mechanisms underlying the physiological responses of the cold-water coral *Desmophyllum dianthus* to ocean acidification. Coral Reefs, 33 (2), pp. 465-476.

Cellura C, Toubiana M, Parrinello N, Roch (2006). HSP70 gene expression in *Mytilus galloprovincialis* hemocytes is triggered by moderate heat shock and *Vibrio anguillarum*, but not by *V. splendidus* or *Micrococcus lysodeikticus*. (2006) Developmental and Comparative Immunology, 30, pp. 984-997.

Cummings V, Hewitt J, van Rooyen A, Currie K, Beard S, Thrush S, Norkko J, Barr N, Heath P, Jane Halliday N, Sedcole R, Gomez A, McGraw C, Metcalf V (2011). Ocean acidification at high latitudes: Potential effects on functioning of the antarctic bivalve *Laternula elliptica*. PLoS ONE, 6 (1).

De Zoysa M, Whang I, Lee Y, Lee S, Lee J-S, Lee J (2009). Transcriptional analysis of antioxidant and immune defense genes in disk abalone (*Haliotis discus discus*) during thermal, low-salinity and hypoxic stress. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 154, 387-395.

Dineshram R, Chandramouli K, Ko GWK, Zhang H, Qian P-Y, Ravasi T, Thiyagarajan V (2016). Quantitative analysis of oyster larval proteome provides new insights into the effects of multiple climate change stressors. Global Change Biology 22(6), pp. 2054-2068.

Dondero F, Piacentini L, Banni M, Rebelo M, Burlando B, Viarengo A (2005). Quantitative PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation. Gene, 345, pp. 259-270.

Dorey N, Martin S, Oberhänsli F, Teyssié J-L, Jeffree R, Lacoue-Labarthe T (2018). Ocean acidification modulates the incorporation of radio-labeled heavy metals in the larvae of the Mediterranean sea urchin *Paracentrotus lividus*. Journal of Environmental Radioactivity 190-191, pp. 20-30.

Fattorini D, Notti A, Di Mento R, Cicero AM, Gabellini M, Russo A, Regoli F (2008). Seasonal, spatial and inter-annual variations of trace metals in mussels from the Adriatic sea: a regional gradient for arsenic and implications for monitoring the impact of off-shore activities. Chemosphere 72, 1524-1533.

Feidantsis K, Pörtner H-O, Antonopoulou E, Michaelidis B (2015). Synergistic effects of acute warming and low pH on cellular stress responses of the gilthead seabream *Sparus aurata.* Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 185 (2), pp. 185-205.

Franzellitti S, Fabbri E (2005). Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. Biochemical and Biophysical Research Communications. 336 (4), pp. 1157-1163.

Freitas R, De Marchi L, Bastos M, Moreira A, Velez C, Chiesa S, Wrona FJ, Figueira E, Soares AMVM (2017). Effects of seawater acidification and salinity alterations on metabolic, osmoregulation and oxidative stress markers in *Mytilus galloprovincialis*. Ecological Indicators, 79, pp. 54-62.

Giuliani ME, Benedetti M, Arukwe A, Regoli F (2013). Transcriptional and catalytic responses of antioxidant and biotransformation pathways in mussels, *Mytilus galloprovincialis*, exposed to chemical mixtures. Aquatic Toxicology, 134-135, pp. 120-127.

Glorieux C, Zamocky M, Sandoval JM, Verrax J, Calderon PB (2015). Regulation of catalase expression in healthy and cancerous cells. Free Radical Biology and Medicine, 87, pp. 84-97.

Goncalves P, Jones DB, Thompson EL, Parker LM, Ross PM, Raftos DA (2017). Transcriptomic profiling of adaptive responses to ocean acidification. Molecular Ecology 26(21), pp. 5974-5988.

Götze S, Matoo OB, Beniash E, Saborowski R, Sokolova IM (2014). Interactive effects of CO2 and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Aquatic Toxicology, 149, 65-82.

Gourgou E, Aggeli I-K, Beis I, Gaitanaki C (2010). Hyperthermia-induced Hsp70 and MT20 transcriptional upregulation are mediated by p38-MAPK and JNKs in *Mytilus galloprovincialis* (Lamarck); a pro-survival response. Journal of Experimental Biology, 213 (2), pp. 347-357.

Hansen J, Satoa M, Ruedy R, Schmidt GA, Lo K (2016). Global Temperature in 2015. In: NASA News Release 16-008. New York City, NY, NOAA GISS.

Hernroth B, Baden S, Thorndyke M, Dupont S (2011). Immune suppression of the echinoderm *Asterias rubens* (L.) following long-term ocean acidification. Aquatic Toxicology, 103 (3-4), pp. 222-224.

Hewitt JE, Ellis JI, Thrush SF (2016). Multiple stressors, nonlinear effects and the implications of climate change impacts on marine coastal ecosystems. Global Change Biology, 22, pp. 2665-2675.

Hoffmann LJ, Breitbarth E, Boyd PW, Hunter KA (2012). Influence of ocean warming and acidification on trace metal biogeochemistry. Marine Ecology Progress Series, 470, 191-205.

IPCC, 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Ivanina AV, Hawkins C, Sokolova IM (2014). Immunomodulation by the interactive effects of cadmium and hypercapnia in marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Fish and Shellfish Immunology, 37, pp. 299-312.

Izagirre U, Errasti A, Bilbao E, Múgica M, Marigómez I (2014). Combined effects of thermal stress and Cd on lysosomal biomarkers and transcription of genes encoding lysosomal enzymes and HSP70 in mussels, *Mytilus galloprovincialis*. Aquatic Toxicology, 149, 145-156.

Kimura T, Itoh N, Andrews GK (2009). Mechanisms of heavy metal sensing by metal response element-binding transcription factor-1. Journal of Health Science, 55, pp. 484-494.

