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Cholinesterase activity in gilthead seabream (*Sparus aurata*) larvae: Characterization and sensitivity to the organophosphate azinphosmethyl

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Abstract

Assessment of cholinesterase (ChE) inhibition is widely used as a specific biomarker for evaluating the exposure and effects of non-target organisms to anticholinesterase agents. Cholinesterase and carboxylesterase activities have been measured in larvae of gilthead seabream, *Sparus aurata*, during the endogenous feeding stage, and ChE was characterized with the aid of diagnostic substrates and inhibitors. The results of the present study showed that whole-body ChE of yolk-sac seabream larvae possesses typical properties of acetylcholinesterase (AChE) with a apparent affinity constant (K_m) of 0.163 ± 0.008 mM and a maximum velocity (V_{max}) of 332.7 ± 2.8 nmol/min/mg protein. Moreover, sensibility of this enzyme was investigated using the organophosphorus insecticide azinphosmethyl. Static-renewal toxicity tests were conducted over 72 h and larval survival and AChE inhibition were recorded. Mean mortality of seabream larvae increased with increasing concentrations of azinphosmethyl and exposure duration. The estimated 72-h LC50 value to azinphosmethyl was 4.59 μg/l (95% CI = 0.46–8.71 μg/l) and inhibition of ChE activity gave an IC50 of 3.04 μg/l (95% CI = 2.73–3.31 μg/l). Larvae exposed to azinphosmethyl for 72 h showed a 70% inhibition of the whole-body acetylcholinesterase activity at approximately the LC50. In conclusion, the results of the present study suggested that monitoring ChE activity is a valuable tool indicating OP exposure in *S. aurata* larvae and that acetylthiocholine is the most appropriate substrate for assessing ChE inhibition in this early-life stage of the fish.

Keywords: Cholinesterase activity; Seabream larvae; Fish; Biomarker; Organophosphate insecticides; Azinphosmethyl

1. Introduction

One of the most popular classes of pesticides currently in use in agriculture are the organophosphorus (OP) insecticides (Wheeler, 2002) as they are highly effective and possess relatively nonpersistent characteristics. However, they lack specificity and it has been demonstrated that they are also highly toxic to non-target species, including mammals, birds and aquatic organisms. Eventually, coastal and marine environments seem to be at risk (Galgani et al., 1992; Payne et al., 1996) because of runoff of these contaminants from agriculture and urban sources.

The mechanism of toxic action of organophosphorus insecticides, or their metabolites in the case of those requiring bioactivation, is based on the irreversible inhibition of the enzyme acetylcholinesterase (AChE), which hydrolyzes the neurotransmitter acetylcholine (ACh) to end the cholinergic neural transmission in both the central and peripheral nervous systems of vertebrates, including fish (Carr and Chambers, 2001). Accumulation of ACh in synapses, arising as a consequence of the inhibition of AChE, results in overstimulation of neurotransmission followed by depression or paralysis and eventual death. At sublethal exposures, such toxicants may cause impairment of multiple functions, including respiration (Pavlov et al., 1992), reproduction (Bhattacharya, 1993), and behaviour (Beauvais et al., 2000).

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Carboxylesterases (CbE) are other type of "B" esterases that are also inhibited by OP pesticides. These esterases are assumed to provide protection against organophosphorus pesticide intoxication via two main mechanisms: (1) detoxication by hydrolysis of ester bonds in some of these pesticides and (2) providing alternative sites of OP binding so that one molecule of insecticide is scavenged by stoichiometric phosphorilation, which reduces the amount of pesticide available for AChE inhibition (Jokanovic, 2001).

OPs have a rapid metabolism in biota (WHO, 1986) and a relatively low persistence in the environment, of the order of days (Lacorte et al., 1995; Shaw, 1998). In contrast, ChE inhibition in tissues may persist for days to weeks (Morgan et al., 1990; Ferrari et al., 2004b; Wijeyaratne and Pathiratne, 2006), even if they no longer contain detectable traces of pesticides. This may offer an outstanding advantage in monitoring OPs over the use of chemical analysis alone. As a result, the measurement of cholinesterase (ChE) activity has been well accepted as a biomarker of exposure and effects of OP pesticides and other anticholinesterase compounds and has become a tool of biomonitoring in continental (Payne et al., 1996; Sturm et al., 1999a), estuarine and marine waters (Galgani et al., 1992; Bocquené et al., 1993; Fulton and Key, 2001; Quintaneiro et al., 2006). At this respect, acetylcholinesterase (AChE) activity has been proposed to be included in a battery of biomarkers of contaminant exposure and effects that could be incorporated into programmes monitoring the quality of the coastal environment in the Iberian Peninsula (Cajaraville et al., 2000). Nevertheless, unlike the large literature on the lethal and sublethal effects of OP compounds on freshwater organisms, there are fewer data on marine fish, especially those on early-life stages. Such data are scarce for, e.g., the gilthead seabream (*Sparus aurata*), a commercially important fish species, which is widely distributed along the Mediterranean Sea and the Eastern Atlantic Coast.

