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Tissue-Specific Variations of Esterase Activities in the Tadpoles and Adults of *Pseudis paradoxa* (Anura: Hylidae)

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Abstract We determined basal levels of cholinesterase (ChE) and carboxylesterase (CbEs; two substrates: α-naphthyl acetate and 4-nitrophenylvalerate) in different tissues of tadpoles and adults of the frog *Pseudis paradoxa* and evaluated their use as complementary biomarkers of anti-cholinesterase pesticide exposure. ChE and CbEs sensitivity to malaoxon was also evaluated. Adults and tadpoles were collected with sweep net from temporary ponds located in natural riparian forests along the Paraná River (Garay Department, Santa Fe province, Argentina). We found significant differences in B-esterase activities between adults and tadpoles and among different tissues. The in vitro inhibition tests indicated that ChE is more sensitive to inhibition than CbEs. Our results suggest that basal ChE and CbE (α-NA and 4-NPV) activities in different tissues of adult and tadpoles *P. paradoxa* would be suitable biomarkers of pesticide exposure, and this amphibian species could be used as sentinel in field monitoring.

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1 Introduction

Organophosphorus (OP) pesticides are still important agrochemicals used in modern agriculture all over the world (Abhilash and Singh 2009). Although OP insecticides persist in the environment for a relatively short time, they show a high acute toxicity that may pose a serious hazard to nontarget species, such as species of mammals, birds and aquatic organisms. Organisms exhibit a great variety of pathways that rapidly degrade or transform OP compounds (Chambers and Levi 1992; Geiger et al. 2010), hindering their detection by chemical analysis. The mechanism of acute toxicity, i.e. inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) in the nervous system is also the basis for the most popular biomarker of OP pesticide exposure in many vertebrate species (Van der Oost et al. 2003). AChE and carboxylesterases (CbEs, EC 3.1.1.1) belong to the group of hydrolases that Aldridge (1953) classified as B-type esterases. These esterases are inhibited by OP pesticides (Wheelock et al. 2008) and can be used as biomarkers to monitored pesticide exposure in wild species (McCarthy and Shugart 1990).

Studies that integrate biochemical responses at higher levels of biological organization have great ecotoxicological relevance (Walker 1998) because they provide a measurement endpoint, which is an aim of environmental risk assessment (Forbes et al. 2006). Most of the studies on inhibition of cholinesterase (ChE) and CbE activities

by pesticides involve different aquatic or terrestrial organisms (Wheelock et al. 2008). However, such studies are scarce in amphibians, despite their high vulnerability to environmental stressors such as OP pesticides (Mann et al. 2009; Robles-Mendoza et al. 2011).

Pseudis paradoxa is widely distributed in the Paraguay and Paraná rivers, in Paraguay, Bolivia, Brazil and Argentina (Cei 1980). In Argentina, this frog is categorized as "not threatened" (Vaira et al. 2012) and distributed in the provinces of Buenos Aires, Formosa, Chaco, Corrientes, Santiago del Estero, Entre Ríos and Santa Fe (Cei 1980; Frost 2009). Adults are very aquatic, occurring in semipermanent and permanent ponds. Tadpoles are greater than adults that reach up to 168 mm in length. Because of the expansion of the agricultural frontier over the last years, many areas of the Argentinean geographical distribution of *P. paradoxa* and other cohabitant native amphibian species currently overlap with regions of intensive agricultural activity, where agrochemicals (e.g. OP and carbamates) are extensively used for pest control (CASAFE 2007; Lajmanovich et al. 2010). In addition, the persistence of this species in a variety of habitats (wetlands, rice agroecosystems and suburban areas; Peltzer and Lajmanovich 2007; Duré et al. 2008) make them appropriate bioindicator species of pesticide contamination.

The aims of this study were to increase our knowledge of the activity of B-esterases (ChE and CbEs; two substrates: α-naphthyl acetate and 4-nitrophenylvalerate) in tadpoles and adults of the frog *P. paradoxa* and to evaluate their use as complementary biomarkers of anti-ChE pesticide exposure. For these purposes, we determined basal levels of B-esterase activity in different tissues, the effect of life stage (adults and tadpoles) on enzymatic activity and in vitro sensitivity of B-esterases to the OP insecticide malaoxon, the main active metabolite of malathion.

