Journal of Experimental Marine Biology and Ecology 465 (2015) 49–55

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

The potential of cholinesterases as tools for biomonitoring studies with sharks: Biochemical characterization in brain and muscle tissues of *Prionace glauca*

Luís M. Alves [a,b], Marco F.L. Lemos [a,b], João P.S. Correia [a,b,c], Nuno A.R. da Costa [a], Sara C. Novais [a,b,*]

[a] ESTM, GIRM, Polytechnic Institute of Leiria, 2520-641 Peniche, Portugal
[b] MARE, Polytechnic Institute of Leiria, Portugal
[c] Flying Sharks, 9900-361 Horta, Portugal

ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form 12 January 2015
Accepted 13 January 2015
Available online 29 January 2015

Keywords:
Biomarker
Blue shark
Pollution
Chlorpyrifos-oxon

ABSTRACT

Cholinesterases (ChE) are a family of enzymes that play an essential role in neuronal and motor functions. Because of the susceptibility of these enzymes to anticholinergic agents and to other contaminants, their activity is frequently used as biomarker in pollution monitoring studies. The three known types of ChE in fish are acetilcholinesterase (AChE), butyrylcholinesterase (BChE) and propionylcholinesterase (PChE). The presence of these enzymes in each tissue differs between species, and thus their usage as biomarkers requires previous enzyme characterization. Sharks, mostly acting as apex predators, help maintain the balance of fish populations performing a key role in the ecosystem. Blue sharks (*Prionace glauca*) are one of the most abundant and heavily fished sharks in the world, thus being good candidate organisms for ecotoxicology and biomonitoring studies. The present study aimed to characterize the ChE present in the brain and muscle of the blue shark using different substrates and selective inhibitors, and to assess the *in vitro* sensitivity of these sharks' ChE to chlorpyrifos-oxon, a metabolite of a commonly used organophosphorous pesticide, recognized as a model anticholinesterase contaminant. The results suggest that the brain of *P. glauca* seems to contain atypical ChEs, displaying mixed properties of AChE and BChE, and that the muscle tissue seems to contain mainly AChE. *In vitro* exposures to chloropyrifos-oxon inhibited blue shark's ChE in both tissues, the brain being the most sensitive tissue and therefore the most suitable for detection of exposure to low concentrations of anticholinergic compounds in the environment. This study indicates that ChE activity in blue sharks has the potential to be used as a sensitive and reliable biomarker in marine biomonitoring programs.

1. Introduction

Cholinesterases (ChE) are a family of enzymes that catalyze the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, an essential process for both neuronal and motor capabilities (Nachmansohn and Wilson, 1951; Pohanka, 2011). The measurement of ChE activity is widely used in pollution monitoring as a biomarker of effect, mainly due to their high sensitivity to anticholinergic chemicals, such as organophosphate and carbamate pesticides, (Arufe et al., 2007; Chambers et al., 2002; Fulton and Key, 2001; Galloway et al., 2002; Kirby et al., 2000; Van der Oost et al., 2003) but also to other contaminants often simultaneously present in marine environments like oils and industrial run-offs (Galgani et al., 1992; Payne et al., 1996). Additionally, ChEs are among the less variable biomarkers, making them suitable for environmental pollution biomonitoring studies (Solé et al., 2008).

Currently there are three known types of ChE in fish: acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and propionylcholinesterase (PChE) (Kirby et al., 2000; Solé et al., 2008; Sturm et al., 1999a, 2000). AChE is a key enzyme of the nervous system, and a well-accepted biomarker of exposure to neurotoxic chemicals, existing predominantly in brain tissue but also, for example, in muscle, liver and blood (Bresler et al., 1999; Burgeot et al., 2001; Lionetto et al., 2004; Solé et al., 2008; Stien et al., 1998). BChE and PChE, the other types of cholinesterases frequently named pseudocholinesterases, have also been found, for example, in plasma, liver and muscle (Chambers et al., 2002; Kirby et al., 2000; Solé et al., 2010; Sturm et al., 1999a, 2000). The roles and physiological functions of pseudocholinesterases are still not completely understood but in humans they seem to be involved in detoxification processes, cell regeneration, lipid metabolism, neurogenesis, and neural development (Mack and Robitzki, 2000).

* Corresponding author at: ESTM, GIRM, Polytechnic Institute of Leiria, 2520 – 641 Peniche, Portugal. Tel.: +351 262 783 607; fax: +351 262 783 088.
E-mail address: sara.novais@ipleiria.pt (S.C. Novais).

http://dx.doi.org/10.1016/j.jembe.2015.01.006
0022-0981/

Activity levels, and types of ChE present in each tissue, vary among species (Chuiko, 2000). Given the lack of ChE characterization in several organisms, studies reporting screening for AChE may, in fact, be dealing with other ChE types that could have distinct sensitivities to contaminants. Different types of ChE can be distinguished using both different substrates and specific inhibitors (Silver, 1974).

In recent years, fish have become very useful for the quality assessment of aquatic environments, acting as bioindicators of environmental pollution (Dautremepuits et al., 2004; Lopes et al., 2001). Like all animals at the top of the food chain, sharks play a very important role in ocean population dynamics, by maintaining other fish populations in balance. Thus, if the number of predators is significantly altered, the consequences for the ecosystem structure, functioning, and resilience can be significant (Baum and Worm, 2009; Duffy, 2002; Paine, 1996). According to Bonfil (1994) and Stevens (2009), blue sharks are one of the most abundant and heavily fished sharks in the world, with an estimated 20 million individuals caught annually as target or by-catch species, making them suitable organisms for ecotoxicology and biomonitoring studies. To our knowledge, and to date, ChE of shark species have only been characterized in the muscle tissue of *Scyliorhinus canicula* and *Galeus melastomus* (Solé et al., 2008).

