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Ecotoxicological Biomarkers in Multiple Tissues of the Neotenic *Ambystoma* spp. for a Non-lethal Monitoring of Contaminant Exposure in Wildlife and Captive Populations

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Abstract Amphibians are the most threatened vertebrate group with a third of currently known species endangered with extinction, as a result of climate change, habitat loss, disease-introduced exotic species, and pollution. Because of their vulnerability, they have often been used as environmental quality indicators, as well as laboratory models for toxicological research. Given the sensitivity of amphibians to changes in their surrounding environment, including pollution, it was deemed important to define a non-lethal technique based on the evaluation of a set of biomarkers in different tissues of neotenic individuals of *Ambystoma velasci*. The levels of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), carboxylesterase (CaE), alkaline and acid phosphatases (ALP, ACP), glutathione s-transferase (GST), 7-ethoxyresorufin-O-deethylase (EROD), and superoxide dismutase (SOD) activities, as well as the oxygen radical absorption capacity (ORAC) were measured in tail, gills, liver, plasma, and brain samples. Significant tissue-specific differences were observed for all biomarkers with the exception of ACP. The highest values of specific activity for most biomarkers were detected in the liver. However, the levels measured in gills were very close to those observed in the liver and showed fewer variations than other tissues. These findings suggest that the sampling of gills could be used to evaluate pollution biomarkers in salamanders without apparent harm, as this tissue quickly regenerates.

Keywords *Ambystoma spp.* · Esterases · Detoxifying enzymes · ORAC

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1 Introduction

One third of all extant amphibian species are threatened with extinction, which makes frogs, salamanders, and caecilians the only major group currently at risk globally (Chanson et al. 2008). Unfortunately, this trend is likely to increase and, among amphibians, the group of salamanders inhabiting the tropical area is particularly at risk, as their small geographical ranges make them especially vulnerable to man-induced habitat changes (Wake and Vredenburg 2008). In Mexico, a country ranked fifth for species richness, but second only in terms of number of threatened species, more than 50%

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of amphibians are threatened with extinction (Chanson et al. 2008). Among these, the order Caudata has the highest number of species listed as threatened by the IUCN (Frías-Alvarez et al. 2010). Additionally, only 2 out of 19 species of salamanders belonging to the Ambystomatidae family are not categorized as endemic (Zambrano et al. 2007). Within this family, *Ambystoma mexicanum* and *A. velasci* are the species that have been most affected by alterations of their habitats in the center of Mexico, highlighting the pollution as one of the main environmental stressors (Frías-Alvarez et al. 2010). *A. velasci* and *A. mexicanum* are listed under special protection and under risk of extinction, respectively, in the national legislation NOM-059-SEMARNAT-2010 (Diario Oficial de la Federación - DOF, 2010). However, the present status of their populations and the possible effects caused by anthropogenic activities are unknown because of the scarcity of studies in the areas of their natural distribution (Frías-Alvarez et al. 2010).

Pollutants induce changes in biochemical and physiological processes, hematological parameters, and behavior of amphibians (Henson-Ramsey et al. 2008; Lajmanovich et al. 2015; Mann et al. 2009). A wide range of environmental pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, anti-cholinesterasic pesticides, heavy metals, and endocrine disrupters have been detected in amphibians (Spraling et al. 2010). Some studies have described detrimental effects from pollutant exposure on wild amphibian populations due to their inherent specific characteristics. For example, their permeable skin, which is critical for both gas exchange and osmoregulation, makes them particularly sensitive to changes in hydric conditions as well as contaminants and certain skin diseases (Frías-Alvarez et al. 2010). In some cases, cessation of reproduction, impaired larval development and even changes in swimming capacity have been noticed (Mann et al. 2009). These effects do not arise without previous alterations of the enzymatic processes, so the monitoring of these changes may be useful to anticipate damage on any physiological processes that compromise its survival, growth, or reproduction. Biomarkers commonly used in wildlife toxicology include the B-type esterases acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CaE) which are sensitive to inhibition by organophosphorus and methyl carbamate pesticides (Attademo et al. 2014). In the same way, enzymes used as markers of renal and hepatic damage or alteration such as alkaline or acid phosphates (ALP, ACP) and others involved in detoxification processes like glutathione s-transferases (GST) and ethoxyresorufin-o-dethylase (EROD) are also included, the response of which is often an increase of their activity in pollutant-exposed organisms (Johnson et al. 2000; Mazorra et al. 2002). Similarly, monitoring of wildlife exposure to contaminants also include the measurement of some oxidative stress biomarkers such as superoxide dismutase (SOD) activity (Costa et al. 2008), or levels of non-enzymatic antioxidants compounds (Gu et al. 2014).

