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Effects of Dietary Pb and Cd and their Combination on Acetyl Cholinesterase Activity in Digestive Gland and Foot of the Green Garden Snail, *Cantareus apertus* (Born, 1778)

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ABSTRACT: The present study was focused upon the assessment of acetyl-cholinesterase (AChE) activity in the digestive gland (main metabolic center) and foot (highly innervated organ) of the green garden snail, *Cantareus apertus* (Born, 1778), exposed to different nominal dietary concentrations of Pb (25 and 2500 mg Pb/Kg), Cd (10 and 100 mg Cd/Kg) and their combination (25 mg Pb + 10 mg Cd/Kg and 2500 mg Pb + 100 mg Cd/ Kg) for 60 days. AChE activity was lower in the foot than in the digestive gland (~50%) and decreased with experimental time in both tissues. In metal treated snails, AChE activity was significantly decreased in both tissues to a 50-60% of the values recorded in control snails. This decrease followed a dose depending trend at each exposure time, albeit the response was attenuated at the long-term (60 d) in comparison with the short-term (7 d). Besides, the combination of both metals provoked interactive effects not seemingly related with the tissue levels of the metals. Thus, it was concluded that model toxic metals such as Pd and Cd cause a reduction in AChE activity in both studied tissues, more markedly at the short-term, although antagonistic effects were elicited by both metals in combination. As a whole, lowered AChE activity in *C. apertus* can be considered as a useful biomarker of the effects provoked by metals on cell signaling and therefore it may be suitable for ecosystem health assessment in metal polluted soils using this species as sentinel organism.

Key words: *Cantareus apertus*, Metals, Mixture, Biomarker, Neurotoxicity, AChE, Pollution biomonitoring

INTRODUCTION

The potential use of cholinesterase activity in sentinel species for monitoring both, environmental quality and the health of organisms inhabiting polluted ecosystems has received increasing attention during the recent years. Cholinesterases are widely distributed enzymes in microorganisms, protozoa and animals. In vertebrates, acetyl cholinesterases (AChE) and butyryl cholinesterases (BuChE) are distinguished; most invertebrates, however, have only AChEs (Massoulie *et al.* 1993). AChEs have been extensively studied because they play an important role in the transmission of nervous influx and are specific target for drugs and insecticides. The inhibition of AChE activity by neurotoxic chemicals causes accumulation of the transmitter (acetylcholine) in the synaptic space, which leads to the permanent transmission of the nerve impulse and may result fatal (Bocquene *et al.* 1996). BuChE is a soluble enzyme observed at high concentrations in plasma, liver, lung and intestine that serves as an effective sequestering agent against toxins or toxicants before they arrive at their target sites (Massoulie *et al.* 1993).

Some invertebrate AChEs have classical neuronal function of synaptic transmission whereas others appear to have a non-neuronal function of chemical defense, analogous to that of vertebrate BuChEs, being involved also in the modulation of the immune

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response through catalyzing the hydrolysis of acetylcholine (Kang *et al.* 201; Shi *et al.* 2012). AChEs, of variable amino acid sequences, and AChE mRNA transcripts have been so far identified in a variety of non-neural tissues of invertebrates (e.g., gills, digestive gland, mantle and blood in molluscs) and specific acetylcholine receptors are found in neurons but also in haemocytes (Moore, 1991; Kang *et al.* 2011; Shi *et al.* 2012). Moreover, in a marine gastropod, it has been reported that a AChE localised in the foot shares functional properties of both neuronal AChEs and soluble BuChE (Cunha *et al.* 2007).

