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Molecular and cellular processes of the innate immune response in insects: investigation on the immune modulation induced by entomopathogenic nematodes.

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ABSTRACT

Aim of this project was to investigate relationships between *Rhynchophorus ferrugineus* or *Galleria mellonella* and the entomopathogenic nematode *Steinernema carpocapsae*. In particular, the work was focused on the immune response of the insect host either in naïve larvae or in larvae infected with entomoparasites. We analyzed different immunological processes: the activity and modulation of prophenoloxidase-phenoloxidase (proPO) system, the cell-mediated encapsulation, the antimicrobial peptides (AMPs) inducible response and finally the phagocytosis activity of the host hemocytes. Furthermore, we investigated the immune depressive and immune evasive strategies of the parasite. Our results indicated that *R. ferrugineus* has an efficient immune system; however, in the early phase of infection, the presence of *S. carpocapsae* induces a strong inhibition of the host proPO system. In addition, the parasite does not seem to be susceptible to the encapsulation by host hemocytes; the parasite *mimetic* properties seem to be related to the structure of its body surface. *S. carpocapsae,* before the release of its symbiotic bacteria (*X. nematophila*), depresses and elude the host immune defenses, with the aim to create a favorable environment for its symbionts responsible of the septicemic death of the insect host. Besides, our results have demonstrated that *X. nematophila* is able to inhibit the synthesis and the activity of antimicrobial peptides. *X .nematophila* elude the recognition by hemocytes since it is not engulfed by the host cells. It is evident that the nematode and its symbiotic bacteria cooperate to elude and inhibit immune responses of the insect host. This study provides data that can help to a better understand of the relationships between parasites and their hosts.

1 INTRODUCTION

The research is part of a project for the study, at the physiological, biochemical, cellular and molecular level, of some aspects of the immune response in insects. The choice of insects as biological models is due to the high impact of these invertebrates on environment, health and biotechnology. Many species, particularly insect vectors, may represent a concrete threat to human and animal health; moreover, several insect pests damage crops and urban green. Then, to study the physiology and immune defense processes of these animal models could provide alternative ways to control their diffusion, improving biological control methods that could allow a significant reduction of the use of pesticides. In many cases, the use of bio-insecticides to control insect species did not provide suitable results due to the scarcity of exhaustive studies on the efficacy of the infection by natural insecticides related to the defense processes of the host.

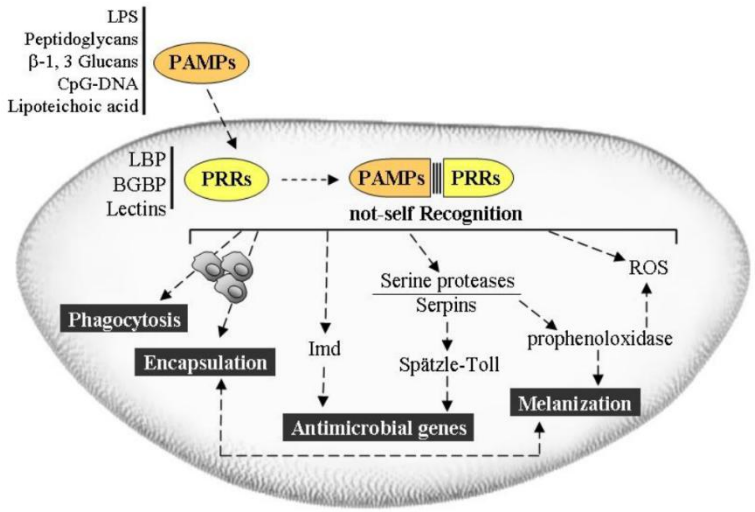
Multicellular organisms, as vertebrates, invertebrates and plants are continually under attack by a great variety of pathogens including bacteria, fungi, viruses, protozoa and multicellular parasites.

Generally, the access of these infectious agents into the host body is prevented by physical, chemical and biochemical barriers. Once the barriers are breached, pathogens are able to spread rapidly and eventually kill the host. In order to survive, they have developed several defense mechanisms, including the immune system. Vertebrates have developed two interconnected powerful defence mechanisms, known as innate and acquired immunity. The acquired immune system is mediated by somatic recombinatory mechanism and clonal expansions involved in production of antibodies and T-cell receptors, which give rise to the specificity and memory of vertebrate adaptive immunity. Despite the immune responses of invertebrates are not based on adaptive immunity, they have a well-developed innate immune system that compared with adaptive immunity is more effective and rapid in the initial defense. Insect immunity more closely resembles the innate immune system of

vertebrates. This system involves rapid, early and relatively not specific responses both cellular and humoral (Beckage, 2008).

1.1 The immune system of insects

The innate immune system of insects was arbitrarily divided into humoral and cellular defenses. Humoral responses refer to soluble molecules such as antimicrobial peptides, Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) and the complex enzymatic cascades that regulate melanin formation and clotting, compounds that are present in the hemolymph; in insects, blood and interstitial fluid are indistinguishable and are referred as hemolymph, which moistens all internal tissues, organs, hemocytes, and facilitates the transport of nutrients, waste products and metabolites. Cellular defense, in contrast refers to hemocyte-mediated immune responses like phagocytosis, nodulation and encapsulation (Nappi *et al.*, 2004; Shia *et al.*, 2009; Sideri *et al.*, 2008).



Scheme resume the insect immune defences (Brivio MF *et al.*, 2005)

1.1.1 Hemocytes

Insect hemocytes have historically been identified and classified using morphological, histochemical and functional features. The most described blood cells derived from *Drosophila* whose hemocytes are named differently from most other species. *Drosophila* larvae contain three differentiated types of hemocytes in circulation named: plasmatocytes, crystal cells and lamellocytes (Lavine and Strand, 2002). Plasmatocytes represent 90-95% of all mature hemocytes and are professional phagocytes that engulf pathogens, dead cells and other bodies. Crystal cells express components of the phenoloxidase (PO) cascade. Lamellocytes are essentially absent in healthy larvae but rapidly differentiate from prohemocytes (hemopoietic precursors) when exposed to intruders in order to encapsulate parasitoids and other foreign entities. Prohemocytes are small cells that are round or oval (4 to 10 μm wide by 4 to 22 μm long) (Gupta, 1985; 1991; Brehelin and Zachary 1986; Butt and Shields, 1996; Chapman, 1998; Silva *et al.*, 2002; Giulianini *et al*. 2003). These hemocytes are characterized by possessing a relatively large nucleus that nearly fills the cytoplasmic space. Other organelles (e.g. smooth and rough endoplasmic reticulum, mitochondria, Golgi bodies) are generally low in number. Prohemocytes are often found in small groups and constitute 1% to 7% of the population of hemocytes (Gupta, 1985; 1991; Brehelin and Zachary 1986; Chapman,1998).

Differentiated hemocytes in Lepidoptera, Diptera, Coleoptera, Orthoptera, Blattaria, Hymenoptera, Hemiptera and Collembola (Lavine and Strand, 2002; Ribeiro e Brehelin 2006) are usually named granulocytes, plasmatocytes, spherule cells and oenocytoids. In Lepidoptera, granulocytes are usually the most abundant cells that strongly adhere to foreign surfaces, spread simmetrically and function as the professional phagocytes (Strand, 2008). Granular cells include the possession of numerous lysosomes, free ribosomes and well developed ER and RER, but only a few mitochondria (Gupta, 1985; Chapman, 1998). The nucleus is round or elongate and located centrally. Nardi *et al*. (2003) showed that these cells are diploid. Granular cells may or may not have filopodia (Gupta, 1985). Granular cells

comprise over 30% of the hemocyte count and, along with plasmatocytes, are together the two most numerous of hemocytes (Chapman, 1998). In *Manduca sexta*, larger phagocyte-like hemocytes called hyperphagocytic cells that differentiate following immune challenge have also been described. Plasmatocytes are polymorphic cells of variable size (3 μm to 40 μm) (Gupta, 1985; 1991; Götz and Boman, 1985; Brehelin and Zachary 1986; Butt and Shields, 1996; Chapman, 1998; Silva *et al.*, 2002). While free-floating in the hemolymph, plasmatocytes are round or oval. Up on contact with certain surfaces, plasmatocytes attach, spread by sending out filopodia, and become very flat. The cytoplasmic space contains many organelles, including a well-developed rough endoplasmic reticulum, Golgi bodies, and numerous mitochondria. The nucleus is most often positioned centrally. Plasmatocytes are among the most numerous of hemocytes and generally comprise between 30% to 60% of the total hemocyte count. These hemocytes possess the ability to adhere to foreign surfaces and play an important role in wound healing and in the immune responses of phagocytosis, nodule formation and encapsulation (Gupta, 1985; 1991; Götz and Boman, 1985; Brehelin and Zachary 1986; Chapman, 1998; Lavine and Strand, 2002). Oenocytoids are opaque hemocytes that range in size from 16 μm to over 54 μm and have been observed in oval, spherical, elongated and crescent shape. They comprise over 5% of the hemocyte count. In the Lepidoptera, oenocytoids are the largest in size of all hemocytes (Gupta, 1985; 1991; Brehelin and Zachary, 1986; Butt and Shields, 1996; Chapman, 1998). The nucleus is small and located eccentrically. Sometimes oenocytoids possess two nuclei. The cytoplasm appears homogeneous. In oenocytoids from most insect species observed, the organelles (e.g. mitochondria, Golgi bodies, RER) are generally underdeveloped, however, the cytoplasm is known to contain numerous free ribosomes. A few oenocytoids have inclusions that are rod, filament, needle-like, or crystal in shape. In D.melanogaster, the oenocytoids are referred to as crystal cells due to the shape of the inclusions (Gupta, 1985; Götz and Boman, 1995; Tepass *et al.* 1994). Oenocytoids have also been shown to contain pro-phenoloxidase (pro-PO) which, when released into the hemolymph and activated to phenoloxidase, plays an important role in the melanization response associated with wound healing, protein cross-linking and immunity (Jiang *et al.*, 1997; Gillespie *et al.*, 1997; Hillyer and Christensen,

2002; Hillyer *et al.*, 2003; Da Silveira *et al.*, 2003; Kanost *et al.*, 2004). Spherulocytes are round or oval and variable in size with diameters from 5 μm to 25 μm (Gupta, 1985; 1991; Butt and Shields, 1996; Chapman, 1998). The defining characteristic of these hemocytes is the membrane-bound spherules in the cytoplasmic space. The number of spherules varies and range in diameter from 1 μm to 5 μm. These spherules appear to enclose some sort of granulated material. Histochemical analysis of the spherules has shown that they contain mucopolysaccharide and glucomucoproteins (Gupta, 1985; 1991). The cytoplasm also contains ribosomes, Golgi bodies, lysosomes, mitochondria and rough endoplasmic reticulum. Although many have speculated as to the function of these hemocytes (e.g. silk production, melanization, phagocytosis, regulation of clotting and cell adhesion), a definitive answer to this question remains elusive (Gupta, 1985; 1991; Brehelin and Zachary 1986; Chapman, 1998).

1.1.2 Hematopoiesis

The hemocytes described above arise during two stages of insect development. The first population oh hemocytesis produces during embryogenesis from head or dorsal mesoderm while the second is produced during the larval or nymphal stages in mesodermally derived hemopoietic organs. The hematopoietic organs of *Drosophila* are called lymph glands that form bilaterally along the anterior part of the dorsal vessel during embryogenesis. By the third instar, each lymph gland consists of an interior primary lobe and several posterior secondary lobes. The primary lobe is further divided into a posterior signaling center (PSC) that contains PSC cells, a medullary zone containing primarily prohemocytes, and a cortical zone containing primarily plasmatocytes (Akai and Sato, 1971; Holz *et al.*, 2003; Jung *et al.*, 2005).

1.1.3 Sensing infection

The mechanisms and molecular effectors of pathogen recognition systems in diverse hosts are highly conserved. Both plant and animal recognition of pathogens relies on sensing of Pathogen-Associated Molecular Patterns (PAMPs) by Pattern Recognition Receptors (PRRs), these factors are able to interact specifically with a broad range of foreign antigenic surface compounds.

The most characterized PRRs are C type lectins, peptidoglycan recognizing proteins (PGRP), β- 1,3- glucan recognition proteins, the hemolin and integrins. PAMPs consist of various compounds, including oligosaccharides, proteins, glycoproteins, lipids and distinct nucleic acid motifs that are unique and essential for microorganism's survival. An important feature of PAMPs is their strongly conserved structures, which are invariant between organisms of a given class (Medzhitov and Janeway, 1997; 2002).

The name Peptidoglycan Recognition Protein was first introduced by the Ashida's group when working on Bombyx mori (Yoshida *et al.*, 1996). They purified a 19 kDa protein present in the silkworm hemolymph that binds Gram positive bacteria and more specifically the bacterial PGN. PGRPs have been now identified in mollusks, echinoderms, and in several groups of vertebrates (fish, amphibians, birds and many mammals). However, plants and lower metazoan, such as nematodes, do not have PGRPs (Li *et al.*, 2007).