2 Materials and Methods

2.1 Reagents

Sodium dodecyl sulphate (SDS) was purchased from Calbiochem® (Canada). Acetylthiocholine (AcSCh), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), α-naphthyl acetate (α-NA), 4-nitrophenyl valerate (4-NPV), and Fast Red ITR salt were obtained from Sigma-Aldrich® (Germany). The pesticide malaoxon (CAS no: 1634-78-2) was purchased from Applied Science® (USA). All the other chemicals used in this study were obtained from Biopack® (Argentina).

2.2 Experimental Animals

Adults (N=12; eight males and four females) were handily collected whereas tadpoles (N=8, stages 33–36; Gosner 1960) were obtained by sweep net both from temporary ponds located in riparian forests belonging the Espinal and Deltas e Islas del Río Paraná ecoregions (Burkart et al. 1999), influence by Paraná River (31° 10′ 21.10″ S, 60° 15′ 31.73″ W Cayastá, Garay Department, Santa Fe province, Argentina) in January 2012. Despite the wide distribution of soybean and rice crops in these regions, all the adults and tadpoles were collected from nonagricultural areas, so they likely had no exposure to pesticides. All individuals were collected with authorization of the Ministerio de Aguas, Servicios Públicos y Medio Ambiente, Santa Fe province, Argentina. The number of amphibian collected was similar to previous studies where characterization of enzymatic activities is done (Attademo et al. 2012; Basso et al. 2012), and they were sufficiently to performed such basal analysis (Sánchez et al. 1997). Snout–Vent length (SVL) and body weight were recorded in each individual with a digital calliper (±0.1 mm precision) and balance (0.01 g), respectively.

2.3 B-esterase Assays

Adults and tadpoles of *P. paradoxa* were euthanized by immersion in a buffered solution of 0.1 % tricaine methanesulfonate (MS-222) with approval of Facultad de Bioquímica y Ciencias Biológicas animal ethics committee following ASIH et al. (2002) guidelines.

Dissection of each adult frog was initiated in the midventral line by a longitudinal incision, and the digestive organs (stomach, intestine, and liver) and hindlimb muscle were removed. In frog tadpoles, the same protocol was followed to obtain digestive organs (coiling gut and liver) and tail muscle.

The tissues were washed in distilled water and placed on a filter paper to remove excess fluids. Whole tissues were homogenized (on ice) in 20 % (*w*/*v*) buffer containing 0.1% t-octylphenoxypolyethoxyethanol (tritonX-100) in 25 mM Tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0) using a polytron. The homogenates were centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was collected. ChE activity was determined colorimetrically following Ellman (Ellman et al. 1971).

The reaction mixture (final volume [FV=930 μl]) consisted of 25 mM Tris-HCl containing 1 mM $CaCl_2$ (pH=7.6), 10 μl 20 mM acetylthiocholine iodide (AcSCh), and 50 μl DTNB (3×10^{-4} M, final concentration). Variation in optical density was measured in duplicate at 410 nm at 25 °C for 1 min using a Jenway 6405 UV-VIS spectrophotometer. Protein (PT) concentrations in the supernatants were determined according to the Biuret method (Kingbley 1942). The activity of ChE was expressed in nanomole of hydrolyzed substrate per minute per milligram of PT using a molar coefficient extinction of 13.6×10^3 M^{-1} cm^{-1}. Carboxylesterase was determined using two substrates: α-naphthyl acetate (α-NA) and 4-nitrophenyl valerate (4-NPV), and specific enzyme activity was expressed as nanomole per minute per milligram of PT. The hydrolysis of α-NA by CbE was measured as described by Gomori (1953) and adapted by Bunyan and Jennings (1968). The reaction medium (FV=1950 μl) contained 25 mM Tris-HCl, 1 mM $CaCl_2$ (pH=7.6) and sample. The reaction was initiated by adding 50 μl α-naphthyl acetate (1.04 mg ml^{-1} in acetone) after a preincubation period of 5 min at 25 °C. The formation of naphthol was stopped after 10 min by adding 500 μl 2.5 % sodium dodecyl sulphate and subsequently 0.1 % of Fast Red ITR in 2.5 % Triton X-100 in deionizer water (prepared immediately before use). The samples were left in the dark for 30 min to develop, and the absorbance of the complex was read at 530 nm (using a molar extinction coefficient of 33.225×10^3 M^{-1} cm^{-1}). Determination of CbE activity towards 4-NPV followed the methods of Carr and Chambers (1991). Samples were preincubated in 50 Mm Tris-HCl (pH 7.5) at 25 °C for 5 min (FV=1980 μl), and the reaction was initiated by adding 20 μl 4-NPV (5×10^{-4} M, final concentration). After 10 min, the reaction was stopped by adding of 1 ml of an aqueous solution containing 2 % (*w*/*v*) SDS and 2 % (*w*/*v*) Tris base. The formation of 4-nitrophenolate was monitored at 405 nm and quantified using an external calibration curve (5–100 nmol 4-nitrophenolate/ml).

