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Effects of methamidophos on acetylcholinesterase activity, behavior, and feeding rate of the white shrimp (*Litopenaeus vannamei*)

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Abstract

The toxicity of methamidophos on the shrimp *Litopenaeus vannamei* was evaluated using acetylcholinesterase (AChE) activity, behavior, and feeding rate as effect criteria. The biochemical characterization of the soluble cholinesterase (ChE) present in both muscle and eye tissues of *L. vannamei* was performed in a first phase of the study. In both tissues, almost full inhibition of enzyme activity by eserine sulfate was found, indicating that the measured activity is mainly from ChE and not from other esterases. The highest rate of substrate hydrolysis was found when acetylthiocholine was used as substrate. To evaluate the effects of methamidophos on *L. vannamei* AChE, behavior, and feeding rate, shrimps were exposed for 24 h to several sublethal concentrations of methamidophos. Significant effects of the pesticide on behavior and AChE were found, with behavior being a more sensitive endpoint than AChE inhibition. Feeding rate was not a sensible endpoint under conditions tested.

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Keywords: Acetylcholinesterase; ChE characterization; Methamidophos; Shrimp; *Litopenaeus vannamei*; Behavior; Feeding rate

1. Introduction

Nowadays, the most used insecticides are organophosphorus (OPs) and carbamates (CB), which are considered cholinesterase (ChE) inhibiting pesticides. The relative lack of target specificity of these compounds and their high acute toxicity to nontarget organisms were devaluated in favor of other characteristics such as short-term environmental persistence and low accumulation in organisms (Mineau, 1991). OPs and CB pesticides inhibit acetylcholinesterase (AChE). This inhibition leads to the accumulation of acetylcholine, interfering with the function of the nervous system. Depending on the doses, this may lead to a

range of deleterious effects which may culminate in respiratory failure and death (WHO, 1986).

The enzymes designed by cholinesterases are typically subdivided into two classes: acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (BuChE; EC 3.1.1.8) or pseudocholinesterase. They are strongly inhibited by eserine sulfate (Eto, 1974). These enzymes can be distinguished by their substrate preferences and behavior toward selective inhibitors. AChE hydrolyzes acetylcholine at higher rates than other choline esters, whereas BuChE prefers butyrylcholine as substrate but also hydrolyzes propionylcholine and acetylcholine at appreciable rates. The behavior of the two types of enzymes toward the compounds 1,5-bis-(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide (BW284c51) and tetraisopropylpyrophosphoramidate (iso-OMPA) is also different: AChE is highly sensitive to BW284c51 and relatively insensitive to

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iso-OMPA, while the opposite is true for BuChE (Fairbrother et al., 1991; Barahona and Sanchez-Fortun, 1999).

ChEs properties show variations among and within species with different forms and sensitivities to anticholinesterase agents (Bocquené et al., 1990; Galgani et al., 1992). Furthermore, recent studies indicate that ChEs of some species cannot be classified as AChE or BChE since they show characteristics of both enzyme types (Bocquené et al., 1997; Varó et al., 2002). Therefore, the determination of the type of ChE predominating in a particular tissue or species is critical for deciding which substrate and concentration are the most appropriate to use for assaying cholinesterase activity as a biomarker for pesticide exposure.

Several studies have successfully used the inhibition of ChE activity as a tool to diagnose OPs and CB exposure and/or poisoning in vertebrates and invertebrates, both in the field (Bocquené et al., 1990, 1993; Escartin and Porte, 1996a; Huang et al., 1997) and in the laboratory (Day and Scott, 1990; Guilhermino et al., 1996; Doran et al., 2001). Behavioral, biochemical/molecular, and physiological alterations represent responses at different levels of organization, some of which are determinant for the survival of the organism and for population fitness. Therefore, the utilization of a suite of parameters at several levels of biological organization can be very useful in assessing the effects of chemical pollution on the viability of a population (Schlenk et al., 1996; Schlenk, 1999).

Behavioral responses may be useful for measuring injury resulting from the release of hazardous materials and can be used to compare the sensitivities of different responses of the same organism (ASTM, 1995). These responses measured during toxicity tests are highly sensitive to sublethal exposure. Behavior alteration may interfere with feeding, survival, and reproduction, thus having implications at a population level. Feeding is also an important response since a reduction in feeding rate may also have considerable implications at the population level (McWilliam and Baird, 2002; Maltby et al., 2002).

The white shrimp *Litopenaeus vannamei* plays an important ecological role in estuarine environments along the Pacific coast of Mexico and Central and South America (Wyban and Sweeney, 1991), while supporting one of the most important fisheries in Mexico. Nowadays, only the state of Sinaloa produces around 11,000 tons by fisheries and 20,737 tons cultured (INEGI, 2003). However, Couch (1978) reports that, after several years of testing, the US Environmental Protection Agency has found that penaeid shrimps are generally far more sensitive to most pesticides than fish and mollusks and they have been proposed as indicators of estuarine health due to their world wide distribution throughout temperate, subtropical, and tropical regions (Couch, 1979). Considering the above arguments, the white shrimp *L. vannamei* was selected as test organism in this work. The main objectives of this study were (a) to characterize the soluble ChE present in muscle and eye homogenates of *L. vannamei* using different

substrates and selective inhibitors, (b) to determine LC50 values of methamidophos on juvenile *L. vannamei*, and (c) to assess the effects of sublethal concentrations of methamidophos to this species using AChE activity, behavior, and feeding rate as indicative parameters.

