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

2 Tambaqui (*Colossoma macropomum*)

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Abstract

Here we explore the dose-dependent response of the tropical fish tambaqui (*Colossoma macropomum*) to intraperitoneal injection of benzo[a]pyrene (BaP) at doses of 0 (carrier control), 1, 10, 100 and 1000 µmolar BaP Kg^{-1} Hepatic ethoxyresorufin-*O*-deethylase (EROD) activity showed a bell-shaped dose-dependent response curve, where the highest injected BaP dose caused enzyme inactivation. Activities of hepatic catalase (CAT) and superoxide dismutase (SOD) increased at the highest dose relative to the carrier control group. Lipid peroxidation (LPO), serum-sorbitol dehydrogenase (s-SDH) and DNA damage in blood cells were higher for all BaP doses when compared to the carrier control group. At high dosage, the production of BaP metabolites was paralleled by induced activity of the antioxidant enzyme SOD, and high levels of DNA damage in blood cells. In a similar way, high LPO was concomitant to elevated s-SDH in the bloodstream, suggesting that lipid peroxidation caused the loss of membrane integrity and leakage of s-SDH from hepatocytes into the bloodstream. These biomarkers were also positively co-correlated. The results demonstrate the potential use of a suite of biomarkers for tambaqui living in contaminated tropical aquatic environments. In particular, we recommend the analysis of DNA damage in blood cells, as this was highly correlated with all other biomarkers.

Keywords:

Tambaqui; BaP; Biomarkers; CAT; SOD; EROD activity; s-SDH; GST; LPO

51 1. Introduction

52 Polycyclic aromatic hydrocarbons (PAHs) are of major concern in contaminated aquatic
53 environments (Abdel-Shafy and Mansour, 2015; Manoli and Samara, 1999), particularly
54 tropical environments which have received urban and industrial contamination due to
55 uncontrolled population growth and rapid industrialization (United Nations, 1987). PAHs
56 are highly lipophilic and tend to adsorb to suspended particulate material or accumulate in
57 sediment, increasing bioavailability to fish inhabiting contaminated environments (Hylland,
58 2006; Paine et al., 1996). While many studies have identified biomarker tools in an array of
59 bioindicator species in well-developed countries from temperate regions (Almeida et al.,
60 2012; Dû-Lacoste et al., 2013), very few studies are available for tropical environments.
61 There is a scarcity of recognized bioindicator species for tropical ecosystems and,
62 consequently, few biomarkers have been validated to study the impacts of PAH
63 contamination in organisms living in these environments.

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

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

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 ± 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 ($(NH_4)_2Fe(SO_4)_2$•
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_2PO_4, 0.05 M K_2HPO_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}.

210 *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.

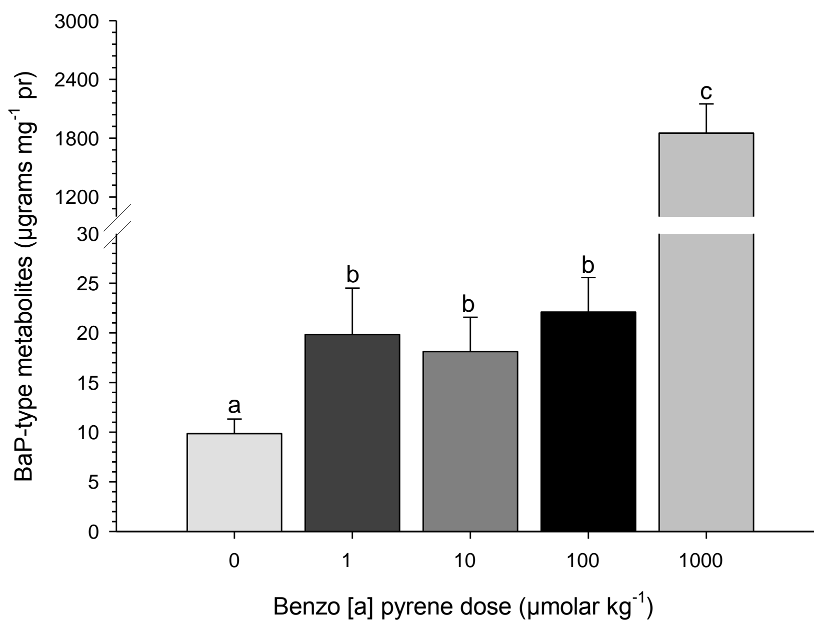
293 Increases ($p < 0.05$) of 189, 92, 102 and 84 fold were measured in tambaqui IP-injected

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.

