1 Validation of a Suite of Biomarkers of Fish Health in the Tropical Bioindicator Species,

2 Tambaqui (*Colossoma macropomum*)

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29   Abstract

30   Here we explore the dose-dependent response of the tropical fish tambaqui (*Colossoma*

31   *macropomum*) to intraperitoneal injection of benzo[a]pyrene (BaP) at doses of 0 (carrier

32   control), 1, 10, 100 and 1000 µmolar BaP Kg^{-1} Hepatic ethoxyresorufin-*O*-deethylase

33   (EROD) activity showed a bell-shaped dose-dependent response curve, where the highest

34   injected BaP dose caused enzyme inactivation. Activities of hepatic catalase (CAT) and

35   superoxide dismutase (SOD) increased at the highest dose relative to the carrier control

36   group. Lipid peroxidation (LPO), serum-sorbitol dehydrogenase (s-SDH) and DNA damage

37   in blood cells were higher for all BaP doses when compared to the carrier control group. At

38   high dosage, the production of BaP metabolites was paralleled by induced activity of the

39   antioxidant enzyme SOD, and high levels of DNA damage in blood cells. In a similar way,

40   high LPO was concomitant to elevated s-SDH in the bloodstream, suggesting that lipid

41   peroxidation caused the loss of membrane integrity and leakage of s-SDH from hepatocytes

42   into the bloodstream. These biomarkers were also positively co-correlated. The results

43   demonstrate the potential use of a suite of biomarkers for tambaqui living in contaminated

44   tropical aquatic environments. In particular, we recommend the analysis of DNA damage in

45   blood cells, as this was highly correlated with all other biomarkers.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are of major concern in contaminated aquatic environments (Abdel-Shafy and Mansour, 2015; Manoli and Samara, 1999), particularly tropical environments which have received urban and industrial contamination due to uncontrolled population growth and rapid industrialization (United Nations, 1987). PAHs are highly lipophilic and tend to adsorb to suspended particulate material or accumulate in sediment, increasing bioavailability to fish inhabiting contaminated environments (Hylland, 2006; Paine et al., 1996). While many studies have identified biomarker tools in an array of bioindicator species in well-developed countries from temperate regions (Almeida et al., 2012; Dû-Lacoste et al., 2013), very few studies are available for tropical environments. There is a scarcity of recognized bioindicator species for tropical ecosystems and, consequently, few biomarkers have been validated to study the impacts of PAH contamination in organisms living in these environments.

Among PAHs, benzo[a]pyrene (BaP) is the most extensively studied compound and has been used as a model contaminant for ecotoxicological studies (Almeida et al., 2012). This compound is known for its carcinogenic and mutagenic effects. However, these effects only become apparent after its metabolization, in a process called bioactivation. Epoxide, diol epoxide and hydro metabolites are primary and secondary metabolites generated after BaP oxidation by cytochrome P450, and monooxygenases are formed in Phase I of the biotransformation processes (Miller and Ramos, 2001; Shimada and Fujii-Kuriyama, 2004; Shimada, 2006). One way of measuring the catalytic activity of cytochrome P450 is via ethoxyresorufin-O-deethylase (EROD) activity. Several authors have reported the induction of hepatic EROD activity in fish acutely exposed to different types of contaminants, such as

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74 the soluble fraction of diesel fuel (Simonato et al., 2011), crude oil (Ramachandran et al.,

75 2004) and chemically dispersed crude oil (Ramachandran et al., 2006). Several field studies

76 have reported increased activity of hepatic EROD in fish collected from recently

77 contaminated areas (Jung et al., 2012; Martínez-Gómez et al., 2006) or that have a history

78 of contamination by oil and oil products (Buet et al., 2006; Dévier et al., 2013; Gagnon and

79 Holdway, 2002; Trisciani et al., 2011).

80 Phase II of biotransformation occurs via conjugation reactions of the BaP primary and

81 secondary metabolites with polar molecules, such as glutathione, facilitating excretion and

82 elimination of the final product (Timbrell, 2004). Glutathione S-transferase (GST) plays a

83 fundamental role in Phase II of biotransformation, participating in conjugation reactions of

84 the xenobiotic with the exogenous tripeptide reduced glutathione (GSH) and resulting in the

85 efficient elimination of the metabolites from the organism (Rinaldi et al., 2002). GST has

86 been considered a reliable biomarker for a variety of fish species exposed to PAHs in both

87 laboratory studies (Simonato et al., 2011) and field investigations (Tim-Tim et al., 2009).

88 Glutathione peroxidase (GPx) is another antioxidant enzyme that catalysis the metabolism

89 of oxidizing compounds, a process which involves the oxidation of reduced GSH to its

90 oxidized form (van der Oost et al., 2003). GPx is also involved in reducing lipid

91 hydroxyperoxides to their corresponding alcohols, making them more water soluble as well

92 as reducing free H_2O_2 to water (van der Oost et al., 2003).

93 A continuous production of reactive oxygen species (ROS) is an inevitable result of

94 NADPH consumption by the cytochrome P450 during the metabolism of BaP

95 (Wiernsperger, 2003). ROS, such as OH, peroxyl and hydroxyl radicals, can oxidize

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96   essential biological molecules such as lipids, proteins, carbohydrates and DNA (Halliwell,

97   2011). The interaction of these free radicals with biomembranes disturbs the membranes'

98   delicate structure, integrity, fluidity and permeability, as well as resulting in a loss of

99   functionality as a consequence of lipid peroxidation (LPO) (Niki, 2009; Wiernsperger,

100 2003). Damage to the lipid bilayer, i.e. lipoperoxidation, is considered a significant cause of

101 cell injury and death following exposure to contaminants (Modesto and Martinez, 2010).

102 Antioxidant enzymes play an important role in neutralizing the effects of ROS molecules in

103 biological membranes. For example, superoxide dismutase (SOD), the first enzyme in the

104 line of antioxidant defense, acts in neutralizing the superoxide radicals, generating

105 hydrogen peroxide which, in turn, is neutralized by catalase (CAT).

106 The measurement of biomarkers of exposure, such as PAH biliary metabolite levels,

107 activity of the enzymes involved in the biotransformation of contaminants (EROD and

108 GST), and neutralization of ROS (SOD, CAT and GPx), along with the analysis of the

109 failures of these systems, such as oxidation of lipids and carbohydrates (which can be

110 measured via LPO), and the oxidation of proteins, such as DNA (which can be measure by

111 DNA damage), are commonly used as biomarkers of water contamination (van der Oost et

112 al., 2003). The responses of these biomarkers can be species-specific and vary depending

113 on the type of contaminant, the dose and the time of exposure. Thus, the validation of

114 biomarkers in a relevant bioindicator species improves the understanding of toxic effects of

115 PAHs on aquatic organisms.

116 Considering the widespread contamination of tropical aquatic environments by petroleum

117 hydrocarbons and the rarity of bioindicator species relevant to tropical waterbodies, this

118 study aims to (i) validate the dose-dependent responses of the fish tambaqui (*Colossoma*

119 *macropomum*) to varying intraperitoneal doses of BaP; and (ii) quantify biomarker

120 responses to select the most relevant suite of biomarkers for monitoring tropical aquatic

121 environments contaminated with PAHs. The tambaqui was chosen as model fish species

122 due its high economic and scientific importance in tropical regions including South

123 America and Asia (Campos-baca and Kohler, 2005; Food and Agriculture Organization of

124 the United Nations - FAO, 2016; Liao et al., 2001; Val and Almeida-Val, 1995; Val et al.,

125 2005), its widespread availability from aquaculture ventures, and because of its resistance

126 to environmental challenges (Val and Almeida-Val, 1995; Val et al., 2005).

127 2. Material and Methods

128 *Fish and experimental protocol*

129 All procedures followed the Brazilian Animal Care Guidelines and were approved by

130 INPA's Animal Care Committee (Protocol number: 022/2012). Juvenile tambaqui

131 (Teleostei, Characiformes, Serrasalmidae) were used to avoid the confounding effect of

132 reproduction on some biomarkers e.g. EROD activity (Goksayr and Firlin, 1992). Juvenile

133 tambaqui are classified as juvenile (4-6cm); advanced juvenile (6-8cm); juvenile II (8-

134 10cm); and advanced juvenile II (10-12 cm) (Gomes et al. 2003). Our experiment used the

135 latest stages of advanced juvenile II to provide sufficient biological material for all

136 biomarker analyses. Tambaqui (n= 75; weight: 50.73 ± 2.12g and length: 13.32 ± 0.21 cm;

137 mean $\pm$ SD), were purchased from a local fish farm (Fazenda Santo Antônio, Amazonas,

138 Brasil) and transported to LEEM/INPA (Instituto Nacional de Pesquisas da Amazônia) in

139 purpose-designed fish transport bags filled with the water from the fish farm and supplied

140 with pure oxygen. The bags were kept in styrofoam boxes to reduce temperature variation

141   and to maintain a dark environment, to minimise transport stress. Fish were stepwise

142   acclimated to laboratory tap water and were then kept in the laboratory for at least 60 days

143   before the experiments to ensure full acclimation to laboratory conditions. No fish died

144   during the transport and the acclimation period. Fish were kept outdoors for at least 60 days

145   in 3000-L polyethylene tanks with INPA's ground water, continuously aerated (average

146   composition: Na^+: 43µmol L^{-1}; Cl^-: 31µmol L^{-1}; K^+: 10 µmol L^{-1}; Ca^{2+}: 9µmol L^{-1}; Mg^{2+}:

147   4µmol L^{-1}; pH 6; 28°C; 6.40 mg of dissolved O_2 L^{-1} and temperature of 29°C) with a flow-

148   through rate of 1200 mL min^{-1}. Fish were fed with dry food pellets (26% protein content,

149   Nutripeixe, Purina®) twice a day and feeding was suspended 2 days prior to experiments.

150   Fish were anesthetized in aerated water containing neutral MS-222 (0.5 g L^{-1} MS-222 and 1

151   g L^{-1} NaHCO$_3$, Sigma Aldrich), before being given an intraperitoneal (IP) injection of one

152   of five BaP concentrations; 0 (carrier controls), 1, 10, 100 or 1000 µmolar BaP Kg^{-1}.

