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Expression of *cyp1a* induced by benzo(A)pyrene and related biochemical and genotoxic biomarkers in the neotropical freshwater fish *Prochilodus lineatus*

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ABSTRACT

The goal of this work was to design specific *cyp1a* primers for the fish *Prochilodus lineatus* to study the expression of this gene and its relation to the activity of biotransformation phase I enzyme (ethoxyresorufin O-deethylase - EROD) and genotoxic damage after 6 and 24 h of benzo(a)pyrene (B(a)P) intraperitoneal injection. In comparison to fish injected only with canola oil (vehicle), the expression of *cyp1a* and EROD activity both in the liver and gills were significantly higher after 6 and 24 h of B(a)P injection. A significant increase in DNA damage was detected in liver and blood cells after 6 h of B(a)P injection and in the gill cells after both times, probably caused by intermediate metabolites of B(a)P. Thus, the expression of *cyp1a* and its relationship with the corresponding enzyme activity is a potential biomarker for evaluation *P. lineatus* exposure to organic compounds.

1. Introduction

Classical approaches in ecotoxicological studies have focused on enzymatic assays and protein quantification. However, fish mRNA levels have also been successfully evaluated using reverse transcription followed by real-time PCR (RT-qPCR) (Costa et al., 2012; Chivittz et al., 2016). This approach differs from other available methods for analyzing gene expression in terms of accuracy, sensitivity and rapid results (Derveaux et al., 2010). But, RT-qPCR requires the design of high-specificity primers from the gene nucleotide sequence of the organism to be studied (An et al., 2011).

The mRNA of most of the genes that have been used as biomarkers encodes proteins or enzymes well-established as biochemical biomarkers, based on the assumption that changes in biochemical parameters run parallel with changes in the expression of the corresponding genes (Piña et al., 2007). Thus, in the induction process, the compound increases the expression of some genes, enhancing their mRNA levels and resulting in new proteins, culminating in increased enzymatic activity (Cousinou et al., 2000). Since gene expression profiles represent the first level of integration between environmental factors and corresponding response mechanisms (Cossu-leguille and Vasseur, 2013), an integrated biomarker approach is required to elucidate the processes involved in cellular responses (Janz, 2013).

The expression of genes involved in the biotransformation of xenobiotics is an important tool for establishing the dynamics of environmental influence on cellular expression patterns (Kassahn et al., 2007). Phase I biotransformation reactions are catalyzed by the subfamily of cytochrome P450 1 A (CYP1 As). Vertebrate CYP1 A is generally involved in the metabolism of drugs, hydrocarbons, steroids and fatty acids (Goldstone et al., 2010). The induction of CYP1 A in fish has been extensively studied and used as a biomarker of exposure to organic contaminants in environmental monitoring and ecotoxicological studies using enzymatic assays (e.g., activity of ethoxyresorufin-O-deethylase; EROD) (Whyte et al., 2000; Billiard et al., 2004; Costa et al., 2011; Karami et al., 2011). Futhermore, *cyp1a* mRNA levels in fish have been successfully evaluated using RT-qPCR (Piña et al., 2007; Lee et al., 2015). Phase II reactions conjugate endogenous polar groups, especially glutathione (GSH), with the xenobiotic or related metabolites resulting from phase I, making them easier to excrete and preventing these products from binding to other cellular components (Van der Oost et al., 2003). Glutathione S-transferases (GSTs) are one type of enzyme mediating phase II biotransformation (Glisic et al., 2015).

During the metabolism of the xenobiotics reactive oxygen species (ROS) are produced. Once the balance between ROS production and antioxidant defense is broken, ROS concentration is acutely or chronically enhanced leading to oxidative modification of cellular

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constituents, resulting in disturbance of cellular metabolism and regulatory pathways (Lushchak, 2016). The oxidation of lipids through the formation of peroxides (lipid peroxidation) is of especial concern in aquatic organisms, as they contain high amounts of lipids with polyunsaturated fatty acid residues, a substrate for oxidation (Lushchak, 2011). Oxidation of DNA is another effect of increased ROS in the cell and the comet assay has been frequently applied to monitor DNA damage in aquatic organisms (Jha, 2008).

Polycyclic aromatic hydrocarbons (PAH) are organic compounds containing two or more fused carbon rings (WHO, 2011) and one of the most widely studied PAHs is benzo(a)pyrene (B(a)P). It originates mainly from the incomplete combustion of fossil fuels, and industrial/domestic waste, reaching aquatic environments mainly by atmospheric deposition. In fish and other vertebrates, exposure to B(a)P results in the induction of enzyme systems involved in metabolizing xenobiotics (El-Kady et al., 2004) due to its high affinity to the aryl hydrocarbon receptor (AhR), which is responsible for activating a series of genes encoding biotransformation enzymes (Costa et al., 2011; dos Anjos et al., 2011).

The neotropical biogeographic region comprises Central America (including the southern part of Mexico and the peninsula of Baja California), the south of Florida, the Caribbean and South America and shows some of the highest numbers of fish families (Lévêque et al., 2008). Neotropical fish comprise approximately 30% of all fish species in the world and are currently being threatened by anthropogenic activities that are showing visible effects on freshwater ecosystems (Mastrochirico Filho et al., 2018). The neotropical freshwater teleost, *Prochilodus lineatus* (Valencienes, 1836), has already been shown to be sensitive to various classes of contaminants, including petroleum derivatives that contain large quantities of PAHs (Simonato et al., 2011). Unfortunately, for this neotropical fish species, there is to date no biotransformation-related DNA gene sequence whose expression could be analyzed after exposure to a xenobiotic. Considering this gap in the knowledge of the effects of contaminants in the expression of biotransformation enzymes of neotropical fish species, this paper standardizes the use of the RT-qPCR technique to access the *cyp1a* gene expression of *P. lineatus*. CYP1 A was induced by B(a)P, and its expression was analyzed, together with its relation to the activity of biotransformation enzymes, oxidative stress and the genotoxic parameters of *P. lineatus,* 6 and 24 h after hydrocarbon injection.

