

A typical enzyme activity for glutathione conjugation indicates exposure of pacu to pollutants

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Functional enzyme assays to detect sublethal poisoning of Neotropical fish are paramount. Accordingly, we assayed a glutathione S-transferase (GST) activity in liver and kidney cytosols from *Piaractus mesopotamicus* injected with methyl parathion or benzo[a]pyrene using the substrate 1-chloro-2,4-dinitrobenzene (CDNB), which is the usual substrate for assaying a known general activity of GST. Since the most reactive substrate is required to reveal specific changes in enzyme activity, we also used two alternative substrates, 1,2-dichloro-4-nitrobenzene (DCNB) and ethacrynic acid (ETHA). Activities with CDNB or ETHA did not change. However, assays with DCNB showed that methyl parathion caused a decrease in GST activity in the liver on the 24th, 48th and 96th hour after the injection. DCNB also revealed that GST activity in the liver increased seven days after benzo[a]pyrene injection, coming down to normal after fourteen days. Benzo[a]pyrene, but not methyl parathion, increased the activities with DCNB in cytosol from the kidney seven and fourteen days after the injection. Thus, a decreased liver GST activity assayed with DCNB corresponded to contamination of *P. mesopotamicus* with methyl parathion. The increase of this GST activity in the liver and the kidney correlates to pacu contamination with polycyclic aromatic hydrocarbons.

Keywords: Contamination, Fish, GST, *Piaractus mesopotamicus*, Serrasalminidae.

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Ensaio prático de enzimas para detectar contaminação subletal de peixes neotropicais são da maior importância. Assim, ensaiamos a atividade da glutathione S-transferase (GST) em citosóis de fígado e de rim de *Piaractus mesopotamicus* injetados com metilparation ou benzo[a]pireno usando 1-cloro-2,4-dinitrobenzeno (CDNB), o substrato usual para ensaiar uma denominada atividade geral de GST. Desde que para indicar alterações na atividade de uma isoenzima, é necessário o substrato mais reativo, também usamos dois substratos alternativos, o 1,2-dicloro-4-nitrobenzeno (DCNB) e o ácido etacrínico (ETHA). As atividades com CDNB ou ETHA não mudaram. Entretanto, ensaios com DCNB mostraram que metilparation decresceu a atividade de GST no fígado em 24, 48 e 96 horas depois da injeção. O DCNB também revelou que sete dias depois da injeção de benzo[a]pireno a atividade da GST aumentou no fígado, normalizando depois de 14 dias. Benzo[a]pireno, mas não metilparation, aumentou as atividades com DCNB no citosol dos rins sete e 14 dias depois da injeção. Assim, uma atividade de GST ensaiada com DCNB diminuída no fígado correspondeu à contaminação de *P. mesopotamicus* com metilparation. O aumento dessa atividade de GST no fígado e nos rins está correlacionada com contaminação do pacu por hidrocarbonetos policíclicos aromáticos.

Palavras-chave: Contaminação, GST, Peixe, *Piaractus mesopotamicus*, Serrasalmidæ.

INTRODUCTION

The longer a xenobiotic stay in an organism, the more severe its effects are. Accordingly, to reduce the toxic effects of an absorbed xenobiotic its amount and permanency in the body must be reduced. The lipophilicity of several xenobiotics molecules hinders their excretion because lipophilic compounds are readily adsorbed to lipids of biomembranes. Therefore, the efficient elimination of xenobiotics from the body depends on increasing their solubility in water, which happens through biotransformation reactions (DeBethizy, Hayes, 1994). Many of these occur frequently by combination of xenobiotics with glutathione, amino acids, or glucuronic acid, which are intracellular molecules that bear polar moieties. As so, biotransformation renders more hydrosoluble polar conjugates, which are more easily excretable, mainly by the kidneys. Glutathione S-transferases (GST) are isoenzymes that catalyze the conjugation of glutathione to xenobiotics. Therefore, play an important role in turning more soluble many xenobiotics (Habig *et al.*, 1974; Rioli *et al.*, 2001; Mannervik *et al.*, 2008).

Since activity levels of GST isoenzymes could change when vertebrates are exposed to chemicals (Hayes, Pulford, 1995), alteration of GST activity has been used as a biomarker of sublethal effects of xenobiotics in fish (Rodríguez-Ariza *et al.*, 1993; Gallagher *et al.*, 1996; Lenartova *et al.*, 1997; van der Oost *et al.*, 2003; Amado *et al.*, 2006; Farombi *et al.*, 2007; Arakawa *et al.*, 2010; Li *et al.*, 2011; Hellou *et al.*, 2012; Bastos *et al.*, 2013a; Blasco *et al.*, 2021). The activities of GST isoenzymes can be detected *in vitro* with synthetic substrates (Danielson, Mannervik, 1985; Sheehan *et al.*, 2001; Mannervik

et al., 2008). To sort out GST isoenzymes' activities, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and ethacrynic acid (ETHA) have been substrates of choice. The substrate CDNB has been the most frequently used for assaying GST activity because it was shown that many GST isoenzymes are capable of conjugating GSH with this synthetic substrate (Habig *et al.*, 1974; Sheehan *et al.*, 2001; Mannervik *et al.*, 2008).

