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Abstract: In fish, the role that cholinesterases (ChEs) play in tissues other than those with neural activity, as well as the involvement of carboxylesterases (CbEs) and cytochrome P450 isoenzymes (CYPs) in drug metabolism needs investigation. For that, Senegalese sole (Solea senegalensis) specimens were selected for characterization of several type B esterases and hepatic CYPs in order to further use this fish as sentinel. ChEs (acetylcholinesterase (AChE) and pseudocholinesterases (butyrylcholinesterase-BuChE and propionilcholinesterase-PrChE) and CbEs were measured in brain, plasma, kidney, liver, gonad, muscle and gills. Moreover, seven fluorimetric substrates were selected to study CYP related activities in fish liver.

The results showed that AChE was the dominant ChE form in brain whereas pseudocholinesterases were absent in most tissues, as demonstrated by a low enzymatic activities using specific substrates and the lack of inhibition by iso-OMPA. Plasma exhibited trace activities of all the esterases assayed and no BChE activity. CbEs were dominant in liver, but they were also present in kidney and brain. For CbE determination, ⍺-naphtyl acetate (⍺NA) was seen as the most adequate substrate as it displayed higher enzymatic activities and showed more in vitro sensitivity to the carbamate eserine and the organophosphate pesticide dichlorvos. Alkoxyresorufin-O-dealkylase (EROD and BFCOD) activities, indicative in mammals of CYP1A and CYP3A subfamilies, respectively, were the highest microsomal CYP-related activities in liver. The results of this preliminary work allow us to select the most adequate esterase substrate, tissue and hepatic CYP substrate for further monitoring studies.

Barcelona 22nd september 2011

Dear Editor,

We are pleased to submit the manuscript entitle "Characterization of type "B" esterases and hepatic CYP450 isoenzimes in Senegalese sole for their further application in monitoring studies".

In this work we have fully characterised B type esterases in a benthic species that has been proposed as sentinel in Southern Iberian Peninsula. With its currently expansion to other areas (NW Mediterranean) and Northern Atlantic waters, we suspect it will be a candidate for future field studies. Moreover, due to its great adaptability to laboratory conditions, it has a great potential to be used in toxicological studies.

We are not aware of any former characterisation on the parameters we propose in this species; therefore we feel are findings and proposals can benefit the scientific community interested on this research topic.

We are looking forward to a positive answer from you.

Sincerely,

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- Brain is the most adequate tissue for neurotoxic determinations in *Solea senegalensis*.
- Hepatic carboxylesterase using α naphtyl acetate are proposed as a potential biomarker.
- Plasmatic B type esterases are not suitable biomarker candidates.
- ER and BFC are good fluorimetric substrates for measuring hepatic CYP1A and CYP3A disturbances.

CHARACTERIZATION OF TYPE "B" ESTERASES AND HEPATIC CYP450 ISOENZIMES IN SENEGALESE SOLE FOR THEIR FURTHER APPLICATION IN MONITORING STUDIES

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1

Abstract

In fish, the role that cholinesterases (ChEs) play in tissues other than those with neural activity, as well as the involvement of carboxylesterases (CbEs) and cytochrome P450 isoenzymes (CYPs) in drug metabolism needs investigation. For that, Senegalese sole (*Solea senegalensis*) specimens were selected for characterization of several type B esterases and hepatic CYPs in order to further use this fish as sentinel. ChEs (acetylcholinesterase (AChE) and pseudocholinesterases (butyrylcholinesterase-BuChE and propionilcholinesterase-PrChE) and CbEs were measured in brain, plasma, kidney, liver, gonad, muscle and gills. Moreover, seven fluorimetric substrates were selected to study CYP related activities in fish liver.

The results showed that AChE was the dominant ChE form in brain whereas pseudocholinesterases were absent in most tissues, as demonstrated by a low enzymatic activities using specific substrates and the lack of inhibition by *iso*-OMPA. Plasma exhibited trace activities of all the esterases assayed and no BChE activity. CbEs were dominant in liver, but they were also present in kidney and brain. For CbE determination, α-naphtyl acetate (αNA) was seen as the most adequate substrate as it displayed higher enzymatic activities and showed more *in vitro* sensitivity to the carbamate eserine and the organophosphate pesticide dichlorvos. Alkoxyresorufin-O-dealkylase (EROD and BFCOD) activities, indicative in mammals of CYP1A and CYP3A subfamilies, respectively, were the highest microsomal CYP-related activities in liver. The results of this preliminary work allow us to select the most adequate esterase substrate, tissue and hepatic CYP substrate for further monitoring studies.

Key words: characterization, cholinesterases, carboxylesterases Solea senegalensis, CYPs, fluorimetric substrates.

Introduction

The Senegalese sole (*Solea senegalensis*, Kaup 1858) is considered an invasive species in the western Mediterranean Sea (Zenetos et al., 2010). Although the original distribution of the species is Eastern Atlantic from Senegal to La Rochelle (France), now it has expanded with a self-maintaining population in the western Mediterranean Sea. In the Iberian Peninsula has a great commercial interest as a fisheries prey as well as in aquaculture, due to its good adaptation to captivity conditions (Dinis et al., 1999). In recent years, it has also been selected as sentinel in pollution monitoring and toxicity experiments in the Southern Mediterranean region (Alvarez-Muñoz et al., 2009; Costa et al., 2009, Kalman et al., 2010; López-Galindo et al., 2010 a; b; Oliva, 2011; Fonseca et al., 2011). However, in the NW Mediterranean region, and to the best of our knowledge, it has not been subject of study from an ecotoxicological perspective, although it is not unlikely to expect that in a context of climate change, over increases on water temperature, its population might increase and expand.

