Ecotoxicology (2011) 20:1400–1410
DOI 10.1007/s10646-011-0697-y

Gasoline effects on biotransformation and antioxidant defenses of the freshwater fish *Prochilodus lineatus*

Juliana D. Simonato · Marisa Narciso Fernandes · Cláudia B. R. Martinez

Accepted: 30 April 2011 / Published online: 11 May 2011

Abstract Biochemical biomarkers in the Neotropical freshwater fish *Prochilodus lineatus* were evaluated following acute exposures to the water-soluble fraction of gasoline (WSFG). Fish were exposed to the WSFG diluted to 5% in water (WSFG group) or only to water (Control group) for 6, 24 and 96 h and the gills and liver were removed for the biochemical analyses. Fish exposed to WSFG for 24 and 96 h showed significant increase in the activity of 7-ethoxyresorufin-*O*-deethylase (EROD) and glutathione-S-transferase (GST) both in liver and gills, pointing toward phase I and phase II biotransformation of the compounds present in the WSFG. The results also indicated the activation of antioxidant defenses in both the liver and gills after fish exposure to WSFG. The liver showed activation of catalase (CAT) and glutathione peroxidase (GPx) after 96 h exposure. An increase in hepatic content of reduced glutathione (GSH) together with decreased glutathione reductase (GR) activity was observed after 24 and 96 h of exposure to WSFG. In the gills, only catalase (CAT) activity augmented after 6 and 24 h of exposure and GSH content increased after 24 h of WSFG exposure. However, in both the organs, activation of the antioxidant defenses was not enough to prevent oxidative damage since they showed lipid peroxidation (LPO) at one of the experimental times: the liver after 6 h and the gills only after 96 h of exposure to WSFG. This may indicate better adaptation of the liver to longer exposures, starting from 24 h. As the gills are the first organ to be exposed to xenobiotics, the antioxidant defenses were triggered immediately upon exposure to WSFG and were able to prevent the occurrence of LPO during the initial times.

Keywords Biomarkers · CYP1A · Hydrocarbons · Lipid peroxidation · Neotropical fish · Oxidative stress

J. D. Simonato · C. B. R. Martinez (✉)
Department of Physiological Sciences, State University of Londrina, CP 6001, Londrina, PR 86051-990, Brazil
e-mail: cbueno@uel.br

M. N. Fernandes
Department of Physiological Sciences, Federal University of São Carlos, CP 676, São Carlos, SP 13565-905, Brazil

Introduction

Gasoline is the most common automotive fuel and is widely used throughout the world. The contamination of water by this petroleum derivative is mainly attributed to small but continuous leaks in storage tanks. These leaks reach water bodies, thus it is important to understand the toxic potential of gasoline for aquatic organisms which is directly related to the most water-soluble aromatic hydrocarbons, i.e., benzene, toluene, ethylbenzene and xylene (BTEX hydrocarbons), and to some polyaromatic hydrocarbons such as naphthalene, phenanthrene (Petrobrás 2003; Tiburtius et al. 2004). Several studies have demonstrated the effects of exposure to petroleum and its derivatives on the antioxidant defense pathways of fish (Achuba and Osakwe 2003; Pacheco and Santos 2001a; Zhang et al. 2004). Fish exposed to petroleum hydrocarbons may display several types of oxidative damage, such as lipid peroxidation (LPO), enzyme inactivation, and DNA chain breakage, among others (Vanzella et al. 2007; Zhang et al. 2003). However, despite the relatively large number of studies about the toxicity of petroleum hydrocarbons, there is a paucity of data about the effects of gasoline on freshwater fish species. Therefore, experimental studies of the effects of this contaminant on ecosystems and on Neotropical freshwater fish are needed.

Biotransformation is important in altering the biological activity of toxic compounds and preventing them from causing cell damage. The biotransformation process includes numerous different enzyme systems and several types of substrates. The function of biotransformation enzymes is to render compounds more water-soluble, thus facilitating their excretion. The biotransformation of xenobiotic compounds usually consists of two phases. Phase I reactions involve oxidation, reduction and hydrolysis reactions, catalyzed mainly by cytochrome P450, that facilitate the excretion of compounds by phase I metabolism, transforming them into more water-soluble compounds and also serving as a substrate for phase II reactions that increase the excretion rate (Stegeman et al. 1992). In fish, the isoenzyme class of the cytochrome P450 family responsible for the biotransformation of a broad range of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) is the CYP1A subfamily (Benedetti et al. 2007). Although it has been studied mainly in the liver, CYP1A induction has also been observed in other organs such as kidneys and gills (Jönsson et al. 2009). The most common method to determine the catalytic activity associated with CYP1A is the assay that measures the activity of the enzyme EROD (7-ethoxyresorufin-*O*-deethylase) (Whyte et al. 2000). Glutathione-S-transferase (GST) is related principally with the conjugation and excretion of toxic compounds in the so-called phase II of biotransformation. GST participates in the detoxification of lipophilic contaminants by catalyzing conjugation reactions with endogenous substrates, such as the tripeptide glutathione (GSH). This conjugation enhances the water-solubility of contaminants and therefore their elimination rates, reducing the probability of toxic compounds to bind to other cellular macromolecules such as DNA. The toxicity of many pollutants can be modulated by the induction of GSTs (Carletti et al. 2008). On the other hand, many environmental contaminants or their metabolites produced during biotransformation have been shown to exert toxic effects related to oxidative stress (Van der Oost et al. 2003).