Some reports have indicated that differentiating which substrate would reveal a GST activity affected by a particular pollutant may be more effective in evaluating whether a species is suffering from sublethal exposition (Martínez-Lara *et al.*, 1996; Camacho *et al.*, 2000). For instance, hepatic GST assayed with ETHA in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) liver was the principal activity induced in response to polychlorinated biphenyls (Pérez-López *et al.*, 2002). An elevated activity assayed with ETHA was also reported in the liver of gilthead sea bream (*Sparus aurata* Linnaeus, 1758) injected with Aroclor 1254 and dieldrin (Martínez-Lara *et al.*, 1996). Other stressors can change the level of GST activity. Hypoxia, for instance, caused a decrease in a GST activity assayed with 4-hydroxy-nonenal (4-HNE) in erythrocytes of a Neotropical species (Rocha-Santos *et al.*, 2018).

Agricultural expansion has been pushing up the number of pesticides released in the South American river basins. Fish may absorb organophosphorus pesticides and polycyclic aromatic hydrocarbons from water. Vast territories in Brazil should be preserved as biodiversity sanctuaries, where more than 200 native fish species dwell; tens of them with a still unknown pisciculture potential. Noteworthy, it has been recently brought to the fore how highly important Neotropical fishes are by showing tambaqui *Colossoma macropomum* (Cuvier, 1816) intensive and extensive production as highly productive with minimal environmental impact (Hilsdorf *et al.*, 2022). According to the Intelligence and Market Centre in Aquiculture (CIAqui) of the Brazilian Agricultural Research Corporation (Embrapa) more than 11,000 tons of pacu *Piaractus mesopotamicus* (Holmberg, 1887) and patinga were produced in 2020. Patinga is a hybrid of pirapitinga *Piaractus brachypomus* (Cuvier, 1818) and pacu. Owing to *P. mesopotamicus* steadily increase as a commercially valuable species, we considered of great importance to establish whether GST conjugation activity could be assayed as a pollutant biomarker in some of its tissues. Because the level of activity of GST isoenzymes varies in fish from organ to organ, we considered establishing if GST isoenzymes activities would change in the liver and the kidney in response to exposing pacu to methyl parathion, as an organophosphate pesticide archetype, and benzo[a]pyrene, as a polycyclic aromatic hydrocarbon archetype. To detect changes in hepatic and renal GST activities as alarm biomarkers we had also to establish which of the three substrates, CDNB, DCNB or ETHA, should be the most suitable.

MATERIAL AND METHODS

Reagents. 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), ethacrynic acid (ETHA), beta-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), glutathione reductase, potassium cyanide (KCN), Folin and Ciocalteu's reagent, bovine serum albumin (BSA), Tris(hydroxymethyl)aminomethane

hydrochloride, benzo[a]pyrene and methyl parathion (dimethyl *p*-nitrophenyl phosphate) were obtained from Sigma Chemical Co. The substrate 1,2-dichloro-4-nitrobenzene (DCNB) was purchased from Fluka. All other chemicals were of analytical grade.

Fish specimens. Specimens of pacu, *Piaractus mesopotamicus*, were supplied by the Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental (CEPTA) located in Pirassununga, São Paulo, Brazil. Two specimens were deposited as vouchers in the ichthyological collection of the Universidade do Estado do Rio de Janeiro (UERJ 2254). Males and females, weighing approximately 250 g and measuring about 22 cm, were acclimatized for three weeks in tanks with 500 L of aerated dechlorinated tap water. A total of 33 fish were acclimatized in two 500 L tanks. Oxygen in water was $5.0 \pm 0.38 \text{ mg L}^{-1}$ measured with a Hanna portable oximeter equipped with a polarographic probe HI-76407A/4. Fish were fed daily with a commercial fish diet for omnivore (Snatural Ambiente) and submitted to a 12 h photoperiod. Feeding was suspended 48 h before injections.

Xenobiotic treatments. For methyl parathion treatment, 15 pacus kept in a 500 L tank were given a single intracoelomic injection of methyl parathion (8 mg per kg body weight) previously diluted in corn oil (assay group). Another 15 fish (control group) in another 500 L tank were injected with corn oil only. Five animals from each group were euthanatized at 24, 48 and 96 h after the injection. For the benzo[a]pyrene treatment, 18 pacus kept in a 500 L tank were given a single intracoelomic injection of benzo[a]pyrene (15 mg per kg of body weight) previously diluted in corn oil (assay group). Another 18 pacus in another 500 L tank were injected with corn oil only (control group). Nine fish of both assay and control groups were euthanatized 7 and 14 days after the injections.