2. Material and methods

2.1. Chemicals

Acetylthiocholine (ATC) iodide, butyrylthiocholine (BTC) iodide, propionylthiocholine (PTC) iodide, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), bovine serum albumin, 1,5-bis (4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51), *N,N'*-diisopropylphosphorodiamic acid (iso-OMPA), eserine sulfate, monobasic potassium phosphate, and dibasic potassium phosphate were purchased from Sigma (USA). Tamaron 600 (commercial grade, Bayer, Mexico) was obtained from an agrochemical store and a standard of methamidophos was obtained from SUPELCO (USA). Solvents including acetone, cyclohexane, hexane, and ethyl acetate were pesticide grade (Fisher Scientific, USA).

2.2. Chemical analysis

Tamaron 600 was used as a toxicant in lethal and sublethal experiments. The nominal concentration of the active ingredient in Tamaron 600 corresponded to 600 g of methamidophos per liter of solution. The actual concentration of methamidophos in Tamaron 600 was determined by gas chromatography (HP 6890 equipped with a nitrogen-phosphorous detector), using a capillary column (HP 5% phenyl methyl siloxane) of 30 m \times 0.25 mm and 0.25 μ m film thickness. Helium was used as carrier and nitrogen as auxiliary gas. Analytical-grade methamidophos (SUPELCO, USA, 77% purity) was used as a standard, with the actual concentration 12.6% lower than the nominal. All Tamaron 600 concentrations are hereinafter expressed as actual concentrations of methamidophos (mg/L).

2.3. Biological material

Juveniles of the white shrimp *L. vannamei* were obtained from a commercial source (Maricultura del Pacifico, S. A., Mexico). Organisms were maintained in a 50-L holding tank with filtered seawater and constant aeration. The experimental conditions were established at $25 \pm 1^\circ\text{C}$, $32 \pm 2\%$ of salinity, and 12 h light: 12 h dark photoperiod. The organisms were fed with *Artemia* sp. nauplii and brine shrimp flakes (Salt Creek, USA) until used in experiments.

2.4. ChE characterization

Eight shrimp (3.2 ± 0.9 g) were sacrificed. Muscles and eyes (including peduncle) were immediately removed and put in ice-cold phosphate buffer (0.1 M, pH 7.2) in 1:5 and 1:50 (w/v) proportion, respectively. Muscles and eyes were homogenized separately on ice using a tissue homogenizer (Polytron PT 1300 D). The homogenates were centrifuged at 6000 g for 30 min at 4°C (Beckman GS-15R) and the supernatants were collected and diluted with phosphate buffer 1:15 (muscle) and 1:1 (eyes) prior to ChE determinations.

The characterization of ChEs was performed using three different substrates and three selective inhibitors. The substrate preferences of *L. vannamei* were investigated by determining, in independent experiments, the enzyme activity using the substrates acetylthiocholine iodide, propionylthiocholine iodide, and *s*-butyrylthiocholine iodide at concentrations ranging from 0.005 to 25.6 mM. The activity of ChE was immediately determined after the preparation of homogenates. Eight replicates per treatment were used and four enzymatic determinations were done per sample. Three inhibitors were used: eserine sulfate

(physostigmine, 1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo-[2,3-b]indol-ol methylcarbamate), iso-OMPA, and BW284c51. Stock solutions of the inhibitors were prepared in ethanol. The effect of both specific inhibitors on the ChE activity was determined immediately after an incubation period of 30 min at room temperature using 0.008 ml of each stock solution and 0.492 ml of *L. vannamei* homogenate. For eserine sulfate and BW284c51, the concentrations used were 0.00625, 0.0125, 0.025, 0.05 and 0.1 mM, while for iso-OMPA the concentrations were 0.5, 1, 2, 4, and 8 mM. In each experiment, two controls were included: the first was incubated with 0.008 ml of bidistilled water and the other with 0.008 ml of ethanol. Eight organisms were tested for each inhibitor concentration and four replicates were done per sample.

The effect of substrates and specific inhibitors on ChE activity in muscle and eye of *L. vannamei* were determined according to the Ellman technique (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). Briefly, 0.250 ml of a mixture reaction (phosphate buffer 0.1 M + DTNB + substrate) was added to 0.050 ml of homogenate. After 10 and 15 min of incubation, the absorbance was read at 414 nm in a microplate reader (Labsystems Multiskan Ascent). The enzyme activity was calculated from the increase of absorbance during the reading period. Four enzymatic determinations per replicate were performed. The enzyme activity was expressed in nmol/min/mg protein. The concentration of protein in samples was determined at 595 nm by the Bradford method (Bradford, 1976) adapted to microplate, using bovine serum albumin as standard. All assays were conducted at 23–25 °C.