153   Injection solutions were freshly prepared and each fish received the same volume of carrier

154   solution (0.5 mL of corn oil, Sigma Aldrich). After injection, fish were held in 2000 L

155   polyethylene tanks, in the same water with continuous aeration for 96 hours. Thereafter,

156   blood samples were drawn from the caudal vein of fish anesthetized in neutral buffered

157   MS-222 (1 g L^{-1} MS-222 and 2 g L^{-1} NaHCO$_3$, Sigma Aldrich). Fish were then killed by

158   severing the spine, measured, weighed and the spleen and liver removed and stored at -

159   80°C until analysis.

160   *Chemicals*

161   All chemicals were purchased from Sigma Aldrich Brazil: Corn oil; tricaine mesylate (MS-

162   222); Benzo [a] pyrene; 1-OH-pyrene; HEPES (2-[4-(2-hydroxyethyl)piperazin-1-

163   yl]ethanesulfonic acid); potassium chloride (KCL); magnesium sulphate (MgSO$_4$);

164   nicotinamide adenine dinucleotide phosphate (NADPH); bovine serum albumin (BSA);

165   ethoxyresorufin; resorufin; methanol; 1-chlore-2,4-dimitrobenzene (CDNB); TRIS-Base

166   (tris(hydroximethyl)aminomethane); dithiothreitol (DTT); sucrose; monopotassium

167   phosphate (KH_2PO_4); dipotassium phosphate (K_2HPO_4); L-glutathione reduced (GSH);

168   hydrogen peroxide (H_2O_2); sodium phosphate monobasic (NAH_2PO_4); xanthine; sodium

169   hydroxide (NaOH); cytochrome c; xanthine oxidase; sodium azide (NaN_3); glutathione

170   reductase (GR); trichloroacetic acid (TCA); xylenol orange; sulfuric acid (H_2SO_4); butyl

171   hydroxyl toluene (BHT); ammonium iron (II) sulfate hexahydrate ((NH_4)$_2$Fe(SO_4)$_2\bullet$

172    $6H2O$); Bradford reagent; fructose; normal melting agarose; low melting point agarose;

173   sodium chloride (NaCl); triton X-100; dimethyl sulfoxide (DMSO); sodium carbonate

174   (Na_2CO_3); ammonia nitrate (NH_4)(NO_3); silver nitrate ($AgNO_3$); silicotungstic acid

175   ($H_4[W_{12}SiO_{40}]$); formaldehyde (H_2CO).

176   *BaP-type metabolites in fish bile*

177   The term 'type of metabolites' is used because the fixed fluorescence technique detects

178   groups of metabolites originating from a common parent compound fluorescing at a

179   common wavelength (Lin et al., 1996). The BaP-type metabolites concentration in fish bile

180   was determined by fixed fluorescence (FF) measurements according to Lin et al. (1996).

181   Bile samples were diluted in 50/50 HPLC-grade methanol-water in the proportion of

182   1:1000 (bile:methanol) and read at EX 380 nm and EM 430 nm. The reference standard for

183   BaP-type metabolites was 1-OH pyrene (also known as 1-pyrenol). Samples were read on a

184   spectrofluorimeter with slit width of 10 nm, against a standard curve. The BaP-type

185   metabolites are reported in µg BaP-type met mg^{-1} protein.

186 *Biotransformation enzymes*

187 Hepatic EROD activity was assessed by the fluorimetric method described by Webb et al.

188 (2005). The post mitochondrial supernatant (PMS) was obtained by homogenizing the liver

189 (1:4 w:v) in HEPES homogenization buffer pH 7.5 (0.02 M HEPES and 0.015 M KCL)

190 before centrifuging at 12,000g for 20 min at 4°C. The PMS (50 µl) was added to the

191 reaction mixture (1250 µl 0.1 M HEPES buffer; 10 µl of 1.28M MgSO4; 30 µl of 0.5 mM

192 NADPH and 50 µl of 40 mg/mL bovine serum albumin-BSA). The reaction started with the

193 addition of 20 µl of 0.12 mM ethoxyresorufin; after 2 min, the addition of 2500 µl of

194 HPLC-grade methanol stopped the reaction. The protein precipitate resulting from the

195 addition of methanol was spun down and the amount of resorufin produced was measured

196 on a spectrofluorimeter at EX wavelength 530 nm and EM wavelength 585 nm. The

197 amount of resorufin was calculated from a resorufin standard curve varying from 0.000 to

198 0.085 M, and readings were relative to a blank fluorescence. EROD activity is expressed as

199 picomol of resorufin produced per milligram protein per minute (pmol mg^{-1} protein min^{-1}).

200 Hepatic GST activity was determined as described by Keen et al. (1976) using 1-chloro-

201 2,4-dinitrobenzene (CDNB) as a substrate. The supernatant was obtained by homogenizing

202 the liver (1:10 w:v) in a cold buffer solution (20 mM Tris-Base, 1 mM EDTA, 1 mM

203 dithiothreitol, 500 mM Sucrose, 150 mM KCL, pH 7.6) and then centrifuging at 9,000g for

204 30 min at 4°C. The homogenates were pipetted (15 µl) into microplate and 235 µl of

205 reaction media (3.8 mL of 0.05 M KH$_2$PO$_4$, 0.05 M K$_2$HPO$_4$, pH 7.0, 25°C plus 80 µl of 50

206 mM CDNB) was added. GSH (10 µl of 25 mM) was added to start the reaction. The change

207 in absorbance was recorded on a spectrophotometer at 340 nm. The enzymatic activity was

208 calculated as nmol CDNB conjugate formed per milligram protein per minute (nmol CDNB

209 mg^{-1} protein min^{-1}) using a molar extinction coefficient of 9.6 mM cm^{-1}.

Oxidative stress enzymes

211 The protocol to obtain the supernatant for measuring CAT, SOD and GPx activities was the

212 same as described for GST supernatant. Hepatic CAT activity was determined as described

213 by Beutler (1975). The homogenates were pipetted (10 µl) into a quartz cuvette and 990 µl

214 of reaction media, consisting of 9 mL of the catalase buffer (1 M Tris-Base, 5 mM EDTA,

215 pH 8.0, 25°C) diluted 1.8 times plus 90 mL of H_2O_2 solution (100 mL distilled water plus

216 100 µl of H_2O_2 30%), was added. The rate of enzymatic decomposition of H_2O_2 was

217 measured in a spectrophotometer at an absorbance of 240 nm. Enzyme activity was

218 expressed in µmol H_2O_2 mg^{-1} protein min^{-1}.

219 Hepatic copper–zinc SOD (SOD) activity was determined according to McCord and

220 Fridovich (1969). The homogenates were pipetted (5 µl) into plastic cuvettes and 1 mL of

221 reaction media was added. The reaction media consisted of 47.5 mL of phosphate buffer

222 (50 mM NaH_2PO_4, 50 mM K_2HPO_4, 0.1 mM EDTA, pH 7.8, 25°C), 2.5 mL of 1 mM

223 xanthine (diluted in 1 mM NaOH) and 20 mM cytochrome c. The reaction was initiated by

224 the addition of 20 µl of 0.2 U mL^{-1} xanthine oxidase. This method measures the inhibition

225 of the reduction rate of cytochrome c by the superoxide radical, read on a

226 spectrophotometer at 550 nm and 25°C. SOD activity was expressed in units of SOD per

227 mg of protein (U mg^{-1} protein), with one U of SOD corresponding to the quantity of

228 enzyme that caused 50% inhibition of the reduction rate of cytochrome c.

