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1 **Protein carbonyls and antioxidant defenses in corkwing wrasse (*Symphodus***
2 ***melops*) from a heavy metal polluted and a PAH polluted site**

3

4

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19 **Abstract:**

20 The use of fish in environmental monitoring has become increasingly important in recent
21 years as anthropogenic substances, many of which function as prooxidants, are accumulating
22 in aquatic environments. We have measured a battery of antioxidant defenses as a measure of
23 oxidative status, as well as protein carbonylation as a measure of oxidative damage, in
24 corkwing wrasse (*Symphodus melops*) captured near a disused copper mine, where water and
25 sediment are contaminated with heavy metals, and an aluminum smelter, a site contaminated
26 with PAHs. Results were compared to two different reference sites. Fish at the heavy metal
27 site had lower glucose-6-phosphate dehydrogenase activity and elevated protein carbonyls
28 (1.8 times) compared to fish from the reference site. At the PAH site, EROD was increased 2-
29 fold, while total glutathione and methemoglobin reductase concentration, were decreased. No
30 differences were seen in protein carbonyl levels at the PAH site. Measures of both antioxidant
31 defenses and oxidative damage should be used when assessing effects of xenobiotics on
32 oxidative stress in fish species.

33

34 **Keywords:** fish; oxidative stress; antioxidant enzymes; biomarker; protein carbonyls; PAHs;
35 heavy metals

36 1. Introduction

37

38 Coastal marine environments are often exposed to the influences of anthropogenic activities
39 and consequentially, toxic chemicals. Many of these xenobiotics exert their effects through
40 redox-cycling, resulting in the production of reactive oxygen species (ROS). ROS production
41 resulting from xenobiotic redox-cycling is an important mechanism of pollutant toxicity and
42 can lead to oxidative damage to proteins, lipids and DNA (Stadtman and Oliver 1991). This is
43 linked to diseases such as carcinogenesis in fish (Livingstone 2001). Protein damage resulting
44 from oxidative stress may be useful as a biomarker for exposure of fish to environmental
45 contaminants (Carney Almroth et al., 2005, Shi et al., 2005, Bagnyukova et al., 2006). The
46 form of oxidative damage to protein molecules that is most widely accepted as a biomarker is
47 protein carbonylation (Shacter et al., 1994). The formation of carbonyl derivatives is
48 irreversible and increases the susceptibility of proteins to proteases (Stadtman and Oliver
49 1991). A number of enzymes and molecules play important roles in detoxifying xenobiotics
50 and in protecting the cell against the harmful effects of ROS. Many of these enzymes and
51 molecules are well studied in mammals (Halliwell and Gutteridge 1999) and have been
52 applied as biomarkers for environmental risks in fish (van der Oost, et al. 2003, Valavanidis
53 2006).

54

55 Oxidative stress is a very complex phenomenon, starting with the production of ROS and
56 including oxidative damage or disruption as well as adaptive responses of antioxidant defense
57 components. Thus, instead of choosing a single biomarker for oxidative stress, use of a
58 battery of biomarkers will provide a more complete picture of the effects of xenobiotics on
59 oxidative stress in the cells of an organism. This battery should preferably include both
60 oxidative damage as well as changes to the antioxidant defense system. In the current study a

61 large battery of biomarkers for pollutant exposure was assessed in corkwing wrasse
62 (*Symphodus melops*) caught at a PAH contaminated site and a heavy metal polluted site near
63 Stavanger, Norway. The biomarkers included were the phase I enzyme CYP1A and the phase
64 II enzyme glutathione S-transferase as were the antioxidant enzymes superoxide dismutase
65 (SOD), catalase (CAT), glutathione reductase (GR) and DT-diaphorase (DTD) in liver, and
66 methemoglobin reductase (MtHB) and glucose-6-phosphate dehydrogenase in blood. In
67 addition to these enzymes, total and oxidized glutathione and metallothionein
68 concentrations in liver were measured. Also, as an example of oxidative damage, protein
69 carbonyl formation was measured. Though protein oxidation products have been used as
70 biomarkers for oxidative stress and damage in humans (Dalle-Donne et al., 2003), their
71 usefulness has not been fully explored as a potential biomarker in fish.
72 The species of wrasse used in the current study is known to possess a limited home range
73 within the littoral zone, as it is a territorial species. Its benthic behavior should make it a
74 suitable sentinel species for studies involving contaminants (Aas, et al. 2001). In addition,
75 corkwing wrasse are ubiquitous throughout the northeastern Atlantic and Mediterranean
76 (Quignard and Pras 1986), making them suitable for European biomonitoring campaigns.

