Universidade de Aveiro Departamento de Biologia
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Doutor Marco Filipe Loureiro Lemos, Professor Adjunto do Instituto Politécnico de Leiria, e do Professor Doutor Bart Devreese, *Full Professor, Department of Biochemistry and Microbiology, Ghent University*

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palavras-chave

Invertebrados de água doce, *Chironomus riparius*, pesticidas, parâmetros da história de vida, marcadores bioquímicos, ecotoxicoproteómica, diferentes níveis de organização biológica, efeitos sub-letais.

resumo

O uso de pesticidas em campos agrícolas resulta na inevitável contaminação dos sistemas de água doce adjacentes, representando uma séria ameaça para as comunidades de invertebrados aquáticos não alvo. O estudo do impacto destes compostos em espécies ecologicamente relevantes é crucial para a avaliação de risco. Tradicionalmente, os testes ecotoxicológicos baseiam-se em respostas ao nível do organismo e da população (ex. mortalidade, crescimento, comportamento e reprodução). No entanto, estas respostas observadas ao nível do organismo e população são usualmente precedidas por alterações nos níveis mais baixos de organização biológica. Nesse sentido, existe a necessidade de desenvolver ferramentas sensíveis que possam ser usadas para prever potenciais efeitos adversos ecológicos de concentrações sub-letais de inseticidas. A avaliação de efeitos ao nível subindividual pode assim fornecer informação prévia da exposição a pesticidas e os seus possíveis impactos em populações naturais.

Nesta tese, larvas da espécie modelo em ecotoxicologia *Chironomus riparius* (Meigen) foram expostas a quatro inseticidas com diferentes modos de ação: amitraz, spinosad, indoxacarb e fipronil, e os seus efeitos avaliados em termos de respostas do ciclo de vida utilizando testes ecotoxicológicos padronizados, e ao nível bioquímico monitorizando biomarcadores específicos de *stress* oxidativo, neurotoxicidade e metabolismo energético. Além disso, os efeitos do spinosad, indoxacarb e fipronil ao nível molecular foram avaliados usando ferramentas de proteómica, com o objetivo de determinar se a proteómica e os marcadores bioquímicos podem ser ferramentas sensíveis na avaliação de risco ecológico.

Os resultados aqui apresentados indicam que concentrações ambientalmente relevantes dos pesticidas testados, podem comprometer significativamente vários indicadores do ciclo de vida de *C. riparius*. Foram observadas reduções no crescimento larval e alterações nos parâmetros relacionados com a emergência dos insectos em resposta à exposição a todos os inseticidas testados, o que pode comprometer a integridade ecológica dos ecossistemas de água doce.

resumo (cont.)

Ao nível bioquímico, foram observadas respostas muito distintas para cada pesticida, provavelmente devido aos seus diferentes modos de ação. No entanto, foram observados indícios de elevados custos metabólicos (indicados pelo aumento das atividades da cadeia transportadora de eletrões (ETS) e/ou da enzima lactato desidrogenase (LDH) para todos os inseticidas. Estes aumentos estão provavelmente relacionados com a ativação de mecanismos de defesa antioxidantes e de processos de destoxificação. Além disso, foram observados indícios de dano oxidativo em larvas expostas a amitraz e spinosad, indicado pelo aumento nos níveis de peroxidação lipídica (LPO).

Ao nível do proteoma, não foram observadas alterações significativas nas larvas expostas a indoxacarb em comparação com larvas não expostas. A exposição ao fipronil causou alterações na expressão de globinas, de proteínas motoras e do citoesqueleto, bem como em proteínas envolvidas na síntese proteica. A exposição ao spinosad resultou em alterações na expressão de globinas, actinas e de proteínas da cutícula. Estas alterações observadas ao nível do proteoma revelaram potenciais mecanismos de ação que levam aos efeitos observados ao nível do organismo. O potencial da expressão das globinas de *C. riparius* em estudos de monitorização ambiental foi previamente afirmado e é aqui sustentado. O decréscimo generalizado observado na expressão destas proteínas sob exposição ao spinosad e ao fipronil pode estar relacionado com os efeitos tóxicos destes inseticidas.

Esta tese destaca a importância de complementar de uma forma integrada os ensaios ecotoxicológicos padronizados com ferramentas bioquímicas e moleculares. A análise de marcadores bioquímicos e do proteoma pode ser útil na avaliação de risco, contribuindo para o conhecimento dos efeitos sub-letais dos pesticidas, auxiliando na compreensão dos mecanismos que conduzem às respostas observadas nos níveis mais elevados de organização biológica. Este estudo revela também que os pesticidas testados representam um risco para os invertebrados aquáticos não alvo, e, portanto, a sua aplicação próxima de sistemas de água doce deve ser revista. *Chironomus riparius*, um organismo modelo em toxicologia aquática, é também aqui apresentando como um modelo promissor em estudos de proteómica ambiental.

keywords

Freshwater invertebrates, *Chironomus riparius*, pesticides, life-history endpoints, biochemical biomarkers, ecotoxicoproteomics, different levels of biological organization, sub-lethal effects

abstract

The application of pesticides in agricultural fields leads to inevitable contamination of adjacent freshwater systems, representing a serious threat to non-target aquatic invertebrate communities. The study of the impact of these stressors on ecologically relevant species is crucial for risk assessment. Traditionally, toxicity testing focuses on organism and population-level responses (e.g. mortality, growth, behavior, and reproduction). However, these responses are often preceded by changes at lower levels of biological organization. In this sense, there is a need to develop sensitive tools that can be used to predict ecological adverse effects of sub-lethal concentrations of pesticides. Assessing sub-organismal endpoints may therefore provide early indicators of pesticide exposure and their possible impacts on natural populations.

In this thesis, larvae of *Chironomus riparius* (Meigen) were exposed to four insecticides with distinct modes of action: amitraz, spinosad, indoxacarb, and fipronil, and their effects evaluated in terms of life-history responses using standard laboratory ecotoxicological tests, and at biochemical level by monitoring specific oxidative stress, neuronal, and energy metabolism biomarkers. Moreover, the effects of spinosad, indoxacarb, and fipronil were assessed at the molecular level using proteomic tools, to determine if proteomics and biochemical biomarkers can be used as reliable and sensitive tools in ecological risk assessment.

The results presented here indicate that environmentally relevant concentrations of the insecticides tested can significantly affect several *C. riparius* life-history traits, with reductions in the larval growth and impairment of emergence endpoints observed for all compounds tested, which ought to compromise the ecological integrity of freshwater ecosystems.

At the biochemical level, very distinct responses were observed for each pesticide, probably due to their distinct modes of action. Nonetheless, evidences of high metabolic costs (as indicated by the increase of electron transport system (ETS) and/or lactate dehydrogenase (LDH) activities) were observed for all insecticides, which are probably associated with the activation of antioxidant defenses and detoxification processes. Additionally, evidences of oxidative damage were found in *C. riparius* larvae under exposure to amitraz and spinosad, as indicated by the increase in lipid peroxidation (LPO) levels.

abstract (cont.)

At the proteome level, no significant changes were found in *C. riparius* proteome between exposed and non-exposed larvae for the concentrations of indoxacarb tested. Fipronil exposure induced alterations in the expression of globins, cytoskeleton and motor proteins, as well as in proteins involved in protein synthesis. Exposure to spinosad resulted in alterations in globins, actin, and cuticle proteins' expression. These changes observed at the proteome level revealed potential mechanisms of action that lead to the effects observed at the individual level. The potential of *C. riparius* globins expression in environmental monitoring studies has been previously stated and are here sustained. The generalized downregulation of these proteins observed under exposure to spinosad and fipronil may be related to the toxic effects of these insecticides.

This study highlights the importance of complementing standard ecotoxicological approaches with biochemical and molecular tools in an integrative manner. The analyses of biochemical biomarkers and of the proteome can be useful in risk assessment, contributing to the knowledge of the sub-lethal effects of pesticides, thus aiding the comprehensive and mechanistically understanding of the mechanisms that lead to higher level responses. It is also demonstrated that the pesticides tested here pose a potential risk to non-target aquatic invertebrates, and therefore their application near freshwater systems should be reviewed. *Chironomus riparius*, a model organism in aquatic toxicology, is also presented as a promising model organism for environmental proteomics.

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Chapter I

General introduction

General Introduction

1. Chemical contamination of freshwater ecosystems

Water is one of our most precious natural resources. Although freshwater lakes and river systems account for just roughly 0.26% of the total freshwater on earth (Scheffers and Kelletat, 2016; Shiklomanov, 2000), they represent a large source of biological diversity (Balian et al., 2008; Strayer and Dudgeon, 2010) being highly sensitive to contamination (Mensah et al., 2014; Schäfer et al., 2011; Villeneuve et al., 2011). In fact, contamination of aquatic environments goes back as far as the geologic formation of the planet, nonetheless industrial and anthropogenic activities have recently generated great contaminant inputs to these ecosystems (Mensah et al., 2014; Strayer and Dudgeon, 2010; Villeneuve et al., 2011; Vörösmarty et al., 2010).

In order to cope with population growth and production losses, there has been an increase of the use of pesticides in agriculture over the last decades (Aktar et al., 2009; Tilman et al., 2002; 2001). The Food and Agriculture Organization of the United Nations (FAO) estimates that almost 10 thousand tons of pesticides were used in Portugal in 2016, of which 570 tons were insecticides (FAO, 2018). The application of these compounds in agricultural fields often culminates in the contamination of nearby lakes or river systems due to runoff, spray drift, or leaching events (Cerejeira et al., 2003; Schäfer et al., 2011; Schulz, 2004). However, the potential risks posed by pesticides to non-target organisms and ecosystem functioning does not get much attention from the public until adverse consequences are evident (ex. losses of habitat) with potential impacts on human health and activities.

One major step towards raising awareness to the risks that agrochemicals pose to the environment and consequently to human health was the publishing of the book “Silent Spring” by Rachel Carson in 1962 (Bonaventura and Johnson, 1997; Carson, 1962; Matthews, 2017; Mnif et al., 2011), which led to the ban of Dichlorodiphenyltrichloroethane (DDT) in the United States (Grung et al., 2015; Mnif et al., 2011; Paull, 2013), and ultimately started a movement that led to the foundation of United States Environmental Protection Agency (EPA) (Paull, 2013). In recent years, several organizations raised awareness about the need on actions to halt biodiversity loss (e.g. the European Union (EU) biodiversity strategy to 2020 (EC, 2011a) and the World Wildlife Fund). Consequently, regulations were implemented to prevent impacts of “substances of very high concern” (eg. REACH regulation (EC, 2006a)). In 2000, the European Union established the Water Framework Directive (EC, 2000) for the protection of all ground and surface waters to reach a good ecological status" and "good chemical status", calling for the need to develop biomonitoring tools for the rapid assessment of the ecological status of water ecosystems. Moreover, the integrated pest management (IPM) concept became widespread to create a balance between economic threshold and

environmental safety (Koul and Cuperus, 2007; Matthews, 2017; Young, 2017). According to EPA, IPM programs "…use current, comprehensive information on the life cycles of pests and their interaction with the environment. This information, (…)is used to manage pest damage by the most economical means, and with the least possible hazard to people, property, and the environment" (Leslie, 1994). From an ecological safety point of view, one of the ultimate goals in IPM is to develop more pest-specific pesticides with minimum or no risk to non-target species, although this is challenging as most pesticides target biological mechanisms that are conserved in closely related species (Blümel et al., 1999; Schäfer et al., 2011). Nonetheless, even closely related species have different susceptibilities to the same chemical and the chemical may have secondary targets within the organism. Another key point is to determine the environmental fate of the pesticides, as some species regarded as pests in agricultural fields, may be crucial in the equilibrium of other ecosystems. In this sense, it is of extreme importance to assess the effects of novel pesticides on non-target key species, not only to evaluate their selectivity but also to determine the risk they pose to invertebrate freshwater communities and to the environment.

2. Eco(toxico)logical Risk Assessment and biomarkers

One of the most important stages in risk assessment is the estimation of the possible harmful or damaging effects of stressors on ecosystems (Chen et al., 2013). After the identification of a potential hazard (stressor), it is imperative to evaluate to what extent a particular ecosystem and the organisms living therein are exposed and affected by it. While it is important to determine the levels of a stressor in a particular ecosystem, analytical chemistry does not provide information on its toxicity. In this sense, ecotoxicological testing is essential for risk assessment to anticipate the actual biological effects and ecological damage due to the presence of a xenobiotic compound.

Traditionally, these effects are assessed in standardized laboratory conditions using test species cultured in laboratory. In aquatic toxicology, common test species include algae, invertebrates (e.g. crustaceans or insects) and vertebrates (e.g. fish or amphibians) that are representative for the receiving waters communities.

2.1 Chironomus riparius as a model organism in ecotoxicology and biomonitoring

Chironomidae (Insecta, Diptera), regularly referred to as non-biting midges, are particularly relevant invertebrates in lotic and lentic freshwater ecosystems. They are ubiquitous and often dominate freshwater communities in both number and biomass (Armitage et al., 1995; Berg and Hellenthal, 1992; Ferrington, 2008). The chironomidae family is of great ecological interest, specifically in the ecosystems' equilibrium: they play a key role in organic matter recycling (Péry and Garric, 2006; Rasmussen, 1984) while serving as a major food source for predators such as fish, other invertebrates, and

aquatic birds (Armitage et al., 1995; Berg and Hellenthal, 1992; Rieradevall et al., 1995).

In this work, *Chironomus riparius* (Meigen, 1804) was used as a model organism. This species is easy to culture, maintain and handle in laboratory, and has a relatively short life-cycle under laboratory conditions that includes a complete metamorphosis (Péry et al., 2002; Taenzler et al., 2007). The first three life stages are aquatic and comprise the egg stage, four larval stages, and a pupal stage, while the adult stage is aerial (Armitage et al., 1995; Ferrington, 2008; Lopes et al., 2005) (Fig. 1). Larval stage is divided in four instars that can be distinguished by the head capsule size (EPA, 2000a; Oh et al., 2014; Watts and Pascoe, 2000) being red colored due to high levels of hemoglobin (Hb) (Bergtrom et al., 1976; English, 1969), leading to their common name "bloodworm"(Grazioli et al., 2016). Another interesting characteristic is the sediment-dwelling behavior of the larvae to seek food or shelter, meaning they live in the water-sediment interface (Crane et al., 2002; Taenzler et al., 2007; Weltje et al., 2010). The above mentioned features make *C. riparius* a widely used model species in freshwater ecotoxicology, for both water and sediment toxicity assessment and a suitable organism for biomonitoring studies (Choi and Roche, 2004; EFSA, 2013a; Weltje et al., 2010).

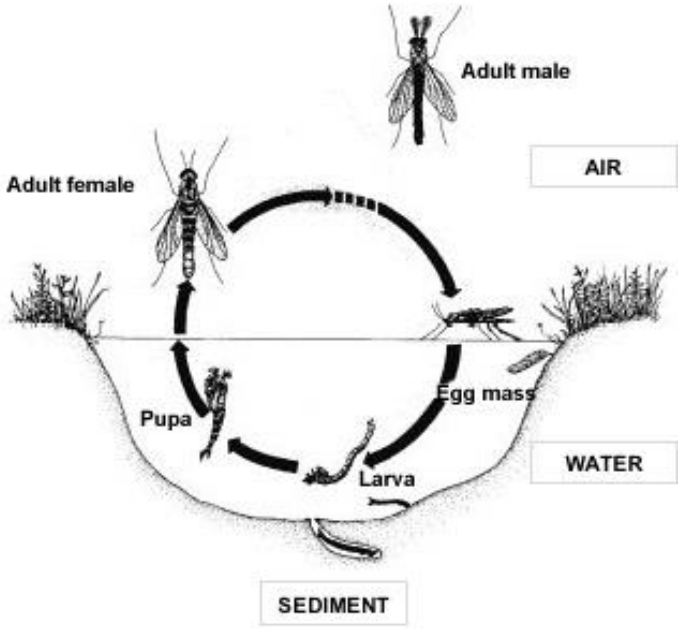

Figure 1 – *Chironomus riparius* life cycle. Adapted from Lopes et al. (2005).

2.2 Ecotoxicological endpoints at the organism level

Until recently, the assessment of pesticide effects on invertebrates has been focusing on phenotypical observations using relatively high concentrations. For this purpose, several guidelines were proposed for the assessment of lethal and sublethal effects of stressors on *C. riparius* in laboratory conditions (ASTM, 2005; EPA, 2000a; OECD, 2011; 2004a; 2004b) (Weltje et al., 2010). Traditionally, measured endpoints include survival, larval growth, percentage and development time of emerged adults, and adult sex ratio, while

fecundity and fertility, despite being less used, are also seen as endpoints. Several other endpoints have been studied for this species at the organism level including burrowing behavior of the larvae and adult body size or weight (Campos et al., 2016; Pestana et al., 2009; Sibley et al., 2001). These lethal and sublethal endpoints are ecologically relevant in the sense they provide sensitive information of the organism performance that can be easily used to predict possible outcomes at the population level. However, these assays are very time consuming. For example, larval growth is monitored for 10 days and emergence is monitored for 28 days. Reproductive output and multigenerational effects take even longer time to analyze. Additionally, one point that is often overlooked, is that apart from a few pesticide contamination pulses, the concentrations regularly found in the environment are not high enough to cause an observable organismal level response. Nevertheless, low concentrations of pesticides can cause physiological alterations that might lead to long-term adverse outcomes for populations and communities. There is thus a need to develop sensitive and early warning tools that can be used to predict ecological adverse effects of pesticides. Assuming that the effects observed at higher levels of biological organization are a consequence of alterations that occur initially within the organism, the study of sub-organismal endpoints may disclose early indicators of stressors' exposure and/or effects (Lemos et al., 2010) (Fig.2).

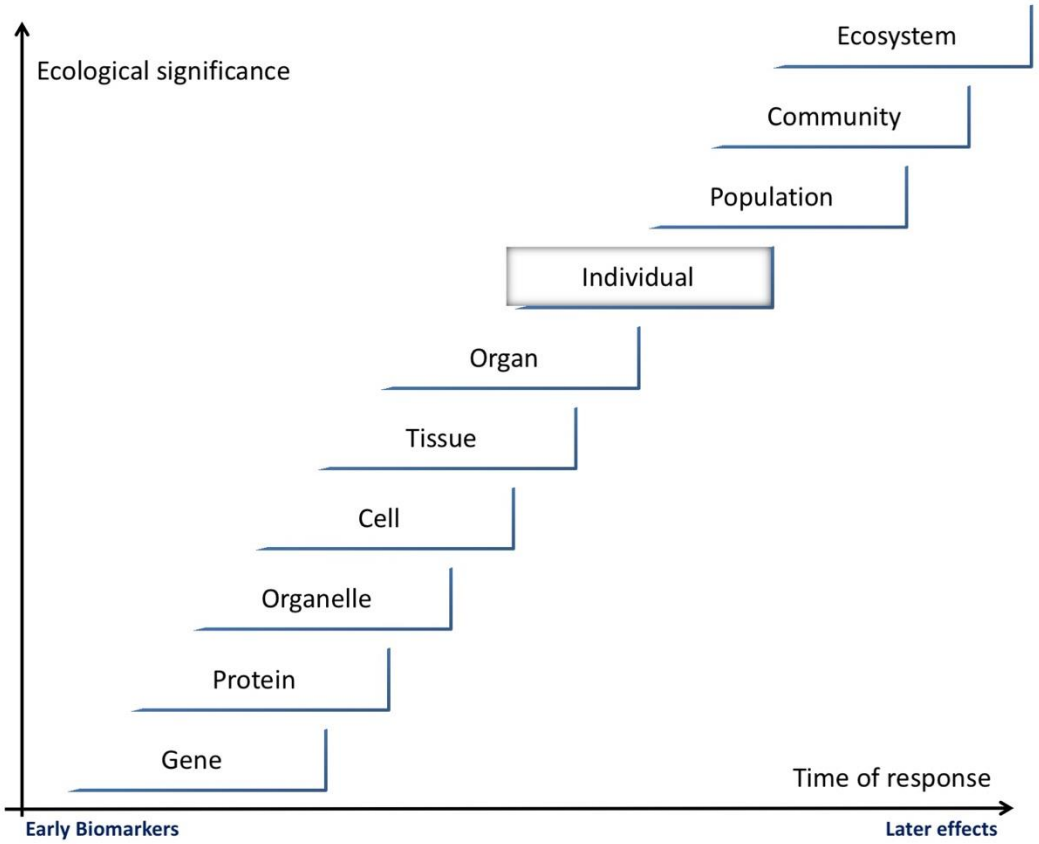

Figure 2 – Relationship between levels of biological organization, response time and ecological relevance.

2.3 Endpoints at sub-organismal level

2.3.1 Biochemical Biomarkers

From an environmental perspective, a biomarker can be defined as any (measurable) biological alteration that can be indicative that an organism has been exposed to a stressor. Although there is still some debate regarding biomarker definition, traditionally the term has been used to include only measurements at the cellular, biochemical or molecular levels, but not to measurements at the individual level (van Gestel and Van Brummelen, 1996).

In recent years, the biomarker approach has been extensively used in aquatic ecotoxicology and several experimental procedures are now optimized for model organisms. This upsurge of biochemical biomarkers is attributed to their potential as early-warning tools, i.e. a quick, simple and early indication of the exposure and the sub-lethal effects of a given stressor or contamination scenario (Picado et al., 2007; Prabhakaran et al., 2017; van Gestel and Van Brummelen, 1996). Additionally, biomarkers may provide insights on the mode of action of the chemicals, and their molecular targets within the organism (Forbes et al., 2006; van Gestel, 2012; van Gestel and Van Brummelen, 1996). However, one of the challenges of the biomarker approach in ecotoxicology is still to ascertain a straightforward relationship between biochemical data and their ecological relevance (De Coen and Janssen, 2003; Forbes et al., 2006). Taking this in consideration, integrating results obtained at different levels is of utmost importance to determine the mode of action of toxicants and understand the alterations that lead to higher levels responses (Lemos et al., 2010; Maltby, 1999).

As stated above, biochemical biomarkers have been frequently considered in aquatic ecotoxicology studies. While most protocols were initially developed using *Daphnia magna* as model organism, more recently, several studies have been published with *C. riparius* or closely related species. Most of currently used protocols rely on enzymatic activities or direct measurements of a damaging effect (ex. lipid peroxidation or DNA damage).

When dealing with xenobiotic exposure, a set of defensive mechanisms inside the organism are activated to protect it from any harmful effects. One of the key defensive enzymes is the glutathione *S*- transferase (GST). This enzyme is involved in the detoxification pathway, by conjugating reduced glutathione to target xenobiotics, largely improving their solubility and therefore facilitating their removal (Pickett and A. Y. Lu, 1989; van der Oost et al., 2003; Ziglari and Allameh, 2013).

Several stressors induce or increase the production of reactive oxygen species (ROS) (M. Ferreira et al., 2005; N. G. C. Ferreira et al., 2015b; Livingstone, 2003; Torres et al., 2002; Yousef et al., 2017). To cope with ROS, antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD) are readily activated to prevent oxidative stress. The activity of these

enzymes has been frequently assessed as biomarkers of effect in ecotoxicology (Campos et al., 2016; N. G. C. Ferreira et al., 2015a; Gonzalez-Rey and Bebianno, 2014; Novais et al., 2014; Rodrigues et al., 2015a; 2015b). If these mechanisms fail to eliminate ROS, oxidative stress can turn into oxidative damage and result in increased lipid peroxidation (LPO) and/or DNA damage (Halliwell and Gutteridge, 2015; Sies, 1997; Valavanidis et al., 2006). LPO and DNA damage can cause several alterations in cellular processes, injuries in cell membranes and tissues, and ultimately cell death (Liu et al., 2015; Livingstone, 2003; Novais et al., 2013; Valavanidis et al., 2006; Roos and Kaina, 2006). These effects can lead to long-term consequences including delayed development, growth impairment, reduced reproductive output and behavioral changes (Gravato and Guilhermino, 2009; Matić et al., 2016; Novais et al., 2013). Moreover, DNA damage and the increase of LPO levels in *C. riparius* under exposure to the xenobiotics have been previously reported (Martínez-Paz et al., 2013; Morales et al., 2013; Rodrigues et al., 2015a).

Energy metabolism parameters have also been historically used as biomarkers in ecotoxicology. Some examples include the measurement of lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) activities. Theses enzymes are involved in several metabolic processes, playing important roles in energy production through aerobic (LDH driven pathway) and anerobic (IDH driven pathway) metabolism (De Coen et al., 2006; Forcella et al., 2007; Luís and Guilhermino, 2012; C. Silva et al., 2013). Another commonly used approach is the estimation of the energy (oxygen) consumption of the organisms by measuring the electron transport system activity (ETS). An increase of ETS activity is an indicator of high cellular consumption rate and is recognized as a general indicator of stress (Choi et al., 2001; Rodrigues et al., 2015b; 2015a; C. S. E. Silva et al., 2016). Acetylcholinesterase (AChE) activity is a measurement of cholinergic neurotransmission associated with neuromuscular toxicity (Ellman et al., 1961) and has been correlated with behavioral alterations (Azevedo-Pereira et al., 2011, Campos et al., 2016; Pérez et al., 2013) and developmental and reproductive effects (Crane et al., 2002) that may lead to population level effects.

2.3.2 Fatty Acid profile

Conventionally, fatty acid (FA) profiling is applied in ecology for taxonomic purposes (Arts et al., 2009; Dussert et al., 2008; Sahu et al., 2013; Shukla et al., 2012). However, alteration of FA profiles is considered as indicative for environmental stress (Filimonova et al., 2016; Gonçalves et al., 2017; N. Lu et al., 2012; C. O. Silva et al., 2017). FAs play a number of essential roles in living organisms, serving as fuel for many metabolic processes and mediating cell signaling and maintenance of membrane fluidity (Arts et al., 2009; Nelson and Cox, 2013), among many others. FAs in particular, and lipids in general, are sensitive to environmental stress (Arts et al., 2009; Gonçalves et al., 2016): as stated above, many xenobiotics induce the production of ROS and can lead to the

oxidation of lipids, particularly of membrane lipids, and thereupon result in impairment of cell membrane functions, tissue damage and ultimately disrupt vital functions (Harris, 1992; Parrish, 2013; Rikans and Hornbrook, 1997; Stohs et al., 2001; Valavanidis et al., 2006). Thus, studying FA profile may help to gain insight on how stressors act on exposed organisms and their potential implications. C. O. Silva et al., (2017) reported changes in the abundance and composition of some fatty acids of the sea snail *Gibbula umbilicalis* exposed to metals, at levels that did not induce LPO. These alterations may be related to the involvement of some FAs in immune response or homeoviscous adaptation (C. O. Silva et al., 2017) and could be an indication that, in some cases, FAs may provide a distinct and/or possibly earlier indication of stress than LPO. Nonetheless, integrating FA profiles with LPO data, can give a better interpretation on the physiological status of the organisms.

To this date some studies assessed FA profile in chironomidae but, to our knowledge, none of these available studies assessed the potential of using FAs as biomarkers in ecotoxicology. Nevertheless, by analyzing the FA profile, (Akerblom and Goedkoop, 2003) made an interesting discovery that *C. riparius* larvae feed mainly on added food rather than on the organic fraction of artificial sediment in long-term standard toxicity tests, thus influencing the toxicity test results. Other available studies on chironomids FAs focused on dietary habits or on phylogenic relationships (Gladyshev et al., 2015; Goedkoop et al., 2000; Happel et al., 2016; Kiyashko et al., 2004; Makhutova et al., 2011; Zinchenko et al., 2013). Due to their roles and sensitivity to stressors, FAs may be promising biomarkers of pesticide-induced stress.

2.3.3 Protein differential expression

2.3.3.1 Historical background

The term “proteome” was coined by Marc Wilkins and initially defined as the protein complement of a genome (Wasinger et al., 1995; M. Wilkins, 2014; M. R. Wilkins et al., 1996; 1995). The development of two-dimensional electrophoresis (2D-GE) in the late 60’s and early 70’s (Kenrick and Margolis, 1970; Margolis and Kenrick, 1969) is a crucial achievement in protein separation, and the refinements made by Klose (1975), O'Farrell (1975), and Scheele (1975) to improve the resolution and reproducibility are regarded as the true beginning of proteomics, i.e. the study of the proteome (N. L. Anderson and N. G. Anderson, 1998; Graves and Haystead, 2002; Patterson, 2003). However, only two decades later two-dimensional gel electrophoresis (2DE) started to be applied at a larger scale in proteomics, due to advances in mass spectrometry (MS) and bioinformatics tools that improved identification and quantification of proteins (Cai et al., 2004; Schneider and Riedel, 2010).

The term proteome currently refers to the proteins that are being expressed in an organism, tissue, or cell, in a particular moment (Cai et al., 2004; Yoithapprabhunath et

al., 2015). In other words, proteomics goal is not limited to the identification of proteins, but is to obtain an integrative view of all proteins, including their abundance, activity, function, modifications and how they interact at a given time, under a given condition (Alzate, 2010; Cai et al., 2004; Graves and Haystead, 2002; Van Oudenhove and Devreese, 2013). The concepts of "time" and "condition" are included in the sense that not all protein coding genes in the genome are expressed at the same time: the proteome is highly dynamic and reflects the organism's physiological state (Lemos et al., 2010; Nature America, 2000).

The study of the proteome offers some advantages over transcriptomics, since proteins are main functional units within the cell and responsible for several biological functions, having a direct effect on organisms' physiology and fitness (Feder and Walser, 2005). Proteins are final products of gene expression, and due to several regulatory steps, mRNA degradation, and translational inefficiencies, only a limited amount of mRNA is translated into proteins (Feder and Walser, 2005; Garcia-Reyero and Perkins, 2010; Sanchez et al., 2011), and thus mRNA abundance is often a poor proxy for protein abundance (Feder and Walser, 2005). Additionally, post-translational modifications, and protein degradation have an impact on protein abundance, activity, and function (Garcia-Reyero and Perkins, 2010). Therefore, the actual protein content depends on the balance of protein synthesis and degradation and may substantially differ from the one predicted by the transcriptome (Feder and Walser, 2005). In this sense, proteomics may provide a more accurate physiological state than transcriptomics, as it measures the actual protein abundance, instead of providing estimates. Moreover, the proteome is highly dynamic, and can change in response to environmental stress and reflect the organisms' current state, sometimes independently of transcriptional changes (Tomanek, 2014).

Proteomics has currently a wide range of applications, mainly in biomedical and health sciences. For instance, a lot of work has been carried out to identify disease specific proteins (biomarkers) (Corbo et al., 2017; de Wit et al., 2014; Sallam, 2015; Sepiashvili et al., 2012; Tsai et al., 2015). This can be achieved by identifying proteins that are differentially produced in samples from patients versus healthy controls, or by identifying defective proteins implicated in disease. At the same time, proteomics may assist in the discovery of new drug targets and in the understanding of secondary effects of a particular drug (M. H. Dias et al., 2016; Kumar et al., 2016; Mishra, 2010; Page et al., 1999; Walgren, 2004; Zhou et al., 2010). One other major field of application of proteomics is in the environmental sciences. The next section addresses current and potential applications of proteomics in environmental sciences, particularly in the ecotoxicology field.

2.3.3.2 Environmental proteomics

The advances in functional and expression proteomics opened new doors for

potential applications of proteomic technologies within environmental sciences.

Due to high energy demands, there is a global effort to search for renewable and sustainable energy sources (Bakhtiari et al., 2016; Mao et al., 2012; Mishra, 2010). Scientists are currently investigating proteins involved in growth, development, and metabolism of biofuel sources such as algae or plants (V. Anand et al., 2017; Paudel et al., 2016; Terashima et al., 2010). This knowledge can be fundamental to understand metabolism of biofuel production or to identify genetically and phenotypically superior species for biomass production (Boaretto and Mazzafera, 2013; Mao et al., 2012).

The "omics" may also be noteworthy tools in microbial community analyses. Metaproteomic analysis of a community does not require organisms to be isolated and cultured, making it possible to study the entire community, including uncultivable microorganisms, which are estimated to account for more than 99% of the total species (Amann et al., 1995; Chovanec et al., 2011; Schneider and Riedel, 2010; Streit and Schmitz, 2004). Besides giving information on the structure and function of microbial communities, proteome analyses may also provide valuable knowledge for bioremediation research, offering insights on molecular pathways involved in remediation of toxic compounds (Chovanec et al., 2011; Singh, 2006; M. J. Wilkins et al., 2009).

Other subjects of interest where proteomics can have a promising role include the screening and characterization of bioactive compounds from biota (Evans et al., 2007; Hartmann et al., 2014; Imhoff et al., 2011) or the identification and understanding of the proteins and mechanisms involved in resistance/susceptibility of plants to insect pests (Sangha et al., 2013). Nonetheless, most of current proteomics studies in environmental sciences are focused on ecotoxicological research.

Advances in human and clinical proteomics, particularly in the search for biomarkers of disease, has allowed researchers to explore applications of proteomics in other fields. In the same framework, ecotoxicoproteomics aims to identify biomarkers associated with the toxicological effects of stressors (Lemos et al., 2010). Moreover, it can aid in the characterization of molecular mechanisms related to the toxic response that may or may not result in physiological responses (Lemos et al., 2010; Ralston-Hooper et al., 2013). The term "ecotoxicoproteomics" was first used in literature in 2006 (Bjørnstad et al., 2006), although the first studies in the field were conducted a few years earlier. Earliest applications of proteomics in aquatic toxicology were accomplished by Shepard and Bradley (2000) and Shepard et al. (2000). The authors monitored alterations in the proteome of the mussel *Mytilus edulis* when exposed to a polychlorinated biphenyl (PCB) compound, to copper, and to salinity stress. Using 2DE as separation technique the authors identified distinct protein expression signatures (presence/absence) between exposed and non-exposed organisms. Since then, many proteomic techniques have been applied, particularly using fishes as test species (reviewed in (Sanchez et al., 2011)). Although invertebrates are estimated to account for roughly 96% of animal species (R. C. Brusca and G. J. Brusca, 2003), there is still very limited proteome information on aquatic

invertebrate model species, with the lack of sequence databases usually considered as a major drawback when compared to vertebrates. Earlier studies with aquatic invertebrates relied on the data available for the species and closely related species or on the protein sequence databases available, with moderate success (López et al., 2002; Manduzio et al., 2005). This strategy would often result in a relatively low number of protein matches (Martyniuk and Simmons, 2016; Sanchez et al., 2011) and if not carefully applied, a lower cut-off level for protein matching could result in false positive matches (Martyniuk and Simmons, 2016). Nevertheless, with the advancements in analytical technologies, the increase of available genome sequences, accompanied with the advances in bioinformatics tools, such as new software packages for protein identification and quantification in complex mixtures (Monsinjon and Knigge, 2007; Ralston-Hooper et al., 2013; Sanchez et al., 2011; Schneider and Riedel, 2010), the number of studies with invertebrates has been increasing in recent years. Some recent studies focused on proteome changes in the model ecotoxicological species *Daphia sp.* (Borgatta et al., 2015; Schwerin et al., 2009; Zeis et al., 2009), especially after the release of *Daphnia pulex* genome (Colbourne et al., 2011). Molluscs have also been used as test organisms, mainly due to their ecological role in biomonitoring (Chora et al., 2009; Ji et al., 2014; E. L. Thompson et al., 2011).

Regarding *C. riparius*, while there are some studies addressing changes of gene expression as response to stress (Morales et al., 2013; Nair and Choi, 2011; Nair et al., 2013a; 2013b; 2012; 2011; 2013c; S. Y. Park et al., 2012), only few ecotoxicoproteomic studies are available. Those studies include the works of S.-E. Lee et al. (2006) who assessed changes in protein expression after cadmium exposure, and Choi and Ha (2009) who, using the same metallic element, focused on the alterations in globin protein expression. Another work, worth mentioning, was published by Ha and Choi (2008), where the authors report a preliminary characterization of *C. riparius* Hb expression after exposure to several chemical contaminants. As mentioned above, the protein matching was relying on the limited data for *C. riparius* and closely related species available at that time. Although this was the best alternative to cope with the lack of a genome sequence, this approach would result in a limited protein coverage since it would be restricted to the most conserved proteins (López et al., 2002; Sanchez et al., 2011; Trapp et al., 2014). Nevertheless, the work of Marinković et al. (2012) on the *C. riparius* transcriptome and the more recent releases of the genome draft of *C. riparius* (Oppold et al., 2017; Vicoso and Bachtrog, 2015) should contribute to a more rapid and reliable analysis of proteomic data of this species.

The increase in the number of proteomic studies in the search for new biomarkers is not surprising. In contrast to other conventional approaches, proteomics can be non-hypothesis-driven but rather discovery-driven (Speicher, 2004). In other words, proteomics can generate high amounts of data, revealing stress-response molecular mechanisms, as well as possible biomarkers or biomarker patterns associated with the

mode of action of a toxicant (or group of toxicants), that are not initially foreseen (Lemos et al., 2010; Simmons et al., 2015).

2.3.3.3 Quantitative tools in proteomics

Since the dawn of ecotoxicoproteomics, scientists used state-of-the-art methodologies in protein profiling. When it comes to protein separation, two main approaches have been utilized: gel-based and gel-free proteomics. The most widespread technique used for protein separation in early years was 2D-GE, followed by MS for protein identification (Chevalier, 2010; Patterson, 2003; Sanchez et al., 2011). Traditionally, in the first dimension proteins are separated by their isoelectric point, using the isoelectric focusing (IEF) technique, and in the second dimension proteins are separated by their molecular weight through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Chevalier, 2010). This is well-established and still regularly used in ecotoxicoproteomic studies today and is a valuable technique to determine post-translational modifications (PTM's) of proteins (Chevalier, 2010; Martyniuk and Simmons, 2016; Wright et al., 2012). However, when considering ecotoxicology goals, this technique has some technical limitations and many of those limitations are overcome by gel-free approaches.