Cholinesterases (ChEs) and carboxylesterases (CbEs) are type B esterases both strongly inhibited by organophoshorous compounds (OPs). Whereas AChE (acetylcholinesterase) is mainly involved in neurotransmission breaking of acetylcholine in neuromuscular junctions, pseudocholinesterases (propionilcholinesterase-PrChE and butyrylcholinesterase-BuChE) have a less clear (or have multiple) physiological role (Karczmar, 2010). In turn, CbEs are involved in the metabolism of a broad range of endogenous as well as man made chemicals and pharmaceuticals (Wheelock et al., 2008). Besides to their physiological role(s), a great sensitivity towards an increasingly broader range of compounds, in addition to pesticides, has been reported for ChEs in invertebrates as well as vertebrates (Guilhermino et al., 2000, Alpuche-Gual and Gold Bouchot, 2008) including *S. senegalensis* (López Galindo et al., 2010 a; b), and for

CbEs (Al Ghais et al., 2000; Wheelock et al., 2008). Moreover, a role in detoxification and protection towards AChE inhibition in neural tissues has been demonstrated by the action of hepatic CbEs (Maxwell, 1992; Laguerre et al., 2009) and plasmatic BChE (Salles et al., 2006). Thus their combined inclusion in pollution monitoring programs has been highly recommended (Küster 2005; Wheelock et al., 2008; Laguerre et al., 2009).

ChEs and CbEs have a particular tissue distribution in vertebrates. While AChE (EC 3.1.1.7), is mostly located in brain and muscle, as they are the most innerved tissues, pseudocholinesterases (EC 3.1.1.8) are mainly present in liver and blood but also in fish muscle (Solé et al., 2010). CbEs (EC 3.1.1.1) consists of multiple isoenzymes and although they are dominant in the liver, they are also present in many other tissues, including kidney, blood and brain (Wheelock et al., 2008). ChEs tissue distribution determines that, in most pollution monitoring studies, brain (Oliveira et al., 2007, Assis et al., 2010), head in the case of smaller fish (Varó et al., 2008; Moreira et al., 2010) or muscle (Solé et al., 2008b; 2010) were the tissues chosen for neurotoxicity determination using AChE as biomarker. Other tissues/organs such as blood (Barrón et al., 1999; Valbonesi et al., 2011), kidney (Üner et al., 2009), gill (Barrón et al., 1999; López-Galindo et al., 2010a,b) and gonad (Galgani et al., 1992) are particularly considered in some fish studies. In the present characterisation, they were included for their involvement as a first line defence (blood and gills) and physiological role in the immune (kidney) and reproductive (gonad) systems.

In marine fish muscle, three ChE forms are present being AChE predominant, followed by PrChE and BChE to different degrees; whereas in brain, only AChE is present. Pseudocholinesterases sensitivity to chemicals was reported in some fish species as to be greater than that of AChE (Sturm et al., 1999; 2000; Kirby et al., 2000).

Thus, formerly to esterases inclusion as biomarkers, a characterisation of their tissue location and their relative activities needs to be performed (Varó et al., 2003; Alpuche-Gual and Gold Bouchot 2008; Solé et al., 2010). CbEs determinations can be done using several substrates, which differ in their isoenzyme specificity (Wheelock et al., 2008, Laguerre et al., 2009). In this study ρ-nitrophenyl acetate (ρNPA) was selected as a more unspecific substrate, whereas αNA has been recognized to have a higher affinity for OPs (Chanda et al., 1997).

As seen for with type B esterases (ChEs and CbEs), monooxigenases (MO) are a family of enzymes also involved phase I metabolism. The cytochrome P450 is a superfamily of MO proteins that consist of several isoforms involved in the biotransformation of endogenous and exogenous compounds (detoxification step) either by introduction of a polar group (e.g hydroxylation) or reduction of hydrophilic compounds in order to make them more readily excretable (van der Oost et al., 2003). In fish, the most studied isoform is the CYP1A-dependent ethoxyresorufin *O*-deethylase (EROD) activity currently associated to dioxin-like chemicals exposure (White et al 2000). Recent pollution monitoring studies have evidenced the importance of the combined action of anthropogenic chemicals over this enzymatic activity. Thus, mixtures of chemicals in the environment can act over this enzyme (either enhancing or decreasing its activity). Therefore the use of EROD activity as an indicator of anthropogenic impact has to be more carefully examined. In this sense, the application of several fluorimetric substrates to measure CYP activities in fish, adapted from mammalian studies on specific CYP families, will allow a closer insight into xenobiotics' metabolism and chemicals interactions over the detoxification system of fish (Smith and Wilson, 2010). As suggested for esterases, previous to CYPs inclusion in monitoring, the characterisation of its basal activities is recommended. Moreover the

combined use of B esterase activities and CYP1A levels has already been applied in assessment of pesticide exposure in fish (Wheelock et al., 2005, Fasulo et al., 2010).

The aim of this study was to characterise, and measure, AChE, PrChE, BChE and CbE activities in different tissues/organs (brain, muscle, liver, kidney, gills, gonad and plasma) of *S. senegalensis* using specific substrates and selective inhibitors. Even though several studies have already applied these biomarkers, a detailed characterization has not yet been published for this specie. Moreover, CYPs distribution in liver of adults was made for its further application in pollution monitoring studies.