77

78

79 **2. Materials and methods**

80

81 **2.1. Chemicals**

82

83 Biotinylated anti-dinitrophenyl-KLH rabbit IgG fraction was purchased from Molecular
84 Probes, streptavidin-biotinylated horse radish peroxidase (HRP) from Amersham Bioscience,
85 UK, and the BCA Protein Assay Reagent Kit from Pierce. Tween 20 was obtained from BIO-

86 RAD. Copper (II) chloride dihydrate and EDTA (Titriplex III) were purchased from Merck,
87 heparin from LEO Pharma AB, Sweden, and trasyolol from Bayer, Germany. Trifluoroacetic
88 acid, oxidized and reduced glutathione, glutathione reductase, reduced glutathione (GSSG),
89 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), dichlorophenol indophenol (DCPIP), 2, 4-
90 dinitrophenol hydrazine (DNPH) and rabbit metallothionein were obtained from Sigma.
91 NADPH and NADH were purchased from Boehringer Mannheim and dicoumoral (DIC) was
92 purchased from Aldrich. All other reagents were of analytical grade.

93

94 2.2. Field study sites

95

96 Corkwing wrasse (*Symphodus melops*) were caught at 4 sites using fyke nets during field
97 sampling near Stavanger, Norway, in the fall of 2001. Fish were collected from the heavy
98 metal site Visnes and the PAH site Høgvarde and the respective reference sites, Salvøy and
99 Bokn. See figure 1 for site locations. The two polluted sites are well known as sinks for
100 industrial pollution. Visnes is a village located outside Stavanger and is home to a copper
101 mine which was in operation during several periods between 1865 and 1965. Tailings and
102 slag were dumped into the sea and heavy metals, including copper, zinc and iron,
103 contaminated the sea water and sediment as a result of dumping and land run off. There was
104 no evidence of higher organisms close to the mine, where copper concentration in the water
105 was $53.6 \mu\text{g l}^{-1}$. Andersen et al. (2003a) reported the contaminant levels in water, sediment
106 and biota at Visnes as follows. The sediment content of metals at Visnes reaches up to 20000
107 mg Zn kg^{-1} , $13000 \text{ mg Cu kg}^{-1}$, $1500 \text{ mg Pb kg}^{-1}$ and 35 mg Cd kg^{-1} . The site displays a sharp
108 gradient in copper concentration, from the mouth of the mine to the sea. Copper concentration
109 in the water at the catch site during this study was $8.59 \mu\text{g l}^{-1}$, zinc was $33.6 \mu\text{g l}^{-1}$, iron $96 \mu\text{g}$
110 l^{-1} and lead $0.135 \mu\text{g l}^{-1}$. Analyses of blue mussel (*Mytilus edulis*) tissue from Visnes indicated

111 48 mg Cu kg dry weight (d.w.)⁻¹, 257 mg Zn kg⁻¹ d.w., 470 mg Fe kg d.w.⁻¹ and 1.3 mg Pb kg
112 d.w.⁻¹, levels which were 9.6, 4.0 and 4.2 and 2.6 fold higher than at the reference site.

113 Høgvarde is located outside an active aluminum smelter and is contaminated with a mixture
114 of primarily pyrogenic PAHs (from the smelter) (Aas et al., 2001). In 2001, 0.6 tons of PAHs
115 were discharged from the aluminum smelter into the sea. At the PAH site, both blue mussels
116 and shore crabs (*Carcinus maenas*) were shown to contain high levels of PAHs in their
117 tissues, totalling 38.3 mg kg⁻¹ d.w. in mussels and 7.8 mg kg d.w.⁻¹ in crabs. The levels in
118 mussel were 1158 fold higher than at the control site and 16 fold higher in crabs (Andersen et
119 al., 2003b).

120

121 The two polluted sites differed with respect to water salinity and current velocity (personal
122 communication with Odd-Ketil Andersen, IRIS – Biomiljø, International Research Institute of
123 Stavanger, Norway) and consequently required comparison with different reference stations.
124 Water temperature at respective depths differed by no more than 0.5°C. Samples taken from
125 Visnes were compared to the reference site Salvøy since both sites are exposed to the same
126 water mass, the open ocean with a salinity of approximately 33‰. Samples taken at Høgvarde
127 were compared to the reference site Bokn as these sites are located inside the archipelago with
128 a lower average salinity of approximately 29.5‰.

129

130 2.3. Sampling

131

132 In order to reduce the risk of measuring changes due to capture-related stress, fish were caged
133 on site 2-3 days after capture before being sacrificed. After acclimatization, fish were killed
134 by a blow to the head, measured and weighed. A blood sample was taken from the caudal
135 vein using a syringe treated with heparin. Blood was centrifuged at 6 000 g for 2 minutes and

136 plasma samples were immediately frozen at -80°C where they were stored until measurement.
137 The liver was excised, weighed, divided into pieces, and stored in liquid nitrogen until
138 analysis. The numbers of fish sampled at each site were as follows: Salvøy – 10 males, 8
139 females; Visnes – 10 males, 10 females; Bokn – 10 males; Høgvarde – 10 males, 6 females.
140 No female fish were caught at Bokn.

