RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2007.33.301

ScienceAsia 33 (2007): **301-305**

Variability in Acetylcholinesterase upon Exposure to Chlorpyrifos and Carbaryl in Hybrid Catfish

Chawanrat Somnuek,[a] Voravit Cheevaporn,[a*] Chutarat Saengkul[a] and F.William H. Beamish[b]

[a] Graduate School of Environmental Science, Faculty of Science, Burapha University, Chonburi 20131, Thailand.
[b] Department of Biology, Faculty of Science, Burapha University, Chonburi,20131, Thailand.

* Corresponding author, E-mail: voravit@buu.ac.th

Received 23 Aug 2006
Accepted 9 Feb 2007

ABSTRACT: AChE was measured in brain, liver, muscle and gill tissues of hybrid catfish (*Clarias macrocephalus* x *Clarias gariepinus*) exposed to a sublethal concentration of an organophosphate, chlorpyrifos and a carbamate, carbaryl, for 4 days. AChE inhibition increased rapidly with insecticide concentration. Relative inhibition of AChE was higher in larger fish but did not differ significantly with sex. Relative inhibition of AChE accompanying insecticide exposure was highest in the brain tissues and progressively less in the liver, muscle and gill tissues. Insecticide concentrations and AChE inhibition in the brain increased over the 4-day sublethal exposure. After transfer to insecticide-free water, AChE inhibition and insecticide residue in the brain decreased but remained above control values over the 4-day recovery period.

KEYWORDS: acetylcholinesterase; chlorpyrifos ; carbaryl; cholinergic insecticide; hybrid catfish.

INTRODUCTION

Organophosphates and carbamates have become the most widely used classes of insecticides in the world replacing the persistent and problematic organochlorine compounds. Both are anticholinergic agents that bind to the esteric site of the enzyme acetylcholinesterase (AChE). Because of the specificity of this relationship, inhibition of AChE is widely used as a specific biomarker for these insecticides.[1] Organophosphates and carbamates can enter aquatic systems that drain agricultural watersheds through aerial overspray and with runoff. Exposure of aquatic ecosystems to these insecticides is difficult to assess because of their short persistence in the water column due to low solubility and rapid degradation.[2] However, monitoring of these insecticides is important because they are highly toxic to aquatic organisms.[3] Thus, a reliable bioindicator of exposure would be useful.

Measurement of AChE activity in aquatic organisms has been suggested as a surrogate of organophosphate and carbamate exposure but has shown high variability in nearly all species on which measurements have been made.[4-5] AChE activities in relation to exposure to these insecticides differs between and within species. Potential sources of variation within species include biotic and abiotic factors.[4]

This study was performed to investigate the influence of Chlorpyrifos and Carbaryl concentration and exposure time on AChE activity in hybrid catfish (*Clarias macrocephalus* x *Clarias gariepinus*) as well as the biotic factors, body mass and sex. Hybrid catfish, is an indigenous and widely distributed fresh water fish in southeast Asia as well as being intensively cultured in ponds for food.

MATERIALS AND METHODS

Testing Organisms

Adult catfish weighing 130-150 g (2.5 months old) and juveniles of 10-15 g (0.5 months old) were used throughout the study. Fish were kept in a cage submerged a larger tank. Ambient water temperature was 23 ± 2 °C, pH 7.4 ± 0.5 and dissolved oxygen, 5 ± 1.5 mg/l. Fish were held for at least 15 days before exposure to the insecticide.

Test Conditions

Experiments were carried out in a continuous flow system in 50 litre test tanks with the same water characteristics described above. Chlorpyrifos and Carbaryl (95.0% purity) were purchased from Gharda Chemicals Limited, Maharashtra, India and Hunan Haili Chemical Industry Co. Ltd., Hunan, China, respectively. Aqueous solutions of the insecticide were prepared by dissolving the insecticides in acetone, diluted with an appropriate amount of water, and added to the aquaria. The concentration of acetone was 0.05 % in all test and control solutions.