Against this background, AChE inhibition in sentinel species, both in invertebrates and vertebrates, has been employed as a biomarker to detect environmental exposure to chemicals such as organophosphates, carbamates, detergents and metals (e.g., Cd, Cu, Zn and Hg) (Bocquene *et al.* 1990; 1993; 1995; Schmidt and Ibrahim 1994; Guilhermino *et al.* 1998; Payne *et al.* 1996; Najimi *et al.* 1997; Dellai *et al.* 2001; Diamantino *et al.* 2003; Romeo *et al.* 2003; Panda and Sahu 2004; Frasco *et al.* 2005; Matozzo *et al.* 2005; Gambi *et al.* 2007; Gagnaire *et al.* 2008; Itziou and Dimitriadis, 2011). Nevertheless, it is worth noting that the concentration of metals required to cause a significant AChE inhibition is considerably higher than the effective concentration of organophosphates or carbamates (Cunha *et al.* 2007).

Terrestrial gastropods have been proposed as biomonitors and sentinels in soil pollution monitoring programmes (Berger and Dallinger 1993; Marigómez et al. 1998; Piham *et al.* 2000; Regoli *et al.* 2006; Radwan *et al.* 2010; Itziou and Dimitriadis 2011; Abdel-Halim *et al.* 2013; Larba and Soltani 2014). Amongst them, the green garden snail, *Cantareus apertus* has been proposed as potential biomonitor and sentinel species for metal pollution monitoring in Tunisia and neighboring regions (Mleiki *et al.* in press). Slugs and snails accumulate metals mainly in the digestive gland which also highly responsive to determine the severity of the biological effects exerted by pollutants (Marigómez *et al.* 1998; 2002; Mleiki *et al.*in press).

Lead and Cd are relevant pollutants in soils that represent different models of metal toxicity. Chronic exposure to low-levels of Pb, which is recognised as a soil contaminant at global scale, may results in adverse neurotoxic effects (Markus and McBratney 2001) but toxic effects other than neurotoxicity are produced only at high exposure concentrations (Marigómez *et al.* 1986). In contrast, Cd exhibits both acute and chronic toxicity at very low exposure levels but it is not necessarily directly related to neurotoxic effects (NCM-WHO 2003).

In this context, the present contribution was aimed: (a) at determining whether model toxic metals such as Pd and Cd elicit changes in AChE activity in the digestive gland (main metabolic center) and the foot (highly innervated organ) of snails, *C. apertus*; (b) at elucidating whether the exposure to the combination of both metals provokes interactive effects on this enzyme activity; and (c) at comparing the short-term response (7 d exposure) with the chronic response (60 d exposure).

MATERIALS & METHODS

Adult snails, *Cantareus apertus* (weight: 1.56±0.18 g; shell height: 18.9±0.23 mm), were collected from a pristine area in Tabarka (37:13:16.05N, 9:56:04.58E; Tunisia) and acclimated to laboratory conditions for 7 d before starting the experiment. Then, snails were left without food supply for additional 2 days in order to void their digestive tracts. During the experiments, snails (N=90) were maintained in transparent polystyrene boxes (25×20×10 cm) with perforated lid, under optimal laboratory conditions (light:dark-photoperiod = 14h:10h; temperature= 20±2°C, humidity= 85-95%) and fed artificial food (mixture of wheat flour, barley, cabbage and calcium carbonate) *ad libitum*. After daily examination, food was supplied as required and the boxes were carefully cleaned to remove mucus and feces. Seven experimental groups, including one control, were established. Metal solutions were prepared using cadmium chloride ($CdCl_2$) and lead nitrate ($PbNO_3$). Treated groups were exposed to two different concentrations of Pb and Cd and their combination during 7 and 60 d. The exposure concentrations were selected based on earlier studies on *Cantareus aspersus* (syn. *Helix aspersa*) carried out by Laskowski and Hopkins (1996): 25 and 2500 mg Pb/Kg food dry-wt; 5 and 100 mg Cd/Kg food dry-wt; and 25 mg Pb + 5 mg Cd/Kg food dry-wt and 2500 mg Pb + 100 mg Cd/ Kg food dry-wt. For all metal treatments, as well as for controls, three replicates were used. Before the treatment, snails were left for another 2 d without food to void their digestive tracts. The quantitative analysis of Pb and Cd in the food as well as in digestive gland tissue and foot was performed in a parallel study by using ICP-MS, detailed by Mleiki et al. (in prep); through which it was confirmed that the concentrations of the metals (in mg metal/Kg food dry-wt) measured in food reasonably approached the nominal ones: 9.2±2.3 and

94.7±9.3 for 5 and 100 mg Cd/Kg food dry-wt, and 24.7±2.7 and 2491.1±24.2 for 25 and 2500 mg Pb/Kg food dry-wt, respectively (LOD_{Cd}=0.02 mg/L; LOD_{Pb}=0.05 mg/L).