2.5. *LC*₅₀ bioassay

A 96-h static renewal bioassay was conducted with *L. vannamei* juveniles (0.48 ± 0.04 g) according to APHA (1998). Before the test, 210 organisms were randomly placed into 21 glass aquaria with 7 L of seawater and constant aeration to be acclimated for 2 days to the experimental conditions. Six different concentrations of Taramon 600 (a commercial formulation of methamidophos) were prepared in seawater to obtain the following actual concentrations of methamidophos: 0.26, 0.46, 0.82, 1.09, 1.45, and 2.59 mg/L. One control without toxicant was included and three replicates for each concentration (with 10 shrimps each) were tested. During exposure, water renewals were performed every 24 h and no food was offered to organisms. Mortality was recorded at 0.5, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h, and dead shrimps were removed as soon as they were detected.

2.6. Sublethal effects of methamidophos on AChE activity, feeding rate, and behavior

Based on the results of the acute toxicity test, a sublethal bioassay was carried out with shrimp juveniles (0.608 ± 0.14 g). One hundred and twenty organisms were randomly placed into 12 aquaria with 7 L seawater and constant aeration and left for 24 h for acclimation. After this period, they were exposed to the pesticide for 24 h, in groups of 10 shrimps under the conditions described above. Five concentrations of methamidophos (0.66, 0.83, 1.01, 1.18, and 1.35 mg/L) and one control were tested in duplicate. After exposure, one set of organisms (10 per treatment) was used to assess the effects on feeding rate. The other set (10 per treatment) was used to measure behavioral parameters and acetylcholinesterase activity.

Feeding rate was assessed by randomly transferring the shrimps of each concentration to 10 individual chambers (transparent polyvinyl chloride jars) with 650 ml of clean seawater to be acclimated for 30 min. After this, each shrimp was offered 0.1 g of feed pellets (Camaronina, 35% protein). Pellets were previously freeze-dried (Labconco, FreeZone 6 Liter-79340) and sieved through 1.73 and 1.3-mm-diameter stainless steel sieves to obtain homogeneous-sized pellets. Shrimps were allowed to feed for 90 min and were then withdrawn from the chamber and wet weighed. The remaining food pellets were obtained, rinsed with distilled water, freeze-dried, and weighed. The difference between initial and final food weight divided by the wet weight of the organism was used to calculate the individual feeding rate (g dry weight/g wet weight).

The evaluation of behavioral parameters was determined by video recording, following the general recommendations of ASTM (1994, 1995). After the exposure period, a mesh dividing each aquarium in halves was placed so that each side would contain a group of five organisms that allowed a closer video recording and thus better determination of the behavioral endpoints. All aquariums were randomly filmed using a digital video camera (Handycam-Digital 8, Sony). Each aquarium side was filmed for 5 min, using the last 2 min of each video recording to measure three behavioral variables for each recorded shrimp: (a) the cumulative time measured with a chronometer, stopping each time that the shrimp was immobile, (b) the frequency of initiation of movements using a manual counter, and (c) the frequency of grid crossing (a grid dividing the aquarium image in four quadrants was used to count each time that the shrimp crossed each quadrant). Qualitative behavioral observations such as location in aquarium (upper, bottom, middle), swimming mode (stationary, sideways, spiral, head-up, and frequent sinking and rising), and activity/excitability (hyperactive, lethargy, and spasms) were also registered. AChE activity was determined after the video recording by sacrificing 10 organisms of each treatment in ice-cold water to extirpate both eyes (including peduncle) to individually measure enzyme activity and proteins, as previously explained under ChE characterization.

2.7. Data analysis

In the characterization experiments, no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were calculated for each inhibitor using a one-way analysis of variance (ANOVA) followed by Dunnett's pairwise comparisons. The median effect concentration (*EC*₅₀) and its 95% confidence intervals were calculated using a linear interpolation method (Norberg-King, 1993). Results of the acute bioassay were analyzed using Probit analysis (Finney, 1971) to determine the median lethal concentration (*LC*₅₀). In the sublethal pesticide bioassay, and for each endpoint, different treatments were compared using a one-way ANOVA after checking for normality and homogeneity of variances. If data failed to show homoscedasticity, a Kruskal–Wallis analysis of variance was applied. If the analysis was significant, Dunnett's tests were performed to identify NOEC and LOEC values. When data showed a concentration-dependent relationship, the median effect concentration (*EC*₅₀) and its 95% confidence intervals were calculated using a linear interpolation method (Norberg-King, 1993). The significance level was 0.05 for all tests. The statistical analyses were performed using SigmaStat (version 3.0) software.