2. Material and methods

2.1. Tissue preparation

Juveniles (females: 65.16 ± 51.9 g and males: 62.04 ± 38.4 g) and adult females (384.84 ± 91.2 g) of Senegalese sole were provided by the aquaculture facilities of Institute of Aquaculture Torre la Sal (IATS-CSIC, Castellón, Spain) and IRTA-Generalitat de Catalunya (Sant Carles de la Rápita, Spain) respectively. Fish were anesthetised using 2-phenoxy ethanol before blood was taken using a heparinized syringe and, immediately after, the tissues (gills, brain, liver, kidney, gonad and muscle) were dissected and quick frozen in liquid nitrogen. Plasma was obtained after blood centrifugation at 3,000 rpm x 15 min at 4ºC. Tissues and plasma were stored at -80ºC until analyses were performed.

A portion of tissue (0.05-0.2 g) was used for ChEs and CbEs determinations. Tissues were homogenised in ice-cold buffer phosphate (50 mM pH 7.4) in a 1:5 (w:v) ratio using a polytron® blender. The obtained homogenate was centrifuged at 10,000 g x 30' at 4ºC and the supernatant (S10) used for enzymatic determinations.

Individual livers of adult females were also selected for CYPs-related activities determinations. Livers (≈2 g) were homogenized in ice-cold phosphate buffer (100 mM pH 7.4) containing 150 mM KCl, 1 mM DTT, 0.1 mM PMFS and 1 mM EDTA in a 1:4 ratio (w:v). After a 10,000g x 30' and a 10,000g x 60'centrifugation steps at 4ºC, the microsomal pellet obtained was dissolved in the homogenization buffer containing also 20% glycerol in a 2:1 (w:v) ratio according to the method published by (Förlin and Andersson, 1985).

2.2. Type B esterases characterization

ChE activities were characterized in several tissues (brain, muscle, liver, kidney, gills, gonad and plasma) using eserine (physostigmine) sulphate, BW253c51 (1,5-bis(4-allyldimethyl-ammonimphenyl)penta-3-one dibromide and iso-OMPA (tetraisopropyl pyrophosphoramide) as specific inhibitors of the true ChE, AChE and BChE, respectively. The inhibitor tested concentrations ranged from 0.64 to 800 μM for eserine sulphate and BW253c51, and from 0.08 to 16 mM for iso-OMPA. In all cases, 120 μl of adequately diluted S10 supernatant (depending on the tissues) was incubated for 30 minutes at room temperature with 5 μl of the inhibitor at a specific concentration within the selected test concentrations. At least three independent replicates of each assay were done. A blank without inhibitor and with either bi-distilled water (in the case of BW284c51) or ethanol (solvent used for eserine sulphate and iso-OMPA) was also done. After the incubation, ChEs activities were determined according to the principle of Ellman et al. (1961) with appropriate modifications for microplate (Solé et al., 2008a), using 1 mM ASCh (acetylthiocholine iodide) as substrate. Reading was performed at 405 nm in kinetic mode using a TECAN Infinite 200 microplate reader at 25ºC for 5 minutes. Enzymatic determinations were always performed in triplicate.

The apparent Vmax and Km values for AChE activity in brain, muscle, kidney and gills, were measured using several ASCh concentrations (0.25, 1, 2.5, 5 and 20 mM) with appropriate S10 fraction dilutions, so linearity of the measure was always achieved, following the ChE assays conditions described above. The values were calculated in this range from Michaelis and Menten equation (V = Vmax [S]/Km+[S]), using the linearity transformation of Lineweaver-Bruk plot.

Hepatic CbE activity using either αNA or pNPA as substrates was also tested. Moreover, its inhibition by the carbamate serine (from 0.64 to 800 μM) or the OP pesticide dichlorvos (*O*-(2,2-dichlorovinyl)-*O,O*-dimethylphosphate (DDVP) from PESTANAL® CAS 62-73-7) in the range 0.256 to 320 μM was assessed. Furthermore, the apparent Vmax and Km values for CbE activity were determined in liver and kidney using five concentrations of αNA (0.0625, 0.125, 0.25, 0.5 and 1 mM) as substrate as described above for AChE.

2. 3. Type B esterases measurements

For ChEs measurements three different substrates ASCh, S-butyrylthiocholine iodide (BSCh) and propionylthiocholine iodide (PrSCh) at a concentration of 1mM were used. In each microplate well, 150 μl of 270 μM DTNB (5,5'-dithio-bis-2-nitrobenzoat) were mixed with 25 μl of sample (undiluted or diluted appropriately) and after 2 minutes pre-incubation, the reaction was started adding 50 μl of the substrate. ChE activity was determined in triplicate as previously described (point 2.2) and was expressed as nmol/min/mg prot.

CbE activity was measured using either αNA or ρNPA as substrates. αNA was assayed following an adaptation to microplate from an UV method (Mastropaolo and Yourno, 1981). Briefly, 25 μl of sample and 200 μl of αNA as substrate (250 μM final

concentration in well) were followed up over 5 min at 235 nm. The other assayed substrate, ρNPA, was used as indicated by Hosokawa and Satoh (2001) with some modifications. That is, 25 µl of sample and 200 µl of ρNPA as substrate (1 mM final concentration in well) were followed up for 5 min at 405 nm over ρ-nitrophenol formation using the same kinetic method as for ChEs in a TECAN Infinite 200 microplate reader. CbE activity was also measured in triplicate and expressed as nmol/min/mg prot.

2.4. CYP determinations

Catalytic activities of liver CYPs were determined using seven fluorescent CYP-mediated substrates with assay conditions optimized in our lab based on the method described in Smith and Wilson (2010) as listed in Table 1. Ten microliters of microsomes were incubated for 10 min at 30ºC and the fluorimetric metabolite formed was recorded at their specific wavelengths (listed in Table1). A calibration curve for each specific metabolite was done (range from 0-160 nM). CYPs assays were run in 100 mM phosphate buffer (pH 7.4), except ECOD determination, which was done in 100 mM Tris buffer pH 7.4. All reactions were run in transparent 96-well format and were linear for the period recorded using kinetic assays mode (Magellan v6.0) in a TECAN Infinite 200 microplate reader. CYP-related activities were expressed in pmol/min/mg prot.