The target tissues for biomarker determination are frequently the liver, brain, and occasionally the stomach and intestine. However, collection of these tissues implies to kill the animal (Attademo et al. 2014; Henson-Ramsey et al. 2008; Robles-Mendoza et al. 2011). Alternatively, other tissues such as plasma or muscle have been used to determine the activity of esterases and oxidative stress enzymes without sacrificing animals (Aguilera et al. 2012). In relation to this issue, non-lethal techniques are of paramount importance when working with endemic and/or threatened species (Barriga-Vallejo et al. 2015). As research models, axolotls are best known for their ability, unique among vertebrates, to regenerate missing body parts. Many studies have investigated the mechanisms controlling growth and patterning during limb regeneration. At the amputation site, a clump of progenitor cells forms and then regenerates the missing tissues (Kragl et al. 2009).

Therefore, taking advantage of the regeneration capabilities of individuals of the genus *Ambystoma*, the present work was aimed to compare the levels of activity of selected biomarkers of pollutant exposure in different regenerative tissues of *A. velasci* in order to select a tissue that could be sampled by a non-lethal and minimally invasive technique that would allow a time-framework monitoring of pollutant impact on wildlife populations using the same individuals.

2 Materials and Methods

2.1 Animals

The neotenic *A. velasci* were captured in May 2013 using a cast net in a cattle pond in Galeana (Nuevo León, México). Animals were transported to the Ecophysiology laboratory of the Faculty of Biological Sciences of the Autonomous University of Nuevo León.

The collection was done under the scientific collecting permit granted by SEMARNAT with the official number SGPA/DGVS/02418/13. Three organisms were killed by hypothermia and dissected to collect tail, gills, plasma, liver, and brain samples. Moreover, we collected samples of tails and gills from 8 live animals (totalling 11 samples), and plasma from 7 live individuals (totalling 10 samples) by a non-lethal procedure. Afterwards, these animals were kept in captivity to observe the recovery of the tissues. Approximately, 5-mm of gill tissue was taken from the third right branchial arch of the animals. Likewise, a 5-mm tail sample was taken as indicated in Fig. 1. Plasma samples (up to 1 ml) were collected by puncturing of the caudal vein using a heparinized syringe following the protocol described by Homan et al. (2003). This experimental protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Biological Sciences (approval number FCB-CB-701).

2.2 Sample Preparation

Tail, gills, liver, and brain samples were weighted and finely cut in pieces using scissors and a bistoury in a petri dish sitting on ice. These tissues were homogenized whit double distilled water in a proportion of 1:10 (*w*/*v*) at 4 °C using a Wheaton-glass homogenizer (GlasCol™) at 333 rpm during 4 min. Homogenized tissues were centrifuged at 15,300 g at 4 °C for 10 min. The supernatant was separated from the superior lipid layer and from the precipitate. Blood samples were centrifuged at 1300 g at 4 °C for 10 min to separate plasma from blood cells. Plasma samples and tissue homogenate was stored in 0.1 mL aliquots at − 70 °C to be later used as enzymatic extracts. Total soluble protein concentrations in the extracts were determined by the Bradford (1976) method using bovine serum albumin (BSA) as a standard.

2.2.1 B-type Esterase Activity Assays

Cholinesterase (AChE and BChE) activities were determined according to Huang et al. (1997). The reaction mixture consisted of 280 μL of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in a PBS 0.1 M (pH 7.8) buffer and 10 μL of enzymatic extract. The reaction was initiated adding 10 μL of 15 mM acetylthiocholine chloride (AChE activity) or s-butyrylthiocholine chloride (BChE activity). To determine the activity of CaE, the reaction mixture consisted of 200 μL of buffer Tris–HCl 50 mM (pH 7.1), 10 μL of enzymatic extract, and 100 μL of 2 mM p-nitrophenyl acetate as the substrate. For the three enzymatic activities, absorbance was immediately registered at 405 nm in intervals of 120 s up to 30 min in a microplate reader (ELx800, Biotek).

The linearity of the enzymatic reaction was verified using the Gen5™ Software (BioTeK). Three analytical replications were conducted for each sample. The samples were replaced with buffer in the case of controls. Activities were expressed as $\mu mol^{-1}\ min^{-1}\ mg\ protein^{-1}$ using a molar extinction coefficient of 13.6 $mM^{-1}\ cm^{-1}$ for TNB in AChE and BChE (Ellman et al. 1961) or 18.5 $mM^{-1}\ cm^{-1}$ for p-nitrophenol in CaE (Mazorra et al. 2002).

