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Evaluation of Liver and Brain Esterases in the Spotted Gar Fish *(Lepisosteus oculatus)* as Biomarkers of Effect in the Lower Mississippi River Basin

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The responses of various xenobiotic metabolizing enzymes in fish models are rapidly evolving as important biomarkers for monitoring unacceptable levels of environmental contaminants. Ethoxyresorufin O-deethylase, a specific cytochrome P_{450}-dependent monooxygenase, is often used as an indicator of polycyclic aromatic hydrocarbon pollution. (Payne et al. 1987). Another class of enzymes which are potential biomarkers are the B-type esterases (Huang et al. 1993a; Labrot et al. 1996). These enzymes are sensitive to inhibition by organophosphates, and include the cholinesterases (ChE) and carboxylesterases. ChEs are further subdivided into acetylcholinesterase (AChE; EC 3.1.1.7) and butyryl cholinesterase (BuChE; EC 3.1.1.8). Among fish, AChE is predominantly localized in the brain and muscle, whereas, BuChE activity is found mainly in liver and plasma (Habig and Di Giulio, 1991). AChE plays a vital role in maintaining the normal neural functioning of the sensory, integrative, and neuromuscular systems. The precise physiological role of BuChE is unknown, although it has been regarded as a marker enzyme for glial or supportive cells or other non-neuronal elements (Peakall, 1992). Inhibition of ChE activity has often been associated with exposure to organophosphate and carbamate insecticides and other neurotoxic xenobiotics (Ludke et al. 1975; Habig and Di Giulio, 1991). Chemicals other than carbamates and organophosphates that are environmental contaminants can also affect the activity of ChEs (Olson and Christensen 1980; Devi and Fingerman 1995; Labrot et al. 1996). Carboxylesterases (CaE; EC 3.1.1.1) represent a heterogeneous group of isozymes that can catalyze the hydrolysis of a wide range of xenobiotic esters, amides and thioesters (Huang et al. 1993b, 1996). For most CaE, their natural substrates are unknown, therefore, their physiological functions remain to be elucidated. These enzymes (CaE) occur widely in most tissues and are generally found in high levels in the liver.

The purpose of this research was to evaluate the liver and brain esterases in the spotted gar fish *(Lepisosteus oculatus)* as biomarkers of effect to multiple contaminants in the lower Mississippi River basin. The study sites were Devil's Swamp (DS) which is situated on the eastbank of the Mississippi River just northwest of Baton Rouge, Louisiana, and a pristine control site, Tunica Swamp (TS) which is approximately 30 miles up river from DS near St. Francisville, Louisiana (Fig. 1). DS is an ecosystem contaminated with chlorinated hydrocarbons such as hexachlorobenzene (HCB) and hexachlorobutadiene (HCBD), and a variety of heavy metals.

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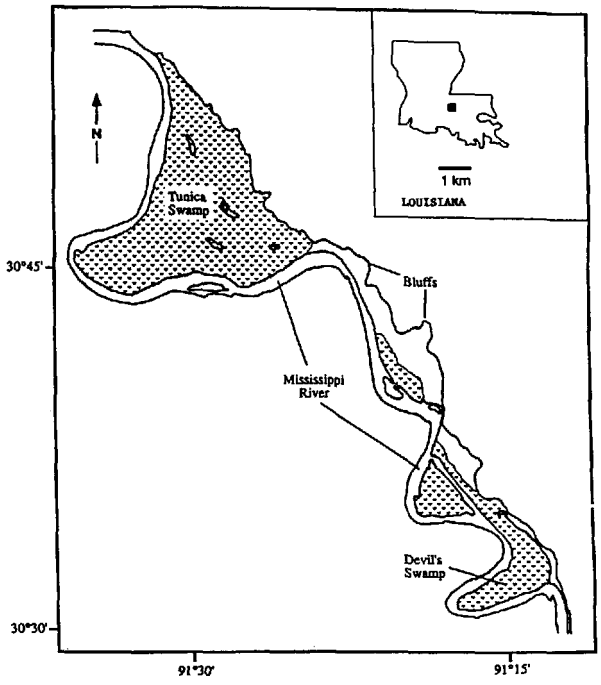

Figure 1. Map of study area showing Tunics Swamp and Devil's Swamp.