2.4 In Vitro Inhibition of B-esterase Activity by Malaoxon

Sensitivity of ChE and CbE (α-NA and 4-NPV) activity to OP pesticides was tested using the oxon metabolite of malathion, i.e. malaoxon (Mx). Insecticide solutions were initially prepared in dimethyl sulfoxide, and the solvent concentration in the reaction medium was kept below 0.1 %. Pools including equal amounts of tissue from six randomly selected adults and tadpoles belonging to the same group were preincubated at 25 °C for 30 min with multiple malaoxon concentrations (1.2×10^{-4} to 1.2×10^{-13} M) to generate a range of esterase inhibition. Samples were used to measure the activity of B-esterase, and the inhibition percentage generated by Mx was calculated with respect to the corresponding controls, which received an equal volume of deionized water. The molar concentration of Mx causing 50 % inhibition of the observed maximum enzyme activity (IC_{50}) was estimated by plotting the percentage of remaining esterase activity against the molar inhibitor concentration (Lajmanovich et al. 2008). To obtain comparable IC_{50} values among tissues, the incubation conditions (temperature, time, pH and substrate concentration) were kept equal for all assays. All the incubations were run in triplicate. The inhibition curves were fitted to the two- [$f=a\times\exp(-b\times x)$] and three-parameter [$f=y0+a\times\exp(-b\times x)$] exponential decay model (Estevez and Vilanova 2009) from the library of nonlinear regressions of the SigmaPlot software (SPSS Science, v. 9.01, Chicago, IL, USA). A level of probability below 0.05 was considered statistically significant.

2.5 Data Analysis

Data are presented as mean±standard error (SE). Significant differences in the B-esterase activities among tissues were tested using the Kruskal–Wallis test followed by the Dunnett's test for post hoc, whereas differences in B-esterase activities between tadpoles and adult frogs (only liver, intestine/gut and muscle) were tested with the Mann–Whitney *U* test. Because there were no differences between sexes in the enzymatic activities, we pooled the data as adults in all the analyses. Data were tested for variance homogeneity and normality (Kolmogorov–Smirnov test and Levene test). The tests were performed using the statistical software InFostat 1.1 for Windows (Grupo InfoStat Professional, FCA, Universidad Nacional de Córdoba, Argentina). A value of $p<0.05$ was considered significant.

3 Results

3.1 Tissue Distribution of B-Esterase

Mean (±SE) SVL and body weight were 43.51±0.85 mm and 9.70±0.79 g for adults and 38.83±0.93 mm and