Drosophila genome contains 13 PGRP genes of which at least one (PGRP-LC) can be further diversified by alternative splicing. Family members share a common PGRP domain, which is evolutionarily related to the bacteriophage type II amidases, and some members have retained this enzymatic activity (these are referred to as catalytic PGRPs). By contrast, other PGRPs have lost crucial amino-acid residues that are essential for catalysis and they serve as microbial sensors (these are referred to as recognition PGRPs) (Werner *et al.*, 2000).

The PGRP transcripts are classified into short (S) and long (L) subfamilies and are often alternatively spliced to generate up to 19 peptides in flies. All PGRPs across species have at

least one C-terminal PGRP domain (165 amino-acid residues) homologous to bacteriophage and bacterial type 2 amidases (Kang *et al.*, 1998). This suggests that animal PGRPs and prokaryotic type 2 amidases may have evolved from a common primordial ancestor gene. PGRP-S are about 200 amino-acids long, have a signal peptide and one PGRP domain, whereas most PGRP-L are at least twice as large and possess one or two C-terminal PGRP domains. Both PGRP-S and PGRP-L contain an N-terminal sequence that is unique for a given PGRP and has no homology with other PGRPs or any other proteins. In almost all PGRPs, two closely spaced conserved Cys can be found in the middle of their PGRP domain forming a disulfide bond, which is needed for the structural integrity and activity of PGRPs (Michel *et al.*, 2001; Chang *et al.*, 2004). Finally, some Drosophila PGRPs, e.g., PGRP-LC or PGRP-LF, are transmembrane (Gottar *et al.*, 2002; Maillet *et al.*, 2008) or intracytoplasmic (PGRP-LE) molecules (Kaneko *et al.*, 2006), whereas most other PGRPs have a signal peptide and are secreted proteins. In Drosophila, PGN and derived muropeptides are, so far, the only identified PGRP ligands. PGN is an essential cell wall component of virtually all bacteria and is a well-known target for recognition by pattern recognition receptors. PGN is a polymer of β-(1-4)-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), cross-linked by short peptide chains, called stem peptides. The nature of the third residue of the stem peptide is an important distinctive feature of Gram positive versus Gram negative bacteria: whereas most Gram positive bacteria have a Lysine in this position, it is replaced by an m-DAP (meso-Di-aminopimelic) residue in Gram negative bacteria. The minimum peptidoglycan fragment that binds to PGRPs is a muramyl-tripeptide. Crystallographic studies also revealed how PGRPs discriminate between the Lys and DAP-type PGN. The only difference between Lys and DAP-type is the presence of an additional carboxylate at the carbon 1 of DAP. Discrimination between Lys- and DAP-type peptidoglycan is based on three aminoacid residues in the peptidoglycan-binding groove of Drosophila PGRPs (Swaminathan *et al.*, 2006).

In *Drosophila melanogaster*, the expression of the genes encoding most immune proteins is under the control of two NF-kB-dependant signaling pathways. The Toll cascade responds to Gram positive bacteria and fungal pathogens while the Immune Deficiency (IMD) pathway preferentially recognizes Gram negative bacteria. The Toll signalling pathway involves several factors that were initially discovered in the control of dorso ventral patterning in the embryo (Moussian and Roth, 2005) and it has some parallels to the mammalian signalling cascades downstream of the interleukin-1 receptor (IL-1R) and the Toll-like receptors (TLRs). By contrast, the IMD pathway is similar to the tumor-necrosis factor-receptor (TNFR) pathway in mammals.

Recognition of DAP-type Gram negative bacteria

The *D. melanogaster* immune system can discriminate between distinct classes of microorganisms. Thus, the IMD pathway is preferentially induced by Gram negative bacteria (and some Gram positive bacilli) and this pathway controls the host defence against these infections. Although lipopolysaccharides (LPS) form the outer cell layer of Gram negative bacteria, they do not activate the IMD pathway (Hoffman, 2003; Leulier *et al.*, 2003). Beneath the external LPS coat and outer membrane of Gram negative bacteria, peptidoglycan (PGN) forms an inner layer of polymeric glycan chains that are cross linked by peptidic stems. PGRP-LC and PGRP-LE, two non-catalytic members of the PGRP family, mediate the detection of Gram negative bacteria and activation of the IMD pathway (Gottar *et al.*, 2002; Takehana *et al.*, 2004).

PGRP-LC is the main transmembrane (type II) receptor of the IMD pathway, whereas PGRP-LE is a cleaved, secreted PGN sensor in the hemolymph. During septic (or intestinal) infection, bacteria release short PGN fragments as a result of cell-wall remodelling during growth and division. These PGN fragments are detected by PGRP-LCx–PGRP-LCa (the gene encoding the transmembrane PGN sensor PGRP-LC produces three distinct splice isoforms, a, x and y that each code for a distinct extracellular PGRP domain), and possibly

PGRP-LCx–PGRP-LE, heterodimers, and these in turn activate the IMD pathway (Mellroth *et al.*, 2005).

As a consequence of this early activation, effectors of the humoral immune response (such as AMPs and lysozymes) attack the invading bacteria, and this leads to the release of large fragments of polymeric DAP-type PGN that were initially hidden under the LPS outer coat. These fragments can then be sensed directly by an array of membrane-bound PGRP-LCx receptors. The intensity of the stimulation of the IMD pathway can be modulated by the catalytic PGRPs (PGRP-SC1, PGRP-SB1 and PGRP-LB), which cleave the amide bond between the muramic acid of the glycan chain and the DAP-containing peptide stem of PGN. PGN that has been digested by PGRP-SC1 or PGRP-LB is barely immunostimulatory and these catalytic PGRPs thereby function as scavengers PGRP-SC1, PGRP-SB1, and PGRP-LB can all cleave DAP-type PGN. It is striking that PGRP receptors can bind the PGN of Gram negative bacteria, as PGN is not directly accessible for binding. Two models have been proPOse for the immune system to circumvent this apparent difficulty. In the first, bacteria could be attacked by hemocytes or by AMPs, which might basally be expressed at low levels, and this would result in the release of PGN fragments in the hemolymph. Phagocytosis of bacteria by hemocytes could also lead to indirect activation of the IMD pathway by an unknown signal. The second postulates that bacteria could release short PGN fragments during proliferation and growth as a result of cell-wall remodelling (Lim *et al.*, 2006; Zaidman-Rémy *et al.*, 2006).

Recognition of Lys-type Gram positive bacteria

Lys-type Gram positive bacteria are also sensed by members of the PGRP family, namely PGRP-SA and PGRP-SD 32,33and by GNBP1, a member of the Gram negative binding proteins (GNBPs; also known as β-glucan recognition proteins (βGRP)) family.

Sensing of Gram positive bacteria (and fungi) results in the activation of proteolytic cascades that culminate in cleavage of the cytokine Spätzle, which is the ligand for the transmembrane receptor Toll (Michel *et al.*, 2001; Gobert *et al.*, 2003). PGRP-SA binds preferentially to

Lys-type PGN in keeping with the absence of the arginine residue that binds specifically to the carboxyl group of DAP-type PGN. GNBP1 binds to a more restricted range of Lys-type PGN. GNBP1 functions together with PGRP-SA in sensing some Gram positive bacterial strains. GNBP1 cleaves PGN into shorter dimeric or tetrameric muropeptides that bind to PGRP-SA (Wang *et al.*, 2006). An important property of *D. melanogaster* pattern-recognition receptors (PRRs) is their ability to form many complexes to detect various microbial species. The combination of several PRRs can expand the repertoire of microorganisms that are detected by the *D. melanogaster* immune system (Lemaitre and Hoffmann, 2007).

1.2 The Toll and IMD pathways

The insect and mammalian innate immune responses exhibit a great deal of evolutionary conservation. One of the best examples was provided by the discovery of the Toll pathway as a key component of the *Drosophila* immune response and the subsequent identification of the mammalian Toll-like Receptors (TLRs). In addition, the insect immune response relies on evolutionarily conserved NF-κB signaling cascades for the control of immune-induced gene expression (Hetru and Hoffmann, 2009).

The Toll pathway responds to Gram positive bacterial and fungal infections. Unlike human Toll-like receptors (TLRs), *Drosophila* Toll does not directly bind pathogens or pathogen-derived compounds. Toll is a cytokine receptor, activated by the cytokine Spätzle (Aggarwal and Silverman, 2008), Spätzle is produced as a pro-protein, with a disulfide-linked dimeric structure. In order to activate the Toll pathway, pathogens must first activate serine protease cascade that culminates in Spätzle cleavage, liberating the mature Toll ligand (C-terminal 106 amino acids of Spätzle). Recognition of Gram positive bacteria is mediated through the detection of lysine-containing peptidoglycan (PGN) by PGRP-SA and PGRP-SD (peptidoglycan receptor proteins) (Bischoff *et al.*, 2004). Bacterial recognition in the Toll pathway also requires the serine protease Grass, which is thought to function downstream of these PGN receptors. On the other hand, detection of fungal infections relies on two

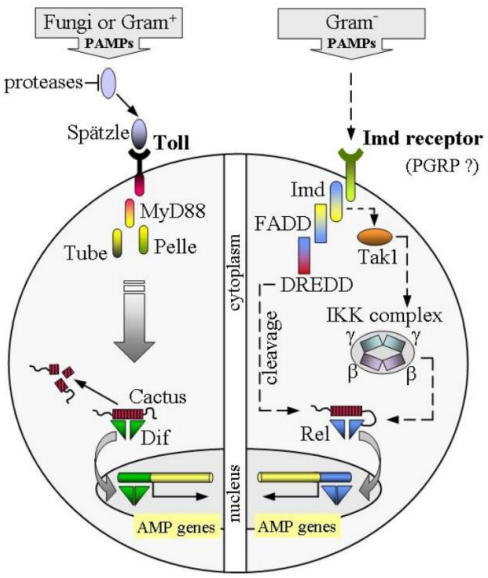
additional detection systems. In one pathway, fungal beta-glucans are recognized by the receptor GNBP-3 (Gottar *et al.*, 2006). Another pathway involves a serine protease known as Persephone (PSH). Once activated, PGRP-SA/GNBP1, PGRP-SD, GNBP3 or PSH lead to Spätzle cleavage by activating serine protease cascades that converge on two chymotrypsin-like serine proteases: Spirit and the Spätzle-processing enzyme (SPE) (Jang *et al.*, 2006). SPE cleaves Spätzle directly while Spirit is thought to cleave and activate SPE. Once cleaved, Spätzle binds the Toll receptor and induces dimerization. This causes the recruitment of three intracellular Death domain-containing proteins, MyD88, Tube and Pelle. Activation of the Pelle kinase leads to the phosphorylation and ubiquitin/protesome-mediated degradation of Cactus, the Drosophila IκB homolog. Degradation of Cactus frees DIF (and Dorsal) to translocate to the nucleus (Towb *et al.*, 1998; Wu and Anderson, 1998), and leads directly to the transcriptional induction of many immune responsive genes like the antimicrobial peptides genes.

Whereas activation of the Toll pathway is initiated by interaction of microbial ligands with circulating proteins, the Imd pathway is triggered by the direct interaction of the transmembrane receptor PGRP-LC (Choe *et al.*, 2002; 2005) with Gram negative bacterial peptidoglycan (diaminopimelic peptidoglycan-DAPPGN). The binding of monomeric and multimeric DAP-PGN to receptors induces their dimerization or oligomerization and leads to signaling (Mellroth *et al.*, 2005; Lim *et al.*, 2006).

The intracytoplasmic cascade of the Imd pathway starts with the recruitment of the 25 kDa death domain protein Imd (of note, the sequence of this particular death domain is closest to that of mammalian RIP1 that is TNF-receptor interacting protein) (Georgel *et al.*, 2001; Myllymäki *et al.*, 2014). Both the intracytoplasmic domain of PGRP-LC and the adaptor protein Imd contain a so-called RHIM domain (for receptor interacting protein [RIP] homotypic interaction motif) required for signaling Imd further associates with the mammalian homolog of FADD and with the caspase-8 homolog DREDD. Through mechanisms not fully understood at present, but which are likely to involve K63 ubiquitination, this upstream receptor–adaptor complex activates the MAP3 kinase TAK1.

TAK1 is associated with the homolog of mammalian TAB2, which has a conserved sequence domain known to interact with K63 polyubiquitin chains. Downstream of the TAK1/TAB2 protein complex, the Imd pathway branches into a signaling cascade, leading to Relish activation and, a second, to JNK activation. Once activated by TAK1, the IKK complex phosphorylates the NF-kB protein Relish on specific serine residues and phosphorylated Relish is cleaved into an amino-terminal transcriptional regulatory domain, which translocates to the nucleus where it binds to Relish response elements and directs expression of dedicated genes (Hetru and Hoffmann, 2009).

