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1 The effect of long-term depuration on levels of oxidative stress biomarkers in mullets
2 (*Mugil cephalus*) chronically exposed to contaminants.

3

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15 *Keywords:* Oxidative stress, biomarkers, Mullet (*Mugil cephalus*), pollutants,
16 depuration.

17 **Abstract**

18 The present study aimed to obtain additional data on the effect of long-term
19 depuration on the levels of oxidative stress biomarkers, and to clarify the role of
20 mullets for monitoring pollution in River Douro estuary. Mulletts chronically exposed
21 to a mixture of contaminants in Douro estuary were captured in Spring of 2001, 2002
22 and 2003. The activities of antioxidant enzymes catalase superoxide dismutase
23 (SOD) and glutathione peroxidase (GPx); and oxidative damages in lipids (lipid
24 peroxidation) and in proteins (protein carbonyl content) were assessed at capture day
25 and after transfer to unpolluted seawater for one, four and eight months. An overall
26 decrease in the activities of the antioxidant enzymes was detected, except for the
27 GPx after 4 months depuration. CAT activity exhibited the more significant decrease
28 at the end of the long-term depuration. The decrease in SOD activity after one month
29 of depuration was then maintained during the remaining depuration period.
30 Regarding oxidative damages, a decrease in lipid peroxidation as well as the content
31 of oxidised proteins was observed during depuration. Indeed, at capture the activities
32 of antioxidant defences were higher as a result of the formation of Reactive Oxygen
33 Species (ROS) from the metabolism of pollutants. The oxidative damaged molecules
34 were repaired or degraded during the depuration period, supporting the use of such
35 damages as indicators of exposure to pro-oxidant pollutants.

36 ***Introduction***

37 Environmental contaminants such as metals, polychlorinated compounds (PCBs),
38 polycyclic aromatic hydrocarbons (PAHs) are known to enhance intracellular
39 formation of reactive oxygen species (ROS) with subsequent oxidative damage to
40 macromolecules (Livingstone, 2001; Ferreira et al., 2005). The response of
41 organisms to the increase in levels of ROS production involves up-regulating
42 antioxidant defences, such as the activities of antioxidant enzymes, and of
43 scavenger's compounds. The level of antioxidants against ROS overproduction
44 associated with tissue damage can be considered as a toxicity measure in aquatic
45 organisms exposed to pollution (Ahmad et al., 2000; Di Giulio et al., 1989; Ferreira et
46 al., 2005). The importance of oxidative stress response as potential biomarkers of
47 environmental pollution has been addressed by different experimental approaches
48 (Ferreira et al., 2005; Orbea et al., 2002; Rodriguez-Ariza et al., 2003). The
49 biomarkers utilized include components of oxidative adaptative responses, such as
50 antioxidant enzyme activities (catalase (CAT), superoxide dismutases (SODs) or
51 glutathione peroxidase (GPx)), or the estimation of oxidative damages in lipids,
52 proteins and DNA (Filho, 1996; Winston and Di Giulio, 1991). When the defences
53 mechanisms are unbalanced regarding the increased presence of ROS generated
54 compounds, e.g. by the presence of pollutants, oxidative damage will occur,
55 indicating a mechanism of toxicity in aquatic organisms. It has been reported that
56 despite an increase in antioxidant enzymes, oxidative damage occurs in animals
57 living in polluted sites (Van der Oost et al., 2003).

58 It has been reported previously the presence of several contaminants, in River Douro
59 Estuary, namely polychlorinated biphenyls (PCBs) and DDT (Ferreira et al., 2004),
60 xenoestrogens (Ferreira et al., 2002), metals from anthropogenic origin (Mucha et al.,

61 2003), and polyaromatic hydrocarbons (PAH) (Ferreira et al., 2006). An earlier
62 report has shown that mullets (*Mugil cephalus*) from Douro estuary had elevated
63 levels of hepatic SODs and CAT activity that decreased after one month depuration
64 in captivity conditions, and also showed lipid peroxidation in liver (Ferreira et al.,
65 2005). When comparing these results, after depuration, with phase I biotransformation
66 enzyme, namely ethoxyresorufin O-deethylase (EROD) (Ferreira et al., 2004, 2006),
67 the antioxidant enzyme (SOD and CAT) responses were not so evident, showing less
68 marked decreases in their activities during depuration (Ferreira et al., 2005). Lipid
69 peroxidation (LPO) in mullet liver, after one month depuration, have decreased in
70 spring and winter; and regarding oxidised proteins there was an increase after the
71 depuration (Ferreira et al., 2005).