8.67±0.88 g for tadpoles. Table 1 shows the basal levels of B-esterase activity in different tissues of adults and tadpoles of *P. paradoxa*. ChE activity responded differently (Kruskal–Wallis, KS=14.66; p=0.0001) to each substrate tested. The highest ChE activity was observed in the stomach and intestine (21.25±1.10 and 20.67±1.38 nmol min^{-1} mg^{-1} of PT, respectively) of adult frogs and the in liver and muscle (16.57±1.39 and 26.64±3.57 nmol min^{-1} mg^{-1} of PT) of tadpoles. There were significant differences in ChE activity in the intestine/gut and muscle tissues between adult frogs and tadpoles (Mann–Whitney U test W=21.00, p=0.0004 and W=70.00, p=0.0076, respectively). Significant differences in CbE activity were observed using α-NA. Adults showed the lowest esterase activity in the muscle (5.33±0.38 nmol min^{-1} mg^{-1} of PT), whereas the highest CbE activity using this substrate was found in the intestine (56.39±6.94 nmol min^{-1} mg^{-1} of PT) and stomach (46.31±5.67 nmol min^{-1} mg^{-1} of PT) (Kruskal–Wallis KS=23.78; p=<0.0001). In tadpoles, the highest CbE (α-NA) activity was recorded in the liver (6.03±0.60 nmol min^{-1} mg^{-1} of PT; Kruskal–Wallis KS 10.75; p=<0.004). CbE (α-NA) activity was significantly higher in the liver, intestine and muscle of adult frogs than in the liver, gut and muscle of tadpoles (Mann–Whitney U test, p=<0.005). CbE activity using 4-NPV, differed significantly among the tissues of tadpoles and adults individuals (Kruskal–Wallis KS=31.00, p=<0.0001 and 14.00, p=0.0009, respectively). The highest CbE activity (4-NPV) was observed in liver (802.39±90.06 and 44.46±6.63 nmol min^{-1} mg^{-1} of PT) and intestine/gut (481.27±71.44 and 81.50±6.27 nmol min^{-1} mg^{-1} of PT, respectively) of adults and tadpoles. CbE (4-NPV) activity was significantly higher in all tissues in adult frogs than in those of tadpoles (Mann–Whitney U test, p=<0.005).

3.2 B-esterase Inhibition

The sensitivity of ChE and CbE (α-NA and 4-NPV) to inhibition by malaoxon (Mx) as model OP pesticides both in different tissues on adult and tadpoles was studied. ChE and CbE (α-NA and 4-NPV) activities followed an exponential decay model (p<0.0001) when exposed to Mx in vitro (Figs. 1, 2, and 3). Mx caused an inhibition on esterase activities with IC_{50s} varying from 10^{-5} to 10^{-8} M. In general, ChE was more sensitive to OP (IC_{50s}) than CbE (α-NA and 4-NPV) activities (Table 2). The rank order for ChE sensitivity to Mx in adults of *P. paradoxa* among tissues was liver>muscle>stomach>intestine, and for CbE (α-NA), it was muscle>stomach>liver>intestine. For CbE (4-NPV), the rank order was liver>intestine>stomach>muscle (Table 2). In tadpoles, rank order was coiling gut>muscle>liver for ChE, liver>coiling gut for CbE (α-NA) and coiling gut>muscle>liver for CbE (4-NPV). In general, CbE activity using α-NA was more sensitive to Mx inhibition than CbE activity using 4-NPV.

4 Discussion

Basal levels of ChE activity measured in *P. paradoxa* were within the same range of variation reported in liver of a native frog species *Leptodactylus latrans* adult (~6 nmol min^{-1} mg^{-1} protein; Brodeur et al. 2011)

Table 1 Mean cholinesterase (ChE) and carboxylesterases (CbE; substrates α-NA and 4-NPV) activities in adults and tadpoles of *P. paradoxa*. Enzymatic activities are expressed as mean±SE (nmol min^{-1} mg^{-1} of PT)

Stage	Tissue	ChE	CbE (α-NA)	CbE (4-NPV)
Adults	Liver	16.95±1.10a	32.53±2.91b*	802.39±90.06c*
	Stomach	21.25±1.10b	46.31±5.67b	147.52±8.17b
	Intestine	20.67±1.38b*	56.39±6.94c*	481.27±71.44b*
	Muscle	13.55±0.91a*	5.33±0.38a*	4.89±2.63a*
Tadpoles (33–36, Gosner)	Liver	16.57±1.39b	6.03±0.60b	44.46±6.63b
	Coiling gut	5.16±0.75a	1.89±0.13a	81.50±6.27b
	Muscle	26.64±3.57b	2.67±0.47a	9.52±1.11a