229   Hepatic selenium-dependent glutathione peroxidase (GPx) activity was determined by the

230   method of Hopkins and Tudhope (1973). The homogenates were pipetted (10 µl) into

231   quartz cuvettes and 1 mL of reaction media, consisting of 25 mL of the phosphate buffer

232   (100 mM NaH_2PO_4, 100 mM K_2HPO_4, 2 mM EDTA, pH 7.0, 25°C), 0.2 mM NADPH, 10

233   mL of 5 mM NaN_3, 1 mM GSH and 15 mL distilled water) was added. The reaction was

234   initiated by the addition of 20 µl of 1 U mL^{-1} glutathione reductase and 20 µl of 20 mM

235   H_2O_2. This method is based on NADPH oxidation in the presence of GSH and H_2O_2,

236   measured in a spectrophotometer at 340 nm. GPx activity was expressed in µmol oxidized

237   NADP mg^{-1} protein min^{-1} using a molar extinction coefficient of 6.22 mM cm^{-1}.

238   *Lipid peroxidation* (LPO)

239   The LPO concentration in the liver was quantified after Jiang et al. (1991). The

240   homogenates were transferred to plastic tubes and treated with TCA 12% (1:1 v:v) then

241   centrifuged at 5,000g for 10 min at 4°C. The treated homogenates were pipetted (30 µl)

242   into a microplate before 270 µl of reaction media (50 mL methanol 90%, 0.1 mM xylenol

243   orange, 25 mM H_2SO_4, 4 mM BHT, 0.25 mM ferric ammonium sulfate $((NH_4)_2Fe(SO_4)_2•$

244   $6H2O)$ was added. This method is based on the oxidation of the Fe^{+2} to Fe^{+3} by

245   hydroperoxides in an acid medium in the presence of ferrous oxidation-xylenol orange,

246   read at 560 nm. Cumene hydroperoxide (CHP) was used as a standard. LPO concentration

247   was expressed in µmol of CHP mg^{-1} protein.

248   *Protein determination*

249   Liver supernatant, as used for the enzyme analyses, was also used to quantify total protein

250   content according to Bradford (1976) using a spectrophotometer at 595 nm and bovine

251   serum albumin as a standard.

252   *Serum Sorbitol Dehydrogenase (s-SDH) activity*

253   Following blood collection, serum was isolated by centrifuging at 5000 rpm for 10 min.

254   The serum was kept at -80°C until the s-SDH determination, after Webb and Gagnon

255   (2007). 50 µl of serum was placed in 450 µl of 0.1 M Tris buffer pH 7.5 and incubated for

256   10 min at room temperature, after which 100 µl of 4 M fructose solution was added and the

257   linear rate of decrease in absorbance over one minute at 340 nm was measured with a

258   spectrophotometer. The enzymatic activity of the s-SDH is expressed as milli-international

259   Units (mIU) of s-SDH activity.

260   *DNA damage in blood cells (comet assay)*

261   DNA damage in blood cells was assessed via a comet assay (alkali method) as described by

262   Singh et al. (1988) for lymphocytes and modified by Da Silva et al. (2000) for peripheral

263   blood cells. Microscope slides were dipped into 1.5% normal melting agarose (NMA)

264   prepared in phosphate-buffered saline (PBS). Each slide was coated with 1.0% NMA in

265   PBS, and then covered with a coverslip. Subsequently, blood (7-10 µl) mixed with 95 µl of

266   0.75% low melting point agarose (LMA) (Gibco BRL) was spread on the slide and allowed

267   to solidify. After removal of the coverslip, the slides were immersed in freshly prepared

268   cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris; pH 10-10.5; 1% Triton X-

269   100 and 10% DMSO) overnight. Then, the slides were placed in an electrophoresis

270   chamber, filled with freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH

271   12.6) for 20 min at 300 mA and 25 V in an ice bath. After electrophoresis, the slides were

272   washed with 0.4 M Tris buffer, pH 7.5 and submerged in silver solution (5% sodium

273   carbonate; 0.1% ammonia nitrate; 0.1% silver nitrate; 0.25% Silicotungstic acid; and 0.15%

274   formaldehyde). Images of 100 randomly selected cells (50 cells from each of two replicate

275   slides) were analyzed using an optical microscope (Leica DM205) at 100x magnification.

276   Cells were scored into five classes, according to tail size (from undamaged -0 to maximally

277   damaged -4) and a value was assigned to each comet according to its class. The final

278   overall rating for the slide i.e. the DNA damage score (Genetic Damage Index, GDI), was

279   between 0 (intact) and 400 (maximum damage), and was obtained by summation according

280   to Kobayashi et al. (1995).

281   *Statistical Analysis*

282   Data are presented as mean ± standard error of the mean (mean ± SEM). Prior to

283   comparative statistical analyses, data were assessed for normality and homogeneity of

284   variance. A one-way analysis of variance (ANOVA) with a Holm-Sidack post hoc test was

285   applied to verify differences in all analyzed biomarkers between fish treated with different

286   BaP doses (0 (corn oil), 1, 10, 100 and 1000 µMolar Kg^{-1}). When data violated the

287   ANOVA assumptions of normality and homogeneity, a non-parametric Kruskall–Wallis

288   test was used. A Pearson correlation was used to identify interactions between all analyzed

289   biomarkers. Statistical significance was accepted at the level of $p \leq 0.05$.

290   3. Results

291   *BaP-type metabolites in fish bile*

292   BaP-type metabolites in the bile varied from 10 to 1850 µg metabolite mg^{-1} protein.

293   Increases ($p < 0.05$) of 189, 92, 102 and 84 fold were measured in tambaqui IP-injected

294   with 1000 µmolar BaP Kg^{-1} relative to the carrier control group, 1, 10 and 100 µmolar BaP

295   Kg^{-1}, respectively. Moreover, increases ($p < 0.05$) of 2; 1.8; and 2.2 fold, respectively, were

296   observed for tambaqui treated with 1, 10 and 100 µmolar BaP Kg^{-1} relative to the carrier

297   control group (Figure 1).
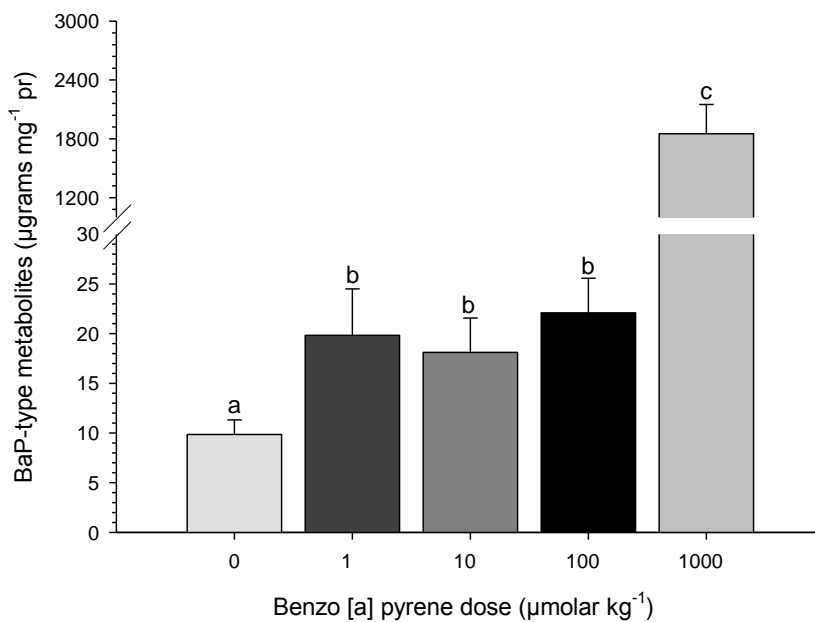
298

299   Figure 1. BaP-type metabolites in bile of tambaqui treated with an intraperitoneal injection

300   of one of five BaP concentrations; 0 (carrier control), 1, 10, 100 and 1000 µmolar BaP Kg^{-1}

301   . Columns represent means and vertical lines represent SEM (n= 15 per treatment).

302   Different letters indicate statistical differences ($p < 0.05$) between doses.

303   *Biotransformation enzymes*

304   Hepatic EROD activity varied from 47 to 75 picomol mg^{-1} protein min^{-1} (Figure 2A).

305   Increases ($p < 0.05$) of 1.6 fold relative to the carrier control group were measured only in

306   tambaqui treated with 100 µmolar BaP Kg^{-1}. The group injected with the highest dose of

307   1000 µmolar BaP Kg^{-1} had similar EROD activity to the carrier control (p = 0.224).

308   Hepatic GST activity varied from 2.9 to 6.2 µmol CDNB mg^{-1} protein min^{-1} (Figure 2B).

309   Increases (p < 0.05) of 1.9 fold over the carrier control group were measured only in

310   tambaqui injected with 1000 µmolar BaP Kg^{-1}.

311
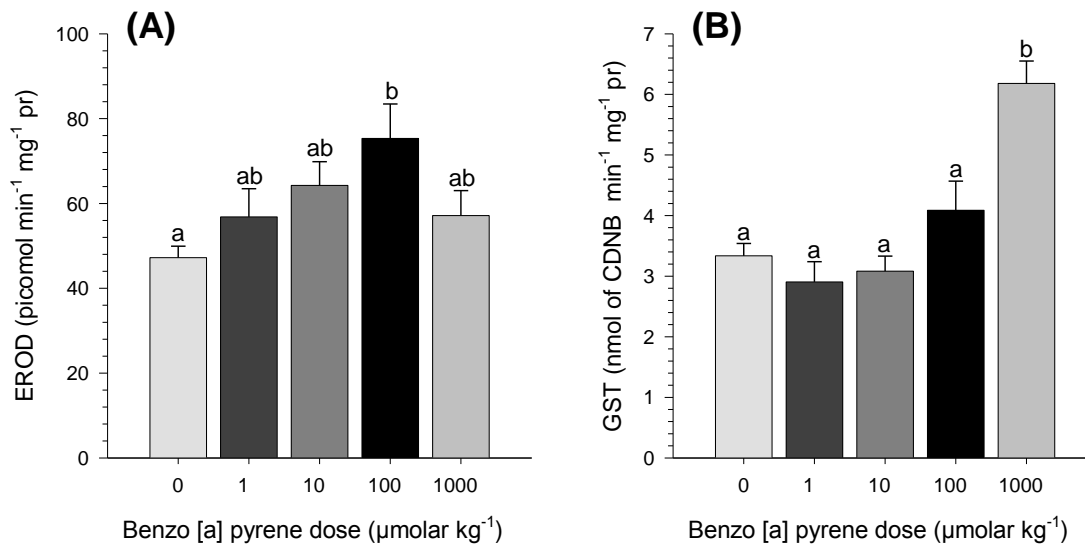
Figure 2. Hepatic **(A)** ethoxyresorufin-*O*-deethylase (EROD) and **(B)** glutathione S-
transferase (GST) of tambaqui treated with an intraperitoneal injection of one of five BaP
concentrations; 0 (carrier control), 1, 10, 100 and 1000 µmolar BaP Kg^{-1}. Columns
represent means and vertical lines represent SEM (n= 15 per treatment). Different letters
indicate statistical differences (p < 0.05) between doses.

