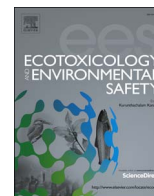Contents lists available at ScienceDirect

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Effect of diet on carboxylesterase activity of tadpoles (*Rhinella arenarum*) exposed to chlorpyrifos

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ABSTRACT

An outdoor microcosm was performed with tadpoles (*Rhinella arenarum*) exposed to 125 µg L^{-1} chlorpyrifos and fed two types of food, i.e., lettuce (*Lactuca sativa*) and a formulated commercial pellet. Acetylcholinesterase (AChE) and carboxylesterase (CbE) activities were measured in liver and intestine after 10 days of pesticide exposure. Non-exposed tadpoles fed lettuce had an intestinal AChE activity almost two-fold higher than that of pellet-fed tadpoles. No significant differences were observed, however, in liver AChE activity between diets. Likewise, intestinal CbE activity – measured using two substrates, i.e. 1-naphthyl acetate (1-NA) and 4-nitrophenyl valerate (4-NPV) – was higher in tadpoles fed lettuce than in those fed pellets. However, the diet-dependent response of liver CbE activity was opposite to that in the intestine. Chlorpyrifos caused a significant inhibition of both esterase activities, which was tissue- and diet-specific. The highest inhibition degree was found in the intestinal AChE and CbE activities of lettuce-fed tadpoles (42–78% of controls) compared with pellet-fed tadpoles (< 60%). Although chlorpyrifos significantly inhibited liver CbE activity of the group fed lettuce, this effect was not observed in the group fed pellets. In general, intestinal CbE activity was more sensitive to chlorpyrifos inhibition than AChE activity. This finding, together with the high levels of basal CbE activity found in the intestine, may be understood as a detoxification system able to reduce intestinal OP uptake. Moreover, the results of this study suggest that diet is a determinant factor in toxicity testing with tadpoles to assess OP toxicity, because it modulates levels of this potential detoxifying enzyme activity.

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1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) and carboxylesterase (CbE, EC 3.1.1.1) are two serine hydrolases actively involved in the toxicity of organophosphorus (OP) pesticides (Thompson and Richardson, 2004). The former is the main molecular target in the mechanism of acute toxicity of OPs, particularly when the 'enzyme-pesticide' interaction takes place in the nervous system (Fukuto, 1990), whereas CbE activity detoxifies OP compounds by phosphorylation of serine residues in the active site of CbEs (Sogorb and Vilanova, 2002). Nevertheless, the affinity of OPs for the active centre of AChEs or CbEs increases several orders of magnitude with the oxon-analog metabolites of OPs (Chambers et al., 2010). Many studies including a wide range of organisms have evidenced that CbE activity displays a higher sensitivity to inhibition by OPs than AChE activity (reviewed in Wheelock et al., 2008), which has led some authors to postulate that CbE activity plays a protective role against OP toxicity, acting as a molecular sink.

Both AChE and CbE activities have been long used as biomarkers of OP exposure in wildlife and laboratory toxicity testing (Nunes, 2010; Wheelock et al., 2008). In this context, some studies have examined the impact of biological variables on baseline activity of cholinesterases (ChEs) and CbEs in non-exposed organisms. In general, body size and diet have a significant impact in the inter-specific variations of plasma ChE and CbE activities in birds (Goldstein et al., 1999; Sogorb et al., 2007; Narvaez et al., 2015). For example, Roy et al. (2005) examined intra- and interspecific variations of plasma ChE and CbE activities in 729 European raptors covering 20 species. They found that normal activity of plasma esterases in some of the studied species varied with sex, age and body size. Likewise, sex-related differences in blood ChE activity were reported for the Australian agamid *Pogona vitticeps* (Bain

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et al., 2004), and the tegu lizard *Tupinambis merianae* (Bassó et al., 2012). In birds, a relationship was determined between diet preference and CbE activity levels; esterase activity was frequently found to be higher in omnivores than in carnivores (Thompson et al., 1991). Similarly, an inverse relationship was observed between body size and plasma esterase activities in birds (Roy et al., 2005) and amphibians (Lajmanovich et al., 2008). Dietary lipids are also important inducers of CbE activity in mammals (van Lith et al., 1992), passerine birds (Ríos et al., 2014), and some invertebrate species (Mommsen, 1978; Prento, 1987); accordingly, this esterase activity seems to be actively involved in lipid metabolism.

