Universidade de Aveiro Departamento de Biologia

Ano 2017

Sónia Rafaela Beleza Fernandes

Deteção e caracterização de fenoloxidase em *Chironomus riparius*

Detection and characterization of phenoloxidase in *Chironomus riparius*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Carlos Alexandre Sarabando Gravato, Professor Auxiliar da Faculdade de Ciências e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Lisboa e coorientação do Doutor João Luís Teixeira Pestana, Investigador auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro.

Dedico este trabalho à minha mãe, Adélia Pinto, cuja força, bondade e resiliência são incalculáveis.

“O caminho faz-se caminhando.”

António Machado

o júri

Presidente	Doutora Maria Paula Polónia Gonçalves Professora Associada com Agregação, Departamento de Biologia, Universidade de Aveiro
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palavras-chave

Sistema imunitário inato, fenoloxidase, zimosan, lipopolissacarídeos , 1-fenil-2-tioureia, bio inseticidas, insetos aquáticos.

resumo

Os insetos estão expostos, diariamente, a uma ampla variedade de microrganismos e parasitas, necessitando, assim, de um sistema imunitário extremamente eficiente que seja capaz de os proteger de possíveis infeções causadas por esses agentes externos. Contudo, estes organismos não possuem sistema imunitário adaptativo, pelo que recorrem a outros tipos de defesa igualmente eficazes, como as reações imunitárias inatas. Estas reações constituem a primeira linha de defesa do hospedeiro contra bactérias, fungos e vírus. O estudo do sistema imunitário inato tem ganho grande relevância em imunologia e ecologia de vários tipos de invertebrados, dada a sua importância na defesa destes hospedeiros. Desta forma, um dos processos imunológicos mais importantes nos insetos é a melanogénese, que leva a um encapsulamento dos agentes invasores seguido de melanização. A melanização requer a ativação de uma enzima chave – fenoloxidase (PO), principal agente do sistema humoral, que se torna ativa através da cascata de ativação de proPO. Assim, e tendo em conta o papel fundamental que esta enzima tem no sistema imunitário dos invertebrados, o presente estudo teve como objetivos caracterizar a atividade de PO em amostras de *C. riparius* e avaliar a sensibilidade da atividade de PO como indicador de efeitos causados por dois bio inseticidas muito usados - Naturalis®-L e VectoBac® 12AS.

Os resultados demonstraram que a atividade de proPO presente nas amostras de *C. riparius* foi prontamente ativada pela presença de quimotripsina, zimosan e lipopolissacarídeos. Por outro lado, a atividade de PO sofreu grande inibição na presença de 1-fenil-2-tioureia. A atividade de PO aumentou significativamente para ~ 5 and 6 abs/mg de proteína em amostras expostas a 2 and 5 mg/L de Naturalis®-L, e para ~ 4 abs/mg de proteína em amostras expostas a 20 ng/L de VectoBac® 12AS, comparativamente a organismos não expostos (~ 2 abs/mg de proteína), revelando a sensibilidade deste parâmetro à exposição a agentes patogénicos. A atividade de PO aumentou na presença de quimotripsina. Além disso, os resultados sugerem síntese de novo de proPO em organismos expostos a bio inseticidas.

Assim, este estudo permitiu confirmar que estamos na presença de uma verdadeira atividade de PO, dada a acentuada inibição causada por PTU. E concluiu-se que a medição da atividade de PO e ativação de proPO pode e deve ser utilizada como um biomarcador em insetos, providenciando informação acerca da condição imunológica do organismo e a respostas a stressores ambientais.

keywords

Innate immune system, phenoloxidase, zymosan, lipopolysaccharide, 1-phenyl-2-thiourea, bio insecticides, aquatic insects.

abstract

Insects are frequently exposed to a large variety of microbes, microorganisms and parasites which requires an effective immune system of defence against infections. However, insects lack an adaptive immune system, so they have developed other highly effective systems of host defence, such as innate immune reactions. These innate immune reactions refer to the first-line of defence of hosts against bacterial, fungal, and viral pathogens. Research on invertebrate model systems have been increasing and becoming very important in ecological immunology research. One of the most important immune component used by insects is melanogenesis, which is responsible for encapsulation followed by melanization of the pathogens. Melanization requires activation of a key enzyme – phenoloxidase (PO), during PO cascade which is the major humoral immune effector system. Due to the importance of PO on insect immune system, the aims of the present study were to characterize PO activity in samples of *C. riparius* larvae whole body homogenates, and evaluate the sensitivity of PO activity as an indicator of effects of two widely used bio insecticides - Naturalis®-L and VectoBac® 12AS.

The results demonstrated that proPO present in *C. riparius* samples was readily activated by the presence of chymotrypsin, zymosan and lipopolysaccharide. On the other hand, 1-phenyl-2-thiourea inhibited PO activity. *In vivo* exposures, PO activity was significantly increased ~ 5 and 6 abs / mg protein in samples exposed to 2 and 5 mg/L of Naturalis®-L and ~ 4 abs / mg protein in samples exposed to 20 ng/L VectoBac® 12AS, in comparison with unexposed organisms (~ 2 abs/mg of protein). This activity increased even more with the presence of chymotrypsin. Also the results suggest *de novo* synthesis of proPO under exposure to bio insecticides.

Thus, this study confirmed the presence of a true PO, due to the inhibition caused by the PTU. We concluded that the measurement of PO activity and activation of proPO can and should be used as an immunological biomarker, providing information on the immunological status of the organism and on sub-lethal responses to stress.

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Chapter 1

General Introduction

1. General introduction

1.1 PO role in insect immune system

Insects are frequently threatened by a large range of pathogenic microorganisms and parasitoids. In such cases, their primary internal defence is the immune system. Research on invertebrate model systems have been increasing and becoming very important in ecological immunology research (Rolff, 2003) and various methods have been developed to estimate their capacity to resist injury.

The response of insects against infection and pathogenic microbes rely on innate immune system (Gillespie et al., 1997; Kanost et al., 2004; Ling and Yu, 2006) which is a universal and an ancient form of host defence, based on cellular and humoral processes (Söderhäll and Cerenius, 1998). Thus, invertebrates do not possess somatic recombinatory mechanisms, antibodies and T-type cells that occur in vertebrates (Schmidt et al., 2008).

It is suggested that the primary components of innate immunity fall into three categories of an immune response: (1) the organism begins by distinguishing between self and nonself; (2) once recognised as non-self, defensive responses are stimulated and the invader may be phagocytosed by circulating haemocytes; (3) larger organisms are encapsulated, i.e., the parasite is covered by a capsule of haemocytes, and killed by a combination of cytotoxic compounds, such as quinones, semiquinones and free radicals (Gillespie and et al., 1997; Mydlarz et al., 2006). This later process is more complex than phagocytosis in several ways, demanding cooperation between several types of haemocytes (Gillespie and et al., 1997).

One of the branches of insect immunity is melanogenesis, which is the rapid synthesis and deposition of a darkly pigment (melanin) around encapsulated objects or at sites of pathogen infection (González-Santoyo and Córdoba-Aguilar, 2012) (Fig.1). Melanin possess cytotoxic activity against microorganisms that allows it to fight the invasion of pathogens and support wound healing (Jiravanichpaisal et al., 2006; Nappi and Christensen, 2005). Moreover, during melanin formation highly reactive and toxic quinone intermediates are also produced (González-Santoyo and Córdoba-Aguilar, 2012).

Melanization requires the activation of a key enzyme – phenoloxidase (PO), during PO cascade which is the major humoral immune effector system (Lilley et al., 2012).

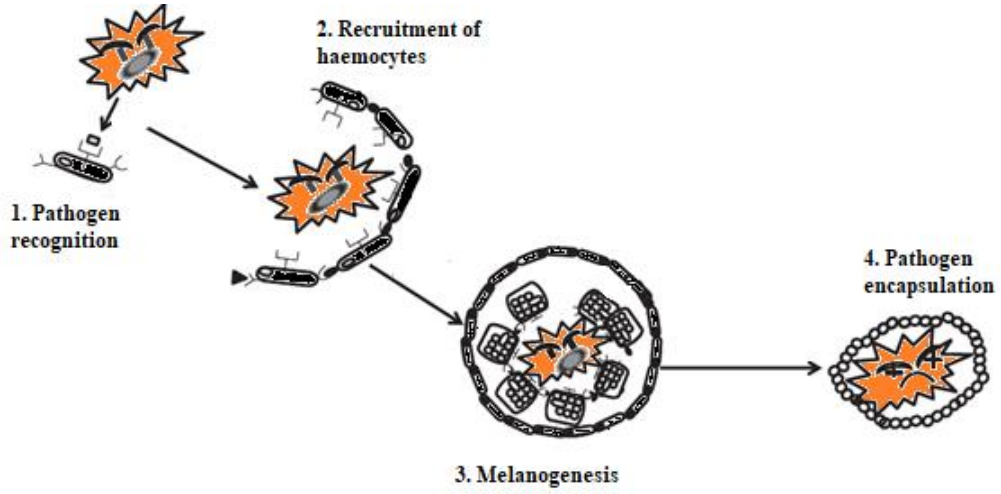

Figure 1 Four steps of melanization: (1) the recognition of pathogens through specific molecules [e.g., lipopolysacharides (LPSs)]; (2) the recruitment of haemocytes to surround the pathogen; (3) melanogenesis within haemocytes; (4) melanin release and pathogen encapsulation (Adapted from González-Santoyo and Córdoba-Aguilar (2012))

Phenoloxidase (PO) is a bifunctional copper containing enzyme which catalyzes two successive reactions: hydroxylation of monophenol to o-diphenol (monophenoloxidase activity) and the oxidation of the o-diphenol to o-quinone (diphenoloxidase activity) (Nappi and Christensen, 2005; Söderhäll and Cerenius, 1998). This production of o-quinones leads to spontaneous polymerization to form soluble melanin (Söderhäll and Cerenius, 1998), being the initial step of the biochemical cascade that results in melanin formation (Söderhäll and Cerenius, 1998) and encapsulation of pathogens (Cerenius et al., 2008; Cerenius and Söderhäll, 2004). Both mono- and diphenols can be oxidized by phenoloxidase and the intermediary products formed during this process are toxic to micro-organisms.

