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Development of sensitive molecular markers for detecting the genotoxicity induced by two pyrethroids insecticides, Cypermethrin and permethrin, to the Meditnerranean sole, *Solea senegalensis* (Kaup 1858)

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ABSTRACT

The effects of two pyrethroid insecticides, cypermethrin and permethrin, on juvenile Senegalese sole, *Solea senegalensis*, were assessed. For this purpose, LC_{50} at 24 h and 72 h were determined as 500 µg L^{-1} and 900 µg L^{-1}, respectively. The specimens were divided into 5 experimental groups exposed to: i) ethanol vehicle in sea water (control), ii) 1/25 of cypermethrin LC_{50} (20 µg L^{-1}), iii) 1/10 of cypermethrin LC_{50} (50 µg L^{-1}), iv) 1/25 of permethrin LC_{50} (36 µg L^{-1}) and v) 1/10 of permethrin LC_{50} (90 µg L^{-1}) during 10 days. At the end of the experiment, gill and hepatic samples were obtained for studying the expression patterns of different enzyme genes related to toxicity and osmoregulation, namely glyceraldehyde-3-phosphate dehydrogenases1 and 2 (GAPDH-1 and 2), and Na^+, K^+-ATPase subunits α and β (NKA α and β). Both pyrethroid insecticides enhanced gill GAPDH-1, NKA-α and NKA-β expressions. However, hepatic responses were less prominent. The low dose of cypermethrin decreased GAPDH-2 expressions. Also, the lowest permethrin dose decreased GAPDH-2 expression. These results indicate that pyrethroids induce some degree of oxidative stress in *Solea senegalensis* specimens led to an osmotic imbalance, activating -mainly at branchial level- different antioxidant and osmoregulatory enzyme genes.

INTRODUCTION

Pyrethroids are among the most used insecticides nowadays, due to their low environmental persistence and toxicity, successful substitutes for organochlorines and organophosphates in pest-control programs. Several pyrethroid compounds have been developed, such as cypermethrin and permethrin. In aquaculture they are applied to control ectoparasites, especially lice, as well as insects in nursery and grow-out systems as alternatives for the more toxic organophosphates. However, fish are hypersensitive to pyrethroids due to the sensitivity of their nervous systems to these pesticides (Das and Mukherjee, 2003; Sayeed et al., 2003; Begum, 2005). Cypermethrin [α-cyano-3-phenoxybenzyl ester of 2, 2-dimethyl-3-(2,2-dichlorovinyl)-2-2-dimethyl cyclopropane carboxylate] is a potent type II pyrethroid pesticide that produces long delay in sodium channel inactivation leading to persistent depolarization of the nerve membrane.

It was proven to be a potential danger to several aquatic organisms (Greulich and Pflugmacher, 2004). Permethrin [3-phenoxyphenyl-methyl (+) cis-Trans-3-(2,2-dicloroethenyl)-2, 2-dimethylcyclopropane carboxylate] is a type I pyrethroid pesticide whose neurotoxicity is induced through repetitive neuronal discharge and prolonged negative after potential. It is also applied against many insect pests all over the world (Shashikumar and Rajini, 2010).Oxidation and hydrolysis are the main biodegrading processes for pyrethroids (Sogorb and Vilanova, 2002).

The oxidative stress induced by pyrethroids is mainly attributed to their cleavage and production of reactive oxygen species (ROS) during the course of their metabolism, especially the

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pyrethroid insecticide cypermethrin (Agrawal and Sharma, 2010). ROS are continuously produced as side-products of certain metabolic pathways. Meanwhile, they are degraded via several mechanisms. Generation and degradation of ROS are usually under delicate cellular control and very low (< 10^{-8} M) steady-state ROS concentrations are maintained. However, under some circumstances, the balance between ROS production and elimination is disturbed leading to their enhanced steady-state level, which is called "oxidative stress". This leads to, or indicates, an abnormal or pathological state. Increased ROS production enhances the activity of certain enzymes that degrade them, that are usually known as the antioxidant enzymes (Lushchak, 2011).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has recently been proven to be a multifunctional protein involved in diverse non-glycolytic activities (Cho et al., 2008), even it exhibits diverse functions extending beyond traditional aerobic metabolism of glucose. In particular, GAPDH was reported to bind DNA and RNA and regulate transcription, catalyze microtubule formation and polymerization, facilitate vesicular transport, and bind integral membrane ion pumps associated with Ca^{2+} release (Butterfield et al., 2010). It also interacts with a number of small molecules, including tumor necrosis factor, ribozymes, glutathione, p53, and nitric oxide (Butterfield et al., 2010).