This research had three main goals: 1) to characterize the ChE present in the brain and muscle of blue shark (*Prionace glauca*), using different substrates and specific inhibitors, in order to optimize tissue selection and experimental procedure; 2) to assess the *in vitro* sensitivity of these sharks' ChE to chlorpyrifos-oxon, a metabolite of a vastly used organophosphorous pesticide, recognized as a model anticholinesterase contaminant; and 3) to address the blue shark ChE as a tool for future biomonitoring studies in marine ecosystems.

2. Materials and methods

2.1. Test organisms

Muscle and brain tissues of eight juvenile blue sharks (*P. glauca*) were sampled aboard a commercial fishing boat on November 2013, off the Atlantic coast of Portugal at 36°43′11.2″N 13°09′30.0″W. The organisms used in this study consisted of four males and four females ranging 105 to 157 cm and 113 to 167 cm, respectively. Tissues from each shark were collected immediately after capture and landing on the vessel, after which all samples were stored on ice until they were deep-frozen in the lab at −80 °C for further biochemical measurements.

2.2. Chemicals

The substrates acetylthiocholine iodide (ATCh), S-butyrylthiocholine iodide (BTCh) and propionylthiocholine iodide (PTCh), as well as the inhibitors eserine hemisulfate, 1,5-bis[4-allyl dimethyl ammonium phenyl] pentan-3-one dibromide (BW284C51) and tetra[monoisopropyl]pyrophosphortetramide (iso-OMPA), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Chlorpyrifos-oxon (CPF-oxon) was obtained from Greyhound Chromatography (Birkenhead, Merseyside, UK).

2.3. Tissue preparation

Brain and muscle tissues from each shark were homogenized in potassium phosphate buffer (0.1 M, pH 7.2) in a 1:5 proportion. The homogenates were centrifuged at 3000 *g*, for 3 min (4 °C), and the supernatant of each sample was transferred to new microtubes and stored at −80 °C.

2.4. Cholinesterase characterization

Before the enzymatic assays, total protein concentration in the supernatant was quantified according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford microassay set up in a 96 well flat bottom plate and using bovine γ-globuline protein standard.

The ChE activity of each muscle and brain sample was determined in quadruplicates in the previously diluted supernatant (final protein concentration of 0.8 mg/ml) by the method proposed by Ellman et al. (1961) adapted to microplate (Guilhermino et al., 1996). For the determinations, 250 μl of the reaction solution [30 ml potassium phosphate buffer (0.1 M, pH 7.2), 1 ml of reagent 5,5-dithiobis-(2-nitrobenzoic acid) 10 mM (DTNB) and 200 μl of substrate] was added to 50 μl of the diluted supernatant. The absorbance was measured every 20 s for 5 min at 414 nm (25 °C). All spectrofotometric measurements were performed using a microplate reader Synergy H1 Hybrid Multi-Mode (BioTek® Instruments, Vermont, USA).

2.4.1. Substrates

Cholinesterases substrate preferences were assessed in both muscle and brain tissues by determining the enzyme activity at 12 increasing concentrations of the substrates ATCh, BTCh, and PTCh: 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24 and 20.48 mM. Cholinesterases activity in the presence of these substrates was determined as described in the previous section, with 200 μl of each substrate being dissolved in the reaction buffer. Blank reactions were made for each substrate concentration using the same volume of potassium phosphate homogenization buffer (0.1 M, pH 7.2) instead of sample.

2.4.2. Inhibitors

Eserine sulfate, BW284C51, and iso-OMPA were used as selective inhibitors of total ChE, AChE, and BChE, respectively. Cholinesterases activities were measured in all muscle and brain samples as described in Section 2.4, using 200 μl of ATCh 0.075 M solution as substrate, at six increasing concentrations of each inhibitor dissolved in the reaction buffer.

Final concentrations of the inhibitors were 0.781, 3.125, 12.5, 50, 200 and 800 μM for eserine sulfate and BW284C51, and 0.0156, 0.0625, 0.25, 1, 4 and 16 mM for iso-OMPA.

Blank reactions were specifically made for each inhibitor concentration using the same volume of potassium phosphate buffer (0.1 M, pH 7.2) instead of sample. Controls of ChE activity in the absence of inhibitors in the reaction buffer were also made.

2.5. In vitro effects of chlorpyrifos-oxon on ChE activity

An *in vitro* test was performed using chlorpyrifos-oxon, a metabolite of a widely used organophosphorous pesticide. The ChE activity was measured as described for the inhibitors using ATCh 0.075 M as substrate and dissolving the different pesticide metabolite concentrations in the reaction buffer. A stock solution of the insecticide was prepared in ethanol, and reactions were done using final concentrations ranging from 0.0365 to 2400 nM.

Blank reactions were made for each contaminant concentration using the same volume of potassium phosphate buffer (0.1 M, pH 7.2) instead of sample. A control of ChE activity without chlorpyrifos-oxon in the reaction buffer was also done as well as an extra solvent control (same solvent concentration as in the maximum tested pesticide metabolite concentration).

2.6. Statistical analysis

To calculate the catalytic efficiency of the enzyme with each substrate, experimental curves were fitted (monotonic increase part of the curve) using the Michaelis–Menten equation, in order to determine the ChE kinetic parameters: maximal velocity (Vmax), Michaelis–Menten constant (Km), and their ratio (Vmax/Km), indicating the catalytic efficiency of the enzyme.

Data from the ChE activity with the specific inhibitors, as well as from the *in vitro* exposures, were analyzed using one-way analysis of

variance (ANOVA) followed by Dunnett's multicomparison test to evaluate significant differences between tested concentrations and the control/solvent control at a significance level of 0.05. *In vitro* inhibition concentration values for chlorpyrifos-oxon (IC_{50}) were calculated using a nonlinear four parameter logistic curve. To address effects of gender and size on the inhibitory capacity of chlorpyrifos-oxon *in vitro* at the different concentrations, a two-way ANOVA was performed, followed by Holm–Sidak test to discriminate statistical significant differences between groups.