Le TTY, Zimmermann S, Sures B (2016). How does the metallothionein induction in bivalves meet the criteria for biomarkers of metal exposure? Environmental Pollution, 212, pp. 257-268.

Lee Y-E, Hong C-Y, Lin Y-L, Chen R-M (2015). MicroRNA-1 participates in nitric oxide-induced apoptotic insults to MC3T3-E1 cells by targeting heat-shock protein-70. International Journal of Biological Sciences, 11, pp. 246-255.

Liao H, Yang Z, Dou Z, Sun F, Kou S, Zhang Z, Huang X, Bao Z (2019). Impact of ocean acidification on the energy metabolism and antioxidant responses of the Yesso scallop (*Patinopecten yessoensis*). Frontiers in Physiology, 10, art. no. 1967.

Liu W, Huang X, Lin J, He M (2012). Seawater acidification and elevated temperature affect gene expression patterns of the pearl oyster *Pinctada fucata*. PLoS ONE 7(3), e33679.

Logan CA, Somero GN (2011). Effects of thermal acclimation on transcriptional responses to acute heat stress in the eurythermal fish *Gillichthys mirabilis* (Cooper). American Journal of Physiology - Regulatory Integrative and Comparative Physiology 300(6), pp. 1373-1383.

Madeira D, Araújo JE, Vitorino R, Costa PM, Capelo JL, Vinagre C, Diniz MS (2017). Molecular plasticity under ocean warming: Proteomics and fitness data provides clues for a better understanding of the thermal tolerance in fish. Frontiers in Physiology, 8, art. no. 825.

Mayer MP, Bukau B (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. Cellular and Molecular Life Sciences, 62, pp. 670-684.

Miao Y, Laun T, Zimmermann P, Zentgraf U (2005). Targets of the WRKY53 transcription factor and its role during leaf senescence in *Arabidopsis*. Plant Molecular Biology, 55, pp. 853-867.

Mlouka R, Cachot J, Boukadida K, Clérandeau C, Gourves P-Y, Banni M (2019). Compared responses to copper and increased temperatures of hybrid and pure offspring of two mussel species. Science of the Total Environment, 685, pp. 795-805.

Moya A, Huisman L, Forêt S, Gattuso J-P, Hayward DC, Ball EE, Miller DJ (2015). Rapid acclimation of juvenile corals to CO_2-mediated acidification by upregulation of heat shock protein and Bcl-2 genes. Molecular Ecology, 24 (2), pp. 438-452.

Moreira A, Freitas R, Figueira E, Volpi Ghirardini A, Soares A M V M, Radaelli M, Guida M, Libralato G (2018). Combined effects of arsenic, salinity and temperature on *Crassostrea gigas* embryotoxicity. Ecotoxicology and Environmental Safety, 147, pp. 251-259.

Múgica M, Izagirre U, Marigómez I (2015). Lysosomal responses to heat-shock of seasonal temperature extremes in Cd-exposed mussels. Aquatic Toxicology, 164, 99-107.

Nardi A, Mincarelli LF, Benedetti M, Fattorini D, d'Errico G, Regoli F (2017). Indirect effects of climate changes on cadmium bioaccumulation and biological effects in the Mediterranean mussel *Mytius galloprovincialis*. Chemosphere, 169, 493-502.

Nardi A, Benedetti M, Fattorini D, Regoli F (2018a). Oxidative and interactive challenge of cadmium and ocean acidification on the smooth scallop *Flexopecten glaber.* Aquatic Toxicology, 196, pp. 53-60.

Nardi A, Benedetti M, d'Errico G, Fattorini D, Regoli F (2018b). Effects of ocean warming and acidification on accumulation and cellular responsiveness to cadmium in mussels *Mytilus galloprovincialis*: Importance of the seasonal status. Aquatic Toxicology, 204, pp. 171-179.

Neff JM (2002). Bioaccumulation in marine organisms: effects of contaminants from oil well produced waters. Elsevier Science, Oxford, UK.

Pierrot D, Lewis E, Wallace DWR (2006). MS Excel Program Developed for CO2 System Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee. doi:509 10.3334/CDIAC/otg.CO2SYS_XLS_CDIAC105a

Regoli F (1998). Trace metals and antioxidant mechanisms in gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. Arch. Envir. Contam. Toxicol. 34(1), 48-63.

Regoli F, Giuliani ME (2014) Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine Environmental Research, 93, pp. 106-117.

Regoli F, Nigro M, Bertoli E, Principato G, Orlando E (1997). Defenses against oxidative stress in the Antarctic scallop *Adamussium colbecki* and effects of acute exposure to metals. Hydrobiologia, 355, 139-144.

Regoli F, Hummel H, Amiard-Triquet C, Larroux C, Sukhotin A (1998). Trace metals and antioxidant enzymes in Arctic bivalve populations. Arch. Envir. Contam. Toxicol. 35(4), 594-601.

Regoli F, Nigro M, Chiantore M, Winston GW (2002). Seasonal variations of susceptibility to oxidative stress in *Adamussium colbecki*, a key bioindicator species for the Antarctic marine environment. Sci. Total Envir. 289, 205-211.

Regoli F, Giuliani ME, Benedetti M, Arukwe A (2011). Molecular and biochemical biomarkers in environmental monitoring: A comparison of biotransformation and antioxidant defense systems in multiple tissues. Aquatic Toxicology, 105, pp. 56-66.

Regoli F, Pellegrini D, Cicero AM, Nigro M, Benedetti M, Gorbi S, Fattorini D, D'Errico G, Di Carlo M, Nardi A, Gaion A, Scuderi A, Giuliani S, Romanelli G, Berto D, Trabucco B, Guidi P, Bernardeschi M, Scarcelli V, Frenzilli G (2014). A multidisciplinary weight of evidence approach for environmental risk assessment at the Costa Concordia wreck: Integrative indices from Mussel Watch. Marine Environmental Research, 96, pp. 92-104.