Oxidative damage emerges when the animal's defense mechanisms responsible for removing reactive oxygen species (ROS) are inadequate or insufficient, promoting oxidative stress, which is associated with several pathological processes, including LPO and DNA damage. These damages are considered important biomarkers in the assessment of the response of organisms exposed to xenobiotics. ROS such as superoxide anions, hydroxyl radicals and hydrogen peroxide are molecules formed from the normal compound metabolism of organisms or from the xenobiotic metabolism (Winterbourn and Hampton 2008). Aerobic organisms present different antioxidant defense mechanisms that can prevent the formation of ROS, react with these reactive intermediaries, and repair the damages they cause (Storey 1996). GSH is an endogenous tripeptide that can be considered one of the most important agents of the cell's antioxidant defense system, protecting it against lesions caused by exposure to oxidant agents (Halliwell and Gutteridge 2005). Furthermore, GSH is a co-factor of glutathione peroxidase (GPx) and GST activity and thus participates in the detoxification of chemical agents and in the elimination of products of LPO (Di Giulio et al. 1995; Hermes-Lima 2004). Glutathione reductase (GR) is a key enzyme in reduced GSH metabolism, which is essential for maintaining GSSG/GSH homeostasis under conditions of oxidative stress, since it catalyzes the oxidized form of GSH (GSSG) to the reduced form (GSH) through the oxidation of NADPH to NADP, promoting the recycling of this tripeptide (Tekman et al. 2008). The superoxide dismutases (SOD) are a family of metalloenzymes responsible for catalyzing the transformation of the reactive superoxide anion ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2). CAT is an enzyme that catalyzes the reduction of H_2O_2 to oxygen and water. Hydrogen peroxide is a potentially harmful compound given its high stability and its ability to pass through biological membranes. The enzyme GPx is also responsible for the metabolism of numerous organic hydroperoxides, involving the concomitant oxidation of GSH to its oxidized form (GSSG). This enzyme plays a very important role in the inhibition and/or prevention of cellular oxidative damage (Dorval and Hontela 2003).

In view of the above, the present study focused on an evaluation of the effects of gasoline on the antioxidant defense system of the Neotropical fish *Prochilodus lineatus* (Characiformes, Prochilodontidae). This species was chosen because it is a Neotropical species of well-known biology found in the Southeast and South of Brazil and widely used for human consumption. Several studies have shown that *P. lineatus* is sensitive when exposed to pollutants, making it suitable for environmental monitoring (Camargo et al. 2009; Carvalho and Fernandes 2008; Cazenave et al. 2009; Modesto and Martinez 2010; Simonato et al. 2008).

Materials and methods

Experimental animals

Juveniles of *P. lineatus* were supplied by the Fish Hatchery Station of the State University of Londrina, with a body mass of 11.23 ± 4.67 g and total length of 10.76 ± 1.98 cm (mean ± SD, N = 96). The animals were acclimated for 7 days in a 300-L tank containing dechlorinated water, with constant aeration, pH, photoperiod, and dissolved oxygen. During acclimation fish were fed with commercial fish food

at 48-h intervals, feeding was suspend 48 h before and during toxicity tests.

Preparation of the water-soluble fraction of gasoline (WSFG)

Gasoline was purchased from a specific gas station. The methodology employed to simulate fuel leakage followed that proposed by Nicodem et al. (1998). The gasoline was mixed with water in a proportion of 1 part gasoline to 4 parts water (forming a film); the mixture was allowed to rest for 24 h. During this period, the mixture was exposed outdoors to direct sunlight for 6 h (from 10 AM to 4 PM) on a cloudless day. Organic and aqueous phase extractions were performed immediately following this exposure. The aqueous phase was separated (WSFG 100%), diluted to 5% (WSFG 5%) and used in the exposure assays, while the organic phase (insoluble) was discarded.

Samples of WSFG before dilution was quantitatively analyzed for BTEX and some PAHs. The PAHs were analyzed according to USEPA 8310 method (2010), using high performance liquid chromatography with UV fluorescence detector. The BTEX were analyzed according to USEPA 5021 method (2010), using gas chromatograph with a flame ionization detector. Total phenol was determined spectrophotometrically with 4-aminoantipyrine (Standard Methods—Method 5530 D, APHA, 1992).

Toxicity tests

The fish were submitted to acute static toxicity tests for 6, 24 and 96 h. A group of fish exposed to WSFG (EXP), and a control group exposed only to water (CTR), were used for each experimental time. The tests were carried out in 100-L glass aquaria containing 8 fish each. The fish were exposed to WSFG diluted to 5% (4 L of WSFG: 76 L of water) and sampled after each period of exposure. The control group was sampled simultaneously to the group exposed to WSFG. Replications were carried out for each aquarium (EXP and CTR). The physicochemical characteristics of the media water were monitored continually for temperature, dissolved oxygen, pH and conductivity. Samples of water from the experimental groups were examined qualitatively for the presence of mono- and polyaromatic hydrocarbons as described above.

Immediately after removing the fish from the aquaria, they were anesthetized with benzocaine (0.1 g L^{-1}), killed by medullary section, measured and weighed. The gills and liver were removed and frozen at −80°C up to the moment of the assays. The gills and liver were weighed, homogenized (5× volume for the gills and 10× volume for the liver) in potassium phosphate buffer (0.1 M; pH 7.0), centrifuged (20 min, 13,200×*g*, 4°C) and the supernatant separated for analyses of the biochemical parameters. The protein concentration of the supernatant was determined according to the method described by Lowry et al. (1951), using bovine serum albumin as standard.

Biotransformation enzymes

CPY1A induction was determined from the analysis of the EROD activity. The method adapted from Eggens et al. (1992) consists of fluorometric measurements on a 96-well plate. The reaction mixture contained potassium phosphate buffer 0.1 M; pH 7.6; NADPH 2 mM and 7-ethoxyresorufin 0.1 mM. The reaction was initiated by the addition of the sample (50 µL of gill or liver supernatant) to 200 µL of the reaction mixture. The progressive increase in fluorescence resulting from the formation of resorufin was measured at 1-min intervals for 10 min (at an excitation wavelength of 530 nm and emission wavelength of 590 nm). The initial linear portion of the curve was used to evaluate the reaction rate and the EROD activity was expressed in pmol of resorufin min^{-1} mg of $protein^{-1}$, based on a resorufin standard curve.

GST activity was determined as described by Keen et al. (1976) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} mg^{-1} protein using a molar extinction coefficient of 9.6 mM cm^{-1}.

