Response of hepatic biomarkers in *Physalaemus nattereri* (Anura) to different benzo(α)pyrene exposure routes

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Abstract

For over 40 years, anurans have been used as a study model to assess the adverse effects of benzo(α)pyrene (BαP), which include genotoxic, hepatotoxic, and immunotoxic effects. In these studies, BαP is administered cutaneously or by injection, with no comparison between two or more routes. The purpose of this study is to assess whether the effect of BαP is influenced by its route of administration, using the response of hepatic biomarkers of *Physalaemus nattereri*. Specimens ($n = 108$) were collected and divided into three experimental treatments (cutaneous, injection, and oral) and three experimental times (one, three, and seven days). Specimens received 0.02 ml of pure mineral oil (control) or mineral oil containing 2 mg/kg of BαP. The BαP causes changes in morphological (melanin, hemosiderin, lipofuscin, and mast cells) and biochemical (superoxide dismutase and glutathione S-transferase) hepatic biomarkers. Compared to biochemical, morphological biomarkers underwent a greater number of significant changes due to the treatment with BαP. The route of exposure alters the effects of BαP, mainly seen in morphological biomarkers, especially the pigments melanin, hemosiderin, and lipofuscin. In these pigments, the effect of the exposure pathway changes according to the analyzed biomarker, and the exposure time modulates the exposure pathway effect. These results are unprecedented for anurans and contribute to the field of herpetology and ecotoxicology.

Keywords Glutathione S-transferase · Liver · Melanomacrophage · Polycyclic aromatic hydrocarbons · Superoxide dismutase

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of chemical compounds, formed during the incomplete burning of organic matter (Agency for Toxic Substances and Disease Registry ATSDR 1985) – having natural origin (e.g., volcanic emissions and forest fires) or due to anthropogenic activity (e.g., exhaustion of cars and trucks, garbage incineration, and fossil fuel burning) (Agency for Toxic Substances and Disease Registry ATSDR 1985; Li and Chen 2002; Madureira et al. 2014). Among the more than 100 types of PAHs, 17 compounds stand out, as they are possibly more harmful than the others (Agency for Toxic Substances and Disease Registry ATSDR 1985), including benzo(α)pyrene (BαP).

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BαP is a group 1 carcinogen—carcinogenic to humans (International Agency for Research on Cancer IARC monographs 2012), found in all environmental compartments—aquatic (e.g., dissolved in water or adhered to organic matter) (Nasr et al. 2010) and terrestrial (e.g., air, soil, plants, and termites) (Wilcke et al. 2003, Gupta et al. 2011). To bring about carcinogenesis, BαP requires metabolic activation (Sims et al. 1974; Penning 2004). This activation occurs after its absorption by the body, which can be through the skin, by ingestion, or by inhalation (Netto et al. 2000), and generates secondary metabolites (Gelboin 1980) as well as reactive oxygen species (ROS) (Penning 2004; Saunders et al. 2006).

Since the 1980s, anurans have been used as an experimental model to assess the adverse effects of BαP. Until the 2000s, these studies focused on genotoxic damage (Höhn-Bentz et al. 1983; Van Hummelen et al. 1989; Sadinski et al. 1995; Mouchet et al. 2005). From 2010 onwards, studies began to emphasize physiological and morphological changes in the digestive system, especially in the liver (Reynaud et al. 2012; Regnault et al. 2014, 2016; Fanali et al. 2017, 2018). In these studies, anurans (tadpoles or adults) were exposed by a single route: cutaneous by aqueous exposure (Van Hummelen et al. 1989, Sadinski et al. 1995; Mouchet et al. 2005; Reynaud et al. 2012; Regnault et al. 2014, 2016) or injectable (Höhn-Bentz et al. 1983; Fanali et al. 2017, 2018), with no comparisons between two or more routes which represents an information gap for this group of vertebrates.