It has been pointed out that using the early developmental stages of animals in bioassays has several advantages. Early-life stages have, e.g., relatively high sensitivity to toxins in comparison with other life stages, and thus they give a good estimate of chronic toxicity of chemicals to fish without the need to carry out a complete life cycle test (McKim, 1985). As a result, duration of the tests, their cost and produced toxic wastes are reduced. We have previously shown that yolk-sac seabream larvae are more sensitive to s-triazine herbicides than the marine bacteria *Vibrio fischeri* as well as adult and larval stages of other marine fish and invertebrate species (Arufe et al., 2004a,b).

Cholinesterases are a family of enzymes that hydrolyze choline esters. In addition to AChE present in brain (Habig and Di Giulio, 1991), fish plasma and muscle tissues may contain AChE as well the related enzyme butyrylcholinesterase (BChE) (Sturm et al., 1999a; Chuiko et al., 2003). In fact, BChE is more sensitive to anticholinesterase compounds than AChE in vertebrates (Ecobichon and Comeau, 1973), including fish (Galgani and Bocquené, 1990; Magnotti et al., 1994; Wogram et al., 2001) and, therefore, it is important to characterize the enzymes present in the tissues and species which are to be used in bioassays or aquatic pollution monitoring. Functionally, ChEs are distinguished on the basis of their substrate specificity and also by their susceptibility to diagnostic inhibitors (Silver, 1974; Fairbrother et al., 1991). AChE is highly selective for acetyl esters as substrates and BChE preferentially hydrolyzes butyryl and propionyl esters, although it also hydrolyzes a wider range of esters, including ACh. The selective inhibition of BChE is usually achieved by OP compounds such as DFP (diisopropylphosphorofluoridate) and *iso*-OMPA (tetraisopropylpyrophosphoramide), and AChE can be selectively inhibited using BW284c51 (1:5-bis[4-allyldimethylammonium phenyl]-pentan-3-one dibromide).

The aims of our work were (a) to establish whether yolk-sac gilthead seabream larvae possessed detectable CbE and ChE activities, (b) to determine the characteristics of ChE activity present, (c) to determine the acute toxicity of the phosphorothionate insecticide azinphosmethyl on yolk-sac *S. aurata* larvae and (d) to study the relationship between cholinesterase inhibition and mortality (72-h exposure) for this organophosphorus insecticide in this early-life stage of the fish.

Azinphosmethyl was selected as a model compound because of its importance in agriculture for the control of many insect pests, the significant number of reported cases of aquatic incidents due to its use (US EPA, 2001) and its prior use by other investigators. The selection of the test species was based on its wide geographic distribution, ready availability through culture or from field collection, commercial significance because of its importance for human consumption as a major source of fish protein, and an adequate background information on the biology of this marine species. These are important criteria that should be considered in selecting organisms for toxicity testing (Rand et al., 1995).

2. Materials and methods

2.1. Chemicals

Azinphosmethyl (99%), Guthion^{TM}, CAS RN [86-50-0], was obtained from Chem Services (West Chester, PA, USA). Acetylthiocholine iodide (ASCh), CAS RN [1866-15-5], propionylthiocholine iodide (PSCh), CAS RN [1866-73-5], *S*-butyrylthiocholine iodide (BSCh), CAS RN [1866-16-6], eserine hemisulfate, CAS RN [64-47-1], 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51), CAS RN [402-40-4], tetraisopropylpyrophosphoramide (*iso*-OMPA), CAS RN [513-00-8], *S*-phenylthioacetate (PSA) [934-87-2] were supplied by Sigma–Aldrich Química (Madrid, Spain), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), CAS RN [69-78-3] was from Merck (Darmstadt, Germany), and bovine serum albumin (BSA) and the BioRad Protein Assay reagent were purchased from BioRad (Madrid, Spain). All other reagents and solvents were of analytical grade.

2.2. Experimental system

Newly hatched larvae of gilthead seabream for definitive and range-finding tests were obtained from Cupimar, S.A. Fisheries (San Fernando, Cádiz, Spain) and from the Laboratory of Marine Culture at the Marine and Environmental

Sciences Faculty (University of Cádiz) and transported to the Institute of Marine Sciences of Andalucía (ICMAN-CSIC) facilities.

Static-renewal toxicity tests were conducted in triplicate for yolk-sac seabream larvae during 72 h. At 3–5 h post-hatching, defined as day 0 post-hatching (0-dph), the yolk-sac larvae were collected and exposed for 3 days, under static-renewal conditions without aeration, to the organophosphate insecticide azinphosmethyl in natural filtered seawater (0.45 mm pore size) at a temperature of 19–20 °C, salinity of 37 ± 1‰, pH 8 ± 0.1, under 12-h light/12-h dark exposure, and a oxygen content higher than 85% saturation. A test consisted of five nominal concentrations of azinphosmethyl (0.04, 0.2, 1.0, 5 and 25 μg/l), a control and a solvent control (0.05% acetone). In each beaker, 30 larvae and one litre of test solution were added. In all insecticide treatments, acetone concentration was 0.05%. Test solutions were freshly prepared and renewed at 24-h intervals over the testing period. Dead larvae were removed each day and, at the end of the experiment, surviving larvae were anesthetized by thermal shock with ice and stored at −80 °C until enzyme analysis.

To characterize ChE activity and to document age-dependent changes in enzyme activity in developing yolk-sac larvae, different pools of larvae were isolated daily from the rearing tanks from day 0 to day 3 (3 days post-hatching, 3-dph), anesthetized and used for the enzyme assays.