All the referred tests were made using the Sigma Plot software for Windows, Version 11.0 (SigmaPlot, 1997).

3. Results

3.1. Cholinesterase characterization

The results of ChE substrate preference in the brain and muscle tissues are shown in Fig. 1.

In brain tissue (Fig. 1A), the substrate with higher hydrolysis rate was ATCh (19.2 nmol/min/mg protein), followed by PTCh (10.8 nmol/min/mg protein) and BTCh (8.1 nmol/min/mg protein). The enzymatic catalytic efficiency indicated by the parameters of the Michaelis–Menten equation (Table 1) also demonstrates the preference for the substrate ATCh (higher Vmax/Km values). Furthermore, a decrease in ChE activity caused by excess of the substrates ATCh and BTCh was also verified with concentrations higher than 2.56 mM and 5.12 mM, respectively.

In muscle tissue (Fig. 1B), there was a clear preference for the substrate ATCh, as seen by the higher hydrolysis rates and greater catalytic efficiency of ChE with this substrate (Table 1). Indeed, there were higher hydrolysis rates in the muscle than in the brain tissue with maximum ChE activities of 29.5 nmol/min/mg protein at 0.64 mM of ATCh when compared with the 19.2 nmol/min/mg protein maximum activity in the brain at 2.56 mM of the same substrate. Almost no ChE activity was observed in muscle when using the substrate BTCh. In this tissue there was also an inhibition of hydrolysis by excess of ATCh at concentrations higher than 0.64 mM.

Regarding the results with the specific inhibitors, incubation with eserine, a generic inhibitor of ChE, significantly inhibited ChE activity in brain tissue already at the lowest concentration tested of 0.781 μM ($F_{6,49} = 115.1$, $p < 0.001$) whereas in muscle significant inhibitions only occurred at concentrations higher than 12.5 μM ($F_{6,49} = 179.5$, $p < 0.001$) (Fig. 2A). However, at concentrations higher than 50 μM almost complete inhibitions were observed in both tissues (over 95% inhibition). Concerning the specific inhibitor for AChE (Fig. 2B), incubation with BW284C51 in brain only significantly inhibited the enzyme activity at concentrations higher than 50 μM ($F_{6,49} = 35.8$, $p < 0.001$) and inhibitions above 90% were observed only at 800 μM, whereas in muscle a significant inhibition of 93% occurred already in the lowest concentration tested ($F_{6,49} = 122.8$, $p < 0.001$). No effects on enzyme activity were observed with iso-OMPA incubations, a specific inhibitor of BChE, either in brain ($F_{6,49} = 9.69$, $p = 0.138$) or muscle ($F_{6,49} = 0.47$, $p = 0.825$) tissues (Fig. 2C). However, there was a dose–response inhibition in the brain, albeit non-significant, reaching 40% inhibition in the highest iso-OMPA concentration tested.

Table 1
Values of the Michaelis–Menten constant (Km), maximal velocity (Vmax) and the catalytic efficiency (Vmax/Km) of *Prionace glauca* cholinesterases for the three tested substrates. Values of the Michaelis–Menten equation are expressed as the mean ± standard error.

	Km (mM)	Vmax (nmol/min/mg protein)	Vmax/Km
Brain			
ATCh	0.045 ± 0.02	18.82 ± 1.65	422.90
PTCh	0.056 ± 0.03	9.09 ± 0.73	163.26
BTCh	0.027 ± 0.01	7.50 ± 0.56	279.76
Muscle			
ATCh	0.053 ± 0.01	31.62 ± 2.09	598.87
PTCh	0.034 ± 0.01	4.97 ± 0.34	147.36
BTCh	–	–	–

3.2. In vitro effects of chlorpyrifos-oxon

The effect of ethanol, the solvent used for chlorpyrifos-oxon stock solution, on ChE activity was tested and compared with the control and no statistical difference was observed either in brain ($t_{(14)} = 0.403$, $p = 0.693$) or muscle ($t_{(14)} = 0.420$, $p = 0.681$) tissues.

Regarding the effects of the *in vitro* exposure to chlorpyrifos-oxon, there was a dose–response pattern showing lower ChE activities with increasing pesticide metabolite concentrations, in both tissues tested, with almost complete inhibitions (over 97%) with the highest pesticide metabolite concentration (Fig. 3).

Although significant inhibitions in relation to control were only detected at 600 nM of pesticide metabolite in both tissues (brain: $F_{6,49} = 40.38$, $p < 0.001$; muscle: $F_{6,49} = 37.77$, $p < 0.001$), the lower concentrations of chlorpyrifos-oxon (until 150 nM) caused statistically higher ChE inhibitions in the brain than in the muscle (two-way ANOVA, $p < 0.001$).

This higher sensitivity to chlorpyrifos-oxon in the brain tissue can also be seen by the estimated IC_{50} (±SE) values of 48.97 ± 3.79 nM (i.e. 16.39 μg/l) in brain and 204.97 ± 94.32 nM (i.e. 68.57 μg/l) in muscle.