MATERIALS AND METHODS

Fish were caught from Tunica and Devil's Swamps using electroshocker, crab trap or rod and line. A total of thirteen (8 from TS and 5 from DS) were captured between November 8, 1994 to October 31, 1995. The fish were placed on ice and transported to the laboratory on the same day. Fish were necropsied within 14 hr of capture. Livers and brains were excised and quickly frozen at -80 °C until enzymes analysis. Results of liver histopathology and direct analysis for metals and organics in the muscle of the same gar fish have been reported (Hartley et al. 1996).

The microsomal and cytosolic fractions of liver and brain were isolated by differential centrifugation at 0-4 °C. Each liver and brain sample was weighed, minced and homogenized in 10 volumes of 0.25 *M* sucrose-0.1 *M* sodium phosphate buffer (pH 7.4). The homogenates were centrifuged at 13,000 g for 13 min, and the supernatant fractions were centrifuged at 105,000 *g* for 65 min. The resulting supernatants were collected as the cytosolic fractions and the pellets representing the microsomal fractions were washed and resuspended in the same buffer.

The cholinesterase activity on acetylthiocholine or butyrylthiocholine were assayed by a modification of the method of Ellman et al. (1961) adapted for use on a microtitre plate. In a typical assay, 278 µL of 0.015% 5,5'-dithiobis-2-nitrobenzoic

acid (DTNB) in 0.1 *M* sodium phosphate buffer (pH 8.0) and 20 µL enzyme solution were added to individual wells. The reaction was initiated by the addition of 2 µL acetylthiocholine or butyrylthiocholine (in water) to give a final concentration of $5x10^{-4}$ *M* and the rates were recorded at 405 nm for 2-5 min. Reagent blanks containing no enzyme were used as controls. The CaE activity on *p*-nitrophenyl acetate was assayed as described previously (Huang et al. 1993 b). The incubation mixture contained 20 µL enzyme solution in 278 µL of 0.1 *M* sodium phosphate buffer at pH 7.4. The reaction was initiated by injecting 2 µL of the substrate (in acetone) to give a final concentration of $5x10^{-4}$ *M*. The liberation of *p*-nitrophenol was monitored for 2-5 min at 405 nm. The enzyme assays were earned out under conditions where the initial hydrolysis rates were linear with time and protein concentration. All assays were carried out at 23 °C.

The *in vitro* inhibitory effects of several chemicals (HCBD, cadmium chloride, mercuric chloride and lead nitrate) representative of contaminants in DS, were tested on fish brain AChE and liver CaE activities. Stock solutions of HCBD and cadmium chloride (99% pure) were prepared in acetone. Mercuric chloride (99% pure) was dissolved in 50% acetone and deionized water. Lead nitrate was dissolved in deionized water containing 1% nitric acid. For determining the effects of these chemicals on esterase activities, the chemicals were added in 2 µL to wells containing 2% µL enzyme solutions diluted in 0.1 *M* sodium phosphate buffer, pH 7.4, or 0.1 *M* sodium phosphate buffer, pH 8.0, containing 0.015% DTNB reagent, respectively. After preincubation of the enzyme with the chemical for 20 min at 23 °C, the substrate acetylthiocholine or *p* -NpAc in 2.0 µL was added and the hydrolytic rates were monitored for 2 min at 405 nm. Controls containing the appropriate solvents, but without addition of inhibitors were also determined and used in calculations to represent 100% activity. Separate experiments indicated that the solvent used (<1% of acetone or 1% nitric acid) had little effect on enzyme activity.

Isoelectric focusing (IEF) was performed on a multiphor II electrophoresis unit using Ampholine PAGplates, pH 3.5-9.5 and pH 4.0-6.5. The gels were prefocused for 30 min at 4 °C and 12 watts without samples. The liver samples were added to their respective lanes at 75 µg of protein, and the samples were focused across 90 mm for 2.5 hr at a constant power of 12 watts. The gel was then sliced into eighteen 5 mm pieces and eluted overnight at 4 °C in 0.3 mL of 0.1 *M* sodium phosphate buffer (pH 7.4) containing 10% glycerol for measurement of enzyme activity or in 0.5 mL of 20 mm KC1 solution for measurement of pH (Fig. 3). Liver samples from TS and DS were pooled from eight and four fish respectively. In addition, adjacent gel lanes were stained with α-napthyl acetate (α-NA) in order to accurately assess the number and positions of esterase isozymes (Fig. 2). Protein concentrations were determined using the standard protocol version of the Pierce BCA assay. Bovine serum albumin was used as a protein standard.

