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# Evaluation of caffeine effects on biochemical and genotoxic biomarkers in the neotropical freshwater teleost *Prochilodus lineatus*

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ABSTRACT

Caffeine is often found in aquatic environments, leading to concerns regarding its adverse consequences for aquatic biota. Biochemical and genotoxic biomarkers were analysed in juveniles of *Prochilodus lineatus* to evaluate the effects of caffeine. Fish were exposed to caffeine (0.3, 3 and 30 μg $L^{-1}$) for either 24 h or 168 h. Longer exposure to caffeine resulted in a significant reduction in the activity of the phase I biotransformation enzyme ethoxyresorufin-O-deethylase (EROD) in the brain but a significant increase in the liver. Changes in glutathione content (GSH), glutathione S-transferase (GST) activity, and lipid peroxidation were not found in the liver and brain of fish exposed to caffeine. DNA damage in erythrocytes were also not found. These results show that caffeine may interfere with the biotransformation mechanism of *P. lineatus* after 168 h exposure, but it does not generate sufficient changes to trigger a state of oxidative stress.

## 1. Introduction

Caffeine (1,3,7-trimethylxanthine) is a bitter, white, crystalline substance found naturally in the leaves, seeds and fruits of several plants. Its pure form was first isolated in 1819 from the coffee plant, but today, its presence is known in teas, chocolates, the mate herb and guarana (Banerjee et al., 2014). Its high consumption is mainly due to its stimulating effect on the central nervous system. This effect reduces fatigue and increases concentration, alertness and physical endurance, and recent studies have demonstrated its contribution to weight loss (Heckman et al., 2010).

Despite its benefits to human health, this high caffeine consumption can lead to the serious problem of aquatic contamination. Approximately 5% of caffeine is not metabolised but instead excreted in the urine and reaches water bodies through the sewage system besides the disposal of food, beverages and drugs containing caffeine (Montagner et al., 2014). Monitoring studies on aquatic environments show a high frequency of samples containing caffeine with concentrations ranging from 0.001 μg $L^{-1}$ to 753 μg $L^{-1}$ (Petrovic et al., 2003; Canela et al., 2014). Caffeine can thus be classified as an emerging contaminant, a substance that is not included in monitoring programmes (USEPA, 2016), but should be added in future legislation and is being widely detected and can harm aquatic biota (Stuart et al., 2012).

The constant presence of caffeine in aquatic ecosystems creates interest in understanding its effects on aquatic animals, that majority are not the target organisms of this substance. Many published studies have already demonstrated the effects of caffeine on fish, with emphasis on research related to behavioural changes (Richendrfer et al., 2012; Ladu et al., 2015; Tran et al., 2017). However, exploring other possible effects of caffeine is necessary, especially changes at the subcellular level, which can be used as an important tool to evaluate early caffeine effects on other species, communities or ecosystems (Lam, 2009). Some studies involve the investigation of caffeine effects on biotransformation enzymes and antioxidant defences in aquatic invertebrates (Aguirre-Martínez et al., 2015, 2016; Cruz et al., 2016), but few include fish species (Li et al., 2012).

In mammals, the phase I biotransformation enzymes of the cytochrome P450 (CYP) complex are extremely important for the metabolism of caffeine. Enzymes of the CYP1A family are mainly responsible for transforming caffeine into paraxanthine, with other CYPs converting caffeine into theobromine and theophylline. In addition to the phase I

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pathways, another important step in the biotransformation of caffeine is acetylation (phase II), through the action of N-acetyltransferase. These pathways act together to facilitate the excretion of caffeine and its metabolites (Sawynok and Yaksh, 1993; Burdan, 2015). In fish, phase I and II biotransformation processes are mainly mediated by enzymes of the CYP1A family and glutathione S-transferases, respectively (Van der Oost et al., 2003). However, the metabolism of caffeine in these animals is still not well described.

Biotransformation processes can generate reactive oxygen species (ROS) that, in excess, become toxic to the body by inducing a state of oxidative stress, which can damage lipids, proteins and DNA (Lesser et al., 2012). Despite the already known antioxidant effects for mammals (George et al., 1999; Azam et al., 2003), studies with bivalves have already demonstrated that caffeine may lead to increased lipoperoxidation (Cruz et al., 2016) and DNA damage (Aguirre-Martínez et al., 2015), demonstrating the need for further investigation into the prooxidant effects of caffeine on aquatic organisms.

The fish *Prochilodus lineatus* is an adequate experimental model for investigating the effects on enzymes related to biotransformation and oxidative damage promotion by contaminants since this species has a well-known physiology, and its sensitivity to various aquatic pollutants has already been demonstrated (Cazenave et al., 2009; Santos and Martinez, 2012; Palermo et al., 2015). Therefore, considering that studies describing the metabolism of caffeine in fish are scarce and that this substance was already related with oxidative damage in aquatic species, the objective of the present study was to evaluate the effects of caffeine on *P. lineatus* after 24 and 168 h of exposure using biomarkers related to biotransformation and oxidative stress.

