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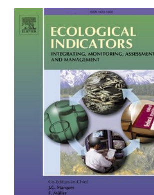

In vivo cholinesterase sensitivity of gilthead seabream (*Sparus aurata*) exposed to organophosphate compounds: Influence of biological factors

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A R T I C L E I N F O

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A B S T R A C T

Two cholinesterases have been found in vertebrates, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). These enzymes are present in the gilthead seabream, AChE in the brain and muscle and BChE in the muscle. Cholinesterases have been used as biomarker of effect in environmental monitoring studies. However, there are few studies about the influence of biometric parameters on ChE. This paper studies the possible influence of biological factors on brain and muscle cholinesterase (ChE) in *Sparus aurata.* Our results show that ChE activity in brain and muscle tissues changes depending on several biological variables. ChE activity in these tissues decreased when the age (48–152 week), body length (14.15–28.95 cm) and body weight (42.73–380.74 g) of the fishes studied increased. The relationships between brain and muscle ChE activity and several biometric factors were curvilinear.

On the other hand, *in vivo* sensitivity of cholinesterase in the gilthead seabream exposed to organophosphorus pesticides (azinphosmethyl, dimethoate and dichlorvos) was studied in order to learn about recovery from cholinesterase present (brain and muscle) after exposure to a sublethal dose. The recovery of muscle AChE was similar to cerebral AChE, while muscular BChE showed a slower recovery.

1. Introduction

Organophosphorus insecticides (OPs) are a classes of pesticides used to control a wide range of invertebrate pests in domestic and natural environments. Most OPs are highly toxic but relatively short-lived in nature (Hill, 2003; Ray and Ghosh, 2006). However, they are also toxic to other non-target species such as mammals, birds, and aquatic organisms. These pesticides can arrive in aquatic systems because they are directly applied into the aquatic environment to control aquatic pests or from runoff from field treatment with OPs after rain events.

Monitoring exposure of this kind of ecosystem to OPs is complicated because they generally do not have a long period of persistence in the water due to their low water solubility and rapid degradation (Kamrin, 2000). For these reasons, a reliable biomarker of exposure would be useful.

Cholinesterases are enzymes inhibited by OPs, and they have been used for monitoring these compounds for a long time. This family of enzymes hydrolyses choline and is present in all animals. Vertebrates have two types of ChEs: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase or pseudocholinesterase (BChE, EC 3.1.1.8). In fish, AChE is predominant in brain and muscle tissues, whereas BChE is present mostly in the liver and plasma (Habig and Di Giulio, 1991). In addition, atypical ChE has been found in some teleost marine fishes (Sturm et al., 1999; Rodríguez-Fuentes and Gold-Bouchot, 2004). The main function of AChE is to hydrolyse the neurotransmitter acetylcholine (Silver, 1974). However, the physiological function of BChE is not well known, although its broad distribution might suggest that BChE has various roles across tissues in an organism (Mack and Robitzki, 2000). Besides, BChE can hydrolyse hydrophobic and hydrophilic compounds with carboxylic and phosphoric acid esters, working as a possible endogenous scavenger of anticholinergic compounds. In fact, BChE is more sensitive to anticholinesterasic compounds than AChE in fish (Magnotti et al., 1994; Wogram et al., 2001).

ChE inhibition is essentially non-reversible with most OPs, and it has been shown to persist for at least two to four weeks in fish (Carr et al., 1995; Ferrari et al., 2004a, 2004b). Therefore, ChE inhibition may be a reliable biomarker for exposure to OPs, but it is very important to know the baseline ChE activity of an organism used as a biomarker and the

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potential sources of variation of cholinesterase enzymes (age, body length, body weight, brain weight, condition factor, and somatic indices for brain and liver).

This study was performed to investigate these potential factors of ChE variation, using gilthead seabream (*Sparus aurata*), a teleost fish in which ChE activity has been shown to be sensitive to inhibition by OPs (Varó et al., 2007; Albendín et al., 2017; Soto-Mancera et al., 2020). In terms of ecotoxicological assessment, *Sparus aurata* is successful because of its wide tolerance to environmental conditions (i.e., saltmarshes, aquaculture systems, etc.), and it is commercially important along the Atlantic and Mediterranean coasts.

The main aims of the present study were the following: 1) to investigate the potential factors for variation of ChE such as age, size, weight, brain somatic index/rate, and condition factors. 2) to study *in vivo* effects of ChE activities in seabream (*Sparus aurata*) exposed to azinphosmethyl, dichlorvos, and dimethoate.

2. Material and methods

2.1. Chemicals

Azinphosmethyl and dimethoate were obtained from Chem Services (West Chester, PA). Dichlorvos from Sigma Chemical Co. ASCh, BSCh, BW284c51, iso-OMPA were supplied by Sigma-Aldrich Química (Madrid, Spain); 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) was from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) and the BioRad Protein Assay reagent were purchased from BioRad (Madrid, Spain).

2.2. Experimental animals and sample preparation

Specimens of gilthead seabream were provided by Marine Cultive Laboratory (CASEM, University of Cádiz, Puerto Real, Cádiz) (Registration number ES110280000312). Fish were fasted for 24 h before sampling. Animals were treated in accordance with ethical guidelines of the European Union Council (Council Directive 86/609/EEC) and the Bioethical Committee from the University of Cádiz (Spain).

The animals were anaesthetised with MS-222 (0.1 g/L) and were euthanized via decapitation. Whole brain and dorsal muscle were excised and homogenized in ice-cold phosphate buffer (0.1 M, pH 7.4) using an Ultra-Turrax homogenizer (Schott Ibérica, Spain) maintaining a ratio of 20 mg of tissue per ml of buffer. The homogenates were centrifuged at 10 000 rpm (9000 × g) at 4 °C for 30 min and the supernatants were collected from what will be called crude extracts. All samples were frozen and stored at −80 °C until further analysis.

2.3. Cholinesterase activity

The procedure used to determine the ChE activity is based on the colorimetric method reported by Ellman et al. (1961) and adapted to microplates as described by Albendín et al. (2017). A 50 μL aliquot of the enzyme source (crude extract of brain or muscle) was mixed with 250 μL of a substrate mix containing Ellman's reagent (DTNB). The substrate mix was prepared by adding 30 mL of phosphate buffer, 1 mL of DTNB 0.01 M in phosphate buffer, and 0.2 mL of substrate (0.2 M unless stated otherwise) in Milli-Q water, resulting in a final substrate concentration of 1.07 mM. Blanks for spontaneous substrate hydrolysis and reaction of DTNB with other thiol groups were determined in the same way in the absence of enzyme or substrate, respectively. Enzyme activity (in triplicate) was determined by following the change of optical density with time at 415 nm for 3 min at room temperature with a microplate reader (BioRad Model 680).

Protein contents were estimated by the method of Bradford (1976) adapted to microplates using bovine serum albumin as the standard, and the enzyme activity was expressed as specific activity (nmol min^{-1} mg $protein^{-1}$).

2.4. Biological factors (effects of some variables on brain and muscle ChE activities)

Effects of age, body size, body weight, brain weight, liver weight, condition factor, and somatic indices for brain and liver on brain and muscle ChE activities were studied. Sixty animals (weight: 28.4–442.6 g and length: 13.5–32.0 cm) of different ages (48, 60, 72, 84, 96 and 152 weeks) were used. The body size and weights of whole fish, brain, and liver were measured in the laboratory. The condition factor was determined as (total weight (g)/$length^3$ (cm^3)) × 100. While Somatic indices for brain and liver were calculated: ISH = (Ph/P) x 100; ISC = (Pc/P) x 100.

2.5. In vivo preliminary toxicity test

The specimens used (403 ± 125 g) were fasted from 24 h before the treatment. Each experimental group (n = 3) received three sublethal intraperitoneal (i.p.) doses of different insecticides: 2, 3.2, and 4 mg/kg azinphosmethyl; 8.8, 11.7, and 23.3 mg/kg dichlorvos; and 2.3, 3.5, and 7.5 mg/kg dimethoate. Due to their low solubility, azinphosmethyl, dichlorvos, and parathion insecticides were administered using corn oil as a vehicle; while dimethoate was dissolved in physiological serum (0.9% solution of NaCl). Two groups of fish (n = 3) were used as control groups; one was injected with corn oil and the other with ClNa 0.9%. The organisms were anaesthetized by immersion in methanosulfonate of tricaine or MS-222 (0.1 g/L seawater) before i.p. injection. This route of administration was chosen to avoid unequal absorption of the insecticides from the aquatic environment which could produce differences in the results (Murphy et al., 1968; Benke et al., 1974).

After 24 h in different tanks of seawater, the fish were anaesthetised with MS-222 and sacrificed by decapitation. Subsequently, the brains were removed, homogenized, and stored at −80 °C for later analyses as described in Section 2.2. The measurement of AChE activity in the brain was performed using a final ASCh concentration of 0.40 mM, as detailed in Section 2.3. Likewise, the percentages of inhibition of brain cholinesterase activity were calculated for the three selected doses of each compound after 24 h of exposure.

The doses used were taken from tabulated values of LD_{50} oral and i.p. in rat, previous studies via i.p. in fish, and unpublished data. (TOXNET).

2.6. Recovery tests: cholinesterase activity

Fifteen specimens (300–500 g (407 ± 91 g), 27–32 cm (29.2 ± 1.6 cm)) were treated with a sublethal dose: azinphosmethyl (4 mg/kg), dichlorvos (23.3 mg/kg) in corn oil, and dimethoate (20 mg/kg) in ClNa 0.9%.