141

142 2.4. Protein carbonyl measurements

143

144 An ELISA method described by Winterbourn et al. (1999) was used to measure protein
145 carbonyls in blood plasma of corkwing wrasse. In brief, plasma samples were derivatized
146 with DNPH (10 mM DNPH in 6 M guanidine hydrochloride, 0.5 M potassium phosphate
147 buffer, pH 2.5) which binds selectively to protein carbonyl groups. Detection was achieved
148 using biotinylated anti-dinitrophenyl (anti-DNP) and streptavidin-biotinylated HRP. A
149 standard curve of fully reduced and oxidized bovine serum albumin was used to quantify
150 results, according to a colorimetric method described by Levine et al. (1990).

151

152 2.5. Enzymatic activities

153

154 Preparation of liver microsome and cytosol fractions was performed according to Förlin
155 (1980). Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in the microsomal
156 fraction using ethoxyresorufin as a substrate and NADPH to provide reducing equivalents
157 (Förlin et al., 1994). Hepatic antioxidant enzyme activities were measured in the cytosolic
158 fraction. Superoxide dismutase (SOD) activity was measured using 6-hydroxy dopamine as a
159 substrate. The assay was run with and without sample to distinguish between SOD-catalyzed
160 activity and auto-oxidation reactions (Heikkila and Cabbat 1976). Catalase (CAT) activity

161 was measured in liver cytosol with hydrogen peroxide as a substrate, according to Aebi
162 (1985). Glutathione reductase (GR) activity was measured using GSSG as a substrate, with
163 NADPH acting as an electron donor. The resulting reduced GSH then reacts with 5,5'-Dithio-
164 bis-(2-nitrobenzoic acid) (DTNB), forming a glutathione-TNB conjugate and free TNB, the
165 production of which is measured over time (Cribb et al., 1989). Glutathione *S*-transferase
166 (GST) was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate as described
167 by Habig et al. (1974) and adapted to a microplate reader by Stephensen et al. (2002). The
168 formation of GST-catalysed CDNB-glutathione conjugate is measured
169 spectrophotometrically. DT-diaphorase activity (DTD) was measured according to Sturve et
170 al. (2005). Here, 2,6-dichloroindophenol (DCIP) was used as a substrate, and samples were
171 analysed with and without the addition of dicumarol, a DTD inhibitor, to distinguish between
172 DTD-catalysed reduction and the action of all other reductases. Red blood cells were lysed
173 and cytosolic fractions prepared according to Beutler (1984). SOD activity in the red blood
174 cells (rbc) was measured following the same method as was used in liver samples (Heikkila
175 and Cabbat 1976). Methemoglobin reductase activity (MetHb) in the rbc was measured using
176 $K_3Fe(CN)_6$ as a substrate according to Beutler (1984). Glucose-6-phosphate dehydrogenase
177 (G6PDH) activity in the rbc was measured using glucose-6-phosphate and NADP⁺ in the
178 reaction buffer, according to Deutsch (1987).

179

180 2.6. Molecular antioxidant measurements

181

182 Total glutathione (tGSH) and oxidized glutathione (GSSG) were measured according to
183 Baker et al. (1990), adapted to a microplate reader by Vandeputte et al. (1994). This is an
184 indirect method in which all GSH is reduced through the action of GR, followed by
185 conjugation to DTNB as described above in section 2.5. To measure the GSSG portion of the

186 total glutathione pool in the sample, GSH was first derivitized by a reaction with 2-
187 vinylpyridine.

188

189 2.7. Metallothionein

190

191 The concentration of metallothionein was measured using differential pulse polarography
192 (DPP) according to Olafson and Olsson (1991). Briefly, cytosolic fractions of liver were
193 prepared according to Förlin et al. (1980), diluted 10x in 0.9% NaCl and heat denatured at
194 95°C for 4 min. Heat-denatured samples were centrifuged 15 min at 10 000 g after which the
195 supernatant was analysed with DPP electrolyte (2 mM hexamine cobalt chloride, 1M NH₄Cl,
196 1M NH₄OH 300 µl 0.025% Triton X-100 added per 10 ml electrolyte). The purging time with
197 N₂ before measurements was 60 sec. A standard of 50.8 µg/ml rabbit MT in 0.9% NaCl was
198 used to prepare a standard curve.

199

200 2.8. Morphological indices

201

202 Morphological indices were calculated as described here. Condition factor (CF) was
203 calculated according to the formula: $CF = (\text{weight (g) length (cm)}^{-3}) \times 100$. Liver somatic
204 index (LSI), gonadosomatic index (GSI) and spleen somatic index (SSI) were calculated
205 according to the formula: $(\text{tissue weight (g) body weight (g)}^{-1}) \times 100$.

206

207 2.9. Statistics

208

209 All data values are given as means \pm standard error. Data from contaminated site and the
210 respective reference site were tested for statistically significant differences using the t-test. All

211 data that did not meet requirements for heterogeneity of variance and normality were log-
212 transformed prior to testing. Significance limit was set at $p < 0.05$. Data were tested using
213 SPSS® 12.0 for Windows. False discovery rate (FDR) was calculated for each p-value. This is
214 a correction for multiple testing, accepted values are normally set at <0.1 or <0.2 .