The advantages and limitations of the two approaches have been a matter of extensive review over the years (Abdallah et al., 2012; Baggerman et al., 2005a; Chevalier, 2010; Scherp et al., 2011). In brief, major limitations of gel-based systems include low sensitivity, low reproducibility, and low sample throughput. Due to restrictions in loading amount of proteins and to the dynamic range of proteome, less abundant proteins are often not detected in 2D-GE systems (Gygi et al., 2000; Ong and Pandey, 2001; Wright et al., 2012). Additionally, standard 2D-GE systems are not suitable to (simultaneously) visualize very high or very low molecular weight proteins (Chevalier, 2010; Görg and Weiss, 2004). The same applies for proteins with extreme isoelectric points, where separation can be challenging, mainly for highly alkaline proteins (Dépagne and Chevalier, 2012). Moreover, due to their low solubility in IEF compatible buffers, hydrophobic proteins (particularly membrane proteins) are often excluded from the analysis (Baggerman et al., 2005b; Chevalier, 2010; Görg and Weiss, 2004; Rogowska-Wrzesinska et al., 2013). Nonetheless, several improvements to overcome these limitations were made including the development of immobilized pH gradient strips up to pH 12 for isoelectric focusing to resolve highly alkaline proteins and the use of agarose gel electrophoresis to visualize high molecular weight proteins (Chevalier, 2010; Görg et al., 1997). Regarding membrane proteins, strategies such as 16-BAC/SDS-PAGE (Moebius et al., 2005; Zahedi et al., 2005), Blue Native-PAGE (Wittig et al., 2006), or one-dimensional SDS-PAGE (Galeva and Altermann, 2002; Moebius et al., 2005) have been successful in separating membrane proteins, although providing limited resolution when compared to the conventional IEF/SDS-PAGE (2D-PAGE) approach (Braun et al., 2009; Kota and Goshe,

2011; Moebius et al., 2005; Zahedi et al., 2005). Indeed, most of gel-based alternative approaches to 2D-PAGE require additional sample processing steps and/or sample fractionation, making them selectively effective for a specific group of proteins and not for a broader analysis of the proteome, thus requiring running of several different gels.

Reproducibility is also a major issue in gel-based proteomics. Even the most experienced operator, using the same sample, will possible generate slightly different protein profiles due to inter-run and inter-gel technical variations (Chevalier, 2010; Sanchez et al., 2011; Wright et al., 2012). This is also a critical issue when quantification is of interest and when poor reproducibility may lead to reduced efficiency in matching proteins spots between different gels (Baggerman et al., 2005a; Wright et al., 2012). Additionally, since quantitative analysis is usually based on the relative intensity of protein spots, the selection of a staining technique is also fundamental for the quantification success. For instance, one of the most common staining techniques is Coomassie blue staining, although the detection limit of this dye is relatively low (Baggerman et al., 2005b; Weiss et al., 2009) and may leave out from the analysis less abundant (and potentially physiologically relevant) proteins. One common alternative is the use of silver staining, which is more sensitive, but in contrast offers a lower dynamic range of detection and some incompatibilities with MS systems for posterior identification (Baggerman et al., 2005b; Weiss et al., 2009). Also, it must be noted that one spot may contain more than one protein, and when that is the case, accurate quantification is very challenging (Trapp et al., 2014; Wright et al., 2012).

Difference gel electrophoresis, or DIGE, was introduced in 1997 (Unlü et al., 1997). It allows for two or three samples, prelabeled with different cyanine dyes, to be simultaneously separated in the same run (Timms and Cramer, 2008; Unlü et al., 1997). After the run, each sample can be visualized by fluorescence imaging and compared, thus reducing the number of runs in an experiment and overcoming the inter-gel variation of traditional 2D-GE systems (Unlü et al., 1997). Additionally, DIGE enables a more accurate spot matching and quantitation, particularly when using an internal standard (Alban et al., 2003; Marouga et al., 2005). This technique has been recently applied in invertebrate aquatic toxicology, in the search for biomarkers of metal contamination in the amphipod *Gammarus pulex* (Vellinger et al., 2016), or in the assessment of the effects of endocrine disrupting chemicals on the proteome of the gastropod *Lymnaea stagnalis* (Giusti et al., 2013). Despite the improvements in reproducibility and quantification, DIGE still presents some of the gel systems limitations (Minden, 2007; Xiangdong Wang, 2013), is expensive and time consuming as it still requires several gels to be performed when accessing multiple conditions and/or biological replicates (Vellinger et al., 2016). This setting, along with the advances in analytical chromatography and high resolution of mass spectrometry quantitative analysis, led to the development of alternative gel-free approaches.

In recent years, various approaches have been developed for MS-driven quantitative analysis. The majority of these approaches entail the isotopic labeling of

proteins or peptides, either metabolic or chemical, allowing to determine identical proteins' abundance in separate samples by MS (Abdallah et al., 2012; Corbo et al., 2017; Wiese et al., 2007). Chemical labeling techniques include isotope-coded affinity tag (ICAT) (Gygi et al., 1999), isotope-coded protein label (ICPL) (Schmidt et al., 2005), isobaric tags for relative and absolute quantitation (iTRAQ) (Ross et al., 2004), and tandem mass tags (TMT) (A. Thompson et al., 2003), while stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002) is an example of a metabolic labeling technique. Conversely, other approaches are based on high resolution MS instruments, and quantification is made by comparison of signal intensity or on spectral counting (label-free quantification) (Neilson et al., 2011). The pros and pitfalls of these techniques were recently reviewed in some detail elsewhere (Abdallah et al., 2012; S. Anand et al., 2017; Corbo et al., 2017; Nikolov et al., 2012; Van Oudenhove and Devreese, 2013). In this study, we will focus on the iTRAQ methodology and its applicability in ecotoxicoproteomics.

Introduced in 2004 as a 4-plex protein quantitation approach (Ross et al., 2004), and later as 8-plex (Choe et al., 2007), a typical 8-plex iTRAQ (Peptide Labeled) workflow is shown in figure 3. Starting with the same protein amount, each protein sample is denatured, reduced, alkylated, and trypsin digested (Choe et al., 2007). Afterwards, each digest is differentially labeled with an amine-modifying labeling reagent with an isobaric mass tag containing a reporter group and a mass balance group (Corbo et al., 2017; Martyniuk et al., 2012a). The 8-plex iTRAQ reagents have reporter ion masses at *113, 114, 115, 116, 117, 118, 119, and 121 m/z* (Choe et al., 2007) and a corresponding balance group to ensure that labeled identical peptides from different samples have identical mass, co-elute and therefore are chromatographically indistinguishable (DeSouza et al., 2005; Ross et al., 2004). After labeling, samples are pooled and subsequently fractionated. Most common fractionation strategies use a two-dimensional liquid chromatographic separation of peptides, typically with strong cation-exchange (SCX) chromatography in the first dimension followed by reversed-phase (RP) chromatography in the second (Choe et al., 2007; Ross et al., 2004). However, alternative effective strategies have been used for peptide separation, for instance, using a high-pH RP chromatography in the first dimension followed by a low-pH RP chromatography (RP/RP-LC) (Dowell et al., 2008; Gilar et al., 2005; Yang et al., 2014). RP/RP-LC has been successfully used for the separation of 8-plex iTRAQ labeled peptides (Van Oudenhove et al., 2012).

After fractionation, samples are subjected to tandem mass spectrometry analysis. As the name suggests, tandem mass spectrometry, or MS/MS, consists in two (or more) sequential rounds of MS. In the first round of MS, peptides are ionized and produce a

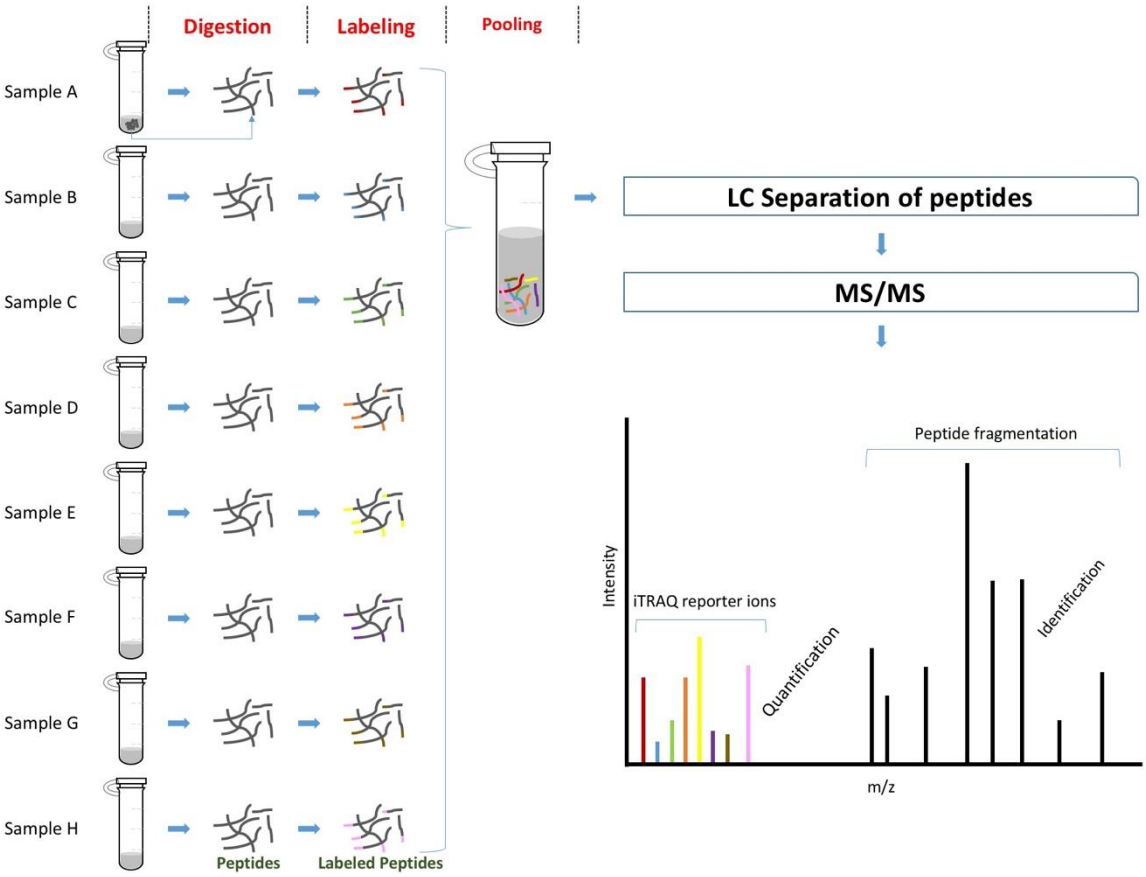

Figure 3 – Schematic representation of a typical 8-plex iTRAQ workflow.

peptide (precursor) ion spectrum, and in the second MS selected precursors are individually fragmented to produce fragment ions for peptide identification (Trapp et al., 2014; Van Oudenhove and Devreese, 2013). Concurrently, during this second round of MS the isobaric mass tags are cleaved to produce distinct reporter ions in the 113–119 and 121 m/z range (Choe et al., 2007; Martyniuk et al., 2012a) and quantification is done by measuring the relative intensity of the reporter ions (Ross et al., 2004).

The possibility of multiplexing up to 8 samples in a single run is one of the major advantages of this quantitative method (Martyniuk et al., 2012a). This feature allows the simultaneous assessment of multiple conditions, making iTRAQ a practical tool in ecotoxicology. Thus, in addition to the input given on stressor-specific targets, iTRAQ can aid in the discovery and identification of potential mechanisms or protein biomarkers of toxicity (Glückmann et al., 2007; Martyniuk et al., 2012a).

One of the early iTRAQ-based studies in aquatic ecotoxicology was conducted by Martyniuk et al. (2009), who investigated the effects of an androgen and an antiandrogen in the liver of female fathead minnows (*Pimephales promelas).* In addition to altered proteins putatively regulated through androgen receptor signaling, the authors identified additional non-androgen receptor signaling pathways regulated by the antiandrogen chemical (flutamide). Other works performed on fish species include the work by Malécot

et al. (2011), who studied the effects a cyanotoxin (Microcystin-LR) on the hepatic proteome of the ecotoxicological model medaka fish (*Oryzias latipes*). The authors found proteins that were altered in accordance to previous gel-based studies, but also found new and yet unreported protein expression changes in response to microcystin-LR exposure.

Some aquatic invertebrates have also been the subject of iTRAQ-based proteomic studies. Ji et al., (2014; 2016) assessed the effects of tetrabromobisphenol A exposure in the proteome of the mussel *Mytilus galloprovincialis*. Using an 8-plex approach, the results suggested that the exposure to this brominated flame-retardant caused distinct responses at protein level in males and females, which may aid in the interpretation of gender-*specific* stress *responses.*

A recent work by Zheng et al. (2017) investigated the changes in the proteome of the chironomid *Propsilocerus akamusi* after exposure to cadmium. Changes in protein expression provided valuable insights on the mechanisms of cadmium resistance in this highly tolerant species. To the best of our knowledge, this is the only published iTRAQ-based ecotoxicoproteomics study using a chironomid as test species.

The applicability of iTRAQ in environmental proteomics is not limited to the assessment of proteome changes under stress. For instance, iTRAQ labeling has been used as a tool to identify proteins that may be associated with deltamethrin (pyrethroid) resistance in the dipteran *Culex pipiens pallens* (Weijie Wang et al., 2015), to determine proteins and/or mechanisms involved in growth regulation in the crab *Portunus trituberculatus* (Ren et al., 2017), to investigate expressional changes during estivation of the gastropod *Pomacea canaliculata* (Sun et al., 2013), or to understand the mechanisms underlying the thermotolerance of Spirulina (Chang et al., 2016).

2.3.3.4 Dose-response in ecotoxicoproteomics

The concept of dose-response is of upmost importance in ecotoxicology. When assessing classical organism-level endpoints, such as survival or growth, setting up an experimental design with numerous experimental treatments will lead to a more precise estimation of an effective concentration (ECx, LCx, etc...) (OECD, 2014). To achieve this, guidelines have been developed for model organisms in ecotoxicology (OECD, 2012; 2007; 2004b). When analyzing emergence-related endpoints in *C. riparius*, an adequate number of replicates is required since, for instance, males emerge slightly before females (a phenomenon termed protandry) (Armitage et al., 1995; Péry et al., 2002; Taenzler et al., 2007), which most of the time requires male and female data to be processed separately in terms of emergence-related endpoints. Thus, without a proper number of replicates, great discrepancies between the number of males and females amongst treatments may occur, making data analysis very challenging.

Conversely, ecotoxicoproteomics is still in a building stage and most studies are

limited to a low number of samples and replicates. This is in great part due to labor, time, and financial cost of most techniques. As discussed above, one of the major advantages of iTRAQ over other tools is the possibility of multiplexing up to 8 samples within a single run. However, setting an "acceptable" number of experimental treatments and the number of replicates in a single run may still be an issue when investigating dose-response relationships. Some authors' experimental designs favored the testing of more exposure concentrations and limited the study to one replicate per treatment (Fong et al., 2014; Zheng et al., 2017). In turn, Carvalho and Lettieri (2011) restricted their study to one exposure concentration, hence favoring the use of more biological replicates. The relevance of assessing dose-response relationships in ecotoxicoproteomics has been addressed before. Gündel et al. (2012) advocate for the use of more concentrations and less replicates in ecotoxicoproteomics, under the basis of that the use of more test concentrations will lead to a better coverage of the actual response variation, resulting in a better fit of dose-response models, and thus potential nonconformities attributed to a low number of replicates in any of the data groups are alleviated by adjacent data groups in the model (OECD, 2014). Using this approach in zebrafish (*Danio rerio*) embryos exposed to six concentrations of phenanthrene, the authors observed different expression profiles at different exposure concentrations and thus endorsing that protein expression levels are concentration-dependent and this should be taken into account when assessing the molecular response to toxicants and in biomarker selection (Gündel et al., 2012). On the other hand, the importance of having biological replicates in an iTRAQ experiment has been demonstrated before. Using three biological replicates of the liver of the medaka fish *Oryzias latipes*, (Malécot et al., 2011) reported that out of the 32 statistically significant protein variations, only 17 were shared between all biological replicates.

A different approach was used by Martyniuk et al. (2009; 2012b). Their experiments entailed three separate 4-plex iTRAQ labeling reactions (three iTRAQ replicates), each consisting on a control sample and three different treatments. Although this setup may still not be ideal, since there are still some issues with variability of protein identification between LC-MS runs (Malécot et al., 2011; Martyniuk et al., 2009), this presents a promising setup to assess dose-response relationship and include biological replicates in the same analysis. In the present work, an 8-plex iTRAQ approach was used, consisting of two biological replicates of each experimental treatment (1 control treatment and 3 experimental concentrations), in order to explore dose-response relationships without disregarding the use of biological replicates.

3. Test chemicals

In this work, the effects of exposure in *C. riparius* were evaluated at different levels of biological organization, using as model compounds four neurotoxic insecticides

with distinct modes of action: amitraz, spinosad, indoxacarb, and fipronil.

3.1 Amitraz

3.1.1 Uses and mechanism of toxicity

Amitraz is a very effective synthetic acaricide-insecticide for the control of cotton and fruit tree pests, and for the management of ectoparasites in livestock, pets and beekeeping (EFSA, 2016; EPA, 2010a; Gurgulova et al., 2015). This non-systemic formamidine pesticide started being used in the mid 1970's (Moser, 2014) as an alternative to conventional pesticides, due to increases in resistance (del Pino et al., 2015). Amitraz exerts neurotoxic effects in ectoparasites through the activation of octopamine receptors, resulting in increased nerve activity, abnormal behavior, detachment, and ultimately death (EPA, 2010a; Hollingworth and Murdock, 1980).

3.1.2 Regulatory Background

Amitraz was not included in the Annex I of EU Council Directive 91/414/EEC of 15 July 1991 concerning active substances authorized for incorporation in plant protection products (EC, 1991). This decision was adopted by means of Commission Decision 2004/141/EC of 12 February 2004, that determined that plant protection products containing amitraz had to be withdrawn by 12 August 2004, with the exception of few uses, including pear trees after harvest in Portugal, for which any authorizations had to be withdrawn by 30 June 2007 (EC, 2004a). Amitraz was again not included in the list of approved plant protection active substances in the Commission Implementing Regulation (EU) No 540/2011 of 25 May 2011 (EC, 2011b). This decision was based on the potential neurological effects of amitraz and insufficient data concerning consumers oral exposure to the compound (EC, 2004a).

These regulations, however, are only applied for the uses of amitraz in plant protection, and do not apply to the use of amitraz in veterinary medicine. Amitraz is listed in the allowed substances in foodstuffs of animal origin in Commission Regulation (EU) No 37/2010 of 22 December 2009 (EC, 2010a). In Portugal, there are currently six veterinary products with amitraz as active ingredient: two for the control of *Varroa destructor* mite in honey bees, marketing authorizations (AIM): 667/01/13DFVPT and 564/01/12NFVPT); two for the control of ticks, fleas or lice in dogs, (AIM: 472/01/12NFVPT and European medicines agency product number: EMEA/V/C/002002); two for the control of ticks, mites, lice in livestock, AIM: 460/01/12NFVPT and 453/01/12NFVPT (DGAV, 2018).
More information on the legislative background of amitraz in Europe can be found in European Food Safety Authority (EFSA) journal "Reasoned opinion on the setting of maximum residue levels for amitraz, coumaphos, flumequine, oxytetracycline, permethrin and streptomycin in certain products of animal origin", EFSA Journal 2016 (EFSA, 2016).

3.1.3 Environmental fate and risk to aquatic invertebrates

Amitraz is expected to be very unstable in aquatic ecosystems and quickly degraded in several transformation products (EPA, 2010b; Moser, 2014). Amitraz has a log Kow of 5.34 - 5.5 (EPA, 2010b; Osano et al., 2002), and its main degradation products have been identified as N-(2,4-dimethylphenyl)-N'-methylimidoformamide (BTS 27271, log Kow <1.63, (EPA, 2010b)), N-(2,4-dimethylphenyl)formamide (BTS 27919, log Kow <1.63 (EPA, 2010b)), and 2,4-Dimethylaniline (BTS-24868, log Kow = 2.20 (EPA, 2010b; Osano et al., 2002)) (Corta et al., 1999; EPA, 2010b). Due to its rapid degradation, the parent compound does not pose a major concern for aquatic environments, as opposed to some more stable and toxicologically relevant transformation products that retain toxic activity (Corta et al., 1999; del Pino et al., 2015; EPA, 2010a; Osano et al., 2002). For this reason, in the EU, the marker residue of amitraz is the sum of amitraz and all metabolites containing the 2,4- dimethylaniline moiety, expressed as amitraz (EC, 2017a; EFSA, 2016).

Since parent amitraz is short-lived in the environment, effects to aquatic invertebrates are expected to be minimal. However, one of the amitraz degradates, BTS-27271, may be of concern because it is more persistent in aquatic environments (EPA, 1996a). Using *Daphnia magna* acute toxicity studies, EPA described BTS-27271 as moderately toxic, while parent amitraz described as very highly toxic to aquatic invertebrates (EPA, 1996a). In the EU, amitraz is classified as very toxic to aquatic life with long lasting effects (EC, 2008a).

3.1.4 Human exposure and poisoning

According to Dhooria and Agarwal (2016) research, as of 2016 over 300 cases of amitraz poisoning in humans have been recorded. Of those cases, central nervous system depression, in the form of drowsiness, confusion, loss of consciousness and coma, were the most common poisoning symptoms (Dhooria and Agarwal, 2016; Veale et al., 2011). Other common clinical signs included hyperglycaemia, bradycardia, vomiting, respiratory depression, followed by less common hypotension and hypothermia (Dhooria and Agarwal, 2016), and six cases resulted in death (Dhooria and Agarwal, 2016). It must be noted that accidental exposures accounted for the majority of the poisoning cases and were more common in children, while a great number of the remaining cases consisted of intentional ingestions by adults with suicidal intents (Dhooria and Agarwal, 2016). There are no reports of amitraz poisoning as consequence of its use in veterinary medicine or plant protection. The only cases of poisoning that may be attributed to direct application of amitraz occurred in Turkey, where amitraz was used to treat scabies and pediculosis in humans, resulting in dermal exposures (Kalyoncu et al., 2002).

To the best of our knowledge, the most recent reported case of amitraz exposure in the EU was in 1997 in Belgium, where a 45-year-old man accidentally ingested 250 mg

of amitraz (Jorens et al., 1997). EFSA's risk assessment concluded that the exposure to products containing amitraz residues at the proposed maximum residue limits (MRLs) set under Regulation (EU) No 37/2010 (and later set in Commission Regulation (EU) 2017/623 of 30 March 2017 (EC, 2017b)) is not expected to pose a risk to consumers (EFSA, 2016). In 2009 the EU adopted emergency measures to control pears originating from Turkey, due to high levels of amitraz found (EC, 2009a). One year later it was reported that Turkish authorities took action on their side (EC, 2010b). Nonetheless, Turkey is the country with the higher number of recorded cases of amitraz intoxication, with over 70% of the cases reported globally (Dhooria and Agarwal, 2016).

3.2 Spinosad

3.2.1 Uses and mechanism of toxicity

Spinosad is a naturally-occurring pesticide mainly used in agricultural purposes, but also in veterinary and sanitary settings (Bacci et al., 2016; EPA, 2016; Kollman, 2003). This translaminar insecticide is very effective again many crop pests, including Lepidoptera, Diptera, Coleoptera, Orthoptera, Tephritidae, and Thysanoptera (Kollman, 2003; Majoni and Munjanja, 2015; Ujváry, 2010). It is also used to kill pet fleas (Blagburn et al., 2010) and in fire ant control (Kollman, 2003; Ujváry, 2010). Spinosad is comprised of spinosyn A and spinosyn D, (tipically 85% A and 15% D, although ratio may vary between products) (EPA, 2016; Ujváry, 2010). These two active components are fermentation products of *Saccharopolyspora spinosa*, a soil actinomycete) (Mertz and Yao, 1990; G. D. Thompson et al., 2000). Their biological activity was first described in the 80's and spinosad was first commercialized in 1997 (Salgado and Sparks, 2005; G. D. Thompson et al., 2000). Evidence suggest that spynosins are nicotinic acetylcholine receptor (nAChR) allosteric modulators (Orr et al., 2009), causing hyperexcitation of the nervous system and consequently lead to involuntary muscle contractions and tremors, followed by paralysis and death (Salgado, 1998; Salgado et al., 1998; Ujváry, 2010). Due to spinosyn's unique mode of action, as they act on a different site of nAChR competitive modulators such as nicotine or neonicotinoids, cross-resistance to spinosad is uncommon (Bacci et al., 2016; Sparks et al., 2012).

3.2.2 Regulatory Background

Spinosad is currently approved in the EU as insecticide in plant protection products under under Regulation (EC) No 1107/2009 (EC, 2009b). It was first included in the list of authorized substances by the Commission Directive 2007/6/EC of 14 February 2007 (EC, 2007a). Spinosad was again included in the list of active substances approved for use in plant protection products by the Commission Implementing Regulation (EU) No 540/2011 of 25 May 2011 (EC, 2011b). The approval was extended on 12 May 2014, by

the Commission Implementing Regulation (EU) No 487/2014 (EC, 2014a). Moreover, spinosad authorization as a plant protection active substance extends to organic farming (EC, 2008b).

Regarding the veterinary use of spinosad, authorizations are issued in accordance to Regulation (EC) No 726/2004 (EC, 2004b). Spinosad is approved as biocide in animal housing against houseflies under Directive 98/8/EC. Additionally, spinosad is approved as biocide for outdoor application to ant nests (EC, 2016a).

In Portugal, there are currently four plant protection products containing spinosad (AV0118, AV0288, AV0557 and AV0558) (DGAV, 2016) and four products for veterinary use: one to control *Ctenocephalides felis* fleas in dogs (EMEA/V/C/002635); one (EMEA/V/C/002233) for the control of *C. felis* on cats and dogs; and two as insecticides for livestock facilities (marketing authorizations (ACM) 174/00/14RBVPT and 044/00/10NBVPT) (DGAV, 2018).

3.2.3 Environmental fate and risk to aquatic invertebrates

Spinosyns A and D have a log Kow of 3.91 and 4.38, respectively (EC, 2006b). There is some contradictory information regarding the fate and persistence of spinosad in aquatic environments. While early studies indicated that spinosad is non-persistent (Cleveland, 2007; Cleveland et al., 2002; 2001; Kollman, 2003; Salgado and Sparks, 2005; G. D. Thompson et al., 2000), recently EPA considered spinosad (and its transformation products) highly persistent in aquatic environments (EPA, 2016). This recent classification was essentially given due to the high affinity of spinosad to the organic matter in sediments, where degradation under anaerobic dark conditions is slower (Cleveland et al., 2002; 2001; EPA, 2016). Laboratory, and microcosm studies indicate that photolysis is the primary route of degradation of spinosad (Cleveland et al., 2002). Microbial breakdown is also an important route of degradation (Cleveland, 2007), with the major degradates being identified as spinosyn B and N-Demethylated spinosyn D for spinosyns A and D, respectively (Cleveland et al., 2001; EC, 2006b; EPA, 2016). Evidence suggest that hydrolysis of spinosad is minimal (Cleveland et al., 2002; Ujváry, 2010).

The European Commission concluded that at the proposed and current use of spinosad, no harmful effects on the environment are to be expected. Nonetheless, EU member states were asked to pay particular attention to the protection of aquatic ecosystems when issuing authorizations for plant protection products containing spinosad (EC, 2006b). In accordance with regulation (EC) No 1272/2008 (EC, 2008a), spinosad is classified as very toxic to aquatic life with long lasting effects, and products containing spinosad as active ingredient should be labeled accordingly.

According to *Daphnia magna* acute toxicity results , EPA classified spinosad as slightly toxic to freshwater invertebrates, even though no acute risk concerns were identified for water column invertebrates (EPA, 2016). On the other hand, chronic risk

concerns were identified for both water column and benthic invertebrates based on agricultural crop and non-crop uses (EPA, 2016). Previous data from *C. riparius* toxicity testing revealed effects of spinosad on emergence at 3.2 μg L^{-1} (Cleveland et al., 2001), a value below the 21-day pore water estimated environmental concentration (EEC) for most of agricultural uses of spinosad (EPA, 2016). Moreover, due to the high affinity to the sediment exhibited by spinosad and its degradation products, who also appear to retain some of the parent compound toxicity, the risks to sediment-dwelling invertebrates are of primary concern (EPA, 2016).

3.2.4 Human exposure and poisoning

EFSA's risk assessment concluded that the exposure to products containing spinosad residues at the proposed MRLs set under Regulation (EU) No 396/2005 is not expected to pose a risk to consumers (EFSA, 2013b). To our knowledge, only one case has been reported of acute exposure to spinosad, in Taiwan. The report describes a suicidal attempt by drinking a mixture of spinosad and other insecticide, flonicamid (Su et al., 2011). The patient initially exhibited loss of consciousness, shock and respiratory depression. Esophageal injuries, oral ulcerations, lung infections, leukocytosis and urinary retention were also detected. After 5 weeks, the patient fully recovered and was discharged from the hospital. The authors point out that although there were no previous reports on the clinical toxicity of both pesticides in humans, the symptoms were not expected to be so severe, as both pesticides are considered safe. Although the solvent composition of the insecticide formulation may have contributed to the observed effects, spinosad was the main compound ingested.

3.3 Indoxacarb

3.3.1 Uses and mechanism of toxicity

Indoxacarb is a syntethic oxadiazine pesticide that was first registered in the United States in 2000 and emerged as a reduced-risk alternative to organophosphates (EPA, 2000b; McCann et al., 2012). It is a broad-spectrum insecticide particularly effective against lepidopteran larvae, but also to other agricultural crop pests (McCann et al., 2012; Wing et al., 2010; 2000) and nonagricultural pests such as termites, fleas, cockroaches, and ants (McCann et al., 2012; Wing et al., 2010). It is non-systemic, yet it does penetrate leaves and exhibits translaminar movement, which contribute to its efficacy against sucking pests (Wing et al., 2010). Indoxacarb is composed by a mixture of two enantiomers: 75% of biological active enantiomer S and 25% of biological inactive enantiomer R (Wing et al., 2000). It acts on voltage-dependent sodium channels, blocking nervous system action potentials (Lapied et al., 2001; McCann et al., 2001; Wing et al.,

2000). This action causes nervous system shutdown, resulting in feeding inhibition and paralysis (Wing et al., 2000; 1998).

The strong activity of indoxacarb is attributed to the conversion of the enantiomer S to the more active metabolite N-decarbomethoxyllated DPX-MP062 or DCMP (also referred to as IN-JT333 in the literature, or to as DCJW in studies with the racemic compound) (J. L. Dias, 2006; EPA, 2017; Wing et al., 2010; 2000). Evidence suggests that this bioactivation of indoxacarb is performed by esterase and/or amidase enzymes present in the midgut and fat body tissues of insects (Wing et al., 2010).

Interestingly, due to overproduction and activation of esterases in pyrethroid-resistant insect species, a negative cross-resistance between indoxacarb and pyrethroid has been observed in the lepidopteran *Helicoverpa armigera* (Gunning et al., 2002; Ramasubramanian and Regupathy, 2004). On the other hand, it was recently reported the detection of an indoxacarb resistant strain of *H. armigera*, with a possible involvement of detoxification enzymes in the mechanism of resistance (Bird, 2017).

3.3.2 Regulatory Background

Indoxacarb is currently approved as a plant protection insecticide under regulation (EC) No 1107/2009 (EC, 2009b). Indoxabarb was first approved as an insecticide the Commission Directive 2007/6/EC of 14 February 2007, regarding plant protection products (EC, 2007a). It was again included in the list of active substances approved for use in plant protection products by the Commission Implementing Regulation (EU) No 540/2011 of 25 May 2011 (EC, 2011b). This approval was extended on 30 May 2017, by the Commission Implementing Regulation (EU) No 2017/1511 (EC, 2017c).

There are currently three market authorizations issued in Portugal for plant protection products containing indoxacarb as active ingredient (AV0093; AV0094; AV0321) (DGAV, 2016). Regarding marketing authorizations for veterinary use, there are currently two products in the market (EMEA/V/C/000163 and EMEA/V/C/002234), one for the control of *Ctenocephalides felis* fleas in cats, and the other for the control of *Ctenocephalides felis* fleas, and *Ixodes ricinus* and *Rhipicephalus sanguineus* ticks in dogs (DGAV, 2018; European Medicines Agency, 2018). These marketing authorizations are issued in accordance to Regulation (EC) No 726/2004 (EC, 2004b). Additionally, indoxacarb is approved as biocide to control cockroaches and ants under Directive 98/8/EC (EC, 2013a).

3.3.3 Environmental fate and risk to aquatic invertebrates

Photolysis, (alkaline) hydrolysis and microbial degradation are the primary mechanisms of degradation of indoxacarb in aquatic ecosystems (EFSA et al., 2018; EPA, 2017). Partitioning to sediment is also an important route for the removal of indoxacarb from the water column. Indoxacarb is somewhat hydrophobic, with a low water solubility

and a relatively high log Kow of 4.65, indicating low persistence in the water and a high tendency to sorb to sediment (J. L. Dias, 2006; EFSA et al., 2018; EPA, 2017). Additionally, from the degradation of indoxacarb in water systems, several metabolites are produced, including the bioactive metabolite DCMP, indicating a potential risk to aquatic organisms (EFSA et al., 2018; EPA, 2017).

According to the regulation (EC) No 1272/2008 of the European Parliament, indoxacarb is classified as very toxic to aquatic life with long lasting effects (EC, 2008a). A similar conclusion was reached by EPA, with growth and reproduction being identified as the most sensitive endpoints for freshwater invertebrates (EPA, 2017).

Based on several crop scenarios and dissipation in the environment, both agencies considered the risk of indoxacarb to most aquatic organisms to be low. However, EPA identified risk concerns for benthic invertebrates due to sediment sorbing of indoxacarb and its degradates (EPA, 2017). On the other hand, based on the current application rates in Europe, and on the available toxicity data, EFSA considered the risk to sediment dwellers to be low (EFSA et al., 2018). Nonetheless, EFSA's report points out that for the major aqueous photolysis metabolites, there is none or insufficient toxicity data to aquatic organisms, particularly to sediment dwellers (EFSA et al., 2018). These metabolites should be taken into consideration, as some indoxacarb metabolites have demonstrated higher toxicity to aquatic organisms than the parent compound (EPA, 2017).

3.3.4 Human exposure and poisoning

For the current uses in plant protection, indoxacarb residues are not expected to pose a risk or have harmful effects on human health (EFSA et al., 2018). Exposure to indoxacarb may occur during the application of the pesticide or through direct contact with treated crops or pets. Nevertheless, indoxacarb demonstrates a high degree of safety to mammals (McCann et al., 2012; Wing et al., 2010). The bioactive metabolite of indoxacarb has higher affinity, and consequently more potency against insect sodium channels than for their mammal counterparts (Silver et al., 2010). Moreover, while indoxacarb is primarily converted to its bioactive metabolite in insects, in mammals this conversion is minimal and indoxacarb is degraded through alternative routes, generating less active metabolites (McCann et al., 2012; Wing et al., 2010). This mammalian safety enables the use of indoxacarb in spot-on flea treatments.

In spite of this, several cases of indoxacarb poisoning in humans have been reported, but all of them denoting deliberate ingestions (Chhabra et al., 2010; Jin, 2012; J. S. Park et al., 2011; Prasanna et al., 2008; Shashibhushan et al., 2015; Shih and Tsai, 2011; Viswanathan et al., 2013; Yen et al., 2017). Methemoglobinemia was the most common clinical manifestation of indoxacarb poisoning, followed by seizures and renal injuries. None of these cases resulted in death.

3.4. Fipronil

3.4.1 Uses and mechanism of toxicity

Fipronil is very effective against a wide range of insect pests. This synthetic phenylpyrazole insecticide acts by blocking the GABA-activated chloride channel (Cole et al., 1993; Gant et al., 1998; Xu Wang et al., 2016). GABA (γ-Aminobutyric acid) is a major inhibitory neurotransmitter in insects, and fipronil action increases neural activity, thus leading to overexcitation and result in paralysis and death (Fent, 2014; Gunasekara et al., 2007; Xu Wang et al., 2016).

Fipronil was developed in 1987, and introduced in the market in 1993 (Gupta and Anadón, 2018; Tingle et al., 2003). It was registered as pesticide by EPA in the United States in 1996 (EPA, 1996b) and in the EU in 2007 (EC, 2007b) as an alternative to organophosphates and pyrethroids (Weston and Lydy, 2014). Fipronil has a wide range of activity against insect pests in both agricultural and residential settings (Salgado et al., 2012). It is particularly effective against crop pests, such as Orthoptera, Isoptera, Diptera, and Lepidoptera (Fent, 2014; Salgado et al., 2012). Additionally, is also widely used in seed treatment and as an urban pest control agent (Fent, 2014; Salgado et al., 2012). Currently, fipronil is the main active ingredient of several ectoparasiticide products for domestic animals (Fent, 2014; Gupta and Anadón, 2018; Salgado et al., 2012).

The effectiveness of fipronil as an insecticide may be explained by its high selective action, by having a much greater affinity for insect GABA receptors, compared to mammalian GABA receptors (Gant et al., 1998; Hainzl et al., 1998; NARAHASHI et al., 2007). Additionally, fipronil has been demonstrated to block Glutamate-activated chloride channels, which are insect specific (Narahashi et al., 2010; Zhao, 2004).

