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Tissue-specific inhibition and recovery of esterase activities in *Lumbricus terrestris* experimentally exposed to chlorpyrifos

Sandra González Vejares [a,b], Pablo Sabat [b], Juan C. Sanchez-Hernandez [a,*]

[a] Laboratory of Ecotoxicology, Faculty of Environmental Science, University of Castilla-La Mancha, Avda. Carlos III s/n, 45071, Toledo, Spain
[b] Department of Animal Ecophysiology, Faculty of Sciences, University of Chile, Chile

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ABSTRACT

Exposure and effect assessment of organophosphate (OP) pesticides generally involves the use of cholinesterase (ChE) inhibition. In earthworm, this enzyme activity is often measured in homogenates from the whole organism. Here we examine the tissue-specific response of ChE and carboxylesterase (CE) activities in *Lumbricus terrestris* experimentally exposed to chlorpyrifos-spiked field soils. Esterases were measured in different gut segments and in the seminal vesicles of earthworms following acute exposure (2 d) to the OP and during 35 d of a recovery period. We found that inhibition of both esterase activities was dependent on the tissue. Cholinesterase activity decreased in the pharynx, crop, foregut and seminal vesicles in a concentration-dependent way, whereas CE activity (4-nitrophenyl valerate) was strongly inhibited in these tissues. Gizzard CE activity was not inhibited by the OP, even an increase of enzyme activity was evident during the recovery period. These results suggest that both esterases should be determined jointly in selected tissues of earthworms. Moreover, the high levels of gut CE activity and its inhibition and recovery dynamic following OP exposure suggest that this esterase could play an important role as an enzymatic barrier against OP uptake from the ingested contaminated soil.

1. Introduction

Biomarkers are any biological responses at sub-individual level that indicates exposure to, and toxic effects from, one or more contaminants (Peakall, 1992; van Gestel and van Brummelen, 1996). A set of biomarkers covering multiple levels of biological organization is often used in the assessment of pollutant effects in order to 1) distinguish short-term from chronic exposure, 2) to provide an accurate interpretation of pollutant-linked biomarkers, because their responses may be influenced by confounding environmental and biological factors and 3) to establish a cause–effect relationship between pollutants and ecological effects (Hagger et al., 2006). Some reviews have exhaustively examined the knowledge of biomarkers in a group of terrestrial organisms of notable ecotoxicological concern, i.e., earthworms (Kammenga et al., 2000; Scott-Fordsmand and Weeks 2000; Sanchez-Hernandez 2006). One of the main conclusions from these works is the limited research on biomarkers of organic pollutants (e.g., pesticides) compared to those of metal pollution.

Exposure to agrochemicals such as organophosphate (OP) and carbamate (CB) insecticides is generally evaluated by the determination of acetylcholinesterase (AChE, EC 3.1.1.7) inhibition in the nervous tissue (Sultatos, 2006). As with other many organisms, earthworm AChE activity is very sensitive to inhibition by both OPs (Booth et al., 2000) and CBs (Ribera et al., 2001; Gambi et al., 2007). Moreover, earthworm AChE activity displays an extremely slow recovery to full normal activity when it is drastically inhibited by OPs (Aamodt et al., 2007; Rault et al., 2008). Likewise, some mesocosm and field studies have documented that AChE inhibition is a workable biomarker of pesticide exposure in earthworms (Booth et al., 2000; Reinecke and Reinecke, 2007; Denoyelle et al., 2007).

Carboxylesterases (CEs, EC 3.1.1.1) are another group of serine hydrolases used as biomarkers of OP exposure. Although scarcely investigated in non-target organisms, these enzymes are frequently more sensitive to OP inhibition than cholinesterases (ChEs) (revised in Wheelock et al., 2008). These esterases are not directly involved in the acute OP toxicity as AChE is, and this could explain the reduced amount of ecotoxicological investigations with CEs. However, these enzymes play an important role in pesticide detoxification. They hydrolyze efficiently synthetic pyrethroids and CBs (Crow et al., 2007; Wheelock et al., 2008), and further they irreversibly bind (1:1 ratio) to OP insecticides (Maxwell, 1992). This function has led CEs to be one of the most important biochemical determinants for pesticide resistance in some pest species (Hemingway and Ranson, 2000). Multiples CE isozymes are present in earthworms (Haites et al., 1972), and they show a tissue-dependent sensitivity to OP insecticides such as chlorpyrifos-oxon (CPOx) (Sanchez-Hernandez and Wheelock, 2009). These esterases are particularly abundant in the earthworm gut (Prento, 1987); even a secretion of CEs has been recently

* Corresponding author. Tel.: +34 925 268800; fax: +34 925 268840.
E-mail address: juancarlos.sanchez@uclm.es (J.C. Sanchez-Hernandez).

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documented in the gastrointestinal tract of *L. terrestris* (Sanchez-Hernandez et al., 2009). Despite these investigations, more laboratory and field studies are still necessary to understand the role of CEs in pesticide toxicity in earthworms, as well as to investigate their usefulness as biomarkers of pesticide exposure in biomonitoring programs.

This study constitutes the concluding step of a laboratory investigation addressed to propose a biochemical multibiomarker scheme based in the esterase (ChEs and CEs) responses of *L. terrestris*, with the scope of using them in either soil toxicity testing or field monitoring of OP contamination. The aim of this work was to examine the time-course profiles of CE and ChE inhibition and recovery following acute exposure to chlorpyrifos-spiked field soils. Esterase activities were measured in different gut segments and in the seminal vesicles (male reproductive system) of *L. terrestris*. We focused on these tissues because of their potential implications in pesticide uptake and reproductive success. Earthworm gut is an important uptake route of hydrophobic OP pesticides present in ingested soil (Yu et al., 2006), whereas the occurrence of CE activity in the male reproductive system could be associated to spermatogenesis and male reproductive health (Jewell and Miller, 1998; Mikhailov and Torrado, 1999).

2. Materials and methods

2.1. Reagents

Acetylthiocholine iodide (AcSCh), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), α-naphthyl acetate (α-NA), 4-nitrophenyl valerate (4-NPV), *p*-nitrophenol, Fast Red ITR salt, Fast Blue RR salt and lipase type II from porcine pancreas (100–400 U mg^{-1} protein) were purchased from Sigma-Aldrich (Madrid, Spain). Dursban 5G (5% w/w of chlorpyrifos, granulated formulation) was obtained from Compo Agricultura S.L. (Barcelona, Spain). All other chemicals were obtained from commercially available sources.