2.3. Enzyme assays

Frozen larvae (−80 °C) were defrosted and homogenized in ice-cold 0.1 M sodium phosphate buffer (pH 7.4), at a density of 10–15 larvae/0.5 ml buffer, using an Ultraturrax homogenizer (Schott Ibérica, Spain) (1 min, 20,500 rpm) and kept on ice during the homogenization. The crude extracts from pools of whole-body homogenates were used directly as the enzyme source without centrifugation.

Cholinesterase activities in the homogenates were measured by the method of Ellman et al. (1961), as adapted to microplates by Guilhermino et al. (1996). In the method, acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh) and propionylthiocholine iodide (PSCh) were used as substrates (0.401 mM final concentration). Briefly, 50 μl of larval homogenate was mixed with 250 μl of a mixture containing DTNB and substrate (30 ml of phosphate buffer, 1 ml of DTNB 0.01 M and 0.2 ml of substrate 0.075 M). The change of optical density with time (mOD/min) as a result of TNB production was recorded at 415 nm for 3 min at room temperature with a microplate reader (BioRad Model 680) to estimate substrate hydrolysis. The spontaneous substrate hydrolysis was determined in the same way in the absence of enzyme. Carboxylesterase activity was determined using a similar protocol with the substitution of *S*-phenylthioacetate (PSA; 1.068 mM, final concentration) as substrate. PSA was dissolved in absolute ethanol.

The protein content of the enzyme extracts was measured by the method of Bradford (1976) adapted to microplates (BioRad microassay procedure for microtiter plates) using bovine serum albumin as standard. The measurement was performed with the microplate reader at 595 nm. All enzyme analysis and protein determinations were carried out in triplicate per sample. The enzymatic activity was expressed as nmol hydrolyzed substrate/min/mg protein (specific activity).

2.4. Catalytic properties and characterization of cholinesterases using specific inhibitors

Esterases from the whole-body homogenates from 3-dph larvae (three different experiments) were characterized using specific substrates and inhibitors. ASCh, BSCh, PSCh and PSA were used as substrate analogues to determine acetyl-, butyryl- or propionyl-cholinesterase or carboxylesterase activities, respectively. Additionally, the effect of substrate concentration on ChE activity was assayed using 10 different concentrations of ASCh, ranging from 0.027 to 3.205 mM.

The inhibition study was performed in the presence of ASCh (0.401 mM final concentration) using three distinct specific inhibitors in the 10^{-4} to 10^{-7} M range (final concentrations in incubation mixture): eserine sulphate as the total ChE inhibitor, and BW284C51 and *iso*-OMPA as specific inhibitors of AChE and BChE, respectively. Stock solutions of eserine and *iso*-OMPA were prepared in methanol as inert vehicle. BW284C51 was prepared in ultra-pure water. Residual enzyme activity was measured on larval homogenates (0.45 μl) after 30 min incubation at room temperature (25 °C) with 5 μl of each selected inhibitor (10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M eserine, *iso*-OMPA or BW284C51) by adding substrate solution. To serve as controls, one sample received only 5 μl of methanol and other 5 μl of water.

2.5. Statistical analysis

Cholinesterase results were analyzed by One-way analysis of variance (ANOVA). When the *p*-value obtained from ANOVA was significant, Tukey's *post hoc* test was applied to test for differences among groups (treatment levels or age groups) and to compare ChE activities from control and exposed animals. Differences were considered significant at $p < 0.05$. Prior to analysis, all data were subjected to the Shapiro–Wilk test for normality and to Barlett's test for homogeneity of variance. The median inhibitory concentration (IC50) with 95% confidence interval for cholinesterase activity was estimated using a linear interpolation method for sublethal toxicity (Ic*p*) according to Norberg-King (1993). In order to determine the kinetic parameters, i.e., the apparent Michaelis–Menten constant (K_m) and the maximum substrate hydrolysis velocity (V_{max}), substrate (ASCh) concentration versus reaction velocity curves were analyzed using Enzyme Kinetics Pro version 2.36 software by fitting experimental data to the Michaelis–Menten equation. For mortality data, TOXSTAT statistical analysis software (WEST Inc. and Gulley, 1994) was used to calculate the median lethal concentration (LC50) values and 95% confidence intervals (CI) by the trimmed Spearman–Karber method (Hamilton et al., 1977).

The results of the triplicate tests were pooled together and then the LC50 value was determined.

3. Results

3.1. Substrate specificity and kinetic parameters

In order to determine the distribution of cholinesterase and carboxylesterase activities in whole-body homogenates of 3-dph seabream larvae, the relative contribution of the esterases and their preferences for the different substrates were determined. Four substrates have been assayed: ASCh, PSCh and BSCh for cholinesterases and PSA for carboxylesterases. Results of this initial screening are displayed in Fig. 1. Results showed that ASCh was the substrate cleaved at highest rate (241.9 ± 28.2 nmol/min/mg protein), whilst the hydrolysis of PSCh was noticeably lower, just 10.8% of the activity measured in the presence of ASCh under the same conditions. A very low activity was measured for BSCh, only 0.8% of the overall activity seen with ASCh. The CbE activity detected by the hydrolysis of phenylthioacetate was also lower than the activity measured on ASCh (23%), but higher than each of the ChE activities measured on BSCh and PSCh.