Oxidative stress enzymes

318   Hepatic SOD activity varied from 333 to 493 U SOD mg^{-1} protein min^{-1}. Increases (p <

319   0.05) of 1.5 and 1.4 fold were observed only in tambaqui injected with 100 µmolar BaP Kg^{-}

320   ^{1} relative to the carrier control group and to the 10 µmolar BaP Kg^{-1} group, respectively

321   (Figure 3A).

322 Hepatic GPx and CAT activity varied from 4.7 to 6.6 µmol NADPH mg^{-1} protein min^{-1} and

323 28.4 to 47.2 H$_2$O$_2$ mg^{-1} protein min^{-1} respectively. No differences ($p > 0.05$) were observed

324 between the treatment groups for either biomarker (Figures 3 B and C).
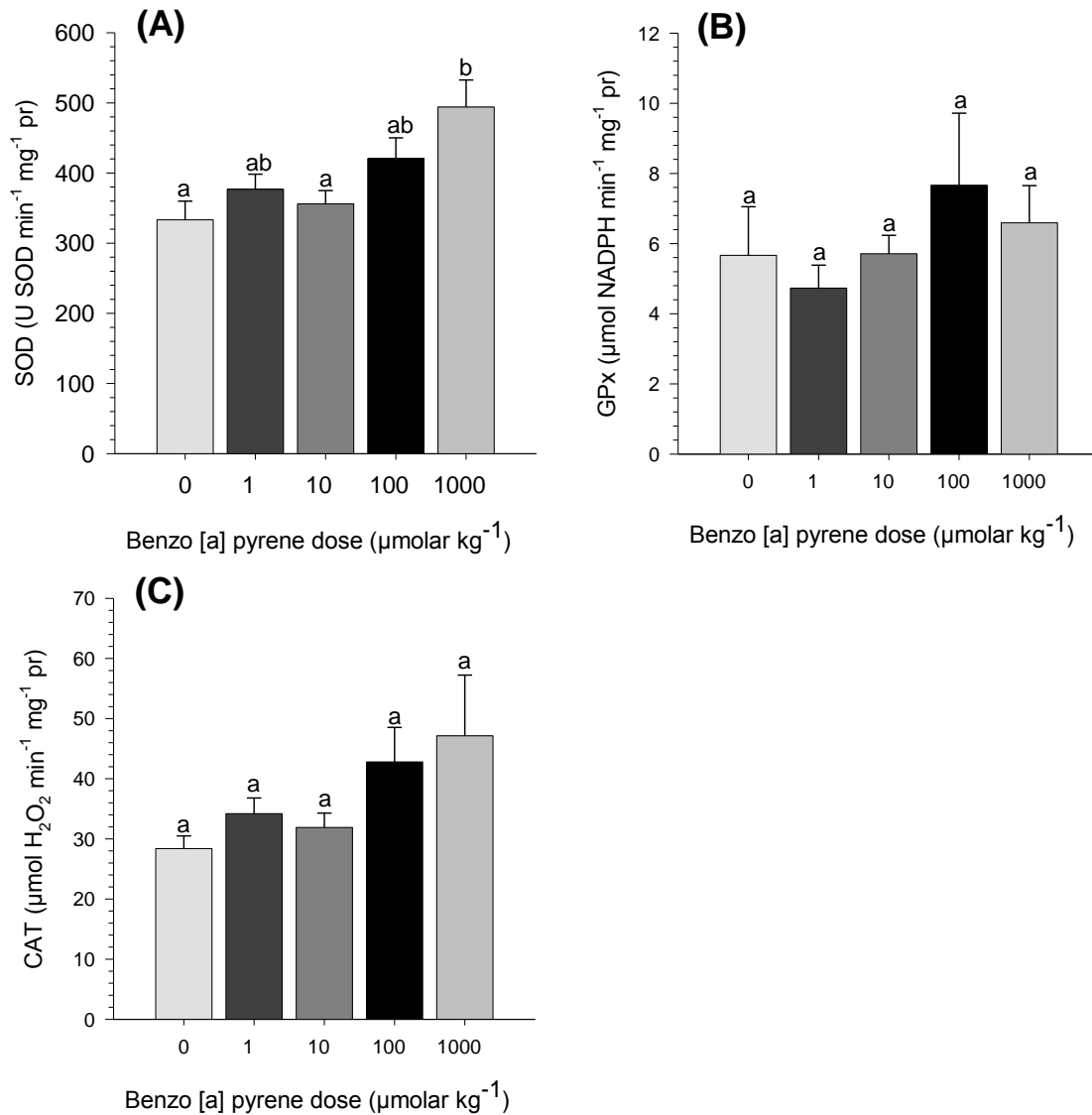
325

326 Figure 3. Hepatic **(A)** superoxide dismutase (SOD), **(B)** glutathione peroxidase (GPx) and

327 **(C)** catalase (CAT) of tambaqui treated with an intraperitoneal injection of one of five BaP

328 concentrations; 0 (carrier control), 1, 10, 100 and 1000 µmolar BaP Kg^{-1}. Columns

329 represent means and vertical lines represent SEM (n= 15 per treatment). Different letters

330 indicate statistical differences ($p < 0.05$) between doses.

331   *Lipid peroxidation (LPO)*

332   LPO concentrations in the liver varied from 566 to 802 µmol of CHP mg^{-1} protein.

333   Increases (p < 0.05) were observed for tambaqui treated with 10, 100 and 1000 µmolar BaP

334   Kg^{-1}, with CHP mg^{-1} pr levels being 1.6, 1.5 and 1.5 fold higher relative to the carrier

335   control group (Figure 4A).

336   *Serum sorbitol dehydrogenase (s-SDH) activity*

337   s-SDH activity in the plasma varied from 34 to 102 mIU and appeared to follow a dose-

338   dependent increase, with progressively higher s-SDH activities with higher BaP doses.

339   Increases (p < 0.05) of 1.2; 2.8 and 3 fold were observed in fish treated with 10, 100 and

340   1000 µmolar BaP Kg^{-1} respectively, relative to the carrier control group (Figure 4B).
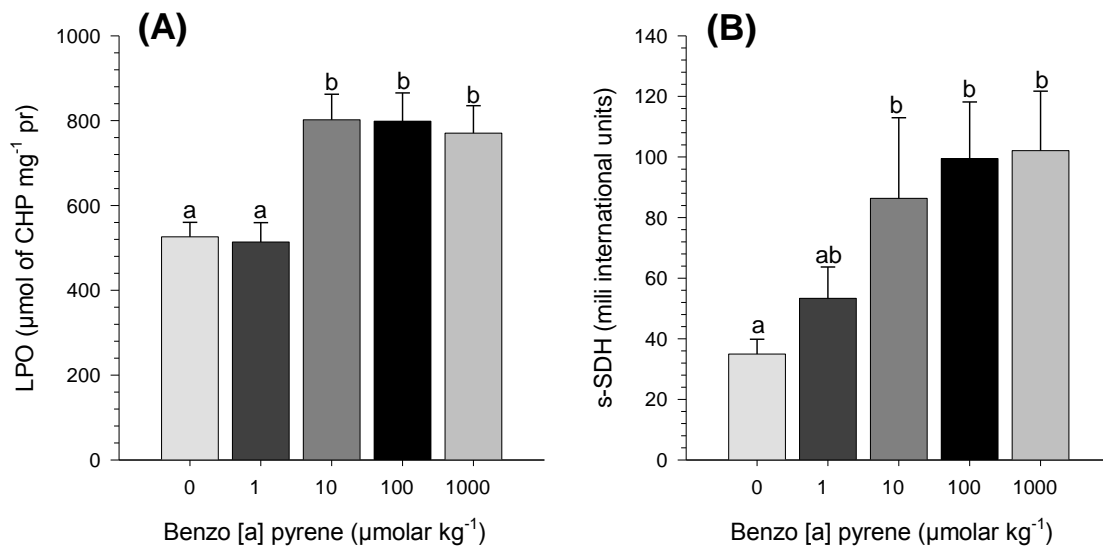

341

342   Figure 4. **(A)** Hepatic lipid peroxidation (LPO) and **(B)** serum sorbitol dehydrogenase (s-

343   SDH) of tambaqui treated with an intraperitoneal injection of one of five BaP

344   concentrations; 0 (carrier control), 1, 10, 100 and 1000 µmolar BaP Kg^{-1}. Columns

345   represent means and vertical lines represent SEM (n= 15 per treatment). Different letters

346   indicate statistical differences (p < 0.05) between doses.