72 The aim of the present work was to evaluate changes in antioxidant defences,
73 measured as hepatic CAT, SOD and GPx activity, associated with a longer
74 depuration period, when animals are transferred from a polluted environment to a
75 laboratory setting. In addition, we assessed the oxidative damages after long-term
76 depuration, to check if mullets are able to repair mainly the damages observed in
77 liver proteins.

78

79 ***Material and methods***

80 2.1. Study area

81 The present work was carried out in the lower Douro estuary. The Douro is one of
82 the longest rivers in the Iberian Peninsula (930 Km), sharing its 98,000 Km² of
83 watershed with Spain and Portugal. It drains into the Atlantic Ocean at 41°08' N and
84 8°42' W, near Porto (fig. 1). Domestic sewage as well as industrial effluents are still
85 discharged, mostly without treatment, directly into the estuary and its tributaries.

86 2.2. Sampling

87 Mulletts (N=66, mean wet weight, 406.4 ± 23.0 g, and mean length, 33.7 ± 1.6 cm) were
88 netted in an established polluted site in Douro estuary in the spring of 2001, 2002
89 and 2003. In each sampling campaign, twenty two mulletts were used. For the first
90 group six mulletts (Group I) were sacrificed within 24 hours after capture. Mulletts
91 were measured and weighed to determine condition factor (CF) ($\text{body weight (g)} \times$
92 $100 / (\text{length (cm)})^3$). After dissection livers were weighed to determine hepato-
93 somatic index (HSI) ($\text{liver weight (g)} / \text{body weight (g)} \times 100$). Livers were frozen in
94 liquid nitrogen and stored at -80°C until further use. At each sampling occasion, the
95 remaining mulletts were allowed to depurate separately for one month (Group II;
96 $n=6$), four months (Group IV; $n=5$) and eight months (Group VIII; $n=5$) in 3000 L
97 tanks at a salinity of 20 ‰ (similar to the salinity in the estuary where the mulletts
98 were captured) with a flow rate of 5 L/min. Water was continuously filtered through
99 an extensive biological filter, and a charcoal filter before being recycled. Aeration
100 was provided in the tanks to maintain 100% oxygen saturation. Fish were kept under
101 natural photoperiod and temperature. Mulletts were fed with uncontaminated frozen
102 fish (hake), during the first 10 days the added food was not consumed probably due
103 to the stress of captivity. At day 15 it was observed that all fish were eating normally.

104 2.3. Antioxidant enzyme activities

105 Livers were homogenized in ice-cold sodium phosphate buffer 50 mM, Na_2EDTA
106 0.1 mM, pH 7.8. Mitochondrial fractions were obtained after centrifugation at 15 000
107 g for 20 min. Catalase activity was determined by measuring the consumption of
108 H_2O_2 at 240 nm (ext. coef. $40 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) according to Aebi (1974). The reaction
109 volume was 1 ml and contained 67.5 mM potassium phosphate buffer, pH 7.5, and
110 12.5 mM H_2O_2 . The reaction was started by the addition of the sample. CAT activity

111 is expressed as mmol/min/mg protein. SOD activity was determined by an indirect
112 method involving the inhibition of cytochrome *c* reduction. In this method SOD
113 competes with cytochrome *c* for the superoxide anion generated by the hypoxanthine
114 and xanthine oxidase reaction. SOD activity was determined in the mitochondrial
115 fraction as the degree of inhibition of cytochrome *c* reduction at 550 nm (McCord
116 and Fridovich, 1969). The concentration of the reactives was: potassium phosphate
117 buffer 50 mM, pH 7.8, hypoxanthine 50 μ M, xanthine oxidase 1.98 mU/ml and
118 cytochrome *c* 10 μ M (Orbea et al., 2002). The activity of MnSOD was evaluated
119 adding to the reaction KCN 2 mM. The activity is given in SOD units (1 SOD unit =
120 50 % inhibition of the xanthine oxidase reaction). GPx activity was measured with
121 H_2O_2 as substrate, i.e. the activity of the selenium(Se)-containing GPX (Halliwell
122 and Gutteridge, 1999). The activity was monitored by following the decrease in
123 NADPH concentration at 340 nm (ext. coef. $6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$), which is consumed
124 during the generation of GSH from oxidized glutathione, according to Günzler and
125 Flohe (1985). The concentration of the reactives was: potassium phosphate buffer 50
126 mM, pH 7.8, 2.5 mM GSH, 0.6 mM sodium azide, 1.25 U glutathione reductase,
127 0.15 mM NADPH, 0.2 mM H_2O_2 . GPx activity is expressed as nmol/min/mg protein.