2.2. Exposure to chlorpyrifos-spiked soils

Adult and clitellated earthworms (*L. terrestris*) were purchased from a local supplier (Armeria20, Toledo, Spain), who imported them from a commercial vermiculture supplier (Vivastic, Elsenheim, France). Animals (3.73 ± 0.87 g fresh mass, $n = 144$ earthworms) were exposed for 2 d to field soils (<4 mm particle size; collected from a non-contaminated area in Toledo, Spain) spiked with Dursban 5G to yield 3, 12 and 48 mg a.i. kg^{-1} dry wt. Dried soil subsamples (105 °C for 48 h) were used for determining physicochemical properties ($pH = 7.6 \pm 0.08$; 249 ± 22 μS cm^{-1} and 2.26 ± 0.24% total organic carbon). Chlorpyrifos concentrations were chosen based upon sublethal effects of this OP in earthworms (Ma and Bodt, 1993; Booth et al., 2000; Reinecke and Reinecke, 2007), and the predicted environmental concentration (PEC) of 3.3 mg a.i. kg^{-1} dry soil. The PEC was calculated considering the lower recommended application rate by the manufacturer (5 g of Dursban $m^{-2} = 2500$ g a.i. ha^{-1}), a depth soil layer of 5 cm of pesticide penetration, no crop interception and a bulk soil density of 1.5 g cm^{-3} (Rault et al., 2008). The concentrations of chlorpyrifos in test soils were therefore equal to the PEC (3 mg a.i. kg^{-1} dw), 4× PEC (12 mg a.i. kg^{-1} dw) and 16× PEC (48 mg a.i. kg^{-1} dw). Soil samples containing chlorpyrifos were mixed thoroughly for 2 min using plastic containers to ensure uniform distribution of pesticide granules in the soil. Three replicates (1.2 kg soil wet weight, soil moisture adjusted to 25% w/v) of each test concentration, including controls, were prepared and placed in plastic containers (14.5×14×12 cm) which were left for 48 h at 12 °C in darkness for equilibration. Earthworms were placed in petri dishes on damp filter paper (24 h at 12 °C and darkness) to void their gut. Groups of 12 individuals were released into the testing containers and maintained at 12 °C and darkness throughout the experiment (Lowe and Butt, 2005). Following 2 d of chlorpyrifos exposure, individuals (OP-exposed and control earthworms) were transferred to clean soils (1.2 kg fresh soil each replicate) and kept there for 35 d. We collected two animals from each replicate ($n = 6$ individuals per treatment) at $t = 0$ (immediately before to release earthworms in clean soil), 2, 4, 8, 18 and 35 d after transferring earthworms to clean soil. Periodically, testing containers were weighted to adjust soil moisture, although they were sealed with perforate lids to minimize water loss. Earthworms were not fed during the 37-d toxicity experiment (OP exposure plus recovery).

2.3. Tissue preparation

Earthworms were introduced in a fridge (4–5 °C) for 10–15 min to allow easy dissection because of muscle relaxing. Organisms were killed by the dissection process, which was performed on the dorsal side. Body wall muscle, seminal vesicles, pharynx, crop, gizzard and foregut (the gut segment between the gizzard and the clitellum) were dissected and washed to remove soil particles with an isotonic buffer balanced for *L. terrestris* (Stein and Cooper, 1981) containing (mM): 71.5 NaCl, 4.8 KCl, 3.8 $CaCl_2$, 1.1 $MgSO_4$, 0.4 NaH_2PO_4, 0.4 Na_2HPO_4, and 4.2 $NaHCO_3$. Tissues were immediately immersed in liquid nitrogen and stored frozen at −80 °C until esterase assays. Weighted samples were added (1:10 w/v ratio) to ice-cold 25 mM Tris–HCl buffer ($pH = 8.0$) containing 0.1% Triton X-100. We used this concentration of Triton X-100 to ensure extraction of membrane-bound esterases (Huang and Hammock, 1997) and, simultaneously, to avoid inhibition of ChE or CE activities by this non-ionic detergent (Vioque-Fernández et al., 2007). Samples were homogenized at 4 °C using a glass-Teflon Potter-Elvehjem homogenizer. The homogenates were centrifuged at $9000 \times g$ at 4 °C for 10 min to obtain a post-mitochondrial fraction. Body wall muscle was used for esterase inhibition and recovery analysis and, further, to test for chemical reactivation of phosphorylated muscle ChE activity using oximes (results with this tissue are not reported in this paper).

2.4. Carboxylesterase activity

Carboxylesterase (EC 3.1.1.1) activity was assayed in 96-well flat bottom plates (Ratiolab, Dreieich, Germany), and measured on an Asys HiTech UVM340 plate reader (Asys HiTech GmbH, Eugendorf, Austria). This esterase activity was determined using two different substrates, i.e., α-NA and 4-NPV because previous in vitro studies with *L. terrestris* suggest that these two substrates are hydrolyzed by different groups of CE isozymes, and furthermore they evidence isozymes with different sensitivities to OPs (Sanchez-Hernandez and Wheelock, 2009). Carboxylesterase activity using α-NA (α-NA-CE) was determined according to Bunyan et al. (1968) as adapted from Gomori (1953). The reaction medium (200 μL, final volume) contained 25 mM Tris–HCl (pH 7.6) and 2 mM α-NA, and it was incubated for 10 min at 22 °C with the enzyme sample. The enzymatic formation of α-naphthol was stopped by the addition of 75 μL of 2.5% SDS in 0.1% Fast Red ITR/2.5% Triton X-100. Solutions were allowed to stand for 30 min at 22–23 °C in the dark. The absorbance of the naphthol–Fast Red ITR complex was read at 530 nm, and the specific activity was calculated using a molar extinction coefficient of 33.225×10^3 M^{-1} cm^{-1} for the naphthol–Fast Red ITR complex. Hydrolysis of 4-NPV by CE (4-NPV-CE) was determined as described by Carr and Chambers (1991). The reaction mixture (200 μL, final volume) contained 1 mM 4-NPV, 50 mM Tris–HCl (pH 7.5) and the sample. Reaction was initiated by addition of 10 μL of 50 mM 4-NPV and stopped after 15 min with 75 μL of a solution 2% (w/v) SDS and 2% (w/v) Tris base. The 4-nitrophenol liberated was read at 405 nm and quantified by a calibration curve (5–100 μM).