Recently, several authors have categorized fipronil as systemic insecticide, a statement that was promptly contested (Mortensen et al., 2015). Nonetheless, evidence suggest that some uptake by plants is likely and may provide some protection against susceptible foliage feeding insects (EFSA, 2013c; Salgado et al., 2012).

3.4.2 Regulatory Background

Fipronil was included in the list of authorized active substances in plant protection products by Commission Directive 2007/52/EC of 16 August 2007 (EC, 2007b). Fipronil was again approved for use in plant protection products by the Commission Implementing Regulation (EU) No 540/2011 of 25 May 2011 (EC, 2011b) under Regulation (EC) No 1107/2009, however the authorized use of fipronil as insecticide was limited to the use as seed treatment only, and only to be performed in professional seed treatment facilities, as established by commission directive 2010/21/EU (EC, 2010c). Further restrictions on the use of fipronil were implemented by the Commission Implementing Regulation (EU) No 781/2013 of 14 August 2013, with the use of fipronil being limited to

"…uses as insecticide for use as seed treatment may be authorized. Uses shall only be authorized for seeds intended to be sown in greenhouses and seeds of leek, onions, shallots and the group of Brassica vegetables intended to be sown in fields and harvested before flowering." (EC, 2013b). On the basis of this decision was the high acute risk identified for bees exposed to the product when used as treatment for maize seed (EC, 2013b; EFSA, 2013c). The approval of fipronil as a plant protection product expired on 30 September 2017 (EC, 2016b) and fipronil is now on the list of candidates for substitution (EC, 2015).

Concerning the veterinary use of fipronil, there are currently twenty products containing fipronil as sole active ingredient being marketed in Portugal, mainly used to control fleas, ticks and lice in cats and dogs (DGAV, 2018). Additionally, formulations combining fipronil with other active ingredients are available (DGAV, 2018). For instance, the combination of fipronil with permethrin has demonstrated great efficacy against *Phlebotomus perniciosus*, one of the main vectors of canine leishmaniasis (Dumont et al., 2015; Franc et al., 2015). Selling authorizations are issued in accordance to Regulation (EC) No 726/2004 (EC, 2004b).

3.4.3 Environmental fate and risk to aquatic invertebrates

Fipronil may contaminate water bodies through agricultural or urban runoff. Fipronil (and its degradation products) have been detected in urban streams near agricultural areas at concentrations high enough to threaten aquatic life.

Once in the environment, fipronil is subjected to hydrolysis, photolysis and biotic degradation (Gunasekara et al., 2007) to produce four major degradation products: fipronil-amide, formed mainly via hydrolytic pathway; fipronil-sulfide, a product of the reductive pathway (mainly in soils); fipronil-sulfone, a product of oxidation; and fipronil-desulfinyl, a photo-degradation product (Bobé et al., 1998a; 1998b; Gunasekara et al., 2007). Regarding aquatic environments, fipronil is relatively stable to hydrolysis at typical environmental pHs (Bobé et al., 1998b; Gunasekara et al., 2007; Ramesh and Balasubramanian, 1999), as opposed to photolysis, which is a more relevant degradative pathway of fipornil in water (Bobé et al., 1998b; Gunasekara et al., 2007). Additionally, with a high log kow of 4.01 (EPA, 1996b) fipronil has a tendency to sorb to sediment. This partition to sediment plays a role in the removal of fipronil from water column, however due to the shielding effect of sediment, the photodegradation rates are slower, and fipronil is more persistent (Gunasekara et al., 2007; Lin et al., 2009; 2008; Oliver et al., 1979; Tingle et al., 2003). Moreover, fipronil degradates, in general, have a higher tendency to sorb to soil and are more persistent than the parent compound (Lin et al., 2009). This constitutes an alarming scenario, since many studies point out that fipronil degradates have comparable or higher toxicity than fipronil itself (EPA, 1996b; Fent, 2014; Gunasekara et al., 2007; Lin et al., 2009; Weston and Lydy, 2014).

Fipronil is very toxic to aquatic life (EC, 2008a; EPA, 1996b; Weston and Lydy, 2014), with chironomids being one of most susceptible aquatic invertebrates (Ali et al., 1998; Stevens et al., 2011; Tingle et al., 2003; Weston and Lydy, 2014). *Chironomus tepperi* (4^{th} instar) has a reported 24h LC_{50} of 0.43 μg L^{-1} (Stevens et al., 1998); *Chironomus crassicaudatus* and *Glyptotendipes paripes* have both an estimated 48h LC_{50} of 0.42 μg L^{-1} for 4^{th} instar larvae (Ali et al., 1998); *Chironomus annularius* (instar not specified) has a reported 48h LC_{50} of 2.45 μg L^{-1} (Chaton et al., 2002). While assessing the toxicity of Fipronil to benthic macroinvertebrates, Weston and Lydy, (2014) determined an EC_{50} (thrashing response when prodded) of 0.030-0.035 μg/L for *Chironomus dilutus*. Of all fourteen species tested, *C. dilutus* was the most sensitive (Weston et al., 2013). The authors emphasize that measured environmental concentrations in some locations exceed the EC_{50}'s for fipronil for *C. dilutus* and that other benthic invertebrates may be at risk (Weston et al., 2013). Fipronil levels as high as 5.29μg L^{-1} were detected on a small bayou surrounded by rice agriculture in 2000 in Louisiana, USA (Demcheck and Skrobialowski, 2003). More recently, fipronil concentrations of up to 10 μg L^{-1} were detected in runoff water from residential areas during 2006−2008 (California, USA) (Gan et al., 2012) and up to 0.049 μg L^{-1} in urban waterbodies in 2012 (California, USA) (Weston and Lydy, 2014). Additionally, sediment dwelling organisms may be at a higher risk of exposure (EPA, 2007; Weston and Lydy, 2014). Through 2007-2008, total fipronil levels (measured as the sum of fipronil, fipronil desulfinyl, sulfide, and sulfone) were detected at up to 17 ng g^{-1} in Ballona estuary (California, USA) sediments (Lao et al., 2010). In river basin sediments, fipronil-sulfide has been detected as up to 24.8 ng g^{-1} in 2000 Louisiana, USA (Demcheck and Skrobialowski, 2003). These reported fipronil concentrations are above the LC_{50} estimated for *Chironomus tentans* (Maul et al., 2008).
It has been demonstrated that fipronil degradates are, generally, just as or more toxic to aquatic invertebrates than the parent compound, and often found in the environment at levels comparable to those of fipronil (Gan et al., 2012; Weston and Lydy, 2014). For instance, for *Chironomus dilutus* EC_{50}'s of 0.0093-0.0105 μg L^{-1} and 0.0075-0.0079 μg L^{-1} were determined for the sulfide and sulfone metabolites, respectively (Weston and Lydy, 2014). Fipronil-desulfinyl, fipronil-sulfide and fipronil-sulfone levels as high as 1.13 μg L^{-1} (Louisiana, USA; (Demcheck and Skrobialowski, 2003)), 0,33 μg L^{-1} and 1.96 μg L^{-1} (California, USA; (Gan et al., 2012)) respectively, were detected in the environment.

Moreover, fipronil is highly toxic to several other aquatic invertebrates, including other dipterans, and arthropods such as mysids, cladocerans, among others (Ali et al., 1998; EPA, 2007; Overmyer et al., 2007). Measured levels, along with the dissipation and persistence of fipronil and its metabolites in the environment, suggest that fipronil is a threat to aquatic invertebrate communities and particularly to chironomids (Weston and Lydy, 2014).

3.4.4 Human exposure and poisoning

In 2014, EFSA's risk assessment concluded that fipronil residues from crop and livestock authorizes uses at the time, were unlikely to pose a consumer health risk (EFSA, 2014). With recent restrictions on authorizations and the withdrawal of fipronil-containing products for plant protection, agricultural use of fipronil should no longer be of concern.

Occupational exposure to fipronil is expected to be negligible, when safety precautions are taken (EPA, 2011a; 2011b; S.-J. Lee et al., 2010). Conversely, many poisoning incidents involving fipronil have been reported. According to EPA, 4243 incidents involving fipronil were reported in the USA from 2002 to 2010, and one incident resulted in death due to an allergic reaction (EPA, 2011b; 2011c). Accidental exposure and suicide attempts are the main causes of fipronil poisoning (Gupta and Anadón, 2018). A detailed study on acute illnesses associated with fipronil exposure in the United States between 2001 and 2007 revealed that Neurological symptoms (50%) such as headache, dizziness, and paresthesia were the most common clinical manifestations, followed by ocular symptoms, such as irritation, pain, inflammation and lacrimation (S.-J. Lee et al., 2010). Nausea, vomiting, respiratory and dermatologic symptoms were also common among patients (S.-J. Lee et al., 2010). Most cases occurred in private residences, and the main contributing factors to fipronil exposure were unintentional release of the product and inappropriate precautionary actions (S.-J. Lee et al., 2010).

In July and August 2017, millions of chicken eggs were withdrawn from the market in several European countries, as they were found to contain elevated fipronil levels (Bratinova et al., 2017). As fipronil is not authorized for use in food-producing animals, a MRL of 0.005 mg Kg^{-1} is set for fipronil (measured as fipronil plus its sulfone metabolite) in eggs by the regulation Commission Regulation (EU) No 1127/2014 of 20 October 2014, the limit of analytical quantification (EC, 2014b; EFSA, 2014). Levels of fipronil in eggs were measured as high as 1.2 mg Kg^{-1}, which exceeds the acute reference dose for children (BfR, 2017a). Besides this possible risk for children through the acute intake of Fipronil, this incident was very unlikely to pose a risk to public health and there were no reported clinical cases (ANSES, 2017; BfR, 2017b).

4. Objectives and outline of the thesis

The work presented here focused on evaluating the effects of pesticides on the freshwater midge *C. riparius* at different levels of biological organization. To investigate that, *C. riparius* larvae were exposed to four insecticides with distinct modes of action to address the following questions:

- What are the biochemical and organismal-level responses to these insecticides' exposure?

- Which proteins are differentially expressed under exposure to different classes of insecticides?

- Is there a relation between the protein differential expression, biochemical biomarkers and higher-level responses?

Answering these questions will provide new insights on the mechanisms that trigger individual responses and determine if proteomics and biochemical biomarkers can potentially be used as reliable and sensitive tools in ecological risk assessment.

In order to answer these questions, *C. riparius* larvae were initially exposed to different neurotoxic insecticides, and their effects evaluated using survival, larval growth, emergence, development time, and imagoes weight as endpoints. Additionally, biomarkers related to oxidative stress, oxidative damage, neurotoxicity, and energy metabolism were used to assess sub-lethal responses to each compound at the biochemical level. Effects of amitraz at individual and biochemical levels are presented in **chapter II**, while the effects of spinosad and indoxacarb are discussed in **chapter III**, and finally the effects of fipronil on these levels are presented in **chapter V**. In **chapter IV**, the potential of proteome as an early warning indicator of spinosad and indoxacarb exposure was evaluated. *C. riparius* larvae were exposed to sub-lethal concentrations of spinosad and indoxacarb in order to assess proteome changes that could possibly lead to the effects observed at the biochemical and organismal level. Besides the effects of fipronil at individual and biochemical levels, **chapter V** offers an integrative approach combining life-history responses, biochemical biomarkers, fatty acid profiling, and proteomics, to assess the effects of this insecticide in different levels of biological organization. This chapter underlines the usefulness of proteomics in risk assessment studies to explore early events associated with the individual response, providing a better interpretation of the effects of insecticides. Finally, in **chapter VI**, the major findings from the previous chapters are highlighted and discussed. Common effects and pesticide-specific responses are examined, and the potential use of proteomics in risk assessment is further discussed.

5. Relevance of the thesis

The present work intends to provide new data and information on two main topics:

Information of sub-lethal effects of insecticides on *Chironomus riparius* – The information of sub-lethal effects of pesticides on non-target aquatic invertebrates, is still scarce, especially for novel pesticides. Traditional ecotoxicological testing and risk assessment is based on mortality data, which may underestimate the risk that pesticides pose to natural environments. In freshwater systems, organisms may be subjected to low concentrations

of insecticides that can produce long-term effects on ecological integrity of the ecosystems. This work focused on sub-lethal effects of exposure to four different insecticides at the individual level to determine possible long-term effects on population dynamics, and at sub-individual levels to determine alterations that could lead to individual responses.

The potential of proteomics in environmental risk assessment – The use of proteomic tools in ecotoxicology is rapidly increasing. It has been demonstrated that proteomics may help Identifying of mechanisms and molecular targets involved in toxic processes, reveal modes of action of toxicants, and be valuable in biomarker discovery. Nonetheless, one of the challenges in ecotoxicoproteomics is still establishing a link between a molecular event and adverse outcomes observed at higher levels of biological organization. Although identifying molecular responses associated with xenobiotics' exposure may provide some important information regarding their toxicity, it does not necessarily mean that these responses are accurate predictors of higher-level impacts. The present study intended to evaluate responses of *C. riparius* larvae to insecticide exposure at the proteome level and interpret them in the light of the responses observed at biochemical and individual levels, and thus contribute to the understanding of early molecular events that lead to higher level responses and how they impact freshwater ecosystems.

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Chapter II

Amitraz toxicity to the midge *Chironomus riparius*: Life-history and biochemical responses

Amitraz toxicity to the midge *Chironomus riparius*: Life-history and biochemical responses. [1]

Abstract

Acute and chronic toxicity of the formamidine pesticide amitraz to the midge *Chironomus riparius* was assessed using conventional ecotoxicological tests and biochemical approaches (biomarkers). Amitraz is mainly used as an ectoparasiticide in veterinary medicine, but also in agriculture and apiculture. However, information of amitraz toxicity to non-target invertebrates is limited. Besides the impairment of developmental and emergence rates (reduced larval growth, emergence, and delayed development time) caused by chronic exposure to amitraz, acute exposures induced alterations in the antioxidant enzymes glutathione peroxidase (GPx) and catalase (CAT), and in energetic metabolism biomarkers, lactate dehydrogenase (LDH) and electron transport system (ETS) activities. Moreover, lipid peroxidation (LPO) increased by amitraz exposure. Our results reveal potential secondary effects of amitraz to invertebrates and biomarkers that may aid in the interpretation of sub-lethal toxic responses to amitraz. These results add information concerning the potential outcomes of amitraz exposure to freshwater invertebrates underlining the importance of risk assessment studies of formamidine pesticides.

Keywords: Formamidine Pesticides; Freshwater invertebrates; Biomarkers; Oxidative Stress; Life-history responses.

[1] Hugo R. Monteiro, Marco F.L. Lemos, Sara C. Novais, Amadeu M.V.M. Soares and João L.T. Pestana, published in *Chemosphere*, doi: 10.1016/j.chemosphere.2019.01.018

1. Introduction

Amitraz is a widely used and effective insecticide and acaricide, mainly used in veterinary medicine to control ticks, mites and lice on animals (Farmer and Seawright, 1980; Veale, et al., 2011; Mueller et al., 2012), and in agriculture to control fruit tree and cotton pests (Bonsall and Turnbull, 1983; Peter et al., 2006; Gholamzadeh, et al., 2012). In some countries it has been also registered for the use in apiculture to control the varroa mite (*Varroa destructor*) (Kayode, et al., 2014; Gurgulova et al., 2015). Its effectiveness and wide spectrum) can be explained by its several biochemical targets, including the inhibition of monoanine oxidases (Aziz and Knowles, 1973; Beeman and Matsumura, 1978; Atkinson et al., 1974), and the activation of octopamine receptors (Evans and Gee, 1980; Dudai et al., 1987; EPA, 2010a; Ahmed et al., 2015).

Once in the environment, due to its high log Kow (5.34 - 5.5), amitraz is expected to adsorb to soil and sediment (Osano et al., 2002; EPA, 2010b), and to quickly metabolize into persistent and more water soluble products (EPA, 1996; Osano et al., 2002; Wexler, 2014). However, in several countries, due to its widespread use and high direct application rate (Veale et al., 2011; Mueller et al., 2012; Kayode et al., 2014; Maciel et al., 2015), there is an elevated risk of run-off and contamination of adjacent aquatic ecosystems (EPA, 2010b). Since parent amitraz is short-lived in the environment, it is not expected to pose a major concern for aquatic invertebrates, as opposed to some more stable and toxicologically relevant metabolites that retain toxic activity (Corta et al., 1999; del Pino et al., 2015; EPA, 2010b; Osano et al., 2002). One of the main amitraz metabolites, BTS-27271, may be of particular concern due to its persistence in aquatic environments (EPA, 1996). However and based on *Daphnia magna* ecotoxicity studies, the United States Environmental Protection Agency (EPA) described this metabolite as moderately toxic, (based on a 48h EC_{50} of 2.59 mg L^{-1}), while parent amitraz was described as very highly toxic to aquatic invertebrates (based on a 48h EC_{50} of 35 μg L^{-1}) (EPA, 1996). In the European Union (EU), amitraz is classified as "very toxic to aquatic life with long lasting effects" due to its acute toxicity to aquatic organisms ($EC_{50} \leq 1$ mg L^{-1}) and an experimentally determined bioconcentration factor of > 500 (EC, 2008; EPA, 2010b). These conflicting views regarding amitraz toxicity and its potential ecological effects, support the need of risk assessment studies of amitraz and its metabolites.

Due to its widespread distribution and key role in aquatic ecosystems (Péry, et al., 2002), Chironomidae are of great ecological relevance and have been used as standard invertebrate models for toxicity testing and risk assessment of aquatic contaminants (Taenzler et al., 2007; Weltje et al., 2010). Moreover, its position in the aquatic food chain, short-life cycle including growth through a molting regime and a complete metamorphosis, the presence of hemoglobin as respiratory pigment and its sensitivity to many pollutants, make *Chironomus riparius* a suitable test species for water quality

monitoring (Osmulski and Leyko, 1986; Choi et al., 2001; Lee et al., 2009; Azevedo-Pereira et al., 2012).

When dealing with xenobiotic exposure, a set of defensive mechanisms inside the organism are activated to protect it from harmful effects. The effects of amitraz were assessed on the phase II biotransformation enzyme glutathione-S- transferase (GST), one of the key enzymes involved in the detoxification pathway, responsible for facilitating the removal of xenobiotics (Pickett and A. Y. Lu, 1989; van der Oost et al., 2003; Ziglari and Allameh, 2013). Several stressors, such as pesticides, are known to increase the production of reactive oxygen species (ROS) (Novais et al., 2014). Antioxidant defense enzymes such as catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) are essential in the control and protection against ROS, and therefore their activities were also assessed. If these defense mechanisms fail, an excess of ROS, can cause oxidative damage (Livingstone, 2003; Winston and Di Giulio 1991). In this work, lipid peroxidation (LPO) was determined as an indicator of oxidative damage.

The activation of detoxification processes and antioxidant defenses are very energy-demanding. An increase in energy consumption, evaluated through the measurement of the electron transport system (ETS) activity, may reveal if extra energy is being required for these defensive mechanisms, which may compromise organisms' homeostasis, performance and development. Other energy metabolism related enzymes, such as lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH), involved in the anaerobic and aerobic metabolism, respectively, that play an important role in energy production (Diamantino et al., 2001; Lima et al., 2007; Silva et al., 2016) were also assessed.

Chironomus riparius acetylcholinesterase (AChE) activity, related to cholinergic neurotransmission, was also used as biomarker of neurotoxicity due to the neurotoxic nature of amitraz and the relationship between AChE acitivity and behaviour (Xuereb et al., 2009), despite previous research indicating negligible effects of amitraz on AChE activity in vertebrates (Moser and MacPhail, 1989).

In this study, the ecotoxicological response of the non-target aquatic midge *C. riparius* to amitraz was thus assessed using standard ecotoxicological tests, with survival, larval growth, emergence, development time, and imagoes weight used as endpoints (OECD, 2004; OECD, 2011). The sensitivity of the above mentioned biochemical biomarkers to amitraz exposure on *C. riparius* and their potential for use in biomonitoring studies of this pesticide in aquatic systems were also evaluated.

2. Material and Methods

2.1 Test Chemical

An 8 g L^{-1} stock solution of amitraz (analytical standard, CAS number 33089-61-1 Sigma-Aldrich, USA) was prepared in dimethyl sulfoxide (DMSO) and stored in the dark at 4°C until further use. Experimental solutions were prepared by diluting this stock solution in American Society for Testing and Materials (ASTM) hard water (ASTM, 1980). The DMSO was kept below 0.01% in all experimental solutions, except in the acute toxicity experiment in which the DMSO concentration was 0.1%. Previous research has shown that this concentration of DMSO poses no effect in aquatic invertebrates (Bowman et al., 1981).

Chemical analyses were made using an API 5000 triple quadrupole mass spectrometer from SCIEX, USA) coupled to a LC system (Agilent, USA) using a LC gradient method with water and methanol. Measurements were made in positive electrospray ionization mode. Amitraz standard was prepared in pure water. Water samples were filtered and measured directly. Matrix effects are compensated by using internal standards and quantification is done by using the method of standard addition. Since amitraz is very unstable in water, metabolites were also analyzed, and results reported here correspond to the current residue definition for amitraz in the EU: "Amitraz (sum of amitraz and all metabolites containing the 2,4- dimethylaniline moiety, expressed as amitraz)" or simply "Amitraz (sum)" (EC, 2017). The limit of quantification for amitraz, 2,4'-Formoxylidid (amitraz metabolite), and amitraz (sum) was 0.15 µg L^{-1}.

2.2 Chironomus riparius culture conditions

C*hironomus riparius* were obtained from a laboratory culture that has been established at the University of Aveiro for over a decade. Briefly, this culture is maintained in plastic containers filled with ASTM hard water medium and inorganic commercial sand (<1 mm) at 20 ± 1°C and a 16:8 h light:dark cycle. Organisms are fed with a suspension of macerated fish food, Tetramin® (Melle, Germany), and continuous aeration is provided. Prior to a test, freshly laid egg masses are collected from the culture and larvae are maintained in previous stated conditions until reaching the desired age for bioassays.

2.3 Acute bioassays

Acute toxicity of amitraz was assessed following OECD guideline 235 with water only exposures (OECD, 2011). Less than twenty-four hours old *C. riparius* larvae (1st instar) were exposed to concentrations of amitraz ranging from 0 to 8000 µg L^{-1} (0, 31.25, 62.5, 125, 250, 500, 1000, 2000, 4000, and 8000 µg L^{-1}; nominal concentrations) in crystalizing dishes, and three replicates, consisting of 5 organisms in 10 mL of

experimental media were used. After 48 hours, mortality (registered as immobilization) was assessed by mechanical stimulation. These tests took place at 20 ± 1°C and in the dark, to prevent photodegradation of the compound. No food was provided during the exposure period.

2.4 Chronic bioassay

To evaluate the chronic effects of sub-lethal concentrations of amitraz, a 28-days chronic test was performed according to OECD guideline 219 (OECD, 2004), with growth, emergence, development time, and adult weight as measured endpoints. *Chironomus riparius* 1st instar larvae (2 days old) were exposed to four amitraz treatments (8.2, 18.9, 25.8, and 29.4 µg L^{-1}, measured concentrations) and, in parallel, to two controls, ASTM hard water medium and solvent control. A total of 13 replicates were used per treatment. Each replicate consisted of 5 larvae in 200 mL glass vessels containing 150 mL of the media and a 1.5 cm layer of sterile sediment (commercial river sand washed, sieved and burnt). Test was conducted in the same conditions as the culture. Organisms were fed every other day with a ration of 0.5 mg Tetramin® per larvae per day, and physicochemical parameters were monitored throughout the test (temperature, pH, dissolved oxygen, and conductivity). After 10 days of exposure, larvae from 5 replicates in each treatment were collected and stored in 70% ethanol to determine larvae growth by subtracting their final length with the average body length of larvae from day -1 of the experiment (stored in ethanol). Measurements were made with a dissecting stereomicroscope fitted with a micrometer. The eight remaining replicates were used to determine emergence endpoints. Emerging *C. riparius* adults were counted on a daily basis and collected and stored in 70% ethanol to determine their gender based on antenna morphology. Afterwards, collected adults were dried at 50°C for 24 h and weighed in a microbalance (RADWAG® MYA 2.3Y). An additional replicate of each treatment was prepared under the same conditions as described above, and 24 hours after the beginning of the experiment, water samples were collected for chemical analyses.

2.5 Biomarkers exposure experiment

Eight-day old larvae (3rd instar) were used in a sub-lethal exposure for biomarker determination. The test consisted ten organisms per replicate in a crystallizing dish containing 80 mL of experimental solution (positive control, 8.2, 18.9, and 29.4 µg L^{-1}) and a sand layer about 1:4 of overlying water. After 48 hours of exposure, organisms from two replicates of the same treatment were pooled, giving a total of twenty organisms per pooled replicate for biomarker determination. A total of seven pooled replicates of twenty organisms were used per treatment. Larvae were not fed during this period. After

collection and pooling, larvae were rapidly dried with filter paper, weighed, frozen in liquid nitrogen and stored at -80°C until further analysis.

2.5.1 Sample preparation for Biomarkers

Samples were homogenized in 800 µL of 0.1 M of K-phosphate buffer (pH=7.4) with a Ystral d-79282 homogenizer. Portions of the homogenate were separated for the determination of the electron transport system activity (ETS) as a measure of cellular oxygen consumption and lipid peroxidation (LPO) levels. The remaining homogenate was centrifuged at 10,000 *g* for 20 min at 4°C and the post-mitochondrial supernatant (PMS) was collected and separated into fractions for superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), acetylcholinesterase (AChE), glutathione reductase (GR), glutathione peroxidase (GPx), lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) activities, and for protein quantification. All biomarker assays were measured at 25°C. Blanks were made using K-phosphate buffer instead of the sample and all spectrophotometric measurements were made in quadruplicates in a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek® Instruments, Vermont, USA).

2.5.1.1 Protein quantification

Protein concentration was quantified following the Bradford protocol (Bradford, 1976) adjusted to 96 well plates. Bovine γ-globulin (Sigma-Aldrich, USA) was used as standard. Absorbance was read at 600 nm and results are expressed in mg of protein mL^{-1}. Before enzymatic assays, protein concentration was adjusted in each sample to approximately 0.8 mg L^{-1} except for IDH and SOD where the total protein amount was used. Protein concentration of the dilution was confirmed by the same method at the end of the assays.

2.5.1.2 Oxidative damage

Immediately after separation of sample for LPO analysis, 2,5 µL of 4% 2,6-Di-tert-butyl-4-methylphenol (BHT) in methanol was added to each aliquot to prevent further lipid oxidation (Torres et al., 2002). Lipid Peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) at 535 nm. The method was described by Ohkawa et al. (1979) and Bird and Draper (1984) and protocol was adapted from Torres et al. (2002). Results are expressed as nmol TBARS g^{-1} of wet weight.

2.5.1.3 Detoxification and oxidative stress related enzymes

For the assessment of GST activity, an adaptation of Habig et al. (1974) protocol to microplate was used. GST activity was determined by following the conjugation of reduced glutathione (GSH) with 1-chloro-2, 4-dinitrobenzene (CDNB). The formation of the resulting thioether was measured at 340 nm. The CAT activity was determined by

following the consumption of H_2O_2 at 240 nm (Clairborne, 1985). The GR activity was determined following the oxidation of NADPH at 340 nm, using oxidized glutathione (GSSG) as substrate (Cribb et al., 1989). The GPx activity was determined by following the reduction of GSSG back to GSH performed by GR (added in excess to the reaction), with consequent oxidation of NADPH (measured at 340 nm), using hydrogen peroxide (H_2O_2) as substrate and sodium azide as an inhibitor of catalase (Mohandas et al., 1984). All above enzymatic activities are expressed in nmol min^{-1} mg^{-1} of protein except CAT, which is expressed in μmol min^{-1} mg^{-1} of protein. The SOD activity was determined through the reduction of cytochrome c by the xanthine/xanthine oxidase system measured at 550 nm. Results are expressed as SOD units (U) mg^{-1} protein (McCord and Fridovich, 1969).

2.5.1.4 Neurotoxicity

The AChE activity was measured by the method described in Ellman et al. (1961) adapted to microplate (Guilhermino et al., 1996). Using acetylthiocholine as substrate, the formation of 2-nitro-5-thiobenzoate anion (TNB^{2-}), product of the reaction between 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and thiocholine, is followed at 414 nm. Results are expressed in nmol TNB^{2-} mg^{-1} protein.

2.5.1.5 Energetic metabolism

The LDH (associated with anaerobic metabolism) activity was determined using the methods described by Vassault, (1983) and Diamantino et al. (2001). The oxidation of NADH when pyruvate is converted to lactate is followed at 340 nm and the results expressed in nmol min^{-1} mg^{-1} of protein. The IDH (associated with aerobic metabolism) activity was determined according to Ellis and Goldberg (1971) adapted to microplate (Lima et al., 2007). The increase of NADPH, when isocitrate (DL-isocitric acid) is decarboxylated by IDH, was followed at 340 nm and results expressed in nmol min^{-1} mg^{-1} of protein. The ETS activity was determined according to the method described by De Coen and Janssen (1997) and the protocol followed was described by Rodrigues et al. (2015a), but starting with 150 μL of homogenate sample.

2.6 Statistical analysis

Larval growth data was analyzed by one-way analysis of variance (ANOVA) followed by a post hoc test for trend. All remaining endpoint data were analyzed by ANOVA followed by Dunnett's post hoc test to discriminate differences between solvent control and treatments. Unpaired *t*-tests did not reveal any differences between solvent and negative controls and therefore solvent control was used as control in chronic bioassays data analysis. Prior to all tests, residuals were checked for normality and homoscedasticity of data was checked with Brown-Forsythe test. All statistical analyses were performed using GraphPad Prism® 7 for Mac software and significance level was set

at 0.05 for all statistical tests.

3. Results

3.1 Acute toxicity test

Because of the gradient of concentrations used, the 48 h LC_{50} could not be calculated and thus is estimated to be greater than the highest concentration tested (8000 μg L^{-1}). In the highest tested concentration there was 20% mortality after 48 h of exposure.

3.2 Chronic toxicity test

Growth and emergence related parameters are presented in Table I. Exposure to amitraz for 10 days did not affect larvae survival but the test for linear trend revealed a significant reduction in larval growth with increasing amitraz concentrations ($r^2 = 0.23$, $p < 0.05$). Midge survival at day 28 (measured as percentage of emerged adults) was 42.5% and was significantly reduced at 29.4 μg L^{-1} ($F_{(4,35)} = 2.67$, $p < 0.05$). No additional larvae or pupae were found alive in test vessels at tear down. Moreover, although not significant, there was also a decrease in the number of emergents at 25.8 μg L^{-1} (only 65% adults emerged) compared to 82.5% emergence in the control treatment. Development time of *C. riparius* males was affected by amitraz and a significant delay in the mean time to emergence was observed for the 18.9 μg L^{-1} and 25.8 μg L^{-1} ($F_{(4, 25)} = 4.98$, $p < 0.01$) treatments. Development time of *C. riparius* females was not significantly affected by amitraz at the concentrations tested compared to the control treatment ($F_{(4, 27)} = 1.01$, $p = 0.42$). There were no significant differences in adult dry weight between control and amitraz treatments for male ($F_{(4, 24)} = 1.98$, $p = 0.13$) and female ($F_{(4, 27)} = 1.39$, $p = 0.26$) imagoes (Table II).

Table I – Growth and emergence endpoints of *Chironomus riparius* larvae exposed to Amitraz. All values are presented as mean ± SEM. An asterisk denotes statistically significant differences to the control treatment ($p < 0.05$, ANOVA, Dunnett's test). A dagger denotes a statistically significant linear trend ($p < 0.05$, ANOVA, test for trend)

Amitraz Concentrations (μg L^{-1})	Growth (mm)†	Total emergents (%)	Development time (days)	
			Males	Females
0	11.11 ± 0.36	82.50 ± 7.96	15.46 ± 0.33	18.99 ± 0.82
8.2	10.80 ± 0.37	82.50 ± 9.59	17.19 ± 0.62	19.67 ± 0.79
18.9	10.50 ± 0.26	80.00 ± 7.56	18.80 ± 0.87*	20.98 ± 0.86
25.8	10.47 ± 0.50	65.00 ± 12.39	17.79 ± 0.50*	20.15 ± 0.97
29.4	9.73 ± 0.43	42.50 ± 13.86*	17.17 ± 0.44	20.65 ± 0.60

Table II – Adult weight of *Chironomus riparius* exposed as larvae to Amitraz. All values are presented as mean ± SEM.

Amitraz Concentrations (μg L^{-1})	Males dry weight (μg)	Females dry weight (μg)
0	425.3 ± 16.1	889.1 ± 43.7
8.2	457.1 ± 13.0	932.6 ± 42.9
18.9	471.0 ± 10.2	979.4 ± 23.9
25.8	468.8 ± 12.4	962.1 ± 36.0
29.4	460.7 ± 11.4	994.4 ± 29.0

3.3 Biochemical responses

Levels of LPO significantly increased at concentrations of 18.9 and 29.4 μg L^{-1} of amitraz ($F_{(3, 24)}$ = 12.87, p < 0.001; Fig. 1). A significant increase in GPx was detected at a amitraz concentration of 29.4 μg L^{-1} ($F_{(3, 24)}$ = 5.83, p < 0.01; Fig. 2a), while for GST ($F_{(3, 24)}$ = 0.68, p= 0.57) and GR ($F_{(3, 24)}$ = 0.27, p = 0.84) there were no differences observed after amitraz exposure (Fig. 2b,c). Catalase activity decreased at 18.9 and 29.4 μg L^{-1} treatments ($F_{(3, 24)}$ = 6.41, p < 0.001; Fig. 2d), whereas no significant difference in SOD activity ($F_{(3, 24)}$ = 0.66, p = 0.59; Fig.2e) or AChE activity ($F_{(3, 24)}$ = 2.74, p = 0.07; Fig. 2f) was observed after amitraz exposure. A significant decrease in LDH activity was observed at the highest test concentration ($F_{(3, 23)}$ = 4.77, p < 0.01; Fig. 3a). No difference in IDH activity was observed ($F_{(3, 24)}$ = 1.24, p = 0.32; Fig. 3b), while a significant increase in ETS activity was observed at the 18.9 and 29.4 μg L^{-1} concentrations of amitraz ($F_{(3, 22)}$ = 18.17, p < 0.001; Fig. 3c).

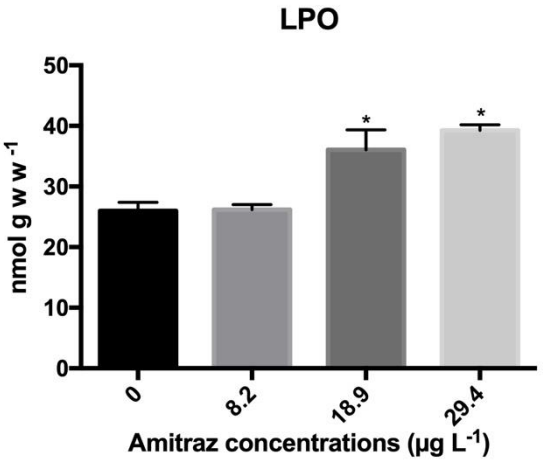

Figure 1 – Lipid Peroxidation (LPO) levels in *Chironomus riparius* larvae after 48h exposure to amitraz. All values are presented as mean + SEM. An asterisk denotes statistically significant differences to the control treatment (p < 0.05, ANOVA, Dunnett's test).

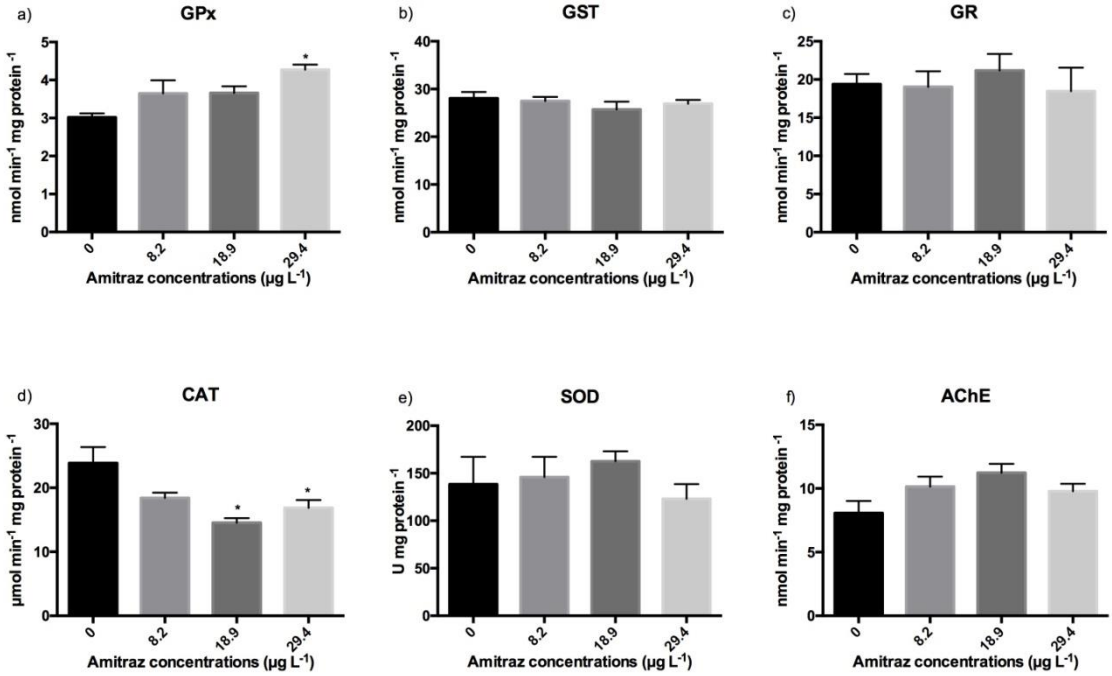

Figure 2 – Oxidative stress and neuromuscular toxicity related biomarkers in *Chironomus riparius* larvae after 48h exposure to amitraz: a) Glutathione Peroxidase (GPx); b) Glutathione S-Transferase (GST); c) Glutathione Reductase (GR); d) Catalase (CAT); e) Superoxide Dismutase (SOD); f) Acetylcholinesterase (AChE). All values are presented as mean + SEM. An astrisk denotes statistically significant differences to the control treatment ($p < 0.05$, ANOVA, Dunnett's test).