2. Material and methods

2.1. Experimental procedures

Juvenile *Prochilodus lineatus* (13.62 ± 3.31 cm, 17.29 ± 4.98 g, n = 80) were obtained from the Fish Hatchery Station at State University of Londrina, Parana, Brazil. The fish were acclimatized in the laboratory for five days in tanks containing 300 L of clean, dechlorinated fresh water with constant aeration at a temperature of 24 ± 1 °C. After anesthesia with benzocaine (0.1 g L^{-1}), a group of fish (B(a)P; n = 6–8) received a single intraperitoneal injection of benzo(a)pyrene ($C_{20}H_{12}$ - Sigma-Aldrich, Brazil) dissolved in canola oil at a dose of 20 mg kg^{-1}. Another group of fish (Oil; n = 6–8) received an equal volume of the canola oil (2 mL kg^{-1}) used as a vehicle for the contaminant, following the work of Oliveira et al. (2013). This procedure was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina (Process 5210.2015.38). Intraperitoneal injection was used to expose the animals to B(a)P because of the direct delivery of the contaminant to the organism while minimizing the risks of B(a)P dispersion into the environment (Karami et al., 2011). The dose of B(a)P of 20 mg kg^{-1} was selected based on the work of Oliveira et al. (2013) who investigate the hepatic metabolism of BaP in the fish *Rhamdia quelen*.

After injection, each group of fish (B(a)P and Oil) was kept in glass aquaria containing 80 L of clean, dechlorinated fresh water for 6 and 24 h. After each experimental period, the fish were anesthetized in benzocaine (0.1 g L^{-1}) and a blood sample taken from the caudal vein using heparinized syringes. Shortly after, the animals were killed by medullar section for removal of the liver and gills. Three gill arches on the right, as well half of the right liver lobe, were immersed in Trizol® (Invitrogen - Life Technologies, USA) in individual cryogenic tubes and immediately frozen in liquid nitrogen for molecular analysis. Three gill arches on the left, as well half of the left liver lobe, were stored in individual tubes for biochemical analysis. Finally, the first gill arches on both right and left, as well the other halves of the right and left lobes of the liver were immersed in saline solution for subsequent cell dissociation for the comet assay.

2.2. Isolation of total RNA

Total hepatic and branchial RNA was extracted using the Trizol protocol, following the manufacturer's instructions (Invitrogen - Life Technologies, USA) but with some changes: the samples were homogenized in Vortex equipment (3 min) and incubated (10 min) at room temperature to allow complete dissociation of the nucleoprotein complex. Then, chloroform (0.2 mL per 1 mL Trizol) was added and the tubes shaken vigorously by hand for 10 s at 10-second intervals. The liquid content was transferred to another tube (separation of whole tissue) and incubated (15 min), shaking manually every 5 min. The samples were centrifuged (12.000 *g*, 20 min, 4 °C) and the aqueous phase (0.4 mL) transferred to another tube. The samples were treated with solutions of phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) in subsequent centrifugation under the conditions described above. After these steps, the total RNA was washed in 75% ethanol, precipitated in absolute ethanol and eluted in DEPC water (Invitrogen - Life Technologies, USA). Total RNA was estimated in a NanoDrop 2000 spectrophotometer (Thermo Scientific) at a wavelength of 260 nm. The respective concentrations of the total RNA (mean ± SD) extracted from the liver and gills were 3081.93 ± 2467.12 ng μL^{-1} and 621.03 ± 308.14 ng μL^{-1}. The value at 260/280 nm (mean ± SD) was 2.01 ± 0.14. To ensure the integrity of the total RNA extracted, the 28S and 18S ribosomal RNA bands were confirmed on 1% agarose gel, according to Aranda et al. (2012).

2.3. Design of specific primers for β-actin and cyp1a

A pair of nonspecific degenerate primers for amplification of the *cyp1a* mRNA of *P. lineatus* was designed based on conserved regions in the sequences of *Carassius gibelio* (GQ353297.1); *Ctenopharyngodon idella* (DQ211095.1); *Cyprinus carpio* (AB048939.1); *Danio rerio* (NM_131879.1) and *Pimephales promelas* (AF232749.1), obtained from the National Center for Biotechnology Information (NCBI: www.ncbi.nlm.nih.gov). The BioEdit Sequence Alignment Editor was used to align the sequence (Hall, 1997) and *cyp1a* primers were designed using GeneRunner software. The β-actin product of *P. lineatus* was amplified using a pair of universal primers with highly conserved sequences encoding the *β-actin* gene (Chaty et al., 2004). All the degenerate primers used are described in Table 1.

A conventional PCR was performed using 50 ng of cDNA synthesized from mRNA samples of *P. lineatus* using degenerate primers. cDNA synthesis is described below. Several amplification conditions were tested to optimize the PCR reaction: initial denaturation for 10 min at 95 °C; 40 cycles of denaturation at 95 °C for 15 s and 30 s; one annealing gradient from 50 to 60 °C for 15 s, 30 s and 1 min; polymerization at 72 °C for 30 s and 1 min; and a final extension of 2 min at 72 °C. After optimization, the PureLink PCR Purification kit (Invitrogen - Life Technologies, USA) was used to purify the amplification products, which were then sent to the Macrogen Incorporation for sequencing. The nucleotide sequences obtained were identified by comparison with databases available at NCBI using the BLAST program (Basic Local

Table 1
Degenerate primer sequences used for amplifying the products of *cyp1a* and *β-actin* and species-specific primers for *P. lineatus* designed for use in the RT-qPCR reaction.