Antioxidants

Copper–zinc SOD (CuZn-SOD) activity was determined according to McCord and Fridovich (1969). This method is based on the measurement of the inhibition of the reduction rate of cytochrome c by the superoxide radical, at 550 nm and 25°C. SOD activity was expressed in U SOD mg of $protein^{-1}$, with one U of SOD corresponding to the quantity of enzyme that promoted the inhibition of 50% of the reduction rate of cytochrome c.

CAT activity was determined according to the technique described by Beutler (1975), by monitoring the H_2O_2 decomposition from the decrease of absorbance at 240 nm. CAT activity was expressed in µmol H_2O_2 min^{-1} mg of $protein^{-1}$.

Selenium-dependent GPx (Se-GPx) activity was determined by the method of Hopkins and Tudhope (1973), based on NADPH oxidation in the presence of GSH (0.95 mM) and H_2O_2 at 340 nm. GPx activity was expressed in µmol oxidized NADPH min^{-1} mg of $protein^{-1}$ using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}.

GR was determined indirectly based on the reduction of NADPH in the presence of oxidized GSH (Carlberg and

Mannervik 1975), at 340 nm. GR activity was expressed in μmol min^{-1} mg of $protein^{-1}$.

Reduced GSH levels were estimated according to Beutler et al. (1963), using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) following Monteiro et al. (2009) and Thomaz et al. (2009). Supernatants of the acid extracts (1:1 v/v with 12% TCA) were added to 0.25 mM DTNB in 0.1 M potassium phosphate buffer, pH 8.0, and thiolate anion formation was determined at 412 nm against a GSH standard curve. The GSH content was expressed in μg GSH mg of $protein^{-1}$, based on a standard curve of 10–200 μmol of GSH.

Lipid peroxidation

A ferrous oxidation xylenol orange (FOX) assay was used to determine lipid hydroperoxide (HP) as described by Jiang et al. (1991). This method is based on the rapid hydroperoxide-mediated oxidation of Fe^{2+} under acid conditions. Fe^{3+} forms a chromophore with xylenol orange which absorbs strongly at 560 nm. Lipid HP levels were expressed in μmol CHP mg of $protein^{-1}$, based on a standard curve of 0.0004–0.1 mM of cumene hydroxyperoxide (CHP).

Statistical analyses

The results obtained for the control and experimental groups at each exposure time were compared to each other, using Student's parametric *t* test or the Mann–Whitney non-parametric test, depending on the distribution of the data. The decision to use parametric or non-parametric tests was based on analysis of normality and homogeneity of variance. Values of $P < 0.05$ were considered significant.

Results

Water parameters

The physicochemical characteristics of the water of the WSFG groups were (mean ± SD): temperature 21.1 ± 0.7°C; pH 7.3 ± 0.5, DO 7.4 ± 0.8 mg O_2 L^{-1}, and conductivity 88.6 ± 2.9 μS cm^{-1}. For the water of the control groups, the values were: temperature 21.8 ± 0.5°C, pH 7.2 ± 0.3, DO 7.1 ± 1.8 mg O_2 L^{-1}, and conductivity 116.6 ± 1.6 μS cm^{-1}. These values remained stable throughout the experimental times for both control and WSFG groups.

The concentrations of BTEX and some PAHs in the WSFG are listed in Table 1. The WSFG showed high levels of BTEX, mainly of ethylbenzene, xylene and benzene. Among the 12 PAHs analyzed, naphthalene was the most significant, followed by anthracene and phenanthrene.

Table 1 Concentration of monocyclic aromatic hydrocarbons (BTEX), PAH and total phenols in the 100% WSFG

Hydrocarbons	WSFG 100% (μg L^{-1}]
Benzene	5,700
Toluene	1,050
Ethylbenzene	10,500
Xylene	7,550
Total BTEX	24,800
Anthracene	53.25
Phenanthrene	12.20
Naphthalene	2,138.25
Total PAH	2,203.70
Total phenols	60.00

Limit of detection: BTEX = 5.0 μg L^{-1}; PAH = 0.05 μg L^{-1}; phenols = 5.0 μg L^{-1}

All the other PAHs analyzed (benzo(a)anthracene, benzo(a)pyrene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)anthracene, fluoranthene, indeno(1,2,3)pyrene and pyrene) showed concentrations below the detection limit (0.05 μg L^{-1}). The fluorescence spectrum of WSFG 100% indicated the presence of aromatic hydrocarbons with emission peaks around 300 nm. The samples of 5% WSFG collected after 6, 24 and 96 h of the onset of the experiments showed a decrease in the relative intensity of these compounds when compared with the WSFG 100%, as already reported by Fedato et al. (2010).

Biochemical alterations of the liver

The animals exposed to WSFG presented a significant increase of EROD (Fig. 1a) at the experimental times of 24 and 96 h when compared with their respective controls. The activity of the GST enzyme (Fig. 1b) showed a significant increase in the animals exposed to WSFG at all the experimental times, when compared with their respective control groups. SOD (Fig. 2a) activity was significantly lower in the animals exposed to WSFG for 24 h. The activity of the antioxidant defense enzymes CAT and GPx (Fig. 2b, c) showed a significant increase in the liver of animals exposed to WSFG for 96 h. The concentration of GSH (Fig. 3a) in the liver of animals exposed to gasoline was significantly higher after 24 and 96 h of exposure than in the CTR group. GR showed a significant increase after 6 h of exposure and a significant decrease at 24 and 96 h in animals exposed to WSFG (Fig. 3b).