This work aims to evaluate the effect of BαP on hepatic biomarkers of *Physalaemus nattereri* (Steindachner, 1863) and whether this effect is influenced by the exposure route, using routes that simulate a natural (cutaneous and oral) or an artificial (injectable) exposure. Because the injectable route ensures the entry of the entire dose of the compound (Pérez-Iglesias et al. 2016), we hypothesize that this route will cause larger effects than the natural exposure routes. To assess the hepatic effects of exposure pathways, we will use biomarkers of different levels, frequently used in anurans, namely: morphological (melanin, hemosiderin, lipofuscin, and mast cells) and biochemical (catalase, superoxide dismutase, glutathione S-transferase, and peroxidation of lipids). Because the biological response occurs initially at the biochemical and molecular levels and then at the physiological and histological levels (Nascimento et al. 2006), we hypothesize that the biochemical biomarkers will be affected by short-term effects from the treatment, while morphological effects will occur after prolonged exposures.

Materials and methods

Collection and experiments

One hundred and eight adult male specimens of *P. nattereri* were collected (License #18573-1, IBAMA) from November 2018 to January 2019 in São José do Rio Preto (coordinates: 20°42'22.6″S, 49°17'25.7″W). After collection, the specimens were sent to São Paulo State University (UNESP), Institute of Biosciences, Humanities and Exact Sciences being kept in acclimatization in plastic boxes ($25 \times 16 \times 14$ cm) containing dechlorinated water. Before the beginning of the experiments, the specimens were weighed and separated into three experimental treatments (cutaneous, injection, oral) and three experimental times (one, three, and seven-day exposure). Each specimen received a single dose (volume: 0.02 ml) of pure mineral oil (control) or mineral oil containing 2 mg/kg of BαP (treatment), administered by cutaneous drip in the dorsal region, subcutaneous injection, or by drip in the oral cavity. Experimental times, as well as concentration, were based on and adapted from the works of Fanali et al. (2017, 2018). At the end of the experiments, the specimens were euthanized in an aqueous solution of benzocaine (5 g/L), weighed, and dissected. Biological liver samples were collected and preserved according to each analysis. All procedures were approved by the Ethics Committee on Animal Use—IBILCE/UNESP-CSJRP (Protocol n° 188/2018), and animals handling followed the NIH Guide for Care and Use of Laboratory Animals.

Morphological analysis

Liver fragments were fixed in Karnovsky's solution (0.1 M Sörensen phosphate buffer, pH 7.2 phosphate buffer with 5% paraformaldehyde and 2.5% glutaraldehyde) for 24 h at 4 °C, with subsequent dehydration in alcoholic series and inclusion in historesin (Leica-Historesin embedding kit, Leica Microsystems). Histological sections of 2 μm were obtained in a rotating microtome (Leica RM2265) and followed the staining process according to the proposed analyses. Each analysis provides information on morphological, physiological (melanin, hemosiderin, and lipofuscin) and immunological (mast cells) changes caused by BαP.

Pigment area quantification

To quantify the area occupied by melanin, hemosiderin, and lipofuscin (Fig. 1), histological sections were stained in hematoxylin-eosin (melanin) or incubated in acidic potassium ferrocyanide solution (hemosiderin – 200 mg potassium ferrocyanide dissolved in 10 ml of hydrochloric acid 0.75 mol/L) or Schmorl's solution (lipofuscin – 38 mg of ferric chloride and 5 mg of potassium ferricyanide dissolved in 5 ml of distilled water). Twenty-five histological fields/animal were photographed under a light microscope (20x), with an image capture system (Leica DMC4500), and analyzed using the Image-Pro Plus 6.0 program (Media–Cybernetics Inc.) following the protocol by