To date, the Toll and Imd pathways are the sole reported intracellular cascades activated by microbial ligands. Recently, bioinformatic analysis has shown that Toll and Imd pathways can synergize to increase the levels of induction of some of the immune-response genes, possibly via the formation of Dif/Relish heterodimers. Another important aspect of the antimicrobial response is the temporal activation of these two pathways. Genes regulated by Imd generally show an acute phase profile, whereas Toll target genes exhibit a late and sustained expression pattern (Lemaitre *et al.*, 1997).



The scheme resume Toll and IMD pathways leading to the synthesis of AMPs in insects

1.3 Humoral defenses

The humoral immune response of insect consists of the processes of melanization, hemolymph clotting and wound healing in response to injury. Humoral factors involved in insect immunity after infection include lysozyme, lectins and the prophenoloxidase cascade and antimicrobial peptides, usually called AMPs (Kavanagh and Reeves, 2004).

1.3.1 ProPO system

The main role in humoral defense against a wide range of pathogens is played by melanization, also responsible for wound healing as well as nodule and capsule formation.

The formation of melanin is catalysed by phenoloxidase-monophenyl L-dopa: oxygen oxidoreductase (PO) (Soderhall and Cerenius, 1998). Phenoloxidase is found in insects in its inactive form pro-phenoloxidase (ProPO) located in the hemocytes as a zymogen. ProPO is a polypeptide of approximately 80 kDa and it is released from hemocytes by rupture and is either actively transported to the cuticle or deposited around wounds or encapsulated parasite. PO catalyses the o-hydroxylation of monophenols and oxidation of phenols to quinones which then polymerise non-enzymatically to form melanin (Nappi *et al.,* 2004). Melanin is deposited within nodules, composed of aggregated hemocytes and microorganism, that form in the hemocoel cavity of heavily infected insects. Deposition of melanin coat is also frequently observed in the encapsulation response of insects to parasites or in the experimental injection of foreign objects that provoke encapsulation. The melanin capsule can block absorption of nutrients by parasites and thus contribute to their killing by starvation. In addition, cytotoxic reactive oxygen and nitrogen intermediates formed during melanin synthesis may help to kill invading organisms.

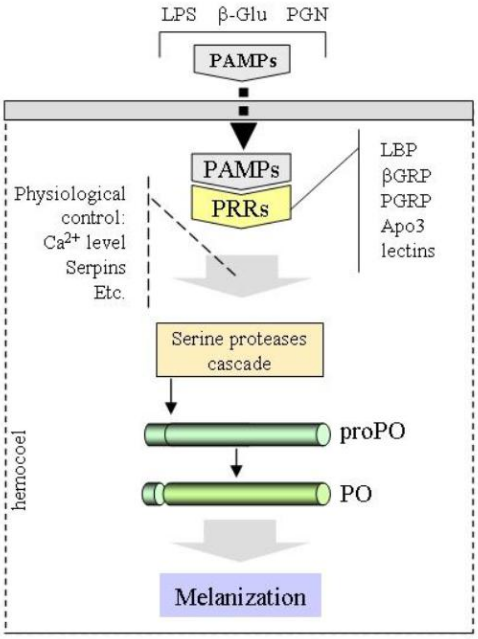
Most reports indicate that proPO is synthetized predominantly by hemocytes. In lepidopterans, oenocytoids are identifies as the cell-type producing proPO, whereas crystal

cells of *D. melanogaster* produce proPO, suggesting that oenocytoids and crystal cells may be homologous cell types (Cerenius and Söderhäll, 2004).

The process of melanisation is initiated by soluble PRRs that bind target surfaces thus initiating the serine protease cascade leading to cleavage of ProPO to PO and ultimately the cross-linking of proteins that produce melanin polymers. This process is frequently referred to as the Prophenoloxidase activating system (ProPO-AS). Several hemolymph molecules (PRRs) are involved in the proPO system activation pathway, among them β-glucans-binding proteins (β-GBP) and LPS-binding proteins (LBPs) seem to play a key role as receptors, triggering proteases cascade that turn on prophenoloxidase into active enzyme (Söderhäll, 1999). In *M. sexta* and perhaps in many insect species, peptidoglycan and β-1,3 glucan are more potent stimulators of proPO activation than lipopolysaccharide. It has been postulated a model of proPO activating pathway in *M. sexta*. An initiating protease, called hemolymph protease-14 (HP14) is able to trigger proPO system in presence of Gram positive bacteria and fungi. HP14 activates a clip domain protease, proHP21, which cleaves and activate proPAP-2 (pro proPO activating proteinase-2) and proPAP-3. SPHs (serine protease homologs) function together with PAPs to form a functional proPO activator, which cleaves proPO to form active PO.

The activation of this system produces several molecules that could harm the host insect if produced in excess. These include proteases that could degrade host proteins, cytotoxic quinones, reactive oxygen and nitrogen species. The system is regulated under most conditions to produce a local melanization response at a specific site and for a limited time. Serine protease inhibitors from several gene families have been identified in hemolymph as a regulators of proPO activation. Serpins are a family of 50 kDa proteins functioning primarily as a serine protease inhibitors. Three serpins from *M. sexta* hemolymph (serpin-1J, serpin-3 and serpin-6) directly inhibit proPO activating proteases. Proteins that directly inhibit PO rather than the activating proteases have been identified in a few insect species. A 4 kDa peptide from hemolymph of *M. domestica* is an efficient PO inhibitor; this peptide contains a

sequence motif similar to a family of toxins from snails and spiders. It has the unusual property of containing a tyrosine residue that is hydroxylated to form DOPA within the peptide (Kanost *et al.*, 2004).



Hemolymph phenoloxidase is found as a proenzyme (prophenoloxidase) that can be converted into its active form by a limited proteolysis; the activation of prophenoloxidase to phenoloxidase physiologically requires different activators, including microorganism lipopolysaccharides (LPS) and -1,3-glucans

1.3.2 AMPs

The last line of defense against the invasion of pathogens is the synthesis of a range of anti-microbial peptides (AMPs), which are produced by fat bodies cells (insect's functional equivalent of the mammalian liver) during systemic response against pathogen, and they are then released into the hemolymph. Antimicrobial peptides are gene-encoded, ribosomally synthetized polypeptides. They usually have common characteristics. They are small peptide from 6 amino acid residues to 60 amino acid residues. Peptides often contain the basic amino acids lysine or arginine, the hydrophobic residues alanine, leucine, phenylalanine or tryptophan and other residues such as isoleucine, tyrosine and valine. They are strong

cationic (pI 8.9-10.7), heat stable (100°C, 15 min) and amphipathic molecules. Since antibiotic resistance to conventional antibiotics is occurring, there is an increasing interest in the pharmacological application of AMPs (Brogden, 2005; Nakatsuji and Gallo, 2012). The important advantage of AMPs resides in the global mechanism of their action which is very different from that of conventional antibiotics. Since the first AMP Cecropin was discovered from Hyalophora cecropia in the 1980s by Boman, more than 200 peptides have been identified in insects. Insect AMPs classification could be summarized into major groups based on their secondary structure, amino acid sequence and antibacterial activity: linear amphipathic α helix-forming peptides (e.g. cecropins), β-sheets or cystine rich and cyclic antimicrobial peptides (e.g. defensin), proline rich peptides and glycine rich peptides (e.g. drosocin and coleoptericin) (Epand and Vogel, 1999; Bulet and Stocklin, 2005; Hull *et al.*, 2012).

Cecropin, an inducible antibacterial peptide, found in the hemolymph of *H. cecropia,* was the first insect peptide discovered. Cecropins are widespread throughout the animal kingdom. In insects, however, cecropins have been described only in the orders of *Diptera* and *Lepidoptera*. Mature cecropin peptides lack cysteine residues, are 35-39 amino acids and form two linear α-helices connected by a hinge, which integrate into the acidic cell membranes of bacteria leading to their disruption.

Defensins form a unique family of cysteine-rich cationic polypeptides with three or four disulfide bridges. They are mainly effective against Gram positive bacteria and also have potent activity against some Gram negative bacteria, fungi, yeast and protozoa (Zasloff, 2002; Bulet *et al.*, 2004).

Mechanism of antimicrobial peptides activity

Different techniques have been used to assess the mechanisms of antimicrobial peptide activity. Specific steps must occur to induce bacterial killing. Antimicrobial peptides must first be attracted to bacterial surfaces, and one evident mechanism is electrostatic bonding between cationic peptides and structures on the bacterial surface. Cationic antimicrobial

peptides are likely to first be attracted to the net negative charges that exist on the outer envelope of Gram negative bacteria (anionic phospholipids and phosphate groups on lipopolysaccharides) and to the teichoic acids on the surface of Gram positive bacteria. Once close to the microbial surface, peptides must pass capsular polysaccharides before they can interact with the outer membrane, which contains LPS in Gram negative bacteria, and pass capsular polysaccharides, teichoic acids and lipoteichoic acids before they can interact with the cytoplasmic membrane in Gram positive bacteria (Scott *et al.*, 1999a; 1999b). Different models have been proPOsed to explain membrane permeabilization. In the "barrel- stave model", peptide helices from a bundle in the membrane with a central lumen, like a barrel composed of helical peptides as the staves. This type of trasmembrane pore is unique ans is induced by alamethicin that adopts an α-helical configuration, attaches to, aggregates and inserts into bilayers that are hydrated with water vapor. The hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore (Yang *et al.*, 2001). In the "carpet model", peptides accumulate on the bilayers surface. This model explains the activity of antimicrobial peptides such cecropin that orientate parallel to the membrane surface. Peptides are electrostatically attracted to the anionic phospholipid head groups at numerous sites covering the surface of the membrane in a carpet-like manner. At high peptide concentrations, peptides are thought to disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles (Oren and Shai, 1998). In the "toroidal-pore model", antimicrobial peptide helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that both the inserted peptides and the lipid head groups line the water core. In forming a toroidal pore, the polar faces of the peptides associate with the polar head groups of the lipids. The toroidal model differs from the barrel-stave model as the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer (Matsuzaki *et al.*, 1996). Although the formation of transmembrane pores, ion channels and membrane rupture eventually leads to the lysis of microbial cells, there is increasing evidence that antimicrobial peptides have other intracellular targets. For example short proline-rich peptides as pyrrhocoricin, drosocin and apidaecin bind specifically to

DnaK, a 70 kDa heat shock proteina and nonspecifically to GroEL, a 60 kDa bacterial chaperone reducing the ATPase activity of DnaK and altering the refolding of misfolded proteins (Otvos, 2000; Kragol *et al.*, 2001). Defensins, proline.arginine rich peptides and α-helical peptides inhibit DNA, RNA and protein synthesis (Yonezawa and Sugiura, 1992; Subbalakshmi and Sitaram, 1998; Patrzykat *et al.*, 2002).

Microorganisms utilize numerous strategies of resistance to bypass antimicrobial peptide killing. In *Staphylococcus aureus*, products of the dlt operon reduce the net negative surface charges by transporting D-alanine from the cytoplasm to the surface teichoic acid (Peschel *et al.*, 1999). This increase of positive net charge on membrane repulse antimicrobial peptides. Gram negative bacteria reduce their sensibility to antimicrobial peptides by hindering peptide attachment to the outer membrane, reducing net negative surface charges by altering the Lipid A moiety of the LPS or by reducing the fluidity by increasing the number of hydrophobic interactions. The increased hydrophobic interaction retards or abolishes peptide insertion and pore formation. Antimicrobial resistance is also associated with the ability to either transport antimicrobial peptides into the cell by the ATP-binding cassette transporter or to export them by efflux pumps (Groisman, 1994). Bacteria produce proteolytic enzymes, which may degrade antimicrobial peptides leading to their resistance. For example, LL-37 is cleaved and inactivated by a *S. aureus* metalloproteinase called aureolysin (Sieprawska-Lupa *et al.*, 2004).

1.4 Cellular defenses

Hemocytes are responsible for a number of defense responses in insects, among which phagocytosis, nodulation and encapsulation.

1.4.1 Phagocytosis

Phagocytosis of foreign particles is universal throughout the animal kingdom, and, in many of the most primitive animals, phagocytosis is predominantly a means of nourishment.

Since the pioneering research of the Russian biologist Elie Metchnikov in the 1880s, the ubiquity of phagocytosis as a means of internal defense has become increasingly well documented. Phagocytosis is the process by which cells recognize, bind and ingest small particles such as bacteria and this process is probably the oldest defense mechanism against microorganism.

