Exposure to the organophosphorus pesticide chlorpyrifos inhibits acetylcholinesterase activity and affects muscular integrity in *Xenopus laevis* larvae

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Abstract

The effect of organophosphate pesticide chlorpyrifos (CPF) on acetylcholinesterase (AChE) activity and on skeletal muscle development in *Xenopus laevis* larvae was studied. To achieve our purpose embryos were exposed to 100, 250 and 3000 µg/l CPF concentrations from late blastula stage (8 h postfertilization, p.f.) to stage 47 (120 h p.f.) and the appearance of AChE activity was monitored every 24 h. Compared with control, CPF treated larvae showed a dose dependent AChE inhibition during the early stages (beginning from 24 h until 120 h p.f.) that are crucial for neuromuscular development.

The amount of AChE activity that can still be measured in treated larvae at stage 47 relative to that of the control, ranged from 28% in CPFs 100 µg/l to 4% in CPFs 3000 µg/l. These low AChE activities were associated with muscular damages such as reduced myotome size and hypertrophies coupled with extensive vacuolated regions in myocytes. The occurrence of this tissue-specific injury was related to CPF concentrations and was most pronounced in CPFs 3000 µg/l which revealed a very severe AChE inhibition during the exposure.

Since AChE is the major neurotransmitter of the neuromuscular system, this initial descriptive study will be an useful starting-point to ongoing and future subcellular/molecular studies that correlate the morphological damage with changes in AChE activity.
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1. Introduction

Acetylcholinesterase (AChE) is an enzyme characteristic of the insect and vertebrate neuromuscular junctions responsible for the hydrolysis of the neuro-transmitter acetylcholine (ACh), so that the activation of cholinergic receptors is transient. Insect AChE is the primary target of organophosphates (OPs) pesticides, which, by phosphorylating the serine hydroxyl group located in the active site of the AChE, inhibit its activity (Ozmen et al., 1999). As a consequence, ACh accumulation in the synapses and neuromuscular junctions leads to overstimulation of cholinergic receptors that results in a general pattern of nerve poisoning worth

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symptoms such as hyperexcitability, tremors and paralysis. The manifestation of cholinergic signs of toxicity in nontarget species exposed to OPs suggests in these organisms a mechanism common to insects. Seasonal use on agricultural fields and intermittent pesticide applications may produce toxic effects affecting different species that are crucial components of many ecological communities, such as amphibians. In the last few years several researches have hypothesized that pesticide applications are a potential cause of amphibian decline (Wake, 1991; Blaustein and Wake, 1995). Amphibians are particularly vulnerable to these compounds for various reasons: they breed and lay eggs in spring when great quantities of pesticides are applied in agriculture, tadpole skin is highly permeable to toxic substances and metamorphosis represents a critical step during larval development that can be disrupted by exogenous compounds.

Although a link between use of pesticides and amphibian population decline has not yet been established, there is experimental evidence demonstrating that exposure to pesticides may be harmful to embryo development (Lien et al., 1997; Harris et al., 1998; Gillilland et al., 2001; Karen et al., 2001; Sahu and Ghatak, 2002).

OPs elicit severe malformations ascribed as abnormal tail flexure, contorted posture and abnormal notochord in various species of frog embryos. In previous studies, the same malformations were observed in association with a serious impairment of motility in OP treated *Xenopus laevis* larvae, a well-known amphibian experimental model (Snawder and Chambers, 1989, 1990, 1993; Richards and Kendall, 2002, 2003; Bonfanti et al., 2004).

In view of these alterations, a deeper investigation into OPs effects other than the well known AChE inhibition was needed. Damage to skeletal muscle for instance could result in motionless larvae unable to forage or escape predation in the environment.

Since literature data (Behra et al., 2002) reveal that the maintenance of muscle cell integrity in zebrafish clearly depends on AChE activity, in the present work we investigated the extent to which chlorpyrifos (*O,O*-diethyl *O*-[3,5,6-trichloro-2-pyridyl] phosphorothioate) (CPF), one of the most used OPs (Barron and Woodburn, 1995; Lemus and Abdelghani, 2000), inhibited AChE activity during the nervous and muscular system development in *X. laevis* and how the axial muscular integrity could be damaged.