The doses of each OP used in these experiments were determined from preliminary bioassays. Batches of three fish were sacrificed following 3, 9, 24, 48, and 168 h of recovery in clean seawater, and brain and muscle samples were obtained for analysis of ChE activity. The fish were not fed during the experiments.

Brain AChE activity was measured directly, while it was determined in muscle after 30′ of inhibition, BChE ($10\ M^{-5}$ iso-OMPA). BChE muscle activity was measured with BSCh. In all cases the substrate concentration used in the reaction mixture was 1.07 mM.

2.7. Statistical analysis

All determinations were performed in triplicate for each sample. Unless otherwise stated, data were expressed as mean ± standard error of the mean (SEM).

The Shapiro-Wilk test and the Levene test were used to assess the normality and homoscedasticity of data. When it was necessary, the data were transformed ($\sqrt{x}$) to comply with normality and homoscedasticity assumptions. The one-way ANOVA test was used to assess the *in vivo* effect of specific inhibitors on ChE activity, and the LSD's post hoc test

was used to discriminate statistically significant differences relative to the control group. Statistical differences between groups were accepted for P-values lower than 0.05. SPSS v.17.0 software was used for statistical analysis.

The association between enzymatic activity (cerebral AChE, AChE and muscular BChE) and the variables age (E), length (L), body weight (P), brain weight (Pc), weight liver (Ph), somatic cerebral index (ISC), hepatosomatic index (ISH) and condition factor (FC) were studied.

The relationship between biometric factors and brain and muscle ChE activities were studied by correlation analysis (Pearson's coefficients) and by linear regression analysis with original data and logarithmic transformation of data. A probability level of 0.05 was chosen as the level of significance. The coefficients of determination (R^2) were calculated for each equation of regression. The least squares method of representing the log–log of the original data ($y = \ln a + b \ln x$) was used to determine the power equation ($y = ax^b$), where b = slope of the log–log representation and a = antilogarithm of the intercept with y. Normal distribution of all data (original and transformed) was verified using the Kolmogorov-Smirnov test (SigmaPlot 10.0).

3. Results

3.1. Relation between biometric parameters and ChE activities

Morphometric parameters in organisms are shown in Table 1. The body length ranged from 13.5 to 32.0 cm, while the body weight ranged from 28.4 to 442.6 g. Cholinesterase activity in brain and muscle tissues ranged from 37.7 to 432.6 nmol/min/mg protein (brain AChE), 38.3–63.4 nmol/min/mg protein (muscle AChE) and 15.9–59.5 nmol/min/mg protein (muscle BChE). During all of the study, muscle ChE activities were lower than brain ChE activities. The relationships between age and morphometric parameters (body length, body weight, brain weight, liver weight, condition factor, and somatic indices in brain and liver) and ChE activities are represented in the Figs. 1–3. The statistic parameters of simple linear regression are shown in Table 2.

The correlation analysis showed a significant negative correlation ($p < 0.05$) between brain AChE activity and body length ($r = -0.3016$) and brain weight ($r = -0.2879$). Muscle AChE had a significant negative correlation with age ($r = -0.7265$), body length ($r = -0.8797$), body weight ($r = -0.8310$), brain weight ($r = -0.8399$) and liver weight ($r = -0.6603$). Muscle BChE had a significant negative correlation with age ($r = -0.3383$), body length ($r = -0.3746$), body weight ($r = -0.3646$) and brain weight ($r = -0.3075$). Brain cholinesterase activity and muscle cholinesterase activity showed a significant positive correlation with brain somatic indices (brain AChE $r = 0.3421$, muscle AChE $r = 0.8974$ and muscle BChE $r = 0.3736$). The rest of the correlation coefficients were not significant. Determination coefficients were low (<0.15) except relationships between muscle AChE activity and age, body length, body weight, brain weight, liver weight, and brain somatic indices (0.53–0.80).

Cholinesterase activity presented a statistically significant relationship with a greater number of morphometric variables. In the majority of cases, it increased the coefficients of determination, when a power model regression was used (Figs. 4–6). The regression parameters and coefficients of correlation are shown in the Table 3. These coefficients indicate the proportion of the total variability of the dependent variable explained by the linear regression model. In the most cases, the power model fits better than a simple linear model. The coefficient of determination was highest for AChE, with values above 0.75 for the following variables: age, body length, body weight, brain weight, liver weight, and brain somatic indices. Statistical analysis showed a higher significance for the correlation ($p < 0.0001$). The factors with a major grade of association with muscle acetylcholinesterase activity (negative correlation) were body length and body weight, $R^2 = 0.8254$ and $R^2 = 0.8541$, respectively. Therefore, these variables explained 83% and 85% of the muscle AChE activity. However, our results showed an important, but relatively weak, significant negative correlation ($p < 0.01$) between these variables and brain AChE and muscle BChE, because none of these explained more than 18% of the activity. A statistically significant correlation was not observed between the following pairs of variables: brain AChE-age, brain AChE-liver somatic indices, brain AChE-condition factor, muscle AChE-liver somatic indices, muscle AChE-condition factor, muscle BChE-age, muscle BChE-liver somatic indices, and muscle BChE-condition factor.

3.2. In vivo preliminary toxicity test

The results of azinphosmethyl assays (Fig. 7A) showed a decrease in enzymatic activity with respect to the control group. Likewise, significant differences were observed for brain AChE activity between lowest doses (2 and 3.2 mg/kg) of azinphosmethyl and the highest (4 mg/kg). Significant differences of brain AChE activity in organisms treated with dimethoate were observed in all of the groups with respect to the controls, as well as among the groups treated with 10 mg/kg and 20 mg/kg (Fig. 7B). In the case of dichlorvos there was not a statistically significant difference between the three concentrations checked and the control group (Fig. 7C).

The doses chosen for subsequent recovery treatments were 4 mg/kg, 23.3 mg/kg, and 20 mg/kg for azinphosmethyl, dichlorvos, and dimethoate, respectively. These doses produced an inhibition of brain AChE activity of 79, 18, and 59%, respectively, in the fish exposed. In the case of azinphosmethyl and dimethoate, the doses chosen were the highest of the three used in the preliminary tests, because these doses affected the cholinesterase activity of the fish at 24 h of treatment, and they did not kill the fish exposed (Table 4). However, the concentration used in the recuperation assays for dichlorvos was 23.3 mg/kg, the highest concentration, too. This is because dichlorvos is a "direct inhibitor" of ChE, and therefore the maximum inhibition of the enzyme

Table 1
Summary of data for the morphometric factors (age, body length, body weight, brain weight, liver weight, condition factor, liver and somatic indices, and mean brain and muscle ChE activities in *Sparus aurata*. Data are shown as mean ± sem.

Age (weeks)	Body length (cm)	Body weight (g)	Brain weight (g)	Liver weight (g)	Brain somatic indices	Liver somatic indices	Condition factor	Brain AChE activity	Muscle AChE activity	Muscle BChE activity
48	14.15 ± 0.29	42.73 ± 3.88	190.56 ± 10.84	0.52 ± 0.08	0.46 ± 0.06	1.22 ± 0.14	1.52 ± 0.18	361.47 ± 28.06	48.00 ± 7.20	35.32 ± 3.71
60	18.21 ± 0.67	92.73 ± 8.63	275.94 ± 14.32	1.15 ± 0.15	0.30 ± 0.02	1.24 ± 0.07	1.53 ± 0.07	316.54 ± 14.85	32.98 ± 4.38	24.00 ± 3.15
72	21.45 ± 1.33	150.4 ± 25.96	346.61 ± 33.82	1.70 ± 0.25	0.25 ± 0.05	1.08 ± 0.04	1.51 ± 0.14	264.82 ± 15.30	24.11 ± 3.80	35.14 ± 9.70
84	24.35 ± 0.78	230.01 ± 29.74	466.11 ± 28.27	2.79 ± 0.54	0.21 ± 0.02	1.19 ± 0.11	1.57 ± 0.09	274.0 ± 25.0	17.16 ± 2.54	29.25 ± 3.86
96	25.75 ± 1.26	276.84 ± 40.64	471.52 ± 35.32	3.25 ± 0.60	0.18 ± 0.02	1.17 ± 0.12	1.59 ± 0.10	335.57 ± 20.81	16.47 ± 3.38	22.62 ± 3.84
152	28.95 ± 0.97	380.74 ± 24.75	581.80 ± 22.50	3.40 ± 0.46	0.15 ± 0.01	0.89 ± 0.10	1.58 ± 0.10	304.08 ± 31.14	10.67 ± 1.14	22.28 ± 4.75