Roberts DA, Birchenough SNR, Lewis C, Sanders MB, Bolam T, Sheahan D (2013). Ocean acidification increases the toxicity of contaminated sediments. Global Change Biology, 19, 340-351.

Rodríguez-Romero A, Jiménez-Tenorio N, Basallote MD, Orte MRD, Blasco J, Riba I (2014). Predicting the impacts of CO2 leakage from subseabed storage: Effects of metal accumulation and toxicity on the model benthic organism *Ruditapes philippinarum*. Environmental Science and Technololgy, 48, 12292-12301.

480 Serrano MAS, Gonzalez-Rey M, Mattos JJ, Flores-Nunes F, Mello ÁCP, Zacchi FL, Piazza CE,
481 Siebert MN, Piazza RS, Alvarez-Muñoz D, Rodriguez-Mozaz S, Barceló D, Bebianno MJ, Gomes
482 CHAM, Melo CMR, Bainy ACD (2015). Differential gene transcription, biochemical responses, and
483 cytotoxicity assessment in Pacific oyster *Crassostrea gigas* exposed to ibuprofen. Environmental
484 Science and Pollution Research, 22, pp. 17375-17385.

485 Somero GN (2010). The physiology of climate change: How potentials for acclimatization and
486 genetic adaptation will determine 'winners' and 'losers'. Journal of Experimental Biology 213(6), pp.
487 912-920.

488 Stoytcheva ZR, Berry MJ (2009). Transcriptional regulation of mammalian selenoprotein expression.
489 Biochimica et Biophysica Acta - General Subjects, 1790, pp. 1429-1440.

490 Sun X, Moriarty PM, Maquat LE (2000). Nonsense-mediated decay of glutathione peroxidase 1
491 mRNA in the cytoplasm depends on intron position. EMBO Journal 19(17), pp. 4734-4744.

492 Thompson EL, Taylor DA, Nair SV, Birch G, Haynes PA, Raftos DA (2012). Proteomic discovery
493 of biomarkers of metal contamination in Sydney Rock oysters (*Saccostrea glomerata*). Aquatic
494 Toxicology, 109, 202-212.

495 Tomanek L, Zuzow MJ, Ivanina AV, Beniash E, Sokolova IM (2011). Proteomic response to elevated
496 PCO2 level in eastern oysters, *Crassostrea virginica*: Evidence for oxidative stress. Journal of
497 Experimental Biology, 214, pp. 1836-1844.

498 Wallace RB, Baumann H, Grear JS, Aller R, Gobler CJ (2014). Coastal ocean acidification: The other
499 eutrophication problem. Estuarine and Coastal Shelf Science, 148, 1-13

500 Wang L, Harris SM, Espinoza HM, McClain V, Gallagher EP (2012). Characterization of
501 phospholipid hydroperoxide glutathione metabolizing peroxidase (gpx4) isoforms in Coho salmon
502 olfactory and liver tissues and their modulation by cadmium. Aquatic Toxicology, 114-115, 134-141.

503 Wang L, Yang C, Song L (2013). The molluscan HSP70s and their expression in haemocytes.
504 Invertebrate Survival Journal, 10, pp. 77-83.

505 Wang X, Wang M, Jia Z, Wang H, Jiang S, Chen H, Wang L, Song L (2016). Ocean acidification
506 stimulates alkali signal pathway: A bicarbonate sensing soluble adenylyl cyclase from oyster
507 *Crassostrea gigas* mediates physiological changes induced by CO2 exposure. Aquatic Toxicology,
508 181, pp. 124-135.

509

summer									
Treatment	measured parameters				calculated parameters			Cd burdens (µg/g dw)	
	S	T (°C)	pH_{NBS}	A_T (µmol/kg)	*p*CO2 (µatm)	Ωc	Ωa	Digestive gland	Gills
CTRL	37 ± 0.5	19.95 ± 0.10	8.21 ± 0.04	2453.6 ±251.5	380.8 ± 25.8	5.3 ± 0.4	3.5 ± 0.2	2.89 ± 1.91	1.04 ± 0.62
Cd	37 ± 0.5	20.00 ± 0.10	8.19 ± 0.04	2390.5 ± 354.1	410.6 ± 30.9	5.1 ± 0.4	3.3 ± 0.3	33.15 ± 3.7	14.22 ± 1.04
A	37 ± 0.5	19.98 ± 0.06	7.42 ± 0.04	2557.3 ± 183.7	2897.6 ± 183.8	1.0 ± 0.1	0.7 ± 0.1	3.01 ± 1.08	0.55 ± 0.26
W	37 ± 0.5	24.80 ± 0.13	8.15 ± 0.06	2325.4 ± 267.7	468.1 ± 47.9	5.4 ± 0.4	3.6 ± 0.3	2.83 ± 1.15	0.38 ± 0.12
A-Cd	37 ± 0.5	19.95 ± 0.06	7.41 ± 0.04	2556.7 ± 479.0	2928.2 ± 144.4	1.0 ± 0.1	0.7 ± 0.1	34.53 ± 9.48	19.47 ± 2.4
W-Cd	37 ± 0.5	24.83 ± 0.08	8.14 ± 0.04	2517.9 ± 206.9	477.4 ± 44.6	5.2 ± 0.4	3.5 ± 0.2	38.03 ± 10.62	18.78 ± 8.93
A-W	37 ± 0.5	24.76 ± 0.18	7.42 ± 0.03	2721.4 ± 215.7	3100.1 ± 241.7	1.2 ± 0.1	0.8 ± 0.1	5.08 ± 0.12	0.33 ± 0.13
A-W-Cd	37 ± 0.5	24.87 ± 0.16	7.43 ± 0.04	2504.2 ± 182	2993.7 ± 186.7	1.3 ± 0.1	0.9 ± 0.1	39.8 ± 5.23	15.99 ± 4.4