For this purpose exposure to CPF (100, 250 and 3000 μg/l) was carried out from late blastula stage (8 h postfertilization, p.f.) to stage 47 (120 h p.f.). During the exposure time AChE activity was monitored every 24 h because of rapid changes of AChE activity in the first 120 h of development and morphological analysis was carried out at histological and ultrastructural level in 47 stage larvae, when the myogenesis process has led to myofibril filled myotubes.

2. Materials and methods

2.1. Chemicals

The organophosphorus insecticide chlorpyrifos (*O,O*-diethyl *O*-[3,5,6-trichloro-2-pyridyl] phosphorothioate; CPF) with over 99% purity was supplied by Labservice Analytica s.r.l., Italy. The reagents for morphological analysis were purchased by Trimital s.r.l., Italy. All analytical grade reagents were obtained from Sigma-Aldrich s.r.l., Italy.

2.2. In vitro fertilization and embryo treatment

Xenopus laevis adult males and females were purchased from the Centre d'élevage de *Xénopes* du CNRS, Rennes, France. The animals were acclimated for at least six weeks in aquaria with dechlorinated tap water at temperature of 22 ± 2 °C and alternating 12 h light/dark cycles and fed a semi-synthetic diet (Mucedola s.r.l., Settimo Milanese, Italy).

In vitro fertilization was performed as previously reported (Vismara et al., 1993). Briefly, females were induced to ovulate by human chorionic gonadotropin injection. Freshly extruded eggs were inseminated in vitro with a testicular homogenate and after 1 min FETAX solution (Dawson and Bantle, 1987) was added to each Petri dish.

Three groups of 156 embryos each were exposed to CPF (100, 250 and 3000 μg/l in FETAX solution) and one group was untreated and used as control. All test solutions were administered 8 h p.f. until 120 h p.f., and embryos were kept in a thermostatic chamber at 23 ± 0.5 °C; each day the test and control solutions were renewed.

For each experimental group, AChE evaluation was performed on three pools of 10 embryos, sacrificed every 24 h until stage 47 (120 h p.f.) whereas morphological analysis was carried out on six embryos at 120 h p.f. All the experiments were repeated three times and the embryo pools were always randomly sampled.

2.3. AChE activity analysis

Control and CPF exposed pools were used for AChE evaluation. Each assay was performed in triplicate using a modified Ellman procedure (Ellman et al., 1961) with acetylthiocholine iodide (0.075 M) as a substrate. The Ellman protocol was adapted to quantify AChE activity in *X. laevis* embryos (Gindi and Knowland, 1979).

Briefly, after being repeatedly rinsed in dechlorinated tap water, whole body embryos were chilled on ice and homogenized in Tris buffer (1% Triton X-100 in 0.05 M Tris–HCl, pH 7.4) at 1:10 (w/v) and centrifuged for 5 min at 15 000*g*. The presence of thiol groups masks low levels of AChE causing an incorrect evaluation of

enzyme activity. For this reason, before adding the substrate, a pre-incubation of the extracts with 0.33 mM 5,5′-dithio-bis (2-nitrobenzoic acid) for 10 min was necessary. The absorbance changes were followed every 15 s for at least 15 min at 412 nm.

Preliminary experiments were conducted to define conditions for sample concentration required for linear rates of substrate hydrolysis. AChE activity was calculated as nmol of acetylthiocholine iodide hydrolyzed per min per g embryos.

Statistical analysis was performed using the program STATGRAPHICS PLUS, version 5.1. Data sets that were normally distributed and possessed equal variances were analyzed using a multifactor analysis of variance (ANOVA) to determine if there were significant ($P \leqslant$ 0.05) differences among treatments, with respect to AChE activities. Tukey's method was used to determine if significantly different AChE activities existed among the different factors: CPF concentrations and time points; Dunnett's method was used to determine which treatments were significantly different from the control.

2.4. Morphological studies

Stage 47 (120 h p.f.) control and exposed larvae were fixed for 2 h in modified Karnowsky's liquid (2% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4), repeatedly rinsed in the same buffer and postfixed for 2 h in 1% OsO_4 in phosphate buffer, pH 7.4. Following rinsing in 0.1 M phosphate buffer, the materials were dehydrated in graded alcohol series and embedded in epoxy resin Epon 812. The Epon blocks were cut on an LKB ultramicrotome. Longitudinal semi thin sections (0.5 μm) were stained with crystal violet and basic fuchsin and examined under a light Zeiss Axioplan MC 100 microscope. Images were taken by a colour digital camera (Image Pro Plus version 4.5.1, Media

Cybernetics). Ultrathin sections were contrasted with uranil acetate and lead citrate according to the standard Reynolds method (Reynolds, 1963), and examined in a TEM Jeol JEM 1220 at an accelerating voltage of 80 kV.