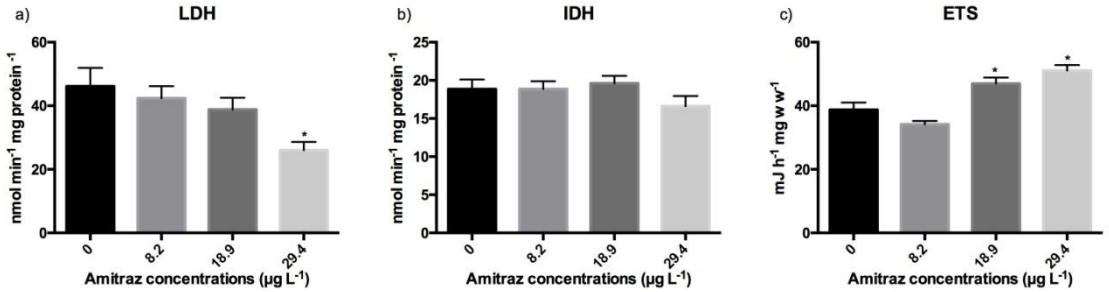

Figure 3 – Energetic metabolism related biomarkers in *Chironomus riparius* larvae after 48h exposure to amitraz: a) Lactate Dehydrogenase (LDH); b) Isocitrate Dehydrogenase (IDH); c) Electron Transport System (ETS) activity. All values are presented as mean + SEM. An asterisk denotes statistically significant differences to the control treatment ($p < 0.05$, ANOVA, Dunnett's test).

4. Discussion

The current study shows that exposure to amitraz affects the non-target freshwater invertebrate *C. riparius*. Additionally, biochemical changes underlying biological endpoint effects and potential secondary targets of amitraz were observed. Reduction of LDH activity, evidences of oxidative stress, oxidative damage and higher energy expenditure may have contributed to the impairment of growth and development of *C. riparius* larvae. At the biochemical level, CAT, ETS, and LPO were the most sensitive endpoints, while male development time was the most sensitive at organismal level.

Regarding the acute toxicity of amitraz, our results are in line with the previous 96h LC_{50} of 3,28 mg L^{-1} estimated by Osano et al. (2002) for *C. riparius* first instar larvae. To our knowledge, available information on acute toxicity of amitraz to other dipterans is limited to the studies conducted by Pridgeon et al. (2009) and Ahmed and Matsumura (2012) on *Aedes aegypti*. Reported 24-hour LC_{50} values are of 660 μg L^{-1} for the first instar larvae (Pridgeon et al., 2009), and 323 mg L^{-1} (24h), 320 mg L^{-1} (48h) and 317 mg L^{-1} (72h) for the fourth instar (Ahmed and Matsumura, 2012). Regarding other aquatic invertebrates, an EC_{50} of 35 μg L^{-1} (immobilization) was calculated for *Daphnia magna* (EPA, 1996).

The emergence of *C. riparius* adults was negatively affected by amitraz, dropping to 42.5% at the highest concentration tested. Development time of males was also impacted while development time of females was not affected. This delay in male development, which may be a direct outcome of the observed reduction in larval growth, can ultimately affect population dynamics of protandrous species like *C. riparius* (Postma and Davids, 1995; Azevedo-Pereira and Soares, 2010). Despite the observed significant delay in C. riparius male development, no changes were observed in terms of imagoes weight which is also directly linked with reproductive fitness (Ponlawat and Harrington, 2007). This delayed emergence in males could also reflect a shift in energy allocation for stress response (as indicated by the increase in ETS activity). The absence of significant differences in the development time of males between control and the highest concentration tested may be explained by the high mortality observed. In turn, female development was not affected by amitraz. This gender-based delay in development has also been observed on *C. riparius* for other pesticides, such as cypermethrin (Goedkoop et al., 2010). It has been postulated that these sex-specific differences in susceptibility may be attributed to the larger size and higher levels of energetic reserves in females, leading to higher tolerance and accumulation capacities (Goedkoop et al., 2010). Still, other mechanisms, such as endocrine disruption, cannot be excluded when considering gender-based effects and can be potentially addressed in later studies (Lemos et al., 2010). After 24h, the metabolite 2,4'-Formoxylidid was detected at higher levels than the parent amitraz in all samples (supplementary data, table I). Notwithstanding, long-term exposure to amitraz (sum) caused sublethal effects to *C. riparius*, being male development time the most sensitive endpoint evaluated (LOEC of 18.9 μg L^{-1}).

Information on effects of amitraz at the biochemical level for aquatic invertebrates is very scarce and the few studies available are mostly made on humans or mice. While there are many recent studies on biochemical responses of *Chirononmus sp.* to pesticides and other xenobiotics' exposures (Lee and Choi, 2009; Park and Choi, 2009; Azevedo-Pereira et al., 2011; Arambourou et al., 2013; Wiseman et al., 2013; Rodrigues et al., 2015a; Rodrigues et al., 2015b; Campos et al., 2016), to our knowledge, this is the first study investigating oxidative stress induced by exposure to amitraz on dipterans. Third

instar larvae were selected for this part of study, as they are big enough to be handled easily, a relatively small number of organisms provide sufficient biomass for biomarker determination and they are not expected to molt during the exposure period – as this event is biochemically catastrophic which may induce difficulties to depict the impact of the pesticide *per se*. The basal levels of biochemical biomarkers determined here are in the same range of the levels previously determined by other authors in *C. riparius* 4th instar larvae (Campos et al., 2016; Campos et al., 2017; Rodrigues et al., 2015a; Rodrigues et al., 2015b) and in other invertebrates (Novais, et al., 2014; Rodrigues et al., 2014; Silva et al., 2013) using similar protocols.

Kruk and Bounias (1992) work indicated that ROS are produced during the oxidation of amitraz, and this increase of ROS can result in several outcomes, such as oxidative damage, including the increase of LPO (del Pino et al., 2015). A previous study reported an increase of LPO in rats and mice after amitraz administration (Kanbur et al., 2016). In the current study, exposure to amitraz induced LPO in *C. riparius* larvae, indicating oxidative unbalance and damage. An increase of LPO was previously observed in *C. riparius* larvae under exposure to the insecticide esfenvalerate (Rodrigues et al., 2015b), while chlorantraniliprole, also an insecticide, does not affect *C. riparius* LPO levels (Rodrigues et al., 2015a).

Catalase and GPx are important oxidative stress defenses and are both involved in detoxification of H_2O_2. Since SOD activity was not altered, the inhibition of CAT activity and probable accumulation of H_2O_2 (as a result of SOD activity) may have led to the increase of the GPx activity that has a higher affinity to H_2O_2 compared to CAT (Lushchak, 2012). Nonetheless, the increased activity of GPx was not sufficient to prevent oxidative damage. The inhibitory effect of amitraz on CAT activity has been previously reported in rats (e.g. Kanbur et al., 2016). Moreover, inhibition of CAT activity appears to be a common effect of chemical stress in *C. riparius* (Rodrigues et al., 2015a; Rodrigues et al., 2015b; Campos et al., 2016), underlining CAT activity as one of the most consistent biomarkers of oxidative stress in invertebrates. By itself, CAT does not provide sufficient information on antioxidant metabolism, and information should always be integrated with other enzymes activities for a better interpretation of indirect effects of stressors at the biochemical level. Based on present results, conjugation by GST does not seem to be a significant pathway for amitraz detoxification. Moreover, despite the increase of GPx activity (and consequent overproduction of oxidized glutathione (GSSG)), GR activity remained unchanged. Since GR is responsible for the recycling of GSSG into GSH (glutathione), the substrate used by GST and GPx, results suggest that the consumption of GSH is not being compensated by GR. These actions may result in the accumulation of GSSG, and a low GSH/GSSG ratio is usually an indicator of oxidative stress (Zitka et al., 2012). However, GSSG, GSH, or total glutathione levels were not measured in this work. Future studies should include these endpoints to ascertain the role of glutathione in detoxification and antioxidant defense against amitraz. Previous studies reported a

decrease in *C. riparius* total glutathione levels under exposure to insecticides (Rodrigues et al., 2015a; Rodrigues et al., 2015b).

Regarding metabolic state indicators, the reduction of LDH activity indicates a decrease of the energy generated through the anaerobic pathway. Usually, when dealing with chemical stress, LDH activity tends to increase in order to respond to higher energy demands for organisms' defenses and homeostasis, and therefore, LDH inhibition observed here may signify that there was not enough energy being generated for an adequate response (Luis and Guilhermino, 2012). One possibility for the decrease in LDH activity, is that the organisms may be favoring more efficient aerobic metabolism at the expense of anaerobic metabolism, as reported in other situations (e.g. Kühnhold et al., 2016). In the current study, despite no significant changes detected in IDH activity, the activity of IDH isoforms was performed in the PMS and therefore it cannot be asserted that mitochondrial IDH activity remained, in fact, unchanged. However, an increase in ETS reveals that there was an increase in cellular metabolism. These higher metabolic costs may be attributed to the energy needed and allocated for antioxidant defenses and repair (ex. GPx), implicating that the amount of energy available for growth, molting, reproduction and other biological functions will be lower. This is in accordance to the reduced growth and developmental impairment of *C. riparius* observed in the present study. Nevertheless, measuring the energy reserves available, in combination with ETS, would give a better overview of the energy status and trade-offs of organism at a cellular level (De Coen and Janssen, 1997; De Coen and Janssen, 2003; Rodrigues et al., 2017).

Despite no effects observed concerning AChE activity, biochemical and life history effects of amitraz might also be related to effects on other neurotransmitters since amitraz has been shown to activate of alpha-adrenergic receptors and inhibit of monoamine oxidases in vertebrates (del Pino et al., 2015), and to interact with octopamine receptors in invertebrates. As such, since biogenic amines (e.g. octopamine, dopamine, tyramine, serotonin, and histamine) are important neurotransmitters and neuromodulators in insects with functions in several physiological and behavioral processes (Blenau and Baumann, 2001), more research should be directed at measuring biogenic amine levels in *C. riparius* larvae under exposure to amitraz and other insecticides.

The data presented here are important since the exact mode of action of amitraz is still unknown (Kayode et al., 2014) and there is still scarce information on the effects of amitraz in invertebrates. Alterations in biological endpoints observed (reduced larval growth, delayed development time, emergence, and surviva) may be direct consequences of the reduction of LDH and CAT activities, increased oxidative stress and damage, and energy allocation for stress response. Although not specific, CAT, GPx, and LDH could be potential indicators of amitraz-induced stress and could provide a better interpretation of sub-lethal toxic responses in aquatic invertebrates. As demonstrated here, LPO and ETS may also be valuable biomarkers to aid in the interpretation of pesticide-induced stress,

as they represent the outcomes of several biochemical processes affected by the pesticide's mode of action.

Measured levels of amitraz in aquatic environments are, as far as our knowledge, absent in the literature. Nonetheless, and although amitraz is currently not approved for agricultural uses in EU countries, EPA estimated an environmental concentration of 4.5 μg L^{-1} for parent amitraz and of 32.4 μg L^{-1} for 2,4'-Formoxylidid for amitraz use on pear orchards (EPA, 1996). The concentrations used in this study are within those estimated levels and it was demonstrated that amitraz exposure can result in adverse outcomes to an ecological key species, which may severely affect the ecological integrity of freshwater ecosystems.

5. Conclusions

Our results show that exposure to amitraz can impact several *C. riparius* biological endpoints, and that alterations in biochemical biomarkers such as CAT, ETS, LPO, LDH, and GPx may aid in the interpretation of sub-lethal toxic responses to amitraz that lead to higher-level responses. This study underlines the importance of complementing standard ecotoxicological data with biochemical approaches in an integrative manner, contributing to the growing knowledge of sub-lethal effects of pesticides thus providing a better interpretation of their outcomes and potential consequences in aquatic insect populations.

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Supplementary data

Supplementary table I – Concentrations of Amitraz measured after 24 hours in chronic exposure. Samples were diluted prior to analysis; values are presented after correction for dilution (*n = 1)*, from the lowest to the highest concentration tested.

	Water (μg L^{-1})		
	Amitraz	2,4'-Formoxylidid	Amitraz (sum)
Chronic exposure	<LQ	2.2	8.2
	<LQ	7.5	18.9
	<LQ	13.8	25.8
	0.174	24.0	29.4

<LQ – Below the limit of quantification: 0.05 μg L^{-1} on measured samples (0.15 μg L^{-1} after correction for dilution);

Chapter III

Toxicity of the insecticides Spinosad and Indoxacarb to the non-target aquatic midge *Chironomus riparius*

Toxicity of the insecticides Spinosad and Indoxacarb to the non-target aquatic midge *Chironomus riparius*. [1]

Abstract

Spinosad and indoxacarb are two relatively new insecticides mainly used in agriculture to control insect pests. However, at their current application rates, non-target aquatic insect species may also be impacted by their use. In this study, larvae of the non-biting midge *Chironomus riparius* were exposed in laboratory to both insecticides and their effects evaluated at the organismal level, using standard ecotoxicological tests, and at the biochemical level, by monitoring specific oxidative stress, neuronal, and energy metabolism biomarkers. Chronic exposure to both insecticides compromised growth and emergence of *C. riparius*. Short-term exposures revealed alterations at a biochemical level that might be related to the toxicological targets of both insecticides. Growth and development time were the most sensitive endpoints at individual level for both pesticides, while at the biochemical level, the electron transport system activity was the most sensitive biomarker for spinosad exposure (LOEC of 0.5 $\mu g\ L^{-1}$), and Glutathione-S-transferase was the most sensitive biomarker for indoxacarb exposure (LOEC of 4 $\mu g\ L^{-1}$), at concentrations within the estimated environmental levels. Additionally, changes in lactate dehydrogenase and glutathione peroxidase activities were observed for both insecticides, and evidences of oxidative damage were found for spinosad. This study contributes to the growing knowledge on sublethal effects of novel insecticides on non-target aquatic invertebrates and strengthens the usefulness of biochemical biomarkers to support the interpretation of their potentially deleterious effects on aquatic insects near agricultural fields.

Keywords: aquatic invertebrates, biochemical biomarkers, insecticides, life-history effects, neurotoxicity

[1] Hugo R. Monteiro, João L.T. Pestana, Sara C. Novais, Amadeu M.V.M. Soares and Marco F.L. Lemos, submitted to *Science of the Total Environment.*

1. Introduction

One of the goals in integrated pest management is to find tailor-made and effective pesticides for specific pests while keeping adverse consequences on non-target species to a minimum (Chitgar and Ghadamyari, 2012; Stara et al., 2010; Wilkinson et al., 1979). Some non-target aquatic insects play vital roles in freshwater ecosystems, and are regularly subjected to significant concentrations of pesticides through runoff, drift, or leaching from adjacent agricultural fields (Cerejeira et al., 2003; Schulz, 2004). Ecotoxicological effects of pesticide exposure seen on higher levels of organization are often preceded by quantifiable alterations at biochemical levels and assessing earlier sub-organismal endpoints on key species may provide insights on the long-term consequences for natural populations (Lemos et al., 2010), hopefully providing regulators with early-warning tools for risk assessment.

Spinosad and indoxacarb are neurotoxic insecticides with distinct modes of action. Spinosad is composed of spinosyns A and D (Crouse et al., 2001), two byproducts of the fermentation of *Saccharopolyspora spinosa* (Actinomycetales: Pseudonocardiaceae) (Mertz and Yao, 1990; Thompson et al., 2000). Spinosad's mode of action targets a unique site in nicotinic acetylcholine receptors (Copping and Menn, 2000; Orr et al., 2009; Watson, 2001), causing hyperexcitation of the nervous system, which leads to exhaustion, paralysis and ultimately death (Salgado, 1998; Salgado et al., 1998; Salgado and Sparks, 2005). It also interferes with gamma-aminobutyric acid receptors, which enhances its toxicity (Sparks et al., 2001; Watson, 2001). Spinosad is very effective against several insect species (Hertlein et al., 2010), including chironomids (Bond et al., 2004; Lawler and Dritz, 2013; Pérez et al., 2007; Stevens et al., 2005). Although some chironomids may be regarded as pest species (Stevens et al., 2005), frequent application rates of pesticides may provoke adverse effects to the integrity of aquatic ecosystems. Spinosad is registered for agricultural use in Europe (European Commission, 2008a). In 2001, Stark and Banks (2001) determined the expected environmental concentration of spinosad in water to be 68 $\mu g\ L^{-1}$ after spray application on a forest at the average foliar application rate and recently, the European Commission assessment report, predicted an (worst case scenario) environmental concentration of 26.28 $\mu g\ L^{-1}$ on surface waters resulting from the applications on leafy and fruity vegetables (EFSA et al., 2018a). Additionally, Cleveland et al. (2002) studied the dissipation of spinosad in an aquatic microcosms simulating the direct overspray of a formulated product (480 $g\ L^{-1}$ suspension concentrate formulation; 42.9% spinosad). At an application rate of 100 $g\ ha^{-1}$, the authors determined an initial concentration of spinosad in water of 37.6 $\mu g\ L^{-1}$ and the concentration remained above the detection limit (0.5 $\mu g\ L^{-1}$) over the following eight days. Moreover, spinosad sorbs to the sediment where it seems to be more persistent (Cleveland et al., 2002), and where many sediment-dwelling organisms, including chironomid larvae, may be affected.

Indoxacarb is an oxadiazine pesticide that acts by blocking voltage-dependent sodium channels (Lapied et al., 2001; Wing et al., 1998; Wing et al., 2000) leading to feeding inhibition, tremors, paralysis, and ultimately death (Gamil et al., 2011; Wing et al., 1998; Wing et al., 2000). It is effective against several insect species (Anikwe et al., 2014; Dryden et al., 2013; Oxborough et al., 2015; Pridgeon et al., 2009), but particularly to lepidopterans (Dias, 2006; Wing et al., 1998; Wing et al., 2000). It is also registered for agricultural use in Europe (European Commission, 2006a). In 2003, indoxacarb estimated environmental long-term average concentrations in surface waters was of 3.7 $\mu g\ L^{-1}$, and peak values of 13.7 $\mu g\ L^{-1}$ were found (EPA, 2003). More recently, levels up to 7.763 $\mu g\ L^{-1}$ resulting from indoxacarb's application in lettuce crops were predicted for surface waters (EFSA et al., 2018b). Additionally, Indoxacarb also has a relatively high log Kow of 4.65 (Dias, 2006) suggesting it has a high tendency to sorb to sediments. Although spinosad and indoxacarb are registered and approved for use, toxicity data for these relatively novel pesticides on aquatic invertebrates is still very limited considering that according to regulation (EC) No 1272/2008 (European Commission, 2008b) they are both classified as very toxic to aquatic life with long lasting effects.

The freshwater midge *Chironomus riparius* Meigen (Diptera: Chironomidae) is a widely used model organism in ecotoxicology testing (Weltje et al., 2010) mainly due to its ecological relevance and easiness to handle in the laboratory. Additionally, *C. riparius* larvae have been previously used as a model to evaluate biochemical responses of insecticide exposure (Rodrigues et al., 2015a; Rodrigues et al., 2015b).

The main goal of this study was to evaluate the toxic effects of indoxacarb and spinosad on *C. riparius*. Survival, growth, emergence rate, development time, and adult (imagoes) weight were selected as endpoints for organism-level effects. Regarding the biochemical responses, the selected endpoints aimed to address effects related with: 1) Antioxidant capacity – activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR); 2) Oxidative damage – DNA damage and Lipid Peroxidation (LPO); 3) Biotransformation processes – activity of glutathione-S-transferase (GST); 4) neuronal activity – acetylcholinesterase (AChE); and 5) energy metabolism – activity of lactate dehydrogenase (LDH) and electron transport system (ETS).

2. Material and Methods

2.1 Test organism

Chironomus riparius larvae were collected from a laboratory culture long established at the University of Aveiro, Portugal. Larvae are kept in plastic aquaria filled with a fine layer of washed and burnt river sand (<1 mm) and ASTM hard water. Cultures

were maintained at 20 °C with a photoperiod of 16:8 h light-dark, with a constant inflow of air. Larvae are fed *ad libitum* with macerated fish food, Tetramin® (Melle, Germany).

2.2 Acute toxicity tests

Acute lethal toxicity was assessed following OECD guideline 235 (OECD, 2011) with water only exposures in crystalizing dishes, using 1st instar larvae. Larvae were exposed to concentrations of spinosad of 0 (solvent control), 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg L^{-1} and to 0, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μg L^{-1} of indoxacarb. After 48 h of exposure, mortality was checked. To halt possible photodegradation of the chemicals, crystalizing dishes were protected from the light during the test. The test was executed at 20 ± 1 °C, and larvae were not fed during the exposure.

2.3 Chronic toxicity tests

A 28-day chronic test was performed according to the OECD guideline 219 (OECD, 2004). First instar larvae of *C. riparius* (2 days old) were exposed to 0 (negative and solvent control), 0.5, 1.28, 3.2, 8, and 20 μg L^{-1} of spinosad in 150 mL of medium and layer of 1.5 cm of sediment in 200mL glass vessels. A similar setup was made for indoxacarb, using 0, 1, 2, 4, and 8 μg L^{-1} treatments. Five larvae were used in each replicate, and five replicates were used for larval growth determination, while eight replicates were used for emergence endpoints. After ten days of exposure larvae growth was determined by measuring body length of the larvae with the aid of a stereomicroscope fitted with a calibrated micrometer and growth was calculated by subtracting the mean body length at the beginning (pool of 25 larvae of initial size). In the eight remaining replicates, adult *C. riparius* were collected daily, their gender determined and stored in 70% ethanol. Afterwards, adult midges were dried at 50 °C for 24 h and weighed with a microbalance (Mettler UMT2).

The tests were performed under the same conditions described for culturing: 20 ± 1 °C with 16:8h light:dark cycle with gentle aeration. Organisms were fed every two days at a ration of 0.5 mg Tetramin® $larvae^{-1}$ day^{-1}, and physicochemical parameters were checked throughout the experiment.

2.4 Biomarkers

For the determination of the biochemical biomarkers, 3rd instar larvae (8 days old) were used. The concentrations used in these bioassays were 0, 0.5, 2, and 8 μg L^{-1} for spinosad and 0, 2, 4, and 8 μg L^{-1} for indoxacarb. Each crystalizing dish contained ten larvae and 80 mL of experimental solution and a fine layer of sediment. After 48 h, organisms from two replicates of the same treatment were pooled to give a total of twenty organisms per replicate. Six pooled replicates were used per treatment for spinosad, and seven pooled replicates for indoxacarb. Afterwards, excess water was

gently removed with a filter paper, and organisms weighed, frozen with liquid nitrogen, and stored at -80 °C until further processing.

Samples were subsequently homogenized in 800 µL of 0.1 M of K-phosphate buffer (pH = 7.4) using a Ystral d-79282 homogenizer. This homogenate was divided into portions for ETS, LPO, and DNA damage determination. To LPO portion, 4% 2,6-Di-tert-butyl-4-methylphenol (BHT) in methanol was added to prevent subsequent lipid oxidation of the samples (Aloísio Torres et al., 2002). These three portions were immediately stored at -80 °C until used. The remaining homogenate was centrifuged at 10000 *g* for 20 min at 4 °C and the supernatant (post-mitochondrial supernatant) was collected and divided into portions for SOD, CAT, GST, GR, GPX, AChE, and LDH activities determination and for protein quantification. In every essay, reaction blanks were performed using K-phosphate buffer instead of the sample and all spectrophotometric measurements were made at 25°C using a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek® Instruments, Vermont, USA).

2.4.1 Protein quantification

Protein concentration was assessed following the Bradford protocol adapted to microplate, using γ-globuline as standard. Prior to AChE, CAT, GR, GPx, GST, and LDH activities determination, protein concentration was adjusted to approximately 0.8 mg L^{-1}. For these biomarkers, the exact protein concentration of the dilution was measured again at the end of the experiment.

2.4.2 Detoxification, oxidative stress and oxidative damage biomarkers

SOD activity was determined by following the method described by McCord and Fridovich (1969) adapted to microplate. Cytochrome c reduction was followed for 5 min at 550 nm, and results are expressed as SOD units (U) mg^{-1} protein. The determination of CAT activity was made according to Clairborne (1985). The consumption of H_2O_2 was assessed at 240 nm for 1 min, and results are expressed in µmol min^{-1} mg^{-1} of protein. For the assessment of GR activity, the method described by Cribb et al. (1989) was used. The oxidation of NADPH was monitored at 340 nm during 1 min, and results are expressed in nmol min^{-1} mg^{-1} of protein. Regarding GPx activity, it was determined by monitoring the oxidation of NADPH at 340 nm for 3 min, as a result of GR conversion of GSSG to GSH (Mohandas et al., 1984). Results are expressed in nmol min^{-1} mg^{-1} of protein. An adaption of Habig et al. (1974) protocol to microplate was used to determine GST activity. The formation of glutathione dinitrobenzene was measured at 340 nm during 3 min, and results are expressed in nmol min^{-1} mg^{-1} of protein. LPO levels were measured using thiobarbituric acid reactive substances (TBARS) assay (Bird and Draper, 1984; Ohkawa et al., 1979). Absorbance was read at 535nm and results are expressed in nmol TBARS g^{-1} of wet weight. DNA damage was assessed following the protocols

described by de Lafontaine et al. (2000) and Olive (1988). Fluorescence was measured using an excitation/emission wavelength of 360/460 nm, and results are expressed as ng of damaged DNA mg^{-1} of wet weight.

2.4.3 Neurotransmission and energy related biomarkers

Effects of spinosad and indoxacarb on cholinergic neurotransmission were evaluated monitoring AChE activity, following Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). The absorbance was read at 414 nm for 5 minutes, and results are expressed in nmol min^{-1} mg^{-1} of protein. To determine the activity of anaerobic metabolism-related enzyme LDH, oxidation of NADH was monitored at 340 nm as proposed by Vassault (1983) and Diamantino et al. (2001). Results are expressed in nmol min^{-1} mg^{-1} of protein. ETS activity was determined following De Coen and Janssen (1997) with some adaptations (Rodrigues et al., 2015b). Absorbance was read at 490 nm for 5 minutes, and results are expressed mJ h^{-1} mg of $protein^{-1}$.

2.5 Statistical analysis

Effects of insecticide exposure on life history and biochemical endpoints were evaluated by one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test to determine statistically significant differences between solvent controls and treatments, and/or by a test for linear trend to discriminate if there is a linear increase or decrease in response as the concentration increases. Data were checked for residual normality using D'Agostino-Pearson and Shapiro-Wilk normality tests and for homoscedasticity with Brown-Forsythe test. Unpaired *t*-tests did not find any differences between negative and solvent controls, therefore solvent control was used as the control for all analysis. Spinonad's DNA damage data were log-transformed to correct for normality. For spinosad LPO data and for indoxacarb percentage of emergence data, transformations did not correct for normality, but since homogeneity of variances was verified, one-way ANOVA was executed. Since all larvae in the spinosad chronic test exposed to 20 μg L^{-1} died, this treatment was excluded from analysis. Statistical analysis was made in GraphPad Prism® 7 for Mac and significance level was set at 0.05.

3. Results

3.1 Spinosad

For spinosad, in the highest concentration tested in the acute toxicity test, there was 40% mortality after 48 h of exposure. Because of the gradients of concentrations used for spinosad, the 48 h LC_{50} could not be estimated and thus is higher than 256 μg L^{-1}. Concerning the chronic bioassay, at day 10 no larvae were alive at the highest concentration tested (20 μg L^{-1}) while 92% of the larvae were recovered from the control.

Additionally, at day 10 of exposure, statically significant differences were found for growth between control and the 8 μg L^{-1} treatment ($F_{(4,20)} = 7.640$, $p < 0.001$) (Table I). Regarding emergence parameters, there was a significant increase in time to emergence at 8 μg L^{-1} for both males ($F_{(4,27)} = 3.831$, $p < 0.05$) and females ($F_{(4,26)} = 3.606$, $p < 0.05$) (Table I). No adults have emerged in the 20 μg L^{-1} treatment, and although overall ANOVA was not significant for the remaining treatments ($F_{(4,34)} = 2.295$, $p = 0.079$), Dunnett's test discriminated differences between control and 8 μg L^{-1} treatments in terms of percentage of emerged adults. No effects were found on adult weight (NOEC = 8 μg L^{-1}) (Table II).

Table I – Growth and emergence endpoints of *Chironomus riparius* larvae exposed to Spinosad. All values are presented as mean ± SEM. An asterisk denotes statistically significant differences to the control treatment (0 μg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test). A number sign denotes statistically significant differences to the control treatment (0 μg L^{-1}; Dunnett's test) when overall ANOVA is not significant ($p = 0.079$).

Spinosad Concentrations (μg L^{-1})	Growth (mm)	Total emergents (%)	Development time (days)	
			Males	Females
0	12.28 ± 0.17	80.00 ± 7.56	15.30 ± 0.41	16.84 ± 0.38
0.5	11.61 ± 0.29	65.00 ± 9.06	15.21 ± 0.31	17.08 ± 0.40
1.28	11.65 ± 0.26	65.00 ± 7.32	15.26 ± 0.34	17.20 ± 0.34
3.2	10.89 ± 0.48	72.50 ± 8.40	15.04 ± 0.26	17.92 ± 0.63
8	8.76 ± 0.90*	45.00 ± 9.82#	17.08 ± 0.71*	19.88 ± 1.09*
20	N.C.	N.C.	N.C.	N.C.

N.C. – not calculated due to 100% mortality

Table II – Adult weight of *Chironomus riparius* exposed as larvae to Spinosad. All values are presented as mean ± SEM.

Spinosad Concentrations (μg L^{-1})	Males dry weight (mg)	Females dry weight (mg)
0	0.5415 ± 0.0123	1.084 ± 0.0366
0.5	0.5404 ± 0.0125	1.084 ± 0.0188
1.28	0.5679 ± 0.0168	1.063 ± 0.0364
3.2	0.5235 ± 0.0146	1.035 ± 0.0334
8	0.5210 ± 0.0269	1.118 ± 0.0913
20	N.C.	N.C.

N.C. – not calculated due to 100% mortality

To what concerns biochemical biomarkers, there was a significant increase in LPO at the two highest concentrations tested ($F_{(3,20)} = 4.87$, $p < 0.05$; Fig. 1a) and, although not significant, DNA damage also increased in the same treatments ($F_{(3,20)} = 2.651$, $p = 0.077$, Fig 1b). LDH activity increased in the 2 μg L^{-1} treatment ($F_{(3,19)} = 8.357$, $p = 0.001$; Fig. 1d), and ETS activity was the most sentitive biomarker, with a significant increase observed for all tested concentration ($F_{(3,20)} = 31.76$, $p < 0.001$; LOEC = 0.5 μg L^{-1}, Fig 1e); this increase was concentration-dependent ($r^2 = 0.83$, $p < 0.001$). Exposure to spinosad significantly increased GPx activity in *C. riparius* larvae in the highest concentration ($F_{(3,20)} = 7.601$, $p <$

0.01; Fig. 2b). No significant alterations were detected for AChE, CAT, GR, GST, and SOD activities (Figures 1-2).

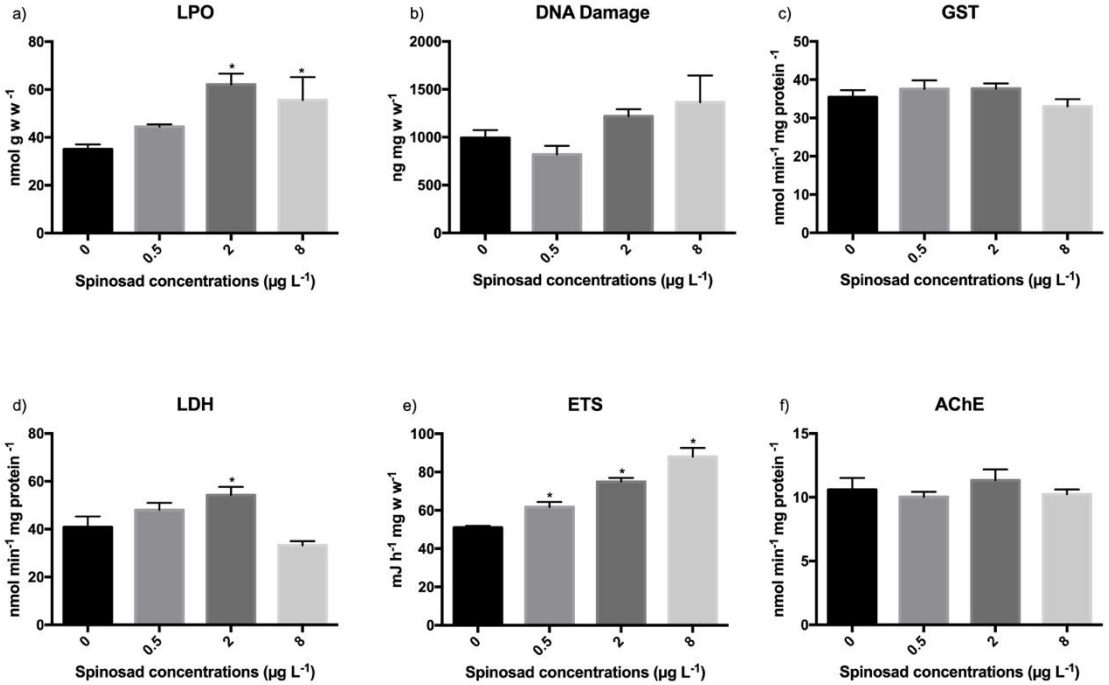

Figure 1 – Oxidative damage, biotransformation, energetic metabolism and neuronal biomarkers in *Chiromomus riparius* larvae after 48h exposure to spinosad: a) Lipid Peroxidation; b) DNA Damage; c) Glutathione-S-Transferase; d) Lactate Dehydrogenase; e) Electron Transport System; f) Acetylcholinesterase. All values are presented as mean + SEM. An asterisk denotes statistically significant differences to the control treatment (0 µg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

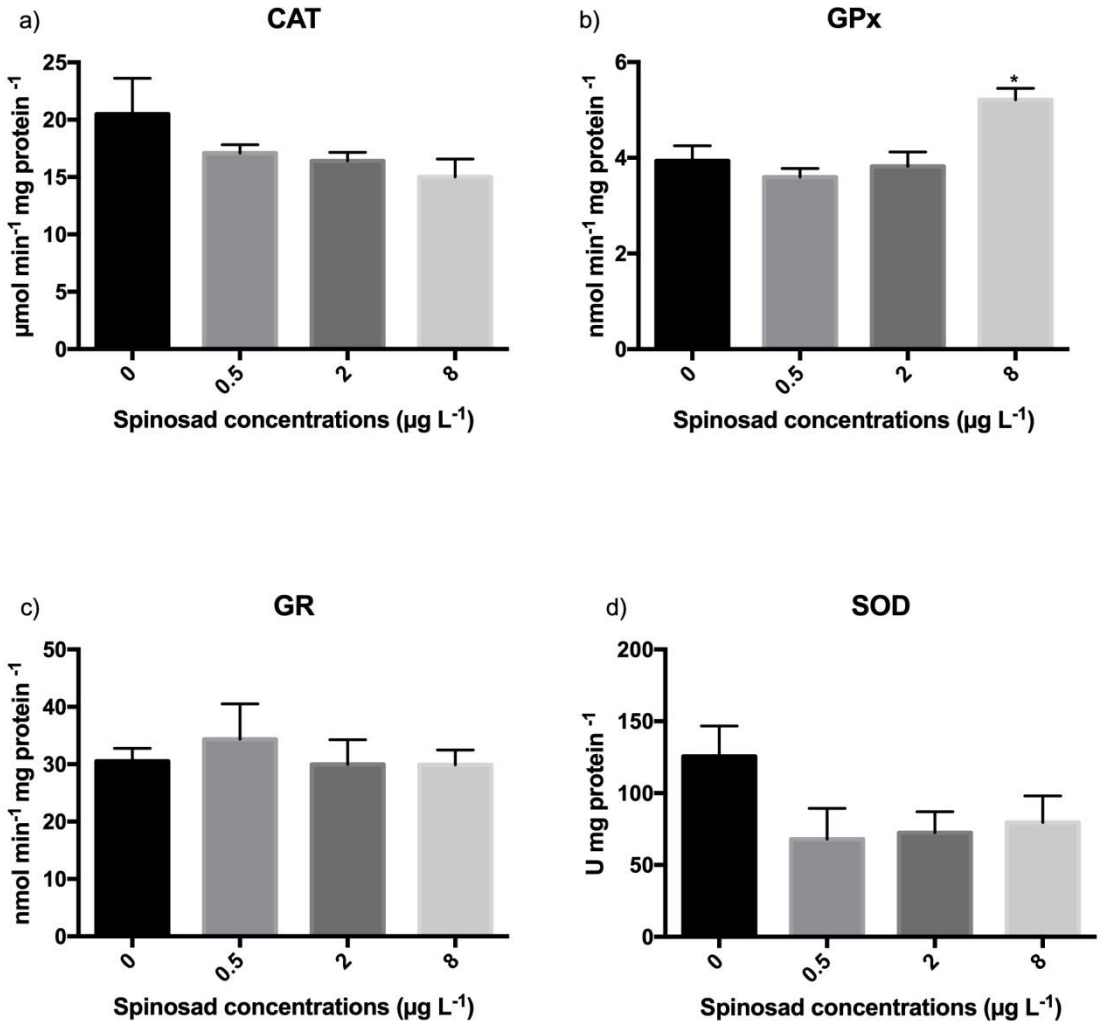

Figure 2 – Oxidative stress biomarkers in *Chironomus riparius* larvae after 48h exposure to spinosad: a) Catalase; b) Glutathione Peroxidase; c) Glutathione Reductase; d) Superoxide Dismutase. All values are presented as mean + SEM. An asterisk denotes statistically significant differences to the control treatment (0 µg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

3.2 Indoxacarb

In acute tests and for the highest concentration of indoxacarb tested, there was 47% mortality after 48 h of exposure. The 48 h LC_{50} of indoxacarb was estimated to be higher than 128 µg L^{-1}. Chronic exposure to indoxacarb led to a decrease in larval growth in the highest concentration tested (8 µg L^{-1}; $F_{(4,19)} = 4.746$, $p < 0.01$) (Table III). Moreover, indoxacarb exposure led to a delay in emergence of males ($F_{(4,32)} = 11.96$, $p < 0.001$) and females ($F_{(4,33)} = 6.031$, $p < 0.001$). No effects were observed for the percentage of emerged adults (Table III) nor for adult weight (NOEC = 8 µg L^{-1}) (Table IV).