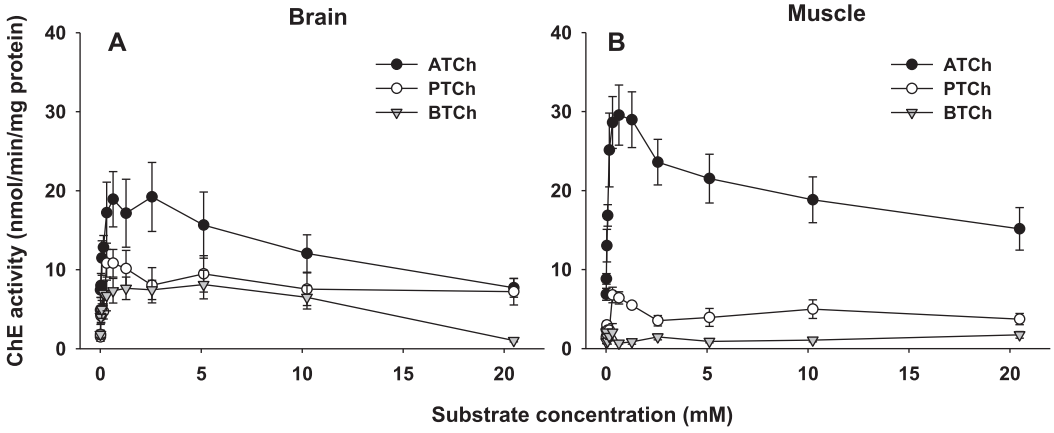

Fig. 1. Cholinesterase substrate preferences in the brain (A) and muscle (B) of *Prionace glauca*. Cholinesterases (ChE) activity is expressed as mean values ± standard error. ATCh = Acetylthiocholine iodide. BTCh = S-Butyrylthiocholine iodide. PTCh = Propionylthiocholine iodide.

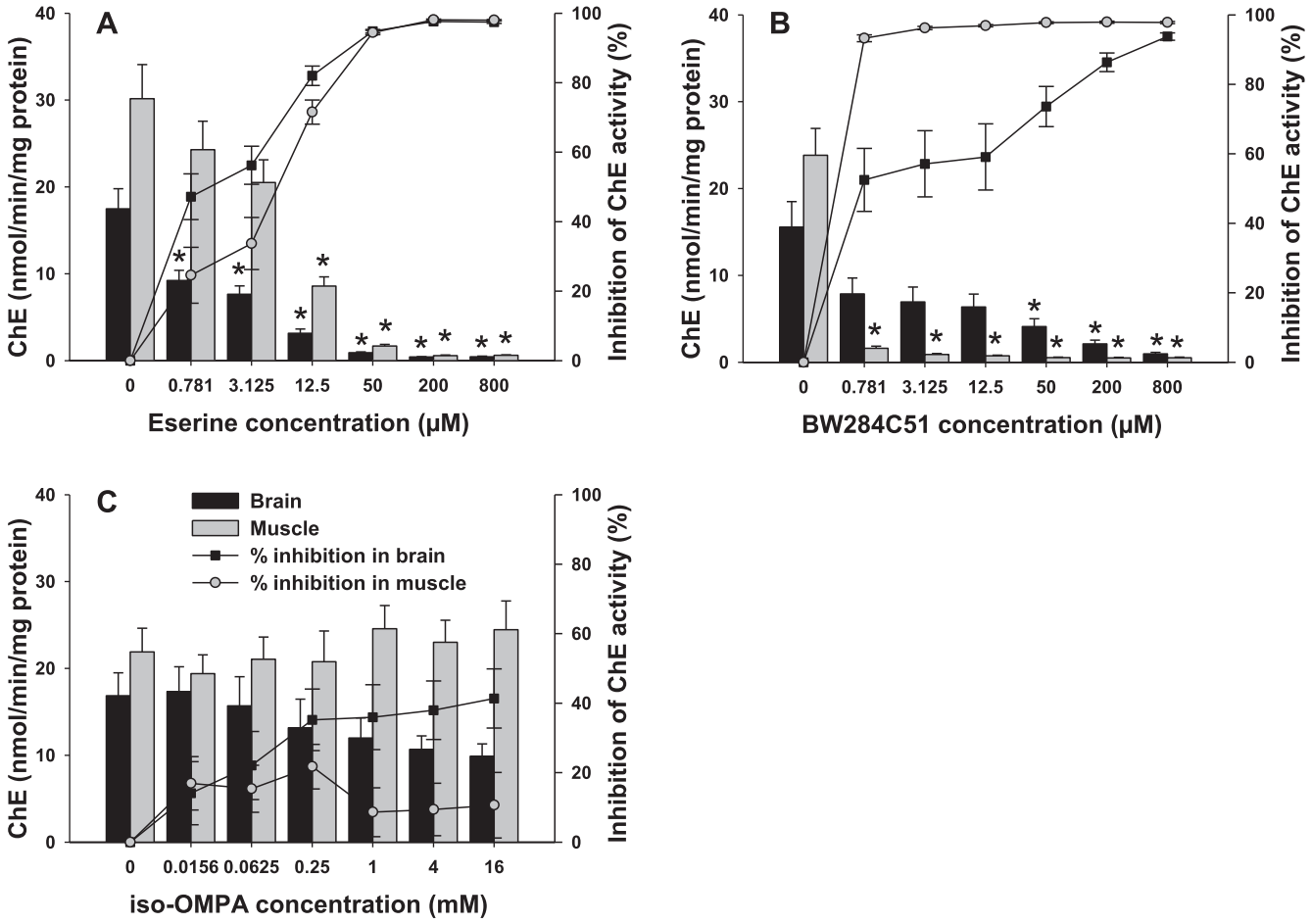

Fig. 2. Effect of the inhibitors eserine (A), BW284C51 (B) and iso-OMPA (C) on *Prionace glauca* cholinesterase (ChE) activities in brain and muscle tissues (expressed as mean values ± standard error) using acetylthiocholine as substrate. Bars correspond to ChE activities and lines correspond to the percentage of ChE inhibition. An asterisk indicates a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's test).

In order to address effects of gender and size on the susceptibility to chlorpyrifos-oxon, samples were divided in two groups of four according to gender (males and females) and size (larger or smaller than 130 cm). The IC_{50} values for these separate groups, in brain and muscle tissues, were calculated and the results can be seen in Table 2.