3. Results

3.1. AChE activity

The effect of CPF on AChE activity was monitored every 24 h in developing embryos during the exposure time. In control no enzyme activity was found at 24 h p.f. A consistent increase was seen starting from 48 h up to 120 h p.f. (Fig. 1), in relation to the development of the nervous and muscular system. The exposure to CPF elicited an inhibition of AChE activity in each treated group and this residual activity was statistically different from control activity starting from 72 h p.f. (Fig. 1). Moreover, the residual AChE activity remained almost unvaried during the stages of development studied. At the end of exposure (stage 47, 120 h p.f.) the enzyme inhibition ranged from 72% at 100 μg/l to 96% at 3000 μg/l in comparison with the control. The AChE mean activities at 3000 μg/l were statistically different from those at 100 μg/l starting from 72 h p.f.

3.2. Morphological analysis

On gross morphological analysis the all treated larvae ($n = 18$ for each treatment group) showed impairment of motility, such as tremors and spasms and, at the highest concentration, paralysis compromising larvae swimming ability. In particular the impairment of motility was associated with physical abnormalities, ascribing to an abnormal tail flexure (Fig. 2B), in six out of 18 larvae in CPFs 100 μg/l, in 12 out of 18 larvae in CPFs 250 μg/l and in 18 out of 18 larvae in CPFs 3000 μg/l.
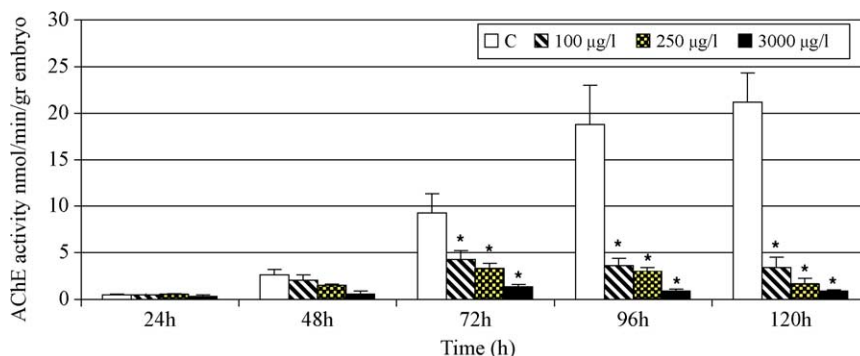

Fig. 1. AChE activity in *X. laevis* larvae control and exposed to CPF 100, 250 and 3000 μg/l. Each value represents the mean ± SD obtained from three independent assays carried out in triplicate on three pools of larvae. Asterisk indicates statistically significant difference from control ($P < 0.05$).
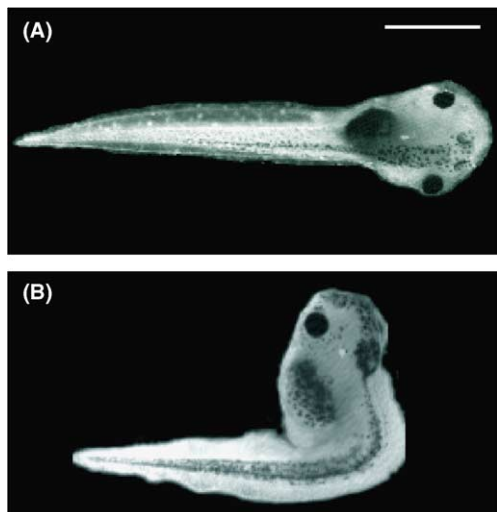
Fig. 2. Stage 47 (120 h p.f.) *X. laevis* larvae. Dorsal view of control larva (A) and abnormal tail flexure of larva exposed to 3000 μg/l CPF (B). Scale bar = 3 mm for A and B.