2.5. Cholinesterase activity

Cholinesterase (EC 3.1.1.7) activity was determined according to the Ellman method (Ellman et al., 1961) as adapted to microplate reader by Wheelock et al. (2005). The reaction mixture was 285 μL of 0.1 M Na-phosphate buffer (pH 8.0) containing 320 μM DTNB, and 5 μL of enzyme sample. After 5 min of equilibration, 10 μL of ATCI (60 mM) was added and the absorbance at 412 nm was read at 1-min intervals for 10 min at 22 °C. All assays were made by duplicate, and esterase activity was calculated using a molar absorption coefficient of $14.15\times10^3\ M^{-1}\ cm^{-1}$ (Eyer et al., 2003). Specific activity was expressed as U mg^{-1} protein (1 U = 1 μmol of substrate hydrolyzed per min under the experimental conditions in this study), and total protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

2.6. Native PAGE

Native polyacrylamide gel electrophoresis (PAGE) was performed on a Bio-Rad Tetra Cell Electrophoresis Unit (Bio-Rad, USA). Samples were loaded on 4% stacking and 12.5% resolving 0.75 mm polyacrylamide gel (25 mM Tris, 192 mM glycine as running buffer), and electrophoresed at a constant voltage of 30 V for 30 min, and subsequently 150 V until the bromophenol blue tracking dye reached the bottom of the gel. Following electrophoresis, in-gel hydrolysis staining was carried out with α-NA/Fast Blue RR salt (Sanchez-Hernandez and Wheelock, 2009). Gels were incubated (5 min at 25 °C) with a solution containing 100 mM Na-phosphate buffer (pH = 6.5), 0.5 mg mL^{-1} α-NA and 0.025 g of Fast Blue RR salt, which was prepared and filtered immediately before use. Stained gels were scanned using the Kodak Digital Science 1D Image Analysis Software, version 2.03 (Rochester, NY, USA).

2.7. Statistical analysis

We tested the assumptions of normality (Shapiro–Wilks *W* test) and homoscedasticity (Levene test) before statistical analysis. General linear models were applied in logarithmic transformed data to examine the impact of both the chlorpyrifos concentration and the time of recovery on ChE and CbE activities. Specifically, changes in normal esterase activity in control animals were analyzed separately through repeated-measures ANOVA using the time of recovery as factor and the tissue as repeated measures. Moreover, a series of ANOVA were performed for each tissue to test the effect of the chlorpyrifos concentration on each esterase activity. Finally, to test the effect of the time of recovery on esterase activities we compared control earthworms with individuals exposed to the higher concentration of pesticide (48 mg kg^{-1} of chlorpyrifos) by means of a factorial ANOVA using the treatment (control and OP exposures) and time of recovery as factors. All ANOVAs were followed by the LSD post-hoc test. Furthermore, linear regression analysis was performed to test for the putative association among biochemical variables.

3. Results

3.1. Normal esterase variation in control earthworms

Analysis of esterase activity in non-exposed (controls) earthworms during the 35-d recovery period provided three main results: (1) esterase activities were highly dependent on the tissue used as source of the enzyme (Fig. 1), (2) variations of CE activity in the tissues were strongly dependent on the substrate (α-NA or 4-NPV) used for measurements (Fig. 1), and (3) α-NA-CE and 4-NPV-CE activities were significantly correlated ($r^2 = 0.39–0.83$, $p<0.001$) for each tissue during the 35-d recovery period (Supplementary Fig. 1).

The ChE activity in control earthworms was significantly affected by the tissue ($F_{(4,\ 68)} = 444.14$, $p<0.0001$) but not by time ($F_{(5,\ 17)} = 1.56$, $p = 0.22$), although we found a significant effect of the interaction between these two factors ($F_{(20,\ 68)} = 2.10$, $p = 0.012$). The LSD post-hoc test revealed that the highest levels of ChE activity were found in the pharynx (Fig. 1A). The α-NA-CE activity was affected by the recovery time ($F_{(5,\ 25)} = 5.92$, $p = 0.001$), the tissue ($F_{(4,\ 100)} = 130.37$, $p<0.0001$) and the interaction between these factors ($F_{(20,\ 100)} = 5.10$, $p<0.0001$). The LSD post-hoc test showed that α-NA-CE activity increased progressively from the pharynx to foregut. Similarly, the 4-NPV-CE activity was higher in the foregut, whereas the lowest activity was found in the gizzard ($F_{(4,\ 100)} = 130.37$, $p<0.0001$; Fig. 1A). The 4-NPV-CE activity varied with time of recovery ($F_{(5,\ 25)} = 5.92$, $p = 0.001$) and by the interaction of time and tissue ($F_{(20,\ 100)} = 510$, $p<0.0001$). Both CE activities showed a maximum enzyme activity at $t = 4$ d of the recovery period.

Multiple protein bands were evidenced by native electrophoresis using α-NA as substrate for in-gel staining activity (Fig. 1B). Relative mobility, isozymes abundance and staining intensity varied with the gut segment and seemed to corroborate the total specific CE activities found in the different gut segment (Fig. 1A).

