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

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20 molecular biomarkers, oxidative stress

21 **Abstract**

22 Continuous anthropogenic inputs of carbon dioxide in the atmosphere are driving ocean warming
23 and acidification. The potential threat represented by these changes for marine species could be
24 amplified in coastal areas, characterized by higher levels of chemical pollutants. In addition,
25 organisms living in temperate areas may exhibit a different seasonal tolerance to stressors influenced
26 by fluctuations of environmental factors and physiological characteristics. In this study, mRNA levels
27 of selected genes related to metal-induced stress response were investigated in the Mediterranean
28 mussel *Mytilus galloprovincialis* collected both in summer and in winter and exposed to combinations
29 of two temperatures (SST, seasonal surface temperature and SST+5 °C), two levels of pH (8.20 and
30 7.40) and two doses of cadmium (0 and 20 µg/L). Selected genes included metal detoxification
31 pathway (metallothionein *mt-20*), cellular stress response (heat-shock protein *hsp70*), and
32 antioxidants network (superoxide dismutase *Cu/Zn-sod*, catalase *cat*, glutathione peroxidase *gpx1* and
33 glutathione S-transferase *gst-pi*). To further elucidate possible differences in responsiveness of
34 various tissues related to metabolic function and physiological characteristics, such analyses were
35 carried out in digestive gland and in gills of exposed mussels. Seasonal- and tissue-specific
36 differences were observed for metallothioneins gene induction after Cd-exposure, while *hsp70* up-
37 regulation was caused by acidification, both alone or in combination with other stressors. A complex
38 array of interactions affected antioxidant genes, with strong differences as a function of analyzed
39 tissue and experimental season and without a clear role of specific stressors and investigated
40 pathways. The overall results highlighted the importance of considering seasonality and
41 responsiveness of different tissues to predict the effects of sudden changes in environmental
42 parameters on responsiveness and toxicity of chemicals to marine coastal organisms.

43 1.Introduction

44 As a consequence of increased anthropogenic carbon dioxide emissions, oceans are threatened
45 by warming and acidification (IPCC, 2013). Since the beginning of the industrial era, ocean has
46 warmed by almost 1 °C because of the greenhouse effect and captured about 30% of anthropogenic
47 CO₂, resulting in a pH drop of 0.1 units (Hansen *et al.*, 2016). By the end of the century, temperature
48 is projected to increase by additional 2°C, while ocean mean pH will further decrease by 0.3 – 0.5
49 units (IPCC, 2013). These changes could be even more pronounced in coastal areas, where large
50 fluctuations and sudden peaks of temperature and pH naturally occur (Wallace *et al.*, 2014). In
51 addition, these ecosystems are characterized by a higher anthropogenic footprint compared to open
52 ocean and synergistic effects can be expected between a wide range of stressors and climate change
53 (Hewitt *et al.*, 2016).

54 Ocean warming and acidification have been described as potential factors affecting trace metals
55 speciation and bioavailability but also their biological effects and toxicity. Such interactions were
56 demonstrated on impaired mitochondrial functioning, energy metabolism, oxidative unbalance,
57 accumulation of lipid peroxidation products, damages to lysosomes, DNA and immune function, as
58 well as to impair larval development (Regoli *et al.*, 2002; Roberts *et al.*, 2013; Rodríguez-Romero *et al.*,
59 2014; Götze *et al.*, 2014; Izagirre *et al.*, 2014; Múgica *et al.*, 2015; Moreira *et al.*, 2018; Cao *et al.*,
60 2019).

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

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

77 Previously published researches of our group (Nardi *et al.*, 2017, 2018b), demonstrated oxidative
78 effects of cadmium in association with temperature and pH stress at biochemical and cellular level in
79 *M. galloprovincialis*. Since the use of molecular biomarkers might represent a sensitive tool to
80 identify early biological responsiveness to environmental stressors, this study aimed to characterize
81 the key transcriptional effects in long-term studies to previously tested multiple stressors such as
82 cadmium (Cd), temperature and acidification. The Mediterranean mussel *Mytilus galloprovincialis*,
83 was chosen as a widely used bioindicator organism (Fattorini *et al.*, 2008; Regoli *et al.*, 2014), while
84 selected target genes reflect pathways of metal-detoxification (the Cd-inducible isoform of
85 metallothioneins, *mt-20*), cellular stress (the heat shock protein 70, *hsp70*) and antioxidants network
86 (cytosolic superoxide dismutase, catalase, Se-dependent glutathione peroxidase and the pi isoform of
87 glutathione S-transferase, *Cu/Zn-sod*, *cat*, *gpx1*, *gst-pi*). Gene expression was analyzed both in
88 digestive gland and gills of exposed mussels due to their different ability to concentrate pollutants
89 and tolerate environmental stressors; further, experiments were performed in summer and in winter
90 to elucidate the potential tissue-dependent and seasonal modulation of observed effects of the
91 investigated parameters. The overall results were expected to provide novel insights on mechanisms
92 underlying the onset of biological and interactions of climate change with other environmental
93 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/pCO₂ (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/ pCO₂=~400 µatm); 2) cadmium exposure (Cd), SST, normocapnia and 20 µg/L Cd; 3) acidification (A), SST, hypercapnia (pH=7.40/ pCO₂=~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 CO₂-saturated ASW, resulting of bubbling pure CO₂ 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