Snails were dissected and digestive gland and foot were homogenized with ultra-turrax in TRIS buffer (50 mM Tris, 150 mM NaCl, pH 7.4). The homogenates were centrifuged at 9000 ×g for 30 min, The resulting supernatants were frozen at -80°C until the biochemical analysis was performed. All procedures were carried at 4°C.

AChE activity was spectrophotometrically determined according to and adaptation of the Ellman's method (Ellman *et al.* 1961) to microwell plates. The increase in absorbance at 405 nm was measured in presence of 1 mM acetylthiocholine as substrate and 0.1 mM 5,5,-dithiobis-2-dinitrobenzoic acid (DTNB) as chromogen. The enzyme reaction rate was conducted against a blank without substrate for each batch of homogenates. Each AChE activity measurement was performed in triplicate and expressed in nmol acetylcholine hydrolysed per min per mg of protein, using a molar extinction coefficient of 13.6 M-1 cm-1. Protein content in the homogenates was estimated by the Bradford's method (Bradford 1976) using bovine serum albumin as standard.

Analysis of variance (ANOVA) was performed using the software STATISTICA® 8.0. After testing ANOVA assumptions, significant differences between groups were analyzed by one way ANOVA and Tukey's HSD test. A probability level of less than 0.05 was considered significant (95% confidence interval).

RESULTS & DISCUSSIONS

AChE activity was lower in the foot tissue than in the digestive gland tissue (~50%), and in both cases decreased after 60 d experimentation to a ~30% of the enzyme activity values recorded at Day 7 (Figs. 1 and 2). AChE activity was inhibited in both tissues after exposure to Pb, Cd and their combination and the degree of inhibition was different depending on the treatment and the exposure time (Figs. 1 and 2).

AChE activity dropped dramatically in the digestive gland of snails exposed to 25 mg Pb/Kg food dry-wt (~60%) for 7 d and little far beyond at higher dietary concentrations of the metal, although all the Pb treatment groups differed amongst them and in comparison with the control group (Fig. 1A). Likewise, AChE activity was significantly reduced at increasing dietary concentrations of Pb after 60 d treatment, the range of differences between groups being attenuated in comparison with those recorded after 7 d treatment (Fig. 1 B). Yet, the enzyme activity was further reduced in snails treated with 2500 mg Pb/Kg food dry-wt (Fig. 1D). AChE activity was markedly reduced in snails exposed to 5 mg Cd/Kg food dry-wt for 7 d and reached low values at a dietary concentration of 10 mg Cd/Kg food dry-wt, which were comparable to those recorded with 25 mg Pb/Kg food dry-wt (Fig. 1C). Like in the case of dietary Pb, the dose-dependent inhibition in AChE activity was somehow attenuated after 60 d Cd treatment; the enzyme activity being further reduced only in snails treated with 100 mg Cd/Kg food dry-wt (Fig. 1D). The combination of both metals also caused AChE activity inhibition in the digestive gland, especially at Day 7; however, the effect of the mixture of 25 mg Pb/Kg food dry-wt and 5 mg Cd/Kg food dry-wt was lower than the effect exerted by these two concentrations of metals separately (Fig. 1 E). As above mentioned for the case of the treatment with individual metals, the dose dependent inhibition in AChE activity was somehow attenuated in the case of the treatment with the mixtures of Pb and Cd after 60 d. In this case, further inhibition of the enzyme activity was recorded only in snails treated with the mixture of 2500 mg Pb/Kg food dry-wt and 100 mg Cd/Kg food dry-wt (Fig. 1D).