Na^+,K^+-ATPase is heterodimeric molecule, consisting of a catalytic α-subunit and a β-subunit, both of which are encoded for by separate genes. Na^+,K^+-ATPase α-subunit contains all of the catalytic domains necessary for a functional enzyme, and the Na^+,K^+-ATPase β-subunit is critical for maintaining the stability and proper membrane orientation of the enzyme. Na^+,K^+-ATPase works as an antiporter, transporting ions in and out of the cell actively. It belongs to the P-type superfamily of transporters (Nelson and Cox, 2005). As all members of its superfamily, Na^+,K^+-ATPase is reversibly phosphorylated by ATP. The most accepted model postulates two forms of the transporter: the phsophorylated form with higher affinity for K^+ than for Na^+, and the dephosphorylated with high affinity for Na^+ and low for K^+ Phosphorylation of the transporter by one ATP molecule moves three Na^+ ions out of the cell, in spite of its low cytoplasmic concentration, and two K^+ ions into the cell, despite its higher cytoplasmic concentration.Pyrethroids inhibit a variety of enzymes, mainly ATPases, as well as the γ-Aminobutyric acid (GABA) receptor, producing excitability and convulsions (Cox, 1998). Also, their neurotoxic effects are mainly through the voltage dependent sodium channel and the integral protein ATPases in the neuronal membrane (Kakko et al., 2003). However, their role in gills is intimately related to osmoregulation and hence the osmotic imbalance produced by these insecticides.

The Senegalese sole, *Solea senegalensis* (Kaup, 1858), is a marine teleost belonging to the Class Actinopterygii, Order Pleuronectiformes, Family Soleidae. It occurs throughout the Northeastern Atlantic Ocean and the Mediterranean Sea. The species is a high-value flatfish reared in intensive aquaculture production in Southern Europe. It was the subject of several works investigating in different life stages its response to different environmental pollutants, such as polluted sediments, benzoapyrene, copper, nitrite, sodium hypochlorite and antifoulant agents, as well as other contaminants (Parra and Yúfera, 1999; Costa et al., 2008, 2009, 2010, 2012; Vicente-Martorell et al., 2009; Kalman et al., 2010; López-Galindo et al., 2010 a,b). However, a general shortage of knowledge on the molecular bases of detoxification processes has been encountered. Therefore, we aimed by this work to shed more light on the role of different antioxidant genes on pyrethroid insecticides (cypermethrin and permethrin) detoxification in the Senegalese sole at the molecular level.

MATERIALS AND METHODS

Fish

Juvenile specimens of *Solea senegalenesis* (n=150, 100-120 g body weight) were provided by Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain). Fish were transferred to the wet laboratories at the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz), where they were acclimated during 7 days to sea water (SW; 40‰, 1090 mOsm kg^{-1} H_2O, 18-20 ºC) in 30-L tanks with a flow through water system. Water quality criteria were checked periodically to affirm their stability. Fish were fed a daily ration of 1 % of their body weight with commercial pellets (Dibaq-Dibroteg S.A., Segovia, Spain). No mortality was observed during experimental time in all tanks used.

Toxicants

Technical grade pyrethroid insecticides, cypermethrin (CAS Number 52315-07-8) and permethrin (CAS Number 52645-53-1) were purchased from Sigma-Aldrich, Madrid, Spain. Stock solutions were prepared in ethanol (cypermethrin: 2 mg mL^{-1}; permethrin: 1 mg mL^{-1}).

Cypermethrin and Permethrin LC_{50} Determination

Acute toxicity tests were conducted to determine 24 h LC50 value of cypermethrin and 72 h LC50 value of permethrin toxicity. Different concentrations of cypermethrin (1.25, 2.5, 5, 10, 40, 50, 60, 120, 150, 180, 400, 500, 600 µg L^{-1}) and permethrin (100, 200, 300, 400, 700, 800, 900 µg L^{-1}) prepared from main stock solutions were assessed. For each concentration, 6 fish were randomly selected and transferred to two separate 30 L tanks (50 cm*40 cm*30 cm). Control aquaria received a dose of ethanol equal to which the maximum dose was dissolved in. The mortality/survival of fish was recorded after 24 h for cypermethrin and after 72 h for permethrin. Feeding proceeded as 1 % of fish weight during the whole time of the experiment. The concentration at which 50 % mortality of fish occurred was taken as the medium lethal concentration (LC50), which in case of cypermethrin was 500 µg L^{-1} while for permethrin was 900 µg L^{-1}. According to the scientific literature, different percentages of LC50 have been used as the sublethal doses for studying the impacts of pyrethroids on fish. Singh and Srivastava (1999) applied 40 % and 80 % of

permethrin LC50 on the snakehead *Channa striatus*. Begum (2005) used about 33.3 % of permethrin LC50 with the catfish *Clarias batrachus*. Suvetha *et al.* (2010) used a 10 % of cypermethrin LC50 for treating the common carp *Cyprinus carpio*. Das and Mukherjee (2003) used 2 % and 10 % of cypermethrin LC50 for studying the insecticide effect on the Rohu *Labeo rohita*. Borges *et al.* (2007) applied 30 % and 45 % of cypermethrin LC50 to the jundiá, *Rhamdia quelen*. In *Solea senegalensis*, and due to the absence of information about the effect of these pesticides, lower doses were chosen from both cypermethrin and permethrin, almost mediating the previously mentioned concentrations: 10 % for the highest dose and 4 % for the lowest one.