Blood measurements. Three-mL syringes equipped with 25 mm long x 0.6 mm gauge needles previously rinsed with heparin were employed to collect blood by puncturing a vessel beneath the spine of the fish's caudal peduncle. Measurements of oxygen partial pressure, carbon dioxide partial pressure, hemoglobin saturation, and blood pH were carried out using 1 mL of blood injected into a blood gas analyzer (AVL Omni 7, Roche Diagnostica, São Paulo, SP, Brazil).

Cytosolic fractions. The rupture of their vertebral column euthanatized fish. Livers and kidneys were excised, rapidly bathed in a cold 0.9% NaCl solution, and immediately frozen in liquid nitrogen until use. After being thawed, they were quickly mopped up with filter paper, weighed, and separately homogenized in four volumes of cold 0.1 M potassium phosphate buffer, pH 7.0, containing 0.25 M sucrose, using a Potter-Elvehjem tissue homogenizer (Potter, 1955). Homogenates were centrifuged at $700 \times g$ for 10 min. The supernatant fractions were centrifuged at $20,000 \times g$ for 20 min, and the resulting supernatant fractions were further centrifuged at $105,000 \times g$ for 90 min. All the steps were carried out at 5°C . The $105,000 \times g$ supernatant, named the cytosolic fraction, was kept frozen in liquid nitrogen until assays of enzyme activities were carried out.

GST isoenzyme assays. Cytosolic fraction protein content was determined according to Peterson (1977) using BSA as standard. All GST assays were carried out with an aliquot of the cytosolic fractions. A spectrophotometer (UV-160, Shimadzu, Kyoto, Japan) was employed to register absorbance continuously at 25 °C, slightly modifying the procedures of Habig *et al.* (1974) and Carmagnol *et al.* (1983). GSH was always available to catalysis in the assay media. Assay concentrations of substrates, buffer solutions and pH values of each assay were those in which initial velocities depended only on the amount of active isoenzymes present in cytosol samples. The GST assay with CDNB was carried out with 2.0 mM CDNB (in 1% ethanol), 15 mg of cytosolic proteins from liver or kidney, 5 mM GSH for the liver and 2.5 mM GSH for the kidney. Assays were carried out in a 0.025 M potassium phosphate buffer solution, pH 7.0, with kidney cytosol, and in 0.1 M potassium phosphate buffer, pH 6.0, with liver cytosol.

The GST assays with ETHA were carried out with 0.25 mM ETHA (in 1% ethanol) and 250 mg of cytosolic proteins from liver or kidney. In cytosol from liver 0.65 mM GSH in a 0.025 M potassium phosphate buffer, pH 7.0. In cytosol from the kidney, the concentration of GSH was 1.0 mM in the same buffer, but at pH 7.5.

The GST assays with DCNB were carried out with 4 mM DCNB (in 1% ethanol), 250 mg of proteins from liver cytosol, or 540 µg of proteins from kidney cytosol. For liver cytosol, the assay medium contained 1.5 mM GSH in 0.1 M potassium phosphate buffer, pH 6.0, and for kidney, it had 10 mM GSH in 0.025 M potassium phosphate buffer, pH 6.5.

Statistical analyses. Analysis of the results was carried out using Instat from GraphPad Software, San Diego, USA. Comparisons among GST activities of cytosolic fractions were made by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons post-test.

RESULTS

No fish died before euthanasia or showed any visible sign of intense poisoning. Measurements of oxygen partial pressure, carbon dioxide partial pressure, hemoglobin saturation and pH in blood were not significantly altered comparing injected pacu with controls (Tab. 1).

The Fig. 1 shows that neither the activity assayed with CDNB as substrate nor that assayed with ETHA changed in pacu liver cytosol after the injection of methyl parathion. Otherwise, the activity assayed with DCNB was reduced approximately to half of the activities measured in liver cytosols from pacu injected with corn oil. The Fig. 2 shows that the activity with CDNB from the kidney did not change significantly after treatment with methyl parathion; neither did the activities with ETHA or with DCNB.

No significant variation in the liver GST activity assayed with CDNB was observed after injecting pacus with 15 mg of benzo[a]pyrene per kg of body weight (Fig. 3). Also, activity measured with ETHA in liver cytosols of benzo[a]pyrene-treated fish did not change compared to the respective controls. On the other side, activity with DCNB increased, becoming about three times higher in the liver of pacus injected than in the liver of controls seven days after the injection. Fourteen days after injecting the fish, it had decreased back to normal levels.