347 *DNA damage in blood cells (comet assay)*

348 DNA damage in blood cells varied from 38 to 300 (comet classes 0-400). Increases (p <

349 0.05) of 5; 5.45; 5.48 and 8 fold, respectively, were observed for tambaqui treated with 1,

350 10, 100 and 1000 µmolar BaP Kg^{-1} relative to the carrier control group (Figure 5).
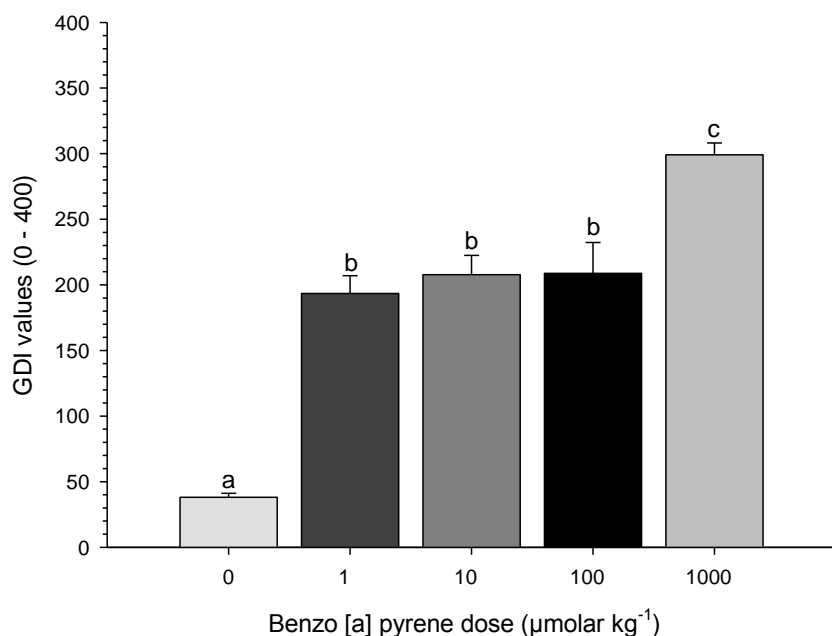
351

352 Figure 5. Genetic Damage Index (GDI) in blood cells of tambaqui treated with an

353 intraperitoneal injection of one of five BaP concentrations; 0 (carrier control), 1, 10, 100

354 and 1000 µmolar Ba Kg^{-1}. Columns represent means and vertical lines represent SEM (n=

355 15 per treatment). Different letters indicate statistical differences (p < 0.05) between doses.

356 *Correlation analysis*

357 Pearson correlations indicated that some biomarkers were positively correlated (p < 0.05).

358 GST was positively correlated with the enzymatic activity of CAT, blood cell DNA damage

359 and biliary BaP-type metabolites. SOD activity was positively correlated with EROD,

360 CAT, GST and s-SDH activity, as well as DNA damage in blood cells. s-SDH and CAT

activity was positively correlated with DNA damage in blood cells, and finally blood cell

DNA damage was positively correlated with BaP-type biliary metabolites (Table 1).

Table 1. Observed Pearson correlations between the biomarkers of tambaqui treated with an intraperitoneal injection of one of five BaP concentrations; 0 (carrier control), 1, 10, 100 and 1000 µmolar BaP Kg^{-1}. Highlighted values represent significant correlations ($p < 0.05$).

	EROD	GST	CAT	SOD	GPx	LPO	s-SDH	DNA damage
GST	-0.094							
CAT	0.147	*0.371*						
SOD	*0.272*	*0.410*	*0.319*					
GPx	0.093	-0.003	-0.072	*0.271*				
LPO	0.167	0.095	0.214	0.148	-0.005			
s-SDH	0.066	0.270	0.097	0.260	-0.002	*0.315*		
DNA damage	0.133	*0.491*	0.272	*0.417*	0.096	*0.464*	*0.387*	
BaP-type metabolites	-0.126	*0.657*	0.175	*0.320*	0.057	0.142	0.190	*0.548*

4. Discussion

This study examined a suite of widely applied aquatic biomarkers (biliary BaP-type metabolites, EROD, GST, CAT, SOD, GPx, LPO, s-SDH and DNA damage in blood cells) in tambaqui, a tropical bioindicator fish species of high economic and scientific importance in South America and Asia (Campos-baca and Kohler, 2005; Food and Agriculture Organization of the United Nations - FAO, 2016; Liao et al., 2001; Val and Almeida-Val, 1995; Val et al., 2005). Intraperitoneal injections of the contaminant BaP were administrated at five different doses to assess if a dose-response can be established in this species 96 h post-injection. Furthermore, a correlation analysis between biomarkers identified which sub-set of biomarkers would be most relevant for environmental monitoring of contaminated aquatic environments.

378   Fish injected with 100 µmolar BaP Kg^{-1} had the highest hepatic EROD activity, indicating

379   that Phase I biotransformation enzymes were activated to metabolize the BaP, as reported

380   by other studies (Almeida et al., 2012; Jönsson et al., 2009; Lu et al., 2009). Fish injected

381   with the highest BaP dose (1000 µmolar BaP Kg^{-1}) presented EROD activity similar to the

382   carrier control group (injected with corn oil). According to Gravato and Santos (2002,

383   2003), the inhibition of the EROD activity following exposure to high BaP doses can be

384   due to increases in the concentration of highly reactive BaP metabolites, similar to our

385   observations here. For some fish species there is a bell-shaped dose-dependent response

386   curve for EROD activity (Bosveld et al., 2002; Lu et al., 2009), where high levels of

387   pollutants cause enzyme inactivation or inhibition. This is an important factor to consider

388   for investigations using EROD activity as a biomarker in this species, and stresses the

389   relevance of coupling other biomarkers such as PAH biliary metabolites and s-SDH activity

390   to measurement of EROD activity.

391   Among treatments, BaP biliary metabolite levels do not follow a similar pattern to the one

392   observed for EROD activity, with the 1000 µmol BaP-treated group producing the highest

393   biliary metabolite levels despite the inhibition of EROD activity at this dose. These results

394   support the concept that EROD is not the sole enzyme responsible for metabolizing BaP. In

395   fact, a myriad of P450 isoenzymes is known to be responsible for the biotransformation of a

396   variety of xenobiotic compounds in fish (van der Oost et al., 2003). Despite different

397   responses at higher BaP doses, the detection of PAHs in fish bile is an extremely sensitive

398   measurement which reflects the absorption and metabolism of these compounds by fish

399   (Aas et al., 2000; Nongnutch et al., 2012). In fact, PAH metablites can be measured in fish

400   bile at levels 1000-times higher than in the surrounding water (Gagnon and Holdway,

401   1998). When injected IP, as in the present study, the biotransformation of BaP is still an

402   extremely efficient process in fish, as demonstrated by all fish exhibiting higher BaP biliary

403   metabolite levels relative to the carrier control fish after 96 h of the injection. These

404   findings are in accordance with other studies which found high levels of PAHs biliary

405   metabolites, including BaP metabolites, in fish experimentally exposed to these substances

406   (Dû-Lacoste et al., 2013; Nongnutch et al., 2012; Telli-Karakoç et al., 2002) or collected in

407   contaminated areas (Neves et al., 2007; Ribeiro et al., 2013; Ruddock et al., 2002; Trisciani

408   et al., 2011). Moreover, the highest IP BaP dose (1000 µmolar Kg^{-1}) was statistically higher

409   than all the other doses which indicate that metabolization activity was not inhibited by the

410   high dose of BaP injected.

411   GST has an important role in Phase II of biotransformation, since this enzyme, together

412   with GSH, acts in conjugation with exogenous compounds derived or not from Phase I

413   biotransformation. It has been suggested that an increase in GST activity is indicative of

414   efficient removal of xenobiotics from the body (Rinaldi et al., 2002). In the present study,

415   the inactivation of the hepatic EROD activity in the highest BaP dose seems to be

416   compensated by increases in GST activity. Other studies also related the GST activity with

417   the metabolism of BaP (Banni et al., 2009; Gravato and Guilhermino, 2009; Gravato and

418   Santos, 2003; Vieira et al., 2008) while some authors suggest that the GST has no role in

419   BaP metabolism by fish (Beyer et al., 1997; Collier and Varanasi, 1990; van Schanke et al.,

420   2001; Willett et al., 2000). In the present study, a positive correlation (Table 1) was

421   observed between GST activity and levels of biliary BaP-type metabolites, suggesting the

422   potential involvement of this enzyme in the BaP metabolism in tambaqui, especially when

423   fish were subjected to high BaP doses.