Acute toxicity was calculated for each insecticide by probit analysis from the results of two replicates for each test concentration.[6] The 96 h LC_{50} for chlorpyrifos

and carbaryl were 33 and 48 mg/l, respectively. The sublethal concentration of 15 and 24 mg/l for chlorpyrifos and carbaryl were estimated from probit regression and were selected as the sublethal concentrations for the study.[6]

Variation in AChE inhibition by sex, male and female catfish was examined in replicate aquaria at the sublethal concentration Sex was examined on 10 fish of each sex. After 4 days exposure, fish were sacrified and AChE activity in brain tissues measured.

To investigate variation in AChE inhibition by fish mass, two groups of 10 fish, 130-150 g and 10-15 g, were used and treated in the same manner. However, to avoid any potential effect from sex, only male fish were used. Following an exposure period of 4 days, fish were then sacrified and brain, gill and muscle tissues excised for subsequent analyses of AChE activity.

The relationship between insecticide concentrations and AChE inhibition were examined over the sublethal ranges, 0 to 15 mg/l and 0 to 24 mg/l for chlorpyrifos and carbaryl, respectively, each for 4 days. In the toxicokinetic study, fish were exposed to 15 and 24 mg/l of chlorpyrifos and carbaryl, respectively, in the continuous flow system for 96 hours. Fish were then moved to a tank supplied with a continuous flow of insecticide-free water for another 96 hours. AChE activity and insecticide concentration in the brain tissues were measured during exposure and after transfer to insecticide-free water.

AChE Activity Analysis

Tissue samples were excised and homogenized (20 mg/ml) in 20 mM Tris/HCl, pH 7.5, plus 0.5 mM EDTA. Aliquots of brain homogenate were taken for AChE analysis. The AChE assay was carried out following the method of Ellman et al [7] and is based on a colorimetric assay employing the reaction of thiocholine with 5,5 – dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction produces the compound 5-thio-2-nitrobenzoic acid and is effective for colorimetric measurement with absorbance at 412 nm.

Protein Analysis

Total protein analysis was conducted with 100 µl of tissue homogenate from each fish using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Life Sciences Group, CA, USA). Colorimetric analysis was performed following the standard procedure outlined in the protein assay instructions. Bovine serum albumin (BSA) was used to obtain a standard curve from which relative measurements of protein concentration in the samples were made.

Carbaryl Analysis

Evaluation of carbaryl concentration in the tissue was carried out according to USEPA (1989) method 531.1.[8] The HPLC system consisted of a Varian (9070) fluorescence detector and PCX 5100 postcolumn derivatization instrument. The fluorescence detector was set at excitation 330 and emission 465 nm. For isolation of carbaryl from animal tissues, 2.5 ± 0.1 g of a pool of tissue was homogenized with an aliquot of ethyl acetate. Tissues were extracted three times with 80 ml of ethyl acetate, in different funnels. The organic layers were mixed and concentrated on a rotary vacuum evaporator. The residue was redissolved in 10 ml of acetonitrile. The acetonitrile extracts were evaporated to 1-1.5 ml and applied to a "Sep Pak" cartridge (Florisil) previously rinsed with acetonitrile. The eluate obtained was evaporated to dryness at 45 °C, and immediately redissolved in 100 ml of methanol. The sample was filtered and then injected into the HPLC system. The mean recovery rate of carbaryl was 95 %.

Chlorpyrifos Analysis

Tissue samples were homogenized with 10 ml of toluene and the mixture agitated for 60 minute at 100 rpm on a horizontal shaker at 25 °C. After separation of layers, the toluene extract was filtered through anhydrous sodium sulfate and evaporated in a rotary evaporator at 40 °C. One ml of toluene was added to the evaporate and was analyzed by Gas Chromatography (Hewlett Packard 7540) equipped with FID detector (2 mm i.d. x 120 cm long borosilicate glass column). Temperatures of the injection block, oven and detector cell were 300, 220, and 300 °C, respectively, during operation. Gas flow rates of 60 ml/min for helium carrier gas, 40-43 ml/min for hydrogen and 325 ml/min for air were used.[9] The mean recovery rate of chlorpyrifos was 97 %.