215

216 **3. Results**

217

218 **3.1. Wrasse captured near the copper mine**

219

220 Data from male and female fish were compared separately since little is known about
221 variations between the sexes in corkwing wrasse in the parameters measured here.

222

223 3.1.1. Morphometric indices

224

225 Male fish caught at Salvøy weighed 126 ± 11 g and females weighed 93 ± 3 g. Males caught
226 at Visnes weighed 93 ± 13 g and females weighed 93 ± 11 g. The mean SSI was significantly
227 lower in all fish, both males and females, captured at Visnes compared to Salvøy. Hematocrit
228 levels were significantly higher (122%) in male wrasse captured at Visnes compared to
229 Salvøy. GSI was significantly decreased by 47% in male fish at Visnes. Both LSI and SSI
230 were significantly higher in males compared to females at Salvøy. See table 1 for results and
231 p values. The FDR for each significant p value in table 1 was <0.1 . The FDR for SSI and LSI
232 comparisons between the two sexes was <0.2 .

233

234 3.1.2. Protein carbonylation ELISA

235

236 Protein carbonylation was significantly higher in the fish captured at Visnes, outside a copper
237 mine, compared to Salvøy (table 2) in both males and females. Levels were 123% higher in
238 males and 49% higher in females caught at Visnes than at Salvøy. FDR was < 0.01 in both
239 sexes.

240

241 3.1.3. Enzyme activities and antioxidants in liver and red blood cells

242

243 Few of the antioxidant enzymes differed between Visnes and Salvøy. G6PDH levels were
244 significantly lower in red blood cells of male fish from Visnes. Males also had significantly
245 higher levels of both G6PDH (FDR <0.01) and SOD (FDR <0.1) than females at Salvøy. No
246 other differences were seen between the two sites. Results and p values are shown in table 2.

247

248 **3.2. Wrasse captured at the PAH contaminated site**

249

250 Only male fish were available for measurements from the reference site Bokn. Data from
251 female fish captured at Høgvarde were therefore not compared to the control site, but were
252 analyzed statistically for sex-related differences at the PAH site.

253

254 3.2.1. Morphometric indices

255

256 Male fish living at Bokn weighed 85 ± 4 g. Male wrasse caught at Høgvarde weighed $107 \pm$
257 10 g and females weighed 67 ± 14 g. CF was significantly higher in male fish captured at
258 Høgvarde compared to Bokn (FDR <0.2). Hematocrit LSI, GSI and SSI were not affected. See
259 table 3 for results and p values.

260

261 3.2.2. Protein carbonylation

262

263 No difference was seen between Høgvarde, the PAH exposed site, and Bokn, the reference
264 site. However, a comparison between males and females at Høgvarde shows that the female
265 fish had higher levels of oxidized proteins in their blood (FDR < 0.2).

266

267 3.2.3. Enzyme activities and antioxidants in liver and red blood cells

268

269 EROD activity was significantly higher in male fish caught at the PAH contaminated site
270 compared to the reference site (FDR<0.1). tGSH was significantly decreased in male fish
271 captured at Høgvarde (FDR<0.02) but GSSG was unaffected. tGSH also correlated
272 significantly to size of the fish (Pearson correlation, $p=0.037$) where levels decreased with
273 increasing weight. GR activity did not differ. Methb levels in red blood cells were
274 significantly lower in fish captured at Høgvarde compared to Bokn (FDR<0.1). No other
275 parameters showed any significant effects when comparing the reference and polluted sites.
276 See table 4 for results and p values.

277

278 4. Discussion

279

280 The definition of oxidative stress states that a disturbance in the prooxidant-antioxidant
281 balance in favor of the former can lead to potential damage. Here, we have investigated a
282 battery of antioxidant defenses, both molecular and enzymatic, and an oxidative damage
283 product, protein carbonyls, and have found that a limited number of biomarkers are affected
284 in corkwing wrasse captured at sites with high levels of heavy metals and PAHs. The
285 information concerning heavy metal and PAH levels in water, sediment and biota, cited in the

286 field site descriptions in section 2.2, indicate that the contaminants are bioavailable and since
287 this species of wrasse is known to feed on molluscs and crustaceans (Quignard and Pras
288 1986), they would have been receiving contaminants both through water exposure and
289 through their food supply. Measurements of metals and PAHs in fish tissue should be
290 conducted in future studies to determine the extent of actual exposure.