2.3 Phosphatases Assays

Alkaline and acid phosphatases (ALP and ACP) activities were measured using p-nitrophenyl phosphate as substrate (Mazorra et al. 2002). The reaction was performed using 200 μL of diethanolamine buffer (1.0 M) with 50 mM MgCl2 (pH 9.8) for ALP or 0.1 M sodium acetate HCl buffer (pH 4.8) for ACP, then 10 μL of the enzymatic extract and 10 μL of the substrate were added at a final concentration of 0.4 mM. Absorbance was immediately registered at 405 nm in the same conditions as the esterases. The enzymatic activity was expressed as $\mu mol^{-1}\ min^{-1}\ mg\ protein^{-1}$, using for p-nitrophenol a molar extinction coefficient of 18.5 $mM^{-1}\ cm^{-1}$ (Mazorra et al. 2002).

2.4 Ethoxyresorufin-O-deethylase (EROD) Assay

EROD activity was measured using fluorescence spectrophotometry as described by Burke and Mayer (1974). The reaction was carried out at 25 °C in a microplate reader (Synergy 2 Biotek). The reaction mixture consisted in 10 μL of the enzymatic extract and 280 μL of 7-ethoxyresorufin (2 μM in 0.1 M Tris–HCl, pH 8, 0.1 M NaCl) and was initiated after adding 10 μl of 25 mg/ml of NADPH (in 50 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA). The fluorescence (530 nm excitation and 585 nm emission), resulting from the resorufin formation, was measured during 3 min. The fluorescence data were calibrated using a standard curve of resorufin. EROD activity was expressed as $pmol^{-1}\ min^{-1}\ mg\ protein^{-1}$.

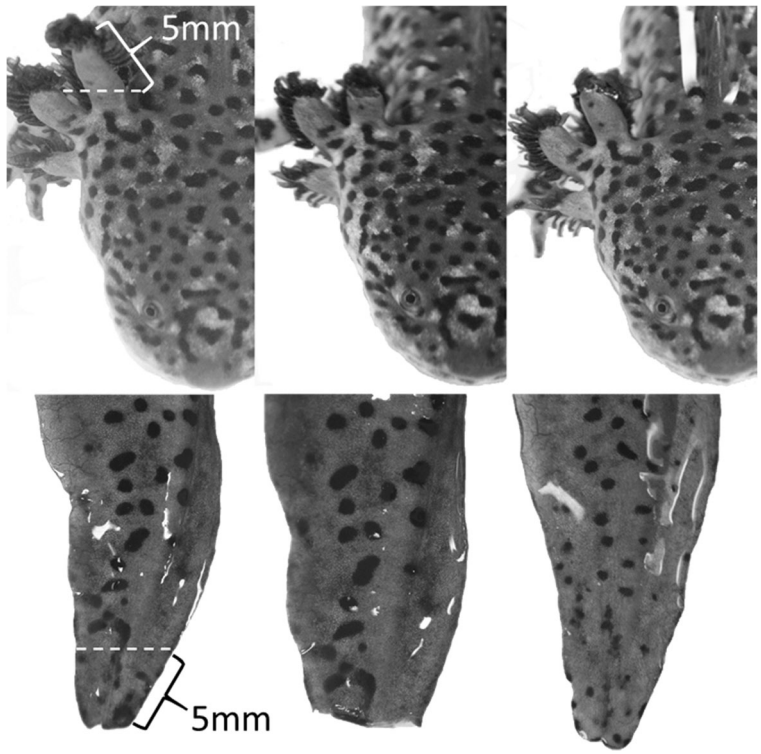

Fig. 1 Pictures of the sampling method and regeneration process of gills and tails from animals kept for 15 days in captivity after tissue extraction

2.5 Glutathione S-transferase (GST) Assay

GST activity was analyzed using the Wilce and Parker (1994) method adapted to microplates. To initiate the reaction, 300 μL of a substrate mixture containing reduced L-glutathione (200 mM) and 1-chloro-2,4-dinitrobenzene (CDNB; 100 mM) in Dulbecco's phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH2PO4, 136.9 mM NaCl and 8.9 mM Na2HPO4•7H2O, pH 7.2), and 10 μL from enzymatic extract were added. Absorbance was immediately registered at 340 nm every minute during a period of 10 min in a microplate reader (ELx800, Biotek). GST activity was expressed as $\mu mol^{-1}\ min^{-1}$ mg $protein^{-1}$, using a molar extinction coefficient of 9.6 $mM^{-1}\ cm^{-1}$ for CDNB (Brodeur et al. 2011).