Fig. 1 Histological sections of liver. **A** Mast cell (arrow). **B** Melanomacrophage with melanin (arrow). **C** Melanomacrophage with hemosiderin (arrow). **D** Melanomacrophage with lipofuscin (arrow). Staining: **A** – Toluidine blue with borax, **B** – Hematoxylin-Eosin, **C** – Ferrocyanide acid solution, **D** – Schmorl's solution. Scale bars: **A** - 5 μm, **B/C/D** - 10 μm
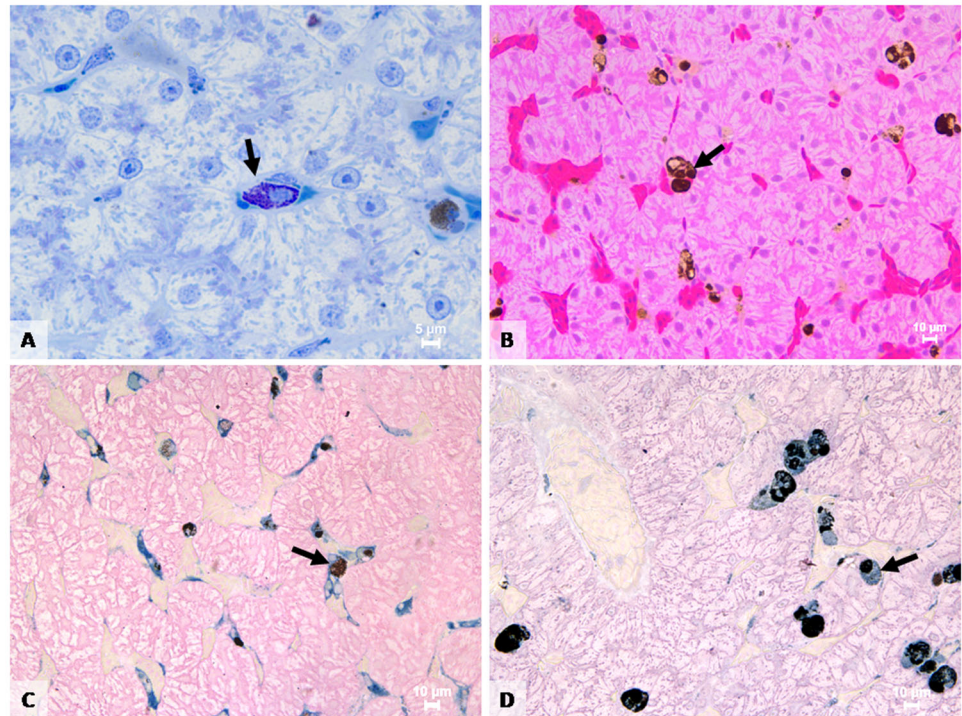
Santos et al. (2014), which determines the measurement of pigments by color intensity.

Mast cells quantification

Sections were stained in toluidine blue with borax and analyzed under a light microscope (40x), counting the mast cells (Fig. 1) present in 10 histological fields/animal. Subsequently, the analyzed fields were photographed under a stereomicroscope (Leica MZ16), with an image capture system (Leica DFC295), and analyzed using the Image-Pro Plus 6.0 program (Media–Cybernetics Inc.) to establish the mast cell density per μm^2 of liver tissue.

Total melanin quantification

Liver fragments were weighed and frozen at $-4\,^{\circ}\mathrm{C}$. To quantify total melanin, the method by Franco-Belussi et al. (2016) was used. The samples were homogenized in a 1 M sodium hydroxide (NaOH) and 10% Dimethylsulfoxide (DMSO) solution, heated at 80 °C for 2 hours, centrifuged at 2500 rotations per minute (rpm) for 15 minutes, and analyzed at 475 nm in an ELISA plate reader. To determine the standard melanin curve, synthetic melanin (Sigma-Aldrich) was prepared and analyzed in the same way as the liver samples. The relationship between absorbance and melanin concentration was used to determine the amount of melanin per milligram of sample.

Biochemical analysis

As the metabolism of BαP can generate ROS, we quantified catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), and lipid peroxidation (LPO), whose responses may indicate possible oxidative stress. For this, biological liver samples were frozen in a biofreezer at $-80\,^{\circ}\mathrm{C}$. The samples were individually homogenized (IKA T10 basic ULTRA-TURRAX®) in a saline-phosphate buffer PBS (137 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4 ($7H_2O$), and 1.8 mM KH_2PO_4, pH 7.2) at 4 °C and centrifuged (Hettich® Universal 320 R centrifuge) at 12,000 g for 20 minutes at 4 °C. The supernatant was collected and used for biochemical determinations.