To our knowledge, there are no data on the response of CbE and AChE activities to OP exposure in animals fed different diets. Because CbE activity seems to have a physiological role in lipid metabolism (Ross and Edelmann, 2012), we hypothesized that lipid-rich diets would cause an increase of CbE activity, resulting in a more efficient detoxification capability against OP exposure. Therefore, the aims of this study were 1) compare CbE activity in the intestinal tissue and liver of tadpoles fed lettuce (*Lactuca sativa*) and commercial pellets, and 2) to examine whether the induction of CbE activity was a mechanism of detoxification in animals exposed to chlorpyrifos. For this purpose, AChE activity was included in this study as an additional molecular target of OP toxicity. We selected tadpoles as test organisms because they are sensitive indicators of ecosystem deterioration by environmental pollutants (Waddle, 2006), given their permeable skin, unshelled (not amniotic) eggs, and a complex life cycle that exposes them to changes in both aquatic and terrestrial environments (Blaustein et al., 1994). Accordingly, some ecotoxicological studies with amphibians have suggested the measurement of certain enzymes involved in the mechanism of contaminant toxicity as important biomarkers to detect potential adverse effects of pesticides (Ossana et al., 2013; Robles-Mendoza et al., 2011; Güngördü and Uçkun, 2015; Güngördü et al., 2016). We expect that our results will provide valuable information about the impact of diet on OP toxicity, which may be of concern in regulatory toxicology (toxicity testing and ecological risk assessment framework).

2. Materials and methods

2.1. Test species

Rhinella arenarum tadpoles were used as model test organisms. This anuran species has an extensive neotropical distribution (Cei, 1980; IUCN, 2010), occurring in forests, wetlands, agricultural lands, and urban territories (Attademo et al., 2005). In Argentina, this toad species is categorized as "not threatened" (Vaira et al., 2012) and is widely distributed in the provinces of Buenos Aires, Formosa, Chaco, Corrientes, Santiago del Estero, Entre Ríos, and Santa Fe. Eggs were collected from temporary ponds in natural floodplains of the Paraná River (31° 11′ 31″ S, 60° 9′ 29″ W, Argentina) with authorization of the Ministerio de Aguas, Servicios Públicos y Medio Ambiente (Santa Fe Province, Argentina). This site has not undergone pollution episodes nor has it been treated with chemical or biological pesticides (Attademo et al., 2014, 2015). The eggs were transported to the laboratory and maintained in dechlorinated tap water (pH$=7.4 \pm 0.05$; conductivity$=165 \pm 12.5$ µmhos cm^{-1}; dissolved oxygen$=6.5 \pm 1.5$ mg L^{-1} hardness$=50.6$ mg L^{-1} CaCO$_3$ at 22 ± 2 °C). They were allowed to develop until tadpoles reached Gosner stage 26–27 (Gosner, 1960; approximately 5 days). A total of 32 tadpoles (total length$=16.67 \pm 1.08$ mm, weight$=0.054 \pm 0.01$ g) were used in this study.