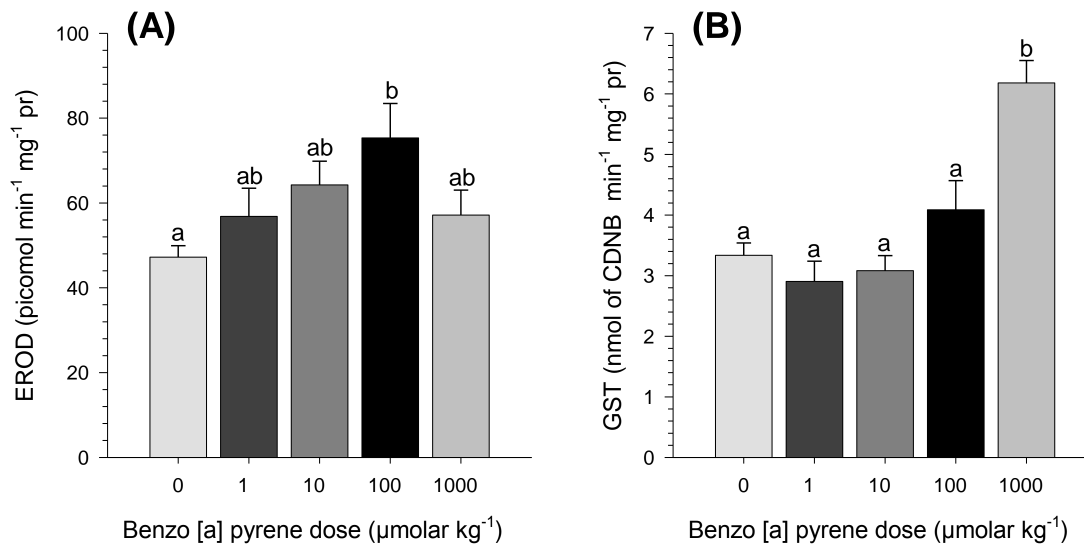
303 *Biotransformation enzymes*

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

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

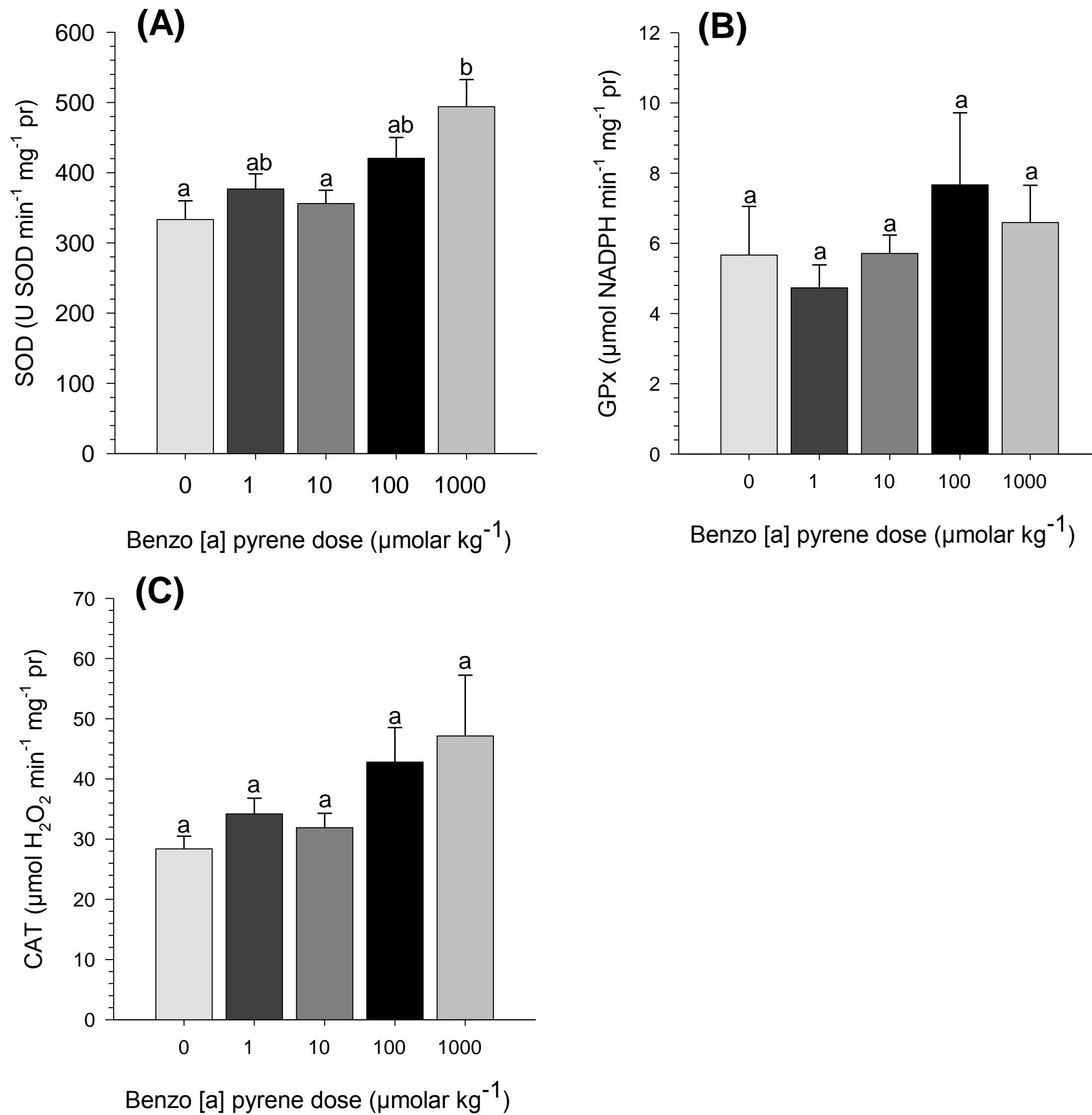
