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Ecological Indicators

Validation of a suite of biomarkers of fish health in the tropical bioindicator species, 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.

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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 Documents, 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; Le 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 the soluble frac-

tion of diesel fuel (Simonato et al., 2011), crude oil (Ramachandran et al., 2004) and chemically dispersed crude oil (Ramachandran et al., 2006). Several field studies have reported increased activity of hepatic EROD in fish collected from recently contaminated areas (Jung et al., 2012; Martínez-Gómez et al., 2006) or that have a history of contamination by oil and oil products (Buet et al., 2006; Dévier et al., 2013; Gagnon and Holdway, 2002; Trisciani et al., 2011).

Phase II of biotransformation occurs via conjugation reactions of the BaP primary and secondary metabolites with polar molecules, such as glutathione, facilitating excretion and elimination of the final product (Timbrell, 2004). Glutathione S-transferase (GST) plays a fundamental role in Phase II of biotransformation, participating in conjugation reactions of the xenobiotic with the exogenous tripeptide reduced glutathione (GSH) and resulting in the efficient elimination of the metabolites from the organism (Rinaldi et al., 2002). GST has been considered a reliable biomarker for a variety of fish species exposed to PAHs in both laboratory studies (Simonato et al., 2011) and field investigations (Tim-Tim et al., 2009).

Glutathione peroxidase (GPx) is another antioxidant enzyme that catalysis the metabolism of oxidizing compounds, a process which involves the oxidation of reduced GSH to its oxidized form (van der Oost et al., 2003). GPx is also involved in reducing lipid hydroxyperoxides to their corresponding alcohols, making them more water soluble as well as reducing free H_2O_2 to water (van der Oost et al., 2003).

A continuous production of reactive oxygen species (ROS) is an inevitable result of NADPH consumption by the cytochrome P450 during the metabolism of BaP (Wiernsperger, 2003). ROS, such as OH, peroxyl and hydroxyl radicals, can oxidize essential biological molecules such as lipids, proteins, carbohydrates and DNA (Halliwell, 2011). The interaction of these free radicals with biomembranes disturbs the membranes' delicate structure, integrity, fluidity and permeability, as well as resulting in a loss of functionality as a consequence of lipid peroxidation (LPO) (Niki, 2009; Wiernsperger, 2003). Damage to the lipid bilayer, i.e. lipoperoxidation, is considered a significant cause of cell injury and death following exposure to contaminants (Modesto and Martinez, 2010). Antioxidant enzymes play an important role in neutralizing the effects of ROS molecules in biological membranes. For example, superoxide dismutase (SOD), the first enzyme in the line of antioxidant defense, acts in neutralizing the superoxide radicals, generating hydrogen peroxide which, in turn, is neutralized by catalase (CAT).

The measurement of biomarkers of exposure, such as PAH biliary metabolite levels, activity of the enzymes involved in the biotransformation of contaminants (EROD and GST), and neutralization of ROS (SOD, CAT and GPx), along with the analysis of the failures of these systems, such as oxidation of lipids and carbohydrates (which can be measured via LPO), and the oxidation of proteins, such as DNA (which can be measure by DNA damage), are commonly used as biomarkers of water contamination (van der Oost et al., 2003). The responses of these biomarkers can be species-specific and vary depending on the type of contaminant, the dose and the time of exposure. Thus, the validation of biomarkers in a relevant bioindicator species improves the understanding of toxic effects of PAHs on aquatic organisms.

Considering the widespread contamination of tropical aquatic environments by petroleum hydrocarbons and the rarity of bioindicator species relevant to tropical waterbodies, this study aims to (i) validate the dose-dependent responses of the fish tambaqui (Colossoma macropomum) to varying intraperitoneal doses of BaP; and (ii) quantify biomarker responses to select the most relevant suite of biomarkers for monitoring tropical aquatic environments contaminated with PAHs. The tambaqui was chosen as model fish species

due its high economic and scientific importance in tropical regions including 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), its widespread availability from aquaculture ventures, and because of its resistance to environmental challenges (Val and Almeida-Val, 1995; Val et al., 2005).