The esterase activities were calculated as means and standard deviations. Levels of statistical significance were determined using the unpaired Student's t-test with $P<0.05$ considered significant.

RESULTS AND DISCUSSION

Significantly lower AChE and BuChE activities were observed in the microsomal and cytosolic fractions of brain and liver of gars from the contaminated site (Table 1). In the same gars from DS, the CaE activity was also significantly lower in the liver microsomal and cytosolic fractions. The inhibition of esterase activities ranged from 30%-49% for AChE, 3l%-52% for BuChE, and 46%-52% for CaE. Greatest decrease in esterase activity was observed with the CaE activity in the liver. Hartley et al. (1996) examined muscle tissue from three species of gars *(L. osseus, L. oculatus and L. platostomus)* from TS and DS collected from September 9, 1993 to January 26, 1995. With the exception of lead, elevated levels of HCBD, HCB, As, Hg, Cd, Cr and Ni were found in the muscle of gar from DS. The muscle of gar from TS had detectable, nonsignificant concentrations of As, Hg and Cr. The remaining contaminants were not detected in gar muscle from TS. We then investigated the *in vitro* effects of some of these chemicals on the esterases.

Table 1. Acetylcholinesterase (AChE), Butyrylcholinesterase (BuChE) and Carboxylesterase (CaE) activities in the brain and liver of *Lepisosteus oculatus* collected from Tunica Swamp (TS) and Devil's Swamp (DS).

Tissues	AChE[a]		BuChE[a]		CaE[a]	
	TS	DS	TS	DS	TS	DS
Brain Microsome	569 ± 25	288* ± 18	91.7 ± 12	49.8* ± 8.0	N.D.	N.D
Cytosol	295 ± 17	188* ± 9.0	38. 3 ± 6.1	26.5* ± 4.3	N.D	N.D.
Liver microsome	153 ± 5.6	94* ± 13	15.5 ± 2.7	8.72* ± 2.06	201 ± 45	108* ± 30
Cytosol	84.0 ± 5.1	59* ± 4.1	11.1 ± 1.5	5.34* ± 1.23	133 ± 23	64.5* ± 17

[a]Values for AChE, BuChE and CaE activities are expressed as nmol of substrate hydrolyzed/min/mg protein. Each value is the meant SD. of 8 and 5 fish from TS and DS respectively.
*Value is significant y different ($p < 0.05$) from corresponding control site (TS). N.D. indicates not determined.

In *in vitro* studies, several of these chemicals such as HCBD, Cd, Hg, and Pb, at varying concentrations, were found to inhibit brain microsomal AChE and liver microsomal CaE activities (Table 2). These chemicals were without effect on the microsomal BuChE from brain or liver. The effects of these chemicals on the cytosolic esterases (AChE, BuChE or CaE) from brain or liver were not tested. Maximum inhibition of AChE was caused by Hg, whereas CaE was most sensitive to the inhibitory effects of Pb. The inhibitory effects of heavy metals such as Hg,

Cd and Pb on enzymes that uses functional sulfhydryl groups such as AChE have also been reported by other workers (Devi and Fingerman 1995; Labrot et al. 1996). Because of the reported sensitivity of AChE to carbamate and organophophorous pesticides, this enzyme has generally been assumed to be a specific biochemical indicator of exposure to these agents (Day and Scott 1990; Galgani and Bocquene 1989). These results support earlier studies indicating that AChE can also be modulated by metals and therefore has potential to serve as a biomarker of heavy metal pollution as well. The HCBD weakly inhibited AChE activity.

Table 2. Effects of selected chemicals on AChE and CaE activities in *Lepisosteus oculatus* collected from Tunica Swamp.

Molar concn of chemical	% inhibition[a]			
	Brain Acetylcholinesterase			
	Cd	Hg	Pb	HCBD
10^{-7}	N.I.	0.64 ± 0.01	N.I.	N.I.
10^{-6}	4.2 ± 0.9	7.4 ± 1.6	N.I.	N.I.
10^{-5}	11.3 ± 2.2	23 ± 2.3	4.5 ± 0.2	0.48 ± 0.06
10^{-4}	30.9 ± 3.2	43.8 ± 2.6	20.5 ± 1.7	9.5 ± 1.1
10^{-3}	46.1 ± 7.7	56.7 ± 6.5	42.9 ± 2.6	23.7 ± 2.4
	Liver Carboxylesterase			
10^{-6}	N.I.	N.I.	0.80 ± 0.01	N.I.
10^{-5}	2.1 ± 0.1	3.6 ± 0.01	17.1 ± 1.0	1.4 ± 0.1
10^{-4}	21 ± 1.9	16.8 ± 0.2	37 ± 1.9	19.5 ± 2.2
10^{-3}	35.3 ± 2.6	31.2 ± 1.5	53.5 ± 3.6	32.7 ± 2.0

