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Acute effects on hepatic biomarkers in the freshwater native fish Aequidens metae exposed to polycyclic aromatic hydrocarbons

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ABSTRACT

BACKGROUND AND OBJECTIVES: Polycyclic aromatic hydrocarbons are present in all environmental matrices. Polycyclic aromatic hydrocarbons-rich wastewater from the oil industry is discharged into natural water bodies. Detritivorous fish shown the effects of pollutants in water. Biomarkers of effect make it possible to demonstrate exposure to xenobiotics such as polycyclic aromatic hydrocarbons. The aim of the present study was to evaluate the hepatic and erythrocyte response in *Aequidens metae*, a detritivorous fish, exposed to polycyclic aromatic hydrocarbons in terms of morphological, biochemical, and genotoxic changes.

METHODS: Juveniles of *Aequidens metae* were exposed to 50 microgram per gram body weight of beta-naphthoflavone, 100 microgram per gram of naphthalene, 50 microgram per gram of phenanthrene and 10 microgram per gram of benzo[a]pyrene, for 72 hours. Water quality variables, total protein content, 7-ethoxyresorufin-O-deethylase activity, liver histopathological changes and genotoxic alterations in peripheral blood were measured during the assay.

FINDINGS: In polycyclic aromatic hydrocarbons-exposed fish, analysis of liver tissue revealed parenchymal lesions and changes in the number and shape of hepatocyte nuclei. On the other hand, only fish exposed to benzo[a]pyrene shown significant increase in the 7-ethoxyresorufin-O-deethylase activity compared to solvent control. In peripheral blood erythrocytes, increased presence of nuclear abnormalities was higher in fish exposed to phenanthrene, followed by benzo[a]pyrene, beta-naphthoflavone, and naphthalene.

CONCLUSION: It is concluded that *Aequidens metae* is a suitable bioindicator for polycyclic aromatic hydrocarbons monitoring in aquatic ecosystems. Phenanthrene reveals for the first time a greater genotoxic effect than benzo[a]pyrene at sublethal concentrations. Juveniles of *Aequidens metae* exposed to concentrations of polycyclic aromatic hydrocarbons close to those found in the environment showed health-compromising damage.

NUMBER OF REFERENCES	NUMBER OF FIGURES	NUMBER OF TABLES
66	4	3

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INTRODUCTION

Oil activity in Colombia plays a decisive role in the country's economic balance and development (Beltrán and Vargas, 2014). In turn, hydrocarbon extraction generates environmental impacts that include contamination of soil, surface water and groundwater, which involves health risks (Ossai *et al*., 2020). Among the compounds associated with oil are polycyclic aromatic hydrocarbons (PAH), which stand out for their recalcitrance and high toxicity; PAH are among the most harmful pollutants (Tariq *et al.,* 2015). As a result, they are the subject of intense research because they are considered priority pollutants by regulatory agencies such as the U.S. Environmental Protection Agency (McGrath *et al*., 2019). They possess two or more benzene rings, with widely varying molecular characteristics that establish their toxicological properties. PAH are recalcitrant organic compounds derived from fossil fuels and coal and word incomplete combustion, among others (Cousin and Cachot, 2014). Despite this scenario, in Colombia, there are scarce studies on the effects of exposure to PAH on biota. This knowledge gap makes it difficult for regulatory agencies to make environmental decisions to identify the impacts generated by anthropogenic activities. Environmental stressors can trigger biochemical and morphological alterations in aquatic organisms, including fish; these tissue modifications may indicate exposure to contaminants such as PAH available in water bodies (Honda and Suzuki, 2020). Histological analysis provides evidence of possible immediate mechanisms of BaP co-exposure and effects on the architecture of organs such as the liver in species exposed to PAH (Briaudeau *et al*., 2021). Histological analysis can detect the effects of sublethal stressors in tissues such as the liver, which is crucial for the detoxification of xenobiotics. (Wolf, 2013). Given the location of fish in food chains, the consequences of exposure to PAH motivate research (Wang *et al.*, 2021). Since fish are used as a food source, the bioaccumulative effect can impact the health of consumers (Javed and Usmani, 2019; Noman *et al*., 2022). Given the varied results of field studies that have the inability to measure all pollutants present in water bodies, studies that individually identify the effects of exposure to each compound are required (Gan *et al*., 2021; Olayinka *et al*., 2019; Yu *et al*., 2019). In this regard, exposure studies to individual PAH such as phenanthrene (PHE) have been performed in cachama blanca (*Piaractus brachypomus*) for 11 days, which showed an increase in hepatic lipid peroxidation in fish exposed to 10 microgram per gram (µg/g) of body weight (BW) (Mora-Solarte *et al*., 2020), in toadfish (*Opsanus beta*) exposed intraperitoneally to 5 µg/g doses of naphthalene (NAP) and PHE for 72 hours (h), increased glucocorticoid to stress response was evidenced (Reddam *et al*. 2017) and, in orange-spotted grouper (*Epinephelus coioides*) exposed intraperitoneally to doses of 2, 20 and 35 milligram per kilogram (mg/kg) of benzo[a]pyrene (BaP) after 4 days showed a decrease in total leukocyte erythrocytes, lysozyme activity and immunoglobulin M (IgM) level (Khaniyan *et al*., 2016). In the Orinoquia region, due to abundant crude oil extraction, PAH including NAP, PHE, and BaP, among others, are deposited on the beds of water bodies, making them accessible to benthic fish (Velasco-Santamaría *et al*., 2019). In this region, *Aequidens metae* is one of the most abundant and easily detected benthic and detritivorous not restricted fish. Furthermore, this fish occupies an intermediate place in the food chain and is a potential vector of biomagnification due to its detritivores feeding habits and it is also adapted to laboratory conditions. In this context, *A. metae* has been proposed as a potential bioindicator of exposure to environmental contaminants (Corredor-Santamaría *et al*., 2016; Corredor-Santamaría *et al*., 2019). In juvenile cichlid *Aequidens metae* native to the Colombian Orinoquia, biomarkers of exposure were used to analyze the effect of three PAH widely distributed in water bodies. The aims of the present study were to determine the suitability of the intraperitoneal route of administration at sublethal concentrations in acute assay to 3 PAH commonly found in the environment and one PAH as a model of induction of 7-ethoxyresorufin-O-deethylase (EROD) activity, histopathological alterations in liver and genotoxic alterations in peripheral blood in the neotropical cichlid *Aequidens metae*. This study was conducted at the Laboratory of Toxicology and Biotechnology of the Universidad de los Llanos in Colombia, between the years 2019 and 2020.

