Universidade de Aveiro

Departamento de Biologia

Ano 2010

Cristiana Andreia Valente Oliveira

Behavioural alterations and biomarker responses of *Palaemon serratus* exposed to pesticides

Alterações comportamentais e respostas de biomarcadores em *Palaemon serratus* exposto a pesticidas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, Ramo de Toxicologia e Ecotoxicologia cuja dissertação foi realizada sob a orientação científica do Doutor Carlos Gravato, Investigador Auxiliar do Laboratório de Ecotoxicologia do Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) da Universidade do Porto, e co-orientação do Professor Doutor Amadeu Soares, Professor Catedrático do Departamento de Biologia e Director Adjunto do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

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palavras-chave

Palaemon serratus, Fenitrotião, Deltametrina, Comportamento, Biomarcadores, Velocidade natatória, Evitamento

resumo

O principal objectivo deste estudo foi investigar os efeitos agúdos do fenitrotião, um pesticida organofosforado, e da deltametrina, um pesticida piretróide, no camarão branco legítimo, *Palaemon serratus*, utilizando parâmetros a diferentes níveis de organização biológica. O estado antioxidante do fígado, o metabolismo energético e a neurotransmissão foram utilizados para avaliar os efeitos do fenitrotião e da deltametrina ao nível sub-individual. A velocidade de natação foi utilizada para avaliar os efeitos de ambos os pesticidas ao nível individual. No caso dos ensaios realizados com o fenitrotião, foi, ainda, realizado um teste de evitamento, para determinar a capacidade do camarão legítimo escapar do pesticida. Neste contexto, também se pretendia desenvolver testes de comportamento com este organismo marinho e perceber se estas alterações comportamentais são sensíveis e se podem ser usadas como um indicador de efeitos em bioensaios ecotoxicológicos. Os resultados demonstraram que o pesticida deltametrina foi mais tóxico para o camarão legítimo do que o fenitrotião, uma vez que o valor de CL_{50} estimado foi 20 vezes inferior ao do fenitrotião. Verificou-se que os dois pesticidas induzidem efeitos opostos nas actividades de AChE no olho e de ChE no musculo e que apenas o pesticida deltametrina induziu stress oxidativo na glândula digestiva do camarão legítimo. Contudo, a velocidade de natação foi reduzida tanto no camarão legítimo exposto a fenitrotião como no exposto a deltametrina, provando que este comportamento pode e deve ser usado como critério de efeito em bioensaios ecotoxicológicos. Para além disso, a velocidade de natação foi tão sensível como a actividade da AchE no olho do camarão legítimo, no caso da exposição ao pesticida deltametrina. No presente trabalho também foi observado que o camarão legítimo consegue evitar baixas concentrações do pesticida fenitrotião. Porém o camarão legítimo perde esta capacidade para escapar quando é exposto a concentrações mais elevadas do fenitrotião, o que poder ser um problema para o teste. Apesar disso, este trabalho mostra que o comportamento do camarão legítimo pode ser usado como critério em bioensaios, uma vez que ambos os pesticidas induzem efeitos a este nível.

keywords

Palaemon serratus, Fenitrothion, Deltamethrin, Behaviour, Biomarkers, Swimming velocity, Avoidance;

abstract

The main purpose of this study was to investigate the acute effects of fenitrothion, an organophosphate pesticide, and deltamethrin, a pyrethroid pesticide, in the common prawn, *Palaemon serratus,* using parameters at different levels of biological organization. Liver antioxidant status, energetic metabolism and neurotransmission were used to assess the effects of fenitrothion and deltamethrin at sub-individual level. Swimming velocity was used to assess the effects of both pesticides at the individual level. To assess the effects of fenitrothion, it was also developed and avoidance behaviour test, to determine the escaping capability of prawns. In this context it was aimed to develop behavioural tests with this marine organism and to understand if those behavioral alterations are sensitive and can be used as an endpoint in ecotoxicology bioassays.

Results showed that the pesticide deltamethrin was more toxic to exposed prawn than fenitrothion, since the LC_{50} value estimated was 20 times lower than the value for fenitrothion. It was also observed that the two pesticides induced opposite effects in the activities of eye AChE and muscle ChE and that only deltamethrin induced oxidative stress in the digestive gland of the common prawn. However, swimming velocity was impaired for prawns exposed to both pesticides, proving that this behaviour can and should be used as effect criteria in bioassays. In addition swimming velocity was as sensitive as eye AChE activity in the case of deltamethrin exposure.

In the present work it was also observed that prawn can avoid low concentrations of fenitrothion. However, common prawn lost this ability to avoid the toxic when it was exposed to high concentrations of fenitrothion, which may be a problem for the avoidance test itself. Despite that, the present work shows that the common prawn behaviour can be used as effect criteria in bioassays, since both pesticides induce effects at this level of biological organization.

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Chapter I

General Introduction

1. Aquatic Contamination

The decline in the quality of the environment is a serious problem for humans, constituting a threat to all living beings. In the 20th century, many thousands of organic trace pollutants have been produced and, in part, released into the environment, and the exposure, fate and effects of chemical contaminants or pollutants in the aquatic ecosystem have been extensively studied by environmental toxicologists (van der Oost *et al.*, 2003).

The aquatic ecosystems are the main recipients of contaminants from industries and agriculture, since they are the target of direct discharges, atmospheric deposition and soil leaching. Most chemical substances either directly or indirectly reach saltwater ecosystems. With saltwater covering over 70% of the earth's surface and the vast diversity of plant and animal life in the estuaries and open sea, toxicological evaluations of effects of chemicals on salt water organisms are essential to humankind (Ward, 1995).

2. Pesticides

Dependence on pesticides for pest control has been increasing since the onset of the green revolution in agriculture (Matson *et al.*, 1997). Pesticides may be introduced into natural aquatic systems by various means: incidentally during manufacture, during their application (i.e., through aerial spray drift), and through surface water runoff from agriculture land after application. In addition, some pesticides are deliberately introduced into aquatic systems to kill undesirable pests such as weeds, algae, and vectors of human disease. In many countries, the significance of pesticides as aquatic contaminants has led to strict regulatory controls on their manufacture, transport, use, and disposal.

Pesticides are a diverse group of widely varying chemical structures ranging from simple inorganic substances to complex organic molecules. Of the later, some are natural derivatives of plants (e.g., pyrethrins) and others are synthetic derivatives of natural products or completely synthetic substances produced in chemical manufacturing facilities.

Unlike most toxic agents, pesticides (insecticides, herbicides, and fungicides) have been selected and synthesized for their biocidal properties and are applied to kill or control organisms. Thus, they are all toxic to some forms of life (Rand *et al.*, 1995). A number of generalizations can be made about pesticides. First, effective pesticides are designed to be selective in their effects; they are extremely toxic to some forms of life and relatively harmless to others. Few are absolutely specific to their target organisms, so other related and unrelated species may be affected. Second, the mode of application of pesticides varies according to the circumstance. Third, in stagnant lentic (i.e., nonflowing) aquatic systems

draining agricultural areas, certain pesticides are more likely to be present at low but persistent concentrations. Such pesticides may be resistant to abiotic and biotic degradation and cause sublethal effects in a wide range of species, including wildlife.

As a result of its ubiquitous use in the environment and the possible exposure of aquatic organisms and consequent effects, as well as regulatory requirements for least hazardous formulations, the literature on pesticides is enormous (Bálint *et al.*, 1995; Escartin and Porte, 1996; Choi *et al.*, 2001, 2002; Frasco *et al.,* 2006; Badiou *et al*., 2008; Sucahyo *et al.*, 2008; Tuzmen *et al.,* 2008; Dorts *et al*., 2009; Elhalwagy and Zaki, 2009; Nørum *et al*., 2010).

Many of the public concerns about pesticides are related to "older" chemicals, these having entered the market in the 1950s and 1960s without the benefit of the extensive toxicity and environmental impact studies demanded prior to the registration of chemicals today.

It must also be pointed out that many of these older pesticides have received little reassessment using the more definitive techniques and protocols required today. Although government agencies and industry have been slow in their reevaluation of a vast array of pesticides in use, reassessment often comes in the wake of or concomitant with some recently disclosed adverse environmental or health effects.

The modern era of chemical pest control began around the time of World War II, when the synthetic organic chemical industry began to develop. The first synthetic organic pesticides were organochlorine compounds, such as dichlorodiphenyltrichloroethane (DDT). Other classes of insecticides include the organophosphorus, carbamates, pyrethroids, and biopesticides.

2.1. Organophosphate compounds

The use of organophosphorus pesticides has increased markedly during the past two decades. The organophosphorus pesticides have the following structure (Figure 1):

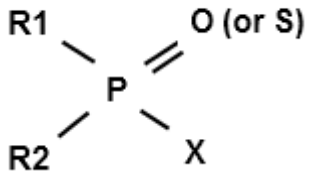

Figure 1- Structure of general organophosphorus

Where the R-groups may be alkoxy, alkyl or amide; the X-group is generally a caboxylating, cyanide, thiocyanate, phosphate, halide, phenoxy or thiophenoxy group (Smith, 1992).

Organophosphorus pesticides act inhibiting important enzymes of the nervous system which play a vital role in the transmission of nerve impulses. Nerve impulses usually travel along neurons (nerve cells) by way of electrical signals. However, at a junction between two neurons (a synapse) and between a neuron and a muscle (neuromuscular junction) the impulse is transmitted in the form of a chemical substance (neurotransmitter). The excitatory neurotransmitter operating in the autonomic nervous system, neuromuscular junctions and parts of the central nervous system is acetylcholine which is released by cholinergic neurons. It is broken down and inactivated in milliseconds by the enzyme acetylcholinesterase. After exposure to organophosphate compounds, the enzyme is unable to function and a build-up of acetylcholine occurs, which causes interference with nerve impulse transmission at nerve endings (Smith, 1992). The target sites of organophosphorus pesticides and/or mechanism(s) of action may be similar in all species: only the dosage (level of exposure and duration) will dictate the intensity of biological effects (Ecobichon, 2001).

2.1.1. Fenitrothion

Fenitrothion is an organophosphorus insecticide that has been in use since 1959, and is highly toxic for aquatic invertebrates in both freshwater and seawater. It has the chemical formula $C_9H_{12}NO_5PS$ and the following structure (Figure 2):

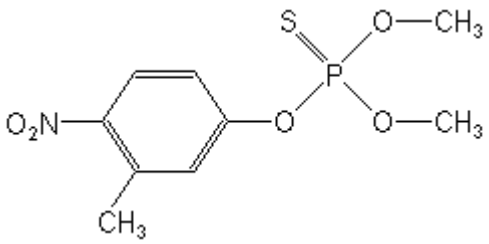

Figure 2- Structure of Fenitrothion

Fenitrothion is mainly used in agriculture for controlling chewing and sucking insects on rice, cereals, fruits, vegetables, stored grains, cotton, and in forest areas. It is also used for the control of flies, mosquitos, and cockroaches in public health programmes and/or indoor use.
Fenitrothion residues are converted in the animal body by oxidative biotransformation to the toxic metabolite, fenitrooxon, which inhibits acetylcholinesterase (AChE) by mimicking the neurotransmitter acetylcholine. This inhibition may lead to paralysis and death of non-target organisms such as crustaceans (Bimerlin *et al.*, 1998).

2.2. Pyrethroids

Pyrethroids are a class of neurotoxic pesticides which their use has continuously increased during the last two decades (Amweg *et al.*, 2005; Oros and Werener, 2005; Meacham *et al.,* 2008; Wolansky and Harrill, 2008).

Synthetic pyrethroids insecticides were derived from pyrethrins, the six insecticidal constituents of extracts from *Chrysanthemum cinerariaefolium* flowers (Figure 3) (Casida, 1980).

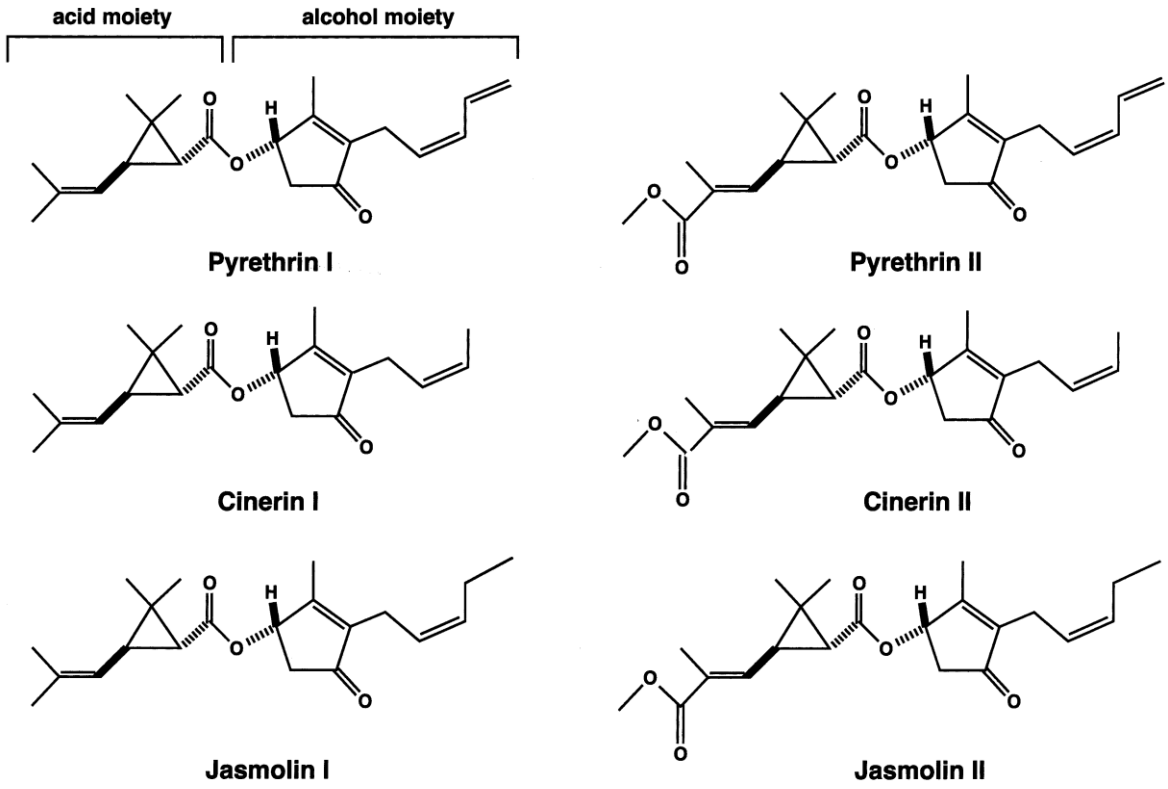

Figure 3- Structures of the six natural pyrethrins. Adapted from Soderlund *et al.* (2002)

Pyrethrins are rapidly degraded by light, and so synthetic analogues, pyrethroids, were developed to improve stability (Anadón *et al.*, 2009). Figure 4 outlines the development of pyrethroid insecticides from natural pyrethrins (e.g. pyrethrin I) and early synthetic derivatives (pyrethroid generations I–II) to modern compounds with improved photostability (III–V). Each successive generation represents an increase in insecticidal activity and mammalian toxicity (Wolansky and Harrill, 2008).