146 7.5 µL of SYBR Select Master Mix (Life Technologies), 5 µL of total cDNA (synthesized as
147 described above and diluted 1:5) and 200 nM of each forward and reverse primers. The real-time
148 PCR program included an enzyme activation step at 95 °C (2 min) and 40 cycles composed by 15 s
149 at 95 °C and 1 min at the annealing temperature (Table 2). The specificity of target cDNA
150 amplification was checked by including controls lacking cDNA template and by a melting analysis
151 (95 °C for 1 min, 65 °C for 10 s and fluorescence detection at increasing temperature between 65 and
152 95 °C). For quantification, serial dilutions of known amounts of plasmid containing the amplicon of
153 interest were used as standards to build a standard plot of Ct versus log copy number (for each target
154 gene). Samples and standards were run in duplicate in the same run. Cycle threshold (Ct) values of
155 unknown samples were converted into mRNA copy number interpolating the standard plot. The
156 values were expressed as log₂ of fold change (FC: exposed samples relative to control samples).

157

158 2.4 Statistical analyses

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

164

165 3. Results

166 3.1 Digestive gland

167 A significant increase of *mt-20* mRNA was observed in the digestive gland of organisms exposed
168 to all experimental combinations containing Cd (Cd, A-Cd, W-Cd and A-W-Cd), with a lower level
169 of induction in summer compared to winter (Fig. 1a and 2a). Acidification increased significantly
170 *hsp70* mRNA levels independently of additional stressors in summer, while in winter no significant
171 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

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

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

The battery of antioxidant genes indicated a certain disturbance of the oxidative balance toward investigated stressors, confirming a generally higher responsiveness during the summer. Acidification, alone or in combination with other stressors, was the main factor promoting the up-regulation of *cat* and *gst-pi* both, suggesting a CO₂-mediated increase of oxidative challenge as already hypothesized by other authors (Tomanek *et al.*, 2011). Interestingly, heat shock proteins were previously suggested to act as redox sensors activating some antioxidant genes (Madeira *et al.*, 2017 and ref. therein): this hypothesis seems to be confirmed by our observations with similar responses

250 to acidification for *cat*, *gst-pi* and *hsp70*. The expression of *gpx1* exhibited a generalized
251 downregulation, with major effects due to Cd-exposure in summer, and by various combinations of
252 temperature with other stressors in winter. Inhibitory effects of Cd on *gpx1* expression have been
253 reported in *Danio rerio* (Banni *et al.*, 2011) and in *Oncorhynchus kisutch* (Wang *et al.*, 2012), while
254 increased temperature was shown to upregulate *gpx1* in the gastropod *Halotis discus discus* (De
255 Zoysa *et al.*, 2009). The downregulation of *gpx1* is mainly due to degradation through the so-called
256 “nonsense mediated decay” mechanism, which may occur e.g. when intracellular Se concentration
257 are limiting (Sun *et al.*, 2000). Although few evidences are available for bivalves, both Cd exposure
258 and changes in pH have been shown to modulate uptake and intracellular levels of Se (Dorey *et al.*,
259 2018).

260

261 4.2 Gills

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

268 Similarly to the digestive tissues, *hsp70* was not induced by warming alone in gills, and
269 acidification was confirmed to provoke a certain up-regulation, although less intense and limited to
270 winter mussels. On the other hand, a marked downregulation of *hsp70* was observed in organisms
271 co-exposed to Cd and acidification during the summer: downregulation of *hsp70* mRNA was
272 observed in the haemocytes of clam *Mercenaria mercenaria* exposed to Cd and hypercapnia (Ivanina
273 *et al.*, 2014), in the haemocytes of oyster *Saccostrea glomerata*, and in the digestive gland of *M.*
274 *galloprovincialis* due to Cd exposure (Thompson *et al.*, 2012; Izagirre *et al.*, 2014); lowered levels
275 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.