3.3. Histological studies

Longitudinal tail tissue sections, obtained from larvae at 120 h p.f., were analyzed from control and each treatment group ($n = 18$). The control tails showed a reg-

ular myotomal structure defined by intersomitic boundaries to which myocytes, oriented in parallel to the notochord, are anchored (Fig. 3A). In each myotome the regular arrangement of striated muscle was evident with succession of A and I bands.

After exposure to CPF, the tail musculature revealed a reduction in myotomal length and alteration in its structure (Fig. 3B–D) due to the appearance of extracellular spaces between myocytes and vacuolated regions. Moreover, the myocytes revealed a disorganization of the contractile apparatus with the presence of hypertrophied areas. These alterations of tail muscle structure increased in a dose dependent manner in all three independent trials. Indeed, the damage, limited in CPFs 100 μg/l (Fig. 3B), was more extensive in CPFs 250 μg/l where myotomal boundaries were undefined (Fig. 3C), whereas in CPFs 3000 μg/l the myotomal structure was completely destroyed and the muscle fibres entirely degenerated (Fig. 3D). In particular in the bent region of CPFs 100 μg/l and 250 μg/l the myotome disorganization was more severe than in nonbent areas.

3.4. Ultrastructural studies

A preliminary analysis of ultrastructural morphology was performed on control larvae and exposed to 3000 μg/l CPF with the attempt to visualize to a greater detail the vacuolated areas. In longitudinal control sec-
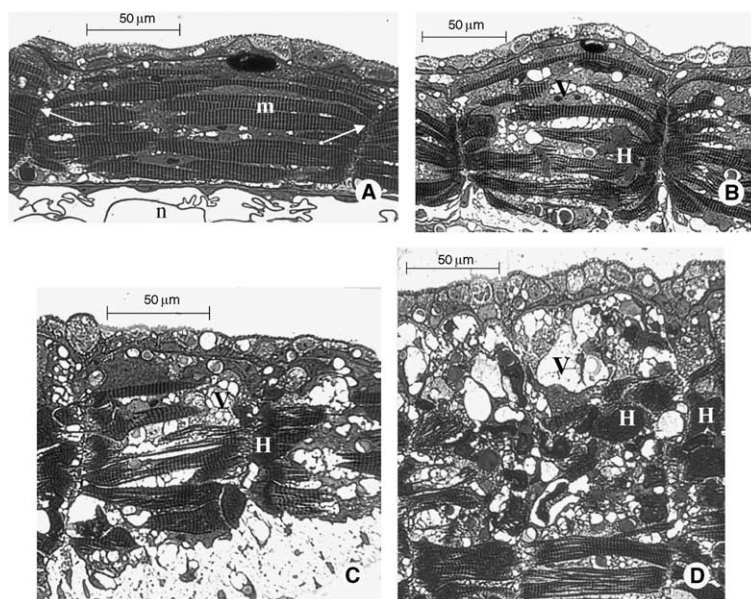