291

292 4.1 Wrasse living at the copper mine

293

294 Although copper and zinc are essential trace elements needed, for example, as cofactors in
295 enzymes (i.e. SOD) they are also toxic heavy metals. Marine teleosts will take up heavy
296 metals directly into the blood stream via the gills (Dethloff et al., 1999) as well as additional
297 amounts via the gut, as they, contrary to fresh water species, drink large amounts of water
298 (Filipovic Marijic and Raspor 2007). Redox cycling of copper and iron species can induce the
299 formation of ROS which can lead to oxidative stress (Stadtman and Oliver 1991, Dean et al.,
300 1997). The levels of protein carbonylation measured in this study were significantly higher at
301 Visnes thereby indicating that the fish were suffering from oxidative stress as a result of
302 heavy metal exposure. Metals are known to directly induce the formation of protein carbonyls
303 via metal catalyzed oxidation reactions (MCO) (Stadtman and Oliver 1991). The resulting
304 damaged protein is more susceptible to degradation and may lose some or all of its function.
305 Interestingly, we did not see an induction of metallothionein in wrasse captured at Visnes,
306 near the copper mine. Measurements of MT proteins have shown mixed results in field
307 studies as levels can be influenced by both biotic factors (age, organ, reproductive status,
308 season) and abiotic factors (temperature, pH, salinity). As a result, MT protein levels will
309 reflect bioavailability, tissue pharmacokinetics of metal uptake or MT synthesis (Handy et al.,
310 2003, Filipovic Marijic and Raspor 2007). Also, we have measured MT levels in liver, while

311 other studies have shown great induction in kidneys and intestines in marine teleost species
312 (Filipovic Marijic and Raspor 2007). To our knowledge, there is no information available on
313 MT levels in corkwing wrasse, but hepatic MT concentrations in a related species, goldsinny
314 wrasse (*Ctenolabrus rupestris*) were lower than those seen here (median concentrations of
315 1.5-2.0 $\mu\text{g}/\text{mg}$ protein). In goldsinny wrasse, there was a clear positive correlation between
316 hepatic concentrations of Zn/Cu and MT (Ketil Hylland, Department of Biology, University
317 of Oslo, Norway, personal communication). Other binding sites may be involved in metal
318 chelation in corkwing wrasse, thereby eliminating the need for MT induction. Kamunde and
319 MacPhail (2007) found indiscriminate Cu binding in all subcellular fractions of rainbow trout
320 liver suggesting that Cu-binding sites are ubiquitous in this organ. Also, base-line levels of
321 MT in corkwing wrasse may be sufficient to counteract the metal exposure at this site.
322

323 G6PDH levels were significantly lower in RBC of male fish captured at Salvøy. Bagnyukova
324 et al. (2006) report a decrease in G6PDH in goldfish exposed to iron. This decrease was
325 negatively correlated to protein carbonyl levels and the authors suggest that this enzyme may
326 be inactivated due to oxidative damaged. The indication that wrasse from the copper mine site
327 might be suffering from oxidative stress, as is evident in the measured increase in oxidative
328 damage to protein molecules, was not paralleled in results from the other measurements; GR,
329 DTD, GST, tGSH, GSSG, metHb and SOD were unaffected. The fact that few of the
330 antioxidant defense mechanisms were increased at the site near the copper mine indicates that
331 these heavy metals may not have been able to induce antioxidant defenses or that fish may
332 have acclimated to the exposure conditions in the field and become more tolerant to acute
333 challenges of metal exposure, via regulation of uptake, detoxification, storage or excretion of
334 metal ions (Kamunde and MacPhail 2007, Rainbow 2007). However, metal exposure that
335 exceeds detoxification capacity may lead to toxicity (Hamilton and Mehrle 1986), i.e.

336 increases in ROS and accumulation of damaged protein molecules. Based on the data found in
337 the current field study, it becomes more evident that oxidative damage products, and not just
338 antioxidant defenses, are of importance to measure in order to determine effects on oxidative
339 stress parameters.

340

341 SSI was lower in both male and female wrasse caught outside the copper mine, results that are
342 mirrored in the higher hematocrit levels measured in these same fish, though this increase was
343 only significant in males. This indicates that wrasse living in this environment were under a
344 certain degree of stress, as release of red blood cells from the spleen is an adaptive response.
345 Cyriac et al. (1989) showed that fish acutely exposed to copper showed an increase in both
346 hematocrit as well as hemoglobin content in blood, possibly due to changes in blood
347 parameters which result in erythrocyte swelling, or by release of large red blood cells from the
348 spleen. GSI was significantly lower in male fish captured at Visnes although this parameter
349 was not altered in female fish. Levesque et al. (2003) found that yellow-perch from metal-
350 contaminated lakes had lower GSI values and that they possessed gonads at less mature stages
351 compared to those from reference stations. Similar results were obtained by Sepúlveda et al.
352 (2002) in a study conducted on largemouth bass. Chronic exposure to metals could alter the
353 physiological functions of fish and delay reproduction.

354

355 4.2 The PAH site

356

357 PAHs are persistent chemicals and many studies have been conducted on their effects on
358 marine animals (van der Oost, et al. 2003). The induction of cytochrome P450, a phase I
359 detoxification enzyme, and its catalytic activity (EROD) have been used as biomarkers of
360 PAH exposure for many years and are known to be sensitive to PAH exposure (Goksøyr and

361 Förlin 1992). An increase in EROD activity was measured at the PAH-contaminated site,
362 Høgvarde, with no differences between the two sexes. EROD induction seen in this study
363 was relatively low, approximately 2-fold. Though there are large amounts of PAHs in the
364 sediment at Høgvarde, water flow is quite high in the strait and this may result in low
365 waterborne PAH exposure for the fish. On the other hand, the fish would also be exposed to
366 PAHs via their diet, as is evident in PAH levels in crab and mussel tissues (Andersen et al.
367 2003b). The somewhat modest elevated EROD activity at the PAH site compared to the
368 reference site might indicate that fish at the PAH site have acclimated to the chronic PAH
369 exposure, as has been demonstrated in brown bullhead (McFarland et al., 1999) and
370 mummichog (Weis 2002).