2.6 SOD and Cellular Antioxidant Capacity Assays

SOD activity was measured with the spectrophotometric method described by Peskin and Winterbourn (2000). The reaction mixture was carried out in microplates, and consisted of 200 μL of tetrazolium salt, 50 mM Tris–HCl buffer (pH 8.0) with diethylenetriaminepentaacetic acid (0.1 mM) and hypoxanthine (0.1 mM), and 10 μL of enzymatic extract. The reaction was initiated by adding 20 μL of xanthine oxidase in 50 mM Tris–HCl buffer (pH 8.0), catalyzing the liberation of superoxide radicals, which were caught by the tetrazolium salt and quantified at 450 nm. The concentration of radicals was inversely proportional to the SOD activity in the samples. To quantify the activity, the percentage of xanthine oxidase inhibition was measured.

The cellular antioxidant capacity was determined by the fluorescence-based oxygen radical absorbance capacity (ORAC-FL) (Huang et al. 2002), which is an indirect method that monitors the antioxidant's ability to protect the fluorescent probe from free radical-mediated damage, and an azo-radical initiator, AAPH (2,2-azobis (2-amidinopropane) dihydrochloride). Protein was eliminated from the extracts by precipitation with perchloric acid (0.5 M) in a 1:1 (*v*:*v*) ratio. The reaction was carried out in a 75-mM phosphate buffer (pH 7.4). The reaction mixture consisted of 150 μL fluorescein (0.004 mM) and 25 μL of antioxidant (Trolox calibration solutions 0–100 μM), test samples or buffer in the case of blanks. The mixture was preincubated for 30 min at 37 °C in a microplate reader (Synergy 2 Biotek). AAPH solution (25 μL; 153 mM) was added rapidly to every well to start the reaction.

Fluorescence (485 nm excitation and 528 nm emission) was recorded every minute for 60 min. Standard curve and samples calculations were performed with the Software Gen5 (Biotek) incorporated in the microplate reader. ORAC-FL values were expressed as Trolox equivalents in μmol^{-1} mg $prot^{-1}$.

2.7 Statistical Analysis

All biomarkers were evaluated in triplicate, with the exception of SOD that was evaluated in duplicate.

Kruskal-Wallis nonparametric test for range comparison and a *post hoc* Conover test were used to compare the biomarkers data observed in the different tissues using the software R.

3 Results

Tail and gill full regeneration in neotenic individuals maintained in captivity lasted nearly 15 days after tissue sampling (Fig. 1). No abnormalities were observed in regenerated tails or gills. After the regeneration period, the animals continued to grow with no apparent signs of disease. Likewise, none of them underwent metamorphosis, suggesting that the technique not only is non-lethal and non-invasive, but also has no negative repercussions on the health or growth performance of these organisms.

Significant differences ($P < 0.05$) in the values of all biomarkers were observed among the tissues analyzed, with the exception of ACP. A statistically higher AChE activity was observed in the brain, tail and gills (Fig. 2a). The BChE activity showed a similar pattern as AChE in the rest of the tissues, with the exception of the brain activity, which was the lowest (Fig. 2b). CaE activity did not follow the former pattern and the significantly highest activity was observed in plasma followed by values observed in liver, gills and tail, while the lowest level was detected in brain (Fig. 2c).

The liver, as expected, showed the highest activities of ALP, EROD, and GST (Fig. 3). ALP activity was similar in most tissues, except for plasma which showed the lowest activity. In the case of ACP, some activities could not be detected in plasma or in brain tissue, and an extremely low ACP activity was only observed in the tail, gills, and liver (Fig. 3b). EROD activity in tail and gills was significantly lower than in the liver; however, no EROD activity could be detected in plasma and brain. GST activity was similar in the liver, gills, and tail, whereas this activity was the lowest in plasma and brain (Fig. 3c).

Liver, brain, and plasma showed the highest values for oxidative stress biomarker SOD, compared to gills and tail (Fig. 4a). The highest levels of ORAC activity was detected in the liver and close values were observed in the gills and brain, while the lowest activity was observed in tail and plasma (Fig. 4b).