2. Material and methods

2.1. Fish and experimental protocol

All procedures followed the Brazilian Animal Care Guidelines and were approved by INPA's Animal Care Committee (Protocol number: 022/2012). Juvenile tambaqui (Teleostei, Characiformes, Serrasalmidae) were used to avoid the confounding effect of reproduction on some biomarkers e.g. EROD activity (Goksayr and Fiirlin, 1992). Juvenile tambaqui are classified as juvenile (4–6 cm); advanced juvenile (6–8 cm); juvenile II (8–10 cm); and advanced juvenile II (10–12 cm) (Gomes et al., 2003). Our experiment used the latest stages of advanced juvenile II to provide sufficient biological material for all biomarker analyses. Tambaqui (n = 75; weight: 50.73 ± 2.12 g and length: 13.32 ± 0.21 cm; mean ± SD), were purchased from a local fish farm (Fazenda Santo Antônio, Amazonas, Brasil) and transported to LEEM/INPA (Instituto Nacional de Pesquisas da Amazônia) in purpose-designed fish transport bags filled with the water from the fish farm and supplied with pure oxygen. The bags were kept in styrofoam boxes to reduce temperature variation and to maintain a dark environment, to minimise transport stress. Fish were stepwise acclimated to laboratory tap water and were then kept in the laboratory for at least 60 days before the experiments to ensure full acclimation to laboratory conditions. No fish died during the transport and the acclimation period. Fish were kept outdoors for at least 60 days in 3000-L polyethylene tanks with INPA's ground water, continuously aerated (average composition: Na^+: 43 μmol L^{-1}; Cl^-: 31 μmol L^{-1}; K^+: 10 μmol L^{-1}; Ca^{2+}: 9 μmol L^{-1}; Mg^{2+}: 4 μmol L^{-1}; pH 6; 28 °C; 6.40 mg of dissolved O_2 L^{-1} and temperature of 29 °C) with a flow-through rate of 1200 ml min^{-1}. Fish were fed with dry food pellets (26% protein content, Nutripeixe, Purina®) twice a day and feeding was suspended 2 days prior to experiments. Fish were anesthetized in aerated water containing neutral MS-222 (0.5 g L^{-1} MS-222 and 1 g L^{-1} NaHCO₃, Sigma Aldrich), before being given an intraperitoneal (IP) injection of one of five BaP concentrations; 0 (carrier controls), 1, 10, 100 or 1000 μmolar BaP Kg^{-1}. Injection solutions were freshly prepared and each fish received the same volume of carrier solution (0.5 ml of corn oil, Sigma Aldrich). After injection, fish were held in 2000 l polyethylene tanks, in the same water with continuous aeration for 96 h. Thereafter, blood samples were drawn from the caudal vein of fish anesthetized in neutral buffered MS-222 (1 g L^{-1} MS-222 and 2 g L^{-1} NaHCO₃, Sigma Aldrich). Fish were then killed by severing the spine, measured, weighed and the spleen and liver removed and stored at −80 °C until analysis.

2.2. Chemicals

All chemicals were purchased from Sigma Aldrich Brazil: Corn oil; tricaine mesylate (MS-222); Benzo [a] pyrene; 1-OH-pyrene; HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid); potassium chloride (KCL); magnesium sulfate (MgSO₄); nicotinamide adenine dinucleotide phosphate (NADPH); bovine serum albumin (BSA); ethoxyresorufin; resorufin; methanol; 1-chlore-2,4-dimitrobenzene (CDNB); TRIS-Base (tris(hydroximethyl)aminomethane); dithiothreitol (DTT);

sucrose; monopotassium phosphate (KH_2PO_4); dipotassium phosphate (K_2HPO_4); L-glutathione reduced (GSH); hydrogen peroxide (H_2O_2); sodium phosphate monobasic (NAH_2PO_4); xanthine; sodium hydroxide (NaOH); cytochrome c; xanthine oxidase; sodium azide (NaN_3); glutathione reductase (GR); trichloroacetic acid (TCA); xylenol orange; sulfuric acid (H_2SO_4); butyl hydroxyl toluene (BHT); ammonium iron (II) sulfate hexahydrate ((NH_4)$_2$Fe(SO_4)$_2$·6H2O); Bradford reagent; fructose; normal melting agarose; low melting point agarose; sodium chloride (NaCl); triton X-100; dimethyl sulfoxide (DMSO); sodium carbonate (Na_2CO_3); ammonia nitrate (NH_4)(NO_3); silver nitrate ($AgNO_3$); silicotungstic acid ($H_4[W_{12}SiO_{40}]$); formaldehyde (H_2CO).

2.3. BaP-type metabolites in fish bile

The term 'type of metabolites' is used because the fixed fluorescence technique detects groups of metabolites originating from a common parent compound fluorescing at a common wavelength (Lin et al., 1996). The BaP-type metabolites concentration in fish bile was determined by fixed fluorescence (FF) measurements according to Lin et al. (1996). Bile samples were diluted in 50/50 HPLC-grade methanol-water in the proportion of 1:1000 (bile:methanol) and read at EX 380 nm and EM 430 nm. The reference standard for BaP-type metabolites was 1-OH pyrene (also known as 1-pyrenol). Samples were read on a spectrofluorimeter with slit width of 10 nm, against a standard curve. The BaP-type metabolites are reported in μg BaP-type met mg^{-1} protein.

2.4. Biotransformation enzymes

Hepatic EROD activity was assessed by the fluorimetric method described by Webb et al. (2005). The post mitochondrial supernatant (PMS) was obtained by homogenizing the liver (1:4 w:v) in HEPES homogenization buffer pH 7.5 (0.02 M HEPES and 0.015 M KCL) before centrifuging at 12,000 g for 20 min at 4 °C. The PMS (50 μl) was added to the reaction mixture (1250 μl 0.1 M HEPES buffer; 10 μl of 1.28 M MgSO4; 30 μl of 0.5 mM NADPH and 50 μl of 40 mg/mL bovine serum albumin-BSA). The reaction started with the addition of 20 μl of 0.12 mM ethoxyresorufin; after 2 min, the addition of 2500 μl of HPLC-grade methanol stopped the reaction. The protein precipitate resulting from the addition of methanol was spun down and the amount of resorufin produced was measured on a spectrofluorimeter at EX wavelength 530 nm and EM wavelength 585 nm. The amount of resorufin was calculated from a resorufin standard curve varying from 0.000 to 0.085 M, and readings were relative to a blank fluorescence. EROD activity is expressed as picomol of resorufin produced per milligram protein per minute (pmol mg^{-1} protein min^{-1}).