This cellular mechanism initiates with the recognition by phagocytic receptors that activate various signaling pathways. These signals lead to a dramatic changes in the dynamics of the plasma membrane and the cytoskeleton of specialized cells. The membrane extends pseudopods around the particle, forming a cup that moves into the cell. Then the membrane closes at the distal end, assembling a new plasma membrane-derived phagosome. The membrane of phagosome fuses with other membranous organelles as lysosome or endosomes to become a mature phagolysosome. This compartment has an acid environment with highly hydrolytic enzyme causing the destruction of the engulfed target. In insects, phagocytosis is performed by a subset of hemocytes in the hemolymph (Strand, 2008). Professional phagocytes in Diptera and Lepidoptera have also been described as plasmatocytes or granulocytes, respectively. Phagocytosis eliminates mainly two types of targets: microorganism and particles represented by apoptotic cells, important during tissue remodeling and embryogenesis (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007).

The process of phagocytosis in insect and mammals appears to be very similar; in both cases, the process is initiated after the interaction of opsonins on the surface of not self with specific receptors on the phagocyte membrane. Phagocytosis can also be triggered, in the absence of opsonins, through the interaction of phagocyte membrane receptors with specific molecules, such as lipids or sugars on the microorganism cell wall (Stuart and Ezekowitz, 2005; 2008). Upon invasion with Gram negative bacteria, specific lectins recognize and bind to peptidoglycans on the bacterial cell surface facilitating the binding to plasmatocytes and the phagocytosis. At the same time the hemolymphatic enzyme, lysozyme, degrades the peptidoglycan layer releasing sugars and exposing teichoic acid and lipomannans that are recognized by lectins. This process gives the insect the ability to discriminate and engulf a range of bacteria despite the changing nature of the exposed bacterial surface.

To date, phagocytosis has been shown to involve several receptors: scavenger receptor family, the EGF-domain protein Eater, the IgSF-domain protein Dscam, proteins related to CD36, PGRP family members. Thioester-containing proteins (TEPs) constitute an important group of proteins that includes the α2 macroglobulin family of protease inhibitors and the C3/C4 complements factors in vertebrates. In insect some of the TEPs are upregulated after a bacterial infection and function as opsonins to promote phagocytosis. This, therefore, suggests how the cellular and humoral arms of the innate immune system co-operate in fighting infection (Lagueux *et al.*, 2000).

1.4.2 Encapsulation

Encapsulation is a dramatic defensive response of hemocytes to large targets to undergo phagocytosis or nodulation such as both biotic objects (e.g. trematodes, cestodes, nematodes, parasitoids, eggs of parasites and parasitoids, fungi and interspecific tissue transplants) and inanimate experimental objects (e.g. Sephadex beads, nylon beads, cotton thread, glass, nylon, latex) (Götz and Boman, 1985; Götz, 1986). Two types of encapsulation are present in insects: cellular encapsulation, mainly described in Lepidoptera,

and melanotic humoral encapsulation more typical for Diptera. In contrast to melanotic encapsulation, which is always associated with PO activity, cellular encapsulation can occur without any sign of melanization. Granulocytes are generally assume to contact a foreign target and then release chemotactic components that attract plasmatocytes, which then form a multilayered capsule. Within the capsule, the parasite is eventually killed, possibly by the local production of cytotoxic products such as ROS and eventually by intermediates of the melanization cascade (Nappi *et al.*, 1995). Based on observations from a number of insects (Gagen and Ratcliffe, 1976; Peck and Strand, 1996; Lavine and Strand, 2001) generalized sequence of events has been constructed: hemocytes contact a foreign object via random movement or directed chemotaxis then the granular cells that contact the foreign object adhere and degranulate. Material discharged from granular cells binds to foreign surfaces and to hemocytes. Substances released from granular cells attract other granular cells and plasmatocytes. In fact, granular cells possess a signal that recruits and activates plasmatocytes into capsule formation. Plasmatocytes attach to the capsule, spread and flatten, forming a multilayer sheath. Granular cells attached to the target object begin to disintegrate and a second thin layer of granular cells covers the capsule that may be melanized.

In 1996, Pech and Strand noted that of all the hemocytes that compose the capsule, only the first layer has contact with the target surface, thus the signal that recruits the cells of the first layer must be different from the signal that recruits the cells that form the subsequent layers. Furthermore, because the capsule does not grow indefinitely, the signal to recruit new hemocytes for encapsulation must decrease with each increased capsule size.

A role for integrins in this process could describe the aggregation of lamellocytes in Diptera that is further supported by studies in Lepidoptera (Lavine and Strand, 2003). Two members of the Rho GTPase family, Rac1 and Rac2 seem to participate in this process regulating many aspects of cytoskeleton remodeling (Williams *et al.*, 2005).

1.4.3 Nodule Formation

Nodules are aggregates of hemocytes and extracellular coagulum that have entrapped large numbers of small-sized foreign entities. The formation of nodules is the immune response to high concentrations of non-living particles or microbes such as bacteria, fungal spores, yeast cells or protozoa (Götz and Boman, 1985; Guzo and Stoltz, 1987; Gillespie, *et al*, 1997; Chapman, 1998). Nodule formation appears to be typical for most Lepidoptera (Ratcliffe and Gagen, 1977). Within 1 minute after injection of heat-killed *Bacillus cereus* in *Galleria mellonella*, the granules within the granular cells that had randomly encountered the bacteria began to swell. These granules migrated out towards the granular cells' periphery and expelled an adhesive substance into the surrounding hemolymph, which then entrapped the bacteria. This resulted in an aggregation of granular cells and bacteria embedded in an extracellular matrix. By 5 minutes, the forming nodules were 50-100 μm in diameter. Granular cells were still undergoing degranulation as the nodule continued to enlarge. The aggregation became more compact and depositions of melanin appeared near entrapped bacteria. The edges of granular cells lost their integrity, followed by the disintegration of the granular cells' nuclei and other organelles. Two to four hours after initiation of nodule formation, plasmatocytes began to attach to the periphery and flatten in a manner similar to encapsulation. Nodule formation was complete by 24 hr and nodules ranged in diameter between 100-150 μm. The completed nodules were comprised of a central flocculent mass consisting of bacteria and the remnants of granular cells and any other hemocytes that had become entrapped, all embedded in a melanized matrix and surrounded by a multilayer of plasmatocytes. Granular cells and plasmatocytes were the only hemocytes observed to play a role in nodule formation; however, one can speculate that oenocytoids play an indirect role since this hemocyte type contains pro-PO, essential for melanization (Ratcliffe and Gagen, 1977; Götz and Boman, 1985; Gillespie, *et al*, 1997; Chapman, 1998).

1.5 Model organisms

Model organisms used in this study are, as mentioned above, insects. Insects are the most represented species all over the world; they have colonized air, water and soil.

Among them, lepidopteran and coleopteran represent two classes very important for the environment; often they are phytophagous and very harmful for various plant species**.**

1.5.1 *Rhynchophorus ferrugineus*

The red palm weevil (RPW) *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) is one of the major pests of palms in all countries where it has been accidentally introduced (Cox, 1993). It is native to southern Asia and Melanesia. This insect is reported to attack more than 20 species of palms but the two main species of concern in the Mediterranean region are date palm (*Phoenix dactylifera*) and canary island date palm (*P. canariensis*) (EPPO 2009). Adults are large, being up to 42 mm and 16 mm wide, with a long rostrum, characteristic for the weevils. They are reddish-brown in color with variable dark markings on the pronotum. Each adult female deposits between 200 to 300 eggs in separate holes or cavities on the host plant. Eggs are whitish-yellow, smooth, shiny, cylindrical with rounded ends, slightly narrower at the anterior end, and about 3 mm long and 1 mm wide. These hatch in two to five days, and larvae bore into the interior of the palms, feeding on the soft succulent tissues, discarding all fibrous material. Larvae are legless, with a creamy-white body and brown hard head capsule, and grow up to 50 mm in length. The larval period varies from one to three months. Pupation occurs in an elongate oval, cylindrical cocoon made of fibrous strands, about 40 mm in length. Adult weevils emerge 2-3 weeks after pupation. Thus, the life cycle is completed in about 4 months. Early symptoms of attack are distinctive but hard to see: egg laying notches; cocoons inserted into the base of the palms; an eccentric growing crown; holes at the base of cut palms; symptoms resembling those caused by lack of water such as wilting, desiccation and necrosis of the foliage; tunnelling

within the stems and trunk. Larvae and adults destroy the interior of the palm tree, often without the plant showing signs of deterioration unless damage is severe. Hollowing out of the trunk reduces its mechanical resistance, making the plant susceptible to collapse and a danger to the public. Pheromone traps, acoustic detection or infrared systems can be used to detect this pest (Malumphy and Moran, 2009). Recently has been verified the effect of a potential biological control against a wide range of insect pests (Ehlers, 2003), the symbiotic complex *Steinernema carpocapsae* associated with the bacterium *Xenorhabdus nematophila*.

1.5.2 *Galleria mellonella*

The larvae of the greater wax moth *G.mellonella* are 1.5-2.5cm in length. Their size enables an easy means of inoculation with specific amounts of drug or pathogen via the pro-leg making *G.mellonella* more amenable to drug pharmacodynamics studies (Fallon *et al.*, 2010). It is also feasible to assess phagocytic cell function and immune responses to determine the virulence of pathogens (Cotter *et al.*, 2000) and the actions of immunosuppressive molecules such as gliotoxin and fumigillin released from the fungus *A. fumigatus* on phagocytic functions (Fallon *et al.*, 2011; Renwick *et al.*, 2007).

The assessment of hemocyte function gives comparable results to those seen in human neutrophils due to the functional homology of phagocytosing hemocytes in *G. mellonella* (Kavanagh and Reeves, 2004). A strong correlation is observed in microbial pathogenicity in *G. mellonella* and mammalian systems (Brennan *et al.*, 2002; Jander *et al.*, 2000). *G. mellonella* are particularly suited as *in vivo* models as they have a high throughput (Cotter *et al.*, 2000) and can be incubated between 30-37°C enabling possible temperature dependent virulence factors to be studied (Glavis-Bloom *et al.*, 2012). The evolutionary conservation of several aspects of the innate immune response between invertebrates and mammals makes the use of these simple hosts an effective and fast screening method for identifying fungal virulence

factors and testing potential antifungal compounds (Kavanagh and Reeves, 2004; Arvainitis *et al.*, 2013).

More recently, the larvae have been employed to study the pathology of brain infections caused by *Listeria* which produces a comparable pathology to that observed in humans (Mukherjee *et al.*, 2013).

1.5.3 *Steinernema carpocapsae* and its symbiont *Xenorhabdus nematophila*

Insect parasitic nematodes *Steinernema carpocapsae* are nematocomplexes associated with mutualistic bacteria *Xenorhabdus nematophila*. The basic life cycle of most entomopathogenic nematodes consists of several stages: an egg stage, four juvenile stages (L1, L2, L3, L4), and a complex adult stage that comprises L5 (early adult stage) and late adult. In general, nematodes moult four times during each life cycle with a moult occurring at the end of each larval stage (Dillman *et al.*, 2012).

Therefore, moults separate the first and second larval stages (L1 and L2), the second and third larval stages (L2 and L3), the third and fourth larval stages (L3 and L4) and also the fourth larval stages and immature adults (L4 and L5). The L5 grows to the size limit of its new cuticle. The third juvenile stage (IJ3) of nematodes is known as the "infective juvenile" and is the only free-living stage (Womersley, 1993). The IJ3 is capable to survive in the soil for extended periods until it is able to find a susceptible host; its function is to locate, attack, and infect an insect host (Akhurst and Dunphy, 1993).

Host infection consists of various steps. Infective juvenile stage enters the host through natural body openings (mouth, anus, spiracles), it reaches the hemocoel of the host, and later on, it releases bacteria spores by defecation or regurgitation. After release, bacteria quickly multiply in the hemolymph; they are mainly responsible for the host mortality

because they produce and release exo- and endotoxins to which the insect succumbs by septicemia within 24-48 hours of infection, furthermore bacteria secrete antibiotics that prevent multiplication of the other microflora. Bacterial cells also express and release proteases and lipases that degrade insect host to issues that can be utilized by the parasite as a food source. After mating, the females lay the eggs that hatch as first-stage juveniles that moults successively to second, third and fourth-stage juveniles and then to males and females of the second generation The adults mate and the eggs produced by these second-generation females hatch as first-stage juveniles that moult to the second stage. The adult nematodes produce hundreds of thousands of new juveniles. The late second stage juvenile ceases feeding, incorporates a small fresh group of bacteria in the bacterial chamber, and moults to the infective juvenile stage (IJ3). When the host has been consumed, the infective juveniles emerge from the exoskeleton of the host, move into the soil and begin the search for a new host (Brivio *et al.*, 2005).