According to the IC_{50} values, the higher sensitivity of brain tissue when compared to the muscle is visible independently of the organisms' gender or size, and the differences between tissues are even more pronounced within females and in larger individuals (Table 2).

Regarding the effects of gender or size within each tissue, there were some differences in the IC_{50} values, e.g. lower IC_{50} for females or larger organisms in the brain (trend not present in the muscle). These differences were however not statistically significant and therefore the response to chlorpyrifos-oxon in brain tissue or muscle was not affected by either gender or size (two-way ANOVA, $p > 0.05$).

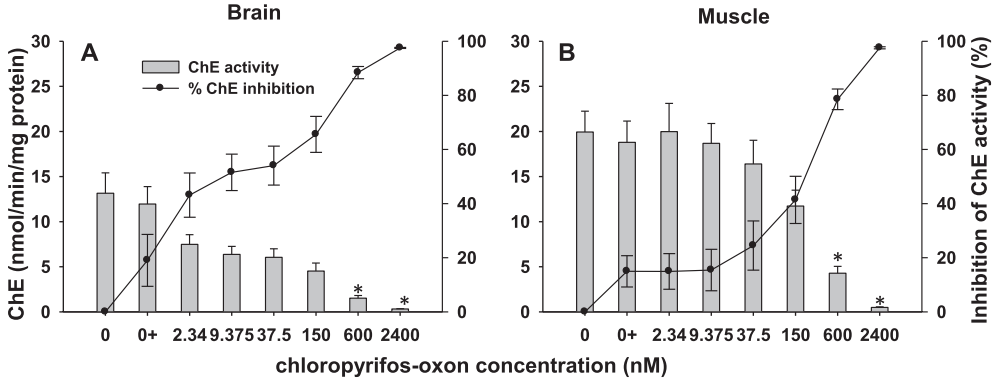

Fig. 3. Cholinesterase (ChE) activity values and percentage of activity inhibition (expressed as mean values ± standard error) in the brain (A) and muscle (B) of *Prionace glauca* exposed *in vitro* to chlorpyrifos-oxon. An asterisk indicates a significant difference from the solvent control (0+) at $p \leq 0.05$ (ANOVA, Dunnett's test).

Table 2
In vitro inhibition concentrations (IC_{50}) of chlorpyrifos-oxon in *Prionace glauca*, depending on gender and size of the organisms.

	IC50 ± SE (nM)	
	Brain	Muscle
Gender		
Males	86.04 ± 6.38	136.49 ± 11.44
Females	21.99 ± 4.57	291.93 ± 73.37
Size		
<130 cm	69.98 ± 6.80	206.36 ± 111.56
>130 cm	29.93 ± 3.69	215.96 ± 40.04

4. Discussion

4.1. Cholinesterase characterization

To use ChE as a biomarker of effect of pollutants in a particular species, it is vital to characterize this enzyme in different target tissues, as they may have several non-specific esterases that can mislead ecotoxicological studies (Gomes et al., 2014; Howcroft et al., 2011; Pestana et al., 2014).

Regarding the brain tissue, incubation with eserine sulfate, an organophosphorus compound well-known as a general inhibitor of ChE at low concentrations, resulted in an almost complete enzyme inhibition (Fig. 2A), meaning that the measured enzymatic activity is mostly due to ChE, and not to other nonspecific esterases (Eto, 1974; Pezzementi et al., 1991). The characterization of the brain's ChE was performed by testing its affinity to different substrates, and response to specific inhibitors. As typical with AChE, ChE in the brain showed a preference for the substrate ATCh, presenting higher hydrolysis rates and greater catalytic efficiencies with this substrate (Table 1). Also, there was an inhibition of hydrolysis by excess substrate (Fig. 1), which is another characteristic of AChE (Toutant, 1989), previously reported in the brain of other fish species (e.g. Monteiro et al., 2005; Rodríguez-Fuentes et al., 2013; Sturm et al., 1999a). Moreover, brain ChE were sensitive to BW284C51, a specific inhibitor of AChE, although significant inhibitions only occurred at concentrations higher than 50 μM (Fig. 2B). Despite all these typical responses of AChE, brain ChE were also able to hydrolyze BTCh (although at a lower rate and with lower catalytic efficiency than with ATCh; Table 1) and showed some sensitivity to iso-OMPA, a specific inhibitor of BChE, at high concentrations (Fig. 2C). Therefore, considering the responses to the different substrates and inhibitors, these findings suggest that the brain of *P. glauca* seems to contain atypical ChE, displaying mixed properties of AChE and BChE. According to the literature, the majority of fish have almost exclusively AChE in their brains, such as *Limanda limanda*, *Platichthys flesus*, and *Serranus cabrilla*, described in a study by Sturm et al. (1999a) or several others described by Solé et al. (2008). However, similar results to the ones obtained in our study were also described for the reef fish *Haemulon plumieri* (Alpuche-Gual and Gold-Bouchot, 2008).

Regarding the muscle tissue, the enzyme preferred the substrate ATCh, presenting much higher hydrolysis rates and catalytic efficiency in relation to other substrates (Fig. 1 and Table 1). Similarly to what was observed in the brain, the incubation with eserine sulfate in the muscle caused an almost complete inhibition of enzymatic activity (Fig. 2A) and therefore suggests that most of the measured activity is related to ChE and not other esterases. The preference for the substrate ATCh and the observed inhibition of activity by excess of this substrate (Fig. 1), along with the high sensitivity to BW284C51 and insensitivity to iso-OMPA (Fig. 2B,C), lead to the conclusion that the ChE present in the muscle of *P. glauca* have characteristics of true AChE and this seems to be the main form present. Although most marine species express both AChE and pseudocholinesterases in their muscle tissues at different levels depending on the species—such as demonstrated by Sturm et al. (1999a) for three teleosts species and by Solé et al. (2008) for two sharks—Garcia et al. (2000) observed that, like in the present blue shark samples, the muscle of *Poecilia reticulate* contains mainly AChE.