424   Metabolization processes can lead to generation of reactive oxygen species (ROS). These

425   ROS have been considered a biochemical challenge for fish exposed to PAHs (Regoli et al.,

426   2011). The antioxidant system consists of enzymes such as SOD, CAT, and GPx, which are

427   able to neutralize the ROS. SOD acts by neutralizing superoxide radicals, generating

428   hydrogen peroxide molecules, which can be further neutralized by CAT. In the present

429   study, both SOD and CAT enzymes were measured at higher levels at the highest BaP dose

430   (1000 µmolar Kg^{-1}) which, together with the GST activity, are induced to metabolize the

431   high IP dose of BaP. Positive correlations occurred between SOD and CAT and between

432   GST and both enzymes (Table 1), suggesting their co-involvement in BaP metabolism.

433   SOD was also positively correlated with biliary BaP-type metabolites, which further

434   suggests that this enzyme contributes to ROS neutralization in tambaqui exposed to PAHs.

435   While GPx activity is also important in ROS elimination and neutralization of peroxides, its

436   activity was not statistically altered by BaP injection in the present study. However, a

437   positive correlation was found between GPx and SOD (Table 1). In fish, enzymatic

438   responses following exposure to organic xenobiotics may have no, little or significant

439   change, with different patterns of responses between the enzymes, contaminant types, and

440   fish species (Lemaire et al., 1996; Livingstone et al., 1993). Here, the combined action of

441   these antioxidant enzymes resulted in metabolism of BaP at all injected doses.

442   The imbalance between the ROS generated and the antioxidant enzymes activity can lead to

443   a ROS overproduction and, as a consequence, may cause oxidative stress at the cellular

444   level (Wiernsperger, 2003). The interaction of ROS with the biological membranes can

445   induce disturbances in the delicate structure, integrity, fluidity and permeability of the cell

446   membrane, as well as loss of functionality through the products of lipid peroxidation (LPO)

447 (Niki, 2009; Wiernsperger, 2003). We observed LPO in tambaqui at most BaP doses (10

448 µmolar BaP Kg^{-1} and higher), suggesting that direct damage of the biological membrane

449 occurred mainly through BaP bioactivation. During BaP metabolism, metabolites such as

450 1,6–quinone and 3,6–quinone, derivates of 1-hydroxybenzo[a]pyrene and 3-

451 hydroxybenzo[a]pyrene, are generated following an increase of SOD at higher doses of

452 BaP. Almeida et al. (2012) found increases of LPO in the liver of *Dicentrarchus labrax,*

453 which also paralleled the increasing of BaP exposure concentrations. Our results support

454 the conclusions of Almeida et al. (2012), who attributed the LPO increases to the oxidative

455 stress experienced by the fish and to the direct action of bioactivated BaP metabolites.

456 Considering that the s-SDH enzyme is a cytoplasmic enzyme, found mainly in hepatocytes

457 (Heath, 1995), its presence in the serum could indicate hepatocellular damage, potentially

458 related to the overproduction of ROS (Webb and Gagnon, 2007). Our results suggest doses

459 of 10, 100 and 1000 µmolar BaP Kg^{-1} caused hepatocellular damage, leading to the release

460 of s-SDH from the cytoplasm of the hepatocyte into the serum. Elevated LPO activity at the

461 three highest doses supports a causative relationship between dysfunctional cellular

462 membranes and elevated s-SDH activity in the serum of tambaqui (Morris and Vosloo,

463 2006). Shailaja and D'Silva (2003) also found hepatocellular damage related to PAHs

464 exposure in the tropical cichlid *Oreochromis mossambicus*, even for fish exposed to the

465 lowest concentration of 0.4µg phenanthrene/g, demonstrating the efficiency of this enzyme

466 for detecting hepatocellular injury. In the present study, a positive correlation (Pearson)

467 between LPO activity and s-SDH activity illustrates the complementary nature of these

468 measurements.

469 BaP bioactivation can also lead to genotoxicity, by leading to the formation of 7,8 D [BaP],

470 which is converted into 7,8 D 9,10-epoxide, a highly carcinogenic and mutagenic

471 xenobiotic (Varanasi et al., 1987). DNA breaks were observed in blood cells of tambaqui at

472 all BaP doses, with the highest dose resulting in the highest levels of DNA damage.

473 Elevated DNA damage in blood cells was paralleled by elevated biliary BaP-type

474 metabolites, suggesting that the bioactivation of the BaP metabolites lead to DNA damage

475 in the blood cells. In addition, positive correlations (Pearson) were found between the DNA

476 damage in blood cells and the BaP-type metabolites (Table 1), which confirms that these

477 two biomarkers co-vary. Dévier et al. (2013) also found positive correlations between

478 biliary PAHs-type metabolites and DNA damage in the blood cells of European seabass

479 *Dicentrarchus labrax* collected in PAHs contaminated sites, further establishing a

480 relationship between PAH exposure and genotoxic damage. Similar to our results, Mu et al.

481 (2012) observed that inhibition of EROD activity was concomitant with DNA damage at

482 high BaP exposure concentrations in medaka (*Oryzias melastigma*). The authors suggested

483 that when EROD activity is inhibited, other CYP1s enzymes may be more likely to produce

484 adduct forming BaP metabolites than CYP1A. Our results are in accordance with this

485 suggestion, considering the inhibition of EROD activity, the high BaP-type metabolite bile

486 accumulation, and the high DNA damage found in the blood cells of fish treated with the

487 highest BaP dose.

488 The suite of biomarkers measured in the present study demonstrates the potential of

489 tambaqui for use as a bioindicator species in tropical environments. Biliary metabolites,

490 GST, SOD, LPO and s-SDH activities, and DNA damage in blood cells were the most

491 sensitive biomarkers in tambaqui treated with BaP. At 1000 μmolar BaP Kg^{-1} by IP

492 injection, inhibition of EROD activity co-occurred with maximum BaP-type biliary

493 metabolites, suggesting the involvement of other CYP1A enzymes in the metabolization of

494 BaP or their inefficiency at higher levels of BaP, since tambaqui showed increased

495 oxidative stress at 1000 μmolar BaP Kg^{-1}. At this dose, metabolic production of reactive

496 BaP metabolites was associated with elevated antioxidant SOD activity and elevated LPO

497 activity. Elevated s-SDH activity indicated hepatocellular damage as a consequence of high

498 LPO, causing cellular membrane dysfunction and release of SDH into the bloodstream. In

499 addition, the production of high levels of BaP metabolites was paralleled with elevated

500 DNA damage in blood cells, suggesting a causal link between BaP metabolites and DNA

501 damage. Finally, the correlations between various biomarkers demonstrate their

502 complementary nature in the evaluation of the effects of PAHs in fish inhabiting

503 contaminated environments and we conclude that this set of biomarkers are appropriate to

504 detect contaminated sites using tambaqui as a bioindicator species.

505 Acknowledgments

513 References

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514 Aas, E., Baussant, T., Balk, L., Liewenborg, B., Andersen, O.K., 2000. PAH metabolites in

515   bile, cytochrome P4501A and DNA adducts as environmental risk parameters for

516   chronic oil exposure: A laboratory experiment with Atlantic cod. Aquat. Toxicol. 51,

517   241–258. doi:10.1016/S0166-445X(00)00108-9

518 Abdel-Shafy, H.I., Mansour, M.S.M., 2015. A review on polycyclic aromatic

519   hydrocarbons: Source, environmental impact, effect on human health and remediation.

520   Egypt. J. Pet. 25, 107–123. doi:10.1016/j.ejpe.2015.03.011

521 Almeida, J.R., Gravato, C., Guilhermino, L., 2012. Biological parameters towards

522   polycyclic aromatic hydrocarbons pollution: A study with *dicentrarchus labrax* L.

523   exposed to the model compound benzo(a)pyrene. Water. Air. Soil Pollut. 223, 4709–

524   4722. doi:10.1007/s11270-012-1227-0

525 Banni, M., Bouraoui, Z., Ghedira, J., Clerandeau, C., Guerbej, H., Narbonne, J.F.,

526   Boussetta, H., 2009. Acute effects of benzo[a]pyrene on liver phase I and II enzymes,

527   and DNA damage on sea bream *Sparus aurata*. Fish Physiol. Biochem. 35, 293–299.

528   doi:10.1007/s10695-008-9210-9

529 Beutler, E., 1975. Red Cell Metabolism: A Manual of Biochemical Methods. Grune and

530   Straton, New York.

531 Beyer, J., Sandvik, M., Sk, J.U., Egaas, E., Hylland, K., Waagbù, R., Goksù, A., 1997.

532   Time- and dose-dependent biomarker responses in flounder (*Platichthys flesus* L.)

533   exposed to benzo[a]pyrene, 2,3,3',4,4', 5-hexachlorobiphenyl (PCB-156) and

534   cadmium. Biomarkers 2, 35–45.

535 Bosveld, A.T.C., De Bie, P.A.F., Van den Brink, N.W., Jongepier, H., Klomp, A. V., 2002.

536 In vitro EROD induction equivalency factors for the 10 PAHs generally monitored in

537 risk assessment studies in the Netherlands. Chemosphere 49, 75–83.

538 doi:10.1016/S0045-6535(02)00161-3

539 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation microgram

540 quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72,

541 248–254.

542 Buet, A., Banas, D., Vollaire, Y., Coulet, E., Roche, H., 2006. Biomarker responses in

543 European eel (*Anguilla anguilla*) exposed to persistent organic pollutants. A field

544 study in the Vaccarès lagoon (Camargue, France). Chemosphere 65, 1846–1858.