3. Results

3.1. ChE characterization

ChE in both muscle and eyes of *L. vannamei* showed preference for ATC relative to the other substrates (ATC > PTC > BTC), which yielded higher activities in eyes than in muscles at all concentrations assayed (Fig. 1). The ChE activities (expressed as nmol/min/mg protein) at the highest concentration of substrate (25.6 mM) were 6.09 ± 1.17 (muscle) and 19.62 ± 4.26 (eyes) for ATC; 5.12 ± 0.89 (muscle) and 15.26 ± 1.4 (eyes) for PTC, and 2.54 ± 0.2 (muscle) and 1.66 ± 0.37 (eyes) for BTC. No substrate inhibition was observed in the range of concentrations tested. The hydrolysis ratios at 25.6 mM in muscle were 1.2:1 for ATC/PTC and 2.4:1 for ATC/BTC, while higher ratios were obtained for eyes: 1.3:1 for ATC/PTC and 12:1 for ATC/BTC.

With regard to the experiments with the inhibitors, eserine sulfate showed the most drastic inhibitory effect on ChE activity of *L. vannamei* for both tissues

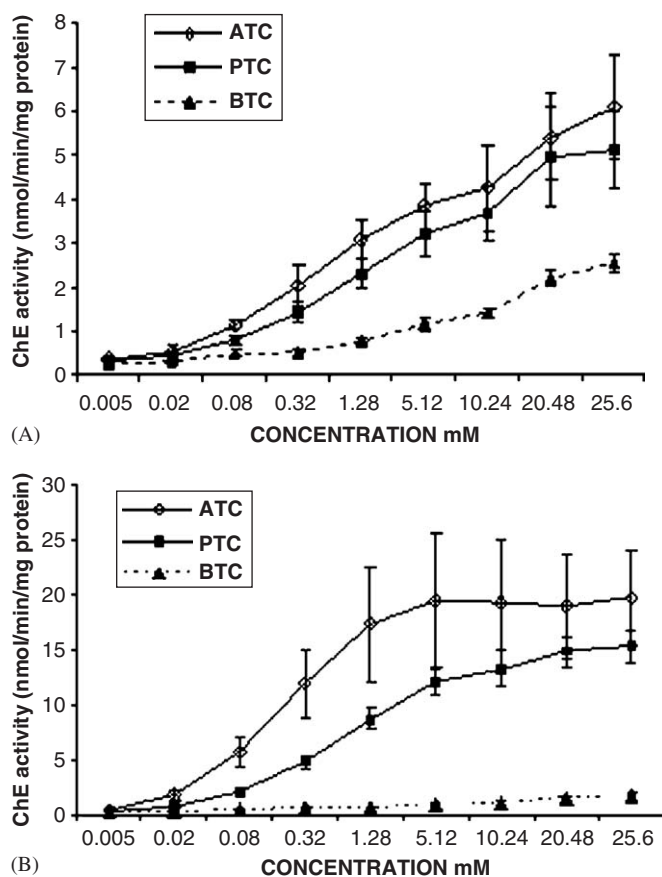


Fig. 1. Cholinesterase (ChE) activity of *Litopenaeus vannamei* in muscle (A) and eye (B) as a function of acetylthiocholine (ATC), propionylthiocholine (PTC), and butyrylthiocholine (BTC) concentration. Values are the mean of eight organisms and standard error bars corresponding to four replicates.

($LOEC_{\text{muscle}} = 12.5 \mu\text{M}$, $LOEC_{\text{eye}} = 6.25 \mu\text{M}$), being more noticeable in eyes with inhibitions from 92 to 98%, while muscle registered inhibitions from 77% to 85% (Fig. 2A). Both tissues were relatively insensible to iso-OMPA, since no appreciable inhibitions were recorded at concentrations up to 8000 μM (NOEC) (Fig. 2B). For BW284c51, both muscle and eyes showed relatively high inhibitions at 6.25 μM (46 and 49%, respectively), increasing gradually to 81.33% in muscle and 87.6% in eye, when the concentration increased to 50 μM ($LOEC_{\text{muscle}} = 12.5 \mu\text{M}$, $LOEC_{\text{eye}} = 6.25 \mu\text{M}$) (Fig. 2C). No significant effect of solvent ethanol was observed for all treatments. The LOEC, NOEC, and EC_{50} results are summarized in Table 1.

The normal range of ChE activity of nonexposed individuals ($n = 40$, w/w of $3.6 \pm 1.14 \text{ g}$) was $6.4 \pm 0.54 \text{ SE nmol/min/mg protein}$ in muscle and $23.3 \pm 1.2 \text{ SE nmol/min/mg protein}$ in eye tissue, using ATC as substrate.

3.2. LC_{50} bioassay

The 72 h LC_{50} value was 2.34 mg/L with a 95% confidence interval from 1.98 to 3.06. The 96 h LC_{50} value was 1.46 mg/L with a 95% interval from 1.25 to 1.73.

3.3. Sublethal effects of methamidophos on AChE activity, feeding rate, and behavior

No mortality was recorded during the sublethal bioassay. Exposed organisms, however, showed several behavioral alterations, such as uncoordinated swimming movements, hyperactivity, and spasms that in general were more apparent with the increasing concentration of pesticide.