Overall, AChE activity was also inhibited in the foot tissue of snails treated with Pb, Cd and their combination, but the degree of the response was less marked than in the case of the digestive gland (Fig. 2). The levels of AChE activity recorded in the foot of Pb-treated snails were not dissimilar to those recorded in the digestive gland (Fig. 2A). A dietary dose of 25 mg Pb/Kg food dry-wt provoked a significant inhibition of this enzyme activity (~50%), which was not more marked at higher dietary concentrations of the metal (all the Pb treatment groups differed with the control group; Fig. 2A). Moreover, although a certain trend to decrease at increasing dietary doses of Pb was seemingly envisaged in foot AChE activity after 60 d treatment, no significant difference was found between the control and the Pb treatment groups (Fig. 2 B). In the foot of snails exposed to 5 mg Cd/Kg food dry-wt AChE activity was markedly reduced but, like in the case of Pb treatment, this effect was not more marked at higher dietary concentrations of the metal (all the Cd treatment groups differed with the control group but not amongst them; Fig. 2C). After 60 d Cd

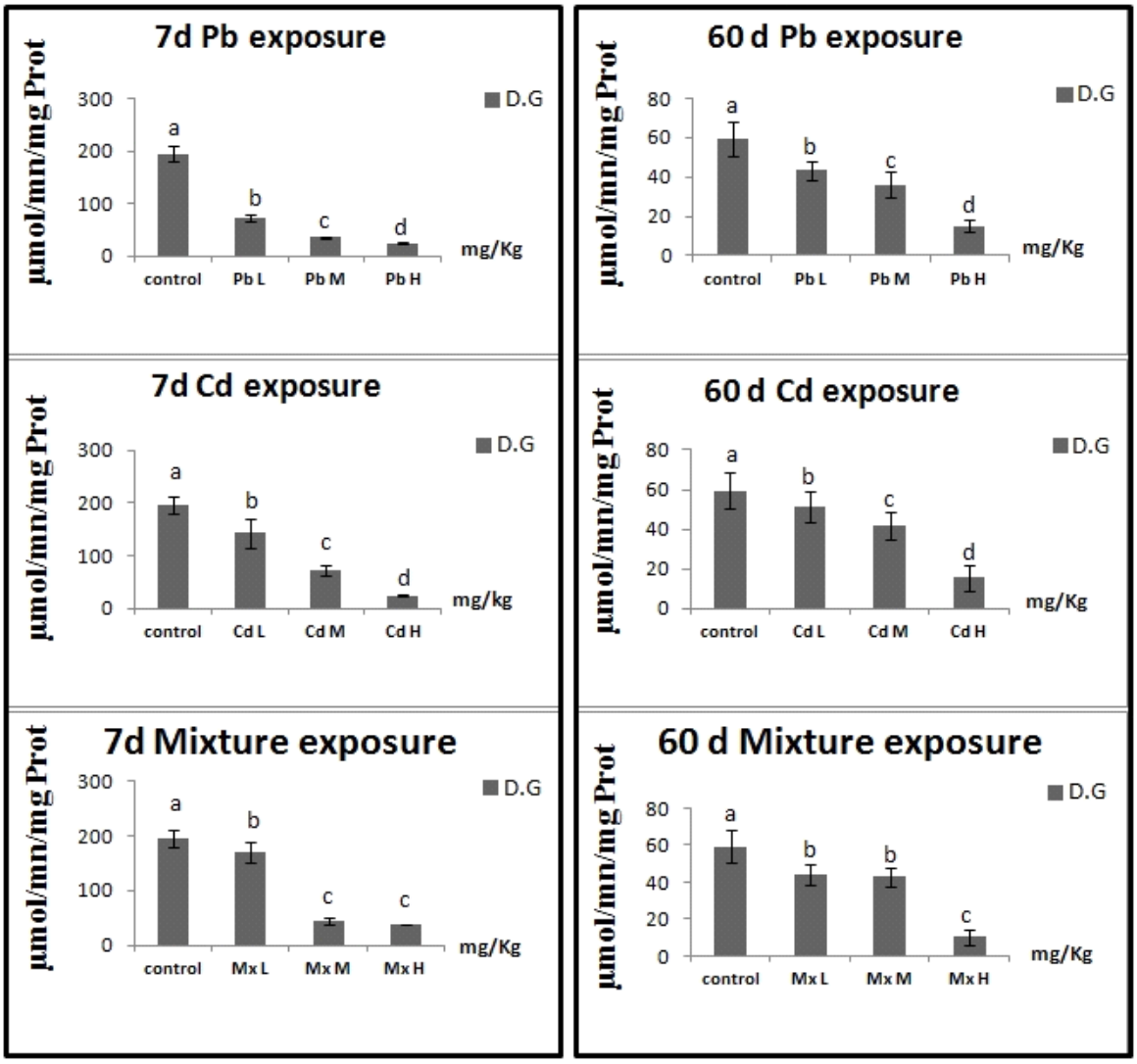

Fig. 1. AChE activity in digestive gland of *C. apertus* after 7 d and 60 d treatment. Values are given as mean ± SD (n=5). Significant differences are indicated by different letters; one way ANOVA followed by Tukey's HSD test. CO, control; PbL, 25 Pb[1]; PbH, 2500 Pb[1], CdL, 5 Cd[1]; CdH, 100 Cd[1]; MxL 5 Cd[1]+25 Pb[1]; MxH, 100 Cd[1]+2500 Pb[1]. Asterisks indicate significant differences with the control group ($p<0.05$). [1] mg/kg food dry-wt.