winter									
Treatment	measured parameters				calculated parameters			Cd burdens (µg/g dw)	
	S	T (°C)	pH_{NBS}	A_T (µmol/kg)	*p*CO2 (µatm)	Ωc	Ωa	Digestive gland	Gills
CTRL	37 ± 0.5	9.95 ± 0.11	8.18 ± 0.03	3283.2 ± 88.8	386.9 ± 26.2	6.2 ± 0.5	3.9 ± 0.3	0.52 ± 0.04	0.22 ± 0.05
Cd	37 ± 0.5	9.97 ± 0.06	8.16 ± 0.03	3334.2 ± 102.6	411.8 ± 35.8	6.1 ± 0.3	3.9 ± 0.2	16.4 ± 1.92	6.79 ± 1.11
A	37 ± 0.5	10.54 ± 0.08	7.40 ± 0.05	3364.1 ± 112.9	2882.2 ± 363.8	1.3 ± 0.2	0.8 ± 0.1	0.44 ± 0.09	0.12 ± 0.08
W	37 ± 0.5	14.95 ± 0.12	8.17 ± 0.03	3378.4 ± 121.2	416.8 ± 34.5	7.2 ± 0.5	4.7 ± 0.4	0.54 ± 0.08	0.25 ± 0.07
A-Cd	37 ± 0.5	10.04 ± 0.15	7.39 ± 0.02	3360.6 ± 36.8	2860.9 ± 207.2	1.2 ± 0.1	0.8 ± 0.1	20.67 ± 3.16	5.21 ± 0.89
W-Cd	37 ± 0.5	15.02 ± 0.11	8.17 ± 0.03	3350.5 ± 164.9	403.5 ± 53.1	7.3 ± 0.5	4.7 ± 0.3	30.49 ± 3.07	16.17 ± 4.3
A-W	37 ± 0.5	14.98 ± 0.05	7.39 ± 0.04	3354.5 ± 80.1	2916.3 ± 288.7	1.5 ± 0.2	1.0 ± 0.1	0.51 ± 0.10	0.31 ± 0.15
A-W-Cd	37 ± 0.5	14.92 ± 0.06	7.39 ± 0.02	3326.5 ± 67.1	2886.4 ± 174.1	1.5 ± 0.1	1.0 ± 0.1	33.4 ± 4.37	19.38 ± 1.26

510

511 **Table 1 -** Summary of water chemistry parameters and Cd burdens (slightly adapted from Nardi *et al.*, 2017,2018b) during experimental exposures
512 in summer and winter. S (salinity), T (temperature), pH_{NBS} (pH calibrated with National Bureau of Standard scale), A_T (total alkalinity), pCO_2 (partial
513 pressure of CO_2), Ωc and Ωa (saturation state of respectively calcite and aragonite). Data are presented as means ± standard deviations.

Gene	Primer pair sequences	Amplicon size	Accession n.	Annealing T, time	References
Cu/Zn-sod	Fwd: AGCCAATGCAGAGGGAAAAGCAGA Rev: CCACAAGCCAGACGACCCCC	177 bp	FM177867	65°C, 1 min	Giuliani *et al.*, 2013
cat	Fwd: CGACCAGAGACAACCCACC Rev: GCAGTAGTATGCCTGTCCATCC	132 bp	AY743716	55°C, 15 sec 72°C, 1 min	Giuliani *et al.*, 2013
Se-dep. gpx	Fwd: AGCCTCTCTCTGAGGAACAACTG Rev: TGGTCGAACATGCTCAAGGGC	166 bp	HQ891311	55°C, 15 sec 72°C, 1 min	Giuliani *et al.*, 2013
gst-pi	Fwd: TCCAGTTAGAGGCCGAGCTGA Rev: CTGCACCAGTTGGAAACCGTC	172 bp	AF527010	55°C, 15 sec 72°C, 1 min	Giuliani *et al.*, 2013
hsp70	Fwd: GGTGGTGAAGACTTTGACAACAG Rev: CTAGTTTGGCATCGCGTAGAGC	295 bp	AY861684	65°C, 1 min	Cellura *et al.*, 2006
mt-20	Fwd: TGTGAAAGTGGCTGCGGA Rev: GTACAGCCACATCCACACGC	80 bp	AY566247	55°C, 15 sec 72°C, 1 min	Dondero *et al.*, 2005

Table 2 - Primer pair sequences, amplicon size, annealing temperatures and accession numbers of genes investigated in quantitative PCR.