2.5. Protein determination

Total protein content of the samples was determined by the Bradford method (Bradford, 1976) adapted to microplate, using the Bradford Bio-Rad Protein Assay reagent and bovine serum albumin (BSA; 0.1-1 mg/ml) as standard, as described (Solé

et al, 2008a). The absorbance was read at 595 nm. All protein determinations were carried out in triplicate.

2.6. Statistical analysis

Data from type B esterase characterization were analysed with one-way analysis of variance (ANOVA), followed by *post hoc* Dunnett´s multiple comparison test to determine which concentrations were significantly different from the control. The homogeneity of variance was determined by Levene´s test. Data expressed in percentages were arcsine transformed to normalize the variable prior to ANOVA analyses. Transformed data were converted back to percentages to calculate means and standard error of the means (Varó et al 2007). In vitro inhibition concentration values (IC50) were calculated using the regression probit module of SPSS Systems Software.

Student *t*-test was used to compare the enzymatic activities between males and females of juveniles, and between juveniles (both sexes) and adult females. Pearson correlation coefficient was used to analyse the relationship between enzyme activities and between activities and fish weigh. The data are presented as means $\pm$ SEM (standard error). Statistical analyses were carried out using SPSS System Software and the significance level for data analyses was 0.05 or 0.01.

3. Results

3.1. Type B esterases characterisation

In addition to the use of selective substrates, the action of selective inhibitors allows to discriminate between esterases. BW284c51, a specific inhibitor of AChE, was assayed in brain and muscle of juveniles as they expressed higher activities. Fig 1 shows the effect of BW284c51 on AChE activity of brain and muscle. They showed inhibition

at all doses; although brain's AChE activity was 10-fold higher than in muscle, and the lowest concentration tested (0.64 μM) caused a 74% and 52 % significant inhibition in brain and muscle, respectively. The IC50 values estimated for AChE activity in brain were 0,076 μM (95% confidence limits: 0,004-0,414) and for muscle 4,17 μM (95% confidence limits: 0,039-175,3). Thus, in *S. senegalensis,* brain seems the most adequate tissue to measure neurotoxicity. Incubations with *iso*-OMPA, BChE inhibitor, were made in kidney and muscle of juveniles, as they were the tissues, in addition to liver, to display a significant measurable BChE activity. In kidney inhibition was already achieved at the lowest dose (0.08 mM) using either ASCh (37 %; p<0.05) or BSCh (54 %; p<0.05) as substrates, and it was maintained thereafter; although in any case it was dose dependent (data not shown). In muscle, the inhibition by iso-OMPA (using ASCh as sustrate) caused at 0.08 mM a significant inhibition (33%; p <0.05) of ChE activity that was maintained thereafter showing a certain dose-dependence (Fig 2). IC50 value estimated for muscle AChE activity was 2,28 mM (95% confidence limits: 1,04-7,58).

The addition of eserine (0.64-800 μM) to kidney and gonad tissue (S10) had no significant inhibitory effect over CbE activity using αNA as substrate (data not shown). However hepatic CbE inhibition was achieved at 800 μM eserine with the two substrates assayed (αNA and pNPA), although it was stronger with αNA (64%; p<0.05) than with pNPA (33%; p<0.05). The OP pesticide dichlorvos was also selected to test hepatic CbE inhibition with the two substrates (Fig. 3). It also showed a greater sensitivity using αNA as substrate, with a significant inhibition (54%; p<0.05) at 2.56 μM. IC50 values for liver CbE activity were 2.32 μM (95% confidence limits: 0.532-8.68) and 15.72 μM (95% confidence limits: 9.63-27.16) using αNA and pNPA substrates, respectively. Therefore, αNA was chosen as a more adequate substrate for future CbE determinations.

Five ASCh concentrations (0.25-20 mM) were assayed to determine AChE Vmax (nmol/min/mg prot) and Km (mM) and for each tissue. The kinetic parameters calculated were 105.8 and 0.189 for brain, 5.03 and 0.141 for muscle, 4.25 and 0.159 for kidney and 3.31 and 0.338 for gills, for Vmax and Km respectively. No inhibition was observed to the highest substrate concentration assayed (20 mM) in any tissue, except brain. In this study and many others using fish species, 1 mM ASCh was also adopted as adequate as it displayed the maximum rate of hydrolysis and it was not rate limiting over the kinetic time. As for CbE activity, five αNA concentrations (0.0625-1 mM) were assayed in liver and kidney to determine the same kinetic parameters (Vmax and Km). In liver Vmax values were 111.61 nmol/min/mg prot and 0.109 mM for Km, whereas corresponding values for kidney were 30.84 nmol/min/mg prot and 0.184 mM. Concentrations over 1 mM of αNA were unrecordable as they overflowed the UV detection system.

3.2. Type B esterase activities

In Table 2 the activities for the different esterases in juveniles (males and females) and adults (females) of *S. senegalensis* are presented. As no sex-related differences in these activities were found in juveniles (t-test, $p>0.05$) in any tissue, except gonad, the data was reported together regardless of sex. In females, enzymatic activities in gonads were generally lower than in males.