treatment, the enzyme activity was only significantly reduced in snails treated with 100 mg Cd/Kg food dry-wt (Fig. 2D). The combination of both metals also caused inhibition in the foot AChE activity all along the experimental time (Figs. 2E and 2F). After 7 d treatment, the lowest effective dose was the mixture of 100 mg Pb/Kg food dry-wt and 10 mg Cd/Kg food dry-wt (Fig. 2E), whereas after 60 d treatment the mixture of 25 mg Pb/Kg food dry-wt and 5 mg Cd/Kg food dry-w was already effective (Fig. 2 F).

AChE activity in digestive gland and foot was relatively high at Day 7 but within the range reported for these tissues in marine gastropods at Day 60 (Roméo *et al.* 2006; Cunha *et al.* 2007). The enzyme activity was lower in the foot tissue than in the digestive gland tissue (~50%), which may be attributed to the presence at each tissue of different isozymes with different affinities for the substrate, as suggested to explain the variation amongst tissues in AChE in fish (Ahammad *et al.* 1980). The activity of foot AChE, which exhibits properties of neuronal AChEs and soluble BuChEs (Cunha *et al.* 2007), is distributed throughout the foot motor nerves (Chiang *et al.* 1972) but also not bound to cells in the blood (Wieser and Fritz 1971). Likewise, AChE activity in