Different letters (a, b, c) denote significant differences among tissues and between stages (Dunn post hoc test; p<0.05)

*p<0.05, statistical significance between tadpoles and adults

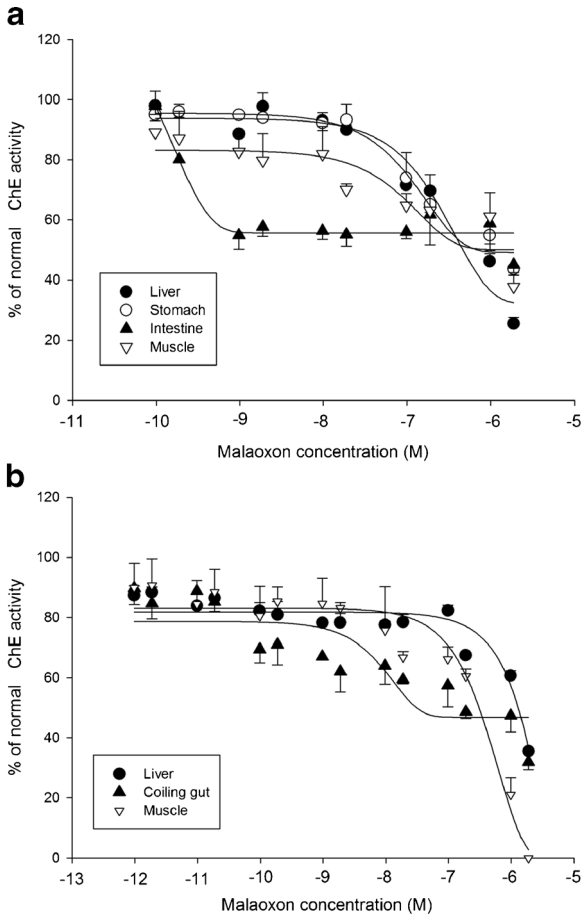

Fig. 1 Inhibitory effects of malaoxon on cholinesterase (ChE) activity in vitro in adults (**a**) and tadpoles (**b**) of *Pseudis paradoxa*. Each point corresponds to the mean of three independent assays (±SE). See Table 2 for nonlinear regression statistics

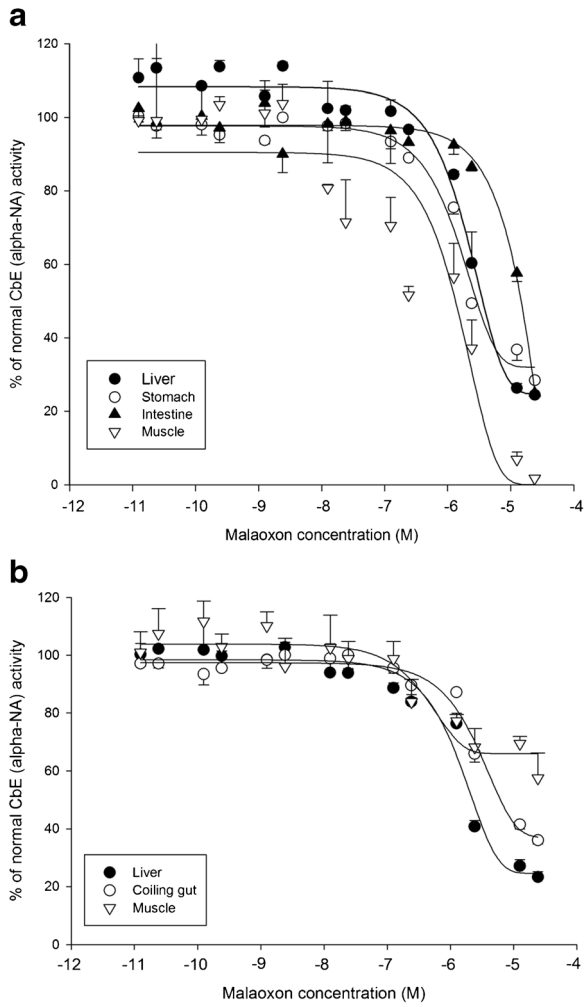

Fig. 2 Inhibitory effects of malaoxon on butyrylcholinesterase (CbE, α-NA) activity in vitro in adult (**a**) and tadpoles (**b**) of *Pseudis paradoxa*. Each point corresponds to the mean of three independent assays (± SE). See Table 2 for nonlinear regression statistics