371

372 Little work has been done on how PAHs affect protein oxidation in fish as most studies have
373 focused on lipid peroxidation as a biomarker for oxidative damage (van der Oost, et al. 2003).
374 A previous study showed increased levels of protein carbonyls in eelpout (*Zoarces viviparus*)
375 exposed to bunker oil containing high levels of PAHs, both in the field following an oil spill
376 and in a laboratory exposure study (Carney Almroth, et al. 2005). In the current study, protein
377 carbonyl levels were not elevated at the PAH site compared to the reference site. The low
378 EROD induction measured in these fish may indicate a low level of PAH exposure which
379 may not have been sufficient to induce an accumulation of protein carbonyls. On the other
380 hand, the fish may have acclimated to the chronic PAH exposure, resulting in mechanisms
381 preventing accumulation of protein carbonyls, for example an increase in activity of the 20S
382 proteasome (Grune et al., 2001). A comparison between male and female wrasse at Høgvarde
383 revealed higher levels of protein carbonyls in the plasma of female wrasse, possibly due to
384 differences in the plasma protein profile. Future work needs to be done in fish to ascertain

385 which proteins are most susceptible to protein carbonylation, the mechanisms by which this
386 damage occurs, and whether baseline variations caused by i.e. season, sex, nutrition, exist.
387

388 We saw a significant decrease in amounts of tGSH at Høgvarde. The GSSG/tGSH ratio
389 remained unchanged as GSSG also decreased, though not significantly. The activity of GR
390 and GST did not differ between the PAH and control site, thereby indicating that the decrease
391 in tGSH may result from reactions other than phase II conjugation, i.e. reduction of lipid
392 peroxides. The method used to measure GST in the current paper measures several isoforms
393 so it is not possible to say whether another isoform may be responsible for the observed
394 decrease in tGSH. This decrease in tGSH could also be due to the action of ATP-dependent
395 efflux pumps that export GSSG and glutathione-conjugates from liver cells during oxidative
396 stress (Keppler 1999). We also found a significant negative correlation between tGSH and
397 weight. These results may reflect changes in synthesis of new glutathione molecules as
398 transcription of these genes can be affected by environmental factors as well as age (Lu 1999,
399 Carney Almroth et al., 2008).

400

401 MetHb levels were significantly lower in the red blood cells of male wrasse captured at
402 Høgvarde compared to those captured at Bokn. This indicates a decreased need for the
403 reparatory activity of the enzyme and therefore a decrease in oxidation of hemoglobin
404 molecules in red blood cells. CF in fish caught at Høgvarde was significantly higher, which
405 probably reflects differences in food availability and nutritional content between sites rather
406 that an effect of PAH exposure.

407

408 **5. Conclusions**

409

410 Results from the current study show that protein carbonyl levels serve as a good biomarker
411 for oxidative perturbations in corkwing wrasse, especially in cases of heavy metal
412 contamination. However, more studies are needed to confirm the mechanisms through which
413 this damage occurs for specific pollutants and if these mechanisms differ between male and
414 female fish. Knowledge concerning which proteins are most susceptible in different exposure
415 situations is also needed. Further studies should investigate toxicological relevance of protein
416 carbonyl accumulation in different species of fish.

417

418 The other parameters measured here, those involved in detoxification and antioxidant
419 functions, were shown to be only lightly affected or unaffected in fish captured at both the
420 heavy metal and PAH contaminated sites. Antioxidant defences do not appear to provide a
421 good biomarker for chronic exposure in corkwing wrasse and other biomarkers should also be
422 measured.

423

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425

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430 statistical analyses.

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607
608

609 Figure legends.

610

611 **Figure 1:** Map of Norwegian west coast, showing site locations. Site 1 = Salvøy, a reference
612 site exposed to the open ocean, Site 2 = Visnes, located outside a copper mine, Site 3 =
613 Høgvarde, a PAH contaminated site, Site 4 = Bokn, a reference site located in a strait.

614

615 **Table 1:** Levels of biometric parameters calculated in corkwing wrasse captured at a heavy
616 metal contaminated site (Visnes) and reference site (Salvøy). Statistical differences between
617 sites are shown in bold. * represents statistically significant difference, $p < 0.05$, between males
618 (M) and females (F) at the same site.