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

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

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summer									
Treatment	measured parameters				calculated parameters			Cd burdens ($\mu\text{g/g dw}$)	
	S	T ($^{\circ}\text{C}$)	pH _{NBS}	A _T ($\mu\text{mol/kg}$)	pCO ₂ (μatm)	Ωc	Ωa	Digestive gland	Gills
CTRL	37 \pm 0.5	19.95 \pm 0.10	8.21 \pm 0.04	2453.6 \pm 251.5	380.8 \pm 25.8	5.3 \pm 0.4	3.5 \pm 0.2	2.89 \pm 1.91	1.04 \pm 0.62
Cd	37 \pm 0.5	20.00 \pm 0.10	8.19 \pm 0.04	2390.5 \pm 354.1	410.6 \pm 30.9	5.1 \pm 0.4	3.3 \pm 0.3	33.15 \pm 3.7	14.22 \pm 1.04
A	37 \pm 0.5	19.98 \pm 0.06	7.42 \pm 0.04	2557.3 \pm 183.7	2897.6 \pm 183.8	1.0 \pm 0.1	0.7 \pm 0.1	3.01 \pm 1.08	0.55 \pm 0.26
W	37 \pm 0.5	24.80 \pm 0.13	8.15 \pm 0.06	2325.4 \pm 267.7	468.1 \pm 47.9	5.4 \pm 0.4	3.6 \pm 0.3	2.83 \pm 1.15	0.38 \pm 0.12
A-Cd	37 \pm 0.5	19.95 \pm 0.06	7.41 \pm 0.04	2556.7 \pm 479.0	2928.2 \pm 144.4	1.0 \pm 0.1	0.7 \pm 0.1	34.53 \pm 9.48	19.47 \pm 2.4
W-Cd	37 \pm 0.5	24.83 \pm 0.08	8.14 \pm 0.04	2517.9 \pm 206.9	477.4 \pm 44.6	5.2 \pm 0.4	3.5 \pm 0.2	38.03 \pm 10.62	18.78 \pm 8.93
A-W	37 \pm 0.5	24.76 \pm 0.18	7.42 \pm 0.03	2721.4 \pm 215.7	3100.1 \pm 241.7	1.2 \pm 0.1	0.8 \pm 0.1	5.08 \pm 0.12	0.33 \pm 0.13
A-W-Cd	37 \pm 0.5	24.87 \pm 0.16	7.43 \pm 0.04	2504.2 \pm 182	2993.7 \pm 186.7	1.3 \pm 0.1	0.9 \pm 0.1	39.8 \pm 5.23	15.99 \pm 4.4

winter									
Treatment	measured parameters				calculated parameters			Cd burdens ($\mu\text{g/g dw}$)	
	S	T ($^{\circ}\text{C}$)	pH _{NBS}	A _T ($\mu\text{mol/kg}$)	pCO ₂ (μatm)	Ωc	Ωa	Digestive gland	Gills
CTRL	37 \pm 0.5	9.95 \pm 0.11	8.18 \pm 0.03	3283.2 \pm 88.8	386.9 \pm 26.2	6.2 \pm 0.5	3.9 \pm 0.3	0.52 \pm 0.04	0.22 \pm 0.05
Cd	37 \pm 0.5	9.97 \pm 0.06	8.16 \pm 0.03	3334.2 \pm 102.6	411.8 \pm 35.8	6.1 \pm 0.3	3.9 \pm 0.2	16.4 \pm 1.92	6.79 \pm 1.11
A	37 \pm 0.5	10.54 \pm 0.08	7.40 \pm 0.05	3364.1 \pm 112.9	2882.2 \pm 363.8	1.3 \pm 0.2	0.8 \pm 0.1	0.44 \pm 0.09	0.12 \pm 0.08
W	37 \pm 0.5	14.95 \pm 0.12	8.17 \pm 0.03	3378.4 \pm 121.2	416.8 \pm 34.5	7.2 \pm 0.5	4.7 \pm 0.4	0.54 \pm 0.08	0.25 \pm 0.07
A-Cd	37 \pm 0.5	10.04 \pm 0.15	7.39 \pm 0.02	3360.6 \pm 36.8	2860.9 \pm 207.2	1.2 \pm 0.1	0.8 \pm 0.1	20.67 \pm 3.16	5.21 \pm 0.89
W-Cd	37 \pm 0.5	15.02 \pm 0.11	8.17 \pm 0.03	3350.5 \pm 164.9	403.5 \pm 53.1	7.3 \pm 0.5	4.7 \pm 0.3	30.49 \pm 3.07	16.17 \pm 4.3
A-W	37 \pm 0.5	14.98 \pm 0.05	7.39 \pm 0.04	3354.5 \pm 80.1	2916.3 \pm 288.7	1.5 \pm 0.2	1.0 \pm 0.1	0.51 \pm 0.10	0.31 \pm 0.15
A-W-Cd	37 \pm 0.5	14.92 \pm 0.06	7.39 \pm 0.02	3326.5 \pm 67.1	2886.4 \pm 174.1	1.5 \pm 0.1	1.0 \pm 0.1	33.4 \pm 4.37	19.38 \pm 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), pCO₂ (partial

513 pressure of CO₂), Ωc and Ωa (saturation state of respectively calcite and aragonite). Data are presented as means \pm standard deviations.