Hepatic GST activity was determined as described by Keen et al. (1976) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The supernatant was obtained by homogenizing the liver (1:10 w:v) in a cold buffer solution (20 mM Tris-Base, 1 mM EDTA, 1 mM dithiothreitol, 500 mM Sucrose, 150 mM KCL, pH 7.6) and then centrifuging at 9000 g for 30 min at 4 °C. The homogenates were pipetted (15 μl) into microplate and 235 μl of 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 mM CDNB) was added. GSH (10 μl of 25 mM) was added to start the reaction. The change in absorbance was recorded on a spectrophotometer at 340 nm. The enzymatic activity was calculated as nmol CDNB conjugate formed per milligram protein per minute (nmol CDNB mg^{-1} protein min^{-1}) using a molar extinction coefficient of 9.6 mM cm^{-1}.

2.5. Oxidative stress enzymes

The protocol to obtain the supernatant for measuring CAT, SOD and GPx activities was the same as described for GST supernatant. Hepatic CAT activity was determined as described by Beutler (1975). The homogenates were pipetted (10 μl) into a quartz cuvette and 990 μl of reaction media, consisting of 9 ml of the catalase buffer (1 M Tris-Base, 5 mM EDTA, pH 8.0, 25 °C) diluted 1.8 times plus 90 ml of H_2O_2 solution (100 ml distilled water plus 100 μl of H_2O_2 30%), was added. The rate of enzymatic decomposition of H_2O_2 was measured in a spectrophotometer at an absorbance of 240 nm. Enzyme activity was expressed in μmol H_2O_2 mg^{-1} protein min^{-1}.

Hepatic copper–zinc SOD (SOD) activity was determined according to McCord and Fridovich (1969). The homogenates were pipetted (5 μl) into plastic cuvettes and 1 ml of reaction media was added. The reaction media consisted of 47.5 ml of phosphate buffer (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 xanthine (diluted in 1 mM NaOH) and 20 mM cytochrome c. The reaction was initiated by the addition of 20 μl of 0.2 U mL^{-1} xanthine oxidase. This method measures the inhibition of the reduction rate of cytochrome c by the superoxide radical, read on a spectrophotometer at 550 nm and 25 °C. SOD activity was expressed in units of SOD per mg of protein (U mg^{-1} protein), with one U of SOD corresponding to the quantity of enzyme that caused 50% inhibition of the reduction rate of cytochrome c.

Hepatic selenium-dependent glutathione peroxidase (GPx) activity was determined by the method of Hopkins and Tudhope (1973). The homogenates were pipetted (10 μl) into quartz cuvettes and 1 ml of reaction media, consisting of 25 ml of the phosphate buffer (100 mM NaH_2PO_4, 100 mM K_2HPO_4, 2 mM EDTA, pH 7.0, 25 °C), 0.2 mM NADPH, 10 ml of 5 mM NaN_3, 1 mM GSH and 15 ml distilled water was added. The reaction was initiated by the addition of 20 μl of 1 U mL^{-1} glutathione reductase and 20 μl of 20 mM H_2O_2. This method is based on NADPH oxidation in the presence of GSH and H_2O_2, measured in a spectrophotometer at 340 nm. GPx activity was expressed in μmol oxidized NADP mg^{-1} protein min^{-1} using a molar extinction coefficient of 6.22 mM cm^{-1}.

2.6. Lipid peroxidation (LPO)

The LPO concentration in the liver was quantified after Jiang et al. (1991). The homogenates were transferred to plastic tubes and treated with TCA 12% (1:1 v:v) then centrifuged at 5,000g for 10 min at 4 °C. The treated homogenates were pipetted (30 μl) into a microplate before 270 μl of reaction media 50 ml methanol 90%, 0.1 mM xylenol orange, 25 mM H_2SO_4, 4 mM BHT, 0.25 mM ferric ammonium sulfate ((NH_4)$_2$Fe(SO_4)$_2$·6H2O) was added. This method is based on the oxidation of the Fe^{+2} to Fe^{+3} by hydroperoxides in an acid medium in the presence of ferrous oxidation-xylenol orange, read at 560 nm. Cumene hydroperoxide (CHP) was used as a standard. LPO concentration was expressed in μmol of CHP mg^{-1} protein.

2.7. Protein determination

Liver supernatant, as used for the enzyme analyses, was also used to quantify total protein content according to Bradford (1976) using a spectrophotometer at 595 nm and bovine serum albumin as a standard.

2.8. Serum sorbitol dehydrogenase (s-SDH) activity

Following blood collection, serum was isolated by centrifuging at 5000 rpm for 10 min. The serum was kept at −80 °C until the s-SDH determination, after Webb and Gagnon (2007). 50 μl

of serum was placed in 450 μl of 0.1 M Tris buffer pH 7.5 and incubated for 10 min at room temperature, after which 100 μl of 4 M fructose solution was added and the linear rate of decrease in absorbance over one minute at 340 nm was measured with a spectrophotometer. The enzymatic activity of the s-SDH is expressed as milli-international Units (mIU) of s-SDH activity.