All pyrethroids contain several common features: an acid moiety, a central ester bound, and an alcohol moiety (Figure 3). In addition, this group of insecticides can be separate into two classes based on the presence or absence of a cyano group and the presence of an α-cyano

group on the alcohol confers increased potency in target and non-target species (Wolansky and Harrill, 2008). Type I compounds lack the α-cyano group while type II compounds contain an α-cyano group (Nasuti *et al.*, 2003; Wolonsky and Harril, 2008) (Figure 4) and this structural difference corresponds to different clinical signs of intoxication (Meacham *et al.*, 2008).

In rats, type I pyrethroids induce a tremor (T) syndrome, while type II pyrethroids induce choreoathetosis (choreiform movements of the forelimbs and trunk with tonic seizures (Anadón, *et al.*, 2009)) and salivation (CS) syndrome (Verschoyle and Aldridge, 1980; Soderlund *et al.*, 2002; Nasuti *et al.*, 2003; Meacham *et al.*, 2008; Anadón *et al.*, 2009).

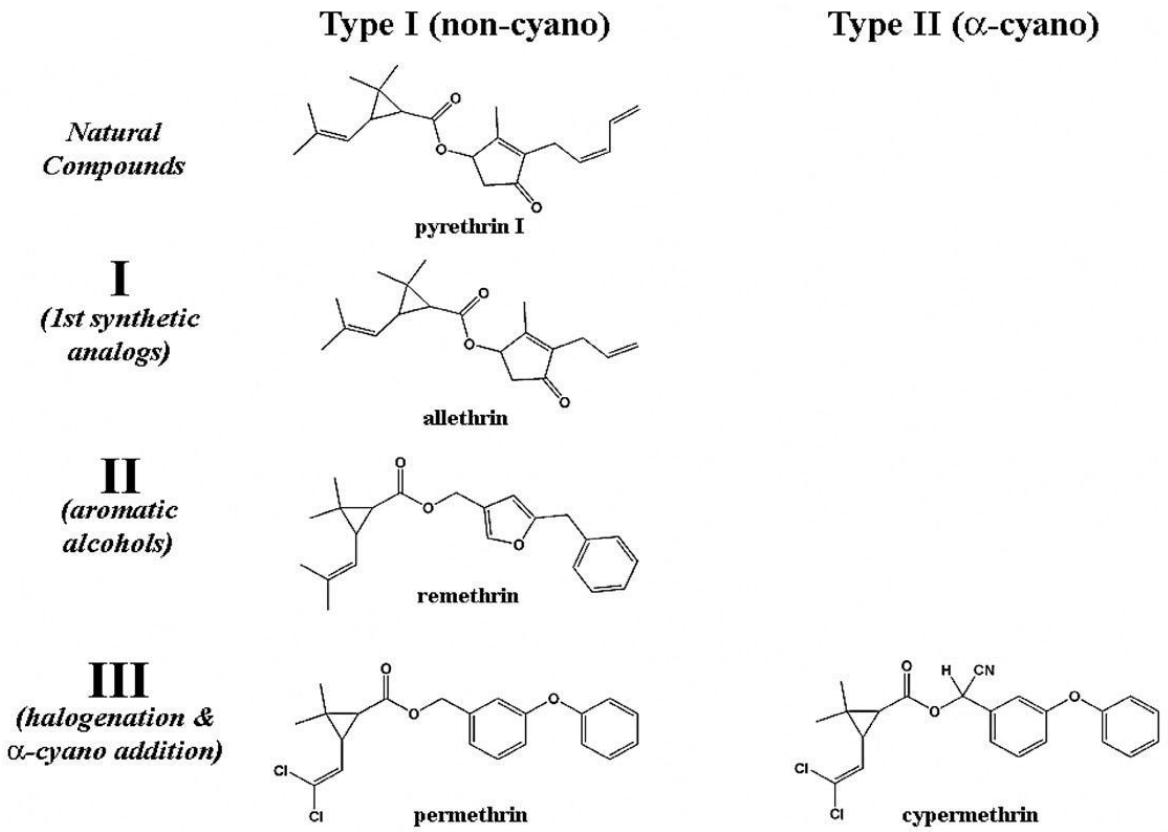

Figure 4- Structures of some representative pyrethroids. Adapted from Wolansky and Harrill (2008).

Despite some differences in the symptoms of poisoning, the primary target for both types of pyrethroids is the nervous system, especially the sodium channels (Narahashi, 1971, 2000; Nasuti *et al.*, 2003; Badiou *et al.*, 2008; Meacham *et al.*, 2008; Wolansky and Harrill, 2008; Soderlund, 2009). Pyrethroids prolong the period that the sodium channels are in the open state, i.e., pyrethroids seem to stabilize gating particles of the sodium channel, resulting in

slowing of the movements of both the activation and inactivation gates, and changing the voltage dependence of the gates in the hyperpolarizing direction and these changes cause a prolonged flow of sodium current into the cell, leading to a sustained membrane depolarization (Nasuti *et al.*, 2003). The result is that sodium channels open at more hyperpolarized potentials (i.e., after smaller depolarizing changes in membrane potential) and are held open longer, allowing more sodium ions to cross and depolarize the neuronal membrane (Shafer *et al.*, 2005). Opening and closing should normally occur in less than a millisecond when an impulse passes, however, when poisoned by a pyrethroid, the closing is delayed and sodium leaks out when the channel should be closed (Jørgen Stenersen, 2004). This interaction with the sodium channel has the effect of slowing the activation and inactivation properties of the sodium channel, resulting in a stable hyper excitable state in all excitable tissues (Lautraite and Sargent, 2009).

In general, type II compounds delay the inactivation of voltage-sensitive sodium channels substantially longer than do type I compounds. Type I compounds prolong channel opening only long enough to cause repetitive firing of action potentials (repetitive discharge), whereas type II compounds hold open the channels for such long periods of time that the membrane potential ultimately becomes depolarized to the point at which generation of action potentials is not possible. These differences in prolongation of channel open times are hypothesized to contribute to the differences in the CS and T syndromes after exposure to type II and I pyrethroids, respectively (Shafer *et al.*, 2005).

2.2.1. Deltamethrin

Deltamethrin is a synthetic type II pyrethroid, which has a wide range of application in industrial and agricultural purposes. This insecticide is one of the most toxic pyrethroids (Sánchez-Fortún and Barahona, 2005) and is used extensively for the control of invertebrate pests; in addition, deltamethrin is acutely toxic to all groups of aquatic organisms (Thomas *et al.*, 2008).

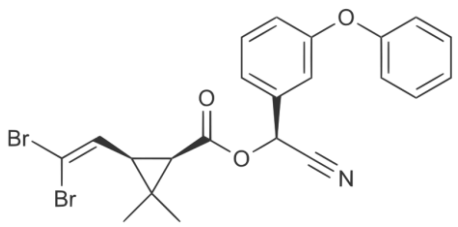

Figure 5- Structure of Deltamethrin

Deltamethrin was developed in 1974 and was the first pyrethroid containing the alphacyano-3 phenoxybenzyl moiety (Elliot *et al.*, 1974). It has the chemical formula $C_{22}H_{19}Br_2NO_3$ and the structure that can be observed in figure 5.

3. Biological responses to contaminants

Analysis of chemical substances in tissues and body fluids, toxic metabolites, enzymes activities and other biochemical variables have frequently been used in assessing the toxin interaction with biological systems (Torre *et al.*, 2007).

Environmental risk assessment (ERA) is defined as the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi, 1994).

The risk assessment process can be divided into a scientifically oriented risk analysis and a more politically oriented risk management. Risk analysis is a process, which comprises some or all of the following elements: hazard identification, effect assessment, exposure assessment and risk characterization. Environmental risk management deals with regulatory measures based on risk assessment. Risk management and risk analysis, are closely related but different processes: in risk analysis the risk of a certain situation is determined, whereas risk management examines solutions to the problem (Van Leeuwen and Hermens, 1995).

In the assessment of risk, biomarkers may be used in hazard identification, exposure assessment and to associate a response with the probability of a disease outcome.

Studies undertaken to identify biomarkers in the aquatic environment are generally performed in animal systems. In particular, extensive studies have been carried out in shrimps (Thebault *et al.,* 1996; Marino-Balsa *et al.,* 2000; Hoguet & Key, 2007; Erk *et al.*, 2008; Key *et al.,* 2008).

3.1 Biochemical Biomarkers

Aiming to develop sensitive and precise diagnostic tools with a predictive capability in assessing the sub lethal effects of common contaminants, specific biomarkers have been selected as endpoints (Bainy, 2000; Callaghan *et al.,* 2001)

Bioassays offer many advantages for comparing the relative toxicity of specific chemicals or specific effluents. However, toxicity tests also have serious limitations for biological monitoring because most do not account for the effect of chemical specification in the environment, kinetics and sorption of chemicals to sediment, accumulation through food

chains and modes of toxic action which are not readily measured as short-term effects (McCarthy and Shugart, 1990). Depledge and Fossi (1994) suggested the use of biomarkers in toxicity tests as an attempt to link biomarker responses to effects on lifehistory characteristics (e.g. survival, reproduction, and behaviour), which will provide a further foundation for the use of biomarkers in environmental assessment.

The main facet of biomarker research is the analysis of a biochemical, cellular, physiological or behavioural organism's responses when exposed to stress conditions (Erk *et al.,* 2008). According to Who (1993) biomarkers can be grouped into three general categories:

- Biomarkers of exposure, an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism. Biomarkers of exposure can be used to confirm and assess the exposure of individuals or populations to a particular substance (group), providing a link between external exposure and internal dosimetry.
- Biomarkers of effect, a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease. Biomarkers of effect can be used to document either preclinical alterations or adverse health effects due to external exposure and absorption of a chemical.
- Biomarkers of susceptibility, an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance. Biomarkers of susceptibility help to elucidate variations in the degree of responses to toxicant exposure observed between different individuals.

The responses of biomarkers can be regarded as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects (van der Oost *et al.,* 2003). Since they give information on the biological effects of pollutants rather than a quantification of their environmental levels, the control of physicochemical profile of the water where the animals are exposed is required. By this way we may establish the possible linkage between both sources of information, i.e. if there are correlations between the magnitude and pattern of the biomarkers response and the environmental pollutants bioavailability.

In this context biomarkers can act as a nexus between the cause and effect of exposure to a multiplicity of stressful environmental chemicals, thus providing the way to carry out an early overall assessment of these effects at the subindividual level. Responses of effect biomarkers are restricted to the lower levels of biological complexity, i.e. molecular, cellular and tissue levels. The stress effect reaches the sub cellular level of an organism, and the basis of biomarker studies is the biological differences between pre- and postexposure to exterior stresses such as chemical pollutants. Thus, they precede alterations at population of communities' levels and, consequently, can provide information on toxic or risk effects and can be used to improve the assessment of biologically significant exposure to chemical contaminants, as early warning systems (Torre *et al.,* 2007). The sequential order of responses to pollutant stress within a biological system is schematically illustrated in figure 6.

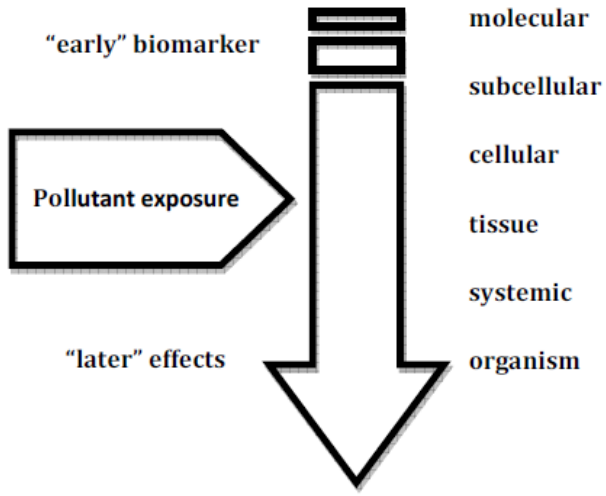

Figure 6- Schematic representation of the sequential order of responses to pollutant stress within a biological system. Adapted from van der Oost *et al.* (2003)

Generally, the most sensitive effect biomarkers are alterations in levels and activities of enzymes, and the activity of these enzymes may be induced or inhibited upon exposure to xenobiotics. Many pollutants (or their metabolites) may exert toxicity related to oxidative stress. Oxygen toxicity is defined as injurious effects due to cytotoxic reactive oxygen species, also referred to as reactive oxygen intermediates, oxygen free radicals or oxyradicals. These reduction products of molecular oxygen (O_2) are the superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$), an extremely potent oxidant capable of reacting with critical cellular macromolecules, possibly leading to enzyme

inactivation, lipid peroxidation (LPO), DNA damage and, ultimately, cell death (Winston and Di Giulio, 1991).

Oxidant-mediated effects with a potential suitability as biomarkers include either adaptive responses, such as increased activities of antioxidant enzymes and concentrations of nonenzymatic compounds, or manifestations of oxidant-mediated toxicity such as oxidations of proteins, lipids and nucleic acids, as well as perturbed tissue redox status (van der Oost *et al.,* 2003). Defence systems that tend to inhibit oxyradical formation include the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathionedependent peroxidase (GPx) and glutathione reductase (GR). SOD, CAT and GPx are critically important in the detoxification of radicals to nonreactive molecules.

In aquatic ecosystems, dissolved oxygen and temperature are environmental variables that are likely to influence oxidative processes (Parihar *et al.,* 1997). These variables must be carefully controlled in laboratory experiments examining oxidative stress, and similarly considered in field studies including this phenomenon in aquatic animals (Winston and Di Giulio, 1991).

3.1.1. Detoxification

In the first step of the biotransformation of toxic compounds, several metabolites are formed, some of which are subject to further transformation by conjugation with endogenous substances. A possible pathway is the reaction catalysed by glutathione Stransferases (GST), that play an important role in the detoxification and excretion of xenobiotics by catalysing the conjugation of the tripeptide glutathione (GSH) with the xenobiotic or its metabolites in the phase II of the biotransformation process promoting its elimination from the organism (Lima *et al.,* 2007). GST are a family of enzymes that is also involved in the prevention of lipid peroxidation (LPO) (Vieira *et al.,* 2008).

3.1.2. Enzymatic Antioxidants

The SODs are a group of metalloenzymes that catalyse the conversion of reactive superoxide anions ($O_2^{-\bullet}$), to yield hydrogen peroxide (H_2O_2), which in itself is an important ROS as well. H_2O_2 is subsequently detoxified by two types of enzymes: CATs and glutathione peroxidases (GPxs). SODs are considered to play a pivotal antioxidant role and their importance is indicated by their presence in all aerobic organisms examined (Stegeman *et al.*, 1992).

Most techniques for the measurement of SOD activity are indirect assays in which an indicating scavenger competes with endogenous SOD for $O_2^{-\bullet}$.