[a]% Inhibition values were obtained as the means ± S.D. of three determinations. Protein concentrations of brain microsomal AChE and liver microsomal CaE were 5.4 µg/mL and 9.5 µg/mL respectively. Tissues from 8 fish from TS were pooled for this study.
N.I. indicates no inhibition was observed.

The histopathology of the liver of the same gar fish from DS showed striking liver lesions (Hartley et al. 1996). These included an abundance of melanin-rich microphage centers (MMC), and ductal proliferation in the exocrine pancreas. Increased levels of hepatocellular and MMC iron, and larger and more numerous MMCs were observed in fish from DS with significant muscle concentrations of HCBD, HCB, and heavy metals. Fish accumulate MMC in the presence of poor environmental conditions, including pollution and stress (Ferguson, 1989). Based on the above observations and the results of this study, it is possible that the contaminants from the DS site may, in part, be responsible for the liver lesions as well as the decrease esterase levels seen in the spotted gar fish from the contaminated site.

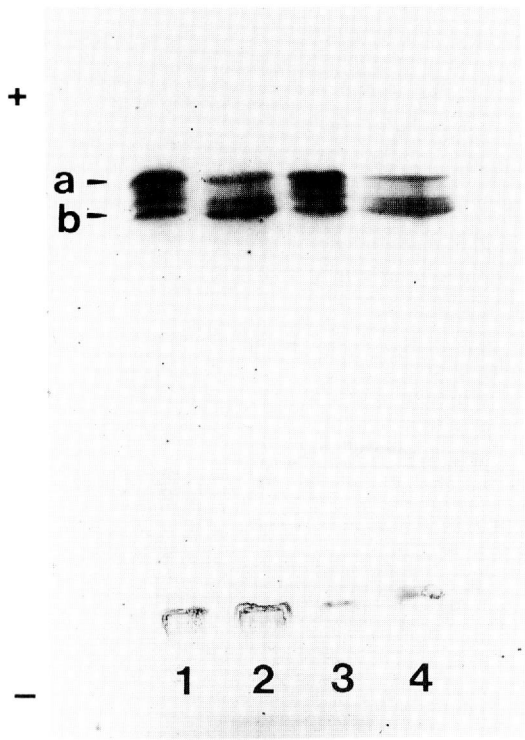

Figure 2. Isoelectric focusing with narrow - range pH gradient gel (pH 4.0-6.5) of liver microsomal (lanes 1 and 3) and cytosolic (lanes 2 and 4) esterases of gar samples stained with α -NA. Lanes 1 and 2 represent samples from TS whereas lanes 3 and 4 represent samples from DS. No other esterases were detected with a wide - range pH gradient gel (pH 3.5-9.5). The amount of proteins applied on the gel for all samples were 75 µg. Isozymes with pI values of 4.84 and 4.90 are represented by a and b respectively.

To investigate possible adverse effects on esterase isozymes, the liver CaE isozymes patterns were characterized by isoelectric focusing. Changes in isozymes patterns may provide important clues regarding the affected organ, tissue or subcellular compartment. Therefore, differences between CaE isozymes in response to specific xenobiotics may provide a more selective indicator of exposure. This approach has been shown to be particularly promising with the response of serum and liver esterase isozymes to specific toxicants in mammals (Huang et al. 1993a). High resolution IEF of liver microsomal and cytosolic CaEs of gars from TS and DS indicated the presence of at least four esterase isozymes (Fig 2.). Two of the four esterase bands were more intensely stained and represent the predominant esterase isozymes which focused at pI values of 4.84 and 4.90 respectively. The esterase bands observed in the gel stained with α -NA (Fig. 2) corresponds to a major peak with a pI of 4.89 detected in the sliced gels (Fig. 3). This major peak probably represents the combined esterase activities of the four isozymes since the four bands were very close to each other and could not be separated in the sliced gels. Although the number of esterase isozymes in gars from the two sites were similar, the intensity of the esterase bands of gar from DS were less intense than from TS. We have observed changes in liver esterase isozymes patterns in other fish species collected from the contaminated site (Jaiswal et al., unpublished data). The amount of proteins applied on the gel were the same (75 µg) for all the samples. Activity assay of sliced gels of liver samples isolated in gar from both sites showed a major esterase peak with a pI of 4.89, with the peak significantly lower (about 40%) in the samples obtained from the contaminated site.