Table III – Growth and emergence endpoints of *Chironomus riparius* larvae exposed to Indoxacarb. All values are presented as mean ± SEM. An asterisk denotes statistically significant differences to the control treatment (0 μg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

Indoxacarb Concentrations (μg L^{-1})	Growth (mm)	Total emergents (%)	Development time (days)	
			Males	Females
0	11.73 ± 0.31	90.00 ± 5.35	17.03 ± 0.33	18.44 ± 0.58
1	11.27 ± 0.10	90.00 ± 3.78	16.00 ± 0.32	19.53 ± 0.49
2	11.55 ± 0.58	85.00 ± 6.27	16.16 ± 0.33	18.53 ± 0.43
4	10.85 ± 0.28	82.50 ± 7.01	17.40 ± 0.39	19.92 ± 0.60
8	9.91 ± 0.36*	95.00 ± 3.27	18.96 ± 0.35*	22.12 ± 0.88*

Table IV – Adult weight of *Chironomus riparius* exposed as larvae to Indoxacarb. All values are presented as mean ± SEM.

Indoxacarb Concentrations (μg L^{-1})	Males dry weight (mg)	Females dry weight (mg)
0	0.4779 ± 0.0122	0.9431 ± 0.0487
1	0.5022 ± 0.0138	0.9979 ± 0.0299
2	0.4614 ± 0.0181	0.9809 ± 0.0511
4	0.5029 ± 0.0220	0. 9988 ± 0.0385
8	0.4692 ± 0.0120	0.9543 ± 0.0489

Regarding biochemical biomarkers, GPx activity increased in the highest concentration tested ($F_{(3,24)} = 5.055$, $p < 0.01$; Fig. 4b). Exposure to indoxacarb significantly increased LDH activity in the highest concentration tested ($F_{(3,23)} = 3.331$, $p < 0.05$; Fig. 3d), and this increase was dose-dependent ($r^2 = 0.30$, $p < 0.01$; Fig. 3d). GST activity increased from concentration 4 μg L^{-1} onwards ($F_{(3,24)} = 4.81$, $p < 0.01$; Fig. 3c). For SOD activity, although ANOVA anova was significant, the post test did not find any significant differences between the control and the experimental treatments. No significant alterations were detected for the remaining biomarkers studied.

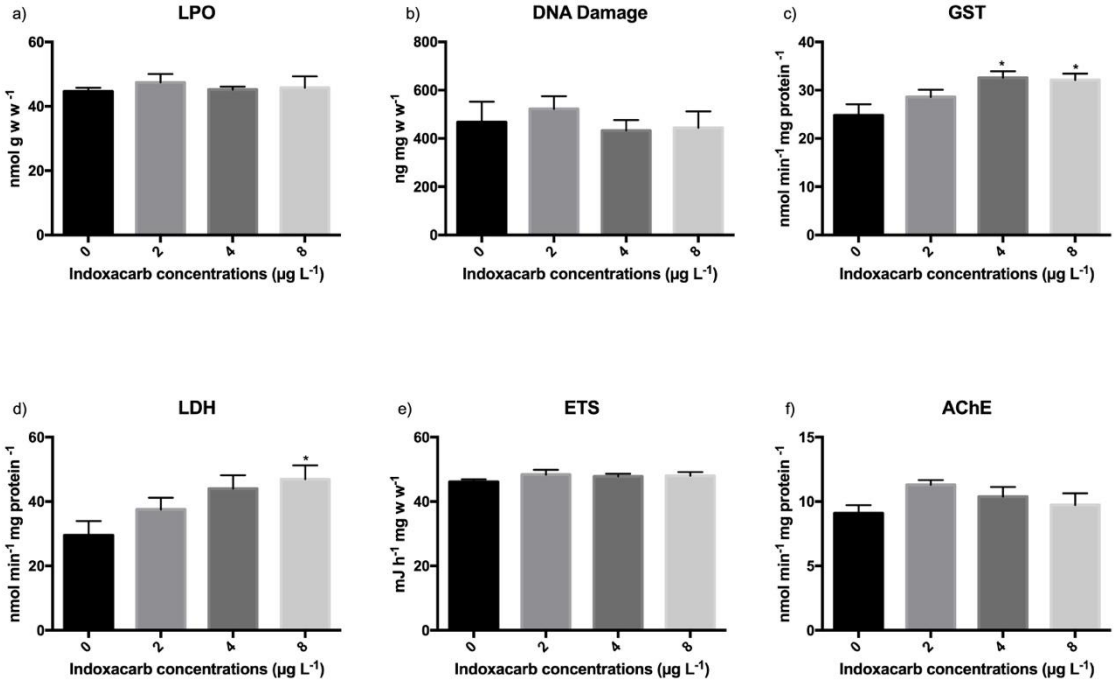

Figure 3 – Oxidative damage, biotransformation, energetic metabolism and neuronal biomarkers in *Chironomus riparius* larvae after 48h exposure to indoxacarb: a) Lipid Peroxidation; b) DNA Damage; c) Glutathione-S-Transferase; d) Lactate Dehydrogenase; e) Electron Transport System; f) Acetylcholinesterase. All values are presented as mean + SEM. An asterisk denotes statistically significant differences to the control treatment (0 µg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

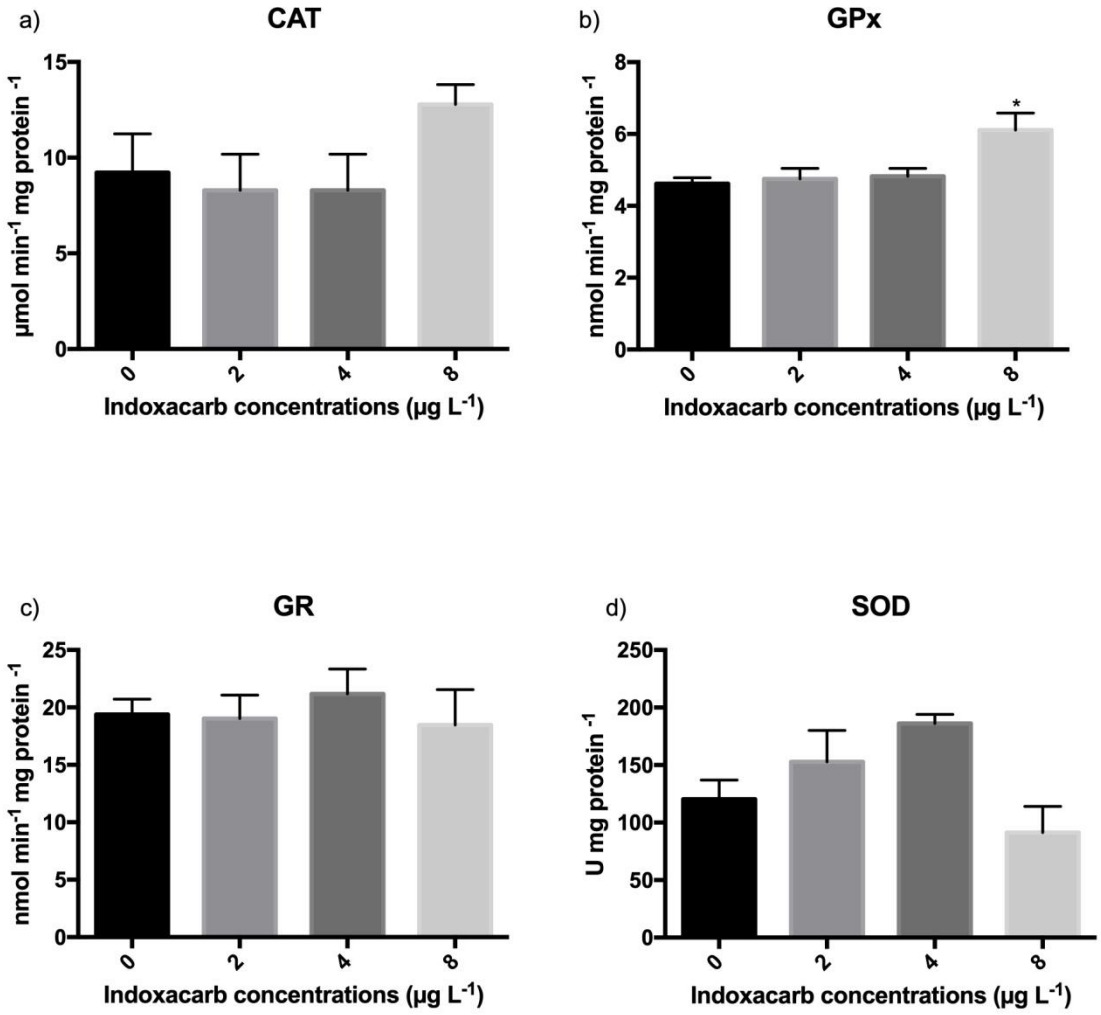

Figure 4 – Oxidative stress biomarkers in *Chironomus riparius* larvae after 48h exposure to indoxacarb: a) Catalase; b) Glutathione Peroxidase; c) Glutathione Reductase; d) Superoxide Dismutase. All values are presented as mean ± SEM. An asterisk denotes statistically significant differences to the control treatment (0 µg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

4. Discussion

Spinosad and Indoxacarb are neurotoxic insecticides highly effective in controlling insect pests which were initially deemed as relatively safe for non-target species (Bacci et al., 2016; Boucher and Ashley, 1999; Jones et al., 2005; Lahm et al., 2000; Liu and Zhang, 2012; Sarfraz et al., 2005). Although there is no recent literature available on measured levels of both chemicals in natural freshwater environments, the concentrations used in this study are within the estimated environmental levels, and clearly impaired *C. riparius* life-history traits with alterations at the biochemical level also observed. Chironomids larvae play a vital role in freshwater ecosystems due to their abundance and food chain position (Péry et al., 2002), and therefore the current rates of spinosad and indoxacarb application near freshwater systems at current rate should be monitored and reviewed.

Available data concerning acute exposures show that spinosad is highly toxic to freshwater insects, including chironomids. Kumar et al. (2011) estimated a 48 h LC_{50} of 9 μg L^{-1} and 32 μg L^{-1} for *Chironomus circumdatus* first and third instars respectively. For *Chironomus tepperi* fourth instar larvae, a 24 h LC_{50} of 61.8 μg L^{-1} was estimated (Stevens et al., 2005). A previous report indicated a 48 h LC_{50} > 32 μg L^{-1} for *C. riparius* (EFSA et al., 2018a), which is in accordance with the data presented here, suggesting that *C. riparius* may be less susceptible to spinosad than other chironomids. Regarding other aquatic dipterans like *Aedes aegypti*, *Aedes albopictus*, *Anopheles albimanus*, *Anopheles stephensi*, and *Culex pipiens* 48 h LC_{50} values ranging from 3.2 to 24 μg L^{-1} have been estimated with *C. pipiens* showing higher sensitivity (Bond et al., 2004; Khan et al., 2011; Romi et al., 2006). Based on acute data, aquatic insects seem to be more susceptible to spinosad than other invertebrates, such as daphnids (Sparks et al., 1998; Stark and Banks, 2001; Thompson et al., 2000), or the grass shrimp (Thompson et al., 2000).

Concerning the exposure to indoxacarb and effects on *Chironomids*, Stevens et al. (2005) using *C. tepperi* as model, estimated a 24 h LC_{50} of 48.8 μg L^{-1} for 4th instar larvae, indicating that *C. tepperi* larvae are less susceptible to indoxacarb than to spinosad. In the present study, at 24 h of exposure, in every concentration tested below 48.8 μg L^{-1}, mortality did not exceed 10%, suggesting that *C. riparius* might be less susceptible to indoxacarb than the pest species *C. tepperi*, even considering that *C. tepperi* larvae are, in turn, less susceptible to indoxacarb than to other insecticides (Stevens et al., 2005). In general, dipterans appear to be more sensitive to indoxacarb than other aquatic invertebrates, with LC_{50}'s available in the literature for *Aedes aegypti*, *Aedes albopictus*, and *Anopheles gambiae* ranging from 22 to 79 μg L^{-1}, with *Aedes aegypti* and *Aedes albopictus* showing higher sensitivity (Khan et al., 2011; N'Guessan et al., 2007; Pridgeon et al., 2009). Regarding other aquatic invertebrates, for *Gammarus pulex*, indoxacarb's 96 h LC_{50} was estimated to be 2520 μg L^{-1} (Beketov and Liess, 2008), while for *Daphnia magna* the 48 h LC_{50} was estimated to be higher than 170 μg L^{-1} (EFSA et al., 2018b).

Regarding chronic toxicity, at the organismal level, larval growth and development rates are presented as the most sensitive *C. riparius* endpoints for both insecticides. The relevance of these endpoints has been extensively addressed in the literature (Azevedo-Pereira et al., 2010; Azevedo-Pereira and Soares, 2010; Faria et al., 2006; Pestana et al., 2009b). Chronic exposures to indoxacarb and spinosad produced comparable outcomes in terms of *C. riparius* life history traits: growth reduction observed after 10 days of exposure at 8 μg L^{-1} of spinosad and at 8 μg L^{-1} of indoxacarb translated into a delay in development of both males and females, but interestingly, did not result in a reduction of imagoes weight. This suggests that *C. riparius* larvae were capable of recovering and reaching the desired weight, at the expense of longer development time. This trade-off is not unusual as body weight is associated with the reproductive output of chironomids (Sibley et al., 2001) - nonetheless, a delay in development time is still an important

ecological driver as it can have direct consequences on population dynamics. The main dissimilarity observed between the effects if the two compounds at the organismal level, was that spinosad exposure also affected *C. riparius* survival: there was a reduction in the number of emerged adults at 8 μg L^{-1}, and at 20 μg L^{-1} no imagoes have emerged. Previous data indicated a NOEC (no observed effect concentration) of 0.62 μg L^{-1} (EFSA et al., 2018a), however, in the present study a NOEC of 3.2 μg L^{-1} and a LOEC (lowest observed effect concentration) of 8 μg L^{-1} for larval growth and emergence were observed under exposure to spinosad. The results present here suggest that *C. riparius* is among the most sensitive aquatic invertebrates to spinosad. Yet, information on the chronic risk to aquatic organisms, including sediment dwellers is lacking (EFSA et al., 2018a). Regarding other dipterans, a spinosad concentration of 17 μg L^{-1} has been demonstrated to decrease the emergence of *Polypedilum nubifer* (Duchet et al., 2015). Concentrations ranging from 3.7 to 45 μg L^{-1} seem to affect *Culex pipiens* emergence (Hertlein et al., 2010), while at 60 μg L^{-1} Cetin et al. (2005) reported a complete inhibition of *Culex pipiens* adult emergence. Tomé et al. (2014) determined that exposure to spinosad compromises swimming behavior of *Aedes aegypti*. Behavioral changes have been demonstrated for many neurotoxic compounds and can lead to a reduction in food intake (Pestana et al., 2009a; 2010; Tomé et al., 2014; Werner and Moran, 2009), which, although not addressed, might have also occurred here with *C. riparius* and may justify the reduced growth and developmental rates. Considering other aquatic invertebrates, impairment of population growth rate by spinosad was described for *Daphnia pulex* and *Daphnia magna* at 8 μg L^{-1} (Duchet et al., 2010) and for *Ceriodaphnia dubia* at 1 μg L^{-1} (Deardorff and Stark, 2011).

Regarding the long-term effects of indoxacarb on chironomids, the information available is very limited. Still, a 28-day EC_{10} of 1.68 μg L^{-1} (endpoint not specified) and a 28-day NOEC (development rate) of 1.8 μg L^{-1} (active substance) were previously determined for *C. riparius* (EFSA et al., 2018b). In the present study, a NOEC of 4 μg L^{-1} was observed for development and emergence endpoints. Ding et al. (2011) investigated the effects of pesticide-contaminated sediments on *C. dilutus*, and the authors concluded that indoxacarb was amongst the most toxic sediment-associated pesticides to *C. dilutus* they tested (10-day LC_{50} of 11.3 μg g_{oc}^{-1}; growth NOEC of 3.2 μg g_{oc}^{-1}). Available information shows that commercial formulations of indoxacarb affect life history traits of some insect pest species (Gamil et al., 2011; Martin et al., 2006; Saryazdi et al., 2012), however present results clearly show that life history of non-target aquatic insects may also be altered.

Short exposures to low concentrations of both insecticides tested induced several biochemical changes in *C. riparius* larvae. As expected, due to their distinct modes of action, different responses were observed at the biochemical level.

GPx, CAT, and SOD are first-line defense antioxidant enzymes against reactive oxygen species (ROS). SOD catalyzes the conversion of superoxide anions to hydrogen peroxide (H_2O_2), which is subsequently detoxified by CAT and GPx (Ighodaro and Akinloye, 2007). The increase in GPx activity induced by spinosad exposure may have occurred to prevent the accumulation of H_2O_2 due to increased oxygen metabolism. GPx has a higher affinity for H_2O_2 than CAT (Lushchak, 2012), which may explain why GPx activity increased while catalase activity remained unchanged. The increase in GPx activity was, however, insufficient to prevent oxidative damage, as indicated by the increase of LPO levels and the perceptible increase of DNA damage. The concomitant increase in LPO and GPx in has been previously observed in the kidney of *Oreochromis niloticus* (Piner and Uner, 2014) and in mammalian cell lines (Pérez-Pertejo et al., 2008) exposed to the same insecticide.

Spinosad also led to the increase in ETS activity, an indicator of cellular oxygen metabolism, and LDH activity, involved in the anaerobic pathway of energy production, indicating high levels of energy consumption and high metabolic demand (Rodrigues et al., 2015a; Silva et al., 2016). This increase in energy demand may be associated with the activation of antioxidant mechanisms, as implicit by the increase of GPx and/or other defense mechanisms that were not addressed here. Moreover, an increase in energy costs of these defense mechanisms may also, in part, explain observed reductions in growth and development.

Spinosad's inhibitory effects on AChE activity have been reported for other insect species (El-Mageed and Elgohary, 2006; Maiza et al., 2013; Rabea et al., 2010; Tine et al., 2015), and as a nicotinic acetylcholine receptor modulator, some alterations in AChE activity were expected. However, the 48h exposure to the tested concentrations did not induce any changes in AChE activity of *C. riparius* larvae. Azevedo-Pereira et al. (2011) work with *C. riparius* larvae have also revealed that a 48h exposure to imidacloprid, an insecticide that also targets nicotinic acetylcholine receptors, did not induce alterations in AChE activity. The authors indicated that inhibitory effects of imidacloprid on AChE were only detected after 96h of exposure and in the post-exposure period, and yet behavioral changes were linked to AChE activity (Azevedo-Pereira et al., 2011). Given the information available in the literature, it is possible that 48h exposure to spinosad was not enough to impair AChE activity. Follow-up tests should be performed with prolonged exposure periods, to evaluate the possible extent of spinosad toxic effect on *C. riparius* AChE. Nonetheless, this short-exposure triggered alterations on other biochemical biomarkers, indicating that secondary mechanisms might also be accountable for spinosad's toxicity to *C. riparius*, such as the interference with gamma-aminobutyric acid receptors or others (Salgado and Sparks, 2005).

Regarding the effects of indoxacarb at the biochemical level, GST was the most sensitive endpoint. GST, an enzyme involved in biotransformation and detoxification (Clark, 1989), has been categorized as an ineffective biomarker of pesticide exposure in *C. riparius* (Hirthe et al., 2001), and some works endorse this assumption due to its disparate

responses to different pesticides (Planelló et al., 2013). Regardless, in this study, an increase in GST activity as a result of indoxacarb exposure was observed in *C. riparius* larvae. An identical response to indoxacarb was observed in *Blattella germanica* and *Spodoptera littoralis* larvae (Gamil et al., 2011; Maiza et al., 2013). Additionally, Nehare et al. (2010) and Pang et al. (2012) postulated that the detoxification by GST might play a relevant role in indoxacarb resistance. GPx activity also increased in larvae exposed to indoxacarb. Since there were no changes in oxidative damage indicators (LPO and DNA damage), it is suggested that GPx activity and detoxification by GST contributed to preventing oxidative damage in a short-term exposure. As opposed to spinosad, only the anaerobic metabolism (LDH) was induced by indoxacarb in *C. riparius* larvae, since no changes were detected in ETS activity. This induction of LDH may occur due to higher and more readily available energy demands for the activation of GPx and GST, and again this might have contributed to the effects observed at the individual level (reduction in larval growth and increase in time to emergence).

5. Conclusion

This study elucidates some biochemical responses to spinosad and indoxacarb exposure that precede the effects observed at the organismal level. The induction of defense mechanisms and higher energy expenditures are most likely direct responses *of C. riparius* larvae to cope with the exposure, while oxidative damage may be a direct consequence of spinosad's mechanism of action and may have contributed to the slightly more severe effects observed. Although not specific, biochemical biomarkers addressed in the present study may be valuable early-warning tools for risk-assessment: ETS was the most sensitive biochemical biomarker for spinosad, as it was responsive to 0.5 $\mu g\ L^{-1}$, while for indoxacarb GST was the most sensitive biomarker (LOEC of 4 $\mu g\ L^{-1}$), underlining the role of GST in the detoxification of indoxacarb.

Our findings revealed that under controlled laboratory conditions, spinosad is slightly more toxic to *C. riparius* than indoxacarb since, besides the reduction of larval growth and the increase in time to emergence, a reduction in emergence rate was also observed. Nonetheless, the use of both insecticides near freshwater systems should be reconsidered, since the spinosad and indoxacarb concentrations used in this work and that elicited clear deleterious effects are within the estimated environmental levels.

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Chapter IV

Proteome responses in *Chironomus riparius* under exposure to the insecticides spinosad and indoxacarb

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Proteome responses in *Chironomus riparius* under exposure to the insecticides spinosad and indoxacarb. [1]

Abstract

The potential of proteome responses as early warning indicators of pesticide exposure was evaluated using the nonbiting midge *Chironomus riparius* (Meigen) as model organism. Larvae of *C. riparius* were exposed to environmentally relevant concentrations of two neurotoxic pesticides, spinosad and indoxacarb, in order to uncover molecular events that may provide insights on the long-term consequences for natural populations. iTRAQ methodology was performed to relatively quantify protein expression changes between exposed and non-exposed organisms. At the proteome level, changes caused by spinosad were more evident than the ones caused by indoxacarb, for which only one identified protein had its expression significantly altered. Data analysis revealed a general decrease in expression of globin proteins as a result of spinosad exposure, which was determined to be dose-dependent. Additionally, the downregulation of actin and a larval cuticle protein were also observed for spinosad exposure, which could be related to previously determined *C. riparius* life-history traits impairment and biochemical responses. Present results suggest that protein profile changes can be used as early warning biomarkers of pesticide exposure and may provide a better mechanistic interpretation of the toxic response of organisms, thus aiding in the assessment of the ecological effects of environmental contamination. This work also contributes to the growing knowledge of sub-lethal effects of pesticides in invertebrates and their molecular targets. *Chironomus riparius*, a model organism in aquatic toxicology, is also presented as a putative model organism for environmental proteomics.

Keywords: *Chironomus riparius*; ecotoxicoproteomics; hemoglobin; iTRAQ; neurotoxic pesticides

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1. Introduction

The study of the impact of stressors on ecological entities is crucial for risk assessment (S. Chen et al., 2013). Most often, toxicity testing is based on organism level responses (e.g. mortality, growth, and behavior) (Sánchez-Bayo and Tennekes, 2017). These tests provide valuable and sensitive information on the organism performance that can be used to predict possible outcomes at the population or community levels. However, xenobiotic concentrations commonly found in the environment may not be high enough to cause an immediate individual level response (Nikinmaa, 2014), and when a response occurs on an ecologically relevant species, it may be too late to set off a successful environmental management. In this sense, there is a need to develop new and sensitive tools that can help determine molecular initiating events that lead to adverse outcomes, and thus be used as early warning tools to predict ecological adverse effects of pesticides.

The recent advances in "omic" technologies and more particularly in proteomics, made it possible to identify and study complex mixtures containing numerous proteins from a particular sample (Hartmann et al., 2014; Yates, 2011). The application of proteomics in ecotoxicology has been expanding in the recent years, and while initially most of the studies available in aquatic toxicology field focused on fish species (Sanchez et al., 2011), many recent studies have been published using aquatic invertebrates (Borgatta et al., 2015; H. Chen et al., 2016; Ji et al., 2014; Oliveira et al., 2016; Vellinger et al., 2016). With the development of methodologies such as iTRAQ (Isobaric tags for relative and absolute quantitation), it is now possible to simultaneously analyze and relatively quantify proteins from up to eight different samples, a great advantage in comparison with traditional gel-based techniques such as two-dimensional difference gel electrophoresis (2D-DIGE) (Martyniuk et al., 2012; Wang et al., 2015). Studying the interaction of a specific chemical with an organism at a molecular level can lead not only to the discovery of potential biomarkers of effect, but also to a better interpretation of its primary and secondary mechanisms of action within the organism (Benninghoff, 2007; Dowling and Sheehan, 2006; Lemos et al., 2010; López-Barea and Gómez-Ariza, 2006; Martyniuk et al., 2012; Sanchez et al., 2011).

Chironomids have a wide distribution around the globe and are frequently the most abundant group in freshwater benthic invertebrate communities (Armitage et al., 1995; Ferrington, 2008; Péry et al., 2003; Weltje et al., 2010). From an ecotoxicological point of view, chironomids exhibit additional interesting features making them model organisms for acute and chronic toxicity tests as they: (1) have a short-life cycle and are relatively easy to culture and handle in laboratory; (2) live in a water-sediment interface; (3) have an important role in organic recycling and are an important prey items for different predators; and (4) usually are not target species for pesticide application (Péry et al., 2003; Taenzler et al., 2007; Weltje et al., 2010). Additionally, from an

(ecotoxico)proteomic perspective, chironomids life-cycle, which includes a complete metamorphosis (Taenzler et al., 2007), and the fact that many species possess hemoglobin in their larval stages are also aspects of interest (Choi and Roche, 2004; S.M. Lee et al., 2006; P. Osmulski and Leyko, 1986). Nonetheless and to the best of our knowledge, studies of protein expression changes in *C. riparius* are limited to the works by Choi and Ha (2009) and S.E. Lee et al. (2006) who assessed the changes in protein expression after exposure to cadmium.

In the present study, the effects of indoxacarb and spinosad in *C. riparius* protein expression profiles are evaluated in order to have a more accurate understanding of the affected biologic pathways that lead to higher level responses. Spinosad is a nicotinic acetylcholine receptor allosteric modulator (Salgado and Sparks, 2005) while indoxacarb acts as a voltage-dependent sodium channel blocker (Lapied et al., 2001). *Chironomus riparius* growth and development rates have been shown to be impaired under exposures to environmentally relevant concentrations of both insecticides.

2. Material and Methods

2.1 Test chemicals

Spinosad (CAS number 168316-95-8) and Indoxacarb (CAS number 144171-61-9) were acquired from Sigma-Aldrich, UK. Stock solutions were prepared in ethanol (spinosad) and acetone (indoxacarb). To prepare working and experimental solutions, stock solutions were diluted with ASTM hard water and the final solvent concentration was kept at 0.01% in all experimental solutions.

2.2 Organism culture and exposure

Chironomus riparius egg masses were collected from a laboratory culture long established in the University of Aveiro. After hatching, larvae were kept in plastic aquariua filled with ASTM and a layer of commercial sterilized sand (>1 mm) at 16:8 h light:dark cycle, and fed with macerated fish food (Tetramin®) until reaching the desired age (8 days old). Larvae were then transferred to glass crystalizing dishes (10.7 cm base diameter) with 200 mL of spinosad (0, 0.5, 2, and 8 $\mu g\ L^{-1}$) and indoxacarb (0, 0, 4, and 8 $\mu g\ L^{-1}$) solutions. Four replicates were used per treatment and each replicate consisted of 20 larvae. After 48 h of exposure, all larvae from each replicate were collected, quickly placed on filter paper to take excess water, and weighed before being transferred to a 2 mL microtube. Larvae were immediately frozen in liquid nitrogen and stored at -80 °C until further use. For both cultures and experiments, temperature was set at 20 ± 1 °C.

The present experimental design was planned to evaluate if there was a dose-dependent relation between pesticide concentration and protein expression. Assuming that the effects at organism level are preceded by changes at molecular level, and these

changes may be assessed earlier and at lower concentrations, two concentrations that did not cause observable long-term effects on *C. riparius* larvae development (exposure of first instar larvae), and one concentration near the lowest observable effect concentration at organismal level were used for each pesticide (Chapter 3). While both pesticides had comparable effects at the organism level for *C. riparius*, distinct responses were detected at the biochemical level (Chapter 3), possibly due to their distinct modes of action and targets within the organisms. Considering this, data obtained for each pesticide was explored separately, in order to look for pesticide specific responses.

2.3 Protein extraction

Protein extraction was performed following a TCA-Acetone extraction method as described by Cilia et al. (2009) with minor modifications. Briefly, samples were homogenized with a mechanical homogenizer (Ystral d-7801, USA). To aid the homogenization process, a few microliters of K-phosphate buffer 0.1 M were added to each sample. Samples were then gently mixed with a solution containing 10 % trichloroacetic acid (TCA) and 2% β-mercaptoethanol (2-ME) in acetone and incubated overnight at -20 °C. After, protein extracts were centrifuged at 5000 *g* during 30 min and the pellets formed were washed in acetone. These two steps were repeated until tissue debris were completely discarded. Acetone used in this protocol was previously stored at -20 °C, and homogenization and extraction steps were performed on ice. The resulting pellets were solubilized in a 0.04 M Tris-HCl buffer with 7 M urea, 2 M thiourea, 0.5 % Triton-X-100, 0.1 % sodium dodecyl sulfate (SDS), 0.05 M $MgCl_2$, protease inhibitor mixture (Roche, Germany), 1 % bovine pancreas DNase I (Roche, Germany), and 1 % bovine pancreas RNase A (Roche, Germany), at pH=8 and stored at -80 °C until further use.

2.4 Protein quantification and sample preparation for iTRAQ®

For each chemical, two iTRAQ runs were made, consisting on two biological replicates of each treatment. To remove potential interfering compounds with iTRAQ labeling, an acetone precipitation was performed according to the manufacturer instructions (iTRAQ Reagents – 8plex protocol; AB Sciex, USA) and proteins were resuspended in 0.5 M triethylammonium bicarbonate (TEAB) buffer. Protein content was determined using Coomassie Plus™ Kit assay (ThermoFisher Scientific, USA), and 5 μg of each sample were loaded onto an SDS-Page gel to verify extraction efficiency and integrity of proteins. Afterwards, 20 μg of each sample were separated, dried in a SpeedVac™ (SC110; Thermo Savant, USA), and resuspended in a total volume of 25 μL of 0.5 M TEAB buffer to initiate iTRAQ labeling protocol. iTRAQ 8-plex labeling protocol was executed according to manufacturer's instructions with slight modifications. Succinctly, 1 μL of denaturant and 2 μL of reducing agents provided with the kit were added to the

sample and incubated at 60 °C for 1 hour. After denaturation, 1 μL of cysteine blocking reagent was added and incubated at room temperature for 10 min, followed by the addition of 10 μL of TEAB buffer. After overnight trypsin digestion (trypsin:protein ratio of 1:50, Sequencing Grade Modified Trypsin, Promega, USA), resulting peptides were labeled as shown in table I and pooled. Before advancing to the separation of peptides, labeling efficiency was checked by MS/MS and 1 μL of each sample was cleaned using Agilent Bond Elut OMIX C18 tips according to manufacturer's guidelines but using 0.1% trifluoroacetic acid (TFA) as washing solution. After pooling, samples were dried and stored at -20 °C.

Table I – iTraq labeling reagents used in each run for spinosad and indoxacarb. T1, T2, and T3 refers to 0.5, 2, and 8 μg L^{-1} for spinosad, respectively, and to 2, 4, and 8 μg L^{-1} for indoxacarb, respectively. iTRAQ 1 refers to the first run, and ITRAQ 2 refers to the second run.

	Labeling reagent used			
	Spinosad		Indoxacarb	
Treatment	iTRAQ 1	iTRAQ 2	iTRAQ 1	iTRAQ 2
Control	121	116	114	115
Control	115	119	118	121
T1	116	121	115	118
T1	119	118	121	117
T2	114	115	117	116
T2	118	117	113	114
T3	117	113	119	113
T3	113	114	116	119

2.5 Two-dimensional reversed phase liquid chromatography

To reduce complexity, fractionation of samples was made using a two-dimensional high-performance liquid chromatography (2D-HPLC) approach, more specifically, a high-pH/low-pH reversed phase (RP) liquid chromatography. This separation method was proposed by Gilar et al., (2005) and has been successfully used in combination with iTRAQ (Van Oudenhove et al., 2012). The first dimension (at high pH) was performed in a ETTAN LC chromatograph (GE Healthcare, UK) using a Gemini® C18 LC Column (100 x 1 mm, 3 μm, 110 Å; Phenomenex, USA) as stationary phase while 2% acetonitrile (ACN), 0.02 M ammonium formate (Buffer A1, pH=10) and 80% ACN, 0.02 M ammonium formate (Buffer B1, pH=10), were used as mobile phases with a flow of 0.05 mL min^{-1}. A total of 100 μg of peptides previously diluted in buffer A1 were injected in each run. The gradient employed was as follows: starting with 5 minutes of 100% buffer A1, it was followed by a 30-minute linear increase of 0 to 50% buffer B1 and then a linear increase from 50 to 100% buffer B1 for 1 minute. The separation gradient remained at 100% for 6 minutes before ending the run with a 7-minute 100% buffer A1. The eluted peptides were monitored at 214, 220, and 280 nm and collected to 8 different fractions for each spinosad run, and to 5 fractions

for each indoxacarb run. After collection, samples were dried, resuspended in a 2% ACN, 0.1% TFA solution and stored at -20 °C when not immediately injected in the second-dimension chromatograph.

The second RP-LC (low pH) was performed in a Dionex™ LC Packings system equipped with a Famos™ autosampler, a Switchos™ switching unit (with a loading pump), an Ultimate™ dual gradient system, an Ultimate™ UV Detector and a Probot™ fraction collector. Five microliters of each sample were first injected and concentrated in an Acclaim™ PepMap™ C18 trapping column (0.3 × 5 mm, 5 µm, 100 Å) using 2% ACN, 0.1 % formic acid as mobile phase at a flow of 0.025 ml min^{-1}. After 5 minutes, samples were eluted off the trapping column and loaded onto an Acclaim PepMap C18 nanoviper analytical column (0.075 x 150 mm, 3 µm, 100 Å). The eluents used for peptide separation were 100% H_20, 0.1% TFA (Buffer A2), 100% ACN, and 0.1% TFA (Buffer B2). The gradient employed was as follows: 3 minutes of 1% B2, followed by a 25 minute linear increase to 50% B2 and a subsequent a linear increase from 50% to 100% B2 for 10 minutes; the gradient remained at 100% B2 for 5 minutes before returning to the initial settings (1% B2). The pump flow was set at 0.3 µl min^{-1}. Eluted peptides were monitored at 214 and 280 nm, and at 3 minutes into the run, Probot fraction collector was turned on and started spotting the samples onto an Opti-TOF™ LC MALDI plate every 30 seconds. Spotted samples were promptly manually mixed with a supporting matrix, consisting of a 70% ACN solution containing 4 mg ml^{-1} of α-Cyano-4-hydroxycinnamic acid, 0.01 M dibasic ammonium citrate, and 0.1 % TFA.

2.6 Mass spectrometric analysis, protein identification and quantification

Mass spectrometric analysis was performed using a 4800 Plus MALDI TOF/TOF Analyser system (AB Sciex, USA). MS spectra were acquired using positive ion reflector mode and the six most intense peaks (minimum S/N ratio of 15) were selected for MS/MS peptide fragmentation. Each spot was analyzed twice, and the masses of peptides fragmented on the first run in each spot were excluded from the analysis on the second run.