Experimental Procedure

Juvenile soles (n=30) were divided into five groups in duplicates, each comprising a minimum of three fish, and placed in 30 L tanks (50 cm*40 cm*30 cm) under low stocking density condition (1.4 kg m^{-2}). The following experimental conditions were assessed: i) control receiving a dose of ethanol equal to which the maximum dose of pyrethroids was dissolved in, ii) exposure to 1/10 value of cypermethrin LC50 (50 µg L^{-1}) (in ethanol), iii) exposure to 1/25 value of cypermethrin LC50 (20 µg L^{-1}), iv) exposure to 1/10 value of permethrin LC50 (90 µg L^{-1}), and v) exposure to 1/25 value of permethrin LC50 (36 µg L^{-1}). Feeding proceeded as 1 % of fish weight during the whole time of the experiment and specimens did not receive any food 24 h before sampling. Specimens were maintained in a closed system under constant temperature (19 ºC) and natural photoperiod (November 2010). According to Willis and Ling (2004), at least 58 % of cypermethrin remains in sea water after 48 h of the initial exposure. A daily replenishment of tank water with 50 % of initial cypermethrin dose does not raise the concentration significantly. Therefore, 50 % of tank water was changed every 2 days. For permethrin, and according to Toynton *et al.* (2009) stating that 50 % of permethrin in water degrades within 19-27 hours, 50 % of permethrin-containing tank water was changed daily and 75 % of the initial pesticide dose was added with the new water. Water quality criteria were checked periodically to affirm their stability.

Tissue Sampling

At the end of experimental time (10 days), fish were netted, anesthetized (2 mL L^{-1} of 2-phenoxyethanol, Sigma), weighed and sampled. All experimental procedures complied with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals. Branchial and hepatic biopsies of each specimen preserved in 5-10x volumes (w/v) of RNAlater® (Ambion, LifeTechnologies), kept overnight at 4 ºC and then transferred to -20 ºC until further analysis.

Total RNA Extraction and QPCR

Total liver and gill RNA was extracted with the NucleoSpin® RNA II kit (Macherey-Nagel) from biopsies weighing 10-15 mg each, that were individually homogenized by an IKA® Ultra-Turrax® T25 with the dispersing tool S25N-8G (IKA-Werke). On-column DNA digestion was carried out using RNase-free DNase that is provided with the kit. RNA quality was checked in the Bioanalyzer 2100 system (Agilent Technologies, Life Sciences), using Agilent RNA 6000 Nano kit (Agilent Technologies, Life Sciences). RNA quantity was measured spectrophotometrically at 260 nm with a BioPhotometer Plus (Eppendorf).

cDNA synthesis was carried out with the qScript™ cDNA Synthesis Kit (Quanta BioSciences). 500 ng of total RNA were reverse transcribed. The cDNAs were stored at -20 ºC until running the quantitative reverse transcription polymerase chain reaction (QPCR) analyses for a period of time that never exceeded a month. For studying expression of target genes the QPCR mix PerfeCTa™ SYBR®Green FastMix™ (Quanta BioSciences) was used. Both target and reference genes were run in duplicate, adjusting the final reaction volume in each to 10 µL. Reaction mixes were applied to twin.tec 96 real-time PCR plates (Eppendorf) capped with Masterclear adhesive real-time PCR-film (Eppendorf). The PCRs were processed using the thermal cycler Mastercycler®ep Realplex^2 (Eppendorf) operated with Realplex 2.2 software (Eppendorf). An automated threshold and walking baseline were used for determining the C_T values. QPCR primers utilized –shown in Table 1– were designed using the software primer3 (http://frodo.wi.mit.edu/primer3/) based on the full cDNA sequences published in GenBank (http://www.ncbi.nlm.nih.gov/nuccore) for Senegalese sole: GAPDH-1 (acc. no.: AB300322.1), GAPDH-2 (acc. no.: AB291587.2), and β-actin (acc. no.: DQ485686.1). All QPCR primers were purified by HPLC and purchased from biomers.net (Germany). Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). β-actin (acc. no.: DQ485686.1) was used as the internal reference gene for normalizing mRNA expression data, owing its low C_T variability in agreement to the results reported before for *Solea senegalensis* (Wunderink et al., 2010;2012) and what we found during the QPCR runs (not exceeding 0.5 C_T differences among different treatments). Optimization of QPCR conditions was made on primers annealing temperature (50 to 60 ºC), primers concentration (100 nM, 200 nM and 400 nM) and template concentration. The best final primer concentration was found to be 200 nM for all tested genes. In all cases, the PCR program consisted of 10 minutes at 95 ºC for initial denaturation and polymerase activation, 95 ºC for 15 seconds for denaturation, and 60 ºC for 30 s for both annealing and extension.