317 *Oxidative stress enzymes*

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_2O_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

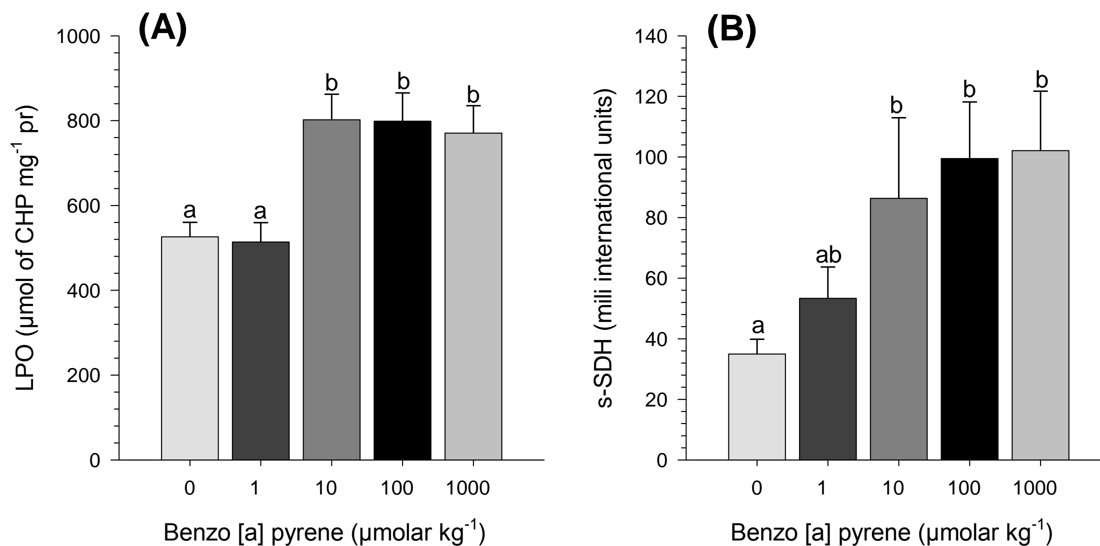
330 indicate statistical differences ($p < 0.05$) between doses.

