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1 **Tidal height influences the levels of enzymatic antioxidant defences in *Mytilus edulis***

2

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11 **Abstract**

12

13 We investigated the potential variability of enzymatic antioxidant activities in blue
14 mussels *Mytilus edulis* from a single intertidal population but living at different tidal heights.
15 Activity levels of antioxidant enzymes (Cu/Zn superoxide dismutase, catalase, glutathione
16 peroxidase, glutathione reductase, glutathione transferase) were measured in the gills and
17 digestive gland of mussels sampled at high shore (HS, air exposure > 6h/12h) and low shore
18 (LS, air exposure < 2h/12h) of an intertidal zone (Yport, Normandie, France) for two
19 consecutive autumns. In both tissues, levels of each enzymatic activity (except GST) were
20 clearly higher in HS mussels than in LS for the two years.

21 These results suggest an ability to acclimate the enzymatic antioxidant defences to the
22 degree of undergone stress, confirming the importance of environmental conditions in the
23 antioxidant responses. Therefore, the location of organisms on the shore should be taken
24 into account in sampling for ecotoxicological studies.

25

26 Key words : *Mytilus edulis*; air exposure; antioxidant defences; intertidal; biomarkers

27 1. Introduction

28 Production of reactive oxygen species (ROS) occurs naturally during cellular aerobic
29 respiration mechanisms as a result of partial reduction of oxygen to water, but also during
30 metabolic processing of certain xenobiotics (Livingstone *et al.*, 1990). Even if cells possess
31 diverse mechanisms to limit the deleterious effects of ROS on biomolecules (oxidation of
32 proteins, lipids, DNA), the generation of oxyradicals can overwhelm antioxidant defence
33 capacities and lead to oxidative stress (Kappus & Sies, 1981; Sies, 1991; Winston & Di
34 Giulio, 1991; Manduzio *et al.*, 2005). For any organism such stress is an important and
35 frequent consequence of exposure to contaminants, that is why measurements of oxidative
36 damage and antioxidant responses are commonly used as biomarkers in ecotoxicological
37 studies. In particular, measurement of antioxidant enzymatic activities is a means to
38 diagnose the adverse effects of environmental pollution on organisms such as the blue
39 mussel *Mytilus edulis*. This filter-feeding and sessile species is widely distributed and known
40 for its ability to accumulate chemical compounds and its high tolerance to diverse
41 environmental stress (Kennedy, 1976; Widdows *et al.*, 1979). With this species, the main
42 antioxidant enzymatic activities described are: copper/zinc superoxide dismutase (Cu/Zn-
43 SOD), catalase (CAT), glutathione-peroxidase (GPx, selenium-dependant or independent),
44 glutathione reductase (GRd) and glutathione transferase (GST) (Ramos-Martinez *et al.*,
45 1983; Livingstone *et al.*, 1990; Fitzpatrick & Sheehan, 1993).

46 Although seasonal variations of antioxidants are well-described in different mussel models
47 (Power & Sheehan, 1996; Regoli *et al.*, 2002; Manduzio *et al.*, 2004; Bocchetti & Regoli,
48 2006), the variability regarding the natural parameters is only partially assessed, which could
49 explain some conflicting results that were obtained in the past (Manduzio *et al.*, 2005). Thus,
50 for intertidal populations of blue mussels which are subjected to wide fluctuations in different
51 abiotic factors (oxygenation, temperature, hygrometry, etc), tidal status represents a
52 supplementary aspect to be taken into account when studying antioxidant capacities.

53 Different laboratory experiments have investigated the specific effects of changes in
54 temperature or aerial exposure on antioxidants in intertidal organisms (Abele *et al.*, 2002;
55 Almeida *et al.*, 2005; Almeida & Bairy, 2006; De Oliveira *et al.*, 2005). However, the
56 acclimation aspects that the chronicity of tidal cycles could induce in the field are not
57 revealed in these cases. Blue mussels can be spatially distributed from high shore, where
58 communities are exposed to air for >50% of the time, to the lowest levels of the shore where
59 the period of water deprivation is short or non-existent. As the length and frequency of air
60 exposure is likely to influence the intensity of stress, the vertical position of mussels on the
61 shore should have some effect on the responses of antioxidant activities. This study aims at
62 determining the possible differences in activities of enzymatic antioxidant defences in the
63 gills and digestive gland of *M. edulis* after 2h of emersion according to its spatial distribution

64 on the shore within a clean site. This represents a preliminary work for the investigation of
65 the influence of tidal condition on enzymatic antioxidants in the blue mussel.