2.2. Experimental design

An in vivo toxicity assay was performed in outdoor microcosms located at the Facultad de Bioquímica y Ciencias Biológicas (Universidad Nacional del Litoral, Santa Fe, Argentina), under an environmental temperature regime (24–26 °C), and a natural light/dark cycle (austral summer). Microcosms consisted of 20-L fishbowl enclosures covered with iron frames and fitted with 2-mm mesh to avoid predation or oviposition by insects or other anuran colonists. Treatment groups (n$=8$ individuals per treatment) were: 1) tadpoles fed lettuce, 2) tadpoles fed pellets, 3) tadpoles fed lettuce and exposed to chlorpyrifos, and 4) tadpoles fed pellets and exposed to chlorpyrifos. Commercial pellets (Vita*Fish* brand, Santa Fe, Argentina) were composed of 44% proteins, 13.4% lipids, 15.2% minerals, 5.3% Ca, and 2.2% P. Lettuce was obtained from a local market; according to data from the USDA National Nutrient Database for Standard Reference (https://ndb.nal.usda.gov/ndb/foods) lettuce is composed of 1.36% proteins, 0.15% lipids, 3.0% minerals, 0.036% Ca, and 0.029% P. Edible portions of lettuce were boiled for a few minutes before use. Both food types were added to microcosms every two days. The amount of food was calculated following mass specific rations provided by Alford and Harris (1988). The OP pesticide used in this study was an emulsified formulation of chlorpyrifos named Tauro® LO (48% w/v chlorpyrifos) at a nominal concentration of 125 µg L^{-1}. This chlorpyrifos concentration was chosen because it is often included in the range of test concentrations in toxicity testing (Vera Candioti et al., 2014), although it was high compared with concentrations detected in streams receiving pesticides from soy-bean crops (Jergentz et al., 2005; Bonansea et al., 2013). Although we are aware that this pesticide concentration is unlikely to occur in surface waters receiving chlorpyrifos input by runoff, it might represent the worst-case scenario (e.g., flooding of agricultural areas or cumulative concentration after consecutive short-term pesticide applications). Tadpoles (5 d-old larvae, Gosner stage 33–35) were exposed to the pesticide and diet regimes for 10 days. Water was replaced with freshly prepared chlorpyrifos solution at the middle of the toxicity assay because of rapid degradation of chlorpyrifos in outdoor macrocosm (Mazanti et al., 2003).

Control and treated larvae were killed according to criteria of ASIH et al. (2011), and with the approval of the animal bioethics committee of the Faculty of Biochemistry and Biological Sciences (Acta 03/12). Each larva was immediately dissected along the midventral line, and digestive organs (intestine and liver) were carefully removed, washed in distilled water and placed on filter paper to remove excess fluids. Tissue samples were stored at −80 °C until biochemical analyses.

2.3. Enzyme assays

Intestine and liver were homogenized (20% w/v) on ice-cold 25 mM Tris-HCl buffer (pH$=8.0$) containing 0.1 Triton X-100 and using a polytron. The homogenates were centrifuged at 10,000 rpm at 4 °C for 15 min, and the supernatants were collected for enzyme assays. AChE activity was determined colorimetrically according to Ellman et al. (1961). The reaction mixture (final volume$=930$ µl) consisted of 25 mM Tris-HCl buffer containing 1 mM CaCl$_2$ (pH$=7.6$), 10 µl 20 mM acetylthiocholine iodide (AcSCh), 50 µl 300 µM DTNB, and 20 µl sample. Variation in optical density was measured in duplicate at 410 nm and 25 °C for 1 min using a Jenway 6405 UV–VIS spectrophotometer. Total protein concentration was determined using the Biuret method (Kingbley, 1942), and AChE activity was expressed in nmol of AcSCh hydrolyzed per minute and milligram of protein using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