4 Discussion

AChE activity in *A. velasci* was higher in brain and tail (skin and muscle) compared to other tissues. High levels of activity of neurotoxicity biomarkers such as cholinesterase enzymes (e.g., AChE) have also been reported in brain and muscle of the flying frog (*Rhacophorus lateralis*) associated with their role in nerve signal control (Padidela and Ravinder 2015). Additionally, the high AChE activity observed in tail samples may be related to the previously reported activity of cholinesterases in the skin and muscle in amphibians. In line with this, the role of acetylcholine as a lightening agent of frog skin melanocytes has been demonstrated (Moellmann et al. 1974) and the role of acetylcholine in muscle fibers of salamanders is well established (Dennis and Ort 1977). It should be noted that a lower AChE activity has been observed in the whole body, brain, gills, dorsal, and lumbar spine of *A. tigrinum* and *A. mexicanum* (Henson-Ramsey et al. 2008; Robles-Mendoza et al. 2011; Scaps et al. 1994), in comparison to tissue activity levels registered in this study. The fact that both *A. tigrinum* and *A. mexicanum* studies were conducted with individuals raised in captivity, in contrast with wild-caught neotenic *A. velasci* used in the present study may explain these differences.

The highest BChE activity was observed in tail and gills of *A. velasci*, followed by those registered in the liver, plasma, and brain. While CaE activity was highest in plasma followed by activities in liver, gills, tail, and brain. In this context, our observations concur with several studies that associate the inhibition of the activity of BChE and CaE, compared to AChE, with the exposure to pollutants, when evaluated in tissues other than the brain (Robles-Mendoza et al. 2011). This selective inhibition has been attributed to the fact that BChE and CaE fix and/or degrade compounds that cause neurotoxicity, preventing them thus from acting

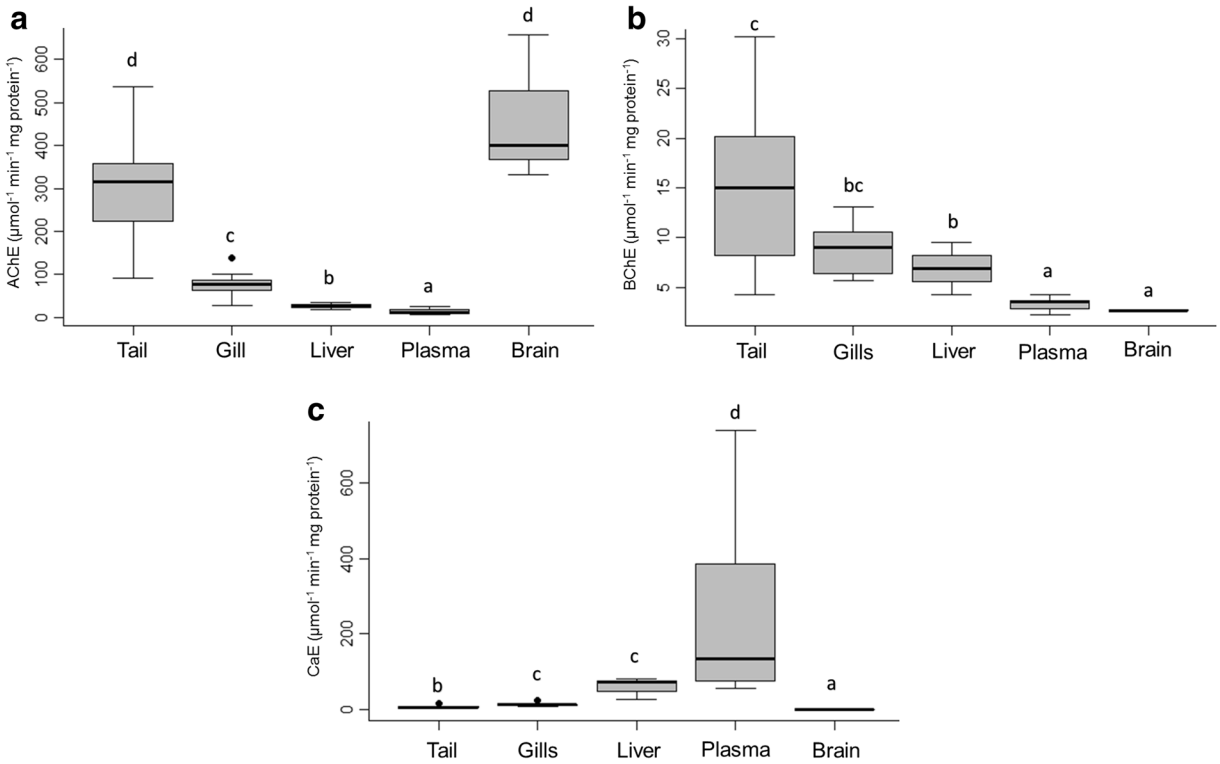

Fig. 2 Enzymatic activity of **a** acetylcholinesterase (AChE), **b** butyrylcholinesterase (BChE), and **c** carboxylesterase (CaE) from different tissues of *A. velasci.* Means with the same superscript letters belong to homogeneous groups (separated by *post hoc* Conover test). (Tail $N = 11$, gills $N = 11$, liver $N = 3$, plasma $N = 10$, brain $N = 3$)

on the nervous system AChE (Sanchez-Hernandez 2007).