2.9. DNA damage in blood cells (comet assay)

DNA damage in blood cells was assessed via a comet assay (alkali method) as described by Singh et al. (1988) for lymphocytes and modified by Silva et al. (2000) for peripheral blood cells. Microscope slides were dipped into 1.5% normal melting agarose (NMA) prepared in phosphate-buffered saline (PBS). Each slide was coated with 1.0% NMA in PBS, and then covered with a coverslip. Subsequently, blood (7–10 μl) mixed with 95 μl of 0.75% low melting point agarose (LMA) (Gibco BRL) was spread on the slide and allowed to solidify. After removal of the coverslip, the slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris; pH 10–10.5; 1% Triton X-100 and 10% DMSO) overnight. Then, the slides were placed in an electrophoresis chamber, filled with freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 20 min at 300 mA and 25 V in an ice bath. After electrophoresis, the slides were washed with 0.4 M Tris buffer, pH 7.5 and submerged in silver solution (5% sodium carbonate; 0.1% ammonia nitrate; 0.1% silver nitrate; 0.25% Silicotungstic acid; and 0.15% formaldehyde). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed using an optical microscope (Leica DM205) at 100 x magnification. Cells were scored into five classes, according to tail size (from undamaged −0 to maximally damaged −4) and a value was assigned to each comet according to its class. The final overall rating for the slide i.e. the DNA damage score (Genetic Damage Index, GDI), was between 0 (intact) and 400 (maximum damage), and was obtained by summation according to Kobayashi et al. (1995).

2.10. Statistical analysis

Data are presented as mean ± standard error of the mean (mean ± SEM). Prior to comparative statistical analyses, data were assessed for normality and homogeneity of variance. A one-way analysis of variance (ANOVA) with a Holm-Sidack post hoc test was applied to verify differences in all analyzed biomarkers between fish treated with different BaP doses (0 (corn oil), 1, 10, 100 and 1000 μMolar kg^{-1}). When data violated the ANOVA assumptions of normality and homogeneity, a non-parametric Kruskall–Wallis test was used. A Pearson correlation was used to identify interactions between all analyzed biomarkers. Statistical significance was accepted at the level of $p \leq 0.05$.

3. Results

3.1. BaP-type metabolites in fish bile

BaP-type metabolites in the bile varied from 10 to 1850 μg metabolite mg^{-1} protein. Increases ($p < 0.05$) of 189, 92, 102 and 84 fold were measured in tambaqui IP-injected with 1000 μmolar BaP Kg^{-1} relative to the carrier control group, 1, 10 and 100 μmolar BaP Kg^{-1}, respectively. Moreover, increases ($p < 0.05$) of 2; 1.8; and 2.2 fold, respectively, were observed for tambaqui treated with 1, 10 and 100 μmolar BaP Kg^{-1} relative to the carrier control group (Fig. 1).
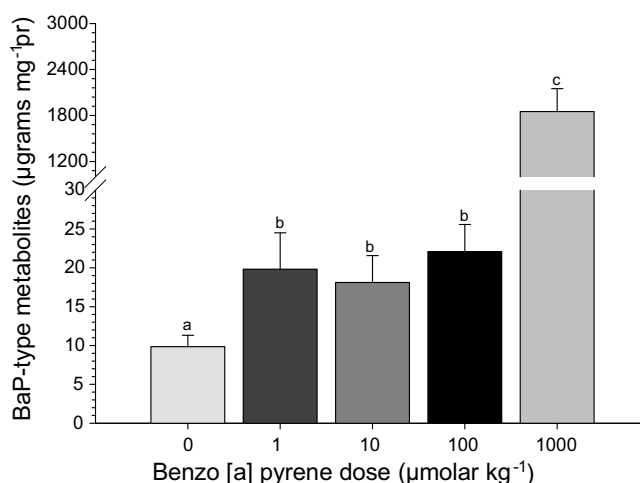

Fig. 1. BaP-type metabolites in bile of tambaqui treated with an intraperitoneal injection of one of five BaP concentrations; 0 (carrier control), 1, 10, 100 and 1000 μmolar BaP Kg^{-1}. Columns represent means and vertical lines represent SEM (n = 15 per treatment). Different letters indicate statistical differences ($p < 0.05$) between doses.

3.2. Biotransformation enzymes

Hepatic EROD activity varied from 47 to 75 picomol mg^{-1} protein min^{-1} (Fig. 2A). Increases ($p < 0.05$) of 1.6 fold relative to the carrier control group were measured only in tambaqui treated with 100 μmolar BaP Kg^{-1}. The group injected with the highest dose of 1000 μmolar BaP Kg^{-1} had similar EROD activity to the carrier control ($p = 0.224$).

Hepatic GST activity varied from 2.9 to 6.2 μmol CDNB mg^{-1} protein min^{-1} (Fig. 2B). Increases ($p < 0.05$) of 1.9 fold over the carrier control group were measured only in tambaqui injected with 1000 μmolar BaP Kg^{-1}.

3.3. Oxidative stress enzymes

Hepatic SOD activity varied from 333 to 493 U SOD mg^{-1} protein min^{-1}. Increases ($p < 0.05$) of 1.5 and 1.4 fold were observed only in tambaqui injected with 100 μmolar BaP Kg^{-1} relative to the carrier control group and to the 10 μmolar BaP Kg^{-1} group, respectively (Fig. 3A).

Hepatic GPx and CAT activity varied from 4.7 to 6.6 μmol NADPH mg^{-1} protein min^{-1} and 28.4 to 47.2 H$_2$O$_2$ mg^{-1} protein min^{-1} respectively. No differences ($p > 0.05$) were observed between the treatment groups for either biomarker (Fig. 3B and C).

3.4. Lipid peroxidation (LPO)

LPO concentrations in the liver varied from 566 to 802 μmol of CHP mg^{-1} protein. Increases ($p < 0.05$) were observed for tambaqui treated with 10, 100 and 1000 μmolar BaP Kg^{-1}, with CHP mg^{-1} pr levels being 1.6, 1.5 and 1.5 fold higher relative to the carrier control group (Fig. 4A).