and muscle for fish species *Scyliorhynus canicula* (12.1±2.7 nmol min^{-1} mg^{-1} protein; Solé et al. 2008) and *Galeus melastomus* (10.3±2.2 nmol min^{-1} mg^{-1} protein; Solé et al. 2008). ChE activity was higher in the stomach and intestine than in the liver and muscle in adults of *P. paradoxa*, whereas in tadpoles, ChE activity was higher in the liver and muscle. Tissue-specific variations in CbE activity have been observed extensively in aquatic invertebrates and vertebrates (Wheelock et al. 2005, 2008). Thus, high levels of CbEs can contribute to pesticide tolerance because this esterase hydrolyses OP, pyrethroids and carbamates (Sogorb and Vilanova 2002). Differences in CbE activities depending on the substrate, tissue and life stage were also observed in *P. paradoxa* specimens. Mainly, CbE activity using 4-NPV was more abundant than when using α-NA in tadpoles and adults. Muscle showed the lowest CbE (α-NA and 4-NPV) activity of the studied liver, stomach and intestine of adults, whereas CbE activity was also lowest in muscle and coiling gut (CbE α-NA) and in muscle (CbE, 4-NPV) in tadpoles. The data obtained showed that CbE activity measured in tissues of *P. paradoxa* with α-NA substrate was of the same order of magnitude as the activity measured in the muscle of axolotl (*Ambystoma mexicanum*; Robles-Mendoza et al. 2011), the liver of juvenile rainbow trout (*Oncorhynchus mykiss*; Barron et al. 1999), in the liver and muscle of juveniles and adults of fish (*Solea senegalensis*; Solé et al. 2012) and in the gastrointestinal tract of invertebrate species such as earthworm (*Lumbricus terrestris*; Sánchez-Hernández et al. 2009).

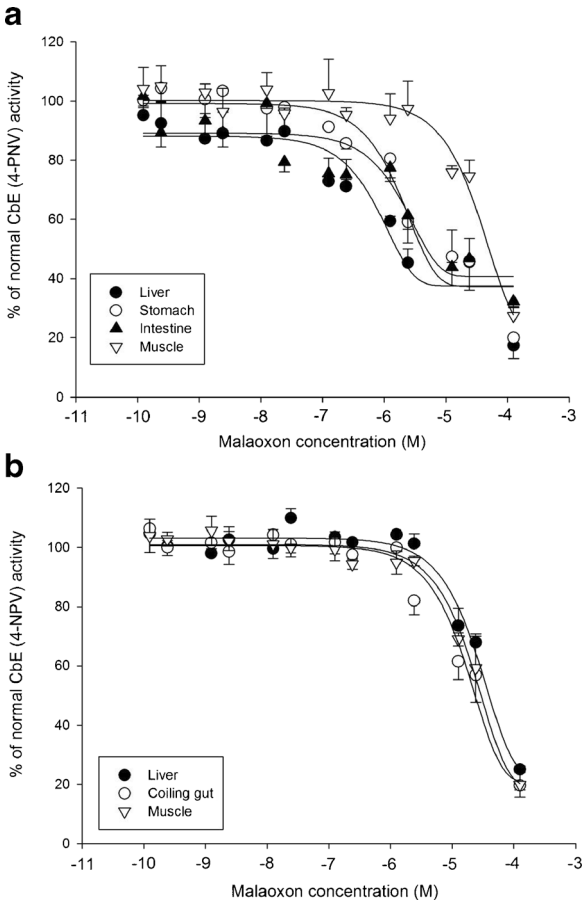

Fig. 3 Inhibitory effects of malaoxon on butyrylcholinesterase (CbE, 4-NPV) activity in vitro in adult (**a**) and tadpoles (**b**) of *Pseudis paradoxa*. Each point corresponds to the mean of three independent assays (±SE). See Table 2 for nonlinear regression statistics