TABLE 1 | Oxygen partial pressure (PO_2), carbon dioxide partial pressure (PCO_2), oxygen saturation of hemoglobin (Hb) and pH in blood from *Piaractus mesopotamicus* injected with parathion or benzo[a]pyrene. Numbers are means \pm standard error of mean from 15 animals. *Concentration of oxygen in water.

Treatments $O_2 = 5.0 \text{ mg L}^{-1}$ *	PO_2 (mm Hg)	PCO_2 (mm Hg)	Hb saturation (%)	pH
Controls (Corn oil)	28.6 ± 5.0	24.3 ± 5.6	59.0 ± 6.2	7.38 ± 0.12
Methyl parathion ($8 \text{ mg kg}^{-1} \text{ b.w.}$)	29.7 ± 3.7	22.1 ± 4.9	57.7 ± 5.5	7.20 ± 0.15
Benzo[a]pyrene ($15 \text{ mg kg}^{-1} \text{ b.w.}$)	27.9 ± 4.8	22.2 ± 4.7	58.2 ± 5.6	7.24 ± 0.17

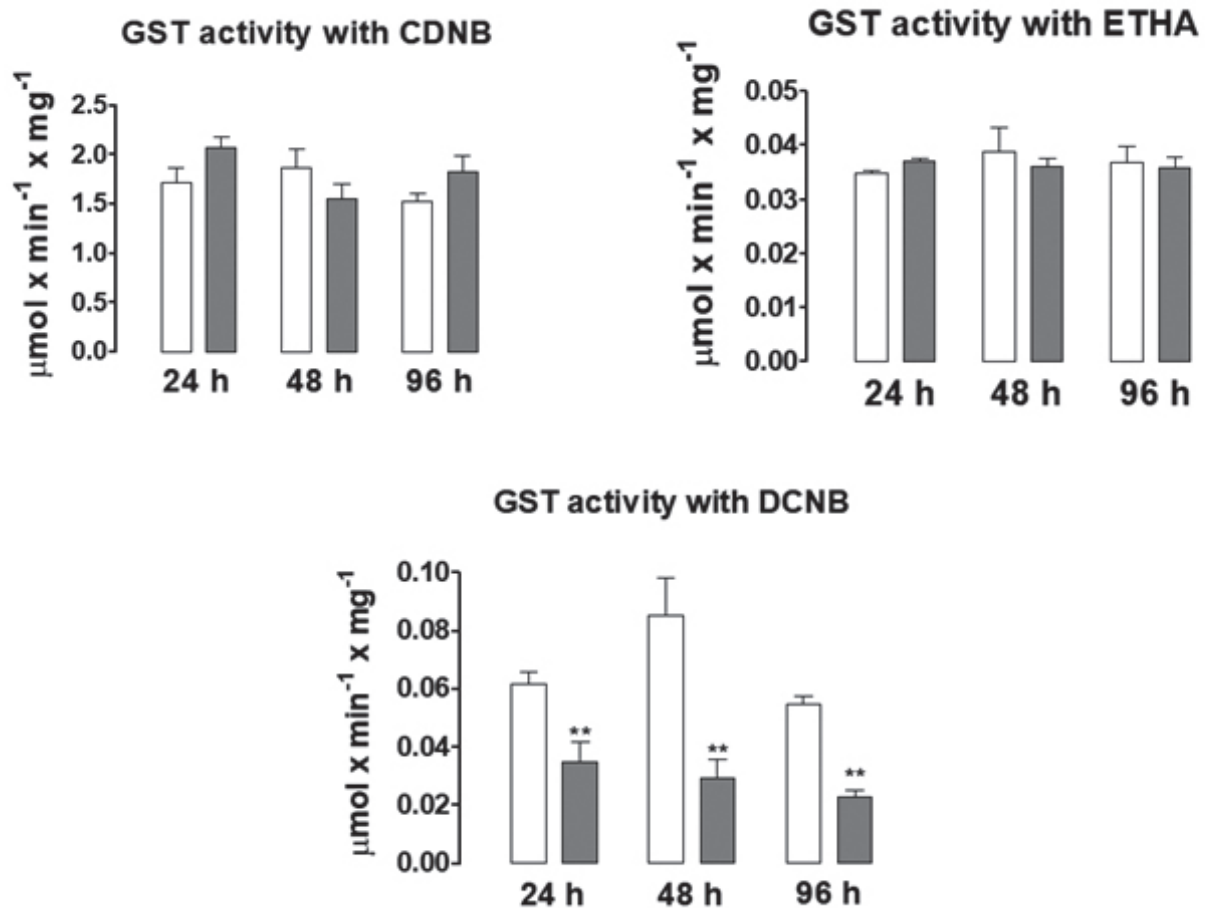


FIGURE 1 | GST specific activities in liver cytosol from *Piaractus mesopotamicus* injected with methyl parathion (8 mg kg^{-1}). Assays were carried out with 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ETHA) or 2-dichloro-4-nitrobenzene (DCNB) at 24, 48 and 96 hours after the injection. Bars represent the means \pm S.E.M. of assays from five fish injected with corn oil (clear) or methyl parathion (dark grey). Significant differences from controls are indicated as ** ($P < 0.01$).