The protein concentration of the samples was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard at 595 nm. CAT activity was determined by the method of Aebi (1984), through the degradation of hydrogen peroxide into water and oxygen. The SOD activity was determined by the method of Crouch et al. (1981), based on the ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT). GST activity was determined by the method of Keen et al. (1976), through the formation of thioether after the reaction of GST with the substrate 1-Chloro-2,4-dinitrobenzene (CDNB). LPO was quantified by the FOX method (ferrous oxidation-xylenol orange) as described by Jiang et al. (1992). This method is based on the oxidation of Fe^{2+} to Fe^{3+} by hydroperoxides in an acidic medium, in the presence of a Fe^{3+} complexing

Table 1 Biometric data of *Physalaemus nattereri*.

Group	Initial Body Mass (g)	Final Body Mass (g)	Liver (g)
CC.1d−CT.1d	$8.25 \pm 0.27 - 8.31 \pm 0.36$	$8.30 \pm 0.37 - 8.21 \pm 0.51$	$0.22 \pm 0.02 - 0.19 \pm 0.01$
IC.1d−IT.1d	$8.11 \pm 0.53 - 8.18 \pm 0.64$	$7.78 \pm 0.55 - 8.06 \pm 0.66$	$0.21 \pm 0.02 - 0.22 \pm 0.03$
OC.1d−OT.1d	$8.10 \pm 0.50 - 8.34 \pm 0.34$	$7.61 \pm 0.48 - 8.29 \pm 0.33$	$0.21 \pm 0.05 - 0.21 \pm 0.01$
CC.3d−CT.3d	$8.02 \pm 0.34 - 7.73 \pm 0.16$	$7.58 \pm 0.37 - 7.57 \pm 0.20$	$0.20 \pm 0.02 - 0.16 \pm 0.01$
IC.3d−IT.3d	$8.19 \pm 0.28 - 8.05 \pm 0.39$	$8.21 \pm 0.36 - 7.73 \pm 0.30$	$0.23 \pm 0.02 - 0.19 \pm 0.02$
OC.3d−OT.3d	$8.46 \pm 036 - 8.38 \pm 0.29$	$7.91 \pm 0.29 - 8.16 \pm 0.28$	$0.23 \pm 0.03 - 0.21 \pm 0.02$
CC.7d−CT.7d	$8.28 \pm 0.28 - 8.48 \pm 0.14$	$7.60 \pm 0.40 - 8.00 \pm 0.29$	$0.19 \pm 0.02 - 0.23 \pm 0.01$
IC.7d−IT.7d	$8.34 \pm 0.60 - 8.35 \pm 0.22$	$7.51 \pm 0.50 - 7.56 \pm 0.29$	$0.19 \pm 0.03 - 0.21 \pm 0.01$
OC.7d−OT.7d	$8.59 \pm 0.44 - 8.34 \pm 0.31$	$8.21 \pm 0.26 - 7.80 \pm 0.40$	$0.22 \pm 0.02 - 0.20 \pm 0.02$

The significant difference (control vs. treatment) is show in bold. Values are mean $\pm$ SE of $N=6$ samples for each group. *CC* cutaneous control, *CT* cutaneous treatment, *IC* injection control, *IT* injection treatment, *OC* oral control, *OT* oral treatment; 1d: one day; 3d: three days; 7d: seven days

pigment (xylenol orange), which has an absorption peak at 560 nm. The spectrophotometric readings were taken in triplicate, being carried out in a microplate reader (SOD, GST, and LPO – SynergyTM HTX Multi-Mode Reader) or in a quartz cuvette (CAT – UV/Vis spectrophotometer, LIBRA S50, BIOCHROM).