128

129 2.4. Oxidative damages

130 The peroxidative damage to lipids that occurs with free radical generation, and
131 results in the production of malondialdehyde (MDA) was assessed by the
132 determination of thiobarbituric acid reactive substances (TBARS). MDA was
133 determined by the thiobarbituric acid method, the liver homogenate was incubated
134 with trichloroacetic acid (TCA) 100%, after centrifugation the supernatant was
135 incubated at 100°C, for 30 min, with thiobarbituric acid (TBA) 1%, NaOH 0.05 M

136 and BHT 0.025 % (Niki, 2000). The absorbance was measured at 532 nm. Lipid
137 peroxidation (LP) is expressed as nanomoles of MDA equivalents per mg of protein.
138 Sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) was
139 performed using minigels consisting of a 4 % stacking gel and 12.5 % running gel.
140 Fish liver samples were prepared to a final protein concentration of 4 $\mu\text{g}\cdot\mu\text{l}^{-1}$ and
141 derivatized with DNPH by incubation at dark for 30 min, a control was incubated
142 with trifluoroacetic acid (TFA) 10% (Levine et al., 1994), without preheating the
143 sample, 5 μl per lane were loaded in the gels. After electrophoresis, separated
144 proteins were transferred onto nitrocellulose membranes using a HOEFER TE 22.
145 Membranes were blocked overnight at 4°C in phosphate buffered saline (PBS) plus
146 0.05 % Tween 20 (TPBS) containing 5 % non-fatty dried milk. Incubations with the
147 diluted antibody rabbit anti-DNP (1:5000) (DAKO) were preformed for 1h at room
148 temperature. Membranes were washed and incubated with the diluted secondary
149 antibody, anti-rabbit IgG-peroxidase (1:5000) (SIGMA) for 1h at 4°C. Activity was
150 visualized with an enhanced chemiluminescence (ECL) kit (Amershan Life Science).
151 In all assays total protein was measured by Lowry method adapted to microplates.

152

153 2.5. Statistical analysis

154 Differences between groups were tested using a one-Way ANOVA with a multiple
155 comparison test (LSD) at a 5% significance level. Some data had to be log
156 transformed in order to fit ANOVA assumptions. All tests were performed using the
157 software Statistica 6.0 (Statsoft, Inc., 2001). No differences between years were
158 observed and therefore the data are presented grouped. Differences between genders
159 were also assessed, and no statistical differences were observed and therefore the
160 data are presented with both sexes grouped.

161

162 **Results**

163 Biological parameters such as HSI and CF can be used to assess the status of the
164 organ and the organism. The values for HSI and CF in mullets after capture and after
165 the three periods of depuration revealed significant decreases after one, four and
166 eight months of depuration for HSI, whereas no differences were observed for CF
167 (Table 1).

168 Hepatic CAT, totalSOD, MnSOD and GPx activities are displayed in fig.2. CAT
169 activity has shown a slightly increase after one month of depuration, however, after
170 four months the activity was lower (18.4 %; $p<0.05$), and were maintained during the
171 following months of depuration. TotalSOD activity decreased after the first month of
172 depuration (15.2 %; $p<0.05$) maintaining similar values in the forthcoming periods of
173 depuration. Hepatic MnSOD activity presented the same pattern as liver totalSOD
174 activity, although more pronounced with significant decreases in group II (16.2 %;
175 $p<0.05$), in group IV (22.5 %; $p<0.05$) and in Group VIII (16.2 %; $p<0.05$). Liver
176 GPx activity decreased after one month of depuration (18.8 %; $p<0.05$), and in
177 contrast with the assessed antioxidant enzymes, an increase was observed at the forth
178 month of depuration, and decreasing after eight months of depuration to values
179 similar to the ones measured in group II.

180 Oxidative damages in lipids evaluated as lipid peroxidation levels (LPO), using the
181 TBA test, showed that after one month of depuration LPO decreased significantly
182 (33.5 %; $p<0.05$), and continued to decrease in group IV (48.8 %; $p<0.05$) (fig. 3). In
183 group VIII however, an increase was observed although below the values measured
184 in mullets after capture. Oxidative damages in proteins were evaluated by
185 immunoblot of carbonyl groups (fig.4). One month of depuration led to an increase

186 in oxidised protein content, in agreement with our previous results and only after
187 eight months of depuration was a reduction of carbonyl groups observed.