This enzyme is present in hemolymph as an inactive proenzyme prophenoloxidase (proPO) which is the zymogen of phenoloxidase (Perdomo-Morales et al., 2007). The proPO activating system is an efficient immune system for non-self-recognition and consists of several proteins that are involved in melanization, cell adhesion and phagocytosis (Gillespie et al., 1997; Söderhäll and Cerenius, 1998). This system is present in many invertebrate groups, such as ascidians, arthropods, echinoderms, bivalves, brachiopods (Gillespie et al., 1997) and is triggered by recognition of certain microbial products – PAMPS (pathogen associated molecular patterns), such as lipopolysaccharides (LPS) or peptidoglycans from

bacteria and β-1,3-glucans from fungi. The proPO activating system involves a proteinase cascade composed of pattern-recognition proteins (PRPs) that are triggering molecules of the system, since they bind microbial components and then induce activation of proteinases (Fig.2). The conversion of proPO to PO is carried out by a proteolytic attack by a serine proteinase named prophenoloxidase activating enzyme (ppA), through limited proteolysis (Cerenius and Söderhäll, 2004; Jiravanichpaisal et al., 2006; Lee and Söderhäll, 2002; Söderhäll and Cerenius, 1998). Serine proteases are hydrolases with a serine amino acid in their active center (Hedstrom, 2002). Trypsin, chymotrypsin and subtilisin are examples of hydrolases, usually used as *in vitro* proPO activators (Adamo, 2004). This serine proteinase cut the polypeptide chain in the carboxyl side of specific amino acids causing peptide bond degradation (Hedstrom, 2002).

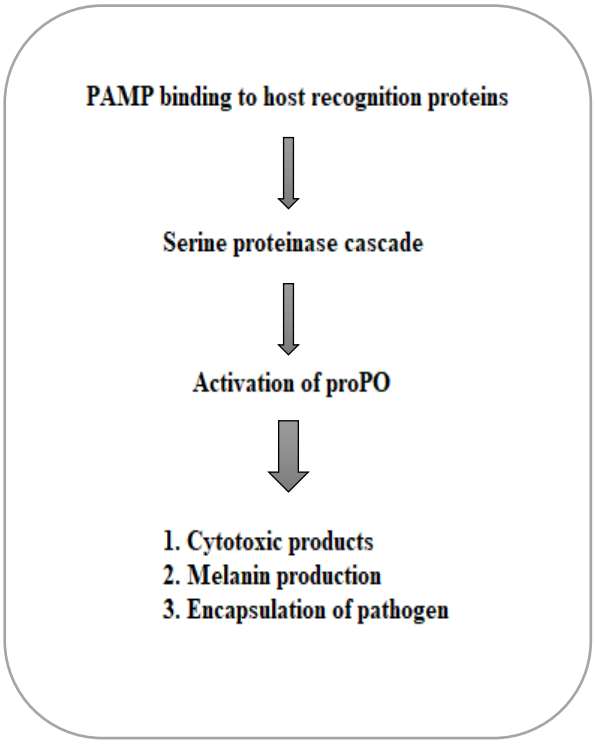

Figure 2 Activation of the prophenoloxidase (proPO) cascade in invertebrate immunity. PAMPS are bound by host recognition proteins, which initiates a serine proteinase cascade that leads to the activation of proPO into PO. This process results in the production of cytotoxic products and encapsulation of the pathogen (Adapted from Cerenius et al., (2008)).

Besides the importance of PO for host defence, this enzyme also produces compounds, like proteases that could promote degradation of host proteins, cytotoxic

quinines, reactive nitrogen and nitrogen intermediates that can produce deleterious consequences for the host if produced in excess (González-Santoyo and Córdoba-Aguilar, 2012). For such reason, both activation and PO activity has to be extremely regulated, by synthesizing the enzyme as an inactive zymogen that requires proteolytic cleavage in order to become active (proPO) and through regulatory mechanisms that involve a phenoloxidase inhibitor (POI) that can directly inhibit PO activity (Daquinag et al., 1995; Sugumaran and Nellaiappan, 2000), and several proteinase inhibitors that prevent over-activation of ppA (Hergenhahn et al., 1987). It is known that 1-phenyl-2-thiourea (PTU) or reduced glutathione inhibits melanization (Pech et al., 1994).

Several reports associated PO activity with decreased pathogen survival both *in vivo* and *in vitro* (Cerenius et al., 2008). Recent and previous data reports associated melanisation cascade, or proPO activating cascade, with coagulation response in insects (Eleftherianos and Revenis, 2011). For instance, PO has been show to play an important role in coagulation in the wax moth *Galleria mellonella* (Rowley and Ratcliffe, 1976). The idea that proPO activating cascade and coagulation system work together during the formation of a hemolymph cot became more clear with the identification of known immune players (such as quinone-like substances, antimicrobial peptides) that included components of both responses (Eleftherianos and Revenis, 2011). Thus, PO promotes cellular defence reactions (e.g. phagocytosis) and increases the efficiency of plasma coagulation as well (Cerenius et al., 2008). PO and melanin formation are also important for other physiological processes involved in host defence, such as, wound healing, sclereotization in many tissues and encapsulation of foreign materials (Nappi and Christensen, 2005; Söderhäll and Cerenius, 1998).

Innate immunity is the remaining mechanism of defence used by invertebrates in limiting infections and destroy infections agents (Cerenius et al., 2010, 2008; Zou et al., 2010). Investigation of this immune system may give new knowledge about the susceptibility, management and control of diseases in animals. Since PO is a key enzyme in the immunological defence of invertebrates, numerous investigations have been carried on the characterization of the enzyme in invertebrates (Cerenius et al., 2008; Johansson and Soderhall, 1989; Pang et al., 2010; Smith and Söderhäll, 1991). Hemolymph PO has been related with resistance to a range of pathogens, and been used as an indicator of immune function (Cotter et al., 2002). It has been shown the effects of several contaminants on PO

activity, such as the effects of TBT and other metals in *Epirrita autumanata* (van Ooik et al., 2008), in *Styela plicata* (Tujula et al., 2001) and in *Chrionomus riparius* (Lilley et al., 2012), and atmospheric pollutants in *Epirrita autumanat*a (van Ooik et al., 2007), increasing the PO enzyme activity.

In this way, the measurement of PO activity could be used as a sensitive indicator of immune response to foreign agents and of detoxification of contaminants (Luna-Acosta et al., 2017). The standardization of the assays and the characterization of this enzyme must be made in order to evaluate the immune *status* of the organism, providing rapid and inexpensive tests for environmental stress (Hellio et al., 2007; Tujula et al., 2001). In this way, the measurement of proPO activation and PO activity becomes a biomarker that can be used in conjunction with other types of biomarkers, such as oxidative stress, neurotoxicity and energy balance/production biomarkers, in order to give additional and important information about the effects that a certain stressor has on the organism.

1.2 *Chironomus riparius* in ecotoxicology

Insects, such as chironomids, are excellent model organisms for studying effects of pollution on immune function due to their immune defence system which is simpler than the vertebrate immune system, even though many components are homologous. *Chironomus riparius* is a species of non-biting midge; their life cycle is holometabolous, with several stages: egg, larvae (4 stages), pupa and the terrestrial adult stage. This species can be found in aquatic sediments during their larval period (Fig.3), usually in organic enriched waters (Péry et al., 2002; Péry and Garric, 2006). The life stages are easy to identify and the life history under laboratory conditions is short, characteristics that make them valuable for toxicity tests. Moreover *C. riparius* also has been used extensively as a model for genome structure analysis in insects and functional developmental genetic studies.

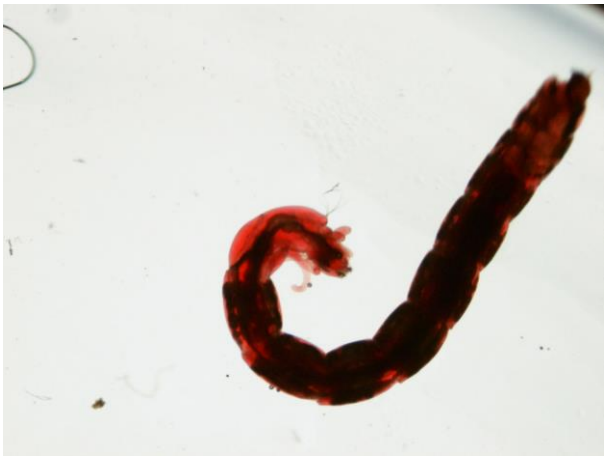

Figure 3 *Chironomus riparius* in its larval stage.

C. riparius was chosen as a model species because of its relevance in terms of biomass and function within benthic communities of diverse freshwater systems (Péry et al., 2002; Péry and Garric, 2006; Pestana et al., 2009), representing non-target organisms in mosquito control scenarios and tested as standard organism in representation of aquatic insects in the environmental risk assessment for chemical pesticides (OECD, 2011; Weltje et al., 2009). In this way, and due to the role of these organisms in the environmental risk assessment, the standardization of the assays and the characterization of PO enzyme in *C. riparius* larvae whole body homogenates will become very useful because it would be possible evaluate, from the same sample, the effects on different types of biomarkers.

1.3 Microbial insecticides

Nowadays, an awareness of the risk and the consequences about the use of chemical pesticides and also the need to reduce their utilization demands for alternatives that are sustainable and environment friendly. These alternatives focus on the search for efficient products in pest control, mainly through the utilization of microorganisms for control of insect pests. The World Health Organization (WHO) and other international institutions in the mid-1970s initiated researches on the development of existing and new biological control agents (Boisvert and Boisvert, 2000). Currently, a variety of entomopathogens are used for the control of invertebrate pests and vector insects, they include bacteria, viruses, fungi protozoa and nematodes (Lacey et al., 2015).

Microbial insecticides, based on live fungi or bacteria and their toxins, are being increasingly used in integrated pest management. Two examples of these alternative products are: 1) the bio insecticide Vectobac® 12AS, based on *Bacillus thuringiensis* subsp. *Israelensis (Bti)* sero. H-14 discovered in the 1970s and very effective against larvae of several species of mosquitoes (Bravo et al., 2011), and 2) the bio insecticide Naturalis®-L, based on *Beauveria bassiana*, an entomopathogenic fungi, widely used for control of several insects pests. These two types of bio insecticides provide a biological alternative to synthetic chemical insecticides.