The postexposure feeding rate evaluation did not show significant differences among treatments ($H = 11.87$, $P < 0.05$) (Fig. 3). In contrast, the postexposure behavioral responses (Fig. 4) showed significant differences at the lowest concentration tested (0.65 mg/L), although they failed to follow a clear concentration-dependent relationship. For instance, locomotory cumulative time was significantly higher (around five times) than control for most methamidophos treatments ($F = 17.89$, $P < 0.05$), except for 0.83 mg/L, where no significant differences with respect to the control group were found. A similar pattern was observed for the frequency of movements initiation and grid crossing, which also registered significantly higher levels for most treatments with respect to control ($H = 12.707$, $P < 0.05$), except for 0.83, 1.18, and 1 mg/L. AChE activity in eyes showed an inhibition related to the methamidophos concentration except for the highest concentration tested (1.35 mg/L), with a minimum of 19.5% at 0.66 mg/L and a maximum of 48.7% at 1.18 mg/L (Fig. 5). The methamidophos EC_{50} value for AChE inhibition was 1.64 mg/L with a confidence interval of 0.98–2.49 mg/L, while NOEC and LOEC values were 0.65 and 0.83 mg/L, respectively.

4. Discussion

In ecotoxicology, the inhibition of ChE has been widely used to assess exposure and/or effects of pesticides. However, variability could be introduced, since distinct enzymatic forms with different sensitivities toward anticholinergic contaminants may exist, and it should be controlled (e.g., by using the appropriated substrate and/or a selective inhibitor), therefore a biochemical characterization study in the species and tissue to be used should be performed as the first step. ChEs may be distinguished from nonspecific esterases by their preference to hydrolyze choline esters rather than other substrates and by their high sensitivity to eserine sulfate (physostigmine) (Fairbrother et al., 1991). In this study, for both eye and muscle tissues, an almost full inhibition of enzymatic activity by eserine sulfate was observed at concentrations equal to or higher than 0.025 mM, suggesting that ChEs are responsible for this activity and not other esterases (Fig. 2).

Both muscle and eye ChE showed preference to acetylthiocholine over propionylthiocholine and butyrylthiocholine. The same trend has been observed in bivalves (Escartin and Porte, 1997; Basack et al., 1998; Valbonesi et al., 2003) and fish (Galgani et al., 1992; García