3.5. Serum sorbitol dehydrogenase (s-SDH) activity

s-SDH activity in the plasma varied from 34 to 102 mIU and appeared to follow a dose-dependent increase, with progressively higher s-SDH activities with higher BaP doses. Increases ($p < 0.05$) of 1.2; 2.8 and 3 fold were observed in fish treated with 10, 100 and 1000 μmolar BaP Kg^{-1} respectively, relative to the carrier control group (Fig. 4B).
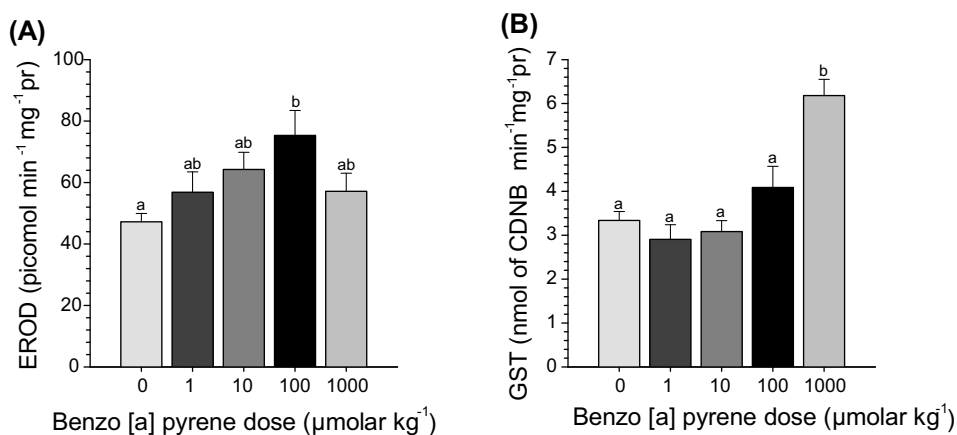
Fig. 2. Hepatic (A) ethoxyresorufin-*O*-deethylase (EROD) and (B) glutathione S-transferase (GST) of tambaqui treated with an intraperitoneal injection of one of five BaP concentrations; 0 (carrier control), 1, 10, 100 and 1000 μmolar BaP Kg^{-1}. Columns represent means and vertical lines represent SEM (n = 15 per treatment). Different letters indicate statistical differences (p < 0.05) between doses.
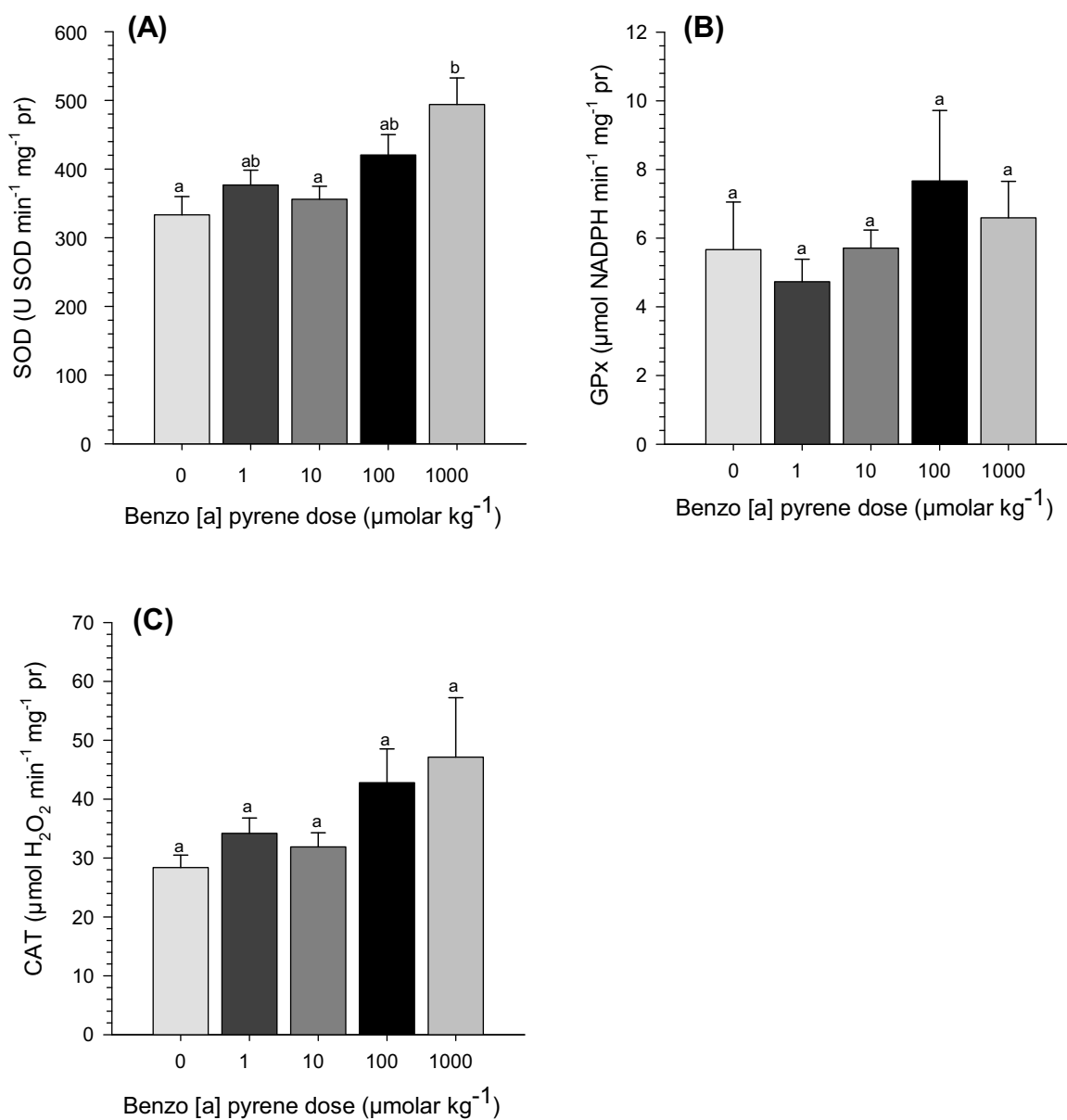