66

67 **2. Materials and methods**

68 **2.1 *In situ* sampling**

69 Mussels were collected on the intertidal rocky-shore of Yport (Seine-Maritime, France). On
70 this shore, the mussel-bed is developed from the upper shore (5.80 m) to the lower shore
71 (1.8 m). Two sampling areas were chosen according to the respective amount of time they
72 were exposed to air (emersion). Twice-daily mussels from high-shore (**HS**) experienced air-
73 exposure of up to 6 hours, whereas mussels from low-shore (**LS**) experienced at the most
74 two periods of 2 - 2.5 hours of air-exposure a few days a month. HS (40-45 mm) and LS
75 mussels (45-50 mm) were collected after 2 hours of air exposure, so that both groups would
76 be exposed to the same emersion period. Gills and digestive glands were dissected out on
77 site immediately after collection, placed in 2 ml sample tubes and directly deep-frozen in
78 liquid nitrogen. In the laboratory, tissues were transferred and kept at -80°C until analysis.
79 Sampling was performed in October 2004 and repeated in November 2005.

80

81 **2.2 Preparation of tissue extracts**

82 The gills and digestive glands were homogenised on ice in 10 mM Tris, 10 mM EDTA, 5 mM
83 2-β mercaptoethanol and 1% Triton X100, pH = 7.5, (1:3 and 1:10 volume respectively) using
84 an electric homogeniser (Ultraturax). Homogenates were treated using ultrasonics at 20 kHz
85 (gills, 2 x 10s periods, digestive gland 2 x 20s periods) and centrifuged for 15 min at 10 000 *g*
86 and 4°C. Supernatants were kept on ice and used immediately for enzymatic assays. Protein
87 concentration of supernatants was measured according to the method of Bradford (Bradford,
88 1976), using bovine serum albumin as a standard.

89

90 **2.3 Measurements of enzymatic activities**

91 **2.3.1 *Catalase* (CAT, EC 1.15.1.1)**

92 CAT activity was measured by the adapted method of Claiborne (1985) according to
93 Livingstone *et al.* (1990). H₂O₂ disappearance was recorded at 240 nm. Activity was
94 expressed as micromoles of H₂O₂ per minute per milligram of protein, or U.mg⁻¹.

95 **2.3.2 *Superoxide dismutase*. (SOD, EC 1.15.1.1)**

96 Isoelectric focusing on high resolution gels (pH 4.0 – 5.0) was performed in a Multiphor II
97 apparatus (Amersham Pharmacia Biotech, France). For each extract, a volume
98 corresponding to 50µg of total protein content was loaded on the gel. Staining of SOD activity
99 was performed immediately after the completion of electrophoresis, using the photochemical
100 method of Beauchamp and Fridovich (1971). Gels were first soaked in 2.5 mM

101 nitrobluetetrazolium (NBT) for 30 min in darkness under gentle agitation. They were then
102 incubated in a solution of 1M phosphate buffer (pH 7.8) containing 0.04 mM riboflavine and
103 0.5% TEMED for 30 min in the dark and under gentle agitation. Detection of SOD isoforms
104 and associated activity was carried out by soaking gels in 10 mM phosphate buffer (pH 7.8)
105 under light conditions. In mussel extracts, using IEF, three isoforms of SOD with acidic
106 isoelectric points were detected: 4.7 for the major band, SOD-1, 4.6 for SOD-2 and 4.55 for
107 the minor one, named SOD-3.

108 Gels were scanned and subsequently analysed using the ImageMaster TotalLab software
109 from Amersham Pharmacia Biotech (France). The activity of each SOD band was
110 determined from the pixel intensity of its volume (Fig. 1). The total activity of SOD was
111 compared to a standard of Cu/Zn-SOD of bovine erythrocyte and expressed in $\text{U}\cdot\text{mg}^{-1}$ prot.
112 The contribution of each isoform was expressed as percentage of the total activity.