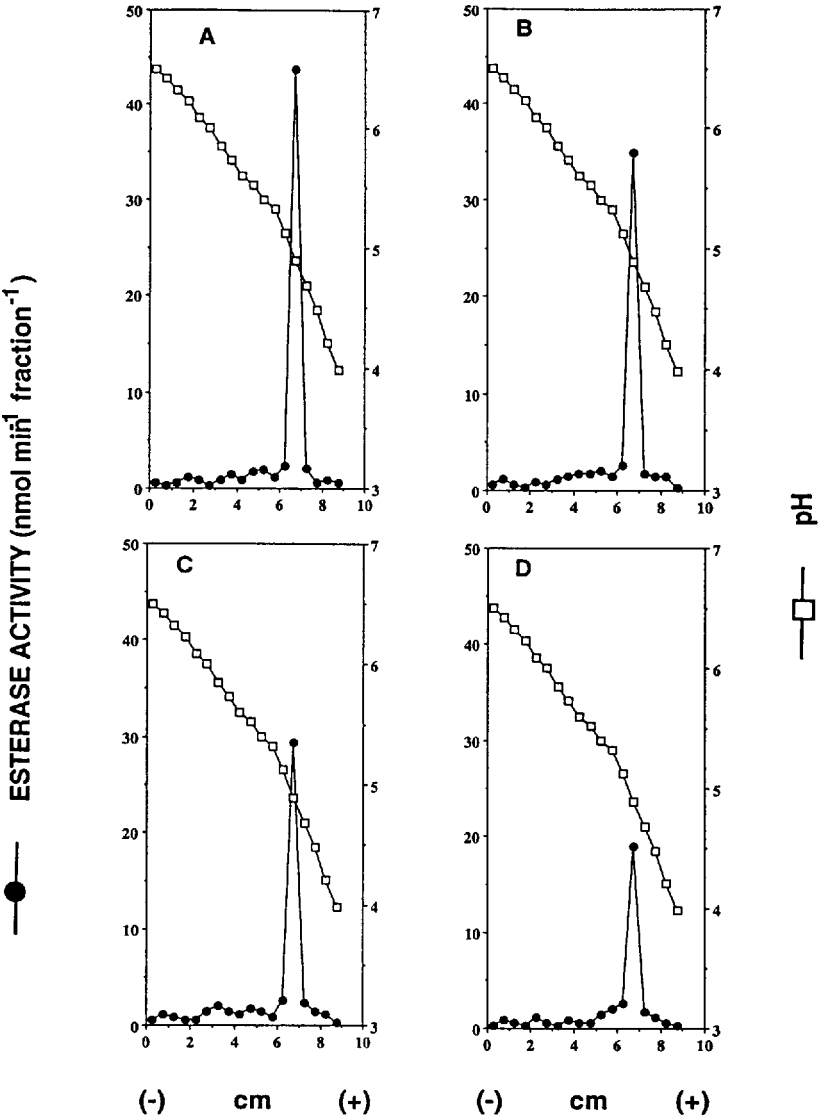

Figure 3. Isoelectric focusing of liver esterases assayed with α-NA in gars from TS and DS. Electrophorectic patterns of liver esterase activities in microsomes (A) and cytosol (B) of gars from TS and in microsomes (C) and cytosol (D) of gars from DS. Conditions of the experiment are described under materials and methods.

Spotted gar is a resident aquatic species common in the Mississippi River Basin and other freshwater environments. They accumulate a wide range of organics and metals in muscle tissue. Our results indicate that the combined pollutants adversely affected the hepatic and neurological systems. Gar with significant body burdens of organics and metals have liver injury at the tissue, cellular and enzymatic

(biochemical) levels. In gar from sites with mixed pollution, concurrent liver injury included large MMC, increased iron pigments, ductal proliferation in the exocrine pancreas and depression of liver esterase activity. The findings of esterase depression may be linked to directly or indirectly to liver lesions due to chemical contamination. Further research is needed to characterize a model of hepatotoxicity in the gar that would be useful in exposure evaluation for ecorisk assessment.

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