Statistic

For the present study, we performed a comparison model between time (one, three, and seven days) and experimental groups (cutaneous control/treatment, injections control/treatment, oral controle/treatment) (time * group). Through this model, multiple comparisons were performed at each experimental time to visualize the effect of BαP (control vs. treatment – Tables 1–3), as well as the effect of exposure pathway (comparison between pathways at each given time – Table S7). For all data, tests of normality (Shapiro) and homogeneity of variance (Bartlett) were performed. For total melanin, CAT, and SOD an ANOVA test were performed. For melanin, hemosiderin, lipofuscin, mast cells, GST, and LPO an GLM were performed, with Gaussian (link function *log*) and binomial distribution (exclusive to mast cells). To compare the mass variation between the beginning and the end of the experiments, a t-test was performed for each experimental time. Subsequently, an ANOVA test was performed to compare the mass variation between groups. All analyzes were performed using the RStudio program (R Core Team).

Results

Biometry

The biometry data are shown in Table 1. BαP did not cause changes in body ($F = 0.849$, $p > 0.05$) or liver mass ($F = 1.048$, $p > 0.05$) of the specimens. There was no variation in the body mass of the specimen between the beginning and the end of the experiments (1d: $t = 0.69542$, 3d: $t = 1.7681$, 7d: $t = 3.064$; $p > 0.05$).

Morphological biomarkers

The morphological data are shown in Table 2. BαP increases the area of melanin in the injectable route (1d: 60%, 3d: 21%, 7d: 41%) and decreases it in the cutaneous route (7d: 31%) and oral routes (1d: 20%, 3d: 21%) ($p < 0.05$, Table S1). The area of hemosiderin has an increase in the cutaneous (3d: 94%), injectable (1d: 24%, 3d: 32%), and oral routes (3d: 27%), and a decrease in the cutaneous route (7d: 20%) ($p < 0.05$, Table S2). For lipofuscin, there was an increase in the cutaneous (1d: 40%, 3d: 94%) and injectable routes (1d: 67%, 3d: 68%) and a decrease in the cutaneous (7d: 57%) and oral routes (3d: 17%) ($p < 0.05$, Table S3). After exposure to BαP, the frequency of mast cells increased in the oral route (3d: 730%) and decreased in the injectable route (7d: 65%) ($p < 0.05$, Table S4).

BαP did not cause changes in total melanin ($F = 5.339$, $p > 0.05$).

Biochemical biomarkers

The biochemical data are shown in Table 3. BαP administered orally causes an increase in SOD (1d: 73%) ($F = 3.837$, $p > 0.05$) and a decrease in GST (3d: 35%) ($p < 0.05$, Table S5).

BαP did not cause changes in CAT ($F = 3.335$, $p > 0.05$) and LPO activities ($p > 0.05$, Table S6).

Discussion

In the present study, we evaluated the effects of BαP on the hepatic biomarkers of *P. nattereri*, and whether these effects are influenced by its exposure pathway. After one, three and

Table 2 Morphologic biomarkers of *Physalaemus nattereri*.