113 **2.3.3 Glutathione peroxidase (GPx, EC 1.11.1.9)**

114 Total GPx activity was measured using the Bioxytech GPx-340TM kit (OxysInternational Inc.)
115 adapted for mussel extracts by Manduzio (2004). Briefly, the enzymatic assay was based on
116 a coupled enzyme system, where the GSSG (glutathione oxidized form) formed in the GPx
117 reaction with a tert-butyl substrate was converted to the reduced form GSH by GRd
118 (Lawrence & Burk, 1976). The consumption of NADPH was monitored at 340 nm and directly
119 proportional to the GPx activity in the sample. Activities were calculated with the extinction
120 coefficient $\epsilon = 6220 \text{ M}^{-1}\cdot\text{cm}^{-1}$. Results were expressed as nanomoles of NADPH per minute
121 per milligram of protein, or $\text{mU}\cdot\text{mg}^{-1}$ prot.

122 **2.3.4 Glutathione reductase (GRd, EC 1.6.4.2)**

123 GRd activity was determined using the Bioxytech GR-340TM kit (OxysInternational Inc.)
124 according to the method of Carlberg and Mannervik (1985), by measuring the oxidation rate
125 of NADPH to NAD^+ by GRd in the presence of oxidized glutathione. One GRd unit was
126 defined as the reduction of one micromole of GSSG per minute per milligram of protein at pH
127 7.6 and 25°C (Manduzio *et al.*, 2004), and activity was expressed as $\text{mU}\cdot\text{mg}^{-1}$ prot.

128 **2.3.5 Glutathione transferase (GST, EC 2.5.1.18)**

129 GST activity was measured according to the method of Habig *et al.* (1974), using CDNB (1-
130 chloro-2,4-dinitrobenzene) and GSH (glutathione reduced form) as substrates. Absorbance
131 was read at 340 nm, and activities were expressed as nanomoles of conjugated product
132 formed per minute per milligram of protein, or $\text{mU}\cdot\text{mg}^{-1}$ prot.

133

134 **2.4 Statistical analysis**

135 Values were expressed as mean values \pm SEM of ten independent individual values.
136 Statistically significant differences between samples were determined with SigmaStat (SPSS,
137 Chicago, USA) using the Student's *t* test. $P < 0.05$ was accepted as significant.

138

139 **3. Results**

140 HS mussels exhibited higher levels of antioxidant activities than LS mussels for both tissues
141 and almost every enzyme. Varying levels were sometimes observed between 2004 and 2005
142 for a single station.

143 In gills, equivalent levels of catalase activity were measured for the two years (HS: 99.0 ± 7.9
144 and 103.6 ± 4.2 U CAT.mg⁻¹; LS: 62.4 ± 4.7 and 68.6 ± 5.4 U CAT.mg⁻¹); in the digestive gland,
145 the difference in CAT levels between HS and LS mussels occurring in 2004 disappeared in
146 2005 (fig. 2a, 2d). Concerning Cu/Zn SOD, the total activity in gills was also higher in HS
147 mussels than in LS especially in November 2005 (fig. 2b, 2e); no significant differences were
148 observed in the relative contribution of isoforms between the two sampling points and
149 between the two stations (around 47% SOD-1, 30% SOD-2, 23% SOD-3). In the digestive
150 gland, HS mussels exhibited a 50% higher level of total Cu/Zn SOD activity in 2004 but not in
151 2005. The contribution of the three isoforms showed a similar pattern to that observed in
152 gills, and in October 2004 SOD-1 presented a 25% higher contribution to the total activity in
153 HS mussels than in LS (Figs. 2c, 2f). Levels of GPx and GRd activities observed for HS
154 mussels were 35% to 132% higher than those observed in LS mussels whatever the tissue
155 (Figs. 3a, 3b, 3d, 3e). GST activity is the one which exhibited the least significant differences
156 between HS and LS mussels, especially in gills (no difference in 2004; Fig. 3c); activity was
157 37% and 50% higher in the digestive gland of HS mussels than in LS respectively in 2004
158 and 2005 (Fig. 3f).