These observations are consistent with current results, demonstrating that high CbE activities are present in the stomach and intestine of *P. paradoxa*. The esterases abundance in the gastrointestinal tract of *L. terrestris* can be explained by their likely involvement in the lipid metabolism. It could be expected that frog exposure to pesticides through consumption of arthropods present in agroecosystem (Attademo et al. 2005) would lead to a lower degree of toxicity than exposure to pesticides by dermal uptake, because the high CbE activity levels and isozyme abundance present in the intestinal and stomach tissues would contribute to a more effective OP detoxification.

Significant differences in ChE and CbE activities were found between tadpoles and adult frogs of *P. paradoxa* in the tissues studied. ChE activity increased in muscle and intestine/gut from tadpoles to adults. CbE measured using either α-NA or 4-PNV (all tissues considered) also showed a positive relationship with life stages. Previous studies also reported that CbE activity varies substantially during organism development. For example, Barron et al. (1999) found that CbE activity of rainbow trout (*O. mykiss*) was up to 12-fold higher in the juvenile and adult than in embryos. In rats, a significant variation in CbE activity using 4-NPA was observed in plasma, liver and lung of groups of different ages (Karanth and Pope 2000). Considering these studies, the increase in CbE activity between tadpoles and adults may be related to transition from feeding on an endogenous to an exogenous food source, or to differences in tissue composition of different life stages (e.g. effects of lipid content or composition on in vitro activity, Barron et al. 1999). Therefore, Phillips et al. (2002) argued that size and age do have an effect on cholinesterase activity in walleye fish.

In the other hand, we found a marked difference in the inhibition of ChE and CbE activities in tissue by Mx, depending on the esterase type and substrate. According to IC_{50} values, ChE was more sensitive than CbE activity in adults and tadpoles of *P. paradoxa.* Similar results were observed in tadpoles of *Scinax fuscovarius* (Leite et al. 2010), where ChE was completely inhibited at lower doses of diazinon (OP pesticide) compared to CbE. Hence, it is possible that events with OP exposure can affect ChE while CbE remains nonresponsive, which indicates ChE activity as a better OP biomarker than CbE in tadpole amphibians. In our study, the resistance of CbE to Mx with respect to ChE should not be taken as a rule for all pesticides. Vioque-Fernandez et al. (2007) observed that ChE was more resistant to chlorpyrifos than CbE in red crayfish, but malathion inhibited ChE completely, whereas no effects were observed in CbE. There are a number of potential causes for the differences observed in the sensitivity of both ChE and CbE to Mx among tissues and between adults and tadpoles. Oliveira et al. (2007) stated that pesticide sensitivity and substrate specificity might be dependent on different mechanisms across species and tissue. Species-specific differences in the sensitivity of B-esterase to inhibition by OP may be due to the affinity of the inhibitor for the active site of the enzyme, differences in the rate of esterase phosphorylation, and even the electrophilicity of the phosphorus P atom in the OP molecule. Among the potential factors influencing in vitro inhibition of esterase by pesticides are

Table 2 Molar concentrations of malaoxon causing 50 % of in vitro enzyme inhibition (IC_{50}) of cholinesterase (ChE) and carboxylesterase (CbE; substrates α-NA and 4-NPV) activities of adults and tadpoles of *P. paradoxa* and statistics for nonlinear regressions