Biochemical alterations of the gills

The animals exposed to WSFG presented a significant increase in EROD at the experimental times of 24 and 96 h

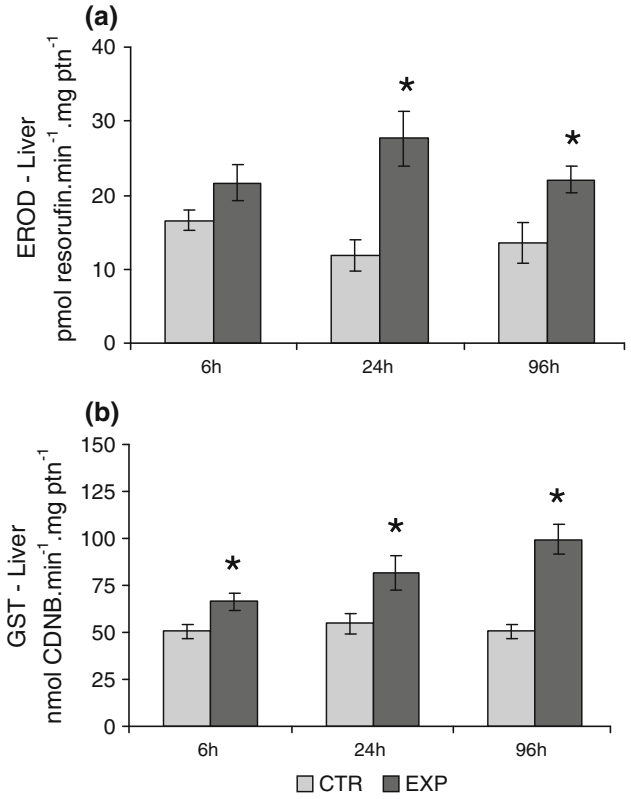

Fig. 1 Hepatic activity of EROD (**a**) and GST (**b**) in *P. lineatus* exposed to WSFG (EXP) or only to water (CTR) for 6, 24 and 96 h. *Bars* represent means and *vertical lines* the SE (number of animals: 7–15). *Indicates significant difference in relation to control at the same time of exposure ($P < 0.05$)

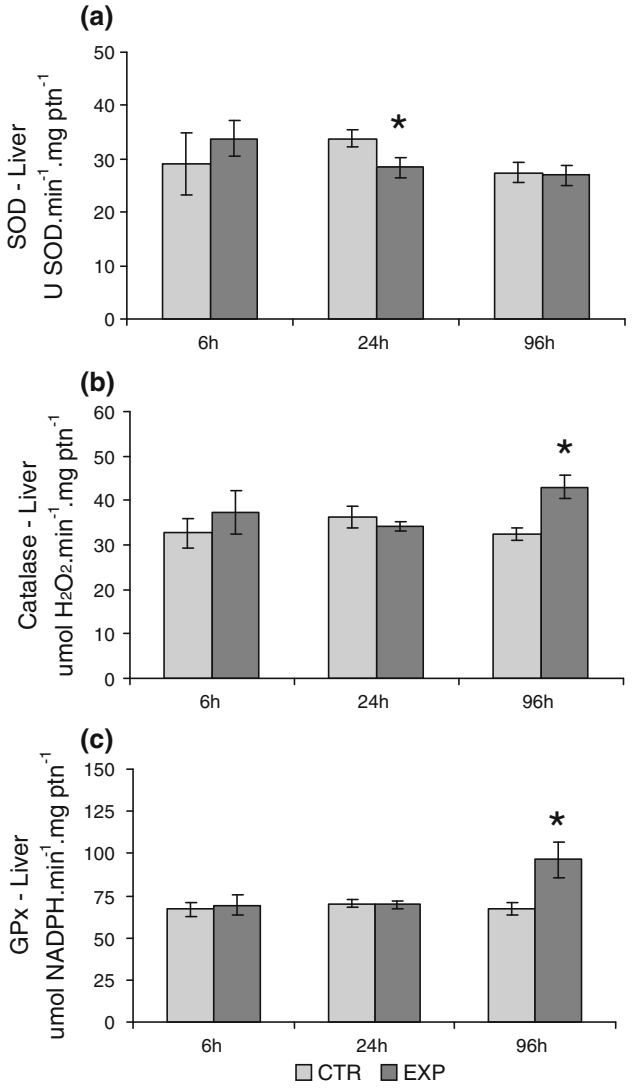

Fig. 2 Hepatic activity of SOD (**a**), CAT (**b**) and GPx (**c**) in *P. lineatus* exposed to WSFG (EXP) or only to water (CTR) for 6, 24 and 96 h. *Bars* represent means and *vertical lines* the SE (number of animals: 7–14). *Indicates significant difference in relation to control at the same time of exposure ($P < 0.05$)

when compared to their respective controls (Fig. 4a). GST enzyme activity increased significantly in the animals exposed to WSFG at all the experimental times, in comparison to their respective controls (Fig. 4b). SOD and GPx activity (Fig. 5a, c) did not show significant alterations among the animals exposed to WSFG when compared to the control groups. The branchial activity of CAT (Fig. 5b) increased significantly in the animals after 6 and 24 h of exposure to WSFG when compared with the CAT activity in the animals of the respective control groups.

The GSH concentration in the gills of animals exposed to WSFG for 24 h was significantly higher than in the CTR group (Fig. 6a). GR activity (Fig. 6b) did not show significant alterations among the animals exposed to WSFG when compared to respective control groups.

Lipid peroxidation in liver and gills

A significant increase in LPO was detected in the animals' liver after 6 h of exposure to WSFG (Fig. 7a). In contrast, the gills showed increased LPO only after 96 h of exposure to WSFG (Fig. 7b).

Discussion

This paper is the first to present results based on the use of biochemical biomarkers to investigate the effects of gasoline on a Neotropical fish. Our data showed that biotransformation enzymes and oxidative stress parameters were effective to evaluate the toxic effects of the components present in gasoline, since low concentrations of hydrocarbons were sufficient to promote significant changes both in the gills and liver of fish exposed to the WSFG, acting as an alert before the commitment of entire populations.