CATs are hematin-containing enzymes that facilitate the removal of hydrogen peroxide

(H_2O_2), which is metabolized to molecular oxygen (O_2) and water. Unlike some peroxidases that can reduce various lipid peroxides as well as H_2O_2, CATs can only reduce H_2O_2 (Stegeman *et al.*, 1992; Filho, 1996). Since CATs are localized in the peroxisomes of most cells and are involved in fatty acid metabolism, changes in activities may often be difficult to interpret (Stegeman *et al.*, 1992).

Peroxidases are enzymes that reduce a variety of peroxides to their corresponding alcohols. While CAT employs one molecule of H_2O_2 as donor in the reduction of another H_2O_2 molecule, peroxidases employ other reductants.

Glutathione peroxidase (GPx) is considered to play an important role in protecting membranes from damage due to LPO. This observation led to the view that the major detoxification function of GPx is the termination of radical chain propagation by quick eduction to yield further radicals. GPx catalyses the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced GSH to its oxidized form (GSSG) (Lauterburg *et al.*, 1983).

3.1.3. Oxidative damage

Lipid peroxidation or the oxidation of polyunsaturated fatty acids is a very important consequence of oxidative stress and has been investigated extensively (Stegeman *et al.*, 1992; Hageman *et al.*, 1992). The process of LPO proceeds by a chain reaction and, as in the case of redox cycling, demonstrates the ability of a single radical species to propagate a number of deleterious biochemical reactions.

3.1.4. Energetic metabolism

The activity of several regulatory enzymes may be altered in order to meet the required energy demands under toxic stress, including the activity of lactate dehydrogenase (LDH), which sustains the continued process of glycolysis under anaerobic conditions (Diamantino *et al.,* 2001). Lactate dehydrogenase (LDH) is a key enzyme in the anaerobic pathway of energy production, being particularly important for muscular physiology in conditions of chemical stress when high levels of energy may be required in a short period of time (De Coen *et al.*, 2001).

The isocitrate dehydrogenases (IDH) catalyze oxidative decarboxylation of isocitrate to 2-oxoglutarate and require NAD^+ or $NADP^+$, producing NADH and NADPH, respectively. $NADP^+$ dependent IDH is one of the enzymes that has the ability to regenerate cellular NADPH, due to its role in the antioxidant system, since NADPH, a cofactor of GR, needs to be regenerated during the maintenance of the cellular redox status (Lima *et al*., 2007).

3.1.5. Neurotoxic effects

With respect to neural functions, enzymes of interest are cholinesterases (CHE) (Payne *et al.*, 1996). Two types of CHE are recognized: firstly, those with a high affinity for acetylcholine (AChE); and secondly, those with affinity for butyrylcholine (BChE), also known as non-specific esterases or pseudocholinesterases (van der Oost *et al.,* 2003).

Acetylcholinesterase (AChE), an important enzyme of the nervous system, hydrolyzes the neurotransmitter acetylcholine (ACh). The inhibition of AChE leads to an accumulation of ACh which, in turn, over stimulates sensitive neurons at the neuromuscular junction which results in spasm and tremors. In invertebrates, the accumulation of ACh can induce the pattern of nerve poisoning with hyperactivity, tremors, convulsions and paralysis, which may finally lead to death (Hoguet & Key, 2007).

Many organophosphate (OP) and carbamate pesticides are reported to be effective AChE inhibitors (Printes and Callaghan, 2004). The inhibition of neurotransmission has been well documented in *Palaemonetes* sp. exposed to various organophosphates (Lund *et al.* 2000; Key *et al.* 2003; Key and Fulton, 2006; Bolten-Warberg *et al.,* 2007).

3.2. Behaviour

Behaviour is the overall outcome of an organism's response to physiological and environmental factors (Dell' Omo, 2002). A change in behaviour can be a direct result of pollutants, but also a method used by the organism to protect itself against the pollutant.

The establishment of links between different levels of biological organization is an important issue when considering the impact of chemical pollutants, because toxicity is induced first at the sub-individual level and then at an individual level before populations being affected (Gravato and Guilhermino, 2009). Therefore, by using parameters at sub-individual level, the so-called biomarkers, deleterious effects can be early diagnose. However, biomarker studies should demonstrate that biomarkers respond in a regular and predictable manner to increasing time and/or concentration exposure and that higher-level effects are predictable from some biomarkers (e.g., cholinesterase, lactate dehydrogenase) are in fact related to ecologically relevant parameters (Gravato and Guilhermino, 2009).

Impairment of locomotion, which is at the basis of nearly all other complex behaviours, is well documented in a number of invertebrates exposed to different classes of contaminants such as oil, pesticides, or metals (Amiard-Triquet, 2009).

Swimming behaviour has been used to assess the toxicity of several pollutants such as polycyclic aromatic hydrocarbons, pharmaceuticals and pesticides (Gravato and Guilhermino, 2009; Gerhardt *et al.,* 2002; Roast *et al.,* 2000a, b; Wallace and Estephan, 2004). The assessment of swimming behaviour is ecologically relevant, because, when an organism lose the swimming velocity he lose the capability to escape from the predators, and he can die (Figure 7).

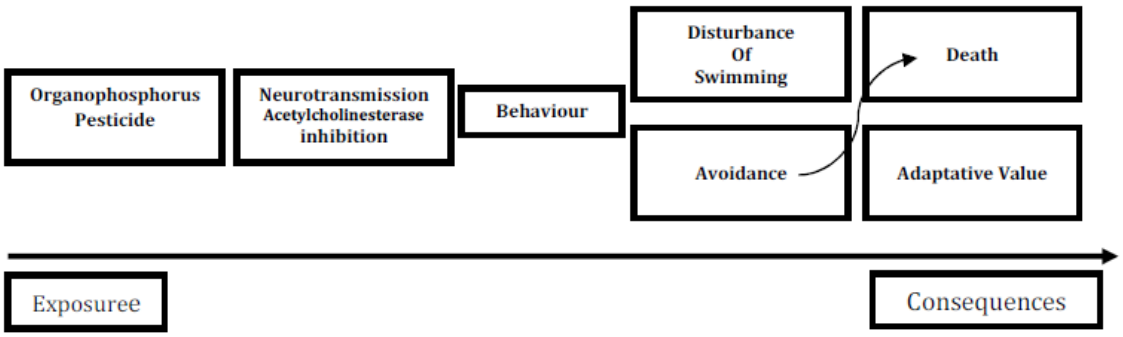

Figure 7- From exposure to a acetylcholinesterase inhibitor to ecological consequences. Adapted from Amiard-Triquet (2009)

Almost toxicity tests involve the forced exposure of organisms to toxicants, however, avoidance behaviour of aquatic organisms to contaminants can be relevant in field situations (Dornfel *et al*., 2009). Avoidance is an ecologically relevant behaviour because it contributes to the defense of the organisms against chemical contaminants (if he is not in contact with the chemical he is not affected), or, on the other hand, it can lead to death (when an organism avoids a contaminated area, he can move for a non optimal habitat and have less food availability and more predators) (Figure 7).

4. *Palaemon serratus*

Due to their ecological and commercial relevance, crustaceans are frequently used as toxicity test species (Marino-Balsa *et al.*, 2000). The common prawn, *Palaemon serratus*, is a large crustacean, about 11 cm long when straightened out. Belongs to the family Palaemonidae and is usually associated with rocks pools. A saber-shaped projection (the rostrum) on its head shield is toothed or serrated, and the eyes are stalked. The outer skeleton is thin and hornlike in texture, and there are two pairs of very long filamentous antennae. The first pair has three branches, one extending forward, the other two upward and backward. The antennae of the second pair are undivided. The prawn has five pairs of limbs on the thorax, the first and second pairs having small but distinct pincer like claws. Five pairs of small swimming legs line the narrow abdomen, which ends in a fan like tail (Burton, 2002).

The crustacean *Palaemon serratus* has a large distribution (occurring along the Northeastern Atlantic coast to the Mediterranean, Black Sea and Mauritanian coast), is abundant and easy to capture, and has a small-scale commercial importance (Frasco *et al.*, 2006). Besides its ecological relevance in European estuaries (some of the most vulnerable and highly productive natural habitats), the common prawn is a suitable model for use in Ecotoxicology (Bocquene and Galgani, 1991; Franchet, 1999; Marino-Balsa *et al.,* 2000).

5. Objectives

The central objective of the present study was to investigate effects of one organophosphorus pesticide, fenitrothion, and one pyrethroid pesticide, deltamethrin, using parameters at different levels of biological organization to compare the effects of the two pesticides. Thus, this research work is divided in three distinct but complementary parts, allowing reach the following specific objectives:

i. Determination of LC_{50} for the common prawn *P. serratus* exposed to fenitrothion and deltamethrin;

ii. Determination of the acute effects of sub-lethal concentrations of fenitrothion and deltamethrin on biomarkers involved in key physiological functions and on swimming velocity. The following biomarkers were used to evaluate sub-individual alterations: LPO, the activity of the enzymes GST, SOD, CAT and GPx, the activity of enzymes involved in energetic metabolism (LDH, IDH) and neurotransmission (ChE);

iii. Development of an avoidance test for the common prawn exposed to sublethal concentrations of fenitrothion for the quantification of the prawns escape capability.

In that context, it was also investigated if the common prawn behaviour can be used as effect criteria in bioassays and if those behavioural endpoints are as sensitive as biochemical biomarkers.

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Chapter II

Behavioural alterations and biomarker responses in *Palaemon serratus* exposed to fenitrothion

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Abstract

The aim of this study was to investigate the acute effects of sublethal concentrations of fenitrothion on the common prawn, *Palaemon serratus,* using biomarkers, swimming behaviour and an avoidance test as endpoints. In a first experiment, prawns were exposed for 96h, and the LC_{50} value was determined to be 1.050 µg/L (IC_{95} 0.77-1.47 µg/L). After 96 h, the swimming velocity and biomarkers of prawns exposed to sub-lethal concentrations were individually measured. Results showed that swimming velocity was significantly reduced at 313 and 625 ng/L and eye' ChE activity was significantly inhibited with a LOEC observed at 78ng/L. In a second experiment a behavioural test was performed using aquaria developed for the tests according to details on Material and Methods section. The common prawn was exposed to sublethal concentrations of fenitrothion in order to determine if animals were able to escape from the aquarium side contaminated with the pesticide. The results showed that animals significantly escape from the aquarium side with low concentrations of this pesticide (78 ng/l) after exposures equal or longer than 60 min. However, animals seem to lose the escaping ability to the highest concentration of fenitrothion tested. In a third experiment the prawns were exposed in aquaria developed for the behavioural test, but forced to stay in the contaminated side by covering all aquaria. Prawns were sampled at different times of exposure (0-120 min) and tissues were sampled to determine ChE, IDH and LDH activities. Results showed that IDH and LDH activities were significantly increased, particularly IDH activity on muscle of prawns exposed 120 min to the highest concentration of fenitrothion tested, and yet prawns lost the ability to avoid the pesticide.

Introduction

In the natural environment the concentrations of contaminants are generally much lower than the median lethal concentration (LC_{50}) values estimated in laboratory bioassays (Roast *et al*., 2000a). Therefore, the acute toxicity tests provide little relevant information on the toxicant effects and so, biomarkers have been widely used in ecotoxicology as early warning signals of contaminant exposure and/or effect (Menezes *et al*., 2006). Thus, to better understand the health status of organisms, biotransformation enzymes, responsible to convert contaminants into more hydrophilic metabolites facilitating excretion (like glutathione-S-transferase) (Van der Oost *et al*., 2003), and antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) involved in the detoxification of reactive oxygen species (Wiston and Di Giulio, 1991) have been widely used.

Enzymes involved in the energy production have also been used as biomarkers to assess the effects of pollutants on aquatic organisms (Sancho, *et al*., 1998a,b; Lee *et al*., 2002; Rao, 2006; Lima *et al*., 2007), since exposed organisms may need additional energy for detoxification to maintain physiological/biochemical functions at normal level (Choi *et al*., 2001). Lactate dehydrogenase (LDH), a key enzyme in the anaerobic pathway of energy production, is particularly important for muscular physiology in stress conditions, when high levels of energy may be required in a short period of time (De Coen *et al*., 2001; Diamantino *et al*., 2001). In fact, Moreira and co-workers (2006) observed an increase of LDH activity in exposed organisms, suggesting that it may be essential to overcome toxic stress. However, decreased LDH activity was also observed in exposed organisms, indicating a possible decrease in muscle anaerobic capacity (Rao, 2006). In this context, the quantification of isocitrate dehydrogenase (IDH) activity seems particularly important, since it is more efficient in ATP production compared to the anaerobic pathway (Moreira *et al*., 2006) and may also be involved in oxidative stress defenses (Jo *et al*., 2001; Lee *et al*., 2002). In fact, increased IDH activity in exposed organisms was previously observed (Lima *et al*., 2007).

Cholinesterase is the most widely used biomarker of exposure and/or effect in studies with organophosphate pesticides, since the main mechanism of toxic action is the inhibition of this enzyme (Choi, *et al.*, 2001, 2002; Frasco, *et al.*, 2006; García-de la Parra *et al.*, 2006; Rao, 2006; Damásio *et al.*, 2007; Amiard-Triquet, 2009).

Traditional toxicological investigations have focused on measuring biochemical biomarkers for perception on the health status of population. However, to further understand the true impacts of aquatic pollution on the whole ecosystem, multidisciplinary research becomes increasingly important (Amiard-Triquet, 2009). Recent results suggest that cholinesterase inhibitors can have sublethal effects on a variety of parameters with implications for fitness of organisms (Hopkins *et al*., 2005). Brewer and co-workers (2001) found correlations between alterations on trout behaviour and cholinesterase activity inhibition. In that context, behavioural ecotoxicology seems to be particularly promising (Amiard-Triquet, 2009) and it have been widely studied (Roast *et al.*, 2000a; Roast *et al.*, 2000b; Roast *et al.*, 2001; De Lange *et al*., 2009; Dornfeld, 2009; Wallace and Estephan, 2004).

Swimming velocity is perhaps the most frequently used "behavioural" measure of an aquatic organisms physiological status (García de la Parra *et al.*, 2006; Gravato and Guilhermino, 2009; Lange *et al.*, 2009; Roast *et al.*, 2000a, b; Roast *et al.*, 2001; Zhang *et al.*, 2006) and has been used to assess the effects of pollutants in fish (Gravato and Guilhermino, 2009; Ballesteros *et al.*, 2009; Vieira *et al.*, 2009). However, there are only a few studies concerning

pollutant-induced alterations in swimming performance of crustaceans (Roast *et al.*, 2000a, b; Wallace and Estephan, 2004).

Locomotor performance of animals is at the basis of practically all other complex behaviours (Amiard-Triquet, 2009). Therefore swimming behaviour is of considerable interest, since it can be considered a main character determining survival in many species of aquatic animals. The best swimming performance may strongly influence the ability of an aquatic organism to obtain food, to reproduction or even to avoid unfavorable conditions (Plaut, 2001).