PCRs were accomplished through 40 cycles. For each gene tested, a melting curve was used to ensure that a single product was amplified and to check for the absence of primer-dimer artifacts and possible genomic contamination. Each primer pair was considered usable when single peak appears in the melting curve, referring to a single amplification product. Amplicon sizes, amplification efficiencies and regression coefficient (r^2) for the different primer pairs used for analyzing the expression patterns of all genes studied are shown in Table 1.

Table. 1: Sequence, Positions, Amplicon Sizes, Amplification Efficiencies and Regression Coefficients of Primers Used for *QPCR* Expression Analysis.

Primer	Orientation	Sequence 5'→3'	Position	Acc. no.	Amplicon Size (bp)	Organ	Amplification Efficiency	r^2
SS αNKA	Sense	TAC GAT GAA GCC AGA AGA TA	3117	JX508624.1	100	Gills	0.998	0.99
	Antisense	GCT CCC TCC GTG ATT GTG	3217					
SS βNKA	Sense	TCT TCT ATC TCG TTT TCT ATGG CTG	337	JX508625.1	129	Gills	0.998	0.99
	Antisense	TTC ACG ATC CTG TTG AGT TTC AC	465					
GAPDH-1	Sense	CAT GAG AGG GAC CCT GCC AAC AT	284	AB300322.1	169	Gill	0.99	0.996
	Antisense	CCA TAA CAA ACA TGG GAG CA	452			Liver	0.97	1.01
GAPDH-2	Sense	TAT GAA GCC ATC CGA GAT CC	274	AB291587.2	166	Gill	0.86	0.999
	Antisense	TCC CAT GAC AAA CAT TGG TG	439			Liver	0.8	0.999
β-actin	Sense	CAG CCA TCT TTC CTC GGT AT	861	DQ485686.1	131	Gill	0.99	1
	Antisense	TAC ATG GTG GTT CCT CCA GA	991			Liver	0.99	1