159

160 **4. Discussion**

161 We found significantly greater antioxidant enzyme activities in mussels living at the
162 upper part of the shore than mussels inhabiting the lower part of the shore. Anthropogenic
163 and natural factors influencing antioxidant defence capacities are contamination of the
164 environment, aging, feeding level and variations in abiotic parameters (Livingstone *et al.*,
165 1990; Viarengo *et al.*, 1991). i) The two sampling stations were located on the same shore,
166 which is used as reference site in biomonitoring studies. Very low levels of organic
167 contaminants in water have been reported, and the body burdens of these contaminants
168 are the same for HS and LS mussels (data not shown). Thus, the levels of contaminants in
169 mussels' environment can not be implicated in the differences of antioxidant activities
170 measured between high and low shore mussels. ii) Estimation of age was performed by
171 counting the annuli on mussel shell surfaces and no significant differences appeared
172 between HS and LS sampled mussels (Haskin, 1954). Hence, a difference in aging between
173 HS mussels and LS mussels could not be involved in the variability observed in antioxidant
174 activities. iii) Mussels inhabiting low shore have a much longer feeding time than those living

175 on the upper part of the shore, but diverse studies suggest that mussels which are submitted
176 to regular and prolonged aerial exposure partially compensate for the loss of feeding time
177 due to this situation through metabolic, physiological and morphological adjustments
178 (Widdows & Shick, 1985; Franz, 1993; Charles & Newell, 1997). Accordingly, access to food
179 seems not to be different between HS and LS mussels in terms of molecular antioxidants
180 provided by the food. However, the enhanced metabolic rate and subsequent ROS
181 production in HS mussels at re-submersion could generate a difference in the oxidative insult
182 between the two groups.

183 Air-exposure implies the isolation from the surrounding environment and consequent
184 decrease in partial pressure of O₂ during low tide. Subsequently, there is a 90% decrease in
185 the initial metabolic level, followed by a switch to anaerobic pathways (De Zwaan, 1991).
186 When recovery occurs, partial pressure of oxygen in tissues rises suddenly and a 2- or 3-fold
187 increase in the respiratory rate is observed during the first hour (Widdows & Shick, 1985).
188 This situation, which would be comparable to an ischemia-reperfusion event, is generally
189 associated with ROS overproduction and oxidative injuries (Ferreira *et al.*, 1999; Abele &
190 Puntarulo, 2004; De Oliveira *et al.*, 2005). The length of air-exposure recurrently endured by
191 HS mussels is likely to lead to a complete tissue anoxia at the end of emersion, adding to
192 accumulation of reducing equivalents; the metabolic burst and drastic increase in oxygen
193 tension at recovery must induce a stronger rise in free radical production than in LS mussel
194 tissues. In an ischemia-reperfusion experiment, it was demonstrated that prolongation of the
195 hypoxic phase increases oxidative damage to molecules and concentration of
196 hydroperoxides during recovery (Venditti *et al.*, 2001). In addition, the intertidal condition
197 induces variations in other abiotic parameters such as temperature, which are known to
198 influence the redox balance (Prakash *et al.*, 1998; Martínez-Álvarez *et al.*, 2002; Khessiba *et*
199 *al.*, 2005; Heise *et al.*, 2006). Fluctuations in the diverse environmental and physiological
200 parameters linked to tidal oscillations are more pronounced for HS mussels than for LS,
201 moreover the cyclic repetition of these conditions probably intensifies the stress endured by
202 HS mussels. Hence, the elevated levels of antioxidant enzymatic activities observed in HS
203 mussels in comparison to those measured in LS mussels may be related to the more
204 demanding conditions imposed by their high location on the shore. Such observations are
205 corroborated by a study on two close species of limpets inhabiting respectively high and low
206 parts of the shore, and where the authors suggest that HS species exhibit high and effective
207 antioxidant defences allowing the limitation of oxidative damage (Malanga *et al.*, 2004). Here,
208 the plasticity of antioxidant capacities evolved by mussels may allow those inhabiting high
209 shore to acclimate to their more extreme habitat, through enhancing their defences in order
210 to minimize the likely oxidative insults due to this fluctuating environment. Measurement of
211 oxidative damages could help to verify this hypothesis.