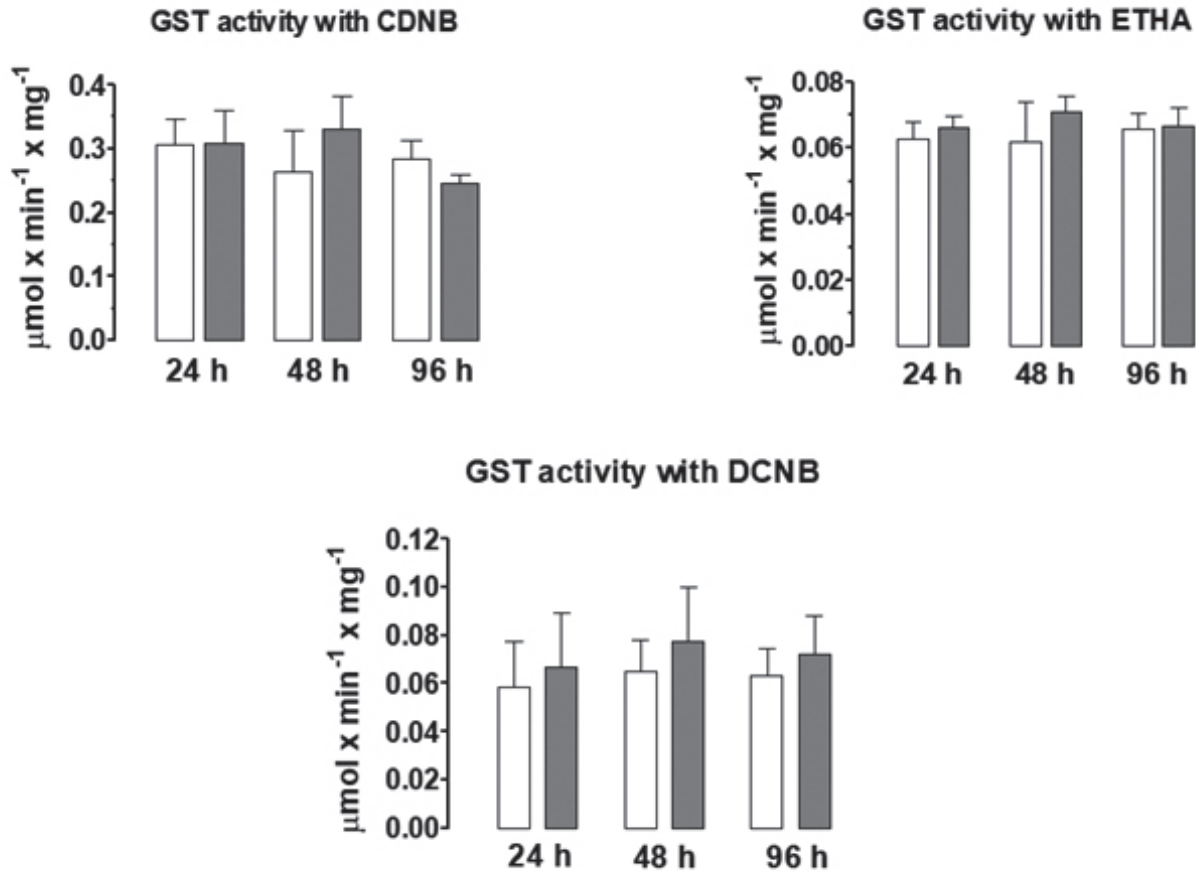


FIGURE 2 | GST specific activities in kidney cytosol from *Piaractus mesopotamicus* injected with methyl parathion (8 mg kg⁻¹). Assays were carried out with 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ETHA) or 2-dichloro-4-nitrobenzene (DCNB) at 24, 48 and 96 hours after the injection. Bars represent the means ± S.E.M. of assays from five fish injected with corn oil (clear) or methyl parathion (dark grey).

No significant alteration in GST assayed with CDNB or ETHA was seen in kidney cytosols after injecting pacu with benzo[a]pyrene (Fig. 4). However, activity with DCNB increased approximately three times on the 7th and 14th days after the injection.

Comparing the levels of activities from non-injected fish shown in Figs. 1 and 3 with the activities in Figs. 2 and 4 it is possible to see that the activities with CDNB were approximately six times higher in the liver than in the kidney of pacu. Non-injected fish activities with ETHA were sixty times lower than those with CDNB in the liver (Figs. 1 and 3) but only five times more down in the kidney (Figs. 2 and 4). GST activities from non-injected fish with DCNB did not present a significant difference comparing liver and kidney.