3.2. Effect of chlorpyrifos concentration on esterase activities

No dead individuals were recorded during the exposure and recovery phases in this work, however, exposure to chlorpyrifos-spiked soils for 2 d had a marked effect on the three esterase activities, although the inhibition potency of the OP depended on the tissue (Figs. 2 and 3A). Compared to controls, chlorpyrifos decreased severely the ChE and CE activities in the pharynx ($F_{(3,\ 19)} = 4.48$, $p = 0.015$ for ChE; $F_{(3,\ 20)} = 7.20$, $p = 0.0018$ for α-NA-CE and $F_{(3,\ 20)} = 39.93$, $p<0.00001$ for 4-NPV-CE), and crop ($F_{(3,\ 20)} = 6.60$, $p = 0.0027$ for ChE; $F_{(3,\ 20)} = 3.32$, $p = 0.0404$ for α-NA-CE and $F_{(3,\ 20)} = 6.13$, $p = 0.0039$ for 4-NPV-CE), whereas only foregut ChE and 4-NPV-CE activities were significantly inhibited by the OP ($F_{(3,\ 20)} = 6.63$, $p = 0.0027$ for ChE, ($F_{(3,\ 20)} = 8.92$, $p = 0.0005$ for 4-NPV-CE and $F_{(3,\ 20)} = 1.69$, $p = 0.201$). Although ChE activity apparently decreased in a concentration-dependent way in the pharynx, crop and foregut, the LSD post-hoc test revealed that this enzyme activity was significantly lower in the pharynx and crop of the earthworms exposed to 48 mg kg^{-1}, in the crop of the group treated with 12 mg kg^{-1} chlorpyrifos, and in the foregut of all OP-exposed groups (Fig. 2). In the pharynx, crop and foregut, 4-NPV-CE activity was more sensitive to chlorpyrifos than α-NA-CE activity, although the responses of this esterase do not show an apparent concentration–response relationship (Fig. 2). Surprisingly, gizzard ChE was affected by the chlorpyrifos concentration ($F_{(3,\ 20)} = 5.34$, $p = 0.0072$), whereas the α-NA-CE ($F_{(3,\ 19)} = 1.03$, $p = 0.400$) and 4-NPV-CE ($F_{(3,\ 20)} = 0.79$, $p = 0.509$) activities were not. The LSD post-hoc test showed that the earthworm groups exposed to 12 and 48 mg kg^{-1} chlorpyrifos had the gizzard ChE activity significantly depressed.

Cholinesterase activity in the seminal vesicles decreased while chlorpyrifos concentration increased ($F_{(3,\ 17)} = 5.93$, $p = 0.0058$). Similar to gut tissues, the 4-NPV-CE activity was more sensitive to inhibition by chlorpyrifos ($F_{(3,\ 18)} = 17.016$, $p = 0.00002$) than α-NA-CE activity ($F_{(3,\ 18)} = 1.815$, $p = 0.1804$), although it did not vary among the treated earthworms (Fig. 3A).

Electrophoretic separation of a crude extract of a representative earthworm exposed to 48 mg kg^{-1} chlorpyrifos showed a reduction of CE isozymes more than 50% compared to control. Protein bands that migrated faster in the gel did not seem to hydrolyse α-NA in the tissue extracts from the OP-exposed earthworm (Fig 1B). Likewise, native electrophoresis of the OP-exposed earthworm extracts support that crop and gizzard CE activities were more resistant to chlorpyrifos inhibition that those in the pharynx, foregut or seminal vesicles.

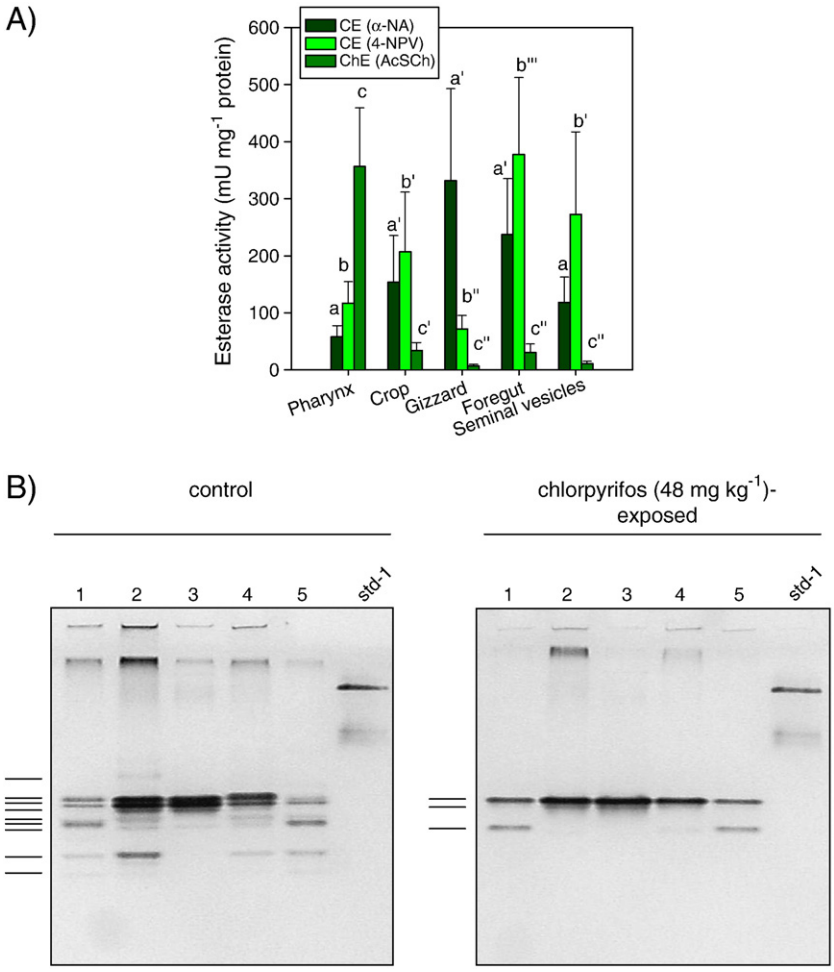

Fig. 1. A) Mean (±SD, $n \geq 29$ earthworms) cholinesterase (ChE) and carboxylesterase (CE) activities in the gastrointestinal tract and seminal vesicles of control earthworms during the 35 d of recovery period. Carboxylesterase activity was determined using the substrates α-naphthyl acetate (α-NA) and 4-nitrophenil valerate (4-NPV). Different letters denote significant differences ($p<0.05$) after a LSD post-hoc test (font "a" for α-NA-CE, font "b" for 4-NPV-CE and font "c" for ChE). B) Native PAGE of gut segments. Protein bands (μg proteins charged on right gel/left gel) were detected and stained using α-NA/Fast Blue RR. Lane 1: pharynx (16 μg/25 μg), lane 2: crop (26 μg/30 μg), lane 3: gizzard (24 μg/37 μg), lane 4: foregut (15 μg/40 μg), lane 5: seminal vesicles (27 μg /42 μg) and std-1: porcine pancreatic lipase (23 μg).