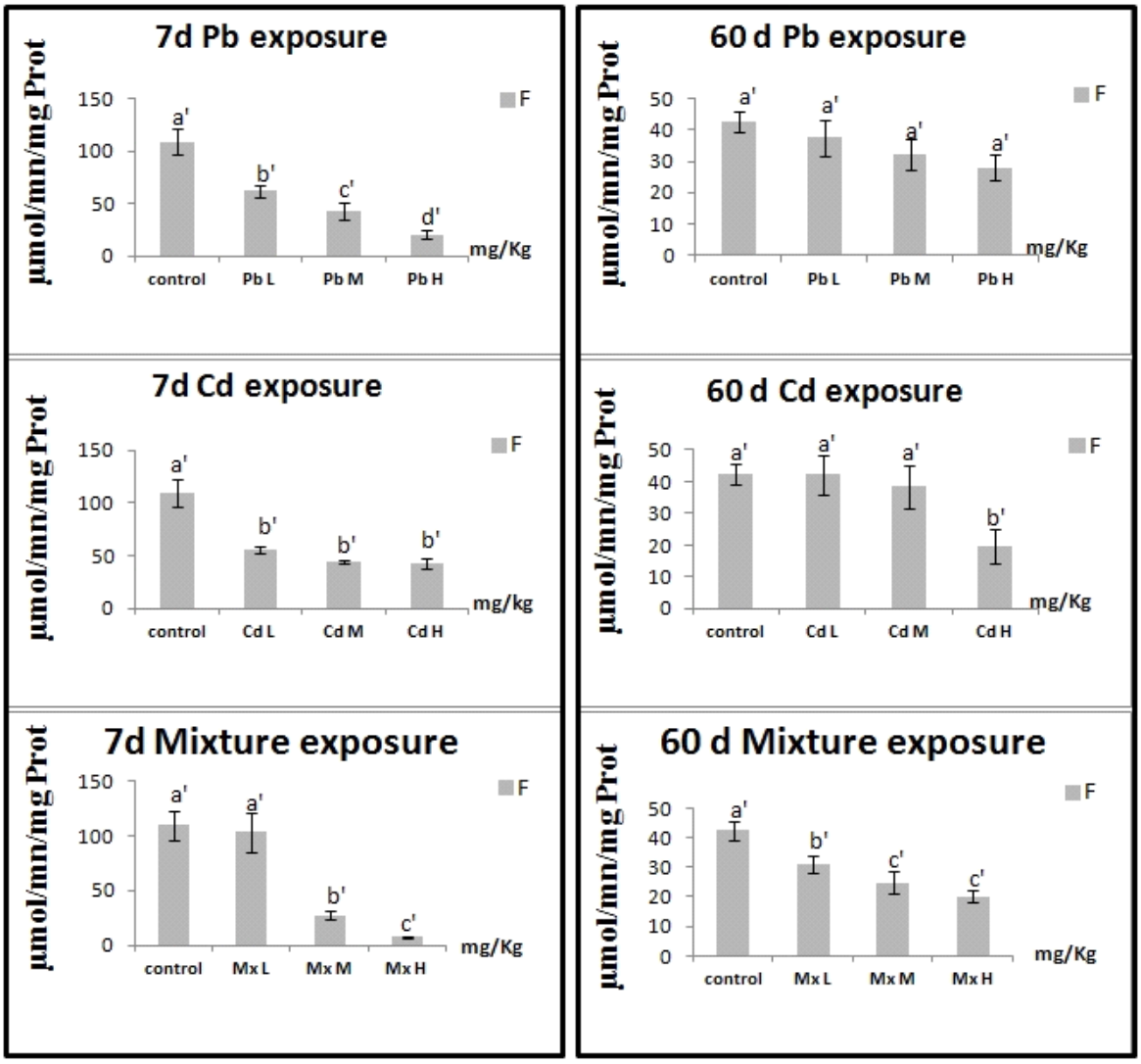

Fig. 2. AChE activity in foot of *C. apertus* after 7 d and 60 d treatment. Values are given as mean ± SD (n=5). Significant differences are indicated by different letters; one way ANOVA followed by Tukey's HSD test. CO, control; PbL, 25 Pb[1]; PbH, 2500 Pb[1], CdL, 5 Cd[1]; CdH, 100 Cd[1]; MxL 5 Cd[1]+25 Pb[1]; MxH, 100 Cd[1]+2500 Pb[1]. Asterisks indicate significant differences with the control group ($p<0.05$). [1] mg/kg food dry-wt.

digestive gland tissue is associated to the intraepithelial nerve plexus of the gut and the digestive gland and to single subepithelial neurons and nervous fibers localized among the muscle cells (Zaitseva and Kuznetsova 2008) but can be associated to haemocytes that occupy blood vessels and interstitial connective tissue (Moore 1991). Consequently, the changes in the AChE activity measured in the present investigation would be indicative of either neurotoxic or immunotoxic effects, or both; which could not be directly linked to neurotoxicity, like in the case of pesticides with brain as the target tissue, but to general impairment of cell signaling (i.e., cholinergic signaling).