Fig. 3. Histological analysis of tail muscle in control and CPF treated *X. laevis* larvae at stage 47 (120 h p.f.). Myotomes of control larva showed contiguous muscle cells (m) across myotomal boundaries (white arrow) oriented in parallel to the notochord (n) (A). Treatment with 100 μg/l (B), 250 μg/l (C) and 3000 μg/l (D) of CPF caused an increasing disorganisation of muscle cells by the appearance of vacuolated (V) and hypertrophied (H) regions leading to the loss of intersomitic boundaries in the bent tail region. Scale bar = 50 μm for A, B, C and D.
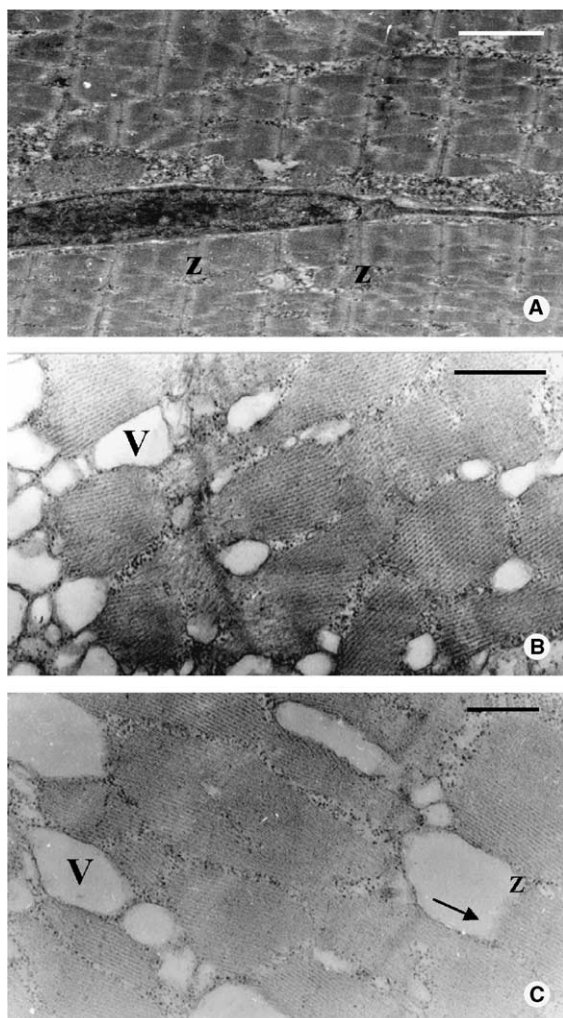
Fig. 4. Ultrastructural morphology of tail muscle in control and 3000 μg/l CPF *X. laevis* larvae at stage 47 (120 h p.f.). Longitudinal section of striated muscle in control larvae showed a regular disposition of sarcomeres (A) (×3000, scale bar = 3 μm). Thin sections of CPFs treated showed the presence of vacuolated regions (V) among the myofibrils (B) (×15000, scale bar = 0.6 μm) and the break down of myofilaments (black arrow) from Z-disk (z) (C) (×20000, scale bar = 0.4 μm).

tions, myocytes exhibited a repeating array of sarcomeres well defined by Z-disks (Fig. 4A). In contrast, the CPF larvae showed extensive regions of vacuolated sarcoplasm interrupting the regular myofibril arrangement (Fig. 4B), although it was hard to make longitudinal sections in the bent tail regions. The morphology of the vacuolated region at high magnification suggested a break down of myofilaments from Z-disk due to the lost of their connection with the Z-disk protein complex (Fig. 4C).

4. Discussion

Many literature data evidenced that OPs elicit AChE inhibition and morphological alterations such as notochordal sheath defects in amphibians (Snawder and Chambers, 1993; Richards and Kendall, 2002, 2003). Our study concerning the effects of the commonly used insecticide CPF on *X. laevis* larvae produced two major findings. First, exposure of embryos to CPF from blastula stage (8 h p.f.) until stage 47 (120 h p.f.) inhibited AChE activity in a dose-dependent manner. Second, a morphological inspection of larvae at 120 h p.f. revealed dose-dependent, tissue-specific damage in the tail musculature, besides the notochordal sheath alteration, which was unknown in nonmammalian vertebrates.

OPs and CPF in particular are heavily used in agriculture and in households because they have replaced the more persistent organochlorine pesticides. Theoretically they should not accumulate in the environment, but unexpectedly high concentrations of CPF in remote areas far from utilization site were found (Chernyak et al., 1996; Garbarino et al., 2002; Muir et al., 2004). This fact increased the importance to add investigations on potential risk deriving from CFP exposure.

The tested concentrations (100, 250 and 3000 μg/l) were experimental ones and were higher than those found in the environment and than LC_{50} values reported for crustacean and insect larvae (<1 μg/l) and for fish (1–100 μg/l) (Barron and Woodburn, 1995); however these concentrations were chosen in order to obtain histological information that may serve as the first descriptive step in determining the molecular mechanisms underlying the effect of AChE inhibition caused by CPF on muscle damage.

The rapid development of the neuromuscular system in *Xenopus* (Seidman et al., 1994) makes it an excellent in vivo model to study OP toxicity and to identify xenobiotics that cause muscular disorders.

In control embryos, the development and ultrastructural maturation of the neuromuscular system give rise to a free swimming tadpole within 4–5 days. Concurrently, in our results quantifiable levels of AChE activity first appeared from 24 to 48 h p.f., and this activity increased with each progressive stage of outgrowth according to Gindi and Knowland (1979).

AChE inhibition in CPF treated larvae proved to be dose-dependent and in contrast to the control, the still measurable AChE activity in CPF treated groups stabilized at values not much higher than those measured at 48 h during the exposure time. This underlines the fact that AChE activities were very low, compared with the controls, during a crucial period for neuromuscular development.