The chemical analysis of WSFG 100% showed high concentrations of the monoaromatic hydrocarbons BTEX and of the polyaromatic hydrocarbon naphthalene. Other

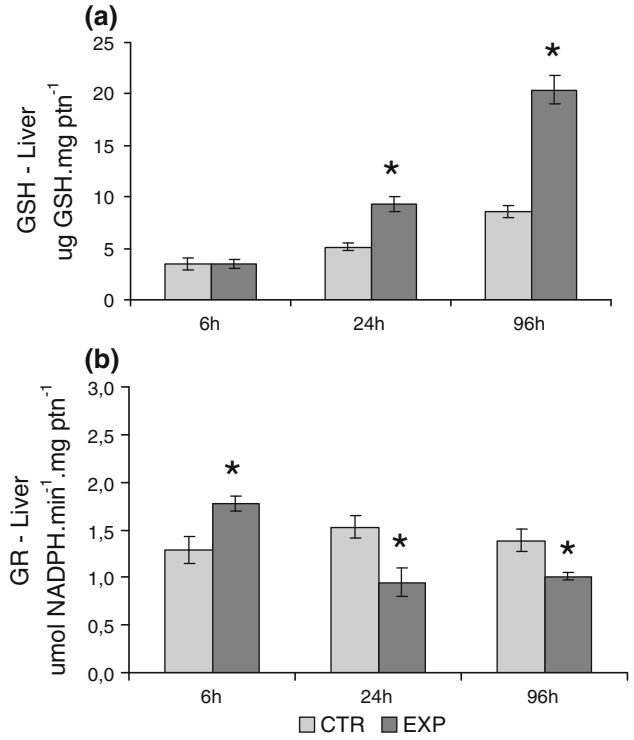

Fig. 3 Hepatic content of GSH (**a**) and hepatic activity of GR (**b**) in *P. lineatus* exposed to WSFG (EXP) or only to water (CTR) for 6, 24 and 96 h. *Bars* represent means and *vertical lines* the SE (number of animals: 8–15).*Indicates significant difference in relation to control at the same time of exposure ($P < 0.05$)

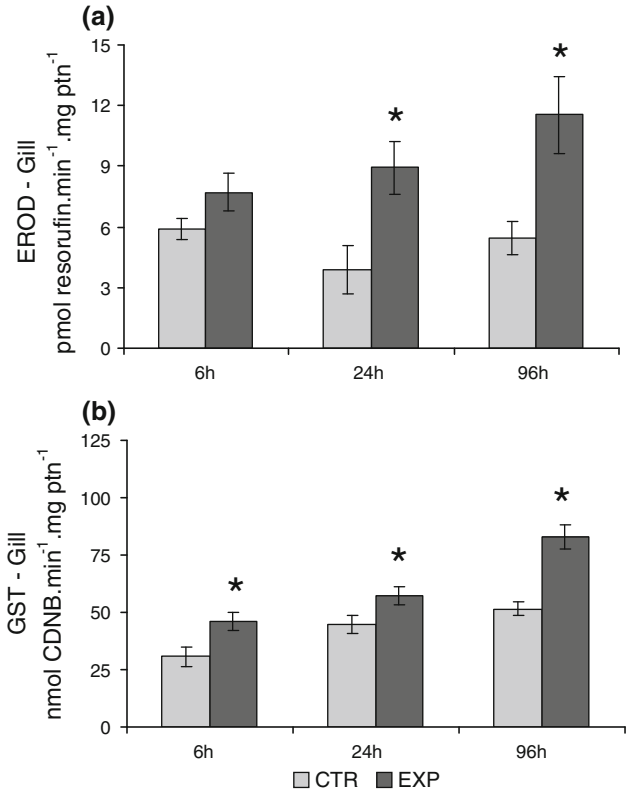

Fig. 4 Branchial activity of EROD (**a**) and GST (**b**) in *P. lineatus* exposed to WSFG (EXP) or only to water (CTR) for 6, 24 and 96 h. *Bars* represent means and *vertical lines* the SE (number of animals: 7–13). *Indicates significant difference in relation to control at the same time of exposure ($P < 0.05$)

PAHs such as anthracene and phenanthrene were also found but in lower concentrations. The high concentrations of BTEX probably reflect the addition of alcohol to Brazilian gasoline (20–25%) which enhances the solubility of these monoaromatics due to co-solvent effect when present in water (Corseuil et al. 2004). The determination of hydrocarbons concentrations was done only in the WSFG 100% considering that the analytical methods available have limitations to measure very small concentrations, as found in WSFG after dilution to 5%. Nevertheless, the qualitative analysis indicated the presence of the hydrocarbons in the 5% WSFG after the toxicity tests. The comparison between 100% WSFG and 5% WSFG after different periods of the tests (6, 24 and 96 h) showed a decrease in the relative intensity of the fluorescence peaks. However, the comparison among the WSFG diluted 5% collected after different exposure times indicated small changes between 6 and 24 h; even in the WSFG collected after 96 h of exposure it is still possible to visualize the fluorescence peak of aromatic compounds. These results emphasize the importance of using sensitive biomarkers in the evaluation and monitoring of sites impacted with gasoline, since low concentrations of oil are sufficient to demonstrate significant changes in the body of exposed animals, serving as a warning before the compromise of entire populations.

Several authors have reported significant changes in biochemical parameters of fish exposed to BTEX and PAH that are present in different types of petroleum and/or their derivatives. In the studies of Pacheco and Santos (2001a, b), eels (*Anguilla anguilla*) exposed to the soluble fraction of gasoline and diesel presented a significant increase in hepatic EROD. Bols et al. (1999) also detected an increase in EROD in hepatocytes of trout exposed to different types de PAHs. Shukla et al. (2007) found PAHs accumulated in gills, liver, muscle and gonads of *Tilapia mossambica*, and Silva et al. (2006) also found naphthalene, phenanthrene, and benzo(a)pyrene in the bile of demersal fish collected in the São Sebastião channel, São Paulo, Brazil.