Gene	Degenerate primers	
cyp1a		
cyp1a forward	5' - CARGTGTCHGATGAGAAGAT - 3 '	
cyp1a reverse	5' - CAGTGTGAGGAATGGTGAA - 3 '	
β-actin (Chaty et al., 2004)		
β-actin forward	5 '- GGAYGAYATGGAGAARATCTGG - 3'	
β-actin reverse	5 '- CCTGYTTGCTGAYCCACATCTG - 3'	
Gene	**Primer**	**Amplified product**
cyp1a		
cyp1a forward	5' - GAGATTCAGGAGAGGCTTC - 3'	197 pb
cyp1a reverse	5' - CCAGGTAGGGCAGGTTG - 3'	
β-actin		
β-actin forward	5' - CTCTGCTATGTTGCCCTTG - 3'	94 pb
β-actin reverse	5' - AGTTGAAAGTGGTCTCGTG - 3'	

Alignment Search Tool). The sequences obtained were used as a basis for designing species-specific *β-actin* and *cyp1a* primers for *P. lineatus*, as shown in Table 1.

2.4. Reverse transcriptase and cDNA synthesis

The cDNA was synthesized in a T100 Thermal Cycler (Bio-Rad). First, an aliquot containing 4 µg of total RNA (125 ng) was taken from all samples and combined with 12 µL of a mix prepared with 4 µL of random primers (20 pmol μL^{-1}) (Invitrogen - Life Technologies, USA), 4 µL of oligo dT (20 pmol μL^{-1}) (Invitrogen - Life Technologies, USA) and 4 µL of dNTPs (2.5 mM - Invitrogen - Life Technologies, USA). The samples were kept for 15 min at 65 °C in the thermal cycler. Then, they were put on ice and the following added to each sample: 4 µL of mix prepared with 1.3 µL of DEPC water, 2 µL of 10X buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl - Invitrogen - Life Technologies, USA), 0.6 µL of $MgCl_2$ (50 mM - Invitrogen - Life Technologies, USA), 0.05 µL of RNase Out (Invitrogen - Life Technologies, USA) and 0.05 µL of M-MLV reverse transcriptase enzyme (Invitrogen - Life Technologies, USA). The reactions were incubated at 25 °C for 2 min, kept at 37 °C for 50 min and then kept at 70 °C for 15 min. cDNA synthesis reactions were performed in triplicate to minimize variations in the efficiency of the reverse transcriptase enzyme, and then combined to obtain a single mixture for each sample. cDNA samples were quantitated by spectrophotometry and adjusted to a final concentration of 50 ng μL^{-1}.

qPCR was conducted using 10 µL Platinum SYBR Green qPCR Supermix - UDG (Invitrogen - Life Technologies, USA) (10 pmol μL^{-1}), 1 µL of each pair of primers, 2 µL of DEPC water and 6 µL of cDNA (50 ng mL^{-1}) from each sample, bringing the final reaction volume to 20 µL. After optimization, qPCR reactions were performed under the following conditions: 50 °C for 2 min, 95 °C for 5 min and 45 cycles (95 °C/15 s, 60 °C/15 s, 72 °C/20 s) in a CFX96 Real-Time System (Bio-Rad). The melting curve was analyzed at the end of each reaction with a temperature gradient of 60 to 95 °C, and a 5-second read every 0.5 degrees. Reactions were performed in triplicate. CFX Manager 3.1 (Bio-Rad) software was used to access the data. The *β-actin* was used as reference gene to normalize expression levels.

2.5. Biochemical biomarkers

Samples of liver and gill were weighed, homogenized in potassium phosphate buffer (0.1 M, pH 7.0) and centrifuged (10000 *g*, 20 min, 4 °C). The supernatant was used for biochemical analysis.

The catalytic activity of CYP1 A was determined by ethoxyresorufin-O-deethylase (EROD) activity based on the conversion of 7-ethoxyresorufin, according to Eggens and Galgani (1992), with some modifications. The reaction was initiated by adding the reactive mixture (0.1 M K phosphate buffer, pH 7.6, 2 mM NADPH, and 0.1 mM 7-ethoxyresorufin) to the sample. The gradual increase in fluorescence resulting from the formation of resorufin was measured at 1-min intervals for 30 min (excitation: 530 nm; emission: 590 nm). EROD activity was expressed in picomoles resorufin per minute milligrams of $protein^{-1}$, based on a resorufin standard curve.

The activity of glutathione S-transferase (GST) was determined using the method described by Keen et al. (1976), based on the GST catalyzed conjugation of reduced glutathione (GSH) with the 1-chloro-2,4-dinitrobenzene (CDNB) substrate. The increase in CDNB conjugate was monitored for 1 min using a spectrophotometer at 340 nm. Enzyme activity was expressed in nanomole CDNB conjugate per minute milligram of $protein^{-1}$.

The concentration of reduced glutathione (GSH) was determined according to the method in Beutler et al. (1975), by the reaction of glutathione with the color reagent 5,5-dithiobis-2-nitrobenzoic acid (DTNB), forming a thiolate anion (TNB), which was measured at 412 nm. The GSH concentration was expressed in microgram GSH per milligram of $protein^{-1}$, based on a GSH standard curve.