Naturalis®-L is based on living conidiospores of the naturally *Beauveria bassiana* and acts primarily by contact when attached to the insect's cuticle (Gillespie et al., 2000; Ladurner et al., n.d.). The conidiospores germinate producing penetration hyphae, which enter and proliferate inside the insect's body (Ladurner et al., n.d.) feeding itself on its host and causing death due to dehydration and depletion of nutrients (Fig.4 a)). Several studies have been shown that PO levels increased when the organism was infected by fungal components such as blastospores and conidiospores (Gillespie and Khachatourians, 1992; Hung and Boucias, 1996). In *Spodoptera exigua, B. bassiana* caused remarkable alterations in the distribution and levels of PO activity (Hung and Boucias, 1996). According to the product label, Naturalis®-L should be applied at a concentration of 0.3% v/v (3 litres in 1000L of water).

VectoBac® 12AS is increasingly used worldwide for effective mosquito control and black flies (Lacey, 2007) and due to a high specificity to mosquito larvae *Bacillus thurigiensis* is considered as the most environmental friendly alternative to chemical pesticides (Kästel et al., 2017). *Bacillus thurigiensis* is a gram-positive bacteria with entomopathogenic properties (Bravo et al., 2007). This bacteria is usually sprayed in breeding sites as a suspension of spores and crystals that kill mosquito larvae by gut disruption after ingestion (Vachon et al., 2012). According to product label, VectoBac® 12AS should be applied 0.5 – 2.5 mg/L. A range of endotoxins are the toxic components of *Bti*, they bound together in stable protoxin molecules in the parasporal inclusion (Larget & de Barjac, 1981; Charles & de Barjac, 1982). During the sporulation phase, *Bti* produces deltatoxins (Cry and Cyt proteins), which are very specific to target insect larvae and that are organized into crystalline inclusions. These inclusions becomes active only when ingested and solubilized in the alkaline larval midgut, being cleavage by proteases (Boisvert

and Boisvert, 2000) (Fig.4 b)). *Bti* is considered non-toxic to most organisms, however, some Dipterans, such as Blephariciдae, Dixidae, Pshychodidae, Tipulidae, Sciaridae, Sciaridae, Tephrtidae and Chironomidae showed to be very sensitive (Boisvert and Boisvert, 2000; Yiallouros et al., 1999).

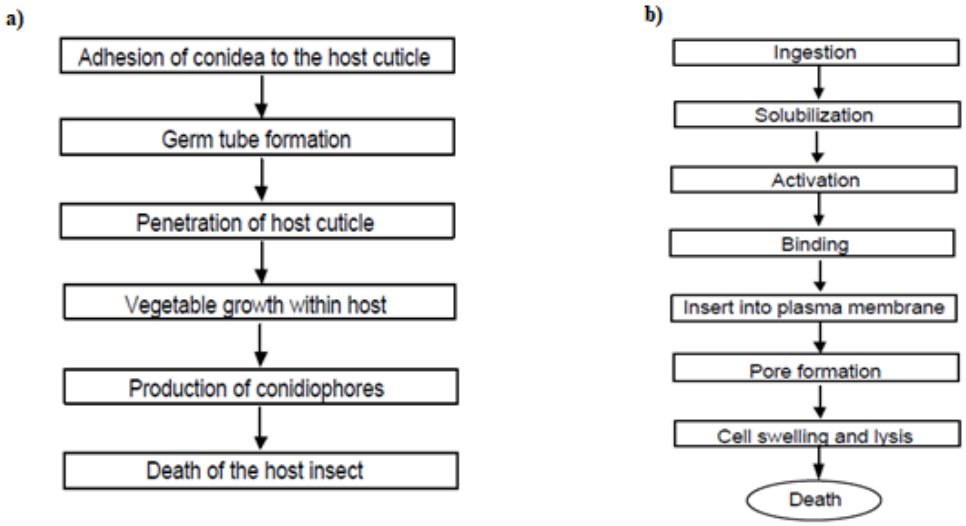

Figure 4 a) *In vivo* developmental cycle of entomopathogenic fungi; b) Scheme of the mode of action of *Bti*. (Adapted from Narayanan, (2004))

In addition of being considered environmentally safe, these bio insecticides also have the potential to replicate and persist in the environment allowing a continued suppression of insect pest populations (Castrillo et al., 2004). However, recent evidences on the ubiquity, persistence and toxicity of microbial insecticides in aquatic ecosystems require a deeper environmental risk assessment (Castrillo et al., 2004). Thus, particular attention should be given on relevant and frequently neglected ecological consequences of these products on aquatic ecosystems (Kästel et al., 2017).

1.4 Main objectives and relevance of the study

The objectives of the present work are: to characterize phenoloxidase activity in whole body samples of larvae of *C. riparius*, in order to standardize a protocol allowing the simultaneous measurement of different biomarkers; and evaluate the sensitivity of PO

activity as an indicator of effects of two widely used microbial insecticides - Naturalis®-L and VectoBac® 12AS.

1.5 Thesis organization

This thesis is divided in four chapters and one annex:

Chapter 1: the general introduction and state of the art regarding insect immune system, PO and proPO activating system in insects and their relevance in the evaluation of effects caused by microbial insecticides Naturalis®-L and Vectobac® 12AS;

Chapter 2: "Detection and characterization of phenoloxidase in *Chironomus riparius*";

Chapter 3: "Effects of microbial insecticides on *Chironomus riparius* immune system";

Chapter 4: a general discussion and conclusions are presented, highlighting the main achievements of this thesis;

Annex: protocol to assess PO and total PO (proPO+PO) activities in whole body samples of *Chironomus riparius* larvae.

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Chapter 2

Detection and characterization of phenoloxidase in *Chironomus riparius*

2. Detection and characterization of phenoloxidase in *Chironomus riparius*

Abstract

Melanogenesis is one of the most important immune processes used by insects and it is responsible for encapsulation followed by melanization of foreign material. The prophenoloxidase (proPO) system is the origin of melanin production and is considered to be an innate defence mechanism in invertebrates. The enzyme phenoloxidase (PO), responsible for melanin production, is present in the hemolymph as an inactive form of proPO in several invertebrate species. The present study focuses on the characterization of PO activity in whole body samples of larvae of *Chironomus riparius*. ProPO was readily activated by chymotrypsin (0.5 mg/mL), zymosan (1 mg/mL) and lipopolysaccharide (1 mg/mL), and extremely susceptible to 1-phenyl-2-thiourea (0.01, 0.1 and 1 mg/mL).

The presence of PO and the activation of proPO system by non-self-molecules in *C. riparius* samples suggests that PO activity can be measured together with other biochemical markers of stress and used as sensitive ecotoxicity endpoint to evaluate indirect effects of contaminants and environmental stressors on immune function in *C. riparius*.

Keywords: Innate immune system, phenoloxidase, zymosan, lipopolysaccharide, 1-phenyl-2-thiourea, *Chironomus riparius.*

2.1 Introduction

Throughout their life-cycle, insects are exposed to a wide range of microorganisms and parasites, requiring an effective defence system against infection (Royet, 2004). However, insects lack adaptive immune systems, so they have developed other highly effective systems of host defence that is based on innate immune reactions (Pang et al., 2010; Perdomo-Morales et al., 2007). Innate immune reactions, refers to the first-line of host defence against bacterial, fungal, and viral pathogens (Iwanaga and Lee, 2005; Pang et al., 2010). Thus, innate immune system, that is also composed of cellular and humoral immune responses, responds to pathogens in a generic way providing an immediate defence against infection, by limiting infection in the early hours after exposure to microorganism (Gillespie et al., 1997; Kanost et al., 2004).

One of the most important immune processes used by insects is melanogenesis, which is responsible for encapsulation followed by melanization of multicellular, bacterial or fungal pathogens (González-Santoyo and Córdoba-Aguilar, 2012).

The most important enzyme involved in this process is phenoloxidase (PO) (González-Santoyo and Córdoba-Aguilar, 2012), which activates melanization in invertebrates (Cerenius and Söderhäll, 2004; Ling and Yu, 2006; Pang et al., 2010; Shao et al., 2012). PO is a copper-dependent enzyme that catalyses the synthesis of *o*-diphenols from *o*-oxygenation of monophenols, which are then dehydrogenated into *o*-quinones (Cerenius and Söderhäll, 2004). This enzyme is expressed as inactive zymogen (proPO) which is mostly synthesized in haemocytes (Cerenius and Söderhäll, 2004). ProPO is a polypeptide that contain two cooper atoms per protein molecule (González-Santoyo and Córdoba-Aguilar, 2012) and it is activated to PO by several microbial surface components, such as, β-1,3-glucan, lipopolysaccharide (LPS) and peptidoglycans (Eleftherianos and Revenis, 2011; Smith et al., 1984) via limited and controlled proteolysis through the action of a serine protease cascade (Eleftherianos and Revenis, 2011). PO can be inhibited by 1-phenyl-2-thiourea (PTU) (Asokan et al., 1997; Hellio et al., 2007; Pech et al., 1994; Xue et al., 2007). Melanin, *o*-quinone like substances and other intermediates of the cascade possess cytotoxic activity towards microorganisms or microbial pathogens and also assist in would healing (Jiravanichpaisal et al., 2006; Nappi and Christensen, 2005). Research on the innate immune systems may give a new understanding about the management and control the ability to resist

disease (Hellio et al., 2007). Haemolymph PO has been implicated in resistance to a range of pathogens, fungi, bacteria and parasitoids (Hung and Boucias, 1996), and PO activity has been used as a proxy for disease resistance in several studies as well as an indicator of the effects of contaminants on several species immune *status* (Aladaileh et al., 2007; Cotter et al., 2002; Lilley et al., 2012; van Ooik et al., 2008, 2007). For such reasons, the characterization of this enzyme in order to become an immunological biomarker would be very useful.

Chironomus riparius is considered a standard species for assessment of sediment contamination (Oecd, 2010). This invertebrate has been widely used in several aquatic toxicological studies including the use of analysis of biochemical and enzymatic biomarkers with important physiological functions, such as detoxification, antioxidant capacity, neurotransmission and cellular energy allocation (Campos et al., 2016; Rodrigues et al., 2015).

This study aimed to characterize phenoloxidase activity in whole body samples of *C. riparius* larvae. For this characterization we started by activation of PO by chymotrypsin in terms of concentrations and incubation period as well as the effects of specific inhibitors (PTU) and exogenous elicitors, such as zimosan (Zs) and lipopolysaccharides (LPS), in order to evaluate the response sensitivity and specificity of this immune assay using such *C. riparius* samples.