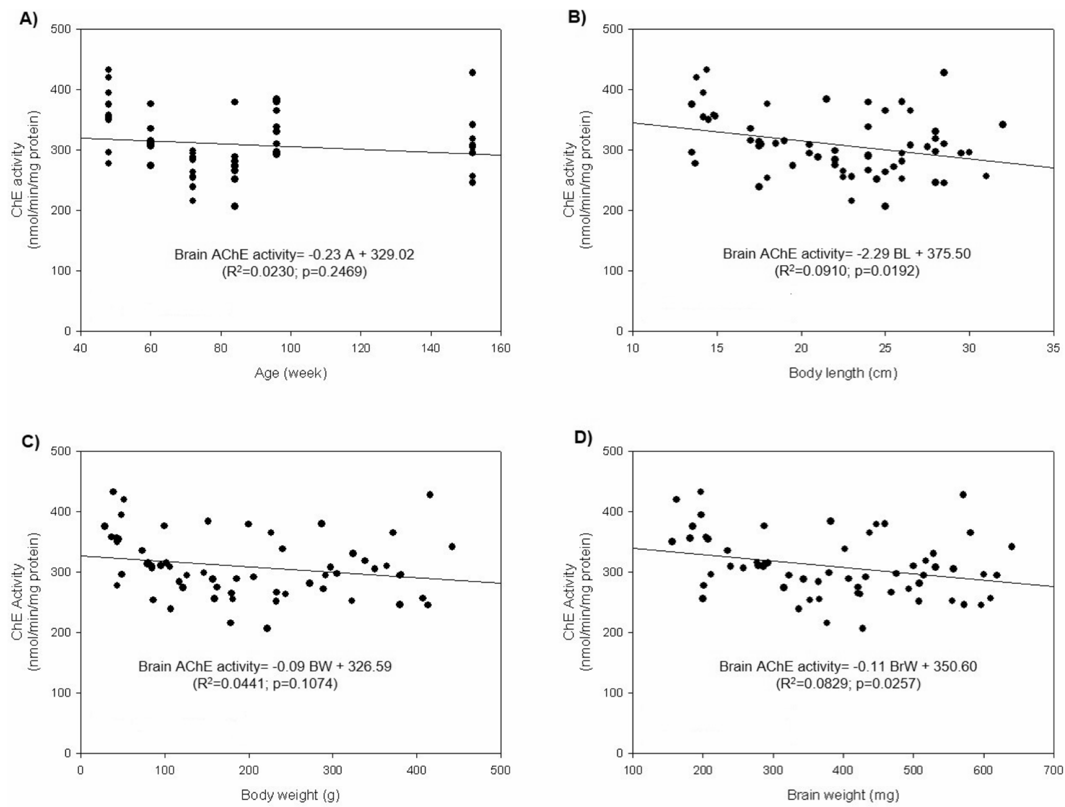

Fig. 1. Scatter diagram and adjustment line of brain cholinesterase activity against A) age, B) length, C) body weight, and D) brain weight in *Sparus aurata* (n = 60). The linear regression equations, the coefficients of determination (R^2), and the corresponding levels of significance (p) have been inserted. The symbol (●) shows the brain cholinesterase activity.

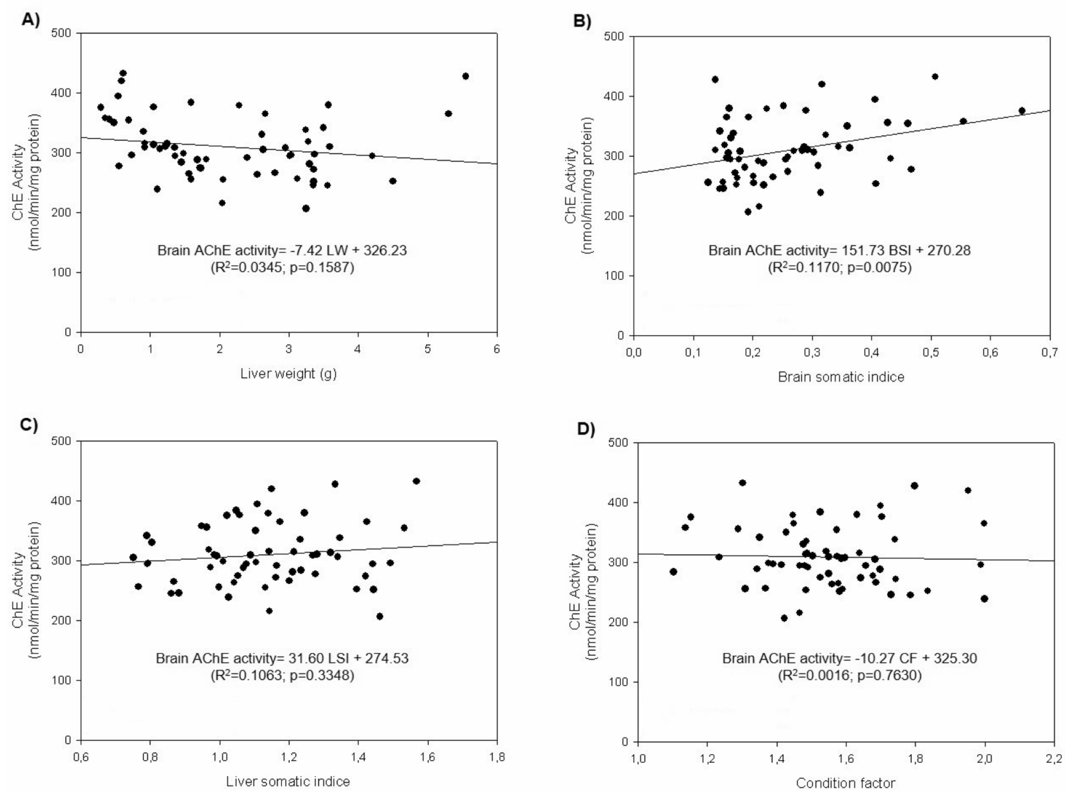

Fig. 2. Relationships between brain AChE activity of *Sparus aurata* and A) liver weight, B) brain somatic indices, C) liver somatic indices, and D) condition factor of fish (n = 60). The symbol (●) shows the brain cholinesterase activity.

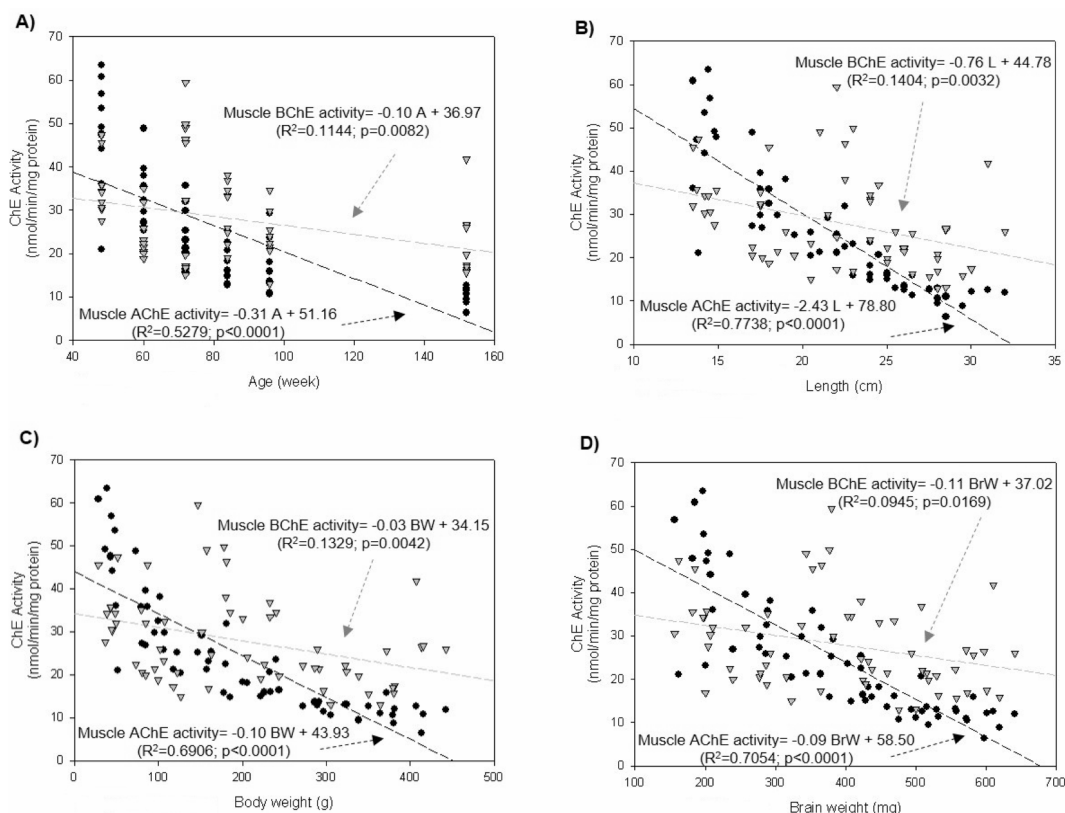

Fig. 3. Relationships between muscle ChE activity of *Sparus aurata* and A) age, B) body length, C) body weight, and D) brain weight of fish (n = 60). The symbols (●) and (▾) show muscle AChE activity and muscle BChE activity, respectively.