3.3. Time-course esterase inhibition and recovery in gut

Time-course dynamic of esterase activities in earthworms following chlorpyrifos exposure varied with the exposure level and the gut segment (Supplementary Table 1). Fig. 4 shows the recovery of gut CE activities in the earthworms exposed to the highest chlorpyrifos concentration. Carboxylesterase activity using α-NA-CE remained significantly inhibited in the pharynx ($F_{(1,\ 59)}=75.70$, $p<0.00001$), crop ($F_{(1,\ 58)}=41.23$, $p<0.00001$), and foregut ($F_{(1,\ 57)}=23.78$, $p=0.00001$) of OP-exposed earthworms compared to controls during the 35-d recovery period (Fig. 4). However the crop α-NA-CE activity recovered its normal levels of variation after 35 d of transferred earthworms into clean soil (LSD test, $p=0.60$). In the foregut, α-NA-CE activity was progressively decreasing as the time of recovery increased, being significantly lower at $t=8$ d (LSD test, $p=0.001$), 18 d (LSD test, $p=0.0001$), and 35 d (LSD test, $p=0.018$) compared to the corresponding controls (Fig. 4). Surprisingly, gizzard α-NA-CE activity was higher in the pesticide-exposed earthworms during the full recovery period ($F_{(1,\ 59)}=8.68$, $p=0.0046$), and the activity was even significantly higher than that of controls after 18 d of recovery period (LSD test, $p=0.003$).

Time-course dynamic of 4-NPV-CE activity in the gut tissues followed a similar pattern than that for α-NA-CE activity (Fig. 4 and Supplementary Table 1). The factorial ANOVA analysis revealed that 4-NPV-CbE activity of the OP-exposed earthworms remained significantly inhibited during the 35 d of recovery period ($F_{(1,\ 59)}=402.95$, $p<0.0001$ for pharynx 4-NPV-CbE; $F_{(1,\ 60)}=163.63$, $p<0.0001$ for foregut 4-NPV-CbE). Crop 4-NPV-CE activity was affected by the treatment ($F_{(1,\ 60)}=125.7$, $p<0.00001$), time ($F_{(5,\ 60)}=15.74$, $p<0.00001$) and the interaction between these factors ($F_{(5,\ 60)}=4.38$, $p=0.0018$). However, this CE activity recovered its basal activity after 35 d of recovery (LSD, $p=0.07$, Fig. 4). Gizzard 4-NPV-CE activity was affected by treatment ($F_{(1,\ 60)}=5.82$, $p=0.018$), time ($F_{(5,\ 60)}=3.93$, $p=0.003$) but not by the interaction between these factors ($F_{(5,\ 60)}=0.93$, $p=0,464$). The LSD post-hoc test revealed that foregut 4-NPV-CbE activity differed between control and exposed groups at $t=35$ d only (Fig. 4 and Supplementary Table 1).

3.4. Time-course esterase inhibition and recovery in the seminal vesicles

Carboxylesterase activity of OP-exposed earthworms was severely affected by chlorpyrifos exposure ($F_{(1,\ 54)}=124.49$, $p<0.00001$ for α-NA-CE; $F_{(1,\ 53)}=230.62$, $p<0.0001$ for 4-NPV-CE). Moreover, a progressive increase of both α-NA-CE activity was observed in the time interval from $t=8$ d to 35 d ($F_{(5,\ 54)}=4.19$, $p=0.0027$), whereas not significant increase of the phosphorylated 4-NPV-CE activity was found during the 35-d recovery period ($F_{(5,\ 53)}=1.83$, $p=0.120$) (Fig. 3B). Despite that CE activities in the seminal vesicles showed a slow recovery of their activity during the recovery period, they were

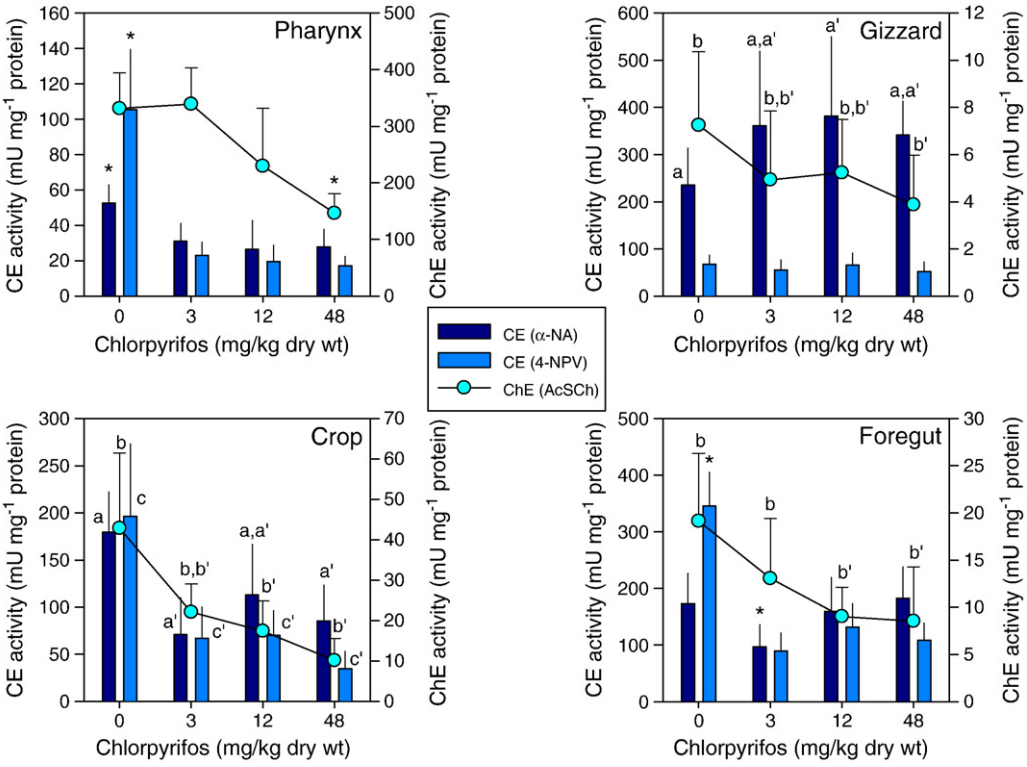

Fig. 2. Mean (±SD, $n \geq 5$ earthworms) of cholinesterase (ChE) and carboxylesterase (CE) activities of *Lumbricus terrestris* sampled after 2 d of chlorpyrifos exposure. Different letters denote significant differences ($p<0.05$) after a LSD post-hoc test (font "a" for α-NA-CE, font "b" for 4-NPV-CE and font "c" for ChE). Asterisks denote significant differences when the mean value of a treatment differs with the rest of the treatments. α-NA and 4-NPV as in Fig. 1.