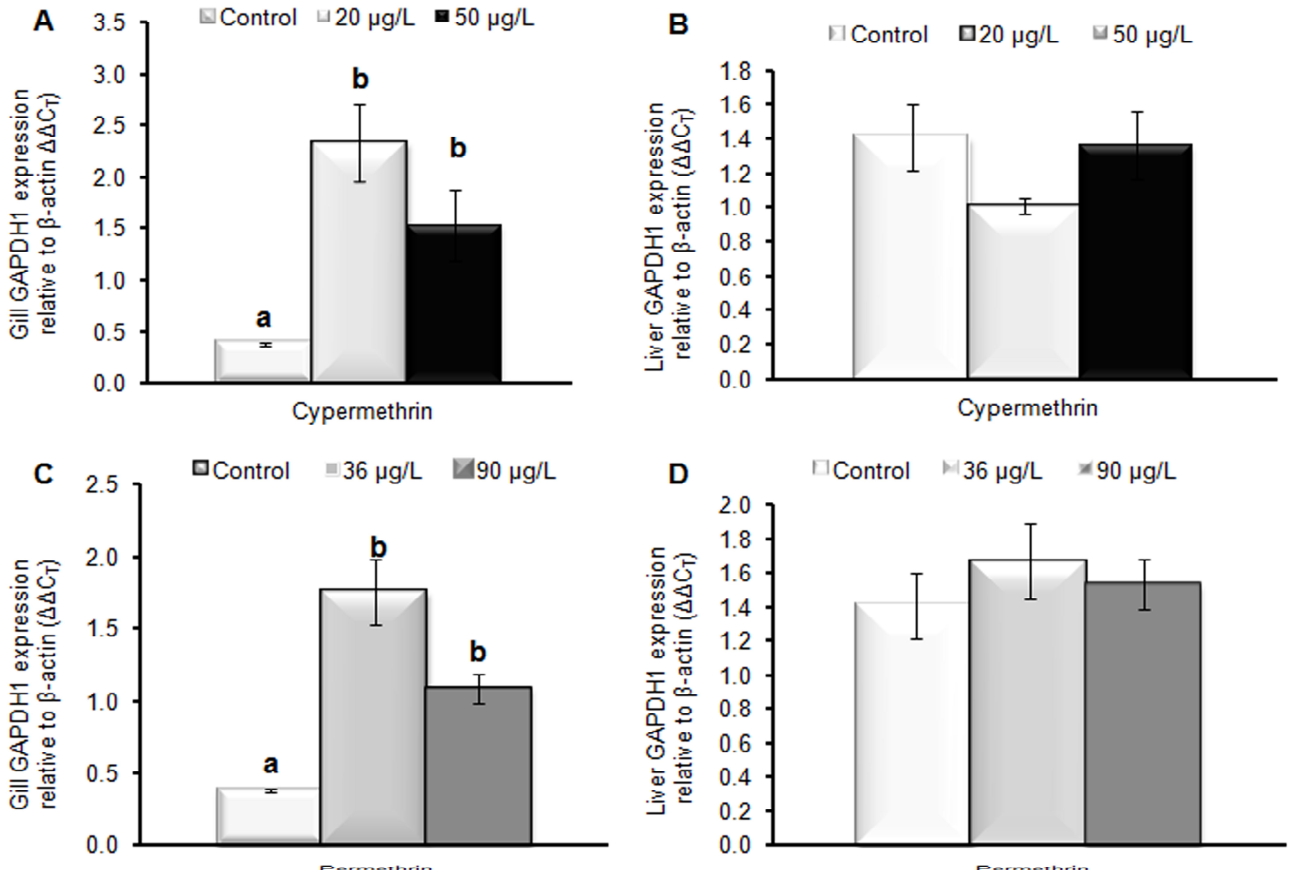

Fig. 1: Expression patterns for GAPDH-1 in gills (A,C) and liver (B,D) of *Solea senegalensis* juveniles exposed to different cypermethrin (A,B) and permethrin (C,D) doses during 10 days. Data (n = 6) are represented as mean ± SEM. Different letters indicated statistically significant differences between experimental groups (P<0.05).

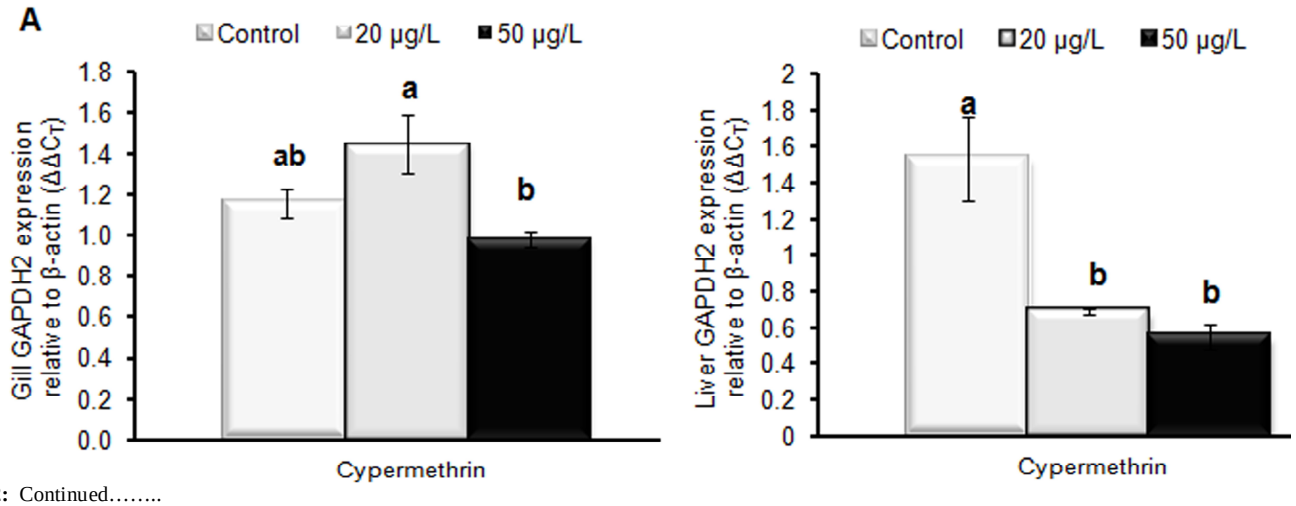

Fig. 2: Continued……..