Table 2
Analysis of linear regression ($y = ax + b$) between ChE activity (nmol/min/mg protein) in brain and muscle samples and different morphometric parameters in *Sparus aurata.*

ChE	Factor	N	a	b	r	R^2	p	S/NS
Brain AChE	Age (A)	60	−0,2301	329,02	−0,1518	0,0230	0,2469	NS
	Body length (BL)	60	−2,9857	375,50	−0,3016	0,0910	0,0192	S
	Body weight (BW)	60	−0,0880	326,59	−0,2100	0,0441	0,1074	NS
	Brain weight (BrW)	60	−0,1060	350,60	−0,2879	0,0829	0,0257	S
	Liver weight (LW)	60	−7,42	326,23	−0,1859	0,0345	0,1587	NS
	Brain somatic indices (BSI)	60	151,73	270,28	0,3421	0,1170	0,0075	S
	Liver somatic indices (LSI)	60	31,60	274,53	0,1278	0,1063	0,3348	NS
	Condition factor (CF)	60	−10,27	325,30	−0,0397	0,0016	0,7630	NS
Muscle AChE	Age (A)	60	−0,3078	51,16	−0,7265	0,5279	<0,0001	S
	Body length (BL)	60	−2,4340	78,80	−0,8797	0,7738	<0,0001	S
	Body weight (BW)	60	−0,0973	43,93	−0,8310	0,6906	<0,0001	S
	Brain weight (BrW)	60	−0,0864	58,50	−0,8399	0,7054	<0,0001	S
	Liver weight (LW)	60	−8,82	43,62	−0,7867	0,6189	<0,0001	S
	Brain somatic indices (BSI)	60	110,99	−3,71	0,8954	0,8018	<0,0001	S
	Liver somatic indices (LSI)	60	20,41	1,59	0,2939	0,0864	0,2939	NS
	Condition factor (CF)	60	−17,41	51,88	−0,2412	0,0582	0,0634	NS
Muscle BChE	Age (A)	60	−0,1044	36,97	−0,3383	0,1144	0,0082	S
	Body length (BL)	60	−0,7550	44,78	−0,3746	0,1404	0,0032	S
	Body weight (BW)	60	−0,0311	34,15	−0,3646	0,1329	0,0042	S
	Brain weight (BrW)	60	−0,0231	37,02	−0,3075	0,0945	0,0169	S
	Liver weight (LW)	60	−2,55	33,28	−0,3192	0,1019	0,0137	S
	Brain somatic indices (BSI)	60	33,83	19,34	0,3746	0,1403	0,0032	S
	Liver somatic indices (LSI)	60	3,98	23,30	0,0804	0,0065	0,5451	NS
	Condition factor (CF)	60	0,8892	26,68	0,0169	0,0003	0,8980	NS

could be produced more quickly than with the other organophosphates used, as this was realised in the recovery assays.

Table 5 shows the average effective dose (ED_{50}) obtained for inhibition of the brain AChE activity of *Sparus aurata*. The brain AChE was 9 times more sensitive to azinphosmethyl than dimethoate. For dichlorvos, this indicator of toxicity was not calculated, because at the doses used there was no significant inhibition of AChE activity after 24 h from the treatment.

3.3. Inhibition and enzyme recovery

The AChE activities were performed in the recovery tests by using the

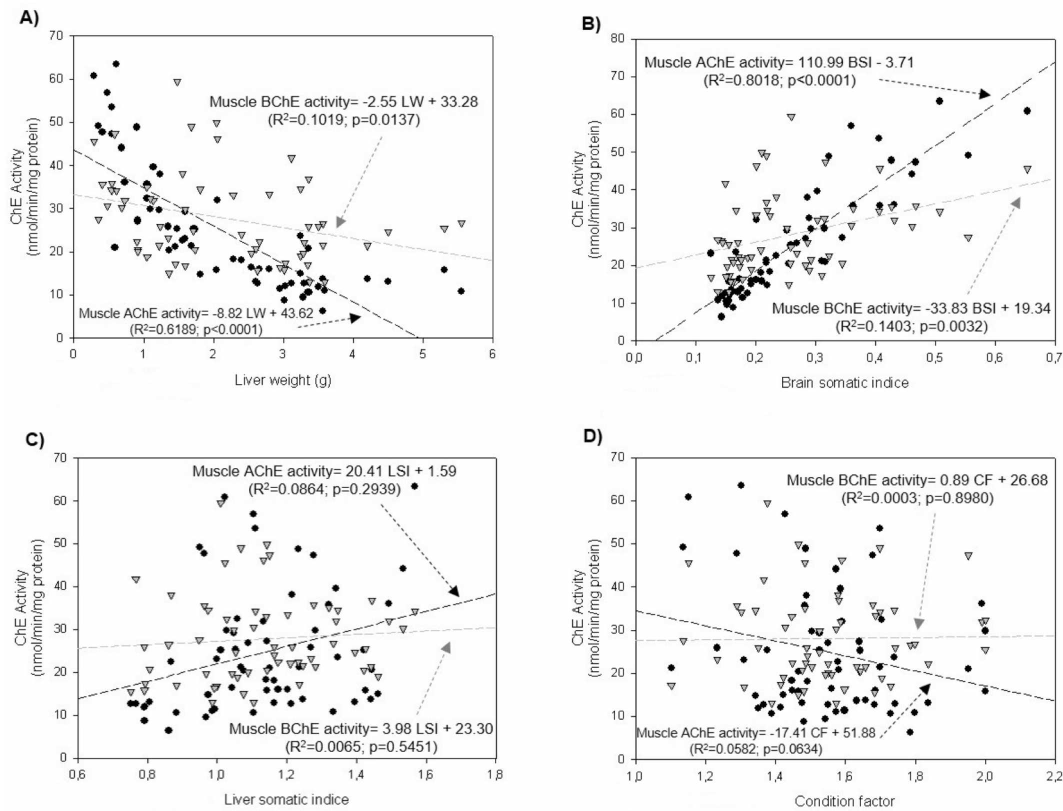

Fig. 4. Relationships between muscle ChE activity of *Sparus aurata* and A) liver weight, B) brain somatic indices, C) liver somatic indices, and D) condition factor of fish (n = 60). The symbols (●) show muscle AChE activity and muscle BChE activity, respectively.

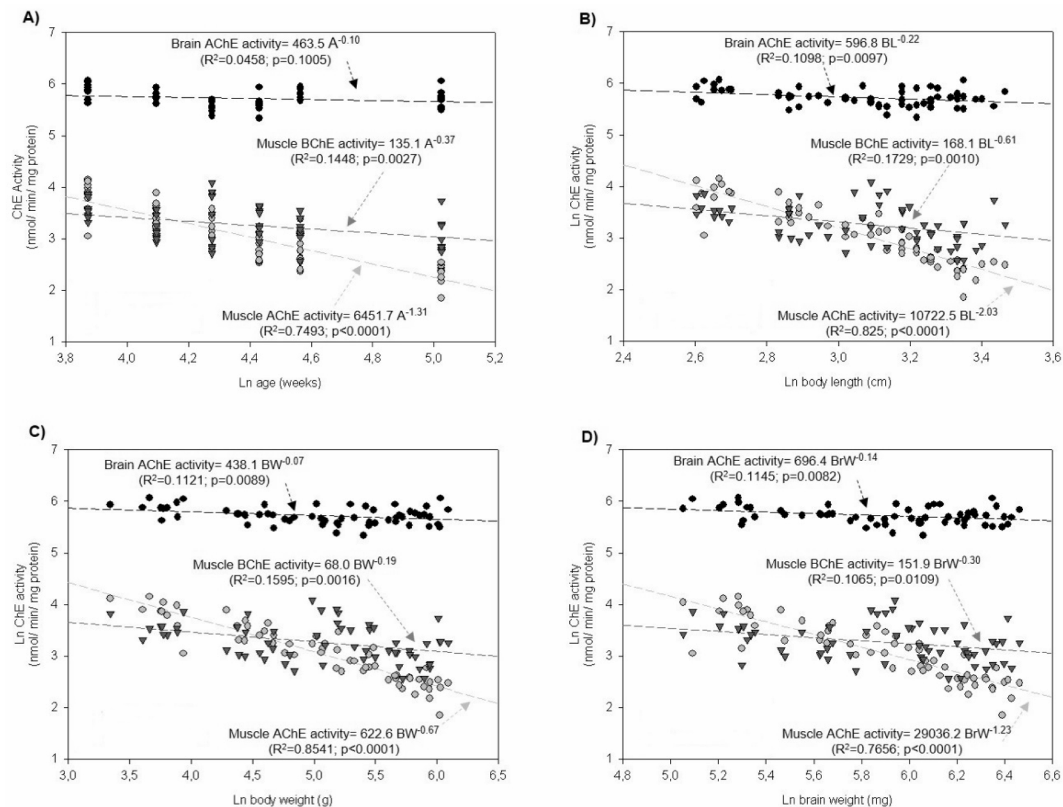

Fig. 5. Relationships between brain and muscle ChE activity of *Sparus aurata* and A) age, B) body length, C) body weight, and D) brain weight of fish (n = 60). The symbols (●) show brain AChE activity, (●) show muscle AChE activity and (▾) muscle BChE activity.