Both in foot and digestive gland, AChE activity decreased with experimental time. In molluscs, this enzyme activity is known to be subject to seasonal variability and fluctuations depending on abiotic factors and on extreme physiological conditions such as aestivation and hibernation (Wieser and Fritz 1971; Najimi *et al.* 1997; Dellali *et al.* 2001; Singh and Singh 2009; Itziou and Dimitriadis 2012). Overall, it seems that AChE activity in land snails is low in summer and high in winter (Wieser and Fritz 1971; Larba and Soltani 2014). Thus, the decay in AChE activity in control snails along the experimental time could be attributed to either the physiological

consequences of stabulation or the progression of the natural life cycle during experimentation (seasonality).

In spite of this time-dependent decrease, AChE activity was reduced clearly in the digestive gland and the foot of snails treated with dietary Pb and Cd, alone and in combination. Accordingly, AChE activity was reduced in snails, *E. vermiculata*, treated with heavy metals, both in the digestive gland and the blood (Itziou and Dimitriadis 2011). AChE inhibition seems to be a widespread response to pollutants in soil organisms, including annelids (Rao and Kavitha 2004), isopods (Engenheiro *et al.* 2005; Stanek *et al.* 2006), and gastropods (Rorke and Gardner 1974; Coeurdassier *et al.* 2001; Radwan and *et al.* 2008; Itziou and Dimitriadis 2011). However, whilst solid results have been obtained after exposure organic chemicals, controversial results have been obtained regarding the effects of metals on AChE activity. Certainly, inhibition of AChE by metals has been often reported (Bocquene *et al.* 1990; Schmidt and Ibrahim 1994; Najimi *et al.* 1997; Guilhermino *et al.* 1998; Dellali *et al.* 2001; Saint-Denis *et al.* 2001; Itziou and Dimitriadis 2011); however, other studies concluded that metals (e.g., Cd and Pb) had no impact on AChE or may even stimulate this enzyme activity at low exposure concentrations (Scaps *et al.* 1997; Richetti *et al.* 2011; de Lima *et al.* 2013). Our results support that Cd and Pb, alone or in combination, provoke a decrease in AChE activity; yet, we cannot conclude whether this is the direct consequence of the inhibition of the enzyme by the metals or is the indirect consequence of general toxicopathic effects of the metals.

Different mechanisms have been proposed to explain the decrease in AChE activity in response to chemicals. This decrease could be either indirectly mediated by acetylcholine accumulation (if the chemicals are agonist of acetylcholine receptors, as in the case of neonicotinoids; Tomizawa and Casida 2005), or result from the direct inhibition of the AChE activity (as shown for other pesticides such as organophosphates and carbamates; Essawy *et al.* 2009). However, since the AChE transcript levels appear to be not affected by heavy metals, as shown in zebrafish, the toxic action of metals would result from their influence in the protein folding (e.g., interacting with cysteine residues) or from metal-mediated oxidative damage (Richetti *et al.* 2011). The latter might explain the effects of Cd, because this metal is known to induce the production of oxygen-free radicals by inhibiting the activity of antioxidant enzymes (Geret *et al.* 2002; Orbea *et al.* 2002; Manzl *et al.* 2004). Likewise, metals could bind to functional groups of the AChE (e.g., imidazole, sulfhydryl and carboxyl groups) and thus compromise its catalytic activity, leading to the loss of enzyme function (de Lima *et al.* 2013). Thus, the mechanisms through which metals affect AChE activity may be different depending on the metal, as well as on the exposure level and the target tissue (Bocquene *et al.* 1990; Dellali *et al.* 2001). Whilst high doses of metals cause direct toxic effects on the metabolism and on the AChE enzyme activity, low ones may stimulate enzyme activity and gene expression as a compensatory response to AChE inhibition and acetylcholine accumulation (de Lima *et al.* 2013). Presently, AChE activity decreased in the digestive gland (~60%) and the foot (~50%) at a dietary concentration of 25 mg Pb/Kg food dry-wt for 7 d and little far beyond at higher Pb concentrations. Likewise, an ~60% decrease in AChE activity was found in the digestive gland after 7 d treatment with 10 mg Cd/Kg food dry-wt but the effect was elicited already by 5 mg Cd/Kg food dry-wt. This dose of Cd also caused AChE inhibition in the foot, which was not more marked at higher concentrations. It seems that there is a threshold activity (50-60%) beyond which AChE cannot be inhibited so far, in agreement with early statements by Wieser and Fritz (1971). Nevertheless, Cd was more toxic than Pb, as previously reported for zebrafish, in which AChE activity was inhibited by 0.5 mg Pb/L after 2 d exposure and by 0.02 mg Cd/L after 7 d exposure (de Lima *et al.* 2013).