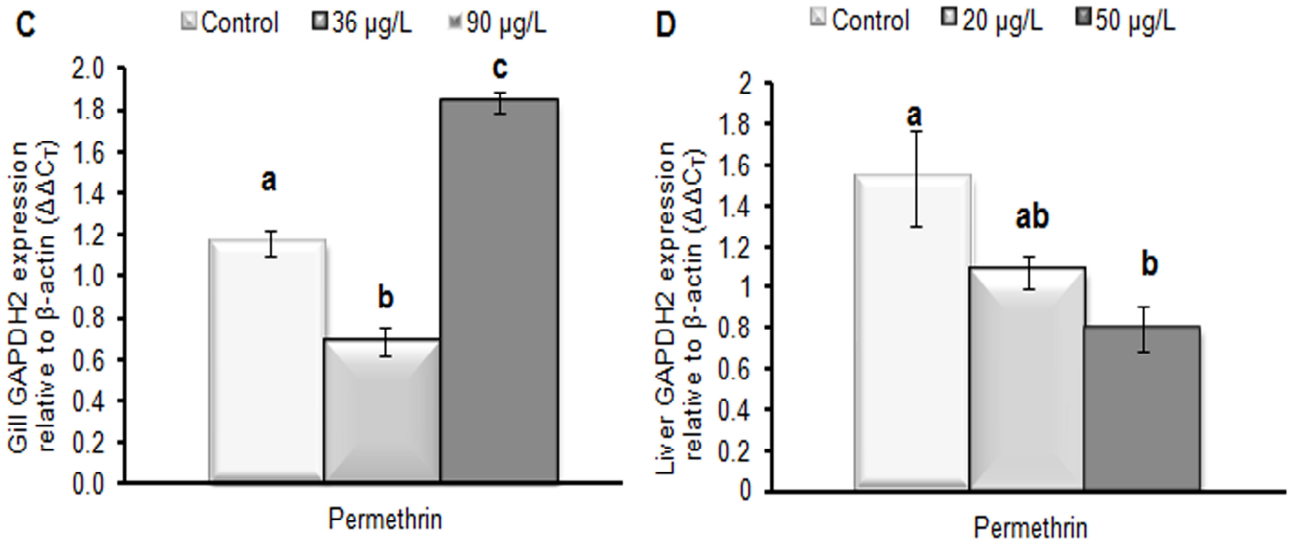

Fig. 2: Expression patterns for GAPDH-2 in gills (A,C) and liver (B,D) of *Solea senegalensis* juveniles exposed to different cypermethrin (A,B) and permethrin (C,D) doses during 10 days. Data (n = 6) are represented as mean ± SEM. Different letters indicated statistically significant differences between experimental groups (P<0.05).

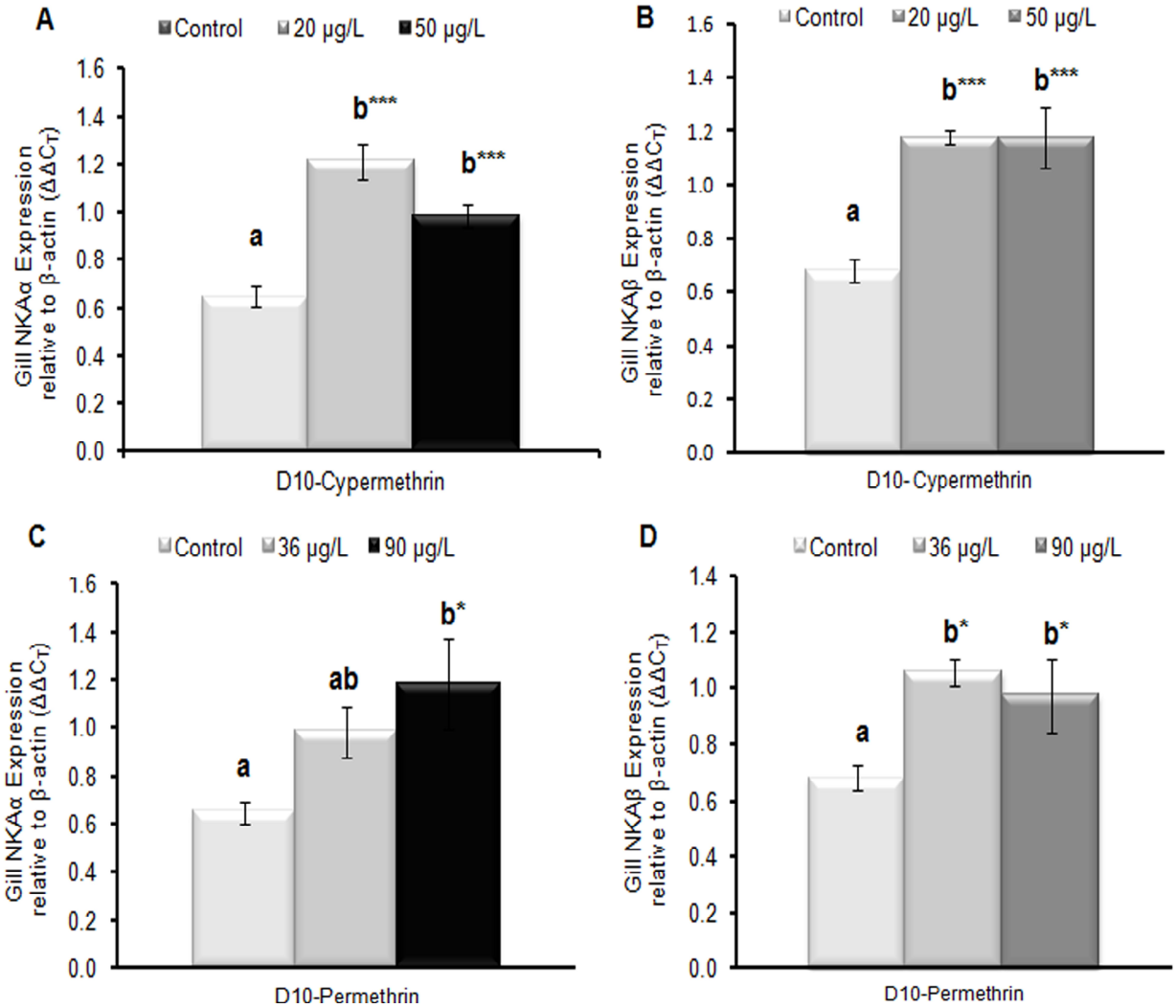

Fig. 3: mRNA expression patterns for Na^+,K^+-ATPase subunits α (A,C) and β (B,D) in gills of *S. senegalensis* juveniles exposed to different cypermethrin (A,B) and permethrin (C,D) concentrations. Data (n = 6) are represented as mean ± SEM. Significant difference were considered at level of significance P<0.05.