MATERIALS AND METHODS

Biological material and exposure experiments

Thirty *Aequidens metae* juveniles with BW of 8.24 ± 0.40 gram (g) and body length of 6.86 ±0.25

centimeter (cm) was acquired from the Laboratory of Toxicology and Biotechnology of the Universidad de los Llanos. The fish were randomly housed in 20 liter (L) capacity glass aquaria with constant aeration. Prior to the exposure period, to evaluate the behavior of the fish, they were monitored for 15 days, fed every 24 h with commercial feed at 3 percent (%) of the biomass. A semi-static system was applied, 30 % of the water volume was restored, and a temperature of 25 Celsius degree (°C) was maintained with a photoperiod of 12 h of light and 12 h of darkness. Physicochemical water quality parameters, including pH, with pH meter (Hanna Waterproof, Mauritius), temperature and dissolved oxygen were measured and a multiparameter equipment (YSI professional plus, Ohio USA), nitrite and ammonium were recorded with a HACH kit, were monitored daily. Fish were injected intraperitoneally with NAP (CAS no. 91-20-3, Sigma-Aldrich) at doses of 100 µg/g BW, PHE (CAS no. 85-01-8, Sigma-Aldrich) at doses of 50 µg/g, BaP (CAS no. 50-32-8, Sigma-Aldrich), at doses of 10 µg/g, and β-Naphthoflavone (BNF) (CAS no. 6051-87-2, Sigma-Aldrich) at doses of 50 µg/g, diluted in canola oil as PAH solvent (Oliveira *et al*., 2013; Santos *et al*., 2018), another group was injected with PAH solvent and another group without injection, each group with five replicates, for 72 h. All fish were injected at a final volume of 5 microliters per gram (µL/g) BW. After the exposure period was completed, the fish were immersed in an anesthetic solution containing 300 milligrams per liter (mg/L) of 2-phenoxyethanol, J. T. Baker, Phillisburg, USA), after losing the swimming axis they were desensitized by cervical dislocation. Fish fork length (ichthyometer) and weight (Ohaus Scout Pro® digital) were recorded. The liver was removed and divided for biochemical and histological procedures, and immediately collected tissues were preserved in vials under freezing at -70°C and in vials containing buffered formaldehyde at room temperature, respectively (Fig. 1). Doses and exposure times were selected according to previous studies of induction of enzymes of intermediary metabolism in the hepatic tissue with NAP, PHE, and BaP in golden tilefish (*Lopholatilus chamaeleonticeps*), yellowfin seabream (*Acanthopagrus latus*), and zebrafish (*Danio rerio*), respectively (Gerger and Weber, 2015; Shirmohammadi *et al*., 2017; Snyder *et al*., 2015). The usefulness of intraperitoneal injection has been proven in various studies of exposure to toxic xenobiotics including PAH since facilitates the direct entry of the compounds into the organism, attenuating losses during administration and the release of residues into the environment (Karami *et al*., 2011). This research project was carried out based in accordance with the norms established in Resolution No. 8430 of 1993 of the Ministerio de Salud, Colombia and those established by the Bioethics Committee of the Universidad de los Llanos.

Hepatosomatic index (HSI)

HSI was calculated based on the ratio of liver weight to the weight of each fish using Eq. 1 (Araújo *et al*., 2017).

$$HSI = \text{Liver weight (g)}/\text{Fish weight (g)} \times 100 \quad (1)$$

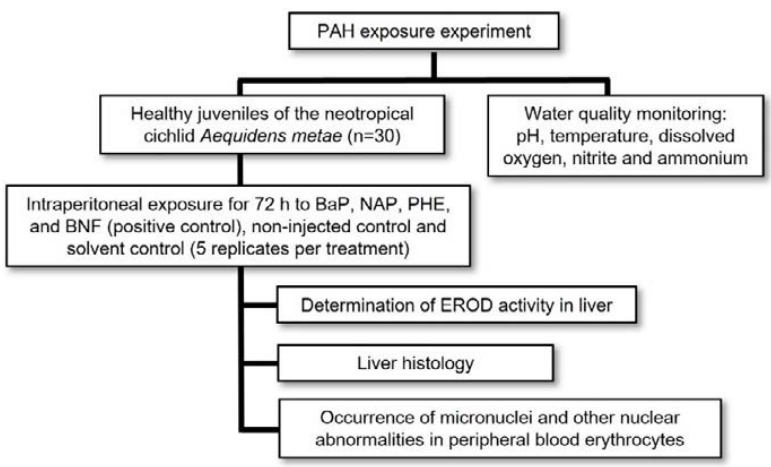

Fig. 1: Summary of the methodology used at the experiment with *Aequidens metae* exposed to PAH during 72 h