545 doi:10.1016/j.chemosphere.2006.03.074

546 Campos-baca, L., Kohler, C.C., 2005. Aquaculture of *Colossoma macropomum* and

547 Related Species in Latin America. Am. Fish. Soc. Symp. 46, 541–561.

548 Collier, T., Varanasi, U., 1990. Hapatic activities metabolizing enzymes and billiary levels

549 of xenobiotics in English Sole (*Parophrys vetulus*) exposed to envinronmental

550 contaminants. Arch. Environ. Contam. Toxicol. 20, 462–473.

551 Da Silva, J., De Freitas, T.R.O., Marinho, J.R., Speit, G., Erdtmann, B., 2000. An alkaline

552 single-cell gel electrophoresis (comet) assay for environmental biomonitoring with

553 native rodents. Genet. Mol. Biol. 23, 241–245. doi:10.1590/S1415-

554 47572000000100042

555 Dévier, M.H., Le Dû-Lacoste, M., Akcha, F., Morin, B., Peluhet, L., Le Menach, K.,

556   Burgeot, T., Budzinski, H., 2013. Biliary PAH metabolites, EROD activity and DNA

557   damage in dab (*Limanda limanda*) from Seine Estuary (France). Environ. Sci. Pollut.

558   Res. 20, 708–722. doi:10.1007/s11356-012-1345-7

559   Dû-Lacoste, M. Le, Akcha, F., Dévier, M.H., Morin, B., Burgeot, T., Budzinski, H., 2013.

560   Comparative study of different exposure routes on the biotransformation and

561   genotoxicity of PAHs in the flatfish species, *Scophthalmus maximus*. Environ. Sci.

562   Pollut. Res. 20, 690–707. doi:10.1007/s11356-012-1388-9

563   Food and Agriculture Organization of the United Nations - FAO, 2016. Introduced species

564   fact sheet [WWW Document]. URL http://fao.org/fishery/introsp/3885/en. (accessed

565   5.20.16).

566   Gagnon, M.M., Holdway, D.A., 2002. EROD activity, serum SDH and PAH biliary

567   metabolites in sand flathead (*Platycephalus bassensis*) collected in Port Phillip Bay,

568   Australia. Mar. Pollut. Bull. 44, 230–237. doi:10.1016/S0025-326X(01)00245-4

569   Gagnon, M.M., Holdway, D.A., 1998. MFO induction in atlantic salmon (*Salmo salar*)

570   during and after exposure to bass strait crude oil. Australas. J. Ecotoxicol. 4, 29–35.

571   Gravato, C., Guilhermino, L., 2009. Effects of Benzo(a)pyrene on Seabass (*Dicentrarchus*

572   *labrax* L.): Biomarkers, Growth and Behavior. Hum. Ecol. Risk Assess. An Int. J. 15,

573   121–137. doi:10.1080/10807030802615659

574   Gravato, C., Santos, M. a, 2002. Juvenile sea bass liver P450, EROD induction, and

575   erythrocytic genotoxic responses to PAH and PAH-like compounds. Ecotoxicol.

576   Environ. Saf. 51, 115–127. doi:10.1006/eesa.2001.2133

577 Gravato, C., Santos, M.A., 2003. Genotoxicity biomarkers' association with B(a)P

578 biotransformation in *Dicentrarchus labrax* L. Ecotoxicol. Environ. Saf. 55, 352–358.

579 doi:10.1016/S0147-6513(02)00070-2

580 Goksayr, A., Fiirlin, L., 1992. The cytochrome P-450 system in fish, aquatic toxicology and

581 environmental monitoring. Aquat. Toxicol. 22, 287–312. doi:10.1016/0166-

582 445X(92)90046-P

583 Gomes, L. de C., Araujo-Lima, C.A.R., Monteiro, R.R., 2003. Alevino – um termo

584 equivocado na piscicultura brasileira com conseqüências no setor produtivo. Cad.

585 Ciência Tecnol. 20, 353–359.

586 Halliwell, B., 2011. Free radicals and antioxidants - Quo vadis? Trends Pharmacol. Sci. 32,

587 125–130. doi:10.1016/j.tips.2010.12.002

588 Heath, A.G., 1995. Water Pollution and Fish Physiology. CRC Press.

589 Hopkins, J., Tudhope, G.R., 1973. Glutathione peroxidase in human red cells in health and

590 disease. Br. J. Haematol. 25, 563–575.

591 Hylland, K., 2006. Polycyclic aromatic hydrocarbon (PAH) ecotoxicology in marine

592 ecosystems. J. Toxicol. Environ. Health. A 69, 109–123.

593 doi:10.1080/15287390500259327

594 Jiang, Z.Y., Woollard, A.C.S., Wolff, S.P., 1991. Lipid hydroperoxide measurement by

595 oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay

596 and an iodometric method. Lipids 26, 853–856. doi:10.1007/BF02536169

597 Jönsson, M.E., Brunström, B., Brandt, I., 2009. The zebrafish gill model: Induction of

598   CYP1A, EROD and PAH adduct formation. Aquat. Toxicol. 91, 62–70.

599   doi:10.1016/j.aquatox.2008.10.010

600 Jung, J.H., Chae, Y.S., Kim, H.N., Kim, M., Yim, U.H., Ha, S.Y., Han, G.M., An, J.G.,

601   Kim, E., Shim, W.J., 2012. Spatial variability of biochemical responses in resident fish

602   after the M/V Hebei Spirit Oil Spill (Taean, Korea). Ocean Sci. J. 47, 209–214.

603   doi:10.1007/s12601-012-0021-7

604 Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for the several activities of the

605   glutathione S transferases. J. Biol. Chem. 251, 6183–6188.

606 Kirby, K.R., Laurance, W.F., Albernaz, A.K., Schroth, G., Fearnside, P.M., Bergen, S.,

607   Venticinque, E.M., da Costa, C., 2006. The future of deforestation in the Brazilian

608   Amazon. Futures 38, 432–453. doi:10.1016/j.futures.2005.07.011

609 Kochhann, D., De Azevedo Brust, S.M., Domingos, F.X.V., Val, A.L., 2013. Linking

610   hematological, biochemical, genotoxic, and behavioral responses to crude oil in the

611   Amazon fish *Colossoma macropomum* (cuvier, 1816). Arch. Environ. Contam.

612   Toxicol. 65, 266–275. doi:10.1007/s00244-013-9894-4

613 Lemaire, P., Förlin, L., Livingstone, D.R., 1996. Responses of hepatic biotransformation

614   and antioxidant enzymes to CYPl A-inducers (3-methylcholanthrene, B-

615   naphthoflavone) in sea bass (*Dicentrarchus labrax*), dab (*Limanda limanda*) and

616   rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 36, 141–160.

617 Liao, I.C., Su, H.M., Chang, E.Y., 2001. Techniques in finfish larviculture in Taiwan,

618   Aquaculture. doi:10.1016/S0044-8486(01)00692-5

619   Lin, E.L., Cormier, S.M., Torsella, J.A., 1996. Fish biliary polycyclic aromatic

620   hydrocarbon metabolites estimated by fixed-wavelength fluorescence: comparison

621   with HPLC-fluorescent detection. Ecotoxicol. Environ. Saf. 35, 16–23.

622   doi:10.1006/eesa.1996.0077

623   Livingstone, D.R., Forlin, L., George, S.G., 1993. Molecular biomarkers and toxic

624   consequences of impact by organic pollution in aquatic organisms, in: Sutcliffe, D.W.

625   (Ed.), Water Quality and Stress Indicators in Marine and Freshwater Ecosystems:

626   Linking Levels of Organisation. Freshwater Biological Association.

627   Lu, G.H., Wang, C., Zhu, Z., 2009. The dose-response relationships for EROD and GST

628   induced by polyaromatic hydrocarbons in Carassius auratus. Bull. Environ. Contam.

629   Toxicol. 82, 194–9. doi:10.1007/s00128-008-9622-3

630   Manoli, E., Samara, C., 1999. Polycyclic aromatic hydrocarbons in natural waters: sources,

631   occurence and. Trends Anal. Chem. 18, 417–428.

632   Martínez-Gómez, C., Campillo, J.A., Benedicto, J., Fernández, B., Valdés, J., García, I.,

633   Sánchez, F., 2006. Monitoring biomarkers in fish (*Lepidorhombus boscii* and

634   *Callionymus lyra*) from the northern Iberian shelf after the Prestige oil spill. Mar.

635   Pollut. Bull. 53, 305–314. doi:10.1016/j.marpolbul.2006.03.010

636   McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: An enzymic function for

637   erythrocuprein (Hemocuprein). J. Biol. Chem. 24, 6049–6055.

638   Miller, K.P., Ramos, K.S., 2001. Impact of cellular metabolism on the biological effects of

639   benzo[a]pyrene and related hydrocarbons. Drug Metab. Rev. 33, 1–35.

640   doi:10.1081/DMR-100000138

641   Modesto, K. a, Martinez, C.B.R., 2010. Effects of Roundup Transorb on fish: hematology,

642   antioxidant defenses and acetylcholinesterase activity. Chemosphere 81, 781–7.

643   doi:10.1016/j.chemosphere.2010.07.005

644   Morris, S., Vosloo, S., 2006. Animals and Environments : Resisting Schisms in

645   Comparative Physiology and Biochemistry. Invit. Perspect. Physiol. Biochem. Zool.

646   79, 211–223.