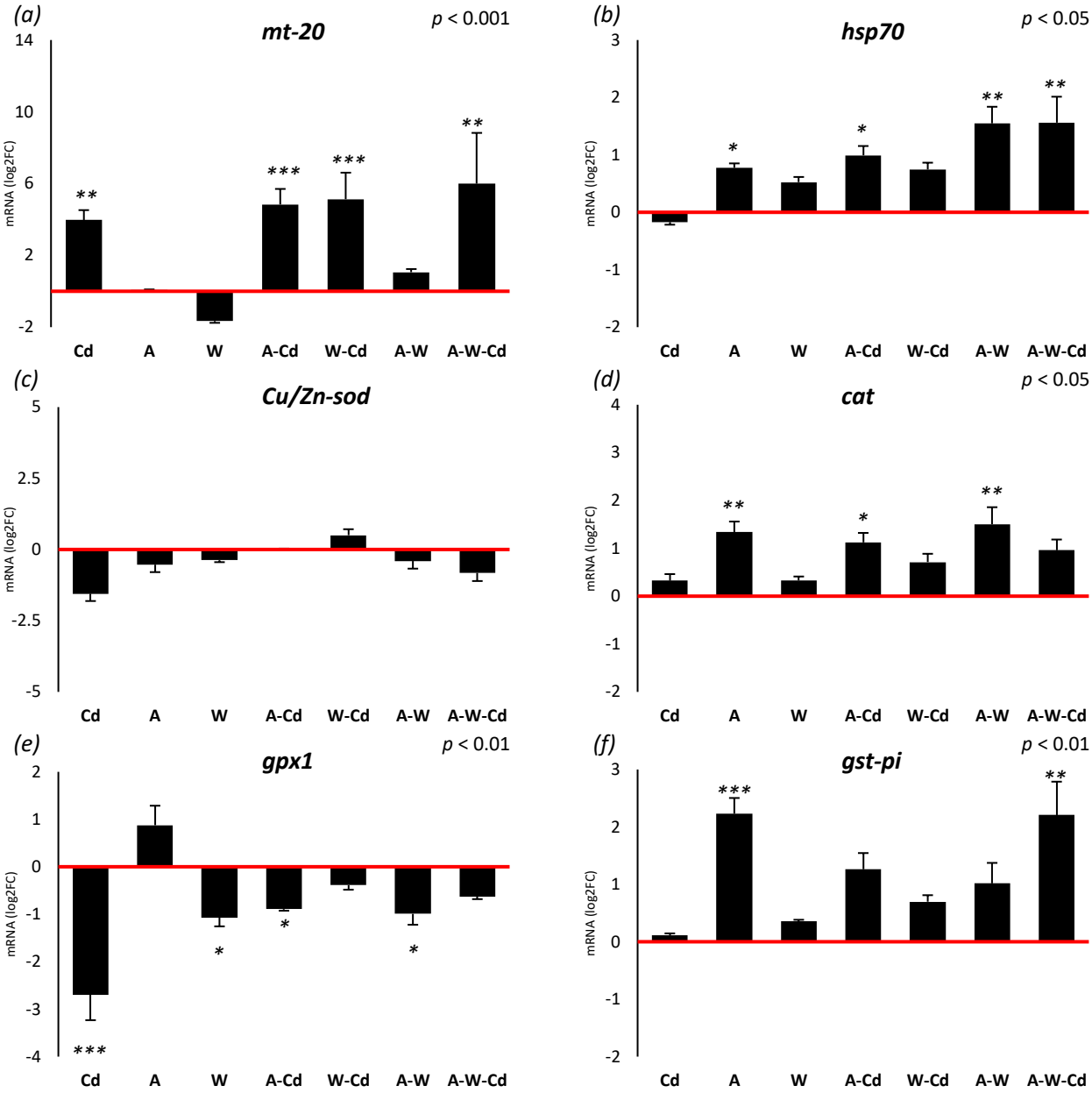


Figure 1 – mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the digestive gland of mussels exposed in summer. Data are given as $\log_2$ of the fold change relative to CTRL treatment (red reference line) ± SEM (n=6). Asterisks indicate significant differences compared to CTRL treatment, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W-Cd= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming + Cd.