188

189 *Discussion*

190 Previous studies reported the presence of different types of contaminants in the river
191 Douro estuary (Mucha et al., 2003; Ferreira et al., 2002, 2004) inducing oxidative
192 stress in fish inhabiting these waters (Ferreira et al., 2005). However, the changes on
193 oxidative stress biomarkers and the ability to recover from damages after different
194 periods of depuration has not been analysed.

195 Four types of PAH metabolites were found in mullet's bile captured in Douro
196 estuary, and a significant decrease was observed after four and eight months of
197 depuration (Ferreira et al., 2006). The presence of PCBs and DDT was also identified
198 and it was observed that these pollutants are more persistent than PAHs. After one
199 month of depuration, the levels of these contaminants in muscle and liver of mullets
200 were maintained (Ferreira et al., 2004), and started to decrease only in the muscle
201 after four months depuration (Antunes et al., *in press*).

202 During the captivity period the mullets CF did not change, indicating that the
203 captivity had no effect at the organism level. In addition, the decreased HSI values
204 after depuration period, also indicate a recovery of liver enlargement due to chemical
205 exposure (Ferreira et al., 2006). However, the smaller HSI observed in Group IV
206 could be a result of the smaller fish used in this particular group.

207 Antioxidant systems can be considered as non-specific biomarkers of exposure to
208 pollutants, and also as an indicator of toxicity. The induction of levels of primary
209 antioxidant defences preventing cell damage can be regarded as an adaptative
210 response to an altered environment; in contrast an inhibition can lead to cell damage

211 and toxicity of bioavailable pollutant in a dose-dependent manner (Vassuer and
212 Cossu-Leguille, 2003). After one month of depuration an increase in hepatic CAT
213 activity was observed, contrary to our previous study (Ferreira et al., 2005). This
214 increase could be related to the period of non-consumption of food during the first
215 days of captivity. Some authors reported the influence of starvation in oxidative
216 stress responses with increases in some antioxidant enzyme activities including CAT
217 (Morales et al., 2004). Nevertheless, after four and eight months of depuration CAT
218 activity had significantly decreased, indicating that a longer period of time is needed
219 to recover from oxidative stress induced by pollutants, directly or indirectly. Hepatic
220 totalSOD and MnSOD activity, contrary to CAT activity, showed significant
221 decreases after one month of depuration and then the levels were maintained in the
222 following period. The decrease observed for MnSOD activity was more evident and
223 so far there are few studies on this enzyme in aquatic organisms although it plays a
224 key role in protecting mitochondria from oxidative stress (Reed, 2001). Interestingly,
225 hepatic GPx activity, revealed a significant decrease after one month of depuration,
226 and then increased in Group IV. This period was concomitant with summer months
227 and the increase in GPx activity can be attributed, probably to the higher
228 temperature, as reported by Bacanskas et al. (2004) for killifish (*Fundulus*
229 *heteroclitus*). These results are in agreement with the ones observed for glutathione
230 S-transferase (GST), an enzyme involved in the defence against oxidative damage
231 and peroxidative products of DNA and lipids (George, 1994). In mullets under
232 similar conditions GST activity also increased in the warmer periods (Ferreira et al.,
233 2006). In addition, the increase in GPx activity in this period may act as a protective
234 mechanism avoiding the increase in LPO in summer observed in mullets during
235 depuration (Ferreira et al., 2005).

236 Oxidative damage can occur when antioxidant and detoxifying systems are deficient
237 and not able to neutralise the active intermediates produced by the metabolism of
238 xenobiotics or their products. The presence of single and mixed contaminants,
239 including metals, PAHs and PCBs led to an increase in oxidative damages in both
240 fish and invertebrates (Livingstone, 2001). Previously we have reported a decrease in
241 LPO in mullet liver, in spring and winter, after one month of depuration, in contrast
242 with the levels of oxidised proteins that were increased (Ferreira et al., 2005). In this
243 study, in summer, LPO levels continued to decrease, which could have been a result
244 of the increased GPx activity observed in summer. Stephensen et al. (2002)
245 speculated that GPx was not an important enzyme in destroying organic peroxides in
246 fish, in mullet liver a possible correlation between GPx activities and LPO was
247 observed. Higher GPx activity in Group IV corresponded to lower levels of LPO and
248 after eight month of depuration the decrease in GPx activity was associated with an
249 increase in LPO.