The assessment of the impacts of contaminants on ecosystems is based largely on ecotoxicological tests measuring survival, growth and reproduction. However, avoidance based toxicity tests may add important ecological information on toxicity assessment and should be considered for routine use, since, in the wild, animals have the ability to escape from contaminated environment before suffering sublethal or even lethal effects (Amiard-Triquet, 2009). The avoidance of aquatic organisms to contaminants is often reflected at population, community, and ecosystem level responses (Moreira-Santos *et al.*, 2008). Thus, the avoidance behaviour should be considered as a complementary sublethal response to improve ecological relevance in the assessment of the environmental risk of contaminants (Moreira-Santos *et al.,* 2008).

The complex behaviours that are necessary for survival are a result of the conjugation of many physiological systems which contribute to the performance of those behaviours (Scott and Sloman, 2004). An avoidance reaction, for example, is based on the fact that organisms have chemoreceptors highly sensitive to chemicals present in the environment, and it influences the energy resources of animals by dispending energy on detecting and escaping from hazard (Loureiro, *et al.*, 2009); and swimming velocity can be influenced by neurotransmission processes, including alterations on AChE activity (Scott and Sloman, 2004).

Thus, the main goal of the present work was to determine whether fenitrothion, a cholinesterase inhibitor, adversely affects swimming performance of the common prawn, *Palaemon serratus*, and if the exposed organisms have the ability to avoid this pesticide. Fenitrothion (O,O-dimethyl O-4-nitro-mtolyl phosphorothiate) is an organophosphate insecticide known to cause neurotoxic effects on target and non-target organisms through inhibition of AChE activity (Choi *et al*., 2002; Garcia-de la Parra *et al*., 2006; Frasco *et al*., 2006; Roast *et al*., 2000), including crustaceans (Escartin & Porte, 1996). Thus, this research work is divided in three distinct but complementary experiments, to attain the following specific objectives: estimate a 96 h median lethal concentration (LC_{50}) of fenitrothion for

prawn and use swimming velocity and biomarkers as effect criteria for sub lethal concentrations of fenitrothion; develop and evaluate a short-term avoidance test for prawn using this behavioural parameter as a response that is sensitive, cost-effective, rapid and easily quantified and that should be considered for routine toxicity assessments; establish possible associations between some biomarkers and the alterations on behavior in order to determine which biomarkers are associated with decreased performance and, thus, if they can be used as early-warning tools with ecological relevance to assess the health status of prawns.

Material and methods

Chemicals

Fenitrothion is of analytical standard grade (CAS number: 122-14-5) purchased from Sigma-Aldrich Chemical Corporation. All the other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Boehringer and Merch.

Test Animals and Acclimatization Conditions

Palaemon serratus specimens were captured at Praia Norte of Viana do Castelo (Northwest of Portugal) using a hand operated net at low tide. Selected specimens were immediately transported to laboratory and allowed to recover for 15 days in 200 L tanks filled with filtered and aerated saltwater. During acclimatization, prawns were kept at controlled conditions with a photoperiod of 16h:8h (light:dark) and fed three times per week. Saltwater was changed 2 times per week and temperature (18 ± 0.2 ºC), salinity (36 ± 0.2 g/L), dissolved oxygen (7 ± 0.5 mg/L), pH (8 ± 0.005), ammonia (2 ± 2 mg/L), nitrates (2 ± 0.5 mg/L), and nitrites (1 ± 0.5 mg/L) were monitored at least once per week.

Acute bioassay

After acclimatization, animals were individually exposed on covered glass flasks during 96 hours to 39, 78, 156, 313, 625, 1250, 2500, 5000, 10000, 20000 and 40000 ng/L fenitrothion in a final volume of 700 mL. Four days before the exposure and during the exposure period animals were not fed. Ten replicates were used per treatment. Test solutions of fenitrothion were obtained by dilution of the stock solution of the pesticide in dimethyl sulfoxide (DMSO) in seawater. Two more conditions were used: a control and a control with DMSO (control+DMSO) by adding the same volume of DMSO used on each exposure flask, i.e. 28 µL.

During the 96 h exposure period, prawns were kept at the same controlled conditions than the acclimatization period.

Animals from sub-lethal concentrations (39, 78, 156, 313 and 625 ng/L) of fenitrothion, control and control+DMSO were used to assess their swimming velocity. Prawns were caught one by one and immediately allowed to swim against a seawater flow of 7 L/min in a 4 m long race track. The distance (m) and time (s) of swimming were determined for each animal. The swimming velocity (m/s) was calculated as the quotient between the covered distance and the time of swimming (Gravato and Guilhermino, 2009). After swimming trials, animals were immediately placed on respective exposure flasks for 2 h until their tissues were sampled for biomarkers' analysis.

Body weight and length of each prawn was determined before being sacrificed by decapitation and dissected. Eye, digestive gland and muscle were rapidly isolated on ice, frozen, and maintained at -80ºC until further analysis.

Each pair of eyes and a portion of muscle were homogenized at 4 °C in 500 µL K-phosphate buffer (pH 7.2; 0.1M) and centrifuged at 3 300 g for 3 min at 4 °C and the resulting supernatant recovered. Eye and muscle supernatants were used to measure ChE by the Ellman's method (Ellman *et al*. 1961), adapted to microplate (Guilhermino *et al*. 1996) using acetylthiocholine as substrate and following the increase of absorbance at 412 nm. Muscle supernatant was also used to determine IDH activity by measuring the increase in NADPH at 340nm, according to Ellis and Goldberg (1971) adapted to microplate (Lima *et al*., 2007).

Another portion of muscle was used to determine LDH activity. For LDH activity, muscle samples were homogenized in 1ml of ice-cold Tris/NaCl phosphate buffer (pH 7.2, Tris 81.3 mM; NaCl 203.3 mM) and centrifuged at 3 300 g for 3 min at 4ºC after 3 frozen/unfrozen cycles. LDH activity was determined by measuring the amount of pyruvate consumed, through the continuous monitoring of the decrease in absorbance due to NADH oxidation at 340nm according to Vassault (1983) adapted to microplate (Diamantino *et al*., 2001).

The digestive gland was homogenized (1:11) in 0.1 M K-phosphate buffer (pH 7.4). Part of this digestive gland homogenate was used to determine the extent of endogenous LPO by measuring the thiobarbituric acid reactive substances, according to Ohkawa (1979) and Bird and Draper (1984), with the adaptations of Filho *et al*. (2001) and Torres *et al*. (2002). LPO was expressed in nmol TBARS per g wt. The remaining digestive gland homogenate was centrifuged for 20 min at 10 000 g (4ºC) to obtain the post-mitochondrial supernatant. The post-mitochondrial supernatant (PMS) was used for determination of GST, GPx and CAT activities. GST activity was determined by the conjugation of reduced glutathione (GSH) with

1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig *et al.* 1974) adapted to microplate (Frasco and Guilhermino, 2002). GPx activity was determined by measuring the decrease in NADPH at 340 nm (Jenway 6405 UV/VIS Spectrophotometer) using H_2O_2 as substrate (Mohandas *et al.* 1984). CAT activity was determined by measuring the H_2O_2 consumption at 240nm according to Clairborne (1985). Protein quantification of all samples was determined according to Bradford (1976), adapted to microplate as indicated in Guilhermino *et al.* (1996), using bovine γ-globulins as protein standard and a wavelength of 600nm. All biochemical analysis were performed at a constant temperature of 25ºC. All enzymatic activities were expressed as nmol/min/mg protein.

Avoidance test

In a second experiment a new behavioural assay was developed to determine the ability of prawns to escape from fenitrothion and, therefore, avoiding the pesticide. After acclimatization, avoidance behaviour was determined by using aquaria divided into 2 equal parts, each one with different solutions (control+DMSO/ treatment). Each part of the aquarium had 1.5 l of saltwater (salinity), one part with the respectively concentration of fenitrothion (39, 78, and 156 ng/L), the other part with the same volume (60µl) of DMSO added to the exposure side (control+DMSO). Three specimens of *P. serratus* were placed on the contaminated side of each aquarium, and the successful jumps to the control side were recorded every 30 min during 2h. Each time a prawn jumped to the control side of the aquarium it was immediately removed. A total of 9 independent tests (with 3 prawns each), were performed for each condition. Nine control tests were also performed by using control+DMSO on both sides of the aquaria. The avoidance behaviour was calculated by the quotient between the number of prawns that jump and the total of exposed prawns (3 for each test).

To test the neurotransmission and energetic status of prawns during the avoidance tests, a third experiment was performed using the same conditions previously adopted on the second experiment, but the prawns were forced to stay in the contaminated side during the 2 hours of exposure. Every 30 min prawns were removed from the experiment and immediately sacrificed by decapitation and dissected. Six individuals were used for each treatment (39, 78 and 156 ng/L) and time (30, 60, 90 and 120 min). Control tests were performed as mentioned on the second experiment. Eye and muscle were rapidly isolated, frozen, and maintained at -80ºC until further analysis. Both tissues were used to measure ChE activity, whereas LDH and

IDH activities were measured in muscle. The enzymatic activities were performed according to the methods mentioned above in this section.

Data Analysis

All data were first analysed by Kolmogorov-Smirnov test and Bartlett's test to check normality and homoscedasticity, respectively, and appropriated data transformation was made whenever necessary. Then, one-way analysis of variance (ANOVA) was used to compare different treatments. Dunnett's test was used to determine differences between control+DMSO and fenitrothion treatments, the values of the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). For all data, outlier values (more than 3.5 standard deviations from the group mean) were removed. Statistical level was 0.05 and SPSS 16.0 package was used for statistical analysis.

Results

The 96h LC_{50} value estimated for common prawn exposed to fenitrothion was 1.05 μg/L with a 95% confidence interval (CI_{95}) from 0.77 to 1.47 μg/L (Table 1).

Tabela 1. Summary of 24, 48, 72 and 96 h LC_{50} values for *Palaemon serratus* exposed to nominal concentrations of fenitrothion (39 – 625 ng/L) and respectively 95% confidence intervals.

Exposure time (h)	LC_{50} (μg/L)	95% CI (μg/L)
24	3.02	2.14 - 4.34
48	1.79	0.61 - 6.55
72	1.38	0.7 - 2.87
96	1.05	0.77 - 1.47

Swimming velocity was significantly decreased ($F_{(6,65)}$=3.598; p=0.004) on common prawn exposed during 96 h to 313 and 625 ng/L fenitrothion (Figure 1), exhibiting about 40% of swimming velocity inhibition compared to control+DMSO. Moreover, fenitrothion induced a

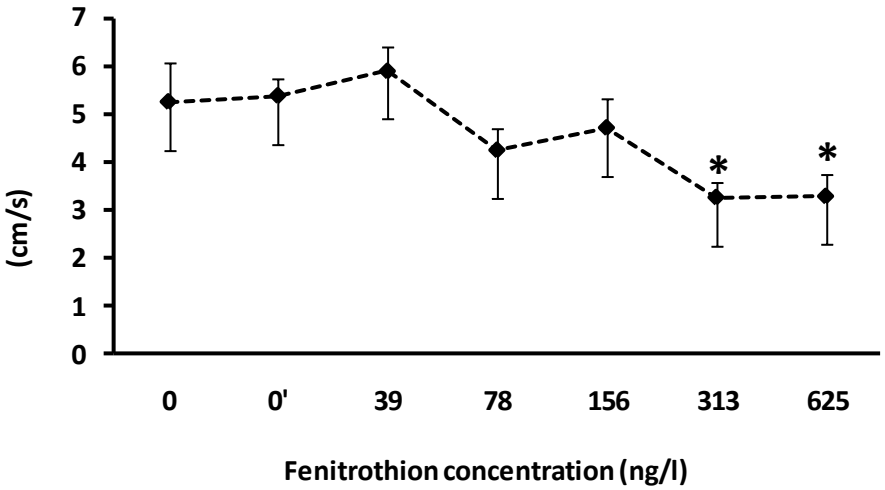

Figure 1. *S*wimming velocity of *Palaemon serratus* exposed to 39 – 625 ng/L fenitrothion for 96 hours. Values are the mean of 10 prawns per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences (p<0.05, Dunnett test) relatively to control (control+DMSO).

significant decrease of eye ChE activity ($F_{(6,69)}$=8.516; p<0.001) on animals exposed during 96 h to 78, 156, 313 and 625 ng/L fenitrothion (Figure 2A), reaching activity inhibitions of 34% in prawns exposed to the highest concentration tested. Muscle ChE activity was also inhibited in prawn exposed to 156, 313 and 625 ng/L of fenitrothion ($F_{(6.66)}$=3.413; p=0.006) (Figure 2B), exhibiting activity inhibitions of 25, 18 and 20%, respectively. Concerning energetic metabolism, muscle LDH ($F_{(6,69)}$=1.335; p=0.255) and IDH ($F_{(6,69)}$=4.960; p=0.000) activities were not significantly changed on common prawn exposed 96 hours to fenitrothion concentrations tested (Table 2). Moreover, CAT ($F_{(6,60)}$=2.833; p=0.018), GST ($F_{(6,63)}$=2.029; p=0.076) and GPx ($F_{(6,45)}$=2.899; p=0.02) activities as well as the levels of LPO ($F_{(6,69)}$=0.842; p=0.542) were not significantly affected on digestive gland of prawn exposed to fenitrothion for 96 h (Table 2).

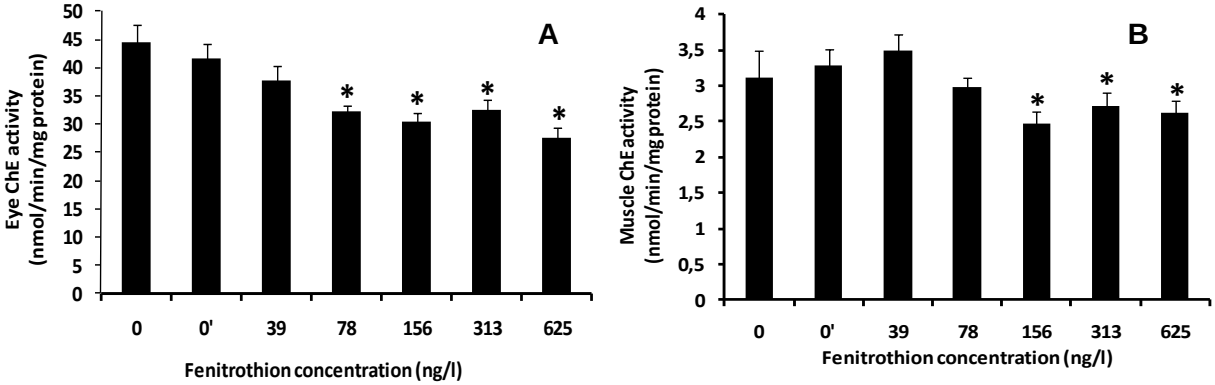

Figure 2. Eye AChE (A) and muscle ChE (B) activities of *Palaemon serratus* exposed to 39 – 625 ng/L fenitrothion for 96 hours. Values are the mean of 10 prawns per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences (p<0.05, Dunnett test) relatively to control (control+DMSO).

Tabel 2. Effects of fenitrothion (39 – 625 ng/L) on different biomarkers of *Palaemon serratus* after 96h of exposure. Values are the mean of 10 prawns per treatment with the corresponding standard error. Units are: nmol min-1 mg protein-1 for gluthathione S-transferase (GST) and gluthathione peroxidase (GPx) activities, umol min-1 mg protein-1 for catalase (CAT), lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) activities and nmol TBARS gwt^{-1} for lipid peroxidation (LPO).