Statistics

Statistical analyses were performed using one way analysis of variance (ANOVA) and Tukey-HSD Post-Hoc test. Significant values were considered when $P<0.05$.

RESULTS

Cypermethrin and Permethrin LC50

The 24 h LC50 of cypermethrin for *Solea senegalensis* was 500 µg L^{-1}, while the 72 h LC50 of permethrin was 900 µg L^{-1}. Based on this, and according to previous experiences with other species (see above), 10 % (50 µg L^{-1} for cypermethrin and 90 µg L^{-1} for permethrin) and 4 %, low dose, (20 µg L^{-1} for cypermethrin and 36 µg L^{-1} for permethrin) of LC50 of both pyrethroids were taken as sublethal test doses.

Glyceraldehyde-3-phosphate dehydrogenase-1 (GAPDH-1) expression

Gill GAPDH-1 expression levels showed a similar pattern of change to cypermethrin and permethrin exposure (Figures 1A and 1C). Both doses of the two pesticides induced a significant increase in the GAPDH-1 expression, although that increase seemed to be higher in response to the lower doses than to the higher doses at this experimental time. At hepatic level GAPDH-1 expression did not show any significant changes at all tested doses of both pyrethroids (Figures 1B and 1D).

Glyceraldehyde-3-Phosphate Dehydrogenase-2 (GAPDH-2) Expression

Cypermethrin did not induce important changes in gill GAPDH-2 expression levels, with higher and lower doses increased and decreased the transcription to both extremities of the control levels (Table 3, Figure 2A). However, it seems that this response to permethrin in gills is dose-dependent, since the lowest dose (36 µg L^{-1}) significantly down regulated GAPDH-2 expression but the highest dose (90 µg L^{-1}) significantly up-regulated it (Table 3, Figure 2C). At hepatic level, cypermethrin significantly down regulated GAPDH-2 expression at both doses (Figure 2B). However, only exposure to the highest permethrin dose could decrease GAPDH-2 expression significantly (Figure 2D).

Proton Pump (Na^+,K^+-ATPase) Subunits α and β

Both proton pump subunits (α and β) exhibited similar expression patterns in gills in response to both tested pyrethroids and their doses (Figure 3 A-D). Cypermethrin significantly up-regulated both α and β subunits with its both doses. However, only the high dose of permethrin could enhance both α and β subunits, meanwhile the low dose induced a significant increase in the β subunit but a non-significant enhancement for the α subunit.

DISCUSSION

The negative effects of pyrethroids on fish physiology are well documented, as recently reviewed by Ali *et al.* (2011) and Maund *et al.* (2012). Their high toxicity and long persistence in the sediment were the causes for massive eel deaths reported before in Europe (Bálint et al., 1997). Cypermethrin is a potent type II pyrethroid pesticide that produces long delay in sodium channel inactivation leading to persistent depolarization of the nerve membrane (Enstey, 2007), presenting an adverse effect, as a potent neurotoxin, on several teleost species (Kumar et al., 2009; Marigoudar et al., 2009; Shashikumar and Rajini, 2010; Suvetha et al., 2010; Shi et al., 2011). Moreover, permethrin, a type I pyrethroid pesticide whose neurotoxicity is induced through repetitive neuronal discharge and prolonged negative after potential (Enstey, 2007), presents high toxicity for aquatic organisms. For example, it increased the time for hatching of grass shrimp *Palaemonetes pugio* embryos, decreased the swimming behavior of larvae, and increased their lipid peroxidation levels (DeLorenzo et al., 2006).

The oxidative stress induced by pyrethroids (e.g. cypermethrin and permethrin) generates free radicals that enhance lipid peroxidation, and may be the underlying molecular mechanism for pesticide-induced toxicity. These free radicals activate several protective enzymatic and non-enzymatic antioxidants that are known to be sensitive indicators of increased oxidative stress (Valavanidis et al., 2006).

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH-1 And -2) Expression

Our results showed a differential pattern of change in GAPDHs expression depending on the transcript assessed (GAPDH-1 *versus* GAPDH-2), pyrethroids used (cypermethrin *versus* permethrin), and tissue analyzed (gill *versus* liver). This differential expression between both GAPDH isoforms has been also previously reported in teleosts and it is believed to be tissue-specific (Cho et al., 2008).