647   Mu, J. li, Wang, X. hong, Jin, F., Wang, J. ying, Hong, H. sheng, 2012. The role of

648   cytochrome P4501A activity inhibition in three- to five-ringed polycyclic aromatic

649   hydrocarbons embryotoxicity of marine medaka (*Oryzias melastigma*). Mar. Pollut.

650   Bull. 64, 1445–1451. doi:10.1016/j.marpolbul.2012.04.007

651   Neves, R.L.S., Oliveira, T.F., Ziolli, R.L., 2007. Polycyclic aromatic hydrocarbons (PAHs)

652   in fish bile (*Mugil liza*) as biomarkers for environmental monitoring in oil

653   contaminated areas. Mar. Pollut. Bull. 54, 1818–1824.

654   doi:10.1016/j.marpolbul.2007.08.013

655   Niki, E., 2009. Lipid peroxidation: Physiological levels and dual biological effects. Free

656   Radic. Biol. Med. 47, 469–484. doi:10.1016/j.freeradbiomed.2009.05.032

657   Nongnutch, K., Nanuam, J., Somnuek, C., 2012. The Efficiency of PAH Metabolites

658   Fluorescence Intensity Measurement to Study Pahs Contamination. Environ. Nat.

659   Resour. Res. 2, 32–37. doi:10.5539/enrr.v2n2p32

660   Paine, R.T., Ruesink, J.L., Sun, A., Soulanille, E.L., Wonham, M.J., Harley, C.D.G.,

661   Brumbaugh, D.R., Secord, D.L., 1996. Trouble on oiled waters: Lessons from the

662   Exxon Valdez Oil Spill. Annu. Rev. Ecol. Syst. 27, 197–235.

663   doi:10.1146/annurev.ecolsys.27.1.197

664   Ramachandran, S.D., Hodson, P. V., Khan, C.W., Lee, K., 2004. Oil dispersant increases

665   PAH uptake by fish exposed to crude oil. Ecotoxicol. Environ. Saf. 59, 300–308.

666   doi:10.1016/j.ecoenv.2003.08.018

667   Ramachandran, S.D., Sweezey, M.J., Hodson, P. V., Boudreau, M., Courtenay, S.C., Lee,

668   K., King, T., Dixon, J. a., 2006. Influence of salinity and fish species on PAH uptake

669   from dispersed crude oil. Mar. Pollut. Bull. 52, 1182–1189.

670   doi:10.1016/j.marpolbul.2006.02.009

671   Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011. Molecular and biochemical

672   biomarkers in environmental monitoring: A comparison of biotransformation and

673   antioxidant defense systems in multiple tissues. Aquat. Toxicol. 105, 56–66.

674   doi:10.1016/j.aquatox.2011.06.014

675   Ribeiro, C.A. de O., Katsumiti, A., França, P., Maschio, J., Zandoná, E., Cestari, M.M.,

676   Vicari, T., Roche, H., de Assis, H.C.S., Filipak Neto, F., 2013. Biomarkers responses

677   in fish (*Atherinella brasiliensis*) of Paranaguá Bay, southern Brazil, for assessment of

678   pollutant effects. Brazilian J. Oceanogr. 61, 1–11. doi:10.1590/S1679-

679   87592013000100001

680   Rinaldi, R., Eliasson, E., Swedmark, S., Morgenstern, R., 2002. Reactive intermediates and

681   the dynamics of glutathione transferases. Drug Metab. Dispos. 30, 1053–1058.

682   doi:10.1124/dmd.30.10.1053

683   Ruddock, P.J., Bird, D.J., McCalley, D. V, 2002. Bile metabolites of polycyclic aromatic

684   hydrocarbons in three species of fish from the Severn Estuary. Ecotoxicol. Environ.

685   Saf. 51, 97–105. doi:10.1006/eesa.2001.2131

686   Shailaja, M.S., D'Silva, C., 2003. Evaluation of impact of PAH on a tropical fish,

687   *Oreochromis mossambicus* using multiple biomarkers. Chemosphere 53, 835–841.

688   doi:10.1016/S0045-6535(03)00667-2

689   Shimada, T., 2006. Xenobiotic-metabolizing enzymes involved in activation and

690   detoxification of carcinogenic polycyclic aromatic hydrocarbons. Drug Metab.

691   Pharmacokinet. 21, 257–276. doi:10.2133/dmpk.21.257

692   Shimada, T., Fujii-Kuriyama, Y., 2004. Metabolic activation of polycyclic aromatic

693   hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. Cancer Sci. 95, 1–6.

694   doi:10.1111/j.1349-7006.2004.tb03162.x

695   Simonato, J.D., Fernandes, M.N., Martinez, C.B.R., 2011. Gasoline effects on

696   biotransformation and antioxidant defenses of the freshwater fish *Prochilodus*

697   *lineatus*. Ecotoxicology 20, 1400–1410. doi:10.1007/s10646-011-0697-y

698   Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for

699   quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175,

700   184–191. doi:10.1016/0014-4827(88)90265-0

701   Telli-Karakoç, F., Ruddock, P.J., Bird, D.J., Hewer, A., Van Schanke, A., Phillips, D.H.,

702 Peters, L.D., 2002. Correlative changes in metabolism and DNA damage in turbot

703 (*Scophthalmus maximus*) exposed to benzo[a]pyrene. Mar. Environ. Res. 54, 511–515.

704 doi:10.1016/S0141-1136(02)00192-7

705 Timbrell, J., 2004. Principles of biochemical toxicology, Third edit. ed. Taylor & Francis

706 Ltd.

707 Tim-Tim, A.L.S., Morgado, F., Moreira, S., Rangel, R., Nogueira, A.J.A., Soares,

708 A.M.V.M., Guilhermino, L., 2009. Cholinesterase and glutathione S-transferase

709 activities of three mollusc species from the NW Portuguese coast in relation to the

710 "Prestige" oil spill. Chemosphere 77, 1465–1475.

711 doi:10.1016/j.chemosphere.2009.10.014

712 Trisciani, A., Corsi, I., Torre, C. Della, Perra, G., Focardi, S., 2011. Hepatic

713 biotransformation genes and enzymes and PAH metabolites in bile of common sole

714 (*Solea solea*, Linnaeus, 1758) from an oil-contaminated site in the Mediterranean Sea:

715 A field study. Mar. Pollut. Bull. 62, 806–814. doi:10.1016/j.marpolbul.2011.01.001

716 United Nations Documents, 1987. Report of the World Commission on Environment and

717 Development: Our Common Future.

718 Val, A.L., Almeida-Val, V.M.F., 1995. Fishes of the Amazon and Their Environment,

719 Zoophysiology. Springer Berlin Heidelberg, Berlin, Heidelberg. doi:10.1007/978-3-

720 642-79229-8

721 Val, A.L., De Almeida-Val, V.M.F., Randall, D.J., 2005. Tropical Environment, in: Fish

722 Physiology. pp. 1–45. doi:10.1016/S1546-5098(05)21001-4

723   van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers

724   in environmental risk assessment: A review. Environ. Toxicol. Pharmacol. 13, 57–

725   149.

726   van Schanke, A., Holtz, F., van der Meer, J., Boon, J.P., Ariese, F., Stroomberg, G., van

727   den Berg, M., Everaarts, J.M., 2001. Dose- and time-dependent formation of biliary

728   benzo[*a*]pyrene metabolites in the marine flatfish DAB (*Limanda limanda*).

729   Environ. Toxicol. Chem. 20, 1641–1647. doi:10.1002/etc.5620200804

730   Varanasi, U., Stein, J.E., Nishimoto, M., Reichert, W.L., Collier, T.K., 1987. Chemical

731   carcinogenesis in feral fish: uptake, activation, and detoxication of organic

732   xenobiotics. Environ. Health Perspect. 71, 155–170. doi:10.1289/ehp.8771155

733   Vieira, L.R., Sousa, A., Frasco, M.F., Lima, I., Morgado, F., Guilhermino, L., 2008. Acute

734   effects of Benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common

735   goby *Pomatoschistus microps* (Teleostei, Gobiidae). Sci. Total Environ. 395, 87–100.

736   doi:10.1016/j.scitotenv.2008.01.052

737   Webb, D., Gagnon, M.M., 2007. Serum sorbitol dehydrogenase activity as an indicator of

738   chemically induced liver damage in black bream (*Acanthopagrus butcheri*). Environ.

739   Bioindic. 2, 172–182. doi:10.1080/15555270701591006

740   Webb, D., Gagnon, M.M., Rose, T., 2005. Interseasonal variability in biomarkers of

741   exposure in fish inhabiting a southwestern Australian estuary. Environ. Toxicol. 20,

742   522–32. doi:10.1002/tox.20141

743   Wiernsperger, N.F., 2003. Oxidative stress as a therapeutic target in diabetes: revisiting the

744 controversy. Diabetes Metab. 29, 579–585. doi:10.1016/S1262-3636(07)70072-1

745 Willett, K.L., Gardinali, P.R., Lienesch, L. a, Di Giulio, R.T., 2000. Comparative

746 metabolism and excretion of benzo(a)pyrene in 2 species of ictalurid catfish. Toxicol.

747 Sci. 58, 68–76. doi:10.1093/toxsci/58.1.68

748