EROD assay and protein quantification

In *A. metae*, hepatic EROD activity was determined based on the protocol proposed by Valdehita *et al.* (2012) after subsequent standardization. During standardization of the determination of EROD activity in liver of *A. metae* after intraperitoneal injection of BaP, the highest level of this biomarker was found at 72 h. Around 30 mg of liver kept at 4°C was homogenized in 1 ml of buffer containing 0 - 1 M tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) pH 7.0 - 25 millimolar (mM) sucrose, 150 mM potassium chloride (KCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0 - 25 mM phenylmethylsulfonyl fluoride (PMSF) and 20 % glycerol). The tissue homogenate was centrifuged for 10 min at 6000 gravities (*g)*. The supernatant was centrifuged for 60 min at 16,000 *g*. The sediment was diluted in 100 microliters (µL) of buffer. EROD activity was measured from total protein concentration (Burke and Mayer, 1974). Fluorescence was recorded on a Cytation 3 spectrophotometer (BioTek®, USA), after the reaction of proteins with Coomassie Brilliant Blue G-250 dye. Finally, a standard curve with bovine serum albumin (BSA) was performed and the absorbance was determined at a length of 595 nanometer (nm).

Histological evaluation

Tissues were fixed in buffered formalin (pH 7.2) at 4 °C for 5 days with increasing concentrations of ethanol between 70 and 100 % the tissues were dehydrated. Then the tissues were embedded in paraffin (MERCK) and sectioned at 3 micrometers (µm) with a CUT 5062 (SLEE Medical GmbH, Germany). Hematoxylin and eosin (H and E) staining was applied. Five sections were examined from each fish under an Eclipse-E100 light microscope (Nikon, Japan) with a Nikon camera connected. The appearance of morphological alterations in each tissue was evaluated following the methodology modified by Abalaka *et al.* (2015) based on the degree of tissue change (DTC), which considers the severity of the alterations. Organ function is not compromised at stage I, at stage II function is altered, and at stage III damage is irreversible. For each fish, DTC = $10^0\sum$I + $10^1\sum$II + $10^2\sum$III, in equation I, II, and III represent the total disruptions observed at stages (n = 5), in 30 fields photographed for each fish. Mean DTC was ranked from 0 to 10 (normal tissue); 11 to 20 (mild to moderate disruption); 21 and 50 (from moderate to severe disruption); 51 and 100 (severe disruption); and higher than 100 (irreparable disruption).

Histometric evaluation

Hepatocyte nuclei were measured in terms of their diameters (µm) and area (µm^2) for each fish using ImageJ. Tissue cell proliferation was determined by counting nuclei in 30 visual fields for each fish in a fixed area of 10,000 µm^2. Values were shown as the mean and standard error of measurements of hepatocyte diameters, area, and nuclei counts for all treatments.

Genotoxicity responses

From the caudal vessels of each fish, 5 µL of peripheral blood were extracted and stained for 10 min with Wright-methanol (Merck®). Micronucleus (MN) frequency was calculated in 2,000 mature erythrocytes from each fish (Al-Sabti and Metcalfe, 1995) and expressed as the total number of MN per 1,000 cells. Following the criteria of Grisolia *et al.* (2002); Carrasco *et al.* (1990), the frequency of MN and other nuclei anomalies were counted, respectively.

Statistical Analysis

All results were recorded as mean ± SE. PAH-exposed groups were compared with control groups by one-way analysis of variance (ANOVA) with subsequent Tukey's post hoc test. P value < 0.05 was the threshold value for recognizing significant statistical differences. Programs such as IBM Statistical Package for the Social Sciences (SPSS) statistic 19, GraphPad v 5.0 and SAS Institute Inc. (Cary, NC, USA).

RESULTS AND DISCUSSION

Physicochemical parameters

During the experiment, no statistical differences were found in the water quality parameters among treatments (Table 1). Water quality parameters were in accordance with the conditions present in the non-polluted natural environment (Corredor-Santamaría *et al.*, 2021).

Hepatosomatic index

HSI under stress conditions can increase and is one of the rates most often associated with exposure

Table 1: Monitoring of physical-chemical water parameters during exposure to PAH in *Aequidens metae*

Treatment	Water quality parameters				
	NH_3 (mg/L)	NO_2 (mg/L)	TEMP (°C)	pH	DO (mg/L)
Control not injected	0,057±0,02[a]	0,01±0,003[a]	25,71±0,10[a]	6,41±0,09[a]	7,5±0,1[a]
Solvent Control	0,048±0,02[a]	0,02±0,001[a]	25,60±0,12[a]	6,21±0,06[a]	7,6±0,0[a]
β-Naphthoflavone	0,051±0,02[a]	0,02±0,001[a]	25,36±0,07[a]	6,23±0,06[a]	7,8±0,1[a]
Naphthalene	0,043±0,04[a]	0,01±0,002[a]	25,42±0,13[a]	6,28±0,11[a]	7,6±0,2[a]
Phenanthrene	0,036±0,03[a]	0,02±0,003[a]	25,31±0,12[a]	6,36±0,10[a]	7,9±0,1[a]
BaP	0,056±0,02[a]	0,03±0,002[a]	25,21±0,11[a]	6,15±0,09[a]	7,5±0,0[a]

[a] Shows no significant differences (Tukey test, p>0.05). Temperature (TEMP), Dissolved oxygen (DO).