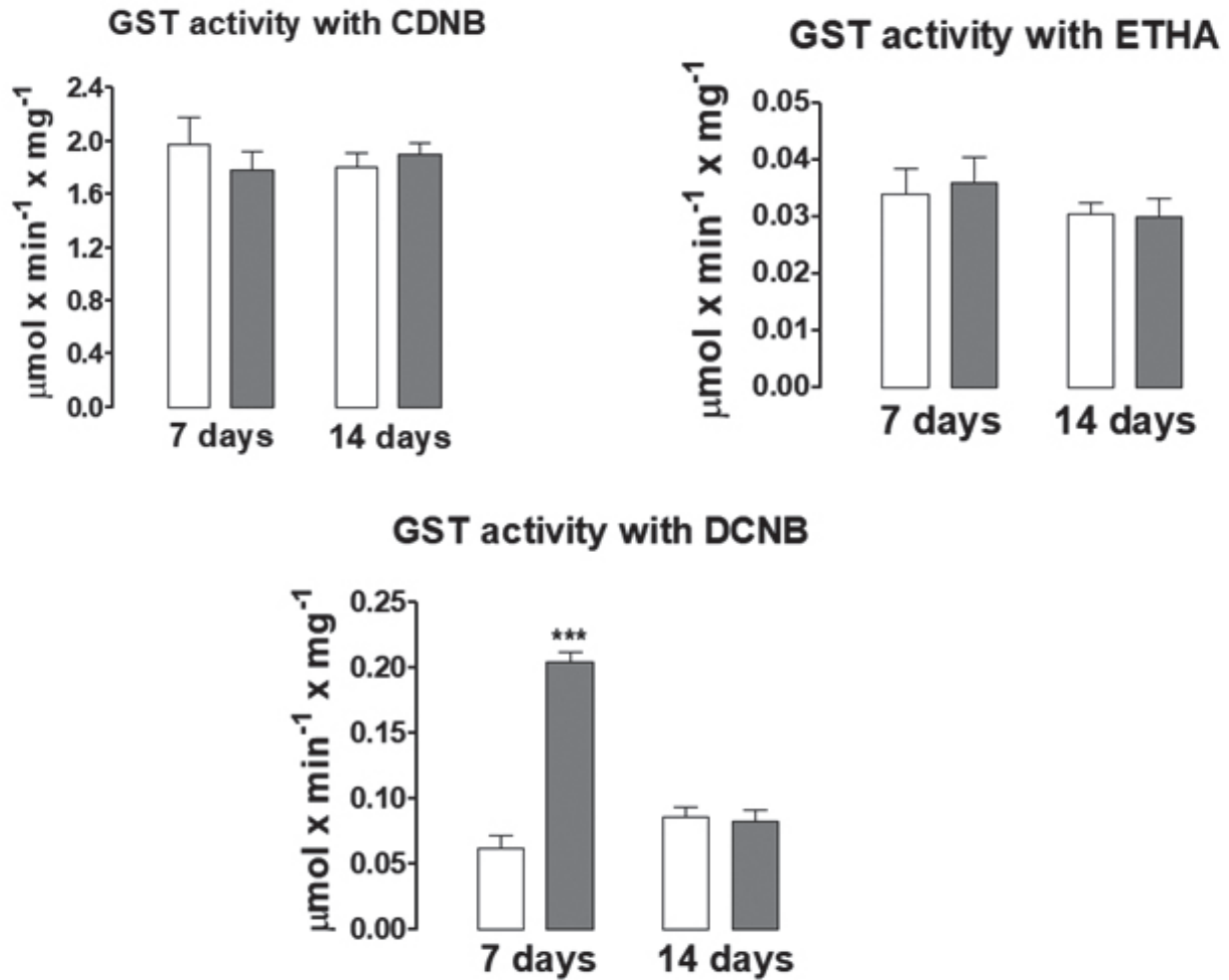


FIGURE 3 | GST specific activities in liver cytosol from *Piaractus mesopotamicus* injected with benzo[a]pyrene (15 mg kg⁻¹). Assays were carried out with 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ETHA) or 2-dichloro-4-nitrobenzene (DCNB) 7 and 14 days after the injection. Bars represent the means ± S.E.M. of assays from nine fish injected with corn oil (clear) or benzo[a]pyrene (dark grey). Significant different from controls are indicated as ***($P < 0.001$).