Data Analysis

Results are expressed as means ± SE. ANOVA were used to test for differences between each treatment. Results were expressed as percentage of inhibition of AChE activity. Percent inhibition of AChE was calculated from:

Percent inhibition of AChE = $(C - T) \times 100/C$

C = Cholinesterase activity in control (nmoles/min/mg protein)

T = Cholinesterase activity in treatment (nmoles/min/mg protein)

Results

Variation in AChE Inhibition by Tissue

Specific AChE activity was highest in muscle followed by the brain, gill and liver, respectively (Table 1).

However, chlorpyrifos and carbaryl-induced reductions in AChE activity were greatest in the brain

Table 1. Acetylcholinesterase (AChE) activity in tissues of catfish (n=10). Exposure time was 96 hours.

	Acetylcholinesterase activity (nmoles/min/mg protein)			
	brain	muscle	liver	gill
Control	234.8 ± 8.9	325.3 ± 6.2	135.8 ± 6.5	138.5 ± 6.8
Chlorpyrifos (15 mg/l)	78.3 ± 5.7	175.7 ± 4.7	55.4 ± 3.7	89.2 ± 1.9
Carbaryl (24 mg/l)	112.3 ±11.3	239.9 ± 9.1	76.9 ± 4.1	123.4 ± 7.5

(67 and 48%, respectively) followed by the liver (61 and 43%), muscle (46 and 26%) and gill (36 and 11%) tissues after a 96-hour exposures to sublethal concentrations (Fig. 1). The reductions in AChE activity in the brain were significantly greater than those for any other tissues, although significant differences were found also among the four tissues ($p<0.05$). Thus, on the basis of its sensitivity to insecticide exposure, brain tissues was selected to examine the effects of sex and body mass.

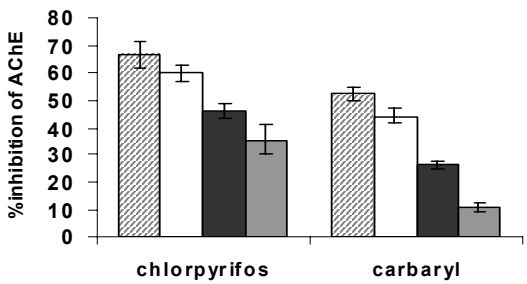

Fig 1. Inhibition of AChE activity as a percentage of total in various tissues. Data are calculated from values shown in Table 1.brain ▨, liver □, muscle ■, gill ▩.

Variation in AChE Inhibition by Sex and Body Mass

Relative inhibition of AChE induced by chlorpyrifos and carbaryl did not differ significantly between male and female catfish ($p>0.05$) (Fig. 2). In sharp contrast, relative inhibition of AChE in the brain tissue differed significantly with mass ($p<0.05$), as small fish was less responsive to the insecticides than large fish (Fig. 3).

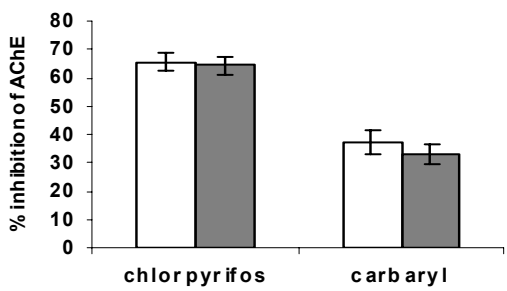

Fig 2. Inhibition of brain AChE activity as a percentage of AChE activity in the control samples from male and female catfish. □ male ▩ female.

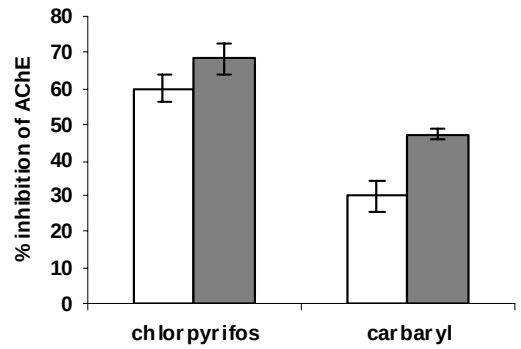

Fig 3. Inhibition of brain AChE activity as a percentage of AChE activity in the control samples from small- and large-sized catfish. □ small ▩ large.