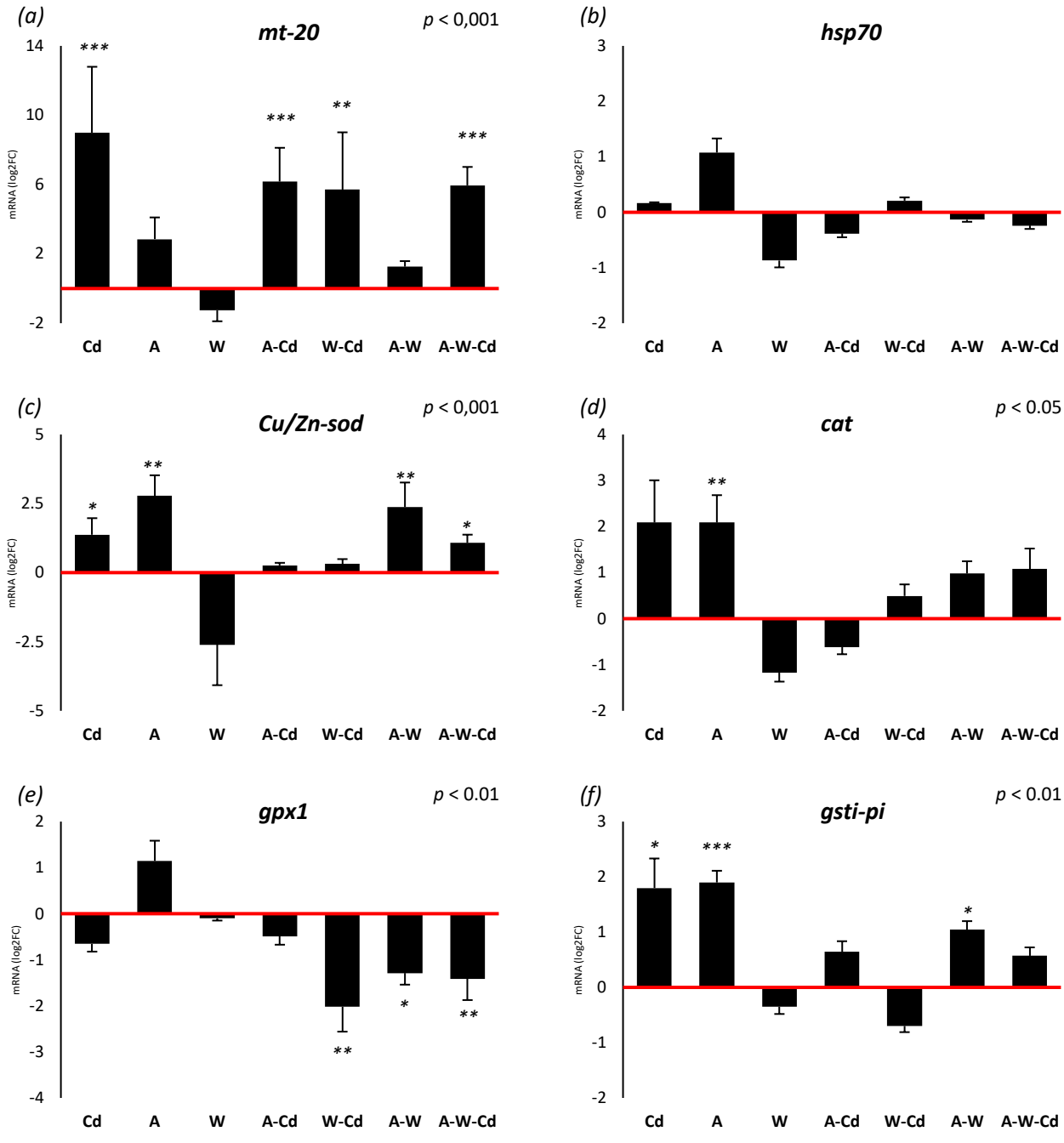
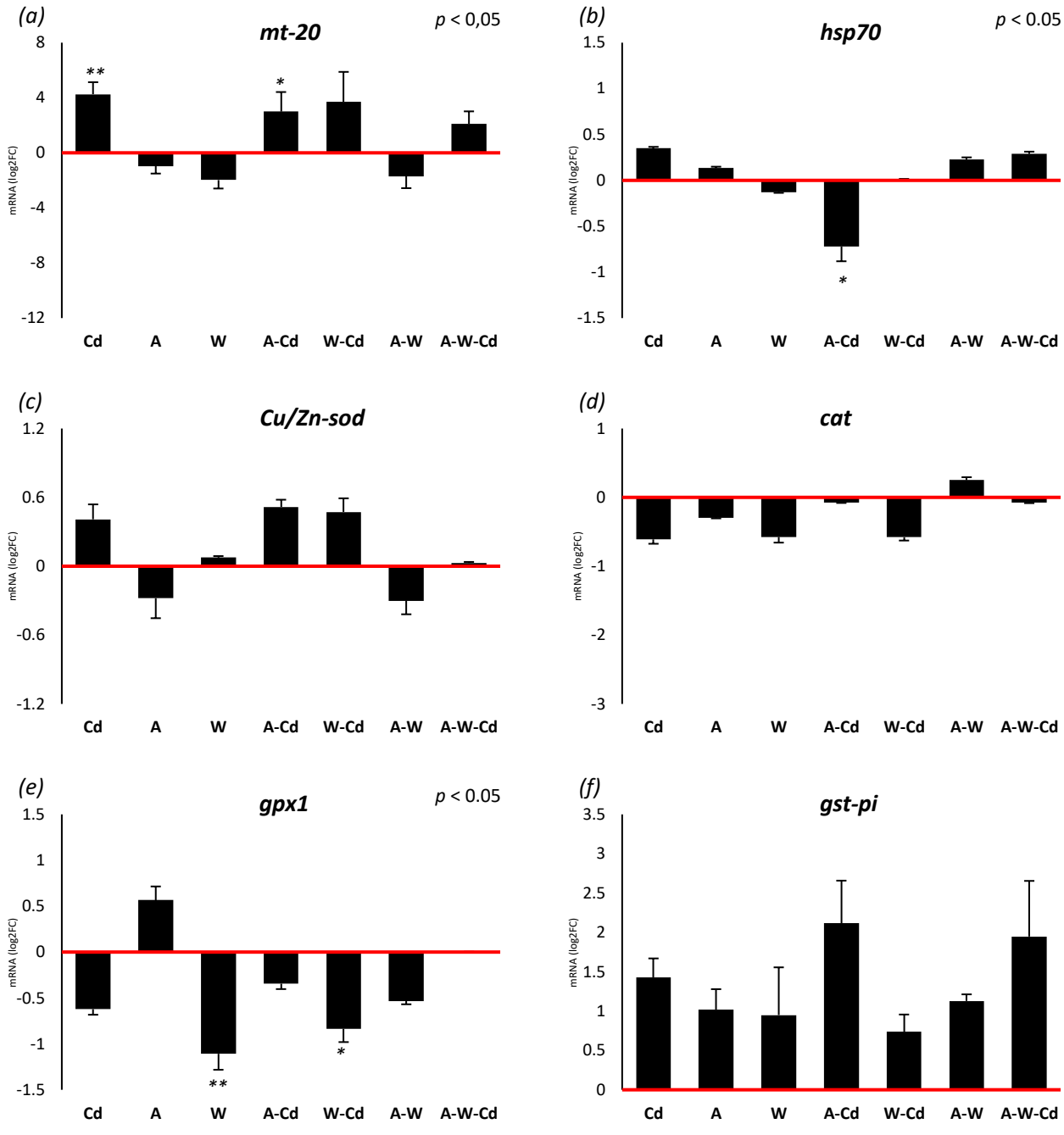


Figure 2 - mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the digestive gland of mussels exposed in winter. Data are given as $\log_2$ of the fold change relative to CTRL treatment (red reference line) ± SEM (n=6). Asterisks indicate significant differences compared to CTRL treatment, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W Cd= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming + Cd.



535

536

537

538 **Figure 3** – mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the gills of mussels

539 exposed in summer. Data are given as $\log_2$ of the fold change relative to CTRL treatment (red reference line) ± SEM

540 (n=6). Asterisks indicate significant differences compared to CTRL treatment, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

541 Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W-Cd= warming + Cd; A-W=

542 acidification + warming; A-W-Cd= acidification + warming + Cd.