Carboxylesterase activity was measured using two different substrates: 1-naphthyl acetate (1-NA) and 4-nitrophenyl valerate (4-NPV). We used two substrates because the multiple isoforms generally occurring in a single tissue show a marked difference in substrate specificity and sensitivity to OP inhibition (Wheelock et al., 2008). The hydrolysis of 1-NA was determined according to Gomori (1953) and adapted by Bunyan and Jennings (1968). The reaction medium (1940 μl) consisted of 25 mM Tris-HCl containing 1 mM $CaCl_2$ (pH=7.6), and 10 μl the supernatant. After a 5-min preincubation period, the reaction was initiated by adding 50 μl of 1-NA (46 μM, in acetone) and incubated at 25 °C for 10 min. The formation rate of naphthol was stopped by adding 500 μl of 2.5% (w/v) SDS and subsequently 0.1% (w/v) of Fast Red ITR dissolved in 2.5% (w/v) Triton X-100. The samples were left in the dark for 30 min for colour development. The absorbance of the naphthol-Fast Red ITR complex was read at 530 nm (using a molar extinction coefficient of $33.225 \times 10^3 \, M^{-1} \, cm^{-1}$). Hydrolysis of 4-NPV was performed following the methods of Carr and Chambers (1991). Samples were preincubated in 50 Mm Tris–HCl (pH=7.5) at 25 °C for 5 min (F.V.=1975 μl), and the reaction was initiated by adding 20 μl 4-NPV (5×10^{-4} M, final concentration) and 5 μl of supernatant. 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/ml).

2.4. Data analysis

The effect of diet and chlorpyrifos on the activity of both esterases was assessed via the non-parametric Mann-Whitney U test. Statistical analyses were performed using the SPSS Statistic software version 21 (IBM® Software, USA). Regression analysis between esterase activities was performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, California, USA).

3. Results

There were no significant differences in total length and body weight between treatment groups (P > 0.05). Mean ($\pm$ SE) total length and weight were 24.70 ± 0.70 mm and 0.19 ± 0.02 g for lettuce-fed control tadpoles; 25.58 ± 0.27 mm and 0.22 ± 0.02 g for pellet-fed control tadpoles; 26.35 ± 0.54 mm and 0.16 ± 0.02 g lettuce- fed tadpoles exposed to chlorpyrifos, and 25.62 ± 0.57 mm and 0.17 ± 0.01 g for pellet-fed tadpoles exposed to chlorpyrifos.

3.1. Acetylcholinesterase activity

Mean ($\pm$ SD, n=8) AChE activity in the treatment groups is shown in Fig. 1. In non-exposed tadpoles, intestinal AChE activity ranged between 2.62 and 12.6 nmol min^{-1} mg^{-1} protein (n=16 individuals), whereas liver AChE activity varied between 11.1 and 24.3 nmol min^{-1} mg^{-1} protein (n=16). Diet had a significant impact on intestinal AChE activity (p=0.002, Mann-Whitney test), but this esterase activity did not change in the liver (p=0.28). Intestinal AChE activity was almost two-fold higher in tadpoles fed lettuce than in those receiving pellets (Fig. 1).

As expected, chlorpyrifos caused a significant inhibition of AChE activity, with the magnitude of this response being highly dependent on target tissue and diet. The hydrolytic activity of this esterase was strongly depressed in liver, and lettuce-fed tadpoles had a higher percentage of AChE inhibition (64% compared with controls) than pellet-fed tadpoles (43%). However, intestinal AChE activity was significantly inhibited (p=0.001, Mann-Whitney test) only in lettuce-fed tadpoles (Fig. 1).

3.2. Carboxylesterase activity

This esterase activity showed a comparable hydrolysis rate between substrates as there was a significant ($p < 0.0001$, $r^2 > 0.65$) linear relationship (Fig. 2). Nevertheless, CbE activity hydrolyzed preferentially the nitrophenyl ester (33.2–158 and 102–533 nmol min^{-1} mg^{-1} protein for intestine and liver, respectively; min-max values, n=16) compared with the naphthyl ester (7.72–39.2 nmol min^{-1} mg^{-1} protein for intestine, and 15.6–54.5 nmol min^{-1} mg^{-1} protein for liver; min-max values, n=16). Diet also had a significant effect on CbE activity, which was tissue-specific (Fig. 3). In pellet-fed tadpoles, intestinal CbE activity decreased in the pesticide-free group ($p < 0.001$), but increased significantly in the liver (p=0.028 for 1-NA and $p < 0.001$ for 4-NPV).