Total % of ChEs activity in the different tissues studied usually followed the order AChE>PrChE>BChE, with a significant positive relationship between AChE and PrChE in muscle ($r=0.633$; $p<0.05$), kidney ($r=0.945$; $p<0.01$), liver ($r=0.918$; $p<0.01$) and plasma ($r=0.745$; $p<0.01$). A positive correlation was also found between AChE and BChE in kidney, liver, plasma and gills ($r=0.677$-0.856; $p<0.05$). Both

pseudocholinesterases (BChE and PrChE) were also positively correlated in kidney, liver and plasma (r=0.728-0.956; p<0.01). In addition, a good agreement was observed between measuring CbE with either αNA or pNPA as substrates in most tissues including muscle, brain, gonad and liver (r=0.766-0.875; p<0.01), but not in gills, kidney and plasma.

Taking into account all ChEs activities per individual tissue and considering the range of both age groups, AChE activity was dominant in brain (72-88%), followed by muscle (58-62%) and gills (47-50%). Whereas per total B type esterases (ChEs and CbE), CbE activity was dominant in liver (82-89%), followed by kidney (52-63%), gills (61-70%), gonad (47-58%) and brain, (24-25%). In plasma, esterase activities were very low. Considering the tissue distribution of each individual esterase activity, a decrease in contribution of muscle AChE (from 15 to 9%) and hepatic CbE (from 59 to 34%) was observed from juveniles to adults. In contrast PrChE contribution in brain increased over age, that is from 20 to 50%. In fact, a negative relationship with fish weight was observed for AChE in muscle (r=-0.760; p<0.01) and liver (r=-0.593; p<0.01). In the case of gills, the negative weight-activity relationship was found with PrChE (r=-0,826; p<0.01). Negative was also the relationship between weight and hepatic CbE (r=-0.566 p<0.01; αNA) as well as in gill and gonad (r=-0.549-0.576; p<0.05; ρNPA).

3.3. CYP determinations

Seven substrates were assayed to measure CYP related activities in the microsomal liver fraction of adult females. Among them the highest enzymatic activity was achieved using ER as substrate followed by BFC>CEC>EC≈DBF≈BR≈PR (Table 1). Thus ER seems to be the most adequate substrate to measure CYP1A related activities while BFC would be an adequate candidate for CYP3A related ones. The

apparent Vmax and Km values determined for EROD activity using six ER concentrations (range 0.1-8 μM) in the hepatic microsomal fraction of adult females were 48.74 pmol/min/mg prot and 0.672 μM, respectively.

4. Discussion.

In many studies, muscle has been the tissue chosen for AChE determinations due to its high sensitivity to neurotoxicants, easiness to obtain and larger amount of tissue available. However, in the case of the species *S. senegalensis*, it expresses a much lower AChE activity in muscle than the observed in other teleosts (Varó et al., 2008; Solé et al., 2008a; 2010), including other flatfish species (Sturm et al., 1999, Minier et al., 2000) although it coincides with values reported for this same species from Southern Spain (Oliva, 2011). Alternatively to muscle, gills have also probed to be an appropriate tissue for neurotoxicity determination in *S. senegalensis* juveniles (López-Galindo et al., 2010a, 2010b). However, the activity detected in the present study was very low (Vmax 3.31 nmol/min/mg prot) in contrast to the reported values by López-Galindo et al., (2010a; b) in which it was 4-times higher, even though all these studies use the same analytical protocol.

In *S. senegalensis*, AChE activity in brain was clearly dominant, it was inhibited to the highest doses of substrate assayed and also by the specific AChE inhibitor BW284c51 in a dose dependent manner, even more strongly than in muscle. All these characteristics taken together were not observed in any other tissue, thus confirming the true nature of AChE in brain and the coexistence of other ChEs in the other tissues. Therefore, in this species brain should be the tissue of choice for neurotoxicity determination.

Another particularity in *S. senegalensis* was the absence of BChE in plasma and a very low catalytic activity in muscle when the specific BSCh substrate is used (Vmax 1.3-2.2 nmol/min/mg prot), although its presence was demonstrated in muscle by a 34% inhibition achieved after *in vitro* incubations with the selective inhibitor *iso*-OMPA. This contrasts with observations in Atlantic flatfish species such as *Platichtys flesus* and *Limanda limanda*, that expressed high BChE activity in muscle (Sturm et al., 1999). However, this particularity is not surprising considering that species differences occur even within the same taxonomic group (e.g. cyprinids). That is, high serum BChE in some fish contrasted with no measurable activity in others (Chuiko, 2000). All these observation stress the need for a former characterisation of esterases in each individual species and tissue before its selection in monitoring programs. In plasma, CbE activity was also very low, despite reports for other fish species. That is, in adult trout (*Oncorhynchus mykiss*) CbE activity in sera (using pNPA as substrate) was 2- and 10-times higher than that recorded in liver and gill microsomes, respectively. Expressed as Vmax (in nmol/min/mg prot), it was: sera(1639)>liver(672)>gill(16.1) (Barron et al., 1999). These results contrast with our observations in *S. senegalensis* using the same substrate, in which CbE activity was in plasma: <2 nmol/min/mg prot, in liver: 6.3-21.7 nmol/min/mg prot and gills: 6.9-8.5 nmol/min/mg prot. Thus, plasmatic CbE activity in *S. senegalensis* does not seem an appropriate marker to include in monitoring studies.