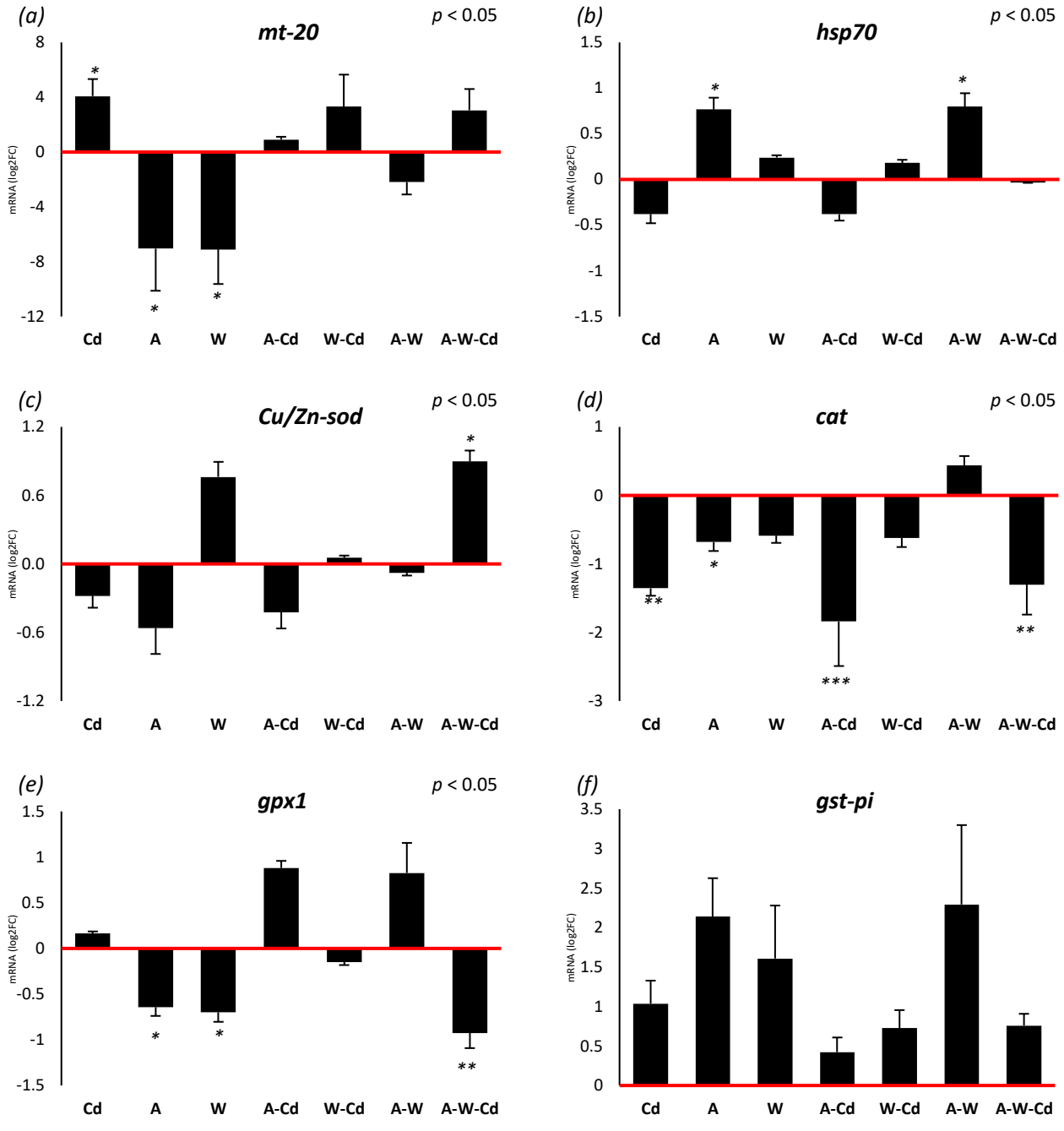
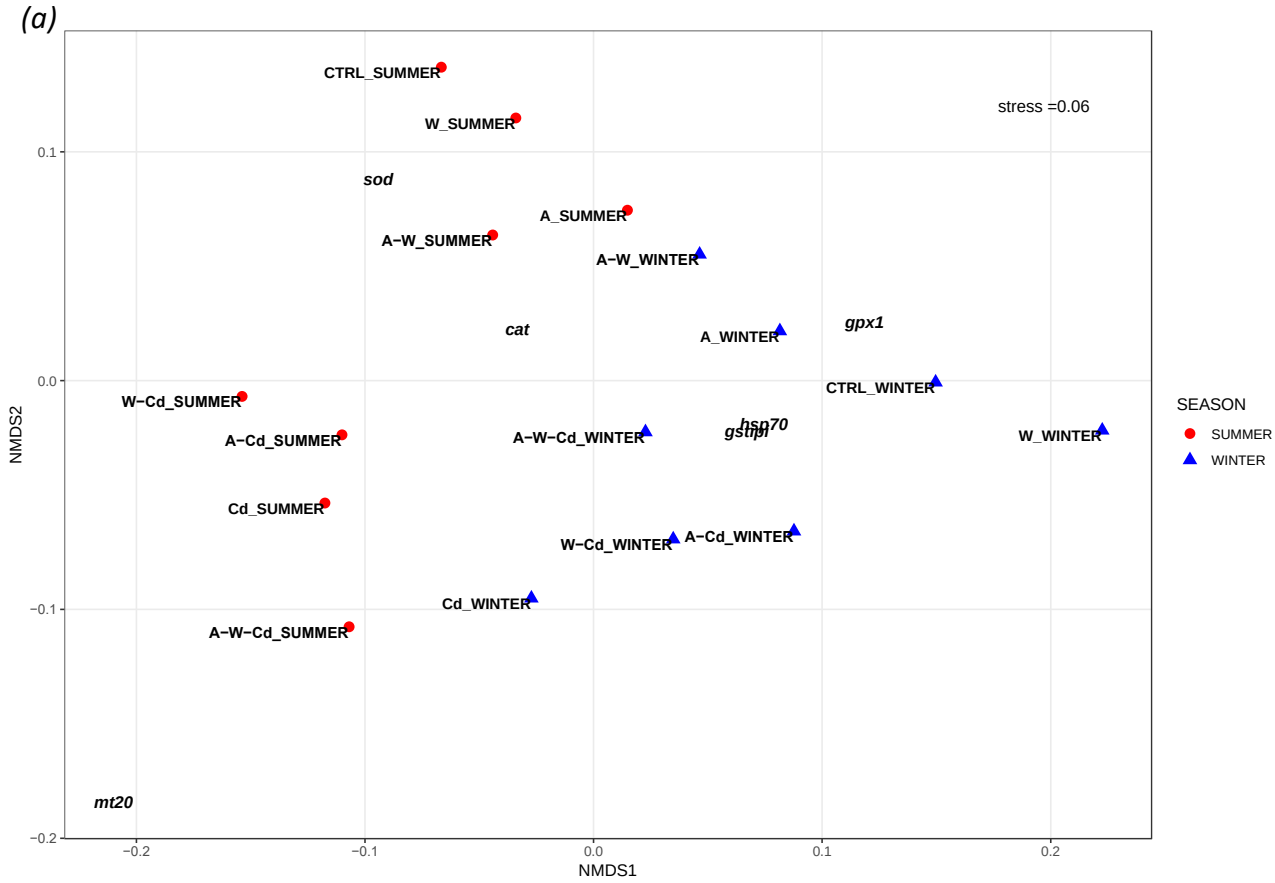
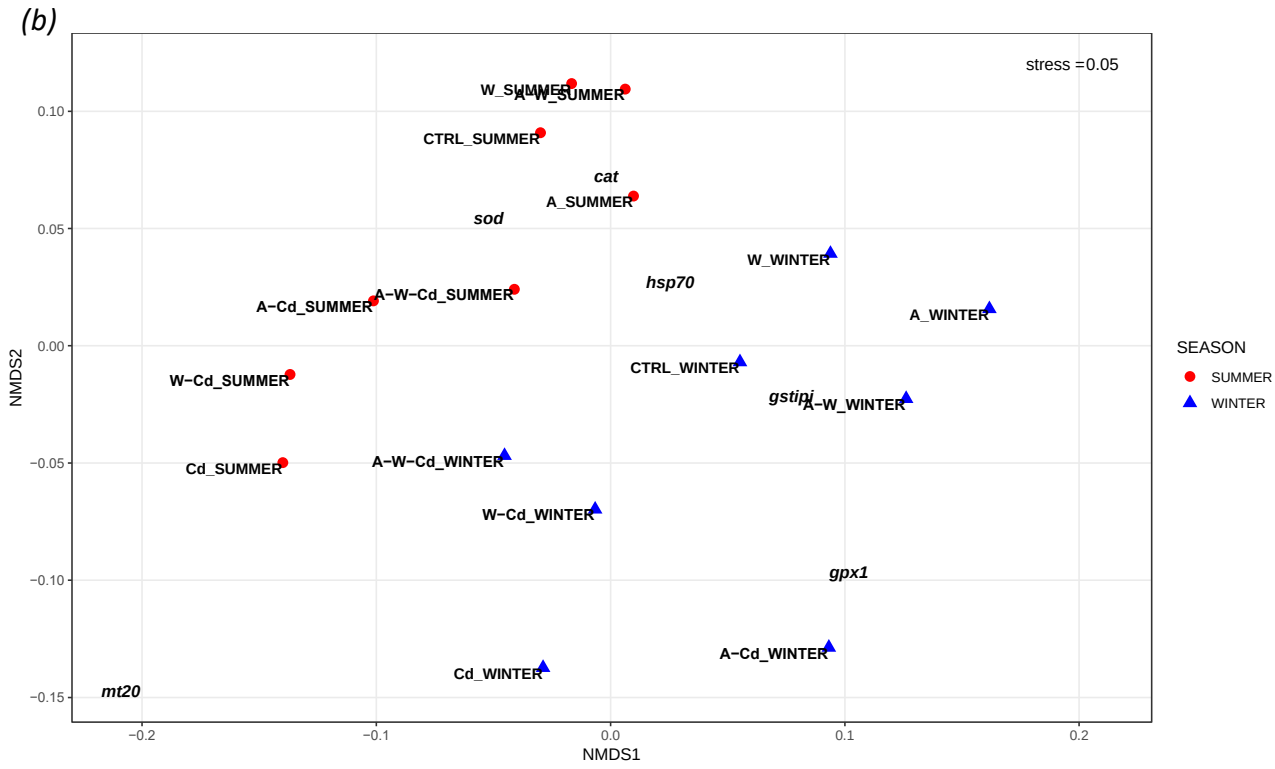


Figure 4 - mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the gills of mussels exposed in winter. Data are given as $\log_2$ of the fold change relative to CTRL treatment (red reference line) ± SEM (n=6). Asterisks indicate significant differences compared to CTRL treatment, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Cd= Cadmium; A= acidification; W= warming; A-Cd= acidification + Cd; W-Cd= warming + Cd; A-W= acidification + warming; A-W-Cd= acidification + warming + Cd.



552



553
554 Fig. 5 – Non-metric multidimensional scaling (NMDS) for digestive gland (a) and gills (b) of exposed mussels.