Lipid peroxidation (LPO) was estimated based on the production of malondialdehyde (MDA), one of the final products of lipid peroxidation, and measured by the TBARS assay (thiobarbituric acid reactive substances), according to Camejo et al. (1998). Butylated hydroxytoluene (BHT 1 M), phosphate-buffered saline (2 mM KCl, 1.4 mM NaH_2PO_4, 357 mM NaCl, 10 mM Na_2HPO_4, pH 7.4), trichloroacetic acid (TCA 50%), and thiobarbituric acid (TBA 1.3%) dissolved in 0.3% NaOH were added to the supernatant and the mixture kept at 60 °C for 1 h. A fluorescence reading was then taken (ex/em: 535/590 nm) and the TBARS content determined in terms of MDA equivalents based on a standard curve for known concentrations of MDA (malonaldehyde bis (dimethyl acetal)) reagent. The concentration of TBARS was expressed in nanomole TBARS milligram of $protein^{-1}$.

Protein concentration was determined according to Bradford (1976), based on the reaction of proteins with Coomassie Brilliant Blue G-250 dye. The bovine serum albumin (BSA) standard curve was used for calibration, and absorbance read at 595 nm in a spectrophotometer. The total protein in each sample was used to express EROD, GST, GSH and TBARS data.

2.6. DNA damage

In order to evaluate DNA damage, the alkaline comet assay developed by Singh et al. (1988), as modified by Cavalcante et al. (2008), was performed using three cell types: erythrocytes, liver cells and gill cells. After sampling, aliquots of blood were added to fetal bovine serum (GIBCO - Invitrogen). Liver and gill cells were dissociated mechanically, followed by chemical dissociation with trypsin (0.1% for gills and 0.05% for liver). The solution was then filtered into a tube containing a 10% fetal bovine serum to stop enzymatic digestion. The resultant solution was centrifuged (10 min, 1000 *g*) and the pellet re-suspended in fetal bovine serum for use in the comet assay. From this point onward, the same procedures were repeated for each cell type. The comet assay was performed exclusively on blood, gill and liver samples with cellular viability greater than 80%, determined by the Trypan blue exclusion test.

The cells were mixed with a solution of 0.5% low melting point agarose and then spread on histological slides previously coated with normal melting point agarose (1%). The basic steps of the comet assay were executed as follows: (a) lysis — slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mLTriton X-100, pH 10.0) at 4 °C, shielded from light, for 1 to 2 h; (b) DNA unwinding — 30 min in electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); (c) electrophoresis — 20 min, 300 mA, 25 V, 1 V cm − 1; and (d) neutralization — three washes of 5 min each in buffer (0.4 M Tris, pH 7.5). The slides were then fixed with absolute ethanol for

10 min and kept under refrigeration pending cytological analysis. After staining with GelRed, the slides were coded and analyzed in a fluorescence microscope. One hundred nucleoids per fish were analyzed, and each nucleoid classified according to the damage exhibited: class 0 = absence of comet tail, class 1 = comet tail smaller than the nucleoid diameter, class 2 = comet tail larger than the nucleoid diameter, and class 3 = comet tail more than twice the nucleoid diameter. To calculate the damage score, the number of cells in each class (0, 1, 2, or 3) was multiplied by the value for each class and the final score calculated using the formula (0 × A) + (1 × B) + (2 × C) + (3 × D), where A, B, C, and D are the number of cells in each class (Collins et al, 2008).

2.7. Statistical analysis

The results are expressed as means, ± standard error. The statistical validation of the reference and relative gene expression were determined by ΔΔCT (Livak and Schmittgen, 2001). The means obtained for mRNA expression, biochemical analysis and the comet assay for the different treatments were compared (Oil x B(a)P) for each experimental period by parametric (Student's t test) or non-parametric (Mann-Whitney) analysis, according to the distribution of the data (normality and homoscedasticity). The significance level was $p < 0.05$.

3. Results

3.1. Expression of cyp1a

The relative expression of *cyp1a* in *P. lineatus* liver tissue was significantly higher in the B(a)P group compared to the Oil group at 6 and 24 h after B(a)P injection (Fig. 1A). The greatest increase in *cyp1a* expression occurred after 24 h (18 times higher). At 6 h after injection, it was 11.46 times higher. The relative expression of *cyp1a* in the gill tissue of *P. lineatus* was also significantly higher in the B(a)P group compared to the Oil group for both experimental periods. In the liver tissue, the greatest increase in expression of *cyp1a* occurred after 24 h (10.89 times higher). After 6 h it was 5.39 times higher (Fig. 1B).

3.2. EROD and GST activities

Liver EROD activity was significantly higher in the B(a)P groups compared to the Oil groups (Fig. 2A), and the highest activity occurred after 6 h. Gill EROD activity in the B(a)P group was significantly higher than that of the Oil group for all experimental periods, but the highest activity was detected at 24 h after injection (Fig. 2B). GST activity showed no significant change in the different organs for both experimental periods (Fig. 3A and B). However, it was higher in the liver than in the gills.

3.3. GSH content

Intraperitoneal injection of B(a)P resulted in a significant decrease in the GSH content of the liver after 6 h compared to Oil group (Fig. 4A). However, no significant change was observed after 24 h. In the gills (Fig. 4B), no significant changes at all were observed.

3.4. Lipid peroxidation

Intraperitoneal injection of B(a)P did not lead to any significant alteration in LPO in the liver or gills of *P. lineatus,* irrespective of the experimental period (Fig. 5).