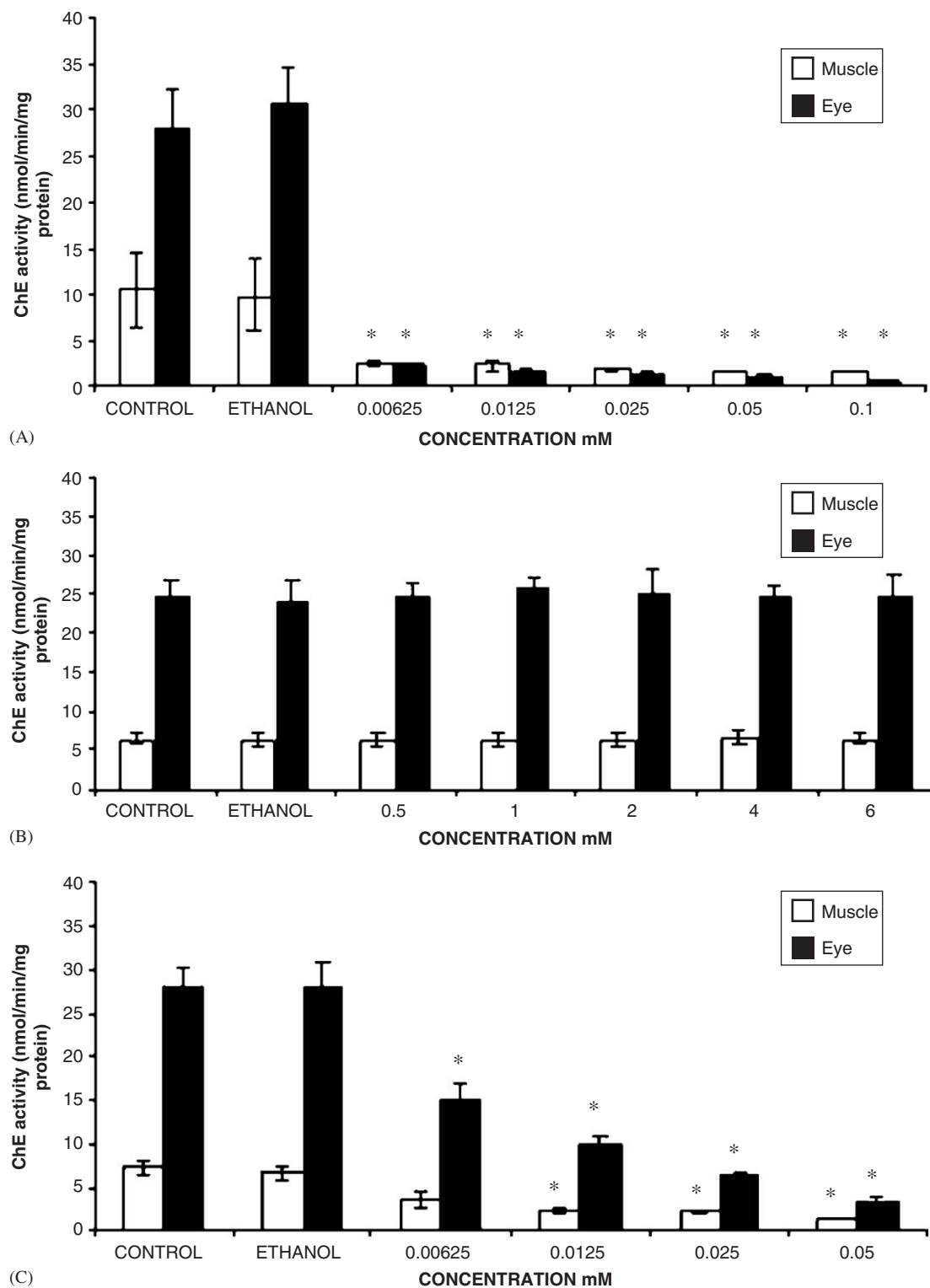


Fig. 2. Effect of eserine sulfate (A), iso-OMPA (B), and BW284c51 (C) on cholinesterase (ChE) activity of muscle and eye of *Litopenaeus vannamei*. Values are the mean of eight organisms and standard error bars corresponding to four replicates. *Statistically significant differences from the control ($P < 0.05$).

et al., 2000). With regard to the AChE/BChE hydrolysis ratio, this was similar to that reported by Escartin and Porte (1997) in gills and digestive gland of the mussel *Mytilus galloprovincialis*. Valbonesi et al. (2003) found hydrolysis ratios ATP/PTC of 2.6:1 and 1.6:1 for gills of

M. galloprovincialis and *Ostrea edulis*, respectively, with ChE activity in *M. galloprovincialis* 4.3 times higher than that in *O. edulis*. Some studies have reported inhibition of ChE activity at high concentrations of substrate in different aquatic species, including fish such as *Poecilia reticulata*

Table 1
Effects of selective inhibitors on ChE of *Litopenaeus vannamei*

	Eserine sulfate		Iso-OMPA		BW284c51	
	Muscle	Eye	Muscle	Eye	Muscle	Eye
NOEC (μM)	<6.25	< 6.25	8000 ^a	8000 ^a	6.25	< 6.25
LOEC (μM)	6.25	6.25	>8000	>8000	12.5	6.25
IC ₅₀ (μM)	4.2	3.4	— ^a	— ^a	8.6	6.8
(95% CL)	(4.1–4.4)	(3.3–3.4)			(6.2–10.4)	(5.6–8.7)

NOEC, no observed effect concentration, LOEC, lowest observed effect concentration, and IC₅₀, 50% in vitro inhibition concentration, 95% confidence interval are shown within parentheses.

^aNo significant inhibition was found up to 8000 μM .

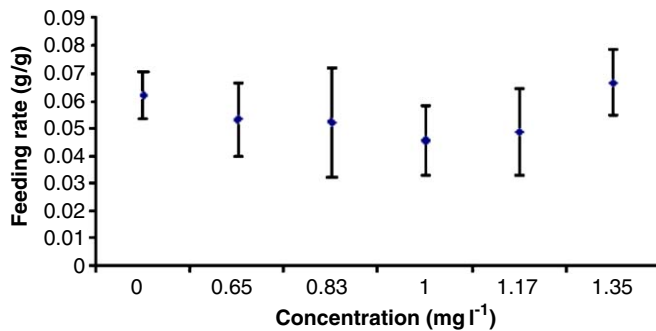


Fig. 3. Feeding rate of *Litopenaeus vannamei* exposed for 24h to methamidophos. The values are the mean of 10 animals with correspondent standard error bars.

(García et al., 2000) and *Dicentrarchus labrax* (Varó et al., 2003) and the marine copepod *Tigriopus brevicornis* (Forget and Bocquené, 1999). In the range of concentrations tested in this study, no inhibition by excess of substrate was observed in both tissues. However, the enzymatic activity in eye remained relatively constant despite the increase of ATC concentration from 5.12 to 25.6 mM. This lack of inhibition has also been observed in the crustaceans *Artemia salina* and *Artemia parthenogenetica* (Varó et al., 2002), in the bivalves *Corbicula fluminea* and *M. galloprovincialis* at concentrations from 0.1 to 20 mM (Mora et al., 1999), and in *Chironomus riparius* at concentrations from 0.05 to 8 mM (Fisher et al., 2000).

In both tissues, no effects of iso-OMPA on ChE activity were found up to 8 mM. In addition, a concentration-dependent ChE inhibition by BW284c51 was found, with the activity reaching almost zero at the highest concentration tested (0.050 mM) in both tissues (Fig. 2). Altogether, the results obtained in the characterization study suggest that both tissues of *L. vannamei* predominantly possess AChE. These results are in good agreement with the findings that have been reported for other shrimps, namely *Palaemon serratus* (Bocquené et al., 1990; Frasco et al., 2003), and mussels such as *M. galloprovincialis* (Escartin and Porte, 1997).