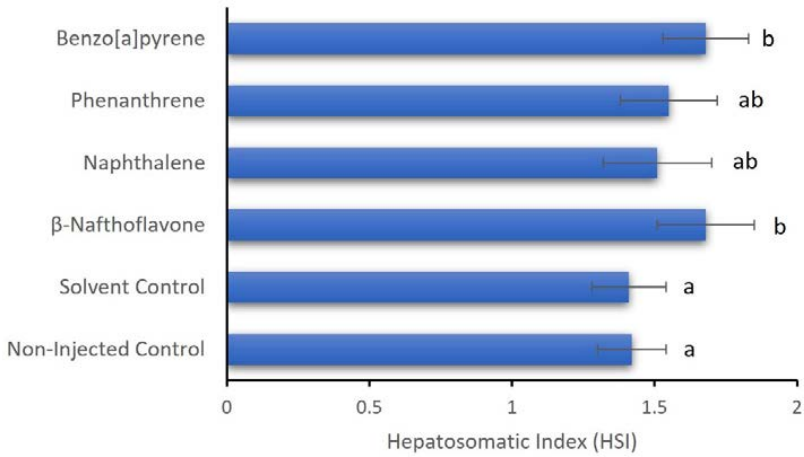

Fig. 2: Hepatosomatic index in *Aequidens metae* exposed to PAH during 72 h. Bars shown the mean ± SE (n = 5). [ab] Bars with different letters shown statistically significant differences (Tukey-Test, p<0.05) between treatments.

to contaminants (Al-Ghais, 2013; De *et al.*, 2016; Aly and Abouelfadl, 2020).

Increased hepatosomatic index in *A. metae* exposed to BNF and BaP (Fig. 2), compared to non-exposed groups, is possibly due to a significant increase in detoxification processes, which was observed in the evaluation of liver tissue, such as vacuolization, hypertrophy, and hyperplasia of hepatocytes, and increased sinusoidal space. In zebrafish (*Danio rerio*) exposed to 6 µg/L BaP for 15 days, Mai *et al.* (2021) reported similar findings along with increased induction of oxidative stress. Also, in fish exposed to produce waters by oil extraction, which are dominated by two-ring PAH, such as naphthalene an increase in the HIS was observed (Meier *et al.*, 2020).

EROD assay and protein quantification

The determination of EROD activity has been routinely employed as an indirect measurement of the induction of cytochrome P450, family 1 (*CYP1)* expression as a biomarker of exposure to different PAH such as BaP (Jönsson *et al.*, 2010). PAH have been shown to induce CYP-related mixed-function oxidases, and that CYP, family 1, subfamily A (CYP1A) activity can be measured in terms of EROD activity, based on the principle that it can catalyze etoxyresorufin into resorufin, whereby the greater the presence of CYP1A the greater the EROD activity (Wincent *et al.*, 2016). In this regard, induction of CYP1 in rainbow trout by BaP after exposure for 12 h has been reported, EROD activity in gills was correlated with the expression level of CYP, family 1, subfamily A, polypeptide 1 (CYP1A1) and CYP, family 1, subfamily A, polypeptide 3 (CYP1A3), they found that it was higher compared to CYP, family 1, subfamily B, polypeptide 1 (CYP1B1) and CYP1. CYP1 mRNA expression levels were induced at a concentration of 1 nM. Whereby, CYP1A was more sensitive than CYP1B and CYP, family 1, subfamily C (CYP1C) to BaP (Gao *et al.*, 2021). It

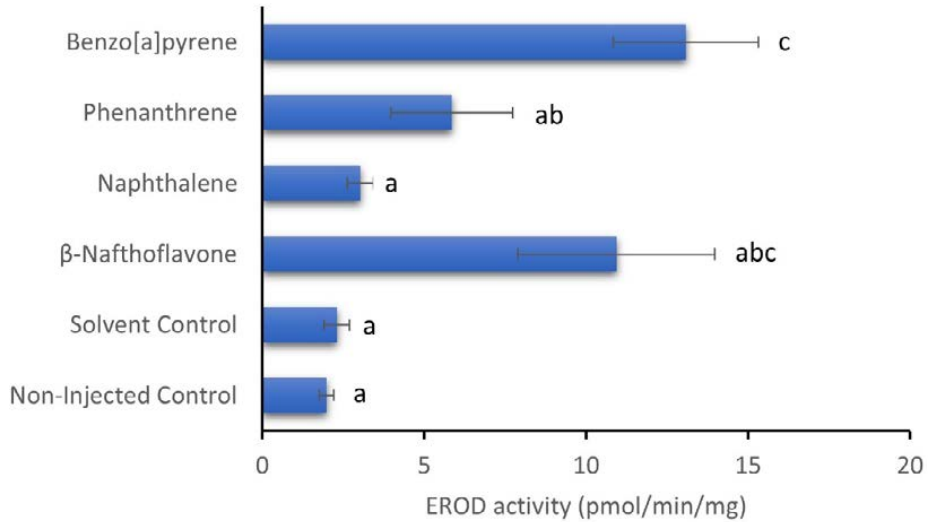

Fig. 3: Hepatic EROD activity in livers of *Aequidens metae* exposed to PAH during 72 h. Bars shown the mean ± SE (n = 5). [abc] Bars with different letters shown statistically significant differences (Tukey-Test, p<0.05)