Phase I (e.g., EROD) and phase II (e.g., GST) detoxification enzymes generally show a higher activity in the liver, as a result of detoxification and oxidative stress functions of this organ (Costa et al. 2008; Mazorra et al. 2002). The first metabolic phase of detoxification of endogenous compounds and xenobiotics may be the activation of the P450 cytochrome enzymatic system (Frasco and Guilhermino 2002), and within this system, the EROD activity is considered particularly sensitive to induction by various pollutants (Dabrowska et al. 2014). In agreement with this statement, the highest EROD activity was observed in the liver followed by values observed in gill and tail samples. These activities were found to be higher than the basal activity reported for the liver and muscle of *A. tigrinum* (Johnson et al. 2000). As mentioned above, these differences may be attributable to the fact that the individuals of *A. tigrinum* were transformed adults raised in terrariums and were less exposed to environmental stressors than wild-caught *A. velasci.* These results are also consistent with the highest GST activity observed in the liver, tail, and gills of *A. velasci*. Indeed, those compounds, modified by phase I enzymes, pass through a second metabolic detoxification phase in which enzymes incorporate polar radicals to the xenobiotics, such as GST with glutathione radical (GSH), thereby facilitating their excretion (Richardson et al. 2010). Even though, GST activity varies among tissues of some amphibians, higher liver activities have also been reported for *A. tigrinum* and *Bufo regularis* (Ezemonye and Tongo 2010; Johnson et al. 2000).

Phosphatases are important in different metabolic processes, as they are implicated in membrane permeability, growth, cell differentiation, and steroidogenesis (Mazorra et al. 2002). Their activity varies depending on the stage of development, and/or tissue analyzed, predominating in the liver, digestive system, and kidneys (Mazorra et al. 2002; Yora and Sakagishi 1986). It has also been shown that their activity in the muscle may increase in the presence of pollutants (Aguilera et al. 2012; Aich et al. 2015). In the present study, the highest ALP activity was registered in the liver, followed by the