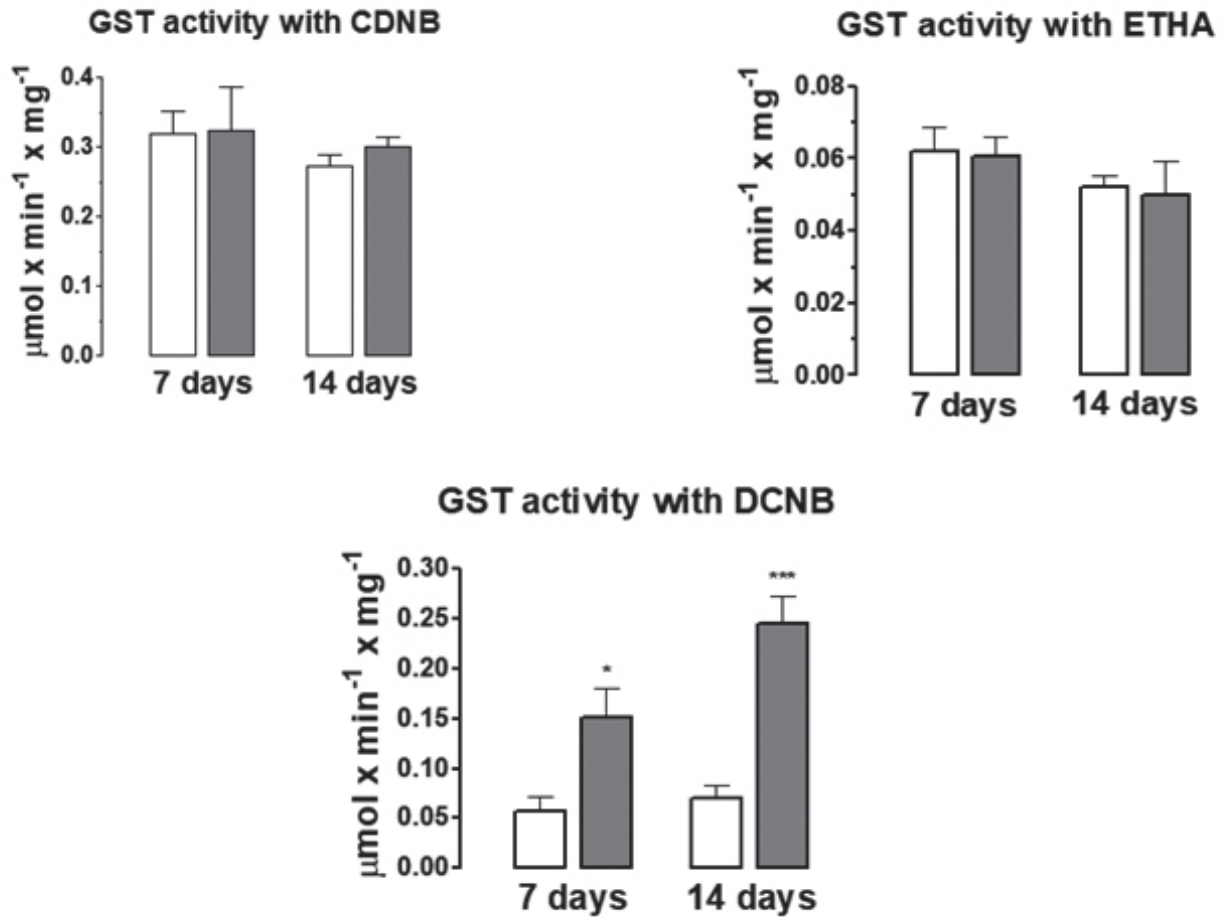


FIGURE 4 IGST specific activities in kidney cytosol from *Piaractus mesopotamicus* injected with benzo[a]pyrene (15 mg kg⁻¹). Assays were carried out with 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (ETHA) or 2-dichloro-4-nitrobenzene (DCNB) 7 and 14 days after the injection. Bars represent the means ± S.E.M. of determinations from nine fish injected with corn oil (clear) or benzo[a]pyrene (dark grey). Significant different from controls are indicated as *(P < 0.05) and ***(P < 0.001).

DISCUSSION

We did not have enough pacus for carrying out tests to establish the best dose of methyl parathion and benzo[a]pyrene. As there was no information on pacu injected with these xenobiotics, we followed a piece of classical information in a previous report (Gerhart, Carlson, 1978). These authors injected doses from 3 μg per kg body weight to 300 mg/kg of benzo[a]pyrene and established as a standard dose 30 mg/kg capable of inducing biotransformation enzymes in liver of trout. Then, we judge that for a challenging dose capable of eliciting a GST response in pacu liver and kidney, 15 mg of benzo[a]pyrene/kg would be enough.