514

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

515

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

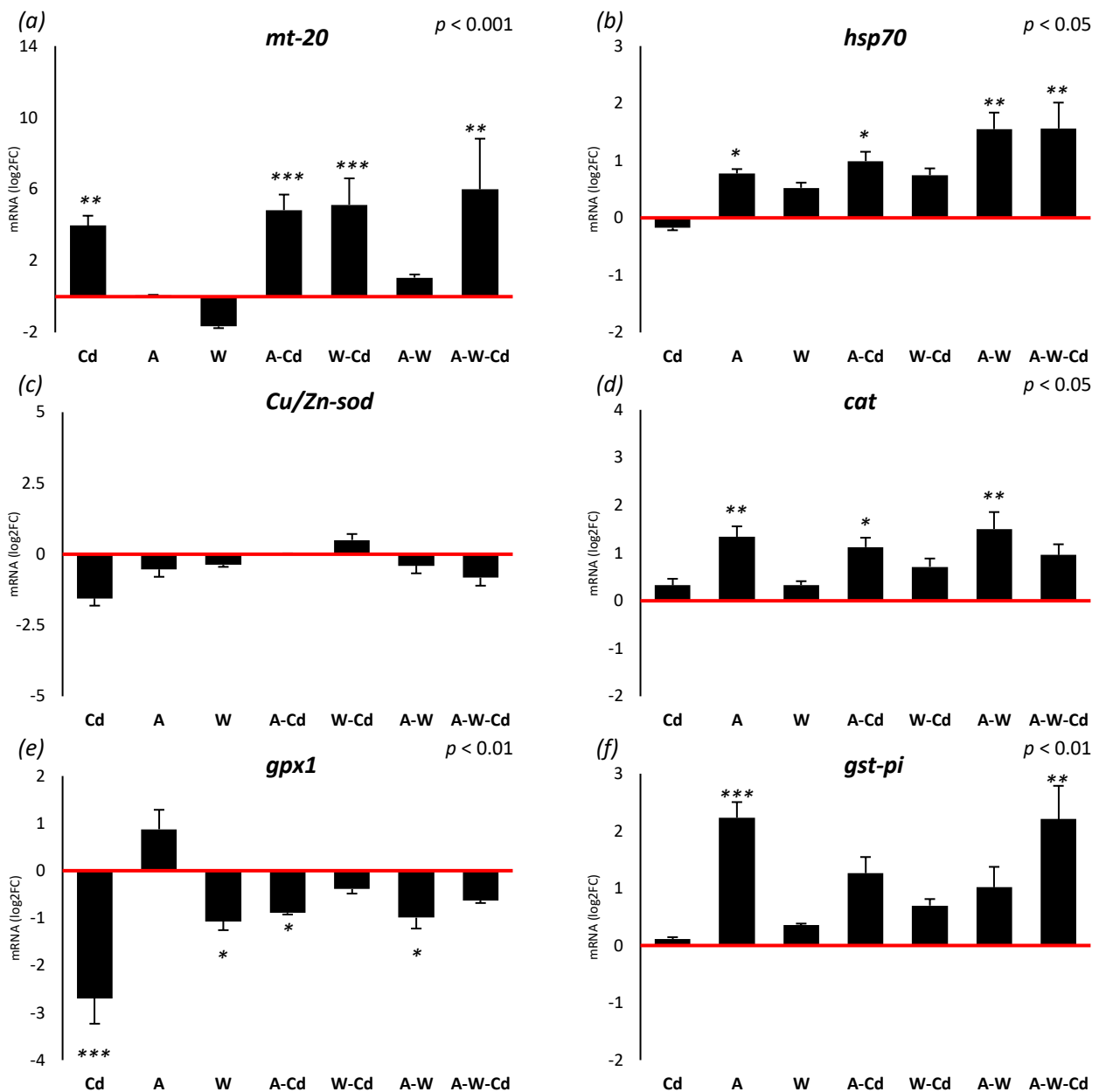


Figure 1 – mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the digestive gland of mussels exposed in summer. Data are given as log₂ of the fold change relative to CTRL treatment (red reference line) ± SEM (n=6). Asterisks indicate significant differences 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.