Virulence factor from S. *carpocapsae* is the key to the success of this bioinsecticide (Shapiro-llan *et al.*, 2003); the letality of the complex is attributed to the bacteria, due to their ability to excrete toxins and proteolytic enzymes (Forst *et al.*, 1997). Then, symbiotic bacteria contribute to the mutualistic relationship actively, by killing insect host, by establishing and maintaining suitable conditions for nematode reproduction and by providing nutrients and microbial substances that inhibit growth of a wide range of other microorganisms. At the same time, the nematode acts as a vector for the symbiotic bacterium. The symbiosis is essential for the efficiency of the biocontrol and it enables nematodes to exploit a diverse array of insect hosts (Dunphy and Thurston, 1990). We have to consider that the parasite does not act simply as a "troian horse" but it must avoid and or suppress the host immune responses (Goodrich-Blair, 2007).

Indeed Vinson (1977; 1990) hypothesized a key role of the body surface of parasites. He suggested that in absence of active suppressive mechanisms the lack of encapsulation could be due to the acquisition of a coat composed of host proteins (mechanism called molecular disguise), the presence of heterophilic antigens or the presence of a not reactive body surface

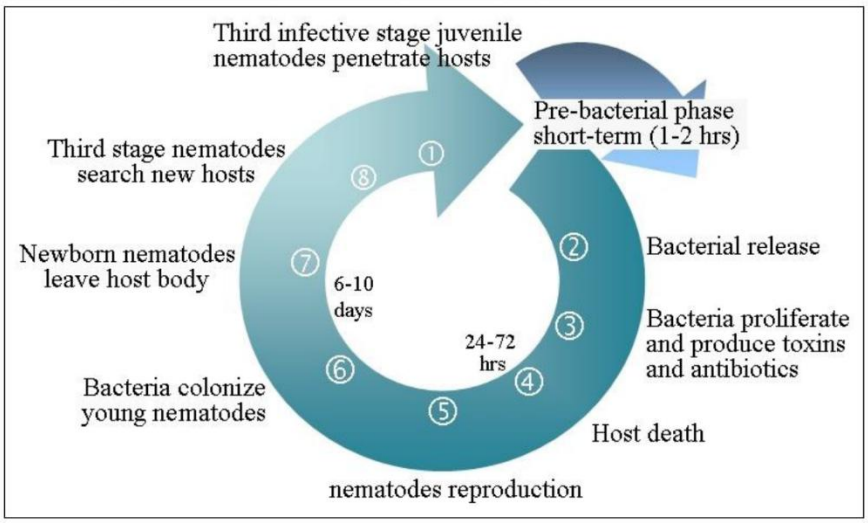
(molecular mimicry). Brivio *et al*. (2004) confirmed this hypothesis demonstrating a key role of the lipids in the interference of the parasite with host defenses.

Xenorhabdus sp. is a motile gram negative bacteria belonging to the family of enterobacteriaceae and is specifically found associated with nematodes of the Steinernematidae group but Xenorhabdus can be grown as free living organism under standard laboratory conditions. As the bacteria enter the stationary phase of their growth cycle, they secrete several extracellular products, including lipases, phospholipases, proteases and several different broad-spectrum antibiotics (Forst *et al.*, 1997; Gualtieri *et al.*, 2009; Massaoud *et al.*, 2010; Richards *et al.*, 2008; Richards and Goodrich-Blair, 2010;Jin *et al.*, 2014; Zhou *et al.*, 2013).

These degradative enzymes break down the macromolecules of the insect to provide the developing nematode with a nutrient supply, while the antibiotics suppress contamination of the cadaver with other microorganisms. Another property of *Xenorhabdus sp.* is the formation of phenotypic variant forms that can be isolated at low and variable frequencies during prolonged incubation under stationary phase conditions. The variant forms or so-called phase II cells are altered in many properties and are not found as natural symbionts in the nematode. Phase I cells represent the form of the bacteria that naturally associates with the infective juvenile nematode (Boemare and Akhurst, 1988; Sicard *et al.*, 2005).

Insects when infected by bacteria as *E. coli* and *M. luteus* usually trigger various cellular and humoral defences, as well as nodulation, phagocytosis and more specifically, the production of antimicrobial peptides directed to foreign microorganisms (Zasloff, 2002; Nappi *et al.*, 2004; Strand, 2008). Instead, when *Xenorhabdus sp.* is released into the hemolymph, seems to be not recognized and it overcomes the host immune responses by secreting toxic factors and degradative enzymes that affect the physiology of the insect leading to a general immunodeficiency (Brillard *et al.*, 2001; Stock and Goodrich-Blair, 2008; Dillman *et al.*, 2012). Several studies that *X. nematophila* impairs humoral and cellular processes (Vallet-Gely *et al.*, 2008) responsible for bacterial clearance. A study described a surface molecule

named rhabduscin on the wall of *X. nematophila* and *Photorhabdus luminescens*; it is a tyrosine derivative with a role as a potent nanomolar-level inhibitor of insect phenoloxidase (Crawford *et al.*, 2012). Moreover, LPS of *Xenorhabdus* act as a hemocyte toxin and may be responsible for the fat-body dissociation (Dunphy and Webster, 1988). *X. nematophila* secretions are able to interfere with eicosanoids pathways, impairing nodulation and phagocytosis and indirectly, AMPs synthesis even if, in this case, the exact mechanism of genes down-regulation remains to be elucidated (Ji and Kim, 2004; Park and Stanley, 2006; Aymeric *et al.*, 2010; Hwang *et al.*, 2013). However, in addition to its depressive capability, we have to consider that some *Xenorhabdus* genera seem not to be susceptible to the action of host immunocompetent factors (Duvic *et al.*, 2012). Finally, as described by Caldas *et al.* (2002), hydrolytic properties of symbionts secretions (protease II) seem to affect antimicrobial peptides. In particular, it reduced 97% of the cecropin A bacteriolytic activity.



The life cycle of entomopathogenic nematodes (Brivio MF *et al.*, 2005)

1.6 Aims of the project

In our work we have focused on the study of the relationship between entomoparasites and insect host, we have examined both host defenses processes and the evasive/depressive strategies of the parasites.

Below is a summary of the topics discussed experimentally in the project:

•To study the basic mechanisms of the innate immune recognition against not-self

•To study physiological and parasites-modulated immune responses in the insect model: investigation of constitutive processes such as ProPO system activity and hemocytic encapsulation, also considering the recognition process of immune sensing

•To study depressive and immune evasive strategies implemented by parasites (entomopathogen nematodes)

•To study the host inducible responses, analyzing the antimicrobial activity by biochemical and microbiological assays against various bacterial strains

•To check the effects of host AMPs on the bacterial cell wall by fluorescence microscopy and scanning electron microscopy

•To study the effects induces by EPNs infections on AMPs activity considering the role played by live parasites, dead parasites and their symbionts in the tripartite interaction.

•Finally we have started a study on host phagocytosis processes.

2 MATERIALS AND METHODS

2.1 Reagents and Instruments

Instruments and reagents were purchased from BioRad Laboratories (Detroit, MI, USA), Sigma Chemicals (St. Louis, MO, USA), Millipore Corporation (Billerica, MA, USA), Merck (Darmstadt, Germany) and Celbio SpA (Milan, Italy). Centrifugations were performed by an Eppendorf MiniSpin® and Eppendorf 5804R (Eppendorf AG, Hamburg, Germany). Spectrophotometric measurements were carried out using a Jasco V-560 (Easton, MD, USA). All materials and buffers were autoclaved.

2.2 Insects and Parasites

R. ferrugineus (Coleoptera, Dryophthoridae) late instar larvae were used to study relationships between insect hosts and the parasites. Larvae were collected from Canary palm trees (*Phoenix canariensis*), in Agrigento and Palermo area (Italy) and transferred (authorization MIPAF prot. 0025254) to our laboratory for experimental tests. RPW at various stages were maintained in a climatic chamber at 30 °C, in dark condition, with a relative humidity of 75%; insects were fed with a formula based on apple slices and only healthy larvae were selected for the experiments.

G.mellonella (Lepidoptera, Pyralidae) larvae were reared on a sterile mixture of food (wax, honey and wholemeal) in a thermostatic chamber, at 27 °C, 70% relative humidity, in the dark. Healthy larvae were selected before assays.

Entomophatogenic nematodes, *S. carpocapsae* (Nematoda, Steinernematidae) were provided by Koppert Biological System (Koppert BV, AD Berkel en Rodenrijs, the Netherlands); parasites are available as commercial preparation (Capsanem®) at the infective juvenile stage L3, in cryptobiosis. To maintain the parasites in infective stage the preparation was kept at 4 °C. Before assays, *S. carpocapsae* were purified from inert material and about 2-3 gr of the

formulation (nematode-clay) was dissolved in dechlorinated tap water. The suspension was layered on a sucrose gradient (75-50-25%) and centrifuged at 100 X g, for 10 min at room temperature; nematodes were recovered at the 25-50% interface then, washed several times with sterile tap water to remove contaminants.

2.3 Isolation and purification of *S. carpocapsae* cuticles

Cuticles were obtained from previously washed nematodes (as describe above). Processed parasites were suspended in 20 volumes of CEB (cuticle extraction buffer: 10mmol/L Tris-HCl, 10 mmol/L EDTA, 1 mmol/L PMSF, pH7.2) and subjected to two cycles of sonication (150 watts for 30 sec), in a Labsonic-L Ultrasonic processor (B. Braun Biotech Inc., Allentown, PA, USA). Parasites body fragments were homogenized using a Potter Dounce (B. Braun, pestle B) to remove tissue and body fluids, finally the cuticles were washed several times with 10 mmol/L of Tris-HCl pH 7.2, to remove tissue debris and contaminants. To verify the purification degree, cuticles fragments were checked by light microscopy.

2.4 Microorganisms to verify effects on host proPO system

Gram negative bacteria (*Escherichia coli* C1a), Gram positive bacteria (*Bacillus subtilis,* ATCC 6051) and yeast (*Saccharomyces cerevisiae*) were used to verify the effects on the host proPO system *in vitro*. Bacterial cultures were grown overnight at 37°C in Luria-Bertani broth(1% tryptone, 0.5% yeast extract, 1.0% NaCl) and the bacteria concentration was estimated by spectrophotometric reading of absorbance (λ = 600nm). Subsequently the cultures were centrifuged at 1700× *g* for 15 min, the bacterial pellet was washed several times with sterile PBS (138mmol/L NaCl, 2.7mmol/L KCl, 10 mmol/L Na2HPO4/KH2PO4, pH 7.4), cells were killed (20min at 95°C), then washed with sterile phosphate buffer. The bacteria strains were used at a final concentration of 103 cfu/mL. *S. cerevisiae* (0.025 g/L) was inoculated in sterile media (1% yeast extract, 2% D-glucose monohydrate, 0.05% peptone) and incubated overnightat 37°C. Cultures were centrifuged

at 1700× *g* for 10min at 20°C, pellets were harvested and washed several times in PBS, yeast cells were counted in a Bürker chamber and suspended to a final concentration of 105 cells/mL. Lastly, yeast cellswereheat-killed (at 95°C for 30 min), centrifuged and washed several times insterile buffer, aliquots of all microorganisms were immediately used or stored at −20°C.

2.5 proPO system activity of *R. ferrugineus*

proPO system relative activity of RPW larvae was monitored by spectrophotometric analysis in hemolymph cell-free samples. The activity was examined *in vitro,* both in the presence of microorganisms (*Bacillus subtilis*, *Escherichia coli*, *Saccharomyces cerevisiae*) and in the presence of isolated PAMPs compounds (Lipopolysaccharides, β-Glucan, Peptidoglycans). Cell-free fraction (CFF) supernatants were obtained by two low-speed centrifugations (200× *g*, for 10 min, at 4 °C) of whole hemolymph, previously flushed out from healthy larvae. Bacteria (2 × 102), yeast cells (2 × 102), lipopolysaccharides (10 μg), 10 μL of a saturated solution of β-Glucans, or peptidoglycans (10 μg), were added to200 μL aliquots of CFF; 30 min after incubation (under nitrogen flow at 25−26 °C) time courses of phenoloxidase activity were recorded. All kinetics were done with 5 μL of hemolymph added to 1 mL of L-Dopa (8 mmol/L L-Dopa in 10 mmol/L Tris-HCl, pH 7.2) as substrate; time courses of absorbance changes were recorded (ΔA 490nm 5 min-1, at 20°C), by evaluating the dopachrome formation from L-Dopa substrate. As control, basal activity of proPO system was analysed in extracted hemolymph samples without activators.