Functions	Parameters	Concentration of fenitrothion (ng/l)						
		0	0'	39	78	156	313	625
Detoxification	Digestive Gland GST	4.57±0.37	4.74±0.62	4.86±0.74	4.10±0,54	6.88±0.85	5.06±0.93	5.83±0.51
Enzymatic Antioxidants	Digestive Gland CAT	1.35±0.28	1.44±0.28	1.15±0.20	1.00±0.15	1.68±0.31	1.36±0.20	0.87±0.14
	Digestive Gland GPx	4.44±0.62	3.54±0.44	3.27±0.38	3.51±0.65	4.82±0.69	4.00±0.70	3.51±0.42
Oxidative Damage	Digestive gland LPO	382.58±31.84	377.14±39.12	432.40±38.05	405.51±40.18	452.98±30.83	346.82±394.86	394.66±54.95
Energetic Metabolism	Muscle LDH	117.08±6.91	113.98±6.46	136.97±6.63	128.58±8.70	118.27±8.78	133.14±10.81	112.85±5.05
	Muscle IDH	15.42±1.12	15.75±1.09	14.60±1.60	13.42±1.36	14.72±1.03	12.65±1.15	14.27±1.23

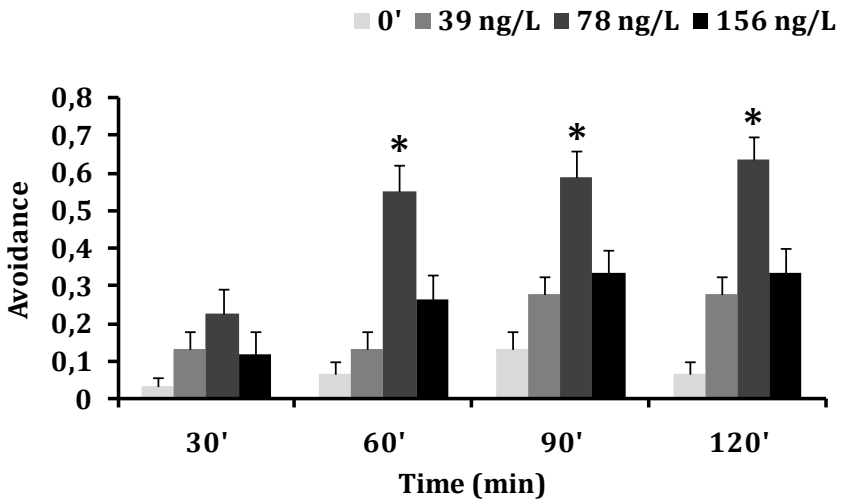

Figure 3. Avoidance behaviour of *Palaemon serratus* after 30, 60, 90 and 120 min exposure to 39 – 156 ng/L fenitrothion. Values are presented as the quotient between the numbers of prawns that jump and the total of exposed prawns per test (3) and are the mean 9 tests per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences (p<0.05, Dunnett test) relatively to control (control+DMSO).

Results of the second experiment showed a significant avoidance behaviour on prawn exposed during 60-120 min to 78 ng/L fenitrothion (60 min: $F_{(3,35)}$=4.626; p=0.008; 90 min: $F_{(3,35)}$=2.975; p=0.046; 120 min: $F_{(3,35)}$=5.682; p=0.003) compared to control (Figure 3). However, this escaping behaviour was not observed neither on common prawn exposed for 30 min ($F_{(3,35)}$=0.955; p=0.426) nor on common prawn exposed to concentrations of fenitrothion higher than 78 ng/L.

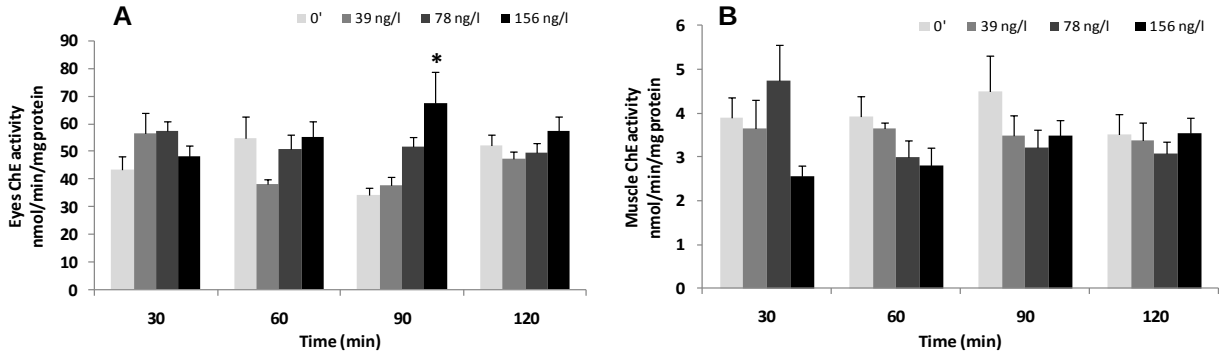

Figure 4. Eye AChE (A) and muscle ChE (B) activities of *Palaemon serratus* after 30, 60, 90 and 120 min exposure to 39 – 156 ng/L fenitrothion. Values are the mean of 9 prawns per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences (p<0.05, Dunnett test) relatively to control (control+DMSO).

The results of the third experiment showed that eye AChE and muscle ChE activities on common prawn forced to be exposed to fenitrothion during 30, 60, 90 and 120 min were not significantly altered when compared to control (Figures 4A and 4B), with the exception of eye AChE activity that was significantly increased on prawn exposed to 156ng/L for 90 min ($F_{(3,23)}$=13.434; p=0000) (Figure 4A). A significant increase of muscle LDH activity was observed on common prawn forced to be exposed to 39, 78 and 156 ng/L fenitrothion during 60 min ($F_{(3,23)}$=6.403; p=0.003) and to 39 and 78 ng/L fenitrothion during 90 min ($F_{(3,23)}$=9.190; p=0.001) (Figure 5A). However, muscle LDH activity was significantly decreased on common prawn forced to be exposed to 156 ng/L fenitrothion during 120 min ($F_{(3,23)}$=4.490; p=0.014) (Figure 5A). Muscle IDH activity was significantly increased in common prawn forced to be exposed to 156 ng/L fenitrothion during 30 min ($F_{(3,23)}$=16.096; p=0.000), 60 min ($F_{(3,19)}$=6.580; p=0.004), 90 min ($F_{(3,23)}$=59.844; p=0.000) and 120 min ($F_{(3,23)}$=6.631; p=0.003) compared to respective control (Figure 5B).

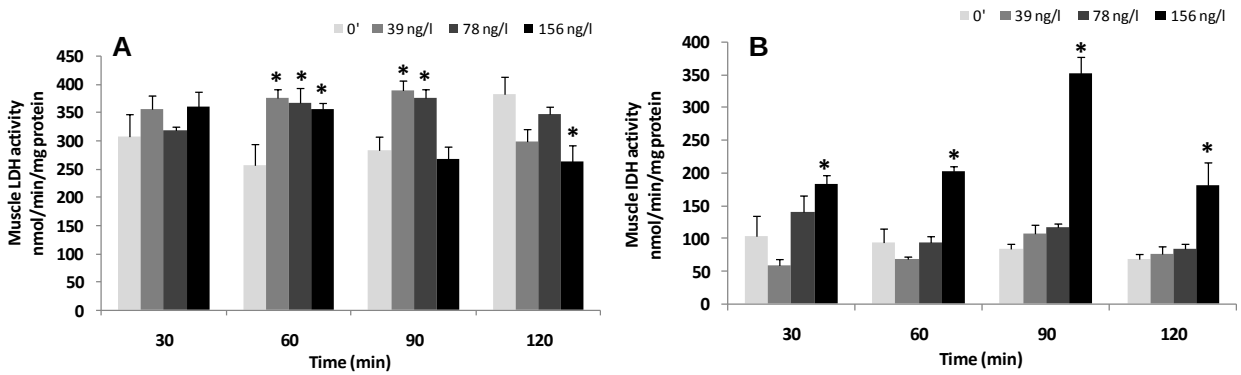

Figure 5. Muscle LDH and IDH activities of *Palaemon serratus* after 30, 60, 90 and 120 min exposure to 39 – 156 ng/L fenitrothion. Values are the mean of 9 prawns per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences (p<0.05, Dunnett test) relatively to control (control+DMSO).

Discussion

The 96h LC_{50} value for *P. serratus* exposed to fenitrothion was estimated in the current research study for the first time on this marine species (1.05 µg/L; CI_{95} 0.77 - 1.47 µg/L). Therefore, comparisons with previous reports, concerning LC_{50}, are only possible considering studies performed with other crustaceans exposed to fenitrothion as shown in Table 3. Moreover, those studies reported LC_{50} values for crustaceans exposed to fenitrothion for 48h or 24h. Considering the LC_{50} values observed for 24h and 48h (Table 1), *P. serratus* seems to be more sensitive to fenitrothion than field resistant clones of *Daphnia magna* (Damásio *et al.*,

2007) and showed a sensitivity similar to *Chironomus riparius* exposed to the same pesticide (Choi *et al*., 2001) (Table 3). In contrast, the common prawn is not as sensitive to fenitrothion as laboratory and field sensitive clones of *Daphnia magna* (Damásio *et al*., 2007).

Table 3. Summary of LC_{50} values for several crustaceans exposed to different pesticides. 95% confidence intervals are given in parentheses.

Specie	Compound	Exposure time (h)	LC_{50} (95% CI) (µg/l)	references
Chironomus riparius	fenitrothion	24	2.86 (1.98-3.76)	Choi *et al.* 2001
Daphnia magna (Laboratory clone)	fenitrothion	48	0.93 (0.27-2.00)	Damásio *et al.* 2007
Daphnia magna (Field sensitive clone)	fenitrothion	48	0.73 (0.34-1.58)	
Daphnia magna (Field resistant clone)	fenitrothion	48	4.82 (3.46-6.71)	
Daphnia magna (Field resistant clone)	fenitrothion	48	4.98 (3.10-7.97)	

Acute toxicity tests have been criticized because they provide little relevant information on the effects of toxicants in the natural environment, where the concentrations of individual toxic contaminants are generally much lower than the LC_{50} values estimated in the laboratory (Roast *et al.,* 2000a). In addition, despite environmental contamination often occurs at concentrations well below those that cause significant mortality and, even though the animals are not clearly affected by a contaminant, they may be unable to function in an ecological context if their normal behaviour is changed (Scott and Sloman, 2004). Thus, behavioural ecotoxicology has been particularly promising (Amiard-Triquet, 2009) and widely studied (Roast *et al.*, 2000a; Roast *et al.*, 2000b; Roast *et al.*, 2001; De Lange *et al*., 2009; Dornfeld, 2009; Wallace and Estephan, 2004), particularly swimming velocity determination (García de la Parra *et al.*, 2006; Gravato and Guilhermino, 2009; Lange *et al.*, 2009; Roast *et al.*, 2000a, b; Roast *et al.*, 2001; Zhang *et al.*, 2006). In the present study, the LC_{50} estimated was more than twice that the lowest concentration that impaired *P. serratus* swimming velocity. This comparison shows the importance of sublethal responses to toxicants and highlights further the flaws of using only mortality as an endpoint in toxicity tests to predict the environmental consequences of pollutant discharges.

The common prawn swimming velocity impairment observed is in good agreement with previous works reporting a reduction in swimming velocity of crustaceans exposed to organophosphate pesticides and other compounds. Roast and co-workers (2000a) observed that *Neomysis integer*, pre-exposed to chlorpyrifos, showed a reduction in the ability to swim against water current. Reduction in crustaceans' swimming ability following toxicant exposure has been also reported on *N. integer* exposed to trace metals (Roast *et al.*, 2000b) and *Gammarus lawrencianus* exposed to cadmium (Wallace and Estephan, 2004). Moreover, a recent study showed that fenitrothion inhibited the running endurance in fat-tailed dunnarts (*Sminthopsis crassicaudata*) (Buttemer at al., 2008).

Locomotor activity is linked to, and illustrates the animal's physiological, metabolic and neurological processes and its anatomical condition (Bayley, 2002). Thus, the impairment of swimming velocity may have consequences for prawns' survival, since their capability to find food, a mate or even to avoid unfavorable conditions may be compromised (Plaut, 2001; Weis *et al.*, 2001; Scott and Sloman, 2004). The inevitable integration of behavior with other levels of biological organization shows that it should be considered not in isolation, but links between different levels of biological organization should be attempted when considering the impact of chemical pollutants (Gravato and Guilhermino, 2009). In fact, behavior has been associated with a variety of stress responses measured on aquatic animals exposed to pollutants (Choi *et al.*, 2002; García de la Parra, *et al.*, 2006; Gerhart *et al.*, 2002; Gravato and Guilhermino, 2009; Moreira *et al.*, 2006; Weis *et al.*, 2001). The current results shown that eye ChE activity was inhibited for concentrations of fenitrothion equal or higher than 78 ng/L and muscle ChE activity for concentrations equal or higher than 156 ng/L. Inhibition of ChE activities was expected for fenitrothion since it belongs to the group of organophosphate pesticides that are known to inhibit AChE activity (García-de la Parra *et al.*, 2006; Frasco *et al.*, 2006). Moreover, current results showed that the inhibition of eye AChE and muscle ChE activities seem to precede the impairment of swimming velocity observed for the highest concentrations of fenitrothion tested. This fact is in good agreement with previous studies reporting associations between AChE or ChE inhibition on animals exposed to organophosphate pesticides and swimming velocity impairments. For example, Brewer and co-workers (2001) found correlations between ChE activity and swimming distance and speed in malathion-exposed rainbow trout (*Oncorynchus mykiss*); Correlations between swimming speed and ChE activity were also observed in larval rainbow trout exposed to carbaryl (Beauvais *et al*., 2001). Sandahl and co-workers (2005) also observed that brain

AChE inhibitions and reductions in spontaneous swimming activity were correlated in coho salmon exposed to chlorpyrifos.

Traditional laboratory toxicity tests involving the forced exposure of test organisms to toxicants may underestimate potentially adverse effects at the population level, since under experimental conditions the test organisms are submitted to a forced exposure, whereas in the wild animals have the ability to avoid a contaminated environment. The avoidance of toxic substances occurs naturally in the animal's habitat and natural populations have the ability to avoid contaminated environments before suffering sublethal or even lethal effects (Amiard-Triquet, 2009). Consequently, there is a need to evaluate avoidance-based toxicity tests as complementary tools to improve ecological realism in the assessment of the environmental risks of contaminants (Dornfeld *et al*., 2009). In the current study an avoidance test was developed and performed with the common prawn. Results showed that prawn, avoided fenitrothion. This avoidance behaviour of *P. serratus* was also observed for a fenitrothion concentration lower than LC_{50} value estimated and in such short period of time (60-120 min). Thus, avoidance proved to be a useful measure of toxicant exposure. In fact, measurement of an organism's behaviour following contaminant exposure may provide a better understanding of the likely environmental consequences of toxic contamination than lethal effects (Roast *et al.*, 2000a). Furthermore, the sensitivity of the avoidance test seems to be similar to the sensitivities of the biomarkers determined, at least for eye AChE activity.