250 Protein oxidation can be increased by xenobiotic exposure (Gibson et al., 1996;
251 Fessard and Livingstone, 1998), and the assessment of individual or groups of
252 proteins can be used as potential biomarkers of contaminant-mediated oxidative
253 damage in fish liver. The formation of carbonyl derivatives is non-reversible, causing
254 conformational changes and decreased catalytic activity in enzymes (Almroth et al.,
255 2005). Our previous study has shown an increase in oxidised protein content in
256 mullet liver after one month of depuration (Ferreira et al., 2005). This increase could
257 be a result of the decrease in antioxidant enzyme activities after the depuration, and
258 because the formation of carbonyl derivatives is a non-reversible process we should
259 expect longer time to replace the damaged proteins. In fact, after eight months of
260 depuration the content in oxidised proteins in liver has decreased.

261 In conclusion, the present study brings key information in the capacity of mullets,
262 after long-term depuration, to recover oxidative damages induced by the presence of
263 pollutants. Antioxidant enzyme activities showed different responses, with CAT
264 activity decrease after only four month of depuration, whereas for total SOD and
265 MnSOD activities the decrease was detected after one month of depuration. GPx
266 activity has shown an increase in activity in the summer period that inhibited the
267 increase observed in LPO. Oxidative damages in LPO returned to normal values after
268 one month of depuration whereas the time for replacing the oxidative damaged
269 proteins was longer.

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ACCEPTED MANUSCRIPT

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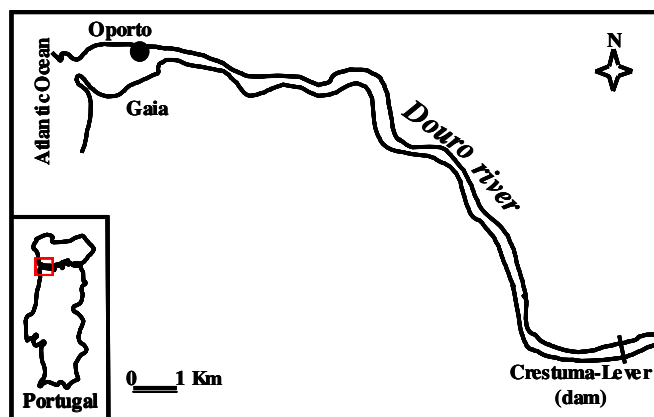
369 Table 1: Mulletts mean weight (g) and length (cm), hepato-somatic index (HSI) (%)
 370 and condition factor (CF) at capture day (Group I), after 1 month (group II), 4
 371 months (Group IV) and after 8 months (Group VIII) in captivity.

	Weight (g)	Length (cm)	HSI (%)	CF
Group I (n=18)	521.5 (29.2)	40.0 (0.8)	1.75 (0.05)	0.80 (0.02)
Group II (n=18)	470.0 (25.1)	39.2 (0.7)	1.27 (0.07)***	0.76 (0.02)
Group IV (n=15)	333.8 (34.4) ***	34.1 (1.6) ***	1.09 (0.06)***	0.84 (0.04)
Group VIII (n=15)	441.7 (37.0) *	37.6 (0.8)	1.23 (0.09)***	0.86 (0.04)

372 Values presented as mean (Standard Error). Statistical differences to Group I : *

373 $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

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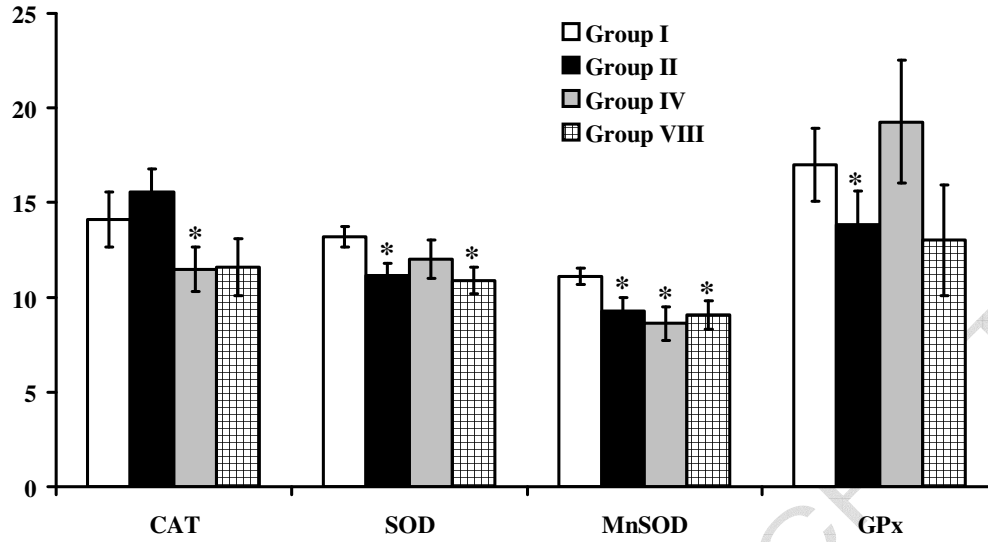