Considering the maximum enzymatic activity levels in both brain and muscle tissues observed for blue sharks, they were lower than those usually found in marine fish (Alpuche-Gual and Gold-Bouchot, 2008; Arufe et al., 2007; Oliveira et al., 2007; Rodríguez-Fuentes et al., 2013; Sturm et al., 1999a), which is in accordance with the study by Solé et al. (2008) where the authors have also reported lower enzymatic activities in the sharks *S. canicula* and *G. melastomus*.

To our knowledge, there is scarce information regarding the enzymatic pathways of sharks, specifically concerning ChE, but generally existing data suggests that activity levels might oscillate seasonally, due to size, sex, and dietary composition (Beauvais et al., 2002; Chuiko et al., 1997, 2003; Flammarion et al., 2002; Kirby et al., 2000; Sturm et al., 1999b). These correlations between ChE activity rates and physiological characteristics are, however, not always observed (Solé et al., 2006; Tortelli et al., 2006). Also in the present work, no correlations were found between the enzymatic activity levels and the physiological parameters sex and size, although it is important to refer that the sampling group was limited and more information could be taken from a larger sampling, preferably using both mature and immature individuals.

4.2. In vitro effects of chlorpyrifos-oxon

Given the important role of AChE in the neuromuscular system and the fact that it is often the main target of toxicity for organophosphate or carbamate insecticides, and might also be affected by other pollutants, the activity of this enzyme can be used as a biomarker of effect after exposure to contaminants, providing extensive applicability for both laboratory and field studies (Alpuche-Gual and Gold-Bouchot, 2008; Whitehead et al., 2005).

Chlorpyrifos-oxon is a metabolite of the organophosphate insecticide chlorpyrifos, widely used for pest control (Sparling and Fellers, 2007). The presence of chloropyrifos in marine ecosystems, as well as other pesticides of similar nature, is well documented making it a suitable candidate for assessing the effects of these pesticides on organism's cholinesterases (Barakat et al., 2002; García-Álvarez et al., 2014; Lund et al., 2000).

The results of the present study show that chloropyrifos-oxon inhibited blue shark's ChE *in vitro*, with brain tissue showing higher sensitivity towards the compound when compared to muscle tissue. Although some authors state that ChE inhibition in muscle is a better predictor of induced mortality than inhibitions using the brain tissue, with brain tissue inhibition being a better tool to foresee alterations in behavior (Fulton and Key, 2001), it is generally assumed that, in order to monitor pesticide accumulation in aquatic environments, one should perform measurements in the tissue proven to be the most sensitive to these compounds (Whitehead et al., 2005). Therefore, as in the present study brain tissue in *P. glauca* showed higher sensitivity to chloropyrifos-oxon than muscle, one may infer that the brain has a greater potential for detection of exposure to low concentrations of this and other similar compounds in the environment.

The lack of statistically significant differences in sensitivity to chlorpyrifos-oxon between genders and sizes gives strength and robustness to this tool. Nevertheless, in order to complement and validate these findings, a greater number of samples should be collected and analyzed, preferably of adult individuals, as the increased sizes and different metabolic responses might influence ChE activity and sensitivity.

When comparing the present *in vitro* results with the ones of vertebrate fish (Carr et al., 1997), it is possible to see that the blue shark has similar IC_{50} values in the brain, but considerable higher values in the muscle. To our knowledge, so far, there are no studies addressing the effects of chloropyrifos, or other pesticides, in elasmobranchs.

However, there are reports of pesticide accumulation in different tissues of several shark species (Schlenk et al., 2005; Shanshan et al., 2013), which enforce the need for studies that provide better understanding on how these compounds affect such marine predators.

With this study's characterization of blue shark ChE in the brain and muscle, together with the determination of its high sensitivity in the in vitro pollutant inhibition assay, the foundation work has been set to further study this enzyme and potentially use it in ocean biomonitoring studies as a biomarker for the effects of anticholinergic contaminants using the widespread and easy to obtain blue shark.

Acknowledgments

Sara C. Novais wishes to acknowledge the financial support given by Fundação para a Ciência e Tecnologia (FCT) through a post-doctoral research grant (SFRH/BPD/94500/2013). **[SS]**

References

Alpuche-Gual, L., Gold-Bouchot, G., 2008. Determination of esterase activity and characterization of cholinesterases in the reef fish *Haemulon plumieri*. Ecotoxicol. Environ. Saf. 71, 787–797.

Arufe, M.I., Arellano, J.M., Garcia, L., Albendin, G., Sarasquete, C., 2007. Cholinesterase activity in gilthead seabream *Sparus aurata* larvae: characterization and sensitivity to the organophosphate azinphosmethyl. Aquat. Toxicol. 84, 328–336.

Barakat, A.O., Kim, M., Qian, Y., Wade, T.L., 2002. Organochlorine pesticides and PCB residues in sediments of Alexandria Harbour Egypt. Mar. Pollut. Bull. 44, 1426–1434.

Baum, J.K., Worm, B., 2009. Cascading top-down effects of changing oceanic predator abundances. J. Anim. Ecol. 78, 699–714.

Beauvais, S.L., Cole, K.J., Atchison, G.J., Coffey, M., 2002. Factors affecting brain cholinesterase activity in bluegill *Lepomis macrochirus*. Water Air Soil Pollut. 135, 249–264.

Bonfil, R., 1994. Overview of World Elasmobranch Fisheries. Food & Agriculture Organization of the UN FAO.

Bradford, M.M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bresler, V., Bissinger, V., Abelson, A., Dizer, H., Sturm, A., Kratke, R., 1999. Marine molluscs and fish as biomarkers of pollution stress in litoral regions of the Red Sea, Mediterranean Sea and North Sea. Helgol. Mar. Res. 53, 219–243.