619

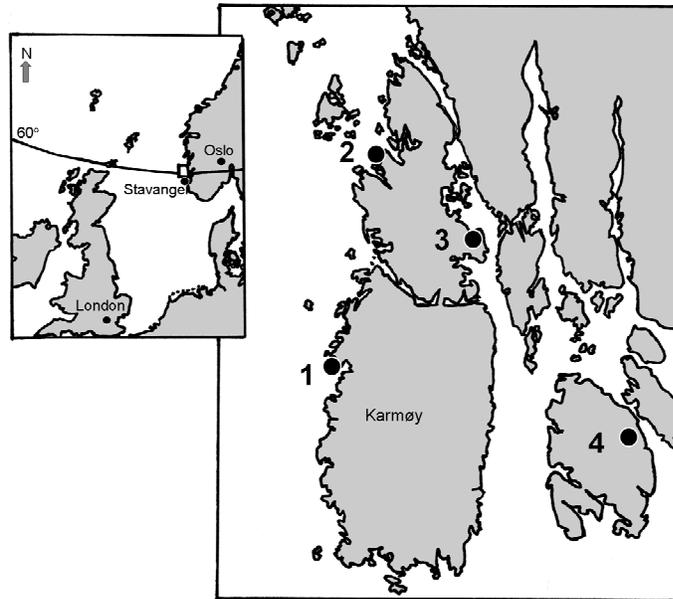
620 **Table 2:** Activities or levels of various liver and red blood cell (rbc) detoxification and
621 antioxidant defenses measured in corkwing wrasse captured at a heavy metal contaminated
622 site (Visnes) and a reference site (Salvøy). Statistical differences between sites are shown in
623 bold. * represents statistically significant differences, $p < 0.05$, between males (M) and
624 females (F). EROD= ethoxyresorufin-O-deethylase, GST= glutathione-S-transferase, GR=
625 glutathione reductase, tGSH= total glutathione, GSSG = oxidized glutathione, DTD= DT-
626 diaphorase, SOD= superoxide dismutase, MT= metallothionein, metHb= methemoglobin
627 reductase, G6PDH= glucose-6-phosphate dehydrogenase.

628

629 **Table 3:** Levels of biometric parameters calculated in corkwing wrasse captured at a PAH
630 contaminated site (Høgvarde) and reference site (Bokn). Statistical differences between sites
631 are shown in bold. * represents statistically significant difference, $p < 0.05$, between males (M)
632 and females (F). Only male fish were available at Bokn.

633

634 **Table 4:** Activities or levels of various liver and red blood cell (rbc) detoxification and
635 antioxidant defenses measured in corkwing wrasse captured at a PAH contaminated site
636 (Høgvarde) and a reference site (Bokn). Statistical differences between sites are shown in
637 bold. * represents statistically significant differences, $p < 0.05$, between males (M) and
638 females (F). Only male fish were available at Bokn. See Table 2 legend for abbreviations.
639



640
641 Fig.1

642

Heavy metal site				
		Salvøy	Visnes	p values
LSI	M	2.39 ± 0.28	1.93 ± 0.21	0.074
(liver weight/body weight) x100	F	1.61 ± 0.15 *	1.87 ± 0.07	0.109
GSI	M	0.19 ± 0.05	0.10 ± 0.01	0.009
(gonad weight/body weight) x100	F	1.079 ± 0.08	0.93 ± 0.03	0.053
CF	M	1.52 ± 0.04	1.59 ± 0.04	0.237
(weight g/(length cm) ³) x100	F	1.58 ± 0.03	1.63 ± 0.03	0.317
SSI	M	0.13 ± 0.01	0.07 ± 0.01	0.0001
(spleen weight/body weight) x100	F	0.10 ± 0.01 *	0.06 ± 0.01	0.019
Hematocrit	M	28.11 ± 0.88	34.44 ± 1.29	0.001
(%)	F	30.71 ± 1.86	32.50 ± 1.81	0.504