2.6 Effects of parasites on host proPO system

The relative activity of host phenoloxidase was recorded by spectrophotometric assays carried out after parasitization (*in vivo*), or by co-incubation of *S. carpocapsae* with host CFF (*in vitro*). To investigate the effects of parasites *in vivo* on host proPO system, 30−50 μL of a suspension containing about 20−30 (living or cold-killed) nematodes in PBS were

injected into the hemocoelic cavity of *R. ferrugineus*. The parasitization was carried out by microinjection into host larvae in order to establish a "time zero", required for a correct kinetic of activation. Injections were performed using micro-syringes Hamilton (mod. Gas-tight), equipped with thin needles (diameter 0.13 mm internal, 0.26 mm external). Thirty minutes after parasites injection, larval hemolymph was collected and the CFF was used for spectrophotometric measurements. For *in vitro* assays, about 30-50 nematodes or cuticles fragments, were added to host CFF aliquots (200 μL), after 30 min of incubation under a gentle nitrogen flow at 25−26 °C, parasites (or cuticles) were pelleted by centrifugation (200× *g* for 10 min) and the relative activity of phenoloxidase was recorded in supernatants as describe above. All kinetics assays were carried out as described in subsection 2.6. As controls in all *in vivo* assays, larvae were injected with sterile PBS.

2.7 RPW hemocytes primary cultures

To investigate *in vitro* the process of cellular encapsulation we established primary cells isolated from the hemolymph of *R. ferrugineus.* Briefly, healthy larvae were sterilized in 70% ethanol, anesthetized on ice and bled by puncturing the dorsal vessel by a sterile needle. Hemolymph was flushed out in a refrigerated sterile Eppendorf tube with anticoagulant buffer (98 mmol/L NaOH,145 mmol/L NaCl, 17 mmol/L EDTA, 41 mmol/L citric acid, pH 4.5), to avoid undesired cells degranulation. Cells were separated by low speed centrifugation (200× *g* for 10 min at 4°C), humoral fraction was discarded and collected hemocytes washed with sterile PBS, the procedure was repeated a few times to avoid any contamination by tissues or cells debris. Hemocytes were suspended in a complete culture medium (10% fetal bovine serum, 1% antibiotic antimycotic, 1% glutamine in Grace's insect medium), and 2 × 105 cells were cultured in 96 microwells plates (Cell cultures cluster, flat bottom, Iwaki) and kept at 25−26 °C, in a humidified incubator (Cellstar) without CO2.

2.8 Encapsulation assays

To examine the ability of host hemocytes to encapsulate foreigners such as nematodes (live, heat-killed and cold-killed), parasites isolated cuticles and synthetic microbeads and we carried out *in vitro* co-incubations of host primary cells with potential targets. Before assays, all the targets samples, were washed in sterile buffer and resuspended in Grace's insect medium, after 30 min from the hemocytes adhesion to the substrate, targets were added to cells cultures at a concentration of about 10−15 units/well. The encapsulation process was examined at two and eight hours after the start of incubation, observations were made under an inverted microscope (Olympus I×51) and images were acquired by a digital system (Nikon digital camera DXM1200F). For *in vivo* assays a slight modification of the procedure described above was used (cold-killed and heat-killed nematodes were suspended in sterile PBS, before injection into larvae); 30 min, or two hours, after hosts infection, larvae were bled and extracted nematodes and hemocytes were observed by microscopy.

2.9 Bacterial strains and culture conditions to induce AMPs synthesis and verify antimicrobial activity

Gram-negative (*E. coli C1a*, *Pseudomonas sp. OX1*) and Gram-positive (*B. subtilis ATCC 6051*, *M. luteus ATCC 4698*) bacterial cultures were used for larval immunizations and AMP activity assays. After inoculation in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), bacterial cultures were grown overnight (16 h) under shaking at 37°C, in a dark room. Bacterial growth was verified by spectrophotometric measurement of biomass (λ = 600 nm). Briefly, for immunization assays, cultures were centrifuged at 1700 g for 10 min at 20°C, the bacterial pellet was recovered and then cells were killed by heating at 65°C for 1 h. Before injections, bacteria were washed several times with sterile PBS (138mM NaCl, 2.7mM KCl, 10mM Na2HPO4/KH2PO4, pH 7.4); finally, bacterial strains

(a mixture 1:1 of *E. coli* and *B. subtilis*) were injected in RPW larvae at a final concentration of 10^5 CFU/ml, or stored at -20°C with glycerol (1:1 v/v).

2.10 AMP synthesis in RPW larvae

In order to induce the synthesis of AMPs in *R. ferrugineus*, larvae were infected with killed or live microorganisms, or a mix of purified LPS and peptidoglycan (PGN). Fifty microlitres of bacterial suspension (10^5 CFU/ml dead bacteria or 10^3 CFU/ml live bacteria), or 20 mg of LPS/PGN mixture, were injected using Hamilton microsyringes (model gas-tight) equipped with thin needles (0.13mm internal diameter, 0.26mm outer diameter); after 24 h of infection, hemolymph was collected. Immunized and naïve larvae were anaesthetized on ice, sterilized with 70% ethanol and bled by puncturing the dorsal vessel by a sterile needle. Hemolymph was flushed out in a refrigerated sterile microfuge tube containing a few 1-phenyl-2-thiourea crystals to avoid activation of prophenoloxidase enzyme. Humoral fractions were collected by increasing centrifugation (200, 400 and 1500 g for 10 min at 4°C) to remove cells and tissue debris. Whole hemolymph was processed to obtain low molecular mass (LMM) fractions (cut-off 30 kDa and 10 kDa) by means of centrifugal filter devices after centrifugation at 1500 g for 4 h at 15°C. Total proteins were estimated by Bradford protein assay, calibrated on BSA (Bradford, 1976). All samples were used immediately or stored at -20°C.

2.11 Antimicrobial activity in hemolymph of *R. ferrugineus* larvae

To evaluate antimicrobial activity in the humoral fraction of RPW hemolymph, bacterial cells were grown overnight (as described above) and then diluted with LB broth to 10^6 CFU/ml for microbroth dilution assays, carried out in 96-microwell plates. Aliquots (20 ml) of whole hemolymph or fractioned hemolymph (<30 kDa and 10 kDa), with different amounts of AMP, were added to 180 ml of bacterial suspension and then incubated for 3 h at 37°C under shaking. After incubation, 100 ml of each sample was placed in a well of a 96-

well microtiter plate, samples were serially diluted with phosphate buffer (61.4mM K2HPO4, 38.4mM H2PO4) and finally, they were plated on solid agar and bacteria colonies counted after incubation at 37°C for 24 h. The antibacterial activity in hemolymph samples was intended as the percentage of bacterial survival compared with the control (bacterial suspension incubated without RPW hemolymph). The final concentration of proteins used for antimicrobial activity tests were as follows: versus *E. coli*, whole hemolymph 500 mg/ml, <30 kDa and <10 kDa fractions, 15, 75, 150 mg/ml; versus Pseudomonas, <30 kDa and <10 kDa fractions, 75 and 150 mg/ml; versus *B. subtilis*, <30 kDa fraction, 15, 75, 150 mg/ml and <10 kDa fraction, 75 and 150 mg/ml; versus *M. luteus*, <30 kDa fraction, 75 and 150 mg/ml, <10 kDa fraction, 75 and 150 mg/ml.

2.12 Effects of lysozyme and lysozyme inhibition on antimicrobial activity versus *B. subtilis*

Hemolymph fractions from RPW larvae were assayed against B. subtilis in the presence of lysozyme or lysozyme inhibitor [L-histidine methyl ester dihydrochloride (LHMED)]. Hemolymph samples, 150 mg/ml of either <30 kDa or <10 kDa fractions, were incubated with 10^6 CFU/ml of *B. subtilis* in the presence of 100mM LHMED or lysozyme (2 mg/ml), respectively, for 3 h at 37°C, under shaking in a dark room. Finally, antimicrobial activity was evaluated by microbroth dilution assay, as described above.

2.13 SDS- and 2D-PAGE analysis of larvae hemolymph

Whole (cell-free) and fractioned hemolymph (<30 kDa and <10 kDa) from non-immunized and immunized larvae were analysed by monodimensional SDS-PAGE 16% (Laemmli, 1970), Tricine-PAGE (Schägger and Von Jagow, 1987) and 2D electrophoresis (O'Farrell, 1975). 2D-page was performed using an Ettan IPGphor II Isoelectric focusing System unit (GE Healthcare), according to the standard manufacturer's protocols. Samples were focused on an immobiline Drystrip (13 cm; pH 3-10), Isoelectric focusing (IEF) strips

were loaded onto a Protean II Cell System (Bio-Rad, Hercules, CA, USA), proteins were separated by Tricine-PAGE (16%) and revealed by silver staining. Molecular mass determination was carried out following the method of Weber and Osborn (Weber and Osborn, 1969).

2.14 RBC lysis in the presence of RPW hemolymph

The lysis of human RBCs was checked by recording hemoglobin release into the extracellular environment after the incubation of blood cells with whole and fractioned hemolymph. Approximately 10 ml whole blood was drawn into K3 EDTA vacuum tubes (BD Vacutainer, Becton Dickinson Inc., USA), from healthy volunteers. Blood testing solution was prepared by washing 3 ml fresh human blood with 7 ml of pyrogen free PBS (137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 2mM KH2PO4, pH 7.4); the suspension was centrifuged at 400 g for 10 min at room temperature (20°C), and washes were repeated until the supernatant turned clear. RBC pellets were diluted to 20 ml with PBS, and 180 ml of diluted blood was added to 20 ml of whole hemolymph from naïve larvae, <30 kDa hemolymph fractions from naïve larvae, and either <30 or <10 kDa hemolymph fractions from immunized larvae. All the samples were incubated for 30 min at 37°C under gentle shaking. Positive or negative controls were performed by adding to blood samples 20 ml of 0.2% Triton X-100 or PBS respectively. Samples were centrifuged at 400 g for 5 min and 100 ml of supernatants were diluted to 1 ml with PBS. Finally, cells lysis was assessed by measuring the OD (λ =404 nm) with a JASCO V-530 UV/VIS spectrophotometer. The percentage of hemolysis was calculated as follows:

$[(A_{peptide} - A_{PBS})/(A_{Triton\text{-}X100} - A_{PBS})] \times 100.$

2.15 Fluorescence microscopy of propidium iodide uptake

Bacteria from overnight cultures were diluted 1:10 (v/v) with LB and cultured until mid-log phase OD 0.6 (λ = 600 nm). Then, AMP samples (hemolymph fractions <30 kDa and <10 kDa) were added to cell cultures to a final concentration of 150 mg/ml. All assays were performed with $2.5x10^7$CFU/ml of *E. coli* and *B. subtilis*, incubated at 37°C in a thermomixer, shaken at 800 rpm. Microscope observations were carried on bacteria after various incubation times (up to 3 h). Propidium iodide (PI; 5 mg/ml) was added at 20°C and samples drops (10 ml) were placed in glass bottom (0.170-mm thick round glass cover slip) observation chambers (Okolab, Ottaviano, Italy) and then covered with a small round glass cover slip in order to trap cells, reduce their mobility and medium evaporation.

2.16 SEM

AMPs treated or not treated with aliquots of *E. coli* or *B. subtilis* suspensions (10^6 CFU/ml) were fixed with 2.5 % glutaraldehyde in 100mM PBS (pH 7.4) overnight at 20°C, and then post-fixed in 1% osmium tetroxide, dehydrated with graded ethanol, dried by the critical point method and coated with gold. Observations were made under a SEM LEO-1430 (Carl Zeiss, GmbH) scanning electron microscope.

2.17 Isolation of symbiotic bacteria and culture conditions

Xenorhabdus nematophila was obtained from the hemolymph of *G. mellonella* infected with IJs of *S. carpocapsae*. About 20 nematodes were surface-sterilized by immersion in 2,5 % sodium hypochlorite for 5 to 10 mins after being washed several times in sterile water, they were suspended in sterile PBS and injected into *G. mellonella* larvae. Dead *G. mellonella* larvae were surface-sterilized in 70% alcohol and hemolymph was streaked onto nutrient agar (NBTA) plates and incubated at 28°C for 48 h. Isolation medium (NBTA) consisted of Nutrient Agar (0.5% Peptone, 0.3% beef extract/yeast extract, 1.5% agar and 0.5% NaCl) supplemented with bromothymol blue (25 mg ml^{-1}) and triphenyl-2,3,5-tetrazolium

chloride (4 mg ml^{-1}). To obtain pure cultures, dark blue colonies were selected and subcultured onto fresh NBTA plates, from which single colonies were picked and plated onto NA. The identity of the bacterial symbiont was confirmed by several morphological and microscopic techniques (Boemare and Akhurst, 1988). To ensure the purity of the sample, a pure colony was sequenced by BMR Genomics, Padova. *X. nematophila* has been grown in Luria-Bertani broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) overnight at 28 °C under shaking in a dark room; growth has been verified by spectrophotometric measures of biomass (λ= 600nm). *X. nematophila* living, heat-killed (65 °C for 2 h), UV-killed (1 h of irradiation), or a mixture of *E. coli*/*B. subtilis* (1:1 v/v) heat-killed at 65°C for 2 h, have been used for immune-challenges. Bacteria have been washed several times with sterile PBS and immediately used.