Moreover, there is a lack of data about the effects of petroleum derivatives, especially of gasoline, on Neotropical freshwater fish; hence, experimental studies of the impact of this contaminant on ecosystems and Neotropical freshwater fish are important. Recent studies have shown that the exposure of *P. lineatus* to the water-soluble fraction of diesel oil activated the detoxification pathways and caused hematologic and metabolic alterations, structural

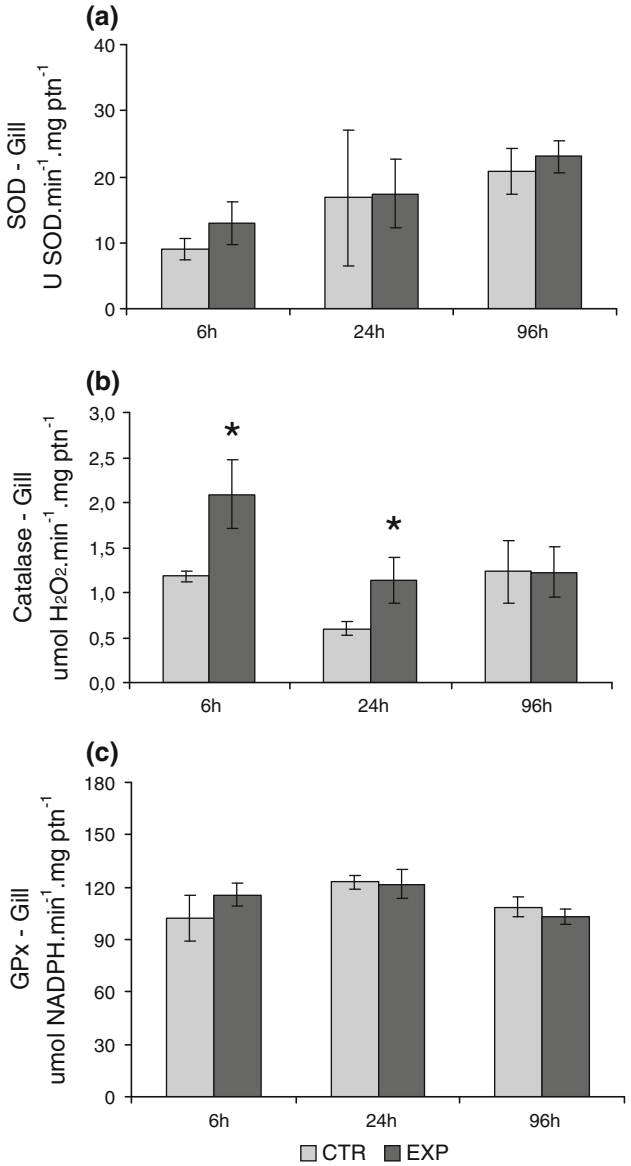

Fig. 5 Branchial activity of SOD (**a**), CAT (**b**) and GPx (**c**) in *P. lineatus* exposed to WSFG (EXP) or only to water (CTR) for 6, 24 and 96 h. *Bars* represent means and *vertical lines* the SE (number of animals: 5–9). *Indicates significant difference in relation to control at the same time of exposure ($P < 0.05$)

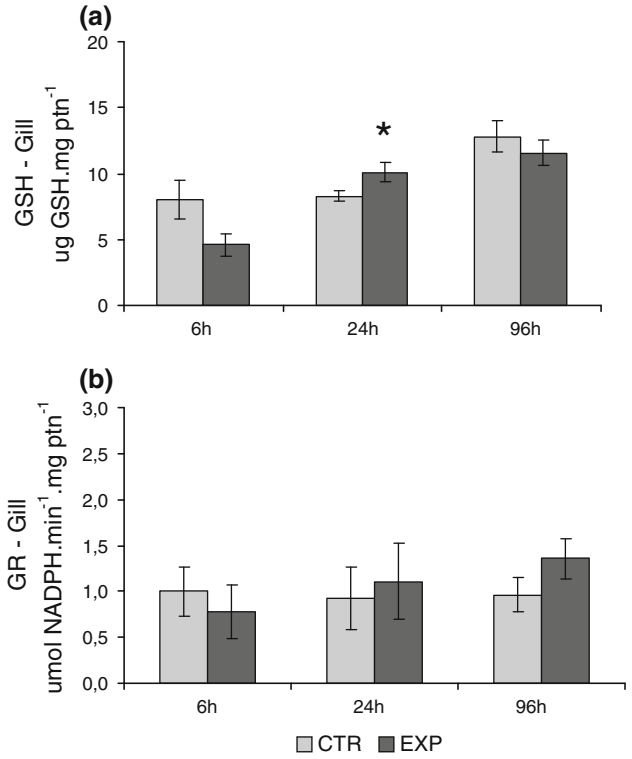

Fig. 6 Branchial content of GSH (**a**) and branchial activity of GR (**b**) in *P. lineatus* exposed to WSFG (EXP) or only to water (CTR) for 6, 24 and 96 h. *Bars* represent means and *vertical lines* the SE (number of animals: 7–14). *Indicates significant difference in relation to control at the same time of exposure ($P < 0.05$)

damages in the gills and liver of fish (Simonato et al. 2006, 2008), and DNA damage in fish erythrocytes (Vanzella et al. 2007). These results demonstrate the sensitivity of this species to petroleum derivatives. The present work is the first to evaluate biochemical parameters of a Neotropical fish exposed to WSFG, and the results indicated that this species is also sensitive to gasoline.

However, there is still a paucity of data about the effects of petroleum derivatives, particularly of gasoline, on Neotropical freshwater fish species, which is why experimental studies of the impact of this contaminant on ecosystems and on these fish are so important. In the present study, the high concentrations of monoaromatic compounds (BTEX) and of some PAHs, such as naphthalene, phenanthrene and anthracene that are found in WSFG, stimulated biotransformation, as indicated by the increase in EROD and GST, and promoted changes in the enzymatic and non-enzymatic compounds of the antioxidant defense system of *P. lineatus*. Moreover, the antioxidant defense system was not entirely effective, since LPO was detected in both gills and liver.