Group	Total Melanin (µg/g)	Melanin (µm²)	Hemosiderin (µm²)	Lipofuscin (µm²)	Mast Cells (n/mm²)
CC.1d–CT.1d	1506.2 ± 217.1 – 1679.4 ± 175.9	562.3 ± 61 – 536.7 ± 103.9	6158.8 ± 1172.8 – 6566 ± 1202.4	2175.4 ± 360.6 – **3037.5 ± 491.6**	0.3 ± 0.11 – 0.31 ± 0.14
IC.1d–IT.1d	2769.5 ± 711.4 – 3688.9 ± 563.1	**439.4 ± 86.4 – 703.1 ± 205.5**	**5098.7 ± 961.7 – 6333.9 ± 806**	1635.9 ± 315.4 – 2733.3 ± 691.3	0.71 ± 0.35 – 0.38 ± 0.1
OC.1d–OT.1d	3316.2 ± 579.9 – 2271.1 ± 471.3	**562.9 ± 107.5 – 451.3 ± 93.5**	5435 ± 984 – 5356.8 ± 739.9	2273.6 ± 452.3 – 1869.6 ± 303.4	0.54 ± 0.16 – 0.42 ± 0.24
CC.3d–CT.3d	2615.8 ± 451.4 – 3192.8 ± 851.1	730 ± 179.9 – 809.2 ± 196.3	**4513.3 ± 1012.2 – 8759.7 ± 1154.4**	**2846.8 ± 574.6 – 5526.9 ± 1756.2**	0.88 ± 0.42 – 0.73 ± 0.23
IC.3d–IT.3d	2015.8 ± 391.2 – 1810.8 ± 366.6	**656 ± 141.3 – 795.4 ± 163.4**	4652.8 ± 607.5 – 6123.5 ± 827.2	2158.9 ± 514.9 – 3631.9 ± 635	0.28 ± 0.13 – 0.8 ± 0.32
OC.3d–OT.3d	1463.1 ± 186.5 – 1033.9 ± 123.9	**859.5 ± 159.5 – 675.2 ± 161.1**	**4982.8 ± 1070.2 – 6341.2 ± 688.5**	**2810.3 ± 418.3 – 2333.8 ± 380.2**	**0.2 ± 0.09 – 1.66 ± 0.62**
CC.7d–CT.7d	914.8 ± 49.5 – 1177.1 ± 169.3	**483.3 ± 100.9 – 334.1 ± 77.6**	**5662.9 ± 1265.6 – 4528 ± 928.8**	**1789.1 ± 343.5 – 754.2 ± 134.1**	0.27 ± 0.13 – 0.43 ± 0.16
IC.7d–IT.7d	1076 ± 109 – 1098.4 ± 150.3	**343.3 ± 75.6 – 484.2 ± 114.4**	6158 ± 1278.3 – 6108.9 ± 892.7	1222.8 ± 260 – 1306.3 ± 396.8	**1.07 ± 0.54 – 0.37 ± 0.08**
OC.7d–OT.7d	1237.9 ± 109.7 – 1812.9 ± 147.8	426.9 ± 131.6 – 420.1 ± 89.9	5113 ± 706.8 – 6062.9 ± 1233.2	981.3 ± 188.7 – 1026.4 ± 264.1	0.45 ± 0.09 – 0.29 ± 0.08

The significant difference (control vs. treatment) is show in bold. Values are mean ± SE of $N = 6$ samples for each group. *CC* cutaneous control, *CT* cutaneous treatment, *IC* injection control, *IT* injection treatment, *OC* oral control, *OT* oral treatment; 1d: one day; 3d: three days; 7d: seven days

seven days changes were observed in the response of morphological and biochemical biomarkers, with morphological ones suffering a greater number of significant changes (*see* Tables 2 and 3). Through a comparative analysis (Table S7), we observed a significant difference between exposure pathways in the treatment group, demonstrating that the pathway alters the effects of BαP, an unprecedented result for anurans. We also report a difference between exposure routes in the control group (Table S7), which may indicate an effect of the mineral oil (which does not compromise our results, since both control and treatment received oil), also influenced by the exposure route.

Among the hepatic morphological biomarkers analyzed, two cell types were used: melanomacrophages and mast cells. Melanomacrophages represent a lineage of pigment cells derived from hematopoietic stem cells (Sichel et al. 1997), with detoxification capacity (Fenoglio et al. 2005, Pérez-Iglesias et al. 2016). Melanomacrophages have three internal pigments (Agius and Roberts 2003): (1) melanin, a biopolymer with antimicrobial (Wolke et al. 1985) and antioxidant (Sichel 1988) properties; (2) hemosiderin, a pigment derived from hemoglobin breakdown (Agius and Agbede 1984; Kranz 1989); and (3) lipofuscin, a product of the degeneration of cellular components (Agius and Agbede 1984).