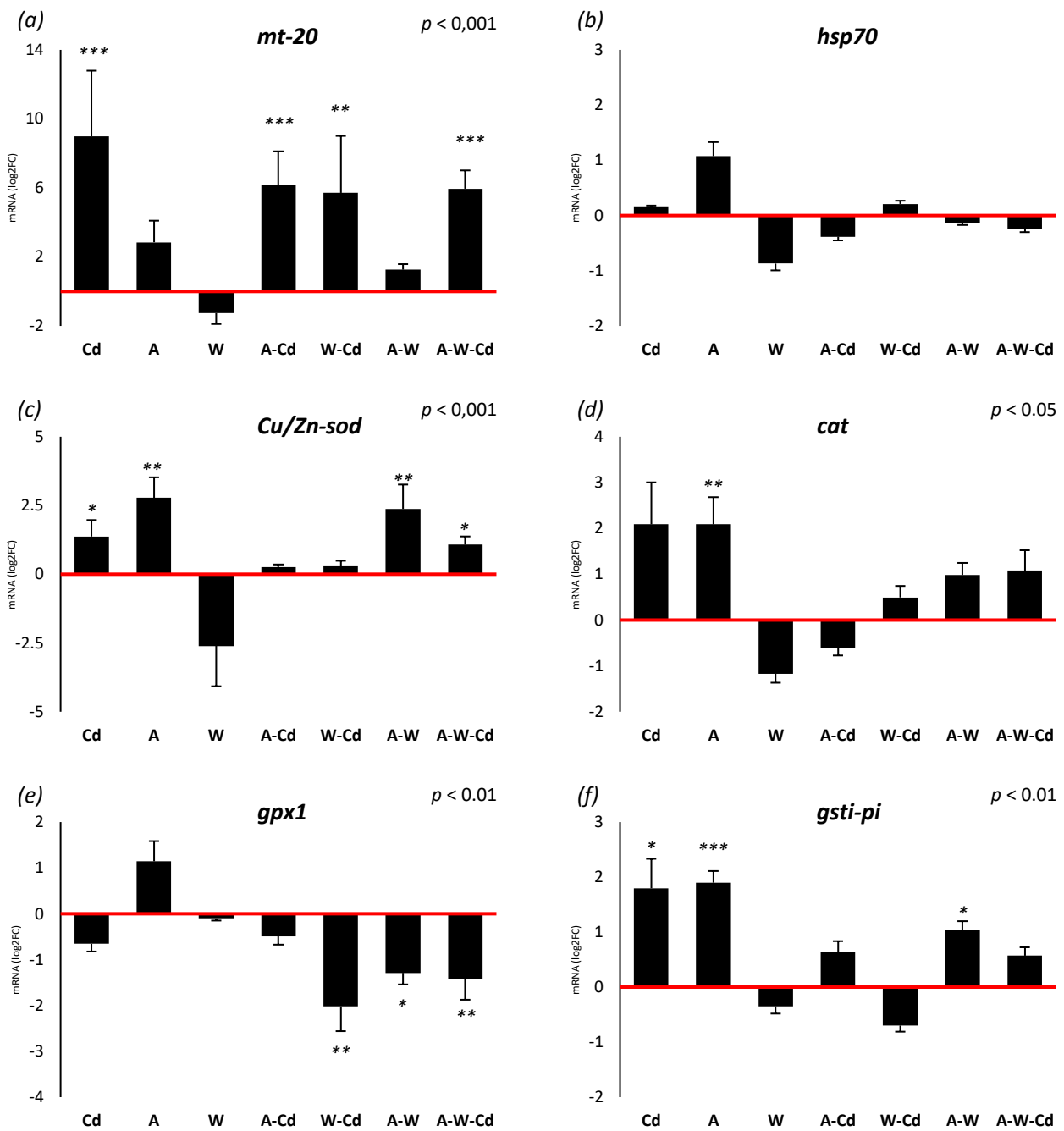


Figure 2 - mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gsti-pi* (f) in the digestive gland of mussels exposed in winter. Data are given as log₂ of the fold change relative to CTRL treatment (red reference line) \pm SEM (n=6). Asterisks indicate significant differences 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.