3.5. DNA damage

The DNA damage score for erythrocytes, liver and gills cells of the B(a)P group was significantly higher than in the Oil group at 6 h after intraperitoneal injection (Fig. 6A, 6B and 6C). Erythrocytes and liver cells showed no significant changes in the damage score at 24 h. However, the DNA damage score for the gill cells of the B(a)P group was significantly higher than that of the Oil groups after 24 h (Fig. 6B).

4. Discussion

RT-qPCR is a robust technique for analysis of gene expression that provides high sensitivity and accuracy (dos Anjos et al., 2011). However, to perform the RT-qPCR assay it is essential to design specific primers (An et al., 2011). The introduction of high-throughput next-generation DNA sequencing technologies (NGS) has transformed the study of transcripts, allowing RNA analysis from cDNA sequencing (Ozsolak and Milos, 2011). Based on cDNA sequencing, it is possible to get only the nucleotide sequence of the gene. In our study, the nucleotide sequence obtained from the synthesized cDNA of the gene of interest was applied to find a region of the *cyp1a* gene for designing species-specific primers for use in RT-qPCR. A similar procedure was described in the literature on *cyp1a* cDNA sequencing in fish (*Sparus aurata* and *Liza aurata*) performed by Cousinou et al. (2000). Since no genome sequence for *P. lineatus* is currently available, this study is the first to present species-specific primers of *β-actin* and *cyp1a*, which allowed the use of RT-qPCR for *cyp1a* gene expression analysis, which means that further studies of this species can be conducted using RT-qPCR.

In our study, *cyp1a* gene expression increased significantly in the liver and gills of *P. lineatus* after intraperitoneal injection of B(a)P (20 mg kg^{-1}) for both experimental periods. In vertebrates, B(a)P induces the expression of CYP1 A and other proteins of cytochrome P450 through binding with the aryl hydrocarbon receptor (AhR) (Hahn, 2002; Tian et al., 2015). AhR is an intracellular transcriptional factor that, once bound with the xenobiotic, allows the xenobiotic-AhR

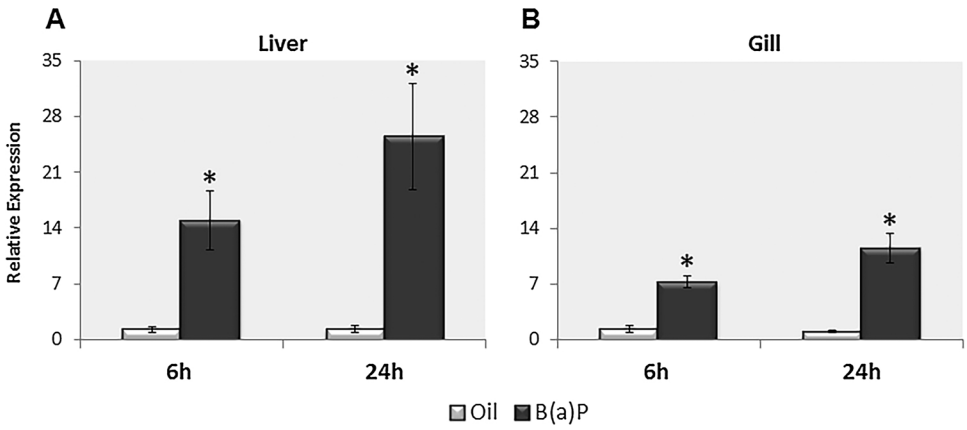

Fig. 1. Values (mean ± SE, n = 6) of *cyp1a* relative gene expression in the liver (A) and gill (B) of *P. lineatus* at 6 and 24 h after intraperitoneal injection of B(a)P (20 mg kg^{-1}) or the vehicle (Oil) only. The values were normalized by reference to the expression of the *β-actin* gene. * Indicates a significant difference between the B(a)P group and the respective Oil group ($p < 0.05$).

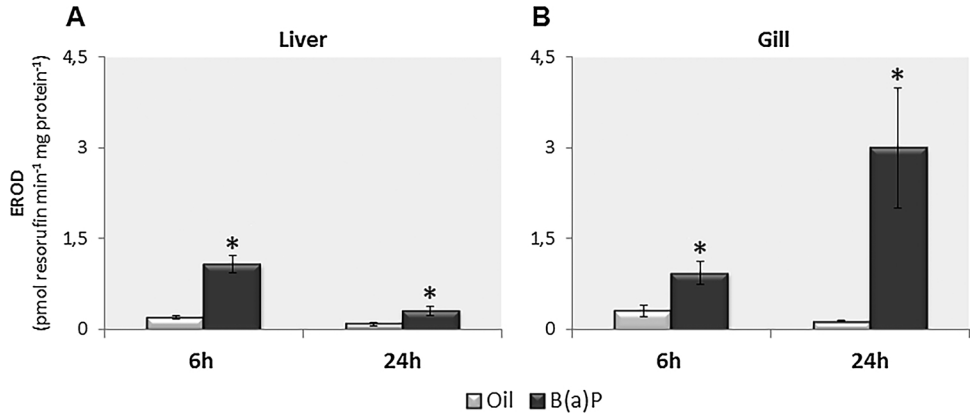

Fig. 2. Activity (mean ± SE, n = 6–8) of 7-ethoxyresorufin-O-deethylase (EROD) in the liver (A) and gills (B) of *P. lineatus* at 6 and 24 h after intraperitoneal injection of B(a)P (20 mg kg^{-1}) or the vehicle (Oil) only. * Indicates a significant difference between the B(a)P group and the respective Oil group ($p < 0.05$).

complex to be translocated to the nucleus and activates xenobiotic response elements (XRES), as the *cyp1a* gene (dos Anjos et al., 2011). Since AhR receptors are inactived in the cytoplasm by a protein complex and need to bind to a xenobiotic to activate XRESs, the complex B(a)P-AhR triggers the AhR signaling pathway, activating the transcription of target gene *cyp1a* in the gills and liver of *P. lineatus*, that persisted even 24 h after injection of the hydrocarbon.