Kinetic experiments were carried out in the presence of ASCh as substrate. ChE activity in 3-dph larvae was a function of increasing substrate concentration within the range 0.027–3.205 mM and followed Michaelis–Menten kinetics (Fig. 2). The reaction rate increased until a maximal velocity was achieved, indicating that a saturating concentration of substrate was reached. An apparent slight reduction of the enzyme activity was observed at the highest concentration of substrate tested (3.205 mM ASCh), but the mean was not statistically different from that of the last three concentrations ($p > 0.05$). The corresponding Lineweaver–Burk plot for ChE activity was linear and is also depicted in Fig. 2. The maximal velocity (V_{max}) and the apparent Michaelis–Menten constant (K_m) were 332.7 ± 2.8 nmol/min/mg protein and 0.163 ± 0.008 mM, respectively.

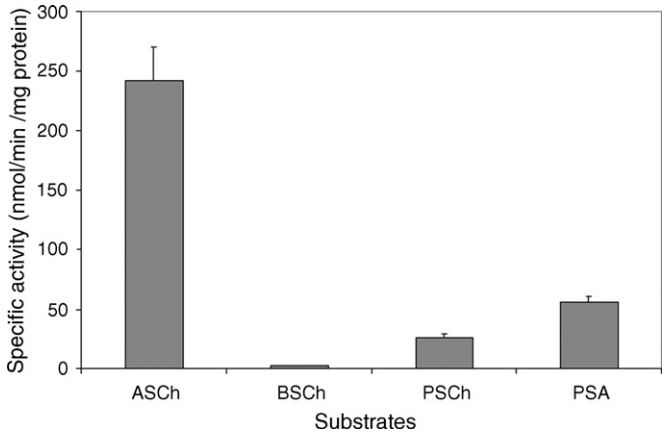

Fig. 1. Substrate specificity of esterase activities in 3-dph seabream larvae. The substrates used were: acetylthiocholine (ASCh), butyrylthiocholine (BSCh), propionylthiocholine (PSCh) and phenylthioacetate (PSA). Data are depicted as mean ± standard deviation ($n = 3$).

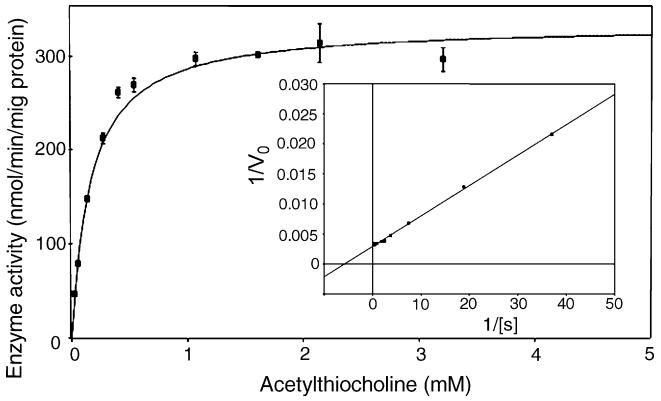

Fig. 2. Michaelis–Menten plot describing seabream ChE activity in the presence of 10 different concentrations of acetylthiocholine. Data are reported as the mean ± standard deviation of three replicates. The insert shows the Lineweaver–Burk plot.

3.2. Cholinesterase characterization

Different ChE-enzyme inhibitors were used to characterize the contribution of the different cholinesterases to the total activity in the seabream larvae. In this study crude homogenate was incubated with 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M of eserine as a general inhibitor of all ChE activities. Also, BW284c51 and *iso*-OMPA, which inhibit acetylcholinesterase and butyrylcholinesterase (pseudocholinesterase), respectively, were used at the same concentrations. The carbamate eserine inhibited ChE activity in a concentration-dependent manner with a maximum effect at 10^{-4} M (Fig. 3); at this concentration ChE hydrolysis was reduced more than 97% with regard to control values. Non-specific esterases that are able to metabolize ASCh might be present in our crude homogenate preparations. However, the high sensitivity to eserine would suggest that alkylthiocholine-cleaving non-specific esterases were not present at significant

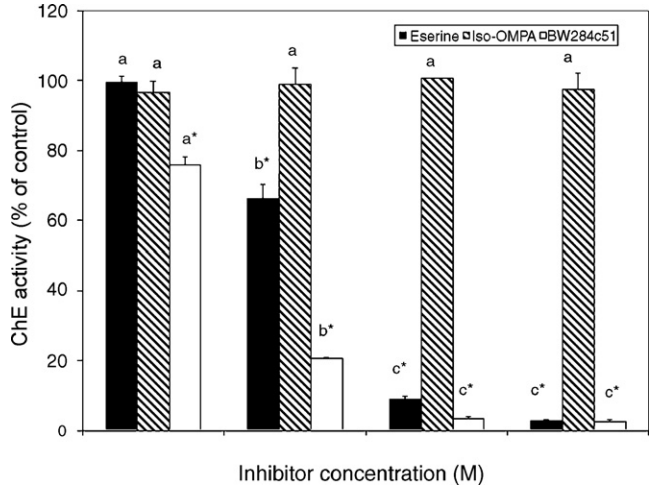

Fig. 3. Percentage of inhibition of cholinesterase (ChE) activity of yolk-sac seabream larvae incubated with different concentrations of eserine, *iso*-OMPA and BW284c51 for 30 min. Each bar represents the mean ± standard deviation ($n = 3$). Bars with the asterisks indicate significant differences from the respective controls at $p < 0.05$. For each inhibitor, bars with a different letter are significantly different from each other ($p < 0.05$).