Regarding melanin, BαP does not change its total amount, but it does change the area occupied by this pigment. This indicates that BαP can alter the movement of melanin granules (i.e., melanosomes) without altering their synthesis—an effect observed in rodent melanoma cells (Joo et al. 2015). *Oncorhynchus mykiss* (rainbow trout) showed a change in actin filaments after seven days of exposure to BαP, leading to an aggregation of melanin granules and consequently a decrease in pigment area (Fanali et al. 2021). This effect may have occurred in *P. nattereri*, but further studies are needed to verify changes in the cytoskeleton and whether these changes are influenced by the route of administration—since we observed a distinct effect between the exposure pathways. The injectable route causes an increase in the area of melanin, while the cutaneous and oral causes a decrease. This variation in the biological response (increase or decrease) between the routes reinforces the influence of the exposure pathway on the effects of BαP. Regarding hemosiderin and lipofuscin, both pigments show an increase in pigment area in the cutaneous, injectable and oral (only hemosiderin) routes, and a decrease in the cutaneous and oral (only lipofuscin). These results indicate possible physiological changes since they are pigments that originated from the catabolic activity of melanomacrophages.

BαP causes an increase in the frequency of mast cells in the oral route (three days) and a decrease in the injectable

Table 3 Biochemical biomarkers of *Physalaemus nattereri*.

Group	CAT (nmol/mg^{protein})	SOD (Units/mg^{protein})	GST (nmol/mg^{protein})	LPO (nmol/mg^{protein})
CC.1d−CT.1d	$1.12 \pm 0.11 - 1.43 \pm 0.07$	$7.45 \pm 0.99 - 10.98 \pm 0.96$	$0.83 \pm 0.04 - 0.96 \pm 0.04$	$0.24 \pm 0.06 - 0.16 \pm 0.02$
IC.1d−IT.1d	$1.24 \pm 0.13 - 1.26 \pm 0.16$	$9.26 \pm 1.09 - 10.26 \pm 0.94$	$1.03 \pm 0.05 - 1.18 \pm 0.07$	$0.19 \pm 0.06 - 0.17 \pm 0.03$
OC.1d−OT.1d	$1.18 \pm 0.06 - 1.55 \pm 0.13$	$\mathbf{5.75 \pm 0.88 - 9.97 \pm 1.07}$	$0.72 \pm 0.04 - 0.94 \pm 0.06$	$0.28 \pm 0.11 - 0.23 \pm 0.06$
CC.3d−CT.3d	$1.26 \pm 0.14 - 1.48 \pm 0.11$	$8.09 \pm 0.82 - 8.36 \pm 1.12$	$0.95 \pm 0.05 - 0.86 \pm 0.05$	$0.2 \pm 0.04 - 0.3 \pm 0.06$
IC.3d−IT.3d	$1.53 \pm 0.06 - 1.67 \pm 0.08$	$7.66 \pm 0.42 - 7.53 \pm 0.5$	$0.77 \pm 0.01 - 0.87 \pm 0.06$	$0.24 \pm 0.03 - 0.33 \pm 0.05$
OC.3d−OT.3d	$1.64 \pm 0.06 - 1.67 \pm 0.09$	$7.78 \pm 0.85 - 7.53 \pm 0.47$	$\mathbf{1.09 \pm 0.06 - 0.71 \pm 0.06}$	$0.39 \pm 0.03 - 0.22 \pm 0.06$
CC.7d−CT.7d	$1.72 \pm 0.16 - 1.81 \pm 0.09$	$6.54 \pm 0.93 - 6.21 \pm 0.16$	$1.1 \pm 0.08 - 1.04 \pm 0.11$	$0.31 \pm 0.04 - 0.2 \pm 0.03$
IC.7d−IT.7d	$1.6 \pm 0.08 - 1.48 \pm 0.17$	$5.68 \pm 0.82 - 2.8 \pm 0.41$	$0.98 \pm 0.1 - 0.95 \pm 0.07$	$0.27 \pm 0.07 - 0.26 \pm 0.09$
OC.7d−OT.7d	$1.55 \pm 0.15 - 1.19 \pm 0.1$	$3.15 \pm 0.3 - 3.96 \pm 0.69$	$0.98 \pm 0.04 - 1.1 \pm 0.03$	$0.08 \pm 0.07 - 0.11 \pm 0.06$