Variation in AChE inhibition by exposure concentrations

AChE inhibition in brain tissues increased rapidly with sublethal concentration of both insecticides (Fig. 4). After 96 hours of exposure to carbaryl (Fig. 4, B), AChE inhibition increased rapidly to about 56% of the control AChE activity at 6 mg/l of the insecticide. AChE inhibition continued to increase with carbaryl concentration but at a slower rate until reaching about 70 % of the control AChE activity at 24 mg/l. The pattern of inhibition change was similar in the brain tissues of fish exposed to sublethal concentrations of chlorpyrifos. Again, inhibition increased rapidly to 73% of the control AChE activity at 10 mg/l of chlorpyrifos but more slowly with further increase in exposure concentration (Fig. 4, A).

Time Course of Brain AChE Inhibition and Recovery

Inhibition of AChE increased rapidly within 24 hours after exposure to the sublethal concentrations of both insecticides, and thereafter remained almost constant (Fig. 5). Chlorpyrifos and carbaryl concentrations in the brain tissues also increased rapidly from 0.2 - 2.2 and 2.0 – 4.7 µg/g, respectively, over the 96-hour exposure period. However, the increase of both insecticides in the brain tissues after 24-hour exposure had no effect on AChE inhibition. After the fish were moved to the tanks supplied with insecticide-free water, the carbaryl concentration in the brain tissues decreased rapidly to almost one half within 30

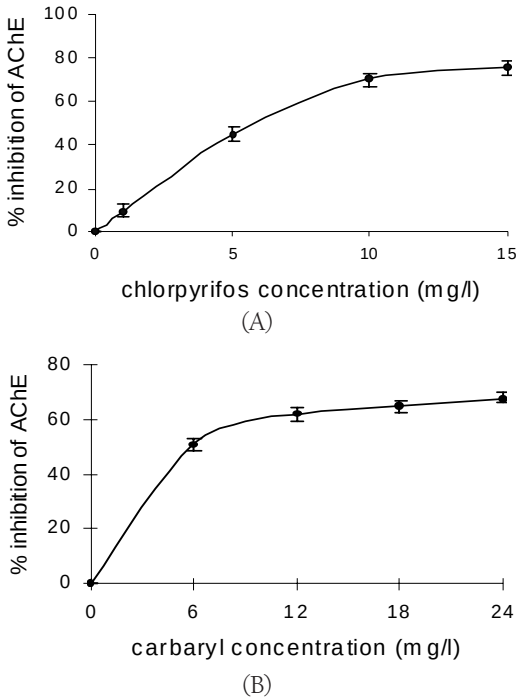

Fig 4. Variability of inhibition of AChE were assessed after a 96-hour exposure to various concentrations of chlorpyrifos (A)and carbaryl (B).

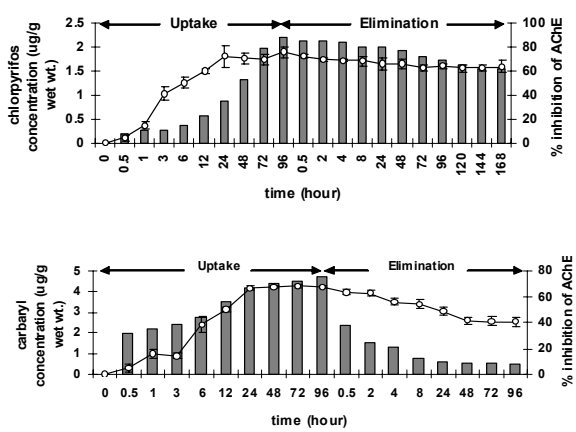

Fig 5. Inhibition and recovery of AChE activity in the brain of catfish during exposure to sublethal concentration of chlorpyrifos and carbaryl and after being transferred to insecticide-free water. Catfish was exposed to sublethal concentration of chlorpyrifos (15 mg/l) and carbaryl (24 mg/l) for 96 hours, then transferred to insecticide-free water. AChE activity and insecticide concentration in the brain tissue were assessed at the indicated time points. Data are means ± SE. Opened circles indicate percent inhibition of AChE activity and closed bars represent tissue insecticide concentration.