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).

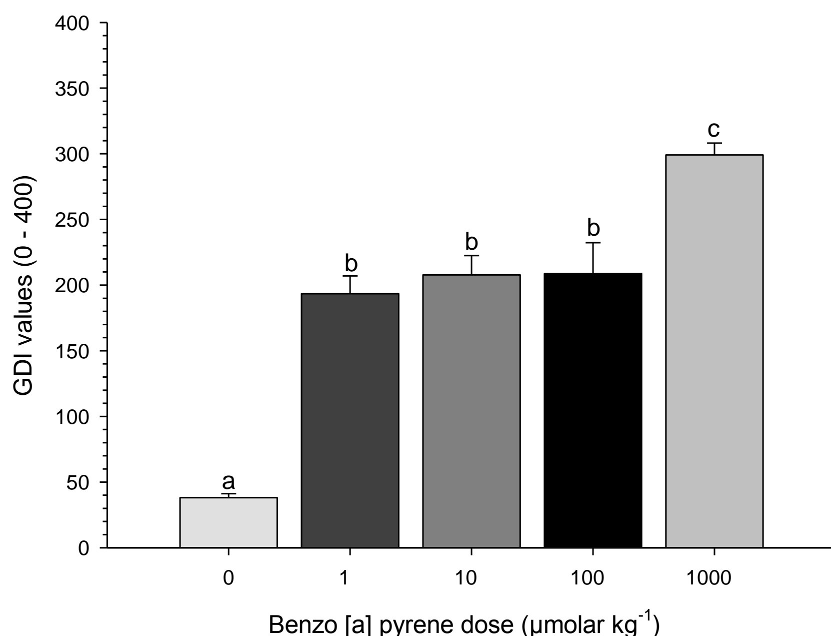


341

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

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

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

363 Table 1. Observed Pearson correlations between the biomarkers of tambaqui treated with an

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

365 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***

366

367 4. Discussion

368 This study examined a suite of widely applied aquatic biomarkers (biliary BaP-type

369 metabolites, EROD, GST, CAT, SOD, GPx, LPO, s-SDH and DNA damage in blood cells)

370 in tambaqui, a tropical bioindicator fish species of high economic and scientific importance

371 in South America and Asia (Campos-baca and Kohler, 2005; Food and Agriculture

372 Organization of the United Nations - FAO, 2016; Liao et al., 2001; Val and Almeida-Val,

373 1995; Val et al., 2005). Intraperitoneal injections of the contaminant BaP were

374 administrated at five different doses to assess if a dose-response can be established in this

375 species 96 h post-injection. Furthermore, a correlation analysis between biomarkers

376 identified which sub-set of biomarkers would be most relevant for environmental

377 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

506 This work was funded by a joint grant, Projeto de inteligência socioambiental da indústria
507 do petróleo na Amazônia (PIATAM), from the FINEP, and INCT ADAPTA
508 (CNPq/FAPEAM). HSH and RMD were recipients of PhD fellowships from CNPq.
509 VMFAV was the recipient of a research fellowship from CNPq. MMG was supported by
510 Curtin University, Australia. Special thanks to Maria de Nazaré Paula da Silva for her
511 continued support. The authors are grateful to Mr Graham Cobby and to Dr Christine
512 Cooper for grammatical improvement of the manuscript.

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