The organism has two main forms of eliminating a chemical compound: either it is excreted in its original form or it is biotransformed by the organism. Biotransformation usually leads to the formation of a more hydrophilic compound in order to facilitate its excretion. The organ most commonly related to the biotransformation of xenobiotic compounds is the liver (Jimenez and Stegeman 1990; Van der Oost et al. 2003). However, some authors point out the importance of other detoxifying organs such as the kidneys, intestines and gills (Jönsson et al. 2002). The studies conducted by Abrahamson et al. (2007) and Jönsson et al. (2009) demonstrated the importance of the gills in the detoxification process of xenobiotics. In the present study, CPY1A induction indicated by the EROD assay revealed the occurrence of phase I

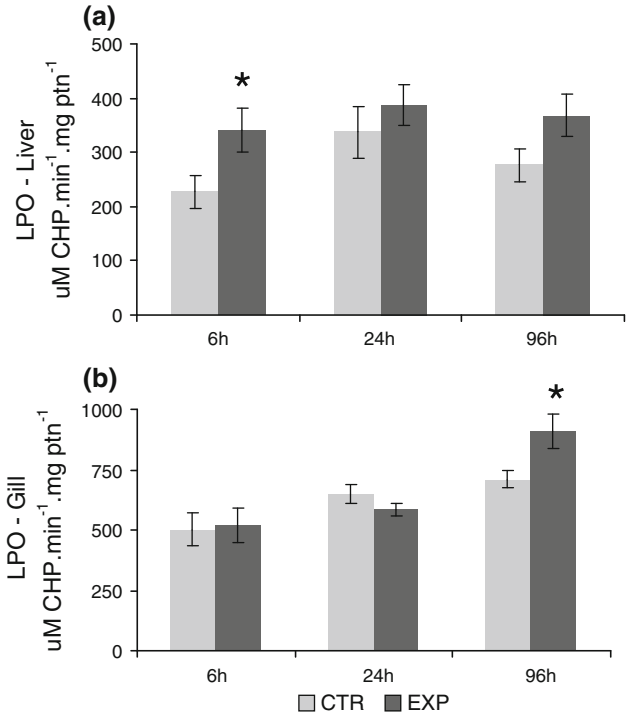

Fig. 7 LPO in the liver (**a**) and gill (**b**) of *P. lineatus* exposed to WSFG (EXP) or only to water (CTR) for 6, 24 and 96 h. *Bars* represent means and *vertical lines* the SE (number of animals: 5–15). *Indicates significant difference in relation to control at the same time of exposure ($P < 0.05$)

biotransformation of the compounds of WSFG at the experimental times of 24 and 96 h in the gills and liver. These results reinforce the induction of CPY1A in the presence of aromatic hydrocarbons. Earlier studies have already reported increased EROD in liver (Benedetti et al. 2007; Pacheco and Santos 2001a, b) and in gills (Abrahamson et al. 2007; Jönsson et al. 2009) of fish exposed to different types of hydrocarbons. In this phase of biotransformation, a molecular oxygen atom is incorporated into the lipophilic substrate, which corresponds to the xenobiotic compound, and although this increases its solubility in water, the most important effect is to render the xenobiotic an adequate substrate for phase II reactions. In phase II reactions, the compound resulting from phase I or the original xenobiotic is conjugated with endogenous compounds, rendering the product more water soluble so that it can be excreted easily (Di Giulio et al. 1995). GST catalyzes conjugation with the GSH of toxic compounds in so-called phase II biotransformation (Pandey et al. 2003). In the present work, both liver and gills presented a significant increase in GST at all the experimental times. These results indicate that induction of phase I and II biotransformation enzymes occurred in both these organs, reinforcing the function of the liver in the xenobiotic metabolism and indicating the gills as an important detoxifying organ. The results also indicate that these two enzymes are good biomarkers of gasoline-impacted sites.

Xenobiotic biotransformation is an essential mechanism for the elimination of toxic compounds, as mentioned earlier, but it is also an important source of ROS (Stegeman et al. 1992). Thus, aerobic organisms present different mechanisms, enzymatic and non-enzymatic, that can react with these reactive intermediates, preventing the formation of these ROS, as well as repairing the damage caused by them (Halliwell and Gutteridge 2005; Storey 1996).

The induction of hepatic CAT in 96 h and branchial CAT after 6 and 24 h suggests an increase in the production of hydrogen peroxide due to its specificity in removing this compound, which causes cell damage (Sturve et al. 2006). According to Hermes-Lima (2004), catalase is more required when the intracellular concentration of H_2O_2 is very high. Therefore, these results are a sign of the animal's efforts to fight the hydrogen peroxide produced during exposure to WSFG. Moreover, the increase in CAT affects the Fenton reaction and diminishes the possibility of LPO (Bagnyukova et al. 2006). Despite the increase in branchial CAT activity, it should be kept in mind that the gills contain much smaller amounts of this enzyme than the liver. According to Wilhelm Filho et al. (1994), the gills may present alternative mechanisms for eliminating hydrogen peroxide. A possible increase in hydrogen peroxide, indicated by the elevation of CAT in both organs, probably reflects an effect of WSFG, since this ROS was not generated by transformation of the superoxide radical anion by SOD. This enzyme showed no variation in the gill, but in the liver it decreased after 24 h, possibly indicating inhibition of SOD. The oxidative stress caused by gasoline may give rise to structural modifications in enzymes and inactivate them (Che et al. 2007). Moreover, ROS cause several pathological conditions that reduce SOD activity (Hermes-Lima 2004). In addition, SOD is also sensitive to hydrogen peroxide and is inactivated in the presence of this compound (Sampson and Beckman 2001), indicating that this enzyme is sensitive to slight alterations in the normal state of the organism. The SOD-CAT system provides an important line of defense against ROS (Pandey et al. 2003), but the SOD and CAT results of this study do not appear to be correlated.