2.2 Material and Methods

2.2.1 Test organism

Chironomus riparius larvae used in the experiment were obtained from a laboratory culture established more than 15 years ago at the Department of Biology, University of Aveiro. The culture is maintained in aquaria with a layer of inorganic fine sediment (<1 mm), previously burnt at 500 °C for 5h, with American Society for Testing and Materials (ASTM, 2002) hard water medium, with a photoperiod of 16 h light:8h dark and room temperature of 20 ± 1 °C, with aeration. A suspension of commercial food fish TetraMin® (Tetrawerke, Melle, Germany) is provided as food every two days. Twelve-day-old, 4th instar, *C. riparius* larvae were used throughout the experiments.

2.2.2 Homogenization and isolation of samples

Five replicates each containing thirty 4th instar larvae were collected and dried on a filter paper, frozen in liquid nitrogen and kept at – 80ºC. Samples were homogenized in ice by sonication using 3200 µL of 0.2 M phosphate buffer pH=7.4. Each sample was centrifuged at 9000 g for 20 minutes at 4 °C and the resulting supernatant was collected and kept on ice.

2.2.3 PO activity and proPO activation with chymotrypsin

Four different concentrations of chymotrypsin (0.25, 0.5, 1 and 2 mg/mL) were used to test proPO activation in samples. Each sample (30 µL) was incubated in the absence (50 µL of ultrapure water) and presence of 50 µL of chymotrypsin (0.25, 0.5, 1 and 2 mg/mL) using 100 µL of 0.2 M phosphate buffer pH=7.4, and left at 25 °C during 10 minutes. After this incubation period, 100 µL of 10 mM sodium cacodylate (CAC) buffer (pH 7.4) and 20 µL of 200 mM L-DOPA (Pang et al., 2004) were added. Blanks of reaction were performed using 30 µL of phosphate buffer and incubated as previously explained for samples. After adding DOPA, the absorbance was immediately read at 490 nm (time 0) and after 15, 30 and 60 min, as well as, 2, 4, 6, 12, 24 and 48 hours. The enzyme activity was expressed as absorbance per mg of protein.

To evaluate the effects of incubation time on proPO activation and PO activity, 30 µL of sample was added in quadruplicate to a microplate and incubated in the absence (50 µL of ultrapure water) and presence of 50 µL of chymotrypsin 0.5 mg/mL using 100 µL of 0.2M phosphate buffer pH=7.4, and left at 25 °C during 10, 20 and 30 minutes. After these incubation periods, 100 µL of 10 mM sodium cacodylate (CAC) buffer (pH 7.4) and 20 µL of 200 mM L-DOPA (Pang et al., 2004) were added. Blanks of reaction were performed using 30 µL of phosphate buffer and incubated as previously explained for samples. After adding DOPA, the absorbance was immediately measured at 490 nm (time 0) and followed after 15, 30 and 60min, as well as, 2, 4, 6, 12, 24 and 48 hours. The enzyme activity was expressed as absorbance per mg of protein.

2.2.4 Inhibition assay of PO activity by PTU

PO activity of samples was assayed in the presence of PTU, a known PO inhibitor (Asokan et al., 1997; Pang et al., 2005). Sample (30 µL) was added in 4 replicates to a microplate and incubated in the absence (50 µL of ultrapure water) and presence of 50 µL of chymotrypsin 0.5mg/mL and each one of these conditions were performed in the absence and presence of 100 µL of PTU solution (0.01, 0.1, and 1 mg/mL in 2 M phosphate buffer pH=7.4), and pre-incubated at 25ºC during 10 min. After this incubation period, 100 µL of 10 mM sodium cacodylate (CAC) buffer (pH 7.4) and 20 µL of 200 mM L-DOPA (Pang et al., 2004) were added. Blanks of reaction were performed using 30 µL of phosphate buffer and incubated as previously explained for samples. After adding DOPA, the absorbance was immediately measured at 490 nm (time 0) and followed after 15, 30 and 60min, as well as, 2, 4, 6, 12, 24 and 48 hours. The enzyme activity was expressed as absorbance per mg of protein.

2.2.5 Effects of Zs and LPS on PO activity and proPO activation

Four replicates of each sample (30 µL) were added to a microplate and incubated with 50 µL of ultrapure water using 100 µL of Zs solution (0.01, 0.1 and 1 mg/mL in 2M phosphate buffer pH=7.4), and pre-incubated at 25 °C during 10 min. After this incubation period, 100 µL of 10 mM sodium cacodylate (CAC) buffer (pH 7.4) and 20 µL of 200 mM L-DOPA (Pang et al., 2004) were added. Blanks of reaction were performed using 30 µL of phosphate buffer and incubated as previously explained for samples. After adding DOPA, the absorbance was immediately measured at 490 nm (time 0) and followed after 4, 6, 12, 24 and 48 hours. The enzyme activity was expressed as absorbance per mg of protein.

Each sample (30 µL) was also added in 4 replicates to a microplate and incubated with 50 µL of ultrapure water using 100 µL of LPS solution (0.01, 0.1 and 1 mg/mL in 2M phosphate buffer pH=7.4), and pre-incubated at 25 °C during 10 min. After this incubation period, 100 µL of 10 mM sodium cacodylate (CAC) buffer (pH 7.4) and 20 µL of 200 mM L-DOPA (Pang et al., 2004) were added. Blanks of reaction were performed using 30 µL of phosphate buffer and incubated as previously explained for samples. After adding DOPA, the absorbance was immediately measured at 490 nm (time 0) and followed after 4, 6, 12, 24 and 48 hours. The enzyme activity was expressed as absorbance per mg of protein.

2.2.6 Protein Determination

The protein concentration of all samples was determined by the Bradford method (Bradford, 1976), using γ-globulins from bovine blood as standard. The samples were diluted 4-8 times to give a final protein concentration of 0.067 ± 0.009 mg/mL, (0.054-0.086), before carrying out the enzymatic analysis.

2.2.7 Data analysis

Data were expressed as mean values ± SD. Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's *pos hoc* test and by *t tests*. These allowed us to identify statistically significant differences between each condition tested and the respective control or between two conditions of assay, respectively. Bartlett's and Brown–Forsythe tests verified the homoscedasticity of data while normality of data was assessed using residual probability plots. For all statistical tests the significance level was set at 0.05. All calculations were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA).

2.3 Results

2.3.1 PO activity and proPO activation by chymotrypsin

Results showed that PO activity increased with the reaction time from 60 up to 1440 min reaching a plateau value of 20.49 ± 4.22 abs/mg protein (Fig.5). However, PO activity measured in the presence of different concentrations of chymotrypsin was significantly increased after 30 min, suggesting an activation of proPO through proteolysis induced by chymotrypsin. Thus, total PO (PO+proPO) activity was significantly increased after 60 min of reaction ($F_{(4, 95)} = 6.98$, $p<0.05$) by 2 mg/mL of chymotrypsin (127.96 ± 24.40%), after 120 min of reaction ($F_{(4,94)} = 23.36$, $p<0.05$) by 0.5 (149.33 ± 36.02%) 1 mg/mL (162.55 ± 15.39%) and 2 mg/mL (151.92 ± 15.11%), and after 240, 360 and 720 min of reaction by all concentrations of chymotrypsin (Fig.5). Nevertheless, after 1440 min ($F_{(4, 94)} = 20,99$,

p<0.05) and 2880 min ($F_{(4,94)}$ = 20,98, p<0.05) of reaction a significant increase of total PO activity induced by chymotrypsin was only observed for concentrations of 0.25 (175.46 ± 62.76% and 166.39 ± 67.59%, respectively) and 0.5 mg/mL (227.66 ± 79.96% and 207.80 ± 73.75%, respectively) showing that high concentrations of chymotrypsin decreased total PO activity (Fig.5). In fact, it was observed that after 360 min of reaction, the highest values of total PO activity were observed in the presence of 0.5 mg/mL chymotrypsin (Fig.5).

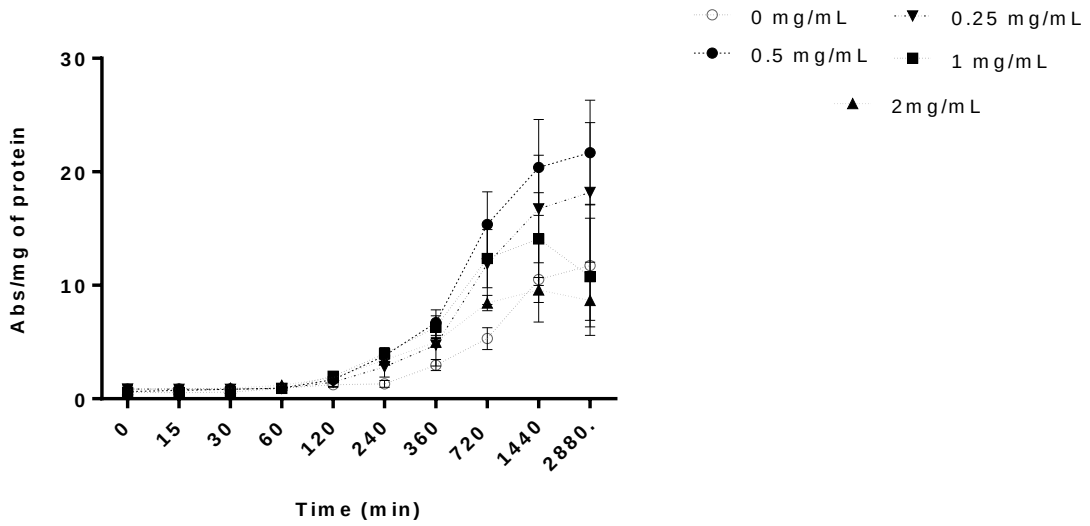

Figure 5 Effects of different concentrations of chymotrypsin on PO activity and activation of proPO of *C. riparius* until 48 hours of reaction in microplate.

2.3.2 Effects of chymotrypsin incubation period on PO activity

Results showed that the optimum incubation period of samples with 0.5 mg/mL chymotrypsin is 10 min, since PO activity was significantly increased 182.61 ± 21.92 %, 273.17 ± 41.14 %, 350.55 ± 64.48 %, 332.61 ± 85.50 % and 248.20 ± 119.61 % at 120, 240, 360, 720 and 1440 min of reaction when compared to respective control treatments performed in the absence of chymotrypsin (p<0.05) (Fig.6). Incubation periods of 20 and 30 min induced a maximum increase of 285.50 ± 65.02 % and 325.34 ± 123.62 % of PO activity at 720 min of reaction, respectively (Fig.6). Moreover, high variability of PO activities was observed for the incubation periods of 20 and 30 min in the absence or presence of chymotrypsin, suggesting degradation of samples during longer incubation periods (Fig.6).