has been reported that in Nile tilapia (*Oreochromis niloticus*) EROD activity was induced following 3 and 5 days of exposure to 300 µg/L of BaP, after which, levels returned to their basal state (Rodrigues *et al.*, 2014). It is also reported in rainbow trout (*Oncorhynchus mykiss*) the intraperitoneal administration of BaP at a dose of 2 mg/kg, which induced an increase in EROD activity and micronucleus frequency after 48 h of administration and a recovery after 7 days of EROD activity levels while MN frequency remained higher than in the control group (Fanali *et al.*, 2021). In the curent study, an induction of EROD activity after exposure via intraperitoneal route for 72 h was reported (Fig. 3), with return to basal levels after 10 days of injection. Increased EROD activity has been reported in *O. niloticus* exposed to PHE at doses of 50, 100 and 400 µg/L for 4, 8 and 14 days, with higher increases on the fourth day of exposure with 50 µg/L (Xu *et al.*, 2009), in the present study a non-significant increase was observed in those exposed to PHE compared to the non-injected and solvent control groups (Fig. 3).

In the case of the exposure of *A. metae* to naphthalene, the fish showed a slight increase in EROD activity (Fig. 3), with no statistical difference (p>0.05). Cichlids such as *O. niloticus* are sensitive to low concentrations of naphthalene, Amutha and Subramanian (2010), reported that fish exposed to concentrations from 6 to 14 mg/L showed a gradual increase in EROD activity after 12 h, but after 24 h of exposure, the activity decline at 14 mg/L and fish die. This was not the case in *A. metae* exposed to NAP, probably due to the function of another organ no identified in this study which can exerted the biotransformation of this PAH.

Histological evaluation

The liver parenchyma of *A. metae* is formed by hepatocytes with granular cytoplasm surrounded by sinusoids, with spherical nuclei and dark and prominent non-concentric nucleoli. The formation of portal triads was not evidenced (Fig. 4).

Displaced nuclei and vacuolization were evident in the perimeter of hepatocytes (Fig. 4D, E and F) from this organ of PAH-exposed fish (Fig. 4D, E, and F). The control group and the exposed treatments presented alterations corresponding to stage I DTC, including nuclear atrophy, mild nuclear and cytoplasmic hypertrophy, and deformation of the nuclear contour. On the other hand, stage II included hyperemia (Fig. 4C-D) and nuclear degeneration (Fig. 4D), which were higher in *A. metae* exposed to β-naphthoflavone,