GAPDH is a classical glycolytic enzyme that was previously considered as house-keeping gene due to its minimum expression changes under different experimental conditions, even in the Senegalese sole (ex. Manchado et al., 2007; Iziga et al., 2007). However, several studies that it is a multifunctional protein with significant activity in a number of fundamental cell pathways. As a result of multiple isoforms and cellular locales, GAPDH is able to come in contact with a variety of small molecules, proteins, membranes, etc. Growing number of studies concerning this protein could detect a prominent response or role of the GAPDH to oxidative stress. For instance, oxidative stress enhanced aggregation GAPDH cells, mediating thus cell death (Nakajima et al., 2009). Furthermore, GAPDH enhancement due to nuclear translocation may be associated with the reactivation of oxidized proteins involved in DNA repair (Schlisser et al., 2010). Even the apoptotic cells enhanced GAPDH expression to more than three times in comparison to normal cells (Dastoor and Dreyer, 2001). GAPDH-1 normally presents a metabolic role, enhancing glycolysis for a local production of energy. In our experiment, the absence of changes in hepatic GAPDH-1 expression could indicate that this enzyme is not involved in the metabolic activation due to

pyrethroids compounds. However, other explanation could arise. Our metabolic data suggested a hepatic metabolism activation with depletion of glycogen stores in *Solea senegalensis* specimens chronically (10 days) exposed to both pyrethroids. At this time lipid and/or amino acid metabolism are more important at hepatic level (data are not shown) and GAPDH-1 activation is not necessary. On the other hand, up-regulation of gill GAPDH expression may refer to a locally enhanced glycolysis for metabolic energy production in order to supply action of detoxifying and osmoregulatory enzymes (Tian et al., 2011), that could be related to the increased production of mRNAs of Na^+,K^+-ATPase enzyme subunits we found. The other ¨apoptotic¨ role of GAPDH-I (see Ishitani et al., 2003) can be abolished due to the concomittent up-regulation of both Na^+,K^+-ATPase subunits presented in gills. Therefore, the role of GAPDH-1 in gills under the oxidative stress should be considered.

Gill GAPDH-2 expression enhanced with the high dose of permethrin significantly, which may further contribute to our ¨local-glycolytic¨ hypothesis. In addition, the decreased hepatic GAPDH-2 expression in response to both doses of cypermethrin and permethrin may suggest either: i) absence of a real role for GAPDH-2 in response to oxidative stress, or ii) a depletion of the enzyme in response to earlier activation in both cases. However, the very scarce data about GAPDH-2 transcription and its relation with oxidative stress processes in fish did not support any of these hypotheses. Therefore, more research is needed to clarify the roles of GAPDH-1 and -2 in different organs of a highly pesticide-resistant fish, like *Solea senegalensis*.

Na^+, K^+-ATPase Subunits α and β

Na^+,K^+-ATPase (NKA) pumps three sodium ions out of the cell while pumping in two potassium ions, making the inside of the chloride cell highly negative and low in sodium. The sodium gradient is then used by the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) to bring chloride into the cell. Chloride subsequently leaves the cells on a favorable electrical gradient through an apical chloride channel homologous of the cystic fibrosis transmembrane conductance regulator (CFTR). The three major ion transport proteins involved in salt secretion, NKA, NKCC and CFTR, have all been co-localized to chloride cells in several species of teleost fishes (McCormick et al., 2003). Na^+,K^+-ATPase is believed to be an accurate and sensitive non-specific biomarker for exposure to different ecotoxins (Suvetha et al., 2010). Pyretrhoid insecticides are known to impact Na^+,K^+-ATPase activities. However, the effect of pyrethroids on Na^+,K^+-ATPase seems to be more species-specific. In the current experiment, the tested doses of both pyrethroids could enhance gill Na^+,K^+-ATPase expression significantly. In *Clarias batrachus*, low doses of deltamethrin enhanced gill Na^+,K^+-ATPase activities than the higher doses (Kumar, 2012). Sublethal concentrations of cypermethrin also increased gill Na^+,K^+-ATPase activity in *Cirrhinus mrigala* (Prashanth and David, 2010). On the other hand, Na^+,K^+-ATPase decreased in *Cyprinus carpio* upon exposure to cypermethrin and fenvalerate, both are of the type II of pyrethroids, and in *Clarias batrachus* upon exposure to cypermethrin (Reddy et al., 1991; Begum, 2009; Suvetha et al., 2010). Sublethal doses of cypermethrin also decreased gill Na^+,K^+-ATPase activity in the Indian major carp *Catla catla* (Vani et al., 2011; 2012). Deltamethrin, a type II pyrethroids, inhibited gill in *Ancistrus multispinis* (Assis et al., 2009). λcyhalothrin, a type II pyrethroids, declined the activity of liver-bound NKA but elevated lipid peroxidation in liver of the tilapia *Oreochromis mossambicus* (Parthasarathy and Joseph, 2011).

CONCLUSIONS

In conclusion, our results indicated that the studied pyrethroid insecticides, cypermethrin and permethrin, could induce some sort of oxidative stress in *Solea senegalensis*. The activation of different enzyme systems synthesis may be indicative of the strength of that oxidative stress. Gills seem to play a comparatively stronger role in the detoxification of these insecticides than liver, at least after 10 days of exposure to pyrethroids, possibly due to the more active synthesis of the studied enzyme genes there. Therefore, gills should receive more attention as a strong marker organ, especially in a resistant fish like *Solea senegalensis*.

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