All MS/MS data retrieved were processed using ProteinPilot™ software v. 4.0. This software allows the inference of proteins by the identification of peptides using the Paragon™ algorithm (AB Sciex, USA) (Shilov et al., 2007), and also the relative quantification of iTRAQ labeled peptides. The following parameters were applied for the analysis: iTRAQ 8 plex (peptide labeled); MMTS (methyl methanethiosulfonate) was set as the cysteine-blocking reagent used during peptide labeling; digestion with trypsin; MALDI 4800 as instrument used. Variable biological modifications and amino acid substitutions were checked for ID purposes. Concerning the quantification analysis, background and bias corrections were applied. All the datasets were blasted against a database resulting from the translated transcriptome of *C. riparius* (Marinković et al., 2012). Transcripts

were obtained from NCBI Transcriptome Shotgun Assembly (TSA) database (Bioproject PRJNA167567) (Marinković et al., 2012) and translated using OrfPredictor tool (Min et al., 2005). To this database, a list of contaminant proteins provided with the software was appended to reduce false positive peptide hits. Additionally, a (reversed) decoy database was used to estimate the false discovery rate (FDR) analysis. Since this database may not cover the full transcriptome of *C. riparius*, datasets were also blasted against a database of dipteran proteins deposited on NCBI using the same settings. Positive matches on this database were manually inspected to discard duplicate protein hits.

Translated protein hits were matched using NCBI BLASTx® tool with non-redundant protein sequences database and the top result was annotated. For quantification analysis, only hits within 5% FDR were considered. Since absolute quantification by iTRAQ would require the use of a standard in each run (Quaglia et al., 2008), and that would be very limiting in terms of experimental design, samples were normalized to one of the control replicates, and average protein ratios determined were used for statistical analysis

2.7 Statistical Analysis

Independent *t*-tests were carried to verify if there were no statistically significant differences between iTRAQ runs for each chemical. One-way analysis of variance (ANOVA) followed by a Tuckey's post-hoc test was performed to discriminate differences among treatments for each insecticide. All data were checked for residual normality and for homoscedasticity. For protein CkMP2, log transformation did not correct for homoscedasticity, therefore Welch's ANOVA was performed instead followed by a Games-Howell post-hoc test. Only proteins identified, and with average protein ratios determined in both runs for the same chemical, were used for statistical analysis. Linear regressions were used to assess the relationship between spinosad concentration and globin expression. Inferential statistical analysis was made in IBM SPSS® 25 and in GraphPad Prism® 7 for Mac with significance level set at $p \leq 0.05$.

3. Results

A total of thirty-six proteins were identified in spinosad-exposed *C. riparius* larvae (supplementary data, table I). From these, fifteen proteins identified in both iTRAQ runs had peptides considered usable for quantification, and six proteins (16,7%) were found to be differentially expressed (Table II): four proteins belonging to globin family (given the codes G1, G2, G3, and G4), one cuticle protein (CB1), and one actin (CkMP2). Proteins G2 ($F_{(3,12)} = 13.39$, $p < 0.001$) and CB1 ($F_{(3,12)} = 10.81$, $p < 0.01$) had their expression significantly decreased in the 8 $\mu g\ L^{-1}$ treatment compared to the control and all other concentrations. Protein CkMP2 expression decreased in the two highest concentrations ($F_{(3,6)} = 18.16$, $p < 0.01$). Protein G1 was significantly upregulated at 0.5 $\mu g\ L^{-1}$ when

Table II – Differentially expressed proteins in *Chironomus riparius* after exposure to spinosad. An asterisk denotes a statistically significant difference to the control treatment. A number sign denotes a significant difference in T1 treatment in comparison with T2 and T3 treatments. A dagger denotes a significant difference between T1 and T3 treatments.

Code	Total score	% Cov.	TSA Accession #	Peptides (95%)	Blast Top Result / Protein Match	Species	Protein Accession #	Significant changes
G2	20.00	59.0	gi|400998655	14	globin VIIA.1	*Chironomus thummi thummi*	AAB58930.1	↘ T3 *
CkMP2	18,29	47.3	gi|401001021	18	actin, partial	*Zygaena filipendulae*	AHW40461.1	↘ T2 and T3*
G1	18.23	89.4	gi|400994540	12	hemoglobin C precursor	*Chironomus thummi*	AAA28251.1	#
G3	16.14	78.9	gi|401009927	11	Globin CTT-VIIB-5/CTT-VIIB-9	*Chironomus thummi thummi*	P84298.1	†
G4	14.00	72.9	gi|401013254	8	Globin CTT-VIIA; Flags: Precursor	*Chironomus thummi thummi*	P02226.2	↘ T3*
CB1	12.00	82.0	gi|401012171	12	predicted: larval cuticle protein 8-like	*Drosophila kikkawai*	XP_017017873.1	↘ T3*

Total score – ProteinPilot total score for the protein;
% Cov. – The percentage of matching amino acids (of translated sequence);
Peptides (95%) - The number of distinct peptides having at least 95% confidence;
T1 – 0.5 μg L^{-1}; T2 – 2 μg L^{-1}; T3 – 8 μg L^{-1}.
↘ decrease; ↗ increase.

compared to the 2 and 8 μg L^{-1} treatments, and although not significant, there was a fourfold increase in the 0.5 μg L^{-1} treatment when compared to the control ($F_{(3,12)}$ = 6.60, p < 0.01; Fig. 1); at the highest concentration, there was a 48% reduction in the expression when compared to the control. A somewhat similar response pattern was observed for protein G3, for which Tuckey's post-hoc analysis revealed differences between 0.5 and 8 μg L^{-1} treatments ($F_{(3,12)}$ = 3,67, p < 0.05; Fig. 1). A significant decrease in expression was observed for protein G4 in the 8 μg L^{-1} treatment when compared to control and 0.5 μg L^{-1} treatments ($F_{(3,12)}$ = 6.24, p < 0.01). Moreover, a significant linear regression was found between spinosad concentration and the expression of the six identified globins (r^2 = 0.17, p < 0.05; Figure 2a). This association is more apparent excluding protein G1 from the analysis (r^2 = 0.64, p < 0.001; Figure 2b).

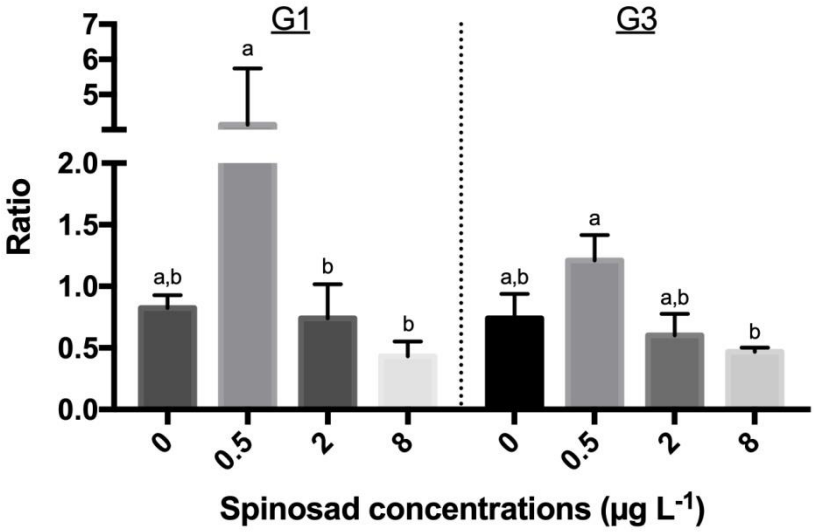

Figure 1 – Protein ratios of the two globins where an increase was observed in the lowest spinosad concentration tested. Values presented as mean + SEM. Bars that do not share a letter are significantly different.

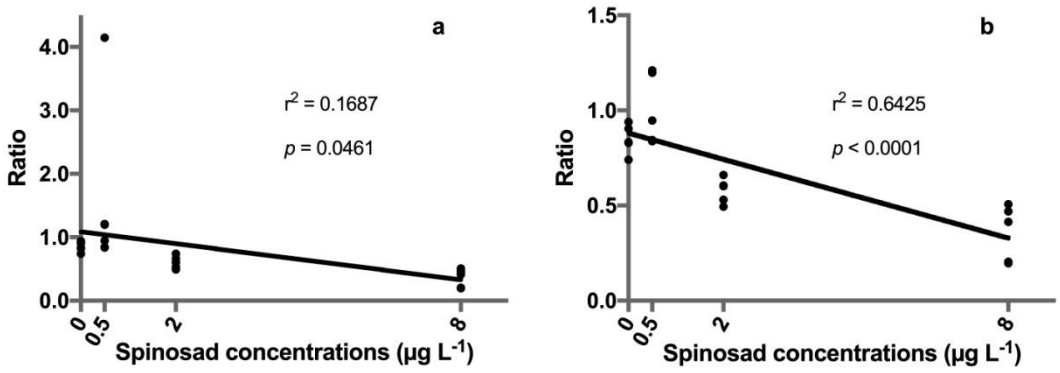

Figure 2 – Linear regression of the ratios of identified globins expression in *C. riparius* after exposure to spinosad. a) includes all identified globins and b) excludes globin G1 expression where a fourfold increase at 0.5 μg L^{-1} was observed. *p* values indicate deviations from zero slope.

Regarding indoxacarb, from a total of thirty proteins identified (supplementary data, table II), fifteen were used for quantification analysis. Only one cuticle protein (CB1) had its expression significantly altered, for the expression levels between 2 and 4 μg L^{-1} treatments ($F_{(3,12)} = 3{,}94$, $p < 0.05$; Fig. 3).

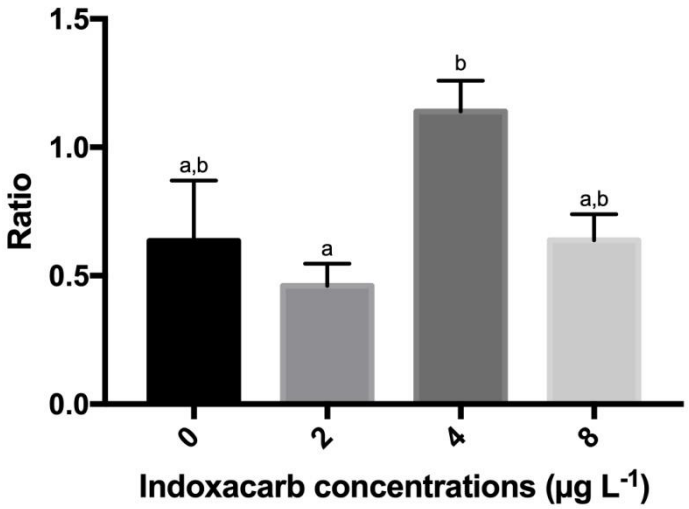

Figure 3 – Protein ratios of protein CB1 (larval cuticle protein) in *C. riparius* under exposure to indoxacarb. Values presented as mean + SEM. Bars that do not share a letter are significantly different.

4. Discussion

The present study shows that environmentally relevant concentrations of spinosad and indoxacarb can cause alterations in the proteome of *C. riparius*. The changes in protein expression observed here can aid to understand the mechanisms involved in spinosad's and indoxacarb's toxic action and reveal indirect effects that together with their neurotoxic mode action, may contribute to the responses at a higher biological level. Changes in the expression of globins, actin, and a cuticle protein were observed as a result of spinosad exposure, while for indoxacarb changes were only observed for a cuticle protein.

An overall analysis of spinosad data revealed that globins, in general, decreased as the concentration of the pesticide increased. The function of hemoglobins (Hb) in *Chironomus* sp. and their ecotoxicological relevance have been extensively studied. These are the most abundant proteins in *C. riparius* larvae (Choi et al., 2001). Hemoglobins perform a respiratory function in *Chironomus*, and due to their high affinity for oxygen (Burmester and Hankeln, 2007; P. Osmulski and Leyko, 1986; Weber et al., 1985) *Chironomus* are capable of maintaining a good oxygen supply for aerobic metabolism even under hypoxic conditions (P. Osmulski and Leyko, 1986; Weber, 1980). It is therefore postulated that freshwater invertebrates containing Hb are very tolerant to adverse environmental conditions (Choi and Ha, 2009; Choi et al., 1999; P. Osmulski and Leyko, 1986), and hemoglobins have been previously proposed as potential biomarkers for

environmental monitoring (Choi and Ha, 2009; Choi and Roche, 2004; Grazioli et al., 2016; Ha and Choi, 2008; Oh et al., 2014). Choi and Ha (2009) reported a generalized decrease in the expression of globins (and subsequently a decrease of total Hb content) as a consequence of exposure to cadmium (Choi and Ha, 2009). These authors also observed a decrease of larval weight as well as a decrease in emergence and reproductive traits and conjectured that these outcomes may be directly related to the alterations of globins expression. The impairment of larval growth and emergence of *C. riparius* by spinosad exposure was previously observed by the present authors (chapter 3), suggesting that these outcomes may not be exclusively associated with the neuromuscular toxicity of spinosad, but also associated with the expression of globin proteins. Since current evidence demonstrates that Hb production in *Chironomus* larvae is regulated by hormones, and Hb levels are only expected to decline during molting periods (Bergtrom et al., 1976; P. Osmulski and Leyko, 1986; Vafopoulou-Mandalos and Laufer, 1984), this suggests that globins may be target molecules of spinosad. Another possibility is that spinosad might be interacting with growth hormones, however it cannot be inferred from this study, and there is no reported evidence of spinosad's endocrine disrupting effects (EPA, 2005; Ewence et al., 2015). Interestingly, for two globins identified, there was an increase in their expression in the lowest concentration tested, with a fourfold increase observed for one of these proteins. This induction at low concentrations, may be associated with hemoglobin roles in oxygen transportation and storage, providing a good oxygen supply for oxygen-dependent detoxification mechanisms (Choi and Ha, 2009; P. A. Osmulski and Leyko, 1991). Moreover, a possible role of Hbs in the detoxification of xenobiotics has been suggested (P. Osmulski and Leyko, 1986; P. A. Osmulski and Leyko, 1991). Despite this increase observed in the lower concentration, expression levels of these proteins in the two highest concentrations decreased to lower levels than the ones observed for non-exposed organisms, similarly to the effects observed for the other identified globins.

A significant decrease was detected for actin (CkMP2). Actin is one of the most abundant proteins in eukaryotic cells (Dominguez and Holmes, 2011; Lodish et al., 2003). This cytoskeleton protein is involved in many physiological processes including cellular motility, muscle contraction, and cytokinesis (Dominguez and Holmes, 2011; Goodson and Hawse, 2002; Nelson and Cox, 2004; Wickstead and Gull, 2011). Several studies have reported alterations of actin state due to oxidative damage (Dalle-Donne et al., 2001; Gómez-Mendikute and Cajaraville, 2003; Gómez-Mendikute et al., 2002; Milzani et al., 1997). A decrease in the expression of *C. riparius´* actins as response to cadmium contamination has been previously observed (S.E. Lee et al., 2006), and the authors suggested a possible association between this decrease and the behavioral changes observed. In this study, behavioral endpoints were not directly assessed, nonetheless changes in growth and survival of the larvae were previously observed for spinosad exposure, as well as evidences of oxidative damage (ex. increased lipid peroxidation)

(Chapter 3). The decrease observed here in actin expression may therefore reflect the spinosad-induced neuromuscular toxicity and consequent oxidative stress on *C. riparius*.

A significant decrease was also observed for a larval cuticle protein (CB1). Insect cuticle is composed of cuticular proteins and chitin, key components of insect exoskeleton and crucial for molting and development (Andersen et al., 1995). Although cuticular penetration of spinosad is expected to be relatively slow (Salgado and Sparks, 2005), alterations on the arthropod *Blattella germanica* cuticle hydrocarbon profile due to spinosad exposure have been reported before (Habbachi et al., 2009). The downregulation of cuticle proteins may interfere with cuticle permeability and molting and consequently with growth and reproduction of arthropods (Poynton et al., 2008). Since chironomids' growth, molting, metamorphosis, and other life traits are controlled by hormones (Dubrovsky, 2005; Taenzler et al., 2007), it is interesting to note that the proteins examined above are, to a certain extent, regulated by hormones (Fretz and Spindler, 1999; P. A. Osmulski and Leyko, 1991; Spindler et al., 1990). More research should be conducted to elucidate if the downregulation of these proteins is a direct effect of the pesticide or if spinosad has an endocrine disrupting activity on *C. riparius* - in any of these cases, hormone direct or indirect impairment might bring up other effects at higher levels of biological organization as these messengers are the cornerstone molecules of a myriad of biological processes.

For spinosad exposure, only two proteins exhibited a monotonic dose-dependent response, globin VIIA.1 (G2) and actin (CkMP2). To our knowledge, the study of concentration-response in environmental proteomics is still very limited, although some studies already addressed this concept (ex. (Choi and Ha, 2009; Gündel et al., 2012)). The non-monotonic responses shown here for some proteins underline the importance of measuring simultaneously proteome alterations at different concentrations (including concentrations that cause no apparent physiological changes in that period).

Concerning indoxacarb exposure, a decrease in larval growth and an increase in development time of *C. riparius* was previously observed, along with changes at the biochemical level (Chapter 3). At the proteome level, none of the proteins identified in both runs showed significant alterations in expression, compared to the control treatment. However, for protein CB1 (larval cuticle protein), there was an evident increase in the expression in the 4 $\mu g\ L^{-1}$ when compared to the other treatments. The increase in the expression of cuticle proteins may reveal a protective adaptation to chemical stress, as previous research indicates that insects protect themselves from insecticides and environmental stress, by thickening their cuticle (Koganemaru et al., 2013; Wood et al., 2010; Zhang et al., 2008). Since increased expression only occurred in the intermediate concentration tested, it might be argued that at higher concentrations, other antioxidant and detoxification mechanisms may be favored for a faster response to indoxacarb exposure, as indicated by the increase in glutathione-S-transferase (GST) and

glutathione peroxidase (GPx) activities previously observed (Chapter 3). This non-monotonic response reinforces once again the importance of accessing multiple conditions in ecotoxicoproteomic studies, since different concentrations of the same chemical trigger different responses at the proteome level. Indeed, one of the major challenges in environmental "omics", is to determine which alterations at molecular level are responsible for the outcomes observed, and which alterations are simply unrelated, adaptive, or even beneficial (Aardema and MacGregor, 2002). For instance, a low dose of a xenobiotic may: (1) not produce any toxic effects, and consequently no alterations in protein expression; (2) merely trigger defense mechanisms, and the changes observed may result from the activation of those processes, and not by the xenobiotic's direct action; and (3) exert toxicity, and alterations at the proteome level may be related to its mechanism of action, to the activation of defense mechanisms, or to other indirect effect (ex. secondary targets or compensatory responses to the action of the xenobiotic).

As expected, the insecticides studied here triggered different responses at the proteome level. Changes caused by exposure to spinosad were more marked than for indoxacarb. Since changes at a biochemical level were previously observed for the same concentrations and exposure time, more evident changes at the proteome level were anticipated for indoxacarb. However, only a part of the complex proteome of *C. riparius* was covered, since only a few highly abundant proteins were identified. The presence of abundant proteins such as hemoglobin, which represents roughly 60% of *C. riparius* total protein content (Choi et al., 2001) or actin, which is also very abundant in eukaryotic cells, may have masked the detection of less abundant proteins, suggesting the requirement of additional sample fractionating steps when studying *C. riparius* proteome. It is possible that other less abundant proteins that were not assessed here may have contributed to the effects observed at higher levels. Additionally, it is important to bear in mind that the abundance of a protein does not necessarily correlate with its activity (Sadaghiani et al., 2007; Schmidinger et al., 2006). This reinforces the requirement of a more integrative ecotoxicological approach, at different levels of biological organization, to discover sensitive and early-warning protein biomarkers (Gündel et al., 2012; Lemos et al., 2010).

5. Conclusions

This work evaluated the effects of three concentrations of two insecticides in *C. riparius* proteome. While most ecotoxicoproteomic studies to this date focus on one single concentration of a stressor, the responses observed here for protein expression under insecticide exposure, support the need of using techniques that allow the simultaneous analysis of several samples – specially in an era of increased awareness about non-monotonic dose-responses and its relevance when considering toxicological studies. Observed effects at the proteome level could be related to the effects observed at higher levels of biological organization, which may be directly and/or indirectly related

to insecticides' modes of action. As also suggested by other authors, globins are very promising general biomarkers of stress in *C. riparius*. The results here presented suggest that globins expression could be a potential biomarker for insecticide toxicity. iTRAQ can be a very valuable tool in environmental proteomics specifically, and in ecotoxicology in general, since this technique allows the evaluation of dose-response relationships without disregarding the use of biological replicates. However, the experimental setup used here may still not be ideal, due to some variability between LC-MS/MS runs – only about half of the proteins identified could be further used for quantification. The development of higher multiplexing capacity methodologies, such as the 10-plex TMT (tandem mass tags) (McAlister et al., 2012), the 12-Plex DiLeu isobaric tags (Frost et al., 2015), or the 18-plex method proposed by Dephoure and Gygi (2012), which allows the simultaneous quantification of eighteen samples in a single run, may be of great use in ecotoxicoproteomics. Extensive research still has to be done, but with the growing information and the techniques available, soon these tools will be available to rapidly screen for environmental stress and/or to uncover mechanisms of action of chemicals that are not yet known. This work also contributed to the knowledge of the effect of neurotoxic insecticides on aquatic insects, highlighting *C. riparius* as a putative good model organism for environmental proteomics.

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Supplementary data

Supplementary table I – Classification of proteins identified in the spinosad exposure

Code	TSA Accession #	Blast Top Result / Protein Match	Species	NCBI Accession #
G1	gi|400994540	hemoglobin C precursor	*Chironomus thummi*	AAA28251.1
G2	gi|400998655	globin VIIA.1	*Chironomus thummi thummi*	AAB58930.1
G3	gi|401009927	Globin CTT-VIIB-5/CTT-VIIB-9	*Chironomus thummi thummi*	P84298.1
G4	gi|401013254	Globin CTT-VIIA	*Chironomus thummi thummi*	P02226.2
G5	gi|400998738	hemoglobin A' precursor	*Chironomus thummi*	AAA28254.1
G6	gi|400998633	globin 1	*Chironomus riparius*	AHV85224.1
EM1	gi|401000383	CLUMA_CG006317, isoform A (ATP synthase subunit beta)	*Clunio marinus*	CRK92903.1
EM2	gi|401002653	glyceraldehyde 3-phosphate dehydrogenase	*Haematobia irritans*	JAV18211.1
EM3	gi|400994789	Creatine kinase U-type, mitochondrial	*Daphnia magna*	JAN91448.1
EM4	gi|401000259	CLUMA_CG003212, isoform A (V-type proton ATPase subunit B)	*Clunio marinus*	CRK89474.1
EM5	gi|662643002	CLUMA_CG017016, isoform C (glutamate dehydrogenase)	*Clunio marinus*	CRL03893.1
EM6	gi|401001179	CLUMA_CG006885, isoform A (Enolase)	*Clunio marinus*	CRK93344.1
EM7	gi|400992509	CLUMA_CG009037, isoform C (Glycogenin-1)	*Clunio marinus*	CRK95573.1
EM8	gi|400996802	CLUMA_CG010689, isoform A (Fructose-bisphosphate aldolase)	*Clunio marinus*	CRK97294.1
EM10	gi|401001977	CLUMA_CG020704, isoform A (isocitrate dehydrogenase)	*Clunio marinus*	CRL07750.1
CB1	gi|401012171	PREDICTED: larval cuticle protein 8-like	*Drosophila kikkawai*	XP_017017873.1
CB2	gi|401010781	CLUMA_CG016256, isoform A (Cuticle Protein)	*Clunio marinus*	CRL02974.1
CB3	gi|400998711	CLUMA_CG012859, isoform A (Pupal cuticle protein)	*Clunio marinus*	CRK99541.1
CB4	gi|401006818	CLUMA_CG013198, isoform A (Larval cuticle protein LCP-17)	*Clunio marinus*	CRK99895.1
CB6	gi|401012720	CLUMA_CG016573, isoform A (Flexible cuticle protein 12)	*Clunio marinus*	CRL02972.1
CkMP1	gi|400991570	myosin heavy chain	*Anopheles darlingi*	ETN57922.1
CkMP2	gi|401001021	actin, partial	*Zygaena filipendulae*	AHW40461.1

CkMP3	gi|400997284	tubulin beta-1 chain	*Aedes albopictus*	XP_019552411.1
CkMP4	gi|401001335	tubulin alpha-1 chain-like	*Dinoponera quadriceps*	XP_019643284.1
CkMP5	gi|400997047	AGAP004877-PA (Myosin)	*Anopheles gambiae* str. PEST	XP_314309.4
CkMP6	gi|400995320	Tropomyosin-2	*Lucilia cuprina*	KNC34186.1
CkMP11	N.A.	tropomyosin	*Chironomus kiiensis*	CAA09938.2
PB1	gi|401000564	elongation factor 1-alpha	*Culicoides sonorensis*	AAV84215.1
PB2	gi|662645425	CLUMA_CG016390, isoform A (40S ribosomal protein S23)	*Clunio marinus*	CRL03238.1
PB3	gi|401013425	CLUMA_CG017633, isoform A (40S ribosomal protein S28)	*Clunio marinus*	CRL04562.1
HSP1	gi|401000218	heat shock cognate 70	*Chironomus yoshimatsui*	AAN14526.1
HSP2	gi|401000032	heat shock protein 70	*Polypedilum vanderplanki*	ADM13382.1
Bin1	gi|401000630	Plasminogen activator inhibitor 1 RNA-binding protein, partial	*Daphnia magna*	JAN92456.1
Bin2	gi|401012772	Histone H2A	*Urechis caupo*	P27325.2
OP1	gi|401001506	LUMA_CG009411, isoform A (Decaprenyl-diphosphate synthase subunit 2)	*Clunio marinus*	CRK95970.1
OP5	N.A.	GK10694 (uncharacterized protein)	*Drosophila willistoni*	XP_002066021.1

Supplementary table II – Classification of proteins identified in the indoxacarb exposure

Code	TSA Accession #	Blast Top Result / Protein Match	Species	NCBI Accession #
G1	gi|400994540	hemoglobin C precursor	*Chironomus thummi*	AAA28251.1
G2	gi|400998655	globin VIIA.1	*Chironomus thummi thummi*	AAB58930.1
G3	gi|401009927	Globin CTT-VIIB-5/CTT-VIIB-9	*Chironomus thummi thummi*	P84298.1
G7	gi|401009251	hemoglobin IA precursor	*Chironomus thummi*	AAA80190.1
EM1	gi|401000383	CLUMA_CG006317, isoform A (ATP synthase subunit beta)	*Clunio marinus*	CRK92903.1
EM2	gi|401002653	PREDICTED: glyceraldehyde-3-phosphate dehydrogenase 2	*Musca domestica*	XP_005176169.1
EM3	gi|400994789	Creatine kinase U-type, mitochondrial	*Daphnia magna*	JAN91448.1
EM4	gi|401000259	CLUMA_CG003212, isoform A (V-type proton ATPase subunit B)	*Clunio marinus*	CRK89474.1
EM7	gi|400992509	CLUMA_CG009037, isoform C (Glycogenin-1)	*Clunio marinus*	CRK95573.1

EM10	gi|401001977	CLUMA_CG020704, isoform A (isocitrate dehydrogenase)	*Clunio marinus*	CRL07750.1
CB1	gi|401012171	PREDICTED: larval cuticle protein 8-like	*Drosophila kikkawai*	XP_017017873.1
CB2	gi|401010781	CLUMA_CG016256, isoform A (Cuticle Protein)	*Clunio marinus*	CRL02974.1
CB3	gi|400998711	CLUMA_CG012859, isoform A (Pupal cuticle protein)	*Clunio marinus*	CRK99541.1
CB4	gi|401006818	CLUMA_CG013198, isoform A (Larval cuticle protein LCP-17)	*Clunio marinus*	CRK99895.1
CB6	gi|401012720	CLUMA_CG016573, isoform A (Flexible cuticle protein 12)	*Clunio marinus*	CRL02972.1
CkMP1	gi|400991570	myosin heavy chain	*Anopheles darlingi*	ETN57922.1
CkMP2	gi|401001021	actin, partial	*Zygaena filipendulae*	AHW40461.1
CkMP3	gi|400997284	n beta-1 chain	*Aedes albopictus*	XP_019552411.1
CkMP5	gi|400997047	AGAP004877-PA (Myosin)	*Anopheles gambiae str. PEST*	XP_314309.4
CkMP7	gi|400993845	Tropomyosin	*Chironomus kiiensis*	CAA09938.2
CkMP8	gi|400992550	PREDICTED: spectrin beta chain-like isoform X1	*Aedes albopictus*	XP_019527004.1
CkMP9	gi|400993929	CG001706, isoform A (Myosin light chain)	*Clunio marinus*	CRK87920.1
CkMP10	N.A.	putative myosin class i heavy chain	*Corethrella appendiculata*	JAB58256.1
PB4	gi|400999761	116 kDa U5 small nuclear ribonucleoprotein component protein	*Daphnia magna*	JAN90818.1
PB5	gi|400996870	CLUMA_CG020686, isoform A (mRNA-capping enzyme)	*Clunio marinus*	CRL07732.1
PB6	gi|400995638	Eukaryotic initiation factor 4A-II	*Daphnia magna*	JAN89802.1
OP2	gi|401001127	CLUMA_CG014022, isoform A (Alpha/beta hydrolase)	*Clunio marinus*	CRL00767.1
OP3	gi|400999405	putative l-2-hydroxyglutarate dehydrogenase mitochondrial	*Culex tarsalis*	JAV34037.1
OP4	gi|401002017	CLUMA_CG015770, isoform A (Serine/threonine-protein kinase SBK1)	*Clunio marinus*	CRL02849.1
OP6	gi|401002758	CLUMA_CG009647, isoform A (hypothetical protein)	*Clunio marinus*	CRK96220.1

Chapter V

Assessment of fipronil toxicity to the freshwater midge *Chironomus riparius*: linking molecular and biochemical endpoints with organismal responses

Assessment of fipronil toxicity to the freshwater midge *Chironomus riparius*: linking molecular and biochemical endpoints with organismal responses. [1]

Abstract

Fipronil is a phenylpyrazole insecticide that entered in the market to replace organochlorides and organophosphates. Fipronil impairs the regular inhibition of nerve impulses that ultimately result in paralysis and death of insects. Because of its use as a pest control, and due to runoff events, fipronil has been detected in freshwater systems near agricultural areas, which might represent a threat to non-target aquatic organisms. In this study, the toxicity of fipronil to the freshwater midge *Chironomus riparius* at different levels of biological organization was investigated in laboratory experiments.

At the individual level, exposure to fipronil resulted in reduced larval growth and emergence. Imagoes weight, which is directly linked to the flying performance and fecundity of midges, was also affected by exposure to fipronil. Additionally, behavioral changes such as irregular burrowing behavior of *C. riparius* larvae and impairment of imagoes flying performance were observed. At a biochemical level, increased cellular oxygen consumption (as indicated by the increase of electron transport system activity (ETS) activity) and a decrease in antioxidant and detoxification defenses (as suggested by the decrease in catalase (CAT) and glutathione S-transferase (GST) activities) were observed. Exposure to fipronil also caused alterations in the fatty acid profile of *C. riparius*: high levels of stearidonic acid (SDA) may be associated with stress response or a consequence of less energy available to fuel its conversion to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A comparison between exposed and non-exposed larvae also revealed alterations in protein expression of globins, cytoskeleton and motor proteins, and proteins involved in protein biosynthesis, disclosing potential mechanisms of action that lead to the effects observed at the organism level.

Present results show that environmentally relevant concentrations of fipronil are toxic to chironomid populations which call for monitoring of phenylpyrazole insecticides and of their ecological effects in freshwaters. Our results also emphasize the importance of complementing ecotoxicological data with molecular approaches such as proteomics, for a better interpretation of the mode of action of insecticides on aquatic invertebrates.

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Keywords: Phenylpyrazole insecticides, Aquatic insects, Sub-lethal toxicity, environmental proteomics, biomarkers.

1. Introduction

The continued use of pesticides in agriculture remains a serious threat to non-target aquatic macroinvertebrate communities. The ban of organochlorine insecticides followed by organophosphates restrictions has led to the increased application of pyrethroids and phenylpyrazoles such as fipronil in residential and agricultural areas (Amweg et al., 2006; Lassiter et al., 2009; Weston and Lydy, 2014). However, ecotoxicological data on fipronil (and other phenylpyrazole insecticides) that can aid to understand its potential impacts in the aquatic environment is scarce.

Fipronil is a neurotoxic insecticide that targets the central nervous system of insects by interfering with γ-aminobutyric acid (GABA)-gated chloride channels (Buckingham et al., 1994; Cole et al., 1993; Hainzl and Casida, 1996) and glutamate-gated chloride (GluCl) channels (Narahashi et al., 2010), resulting in the impairment of the normal transmission of nerve impulses (Gunasekara et al., 2007; Hosie et al., 1997; Raymond-Delpech et al., 2005). This specific mode of action makes fipronil very effective in controlling agricultural insect pests (Gunasekara et al., 2007). However, and due to runoff, spray drift, and leaching events, fipronil may contaminate adjacent aquatic systems, threatening non-target aquatic organisms (Clasen et al., 2012; Gan et al., 2012; Harman-Fetcho et al., 2005; Mize et al., 2008). Fipronil has been detected in water systems near agricultural fields in concentrations of up to 6.41 µg L-1 (Mize et al., 2008) and up to 10 µg L-1 in runoff water from residential areas (Gan et al., 2012). Fipronil is fairly insoluble in water, with a relatively high octanol/water partition coefficient (log Kow = 4.01) (US EPA, 1996), so it adsorbs onto the sediment where it is more persistent (Lin et al., 2008; Tingle et al., 2003).

Chironomids frequently dominate the benthic communities of lotic and lentic environments in terms of number and biomass (Pery et al., 2002; Taenzler et al., 2007), and although they have been described as crop pests (Stevens et al., 2006), they play a key role in freshwater food webs by recycling organic material and representing an important food source for higher trophic level organisms (Hölker and Stief, 2005; Pérez et al., 2010). Chironomids spend a large period of their life in direct contact with the sediment, making them model organisms for water and sediment toxicity testing (e.g. Azevedo-Pereira and Soares, 2010; Faria et al., 2006; Pestana et al., 2009). Toxicity of fipronil was previously described for other chironomidae (Ali et al., 1998; Chaton et al., 2002; Stevens et al., 1998; Weston and Lydy, 2014). However, none of these studies address the sub-lethal toxicity of fipronil in water on Chironomus riparius.

Commonly assessed endpoints in chironomus include survival, larval growth, development time, and sex ratio. Since the effects observed at higher biological levels are preceded by changes within the organism, the study of sub-lethal and sub-organismal endpoints may unveil early indicators of stressors exposure and/or effects (Lemos et al., 2010a).

Current advances in functional and expression proteomics opened new doors for potential applications of these technologies in ecotoxicological research. Since proteins are the main functional units within the cell, any changes in protein expression will reflect organisms' state. In this sense, proteomics may help in the characterization of molecular mechanisms related to the toxic response that may or may not result in physiological responses (Ralston-Hooper et al., 2013). Moreover, proteomics may also reveal possible biomarkers associated with the toxicological effects of stressors (Lemos et al., 2010a; Martyniuk et al., 2012).

The potential of fatty acid (FA) profile as a biomarker for environmental stress has also been recently addressed in some studies (Filimonova et al., 2016; Gonçalves et al., 2017; Lu et al., 2012; Silva et al., 2017). FAs play a number of essential roles in living organisms and are sensitive to environmental stress (Arts et al., 2009; Gonçalves et al., 2017). For instance, metabolic processes stimulate the production of reactive oxygen species (ROS) and xenobiotics exposure further enhances this production (Novais et al, 2014). This action induces lipid oxidation, which may result in the impairment of cell membrane functions or tissue damage (Parrish, 2013). On the other hand, changes in FA profile due to environmental stress, may also be indicative of adaptive responses to sustain membrane fluidity (Fokina et al., 2013; Los and Murata, 2004). In this sense, following FA profile may aid in the understanding of how stressors act on exposed organisms and the potential physiological implications of their actions.

Several biochemical biomarkers have been frequently used in aquatic ecotoxicology to assess sub-lethal effects of pesticides. The activity of phase II biotransformation enzyme glutathione S-transferase (GST) (Ziglari and Allameh, 2013) and the activities of antioxidant defense enzymes catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) can be assessed as oxidative stress biomarkers (Espinosa-Diez et al., 2015; Livingstone, 2003). Lipid peroxidation (LPO) levels and DNA damage can be measured as oxidative damage indicators, and acetylcholinesterase (AChE) as a measure of neuromuscular toxicity (Payne et al., 1996). Moreover, electron transport system (ETS) activity and lactate dehydrogenase (LDH) activity are used as cellular energy metabolism biomarkers (De Coen and Janssen, 1997; Diamantino et al., 2001)

Although biomarkers may provide some insights on the mode of action of the chemicals and their target mechanisms within the organism, most of the time it is still challenging to determine the ecological relevance of a biochemical alteration and a straightforward relationship between biochemical data and higher-level responses. In the present study, the effects of environmentally relevant concentrations of fipronil on *Chironomus riparius* (Meigen) at different levels of biological organization were investigated and integrated to get a hold of the continuum of biological response. The main goal of the study was to determine if there is a link between molecular level and

organismal responses to fipronil, to better understand the pathways involved in its toxic action in *C. riparius*.

2. Material and methods

2.1. Chironomus riparius culture conditions

Chironomus riparius cultures are maintained in ASTM hard water with continuous aeration and sterilized commercial sand (<1 mm) as sediment at 20° C with a 16:8 h light:dark cycle. The larvae in the culture are fed three times a week with macerated fish food, Tetramin® (Melle, Germany), and ASTM is renewed on a weekly basis. To initiate tests, freshly laid egg masses of *Chironomus riparius* were collected from cultures and kept in separate aquaria until hatching and maintained in culture conditions until reaching the desired age.