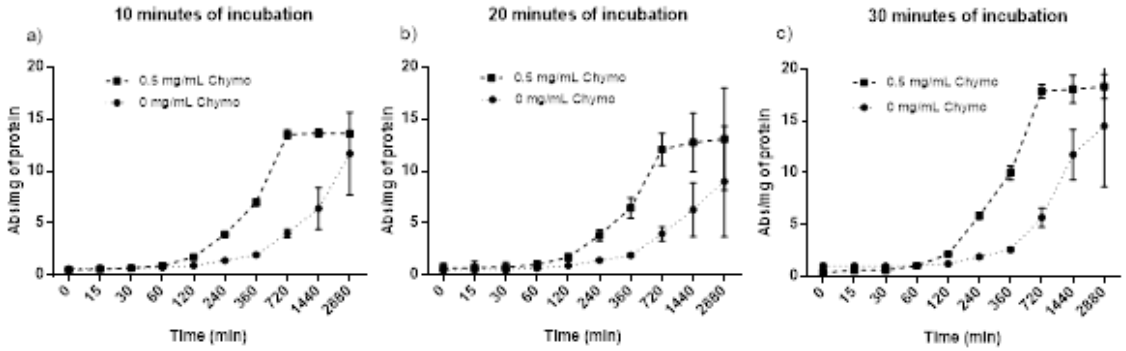

Figure 6 PO activity of *C. riparius* after a) 10 minutes b) 20 minutes and c) 30 minutes of reaction with 0.5 mg/mL chymotrypsin. The values are the mean of five samples performed in quadruplicate ± SD).

2.3.3 Inhibition assay of PO activity by PTU

Results showed that PTU significantly inhibited PO activity compared to the control treatment and both in the presence ($F_{(3,16)}$= 59,81, p<0.05, Fig.7 a)) and absence of chymotrypsin ($F_{(3,\ 16)}$= 7,91, p<0.05, Fig.7 c)). A concentration of 0.01 mg/mL PTU inhibited by 49,50 ± 22.22 % and 82.13 ± 10.28 % the activity of PO measured in the absence and presence (total PO) of 0.5 mg/mL chymotrypsin, respectively (Fig.7). Concentrations of 0.1 and 1 mg/mL of PTU, inhibited total PO activity by 95.51 ± 1.1,6 % and 94.89 ± 2.74%, respectively, suggesting that remaining activities are not due to a true PO activity (Fig.7 c)). Moreover, the remaining activities that were not inhibited by PTU account only for 4.49 ± 1.16 % and 5.11 ± 2.74 % of the total PO activity measured, respectively (Fig.7 c)).

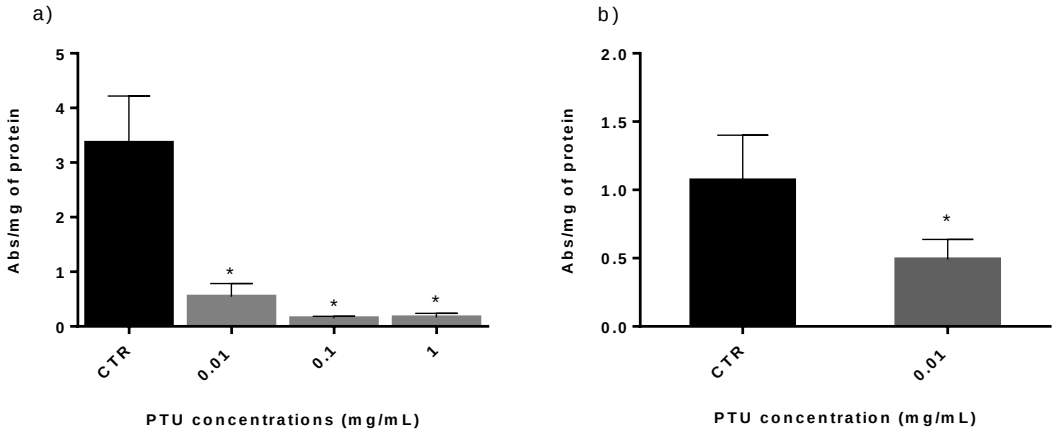

Figure 7 Effect of PTU on PO activity of *C. riparius* after a) 6 h of reaction in the presence of chymotrypsin and b) 0.01mg/mL of PTU after 6 h reaction in the absence of chymotrypsin. Values are the mean of five samples performed in quadruplicate ± SD). * denotes a significant difference compared to control (CTR) treatment at $p < 0.05$.

2.3.4 Activation of proPO by Zs and LPS

Pre-treatment of samples of *C. riparius* with 1 mg/mL LPS and 1 mg/mL Zs significantly increased PO activity *in vitro* with inductions of 213.18 ± 9.88 % ($F_{(3,12)} = 9.20$, $p<0.05$) and 134.97 ± 3.92 % ($F_{(3,14)} = 5.25$, $p<0.05$) , respectively (Fig.8).

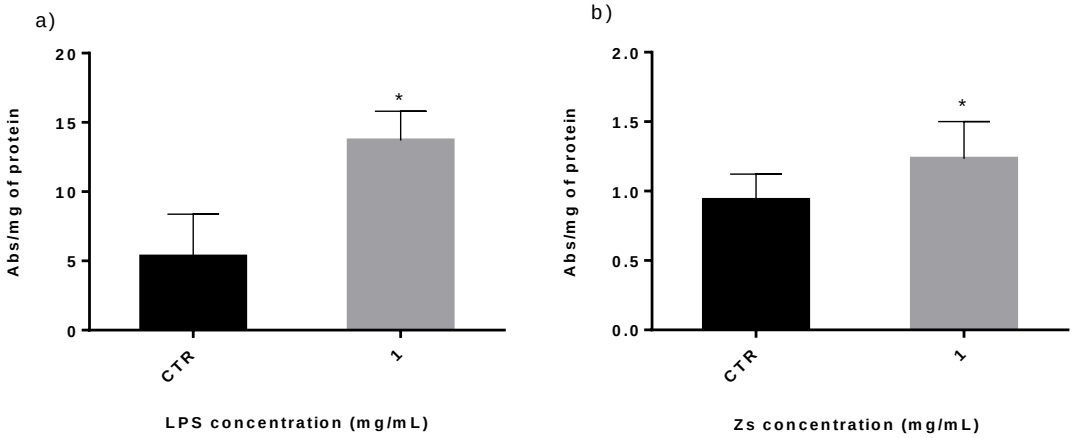

Figure 8 Effect of LPS after 48 h reaction a) and Zs after 4 h reaction b) on PO activity of *C. riparius.* Values are the mean of five samples performed in quadruplicate ± SD). * denotes a significant difference compared to control (CTR) treatment at $p < 0.05$.

2.4 Discussion

In this work, we show the feasibility of measuring PO activity using the whole body of larvae of *C. riparius*, which presents several advantages when simultaneously assessing other biochemical endpoints.

The measurement of PO activity in humoral fluid of *C. riparius* (Lilley et al., 2012) presents technical problems related with the volume of sample, number of organisms to be used, sampling technique procedure thus limiting the concomitant measurements of other biomarkers of interest.

In the current study, when an exogenous proteinase (chymotrypsin) was added, the PO activity increased comparing to non-activated samples. The mechanism of the stimulation of PO activity by chymotrypsin suggests that PO activity in *C. riparius* results from the proteolytic cleavage of the zymogen proPO into an active PO. As in other species, PO of *C. riparius* also exists as a latent pro-enzyme (proPO), which has to be activated by a cascade of enzymatic reactions incorporating pattern recognition proteins, proteinases and regulatory factors (Cerenius and Söderhäll, 2004).

The typical o-diphenoloxidase inhibitor – PTU – drastically inhibited (> 95 %) PO activity which confirms the occurrence of PO system (Hellio et al., 2007) and also demonstrated that measured PO activity of *C. riparius* was most probably a type of o-diphenoloxidase. The inhibition carried out by PTU is based on copper chelation, where sulphur binds to copper at the active site of the enzyme blocking the accessibility of the substrate (Luna-Acosta et al., 2017). These results are similar to those obtained from *Dugesia japonica* (Pang et al., 2010), *Halocynthia roretzi* (Hata et al., 1998), and *Penaeus californiensis* (Gollas-Galván et al., 1999). These previous studies also indicate that PO from *C. riparius* has some common properties with other invertebrate species.

Certain microbial products, such as pathogen associated molecular patterns (PAMPS), which include peptidoglycans or lipopolysaccharides from bacteria and β-1,3-glucans from fungi, will provoke activation of proPO after engaging specific recognition proteins. Thus, the *in vitro* increases of PO-like activity induced by Zs and LPS indicate that PO exists predominantly as an inactive proenzyme (proPO) in larvae of *C. riparius* and also reveals that the activation of this system is a fast and sensitive mechanism towards non-self-recognition and host defence against bacterial and fungal pathogens. However, induction of PO activity by LPS was greater than that promoted by Zs. It is considered that zymosan

needs to affiliate with a complex system of recognition (Hellio et al., 2007) which may influence the rate of activation. There are several studies about this mechanism involving exogenous molecules and it has been shown that in *Perna viridis* the proPO system is 1000 times more sensitive to LPS than to a polymer of p-1,3 glucans (common carbohydrates in fungal cell walls) (Asokan et al., 1997).

Due to the importance of PO in non-self-recognition of foreign biological agents in invertebrates (Cerenius et al., 2008) and due to its suspected participation in resistance to infections caused by pathogens (Aladaileh et al., 2007), it is thought that this enzyme plays a crucial role in immune system when foreign molecules enter an host organism (Luna-Acosta et al., 2017). PO activity in insects has been related to pathogens resistance (Cotter et al., 2002), including fungi, nematodes and nucleopolyhedroviruses (NPVs) (Cotter et al., 2002; Hung and Boucias, 1996).

Because of its oxidoreductive properties, PO might also play a role in metabolism and detoxification of contaminants in invertebrates (Luna-Acosta et al., 2011) and has specificity towards different organic contaminants due to its affinity for a large number of non-phenolic and phenolic compounds (Luna-acosta et al., 2017). In fact, several studies have been conducted in order to evaluate effects of inorganic (metals) and organic contaminants (pesticides, hydrocarbons, phytosanitaries) on PO activity of insects (Lilley et al., 2012; van Ooik et al., 2008, 2007). These studies have shown that chemical exposure increase PO activity (Lilley et al., 2012; van Ooik et al., 2008, 2007). Thus, PO can also be used as sensitive response towards effects of these contaminants (Luna-acosta et al., 2017).