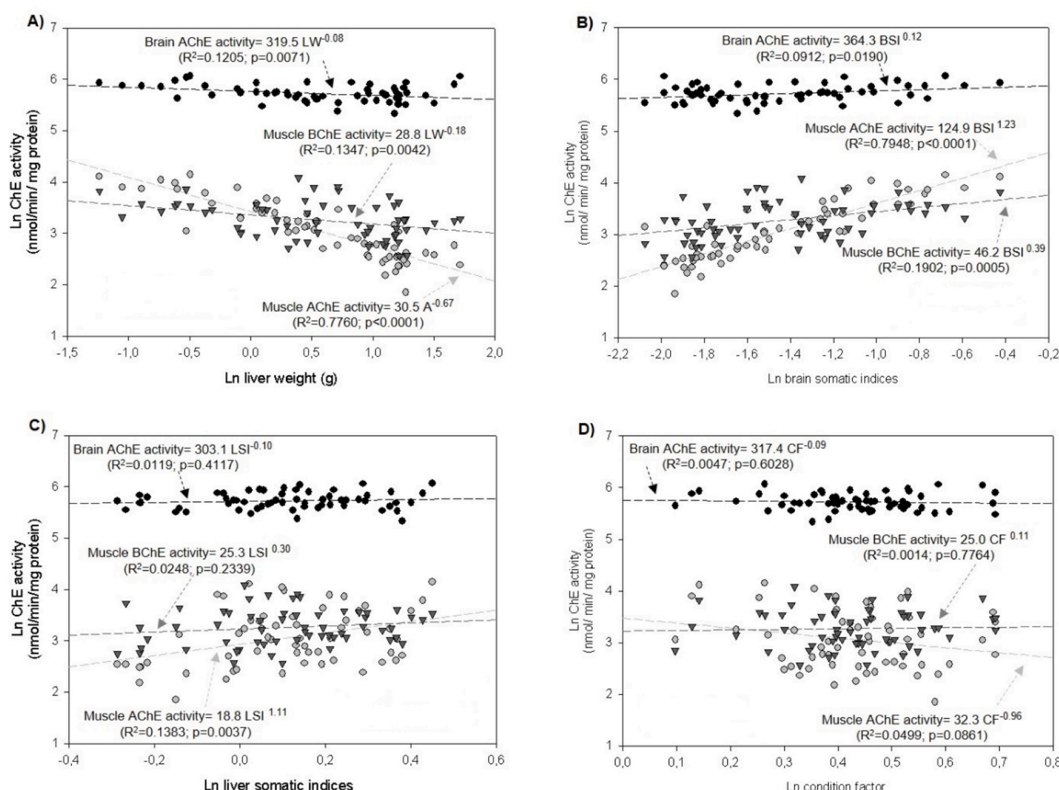

Fig. 6. Relationships between brain and muscle ChE activity of *Sparus aurata* and A) liver weight, B) brain somatic indices, C) liver somatic indices, and D) condition factor of fish (n = 60). The symbols (●) show brain AChE activity, (●) show muscle AChE activity and (▾) muscle BChE activity.

Table 3
Analysis of regression ($y = ax^b$) between ChE activity (nmol/min/mg protein) in brain and muscle samples and different morphometric parameters in *Sparus aurata*.

ChE	Factor	N	b	Ln a	r	R^2	p	S/NS
Brain AChE	Age (A)	60	−0,0954	6,1387	−0,2141	0,0458	0,1005	NS
	Body length (BL)	60	−0,2185	6,3916	−0,3313	0,1098	0,0097	S
	Body weight (BW)	60	−0,0718	6,0824	−0,3349	0,1121	0,0089	S
	Brain weight (BrW)	60	−0,1400	6,5459	−0,3384	0,1145	0,0082	S
	Liver weight (LW)	60	−0,0782	5,7668	−0,3471	0,1205	0,0071	S
	Brain somatic indices (BSI)	60	0,1227	5,8981	0,3020	0,0912	0,0190	S
	Liver somatic indices (LSI)	60	0,0953	5,7141	0,1089	0,0119	0,4117	NS
	Condition factor (CF)	60	−0,0869	5,7586	−0,0686	0,0047	0,6028	NS
Muscle AChE	Age (A)	60	−1,3047	8,7721	−0,8656	0,7493	<0,0001	S
	Body length (BL)	60	−2,0266	9,2801	−0,9085	0,8254	<0,0001	S
	Body weight (BW)	60	−0,6705	6,4339	−0,9242	0,8541	<0,0001	S
	Brain weight (BrW)	60	−1,2247	10,2763	−0,8750	0,7656	<0,0001	S
	Liver weight (LW)	60	−0,6740	3,4184	−0,8809	0,7760	<0,0001	S
	Brain somatic indices (BSI)	60	1,2251	4,8274	0,8915	0,7948	<0,0001	S
	Liver somatic indices (LSI)	60	1,1052	2,9337	0,3719	0,1383	0,0037	S
	Condition factor (CF)	60	−0,9581	3,4735	−0,2235	0,0499	0,0861	NS
Muscle BChE	Age (A)	60	−0,3733	4,9051	−0,3806	0,1448	0,0027	S
	Body length (BL)	60	−0,6047	5,1248	−0,4158	0,1729	0,0010	S
	Body weight (BW)	60	−0,1889	4,2193	−0,3994	0,1595	0,0016	S
	Brain weight (BrW)	60	−0,2977	5,0233	−0,3263	0,1065	0,0109	S
	Liver weight (LW)	60	−0,1804	3,3596	−0,3670	0,1347	0,0042	S
	Brain somatic indices (BSI)	60	0,3906	3,8322	0,4361	0,1902	0,0005	S
	Liver somatic indices (LSI)	60	0,3005	3,2294	0,1574	0,0248	0,2339	NS
	Condition factor (CF)	60	0,1047	3,2244	0,0374	0,0014	0,7764	NS

sublethal doses selected in the preliminary toxicity test. This was carried out in order to observe the evolution of the ChE present in the brain and muscle of *Sparus aurata* during 168 h (7 days) after i.p. administration of each compound or after administration of corn oil or (0.9%) NaCl solution, which were the vehicles in which the compounds were dissolved.

The values of the AChE activity of seabreams treated only with corn oil or 0.9% NaCl were statistically studied using one-way ANOVA. This analysis revealed that for each vehicle, there were no significant differences between the groups slaughtered at different times; so it was decided to consider them in each case as the same group for subsequent statistical analyses. The average values of the ChE activity measured in the brain and in the muscle of the control specimens treated only with vehicle in the different periods of time and the results of the analysis of variance are shown in Table 6.

Table 7 shows the ChE activity in the gilthead's brain and muscle after administration, via i.p., of 4 mg/kg of azinphosmethyl, 23.3 mg/kg

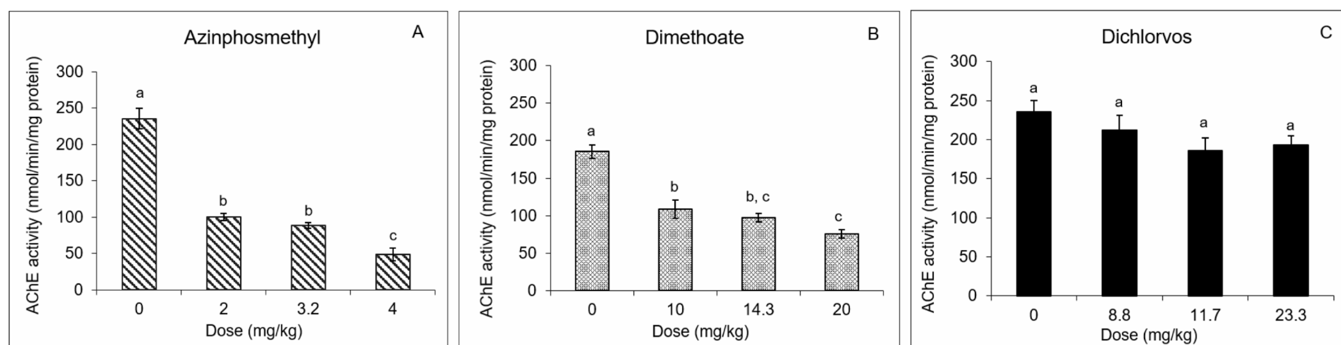

Fig. 7. Different dose effects of each one of three OP on brain AChE activity 24 h after administration by i.p. injection. The values represent the mean ± sem of three individual by group. Different letters indicate significant differences compared with control values (p greater than 0.05).

Table 4
Percentages of inhibition obtained with different doses used.

Insecticides	Dose (mg/kg)	% Inhibition
Azinphosmethyl	2	58
	3.2	63
	4	79
Dimethoate	10	42
	14.3	48
	20	59
Dichlorvos	8.8	10
	11.7	21
	23.3	18

Table 5
Data of ED_{50} for inhibition of brain AChE from *Sparus aurata.*

Insecticides	ED_{50} (mg/kg)	Confidence Intervals 95%
Azinphosmethyl	1.74	1.52–2.23
Dimethoate	16.20	11.80–19.87
Dichlorvos	–	–

of dichlorvos and 20 mg/kg of dimethoate, as well as the corresponding percentage of inhibition with respect to controls.

In the case of treatment with azinphosmethyl, brain AChE activity showed an inhibition of almost 81% with the mean of its respective control group only 3 h after the administration of this compound (Table 7); however, the greatest inhibition of this enzyme (91.5%) was observed at 9 h after exposure. From this moment, the cholinesterase activity in the brain showed a slight increase, although only 52.5% of the enzyme activity of the control group was recovered 7 days after the treatment (Fig. 8A). On the other hand, a slight decrease in the muscular AChE activity was observed at 3 h after the injection of azinphosmethyl with respect to the controls, the inhibition was 31%. This downward trend in enzyme activity was observed after 9 h; however, later a stabilization of activity was observed in the analysed specimens. In this test, the inhibition of AChE activity in the muscle did not reach the 65%.

Likewise, there was a progressive decrease in the activity of the BChE enzyme during the first 24 h, at which time it reached an inhibition of 84%, but 94.1% was recorded at 7 days, after an intermediate phase of partial recovery of the activity at 48 h.

In the same way as azinphosmethyl, brain AChE activity in the gilthead seabream decreased slightly after 3 h exposure to dimethoate; a decrease that became significant after 9 h. Although the highest level of inhibition was reached at 48 h (77.6%), no statistically significant differences were found from 9 h to the end of the assay (Table 7). Similarly, muscle AChE activity in treated gilthead showed a similar trend to that described for brain AChE. In contrast, muscle BChE activity showed a different behaviour after the administration of this compound. In this case, the enzymatic activity presented inhibition throughout the study period, beginning in the first 3 h after administration of this OP (Fig. 8. B).