Although snails accumulated Pb and Cd in their tissues in a dose and time dependent manner (Mleiki *et al.* in prep), AChE inhibition was dose dependent but higher after 7 d than after 60 d, when metal tissue concentrations were the highest. AChE inhibition is known to be a prompt response in land snails exposed to a variety of pesticides including organophosphates, carbamates and neonicotinoids (Coeurdassier *et al.* 2002; Essawy *et al.* 2009; Radwan and Mohamed 2013). The response appears to be dose-dependent but not time-dependent, and may be attenuated or partly reversed at long exposure times, as seen in *C. aspersus* exposed to the pesticide imidacloprid (Radwan and Mohamed 2013) as well as in other animal groups such as earthworms, marine bivalves and fish exposed to different pesticides and heavy metals such as Cd, Pb, and Hg (Ahammad et al. 1980; Gambi *et al.* 2007; Dondero *et al.* 2010; Richetti *et al.* 2011). It has

been suggested that such an attenuated or partly reversed response at the long term is due to the accumulation of acetylcholine substrate once the enzyme is inhibited, which would induce gene up-regulation and enhanced synthesis of AChE (Matozzo *et al*. 2005; Radwan and Mohamed 2013).

The combination of both metals provoked interactive effects not seemingly related with the dietary concentrations of the metals. Though further specific experimentation is needed to obtain a definitive conclusion in this respect, the present results indicate an antagonistic effect between Pb and Cd, at least at the lowest dietary concentrations of metals employed herein, because the mixture of 25 mg Pb/Kg food dry-wt and 5 mg Cd/Kg food dry-wt caused less AChE inhibition than each metal separately at the same concentration, especially in the digestive gland. Conversely, the mixture of Cd and Pb was reported to exert synergistic toxic effects in rainbow trout (Birceanu *et al*. 2008). Interactive effects between metals in mixtures may be of distinct nature depending on the concentration of each individual metal and on the ratio between metal concentrations, among other factors (e.g., species, tissue). For instance, in zebrafish Cd uptake is inhibited in the presence of Pb whilst the uptake of this metal is enhanced by low, but not by high, concentrations of Cd; all this results in complex interactive toxic effects (Komjarova and Blust 2009). In the present study, the concentration of both metals in the digestive gland was higher on exposure to the mixture than to individual metals (Mliki *et al.* in prep); however, toxicity of Pb was apparently hampered in presence of low concentrations of Cd. Further research is needed to determine whether similar interactions would explain why antagonistic effects occur in the green garden snail fed mixtures of Pb and Cd at low concentrations.

CONCLUSIONS

In summary, it is concluded that model toxic metals such as Pd and Cd provoke a decrease in AChE activity in the foot and the digestive gland of the green garden snail, more markedly at the short-term (7 d *vs.* 60 d), although antagonistic effects were elicited by both metals in combination. As a whole, the reduction in AChE activity in *C. apertus* can be considered as a useful biomarker of the effects provoked by metals on cell signaling and therefore it may be suitable for ecosystem health assessment in metal polluted soils using this species as sentinel organism.

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