2.5 Conclusion

This study demonstrated the activity of a true PO since more than 95 % of total PO activity was inhibited by PTU, a known inhibitor of o- diphenoloxidase (Hellio et al., 2007). Furthermore, levels of PO activity in samples were increased 350.55 % after *in vitro* incubation of 10 min with 0.5 mg/L chymotrypsin, showing that total PO measured was mainly due to the activation of proPO. Moreover, PO activity was increased *in vitro* by LPS and Zs which demonstrated the activation of proPO by bacteria and fungi. Thus, as previously observed for other insects, PO activity participates in humoral defence system, which is involved in non-self recognition, bactericidal activity and parasite resistance,

functioning as a defence factor to protect insects from the attack of environmental pathogens and foreign agents. PO activity can be used as a sensitive indicator of immune response to foreign agents and of detoxification of contaminants.

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Chapter 3

Effects of microbial insecticides on *Chironomus riparius* immune system

3. Effects of microbial insecticides on *Chironomus riparius* immune system

Abstract

Several natural products have been proposed as "environment-friendly" insecticides. Entomopathogens, which include bacteria, viruses, fungi protozoa and nematodes, are part of these natural products which are frequently used for the control of invertebrate pests and vector insects. The bio insecticide based on *Bacillus thuringiensis* subsp. *Israelensis (Bti)* sero. H-14 named Vectobac® 12AS, and a mycoinsecticide based on *Beauveria bassiana*, an entomopathogenic fungi - Naturalis®-L, are two examples of these microbial insecticides that are widely used for the control of several insect pests. However, it is very important to determine the potential risks of these control agents to non-target organisms. Phenoloxidase (PO) is the key enzyme in cellular and humoral immune responses of insects playing a very important role in the recognition of self and non-self molecules. Thus, PO activity can be used to understand if these two bio insecticides affect the immune *status* of the aquatic insect *Chironomus riparius*.

PO activity was significantly increased on larvae of *C. riparius* exposed to 2 and 5 mg/L of Naturalis®-L and 20 ng/L of Vectobac® 12AS and total PO activity was significantly increased on larvae exposed to 2 and 5 mg/L of Naturalis®-L and 2 and 20 ng/L of Vectobac® 12AS. Results suggest recognition of both bio insecticides as external agents leading to activation of the immune system of larvae. Moreover, it seems that increased activity of total PO observed on larvae exposed to Naturalis®-L and Vectobac® 12AS was also due to *de novo* synthesis of proPO. However, further studies should be performed in order to determine how this activation induced by bio insecticides will compromise future responses of insects to other infectious events and consequences for their life-history traits.

Keywords: Beauveria Bassiana; *Bacillus thurigiensis*; innate immune system, bio pesticides, phenoloxidase, aquatic insects.

3.1 Introduction

In insects, phenoloxidase (PO) is the key enzyme in cellular and humoral immune responses and also plays a very important role in the recognition of self and non-self molecules (Cerenius and Söderhäll, 2004; González-Santoyo and Córdoba-Aguilar, 2012; Pang et al., 2010), cell adhesion, encapsulation, production of melanin and phagocytosis, being an efficient immune defence system against foreign material (Lee and Söderhäll, 2002). PO is usually present as an inactive zymogen (proPO) that can be activated by limited proteolysis (Cerenius and Söderhäll, 2004). This activating system of proPO is initiated by recognition of peptidoglycans or lipopolysaccharides from bacteria and/or β-1,3-glucans from fungi (Lee and Söderhäll, 2002).

Microbial insecticides such as Naturalis®-L (fungal pathogen *Beauveria bassiana* strain ATCC 74040 based) and VectoBac® 12As (bacterial pathogen *Bacillus thuringiensis* var. *israelensis* H-14 strain AM65-52 based) have been proposed as "environment-friendly" insecticides, in order to overwhelm the problems resulting from the use of pesticides in modern agriculture, such as, their bioaccumulation across the food chain, development of resistance on target organisms and also environmental problems arising from deleterious effects to non-target organisms. They are both widely used for mosquito control, and can be introduced into aquatic ecosystems where mosquito larvae develop (Duchet et al., 2015). This mode of action optimizes the efficiency of control because mosquito larvae cannot escape from the treated water.

Several reports indicate that within the group of Diptera the non-biting midges (Chironomidae) are susceptible to *Bacillus thuringiensis* (Boisvert and Boisvert, 2000; Charbonneau, C.S., Drobney, R.D. & Rabeni, 1994; Pont et al., 1999; Vinnersten et al., 2010). Mosquito larvae are killed by *Bti* through the crystals and cytolitic-proteins that are built-up during sporulation of the bacteria (Bravo et al., 2007). When these proteins are consumed by mosquitos, they are activated in the alkaline milieu of the midgut and form pores in the epitelium causing the disruption of the midgut cells and leading to death of the larvae in few hours (Bravo et al., 2011, 2007; Yiallouros et al., 1999).

Beauveria bassiana is one of the most entomophatogenic fungal species extensively studied and is the active agent in many products currently use worldwide (Taylor and Feng, 2008). It is based on living conidiospores and acts primarily by contact when attached to the insect's cuticle (Gillespie et al., 2000; Ladurner et al., n.d.). The conidiospores germinate producing penetration hyphae, which enter the host by mechanical and enzymatic mechanisms and proliferate inside the insect's body (Ladurner et al., n.d.). When inside, it feeds on its host, causing death due to dehydration and depletion of nutrients. Several studies have been shown that PO levels are increased when the organism is infected by fungal components (Gillespie and Khachatourians, 1992; Hung and Boucias, 1996), particularly in *Spodoptera exigua, B. bassiana* caused remarkable alterations in the distribution and levels of PO activity (Hung and Boucias, 1996).

The aim of this study was to evaluate the sensitivity of PO activity as an indicator of effects of two widely used bio insecticides - Naturalis®-L and VectoBac® 12AS. For that laboratory assays were performed using *Chironomus riparius* larvae as model organisms.

3.2 Material and Methods

3.2.1 Test organisms

Chironomus riparius larvae used in the experiments were obtained from a laboratory culture established more than 15 years ago at the Department of Biology, University of Aveiro. The culture is maintained in aquaria with a layer of inorganic fine sediment (<1 mm), previously burnt at 500 °C for 5h, with American Society for Testing and Materials (ASTM) hard water (16), with a photoperiod of 16 h light:8h dark and room temperature of 20 ± 1 °C, with aeration. A suspension of commercial food fish TetraMin® (Tetrawerke, Melle, Germany) is provided as food every two days.

3.2.2 Exposure conditions

Fourth instar (12-day old) *C. riparius* larvae were exposed to 0.8, 2 and 5 mg/L Naturalis®-L solutions and 2, 20 and 200 ng/L of VectoBac® 12 AS for 48h in 500 mL with sediment and containing experimental solutions diluted in ASTM (ASTM, 2002) hard water medium. These bioassays were performed with a photo-period of 16h light: 8h dark and room temperature of 20 ± 1 °C with aeration. During the exposure period, no food was provided to organisms. Seven replicates with 15 organisms each, were used for each experimental treatment including control treatments (ASTM only).

After 48h of exposure, larvae were collected and quickly dried on a filter paper, frozen in liquid nitrogen and kept at -80 ºC. Samples were homogenized in ice by sonication using 1600 µL of 0.2 M phosphate buffer pH=7.4. Each sample was centrifuged at 9000 g for 20 minutes at 4 °C and the resulting supernatant was collected to a new microtube and kept on ice.

3.2.3 PO activity

PO activity was assayed using 30 µL of each sample that was added in 4 replicates to a microplate and incubated at 25 °C during 10 min with 50 µL of ultrapure water and 100 µL of 0.2 M phosphate buffer pH =7.4. After this incubation period 100 µL of 10 mM sodium cacodylate (CAC) buffer (pH 7.4) and 20 µL of 200 mM L-DOPA were added to each well (Pang et al., 2004). Blanks of reaction were performed using 30 µL of phosphate buffer and incubated as previously explained for samples. The absorbance was immediately measured at 490 nm (time 0) and followed after 6, 12, 24 and 48 hours. The enzyme activity was expressed as absorbance per mg of protein.

3.2.4 Total PO activity (proPO + PO)

Total PO activity (proPO + PO) was assayed using 30 µL of each sample that was added in 4 replicates to a microplate and incubated at 25 °C during 10 min with 50 µL of chymotrypsin 0.5 mg/mL and 100 µL of 0.2M phosphate buffer pH=7.4. After this incubation period, was added to each well 100 µL of 10 mM sodium cacodylate (CAC)

buffer (pH 7.4) and 20 µL of 200 mM L-DOPA (Pang et al., 2004). Blanks of reaction were performed using 30 µL of phosphate buffer and incubated as previously explained for samples. The absorbance was immediately measured at 490 nm (time 0) and followed after 6, 12, 24 and 48 hours. The enzyme activity was expressed as absorbance per mg of protein.

3.2.5 Protein Determination

Protein concentration was determined by the method of Bradford (Bradford, 1976), with bovine serum albumin standard. The supernatant was diluted 4 times to give a final protein concentration of 0.063 ± 0.009mg/mL, (0.050-0.083), to carry the enzymatic test.

3.2.6 Data analysis

Data were expressed as mean values ± SD. Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test. This allowed us to identify statistically significant differences between each condition tested and the respective control. Bartlett's and Brown–Forsythe tests verified the homoscedasticity of data while normality of data was assessed using residual probability plots. For all statistical tests the significance level was set at 0.05. All calculations were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA).

3.3 Results

3.3.1 Effects of microbial insecticide Naturalis®-L on PO activity

Results showed that PO activity was significantly increased to 5 - 6 abs/mg of protein on samples of *C. riparius* larvae exposed to 2 and 5 mg/mL of Naturalis®-L, compared to control treatments (~ 2 abs/mg of protein) ($F_{(3,21)}$=5,87; p<0.05, Fig.9). Moreover, total PO activity of samples of *C. riparius* larvae exposed to 2 and 5 mg/L of Naturalis®-L was also significantly increased reaching 13 - 14 abs/mg of protein due to previous incubation with

chymotrypsin when compared to respective control treatment (~ 8 abs/mg of protein) ($F_{(3, 22)} = 5{,}20$, $p<0.05$, Fig.10).

In vitro exposure of samples to 0.5 mg/L of chymotrypsin increased 430.8 ± 64.4%, 334.5 ± 81.5%, 287.9 ± 179.0% and 289.76 ± 147.77% the total PO activity measured in control, 0.8, 2 and 5 mg/mL of Naturalis®-L, respectively (Fig.11).