The significant difference (control vs. treatment) is show in bold. Values are mean ± SE of $N = 6$ samples for each group. *CC* cutaneous control, *CT* cutaneous treatment, *IC* injection control, *IT* injection treatment, *OC* oral control, *OT* oral treatment; 1d: one day; 3d: three days; 7d: seven days

route (seven days). Mast cells constitute immune cells used as a marker of an inflammatory response (Franco-Belussi et al. 2014, Fanali et al. 2018). In a previous study involving anurans, the proportion of these cells increases in response to BαP (Fanali et al. 2018), demonstrating the ability to have induced an inflammatory response in the liver tissue. In fish, BαP compromises immune function (Carlson et al. 2002), which explains why we observed a decrease in the proportion of mast cells in the liver. The fact that we observed a distinct effect between the oral and the injectable routes reinforces the influence of the exposure route on the effects of BαP.

Regarding biochemical biomarkers, our experimental design did not cause changes in CAT and LPO, but caused an increase in SOD activity in one day and a decrease in GST in three days, both occurring in the oral route. To our knowledge, it is the first time that these biomarkers, CAT, SOD, and LPO, have been used to assess the effects of BαP in anurans. SOD represents an immediate response biomarker (Nascimento et al. 2006), whose activity is related to transforming the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) (Scandalios 2005). As superoxide is a by-product of BαP metabolism (Penning 2004), an increase in SOD activity demonstrates oxidative unbalance induced by BαP. In general, hydrogen peroxide produced by SOD is converted to less reactive species (H_2O) by CAT or by glutathione peroxidase (GPx) (Scandalios 2005). As we did not observe changes in CAT activity, it is possible that GPx had compensatory activity on the excess of hydrogen peroxide. GPx and GST are enzymes dependent on reduced glutathione (GSH), using it as a cofactor in their activities (Balasenthil et al. 2000, Van der Oost et al. 2003). Lower availability of GSH, conjugated by GPx, leads to a decrease in GST activity, as observed in three days. GST, a class of enzymes that acts in the biotransformation of xenobiotics by facilitating their excretion by the organism (Van der Oost et al. 2003), had already been used for the species

Pelophylax esculentus, where an increase in its activity was observed over a period of one and two days, demonstrating that BαP had been cleared by the liver (Reynaud et al. 2012). Although the liver is an important BαP metabolizing organ, other organs can perform this function (Lemaire et al. 1990, 1992). As the pathways used in our study can lead to a differentiated distribution of BαP throughout the body, the metabolism of BαP may have occurred in different organs, requiring further studies on the activity of biochemical biomarkers in other organs.

Conclusion

In the present study, we observed that BαP triggers the response of hepatic biomarkers of *P. nattereri*. This response is influenced by the route of administration, an unprecedented result for anurans, seen mainly in the area of melanin, hemosiderin, and lipofuscin. The results for these pigments demonstrate two characteristics related to the route of administration. (1) Artificial and natural pathways cause distinct effects, depending on the biomarker analyzed, mainly seen in the comparison of melanin *vs*. hemosiderin or melanin *vs*. lipofuscin. (2) The exposure time affects the exposure route, seen in the cutaneous route for hemosiderin and lipofuscin, wherein short exposure times (one and three days) causes an increase in the pigmented area, while in prolonged periods (seven days) causes a decrease. Our experimental design demonstrated that morphological biomarkers were more sensitive to BαP, which reinforces their use as effect biomarkers to assess changes induced by chemical compounds and other xenobiotics.

Data availability

Not applicable.

Code availability

Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval Approval was obtained from the Ethics Committee on Animal Use (IBILCE/UNESP – CSJRP n°188/2018). Animals collection permission (#18573-1, RAN/IBAMA/MMA).

Consent to participate Mr. Bruno Serra de Lacerda Valverde submitted this manuscript via online submission system, and on behalf of all co-authors that have been listed as a member of this submission, I confirm their consent to participate in this study and their consent to be listed as co-authors.

Consent for publication On behalf of all co-authors listed in this submission, I confirm their consent to publish this original research article.

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