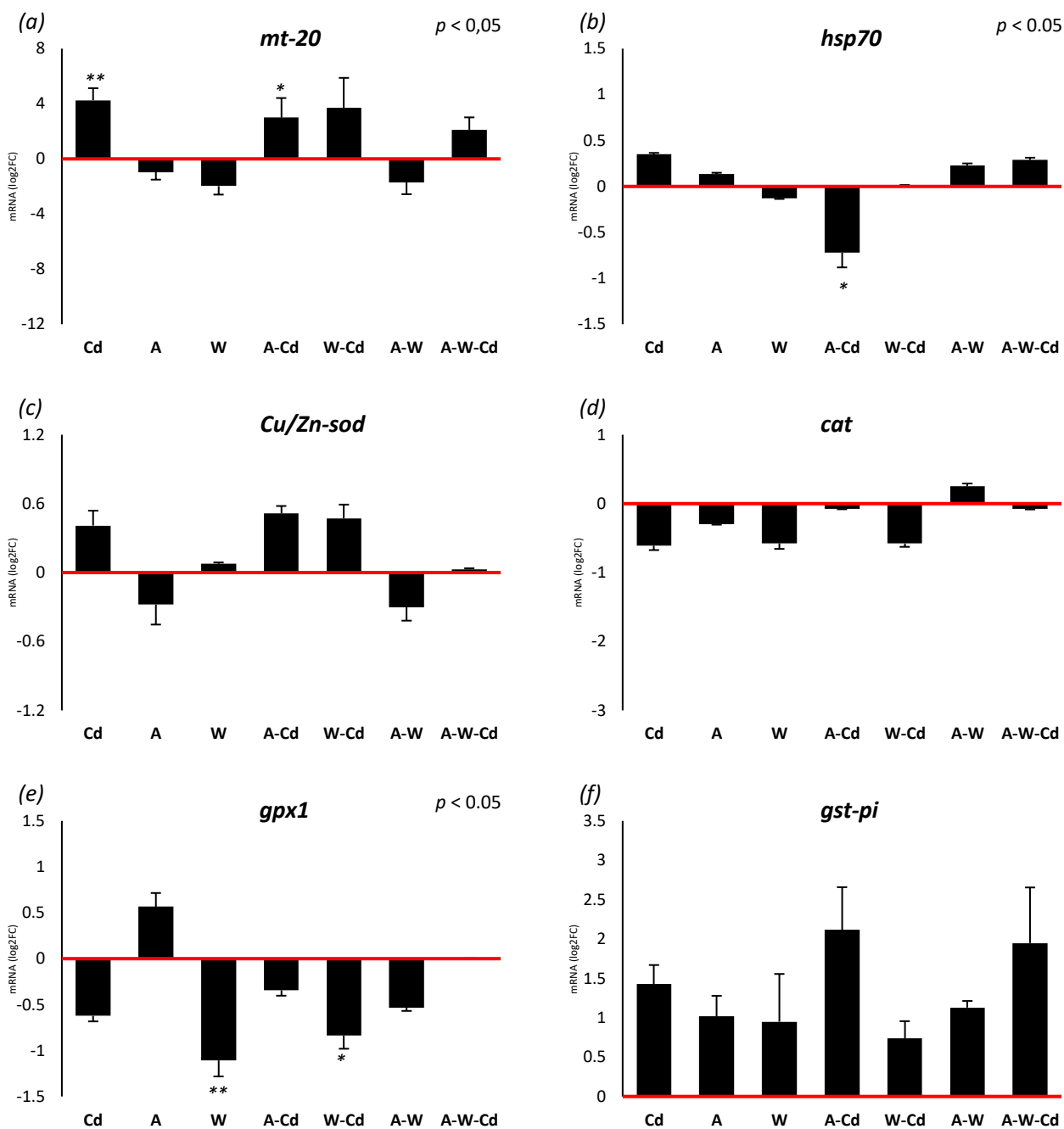


Figure 3 – mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the gills of mussels exposed in summer. Data are given as log₂ of the fold change relative to CTRL treatment (red reference line) \pm SEM (n=6). Asterisks indicate significant differences compared 