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376 Figure 1: Map of the Douro estuary and the sampling station (black dot).

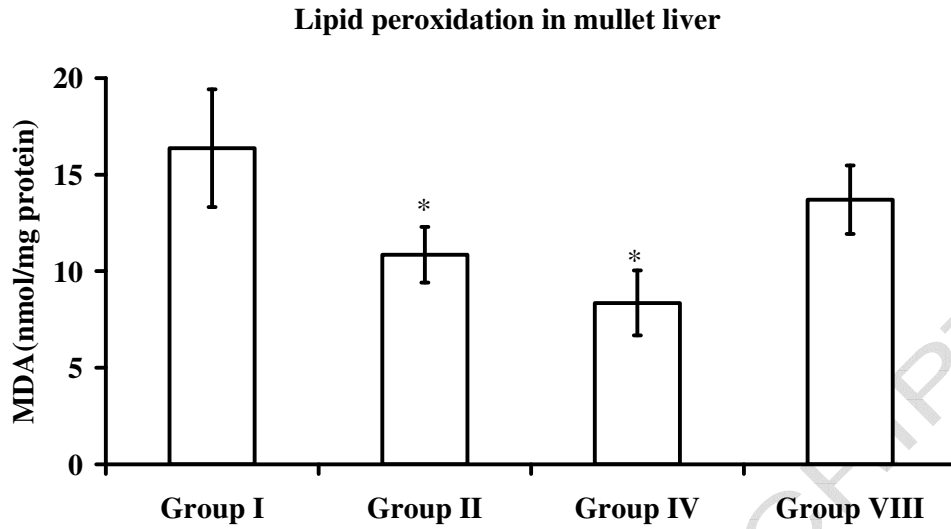
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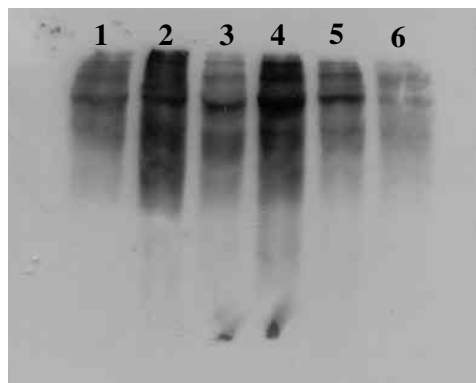
379 Figure 2: Hepatic antioxidant enzymes activities in mullets after capture in Douro
 380 estuary (Group I), and after one month (Group II), 4 months (Group IV) and 8
 381 months (Group VIII) of depuration. Values presented as mean \pm SE. Statistical
 382 differences to Group I: * $p < 0.05$. CAT in mmol/min/mg protein, Total SOD and
 383 MnSOD in U/mg protein and GPx in nmol/min/mg protein.



384

385 Figure 3: Hepatic lipid peroxidation (LP) in mullets after capture in Douro estuary
386 (Group I), and after one month (Group II), 4 months (Group IV) and 8 months
387 (Group VIII) of depuration. Values presented as mean \pm SE. Statistical differences to
388 Group I: * $p < 0.05$. MDA: malondialdehyde. LP in nmol MDA equivalents per mg of
389 protein.

390



391

392 Figure 4: Example of Western blot of carbonyl groups (derivatized with DNPH)
393 from oxidised mullet liver proteins. In lanes 1 and 3 liver proteins from mullets
394 captured in Douro estuary (Group I), in lanes 2 and 4, from mullets kept in captivity
395 for 1 month (Group II) and in lanes 5 and 6 mullet depurated for 4 and 8 months
396 respectively.

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