Chlorpyrifos caused a significant inhibition (46–78% of controls) of both intestinal and liver CbE activities, except for the liver CbE activity of pellet-fed tapoles (Fig. 3). The highest effect of this pesticide on enzyme activity was observed in the tadpoles fed lettuce, irrespectively of the tissue. Indeed, liver CbE activity did not differ significantly between control and chlorpyrifos-exposed animals that received fed pellets (p=0.083 for 1-NA hydrolysis, and p=0.105 for 4-NPV hydrolysis).

4. Discussion

Basal levels of AChE and CbE activities in non-exposed tadpoles were similar to those reported in the literature. For example, mean liver AChE activity of *Pseudis paradoxa* tadpoles was 16.57 ± 1.39 nmol min^{-1} mg^{-1} protein (n=8) and CbE (1-NA=6.03 ± 0.60 and 4-NPV=44.46 ± 6.63 nmol min^{-1} mg^{-1} protein), and AChE had a mean activity of 5.16 ± 0.75 nmol min^{-1} mg^{-1} protein and CbE (1-NA=1.89 ± 0.13 and 4-NPV=81.50 ± 6.27 nmol min^{-1} mg^{-1} protein) in the gastrointestinal tissue (Attademo et al., 2014). Similarly, in *Leptodactylus latrans* adults, liver AChE activity showed a mean of 6 nmol min^{-1} mg^{-1} protein (n=14) (Brodeur et al., 2011). The present results also showed a higher hydrolytic activity towards 4-NPV than that of naphthyl ester in both tissues. Furthermore, the activity of CbE in chlorpyrifos-exposed tadpoles responded differently according to the substrate used in the enzyme assay. These results are consistent with previous results that describe the occurrence of multiple CbE isozymes in many tissues, with a marked isozyme-specific substrate preference and an isozyme-specific sensitivity to OP exposure (Satoh and Hosokawa, 1998; Sanchez-Hernandez and Wheelock, 2009; Kristoff et al., 2010; de Lima et al., 2013). Pending of confirmation using electrophoretic proves, these findings suggest a different abundance of CbE isozymes in the intestine and liver of *R. arenarum* tadpoles, and probably also in both diets.

The present data showed that non-exposed tadpoles fed a lettuce diet had a higher intestinal CbE activity than those fed commercial pellets; the opposite response, however, was observed in the liver. These results leave open the hypothesis that lipid-rich diets induce CbE activity because of its role in the metabolism of short-chain fatty acids (Yan, 2012; Ross and Edelmann, 2012). Indeed, some studies suggest that plant-rich diets may contain CbE-inducer compounds (plant secondary metabolites) that account for a higher CbE activity compared with other dietary categories (Thompson, 1999; Roy et al., 2005; Narvaez et al., 2015). Our results of intestinal CbE activity would support this hypothesis, although such a relationship seems to be tissue dependent.