min and remained more or less constant at about 0.5 μg/g after 24 hours. Thereafter recovery of AChE activity was slower, reaching only about 40% after 96 hours in the carbaryl-free water. In contrast to carbaryl, the chlorpyrifos concentration in the brain tissues decreased much more slowly during the elimination period. Recovery of AChE activity occurred slowly, not being complete after 7 days in the insecticide-free water.

Discussion

The present study found AChE inhibition in the brain of catfish is more sensitive to chlorpyrifos and carbaryl exposure than that in the liver, muscle and gill tissues in accord with earlier observations on common sole (*Solea solea*) and European seabass (*Dicentrachus labrax*).[10-11] In contrast to these observations, *in vitro* studies conducted by Carr, et al[12] on mosquito fish (*Gambusia affinis*) demonstrated that muscle AChE is more sensitive to organophosphates than that in brain. Such a discrepancy might indicate differences among species and could also be due to the effects of chemical impurities in the synthesis of these insecticides.

This study found no significant difference in carbaryl-induced AChE inhibition between male and female catfish, in agreement with the observations by Beauvais[13] who found no significant difference in the brain AChE activity by sex in bluegill (*Lepomis macrochirus*).

The higher percent inhibition of AChE in larger and older catfish was consistent with observations by Flammarion et al.[14] They found inhibition of AChE activity in the brain of large and presumably older rainbow trout (*Oncorhynchus mykiss*) was higher than that of smaller fish. However, the results of this study were in contrast with those reported by Walker and Thompson[15] who found mature and larger bluegill sunfish were less susceptible than juveniles. Such a discrepancy with the present study might indicate differences among species.

Concentration of pesticide affected AChE inhibition. In this study, AChE inhibition increased rapidly with insecticide concentrations reaching slightly more than 70 and 80% of the control AChE activity at the highest sublethal concentrations of carbaryl and chlorpyrifos, respectively. Zinki et al[3] proposed that depressions of 70-90 % brain ChE activity occurs at the LC_{50}. The current data indicate that an inhibition of this magnitude may not be lethal to all species but that it may exercise a deleterious impact on important activities such as swimming and motivation.

AChE inhibition increased rapidly within 24 hours after exposure to the sublethal concentration of both insecticides and remained almost constant thereafter. After the fish was transferred to insecticide-free water,

the percent inhibition of AChE activity by carbaryl decreased from about 60 to 40 % of the control AChE activity after 48 hours. Recovery of carbaryl-inhibited AChE activity may occur through spontaneous reactivation as well as synthesis of the carbamylated enzyme. Kuhr and Dorough[16] reported a half-life for the reactivation of the carbamylated enzyme of 30-40 min, resulting in a complete recovery of enzyme concentration in several hours. In the current experiment, when the catfish was transferred to the carbaryl-free water, the enzyme activity recovered more slowly reaching about 60 % of the control AChE activity after 48 hours. This suggests that both reactivation and new synthesis of enzyme participate in the process for hybrid catfish. Recovery of AChE activity in fish exposed to chlorpyrifos was even slower than that for carbaryl, reaching only about 40 % of the control AChE activity after 7 days. This indicated that aging of the enzyme complex is possible with chlorpyrifos. Structural changes are imposed on the enzyme by chlorpyrifos, such as covalent modifications, making the inhibition of the enzyme activity permanent.[17]

In conclusion, this study demonstrated the effect of chlorpyrifos and carbaryl on AChE activity in catfish. AChE inhibition in the brain appears as an early process in response to sublethal exposures, and could be a more sensitive biomarker than AChE inhibition in the liver, muscle, and gill to characterize toxicological impacts. Duration of exposure and concentration of the insecticide may also play a role, as may the size of the test subjects. At sublethal concentrations, chlorpyrifos causes greater inhibition of AChE activity in the brain tissues of catfish than carbaryl does.