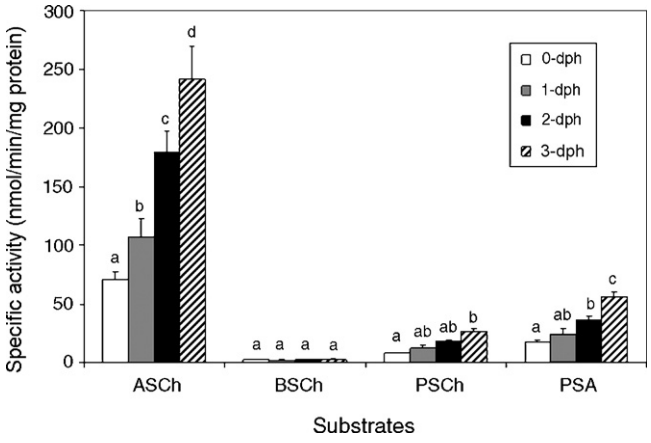

Fig. 4. Specific enzyme activities of cholinesterases and carboxylesterase in yolk-sac seabream larvae from hatching (0-dph) to 3-days post-hatch (3-dph) using ASCh, BSCh, PSCh and PSA as substrates. Data are reported as the mean ± standard deviation of eight different pools of 15 larvae. For each substrate, bars with different letters are significantly different from each other.

levels in 3-dph *S. aurata* larvae. The selective inhibitor of AChE, BW284c51, significantly inhibited ChE activity using ASCh ($p < 0.05$). Results showed that there were progressive inhibitions of the enzyme activity, with values 24%, 79%, 97% and 99% lower than the control at levels of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M of BW284c51, respectively. However, ChE activity remained unaffected ($p > 0.05$) by the selective inhibitor of BChE, *iso*-OMPA, at concentrations up to 10^{-4} M.

3.3. Age-dependent enzyme activities

Cholinesterase and carboxylesterase activity of *S. aurata* varied with larval age during the first 3 days post-hatch, the sac-fry stage or period of endogenous feeding. The baseline esterase activity measured with ASCh, PSCh and PSA as substrates during this early-life stage followed the order of increasing activity, newly hatched < 1 < 2 < 3-dph larvae (Fig. 4), whereas a very low activity was invariably measured for BSCh. ChE activity of 3-dph larvae on ASCh was 3.4-fold higher than that of newly hatched larvae. A similar relative increase in enzyme activity was measured using PSCh and PSA as substrates. The normal range of ChE activity of unexposed 3-dph individuals was 248.9 ± 29.7 nmol/min/mg protein using 0.401 M ASCh as substrate. The value is expressed as the mean ± S.D. for 15 pooled animals from eight independent experiments and three enzymatic determinations per replicate.

3.4. In vivo experiments with azinphosmethyl

3.4.1. Survival/mortality

The organophosphate azinphosmethyl affected post-hatch survival of larvae. Upon completion of the test (72-h exposure), the mean mortalities of the organisms were 46.1%, 50.9%, 55.0%, 62.2% and 87.0% at 0.04, 0.2, 1, 5 and 25 μg/l azinphosmethyl. Mean control mortality was 17.1%, which indicates good experimental conditions, according to the OECD guideline for early-life stage tests (OECD, 1998). The Trimmed

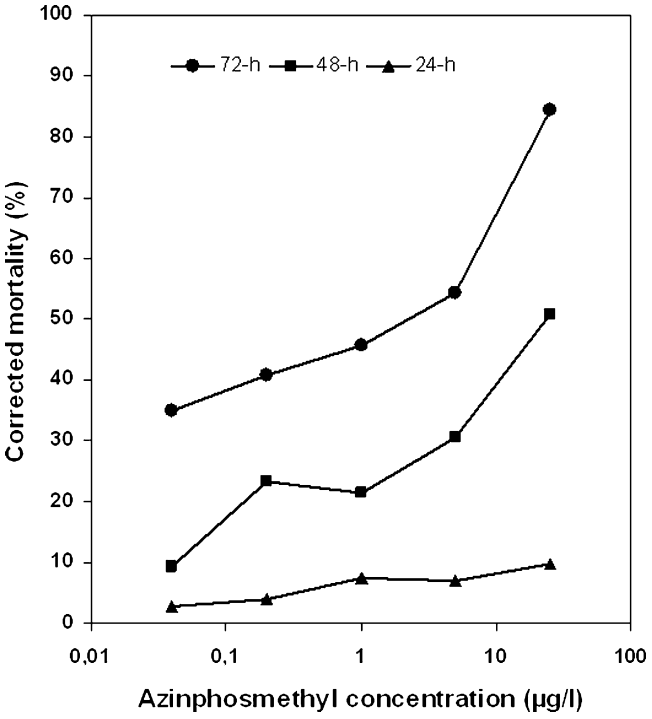

Fig. 5. Mortality of yolk-sac seabream larvae exposed to azinphosmethyl for 72 h (●), 48 h (■), and 24 h (▲). Results are pooled data from three independent experiments expressed as corrected mortalities by Abbott formula.