Fig. 3. Hepatic (A) superoxide dismutase (SOD), (B) glutathione peroxidase (GPx) and (C) catalase (CAT) of tambaqui treated with an intraperitoneal injection of one of five BaP concentrations; 0 (carrier control), 1, 10, 100 and 1000 μmolar BaP Kg^{-1}. Columns represent means and vertical lines represent SEM (n = 15 per treatment). Different letters indicate statistical differences (p < 0.05) between doses.
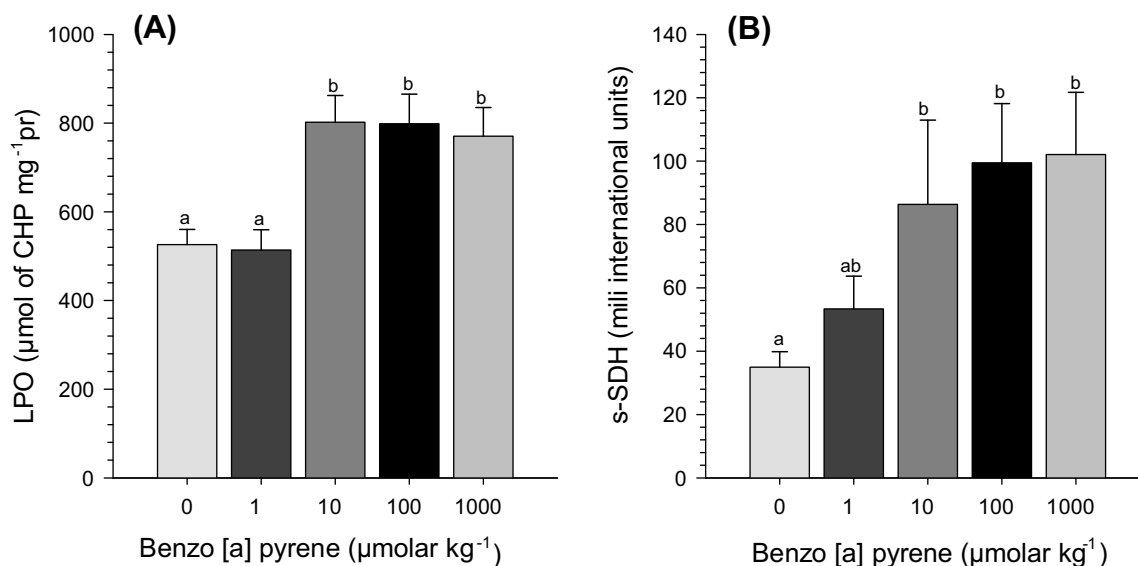
Fig. 4. (A) Hepatic lipid peroxidation (LPO) and (B) serum sorbitol dehydrogenase (s-SDH) of tambaqui treated with an intraperitoneal injection of one of five BaP concentrations; 0 (carrier control), 1, 10, 100 and 1000 μmolar BaP Kg^{-1}. Columns represent means and vertical lines represent SEM (n = 15 per treatment). Different letters indicate statistical differences (p < 0.05) between doses.
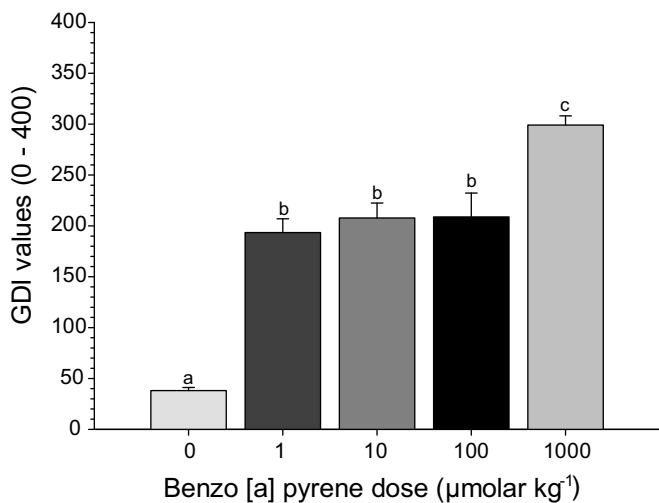

Fig. 5. Genetic Damage Index (GDI) in blood cells of tambaqui treated with an intraperitoneal injection of one of five BaP concentrations; 0 (carrier control), 1, 10, 100 and 1000 μmolar Ba Kg^{-1}. Columns represent means and vertical lines represent SEM (n = 15 per treatment). Different letters indicate statistical differences (p < 0.05) between doses.