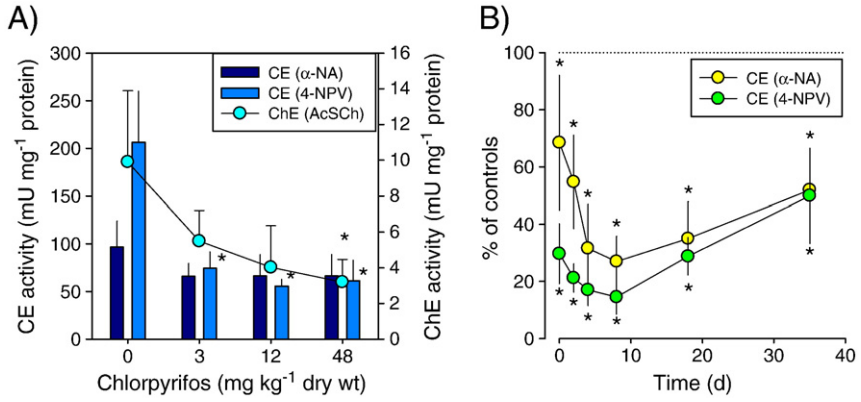

Fig. 3. A) Mean (±SD, $n \geq 5$ earthworms) of seminal vesicle carboxylesterase (CE) and cholinesterase (ChE) activities in *Lumbricus terrestris* following 2 d of exposure to chlorpyrifos-spiked soils. B) Recovery of CE activities of earthworms exposed to 48 mg kg^{-1} chlorpyrifos for 2 d. The symbols are percentages (mean ± SD, $n \geq 5$ earthworms) of residual esterase activity with respect to the mean esterase activities of the corresponding controls at each sampling time, which was set to 100% (horizontal dotted line). Asterisks denote significant differences when the mean value of the treatment differed with respect to the corresponding control. α-NA and 4-NPV as in Fig. 1.

still significantly depressed after 35 d (LSD test, $p<0.04$ for α-NA-CE and $p<0.0003$ for 4-NPV-CE).

4. Discussion

4.1. Variation of normal esterase activity

We found that esterase activities varied significantly in the control earthworms during the 35-d recovery period. Similar results have been also reported by others (Aamodt et al., 2007; Booth et al., 2000). For example, Denoyelle et al. (2007) reported coefficients of variation for ChE activity in the earthworm *Allolobophora chlorotica* collected from a field that ranged between 15 and 30%; which caused difficulties in the identification of OP-exposed individuals. Factors such as temperature, nutritional status, reproductive stage or burrowing activity may affect the esterase activity of earthworms (Scott-Fordsmand and Weeks, 2000; Lowe and Butt, 2007). Particularly, nutritional status could change the CE activity because this esterase seems to metabolize lipids in invertebrates (Geering and Freyvogel, 1974; Mommsen 1978) and vertebrates (Van Lith et al., 1992), and its hydrolytic activity could be altered by ingestion of lipid materials or inanition. In this work, earthworms were not fed throughout the experiment and a nutritional deficit could explain the high variation of esterase activities; particularly gut CE. Indeed, this enzyme activity decreased in the crop and the foregut of control earthworms at the end of the recovery period (Supplementary Table 1). Earthworm activity could reduce substantially the initial concentration of organic matter in the test field soils (Lowe and Butt,

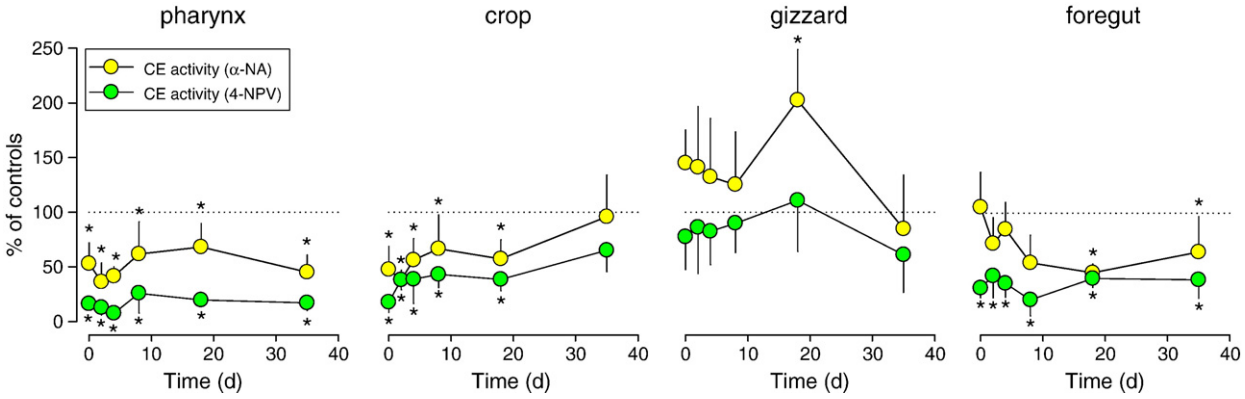

Fig. 4. Recovery of gut carboxylesterase (CE) activities in earthworms following exposure (2 d) to 48 mg kg^{-1} chlorpyrifos. The symbols are percentages (mean ± SD, $n \geq 5$ earthworms) of residual esterase activity with respect to the mean enzyme activity of the corresponding controls at each sampling time, which was set to 100% (horizontal dotted lines). α-NA and 4-NPV as in Fig. 1. Asterisks denote significant differences when the mean value of the treatment differed with respect to the corresponding control.

2007), which would lead to a deficit of lipid material in the ingested soil. Alternatively, triacylglycerol hydrolases or lipases (EC 3.1.1.3) are present in the earthworm gut (Drake and Horn, 2007), and these enzymes are able to hydrolyze the substrates α-NA and 4-NPV commonly used for CE assays (Gilham and Lehner, 2005). However, native PAGE gels of *L. terrestris* crude extracts revealed no protein bands matching the relative mobility of the porcine pancreatic lipase (EC 3.1.1.3) used as the standard. Despite this result, electrophoresis analysis does not totally exclude the impact of nutritional status on CE activity because this esterase seems to play a more important role in lipid digestion in invertebrates than own lipases (Mommsen, 1978).