Burgeot, T., Bocquené, G., His, E., Vincent, F., Geffard, O., Beira, R., 2001. Monitoring of biological effects of pollutants: field application. In: Garigues, Ph., Barth, H., Walker, C.H., Narbonne, J.F. (Eds.), Biomarkers in Marine Organisms: A Practical Approach. Elsevier, Amsterdam, pp. 179–213.

Carr, R.L., Ho, L.L., Chambers, J.E., 1997. Selective toxicity of chlorpyrifos to several species of fish during an environmental exposure: Biochemical mechanisms. Environ. Toxicol. Chem. 16, 2369–2374.

Chambers, J.E., Boone, J.S., Chambers, H.W., Straus, D.L., 2002. Biomarkers as predictors in health and ecological risk assessment. Hum. Ecol. Risk. Assess. 81, 165–176.

Chuiko, G.M., 2000. Comparative study of acetylcholinesterase and butyrylcholinesterase in brain and serum of several freshwater fish: specific activities and in vitro inhibition by DDVP, an organophosphorus pesticide. Comp. Biochem. Physiol. C 127, 233–242.

Chuiko, G.M., Zhelnin, Y.Y., Pod'gornaya, V.A., 1997. Seasonal fluctuations in brain acetylcholinesterase activity and soluble protein content in roach *Rutilus rutilus* L.: a freshwater fish from northwest Russia. Comp. Biochem. Physiol. C 107, 251–257.

Chuiko, G.M., Podgornaya, V.A., Zhelnin, Y.Y., 2003. Acetylcholinesterase and butyrylcholinesterase activities in brain and plasma of freshwater teleost: cross-species and cross-family differences. Comp. Biochem. Physiol. B 135, 55–61.

Dautremepuits, C., Paris-Palacios, S., Betoulle, S., Vernet, G., 2004. Modulation in hepatic and head kidney parameters of carp *Cyprinus carpio* L. induced by copper and chitosan. Comp. Biochem. Physiol. C 137, 325–333.

Duffy, J.E., 2002. Biodiversity and ecosystem function: the consumer connection. Oikos 99, 201–219.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric of acetylcholinesterase determination. Biochem. Pharmacol. 7, 88–95.

Eto, M., 1974. Organophosphorus Pesticides. Organic and Biological ChemistryCRC Press.

Flammarion, P., Noury, P., Garric, J., 2002. The measurement of cholinesterase activities as a biomarker in chub *Leuciscus cephalus*: the fish length should not be ignored. Environ. Pollut. 120, 325–330.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ. Toxicol. Chem. 20, 37–45.

Galgani, F., Bocquene, G., Cadiou, Y., 1992. Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North Sea. Mar. Ecol. Prog. Ser. 13, 77–82.

Galloway, T.S., Millward, N., Browne, M.A., Depledge, M.H., 2002. Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. Aquat. Toxicol. 613–4, 169–180.

Garcia, L.M., Castro, B., Ribeiro, R., Guilhermino, L., 2000. Characterization of cholinesterase from guppy *Poecilia reticulata* muscle and its in vitro inhibition by environmental contaminants. Biomarkers 5 (4), 274–284.

García-Álvarez, N., Boada, L.D., Fernández, A., Zumbado, M., Arbelo, M., Sierra, E., Xuriach, A., Almunia, J., Camacho, M., Luzardo, O.P., 2014. Assessment of the levels of polycyclic aromatic hydrocarbons and organochlorine contaminants in bottlenose dolphins *Tursiops truncatus* from the Eastern Atlantic Ocean. Mar. Environ. Res. 100, 48–56.

Gomes, I.D.L., Lemos, M.F.L., Soares, A.M.V.M., Barata, C., Faria, M., 2014. The use of cholinesterase as potential biomarker: in vitro characterization in the polychaete *Capitella teleta*. Mar. Pollut. Bull. 85, 179–185.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32, 727.

Howcroft, C.F., Gravato, C., Amorim, M.J.B., Novais, S.C., Soares, A.M.V.M., Guilhermino, L., 2011. Biochemical characterization of cholinesterases in *Enchytraeus albidus* and assessment of in vivo and in vitro effects of different soil properties, copper and phenmedipham. Ecotoxicology 20 (1), 119–130.

Kirby, M.F., Morris, S., Hurst, M., Kirby, S.J., Neall, P., Tylor, T., Fagg, A., 2000. The use of cholinesterase activity in flounder *Platichthys flesus* muscle tissue as a biomarker of neurotoxic contamination in UK estuaries. Mar. Pollut. Bull. 409, 780–791.

Lionetto, M.G., Caricato, R., Giordano, M.E., Schettino, T., 2004. Biomarker application for the study of chemical contamination risk on marine organisms in the Taranto marine coastal area. Chem. Ecol. 20, 333–343.

Lopes, P.A., Pinheiro, T., Santos, M.C., da Luz Mathias, M., Collares-Pereira, M.J., Viegas-Crespo, A.M., 2001. Response of antioxidant enzymes in freshwater fish populations *Leuciscus alburnoides* complex to inorganic pollutants exposure. Sci. Total Environ. 280, 153–163.

Lund, S.A., Fulton, M.H., Key, P.B., 2000. The sensitivity of grass shrimp, *Palaemonetes pugio*, embryos to organophosphate pesticide induced acetylcholinesterase inhibition. Aquat. Toxicol. 482–3, 127–134.

Mack, A., Robitzki, A., 2000. The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense-50 butyrylcholinesterase-DNA study. Prog. Neurobiol. 60, 607–628.

Monteiro, M., Quintaneiro, C., Morgado, F., Soares, A.M.V.M., Guilhermino, L., 2005. Characterization of the cholinesterases present in head tissues of the estuarine fish *Pomatoschistus microps*: application to biomonitoring. Ecotoxicol. Environ. Saf. 62, 341–347.