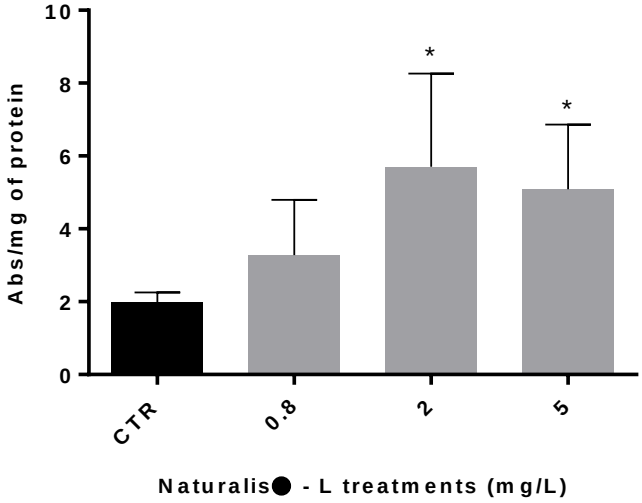

Figure 9 Effects of Naturalis®-L on *C. riparius* PO activity after 720 minutes of reaction in the absence of chymotrypsin. Values are the mean of seven samples performed in quadruplicate ± SD). * denotes a significant difference compared to control (CTR) treatment at $p < 0.05$.

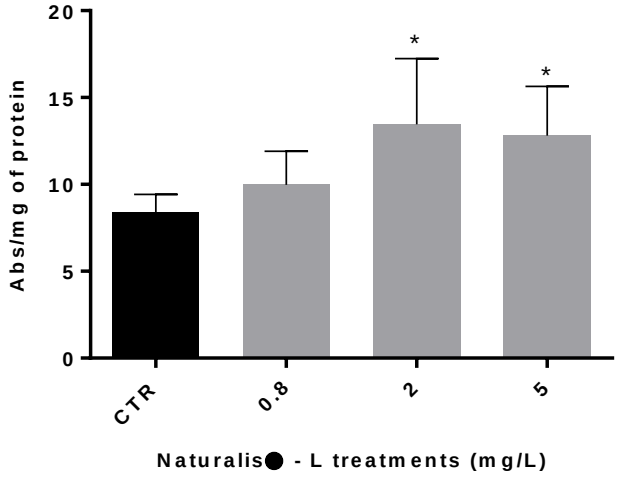

Figure 10 Effects of Naturalis®-L on total PO activity of *C. riparius* after 720 minutes of reaction in the presence of chymotrypsin. Values are the mean of seven samples performed in quadruplicate ± SD). * denotes a significant difference compared to control (CTR) treatment at $p < 0.05$.

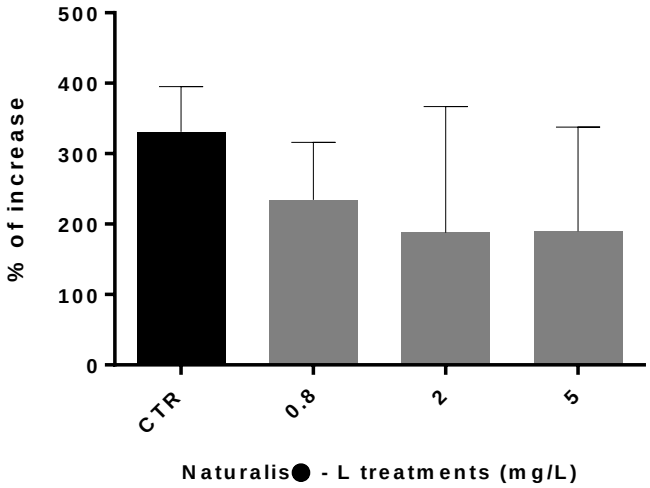

Figure 11 *In vitro* activation of total PO activity by chymotrypsin on *C. riparius* previously exposed *in vivo* to Naturalis®-L. Values are the mean of seven samples performed in quadruplicate ± SD).

3.3.2 Effects of microbial insecticide VectoBac® 12AS on PO activity

Results showed that PO activity was significantly increased to 4 abs/mg of protein on samples of *C. riparius* larvae exposed to 20 ng/L of VectoBac® 12AS, compared to control treatment (~ 2 abs/mg of protein) ($F_{(3,23)}$= 5,31, p<0.05, Fig.12). Furthermore, results showed that total PO activity of samples of *C. riparius* larvae exposed to 2 and 20 ng/L of VectoBac® 12AS was also significantly increased reaching levels up to 10 – 11 abs/mg of protein due to previous incubation with chymotrypsin when compared to respective control (~ 8 abs/mg of protein) ($F_{(3,23)}$= 4,35, p<0.05, Fig.13). *In vitro* exposure to 0.5 mg/mL of chymotrypsin increased 430.8 ± 64.4 % the PO activity measured in control samples and 555.9 ± 76.6%, 336.7 ± 140.5% and 417.4 ± 147.9 % in samples previously exposed to 2, 20 and 200 ng/L of VectoBac®-L, respectively (Fig.14).

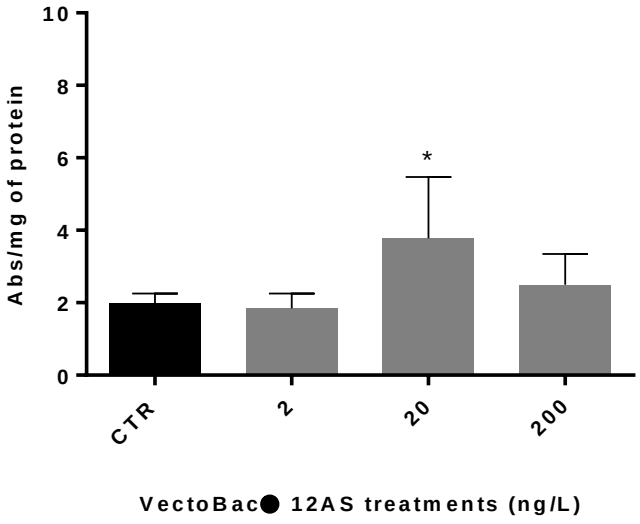

Figure 12 Effects of VectoBac® 12AS on PO activity of *C. riparius* after 720 minutes of reaction in the absence of chymotrypsin. Values are the mean of seven samples performed in quadruplicate ± SD). * denotes a significant difference compared to control (CTR) treatment at $p < 0.05$

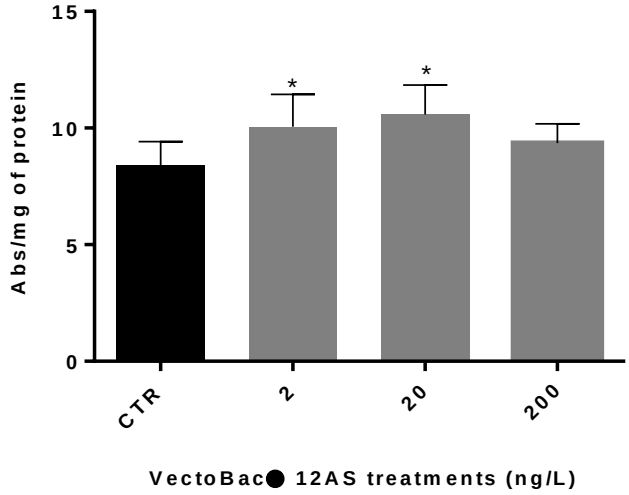

Figure 13 Effects of VectoBac® 12AS on total PO activity of *C. riparius* after 720 minutes of reaction in the presence of chymotrypsin. Values are the mean of seven samples performed in quadruplicate ± SD). * denotes a significant difference compared to control (CTR) treatment at $p < 0.05$.

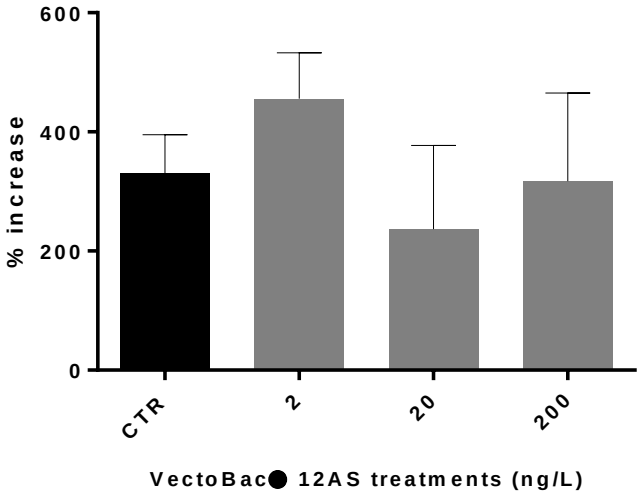

Figure 14 *In vitro* activation of total PO activity by chymotrypsin on samples of *C. riparius* previously exposed *in vivo* to VectoBac® 12 AS. Values are the mean of seven samples performed in quadruplicate ± SD).

3.4 Discussion

Microbial insecticides Naturalis®-L and VectoBac® 12AS promoted an increase of PO activity on larvae of *Chironomus riparius* showing the sensitivity of the immune system towards these compounds.

Several investigations have shown increases of PO activity in different insects species exposed to *Beauveria baussiana* ((Meshrif et al., 2010, Gillespie and Khachatourians, 1992, Hung and Boucias, 1996) and to *Bacillus thuringiensis* subsp. *Israelensis (Bti)* (Jakob and Poulin, 2016).