GPx is an enzyme of the antioxidant defense system that uses GSH as a cofactor, and that is also responsible for catalyzing the reduction of H_2O_2 in water, as well as eliminating organic hydroperoxides (Dorval and Hontela 2003), and is therefore very important for preventing the formation of lipid peroxides (Di Giulio et al. 1995). The gills did not show significant changes in GPx activity after exposure to WSFG, but the liver presented a significant increase of this enzyme in fish exposed to WSFG after 96 h, reflecting the need to eliminate hydrogen peroxide

and/or hepatic organic hydroperoxides. The augmented activity of hepatic GPx and CAT reinforces the notion of an increased quantity of hydrogen peroxide in this organ, probably resulting from exposure to WSFG. The amount of GPx in the gills of *P. lineatus* was also found to be almost twice the amount in the liver. This may indicate that branchial GPx in *P. lineatus* can act as a reinforcement to eliminate ROS, since the quantity of CAT is very small. However, this pattern was not observed in other species of fish, such as *Liza aurata* (Oliveira et al. 2008), *Clarius gariepinus* and *Oreochromis mossambicus* (Siwela et al. 2009) and *Wallago attu* (Pandey et al. 2003), which have consistently shown larger amounts of hepatic GPx than of branchial GPx.

GSH is an endogenous antioxidant tripeptide that represents the first line of defense against ROS (Ahmad et al. 2000), participating in many cellular reactions by directly neutralizing pro-oxidants or acting in enzymatic reactions where it acts as a substrate. The significant increase in the concentration of hepatic GSH after 24 and 96 h and of branchial GSH after 24 h of exposure to WSFG indicates augmented protection of the organism against oxidative stress. Apart from its individual antioxidant capacity, this increase in GSH may also be justified by the needs of the enzymes GST and GPx, which require this tripeptide as a substrate for the reactions of biotransformation and of antioxidant defense, respectively (Halliwell and Gutteridge 2005). According to Oliveira et al. (2008), phenanthrene promoted an increase of GSH in the gills and liver of the fish *L. aurata*. Other studies have also demonstrated augmented concentrations of GSH in animals exposed to PAHs (Ahmad et al. 2003; Pandey et al. 2003). Stegeman et al. (1992) state than an increase in the concentration of GSH may occur through two pathways: by an increase in GR activity, which converts GSSG into GSH, or by an increase of its synthesis. In the present study, an increase in the synthesis of GSH probably occurred, since the branchial GR showed no variation and the hepatic GR presented an increase in the animals exposed to WSFG only after 6 h. Furthermore, the hepatic GR was found to diminish after 24 and 96 h of exposure. The increase in hepatic GR in 6 h reflects the need for recycling GSH through the reduction of oxidized GSH (GSSG) (Tekman et al. 2008). However, the inhibition probably occurred due to the sensitivity of this enzyme to the compounds present in WSFG or to a ROS generated by the presence of this xenobiotic. According to Bagnyukova et al. (2006), GR is sensitive to hydrogen peroxide and to the superoxide radical anion and, as we have seen, it is possible that WSFG may have caused the quantity of hydrogen peroxide to increase, since the hepatic CAT and GPx were activated in 96 h. In addition, the decrease in GR may also be related to diminished availability of NADPH (Stegeman et al. 1992), which is highly required during phase I biotransformation reactions (Di Giulio et al. 1995), and which in this study demonstrated activation, as indicated by the induction of CPY1A (increase of EROD) in the same experimental times (24 and 96 h).

When the antioxidant defense system is insufficient or inactive, oxidative damage may occur, such as LPO, which is responsible for altering membrane permeability, causing cell damage or even death (Hermes-Lima 2004). The FOX assay indicated LPO in the liver after 6 h and in the gills after 96 h in animals exposed to WSFG, demonstrating that these organs suffered oxidative damage. According to Ahmad et al. (2003), exposure to naphthalene also promoted LPO in various organs of *A. anguilla*, and the damage was organ-dependent, indicating the liver as the most well adapted organ, followed by the kidneys and gills, which are considered more vulnerable. An analysis of the findings on the antioxidant line of defense in the liver after 6 h and in the gills after 96 h indicated that the enzymes CAT, GPx and SOD were not activated, nor was there an increase in the GSH content. Thus, it can be inferred that the antioxidant defense system was insufficient for both organs in these experimental times, and that the ROS generated by the presence of WSFG caused peroxidative damage. This may indicate a better adaptation of the liver to longer exposure (after 24 h). According to Oliveira et al. (2008), GSH and GPx are considered the first two lines of defense and very important in preventing LPO, and they were both active in the liver in the experimental times where oxidative damage did not occur. On the other hand, the gills are the first organ of contact with contaminants present in water, and as such, are an entryway for pollutants given their large contact surface and permeability (Wendelaar Bonga 1997). Therefore, their antioxidant defenses were activated right at the beginning of exposure to WSFG and were able to prevent the occurrence of LPO in the initial times (6 and 24 h). Moreover, it is also possible that the gills have different mechanisms for the elimination of ROS, as mentioned earlier.

Conclusions

The results demonstrate that the monoaromatic and polyaromatic hydrocarbons in WSFG 5% promoted the activation of phase I and II biotransformation enzymes in the liver and gills, confirming the important role of the liver in these processes of elimination of toxic compounds and reinforcing the role of the gills as a detoxifying organ. Moreover, these parameters proved to be efficient biomarkers for evaluating the toxic effects of the compounds present in gasoline, even at low concentrations for fish. The antioxidant defense system was more efficient in the liver

than in the gills. Even so, both organs showed the occurrence of LPO in one of the experimental times, i.e., the liver after 6 h and the gills after 96 h of exposure to WSFG. This may indicate a better adaptation of the liver to longer exposures (more than 24 h). In the case of the gills, because they represent the first organ of contact with xenobiotics, the antioxidant defenses were able to prevent the occurrence of LPO in the initial times (6 and 24 h). Our findings also indicate the important role of GSH as an antioxidant defense in both organs, since there was no evidence of oxidative damage when its concentration was augmented.

Acknowledgments The authors thank the Hatchery Station of the State University of Londrina for supplying the fish for this research. This work is part of the doctoral thesis of J. D. Simonato. CNPq, FAPESP and Fundação Araucária provided financial support for this work. M.N. Fernandes and C.B.R. Martinez are research fellows from CNPq and members of the Brazilian Institute of Aquatic Toxicology (INCT-TA, CNPq: 573949/2008-5).

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