212 The greatest spatial variability among the enzymes measured was for GPX, GRd and
213 catalase activities, which play a role in the destruction of peroxides. In general, antioxidant
214 activities are clearly higher in gills than in the digestive gland except for CAT and GPX whose
215 activities are of the same order for both tissues, and therefore consistent with previous
216 reports (Power & Sheehan, 1996; Manduzio *et al.*, 2004). This difference could be related to
217 the position of gill tissue at the interface between external and internal medium, thus being a
218 likely site of major oxidative stress. Absolute levels sometimes vary between October 2004
219 and November 2005, possibly because of a difference in temperature between the two
220 sampling points, but the HS/LS pattern is generally confirmed. Autumn corresponds to a rest
221 period and also to the onset of gametogenesis, which can be influenced by environmental
222 factors (Sprung, 1983). This implies a spatial difference in the distribution of energy between
223 gamete building and stress response (Petes, 2007), with potential adjustments of antioxidant
224 defences. The HS/LS pattern certainly varies with the time of year and thus emphasizes that
225 temporal variations need to be assessed, particularly since seasonal changes in antioxidant
226 activities have already been reported in *Mytilus* sp. (Viarengo *et al.*, 1991; Power & Sheehan,
227 1996; Manduzio *et al.*, 2004; Borkovic *et al.*, 2005; Bocchetti & Regoli, 2006). We already
228 notice changes in the HS/LS difference and in the SOD isoforms' contribution to the total
229 activity between the present work and a previous study, which deals with the tidal variations
230 of Cu/Zn-SOD in tissues of mussels sampled in summer (Letendre *et al.*, 2008). In addition,
231 this study shows that SOD activity is affected by the tidal cycle in a tissue-specific manner.
232 The antioxidant response to the alternation of emersion-submersion could be different
233 according to the vertical location of organisms and the effect of tidal cycle on the HS/LS
234 pattern could itself depend on the season. Future studies are therefore investigating the
235 variations of antioxidant activities exhibited by HS and LS organisms during a whole tidal
236 cycle for different periods of the year.

237

238 5. Conclusion

239 This study shows that in a clean environmental context, antioxidant enzymatic
240 activities are higher for mussels living in the upper intertidal zone, suggesting an ability to
241 adjust their antioxidant defences in response to the stressful situation induced by living in this
242 part of the habitat. These data suggest the importance of the vertical position of animals on
243 the shore in the response of enzymatic antioxidant defences, which is of great interest for
244 sampling in monitoring studies. Nonetheless, further investigations concerning tidal and
245 seasonal variations have to be performed to provide a complete picture of the impact of
246 intertidal condition on antioxidant enzymatic activities.

247

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252

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Legends to figures

Fig. 1: Densitograms of gels after SOD staining. SOD activity was revealed by the NBT method after IEF on pH gradient 4.0-5.0 gels as described in the Materials and Methods.

Fig. 2: Activities of catalase and Cu-Zn SOD and relative contribution of the SOD isoforms to the total activity in gills (A, B, C) and digestive gland (D, E, F) of mussels sampled at high shore (HS) and low shore (LS) after 2h of emersion in October 2004 and November 2005. For each sample, total SOD activity was the sum of the activity of all revealed SOD isoforms. Pie charts represent the percentage of contribution of each SOD isoform to the total activity. Data are expressed as mean \pm S.E.M. of 10 inter-individual measurements. Differences of values between LS and HS mussels were considered as significant from $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)).

Fig. 3: Activities of GPx, GRd and GST in gills (A,B,C) and digestive gland (C,D,E) of mussels sampled at high shore (HS) and low shore (LS) after 2h of emersion in October 2004 and November 2005. All values are means of 10 inter-individual measurements. Data are expressed as mean \pm S.E.M. of 10 inter-individual measurements. Differences of values between LS and HS mussels were considered as significant from $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)).