2.18 Infection of RPW larvae with *S. carpocapsae* nematocomplexes or symbiotic bacteria

To evaluate the interference of *S. carpocapsae* complex or of isolated *X. nematophila* on the synthesis of antimicrobial peptides in RPW larvae, we have carried out various infection assays. Briefly, 30-40 parasites, living or cold-killed (-20°C for 48 h), or 10^3 living or 10^5 killed symbionts bacteria have been injected into the hemocoelic cavity of RPW larvae, using a Hamilton gas-tight syringe (Hamilton, Reno, NE, USA) with 0.21 mm needles.

Twenty-four hours post-infection, host hemolymph samples have been collected in the presence of few PTU (1-Phenyl-2-thiourea) crystals to avoid activation of prophenoloxidase. Hemolymph samples have been centrifuged several times (500 to 1500 x g for 10 min at 4°C) to remove cells, tissue debris and bacteria. Humoral fractions have been processed to obtain low molecular weight protein pools (LMW, *cut-off* 30 kDa) by centrifugation at 1500 x g for 1 h at 15 °C on centrifugal filter devices (Amicon, Merck Millipore Ltd). As positive immunization control, a mixture of *E. coli*/*B. subtilis* has been injected; moreover, to further

evaluate immune stimulation/evasion properties of parasites body-surface, isolated parasites cuticles (about 50 fragments) have been injected into RPW larvae. Total protein content has been estimated by Bradford protein assay, calibrated on bovine serum albumin. Finally, antimicrobial activity in all samples has been evaluated by microbiological assays performed vs. *E. coli* or *X. nematophila*.

2.19 Sequential co-infection of RPW larvae with *S. carpocapsae* and exogenous bacteria, *or with X. nematophila* and exogenous bacteria

The ability of *S. carpocapsae* or *X. nematophila* to modulate AMPs synthesis in the presence of exogenous bacteria (mixture of *E. coli*/*B. subtilis*) has been tested by assays of double-infection. Briefly, after injections with parasites or *X. nematophila*, larvae have been re-infected (30 min, 2 h, 4 h and 5 h after) with exogenous bacteria mix (10^5 *E. coli*/*B. subtilis*). Alternatively, exogenous microorganism infection has been performed before injections of parasites or symbionts. After 24 hours , cell free fractions (CFF) samples were collected and fractioned by centrifugal filter devices (Amicon, *cut-off* 30 kDa), protein concentration has been determined and microbiological assays vs. *E. coli* or *X. nematophila* have been carried out.

2.20 Microbiological assays

To assess antimicrobial activity in humoral fraction of RPW hemolymph, bacteria (*E. coli* and *X. nematophila*) have been grown overnight (as described above), then diluted with LB broth to 10^6 CFU/ml for microbroth dilution assays and carried out in 96 microwells plates. Aliquots of fractioned hemolymph (150 µg/ml) have been added to bacterial suspension, then incubated for 3 h at 37 °C or 30°C under shaking. After incubation, 100 µl of each sample has been serially diluted with phosphate buffer (61.4 mM K_2HPO_4, 38.4 mM KH_2PO_4) and finally, they have been plated on solid agar. Bacteria colonies have been counted after incubation at 37°C or 30°C for 24 h.

2.21 Extraction of LPS from symbiotic bacteria *X. nematophila*

Phenol/water extracts from bacterial have been isolated by LPS extraction kit (iNtRON Biotechnology) according to the manufacturer. Briefly, bacterial cells (1 ml of 10^9 CFU/ml of *X. nematophila*) have been harvested by centrifugation at 10.000 g for 5 min at room temperature and pellets washed twice in PBS. 1 ml of Lysis Buffer has been added to samples, then vortexed until the cell clump disappeared. After addition of 200 µl chloroform, the mixture has been vortexed for 30 s and incubated at room temperature for 5 min. The mix has been centrifuged at 10.000 g at 4°C for 10 min, the upper phase (400 µl) has been transferred to a new test tube. Eight hundred µl of *Purification Buffer* have been added and samples incubated for 10 min at -20 °C.

After centrifugation (10.000 g at 4 °C for 15 min), pellets have been washed with 1 ml of 70% ethanol, air-dried and dissolved in 10 mM Tris-HCl buffer (pH 8.0), solubilisation has been obtained by sonication (60 °C for 5 min).

30 µg of purified *X. nematophila* LPS have been injected into RPW larvae, after 24 hours host hemolymph has been collected, then fractioned as described previously and antimicrobial activity has been assessed. 30 µg of LPS from *E. coli* (Serotype 055:B5, Sigma-Aldrich) have been injected as control.

2.22 Tricine-PAGE analysis of hemolymph peptidic pattern from RPW larvae and SDS-PAGE analysis of purified lipopolysaccharides (LPS)

With the aim to analyse hemolymph proteins and peptides in naïve or treated-larvae, low molecular weights hemolymph samples <30 kDa from RPW larvae have been analysed by monodimensional 16% Tricine-PAGE (Schagger and Von Jagow, 1987) in a Mini

PROTEAN® 3 Cell (Bio Rad). Electrophoresis has been carried out at 120 V (constant voltage) and after separation, proteins have been revealed by conventional silver staining.

For lipopolysaccharides analysis, 5 µg of LPS purified from *X. nematophila* have been separated on 12% SDS-PAGE and visualized by silver staining method modified by a previous oxidation with 7 mg/ml of periodic acid (SIGMA) added to the fixative solution. As control, 5 µg of LPS from *E. coli* (Serotype 055:B5, SIGMA) have been separated and stained as described above.

2.23 Phagocytosis assays

Larvae of G. mellonella were surface-sterilized with ethanol (70%) and hemolymph was collected in eppendorf tubes, by puncturing prolegs of last instar larvae. Hemolymph was immediately diluted (1:1, v/v) with anticoagulant buffer (20 mM EDTA in PBS buffer) and centrifuged at 250×g for 10 min at 4 °C, to separate hemocytes and humoral fraction. Hemocytes were washed two times with anticoagulant buffer by centrifugations (250×g for 5min at 4 °C). Finally, cells were resuspended in 400 µl of Grace's Insect Medium (Sigma) plus 10% fetal bovine serum, 1% glutamine, and 1% antibiotic/antimycotic solution (10,000 units penicillin G, 10mg Streptomycin, 25 g amphotericin B ml^{-1}. 50 µl of hemocytes solution were added to 450 µl of Completed Grace Insect medium and cultured in microwells (24-well culture plates, Flat Bottom, Corning Inc., Costar) and incubated at 26 °C for 24 h. All preparations and assays were carried out in sterile conditions.

To evaluate phagocytosis activity, bacterial visibility was enhanced by a conventional FITC (fluorescein isothiocyanate) or TRITC (tetramethylrhodamine isothiocyanate) labeling method. Briefly, an overnight bacteria culture were pelletted at 2800×g, for 15 min and washed several times with PBS buffer (pH 7.4). *E. coli*, *M. luteus* and *X. nematophila* were heat-killed at 65 °C for 90 min; then bacteria were centrifuged and washed in PBS buffer.

Bacteria were suspended in 0.1 M sodium carbonate-bicarbonate buffer pH 9 with 1mgml^{-1} FITC/TRITC in DMSO, incubated in the dark on a shaker for an hour at 25°C. Finally, bacteria were washed in PBS buffer to remove unbound FITC/TRITC, then immediately used or stored at -20 °C. We also have used pHrodo-labeled *S. aureus* obtained from Invitrogen and resuspended according to the manufacturer's protocol. pHrodo is a dye that is non fluorescent at neutral pH and bright red in acidic environments (e.g., phagolysosomes).

To evaluate in vitro phagocytosis activity, hemocytes of *G. mellonella* were co-incubated with *E. coli*, *M. luteus* or *X. nematophila*. Bacteria were added to a final concentration of 5 x10^5 ml^{-1} and incubated for at least 2h. Trypan-blue quenching (2mg ml^{-1} in Grace medium, for 20 min) was used to discriminate non-phagocytized bacteria. Phagocytosis activity was analized by fluorescence microscope (Olympus IX81)

2.24 Data processing and statistical analysis

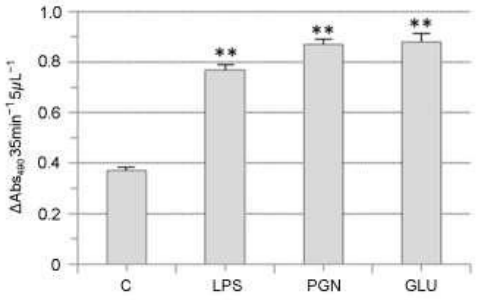
Statistical analyses were performed using the Student's unpaired t-test, the Fisher's exact test and the one-way analysis of variance (ANOVA) followed by post-hoc analysis Dunnet's t-test. the Means and SD were calculated in all assays; a P-Value <0.05 was considered statistically significant. All experiments were replicated at least five times. Data were processed with GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) and R version 3.0.2 (Free Software Foundation, Boston, MA, USA).

3 RESULTS

3.1 The proPO system of RPW

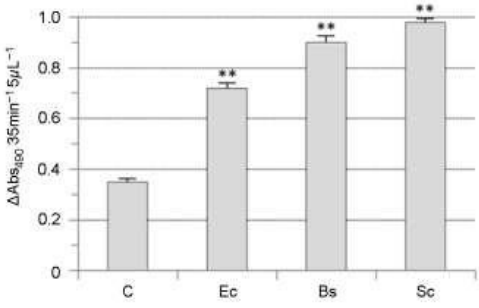
To evaluate the activity of phenoloxidase, in the hemolymph of larvae both in physiological conditions and in the presence of microorganisms or their PAMPs, spectrophotometric analysis was performed.

As shown in Fig. 1A, the presence of lipopolysaccharides (from E. *coli*), peptidoglycans (from *B. subtilis*), or β-glucans (from S. *cerevisiae*), resulted in an evident increase of activity (Fig. 1A, LPS, PGN, GLU); absorbance recorded values were, on average, more than twice as compared to the control (Fig. 1A, C).



A

Sample	Means ± SEM
C	0.37 ± 0.020
LPS	0.77 ± 0.031
PGN	0.87 ± 0.032
GLU	0.89 ± 0.049



B

Sample	Means ± SEM
C	0.35 ± 0.019
Ec	0.72 ± 0.035
Bs	0.90 ± 0.043
Sc	0.98 ± 0.027

Fig. 1(A). *In vitro* host proPO system modulation in the presence of various PAMPs. C:control LPS: lipopolysaccharides, PGN: peptidoglycans, GLU: β-glucans. (B) *In vitro* host proPO system modulation in the presence of various microorganisms. C: control; Ec: *Escherichia coli*; Bs: *Bacillus subtilis*; Sc: *Saccharomyces cerevisiae*. Mean ± SEM, $n = 5$. **p< 0.0001.

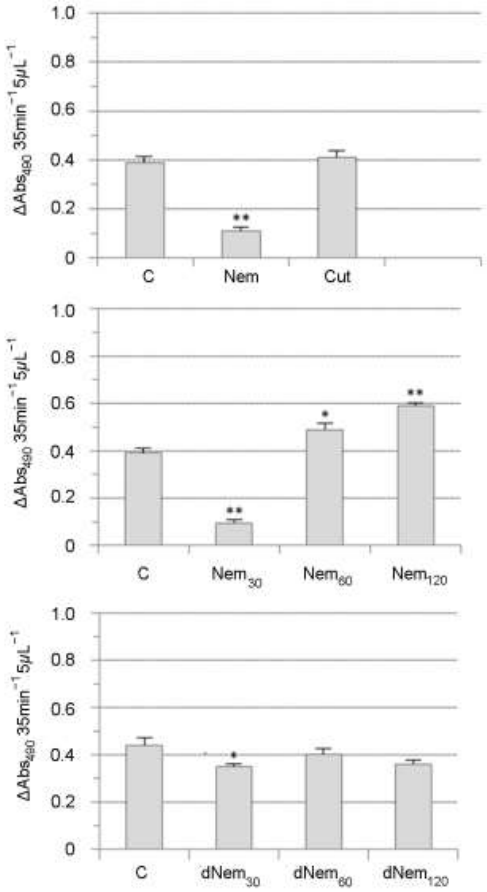
The proPO system triggered by PAMPs was comparable to that obtained in presence of microorganisms (Fig. 1B); graphs Ec, Bs and Sc show an increase of activity in hemolymph samples incubated with *E. coli*, *B. subtilis* and *S. cerevisiae* respectively. Since the extraction procedures induced a basal activity of the phenoloxidase it was considered as control (Fig. 1A and 1B, C).