It is well known that the capability of CbE activity to function as a detoxifying enzyme is largely attributed to its higher reactivity to OPs compared with other serine hydrolases (Yan, 2012). This stoichiometric reaction leads to a reduction of the number of OP
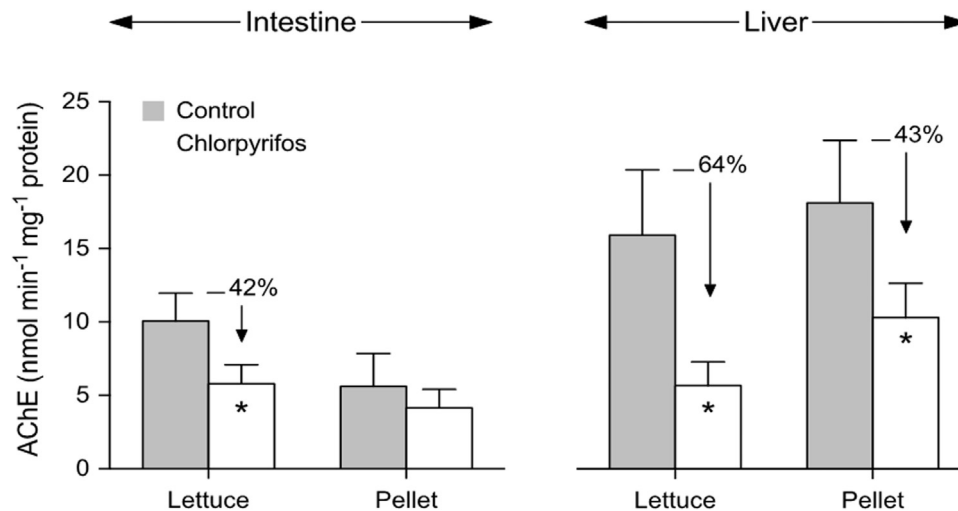
Fig. 1. Intestinal and liver acetylcholinesterase (AChE) activity (mean ± standard deviation, n=8) in *R. arenarum* tadpoles exposed to chlorpyrifos (125 µg L^{-1}) for 10 d and fed two types of diet (lettuce and pellet). Asterisks denote significant differences between treated groups and their corresponding controls ($P < 0.05$, Mann–Whitney test); percentages above bars indicate the associated inhibition percentage of AChE activity.
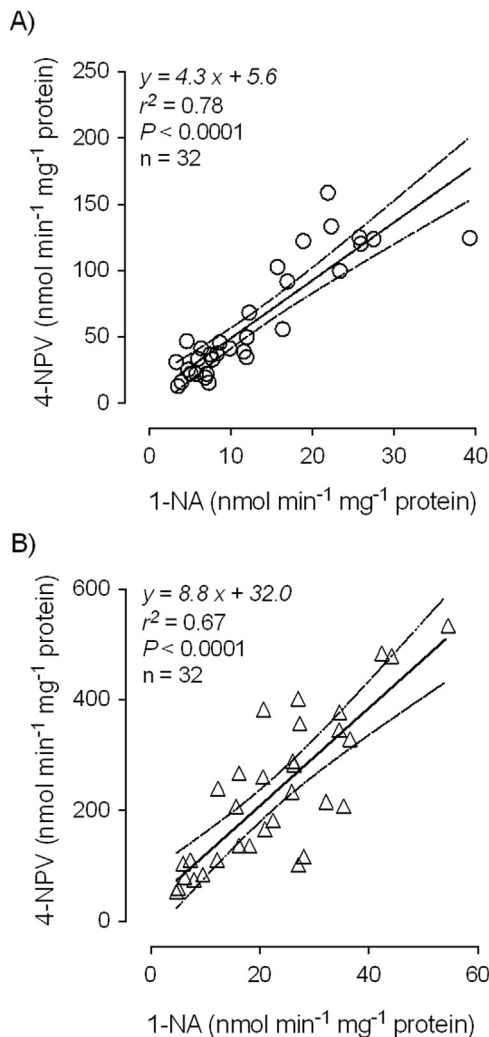

Fig. 2. Relationship between hydrolysis rates of 1-naphthyl acetate (1-NA) and 4-nitrophenyl valerate (4-NPV) in the intestine (A) and liver (B) of non-exposed tadpoles (*R. arenarum*).

molecules able to interact with other esterases, such as AChE. This protective role of CbE activity was described by Chanda et al. (1997) in an in vitro study using homogenates of nervous and hepatic tissues of male and female rats. The authors found that the higher level of CbE activity in the male liver than in female liver was enough to limit the access to brain AChE activity of male rat. Our results with the intestinal tissue corroborate the protective role of CbE activity postulated by Chanda et al. (1997), because AChE activity was less inhibited by chlorpyrifos exposure than CbE activity, irrespective of the tadpole diet. This finding suggests that, as the gastrointestinal tract of tadpoles is a major route of contaminant uptake, levels of CbE activity in this tissue should contribute to a reduction in OP uptake.