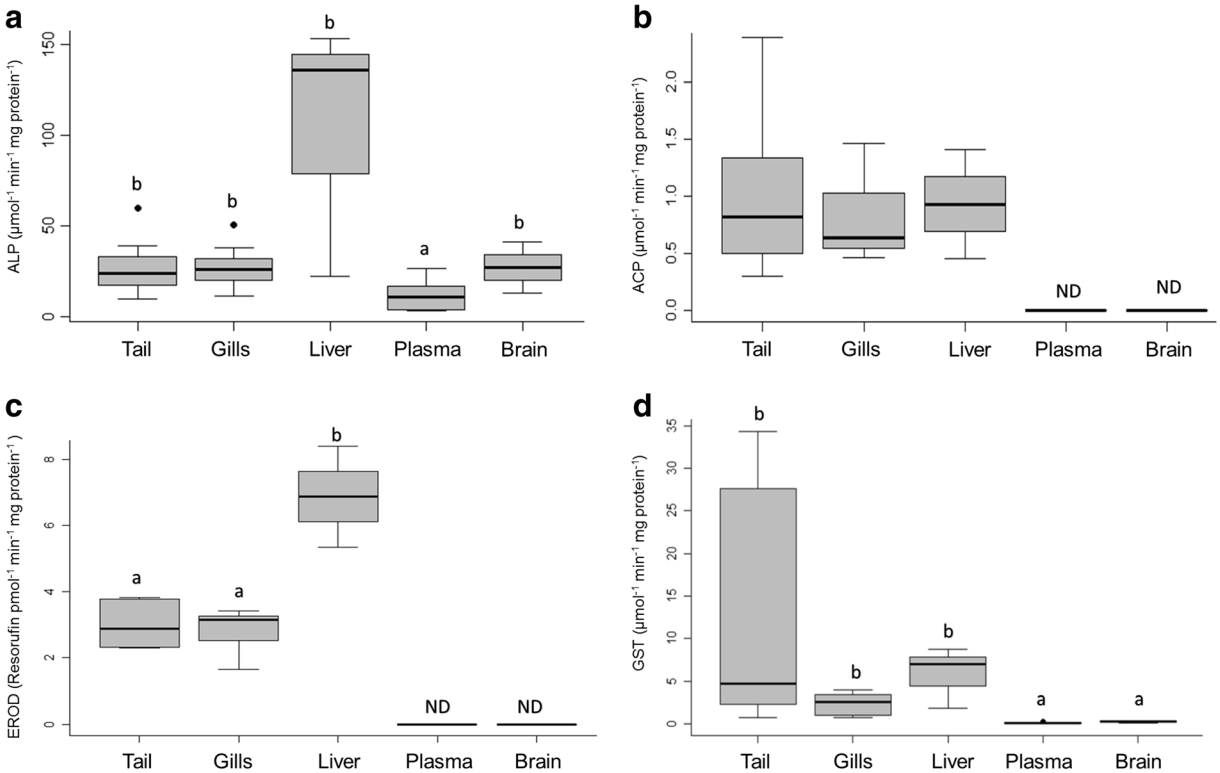

Fig. 3 Enzymatic activity of **a** alkaline phosphatase (ALP), **b** acid phosphatase (ACP), **c** ethoxyresorufin-O-deethylase (EROD) assay, and **d** glutathione S-transferase (GST) assay from different tissues of *A. velasci.* Means with the same superscript letters belong to homogeneous groups (separated by *post hoc* Conover test). (Tail $N = 11$, gills $N = 11$, liver $N = 3$, plasma $N = 10$, brain $N = 3$)

gills, tail, and brain. These results are in agreement with the high liver activity reported in *Rana ridibunda*, and *Xenopus laevis* (Al-Attar 2004; Yora and Sakagishi 1986) and the amplified activity in tail samples of lizards after exposure to pollutants (Aguilera et al. 2012).

SOD enzymatic activity is very sensitive to the presence of pollutants and thus has been commonly used as a biomarker of oxidative stress (Ezemonye and Tongo 2010). The highest SOD enzyme activity in the present study was observed in the liver followed by brain, plasma, gills, and tail samples. This is consistent with the greater SOD liver activity observed in other amphibians like *Rana ridibunda*, compared to heart and skeletal muscle activity (de Quiroga et al. 1984). A similar result was also observed in *Lithobates catesbiana*, which after exposure to glyphosate showed a higher liver activity compared to the muscle activity (Costa et al. 2008). The oxidative stress system also includes non-enzymatic molecules, which can be produced endogenously, such as the GSH, or exogenous antioxidants, such as some vitamins or pigments derived from food (Richardson et al. 2010). The quantification of this kind of molecules, as well as the products generated by free radicals (e.g., lipid peroxidation) have also been used regularly as biomarkers of oxidative stress (Ezemonye and Tongo 2010). In relation to this, the antioxidant activity of non-enzymatic molecules evaluated by the ORAC technique is still underused in ecotoxicology studies. Nevertheless, because of its sensitivity and utility, it has gained relevance in the areas of physiology and food quality (Huang et al. 2002). Recent studies have shown changes in ORAC levels in some crustaceans after exposure to pollutants; however, the eventual differences that may exist in ORAC levels among tissues have not yet been discussed (Wiklund et al. 2014). In the present study, liver, brain, and gills samples showed the highest ORAC values. The high levels observed in gills may be explained in function of the continuous exposure of this respiratory organ to pollutants and fluctuating oxygen levels in the cattle ponds where

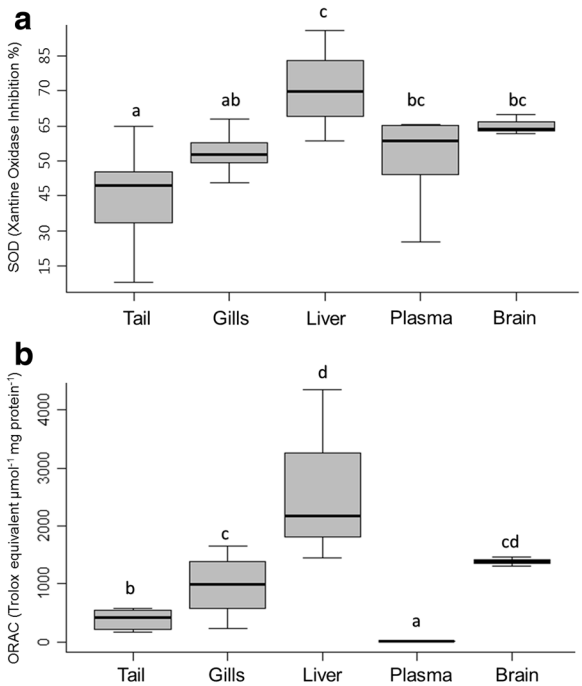

Fig. 4 Enzymatic activity of **a** superoxide dismutase (SOD) and not enzymatic antioxidant activity **b** oxygen radical absorption capacity (ORAC) from different tissues of *A. velasci.* Means with the same superscript letters belong to homogeneous groups (separated by *post hoc* Conover test). (Tail $N = 11$, gills $N = 11$, liver $N = 3$, plasma $N = 10$, brain $N = 3$)

they inhabit. In this regard, it has been shown that the co-exposure to low levels of oxygen and pollutants impairs the adaptive antioxidant increase (ORAC and SOD) in crustaceans and AChE activity declines along with a concomitant increase in antioxidants (assayed by ORAC, SOD) (Gorokhova et al. 2013).