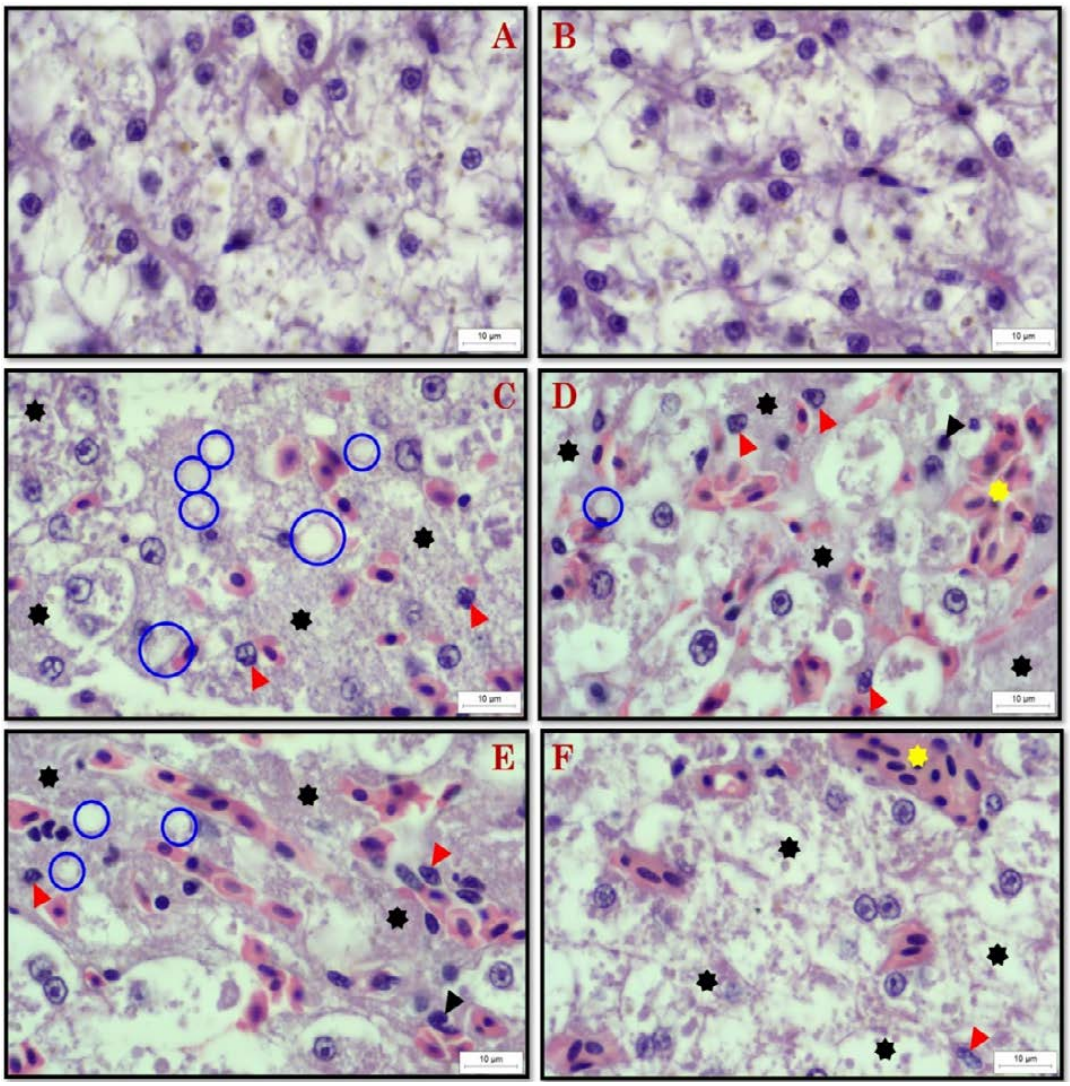

Fig. 4: Disturbances in the liver parenchyma of *Aequidens metae* exposed to PAH during 72 h. A) Non-injected control and B) solvent control show normal tissue with non-concentric nuclei and nucleoli and granular cytoplasm, C) exposed to β-naphthoflavone, D) exposed to naphthalene, E) exposed to phenanthrene and F) exposed to benzo[a]pyrene. Note in the different microphotographs the hyperemia (yellow star), degeneration of the nuclei (red triangle), pyknotic nuclei (black triangle), degeneration of the cytoplasm (black star), vacuolization (blue circle). H and E staining, magnification x 400.

phenanthrene and BaP; in the latter, pyknotic nuclei were more noticeable, together with cytoplasmic degeneration (Fig. 4E-F). It should be noted that the presence of necrosis indicative of stage III was not identified. The increased occurrence of histopathological changes may indicate dysfunctions induced by potentially toxic agents, considering that the liver biotransformation sites are reduced, which may alter the metabolizing functions of xenobiotics and endogenous substances (Gu and Manautou, 2012; Kostić *et al*., 2017). Liver DTC in *A. metae* were not considered severe, as no necrotic tissue was observed, and the lesions evidenced corresponded to stages I and II, as reported in the carp (*Capoeta capoeta*) from the Karasu River, Turkey and in crucian carp (*Carassius auratus*), pale chub (*Zacco platypus*), and Korean chub (*Zacco koreanus*) caught in streams contaminated with wastewater from

treatment plants (Dane and Şişman, 2014; Samanta *et al*., 2018). In *A. metae* exposed to βNF and BaP, the diameter of nuclei and the area of hepatocytes exceeded ($p<0.05$) the values of the control group fish (Table 2). In response to stressors such as toxic metals, an increase in the diameter of hepatocyte nuclei (karyomegaly) has been reported, which may indicate increased biotransformation reactions (Velma and Tchounwou, 2010; Wolf and Wheeler, 2018;). In *A. metae*, exposure to βNF and BaP induced hepatocyte hypertrophy, which could be related to alterations in structure associated with an increase in cellular metabolic rate. More hepatocyte nuclei were counted in *A. metae* exposed to βNF, PHE and BaP than fish in the control groups ($p<0.001$) (Table 2).

In the present study, quantification of the nuclei of hepatocytes made it possible to establish a link with of hepatocyte proliferation which signal possibly the induction of the cell cycle by alterations in liver function produced from PAH such as BaP on the hepatocytes during detoxification of this types of compounds (Regnault *et al.,* 2014). After an acute exposure period of 72 h, and at the concentrations of PAH used, it can be concluded that juvenile *A. metae* develop an early intoxication process, with reversible injuries. The usefulness of histological biomarkers as a tool for assess the sublethal effects of environmental contaminants on a native fish *A. metae* was validated.

Genotoxicity analysis

Regarding the frequency of micronuclei, an increase of 6.7; 4.9; 4.7 and 4.4 times was observed in erythrocytes from fish exposed to PHE, BNF and BaP, all with $p<0.0001$ and NAP (0,042), respectively, compared to the solvent control group. Also, notched abnormalities were more frequent in those exposed to PHE 5.5-fold ($p<0.0001$), NAP 5.1-fold ($p<0.0024$) and BaP 4.1-fold ($p<0.006$) compared to the solvent control group. In turn, fish exposed to PHE ($p<0.0001$) and BaP ($p<0.0019$) exhibited a 6.78- and 5.78-fold increase, respectively, in lobed abnormalities compared to the solvent control group. Blebbed nuclear abnormalities occurred more frequently in the PHE ($p<0.0001$), BaP ($p<0.015$), and NAP ($p<0.0016$) exposed groups, 6; 3.9 and 3.9 times compared to the solvent control group. Likewise, binucleated erythrocytes presented higher occurrence in fish exposed to PHE ($p<0.0001$), BaP ($p<0.013$), and NAP ($p<0.022$), 4.3; 3.3 and 2.6 times compared to the solvent control group (Table 3).