On the other hand, dichlorvos is a direct-acting inhibitor of ChE, thus the decrease of ChE activity measured in the *Sparus aurata* group treated with this compound was expected. An inhibition of brain AChE was observed in the first three hours after exposure (57.3%); at this point brain ChE activity levels had recovered. This recovery was statistically similar to that of the control group after 7 days in seawater (Table 7). The inhibition profile (maximum value 54.5%) and recovery of muscle AChE activity was similar to brain AChE data (Fig. 8C). Nevertheless, muscle BChE activity presented a similar trend to the previous ones; the levels of inhibition were the highest, reaching values of 95.2% at 3 h. The recovery of enzymatic activity was only 53% of the control activity at the end of this assay.

4. Discussion

4.1. Relation between biometric parameters and ChE activities

The present study of *Sparus aurata* showed that brain baseline ChE activity decreased with age; so older (larger) fish presented a lower activity than the younger (smaller) ones. Previous studies indicate that baseline ChE activities in the brains of some fish vary with body weight

Table 6
ChE activity present in the brain and muscle of *Sparus aurata* control group treated with corn oil, and results of the analysis of variance.

	ChE activity	Sampling time (hours)*					Statistical parameters
		3	9	24	48	168	
Corn oil	Brain AChE	245.1 ± 12.1	281.4 ± 15.6	295.0 ± 26.6	282.4 ± 7.2	269.3 ± 26.6	$F_{[14,4]} = 0.956$ (p = 0.472)
	Muscle AChE	9.9 ± 0.6	11.1 ± 3.1	11.5 ± 2.3	12.7 ± 1.4	13.1 ± 1.9	$F_{[14,4]} = 1.084$ (p = 0.415)
	Muscle BChE	24.3 ± 0.1	21.1 ± 3.1	23.1 ± 2.6	27.8 ± 3.0	26.9 ± 5.2	$F_{[14,4]} = 0.689$ (p = 0.616)
NaCl (0.9%)	Brain AChE	226.9 ± 7.6	263.2 ± 62.5	266.6 ± 39.4	259.1 ± 62.0	280.8 ± 28.3	$F_{[14,4]} = 0.194$ (p = 0.936)
	Muscle AChE	14.3 ± 2.6	9.5 ± 0.2	13.8 ± 1.1	13.8 ± 1.9	16.2 ± 2.4	$F_{[14,4]} = 2.036$ (p = 0.165)
	Muscle BChE	23.1 ± 2.7	29.5 ± 6.2	30.1 ± 4.2	35.8 ± 2.1	32.6 ± 3.1	$F_{[14,4]} = 1.436$ (p = 0.292)

Specific activity is the mean ± sem expressed in nmol/min/mg proteins (n = 3 specimens).
* Statistical parameter F and its significance p; degrees of freedom in square brackets. Level of significance required p < 0.05.

Table 7
Specific ChE activity in nmol/min/mg protein present in brain and muscle of *Sparus aurata* after administration by i.p. of 4 mg/kg of azinphosmethyl, 23.3 mg/kg of dichlorvos, and 20 mg/Kg dimethoate and percentage of inhibition with respect to the control.

	Tiempo (h)	Activity*		
		Brain AChE	Muscle AChE	Muscle BChE
Control (corn oil)		274.6 ± 8.56 A[#]	11.65 ± 0.55 A	24.64 ± 1.41 A
Azinphosmethyl	3	53.13 ± 9.02B (80.6)	8.03 ± 1.02B (31.1)	18.43 ± 2.83B (25.2)
	9	23.43 ± 3.04C (91.5)	5.13 ± 0.40 BC (56.0)	9.49 ± 1.16C (61.5)
	24	40.89 ± 8.93 BC (85.1)	4.27 ± 0.78CD (63.3)	3.96 ± 1.28CD (83.9)
	48	82.96 ± 5.88 D (69.8)	5.02 ± 2.30 BD (56.9)	9.14 ± 1.07C (62.9)
	168	144.2 ± 27.41 E (47.5)	4.33 ± 0.71CD (62.9)	1.45 ± 0.90 D (94.1)
Diclorvos	3	117.4 ± 36.68B (57.3)	5.30 ± 0.64B (54.5)	1.19 ± 0.69B (95.2)
	9	169.6 ± 53.40 BC (38.3)	6.71 ± 2.01 BC (42.4)	5.31 ± 2.28 BD (78.4)
	24	238.5 ± 24.17 AC (13.2)	9.31 ± 1.44 ACD (20.1)	12.74 ± 5.88C (48.3)
	48	219.96 ± 8.50 AC (19.9)	8.21 ± 1.37 BD (29.5)	13.42 ± 3.94CD (45.5)
	168	256,6 ± 13,68 A (6.6)	10.70 ± 0.97 AD (8.2)	13.00 ± 0.82CD (47.2)
Control (NaCl)		259.34 ± 17.69 A	13.54 ± 0.88 A	30.24 ± 1.86 A
	3	201.5 ± 12.01 A (22.3)	6.74 ± 1.62B (50.2)	0.02 ± 0.02B (100)
Dimethoate	9	88.7 ± 14.78B (65.8)	3.91 ± 0.72 BC (71.1)	0.60 ± 0.50B (98.2)
	24	64.0 ± 3.08B (75.3)	2.99 ± 0.14C (77.9)	0.01 ± 0.00B (100)
	48	58.1 ± 3.58B (77.6)	3.04 ± 0.36C (77.5)	1.03 ± 0.89B (97.0)
	168	103.9 ± 10.34B (59.9)	3.45 ± 1.49C (74.6)	1.54 ± 0.95B (94.9)

* Cholinesterase activity is expressed as mean ± sem. N = 3 for all groups except the control group in which N = 15. The percentage decrease with respect to the control group is indicated in parentheses.
[#] The different letters located next to the means in each column indicate statistically significant differences between the groups ($p < 0.05$).

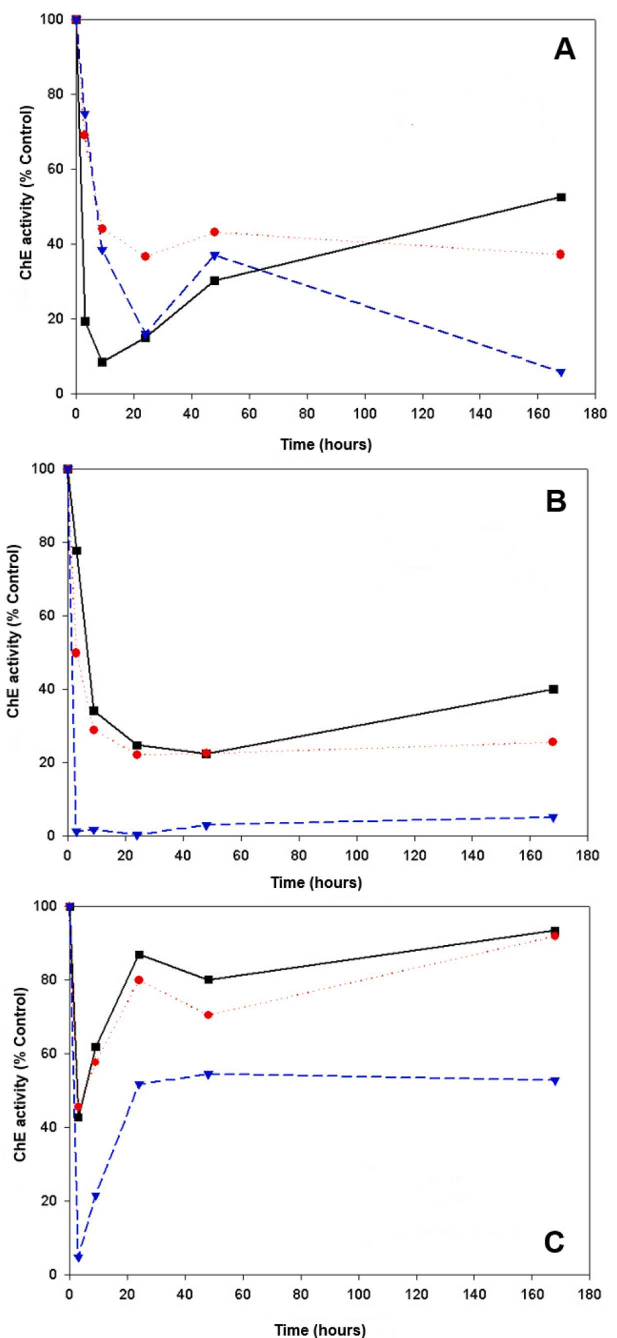

Fig. 8. Time-course of inhibition and recovery of seabream (■) Brain AChE activity (●) muscle AChE and (▼) muscle BChE following a sublethal i.p. injection of each of three studied OP in corn oil for azinphosmethyl (A) and dimethoate (B) and NaCl (0.9%) for dichlorvos (C). Each point represents the mean ± sem of triplicate analysis.

and age. These studies found significant negative correlations between brain ChE activity and variables as body length, body weight, and brain weight. Thereby, Napierska and Podolska (2003) found that brain ChE activity of *Platichthys flesus* decreased with age. Also, a decrease was observed with an increase of body length in several species such as *Oreochromis niloticus* (Chandrasekara and Pathiratne, 2007; Pathiratne et al., 2008), *Pagrus pagrus, Porichthys porosissimos* and *Percophis brasiliensis* (Oliveira et al., 2007), *Morone saxatilis* (Durieux et al., 2011), *Alepocephalus rostratus,* and *Lepidion lepidion* (Koenig and Solé, 2014). In the same way, a negative correlation between body weight and brain ChE activity was found in *Lepomis lucius* and *Pimephales promelas* (Weiss, 1958) and *Oreochromis niloticus* (Pathiratne et al., 2008), as well as between brain weight and brain ChE activity in *Micropterus salmoides*, *Lepomis macrochirus*, *Notemigonus crysoleucas,* and *Carassius auratus* (Weiss, 1961).