Despite the fact that levels of most biomarkers were higher in liver and brain, as expected, the use of these tissues requires the sacrifice of the organisms. In the present study, the biomarkers levels observed in plasma (with the exception of CaE) were lower than in other tissues, however, it is widely known that plasma provides the most sensitive biomarkers with the additional advantage that blood generally reflects recent exposure (Burger et al. 2005). By contrast, gills and tail, which in this study showed higher (AChE, GST, and BChE) or similar (ORAC and CaE) levels of biomarkers to the liver, could be used as sampling primary tissues for ecotoxicology studies in neotenic *Ambystoma* species. With the additional advantage of being non-lethal and easily collectable. Furthermore, these tissues quickly regenerate in amphibians of the genus *Ambystoma* (Nye et al. 2003).

Even though tail sampling has been used to quantify contaminant exposure in terrestrial salamanders (Bergeron et al. 2010; Pfleeger et al. 2016), it has not been used to measure physiological indicators, with the exception of the work of Aguilera et al. (2012) with lizards. An attractive part of tail sampling is the skin portion which, as mentioned above, reflects well the activity of certain biomarkers. Indeed, the permeable skin of amphibians, is in constant contact with water and soil, and is important in respiration and osmoregulation. Consequently, the skin is also continuously exposed to chemical pollution present in aquatic systems. As a result, it has been demonstrated that the skin is a site for toxicant (PCB) accumulation, but also of toxicant elimination, as P450 enzymes were shown to be present in the epithelial cells of mucous glands and dermal vascular endothelium (Huang et al. 2001). In this regard, shed skin from *Ambystoma* sp. has been proposed as suitable alternative models for detecting genotoxic exposures relevant to aquatic environments (Zavala-Aguirre et al. 2007). However, skin alone was not used in this study because larvae and neotenic individuals do not slough their skins suitably. Tail sampling could be fitted for terrestrial salamanders.

On the other hand, one of the main advantages of sampling gills in *Ambystoma*, beyond their continuous exposure to the environment, is their enlargement and reduction in response to changes in oxygen conditions (Bond 1960). Another benefit of using gills is that each gill is supplied with blood by a ventral artery, branches of which supply the filaments in which blood flows continuously as part of normal respiration (Rivera and Davis 2013). In this way, when a part of the gill is severed, blood can be collected at the same time to be used to study the leucocyte profiles (Barriga-Vallejo et al. 2015), micronucleus test (Zavala-Aguirre et al. 2007), or comet assay (Mouchet et al. 2005). It is worth noticing that the values of the different biomarkers observed in the gills were more steady than values from tail samples, and also were closer to those observed in the liver.

In addition to the abovementioned advantages, *A. velasci* has the ability to remain in the aquatic environment in a neotenic stage until adult, allowing long-term studies because these salamanders are active most of the year (Barriga et al. 2016). Ecotoxicological studies have shown that members of the *Ambystoma* genus are sensitive to agrochemicals, hydrocarbons, and other contaminants (Henson-Ramsey et al. 2008; Johnson

et al. 2000; Robles-Mendoza et al. 2011). Therefore, they are considered a good model to investigate the effects of pollutants. On top of this, urodele amphibians are the only vertebrates that can regenerate multiple structures such as limbs, tails, gills, heart, spinal cord jaws, and skin throughout their whole life (Goršič 2007). This great regeneration potential is suitable for seasonal sampling to study the effect of pollutants over time.

In the present study, it could be stated that the regeneration time of gill and tail samples is relatively fast (approximately 2 weeks). Nevertheless, it has been shown that limb regeneration tends to be sensitive to chemical pollution, ultraviolet B radiation, predation, and parasites (Johnson et al. 2006; Bowerman et al. 2010). Therefore, further studies should elucidate the effectiveness of this technique in sites with a high environmental disturbance.

In conclusion, the results of the present research indicate that sampling small portions of gills represent a suitable non-lethal technique to develop toxicological laboratory and field studies to evaluate the susceptibility of neotenic salamanders, as well as other endemic amphibians under some risk category, to the exposure of environmental pollutants.

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