The genotoxic alterations found in fish exposed to PAH have been shown in other studies where the DNA damage induced in the liver of Caspian Kutum (*Rutilus frissi kutum*) has been associated with reactive oxygen species induced by BaP, together with the production of secondary metabolites during the biotransformation

Table 2: Degree of tissue change (DTC), average diameter, area and number of the hepatocytes nuclei in the liver tissue of juvenile *A. metae* exposed to different concentrations of PAH after 72 hours

Treatment	Micronuclei	Notched	Lobed	Blebbed	Binucleated
Control not injected	1,11±0,48^{a}	2,33±0,17^{b}	0,33±0,17^{cd}	1,00±0,33^{c}	0,27±0,02^{c}
Solvent Control	1,89±0,26^{a}	2,00±0,37^{b}	1,00±0,37^{cd}	1,67±0,29^{bc}	1,67±0,37^{bc}
B-Naphthoflavone	9,44±1,89^{b}	5,56±0,56^{ab}	4,89±2,02^{abc}	4,56±0,90^{ab}	3,33±0,41^{ab}
Naphthalene	8,38±1,97^{b}	10,13±2,66^{a}	6,13±2,46^{abc}	6,63±1,48^{a}	4,38±0,63^{a}
Phenanthrene	12,78±1,67^{b}	11,00±1,58^{a}	6,78±0,83^{a}	11,00±1,12^{a}	7,33±1,27^{a}
Benzo[a]Pyrene	9,00±1,46^{b}	8,11±1,25^{a}	5,78±0,98^{ab}	6,67±1,57^{a}	5,67±1,53^{a}

^{abc} Different letters indicate statistically significant differences (Tukey-Test $p<0.05$) between treatments.

Table 3: Frequency of micronuclei and other nuclear abnormalities in peripheral blood of juvenile *A. metae* exposed to different concentrations of PAH after 72 h

Treatment	Micronuclei	Notched	Lobed	Blebbed	Binucleated
Control not injected	1,11±0,48^{a}	2,33±0,17^{b}	0,33±0,17^{cd}	1,00±0,33^{c}	0,27±0,02^{c}
Solvent Control	1,89±0,26^{a}	2,00±0,37^{b}	1,00±0,37^{cd}	1,67±0,29^{bc}	1,67±0,37^{bc}
B-Naphthoflavone	9,44±1,89^{b}	5,56±0,56^{ab}	4,89±2,02^{abc}	4,56±0,90^{ab}	3,33±0,41^{ab}
Naphthalene	8,38±1,97^{b}	10,13±2,66^{a}	6,13±2,46^{abc}	6,63±1,48^{a}	4,38±0,63^{a}
Phenanthrene	12,78±1,67^{b}	11,00±1,58^{a}	6,78±0,83^{a}	11,00±1,12^{a}	7,33±1,27^{a}
Benzo[a]Pyrene	9,00±1,46^{b}	8,11±1,25^{a}	5,78±0,98^{ab}	6,67±1,57^{a}	5,67±1,53^{a}

^{abc} Different letters indicate statistically significant differences (Tukey-Test $p<0.05$) between treatments.

process (Esmaeilbeigi *et al.,* 2021), it is interesting that in the present study at the exposure doses, lesions compatible with genotoxic damage were induced without evidence of irreversible alterations such as the presence of neoplasms, which is corroborated with the findings of Myers *et al.* (2008), in wild English sole (*Pleuronectes vetulus*) exposed *in situ* to sediments with high concentrations of BaP, where the induction of hepatic CYP1A was associated with the occurrence of neoplastic lesions and the formation of DNA adducts. In contrast, it is possible that the absence of neoplastic lesions in *A. metae* exposed to sublethal concentrations of BaP, PHE, and NaP, is related to the short period of exposure. Regarding the greater increase observed with those exposed to PHE, it has been reported in juvenile European bass (*Dicentrarchus labrax*), increases in nuclear abnormalities including the presence of micronuclei in specimens exposed for intraperitoneal route to 10 µg/g of PHE in sea bass after 3 and 6 months, which is related to biotransformation processes, led by CYP monooxygenase enzymes, which results in bioactivation into reactive metabolites that are more toxic than the precursor compounds (Reis-Henriques *et al.,* 2009l; Karbassi and Heidari, 2015). Phenanthrene exposure in the neotropical guppy fish (*Poecilia vivipara*) was reported to alter behavior, at doses above 200 µg/L, that reduced prey capture, by modifications in swimming speed and associated trajectories that could reduce growth rates in the exposed fish (Torreiro-Melo *et al.,* 2015). In the liver of Senegal sole (*Solea senegalensis*), caught in a polluted estuary, EROD activity correlated with metabolites derived from the biotransformation of phenanthrene present in the water (Oliva *et al*., 2014). In the current study, the exposure to PHE induced biotransformation by CYP1A that was evidenced by the increased in EROD activity (although it was not significant); likewise, it is important to highlight that erratic swimming was observed in phenanthrene-exposed fish during the development of the assay, this finding probably is related to the presence of toxic metabolites. In another study, bioactivation of toxic metabolites has been reported in the benthic species southern flounder (*Paralichthys lethostigma*) where relative biotransformation rates were slow for monohydroxylated metabolites of phenanthrene after 72 h intraperitoneal exposure to crude oil from the Deepwater Horizon spill (Pulster *et al*., 2017). In addition, we cannot discard that others organ different that liver may exert biotransformation process in the benthic and detritivorous cichlid *A. metae*. Instead, the induction of nuclear abnormalities in golden grey mullet (*Liza aurata*) during 16 h of exposure to concentrations of 0.1, 0.3, 0.9 and 2.7 µM PHE is reported, which led to a progressive increase in the presentation of abnormalities associated with increasing PHE concentrations (Oliveira *et al.,* 2007). PHE is also reported to induce genotoxicity in marine species such as the gastropod (*Morula granulate*), where DNA damage was demonstrated by comet assay from concentrations of 10 µg/L (Bhagat *et al*., 2016). In turn, the presence of blebbed nuclei in *A. metae* erythrocytes exposed to PHE, BaP, and NAP, can be deduced that these abnormalities may be associated with a clastogenic effect of these PAH (Brzuzan *et al.,* 2006; Matsumoto *et al.,* 2006). Regarding the occurrence of binucleated cells it is considered as a cytotoxicity indicator (Çavaş and Ergene-Gözükara, 2005), due to this abnormality occurs by blocking cytokinesis during abnormal cell division (Yasui *et al*., 2015); as evidenced in the specimens of *A. metae* exposed to PAH.