Most studies of ChE activity in aquatic organisms have been carried out in tissues such as muscle, brain, and gills

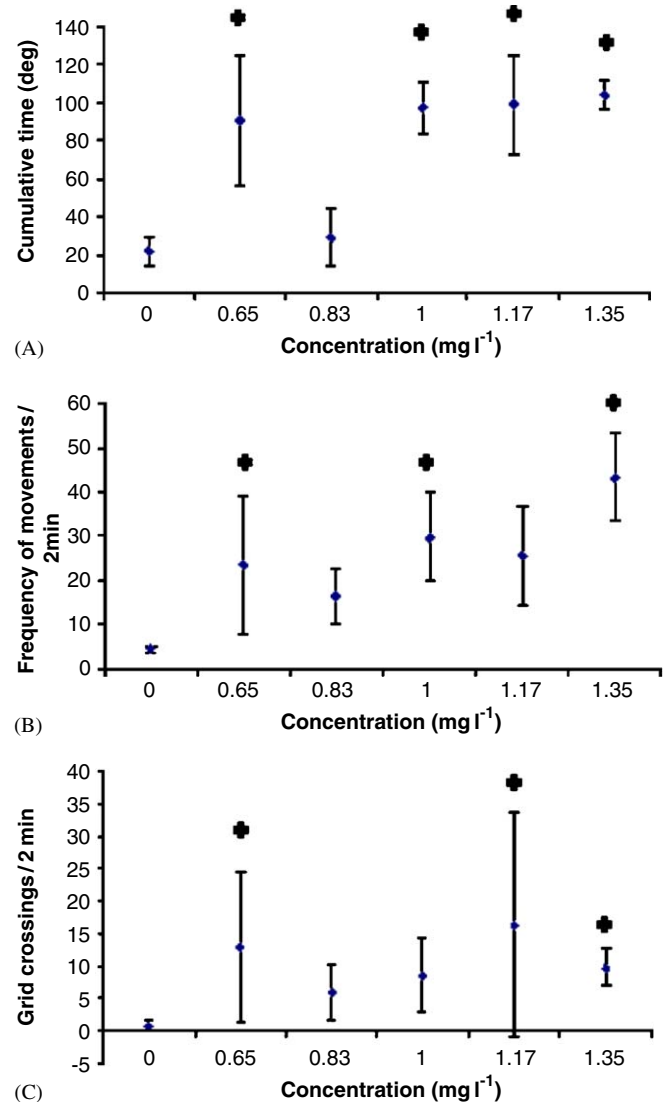


Fig. 4. Behavioral responses: (A) cumulative time, (B) frequency of initiation of movements, and (C) frequency of grid crossing. The values are the mean of five animals with correspondent standard error. * Significantly different from the control ($P < 0.05$).

and only some have used eyes. In this study, we found that eyes are a good alternative for biomarker studies, particularly when using invertebrates such as shrimps,

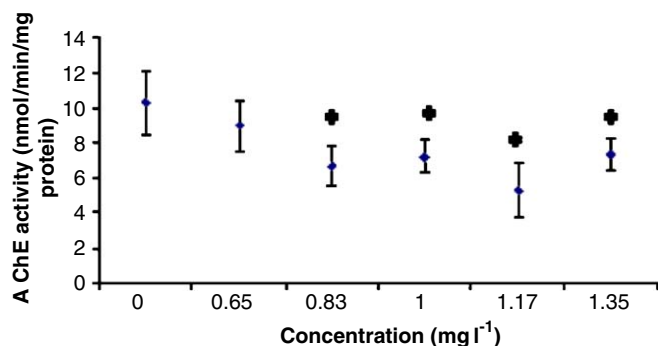


Fig. 5. Acetylcholinesterase (AChE) activity of *Litopenaeus vannamei* exposed for 24 h to methamidophos. Values are the mean of 10 animals with correspondent standard error bars. * statistical significant differences from the control ($P < 0.05$).

where extracting nervous tissue of a considerable number of animals is time consuming and, therefore, impractical. In fact, the high levels of AChE activity in the eye, due to its high nerve activity and acetylcholinesterase content, make it an appropriate tissue for use in studies with this biomarker. This suitability was already pointed out by Ceron et al. (1996), who found higher AChE activity in the whole eye than in the brain in *Anguilla anguilla*.