In former studies, *S*-phenyl thioacetate (SPTA) was the substrate used for hepatic CbE determinations in *S. senegalensis* (Solé et al. 2008b; López-Galindo et al., 2010a; b). However, due to the unespecificity of SPTA, pNPA and αNA have been recommended (Wheelock et al., 2008) and selected for the present study of characterisation. Contrasting the two recommended substrates, a greater sensitivity towards the carbamate eserine and the OP dichlorvos was revealed when using αNA

rather than with pNPA. Moreover, higher catalytic activities were achieved with αNA and a good correlation between both forms was seen in most tissues. Then, it is concluded that in *S. senegalensis*, the UV method using αNA is the most appropriate assay for CbE determinations. The tissues that did not to correlate in CbE activity using either substrate were kidney, plasma and gills, all having an important haematopoietic role. CbEs are constitutive for multiple isoenzymes that are likely to have a different tissue distribution. Moreover, as the substrates used for CbE determinations (SPTA, αNA or pNPA) are all synthetic, the physiological meaning of the discrepancies using either substrate remains unclear (Wheelock et al., 2008)

As far as tissue distribution concerns and regardless of age group, a general trend was found: AChE was dominant in brain (53-65%) followed by kidney (11-13%)≈muscle (9-15%)>gonad (4-8%)≈liver (4-7%) >gills (4%) >plasma (1-2%). PrChE was more equally distributed in all tissues, except plasma, while BChE was better represented in kidney (27-40 %) and liver (13-20%). CbEs were dominant in liver (34-59%) followed up by kidney (18-24%) >brain (9-20%) >gills (7-10%) >gonad (5-7%). In the flatfish dab, *L. limanda,* AChE followed the same trend per tissue as in our study with *S. senegalensis*, although kidney and plasma were not considered in the former study (Galgani et al., 1992). However, in contrast to Senegalese sole, BChE activity was more significant in muscle and liver of dab (Galgani et al., 1992). There are hardly any studies including kidney as target tissue. However, in one conducted on *Oreochromis niloticus*, AChE activity was lower in kidney than brain and liver, but BChE activity was dominant in liver and kidney (Üner et al., 2009). Another study also carried out on *O. niloticus* contrasting AChE activity in kidney, gill and alimentary tract, also confirmed that AChE activity in kidney as the lowest (Durmaz et al 2006). In a recent study conducted on *Anguilla anguilla,* AChE activity in brain and muscle were equally

16

represented while pseudocholinesterases were present in plasma and liver, although to a lesser extent (Valbonesi et al., 2011). Present results with *S. senegalensis,* especially in the adult group, resemble more those observed on carp (*Cyprinus carpio*) in which AChE and pseudocholinesterases are codominant in liver (Sevgiler et al., 2007). Tissues, other than liver and kidney, with a less evident metabolic role (that is gill and plasma) showed low catalytic activities for CbE and ChEs. They were, however, considered in our study, due to the importance of presystemic metabolism taking place in gill and blood. This first catalytic step is crucial, as it would render compounds readily to be excreted and thus, lower their potential to bioaccumulate and act over critical organs such as brain or muscle. Nevertheless, as the esterase activities measured in these two tissues (gills and plasma) were very low in *S. senegalensis*, it seems unlikely that they play a significant role in protecting the species in front of ester chemicals and OP pesticides. B type esterase activities in gonads, although low, should be considered, as any esterases alterations in this organ due to xenobiotics exposure might interact with hormone metabolism (Wheelock et al., 2008).

An attempt to establish if there was a relationship of esterase activities with age/size was made. Muscle AChE activity in Senegalese sole also showed a negative relationship with fish size as seen before in other teleosts (Solé et al, 2006; 2010; Varó et al, 2003; Alpuche-Gual and Gold-Bouchot, 2008). AChE activity contribution, all ChEs considered, decreased in muscle and in brain from juveniles to adults. However, in brain the percentage of PrChE activity increased in adulthood (from 8 to 24%). Hepatic CbE measured using either pNPA or αNA also showed a negative relationship with age. Hepatic CbE contribution (all tissues considered) decreased from juveniles to adults while in brain it increased during adulthood (from 9% to 20%). In a study conducted on 20 Brazilian fish species, three of them showed a negative relationship

between brain AChE and fish size (Oliveira et al 2007). Contrarily, CbE activity in whole body homogenates of *O. mykiss,* did not differ from juvenile and adult life stages despite a 250 fold increase in size. Moreover, similar activities, regardless of fish size, were found across a group of freshwater juvenile fish (Bluegill, Fathead Minnow and Catfish) including trout (Barron et al., 1999). Thus, the allometric theory that predicts a decrease in metabolic parameters over size, was confirmed in *S. senegalensis* for muscle ChEs and liver CbEs but not for any other tissue.

No sex relationship was observed in any of the B type esterases determined in any tissue, except gonad. In fact juvenile males displayed higher esterase activities than females. The reason for this sex particularity remains unclear. Other fish studies have not revealed a sex bias in ChEs activity (Varó et al., 2008; Alpuche-Gual and Gold-Bouchot 2008). However, CbE activity was higher in males of the reef fish *Haemulon plumieri* than in females, in addition and, contrarily to *S. senegalensis,* the relationship between hepatic CbE and size in *H. plumieri* was positive (Alpuche-Gual and Gold-Bouchot 2008). All these evidences stress the need for an individual characterisation of each species and increase the number of individuals and ages contrasted in order to assess the influence of biological parameters.

As applied to B type esterases, previous to their inclusion in monitoring, background CYP-related activities were measured in the hepatic microsomal fraction of *S. senegalensis* adult females by the use of "specific" fluorimetric substrates. ER was the substrate to display higher alkoxyresorufin-O-dealkylase activity evaluated as EROD activity and representing the CYP1A family, which is broadly used as a biomarker of "dioxin-like chemicals" exposure (Whyte et al., 2000). BFC, considered a more specific substrate for the CYP3A family with a physiological role in hormone metabolism, showed high enzymatic hydrolysis in this species. The substrates BR and

CEC are rather unspecific but they have successfully been used in some fish hepatocyte studies evaluating to sewage treatment works discharges (Gagné et al., 2006). In *S. senegalensis* the capacity to metabolite CEC substrate was quite remarkable, therefore it could be a potential substrate to further validate in laboratory experiments. The basal activities measured in this study fall bellow those reported for other teleosts (*O. mykiss* and *Fundulus heteroclitus*), even taking into account the high variability, especially for EROD activity, within species (Smith and Wilson, 2010). They are, however, in the range or even higher, if contrasted to deep-sea fish in a study using the same fluorimetric substrates and methodology (Koenig et al., data not published). Although a lower number of substrates is usually applied to environmental or toxicological studies in the laboratories, among the most frequently selected, it stands out the combined use of ER and BFC as CYP substrates applied to drug metabolism studies in fish (Hegelund et al., 2004; Thibaut et al., 2006).