Spearman–Karber estimate of the 72-h LC50 was 4.59 μg/l (95% confidence interval of 0.46–8.71 μg/l). As one might expect, higher median lethal concentrations were obtained at shorter exposures. Accordingly, the 48-h LC50 value was calculated to be 20.03 μg/l (95% CI: 0.46–8.71 μg/l), whereas a 24-h LC50 could not be calculated because of the low mortality, but was estimated to be greater than the highest test concentration (25 μg/l). Experimental results expressed as corrected mortalities by Abbott formula (Abbott, 1925) are depicted in Fig. 5.

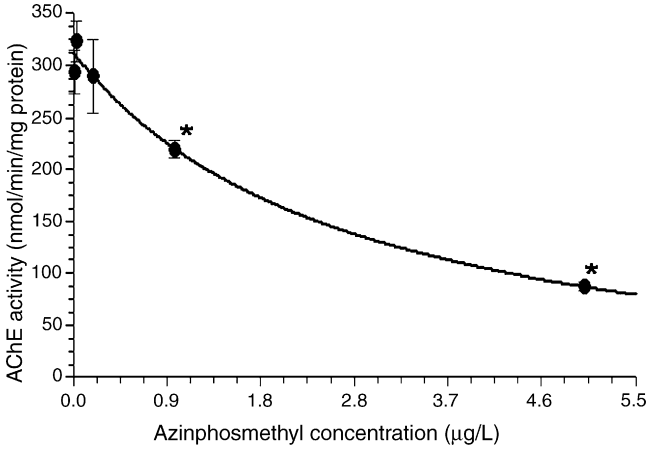

Fig. 6. Acetylcholinesterase (AChE) activity in 3-dph seabream larvae exposed to different concentrations of azinphosmethyl. Each point represents the mean response ± standard deviation of three individual experiments. Asterisks denote responses significantly different from control ($p < 0.05$).

3.4.2. Cholinesterase activity inhibition

Cholinesterase activity in the yolk-sac seabream larvae decreased upon exposure to azinphosmethyl (Fig. 6). However, comparison between azinphosmethyl concentrations showed that ChE activity differed significantly from control only at the 1 and 5 μg/l levels. AChE activity measured in larvae that survived till the end of the experiments (72 h) at 5 μg/l (close to the estimated LC50) was inhibited by 70% of the control values. It was not possible to measure AChE activity in larvae exposed to the highest concentration tested (25 μg/l) because of the lack of an adequate number of alive larvae in replicates. The 72-h IC50 was 3.04 μg/l (95% CI = 2.73–3.31 μg/l).

4. Discussion

Cholinesterases are classified as AChE or BChE with the aid of diagnostic substrates. Because both lack complete selectivity, findings with diagnostic substrates have to be confirmed with diagnostic inhibitors (Silver, 1974). In order to detect the presence of multiple enzymes in yolk-sac seabream larvae, esterase activity was firstly measured in whole-body homogenates with different alkyl-substituted thiocholines (ASCh, PSCh and BSCh) and phenylthioacetate (PSA). PSA hydrolysis mediated by ChEs is negligible (Basack et al., 1998; Galloway et al., 2002) and hence it is considered a good substrate to measure carboxylesterase (CaE) activity using a similar methodology to ChE. Esterase activity decreased in the order ASCh > PSA > PSCh, while BSCh was almost not hydrolysed. Non-specific esterases that are able to metabolize the substrate ASCh might be present in our crude homogenate preparations. However, the enzyme activity was inhibited by more than 90% at two concentrations of the non-selective ChE inhibitor eserine (<9% of control activity at 10^{-5} M and <3% at 10^{-4} M) on 0.401 M ASCh. This confirmed that activity measured in our experimental conditions was due to ChE and not to other types of esterases (Eto, 1974).

Cholinesterases of vertebrates can be further distinguished with enzyme-selective inhibitors such as BW284c51, which is acetylcholine esterase-selective, while *iso*-OMPA affects butyrylcholinesterase. Cholinesterase activity in larval homogenates on ASCh was completely inhibited by 10^{-4} and 10^{-5} M BW284c51 but resistant to *iso*-OMPA (Fig. 3). This behaviour suggests that detectable BuChE activity is not present in these preparations and that the ChE of 3-dph seabream larvae exclusively consists of AChE. In most fish, brain tissue contains exclusively AChE (Kozlovskaya et al., 1993; Sturm et al., 1999a; Chuiko, 2000; Chuiko et al., 2003) and this activity may vary 10-fold among fish species (Chuiko, 2000). On the other hand, plasma and axial muscle tissue may contain exclusively AChE (Augustinsson, 1959; Hogan, 1971; Chuiko, 2000; Chuiko et al., 2003; Chuiko and Podgornaya, 2005), or both AChE and BChE (Chuiko, 2000; Chuiko et al., 2003; Chuiko and Podgornaya, 2005; Sturm et al., 1999a, 2000).