However, results showed decreased avoidance behaviour of prawn exposed to concentrations of fenitrothion higher than 78 ng/L, which suggest that animals cannot avoid higher concentrations of this pesticide. There are some authors that have similar results: Sousa *et al* (2008) found that the earthworm *Eisenia andrei* was not able to avoid the highest concentration of a "FIRE" soil which had a high concentration of pesticides; Garcia-de la Parra *et al.* (2006) investigate the white shrimp (*Litopenaeus vannamei*) behaviour exposed to methamidophos, and also found significant differences at the lowest concentration tested: the locomotory cumulative time (the sum of the time when shrimp is in movement) was significantly higher for the lowest concentration tested. The number of animals that avoided the pesticide seems to stabilize after 90 min exposure except for control indicating that prawn may not be able to avoid the pesticide after that exposure time. This seems to happen also for the highest concentrations of fenitrothion tested. The fact that prawn only avoided lower concentrations of fenitrothion tested may prove that the avoidance behaviour is more environmental realistic than acute tests, showing that animals can avoid contaminated environments before they suffer effects of exposure. However, this also represents a problem

for the avoidance test itself, because it cannot be used for the highest concentrations of fenitrothion tested. Nevertheless, this lost of capacity to avoid seems to be of interest to understand why prawns do not escape the highest concentrations of fenitrothion and further research on this subject seems to be needed. Organophosphate pesticides are known to act on the nervous system causing a loss of the ability to escape (Loureiro *et al.*, 2009), although, contrary to what was expected, eye AChE and muscle ChE activities (Figure 4) had no significant differences among the different periods of time and the different treatments, suggesting that their inhibition is not involved in the impairment of the avoidance behaviour. Contradictory results were found in a previous work with *Chironomus riparius* exposed to fenitrothion showing that ChE activity was inhibited as soon as 1 hour after the beginning of exposure and no recovery was observed during the experiment (Choi *et al*., 2001).

However, significant differences were found in energetic metabolism of prawn exposed to fenitrothion during the different periods of time of exposure (up to 120 min). Changes in organism's energy metabolism may occur due to contaminant exposure, the supply of extra energy from anaerobic sources may be essential to overcome toxic stress (Moreira *et al.*, 2006). Results from the third experiment showed that LDH activity was significantly increased in prawn exposed to all concentrations of fenitrothion tested at the end of 60 min and for 39 and 78 ng/L fenitrothion at 90 min. LDH is involved in the production of energy, being particularly important when a considerable amount of additional energy is rapidly required (Diamantino *et al.*, 2001). However, results showed that muscle LDH activity was decreased in prawn exposed to 156 ng/L of fenitrothion at the end of 120 min, which may indicate that the glycolitic capacity of the tissue was decreased (Rao, 2006). Decreased LDH activity was also found in muscle of fish (*Oreochoromis mossambicus*) exposed to an organophosphate insecticide (Rao, 2006). In addition, current results showed that for prawn exposed to 156 ng/L fenitrothion, muscle IDH activity was increased, suggesting that animals give preference to aerobic pathway to get energy. The aerobic pathway is more efficient in ATP production compared to the anaerobic pathway (Moreira *et al.*, 2006). Although, current results showed a significant increase of muscle IDH activity in prawn exposed to 156 ng/L of fenitrothion but their avoidance behaviour was decreased, which may suggest that the supply of extra-energy may be essential and required to cope with toxic stress due to contaminant exposure. Several authors compared the relative sensitivity of behavioural endpoints to endpoints representative of metabolic disruption, and it has been hypothesized that the ability to resist a toxicant may be expensive in terms of energy, involving a decrease in the

energy available for other biological processes (Diamantino *et al*., 2001; Moreira *et al*., 2006; Amiard- Triquet, 2009).

Conclusions

This research work showed important alterations to several parameters at sub-individual and individual levels of prawn exposed to fenitrothion, such as inhibition of swimming velocity eye AChE activity and muscle ChE activity. In addition, results showed that prawn had the ability to escape from fenitrothion during the avoidance tests developed for this marine species, which is an ecologically relevant parameter. However, prawn seem to lose the ability to avoid the highest concentration of fenitrothion, even when the activity of IDH was increased, suggesting that increased energy production resulting from the aerobic pathwaywas being used for detoxification. Results also showed that energy production resulting from the anaerobic pathway was impaired on prawn exposed to the highest concentration of fenitrothion tested, since muscle LDH was inhibited.

Acknowledgements

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Chapter III

Acute effects of deltamethrin on swimming velocity and biomarkers of the common prawn *P. serratus*

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Abstract

The main purposes of this study were to investigate the effects of deltamethrin on biomarkers and behaviour of *P. serratus*, since this attempt to link different levels of biological organization will allow determine which biomarkers might be ecologically relevant and will be useful to complement the information about the effects of pesticides by using behavioral parameters. Therefore, liver antioxidant status, energetic metabolism and neurotransmission were determined in different tissues of the common prawn, and used to assess the effects at sub-individual level, whereas swimming velocity was used to assess the effects at the individual level. It was also investigated if the swimming velocity can be used as an endpoint in ecotoxicology bioassays and if it can be as sensitive as biomarker endpoints.

Swimming velocity was significantly inhibited in prawn exposed to deltamethrin, showing a LOEC of 0.6 ng/L. Eye AChE activity was significantly increased in prawns exposed to 0.6, 1.2 and 2.4 ng/L deltamethrin, whereas muscle ChE activity was significantly increased in prawn exposed to 19 and 39 ng/L. In the other hand, LDH was significantly increased in muscle of prawns exposed to 0.6, 1.2, 2.4, 4.8 ng/L deltamethrin, showing that animals are requiring additional energy, probably using it for detoxification processes. Glutathione S-transferase activity was significantly increased in digestive gland of common prawn exposed to 19 and 39 ng/L deltamethrin. Catalase (CAT) activity increased in digestive gland of prawn exposed to 19 ng/L deltamethrin. However, CAT activity decreased in digestive gland of prawn exposed to 39 ng/L, suggesting a failure in the antioxidant defense. Moreover, lipid peroxidation (LPO) was significantly increased in digestive gland of prawn exposed to 39 ng/L. Thus, global results showed that decreased swimming velocity was not associated with cholinesterase inhibition. In fact, the impairment of swimming velocity may be due to allocation of energy for detoxification. Swimming velocity can be used as an ecologically relevant tool and sensitive endpoint to assess and complement the study of the effects of pesticides on marine organisms.

Introduction

Pyrethroids are a class of neurotoxic pesticides which use has continuously increased during the last two decades (Amweg *et al.*, 2005; Oros and Werener, 2005; Meacham *et al.*, 2008; Wolansky and Harrill, 2008). These compounds are derivatives and synthetic analogues of natural pyrethrins (neurotoxins) of the flowers of genus Chrysanthemum (Casida, 1980; Soderlund *et al.,* 2002; Hossain *et al.,* 2005; Wolansky and Harrill, 2008). Based on structure-activity and symptomology following acute intoxication, pyrethroids can be categorized in

two groups: type I, which don't have a cyano group, and it's known to induce a tremor syndrome (eg. allethrin, pyrethrin); and Type II, which have a cyano group in the α-position and induce choreoathetosis and salivation (eg. cypermethrin, deltamethrin) (Verschoyle and Aldridge, 1980; Soderlund *et al.,* 2002; Nasuti *et al.,* 2003; Hossain *et al.*, 2005; Meacham *et al.,* 2008; Anadón *et al.*, 2009). Both types act mainly on the nervous system, especially on the voltage-dependent sodium channel of excitable membranes, inducing a prolongation of the sodium current during excitation caused by membrane depolarization (Narahashi, 1992).

Deltamethrin, one of the most toxic type II pyrethroid, (Sánchez-Fortún and Barahona, 2005; Velíšek *et al.,* 2007; Dorts *et al.,* 2009) has been used as an alternative pesticide in animal health, in vector control and in public health (Dorts *et al.*, 2009). Some studies showed behaviour impairments at lower dose ranges of pyrethroids, in fact, Wolansky and Harril (2008) describe a large database of behavioural alterations after pyrethroid exposure, although, mostly of them with mammals. However, there is a need to evaluate the effects of these pesticides in aquatic animals, since pyrethroids are spread to aquatic environments by agricultural and urban runoff from rainstorms (wang *et al*., 2009). According to Wolansky and Harril (2008), motor function is impaired by all pyrethroid compounds regardless of species and that the motor activity is the most extensively characterized neurobehavioural endpoint for pyrethroid effects. Thus, the study of behavioural impairments after exposure is of considerable interest, particularly, swimming behaviour, which is considered a main character determining survival in many species of aquatic animals (Plaut, 2001). Swimming velocity is perhaps the most frequently used behavioral endpoint used on aquatic organisms (García de la Parra *et al.*, 2006; Gravato and Guilhermino, 2009; Lange *et al.*, 2009; Roast *et al.*, 2000a, b; Roast *et al.*, 2001; Zhang *et al.*, 2006). Even at non-lethal concentrations, there are significant behavioral changes in aquatic invertebrates exposed to pyrethroids, which may affect their survival (Sánchez-Fortún and Barahona, 2005). In addition, these compounds are highly toxic to aquatic invertebrates, being the LC_{50} values less than 1 ppb (Sánchez-Fortún and Barahona, 2005). However, only a few studies investigated pollutant-induced alterations in swimming performance of crustaceans (Roast *et al.*, 2000a, b; Wallace and Estephan, 2004).

Other endpoints that may provide specific information on the mechanism of pyrethroid toxicity should be used in conjunction with motor activity when determining the relationship between internal dose, target tissue level, and adverse neurobehavioural effects, to generate relevant information for estimating hazard effects (Wolansky and Harril, 2008). Biomarkers are based on physiological and biochemical parameters of perturbations which persist after

exposure and have been widely used to assess the exposure of organisms to a range of chemicals in the environment (Van der Oost, 2003; Badiou *et al.*, 2008). Among the most commonly used biomarkers, those associated to oxidative stress are important (Dorts *et al.*, 2009), since the mechanisms of toxicity for most pesticides, including pyrethroids, are the stimulation of free radical production, induction of lipid peroxidation, and disturbance of the total antioxidant capability of the body (Mohammad *et al.*, 2004). A suite of biochemical defense mechanisms called the antioxidant defense system is found in aquatic organisms to prevent cellular damage from reactive oxygen species. Both enzymatic and non-enzymatic antioxidants counteract the deleterious action of reactive oxygen species and protect from cellular and molecular damage (Livingstone, 2001).

Energetic metabolism is also used to assess the effects of pollutants on aquatic organisms (Sancho, *et al.*, 1997, 1998; Lee *et al.*, 2002; Rao, 2006; Lima *et al.*, 2007), since additional energy for detoxification may be needed to maintain physiological or biochemical functions at a normal level (Choi *et al.*, 2001).

Acetylcholinesterase (AChE) is an enzyme responsible for the rapid degradation of acetylcholine at the cholinergic synapses and so allowing precise control and modulation of the neural transmission. It is used for identifying exposure to anticholinesterase chemicals, like organophosphate and carbamate pesticides (Badiou *et al*., 2008). However, only a few studies reported effects of pyrethroids on AChE, and there are contradictory results (Bálint *et al*., 1995; Hossain *et al*., 2004, 2005; Velíšek *et al*., 2006, 2007; Badiou *et al*., 2008; Elhalwagy and Zaki, 2009). In addition, inhibition of swimming velocity may be associated with inhibition of AChE activity (Bálint *et al.*, 1995). Therefore, there is a need to evaluate and elucidate the effects of pyrethroids on organisms, namely on marine crustaceans.

Prawns are good indicators of aquatic contamination, because their biochemical stress responses are quite similar to those found in other crustaceans and invertebrates species (Vijayavel and Balasubramanian, 2009). The common prawn, *Palaemon serratus*, is a crustacean with a large distribution (occurring along the Northeastern Atlantic coast to the Mediterranean, Black sea and Mauritanian coast), is abundant and easy to capture (Frasco *et al.*, 2006).

The main purposes of this study were to investigate the effects of deltamethrin on biomarkers and behaviour of *P. serratus*, since this information with marine organisms is still scarce, and establish possible associations between the parameters determined at different levels of biological organization. This approach will allow to determine which biomarkers might be ecologically relevant (if they are linked to alterations of behavior) and to

demonstrate if behavioral parameters can be useful to complement the information obtained with biomarkers about the effects of pesticides In that context, it was also investigated if behavior can be used as endpoint for ecotoxicology bioassays and if this behavioural endpoint can be as sensitive as biomarker endpoints. Swimming velocity was used as behavioral endpoint and eye AChE and muscle ChE activities; muscle isocitrate dehydrogenase (IDH) and lactate dehydrogenase (LDH); and digestive gland gluthathione-S-transferase (GST), gluthathione peroxidase (GPx) and catalase (CAT), were used to assess the effects on biomarkers. Lipid peroxidation was also determined as a marker of oxidative damage.

Material and Methods

Chemicals

(S)-α-cyano-3-phenoxybenzyl (1R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate(deltamethrin, minimum 98%), is an analytical standard grade purchased from Sigma-Aldrich Chemical Corporation. All other chemicals were of analytical grade and were obtained from Sigma-Aldrich and Merck.

Prawn capture and maintenance

P. serratus (common prawn) specimens were captured at Praia Norte of Viana do Castelo (Northwest of Portugal) using an hand operated net at low tide. Selected specimens were transported to laboratory and allowed to recover for 15 days in 200 L tanks with filtered and aerated saltwater. Prawns were maintained at 18°C with a 14 h:10 h (light:dark) photoperiod and fed three times per week with commercial food. Water renewal and determination of abiotic parameters (salinity, temperature, pH and dissolved oxygen) was made once per week.

Acute bioassay

After recovery, the animals were individually exposed to deltamethrin (0.6, 1.2, 2.4, 4.9, 9.8, 19.5, 39, 78, 156 and 313 ng/L) for 96 hours in 700 mL of each test solution. Test concentrations of deltamethrin were prepared by dilution of a stock solution (10 mg/L) prepared in dimethyl sulfoxide (DMSO). Two more conditions were used: a control and a control with the solvent by adding the same volume of DMSO used on each exposure flask, i.e. 28 µL (control+DMSO). Prawn with 0,65±0,03 cm long, weighting 2,78±0,04 g, were used in the bioassay. Animals were not fed 2 days before the exposure and during the exposure

periods. Ten replicates were used per treatment. The bioassay was carried in a room with controlled photoperiod (14 h light: 10 h dark) and temperature (18°C). Abiotic parameters (salinity, temperature, pH and dissolved oxygen) were monitored daily during the exposure period.

Swimming velocity

After 96 h of exposure, each prawn was transferred, one by one, to a 4 m long track race with 12 cm diameter and allowed to swim against a water flow of 7 L/min. The swimming velocity (m/s) was calculated as the quotient between the covered distance and the time of swimming (Gravato and Guilhermino, 2009). After swimming, each prawn was allowed to recover during two hours on the respective exposure aquarium.