2.2. Fipronil and chemical analysis

Fipronil (≥97% purity) was purchased from Sigma-Aldrich (USA, CAS Number 120068-37-3). A fipronil stock solution was prepared in 100% ethanol and stored at 4° C, protected from light. Working and experimental fipronil solutions were prepared from this stock solution in ASTM, keeping the final concentration of ethanol below 0.01% in all treatments.

Concentrations of fipronil in stock solutions (2.0, 3.4 and 5.1 mg L^{-1}) were assessed by GC-MS based on the on information available in published works by Vílchez et al. (2001). Reagents used were of analytical grade and water used was purified by Milli-Q system (Millipore). A solution of 100 µg L^{-1} was prepared in N-hexane and from this solution, intermediate standard solutions were prepared from 1 to 5 ng L^{-1} through successive dilutions in N-hexane. Standard solutions were directly injected to the GC-system to build the calibration curve. The GC-MS system consisted of an Agilent Technologies 6890 N Network GC system, coupled with an Agilent 7683B Series Injector and Agilent 5975 Inert Mass Selective Detector and the software used was MSD ChemStation also from Agilent.

2.3 Acute toxicity tests

Acute tests were performed according to the OECD guideline 235 with spiked water (OECD, 2011). Four replicates, each containing five first-instar larvae (less than one-day old) were used per treatment and the concentrations of fipronil were 0, 0.05, 0.10, 0.20, 0.40, 0.80, 1.60, 3.20, 6.40, 12.80, and 25.60 µg L^{-1} (nominal concentrations). Organisms were exposed in small crystallizing dishes (45 mm diameter) with 20 mL of experimental solutions. No food was provided and the acute tests were performed at 20° C in complete darkness to avoid degradation of the compound. After 24 h and 48 h of

exposure, mortality (registered as immobilization) was checked by mechanical stimulation.

2.4 Chronic toxicity tests

A chronic, 28-days life cycle test was performed according to the OECD guideline 219 with spiked water (OECD, 2004). Briefly, the test was performed with 200 mL glass vessels each containing 150 mL of medium and 1.5 cm layer of fine, previously burnt sediment (sand). Food was given at a ration of 0.5 mg Tetramin® larvae^{-1} day^{-1}. A total of 8 treatments were tested: 6 increasing concentrations of fipronil in water (0.005, 0.010, 0.020, 0.040, 0.081, and 0.162 µg L^{-1}; based on working solution concentration, supplementary table I), a negative control, and a solvent control treatment. Each treatment consisted of 15 replicates with 5 first instar larvae each: 10 replicates were used to collect the adult midges (imagoes) and assess emergence, while 5 replicates were used to evaluate effects of fipronil on larval growth after 10 days of exposure. The test was conducted at 20 ± 1 º C with 16:8 h light:dark cycle and water physicochemical parameters were monitored throughout the whole test. *C. riparius* larvae growth was estimated by subtracting the average body length at the start of the experiment from the body length of the individuals after 10 days of exposure. Measurements were made with a dissecting stereomicroscope fitted with a calibrated micrometer. Mean development time (time until emergence), number of emerged imagoes, and imago size (as dry mass) were also evaluated. Emerging imagoes were checked on a daily basis, collected and stored in ethanol until being dried at 50° C and weighed. Development time and imagoes weight were analyzed separately for males and females, since females emerge later and are heavier (Pery et al., 2002). Burrowing behavior of the larvae was also checked in all replicates after 10 days of exposure, and was expressed as the total number of larvae on the top of the sediment relatively to the initial number of larvae, according to Pestana et al. (2009).

2.5 Exposure for biochemical biomarkers determination

Third instar larvae (8 days old) were used for biomarker determination. The test was performed with twenty organisms per replicate (seven replicates per treatment) in a crystallizing dish containing 200 mL of experimental solution (positive control, 0.007, 0.028, 0.110, and 0.220 µg L^{-1}; based on working solution concentration, supplementary table I) and a sediment layer about 1:4 of overlying water. The test was conducted in the same conditions as the chronic test. After 48 h of exposure, organisms were collected, dried with filter paper, weighed, frozen in liquid nitrogen and stored at -80° C until further analysis. Organisms were not fed during the exposure period.

2.5.1 Biochemical biomarkers

Samples were homogenized in 800 µL of 0.1 M of K-phosphate buffer (pH=7.4) with the help of a Ystral d-79282 homogenizer. The homogenate was separated as follows: 150 µL for determination of the electron transport system (ETS) activity; 150 µL for determination of lipid peroxidation (LPO) levels; 50 µL for determination of DNA damage; the remaining homogenate was centrifuged at 10,000 g for 20 min at 4º C and the post-mitochondrial supernatant (PMS) was collected. Portions of the PMS fraction were separated for protein quantification and for the determination of glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), acetylcholinesterase (AChE), and lactate dehydrogenase (LDH) activities.

Protein concentration was quantified following the Bradford protocol (Bradford, 1976) adjusted to microplate. Using bovine γ-globuline (Sigma-Aldrich, USA) as standard, results are expressed in mg of protein mL^{-1}. Prior to enzymatic assays, protein concentration in each sample was diluted and adjusted to roughly 0.8 mg L^{-1}, with the exception of SOD, where a 50x dilution of the initial protein amount was used. At the end of the assays, protein concentration of the dilutions was confirmed by the same quantification method.

ETS activity, as a measure of cellular oxygen consumption, was determined according to the method described by De Coen and Janssen (1997) with some adaptations (Rodrigues et al., 2015a); LPO levels were determined using the thiobarbituric acid reactive substances (TBARS) assay (Bird and Draper, 1984; Ohkawa et al., 1979; Torres et al., 2002) DNA damage was assessed using the alkaline precipitation assay (de Lafontaine et al., 2000; Olive, 1988).

Regarding enzymatic assays, GST activity was determined by following the formation of glutathione-dinitrobenzene when reduced glutathione (GSH) is conjugated with 1-chloro-2, 4-dinitrobenzene (CDNB) (Habig et al. 1974); SOD activity was assessed using the method described by McCord and Fridovich (1969), following cytochrome c reduction by the xanthine/xanthine oxidase system; CAT activity was assessed following the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen (Clairborne, 1985); The activity of GR was monitored by following the oxidation of NADPH when oxidized glutathione (GSSG) is added as substrate (Cribb et al., 1989); GPx catalyzes the conversion of GSH to GSSG, using H_2O_2 as substrate, and its activity was assessed by following the oxidation of NADPH when GSSG is converted back to GSH by GR (which is added in excess to the reaction) (Mohandas et al., 1984); AChE activity was evaluated by Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996) using acetylthiocholine as substrate; LDH was assessed by following the oxidation of NADH when pyruvate (substrate) is converted to lactate (Vassault, 1983; Diamantino et al., 2001).

All biomarker assays were performed in quadruplicates and blanks were made with K-phosphate buffer. All spectrophotometric measurements were made in a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek® Instruments, Vermont, USA) at 25° C.

2.6 Fatty acid profile determination

The exposure for FA profiling consisted of twenty third instar larvae per replicate (three replicates per treatment) in a crystallizing dish containing 200 mL of experimental solution (0, 0.01, 0.04, and 0.16 µg L^{-1}, based on working solution concentration, supplementary table I) and a sediment layer about 1:4 of overlying water. The test was conducted in the same conditions as the chronic test, and larvae were not fed during the exposure period. After 48 h of exposure, organisms were collected, dried with filter paper, weighed, frozen in liquid nitrogen and stored at -80° C until further analysis.

FA extraction and preparation for identification and quantification was performed according to Silva (2017) with some modifications. Briefly, each sample was homogenized in 200 µL of Buffer K-Phosphate 0.1M, pH 7.4. To the homogenate 200 µL of 0.6 M KOH (67% (v/v) ethanol) and 100 µg of decanoic acid (C10:0, Sigma-Aldrich, USA; used as internal standard) were added. The mixture was saponified overnight at 90° C. Samples were subsequently diluted 1:1 with ultrapure water, and pH adjusted to 1 with HCl. Afterwards, 330 µL of hexane was added to the mixture and samples were centrifuged of 1500g for 5 min. The organic phase was recovered and transferred to new vials for methylation. Some 875 µL of methanol:acetyl chloride (19:1 v/v) were added to the resulting organic phase from the previous step, prior to incubation at 80º C for 60 min. Then, 583 µL of ultrapure water were added before centrifugation at 1500g for 5 min. The organic phase was collected and analyzed by gas chromatography (GC). One µL of each sample was injected into a TR-FAME capillary column (60 m × 0.25 mm ID, 0.25 µm film thickness) on a Finnigan Ultra Trace gas chromatograph (Thermo Scientific, USA) equipped with an AS 3000 auto-sampler and a flame ionization detector. Injector temperature was set to 250° C and detector temperature was set to 280° C. Column temperature was set as follows: 100° C for 1 min followed by a 10° C rise per minute for 6 minutes, held at 160° C for 10 min, raised by 4° C per minute until 235° C, and finally kept at 235° C for 10 min. Helium was used as carrier gas with a flow rate of 1.5 mL min^{-1}, while air and hydrogen were supplied to the detector with flow rates of 350 and 35 mL min^{-1}, respectively. Results are expressed as FA peak area / C10:0 peak area ratio.

2.7 Protein differential expression determination

The exposure for protein differential expression determination was done under the same conditions as described for the exposure for FA profile analysis. After 48 h of exposure, organisms were collected, frozen in liquid nitrogen and mechanically grounded to a fine powder with the aid of a mortar and a pestle.

2.7.1 Protein extraction

Chironomus riparius proteins were extracted following TCA-acetone method described by Cilia et al. (2009), with minor modifications. Briefly, samples were mixed with a solution containing 10 % trichloroacetic acid (TCA) and 2% β-mercaptoethanol (2-ME) in acetone and incubated overnight at -20° C. Proteins extracts were then centrifuged at 5000 g for 30 min and the pellets were washed in acetone until tissue debris was completely discarded. All reagents were previously stored at -20° C and sample handling was done on ice. Resulting pellets were solubilized in a 0.04 M Tris-HCl buffer (pH=8) containing 7 M urea, 2 M thiourea, 0.5 % Triton-X-100, 0.1 % sodium dodecyl sulfate (SDS), 0.05 M $MgCl_2$, protease inhibitor mixture, 1 % bovine pancreas DNase I and 1 % bovine pancreas RNase A and stored at -80° C until use.

2.7.2 Protein quantification and sample preparation for iTRAQ®

Before starting the protocol, an acetone precipitation was performed according to the manufacturer instructions to remove potential interfering compounds with the iTRAQ labeling, and samples were resuspended in 0.5 M triethylammonium bicarbonate (TEAB) buffer. Protein content was determined using Coomassie Plus™ Kit assay, and extraction efficiency and protein integrity were verified by SDS-PAGE. Twenty µg of each sample were separated, dried in the SpeedVac™ (SC110 Thermo Savant) and resuspended in 25 µL of 0.5 M TEAB. iTRAQ 8plex labeling protocol was executed according to manufacturer's instructions with minor modifications. Briefly, 1 µL of denaturant buffer and 2 µL of reducing agent (provided with the iTRAQ kit) were added to the sample and incubated at 60° C for 1 h. After denaturation, 1 µL of cysteine blocking reagent (iTRAQ kit) was added and incubated at room temperature for 10 min, before adding 10 µL of TEAB buffer. Trypsin (Promega, USA) was subsequently added at a 1:50 ratio and samples were incubated overnight at 37° C. After digestion, peptides were labeled with iTRAQ reagents according to the manufacturer instructions (Table I). Samples were then pooled, dried and stored at- 20°C.

Table I - iTraq labeling reagents used for each replicate. T1, T2 and T3 refers to 0.01, 0.04 and 0.16 µg L^{-1}, respectively.

Treatment	iTRAQ 1
Control	113
Control	117
T1	114
T1	118
T2	115
T2	119
T3	116
T3	121

2.7.3 Two-dimensional reversed phase liquid chromatography

To reduce sample complexity, fractionation of samples was made using a high-pH/low-pH reversed phase two-dimensional high-performance liquid chromatography (RP/RP-HPLC). For the first dimension (pH=10), an ETTAN™ LC chromatograph (GE Healthcare, UK) equipped with a Luna C18 column (150 × 2.0 mm, 5 µm, 100 Å; Phenomenex, USA) was used. The mobile phases consisted of 2% (v/v) acetonitrile (ACN), 0.02 M ammonium formate (Buffer A1, pH=10) and 80% ACN, 0.02 M ammonium formate (Buffer B1, pH=10), and the pump operated at a flow rate of 0.05 mL min-1. A total of 200 µg of peptides previously diluted in buffer A1 were injected on a 100 µL loop. The gradient employed was as follows: 7 minutes of 100% buffer A1, followed by a 30-minute increase from 0 to 50% of buffer B1 and a subsequent increase from 50 to 100% of buffer B1 in 15 minutes; the separation gradient remained at 100% for 10 minutes before ending the run with 3 minutes of 100% buffer A1. The eluted peptides were monitored at 214, 220 and 280 nm and collected every 1 minute giving a total of 68 fractions. After visual examination of peak intensity, some fractions were pooled resulting in a total of 15 fractions. After pooling, samples were dried, resuspended in a 2% ACN, 0.1% (v/v) Trifluoroacetic acid (TFA) solution.

The second dimension (pH=3) was performed in a Dionex™ LC Packings system equipped with a Famos™ autosampler, a Switchos™ switching unit, an Ultimate™ dual gradient system, an Ultimate™ UV Detector and a Probot™ fraction collector. Ten microliters of each fraction were injected on an Acclaim™ PepMap™ 100 C18 trapping column (0.3 × 5 mm, 5 µm, 100 Å; Dionex, USA) using 2% ACN, 0.1 % TFA as mobile phase at a flow of 0.01 mL min^{-1}. After 5 minutes, samples were eluted off the trapping column and loaded onto an Acclaim PepMap C18 nanoviper analytical column (0.075 x 150 mm, 3 µm, 100 Å; Dionex, USA). The mobile phases consisted of 2% ACN, 0.1% TFA (Buffer A2) and 80% ACN, 0.1% TFA (Buffer B2). The pump flow was set at 0.3 µL min^{-1} and the gradient started with 3 minutes of 1% buffer B2, followed by a 25-minute increase to 50% of B2 and a subsequent increase from 50% to 100% of B2 in 10 minutes; the gradient was maintained at 100% B2 for 5 minutes before returning to the initial settings. Eluted peptides were monitored at 214 and 280 nm and Probot fraction collector was turned on after 20 minutes into the run (for 30 minutes) to start spotting the samples onto an Opti-TOF™ LC MALDI plate every 40 seconds. Spotted samples were immediately mixed with matrix consisting of a 70% ACN solution containing 4 mg ml^{-1} of α-cyano-4-hydroxycinnamic acid, 0.01 M dibasic ammonium citrate and 0.1 % TFA for mass spectrometric analysis.

2.7.4 Mass spectrometric analysis, protein identification and quantification

A 4800 Plus MALDI TOF/TOF Analyzer system (AB Sciex, USA) was used for acquiring MS spectra in the positive ion reflector mode. The six most intense peaks

(minimum Signal/Noise ratio of 20) in each spot were selected for MS/MS peptide fragmentation. Each spot was analyzed twice, and the peaks selected on the first run in each spot were excluded on the second run.

All MS/MS data were processed using ProteinPilot™ software v. 4.0, with the following parameters applied: iTRAQ 8 plex (peptide labeled); MMTS (methyl methanethiosulfonate) modification; trypsin digestion; MALDI 4800 as instrument used; biological modifications and amino acid substitutions (for ID purposes). Regarding quantification, background and bias corrections were applied. All data were matched against a database of translated transcriptome of *C. riparius* obtained from NCBI Transcriptome Shotgun Assembly database (Bioproject PRJNA167567) (Marinković et al., 2012) and translated using the ORFPredictor tool (Min et al., 2005). To this database, a list of common contaminants was added to reduce false positive peptide hits. Additionally, a reversed database was used as decoy to estimate the false discovery rate (FDR), which was set at 5%. Translated protein hits were matched using NCBI BLASTx® tool with non-redundant protein sequences database, and the top result was noted. One control sample was selected as denominator in ProteinPilot for relative quantification, and expression ratios between samples determined by ProteinPilot software were used for statistical analysis. Only proteins with an "unused score" > 1.0 (90% confidence) were considered for quantification analysis.

2.8 Statistical analysis

Four parameter logistic curves (nonlinear regression) were used to calculate LC_{50} and EC_{50} values. The equation is as follows: $Y=Bottom+(Top-Bottom)/(1+10^{((LogEC50-X)*HillSlope)})$, where *Y* is the response (% mortality for LC_{50} estimation; % larvae on top of the sediment for EC_{50} estimation), *Top* is the maximal response (100%), *Bottom* is the basal response (0%) and *X* is the logarithm of concentration.

Life-history, biochemical biomarkers and FA profile data were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett post-hoc test to discriminate significant differences between experimental treatments and the control. All variables were assessed for normality using residual normality tests, and homoscedasticity checked. GPx data were log transformed to correct for normality. Larval growth data did not meet the assumption of normality even after data transformation, but one-way ANOVA was still used as it is fairly robust against violations of the normal distribution when homogeneity of variances is verified (Blanca et al., 2017). Data transformations did not correct for unequal variances for the percentage of dead emerged imagoes, therefore a Kruskal-Wallis ANOVA on ranks was performed followed by Dunn's pairwise-comparisons test to discriminate significant differences between experimental treatments and the control treatment. No significant differences were identified by independent sample *t* tests between ASTM controls and solvent controls, therefore

solvent control was used as control treatment for all the statistical analysis. All above mentioned analyses were performed using GraphPad Prism® 7 for Mac software and the type I error rate was set at 0.05 for all statistical tests.

Regarding proteomics data, only proteins where no statistically significant differences between control replicates were observed, and statistically significant differences were observed between control and exposed larvae in at least one biological replicate were kept as results (ProteinPilot, p <0.05).

3. Results

The estimated 48h LC_{50} (95% CI) for fipronil was 1.74 µg L^{-1} (0.81-3.75) for first-instar larvae. At the highest fipronil concentration tested (25.6 µg L^{-1}), all larvae were found dead after 48h of exposure.

Regarding the 28-day life cycle test, *C. riparius* larval growth was significantly reduced, with a lowest observed effect concentration (LOEC) of 0.081 µg L^{-1} and a 40% reduction in larval length at the highest tested concentration ($F_{6,25}$ = 14.15, p < 0.001; Fig. 1). Concerning the burrowing behavior of the larvae (Fig. 2), there was an increase in the number of *C. riparius* larvae found on top of the sediment at the highest concentrations tested, with 53.33% ± 5.75 and 77.33% ± 5.47 of total larvae on top of the sediment for 0.081 µg L^{-1} and 0.162 µg L^{-1}, respectively. The EC_{50} for burrowing inhibition was of 0.084 µg L^{-1} (95% CI 0.08-0.09). There were no differences on the development time for either males ($F_{5,47}$ = 2.28, p = 0.06; Table II) or females ($F_{5,47}$ = 1.35, p = 0.26; Table II). However, the percentage of emerged imagoes was significantly reduced, with a LOEC of 0.081 µg L^{-1} (Table III). Some imagoes did not exhibit flying capabilities and were found lying on the top of the fipronil-contaminated water column. Additionally, some imagoes were found dead at 0.081 µg L^{-1}. These imagoes were regarded as non-viable. Considering only viable organisms, the LOEC for emergence was 0.041 µg L^{-1} (H = 26.87, p < 0.001; Table III), as there is a significant increase in non-flying imagoes (H = 43.05, p < 0.001; Table III). No midges have emerged nor found alive in the vessels by the end of the chronic test in the highest concentration tested ($F_{5,47}$ = 25.33, p < 0.001; Table III). Concerning the imagoes' dry weight, significant decreases were detected for both males ($F_{5,44}$ = 4.47, p < 0.01) and females ($F_{5,46}$ = 5.71, p < 0.001; Fig. 3) with a LOEC of 0.041 µg L^{-1}.

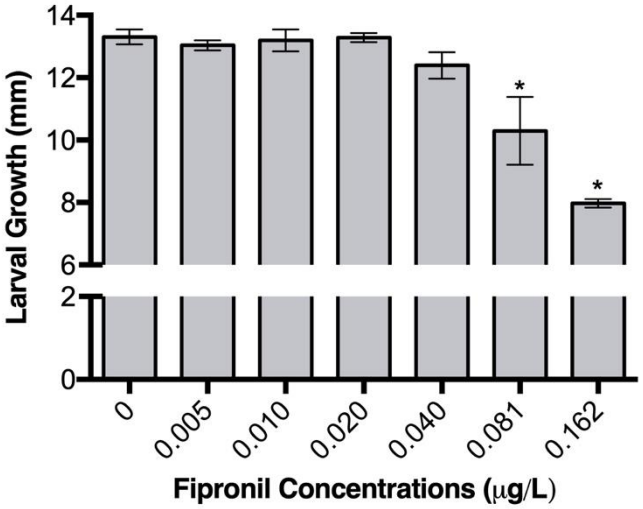

Figure 1 - *Chironomus riparius* larvae growth after 10 days of exposure to fipronil (mean ± SEM). Asterisks indicate statistically significant differences to the control treatment.

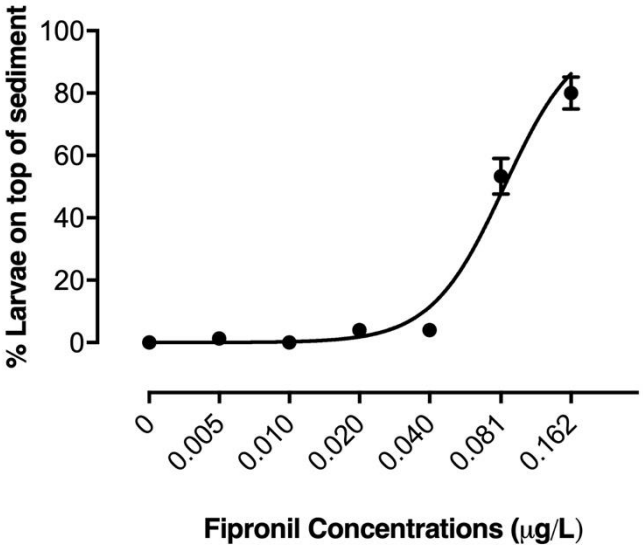

Figure 2 - Percentage of larvae found on the top of the sediment after 10 days of exposure relatively to the initial number of organisms (mean ± SEM).

Table II - *Chironomus riparius* development time after exposure to the insecticide fipronil. Values presented as mean ± SEM).

	[Fipronil] μg L^{-1}	Development time (days)		[Fipronil] μg L^{-1}	Development time (days)
Males	0	18.58 ± 0.75	Females	0	21.15 ± 0.49
	0.005	17.93 ± 0.60		0.005	21.29 ± 0.56
	0.010	17.88 ± 0.46		0.010	19.77 ± 0.72
	0.020	16.85 ± 0.30		0.020	20.45 ± 0.42
	0.040	16.63 ± 0.54		0.040	21.47 ± 0.53
	0.081	16.37 ± 0.61		0.081	20.60 ± 0.75
	0.162	n.e.		0.162	n.e.

n.e. no emerged adults

Table III - *Chironomus riparius* percentage of emerging imagoes after exposure to the insecticide fipronil (Mean ± SEM). Asterisks indicate statistically significant differences to the control treatment ($p < 0.05$, Dunnett's or Dunn's test).

	Emerged imagoes (%)			
[Fipronil] µg L^{-1}	Total	Flying	Non-Flying	Dead
0	94 ± 3.06	100 ± 0	0	0
0.005	90 ± 3.33	100 ± 0	0	0
0.010	88 ± 4.42	97.5 ± 2.5	2.5 ± 2.5	0
0.020	88 ± 4.42	95 ± 3.3	5 ± 3.3	0
0.040	84 ± 6.53	70.7 ± 6.2 *	29.3 ± 6.2 *	0
0.081	28 ± 6,80 *	0	77.1 ± 11.3 *	22.9 ± 11.3
0.162	0	n.d.	n.d.	n.d.

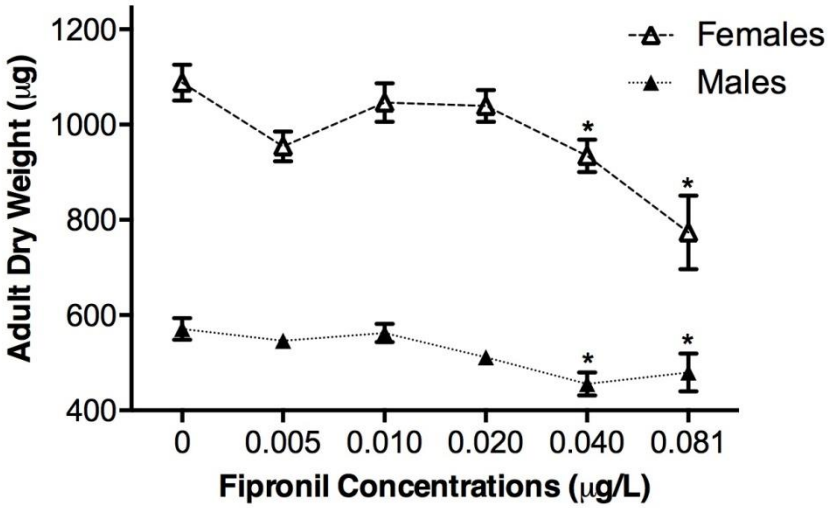

Figure 3 – Imagoes dry weight of male and female *Chironomus riparius* exposed to fipronil as larvae. Asterisks indicate statistically significant differences to the control treatment ($p < 0.05$, ANOVA Dunnett's test). Values presented as mean ± SEM).

Regarding biochemical biomarkers, a decrease in CAT activity was observed at 0.22 µg L^{-1} ($F_{4,22} = 4.27$, $p < 0.05$; Fig. 4a). GST activity decreased in the two highest tested concentrations ($F_{4,22} = 8.31$, $p < 0.001$; Fig. 4b), while ETS activity increased in the same experimental treatments ($F_{4,26} = 13.76$, $p < 0.001$; Fig. 4c). For AChE ($F_{4,25} = 3.12$, $p < 0.05$), GPx ($F_{4,26} = 3.69$, $p < 0.05$), and LPO ($F_{4,26} = 3.52$, $p < 0.05$) data, overall ANOVA's were significant but post hoc test failed to detect significant differences between the control and any of the experimental treatments. No significant changes were detected for DNA damage ($F_{4,26} = 1.69$, $p = 0.182$), and for GR ($F_{4,26} = 1.90$, $p = 0.141$), LDH ($F_{4,25} = 0.23$, $p = 0.916$), and SOD ($F_{4,26} = 2.48$, $p = 0.069$) activities.

To what concerns FA profile, significant differences were only found for stearidonic acid, with an increase observed in the highest tested concentration (C18:4 *n3*; $F_{3,8} = 13.07$, $p < 0.01$; table IV).

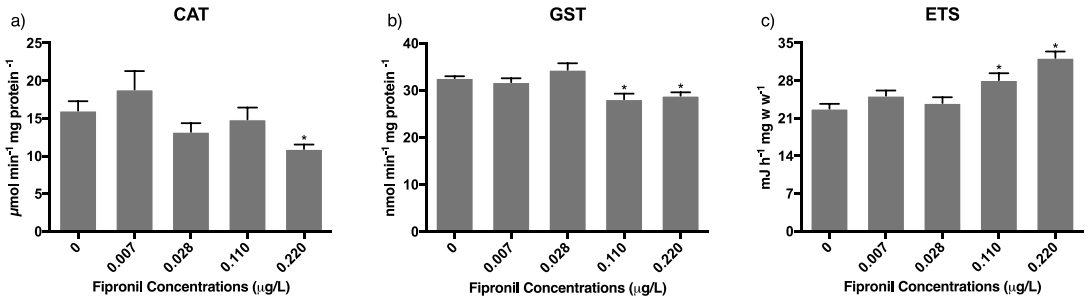

Figure 4 – Significantly altered biochemical biomarkers in *Chironomus riparius* larvae after exposure to fipronil: a) Catalase (CAT; μmol H_2O_2 consumed mg^{-1} protein); b) Glutathione-S-Transferase (GST; nmol CDNB conjugate formed mg^{-1} protein); c) Electron Transport System (ETS; mJ h^{-1} mg^{-1} wet weight). All values are presented as mean + SEM. Asterisks indicate statistically significant differences to the control treatment ($p < 0.05$, ANOVA, Dunnett's test).

Table IV- Fatty acid composition, expressed as fatty acid peak area / C10:0 peak area ratio, of *Chironomus riparius* larvae exposed to Fipronil. Values presented as mean ± SEM). Asterisks indicate statistically significant differences to the control treatment ($p < 0.05$, ANOVA Dunnett's test).

	Fipronil Concentrations				ANOVA
Fatty Acid	0 µg/L	0.01 µg/L	0.04 µg/L	0.16 µg/L	
C12:0	0.0167 ± 0.0006	0.0171 ± 0.0001	0.0180 ± 0.0008	0.0222 ± 0.0041	$F_{(3,8)} = 1.43$, $p = 0.303$
C14:0	0.0706 ± 0.0033	0.0748 ± 0.0028	0.0694 ± 0.0029	0.0727 ± 0.0056	$F_{(3,8)} = 0.39$, $p = 0.762$
C14:1	0.0309 ± 0.0041	0.0282 ± 0.0042	0.0276 ± 0.0023	0.0286 ± 0.0042	$F_{(3,8)} = 0.14$, $p = 0.934$
C15:0	0.0071 ± 0.0017	0.0041 ± 0.0010	0.0049 ± 0.0021	0.0095 ± 0.0005	$F_{(3,8)} = 2.71$, $p = 0.115$
C16:0	2.7754 ± 0.0681	2.9927 ± 0.0620	3.0483 ± 0.2387	3.0836 ± 0.1169	$F_{(3,8)} = 0.97$, $p = 0.456$
C16:1 *n9*	0.0129 ± 0.0007	0.0129 ± 0.0010	0.0134 ± 0.0013	0.0158 ± 0.0015	$F_{(3,8)} = 1.38$, $p = 0.318$
C16:1 *n7*	0.0742 ± 0.0120	0.0815 ± 0.0153	0.0705 ± 0.0003	0.0793 ± 0.0015	$F_{(3,8)} = 0.26$, $p = 0.853$
C16:1 *n5*	0.0104 ± 0.0018	0.0119 ± 0.0023	0.0097 ± 0.0002	0.0102 ± 0.0001	$F_{(3,8)} = 0.40$, $p = 0.756$
C16:2 *n7*	0.0123 ± 0.0005	0.0124 ± 0.0007	0.0047 ± 0.0037	0.0121 ± 0.0007	$F_{(3,8)} = 3.90$, $p = 0.055$
C18:0	1.6508 ± 0.0524	1.7549 ± 0.0286	1.7807 ± 0.1324	1.7097 ± 0.0602	$F_{(3,8)} = 0.52$, $p = 0.677$
C18:1 *n9*	0.1384 ± 0.0140	0.1470 ± 0.0222	0.1496 ± 0.0094	0.1307 ± 0.0041	$F_{(3,8)} = 0.37$, $p = 0.775$
C18:1 *n7*	0.0354 ± 0.0013	0.0357 ± 0.0037	0.0427 ± 0.0043	0.0370 ± 0.0046	$F_{(3,8)} = 0.85$, $p = 0.507$
C18:2 *n6*	0.4242 ± 0.0498	0.4516 ± 0.0719	0.4434 ± 0.0425	0.4026 ± 0.0158	$F_{(3,8)} = 0.20$, $p = 0.896$
C18:3 *n3*	0.0222 ± 0.0027	0.0224 ± 0.0033	0.0212 ± 0.0026	0.0217 ± 0.0015	$F_{(3,8)} = 0.04$, $p = 0.988$
C18:4 *n3*	0.0076 ± 0.0004	0.0072 ± 0.0006	0.0074 ± 0.0003	0.0110 ± 0.0006*	$F_{(3,8)} = 13.07$, $p < 0.01$
C20:3 *n3*	0.0140 ± 0.0019	0.0143 ± 0.0027	0.0132 ± 0.0017	0.0123 ± 0.0008	$F_{(3,8)} = 0.22$, $p = 0.880$
C20:5 *n3*	0.0665 ± 0.0123	0.0663 ± 0.0155	0.0634 ± 0.0086	0.0622 ± 0.0033	$F_{(3,8)} = 0.04$, $p = 0.989$

Twenty-two proteins were selected for quantification (supplementary table I), and nine distinct proteins were revealed to be downregulated by fipronil exposure (table V): a decrease in the expression of proteins CkMP1 and CkMP3 was observed in all tested concentrations. Proteins G2, CkMP12, OP7, OP8, and OP9 expression was reduced at the

Table V - Differentially expressed proteins in *Chironomus riparius* after exposure to fipronil

Code	Total score	% Cov.	TSA Accession #	Peptides (95%)	Blast Top Result / Protein Match	Species	Protein Accession #	Significant changes
G2	12.12	47.2	gi|400998655	6	globin VIIA.1	*Chironomus thummi thummi*	AAB58930.1	↘ T2, T3
G3	6.54	50.9	gi|401009927	5	Globin CTT-VIIB-5/CTT-VIIB-9	*Chironomus thummi thummi*	P84298.1	↘ T3
CkMP1	11.49	8.3	gi|400991570	6	myosin heavy chain	*Anopheles darlingi*	ETN57922.1	↘ T1, T2, T3
CkMP3	1.30	9.8	gi|400997284	1	tubulin beta-1 chain	*Aedes albopictus*	XP_019552411.1	↘ T1, T2, T3
CkMP12	5.24	20.7	gi|400993709	2	troponin t, invertebrate	*Anopheles darlingi*	ETN61955.1	↘ T2, T3
PB7	1.2	12.2	gi|401011791	1	CLUMA_CG017893, isoform A (putative 40S ribosomal protein S30)	*Clunio marinus*	CRL04840.1	↘ T3
OP7	2.29	10.7	gi|401000478	1	Calmodulin, partial	*Cupiennius salei*	CFW94154.1	↘ T2, T3
OP8	2	5.5	gi|401000997	1	CLUMA_CG008972, isoform A (putative Protein disulfide-isomerase)	*Clunio marinus*	CRK95503.1	↘ T2, T3
OP9	1.14	8.2	gi|400997946	1	CLUMA_CG015599, isoform A (similar to Estrogen sulfotransferase)	*Clunio marinus*	CRL02069.1	↘ T2, T3

Total score – ProteinPilot total score for the protein;
% Cov. – The percentage of matching amino acids (of translated sequence);
Peptides (95%) - The number of distinct peptides having at least 95% confidence;
T1 – 0.01 µg L^{-1}; T2 – 0.04 µg L^{-1}; T3 – 0.16 µg L^{-1};
↘ decrease; ↗ increase.

two highest tested concentrations, while a reduction in the expression of G3 and PB7 was observed at the highest tested concentration.

4. Discussion

The present study shows that exposure to fipronil results in several alterations in *C. riparius* at different levels of biological organization. Besides the impairment of life-history traits (reduced larval growth and emergence, reduced imagoes weight, irregular burrowing and flying behavior), at a biochemical level, high energy expenditure (increased oxygen consumption) and a decrease in antioxidant and detoxification defenses were observed. Moreover, fipronil exposure also caused alterations in protein expression, that may have contributed to the effects seen at organismal level and help understand the mechanisms involved in its toxic action in *C. riparius.*

Concerning acute toxicity, changes in behavior of *C. riparius* were noticed from the lowest concentration used in the acute test. *C. riparius* larvae movement was highly impaired, not being able to move in their typical figure-of-eight swimming movements, and only reacted when physically stimulated. Similar effects were observed by Stratman et al. (2013) on *Cricotopus lebetis* and may be explained by the neurotoxic mechanism of action of fipronil, blocking the normal inhibition of nerve impulses which may result in paralysis and ultimately death (Kitulagodage et al., 2011; Lourenço et al., 2012). These outcomes are consistent with the hypothesis that chironomids are amongst the most vulnerable freshwater invertebrates to fipronil (Stevens et al., 2011; Weston and Lydy, 2014). Stevens et al. (1998) reported a 24h LC_{50} of 0.43 µg L^{-1} for *Chironomus tepperi* (4^{th} instar). In their experiments, Ali et al. (1998) estimated a 48h LC_{50} of 0.42 µg L^{-1} for *Chironomus crassicaudatus* (4^{th} instar) and for *Glyptotendipes paripes* (4^{th} instar), while Chaton et al. (2002) calculated a 48h LC_{50} of 2.45 µg L^{-1} for *Chironomus annularius* (instar not specified). Stevens et al. (2011) estimated a 48h LC_{50} ranging from 0.89 to 2.18 µg L^{-1} for *Polypedilum nubiferum*. Considering other dipterans, some species are similarly susceptible, like *Culex quinquefasciatus*, with a 48h LC_{50} of 0.35 µg L^{-1} (4^{th} instar; Ali et al., 1999) while others are less sensitive like *Aedes albopictus* with a 48 h LC_{50} of 646 µg L^{-1} (instar not specified) (Ali et al., 1998). Regarding other aquatic invertebrates, the responses to fipronil appear to be highly variable even on closely related species (Stevens et al., 2011; Weston and Lydy, 2014). For instance, Hayasaka et al. (2011) determined 48h EC_{50} values (immobilization) for five cladoceran species, and values ranged from 0.99 µg L^{-1} for *Ceriodaphnia dubia* to 88.30 µg L^{-1} for *Daphnia magna*. This variability in the susceptibility of aquatic invertebrates reinforces the need to further study the effects on other ecologically relevant species in order to better predict the effects of fipronil on macroinvertebrate communities (Kitulagodage et al., 2011; Stevens et al., 2011). However, the consequences of fipronil exposure may not be restricted only to

marcoinvertebrate communities, but also on higher trophic levels, as available data indicates that fipronil can also have an impact on predators, such as birds, that feed upon contaminated insects (Kitulagodage et al., 2011).