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.

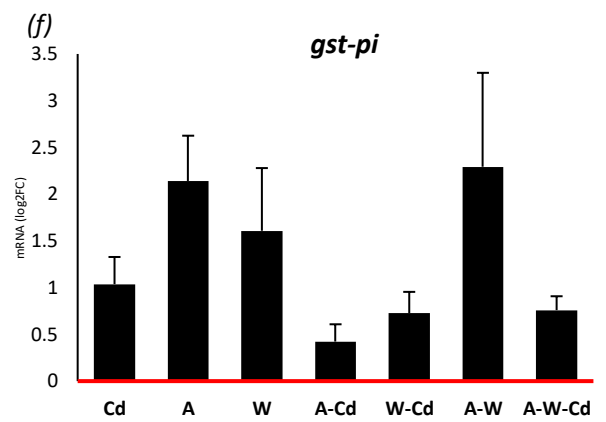
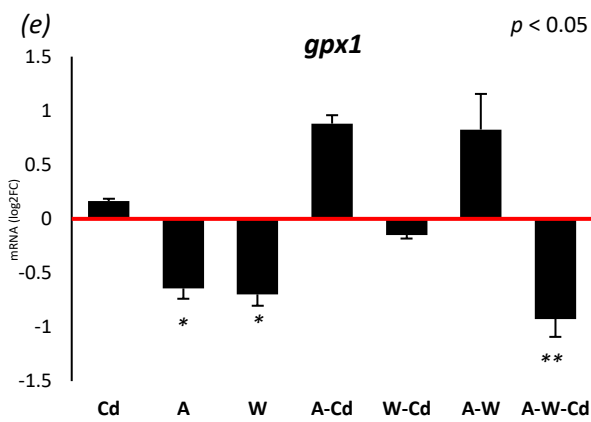
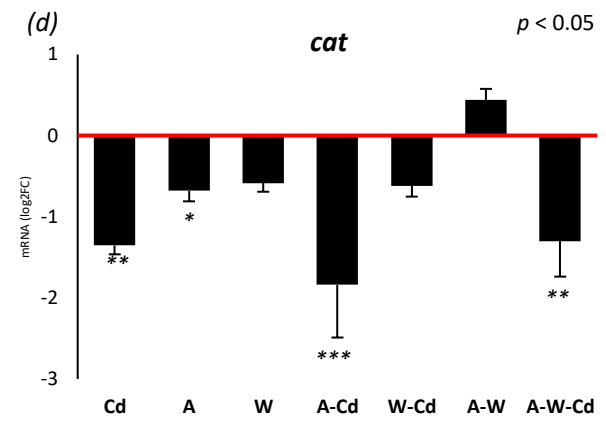
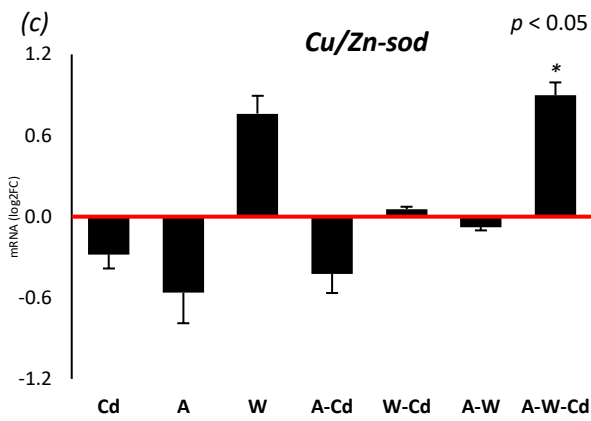
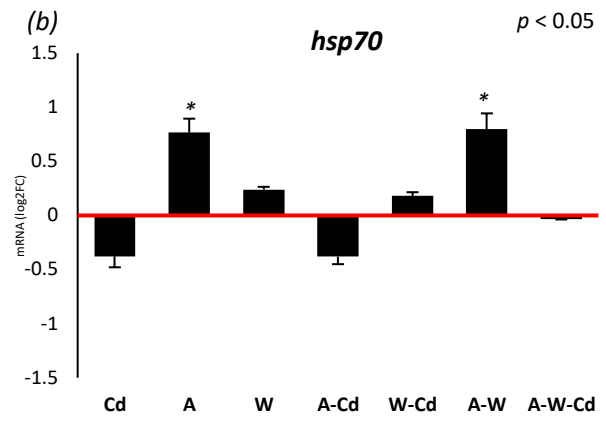
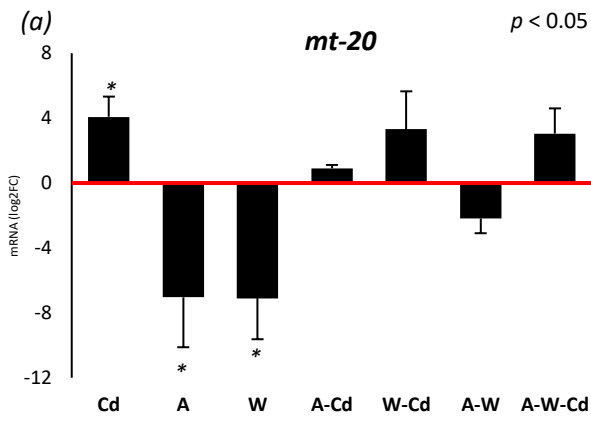