More work is still needed to investigate the impact of confounding environmental and biological variables on earthworm esterase activities. Yet, the high interindividual variation of normal esterase activity in earthworms suggests that both ChE and CE activities should be determined to assess OP exposure; CE activity should be even measured using diverse substrates because of multiple isozymes with marked differences in substrate hydrolysis rate as well as sensitivity to OP inhibition (Wheelock et al., 2008). Indeed, we found little correlation between both CE activities in the gizzard and foregut ($r^2 \sim 0.40$) of control earthworms (Supplementary Fig. 1).

4.2. Tissue-specific esterase responses

Many ecotoxicological investigations use the whole body, or portions, of the earthworm for biomarker measurements (Booth et al., 2000; Aamodt et al., 2007; Rault et al., 2008). In many cases, the worm size is not enough to perform accurately biochemical and molecular analysis in single tissues. However, some studies have evidenced the existence of diverse ChEs with a marked difference in OP and CB sensitivity (Stenersen 1980; Aamodt et al., 2007), whereas others have documented a high tissue-dependent variation of ChE activity in earthworms (Rault et al., 2007). Similarly, earthworm CE activity displays a tissue-specific distribution and sensitivity to OPs (Sanchez-Hernandez and Wheelock, 2009). Taken together, these investigations suggest that earthworm esterases (ChEs and CEs) should be determined in selected tissues.

We have found a notable difference in esterase activity and sensitivity to chlorpyrifos dependent on the target tissue. For example, ChE activity was extremely high in the pharynx compared to the rest of target tissues; although the presence of nervous tissue close to the pharynx would explain this high ChE activity. Likewise, 4-NPV-CE activity was strongly inhibited in the pharynx, foregut and seminal vesicles without apparent recovery of its normal activity after 35 d of OP exposure, whereas no inhibition was observed in the gizzard 4-NPV-CE activity. Moreover, the use of this substrate evidenced a group of isozymes with a higher sensitivity to chlorpyrifos than when α-NA was used.

The tissue-specific sensitivity of CE activity to chlorpyrifos may be explained by two factors. First, the in vitro sensitivity of the enzyme to CPOx could be a determinant of in vivo inhibition response. Indeed, we found an apparent positive relationship between IC50s of CPOx for α-NA-CE activity measured in the gut tissues of *L. terrestris* (data obtained from Sanchez-Hernandez and Wheelock, 2009) and the percentages of CE inhibition after 2 d of exposure to chlorpyrifos-spiked soils (Supplementary Fig. 2). This relationship would mean that toxicokinetic elements for CPOx dissipation such as the OP-detoxification enzymes (phosphotriesterases and glutathione *S*-transferases) have not a notable local significance besides differences in tissue distribution of the OP. Second, the tissue-specific abundance of CE isozymes as revealed by native PAGE gels could also contribute to differences in total CE sensitivity to chlorpyrifos (Fig. 1B).

4.3. Gut carboxylesterases: an enzymatic barrier against pesticide uptake

Mammalian CEs can act as scavengers reducing the concentration of OP pesticide able to interact with AChE (Maxwell, 1992; Chambers et al., 1994; Dettbarn et al., 1999). These enzymes are ubiquitous in the organism (Satoh and Hosokawa, 1998), although they are particularly abundant in the small intestine of mammals (Taketani et al., 2007). Intestinal CE activity of human and rat hydrolyzes efficiently some pyrethroid insecticides such as *trans*-permethrin (Crow et al., 2007). Collectively, it would not be unwise to assume that gut CEs would play an important role in reducing OP and pyrethroid uptake from gut lumen.

We found high levels of CE activity and isozyme abundance in the gut of *L. terrestris*. Furthermore, the pharynx, crop and foregut CE activities were inhibited by chlorpyrifos. The parental OPs usually are weak esterase inhibitors compared to their highly toxic oxon metabolites. The oxidative desulfuration reaction needed to convert chlorpyrifos into CPOx can be performed by soil microorganisms, and it would explain why esterases are strongly inhibited in the first segment of earthworm gut. Conversely, gizzard CE activities were not affected by the pesticide. They even increased their hydrolytic activity during the first two weeks of the recovery phase (Fig. 4 and Supplementary Table 1). The reason for this observation is unclear, although Sanchez-Hernandez et al. (2009) reported a prominent secretion of CEs in the crop/gizzard segment of *L. terrestris*. This luminal CE activity could contribute to chlorpyrifos sequestration, reducing its intestinal uptake and consequently no inhibition of tissue esterase activity should be found. However, the foregut esterases were found inhibited by the OP. If chlorpyrifos residues are progressively sequestered by irreversible inhibition exerted by luminal CEs during the gut passage of soil, then why esterase activities were again significantly inhibited at the foregut level. Bioactivation of

chlorpyrifos by gut symbionts at the foregut level could be a reasonable explanation. Alternatively, the foregut tissue could be contaminated by chloragogenous tissue (a highly metabolic tissue surrounding the intestine) during dissection, and the esterase inhibition found in the foregut could be that from chloragogenous tissue because pesticide uptake through the skin should not be excluded. Further investigations are still needed to explain this marked variation of esterase responses to chlorpyrifos at very small spatial scale in the gut of *L. terrestris*.

According to our results and those previously published by others, we hypothesize that gut CE activity of *L. terrestris* offers an efficient biochemical barrier against pesticide uptake. Three lines of indirect evidence support this assumption. First, gut CE activity is very sensitive to both in vitro and in vivo inhibition by OP insecticides. Second, a secretion of active CEs has been recently reported for this earthworm species (Sanchez-Hernandez et al., 2009). These luminal CEs would irreversibly bind CPOx residues present in the ingested soil reducing therefore its intestinal uptake. Third, our hypothesis would be workable if we find differences in pesticide toxicity related to exposure route (skin or gut), or if there is an inverse relationship between gut CE activity levels and pesticide toxicity. Henson-Ramsey et al. (2007) found that *L. terrestris* exposed to malathion-spiked soils (gut and dermal exposure) were more tolerant to malathion than those exposed to this OP but using the filter paper exposure test (dermal exposure). Moreover, malathion was less toxic to *L. terrestris* compared to other species such as *E. fetida* or *L. rubellus*. They suggested that high levels of CE activity could be a plausible reason for this tolerance of *L. terrestris* to malathion. Our data provides some evidence supporting the findings by Henson-Ramsey et al. (2007). Further investigations are needed to confirm if CE activity, particularly that present in the gut, provides an efficient mechanism of natural tolerance of earthworms to pesticides.