## 2. Material and methods

### 2.1. Experimental design

Juveniles of *P. lineatus* (weight 15.26 ± 1.1 g, standard length: 9.28 ± 0.2 cm, mean ± SD, n = 80) were obtained from a fish farm and acclimated for 7 days in 300 L tanks containing clean, dechlorinated water with constant aeration. During this period, commercial feed (Guabi®, BR) was provided every 48 h, with feed being stopped 24 h before the tests; no feeding occurring during exposure. The physical and chemical parameters of the water were monitored throughout the acclimation and exposure tests (temperature = 22.78 ± 0.065 °C; pH = 8.19 ± 0.015; OD = 8.16 ± 0.128 mg $O_2$ $L^{-1}$; conductivity = 0.116 ± 0.002 mS $cm^{-1}$).

Using a stock solution of caffeine (99% Synth, Brazil) dissolved in ultrapure water (0.48 g $mL^{-1}$), aquaria were prepared with 80 L of water and caffeine at concentrations of 0.3, 3 or 30 µg $L^{-1}$ (CAF 0.3, CAF 3 and CAF 30, respectively) or only water (control). After acclimation, ten fish were transferred to each aquaria and held in these conditions for 24 h or 168 h (with total water renewal every 24 h). The tests were performed for each experimental period (24 and 168 h) in independent experiments and for each exposure time, control (CTR) and caffeine groups (CAF 0.3, CAF 3 and CAF 30) were run simultaneously. Thus, we had four aquaria for each experimental time totaling eight aquaria and 80 fish. The concentrations of caffeine tested are based on concentrations already found in Brazilian surface waters (Sodré et al., 2010; Montagner et al., 2014; Campanha et al., 2015). Samples of water from the aquaria were collected at the beginning of each experiment (t0), after the fish were removed from the 24-h experiment and at each water replacement (24-h intervals) along the 168-h experiment. These water samples were stored at 10 °C for further analysis of the caffeine concentration.

At the end of the exposure periods, specimens were anesthetised in benzocaine (0.1 g $L^{-1}$) to allow blood to be drawn from the caudal vein. And euthanized by medullary section for removing liver and brain. The organs were stored in ultra-freezer (−80 °C) for biochemical analyses, and the blood was stored in fetal bovine serum and refrigerated (10 °C) for the genetic assay. The procedures described were approved by the Animal Use Ethics Committee at the State University of Londrina (CEUA process nº 19,618.2013.57).

### 2.2. Measurement of caffeine in water

The quantification of the caffeine in the water samples was performed without prior extraction using an Agilent 1200 chromatograph coupled to an Agilent 6410 quadruple triple mass spectrometer with an electrospray ionisation source after filtration in a 13mm nylon syringe filter with 0.2 µm pore size. The separation occurred at 25 °C (Zorbax SB-C18 column), with gradient elution from a mobile phase consisting of a solution of formic acid (0.01%) in methanol at a flow rate of 0.3 mL/min. The injection volume was 10 µL. Two transitions of precursor product ions (m/z) were used for the determination: 195–138 m/z EC = 15 eV for quantification and 195 - 110 m/z EC = 20 eV for confirmation. The shear stress was set at 110 volts. A calibration curve was made from a standard, and the limit of quantification was 30 ng $L^{-1}$.

### 2.3. Biochemical biomarkers

Liver and brain samples were homogenized (1:10 mass/vol) in phosphate buffer (0.1 M, pH 7.0) and centrifuged (20 min, 13,000*g*, 4 °C). The supernatants were used for the following analyses:

*CYP1A catalytic activity* - determined by EROD activity (ethoxyresorufin-*O*-deethylase), through the conversion of 7-ethoxyresorufine (provided in the reaction medium) into resorufin during 10 min (ex/em: 530/590 nm), according to Eggens et al., (1992). EROD activity was expressed in pmol of resorufin.minute $^{-1}$ mg of protein $^{-1}$ based on a standard curve of resorufina.

*Activity of glutathione S-transferase (GST)* - measured by the GSH conjugation with the substrate 1-chloro-2,3-dinitrobenzene (CDNB) in a spectrophotometer at 340 nm, according to Keen et al. (1976). GST activity was expressed in nmol of CDNB conjugated.min $^{-1}$ mg of protein $^{-1}$.

*Content of glutathione (GSH)* - measured by the reaction of glutathione with the substrate 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), forming thiolate (TNB), at 412 nm (Beutler et al., 1963). GSH was expressed in µg of GSH.mg of protein $^{-1}$.

*Lipid peroxidation (LPO)* - estimated by the quantification of one of the final products of lipid peroxidation, malondialdehyde (MDA), using the thiobarbituric acid reactive substances test (TBARS) and fluorescence readings (ex/em: 530/590 nm), according to Federici et al. (2007). LPO was expressed in MDA equivalents as µmol of MDA.mg protein $^{-1}$.

*Total protein concentration* - determined by the method of Bradford (1976), based on a standard curve of bovine serum albumin (BSA) in a spectrophotometer at 595 nm.

### 2.4. Genotoxic biomarker

DNA damage was quantified in erythrocytes by the comet assay, based on Singh et al. (1988), with some modifications (Vanzella et al., 2007). Blood samples diluted in fetal bovine serum (1: 100) were mixed in low melting point agarose (0.5%), placed on slides precoated with normal (1%) melting point agarose, covered with coverslips and brought to solidify (10 °C). After 40 min, the coverslips were removed and the slides were subjected to: a) lysis: 2 h at 4 °C, protected from light, in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% lauryl, 10% DMSO, 1 mL Triton X-100, pH 10.0); b) DNA denaturation: 30 min in the dark in an electrophoresis buffer (3M NaOH, 1 mM EDTA,