In fish, the subfamily CYP1 A is the most studied among the P450 cytochrome family, since it is induced by various contaminants, including PAH, and P450 activity is widely used as a biomarker for PAH effects on aquatic organisms (An et al., 2011; dos Anjos et al., 2011; Karami et al., 2011; Ferreira et al., 2014;). The increase observed in EROD activity in the liver and gills of *P. lineatus* lends further support to what is already known about the CYP1 A induction mechanism in fish exposed to PAHs. During this biotransformation phase, an atom of molecular oxygen is incorporated into the xenobiotic molecule, enhancing its solubility in water and transforming it into a substrate for phase II reactions (Di Giulio et al., 1995).

The CYP1 A enzyme is expressed mainly in the liver, but is also found in other tissues, such as the gills (Sarasquete and Segner, 2000). Previous studies suggest that the gills are useful indicators of the health of aquatic organisms, and the induction of *cyp1a* in the liver could be influenced by the gills during exposure to B(a)P (Levine and Oris, 1999; Chen et al., 2012). If the contaminant is dissolved in water, the gills may induce stronger *cyp1a* expression and activity than the liver, and are therefore important target organs for organic pollutants (Lee et al., 2015). Thus, the significant increase in EROD activity observed in the gills after B(a)P intraperitoneal injection may indicate the sensitivity of the gills to organic pollutants, although the exposure route was not water. Simonato et al (2011) showed the induction of CYP1 A in *P. lineatus* exposed to the water-soluble fraction of gasoline; these authors detected a significant increase in EROD activity in liver and gills at 24 and 96 h after exposure.

A simultaneous increase in the expression and activity of *cyp1a* gene was also observed in the liver of *P. lineatus*. The relationship between the expression of *cyp1a* gene and catalytic activity of the enzyme it encodes has already been described in the literature on other species of fish. Zapata-Perez et al. (2002) reported that *Oreochromis niloticus* specimens injected with pyrene (20 mg Kg^{-1}) showed increased hepatic expression of *cyp1a* one day after injection, followed by small decreases on 3rd and 5th days, but no complete return to basal levels. Furthermore, EROD activity also increased during the first day after injection, and reached the maximum activity on the 3rd day, showing only a slight drop on the 5th day, also no complete return to basal levels. In another study Costa et al. (2011) found that, after exposure to water contaminated with B(a)P, Nile tilapia (*Oreochromis niloticus*) showed an increase in EROD activity. The same laboratory conducted a further study on the same species, showing that the hepatic and branchial increase in *cyp1a* expression is reflected in increased functionality of the protein (Costa et al., 2012). In our study, we observed that the gene expression and enzymatic activity of CYP1 A in the liver and gills were significantly higher after 6 h and remained high after 24 h. Although the maximum expression and activity of the enzyme were not of equal magnitude, both parameters remained high without returning to basal levels.

Despite the increase in liver and gill EROD activity during the experimental periods, no significant modifications in GST activity in any of the organs and for any experimental period were observed. The

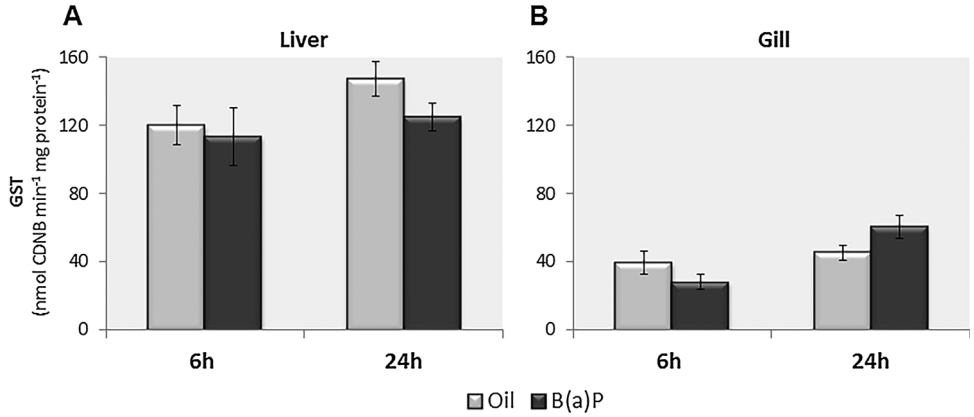

Fig. 3. Activity (mean ± SE, n = 6–8) of Glutathione S-transferase (GST) in the liver (A) and gills (B) of *P. lineatus* at 6 and 24 h after intraperitoneal injection of B(a)P (20 mg kg^{-1}) or the vehicle (Oil) only ($p < 0.05$).