CONCLUSIONS

The strength of the present study consisted in elucidate the effect of three representative PAH of environmental pollution on the response of different biomarkers in a native cichlid, since NAP and PHE have an environmental high frequency and BaP lead to high toxicity. The EROD activity in *A. metae* is a reliable biomarker of exposure to BaP. NAP, PHE, and BNF showed a non-significant increase in EROD activity in *A. metae*, although an elevated trend compared to controls was observed. This pattern is possibly due to two reasons, first, the low number of animals used that lead to the dispersion of the data distribution, and secondly, probably the liver is not the only organ capable of metabolized this PAH in this species. In the field studies, those issues should be considering, given that a complex mixture of contaminants including different PAH are found in the monitored water bodies. This study identified a greater genotoxic effect by PHE, even higher than fish exposed to BaP, this finding is very interesting considering that BaP is a recognized mutagenic agent and although PHE has fewer benzene rings, its biotransformation in this fish was possibly slower compared to BaP, NAP and BNF, in addition, it has been reported that PHE toxic metabolites are produced, which induced a greater cellular interaction

that was evidenced in the higher count of nuclear abnormalities. The behavior erratic swimming in fish exposed to PHE is a possible manifestation of the bioavailability and interaction with those metabolites. Finally, the presence of reversible alterations and the increase in cell proliferation in liver tissue in the groups exposed to PAH are associated to the sublethal doses and the short exposure used; however, it is not possible to discard that longer exposure could induce irreversible alterations that compromised the function of liver. In realist environmental scenarios a mixture of concentrations of xenobiotics such as NAP, PHE, and BaP are commonly found in natural waters that receive discharges of domestic and industrial wastewater. The results indicate that is relevant to evaluate the interaction of biomarkers because responses to individual xenobiotics can be attenuated in ecotoxicological studies when dealing with different classes of PAH. The usefulness of the intraperitoneal exposure route in the neotropical cichlid *A. metae* were confirmed, which in turn ratified this detritivorous fish as a bioindicator species for PAH pollution. From the analysis of the shown data, it is inferred that at concentrations close to those found in natural environments, exposed fish have the potential risk of developing alterations that impact both their health and also the one of the organisms associated with them. These results will serve as input to the environmental authorities for making regulatory decisions on the maximum PAH concentrations allowed in the freshwater bodies.

AUTHOR CONTRIBUTIONS

W. Corredor-Santamaría performed the literature review, experimental design, analyzed and interpreted the data, prepared the manuscript draft and manuscript edition. I.C. Calderón-Delgado performed the experiments, compiled the data and manuscript edition. Z. Arbeli designed the experiments, J.M. Navas performed the manuscript edition. Y.M. Velasco-Santamaría obtained the funding, performed experimental design, interpreted the data and manuscript edition.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

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PUBLISHER'S NOTE

ABBREVIATIONS

°C	Celsius degree
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar

µm²	Square micrometer
%	Percent
A. metae	*Aequidens metae*
ANH	*Agencia Nacional de Hidrocarburos*
ANOVA	*Analysis of variance*
BaP	Benzo[a]Pyrene
BNF	β-Naphthoflavone
BW	Body weight
BSA	Bovine serum albumin
cm	Centimeter
CYP1	Cytochrome P450, family 1
CYP1A	Cytochrome P450, family 1, subfamily A
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1A3	Cytochrome P450, family 1, subfamily A, polypeptide 3
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
CYP1C	Cytochrome P450, family 1, subfamily C
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DTC	Degree of tissue Change
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EROD	7-ethoxy-resorufin-O-deethylase
g	Gram
g	Gravities
h	Hours
H and E	Hematoxylin and eosin
HSI	Hepatosomatic index
KCl	Potassium chloride
Kg	Kilogram
IgM	Immunoglobulin M
L	Liter
M	Molar
mg	Milligram
Min	Minute
mM	Millimolar
MN	Micronucleus
NAP	Naphthalene
nm	Nanometer
PAH	Polycyclic aromatic hydrocarbons
pH	Potential of hydrogen
PHE	Phenanthrene
PMSF	Phenylmethylsulfonyl fluoride
SE	Standard error
SPSS	Statistical Package for the Social Sciences
Tris-HCl	Tris hydroxymethyl aminomethane hydrochloride

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