Concerning chronic toxicity, larval growth has been proven to be a sensitive endpoint for pesticide exposure on *C. riparius* (Crane et al., 2002; Faria et al., 2007; Rodrigues et al., 2015a; Rodrigues et al., 2015b) and may be directly related to overall population biomass and the reproductive output of chironomids (Sibley et al., 1997). Based on the results presented here, reduced larval growth may be a direct consequence of the neurotoxic nature of the pesticide and/or an indirect consequence of the reallocation of energy to other biological processes, as indicated by the increase of ETS. In this study a LOEC of 0.081 μg L^{-1} was observed for C. *riparius* growth, however the effects on larval growth were not translated into delayed emergence, but rather on imagoes' weight. Imagoes body weight can be a relevant life-cycle and reproductive endpoint, as it is directly linked to the flying performance, fecundity and number of eggs in females (Carron, 2007; Sibley et al., 2001) and sperm count in males (Ponlawat and Harrington, 2007). In control conditions, it is expected that longer development time of larvae will result in an increase of imagoes size (Nunney, 2006; Sibley et al., 2001). Here, since adult *C. riparius* exposed as larvae were significantly smaller and there were no statistical differences on development time, this can be an indication that the trade-off between development time and imagoes size was altered by exposure to fipronil. This change, seen for both males and females, is expected to have short-term consequences on the reproductive output of *C. riparius* populations, which may lead to population decline.

Low fipronil levels also caused alterations in the burrowing behavior of larvae. Similarly to the behavioral (movement) changes observed in the acute toxicity tests, this impairment of burrowing behavior might be related to the neurotoxic mechanism of fipronil and has been reported for other neurotoxic insecticides such as imidacloprid (Pestana et al., 2009) and chlorpyrifos (Langer-Jaesrich et al., 2010). An EC_{50} for burrowing inhibition was estimated at 0.084 μg L^{-1} which is proximate to the LOEC for the larval growth. As mentioned above, the alterations of larval activity and consequently the diminished ability to burrow may also interfere with the search for food and could also explain the reduced growth of *C. riparius* larvae. Additionally, these alterations can lead to indirect populational effects, with the larvae being, for example, more vulnerable to predation (Schulz and Dabrowski, 2001).

Exposure to fipronil also caused a reduction in the number of emerged imagoes (survival) and an increase of non-flying imagoes. Gaertner et al. (2012), using an arthropod model, revealed that fipronil triggers the expression of ecdysone receptor. Since ecdysone regulates development and metamorphosis in insects (Ozáez et al., 2014), this could explain why some organisms did not complete metamorphosis nor emerged healthy. This action on the percentage of emerged imagoes may have direct

consequences on population dynamics and thus also impact the community (Agra and Soares, 2009).

Considering these toxicity data and the information derived from these experiments, it is clear that non-target aquatic insect populations dynamics may be severely affected by environmentally relevant concentrations of fipronil, with adverse consequences to the ecological integrity of freshwater ecosystems. Adult weight was the most sensitive organismal endpoint studied here, however our findings revealed that burrowing behavior is also a very sensitive endpoint that could give an earlier indication of the effects of neurotoxic insecticides by non-invasive observations.

CAT and GST are enzymes involved in detoxification after metabolic processes that are further enhanced by stress. The decrease of CAT and GST activities suggest a deficient removal of H_2O_2 and detoxification of fipronil. Interestingly, despite this effect on both enzymes, no evidences of oxidative damage were observed for the oxidative damage indicators DNA damage and LPO. This indicates that other antioxidant and detoxification processes not addressed in this study might have prevented oxidative damage to lipids and DNA. The concomitant decrease of GST and CAT in *C. riparius* has been described for other xenobiotics such as DEET (Campos et al., 2016) and chlorantraniliprole (Rodrigues et al., 2015b). In both studies, a depletion in total glutathione was observed, hinting that non-enzymatic conjugation of glutathione (GSH) with xenobiotics may represent an important mechanism of some xenobiotics detoxification in *C. riparius*. However, in this study, levels of GSH were not measured; therefore, this hypothesis cannot be confirmed. Nonetheless, a reduction of glutathione content, along with decreases in CAT and GST activities, has been observed in rats exposed to fipronil (Mossa et al., 2015; Swelam et al., 2017), and thus the measurement of GSH content should be considered in further studies in response to fipronil exposure. Nevertheless, although no evidences of oxidative damage to lipids or DNA were observed in the present study, it cannot be excluded that oxidative damage to proteins did not occur. Several studies reported that fipronil can induce ROS production (as reviewed in Wang et al. (2016)), and previous research on *Cyprinus carpio* indicated that exposure to fipronil increases protein carbonyl levels, which are indicative of oxidative protein damage (Clasen et al., 2012). Increased ROS production can cause modifications to amino acids of proteins, which generally result in inhibition of enzymatic activity (Sitte, 2003). Therefore, antioxidant status imbalance may be a possible explanation for the decreases observed for CAT and GST, as suggested in previous studies (Clasen et al., 2012; Mossa et al., 2015; Wang et al., 2016).

Additionally, increased ETS activity also contributes to a higher production of ROS (Sanz et al., 2010). ETS activity is a measurement of metabolic state of organisms and provides information on cellular oxygen consumption. An increase in ETS induced by a stressor is usually associated with the activation of the respiratory chain due to increased energy requirements for the activation of detoxification and antioxidant mechanisms

(Choi et al., 2001). However, the increase in ETS observed in the present study may be a direct consequence of fipronil's mode of action, as evidence indicates that fipronil is a powerful uncoupler of oxidative phosphorylation, leading to increased respiration and oxygen consumption rates (Vidau et al., 2011). These higher energy demands can limit the energy available for other physiological functions fundamental to the organism's fitness, such as growth and reproduction (Sokolova et al., 2012). Regarding AchE, present results indicate that fipronil does not affect AchE activity; this is not surprising as fipronil is a known to exert neurotoxicity by blocking the GABA-activated chloride channel.

Stearidonic acid (SDA) is an intermediate in the synthesis pathway of eicosapentaenoic acid (EPA, C20:5 *n3*) and docosahexaenoic acid (DHA, C22:6 *n3*) from a-linolenic acid (ALA, C18:3 *n3*) in humans (Walker et al., 2013). Usually, high levels of SDA in aquatic invertebrates are associated with a phytoplankton-rich diet (Kelly and Scheibling, 2013) however SDA is present in Tetramin (Fujibayashi at al., 2015; Lau et al., 2013), explaining the basal levels observed for this FA in this study. Exposure to fipronil led to an increase of SDA levels in *C. riparius*. To the extent of our knowledge, there is not much information on the literature regarding the ecotoxicological relevance of this FA and its increase, but SDA levels have been associated with immune and oxidative stress responses in *Caenorhabditis elegans* (Nandakumar and Tan, 2008). Additionally, the accumulation of SDA may be possibly related to the effects of fipronil in ETS activity and in oxidative phosphorylation, limiting the energy available for the conversion of SDA to EPA and DHA, thus interfering with the omega-3 biosynthetic pathway. The information available on the effects of fipronil on FA profile is also very scarce, still fipronil has been found to increase lipid content of the shrimp *Farfantepenaeus aztecus* (Al-Badran et al., 2018) and to promote fatty acid synthase expression in a mouse adipocyte cell line (Sun et al., 2016), and therefore the action of fipronil on FA profile should be further investigated.

Regarding protein differential expression, the two hemoglobins (Hb) identified appear to be affected by fipronil, since a decrease was observed with the increase of fipronil concentration. Hemoglobins, in *Chironomus* sp. as in other organisms, are involved in oxygen transport and storage, allowing them to survive in low oxygen environments or more oxygen-demanding situations (Osmulsky and Leyko, 1986). Alterations in Hb expression, particularly underexpression, have been previously reported for *C. riparius* in stress conditions (Choi and Ha, 2009), and have been linked with developmental adverse effects, such as reduced growth and reproduction. Considering these results, an increase in ETS activity observed at the biochemical level is a rather interesting response. Increased ETS activity implies additional oxygen requirements for cellular metabolism, whereas the underexpression of Hb proteins may result in insufficient oxygen supply to the cells and may ultimately lead to hypoxia and translate to organismal level effects observed in the present study.

The downregulation of cytoskeleton and motor proteins also reveals a decline in the condition state of C. riparius (Chora et al., 2009; Manduzio et al., 2005). Myosin, troponin, and calmodulin, proteins involved in muscle contraction and motility (Dominguez and Holmes, 2011; Śliwińska et al., 2008; Walsh, 1994), were downregulated by fipronil exposure, suggesting exposure to fipronil can affect the cytoskeletal structure of C. riparius and, along with the neurotoxic mode of action of fipronil, may have contributed to the lack of movement, abnormal behavior, and reduced growth and development observed in this study. Moreover, an inhibition of calmodulin has been previously found to inhibit metamorphosis of the polychaete *Hydroides elegans* (Chen et al., 2012). Since metamorphosis impairment was also observed in the present study, more research should be conducted to evaluate the potential role of calmodulin in dipterans' metamorphosis. Previous studies have observed differential expression of contractile and cytoskeletal proteins in aquatic organisms under stress (e.g. Gündel et al., 2012; Hook et al., 2014; Li et al. 2009; Manduzio et al., 2005; Muralidharan et al., 2012). Regarding *C. riparius*, Lee et al. (2006) reported a decrease in myosins as response to cadmium contamination. Additionally, previous studies have identified cytoskeleton proteins as major targets of oxidative stress (Dalle-Donne et al 2001; McDonagh et al., 2005), and excess ROS production may result in cytoskeletal damage (Anderson et al., 2015). So, this downregulation may also be related to ROS species produced after fipronil exposure. A similar conclusion can be drawn for tubulins, which are also major targets of oxidative stress (McDonagh and Sheehan, 2007). Alpha and beta tubulins polymerize into microtubules, that are essential components of the cytoskeleton (Wickstead and Gull, 2011) and are involved in several processes, such as cell division and intracellular transport (Cooper and Hausman, 2007). The downregulation of beta-tubulins has been previously reported for other aquatic organisms under chemical stress (Apraiz et al., 2006; Chora et al., 2009; Jaafar et al., 2015). The decrease in the expression of beta-tubulin observed here suggests cellular stress and may compromise polymerization and microtubule assembly and consequently affect cytoskeleton structure. It has been suggested that increases in tubulin expression may lead to the impairment of the reproductive function of invertebrates and therefore may affect the population dynamics (Lemos et al, 2010b). Although in the present study a decrease in tubulin expression was observed, the effects observed on larval growth, development, and imagoes weight suggest a reproductive impairment caused by fipronil exposure, and therefore the effects of fipronil in tubulin (and other cytoskeleton and motor proteins) expression and their potential consequences on the reproductive function of C. riparius should be further explored.

Proteins involved in protein biosynthesis and folding were also affected by fipronil exposure. The expression of protein CLUMA_CG017893, isoform A (putative 40S ribosomal protein S30) decrease in the highest tested concentration, and

CLUMA_CG008972, isoform A (putative Protein disulfide-isomerase) decreased in the two highest tested concentrations. The downregulation of ribosomal proteins has been suggested as metabolic strategy to save energy to increase organism's defenses (Ji et al., 2016). Moreover, the downregulation of RPL15 ribosomal protein gene has been previously reported in *C. riparius* exposed to cadmium and silver nanoparticles (Nair et al., 2011). The results obtained in the present study indicate a possible disturbance of protein biosynthesis induced by fipronil, which is supported by the generalized downregulation of proteins observed – all differentially expressed proteins identified in the present study were downregulated. A decrease in the expression of CLUMA_CG015599, isoform A (predicted estrogen sulfotransferase) was also observed, although there is not sufficient homology to support that it is actually an estrogen sulfotransferase, and the presence and role of this protein in invertebrate species is fairly unknown (Kornthong et al., 2014).

5. Conclusion

Our findings underline the importance of complementing chronic toxicity testing with molecular biomarkers, for a better interpretation of long-term effects of insecticides. Proteomics may be a useful tool in risk assessment to explore early events associated with the individual response. Although not specific, globins, cytoskeleton and motor proteins could be potential biomarkers of fipronil exposure, as they responded earlier and in some cases at lower concentrations, than the ones that caused individual and biochemical responses. Additionally, the above-mentioned proteins have clear and crucial roles inside the organism, and alterations on their expression may not only reveal the organism's current state but can be associated with higher level responses.

One of the challenges in ecotoxicoproteomics is to validate observations in field experiments; It is important to note that in an aquatic ecosystem, organisms are exposed to other natural and chemical stressors and therefore, responses measured in controlled and simplified laboratory conditions may not reflect the responses in a more complex system (Pestana et al., 2010), hence future research should be conducted towards that. Additionally, very few ecotoxicoproteomic studies have addressed the concentration-response concept. The study of one predefined concentration may not be sufficient to identify molecular pathways of toxicity and may lead to false conclusions (Gündel et al., 2012; Lemos et al., 2010a). In the present study several proteins were downregulated in a concentration-dependent manner, demonstrating that this approach can provide some mechanistic understanding of the effects of xenobiotics. This approach may also help understand which alterations are associated with toxic action of xenobiotics and which are adaptive responses (Gündel et al., 2012). Although iTRAQ methodology performed well in terms of accessing dose-response relationships of the identified proteins, in the present study only a few highly abundant proteins were identified. This issue seems to be common in environmental proteomics studies with invertebrate species, possibly due to

their biological complexity (Simões et al., 2018). It has been reported that current label-free methods may perform better than iTRAQ in protein differential expression studies (Latosinska et al., 2015; Trinh et al., 2013) and can be applied in ecotoxicological studies (Ralston-Hooper et al., 2013). The development of these multiplexing and label-free techniques enables the simultaneous study of multiple conditions (e.g. different concentrations and/or different exposure times), thus the assessment of multiple conditions in ecotoxicoproteomics should increase in the near future.

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Supplementary data

Supplementary table I - Concentrations of fipronil in stock solutions

	Fipronil Concentration	
Solution	Nominal	Measured
Chronic toxicity test	5.000 µg L^{-1}	5.055 µg L^{-1}
Exposure for biochemical biomarkers determination	5.000 µg L^{-1}	3.441 µg L^{-1}
Exposure for FA profile and protein expression determination	2.000 µg L^{-1}	1.995 µg L^{-1}

Supplementary table II - Proteins used for quantification

Code	TSA Accession #	Blast Top Result	Species	NCBI Accession #
G2	gi|400998655	globin VIIA.1	*Chironomus thummi thummi*	AAB58930.1
G3	gi|401009927	Globin CTT-VIIB-5/CTT-VIIB-9	*Chironomus thummi thummi*	P84298.1
CkMP1	gi|400991570	myosin heavy chain	*Anopheles darlingi*	ETN57922.1
CkMP2	gi|401001021	actin, partial	*Zygaena filipendulae*	AHW40461.1
CkMP3	gi|400997284	tubulin beta-1 chain	*Aedes albopictus*	XP_019552411.1
CkMP6	gi|400995320	Tropomyosin-2	*Lucilia cuprina*	KNC34186.1
CkMP7	gi|400993845	Tropomyosin	*Chironomus kiiensis*	CAA09938.2
CkMP12	gi|400993709	troponin t, invertebrate	*Anopheles darlingi*	ETN61955.1
CB1	gi|401012171	PREDICTED: larval cuticle protein 8-like	*Drosophila kikkawai*	XP_017017873.1
CB3	gi|400998711	CLUMA_CG012859, isoform A (similar to pupal cuticle protein)	*Clunio marinus*	CRK99541.1
CB6	gi|401012720	CLUMA_CG016573, isoform A (Flexible cuticle protein 12)	*Clunio marinus*	CRL02972.1
EM2	gi|401002653	glyceraldehyde 3-phosphate dehydrogenase	*Haematobia irritans*	JAV18211.1
PB7	gi|401011791	CLUMA_CG017893, isoform A (putative 40S ribosomal protein S30)	*Clunio marinus*	CRL04840.1
Bin3	gi|400995364	hnRNP protein	*Chironomus tentans*	CAA90716.1
Bin4	gi|401013907	PREDICTED: histone H4-like, partial	*Drosophila takahashii*	XP_017008549.1
OP7	gi|401000478	Calmodulin, partial	*Cupiennius salei*	CFW94154.1
OP8	gi|401000997	CLUMA_CG008972, isoform A (putative Protein disulfide-isomerase)	*Clunio marinus*	CRK95503.1
OP9	gi|400997946	CLUMA_CG015599, isoform A (similar to Estrogen sulfotransferase)	*Clunio marinus*	CRL02069.1
OP10	gi|401004132	CLUMA_CG004403, isoform A	*Clunio marinus*	CRK90710.1
OP11	gi|401009339	CLUMA_CG016253, isoform A	*Clunio marinus*	CRL03221.1
OP12	gi|400999706	CLUMA_CG013262, isoform A	*Clunio marinus*	CRK99967.1
OP13	gi|400998081	chymotrypsin-like elastase family member 2A	*Fundulus heteroclitus*	XP_021163427.1

Chapter VI

General Discussion

1. General discussion

The current study shows that amitraz, spinosad, indoxacarb, and fipronil affect the non-target freshwater invertebrate *Chironomus riparius*. Besides the impairment of several life-history traits, at a biochemical level, changes underlying organismal level effects and potential secondary targets of insecticide exposure were observed. Moreover, pesticide exposure induced changes in protein expression that may aid in the understanding of the affected mechanisms involved in their toxic action. This chapter highlights the major results of this study, and review in an integrative manner the effects of amitraz, spinosad, indoxacarb, and fipronil at different levels of biological organization in *C. riparius.* Common effects and pesticide-specific responses are explored in order to verify the usefulness of protein differential expression and biochemical biomarkers in ecotoxicology and environmental monitoring. Table I summarizes the effects of the exposure to amitraz, spinosad, indoxacarb, and fipronil in *C. riparius*.

1.1 Effects of pesticides at the individual level

An overall overview of the results reveals that environmentally relevant concentrations of the pesticides tested impaired several *C. riparius* life-history traits. A reduction in larval growth was observed for all the pesticides. Larval growth has proven to be a very sensitive endpoint in the assessment of xenobiotics' toxicity and can give an early indication of the reproductive output and population dynamics in chironomids. Additionally, it can be measured earlier than other life-history responses, which makes larval growth a valuable endpoint in the assessment of chronic effects of pesticides. Of the four pesticides tested, amitraz was the only that caused a gender-based effect, since only male development time was affected. This response has been observed for other stressors, and it has been suggested that females are less susceptible to chemical contamination due to their larger size and energy reserves (Goedkoop et al., 2010). Spinosad and indoxacarb produced similar effects at the individual level in the same range of concentrations – both pesticides reduced larval growth and increased time to emergence of males and females. However, spinosad's mode of action appears to be slightly more toxic to *C. riparius*, since a reduction in the emergence rate was also observed. For fipronil, reduced emergence rate, reduced imagoes weight, irregular burrowing and flying behavior were observed. Imagoes weight is not a usually assessed endpoint in *C. riparius* toxicity experiments, but the results presented here and in other recent works (Campos et al., 2016; 2017; Rodrigues et al., 2015), indicate that imagoes weight could be a relevant life cycle endpoint, as it is directly associated to the reproductive fitness in chironomids (Ponlawat and Harrington, 2007; Sibley et al., 2001). Additionally, imagoes weight was the most sensitive endpoint (LOEC of 0.040 µg L^{-1}) in

Table I – Summary of the significant alterations observed at different levels of biological organization in *Chironomus riparius* induced by amitraz, spinonad, indoxacarb and fipronil exposure.

	Life-history	Biochemical Biomarkers											Protein Differential Expression / FA profile
		LPO	DNA damage	GST	GPx	GR	CAT	SOD	AChE	LDH	IDH	ETS	
Amitraz	Reduced larval growth Reduced number of emergents Increased time to emergence of males	↗	n.a.	-	↗	-	↘	-	-	↘	-	↗	n.a
Spinosad	Reduced larval growth Reduced number of emergents Increased time to emergence of males and females	↗	-	-	↗	-	-	-	-	↗	n.a.	↗	-General decrease in globins expression -Decrease in actin expression -Decrease in the expression of a larval cuticle protein
Indoxacarb	Reduced larval growth Increased time to emergence of males and females	-	-	↗	↗	-	-	-	-	↗	n.a.	-	-Alterations in the expression of a larval cuticle protein
Fipronil	Reduced larval growth Reduced number of emergents Reduced imagoes weight Impairment of burrowing behavior and flying performance	-	-	↘	-	-	↘	-	-	-	n.a.	↗	-Decrease in the expression of globins -Decrease in the expression of cytoskeleton and motor proteins -Decrease in the expression of proteins involved in protein synthesis - Increase in Stearidonic Acid levels

n.a. – not assessed

↗ denotes a statistically significant increase to the control treatment; ↘ denotes a statistically significant decrease to the control treatment; - denotes that there were no statistically significant alterations.

LPO - lipid peroxidation; GST - glutathione-S-transferase; Gpx - glutathione peroxidase; GR - glutathione reductase; CAT – catalase; SOD - superoxide dismutase; AChE – acetylcholinesterase; LDH - lactate dehydrogenase; IDH - isocitrate dehydrogenase; ETS - electron transport system

both male and females. Burrowing behavior was also found to be a very sensitive endpoint that could give an earlier indication of the effects of neurotoxic insecticides by non-invasive observations.

1.2 Effects of pesticides at the biochemical level

None of the pesticides tested in this study caused alterations in SOD, AChE, and GR activities or induced oxidative damage in the DNA of *C. riparius* - albeit an increase in DNA damage was perceptible under spinosad exposure, which was accompanied by the increase of oxidative damage in lipids. No changes were detected in IDH activity, although this enzyme was only measured in amitraz-exposed larvae. Very dissimilar responses were noted for the pesticides tested, probably due to their distinct modes of action. The most commonly observed effects were (1) the increase of ETS activity observed for amitraz, spinosad, and fipronil exposures, which is an indication of high energy expenditure probably due to activation of defense mechanisms, and this energy allocation may result in less energy available for other functions, here evident by the reductions in the rates of larval growth and development; and (2) increase in GPx activity observed for the exposure to amitraz, spinosad, and indoxacarb. GPx is involved in the prevention of H_2O_2-induced oxidative stress. Nonetheless GPx activity was not sufficient to prevent the oxidative damage to lipids observed in the exposures to amitraz and spinosad. Regarding the other biochemical biomarkers, changes were noted in GST (indoxacarb and fipronil), CAT (amitraz and fipronil), and LDH (amitraz, spinosad, and indoxacarb) activities. CAT, ETS, and LPO were the most sensitive endpoints of amitraz exposure at biochemical level (LOEC of 18.9 $\mu g\ L^{-1}$). For spinosad, ETS activity was the most sensitive endpoint at the biochemical level (LOEC of 0.5 $\mu g\ L^{-1}$). For indoxacarb, GST was the most sensitive biomarker (LOEC of 4 $\mu g\ L^{-1}$), underlining the role of GST in the detoxification of indoxacarb and in the prevention of oxidative damage. For fipronil, GST and ETS were the most sensitive endpoints at the biochemical level (LOEC of 0.11 $\mu g\ L^{-1}$).

1.3 Effects of pesticides at the proteome level

Spinosad and fipronil were responsible for a number of alterations at the proteome level. Both pesticides caused a decrease in globins expression. The presence of hemoglobin is of utmost importance to chironomids, enabling a good supply of oxygen to cells and tissues, which allows them to survive in extreme environmental conditions and accelerate metabolism for rapid removal of xenobiotics (Osmulski and Leyko, 1986). The downregulation of these proteins may increase the vulnerability of *C. riparius* larvae to chemical stress and has been associated with decreased growth and development (Choi and Ha, 2009). Moreover, this general reduction of globins expression, and consequent possible decrease of oxygen uptake, may have caused the larvae to switch to anaerobic metabolism an become more dormant (Armitage et al., 1995), which is supported by the

increase in LDH activity verified under spinosad exposure. Despite this general decrease, an increase in some globins expression was observed in the lowest concentration of spinosad tested. In fact, the most sensitive endpoint at the proteome level was the protein "hemoglobin C precursor", which increased at 0.5 µg L^{-1}. This differential protein expression profiles (up and downregulation) over a concentration range may be explained by processes of adaptation followed by the adverse effects as the concentration increases (Gündel et al., 2012). At lower concentrations, the larvae may attempt to adapt or compensate for the adverse outcomes of the chemical exposure, while at higher concentrations, the effects may cause irreversible damage (Gündel et al., 2012). For fipronil, globin proteins were found to be downregulated in a dose-dependent manner, underlining that these proteins could be good indicators of pesticide-induced stress.

Additionally, the underexpression of cytoskeleton and motor proteins was observed for both pesticides (actin for spinosad; troponin, myosin, calmodulin, and tubulin for fipronil). Together with the neuromuscular toxicity induced by both pesticides – fipronil acts on GABA-gated chloride channels (Cole et al., 1993), causing hyperexcitation of nerves and muscles and leading to convulsions and paralysis (Gunasekara et al., 2007) and spinosad targets the nicotinic acetylcholine receptors, causing hyperexcitation of the nervous system and leading to exhaustion and subsequent paralysis (Salgado and Sparks, 2005) - the downregulation of these proteins may contribute to some of the effects observed in *C. riparius* larvae (e.g. lack of movement and abnormal behavior observe under exposure to fipronil). Moreover, a decrease in the expression of a larval cuticle protein was observed for the exposure to spinosad, which may interfere with growth and development of *C. riparius*. For fipronil, decreases in the expression of proteins involved in protein biosynthesis were also observed. These decreases are in line with the generalized downregulation of proteins observed under exposure to this insecticide, suggesting that fipronil may interfere with protein synthesis.

Regarding indoxacarb, none of the proteins identified showed significantly alterations in their expression in comparison to the control. A significant alteration was, however, observed for a larval cuticle protein between the two lowest tested concentrations, albeit there was an apparent increase in the expression of this protein in the intermediate concentration tested when compared to the remaining treatments. An increase in cuticle proteins expression suggest cuticular thickening, which has been associated with slower and reduced insecticide penetration, thus increasing the efficiency of detoxification (Koganemaru et al., 2013; Wood et al., 2010). At the highest concentration, the expression of this cuticle protein returned to near basal levels, suggesting that other defense mechanisms may be favored to detoxify indoxacarb – at this concentration, increases in the antioxidant enzyme GPx and in the biotransformation enzyme GST activities were observed. Nonetheless, this non-monotonic response supports the importance of studying dose-response relationships in environmental

proteomics, since different concentrations of the same chemical produce different responses at the proteome level.

1.4 Proteome as an early warning indicator of pesticide exposure in C. riparius

One of the goals in ecotoxicoproteomics is to uncover which proteome alterations are associated with higher level responses observed. With this in mind, the effects of pesticides at different levels of biological organization of *C. riparius* were here assessed, in order to get hold of the continuum of biological response: proteome modification (early event) vs. response at the organism level.

The experimental design for the assessment of protein differential expression was planned to determine if there was a dose-dependent relation between pesticide concentration and the responses observed. Short-term (48 h) exposures were used to assess the effects on the proteome level, on the assumption that the effects at the individual level are preceded by changes at lower levels, and these changes may be assessed earlier. Third-instar larvae were used (as opposed to first-instar larvae used in the assessment of organismal level responses). The use of third-instar larvae is due to the fact at this stage they are big enough to be handled easily, a relatively low number of organisms provide sufficient biomass for biomarker and proteomics determination and they are not expected to molt during the exposure period – as this event is expected to cause several biochemical changes which may create difficulties in the assessment of which effects were caused by the pesticides and which were merely related to the molting process. Additionally, for a better determination of the molecular events that lead to higher level responses, the concentration ranges used in the present study were based on the LOEC's and NOEC's determined for *C. riparius* life-history traits. Using higher concentrations would likely result in a higher detection of protein expression changes, but the use of concentrations within environmentally relevant levels was also major aim in this study.

The results derived from the proteomics data revealed that protein differential expression can aid in the interpretation of the mechanism affected that lead to higher level responses. Exposure to different classes of pesticides on *C. riparius* revealed common and pesticide-specific proteins and mechanisms affected, and potential biomarkers of pesticide contamination. Present results also reinforce that integrating the data obtained at different levels of biological organization will provide a better interpretation on the effects observed and how they may translate to population- and community-level effects. For instance, the downregulation of globin proteins observed for spinosad and fipronil was accompanied by the increase of ETS activity – increased ETS activity indicates a higher cellular oxygen consumption, while the downregulation of globin proteins may result in a deficient oxygen supply to cells and tissues, which may on long-term lead to hypoxia and contribute to the effects observed at the individual level.

Present results suggest that proteomic tools may be useful in risk assessment to explore potential molecular initiating events that could lead to individual responses. Proteomic tools may also provide a better mechanistic interpretation of the interaction of a specific chemical with an organism at a molecular level, which can lead to the discovery of potential biomarkers and affected pathways. Although in this study proteomics data was obtained from short-term exposures, they gave a good indication of affected mechanisms that may be involved in pesticides' toxic action that could lead to long-term (chronic) effects.

2. Conclusions and future directions

Studying the effects of xenobiotics on non-target species and how they translate into the ecosystems is of utmost importance in environmental risk assessment. Although assessing lethal and sublethal effects of pesticides at the organismal level provides sensitive information that can be easily used to predict possible outcomes at the population level, there is a need to develop new and sensitive early warning tools for fast detection of ecological adverse effects. In this sense, the main goal of this study was to evaluate if biochemical biomarkers and the proteome can be used as early warning indicators of pesticide exposure.

The array of the very distinct responses observed at biochemical level suggest that, although the biochemical biomarkers addressed in this study may be important to interpret some higher-level responses and unravel some mechanisms behind pesticides' toxicity, they are not specific biomarkers for insecticide exposure and therefore should always be interpreted in an integrative manner with higher level responses. The results presented in this study revealed that protein differential expression can aid in the interpretation of the mechanism affected that lead to higher level responses. Although not specific, globins and cytoskeleton and motor proteins may be potential biomarkers of pesticide exposure under laboratory conditions. The potential of globins expression in environmental monitoring studies has been previously stated and here confirmed. The generalized underexpression of these proteins was observed for the exposure to fipronil and spinosad, being concordant with some effects observed at the biochemical level (e.g. shift to anaerobic metabolism in the exposure to spinosad and decrease of defense mechanisms in the exposure to fipronil) but also to the effects observed at individual level (ex. reduced growth and development), which are expected to have direct consequences on the reproductive output of *C. riparius* populations, leading to population decline and thus impact freshwater ecosystems. Although globins are not primary targets of fipronil and spinosad – not being described as their mode of toxicological action – it is clear that their action on these proteins contribute to their toxic action; globins are responsible for a good oxygen supply in *C. riparius* needed for several metabolic processes, and their downregulation increases larval vulnerability to chemical stress. Additionally, the action

on cytoskeleton and motor proteins could enhance the toxicity of insecticides and may have also contributed to some higher-level responses observed, such as behavioral changes. Furthermore, alterations in the expression of other proteins with relevant roles in *C. riparius* such as cuticle proteins were observed and discussed in the line of the effects observed, which could also be candidate biomarkers for chemical contamination.

These results suggest that the proteome can be a relevant and sensitive early warning indicator of pesticide-induced toxicity. The analysis of proteome changes can reveal primary and secondary targets of the pesticides, as well direct and/or indirect consequences (e.g. activation of defense mechanisms) of their exposure, thus aiding in the assessment of the ecological effects of environmental contamination. Moreover, the non-hypothesis-driven approach used in this study offers the possibility of studying several proteins without looking for specific proteins or mechanisms, and thus can either add new evidences or exclude mechanisms. However, in the present study only a part of the complex proteome of *C. riparius* was covered, so many other proteins that were not assessed here could be involved in the effects observed at higher levels. The low number of proteins identified seems to be a common issue in environmental proteomics when using invertebrate models (Chandramouli, et al., 2014; Simões et al., 2018). In the particular case of *C. riparius*, the presence of hemoglobin, which comprises about 60% of the total protein content (Choi et al., 2001), may mask less abundant but possibly ecotoxicologically relevant proteins. This could be the case of the exposure to indoxacarb, where no significant alterations in protein expression to the control were found, despite the dramatic physiological changes observed. It is suggested that an additional sample fractionation step may be needed to asses less abundant proteins in *C. riparius*. Nonetheless, changes observed at the biochemical level for the same concentrations suggest the activation of defense mechanisms was apparently sufficient to detoxify indoxacarb in the short-term, since no evidences of oxidative damage were found. In this particular case, biochemical biomarkers assessed proved to be more helpful than the proteins assessed for gaining insight regarding the mechanisms causing the effects observed. Nevertheless, as demonstrated in this thesis, the information derived from molecular and biochemical levels can be complementary.

One of the challenges in ecotoxicoproteomics is to validate proteome patterns observed under controlled laboratory conditions, in the complexity of natural environments – in an aquatic ecosystem, organisms are subjected to other natural and chemical stressors and therefore, responses patterns measured in laboratory may not reflect the responses in natural environments (Pestana et al., 2010). Hence future research should be conducted towards that, and evaluating such responses is a necessary step in furthering gaining insight in molecular responses to stress and its effects on aquatic health (Melwani et al., 2016). Still, the information derived from laboratory studies cannot be disregarded as it can provide the basis for the understanding of molecular responses to chemicals, molecular targets, and potential biomarkers for

ecotoxicological research – and thus contribute to the development and application of more targeted and hypothesis-driven methodologies (e.g. study of the impact of pesticides on chironomids' globins and cytoskeleton and motor proteins, as suggested by this study). With the increasing number of ecotoxicoproteomics studies in recent years using aquatic invertebrates as models, the first steps towards a creation of an "ecotoxicoproteomics database" could be taken in order to identify candidate biomarkers or biomarker patterns for environmental monitoring.

It is also demonstrated here, that iTRAQ is a valuable technique for ecotoxicoproteomic studies. The possibility of analyzing simultaneously up to eight samples enables the study of concentration-response relationships, which was particularly relevant, since some of the responses observed revealed to be non-monotonic, while others were dose-dependent. The differential expression profiles over a concentration range of a xenobiotic may be justified by processes of adaptation and/or activation of defense mechanisms on lower concentrations, and by more severe effects on higher concentrations. This phenomenon calls for the need of using multiplex techniques in ecotoxicoproteomics. Nevertheless, the 8-plex approach used here is still not ideal in terms of biological replicates, but the development and improvement of higher multiplexing capacity methodologies such as the TMT 10-plex (tandem mass tag) (McAlister et al., 2012; Werner et al., 2014) which allows the comparison of up to 10 samples single analysis, will certainly attract ecotoxicologists in the near future to explore multiple exposure conditions (e.g. different exposure times). Additionally, an 18-plex approach has been proposed (Dephoure and Gygi (2012). Moreover, current label-free approaches have been demonstrated to perform better than iTRAQ in some protein differential expression studies (Latosinska et al., 2015; Trinh et al., 2013). The main advantage of the label-free approach is the possibility to compare an unlimited number of samples (Lindemann et al., 2017), and the number of ecotoxicoproteomics studies using this approach is expected to increase in the next years.

Another major finding in this study was that, environmentally relevant concentrations of amitraz, spinosad, indoxacarb, and fipronil significantly impaired several *C. riparius* life history traits. It was demonstrated that under laboratory conditions, all insecticides studied may cause adverse outcomes to a non-target aquatic insect, which may severely affect the ecological integrity of freshwater ecosystems, and therefore the use of these pesticides near freshwater systems should be carefully considered or avoided. Fipronil was the most toxic compound to *C. riparius* larvae of the insecticides tested, in the sense that adverse effects at the individual level were observed at lower concentrations. Moreover, fipronil was responsible for a higher number of alterations at the individual and at the proteome level. Besides the impairment of growth and emergence, fipronil affected the trade-off between growth and development time, imagoes weight, and caused behavioral alterations. These changes ought to compromise reproduction and increase larvae vulnerability to predation. At the proteome level,

several mechanisms were affected and all of them were downregulated by fipronil exposure. This is an indication that by its neurotoxic action, fipronil directly and indirectly affects several proteins (and as evidence suggests, affect protein synthesis itself) which will lead to long-term consequences on *C. riparius*. On the other hand, amitraz was the least toxic compound tested, still several life-history traits were affected by its exposure and was the only to cause gender-based effect, which can impact population dynamics. In this sense, the possibility of amitraz to induce endocrine disruption effects on aquatic insects should be addressed in later studies, since amitraz has been shown to disrupt hormones in mammals (del Pino et al., 2015). At the biochemical level, amitraz was responsible for a higher number of alterations than any of the other pesticides tested, which is in accordance with its multiple biochemical targets.

In conclusion, this study contributed to the growing knowledge of sub-lethal effects of neurotoxic pesticides on aquatic invertebrates and their molecular targets. *Chironomus riparius*, a model organism in aquatic toxicology, is also presented as a putative model organism for environmental proteomics. Present findings reveal that biochemical biomarkers and proteome changes have the potential to be used as early warning indicators of pesticide exposure and provide insights on the molecular and biochemical-level alterations underlying life-history responses. Therefore, biochemical biomarkers and proteome changes can potentially be used in ecological risk assessment. This study underlines the relevance of integrative ecotoxicological approaches for a better understanding of the mechanisms of action of pesticides in aquatic invertebrates, and their potential outcomes in aquatic ecosystems. There is still a long road ahead, but with the rapidly growing information and newly developed techniques in the field of proteomics, soon these tools may be applied in natural environments and be used to rapidly screen for environmental and chemical stress and/or to uncover mechanisms of action of xenobiotics that are yet to be determined.

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