643

644 Table 1

645

Heavy metal site		Salvøy	Visnes	p value
EROD (liver)	M	0.021 ± 0.002	0.029 ± 0.003	0.056
	F	0.019 ± 0.002	0.025 ± 0.004	0.307
(nmol mg ⁻¹ min ⁻¹)				
GST (liver)	M	0.860 ± 0.022	0.844 ± 0.037	0.065
	F	0.714 ± 0.074	0.801 ± 0.033	0.233
(μmol mg ⁻¹ min ⁻¹)				
GR (liver)	M	28.35 ± 1.93	20.82 ± 3.28	0.070
	F	28.69 ± 3.73	31.72 ± 1.79	0.446
(nmol mg ⁻¹ min ⁻¹)				
tGSH (liver)	M	2333.7 ± 137.3	2109.6 ± 104.0	0.216
	F	2083.7 ± 71.2	2158.8 ± 56.4	0.414
(nmol g liver ⁻¹)				
GSSG (liver)	M	58.77 ± 5.85	58.01 ± 4.56	0.998
	F	63.92 ± 6.11	60.70 ± 7.67	0.574
(nmol g liver ⁻¹)				
%GSSG (liver)	M	2.55 ± 0.24	2.81 ± 0.27	0.480
	F	3.07 ± 0.28	2.77 ± 0.30	0.479
(%, ratio GSSG/tGSH)				
Catalase (liver)	M	280.10 ± 30.91	291.93 ± 31.78	0.526
	F	289.08 ± 23.50	288.18 ± 26.54	0.981
(nmol mg ⁻¹ min ⁻¹)				
DTD (liver)	M	13.57 ± 1.22	16.49 ± 1.64	0.167
	F	12.38 ± 1.90	18.18 ± 3.11	0.156
(nmol mg ⁻¹ min ⁻¹)				
SOD (liver)	M	94.71 ± 11.01	93.56 ± 19.74	0.470
	F	65.67 ± 8.73	78.40 ± 7.60	0.238
(ng mg ⁻¹)				
MT (liver)	M	5.12 ± 0.24	5.83 ± 0.31	0.210
	F	4.63 ± 0.28	5.63 ± 0.38	0.693
(μg mg prot ⁻¹)				
SOD (rbc)	M	388.38 ± 45.21	419.32 ± 34.57	0.593
	F	376.92 ± 33.41 *	381.52 ± 61.78	0.949
(ng mg ⁻¹)				
metHb (rbc)	M	252.97 ± 8.63	248.00 ± 9.79	0.708
	F	238.32 ± 18.18	225.78 ± 6.35	0.526
(D abs mg ⁻¹)				
G6PDH (rbc)	M	125.72 ± 4.77	97.96 ± 7.41	0.004
	F	84.14 ± 6.86 *	87.21 ± 6.75	0.709
(D abs mg ⁻¹)				
Protein carbonyls	M	1.59 ± 0.17	3.55 ± 0.43	0.001
	F	2.24 ± 0.44	3.34 ± 0.14	0.026
(plasma) (nmol/mg prot)				

646

647

Table 2

648

PAH site				
		Bokn	Høgvarde	p values
LSI	M	1.40 ± 0.09	1.60 ± 0.09	0.118
(liver weight/body weight) x100	F		1.46 ± 0.11	
GSI	M	0.13 ± 0.02	0.23 ± 0.10	0.132
(gonad weight/body weight) x100	F		1.05 ± 0.05	
CF	M	1.47 ± 0.05	1.60 ± 0.03	0.034
(weight g/(length cm) ³) x100	F		1.50 ± 0.05	
SSI	M	0.09 ± 0.01	0.07 ± 0.01	0.200
(spleen weight/body weight) x100	F		0.06 ± 0.01	
Hematocrit	M	30.56 ± 1.00	27.67 ± 1.79	0.178
(%)	F		30.00 ± 2.22	

649

650 Table 3

651

PAH site		Bokn	Høgvarde	p value
EROD (liver) (nmol mg ⁻¹ min ⁻¹)	M	0.013 ± 0.002	0.029 ± 0.006	0.008
	F		0.024 ± 0.009	
GST (liver) (μmol mg ⁻¹ min ⁻¹)	M	0.898 ± 0.043	0.842 ± 0.038	0.371
	F		0.870 ± 0.079	
GR (liver) (nmol mg ⁻¹ min ⁻¹)	M	30.33 ± 1.61	28.35 ± 1.51	0.382
	F		24.57 ± 3.88	
tGSH (liver) (nmol g liver ⁻¹)	M	2530.4 ± 172.0	1855.1 ± 209.2	0.023
	F		2099.9 ± 233.0	
GSSG (liver) (nmol g liver ⁻¹)	M	61.81 ± 4.57	47.86 ± 9.70	0.056
	F		48.97 ± 6.25	
%GSSG (liver) (%, ratio GSSG/tGSH)	M	2.55 ± 0.26	2.53 ± 0.29	0.954
	F		2.38 ± 0.27	
Catalase (liver) (nmol mg ⁻¹ min ⁻¹)	M	376.11 ± 26.04	349.75 ± 37.77	0.293
	F		270.77 ± 22.77	
DTD (liver) (nmol mg ⁻¹ min ⁻¹)	M	14.07 ± 2.00	14.84 ± 1.68	0.823
	F		8.39 ± 3.38	
SOD (liver) (ng mg ⁻¹)	M	134.23 ± 11.50	147.11 ± 20.22	0.918
	F		105.40 ± 25.70	
MT (liver) (μg mg prot ⁻¹)	M	6.15 ± 0.60	5.52 ± 0.32	0.345
	F		5.37 ± 0.45	
SOD (rbc) (ng mg ⁻¹)	M	328.56 ± 39.13	330.42 ± 51.40	0.977
	F		406.99 ± 47.98	
metHb (rbc) (D abs mg ⁻¹)	M	248.35 ± 12.63	192.17 ± 10.35	0.003
	F		222.35 ± 10.71	
G6PDH (rbc) (D abs mg ⁻¹)	M	106.16 ± 13.67	85.21 ± 5.95	0.208
	F		88.57 ± 8.62	
Protein carbonyls (plasma) (nmol/mg prot)	M	2.02 ± 0.14	2.39 ± 0.22	0.207
	F		3.39 ± 0.35*	

652

653

654

Table 4