Figure 4 - mRNA levels of *mt-20* (a), *hsp-70* (b), *Cu/Zn-sod* (c), *cat* (d), *gpx1* (e) and *gst-pi* (f) in the gills of mussels exposed in winter. Data are given as \log_2 of the fold change relative to CTRL treatment (red reference line) \pm SEM (n=6). Asterisks indicate significant differences 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.

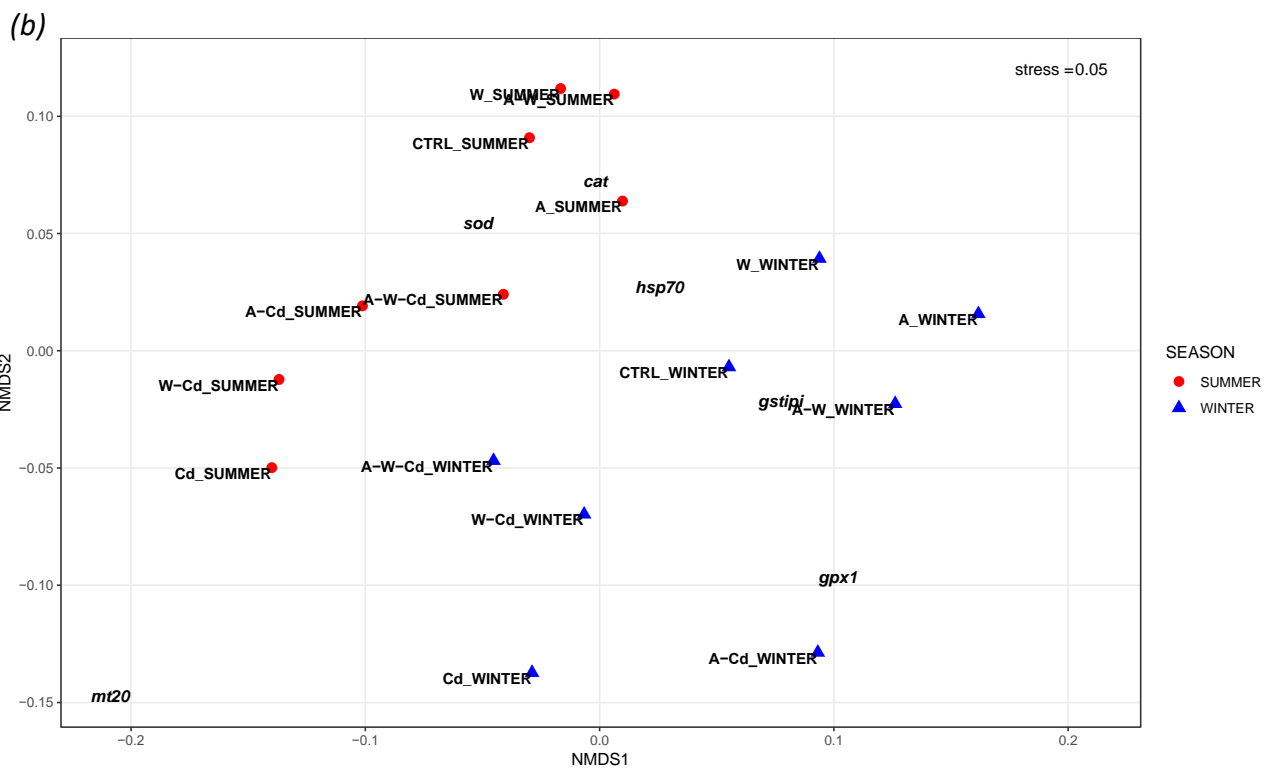
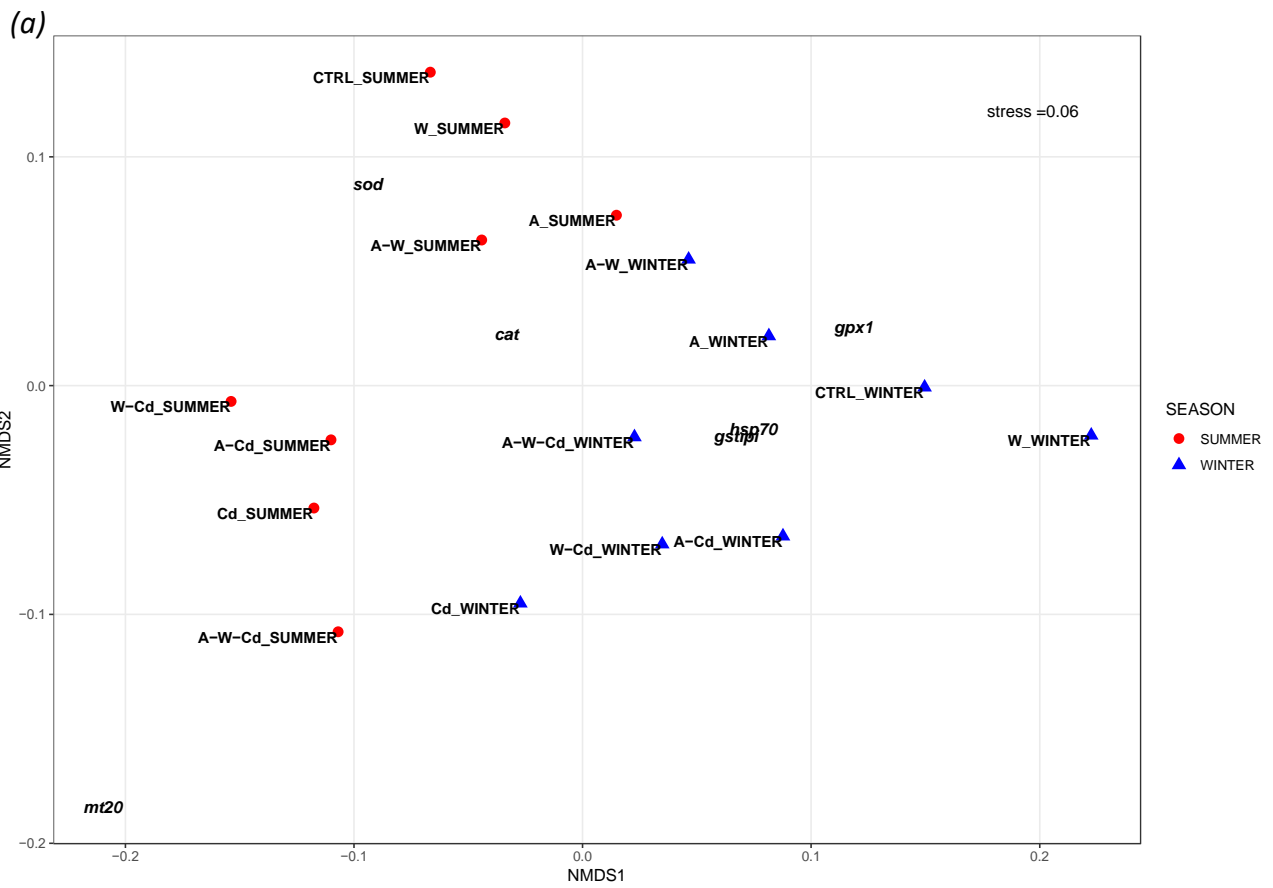


Fig. 5 – Non-metric multidimensional scaling (NMDS) for digestive gland (a) and gills (b) of exposed mussels.