Assessment of biomarkers

After 2 h recovery, body weight and length were determined and prawns were immediately euthanized by decapitation. Digestive gland, liver and muscle were immediately isolated and stored at -80°C until further analysis.

Eye and a portion of muscle were homogenized in 1 ml of ice-cold k-phosphate buffer (pH 7.2, 0.1 M) and centrifuged at 3300 *g* for 3 min at 4°C. The resulting supernatant from eye and muscle samples was used to measure ChE by Ellman's technique (Ellman *et al.*, 1961) adapted to microplate by Guilhermino *et al.* (1996). No distinction was made between different forms of ChE and acetylthiocholine was used as substrate in all the assays. *P. serratus* eye contains mainly AChE activity (Frasco *et al.*, 2006) and thus, the enzymatic activity measured in the eye was considered AChE activity, while in the muscle the activity was designated as ChE activity. The supernatant from muscle samples was also used to determine IDH activity, by measuring the increase of NADPH at 340 nm, according to Ellis and Goldeberg (1971) adapted to microplate (Lima *et al.*, 2007).

Another portion of muscle was used to analyze LDH activity. For LDH determination, samples were submitted to a cycle of 3 freezing and thawing and homogenized in 1 ml of ice-cold Tris/NaCl phosphate buffer (pH 7.2, Tris 81.3 mM; NaCl 203.3 mM) and centrifuged at 3300 *g* for 3 min at 4°C. The resulting supernatant was collected and LDH activity determined by measuring the amount of pyruvate consumed due to NADH oxidation at 340 nm according to Vassault (1983) adapted to microplate (Diamantino *et al.*, 2001).

Digestive gland was homogenized in phosphate buffer (pH 7.2, 0,1M). Part of the homogenate was used to determine the LPO by measuring the thiobarbituric acid reactive species

(TBARS), according to Ohkawa (1979) and Bird and Draper (1984), with the adaptations of Filho *et al.* (2001) and Torres *et al.* (2002). LPO was expressed in nmol TBARS per g wt. The remaining homogenate was centrifuged at 10000 g for 20 min at 4°C to isolate the post-mitochondrial supernatant. The PMS was used for determinations of GST, GPx and CAT activities. GST activity was quantified by the conjugation of reduced gluthathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig *et al.*, 1974) adapted to microplate (Frasco and Guilhermino, 2002). GPx activity was determined by measuring the decrfease of NADPH at 340 nm using H_2O_2 as substrate (Flohe and Gunzler 1984). CAT activity H_2O_2 consumption at 240 nm according to Clairborne (1985) was used to determine CAT activity.
All enzymatic activities are expressed as the amount of substrate hydrolyzed per min per mg protein. Protein quantification of all samples was determined according to Bradford (1976), adapted to microplate as indicated in Guilhermino *et al.* (1996), using bovine γ-globulins as protein standard and a wavelength of 600nm. All proceedings were performed at a constant temperature of 25ºC.

Statistical Analysis

LC_{50} and 95% confidence limits were calculated with Probit Analysis using SPSS 16.0 software.

All data were first analysed by Kolmogorov-Smirnov and Bartlett's tests to check normality and homoscedasticity, respectively. Appropriated data transformations were made whenever necessary. Then, one-way analysis of variance (ANOVA) was used to compare different treatments. When significant differences among treatments were found, the Dunnett's test was used to determine the values of the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). For all data, outlier values (more than 3.5 standard deviations from the group mean) were removed. Statistical level was 0.05 and SPSS 16.0 package was used for statistical analysis.

Results

The 96 h LC_{50} value estimated for common prawn exposed to deltamethrin was 48.40 ng/L ($CI_{95\%}$ 34.05 - 67.44 ng/L).

The swimming velocity of common prawn exposed to deltamethrin was significantly decreased after 96 h exposure compared to control+DMSO ($F_{(8;83)=}15.607$; p=0.000), showing a LOEC value of 0.6 ng/L (-58% inhibition) and reaching 87% of inhibition at 39 ng/L (Figure 1).

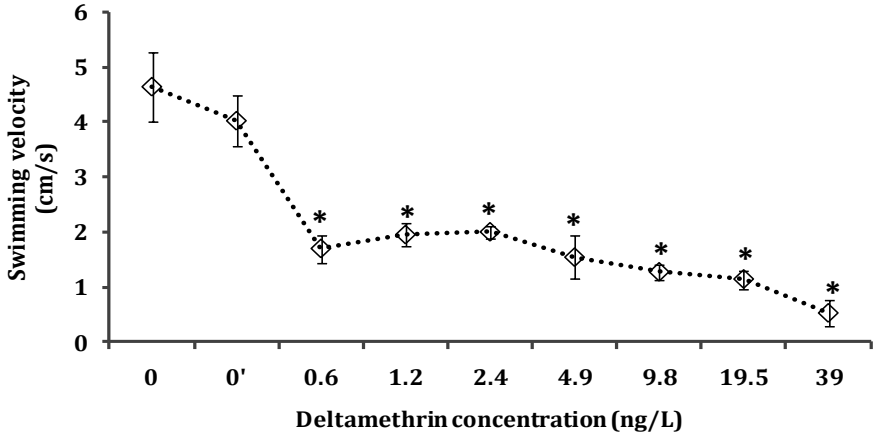

Figure 1. Swimming velocity of *Palaemon serratus* exposed to 0.6 – 39 ng/L deltamethrin for 96 hours. Values are the mean of 10 prawns per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences (p<0.05, Dunnett test) relatively to control (control+DMSO).

Eye AChE activity was significantly increased ($F_{(8;77)}$=3.613; p=0.001) in prawn exposed to 0.6, 1.2 and 2.4 ng/L deltamethrin when compared to control+DMSO, with increases of 78, 66 and 100 %, respectively (Figure 2A). Muscle ChE activity was significantly increased ($F_{(8;80)}$=2.662; p=0.013) in prawn exposed to 19.5 and 39 ng/L deltamethrin compared to control+DMSO, with inductions of 73 and 81%, respectively (Figure 2B).

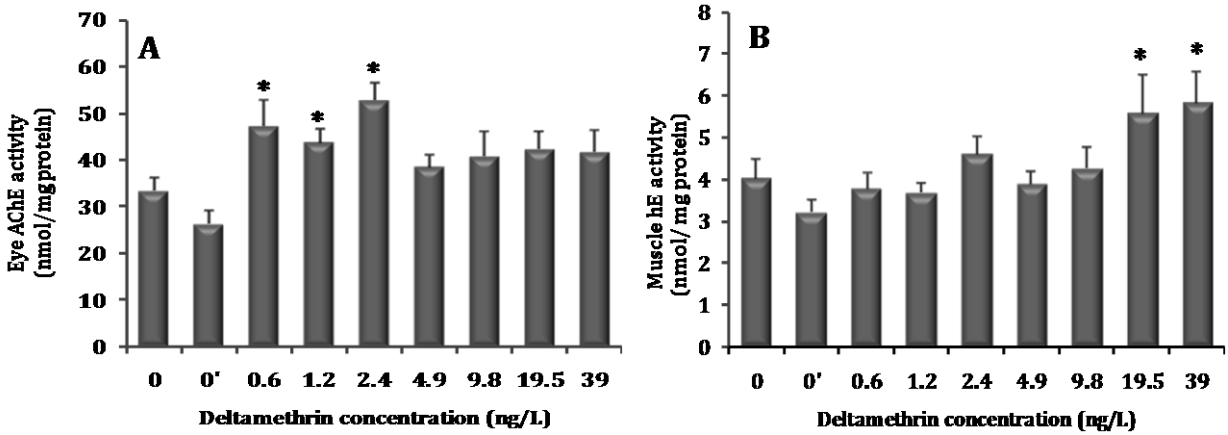

Figure 2. Eye AChE (A) and muscle ChE (B) activities of *P. serratus* exposed to 0.6 – 39 ng/L deltamethrin for 96 hours. Values are the mean of 10 prawns per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences (p<0.05, Dunnett test) relatively to control (control+DMSO).

LDH activity was significantly increased ($F_{(8,80)}$=27.326; p=0.000) in muscle of prawn exposed to 0.6 (283% increase), 1.2 (300% increase), 2.4 (374% increase) and to 4.9 ng/L (340% increase) deltamethrin (Figure 2A) compared to control+DMSO. IDH activity was not significantly altered ($F_{(8,81)}$=2.139; p=0.043) in muscle of prawn exposed to deltamethrin (Figure 2B).

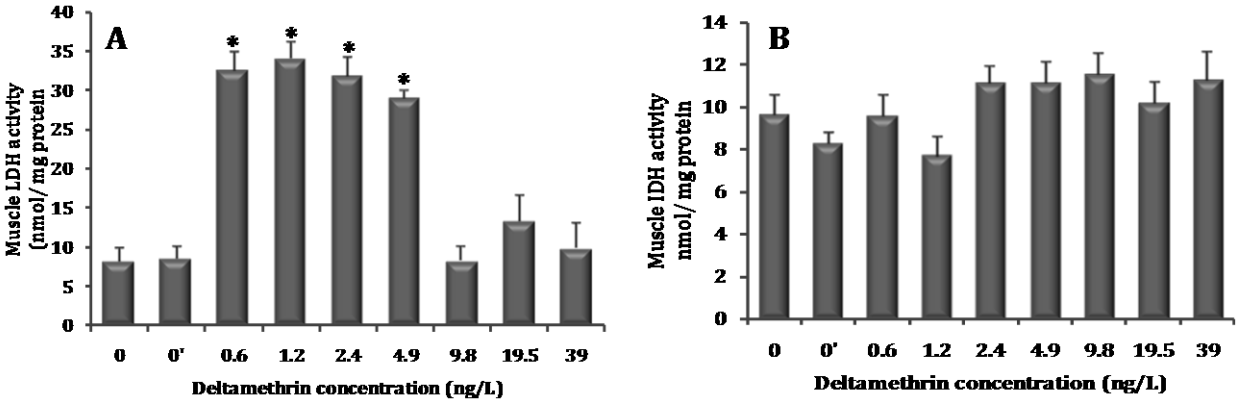

Figure 3. Muscle LDH (A) and IDH (Ba ctivities of *P. serratus* exposed to 0.6 – 39 ng/L deltamethrin for 96 hours. Values are the mean of 10 prawns per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences (p<0.05, Dunnett test) relatively to control (control+DMSO).

GST activity was significantly increased ($F_{(8,82)}$=4.210; p=0.000) in digestive gland of prawn exposed to 19.5 and 39 ng/L deltamethrin compared to control+DMSO, with inductions of 221 and 255% respectively (Figure 3A). CAT activity was significantly increased ($F_{(8,80)}$=2.389; p=0.024) in digestive gland of prawn exposed to 19.5 ng/L deltamethrin, showing an increase of 118% when compared to control group (Figure 3B). No significant differences ($F_{(8,74)}$=0.732; p=0.663) were found for GPx activity in digestive gland of prawn exposed to deltamethrin (Figure 3C). Level of LPO was significantly increased ($F_{(8,73)}$=2.546; p=0.018) in digestive gland of prawns exposed to 39 ng/L deltamethrin, reaching almost 90% increase compared to control+DMSO (Figure 3D).

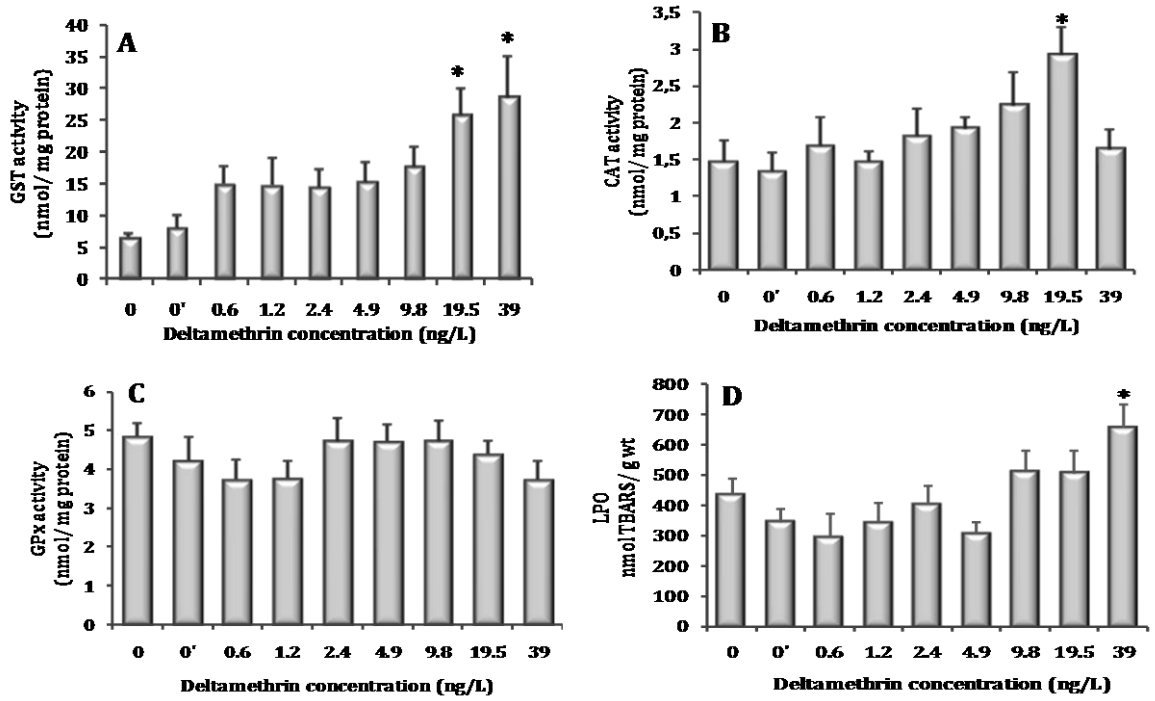

Figure 4. Digestive gland GST (A), CAT (B), and GPx (C) activities and LPO levels (D) of *P. serratus* exposed to 0.6 – 39 ng/L deltamethrin for 96 hours. Values are the mean of 10 prawns per treatment with the corresponding standard error bars. 0 and 0' represents control only with saltwater and control with solvent (control+DMSO), respectively. * indicates statistical significant differences ($p<0.05$, Dunnett test) relatively to control (control+DMSO).

Discussion

The 96h LC_{50} value for *P. serratus* exposed to deltamethrin was estimated as 48.4 ng/L ($CI_{95\%}$ 34.05 - 67.44 ng/L). The range of LC_{50} value estimated in the present work is in agreement with a previous research report showing that 96h LC_{50} for *Paratya australiensis* exposed to deltamethrin was 12 ng/L in artificial saltwater and 37 ng/L in river water (Thomas *et al.,* 2008). Thomas and co-workers (2008) observed that crustacean species, namely *P. australiensis and Ceriodaphnia cf. dubia*, were more sensitive to deltamethrin than larvae of the rainbowfish (*Melanotaenia duboulayi*). Moreover, Viran and co-workers (2003) observed that the 48h LC_{50} value for guppies (*Poecilia reticulata*) exposed to deltamethrin was 5.01 µg/L and Velíšek *and co-workers* (2007) observed that the 96h LC_{50} value for rainbow trout (*Oncorhynchus mykiss*) exposed to deltamethrin was 20 µg/L. Current results showed that *P. serratus* is more sensitive to deltamethrin than fish species. Thus, the present result is in

agreement with the outcomes of the risk assessment by Solomon *et al.* (2001) that identified crustaceans as the most sensitive animals of the aquatic fauna tested.