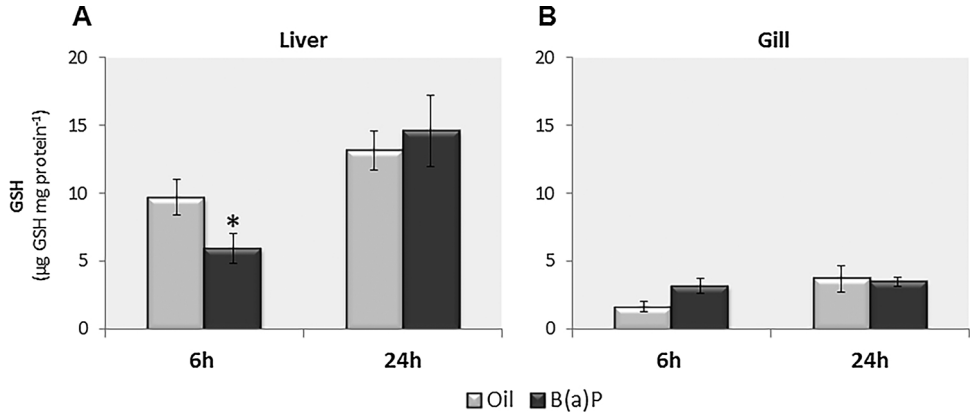

Fig. 4. Values (mean ± SE, n = 6–8) of glutathione content (GSH) in the liver (A) and gills (B) of *P. lineatus* at 6 and 24 h after intraperitoneal injection of B(a)P (20 mg kg^{-1}) or the vehicle (Oil) only. * Indicates a significant difference between the B(a)P group and the respective Oil group ($p < 0.05$).

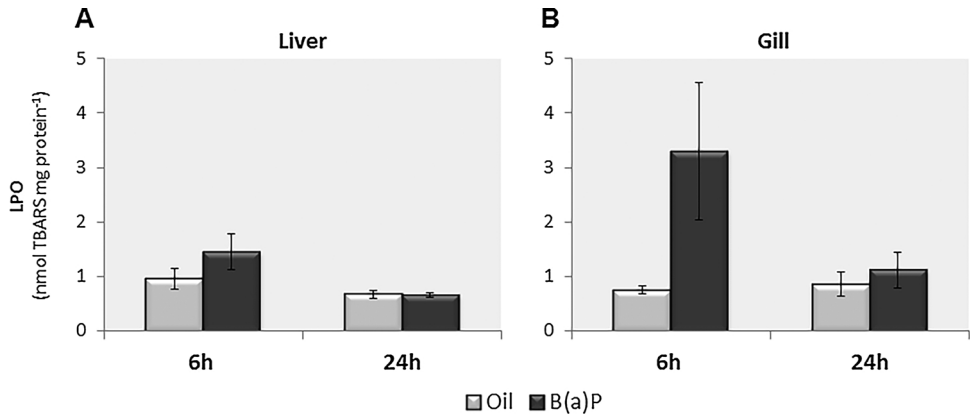

Fig. 5. Values (mean ± SE, n = 4–8) of lipid peroxidation (LPO) in the liver (A) and gills (B) of *P. lineatus* at 6 and 24 h after intraperitoneal injection of B(a)P (20 mg kg^{-1}) or the vehicle (Oil) only.

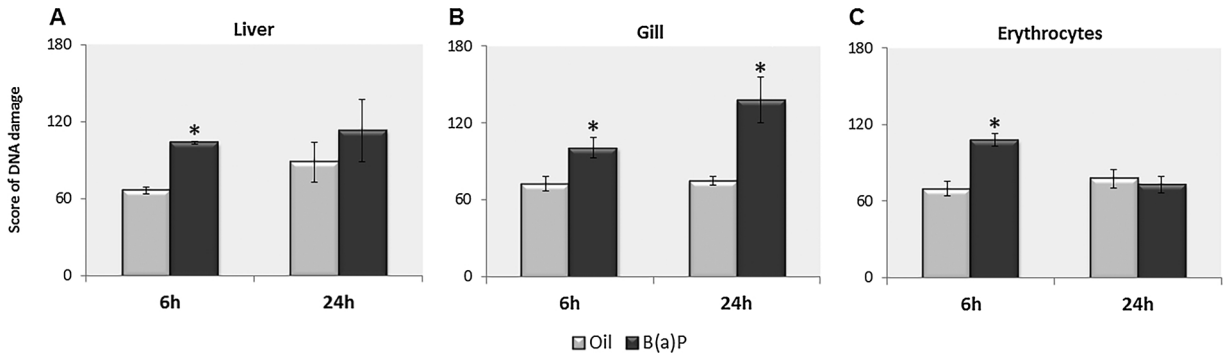

Fig. 6. DNA damage score (mean ± SE, n = 6) in the liver (A), gills (B) and erythrocytes (C) of *P. lineatus* at 6 and 24 h after intraperitoneal injection of B(a)P (20 mg kg^{-1}) or the vehicle (Oil) only. * Indicates a significant difference between the B(a)P group and the respective Oil group ($p < 0.05$).

phase I biotransformation of B(a)P leads to the production of a number of compounds, including electrophilic diol epoxides (BPDE) (Dolcetti et al., 2002; Pisoni et al., 2004), which are considered carcinogens as they bind to DNA and react with proteins (Miller and Ramos, 2001; Miranda et al., 2006). BPDEs can be conjugated with glutathione by GST, reducing its toxicity, or converted to phenols or diols that can be conjugated with glucuronide or sulfuric acid for excretion (Crowell et al., 2011). The last two conjugations (with glucuronic acid and sulfuric acid) are catalyzed respectively by UDP-glucuronosyltransferase (UGT) and PAPS-sulfotransferase (SULT) enzymes (James, 2011), forming alternative routes for phase II biotransformation. Thus, as the data from our study showed no significant alterations in the GST activity in any *P. lineatus* organs after B(a)P injection we might suggest that B(a)P and/or its metabolites were metabolized by the action of UGT or SULT, which do not involve the consumption of GSH or activation of GSTs.