Therefore, if we assume that this enzymatic barrier is efficient against intestinal absorption of chlorpyrifos, then the liver CbE inhibition to chlorpyrifos should be lower than that of the intestine. In fact, our findings support this assumption because a lower effect of chlorpyrifos on liver CbE activity was observed, which was more evident in pellet-fed tadpoles. However, the strongly inhibited liver AChE activity compared with liver CbE activity found was an unexpected result. A plausible reason for this observation may be role of liver in the bioactivation of OP compounds. It is well known that most OP pesticides, such as chlorpyrifos, are metabolized to their "oxon" analog metabolites, thereby gaining affinity for the active site of esterases. This bioactivation or oxidative desulfuration reaction is mainly catalyzed by the liver cytochrome P450-dependent monooxygenases (Hodgson, 2010). Therefore, it is probable that a higher conversion rate of chlorpyrifos into chlorpyrifos-oxon in the liver compared with intestine would account for the high inhibition degree of AChE activity in this organ. Another factor that could contribute to this discrepancy between liver and intestine esterase responses is the likely tissue-specific difference in the abundance of CbE isozymes. Some studies with mammals and invertebrates have evidenced that CbE inhibition by OPs was dependent on the isozyme-specific sensitivity (Chanda et al., 1997; Sanchez-Hernandez and Wheelock, 2009; Kristoff et al., 2010; de Lima et al., 2013). Moreover, a resistant fraction of CbE activity is often observed, thereby indicating that this detoxification system may be saturated, depending not only on the number of CbE molecules, but also on the abundance of isoforms resistant to OP inhibition.

In the last decade, there has been a growing concern on how intestinal microfloras participate in the xenobiotic metabolism
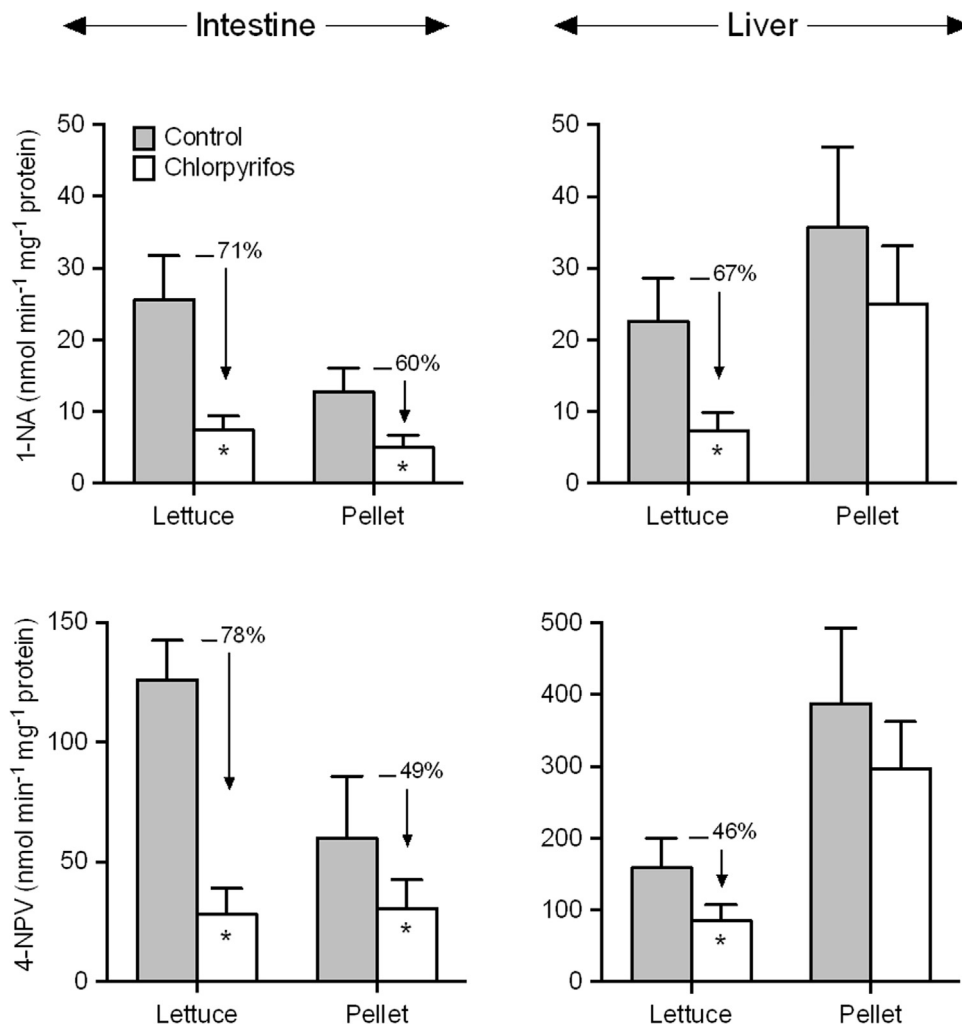
Fig. 3. Intestinal and liver carboxylesterase activity (mean ± standard deviation, n=8) in *R. arenarum* tadpoles exposed to chlorpyrifos (125 µg L^{-1}) for 10 d and fed two types of diet (lettuce and pellet). Asterisks and percentage values above bars as in Fig. 1. 1-NA=1-naphthyl acetate, 4-NPV=4-nitrophenyl valerate.