AChE residual activities in larvae at stage 47, ranging from 28% in CPFs 100 μg/l to 4% in CPFs 3000 μg/l in comparison with the control, were associated with

muscular damage. The occurrence of this tissue-specific injury was related to CPF concentrations and was most pronounced in CPFs 3000 µg/l, which revealed a most severe AChE inhibition during the exposure. In our histological studies a severe histopathological damage, such as reduced myotome size and hypertrophies coupled with extensive vacuolated regions in myocytes, was observed in CPFs 3000 µg/l. According to CPF teratogenic concentration value in 50% of larvae ($TC_{50} = 161.54$ µg/l) found in our previous teratogenesis assay (Bonfanti et al., 2004), tail flexure did not occur in all larvae exposed to CPF 100 and 250 µg/l, whereas it appeared in 100% of the larvae exposed at the highest concentration. Although more severe muscular abnormalities were evidenced in the bent tail region, an initial muscle disorganization was found by histological analysis in all bent and nonbent larvae exposed to lower CPF concentrations.

Other authors have found a quantitative relation between AChE levels and skeletal muscle necrosis in OP treated rats, suggesting that the loss of AChE activity could be the trigger mechanism leading to muscular degeneration (Gupta et al., 1987; De Bleecker et al., 1994). Behra et al. (2002) reported that a mutation in zebrafish *ache* caused impairment of motility and severe muscular system defects during early larval stages similar to those observed by us in *X. laevis* exposed to CPF. According to literature data we hypothesize that the inhibition of AChE, elicited by the pesticide, is involved in the appearance of muscle damage in CPF treated larvae.

Some authors ascribed the musculature impairment in OP treated adult mammals to an excessive calcium influx caused by ACh accumulation and subsequent overstimulation of the postsynaptic membrane (Wecker et al., 1986; De Bleecker et al., 1994). The involvement of calcium as the mediator of muscle necrosis has been described by Leonard and Salpeter (1979) who related the myocyte disorganization to Ca^{2+}-activated proteases which specifically disarray Z-disks in mammalian skeletal muscles. In zebrafish *ache* mutant embryos muscular degeneration was evident starting from 48 h p.f. when increasing and focalized muscle stimulation lead to accumulation of ACh in the synapse and constant occupancy of ACh receptors (Behra et al., 2002).

The loss of characteristic striation and the presence of wide areas with frayed myofibrils, observed in malformed tail regions of *Xenopus* larvae, are in agreement with the above hypothesis. The marked hypertrophies present in myocytes suggest an aggregation of broken myofibrils and a perturbation of the contractile apparatus. The myofibril organization at ultrastructural level in CPFs 3000 µg/l was altered by the presence of vacuolated areas. These areas seemed to be deprived of myofilaments suggesting the loss of a mechanical link between actin filaments and Z-disk proteins, such as α-actinin, or the costameric proteins and the extracellular matrix. The perturbations in the connectivity due to disorganization or proteolysis of protein complexes, involved in the highly ordered sarcomeric structure, could be why CPF induced muscle degeneration. Indeed, similar myocyte ultrastructural alterations are expressed in pathologies where specific proteins involved in sarcomeric structure are modified. The genetic alterations in dystrophin lead, for instance, to muscle fragility, contraction-induced damage and necrosis (Spence et al., 2002). A concomitant perturbation of collagen synthesis (Snawder and Chambers, 1993), could be another factor contributing in the alteration of muscular tissue architecture as well as in the loss of intersomitic boundaries.

In conclusion, to the best of our knowledge the results obtained indicate for the first time the CPF dose-dependent effect on the tail muscle apparatus during the development of anuran larvae.

Although no literature data exist about CPF effects on adult *Xenopus* musculature, the experimental evidences described in adult mammals (Wecker et al., 1986; De Bleecker et al., 1994) suggest that the link between muscular damage and AChE inhibition could be displayed not only during development but in adult animals as well. Moreover a different potential risk could be noticed within amphibian species in relation to their wide range of sensitivity to CPF (Barron and Woodburn, 1995).

Nevertheless, further studies concerning subcellular/molecular mechanisms are called for to clarify the relationship between the AChE inhibition and muscular damage.

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