Nachmansohn, D., Wilson, I.B., 1951. The enzymic hydrolysis and synthesis of acetylcholine. Adv. Enzymol. Relat. Subj. Biochem. 12, 259–339.

Oliveira, M.M., Filho, M.V.S., Bastos, V.L.F.C., Fernandes, F.C., Bastos, J.C., 2007. Brain acetylcholinesterase as a marine pesticide biomarker using Brazilian fishes. Mar. Environ. Res. 63, 303–312.

Paine, R.T., 1996. Food web complexity and species diversity. Am. Nat. 100910, 65–75.

Payne, J.F., Mathieu, A., Melvin, W., Fancey, L.L., 1996. Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper millin Newfound land. Mar. Pollut. Bull. 32 (2), 225–231.

Pestana, J.L.T., Novais, S.C., Lemos, M.F.L., Soares, A.M.V.M., 2014. Cholinesterase activity in the caddisfly *Sericostoma vittatum*: biochemical enzyme characterization and in vitro effects of insecticides and psychiatric drugs. Ecotoxicol. Environ. Saf. 104, 263–268.

Pezzementi, L., Sanders, M., Jenkins, T., Holliman, D., Toutant, J.P., Bradley, R.J., 1991. Structure and function of cholinesterase from amphioxus. In: Massoulié, J., Bacou, F., Barnard, E.A., Chatonnet, A., Doctor, B.P., Quinn, D.M. (Eds.), Cholinesterases: Structure, Function, Mechanism, Genetics and Cell Biology. American Chemical Society, Washington, D.C., pp. 24–31.

Pohanka, M., 2011. Cholinesterases, a target of pharmacology. Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub. 1553, 219–230.

Rodríguez-Fuentes, G., Soto, M., Luna-Ramírez, K., 2013. Characterization of cholinesterases in the damselfish Sergeant major *Abudefduf saxatilis*. Ecotoxicol. Environ. Saf. 96, 99–102.

Schlenk, D., Sapozhnikova, Y., Irwin, M.A., Xie, L., Hwang, W., Reddy, S., Brownawell, B.J., Armstrong, J., Kelly, M., Montagne, D.E., Kolodziej, E.P., Sedlak, D., Snyder, S., 2005. In vivo bioassay guided fractionation of marine sediment extracts from the southern California bight for estrogenic activity. Environ. Toxicol. Chem. 24, 2820–2826.

Shanshan, Z., Lu, T., Qiaozhi, T., Xiaoting, G., Bin, X., Weiping, L., 2013. Residues, sources and tissue distributions of organochlorine pesticides in dog sharks *Mustelus griseus* from Zhoushan Fishing Ground, China. Mar. Pollut. Bull. 73, 374–380.

SigmaPlot, 1997. SigmaPlot for Windows version 11. SPSS Inc., Chicago, IL.

Silver, A., 1974. The biology of cholinesterases. In: Neuberger, A., Tatum, E.L. (Eds.), Frontiers of Biology. Vol. 36.

Solé, M., García de la Parra, L.M., Alejandre-Grimaldo, S., Sardá, F., 2006. Esterase activities and lipid peroxidation levels in offshore commercial species of the NW Mediterranean Sea. Mar. Pollut. Bull. 52, 1708–1716.

Solé, M., Lobera, G., Aljinovic, B., Ríos, J., García de la Parra, L.M., Maynou, F., Cartes, J.E., 2008. Cholinesterases activities and lipid peroxidation levels in muscle from shelf and slope dwelling fish from the NW Mediterranean: Its potential use in pollution monitoring. Sci. Total Environ. 402, 306–317.

Solé, M., Baena, M., Arnau, S., Carrasson, M., Maynou, F., Cartes, J.E., 2010. Muscular cholinesterase activities and lipid peroxidation levels as biomarkers in several Mediterranean marine fish species and their relationship with ecological variables. Environ. Int. 36, 202–211.

Sparling, D.W., Fellers, G., 2007. Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*. Environ. Pollut. 147 (3), 535–539.

Stevens, J.D., 2009. *Prionace glauca*. IUCN 2014 (Available from http://www.iucnredlist.org/apps/redlist/details/39381/0. July 12th, 2014).

Stien, X., Percic, P., Gnassia-Barelli, M., Roméo, M., Lafaurie, M., 1998. Evaluation of biomarkers in caged fishes and mussels to assess the quality of waters in a bay of the NW Mediterranean sea. Environ. Pollut. 99, 339–345.

Sturm, A., da Silva Assis, H.C., Hansen, P.D., 1999a. Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. Mar. Environ. Res. 47, 389–398.

Sturm, A., Wogram, J., Hansen, P.D., Liess, M., 1999b. Potential use of cholinesterase in monitoring low levels of organophosphates in small streams: natural variability in three-spined stickleback *Gasterosteus aculeatus* and relation to pollution. Environ. Toxicol. Chem. 18, 194–220.

Sturm, A., Wogram, J., Segner, H., Liess, M., 2000. Different sensitivity to organophosphates of acetylcholinesterase and butyrylcholinesterase from three-spined stickleback *Gasterosteus aculeatus*: application in biomonitoring. Environ. Toxicol. Chem. 19, 1607–1615.

Tortelli, V., Colares, E.P., Robaldo, R.B., Nery, L.E.M., Pinho, G.L.L., Bianchini, A., Monserrat, J.M., 2006. Importance of cholinesterase kinetic parameters in environmental monitoring using estuarine fish. Chemosphere 65, 560–566.

Toutant, J.P., 1989. Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms. Prog. Neurobiol. 32, 423–446.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 132, 57–149.

Whitehead, A., Anderson, S.L., Ramirez, A., Wilson, B.W., 2005. Cholinesterases in aquatic biomonitoring: assay optimization and species-specific characterization for a California native fish. Ecotoxicology 14, 597–606.