The range of AChE activity in nonexposed *L. vannamei* was 6.4 ± 0.54 SE nmol/min/mg protein in muscle and 23.3 ± 1.2 SE nmol/min/mg protein in the eye. These levels are within the range of values that have been reported in the literature for other crustacean species such as *Daphnia magna* (whole body: 8.2 ± 0.995 nmol/min/mg protein, Guilhermino et al., 1996), *Procambarus clarkii* (muscle: 8.7 ± 1.5 nmol/min/mg protein, Escartin and Porte, 1996b), *A. salina* (whole body: 2.65 ± 0.15 nmol/min/mg protein), *A. parthenogenetica* (whole body: 3.69 ± 0.17 nmol/min/mg protein, Varó et al., 2002), and *Palaemonetes pugio* (embryos, stage V: 5.42 ± 0.91 nmol/min/mg protein, Lund et al., 2000). However, in other crustaceans higher values have been reported. For example, in the head capsules of *Gammarus pulex* an activity of 250 ± 16 nmol/min/mg protein was measured (McLoughlin et al., 2000), while in hemolymph of *Carcinus maenas* a value of 201 ± 95 nmol/min/mg protein was determined (Lundebye et al., 1997).

LC₅₀ values of methamidophos to *L. vannamei* juveniles found in this study (2.34 mg/L at 72 h and 1.46 mg/L at 96 h) are higher than those reported for other crustaceans. For instance, Juárez and Sánchez (1989) found that *Litopenaeus stylirostris* larvae (mysis) exposed to methamidophos had a 36 h LC₅₀ of 8 ng/L, 10⁶ times lower than at found for *L. vannamei*. This difference seems to suggest that methamidophos is more toxic to larval than juvenile crustaceans and possibly that *L. stylirostris* is more sensitive than *L. vannamei*. On the other hand, aquatic organisms exhibit a broad range of toxic responses to OP and CB pesticides, depending on the compound, exposure time, and species (Dembélé et al., 2000). In another study with *L. vannamei* juveniles exposed to fenitrothion, Lignot

et al. (1998) reported 48 h LC₅₀ values of 19 µg/L, while Key et al. (1998) reported a 96 h LC₅₀ to adult *P. pugio* of 38 µg/L exposed to malathion and 1.64 µg/L when exposed to azinphosmethyl.

Some studies have revealed that the feeding rate is a sensitive indicator of toxic stress in both freshwater and marine species (Maltby et al., 1990; Butler et al., 1990). McLoughlin et al. (2000) observed that, in *G. pulex* exposed to the OP pirimiphos-methyl, feeding rate was 13-fold more sensitive than ChE activity. However, in the present study, this response was not a sensitive endpoint, being unable while to discriminate between control and pesticide treatments (Fig. 3), although, it is worth to notice that this could be related to the experimental setup (i.e., inadequate food offered, feeding time, or feeding conditions).

In this study, AChE was a sensitive endpoint with an LOEC of 0.83 mg/L. However, contrary to what was expected, the inhibition did not completely depend on the concentration since a slight increase in activity was observed at the highest concentration tested. The maximum level of inhibition was 48.7% in the group exposed to 1.17 mg/L of methamidophos. Inhibition levels equal to or higher than 50% are generally considered to induce irreversible effects, while inhibitions below this value are considered reversible, assuming that organisms are able to recover their normal functions after the toxic insult (Peakall, 1992). This approach, although practical, has some obvious limitations since it does not consider the nature of the chemical, the sensitivity of the species, and the linkages with other vital functions of the organisms. For instance, it is well documented that the inhibition of AChE activity affects several physiological and behavioral processes and may have consequences on the organism's feeding capability, identification and avoidance of predators, and spatial orientation (Pan and Dutta, 1998). This illustrates the need to associate specific inhibition levels with responses at higher levels of biological organization.

According to our results, behavioral responses seem to be more sensitive than AChE since significant differences compared to control group were found in animals exposed to the lowest concentration tested (0.65 mg/L). Also, it is worth while to notice that, regardless of the behavioral response, treatments consistently showed greater variability than controls, although they failed to follow a clear concentration–response relationship as could be expected, with some concentrations (particularly 0.83 mg/L) showing no significant differences from the control group. Nevertheless, the combination of different types of responses indicates that changes in AChE activities could affect normal behavior, thus potentially affecting the organism's survival capabilities (Pan and Dutta, 1998; Dutta and Arends, 2003). This is not surprising since behavioral criteria integrate many cellular processes vital to an organism's survival and reproduction, thus reflecting both biochemical and ecological consequences of the toxic insult, sometimes quicker than conventional test criteria

such as survival, reproduction, and growth (Janssen et al., 1994). Therefore, toxicity tests using behavioral test criteria and others, such as mortality and biochemical endpoints, could altogether represent excellent tools for evaluating the toxicity of chemicals and effluents. Furthermore, it is important to consider a suite of measurements to elucidate the impact of contaminants on integrated responses of an organism to assess potential consequences for populations.

5. Conclusion

The use of different substrates and specific inhibitors demonstrated that AChE is the main form present in both muscle and eyes of *L. vannamei*. Although it is considered a sensitive endpoint, in this study feeding rate failed to detect effects of methamidophos under the experimental conditions used. Both AChE and behavioral parameters of *L. vannamei* were sensitive to the pesticide, therefore, they are biomarkers suitable to be used in both toxicity tests and biomonitoring studies. Altogether, the results of this study suggest that using different endpoints to evaluate the toxicity of chemicals and effluents could be an excellent tool in environmental studies.

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