From a kinetic point of view, the enzyme detected in whole body extracts of 3-dph seabream larvae displayed an apparent Michaelian behaviour in the 0.027–3.205 mM range (Fig. 2) when ASCh was used as substrate. Linear Lineweaver–Burk plots showed no evidence for the presence of more than one enzyme, because multiple enzymes using the same substrate but with different kinetic parameters generate non-linear Lineweaver–Burk plots (Cornish-Bowden, 1995; Brown et al., 2004). The apparent K_m value of this enzyme in 3-dph seabream larvae (0.163 ± 0.008 mM) is very similar to the value reported previously by Romania et al. (2003) for AChE in high-salt-detergent soluble extracts of brain from specimens of this species 30–40 cm in length (0.183 ± 0.038 mM) and is also in agreement with data obtained in our laboratory for AChE in crude brain homogenates (unpublished data).

Comparative analysis of the K_m value, a measure of the affinity of the enzyme for its substrate, presently measured in seabream larvae and reported for the first time, indicated that it is in the same range as those reported for ChE in other fishes. For example, Michaelis–Menten constant (K_m) for brain acetylcholinesterase of 20 species of marine neotropical fishes varied from 0.104 to 0.291 mM and AChE specific activity varied from 145 to 530 nmol/min/mg of proteins (Oliveira et al., 2007). Tortelli et al. (2006) found K_m values for the estuarine fishes *Cathorops spixii* and *Micropogonias furnieri* to be 0.196 and 0.201 mM, respectively. In particular, for freshwater fishes, the reported affinity constants were as follows: *Cnesterodon decemmaculatus*, 0.17 mM and *Cyprinus carpio*, 0.23 (De La Torre et al., 2002), *Ictalurus punctatus*, 0.376 (Carr and Chambers, 1996), *Pseudorasbora parva*, 0.113, *Carassius auratus*, 0.112 and *Oncorhynchus mykiss*, 0.085 (Shaonan et al., 2004).

To our knowledge no data have been published on the age-dependent increase of cholin- and carboxylesterases in the developing seabream larvae. In rearing conditions, the larvae of *S. aurata* emerging after hatching deplete the yolk-sac after 3–4 days of endogenous feeding. During this stage, significant activity of esterases could be measured at low intensity at newly hatched larvae, and a consistent increase was seen up to 3 days post-hatch, in relation to the development of the nervous and muscular system. ChE activity on ASCh of 3-dph seabream larvae was 3.4-fold higher than that of newly hatched larvae, and a similar relative increase in enzyme activity was measured using PSCh and PSA as substrates.

Data about enzyme activities in developing fish embryos and larvae are sparse. Küster (2005) monitored the activities of cholinesterase and carboxylesterase in the first 48 h post-fertilization (hpf) of zebrafish development. Significant specific activities in the range of 0.5–25 μmol/min/mg protein could be measured from the sixth somite stage (12 h) up to the Long Pec stage (48 h) for different cholinesterases using acetyl-, acetyl-β-methyl-, butyryl- and propionylthiocholine as substrates. The specific activity of carboxylesterase ranged from 4 to 16 μmol/min/mg protein in the respective developmental stages. Phillips et al. (2002) evaluated the relationship of ChE activity through the larval stages (prolarval, post-larval I and post-larval II) of walleye (*Stizostedion vitreum*) and found that the ChE activity increased about 2.5-fold during the first 19 days post-hatch. In the larvae of the amphibian *Xenopus laevis*, no enzyme activity was found at 24 h postfertilization (p.f.), but increasing intensity was measured starting from 48 h up to 120 h p.f. (Colombo et al., 2005).

The normal range of ChE activity of non-exposed 3-dph individuals (248.9 ± 29.7 nmol/min/mg protein) was greater than the value reported by Varó et al. (2007) for AChE activity present in brain (52.69 nmol/min/mg protein) and muscle (18.21 nmol/min/mg protein) of gilthead seabream fingerlings. Apart from the positive correlation between the whole-body ChE activity and the larval age, mentioned above, several studies have shown that brain AChE activity is lower in older (larger) fish than in younger (smaller) fish (Sturm et al., 1999b; Beauvais et al., 2002; Flammarion et al., 2002). Normal values of ChE activity reported in the literature for several species of marine teleost fish, using AcSCh as substrate, were 195.9 ± 56.8, 314 ± 35.5, and 85.8 ± 23.1 S.D. U/mg protein in the brain of *Limanda limanda*, *Platichthys flesus*, and *Serranus cabrilla*, respectively (Sturm et al., 1999a), 43.32 ± 4.42 S.D. U/mg protein in the brain of the European sea bass juveniles (*Dicentrarchus labrax*) (Varó et al., 2003) and 82.0 ± 18.5 S.D. U/mg protein in head homogenates of *Pomatoschistus microps* (Monteiro et al., 2005) (1 U being a nmol of substrate hydrolysed per minute).