We had previously verified the levels of parathion that could be accumulated in the muscle, heart, brain, liver, and serum of pacu (Salles *et al.*, 2015). Based on these data, we estimated that 8 mg of methyl parathion per kg body weight was a challenging dose

capable of eliciting a GST response in pacu liver and kidney. GST isoenzymes found in the cytosol of many vertebrate cells correspond to 90% of all cellular GST (Parkinson, 1996). Cytosolic GST proteins comprise a family of isoenzymes, many capable of detoxifying cells through conjugation of glutathione with an array of electrophile compounds. Among these, the organophosphorous xenobiotics can be biotransformed by some GST isoenzymes (Jokanović, 2001; Abel *et al.*, 2004). Human placenta and fetal liver GST isoenzymes can carry out biotransformation of methyl parathion through O-demethylation producing demethyl parathion (Radulovic *et al.*, 1986; Radulovic *et al.*, 1987; Abel *et al.*, 2004). Di Ilio *et al.* (1995) reported that a GST assayed with DCNB from female mouse liver presented ten times more affinity to bind methyl parathion *in vitro* than a GST assayed with ETHA. Thus, it is plausible that pacu, also a vertebrate, showed a liver GST activity assayed with DCNB affected by methyl parathion (Fig. 1). Interestingly, the fact that this GST activity decreased in the liver of pacu injected with methyl parathion (Fig. 1), but not in kidney (Fig. 2), indicate that some GST isoenzymes in the liver of pacu have structural characteristics which make them capable of suicidal attaching to triesters of phosphoric acid toxicants, as it has been described (Jakoby, Keen, 1977; Jokanović, 2001).

According to what was reported for rats (Hesse *et al.*, 1980) the increased GST activity in kidney cytosol following injection of benzo[a]pyrene (Fig. 4) is an indication that typical isoenzymes may be responsible for preventing the binding of electrophiles derived from benzo[a]pyrene to DNA, RNA and proteins in pacu kidney cells. Noteworthy, pacu has inhabited South America for millions of years before murines, undergoing many climate changes and adapting to a variety of earth's alterations of structure. Thus, this preventive role in pacu, an Osteichthyes, is proof that the versatility of GST isoenzymes, many of them nowadays capable of detoxification, has been evolving for millions of years.

Another three essential findings are also noteworthy. First, results from Tab. 1 show that the experimentation procedures did not produce alterations of GST activities attributable to lower levels of oxygen in the blood of the pacus. This is important because it has been shown that GST activity from liver cells (Bastos *et al.*, 2013b) and erythrocytes (Rocha-Santos *et al.*, 2018) can be diminished in pacus submitted to hypoxia causative of an increase in reactive oxygen species. Second, GST activities in the liver and kidney of pacu assayed with ETHA, contrarily to what was found for other vertebrate species (Di Ilio *et al.*, 1995; Abel *et al.*, 2004) were not affected by methyl parathion or benzo[a]pyrene, what points to the existence of typical GST isoenzymes in pacu's liver and kidney. Third, even though the assays of GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) revealed higher activities, they were ineffectual in indicating contamination of both liver and kidney by methyl parathion or benzo[a]pyrene.

It must be stressed that the absence of significant variation in the levels of GST activity assayed with CDNB could lead one to mistakenly conclude that GST does not respond to polycyclic aromatic hydrocarbons or organophosphate pesticides in pacu and therefore that GST could not be used as a biomarker in this fish species. Such a mistake would only be sorted out whether a GST activity was assayed with DCNB, since it was increased in the pacu kidney by benzo[a]pyrene and inhibited in the pacu liver by methyl parathion. Indeed, as reviewed for aquatic biota (Hellou *et al.*, 2012), using GST activity as a biomarker for xenobiotics in fish must always

consider that GST from different fish species might have different affinities relative to the several substances which have been ordinarily employed to assay their activity. The assay we established here is more straightforward, cheaper, and easier to use than any sophisticated procedures from molecular biology, which detect gene expression but do not unequivocally unveil alteration of an enzyme activity, which is biochemically more relevant (Karaca *et al.*, 2014).

In conclusion, there is a GST activity measurable with 1,2-dichloro-4-nitrobenzene (DCNB) in the liver and kidney of pacu that might help to indicate that organophosphorus or polycyclic aromatic hydrocarbons were absorbed by pacu. However, one should not take pacu as the archetype of Neotropical fishes and conclude that in all Neotropical fish species organophosphorous or polycyclic aromatic hydrocarbons will be unequivocally biomarked using GST activity assayed with DCNB. It will always be required to check GST activities with different substrates in a specific tissue of a fish species in which these relevant detoxifying activities have not been assayed before.

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Frederico F. Bastos: Investigation, Methodology, Validation, Writing–review and editing.

Lin M. de Lima: Methodology, Validation.

Santiago A. L. Tobar: Formal analysis, Investigation.

Vera L. F. Cunha Bastos: Conceptualization, Formal analysis, Investigation, Supervision.

Jayme Cunha Bastos: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing–original draft, Writing–review and editing.



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ETHICAL STATEMENT

These procedures were carried out according to the ethical principles in animal experimentation elaborated by the Brazilian College for Animal Experimentation (COBEA), which follows the uniform requirements for submitting manuscripts to biomedical journals (protocol number CEUA/017/2016).

COMPETING INTERESTS

The authors declare no competing interests.

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