This research study showed that deltamethrin decreased the swimming velocity of exposed prawn. This finding is in good agreement with recent studies. For example, Christensen *et al.* (2005) found that cypermethrin inhibited the ability of *Daphnia magna* to swim. Nørum *et al.* (2010) also observed that 100 ng/L lambda-cyhalothrin induced immobilization in *Leuctra nigra*, *Gammarus pulex* and *Heptagenia sulphurea.*

Reduction in crustaceans swimming ability following toxicant exposure has been reported previously with other chemicals (such as metals, like cadmium and organophosphate pesticides, like chlorpyrifos) (Roast *et al.*, 2000a,b; Wallace and Estephan, 2004). The impairment of swimming velocity may have serious consequences for macroinvertebrates since the ability of individuals to acquire food or mates or to avoid predators may be reduced (Nørum *et al.*, 2010) and, thus, swimming velocity seems to be an ecologically relevant parameter. In addition, high sensitivity, simple testing procedures, and reproducibility across studies, make measurements of motor activity an endpoint with advantages for use in the assessment of pyrethroid neurotoxicity (Wolansky and Harril, 2008).

Pyrethroids is known to act mainly on the voltage-dependent Na+ channels of the nerve cell membrane, although, studies have demonstrated that they may have secondary effects on the cholinergic system, particularly on AChE activity (Hossain *et al.*, 2004, 2005; Velíšek *et al.*, 2007; Badiou *et al.*, 2008). In the present study eye AChE and muscle ChE activities were significantly increased on prawn exposed to deltamethrin. A previous study with bees exposed to deltamethrin (Badiou *et al.* 2008) showed an increased AChE activity in bees head and suggested that deltamethrin increases the release of ACh in hippocampus (Hossain *et al.*, 2004) leading to increased AChE activity. Other studies performed mostly in fish and rats showed contradictory results concerning pyrethroid effects on AChE activity (Bálint *et al.*, 1995; Hossain *et al.* 2004, 2005; Velíšek *et al.*, 2007; Badiou *et al.*, 2008; Badiou and Belzunces, 2008; Elhalwagy and Zaki, 2009). Nevertheless, Bálint *et al.* (1995) suggested that carp (*Cyprinus carpio*) decreased swimming velocity was associated with inhibition of brain and muscle AChE activity. However, the current study showed a decreased swimming velocity of prawn exposed to deltamethrin despite the increased activities of eye AChE and muscle ChE, .

Concerning energetic metabolism, no significant effects were found in muscle IDH activity showing that animals exposed to deltamethrin were not getting energy from the aerobic pathway. However, LDH activity was significantly increased in prawns exposed to 0.6 up to

4.8 ng/L deltamethrin demonstrating metabolic changes induced by the pesticide. LDH activity is particularly important when a considerable amount of additional energy is rapidly required (Diamantino *et al*., 2001), which suggests that in the case of prawn exposed to those concentrations (0.6 up to 4.8 ng/L) need energy for detoxification, because the ability to resist a toxicant may be expensive in terms of energy, a decrease in the energy available for other biological processes might be compromised (Choi *et al*., 2001; Amiard- Triquet, 2009). Therefore, the impairment of swimming velocity of prawn exposed to low concentrations of deltamethrin may be due to insufficient energy. For the concentrations of deltamethrin higher than 4.8 ng/L the results showed that LDH activity was not increased which suggests that energy was not sufficient even for detoxification since oxidative damage was observed in digestive gland of prawn exposed to deltamethrin. Furthermore, the increased activities of GST in digestive gland of prawn were not sufficient to cope with the oxidative stress induced by high concentrations of delatamethrin, since the levels of LPO were significantly increased. In previous works with prawns, Dorts *et al.* (2009) and Vijayavel and Balasubramanian (2009) observed an increased LPO in *P. monodon* exposed to deltamethrin and fenvalenate, respectively. Moreover, in a previous study, it was observed that high doses of a type I (permethrin) and a type II (cypermethrin) pyrethroids induced a significant increase of oxidation index in plasma membrane of erythrocyte from rats (Nasuti *et al.* 2003). Tuzmen *et al.* (2008) and Yousef *et al.* (2006), also observed an increase of LPO levels in liver and plasma of rats exposed to deltamethrin. Therefore, LPO has been suggested as one of the molecular mechanisms involved in general pesticides, particularly pyrethroids induced toxicity (Mohammad *et al.*, 2004; Elhalwagy and Zaki; 2009; Vijayavel and Balasubramanian, 2009).

In fact, it is documented that pesticides may induce oxidative stress due to the generation of free radicals (Yousef *et al.*, 2006; Tuzmen *et al.*, 2008; Dorts, *et al.*, 2009; Elhalwagy and Zaki, 2009). To mitigate and repair damage caused by free radicals, organisms have evolved complex enzymatic antioxidant systems, such as the enzymatic activities of GPx and CAT (referencias). Concerning GPx activity, no significant effects were observed in digestive gland of prawn exposed to deltamethrin. However, it was observed that deltamethrin induced CAT activity in digestive gland of prawn demonstrating an increase of hydrogen peroxide levels. For the highest concentration of deltamethrin tested, CAT activity was not increased in digestive gland of exposed prawn, suggesting a detoxification failure since oxidative damage was observed. Almost similar results were found in a previous work with *C. elegans* exposed

to cypermethrin, where it was observed a significant increased of CAT activity whereas GPx was significantly decreased (Shashikumar and Rajini 2010).

Conclusion

Deltamethrin caused a significant inhibition of the swimming velocity of *P. serratus.* LDH activity was significantly increased to the concentrations of deltamethrin equal or lower than 4.8 ng/L, whereas IDH activitiy were not affected. These results suggest that the impairment of the swimming velocity may be due to an energy default, since energy seems to be used to resist the toxicant. For concentrations of deltamethrin higher than 4.8 ng/L, the results suggest that detoxification failure occurred since oxidative damage was observed, the energy production was impaired and swimming velocity was decreased. Since the swimming velocity is essential for the performance and survival of prawns, this parameter should be considered as an ecologically relevant endpoint. In addition, the LOEC for deltamethrin determined for swimming velocity was similar to the one observed for muscle LDH, which showed that this behavioural endpoint can be used as sensitive tool to assess the effects of pollutants. Furthermore, the present work complemented the knowledge about the effects of deltamethrin on biomarkers and behavior of a marine species.

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Chapter IV

General Discussion and Final Considerations

The present study aimed to investigate the acute effects of fenitrothion, an organophosphate pesticide, and of deltamethrin, a pyrethroid pesticide, in the common prawnusing parameters at different levels of biological organization. Deltamethrin was about 20 times more toxic to prawn than fenitrothion, since LC_{50} values estimated were about 50 ng/L and 1050 ng/L, respectively. This finding is in good agreement with previous results that indicated that pyrethroid pesticides (cypermethrin and resmethrin) were more toxic to *Triops longicaudatus* than organophosphate pesticides (chlorpyrifos and fenthion) (Walton *et al*., 1990). Moreover, current results with common prawn exposed to the sub-lethal concentrations of both pesticides showed several alterations on biomarkers and swimming velocity (Table 1). These results also showed that deltamethrin was more toxic to the common prawn than fenitrothion. In fact, both pesticides impaired the swimming velocity of exposed prawn but the LOECs determined were 313 ng/L for fenitrothion and 0.6 ng/L for deltamethrin. Furthermore, deltamethrin seems to interfere more with the swimming capability of prawn, since it was observed an inhibition of the swimming velocity between 50 and 90%, whereas maximum inhibition observed for fenitrothion was only 40%. The impairment of the swimming velocity may have serious consequences for aquatic organisms, since their capability to escape from predators, capture preys or find a partner may be reduced (Weis *et al*. 2001; Scott and Sloman 2004). Thus, swimming velocity is an ecologically relevant parameter, and this research work showed that it can be used as an endpoint to assess the effects of pesticides.

It was previously reported that several physiological and behavioural processes are affected due to the inhibition of ChE activity, potentially affecting the organism's survival capabilities (García-de la Parra *et al*., 2006). In the present work, fenitrothion inhibited eye AChE and muscle ChE activities in exposed prawns, which was expected, since organophosphate pesticides are AChE inhibitors. The results suggest that inhibition of eye AChE and muscle ChE activities are associated with the impairment of the swimming velocity of common prawn. This is in good agreement with previous studies reporting associations between the inhibition of AChE and ChE activities on animals exposed to organophosphate pesticides and the impairment of swimming velocity, swimming distance or spontaneous swimming speed (Beauvais *et al*., 2001; Brewer *et al*., 2001; Sandahl *et al*., 2005).. Studies concerning pyrethroids' effects in ChE activity were contradictory, since in some cases it was observed an inhibition of this activity (Hossain *et al*., 2004, 2005; Velíšek *et al*., 2007, Badiou *et al*., 2008), whereas in other cases this activity increased (Badiou *et al*., 2008). In the present work, eye AChE and muscle ChE activities of common prawn exposed to deltamethrin were

increased. Badiou and co-workers (2008) suggested two mechanisms that might explain this increase of activity: the release of ACh, may induce a regulatory overcompensation by increasing AChE in the cholinergic system; or the increase of AChE may be due to protein synthesis, which is consistent to an effect of pyrethroids on signal transduction. The present results showed that fenitrothion and deltamethrin had opposite effects on ChE activities of exposed prawn. Thus, swimming velocity inhibition may only be linked to the inhibition of ChE observed on prawn in the case of the fenitrothion exposure, but not deltamethrin.

Tabela 1. Changes in several parameters determined in the common prawn exposed to fenitrothion (39 – 625 ng/L) and to deltamethrin (0.6 – 39 ng/L) for 96 hours

	Fenitrothion concentration (ng/L)					Deltamethrin concentration (ng/L)						
	39	78	156	313	625	0.6	1.2	2.4	4.9	9.8	19.5	39
Lipid peroxidation			+							+	+	++*
Glutathione-S-Transferase			+		+	++	++	++	++	+++	+++*	+++*
Catalase	–	–	+		–	+		+	+	++	+++*	+
Glutathione peroxidase			+									
Acetylcholinesterase		–*	–*	–*	–*	++*	++*	+++*	+	++	++	++
Cholinesterase			–*	–*	–*			+	+	+	++*	++*
Lactate dehydrogenase	+					+++*	+++*	+++*	+++*		+	
Isocitrate dehydrogenase								+	+	+	+	+
Swimming velocity		–		–*	–*	– –*	– –*	– –*	– –*	– –*	– –*	– –*

+++ more than 100% increase; ++ 50–100% increase; + 20–50% increase; no symbol means similar to control with DMSO; – 20–50% decrease; –– more than 50% decrease. Asterisk indicates significant changes between a treatment group and control with DMSO (p <0.05).

Results also showed that only deltamethrin induced oxidative stress in digestive gland of common prawn. This is in agreement with a previous work with *Penaeus monodon* exposed to endosulfan (organophosphate pesticide) and deltamethrin, showing that only deltamethrin induced oxidative damage on shrimps (Dorts *et al*., 2009). Several other studies comparing the effects of organophosphate and pyrethroid pesticides in the responses of antioxidant

enzymes have been performed (Tuzmen *et al*., 2007; Dorts *et al*., 2009; Elhalwagy and Zaki, 2009). However, the results observed showed wide differences in terms of the effects of pesticides. The present results showed that fenitrothion and deltamethrin induced differently the enzymatic activities determined in the digestive gland of *P. serratus*. Deltamethrin significantly increased consistently the activities of GST and CAT, whereas fenitrothion promoted an opposite effect on the activity of CAT despite the increase observed for 156 ng/L fenitrothion. Therefore, the difference in biological responses could allow discriminating both pesticides if these responses are used as biomarkers, but further research will be needed.

In the present work it was also observed that fenitrothion had no significant effects in muscle LDH and IDH activities of common prawn. On the other hand, deltamethrin significantly increased muscle LDH and IDH activities of common prawn, suggesting that organisms are in need of energy to resist the toxicant, because the supply of extra energy may be essential to overcome toxic stress (Moreira *et al*., 2006). It's interesting to note that LDH and IDH activities were increased in prawn exposed to deltamethrin, and still, the swimming velocity was impaired. This suggests that prawn could be using energy to detoxify and not using it for other biological processes as also suggested by Amiard-Triquet (2009).

Concerning the avoidance test, the results showed that prawn avoided the concentration of 78 ng/L fenitrothion as soon as 1 hour after exposure. Avoidance can be detrimental because organisms may be displaced from preferred habitats to suboptimal areas where they may face greater competition and predation pressure or inadequate resource availability (Little, 2002). Since it is a highly relevant response, and is quickly and easily measurable, the avoidance assay should be recommended as a complementary tool in ecological risk assessment. However, prawns seem to lose the capability to avoid the pesticide for the highest concentrations. This represents a problem for the avoidance test itself, because it cannot be used for the highest concentrations of fenitrothion. Nevertheless, this lost of capacity to avoid seems to be of interest to understand why prawn do not escape the highest concentrations of fenitrothion and further research on this subject would be needed and desirable. To understand this lost of capability to avoid the pesticide, prawn were forced to be exposed to fenitrothion using the same experimental design than the avoidance test. Results showed that there were no significant changes in eye AChE and muscle ChE activities, which suggest that those activities were not involved in the inhibition of the avoidance behaviour. However, enzymes involved in energy production were significantly increased in muscle of common prawn exposed to fenitrothion, particularly IDH activity, which was

clearly and significantly increased for the group of prawn that could not avoid the pesticide. This result may confirm the fact observed on prawn exposed to deltamethrin. Organisms may be using the energy and extra energy to resist the toxicant, giving preference to that, instead other biological processes, such as escaping from the pesticide or even swimming.

The results in the present work showed that the common prawn behaviour can be used as effect criteria in bioassays, since both pesticides induced effects on their behaviour, which was not observed for all biochemical biomarkers. In addition, concerning the sensitivity of the parameters tested, the swimming velocity was found to be as sensitive as LDH and eye AChE activities (LOEC =0.6 ng/L) in the deltamethrin bioassay, and thus it can be considered that this behavioural response has an ecologically relevant value and sensitivity. However, results from the fenitrothion exposure showed that eye AChE was more sensitive (LOEC=78 ng/L) than swimming velocity (LOEC=313 ng/L) demonstrating the specificity of this biomarker in relation to exposure anti-cholinesterase agents. In relation to the avoidance behaviour (LOEC=78 ng/L), it seems to be as sensitive as eye AChE activity. Further research comparing the sensitivities of behavioural tests and biomarkers would be of great interest, specially using other classes of environmental contaminants, in order to validate the use of behavioral tests since they give important and complementary information on the effects induced by toxicants on marine organisms.

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