Acknowledgements

Financial support from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0051/2548) to Ms. Chawanrat Somnuek and Associate Professor Dr. Voravit Cheevaporn is acknowledged.

References

1. Matsumura F (1985) Toxicology of Insecticides. Plenum Press, New York,USA.
2. Brock TCM, Crum SJH, van Wijngaarden R, Budde BJ, Tijink J, Zuppeli A and Leeuwangh P (1992) Fate and effects of the insecticide Dursban 4E in indoor *Elodea*-dominated and macrophyte-free freshwater model ecosystems: I. Fate and primary effects of the active ingredient chlorpyrifos. *Arch Environ Contam Toxicol* **23**, 69-84.
3. Zinki JG, Lockhard WL, Kenny SA and Ward FJ (1991) The effect of cholinesterase inhibiting insecticides on fish. In : *Cholinesterase-inhibiting Insecticides.* (Edited by Mineau P), pp. 233-54. Elsevier, New York, USA.
4. Habig C and Di Giulio RT (1991) Biochemical characteristics of cholinesterase in aquatic organisms. In: *Cholinesterase-inhibiting Insecticides.* (Edited by Mineau P), pp. 19-33. Elsevier, New York, USA.
5. Olson DL and Christensen GM (1980) Effects of water pollutants and other chemicals on fish acetylcholinesterase (*in vitro*). *Environ Res* **21**, 327-35.
6. Echobichon DJ (1997) The Basis of Toxicity Testing. CRC Press, NewYork, USA.
7. Ellman GL, Courtney KD, Andres Jr. V and Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**, 88-95.
8. USEPA (1989) Measurement of N- Methyl Carbamoyloximes and N-Methyl Carbamates in Drinking Water by Direct Aqueous Injection LC with Post-column Derivatization.. Method 531.1 (Graves,R.L., Ed.) EPA, Environmental Monitoring and Support Laboratory, Cincinnati, USA.
9. Venkata Mohan S, Sirisha K, Chandrasekhara Roa N, Sarma PN and Jayarama Reddy S (2004) Degradation of Chlorpyrifos contaminated soil by bioslurry reactor operated in sequencing batch mode: bioprocess monitoring. *J Hazard Mater* **B116**, 39-48.
10. Straus DL and Chambers JE (1995) Inhibition of acetylcholinesterase and aliesterase of fingerling channel catfish by chlorpyrifos, parathion, and S,S,S-tributyl phosphorotrithioate (DEF). *Aquat Toxicol* **33**, 311-24.
11. Nemesok J, Nemeth A, Buzas ZS and Boross L (1984) Effects of copper, zinc, and paraquat on acetylcholinesterase activity in carp (*Cyprinus carpio* L.) *Aquat Toxicol* **5**, 23-31.
12. Carr RL , Ho LL and Chambers JE (1997) Selective toxicity of chlorpyrifos to several species of fish during an environmental exposure : Biochemical mechanisms. *Environ Toxicol Chem* 1**6**, 2369-374.
13. Beauvias SL, Cole KJ, Atchison GJ and Coffey M (2002) Factors affecting brain cholinesterase activity in bluegill (*Lepomis macrochirus*). *Water, Air and Soil Pollution* **135**, 249-64.
14. Flammarion P, Noury P and Garric J (2002) The measurement of cholinesterase activities as a biomarker in chub (*Leuciscus cephalus*): the fish length should not be ignored. *Environ Pollut* **120**, 325-30.
15. Walker CH and Thompson HM (1991) Phylogenetic distribution of cholinesterase and related esterase. In: *Cholinesterase-inhibiting insecticides-their impact on widelife and the environment.* (Edited by Mineau P), pp.1-18. Elsevier Science Publishers, Amsterdam, The Netherlands.
16. Kuhr RJ and Dorough HW (1976) Carbamate Pesticides: Chemistry, Biochemistry, and Toxicology. CRC Press, Cleveland, OH, USA.
17. Silver A (1974) The Biology of Cholinesterases. North-Holland Publishing Company, Amsterdam, The Netherlands.