On the other hand, an inverse correlation between muscle ChE activity and body length was confirmed in *Gasterosteus aculeatus* (Sturm et al., 1999) and *Pleuronectes vetulus* (Rodríguez-Fuentes et al., 2008). Likewise, Kirby et al. (2000) found a weak correlation between muscle ChE activity and several factors such as size, sex, somatic indices for liver and gonad, and condition factor in *Platichthys flesus.* However, Alpuche-Gual and Gold-Bouchot (2008) did not find a significant correlation between muscle ChE activity and body weight and body length

in *Haemulon plumieri*. Lundin (1962) showed that the muscle ChE activity and body length relationship follows simple suppositions: cell length is proportional to body length and to the radius of muscle cells, ChE activity is proportional to cell surface; so, in small fish the density of cells is higher than in large fish. Thus, it is possible to relate the inverse ChE activity to the body length of the specimen.

The power equation model ($y = ax^b$) was the one that best fit our data. In the base of the statistic parameters of this model, it was found that values of brain and muscle ChE activity were explicated for body length and body weight, with the highest degree of significance. The power equation model explained about 82% of the variability in muscle AChE-body length and muscle AChE-body weight relationships. However, in the case of brain AChE and muscle BChE activity with the same factors, this model explained <18%. With the power equation model, brain and muscle AChE-weight relationships got higher correlation coefficients that AChE-length relationship. Contrary to this, the linear regression equation showed higher coefficients for the ChE-body length association in all cases. Our results coincide with those of Flammarion et al. (2002). Their study showed that the regression between Ln (muscle-ChE) and Ln (length) in *Leuciscus cephalus* had a higher determination coefficient ($R^2 = 0.62$) than the original regression variables ($R^2 = 0.51$). In the same way, Pathiratne et al. (2008) showed that the relationship between ChE activity in brain and muscle tissues and body length and body weight in *Oreochromis niloticus* are curvilinear and had a highly significant relationship when applying the power equation model. Nevertheless, these authors obtained high correlation coefficients ($r \geq 0,880$) between size and ChE activity in muscle and brain. In addition, the relationship between ChE activity and body length had values of correlation coefficients comparatively above the relationship between ChE activity and body weight. Sturm et al. (1999) found significant and more negative linear relationships between muscle ChE (measure on ASCh) and body length and body weight in *Gasterosteus aculeatus*, with determination coefficients of 0.446 and 0.680, respectively. Likewise, muscle ChE activity in *Pleuronectes vetulus* presented a significant negative coefficient with body length ($r = -0.3271$, $p = 0.011$), although the linear regression model explained only 10.75%of the variability (Rodríguez-Fuentes et al., 2008). Finally, a significant and negative relationship was seen between total length and muscle AChE in *Alepocephalus rostratus* ($r = -0.448$, $p < 0.0001$) and *Lepidion lepidion* ($r = 0.679$, $p < 0.001$) (Koenig and Solé, 2014).

4.2. In vivo preliminary toxicity test

Table 8 shows LC_{50}-24 h values (24 h LC_{50}) of the organophosphorus pesticides for different species of fish, compiled in the ECOTOX database (EPA, 2007). The order of toxicity of these insecticides is observed in Table 9: azinfosmetil > dichlorvos > dimethoate. These data agree with our results for *Sparus aurata* (taking into account the inhibitory potency of the corresponding oxons on cerebral and muscle ChE in vitro tests (Albendín et al., 2017) as well as the ED_{50} for the inhibition of cerebral AChE obtained for azinphosmethyl (1.74 mg/kg) and dimethoate (16.20 mg/kg) administered via i.p.

4.3. Inhibition and enzyme recovery

In the case of azinphosmethyl, a dose of 4 mg/kg in *Lepomis gibbosus* and 8 mg/kg in *Ictaluras melas* produced an inhibition of 14% and 27% of cerebral AChE after 2 h from administration by the i.p. route, respectively. Likewise, a dose of 16 mg/kg in both species reduced the activity by 76% and 57%, respectively (Murphy et al., 1968). In our study, a greater inhibition was observed with a dose four times lower (4 mg/kg) after 3 h from its administration, reaching values of 80.6% of brain AChE activity. Murphy et al. (1968) investigated under limited time conditions; so, the lack of depression in cholinesterase levels in some species, even at higher doses of the pesticides, could be because there was insufficient time to produce adequate amounts of the active metabolite. The relative amount of the oxo analogues that are available to inhibit the cholinesterase enzyme will depend (at least in part) on the relative speed of their formation from the parent compound and the relative rate of metabolic degradation of these compounds by tissue hydrolases or other mechanisms of inactivation. In our study, the beginning of the toxic action of azinphosmethyl was rapid. The maximum inhibition of brain ChE occurred at 9 h after a sublethal dose, while in the case of dimethoate, the minimum value of ChE activity was obtained at 48 h (Table 7). Although dimethoate has been detected in water samples, sediments, and soil (Brunetto et al., 1992; El-Kabbany et al., 2000; Sapozhnikova, et al., 2004) as well as in fish tissues (Abou-Arab et al., 1996; Sapozhnikova et al., 2004), and its effect on metabolism has been studied too (Pant and Singh, 1983; Awasthi et al., 1984), the present results showed an important effect of this compound on brain AChE activity in *Sparus aurata*. No recovery of the enzymatic activity was observed in all assays.

Varó et al. (2007) studied the effect of three concentrations (0.025, 0.05 and 0.1 mg/L) of dichlorvos on gilthead sea bream fingerlings (~0.3 g) exposed for 24 h. Their results showed a reduction of 41% and of 52% in head ChE at the highest concentrations, while the lower concentration did not induce significant inhibition. In a previous study, these authors obtained similar data with juveniles (~5 g) of *Dicentrarchus labrax*, meaning a significant inhibition in the ChE activity of the head (greater than35%) after acute exposure to 0.125 mg/L of dichlorvos, but no dose-dependent relationship was found in the dose range studied (Varó et al., 2003). A study of the temporal evolution of inhibition and recovery of ChE activity was not done in either of these works. However, in a subsequent study, Varó et al. (2008) studied the course-time pattern of ChE inhibition and recovery in the tissues of *Aphanius iberus*, a typical specie of the Spanish east coast, after sublethal exposure to dichlorvos (1 mg/L) for 96 h, and after 96 h in clean seawater (period of recovery). The maximum value of ChE inhibition on

Table 8
Lethal Concentrations-50 (24 h-LC_{50})* of the pesticides used in this study for several species of fish (from the data base ECOTOX).

Species	Azinphosmethyl 24 h-LC_{50} (mg/L)	Dichlorvos 24 h-LC_{50} (mg/L)	Dimethoate 24 h-LC_{50} (mg/L)
Carassius auratus (F)	2.40–11.20	–	–
Cyprinodon variegatus (S)	–	–	–
Cyprinus Carpio (F)	1.24	9.44–20.0	4.23–4.62
Danio rerio (F)	–	–	100
Esox lucius (F)	0.00067	–	–
Fundulus heteroclitus (S)	–	3.41	–
Gambusia affinis (F)	–	17.8	–
Gasterosteus aculeatus (S)	0.007–0.016	–	–
Heteropneustes fossilis (F)	–	8.13	5.14–12.13
Ictalurus punctatus (F)	3.90–4.53	–	–
Leiostomus xanthurus (S)	–	1.00	–
Lepomis cyanellus (F)	0.13	–	–
Lepomis macrochirus (F)	0.011–0.15	0.87–3.08	28.00
Menidia menidia (S)	0.0014	5.70–9.60	–
Oncorhynchus kisutch (F)	0.004–0.023	–	–
Oncorhynchus mykiss (F)	0.005–0.026	0.50	11.20–133.0
Pimephales promelas (F)	0.141–13.7	23.0	–
Poecilia reticulata (F)	0.37–1.80	5.81	25.29–620

S: Seawater species; F: Freshwater species.
* The minimum and maximum values are shown.

Table 9
Summary of the acute toxicity on fish for the pesticides used in the present study (PAN Pesticides Database) [http://www.pesticideinfo.org/].

Compounds	Average toxicity	Range of toxicity
Azinphosmethyl	Highly toxic	Moderate-very high toxicity
Dichlorvos	Moderately toxic	Slight-high toxicity
Dimethoate	Slightly toxic	No toxic-high toxicity

the acetylthiocholine substrate in the head of the fish it was 83.1% (females) and 90.8% (males). In muscle, the inhibition reached values of 79.5% and 82.1% in females and males, respectively. After 96 h in clean water, head ChE activity recovered to 43.3% and 44.3% of the control value for females and males, respectively. In muscle, the recovery of ChE activity was greater– up to 70.5% of the control for females and up to 50.9% of the control for the males. In the present study, a maximum ChE inhibition of 57.3% was achieved in the gilthead at 3 h after administration of 23.3 mg/kg dichlorvos. At the end of this assay (168 h), the recovery of the enzymatic activity (93.4%) was total, no statistically significant difference was observed between the control values and treatment groups ($p = 0.05$).