After exposure of seabream larvae to azinphosmethyl for 72 h, the ChE activity and survival were affected in a concentration-dependent manner. With a 72-h LC50 of 4.59 μg/l, azinphosmethyl can be considered as highly toxic to the seabream yolk-sac larvae. Comparison of our results to published information for marine fish indicates that median lethal concentration (72-h) values for azinphosmethyl higher than those in the present study have been reported by Van Dolah et al. (1997) for adults of *Fundulus heteroclitus* (70–105 μg/l) and juveniles of *Sciaenops ocellatus* (10–12 μg/l), suggesting that seabream larvae are more sensitive to this OP insecticide than those species. Nevertheless, our data are in the range of 96-h LC50s reported in the literature for other species, with values of 6–7 μg/l for *S. ocellatus* (Van Dolah et al., 1997), 3.4 μg/l for *Atherinops affinis* (Hemmer et al., 1992), 2.0 μg/l for *Cyprinodon variegatus* (Morton et al., 1997) and 1.19 μg/l for *Menidia menidia* (Lauth et al., 1996). Concerning fish larvae, the estimated 96-h LC50s for azinphosmethyl for 29-days larvae of *Menidia beryllina* and 35-days larvae of *A. affinis* were 22.8 and 3.4 μg/l, respectively (Hemmer et al., 1992). In freshwater fish species, 72-h mean lethal concentration values of 3.36 and 2.31 mg/l for the goldfish (*C. auratus*) and the fathead minnow (*Pimephales promelas*), respectively, have been reported (Adelman and Smith, 1976), indicating that these species are roughly 800- and 500-fold more tolerant than yolk-sac seabream larvae, respectively. Likewise, *Oreochromis niloticus* (Oruç and Üner, 2000) and *C. carpio* (Mayer and Ellersieck, 1986) were more tolerant than *S. aurata* larvae, showing 96-h LC50 values one and two orders of magnitude, respectively, higher than the 72-h LC50 obtained with seabream larvae. In contrast, other species such as *Salmo salar* (Mayer and Ellersieck, 1986) and *O. mykiss* (Ferrari et al., 2004a), showed a similar sensitivity as seabream larvae to acute exposure with azinphosmethyl, with 96-h LC50s in the same order of magnitude.

Seabream larvae exposed to azinphosmethyl for 72 h showed a 70% inhibition of the whole-body acetylcholinesterase activity at approximately the 72-h LC50. This result is in agreement with that of Zinkl et al. (1991), who stated that most laboratory studies suggest that inhibition of 70–90% of fish brain AChE activity occurs at the LC50. In accordance with this general statement, Coppage (1972) reported that AChE in the brain was inhibited more than 80% in all fish that survive median lethal exposures, when the estuarine fish *C. variegatus* was exposed to selected OP insecticides, including azinphosmethyl, at sublethal and median lethal concentrations (40–60% mortality). Likewise, brain AChE activity inhibition in the spot (*Leiostomus xanthurus*) and the pinfish (*Lagodon rhomboides*) that survived median lethal exposures to azinphosmethyl was 96 and 80%, respectively (Coppage and Matthews, 1974).

On the other hand, the estimated 72-h IC50 for inhibition of AChE activity of the seabream yolk-sac larvae by azinphosmethyl in our study was 3.04 μg/l, 1.5 times lower than its 72-h LC50 (4.59 μg/l). According to the bibliography reviewed, this relationship is variable between species. For instance, the 96-h LC50 for this insecticide in the mummichog (*F. heteroclitus*) was 32.16 μg/l, while the 24-h EC50 for brain AChE inhibition was 0.81 μg/l, as outlined by Fulton and Key (2001). Therefore, the 96-h LC50 was about 40 times higher than the 24-h EC50 for brain AChE inhibition. In another study, the relationship between the inhibition of brain AChE induced by azinphosmethyl and mortality was quite different for juvenile red drum (*S. ocellatus*) and adult mummichogs (*F. heteroclitus*) (Van Dolah et al., 1997). Thus, while in the red drum, the 96-h LC50 (6.2 μg/l) and the 24-h EC50 for brain AChE inhibition (5.2 μg/l) were quite similar, in the mummichog, the 96-h LC50 was 50 times greater than the 24-h EC50 (1.0 μg/l). Likewise, for the goldfish, the 96-h IC50 for brain cholinesterase inhibition was 50 μg/l, more than 140-fold lower than the LC50 of this insecticide (Ferrari et al., 2004b).

5. Conclusion

In summary, this study contributes to the knowledge of yolk-sac seabream cholinesterases and their sensitivity toward organosphosphorus pesticides (OP). A comparison to the lethal effect data published for other fish species indicates that gilthead seabream larvae are more sensitive to azinphosmethyl exposure than are many of the species examined. Although further investigation about the sensitivity of seabream larvae to other anticholinesterase agents is needed, the present results indicate that monitoring ChE activity is a valuable tool indicating OP exposure in *S. aurata* larvae and that ASCh is the most appropriate substrate for assessing ChE inhibition in this early-life stage of the fish. Besides, the species fulfils many of the criteria that should be considered in selecting organisms for toxicity testing: it is readily available throughout the year because it is an economically important species and one of the most important species in the European marine fish hatchery sector, it is easily maintained in the laboratory, and has a broad geographical distribution. Therefore, we suggest that early-life stage toxicity tests with *S. aurata* could be used for risk assessment of anticholinestase agents in laboratory conditions. Nevertheless, further studies are still needed in individuals of different ages (fingerlings and adults) of this marine species, both in natural environments and in laboratory exposed ani-

mals to elucidate its potential use as sentinel species in field studies.

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