3.6. DNA damage in blood cells (comet assay)

DNA damage in blood cells varied from 38 to 300 (comet classes 0–400). Increases (p < 0.05) of 8; 1.5; 1.43; and 1.4 fold, respectively, were observed for tambaqui treated with 1000 μmolar BaP Kg^{-1} relative to the carrier control group, 1, 10 and 100 μmolar BaP Kg^{-1}, respectively. Moreover, increases (p < 0.05) of 5; 5.45; and 5.48 fold, respectively, were observed for tambaqui treated with 1, 10 and 100 μmolar BaP Kg^{-1} relative to the carrier control group (Fig. 5).

3.7. Correlation analysis

Pearson correlations indicated that some biomarkers were positively correlated (p < 0.05). GST was positively correlated with the enzymatic activity of CAT, blood cell DNA damage and biliary BaP-type metabolites. SOD activity was positively correlated with EROD, 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).

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.

Fish injected with 100 μmolar BaP Kg^{-1} had the highest hepatic EROD activity, indicating that Phase I biotransformation enzymes were activated to metabolize the BaP, as reported by other studies (Almeida et al., 2012; Jönsson et al., 2009; Lu et al., 2009). Fish injected with the highest BaP dose (1000 μmolar BaP Kg^{-1}) presented EROD activity similar to the carrier control group (injected with corn oil). According to Gravato and Santos (2002, 2003), the inhibition of the EROD activity following exposure to high BaP doses can be due to increases in the concentration of highly reactive BaP metabolites, similar to our observations here. For some fish species there is a bell-shaped dose-dependent response curve for EROD activity (Bosveld et al., 2002; Lu et al., 2009), where high levels of pollutants cause enzyme inactivation or inhibition. This is an important factor to consider for investigations using EROD activity as a biomarker in this species, and stresses the relevance of coupling other biomarkers such as PAH biliary metabolites and s-SDH activity to measurement of EROD activity.

Among treatments, BaP biliary metabolite levels do not follow a similar pattern to the one observed for EROD activity, with the 1000 μmol BaP-treated group producing the highest biliary metabolite levels despite the inhibition of EROD activity at this dose. These results support the concept that EROD is not the sole

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*

enzyme responsible for metabolizing BaP. In fact, a myriad of P450 isoenzymes is known to be responsible for the biotransformation of a variety of xenobiotic compounds in fish (van der Oost et al., 2003). Despite different responses at higher BaP doses, the detection of PAHs in fish bile is an extremely sensitive measurement which reflects the absorption and metabolism of these compounds by fish (Aas et al., 2000; Nongnutch et al., 2012). In fact, PAH metablites can be measured in fish bile at levels 1000-times higher than in the surrounding water (Gagnon and Holdway, 1998). When injected IP, as in the present study, the biotransformation of BaP is still an extremely efficient process in fish, as demonstrated by all fish exhibiting higher BaP biliary metabolite levels relative to the carrier control fish after 96 h of the injection. These findings are in accordance with other studies which found high levels of PAHs biliary metabolites, including BaP metabolites, in fish experimentally exposed to these substances (Le DÛ-Lacoste et al., 2013; Nongnutch et al., 2012; Telli-Karakoç et al., 2002) or collected in contaminated areas (Neves et al., 2007; Ribeiro et al., 2013; Ruddock et al., 2002; Trisciani et al., 2011). Moreover, the highest IP BaP dose (1000 μmolar kg^{-1}) was statistically higher than all the other doses which indicate that metabolization activity was not inhibited by the high dose of BaP injected.

GST has an important role in Phase II of biotransformation, since this enzyme, together with GSH, acts in conjugation with exogenous compounds derived or not from Phase I biotransformation. It has been suggested that an increase in GST activity is indicative of efficient removal of xenobiotics from the body (Rinaldi et al., 2002). In the present study, the inactivation of the hepatic EROD activity in the highest BaP dose seems to be compensated by increases in GST activity. Other studies also related the GST activity with the metabolism of BaP (Banni et al., 2009; Gravato and Guilhermino, 2009; Gravato and Santos, 2003; Vieira et al., 2008) while some authors suggest that the GST has no role in BaP metabolism by fish (Beyer et al., 1997; Collier and Varanasi, 1990; van Schanke et al., 2001; Willett et al., 2000). In the present study, a positive correlation (Table 1) was observed between GST activity and levels of biliary BaP-type metabolites, suggesting the potential involvement of this enzyme in the BaP metabolism in tambaqui, especially when fish were subjected to high BaP doses.

Metabolization processes can lead to generation of reactive oxygen species (ROS). These ROS have been considered a biochemical challenge for fish exposed to PAHs (Regoli et al., 2011). The antioxidant system consists of enzymes such as SOD, CAT, and GPx, which are able to neutralize the ROS. SOD acts by neutralizing superoxide radicals, generating hydrogen peroxide molecules, which can be further neutralized by CAT. In the present study, both SOD and CAT enzymes were measured at higher levels at the highest BaP dose (1000 μmolar kg^{-1}) which, together with the GST activity, are induced to metabolize the high IP dose of BaP. Positive correlations occurred between SOD and CAT and between GST and both enzymes (Table 1), suggesting their co-involvement in BaP metabolism. SOD was also positively correlated with biliary BaP-type metabolites, which further suggests that this enzyme con-

tributes to ROS neutralization in tambaqui exposed to PAHs. While GPx activity is also important in ROS elimination and neutralization of peroxides, its activity was not statistically altered by BaP injection in the present study. However, a positive correlation was found between GPx and SOD (Table 1). In fish, enzymatic responses following exposure to organic xenobiotics may have no, little or significant change, with different patterns of responses between the enzymes, contaminant types, and fish species (Lemaire et al., 1996; Livingstone et al., 1993). Here, the combined action of these antioxidant enzymes resulted in metabolism of BaP at all injected doses.