Total PO activity was increased by Naturalis®-L and VectoBac® 12AS to activity values (~ 13 - 14 and 10 - 11 abs/mg of protein, respectively) higher than those obtained for control organisms (~ 8 abs/mg of protein), which indicates *de novo* synthesis of proPO. On the other hand, the highest concentration of VectoBac® 12AS used (0.2 µg/L) had no significant effects on PO activity. It is known that in sufficient quantity *Bti* can produce enough toxic proteins to induce cellular damage that could lead to death (Boisvert and Boisvert, 2000), this lack of effect on PO activity may be related to the fact that the organisms were trying to avoid other damages, such as oxidative stress, or other implications. Studies report several trade-offs between immunity and other biological processes, such as reproduction (Adamo et al., 2001), where males of *Gryllus texensis* trade off immunity for reproduction, becoming more sensible to *S. marcescens* at onset off sexual behavior (Adamo et al., 2001). In this way, *C. riparius* larvae could be investing less energy in immunity while trying to allocate all of their energy for other physiological systems that allow its survival (Sokolova et al., 2012). Besides that, according to González-Santoyo & Córdoba-Aguilar, (2012), maintenance of the proPO activating system is dietary-dependent, requiring a protein based diet for the proper function of this system (González-Santoyo and Córdoba-Aguilar, 2012). A possible explanation is that protein content (obtained through diet) directly affects the production of amino acids, which can be used for the synthesis of several intermediates of proPO activating system, including PO (Abisgold and Simpson, 1987). Starvation decreases immunity in many insects (Feder et al., 1997) and during larvae exposure no food was provided which, along with other factors, may interfere with their immune response capacity. The organisms are also dispending their energy in *de novo* synthesis of proPO increasing the levels of total PO. This energy expenditure may increase

the susceptibility of *C. riparius* to infection by other infectious agents, contaminants and abiotic factors. On the other hand, if PO is produced in excess, it can lead to damage in the organism due to the production of proteases that could promote degradation of host proteins, production of cytotoxic quinones and reactive nitrogen and nitrogen intermediates (Daquinag et al., 1995; González-Santoyo and Córdoba-Aguilar, 2012; Sugumaran and Nellaiappan, 2000).

The increase of PO activity of larvae of *C. riparius* in both tests suggests the activation of the immune system upon recognition of bio insecticidal material, combating microbial infection. Our results also suggest that PO activity is altered at concentrations much lower than the ones showing to produce lethal effects (Kästel et al., 2017). Further investigation should focus on possible sub-lethal effects of these concentrations of both compounds in order to assess the sensitivity and relevance of PO as an early warning indicator of stress in *C. riparius* exposed by microbial insecticides as well as potential energetic trade-offs involved in the immunological response.

3.5 Conclusion

PO and total PO activities seem to be very effective physiological parameters to understand and determine the activation of the immune response against entomopathogenic microbial infections (Narayanan, 2004). As shown here, Naturalis®-L and VectoBac® 12AS increased PO activity in *C. riparius* larvae through activation and *de novo* synthesis of proPO. However, further studies should be performed in order to determine the physiological consequences of this response.

Nonetheless, according to Adamo (Adamo, 2004), PO activity is not sufficient to establish a relation between immune response and the actual resistance of the individual, i.e., a decline measured during an immune response is not necessarily related with an overall decrease in disease resistance but could only indicate a changing and/or redistribution of the immune components within the immune system in order to increase the immune capacity against several invaders (Braude et al., 1999). Research involving more than one immune component simultaneously (i.e., evaluate PO activity and numbers of haemocytes; or PO activity and encapsulation) will greatly increase our understanding on how the immune system of invertebrates works to protect them against pathogens infections.

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Chapter 4

General discussion and conclusions

4. General discussion and conclusions

4.1 General discussion and conclusions

Several studies detected and characterized PO activity in invertebrates, such as in insects and crustaceans (Cerenius and Söderhäll, 2004). Concerning *C. riparius*, studies have been done using humoral fluid for PO measurement (Lilley et al., 2012). However, this procedure has technical issues related with the amount of sample, number of organisms to be used and sampling procedure that limit the concomitant measurements of other biomarkers of interest. So, in order to overcome these problems, in this work we showed the feasibility of measuring PO activity in whole body samples of larvae of *C. riparius*.

As in other species, our study shows that PO in *C. riparius* exists as a latent proenzyme (proPO), which is activated by an exogenous protease (chymotrypsin) that increases PO activity, by proteolytic cleavage of the zymogen into an active PO. As in many other invertebrates (Aladaileh et al., 2007), PAMPS (LPS and Zs) increase PO activity of *C. riparius* larvae. The strong inhibition caused by PTU indicates the presence of a true PO (Hellio et al., 2007). This study showed that the immune response of *C. riparius* could be modulated by bio insecticides exposure. The amount of active enzyme increased during an immune challenge (exposure to Naturalis®-L and VectoBac® 12AS). *C. riparius* larvae are dispending energy, considering that the cost of production and maintenance of the PO system (inclusively proPO activating system) appear to be high (González-Santoyo and Córdoba-Aguilar, 2012). This energy expenditure can increase the susceptibility of *C. riparius* when exposed to other pathogens or abiotic factors, lacking on sufficient reserves and energy sources to combat them and avoid other damages, such as oxidative stress. The application of these bio insecticides without evaluating all their implications can lead to disruptions on higher trophic levels within the wetland food web (Kästel et al., 2017).

Estimating disease resistance and immunological response in animals is a very difficult mission (Adamo, 2004). Studies reported some trade-offs between immunity and other biological processes, such as reproduction (Adamo et al., 2001) and growth rate under stress (Stoks et al., 2014). In these cases, immune function may receive less attention because the

organisms are allocating their energy for other biological processes (Adamo et al., 2001; Stoks et al., 2014). PO activating systems in invertebrates have been the object of diverse investigations and now we begin to understand some aspects of non-self recognition and host defence in these organisms.

However, simultaneously assessment of other components of immune defence, such as haemocytes counting, encapsulation and sclereotization will allow an integrated view about immune mechanisms and how they work together to protect the organism giving insights into entomopathogenic microbial infections and the management and control of diseases (Narayanan, 2004) within freshwaters.

A biochemical assay for the measurement of immunological responses such as PO activity and proPO activation can also be used to evaluate the sub-lethal effects of other contaminants and their potential indirect effects (Lilley et al., 2012). In particular, it would be extremely interesting to address effects of nanoparticles and microplastics that are ingested by detritivores such as *C. riparius* (Horton et al., 2016). Further studies are also needed to assess the specificity, robustness and relevance of PO activity as an ecotoxicological endpoint and understand how natural selection, phenology and exposure to contaminants mediate immunological responses of invertebrates.

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ANNEX

Annex

Measurement of Phenoloxidase (PO) and Total Phenoloxidase (proPO+PO) activities in *Chironomus riparius*

Overview:

Phenoloxidase (PO) is a bifunctional copper containing enzyme and is a key enzyme in insect's immune system, being the initial step of the biochemical cascade that results in melanin formation (or melanization). Melanization is associated with antimicrobial activity, host defence and wound healing. PO exists as an inactive zymogen (proPO), which is activated by a limited and controlled proteolysis through the action of a serine protease cascade. PO is quantified through a colorimetric assay by measuring the conversion of L-DOPA substrate in dopachrome, at 490nm. This conversion darkens the test solution and the greater the amount of PO, the faster the conversion of L-DOPA to dopachrome. The objective is to measure both PO and total PO activity. The assays only differ in the addition of chymotrypsin (in total PO measurements). This exogenous protease stimulates the activation of proPO to PO. The general idea is that PO (or proPO) converts L-DOPA to a melanized form. Thus it is critical that the L-DOPA be fresh and protected from light until used.

A) <u>Solution preparation</u>

1. K-phosphate buffer 0,2 M, pH = 7,4

Reagents

K_2HPO4 (M = 174,18 g/mol) **(cabinet 1, reagent nº 63; Sigma-Aldrich)**

KH_2PO_4 (M = 136,09 g/mol) **(cabinet 1, reagent nº 49; Sigma-Aldrich)**

1.1. Method

1.1.1. Prepare 1 L of K_2HPO_4 in ultra pure water (0,2 M; 34,863 g/L);

1.1.2. Prepare 1 L of KH_2PO_4 in ultra pure water (0,2 M; 27,218 g/L);

1.1.3. Add little amounts of basic solution to the acid solution till a pH = 7,4 is reached;

1.1.4. Keep the solution in the cooler.

a) Use at room temperature.

2. Sodium cacodylate buffer solution (10 mM; pH = 7,4)

2.1. Reagents

Calcium chloride ($CaCl_2$•$2H_2O$) (M = 147,01 g/mol) **(cabinet 1, reagent nº 37; Sigma-Aldrich)**

Sodium cacodylate (($(CH_3)_2AsO_2Na$•$3H_2O$)) (M = 214,03 g/mol) **(acid/base cabinet 2, reagent nº 426; Sigma-Aldrich)**

2.2. Method

a) Weight the calcium chloride (0,014702 g);

b) Weight the sodium cacodylate (0,042806 g);

c) Separately dissolve the calcium chloride and sodium cacodylate in K-phosphate buffer (0,2M; pH = 7,4, 25ºC). Mix both solutions and add K-phosphate buffer to get a total volume of 100 mL (≈ 2 microplates);

d) Prepare when needed.

3. L-DOPA solution

3.1. Reagents

L-DOPA (3,4-Dihydroxy-L-phenylalanine) (M = 197,1879 g/mol) **(tupperware cabinet 1, reagent nº 427; Sigma-Aldrich)**

3.2. Method

a) Prepare 10 mL of L-DOPA (1 mg/mL) in cacodylate buffer (≈ 2 microplates);

b) Keep at room temperature and protected from light;

c) Prepare when needed.

4. Bovine chymotrypsin solution

4.1. Reagents

Bovine chymotrypsin **(freezer º 3, reagent nº 428; Sigma-Aldrich)**

4.2. Method

a) Prepare 10 mL of chymotrypsin solution (0,5 mg/mL) in ultra pure water (≈ 2 microplates);

b) Keep on ice and protected from light.

B) <u>Protocol</u>

1. Samples preparation

1.1 Homogenize each sample (15 C. riparius) in 1600 µL of 0.2M phosphate buffer (pH=7.4) with sonicator.

1.2 Centrifuge the tissue homogenate of each sample at 9000 g for 20 minutes at 4 °C to collect the resulting supernatant into new microtubes.

1.3 Keep samples on ice.

2. Method

2.1. Leave the first column empty;

2.2. In the second column, pipette 30 µL of phosphate buffer (whites);

2.3. For the next columns, pipette 30 µL of sample (make 3 to 4 replicates);

2.4. In the **total PO assay (PO + pro-PO)** microplates, add 50 µL of chymotrypsin and 100 µL of 0.2M phosphate buffer (pH=7.4) to each well and leave at room temperature (25 °C) and in the dark for 10 minutes (measures the PO and pro-PO activity as a result of the activation of pro-PO by the chymotrypsin);

2.5. In the **PO assay** microplates, add 50 µL of ultra pure water to each well and 100 µL of 0.2M phosphate buffer (pH=7.4) and leave at room temperature (25 °C) and in the dark for 10 minutes (measures only t1he PO; pro-PO is inactive);

2.6. Add 100 µL of 10mM sodium cacodylate (CAC) (pH=7.4) and 20 µL of L-DOPA to each well;

2.7. Read absorbance at 490 nm immediately, and 6hours after.