In the present study, the production of BDPEs or other metabolites might have occurred as we detected DNA damage, in addition to increased activity and expression of *cyp1a*. It is interesting to note that DNA damage also occurred in the gills, which were not in direct contact with the B(a)P. One possible explanation is that some of the B(a)P metabolites formed in the liver, as well as the non-metabolized compound, were excreted by phase III transporter proteins and therefore could have reached the gills, causing DNA damage, and increased CYP1 A activity and expression. Membrane transporter proteins called ABC (ATP-binding Cassette) are responsible for the efflux of the

biotransformation metabolites, characterizing biotransformation phase III (Ferreira et al., 2014). It is believed that, as in mammals, these ABC transporters in fish promote the extrusion of unmodified xenobiotics and metabolites resulting from phase I or phase II (Epel et al., 2008). Paetzold et al. (2009) investigating the relationship among ABC transporters, phase I (CYP1 A1) and phase II (GST) enzymes in killifish (*Fundulus heteroclitus*), from a heavily polluted area, showed that the up-regulation of phase I and II enzymes and complementary ABC transporters may confer contaminant resistance to this fish.

We did not detect any significant increase in lipid peroxidation, indicated by TBARS levels, in the liver and gills of *P. lineatus* during the experimental periods. However, GSH concentration dropped significantly in the liver at 6 h after B(a)P injection and GST activity remained unchanged. GSH is an effective antioxidant that directly neutralizes pro-oxidant compounds and acts as antioxidant enzyme cofactor, such as GSH-dependent peroxidase (Lushchak, 2011; 2016). Thus, we might suppose that liver GSH was consumed within 6 h as a direct or indirect antioxidant to prevent lipid peroxidation and other antioxidant defense mechanisms, not measured in this study, were effective to prevent liver and gill oxidative damage at 24 h after B(a)P injection.

DNA damage can be directly caused by the xenobiotics themselves, by activated metabolites or by the ROS produced during biotransformation. The PAH biotransformation process routinely converts xenobiotics into highly reactive intermediate toxic substances. The results of the comet assay showed a significant increase in the occurrence of DNA damage in gill, liver, and blood cells at 6 h after B(a)P injection, and as mentioned before this could be related to BPDEs, intermediate reactive metabolites resulting from the phase I biotransformation of B(a)P. The occurrence of DNA damage after exposure to PAHs has been demonstrated in a study conducted by Vanzella et al. (2007), in which *P. lineatus* exposed to the soluble fraction of diesel oil showed a significant increase in damage score after acute and sub-chronic exposures. In our study, despite the fact that hepatic EROD activity remained significantly higher, there was no increase in DNA damage in the liver after 24 h. However, as the B(a)P was injected only once (at the beginning of the experiment) and EROD activity remained high, the concentration of B(a)P dropped, resulting in a drop in the BPDEs formed as time progressed. Moreover, since the comet assay detects repairable DNA breaks, genotoxic damage may have occurred but been repaired during the 24 h experimental period.

Of the organs examined, we expected the liver to exhibit the most intense response to B(a)P exposure, since it was introduced into the fish by intraperitoneal injection. Changes in *cyp1a* expression and EROD activity, as well as the DNA damage observed in the gills might be the result of the circulation of B(a)P and metabolites produced in phase I and II and translocated from the liver into the bloodstream by ABC transporters (Ferreira et al., 2014). The significant increase in blood cell DNA damage at 6 h after B(a)P injection supports this hypothesis.

Gill responses to B(a)P exposure by intraperitoneal injection reflect the sensitivity of this organ to physiological changes in the whole organism. In a study by Lee et al. (2015), four fish species (*Oryzias latipes, Danio rerio, Cyprinus carpio,* and *Zacco platypus*) were exposed to B(a)P dissolved in water and the highest induction of *cyp1a* expression was shown to be in the gills rather than liver. However, the highest levels of *cyp1a* expression in the gills were reached later than those in the liver, which shows a significant but slower response in the gills. In our study, there was a significant increase in DNA damage in gill cells after 6 and 24 h, while in other cell types an increase in DNA damage occurred after only 6 h. The gill response is therefore slower, either as a result of the exposure route or the kinetic response characteristics of the organ itself. In fish the expression of repair genes and the mechanisms activity are specie and tissue specific (Kienzler et al., 2013). A previous work with *P. lineatus* have already suggested that the repair system in gill cells is slower than in erythrocytes and consequently damaged cells could remain longer in the gill tissue (Cavalcante et al., 2008).

5. Conclusions

The cDNA sequencing of the *cyp1a* gene in *P. lineatus* and the specific primers designed were efficient and allowed the use of RT-qPCR for gene expression analysis. Enzyme activity (EROD), as well as the expression of the gene itself, increased in the gills and liver at 6 h after B(a)P injection, and remained high in both organs for 24 h, confirming that they are sensitive biomarkers for exposure of *P. lineatus* to B(a)P. Although there was a transient drop in GSH content in the liver, GST activity and LPO were not significantly modified, suggesting that metabolism of B(a)P involves other biotransformation pathways and antioxidant defenses in the liver and gills of *P. lineatus*. The DNA damage detected in the liver, gill and blood cells of *P. lineatus* at 6 h after the injection of B(a)P was probably caused by reactive metabolites of B(a)P and is indicative of the efflux of these compounds from the metabolizing organs and their circulation through the body. In the near future, we intend to use sequencing techniques to identify the genes involved in phase II (GSTs, UGTs and SULTs) and phase III (ABC transporters) of PAH biotransformation and study their expression and activity in order to elucidate the biotransformation mechanisms in the different organs of Neotropical fish *P. lineatus*.

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