Esterase	Stage	Tissue	Nonlinear regressions					
			Y_0	a	b	R^2	p	IC_{50} (M)
ChE	Adults	Liver	32.07	61.78	2.6×10^{6}	0.95	<0.0001	4.44×10^{-7}
		Stomach	49.14	46.38	5.8×10^{6}	0.97	<0.0001	7.72×10^{-7}
		Intestine	55.66	76.52	6.1×10^{9}	0.90	<0.0001	8.92×10^{-7}
		Muscle	50.10	33.09	7.2×10^{6}	0.95	<0.0001	5.44×10^{-7}
	Tadpoles	Liver	–362.7	444.5	5.7×10^{4}	0.90	<0.0001	1.29×10^{-6}
		Coiling gut	46.83	31.82	7.4×10^{7}	0.68	<0.0001	3.05×10^{-8}
		Muscle	–1.15	84.22	1.6×10^{6}	0.95	<0.0001	3.05×10^{-7}
CbE (α-NA)	Adults	Liver	24.61	83.62	3.3×10^{5}	0.98	<0.0001	3.62×10^{-6}
		Stomach	31.99	65.68	4.5×10^{5}	0.98	<0.0001	2.98×10^{-6}
		Intestine	–105.6	203.4	1.4×10^{4}	0.98	<0,0001	1.42×10^{-5}
		Muscle	–	90.47	4.5×10^{5}	0.86	<0.0001	1.27×10^{-6}
	Tadpoles	Liver	24.55	73.88	4.7×10^{5}	0.97	<0.0001	2.23×10^{-6}
		Coiling gut	37.06	60.37	2.4×10^{5}	0.98	<0.0001	6.14×10^{-6}
		Muscle	65.95	37.94	1.6×10^{6}	0.90	<0.0001	–
CbE (4-NPV)	Adults	Liver	37.56	50.59	8.7×10^{6}	0.88	<0.0001	1.63×10^{-6}
		Stomach	37.38	61.79	3.8×10^{5}	0.93	<0.0001	4.87×10^{-6}
		Intestine	40.73	48.47	3.4×10^{5}	0.87	<0.0001	4.16×10^{-6}
		Muscle	21.57	78.59	2.0×10^{4}	0.96	<0.0001	5.06×10^{-5}
	Tadpoles	Liver	22.61	80.61	2.7×10^{4}	0.97	<0.0001	3.85×10^{-5}
		Coiling gut	20.62	80.02	4.2×10^{4}	0.96	<0.0001	2.22×10^{-5}
		Muscle	18.64	83.02	3.3×10^{4}	0.99	<0.0001	2.93×10^{-5}

The statistics correspond to the exponential curves fitted to the effect of malaoxon concentration on B-esterase activity (see Figs. 1, 2, and 3)

temperature, pH, time of incubation and dilution of the enzyme in the reaction medium (Mortensen et al. 1998; Laguerre et al. 2009). Because there may be a pool of pesticide-metabolizing enzymes (e.g. CbE, gluthatione S-transferase and phosphotriesterases) in a crude homogenate, it is difficult to evaluate the sensitivity of ChE activity to OP pesticides separately only by measuring IC_{50} in tissue. Ideally, reliable IC_{50s} should be obtained from purified ChE or CbE activity from the frog tissue, but this was beyond the scope of this study. As Mortensen et al. (1998) pointed out, IC_{50s} data obtained from incubation of crude homogenates may not indicate the inherent sensitivity of the enzyme to the anti-cholinesterase pesticide, but they are an indicator of the capacity of the tissue or blood to sequester the circulating OP. In vitro IC_{50} values could be useful to determine tissue differences in buffering capacity and indirectly to detect what pesticides can be more toxic in real-life situations (Lajmanovich et al. 2008; Laguerre et al. 2009). Taking into account this assumption, OP could be highly toxic to *P. paradoxa* (adults and tadpoles) because of its high IC_{50s} (10^{-7} M) values for ChE activity, with respect to CbE, which exhibited low inhibitory potential using the α-NA and 4-NPA as substrates. Nevertheless, further experiments are needed to assess the IC_{50} with different pesticides and other amphibian tissues.

5 Conclusion

Overall, our results indicate that basal ChE and CbE (α-NA and 4-NPV) activities in different tissues in adult and tadpoles of *P. paradoxa* would be suitable biomarkers of pesticide exposure. Thus, two main conclusions can be drawn from the present results. Firstly, tissue esterase activity varied between life stages and among tissues of this frog species. Second, the in vitro

inhibition tests indicated that ChE is more sensitive to inhibition than CbE, suggesting that ChE is the best biomarker of susceptibility in this amphibian species. However, the effects of other pesticide classes on B-esterase activity should be investigated.

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