(Jeong et al., 2013). Furthermore, intestinal microbiota may modulate gene expression of detoxifying enzymes at the hepatic level (Björkholm et al., 2009). Our data on intestinal esterase activities in chlorpyrifos-exposed tadpoles feeding on two different diets leave open the possibility of exploring the role of gut microbiota in OP bioactivation and detoxification in this class of vertebrates with drastic changes in their life style during development. Embryos and larval stages of amphibians are commonly used in laboratory toxicity testing to evaluate exposure and toxicity of aquatic contaminants (Linder et al., 2010). The diet of anuran tadpoles is usually diverse, including plant fragments, protozoa, rotifers, anuran eggs, and even other tadpoles, although most of them are primarily herbivorous, consuming a wide variety of algal taxa (Altig et al., 2007). *R. arenarum* tadpoles are classified under the Ranoids group (McDiarmid and Altig, 1999) and are benthic, raspers and herbivorous/detritivorous, with algae and plants being their primary food items in their natural habitat (Lajmanovich and Fernandez, 1995). Because *R. arenarum* tadpoles are omnivorous, diet should be an important factor in contaminant exposure, metabolism and disposition. Indeed, our results reveal that lettuce diet not only increased intestinal CbE activity, but also showed higher sensitivity to chlorpyrifos than esterase in tadpoles fed pellets. This diet-dependent induction of intestinal CbE activity means that plants as food items provide a higher number of molecular binding sites for chlorpyrifos-oxon, therefore reducing its further distribution in the organism and toxicity.

5. Conclusions

Tadpoles of *R. arenarum* fed two different diets exhibited a marked difference in both intestinal and liver esterase activities. The diet based on plant fragments produced higher intestinal esterase activity than a protein-rich diet (pellet); the opposite response was observed in the liver. The presence of secondary metabolites such as carotenoids, R-tocopherols, glutathione, flavonols and phenoloic compounds (Sobolev et al., 2005) in lettuce might be a major CbE inducer in the intestine of tadpoles. Exposure to chlorpyrifos showed that intestinal CbE activity was more sensitive to inhibition by this OP pesticide than AChE activity. Moreover, the lower effect of chlorpyrifos on liver CbE activity suggests that the gastrointestinal tract could play a significant role in reducing chlorpyrifos uptake and toxicity by binding of highly toxic metabolite chlorpyrifos-oxon to the active site of CbEs. Although our studies in this area of pesticide toxicology with amphibians are still in progress, they seem to support that the gastrointestinal tract of tadpoles provides an active microenvironment for xenobiotic metabolism, which greatly should dependent on diet. Accordingly, the capability of intestinal CbE activity to display phenotypic plasticity linked to dietary shifting is an interesting area of future work in amphibian ecotoxicology.

Acknowledgements

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