5. Conclusions.

Enzymes characterisation and its distribution in several tissues of a given species are necessary steps to take previously to consider it as sentinel. The present characterisation in Senegalese sole, (*S. senegalensis*), allowed us to deduce that brain is the most suitable tissue for neurotoxicity determination whereas hepatic CbE, using αNA as substrate, is suited for assessing the action of environmental contaminants over these metabolic system. The fluorimetric substrates ER and BFC, as indicative of CYP1A and CYP3A related activities, respectively, are potentially adequate candidates for pollution monitoring studies using this fish species.

Acknowledgements

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Table 1

Table 1. Assay conditions for fluorimetric substrates to determine microsomal CYP activities and activities recorded for hepatic microsomes in adult females of *S. senegalensis*.

Susbstrate		Metabolite	Ex/Em (nm)	Substrate concentration (µM)[a]	Mammalian CYPs[b]	S. senegalensis (pmol/min/mg prot)
ER	7-ethoxyresorufin			3	1A1 > 1A2, 1B1	32.0 ± 6.1
PR	7-pentoxyresorufin	Resorufin sodium salt	537/583	5	2B	0.8 ± 0.1
BR	7-benzyloxyresorufin			3	1A1, 2B, 3A	0.9 ± 0.2
CEC	3-cyano-7-ethoxycoumarin	3-cyano-7-hydroxy-4-methylcoumarin	408/455	10	1A2, 2C9, 2C19	14.6 ± 2.3
BFC	7-benzyloxy-4-trifluoromethylcoumarin	7-OH-4-trifluoromethylcoumarin	410/538	500	3A4, 2C19	27.7 ± 5.9
DBF	Dibenzylfluorescein	Fluorescein	485/538	1	2C8, 2C9, 2C19, 3A4	1.1 ± 0.2
EC	7-ethoxycoumarin	7-OH-coumarin (umbeliferona)	370/450	100	2A, 2B, 1A	2.0 ± 0.2

[a]NAPDH [200 µM] in plate for all substrates except for BFC [20 µM], [b](Smith and Wilson, 2010)

Table 2

Table 2. Type B esterase activities (in nmol/min/mg prot) for S10 of several tissues of *S. senegalensis* juveniles and adults.

		AChE	PrChE	BChE	CbE (α-NA)	CbE (p-NPA)
Muscle	Juveniles (18)	8.95 ± 0,45	3,35 ± 0,22 (10)	2,17 ± 0,22	1,39 ± 0,13 (16)	1,88 ± 0,10
	Adults (5)	4.88 ± 0,61*	2.17 ± 0,38*	1.28 ± 0,41	1.36 ± 0.12 (4)	1,77 ± 0,16 (4)
Brain	Juveniles (19)	31,5 ± 3,45	2.96 ± 0,33	1,42 ± 0,18 (13)	11,6 ± 1,15 (16)	14,5 ± 1,51 (17)
	Adults (5)	34.4 (4) ± 2.52	11.3 ± 0.80*	2.15 ± 0.20*	14.7 ± 1.7 (4)	12.1 ± 2.06(4)
Kidney	Juveniles (19)	6,75 ± 0,67	3,59 ± 0,4 (16)	2,94 ± 0,33	22,8 ± 2,01 (17)	12,9 ± 0,90 (18)
	Adults (5)	6.87 ± 1.93	4.45 ± 1.30	4.64 ± 1.20	17.6 ± 2.83(4)	7.40 ± 0.57(4)*
Gonad	Juvenile females (12)	2.42 ± 0,18	1,13 ± 0,05	1,00 ± 0,10	4.29 ± 0.58 (11)	3,86 ± 0,41
	Juvenile males (5)	9.95 ± 1.15+	4.04 (1)+	1.31 ± 0,15 (3)	13.4 ± 2.5 (3)+	9.87 (1)+
	Adults (5)	2.04 ± 0.25	1.12 ± 0.11	0.67 ± 0.07	5.30 ± 0,27	1.38 ± 0.07*
Liver	Juveniles (19)	4.11 ± 0.31	2.50 ± 0.19	2.22 ± 0.15	74.08 ± 5.84	21.66 ± 1.51
	Adults (5)	2.11 ± 0.16*	1.81 ± 0.16*	1.45 ± 0.16*	24.26 ± 2.03*	6.31 ± 0.53*
Plasma	Juveniles (19)	1,23 ± 0,20	0.29 ± 0,06 (14)	0,11 ±0,03 (18)	1,05 ± 0,24	1.15 ± 0,19 (12)
	Adults (5)	0.32 ± 0.03*	0.10 ± 0.06	0.06 ± 0.01	1.59 ± 0.20	1.95 ± 0.26*
Gills	Juveniles (12)	2,04 ± 0,24	1.26 ± 0,16	1,02 ± 0,11	9,32 ± 0,41	8,45 ± 0,20
	Adults (4)	2.30 ± 0.34	1.39 ± 0.17	1.25 ± 0.04	7.75 ± 0.82	6.93 ± 0.60*

* denotes statistical significance between juveniles and adults. In the case of gonad of the same sex (females); $p < 0.05$.