On the other hand, Carr and Chambers (1996) did not observe a spontaneous reactivation of the cerebral AChE enzyme of *Ictalurus punctatus*. For their part, Wallace and Herzberg (1988) suggested that the recovery of activity in rainbow trout after exposure to malaoxon and paraoxon depended on *de novo* synthesis of AChE. This inability of the brain AChE of fish to reactivate spontaneously was pointed out as the probable reason for the prolonged AChE inhibition observed after exposure of *Salmo salar* (Morgan et al., 1990), *Ictalurus punctatus* (Carr et al., 1995; Straus and Chambers, 1995), *Gambusia affinis* (Boone and Chambers, 1996) and *Carassius auratus* (Ferrari et al., 2004a) to different organophosphorus insecticides. Specifically, an inhibition of cerebral and muscular AChE in *G. affinis* was observed for 6 weeks after exposure to the insecticides chlorpyrifos, parathion, and methyl parathion (Chambers et al., 2002). Likewise, after exposure to sublethal concentrations of parathion and azinphosmethyl (0.1 mg/L), the activity of the cerebral AChE enzyme in *Carassius auratus* required more than 35 days for its total recovery (Ferrari et al., 2004a). In the case of *Oncorhynchus mykiss* exposed to azinphosmethyl (0.001 mg/L), no significant recovery was observed after 21 days in clean water (Ferrari et al., 2007).

Several studies have suggested that fish mortality occurs when the inhibition of cerebral AChE reaches values between 70 and 90% (Coppage and Matthews, 1974; Zinkl et al., 1987, 1991). According to this, in previous work where gilthead seabream larvae in the endogenous feeding phase were exposed to azinphosmethyl and parathion at concentrations close to its respective LC_{50} for 72 h, they showed a 70% inhibition of body AChE activity (Arufe et al., 2010). However, the relationship between inhibition of cholinesterase and mortality is not clear in fish, because there are species for which an 8% inhibition was lethal (Weiss, 1958, 1961), while others are able to tolerate levels of inhibition greater than 90% (Fulton and Key, 2001; Ferrari et al., 2004a, 2004b). The present results confirm this last point. Unlike the larvae with a yolk sac, adult specimens of *S. aurata* were able to survive with inhibition values of 91.5 ± 1.9% at 9 h after azinphosmethyl administration. Similar results were described by Ferrari et al. (2004a) who pointed out that the species *Carassius aurata* was able to survive after 96 h of treatment with azinphosmethyl and parathion (0.1 mg/L) with an inhibition of cerebral AChE that reached 90% and 95%, respectively. Likewise, these authors indicated that the residual ChE activity in rainbow trout became <5% of the control value after exposure to azinphosmethyl (0.001 mg/L) for 96 h without causing signs of toxicity (Ferrari et al. 2004b). Coppage (1972) observed that individuals of *Cyprinodon variegatus* survived when they were exposed to average lethal concentrations of various organophosphorus insecticides (including azinphosmethyl and dichlorvos) with an inhibition of cerebral AChE greater than 80%. Coppage also stated that given the variability of the AChE levels in control fish, activity should be inhibited more than 13% to indicate exposure to organophosphorus insecticides. As indicated by Fulton and Key (2001), these findings suggest that levels of inhibition of 20–70% cerebral cholinesterase in live fish may be indicative of OP exposure. Attending to the study of Murphy et al. (1968), there is evidence that markedly low levels of brain AChE activity could be tolerated better by fish than mice or hens. According to these authors, this suggests that additional factors should be considered in order to assess the relative toxicities of anticholinesterase insecticides for each species, such as the relative speed of formation and accumulation of acetylcholine and the relative susceptibility to lethal effects of an excess of this neurotransmitter.

In general, muscle AChE has a behaviour similar to cerebral AChE for the studied OPs in this work. On the other hand, the order of sensitivity to the corresponding oxons in *in vitro* tests was analogous (azinphosmethyloxon > dichlorvos > omethoate) (Albendín et al., 2017), and the time course evolution (inhibition and recovery) of ChE activity during the study period after i.p. administration of each OP followed a similar pattern for both enzymes. Fig. 2 shows a decline of cholinesterase activity during the first hours, depending on the compound, and the recovery of enzymatic activity after 48 h. It was at this moment when the recovery profile of acetylcholinesterase in brain and muscle was analogous independent of the compounds. However, muscular BChE presented a different behaviour. Firstly, the order of sensitivity to active metabolites (dichlorvos > azinphosmethyl-oxon > omethoate) was different from that observed for tissue AChE. A direct correlation was not observed between the inhibition of skeletal muscle BChE by the organophosphorus compound studied and the acute toxicity observed for these insecticides in fish (Table 8). Secondly, the depression of the BChE was after 3 h, as in the case of dichlorvos (Fig. 8C) and dimethoate (Fig. 8B) or it was towards the end of the assay (7 days) as observed for azinphosmethyl (Fig. 8A). Finally, the inhibition of BChE after 7 days was almost total (average between 94.1 and 97.1%) for the thiophosphates, which need to be previously bioactivated to exert their maximum potency, independent of the different temporal evolution of enzyme inhibition in the previous stages. Nevertheless, dichlorvos had a different behaviour. This pesticide, which has direct anticholinesterase action, showed a partial recovery of BChE activity; although this recovery was not total, it stabilized after 24 h.

This behaviour of muscle BChE seems to provide a protective mechanism against the toxic impact of an anticholinesterase compound on this species. Although, a higher V_{max} was found for brain AChE for gilthead seabream (243.09 nmol/min/mg protein) than for muscle BChE (16.07 nmol/min/mg protein), the amount of skeletal muscle present in the animal is much greater than the amount of brain mass. Consequently, the amount of BChE present in muscle should be much greater than the amount of AChE in the brain. On the other hand, this enzyme is much more sensitive to oxons than cerebral AChE (azinphosmethyl ~2-fold, omethoate ~100-fold, dichlorvos and paraoxon ~10^4 fold) (Albendín et al., 2017). Thus, it could be possible that once the inhibitor penetrates into body, muscle BChE suffers phosphorylation, capturing the inhibitor and thus reducing the amount available for inhibiting cerebral AChE, which is more resistant to inhibition. During exposure to low concentrations of the inhibitor, skeletal muscle could supply the inhibitor with an alternative place of phosphorylation, thereby protecting the target sites of the brain.

Carr et al. (1997) observed this protective role for muscle AChE in the *Gambusia affinis*, because this enzyme is 20-fold more sensitive to Chlorpyrifos-oxon inhibition than cerebral AChE. The difference of sensitivity between both enzymes was not so pronounced in other species like *Micropterus salmoides*, *Lepomis macrochirus,* and *Notemigonus crysoleucas*. In these species, muscular AChE would not be so efficient for providing an alternative site of phosphorylation, and therefore the inhibitor could reach and act on cerebral AChE. In any case, the authors pointed out that inhibition of cerebral AChE is the most important factor for determining toxicity differences among the most sensitive species (*M. salmoides, L. macrochirus* and *N. crysoleucas*) and the more tolerant species (*G. affinis*) against exposure to insecticide chlorpyrifos. In contrast, Ferrari et al. (2007) indicated that the preponderance of the inhibition of muscular ChE against/versus cerebral ChE (measured on ASCh) was the main cause of mortality in some fish species such as rainbow trout. These authors suggested the following factors: (a) Skeletal muscle ChE, which controls muscle function, is the largest "pool" of body ChE activity (b) ChE specific activity is also higher in muscle than in brain, (c) low concentrations of pesticides that inhibit cerebral ChE,

but not muscle, did not cause mortality and (d) EC50 values for muscle ChE are greater than those corresponding to the brain enzyme and near the LC50. In addition, muscular ChE in this species showed a lower sensitivity than the brain and a slower recovery after exposure to a sublethal concentration of azinphosmethyl (Ferrari et al, 2004a). In this regard, the authors indicated that a different speed of recovery may be associated with a different rate of protein synthesis in both organs, or it may be related to the "pool" of ChE and toxicodynamic aspects.

5. Conclusion

In summary, the results showed that basal ChE activity in brain and muscle decreased significantly when age, body length, and body weight increased in a curvilinear manner that could be described by power equation. This study indicates that ChE activity can change in *Sparus aurata* depending on biological variables. For this reason, one needs take into account these biological variables such as body length and body weight when using brain and muscle ChE as biomarkers for biological monitoring of organophosphates compounds in gilthead seabream. Despite this, we can use these as biomarkers if we limit the size range of sample fish or normalize the observed activity.

Taking into account the ED_{50} for the inhibition of cerebral AChE after administration of the different organophosphates via i.p., azinfosmethyl is the most potent inhibitor followed by parathion and dimethoate. This trend agrees with the acute toxicity of these compounds for other fish species, based on their LC_{50}-24 h. Adult individuals of this species were able to survive with levels of inhibition of cerebral AChE greater than 90%. Thiophosphate type compounds (azinphosmethyl and dimethoate) produced an inhibition of cerebral AChE that reached a maximum value at about 24 h after treatment via i.p., and the normal values were not recovered after a period of 7 days. However, the maximum inhibition for dichlorvos was obtained during the first hours, and after a week, enzymatic activity was recovered. Recovery of muscle AChE followed a profile similar to cerebral AChE; while muscular BChE showed a slower recovery.

Finally, for future use of this biomarker in the seabream, it should be kept in mind that the brain has the advantage that it has enzymatic activity much greater than muscle and only AChE activity is present. However, muscle tissue is easier to collect and more abundant than the brain. In addition, the characteristics of muscular BChE (greater sensitivity and slower baseline activity recovery) would support its usefulness as a biomarker for exposure to organophosphates in this species.

CRediT authorship contribution statement

M.G. Albendín: Data curation, Writing - original draft, Software. **M. P. Mánuel-Vez:** Visualization, Investigation. **J.M. Arellano:** Conceptualization, Methodology, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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