4.4. Carboxylesterases in the male reproductive system

Organophosphate pesticides exhibit (anti)estrogenic and (anti) androgenic effects (Kitamura et al., 2006). For example, Jewell and Miller (1998) found that the herbicide molinate inhibited a rat testis CE activity causing a concomitant reduction of testosterone levels. Previously, Yan et al. (1995) had characterized a CE activity expressed in the rat testis probably involved in tissue protection against OP toxicity. These investigations suggest that CE activity in the male reproductive system may be a secondary target of OP exposure with ongoing reproduction impairment (Mikhailov and Torrado, 1999).

We have also found relatively high levels of CE activity in the seminal vesicles of *L. terrestris*, particularly using 4-NPV as substrate. Moreover, this CE activity showed a stronger inhibitory response to chlorpyrifos compared to that observed in some portions of the gut. Besides, no recovery to normal enzyme activity was observed during the 35 d of recovery period for all treated groups (Fig. 4 and Supplementary Table 1). This high sensitivity of gonadal CE activity to anti-ChE pesticides has been also documented in rats. Testis α-NA-CE and 4-NPA-CE activities were severely inhibited by 400 mg kg^{-1} (i.p. injection) molinate compared to liver CE activities, and furthermore the activity did not return to control values three weeks after pesticide administration (Jewell and Miller, 1998). Organophosphorus and CB pesticides also cause toxic effects on male reproductive system of earthworms. For example, Sorour and Larink (2001) reported severe damage in the morphology and development of spermatozoids of *E. fetida* exposed during one week to 8.3, 56 and 112 mg benomyl kg^{-1} dry weight. Likewise, Espinoza-Navarro and Bustos-Obregón (2005) documented that malathion altered the cell proliferation and DNA structure of spermatogonia in the seminal vesicles of *E. fetida*. On the other hand, Cikutovic et al. (1993) proposed a simple and rapid method for quantifying the impact of pollutant exposure in the reproductive system of *L. terrestris* through the sperm count. They found that sperm count decreased significantly after one week of exposure to Cd and chlordane. Taken together, these works lead us to suggest that if the method by Cikutovic et al. (1993) is also sensitive to OP pesticides, then it would be attractive to investigate whether CE inhibition in the seminal vesicles is really a promising biochemical biomarker of OP exposure with significant meaning at individual and population levels (reproduction impairment).

4.5. Earthworm esterases in field monitoring of pesticide pollution

Measurement of ChE inhibition is a sensitive, rapid, and widely used biomarker of pesticide (OPs and CBs) exposure in many aquatic and terrestrial organisms (Fulton and Key, 2001; Hill, 2003). When used in field monitoring, stability of ChE inhibition is one of the most desirable features to detect pesticide-exposed individuals several days after agricultural pesticide applications. For example, brain AChE activity of fish often takes one (Sancho et al., 1997) or two (Straus and Chambers, 1995) weeks to recover fully its activity following acute exposure to OP pesticides. Similarly, blood ChE activity of reptiles returned to its normal levels within two (Sanchez et al., 1997) or three (Bain et al., 2004) weeks following severe inhibition by OPs. Conversely, recovery of phosphorylated ChE activity seems to occur faster in homeothermic than in poikilothermic organisms. Female mice administrated with 10 and 30 mg kg^{-1} (i.p. injection) dimethoate reversed brain and serum AChE activities within 24 h (Long et al., 2006). In birds, brain ChE activity recovers completely its normal activity in 1–2 weeks after acute OP exposure (Fleming and Grue, 1981), whereas blood ChE activity needs a few hours to reach normal values of activity (Soler-Rodríguez et al., 1998). This rapid recovery of blood ChE activity is a weakness when this non-lethal biomarker is used alone for field monitoring purposes. Contrarily, earthworm ChE activity displays an extremely slow recovery rate after OP inhibition. For example, a carbaryl-resistant form of ChE in *E. fetida* did not return to its normal activity during 84 d following exposure to 240 mg kg^{-1} dry wt chlorpyrifos (Aamodt et al., 2007). Similarly, no recovery of ChE activity was evident during an observation period of 70 d in *A. caliginosa* previously exposed for 14 d to parathion (1 and 10 mg kg^{-1} dry wt)-spiked soils (Rault et al., 2008). Current results also support this slow recovery of phosphorylated ChE activity in earthworms, which makes these organisms suitable candidates for monitoring field exposure of OP contamination in the below-ground system where pesticides such as chlorpyrifos shows high half-live times (4–8 weeks) (Racke, 1993).

Although investigations on recovery of phosphorylated CE activity are still scarce and limited to laboratory mammals, this esterase is an attractive biomarker of pesticide contamination because of its high sensitivity to inhibition by OP agrochemicals (Wheelock et al., 2008). In addition, it shows a slow recovery rate to normal levels in the liver (Chambers and Carr, 1993) and testis (Jewell and Miller, 1998) of OP-exposed rats. As with mammals, gut (except gizzard) and gonadal CE activities of *L. terrestris* remain long time (>1 month) inhibited after severe OP inhibition. The stability of this response to OP pesticides makes this esterase a complementary biochemical endpoint for field monitoring of exposure to ChE-inhibiting agrochemicals.

5. Conclusions

Three main conclusions can be highlighted when esterases of *L. terrestris* are used as biomarkers of OP exposure. First, ChE and CE activities should be jointly used as biomarkers of pesticide exposure because of their differences in sensitivity to inhibition by OPs. This approach would provide a more comprehensive assessment of the impact of OPs on earthworms, reducing the uncertainty that the high interindividual variation of normal esterase activity causes in the identification of OP-exposed individual. Second, CE activity should be

determined using multiples substrates because of marked differences in CE response against OP exposure, probably as a consequence of enzymological differences in CE isozymes. The use of the substrate 4-NPV revealed a group of CE isozymes very sensitive to chlorpyrifos and showing an extremely slow recovery to full normal activity. Third, ChE and CE activities should be determined in multiple tissues of *L. terrestris* because the response of these esterases is highly dependent on the tissue where they are present.

Current results also suggest that the inhibition of CE activity by OPs in the seminal vesicles should be investigated in relation to its potential role in spermatozoid differentiation and maturation. This approach would enable to propose this esterase as a biochemical biomarker of endocrine disruption with ongoing adverse effects at individual and population levels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbpc.2009.12.008.

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