The imbalance between the ROS generated and the antioxidant enzymes activity can lead to a ROS overproduction and, as a consequence, may cause oxidative stress at the cellular level (Wiernsperger, 2003). The interaction of ROS with the biological membranes can induce disturbances in the delicate structure, integrity, fluidity and permeability of the cell membrane, as well as loss of functionality through the products of lipid peroxidation (LPO) (Niki, 2009; Wiernsperger, 2003). We observed LPO in tambaqui at most BaP doses (10 μmolar BaP Kg^{-1} and higher), suggesting that direct damage of the biological membrane occurred mainly through BaP bioactivation. During BaP metabolism, metabolites such as 1,6–quinone and 3,6–quinone, derivates of 1-hydroxybenzo[a]pyrene and 3-hydroxybenzo[a]pyrene, are generated following an increase of SOD at higher doses of Ba Almeida et al. (2012) found increases of LPO in the liver of *Dicentrarchus labrax,* which also paralleled the increasing of BaP exposure concentrations. Our results support the conclusions of Almeida et al. (2012), who attributed the LPO increases to the oxidative stress experienced by the fish and to the direct action of bioactivated BaP metabolites.

Considering that the s-SDH enzyme is a cytoplasmic enzyme, found mainly in hepatocytes (Heath, 1995), its presence in the serum could indicate hepatocellular damage, potentially related to the overproduction of ROS (Webb and Gagnon, 2007). Our results suggest doses of 10, 100 and 1000 μmolar BaP Kg^{-1} caused hepatocellular damage, leading to the release of s-SDH from the cytoplasm of the hepatocyte into the serum. Elevated LPO activity at the three highest doses supports a causative relationship between dysfunctional cellular membranes and elevated s-SDH activity in the serum of tambaqui (Morris and Vosloo, 2006). Shailaja and D'silva (2003) also found hepatocellular damage related to PAHs exposure in the tropical cichlid *Oreochromis mossambicus,* even for fish exposed to the lowest concentration of 0.4 μg phenanthrene/g, demonstrating the efficiency of this enzyme for detecting hepatocellular injury. In the present study, a positive correlation (Pearson) between LPO activity and s-SDH activity illustrates the complementary nature of these measurements.

BaP bioactivation can also lead to genotoxicity, by leading to the formation of 7,8 D [BaP], which is converted into 7,8 D 9,10-epoxide, a highly carcinogenic and mutagenic xenobiotic (Varanasi et al., 1987). DNA breaks were observed in blood cells of tambaqui at all BaP doses, with the highest dose resulting in the highest levels

of DNA damage. Elevated DNA damage in blood cells was paralleled by elevated biliary BaP-type metabolites, suggesting that the bioactivation of the BaP metabolites lead to DNA damage in the blood cells. In addition, positive correlations (Pearson) were found between the DNA damage in blood cells and the BaP-type metabolites (Table 1), which confirms that these two biomarkers co-vary. Dévier et al. (2013) also found positive correlations between biliary PAHs-type metabolites and DNA damage in the blood cells of European seabass *Dicentrarchus labrax* collected in PAHs contaminated sites, further establishing a relationship between PAH exposure and genotoxic damage. Similar to our results, Mu et al. (2012) observed that inhibition of EROD activity was concomitant with DNA damage at high BaP exposure concentrations in medaka (*Oryzias melastigma*). The authors suggested that when EROD activity is inhibited, other CYP1 s enzymes may be more likely to produce adduct forming BaP metabolites than CYP1A. Our results are in accordance with this suggestion, considering the inhibition of EROD activity, the high BaP-type metabolite bile accumulation, and the high DNA damage found in the blood cells of fish treated with the highest BaP dose.

The suite of biomarkers measured in the present study demonstrates the potential of tambaqui for use as a bioindicator species in tropical environments. Biliary metabolites, GST, SOD, LPO and s-SDH activities, and DNA damage in blood cells were the most sensitive biomarkers in tambaqui treated with BaP. At 1000 μmolar BaP Kg^{-1} by IP injection, inhibition of EROD activity co-occurred with maximum BaP-type biliary metabolites, suggesting the involvement of other CYP1A enzymes in the metabolization of BaP or their inefficiency at higher levels of BaP, since tambaqui showed increased oxidative stress at 1000 μmolar BaP Kg^{-1}. At this dose, metabolic production of reactive BaP metabolites was associated with elevated antioxidant SOD activity and elevated LPO activity. Elevated s-SDH activity indicated hepatocellular damage as a consequence of high LPO, causing cellular membrane dysfunction and release of SDH into the bloodstream. In addition, the production of high levels of BaP metabolites was paralleled with elevated DNA damage in blood cells, suggesting a causal link between BaP metabolites and DNA damage. Finally, the correlations between various biomarkers demonstrate their complementary nature in the evaluation of the effects of PAHs in fish inhabiting contaminated environments and we conclude that this set of biomarkers are appropriate to detect contaminated sites using tambaqui as a bioindicator species.

Acknowledgments

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