+ denotes statistical significance between male and female juveniles. Only observed for gonad parameters; $p < 0.05$.

Figure legends.

Figure 1. The *in vitro* effect of BW284c51 on AChE (using ASCh) in brain and muscle S10 of *S. senegalensis*.

Figure 2. The *in vitro* effect of *iso*-OMPA on AChE (using ASCh) in muscle S10 of *S. senegalensis*.

Figure 3. The *in vitro* effect of the OP pesticide dichlorvos on CbE activity (using α-naphtyl acetate or ρ-nitropenyl acetate) in liver S10 of *S. senegalensis*.
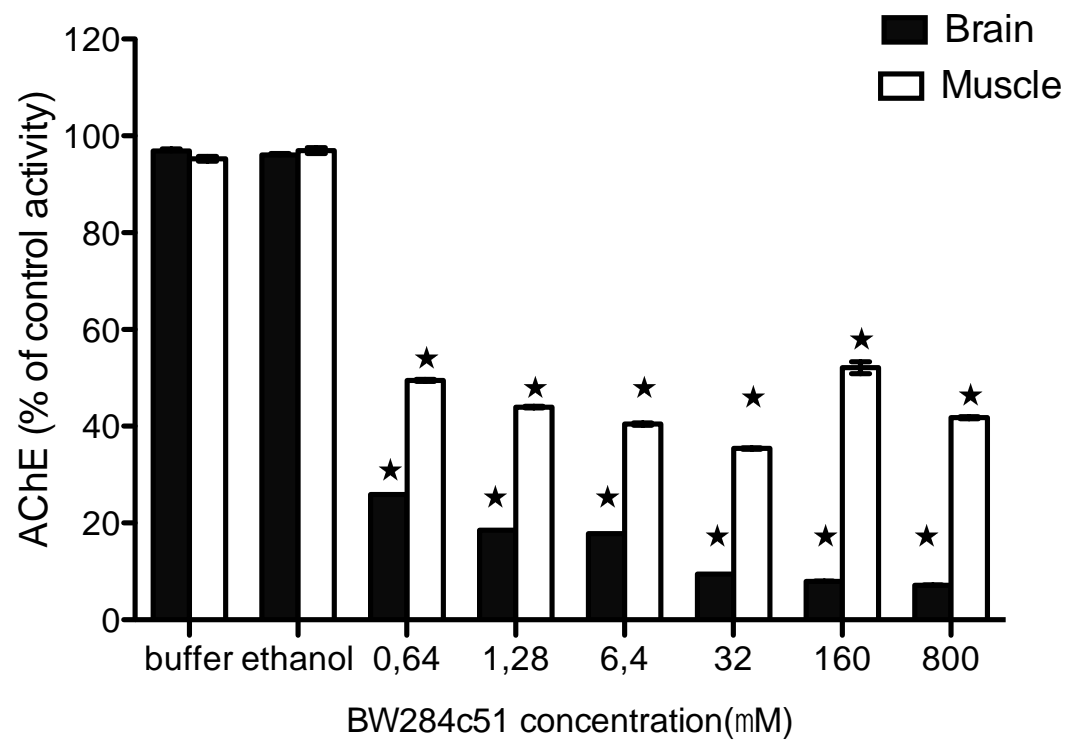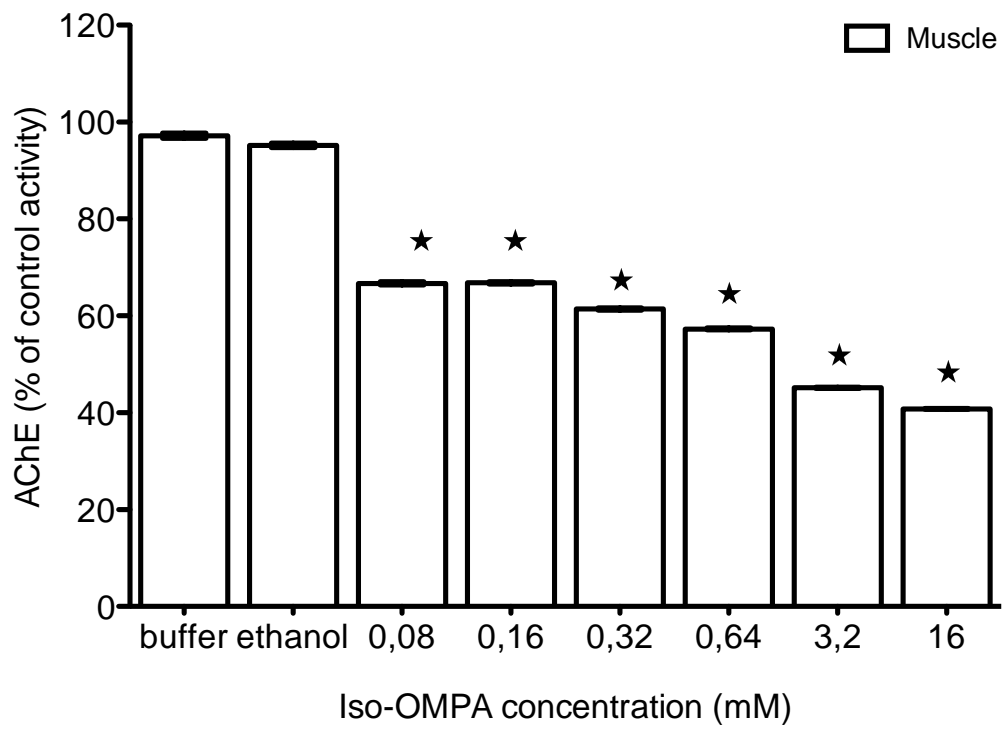
Fig 1

Fig 1

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