3.2 Parasite-induced host proPo System modulation

The effects of *S. carpocapsae* on ProPo system modulation were assessed by *in vitro* and *in vivo* assays. Fig. 2 highlights that the presence of whole parasite reduced significantly the activity of proPo system compared to the value of basal activity in the hemolymph (Fig.2A, C), on the contrary, cuticles do not interfere with the activity of phenoloxidase.

After the *in vitro* investigation of the effects of nematodes, *in vivo* assays were performed by injecting viable parasites inside the hemocoelic cavity of host larvae. In the early phase of infection (Fig. 2B, Nem 30) a level of inhibition comparable to that recorded in the in vitro assay, was observable (Fig. 2B, Nem). After 60 minutes, a marked activation of proPO system was observed (Fig. 2B, Nem 60, Nem 120).

Fig. 2C shows the proPO activity after injection of cold-killed nematodes; in all the analyzed periods (Fig. 2C, dNem 30, dNem 60, dNem 120) a weak inhibition was evident when compared to the control (Fig. 2C, C).



A

Sample	Mean ± SEM
C	0.39 ± 0.024
Nem	0.11 ± 0.021
Cut	0.41 ± 0.033

B

Sample	Mean ± SEM
C	0.39 ± 0.025
Nem_{30}	0.09 ± 0.017
Nem_{60}	0.51 ± 0.034
Nem_{120}	0.58 ± 0.011

C

Sample	Mean ± SEM
C	0.44 ± 0.035
$dNem_{30}$	0.35 ± 0.014
$dNem_{60}$	0.40 ± 0.031
$dNem_{120}$	0.36 ± 0.022

Fig. 2(A): In vitro host proPO system modulation in the presence of parasites and isolated cuticles. C: control; Nem: *S. carpocapsae*; Cut: *S. carpocapsae* isolated cuticles. Mean ± SEM, n = 5, **: $p < 0.0001$. (B) In vivo host proPO system modulation in the presence of live parasites at various times. C: control, Nem 30'-60'-120': *S. carpocapsae*-injected larvae, 30, 60 and 120 min after infection. Mean ± SEM, n = 5,**, $P < 0.0001$, *: $p < 0.05$. (C) In vivo proPO system modulation in the presence of cold-killed parasites. C: control, dNem 30'-60'-120': dead *S. carpocapsae*-injected larvae, 30, 60 and 120 min after infection. Mean ± SEM, n = 5, *p :< 0.05.

3.3. Cellular encapsulation of abiotic not self targets

We initially evaluated the ability of hemocytes to recognize and encapsulate multicellular organisms by performing in vitro interaction assays, so entomopathogenic nematodes were co-incubated with viable hemocytes in culture. Main hemocytes

populations from late stage larvae of *R. ferrugineus* are showed in Fig. 3 (A and B), granulocytes (G), plasmatocytes (P) and larger oenocytoids (Oe) are clearly observable. Granulocytes (Gr) are rounded cells with a small nucleus; in the cytoplasm several granules (detected by focusing through the cells) are present. Plasmatocytes (Pl) are also evident; they are easily recognized by their spreading behavior. Oenocytoids (Oe) are larger round cells, they are generally refractive and occasionally the nucleus in peripheral position can be seen.

Encapsulation properties of RPW larvae hemocytes were assessed *in vitro* in the presence of inert materials, the process was tested versus synthetic microbeads. As shown in Fig. 3, RPW immunocompetent cells are able to react against not self. Agarose beads were co-incubated with hemocytes; after 2 hrs (left), both plasmatocytes and granulocytes move towards and surround the bead, in panel right (8 hrs), beads are enclosed by several cellular layers and melanin within the capsule is evident.

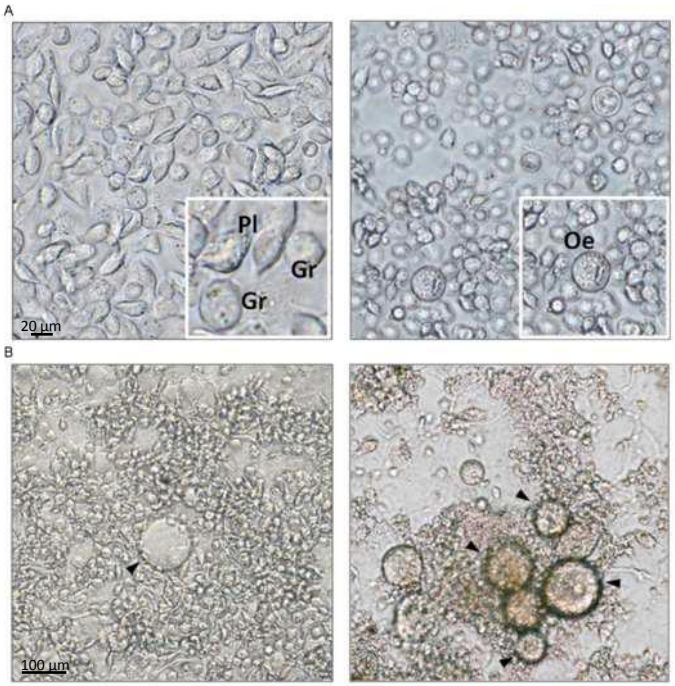


Fig. 3. In vitro assay with *Rhynchophorus ferrugineus* hemocytes.

(A) Micrographs show main cell populations from last instar larvae.
Gr: granulocytes;
Pl: plasmatocytes;
Oe: Oenocytoids

(B) Agarose beads were co-incubated in vitro with larvae hemocytes. Left (2 h), hemocytes are close to the bead and the encapsulation process begins; right, (8 h), various beads are enclosed by the cells and melanin formation is evident inside the capsule.

3.4 Cellular response against parasites (*in vitro*)

Besides reaction against abiotic targets, we tested the host cellular response against live and dead parasites. Co-incubations with *S. carpocapsae* were very useful to investigate the potential ability of the parasite to evade cell immune surveillance, as well as to ascertain the role of immunocompetent cells to recognize and to react against not self.

Fig. 4 shows *in vitro* incubations of host hemocytes with live and dead *S. carpocapsae* (left and middle). After eight hours of incubation, both live and cold-killed parasites were not recognized and not encapsulated. Melanin formation is absent in the culture. In order to verify if the presence of parasites affected cell health and functions, nematodes and microbeads were incubated concurrently with host hemocytes (right); the presence of cold-killed parasites did not interfere with hemocytes encapsulation properties since host cells were able to encapsulate beads (arrowhead).

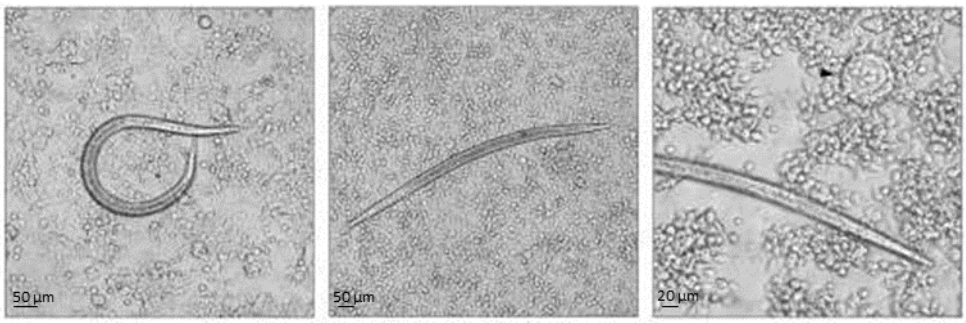


Fig.4. S. *carpocapsae* living (left) and cold-killed (middle) were incubated in vitro with host hemocytes (right). In both cases parasites were not recognized and encapsulation process was not observed, (100x). In right photo, nematodes and microbeads were co-incubated, even if in the presence of parasites cells are able to encapsulate agarose beads (arrowhead).

It is therefore evident that the nematode seems to have mimetic abilities and it is not recognized by the cells, therefore no encapsulation was observed. The elusive role of the

parasite body-surface was further verified by encapsulation assays against isolated cuticles, as shown in Figure 5 (left and middle, 2 hours and 8 hours post incubation, respectively), cuticles were not detected by host cells, no migration and encapsulation were observed. However, when parasite body-surfaces were damaged by heat-treatments, *S. carpocapsae* seems to lose its mimetic properties; as shown in Figure 5 (right), cuticle-damaged parasites are recognized and multilayered capsules of host hemocytes surround the structure, host cells mainly involved in this process are granulocytes and plasmatocytes. Cell migration towards parasites were usually preceded by the formation of several stretched motile pseudopodia (Fig.5 left, arrowheads).

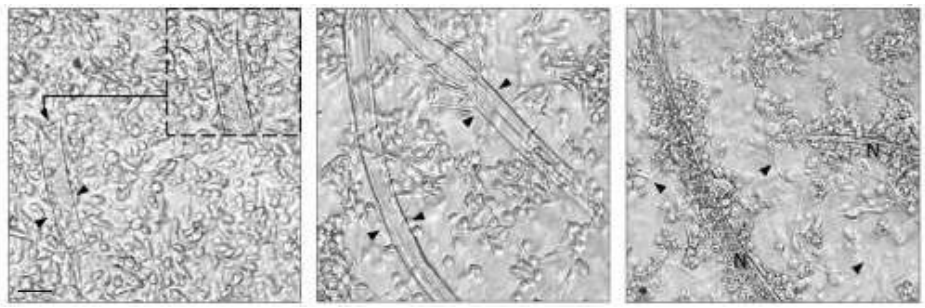


Fig. 5. Immune evasion properties of the parasite body-surface. Isolated cuticles were incubated with host hemocytes for 2 hours (left) and 8 hours (middle), either at short or long in vitro incubations, cuticles seem to avoid cell recognition. In right, heat-induced cuticle damage resulted in the lost of the mimetic properties of the parasite, thus a strong encapsulation was observed. Bar= 30µm

3.5 Cellular response against parasites (*in vivo*)

The *in vivo* host-parasite interaction assays were carried out by microinjection of nematodes into the host hemocoel (Fig. 6). Cold-killed parasites show mimetic properties (Fig. 6, left); the lack of cell responses was comparable to that observed *in vitro*. In contrast, cellular and humoral reactions were faster and stronger respect to *in vitro* assays when heat-killed parasites were injected. After 30 minutes and after two hours (Fig. 6, middle and right

respectively) is, clearly, visible the formation of dark melanin aggregates around a parasite (arrowheads) and hemocytes are enclosed inside the dark capsule.

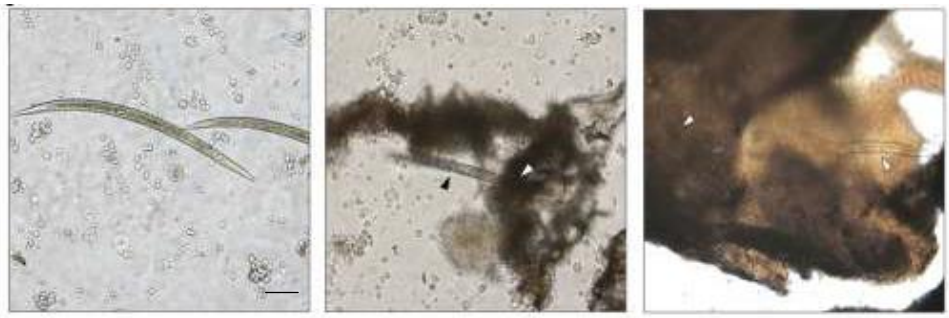


Fig. 6 In vivo humoral and cellular reaction against S. *carpocapsae*: cold-killed (left) and heat-killed (middle and right) nematodes were injected into RPW larvae hemocoelic cavity, then larvae were bleeded, hemocytes and parasites were placed in microwells and observed under a light microscope. In left Figure, cold-killed parasites after 2 hours from injection were not encapsulated. Middle and right Figures show assays carried out with heat-killed nematodes 30 min (middle) and 2 hours (left) after injection, remarkable formation of melanin was evident. Bar= 50µm

3.6 Antimicrobial peptides (AMPs): proteins and peptides analysis in RPW hemolymph

The occurrence of newly synthesized proteins and peptides was studied by electrophoretical methods; the effects of the presence of microorganisms were assessed examining the proteins pool before and after injections (examined samples were from naïve and immunized larvae).

In Figure 7, left panel (SDS-PAGE), patterns of samples from naïve larvae, larvae immunized with a LPS/PGN mixture of PAMPs and larvae immunized with Gram-positive/Gram-negative bacteria mix are depicted. Differences in patterns are evident even if the SDS-PAGE does not provide a clear resolution of low molecular weights compounds. Instead, an analysis by Schagger-PAGE (Figure 7, right panel) shows an evident variation in

protein patterns in the region <30. The right-hand panels in Figure 7 (A and B) show electrophoretic patterns of hemolymph samples <30 kDa and <10 kDa, respectively.

New bands are clearly evident in the region between 5 kDa and 20 kDa of immunized sample (arrowheads).

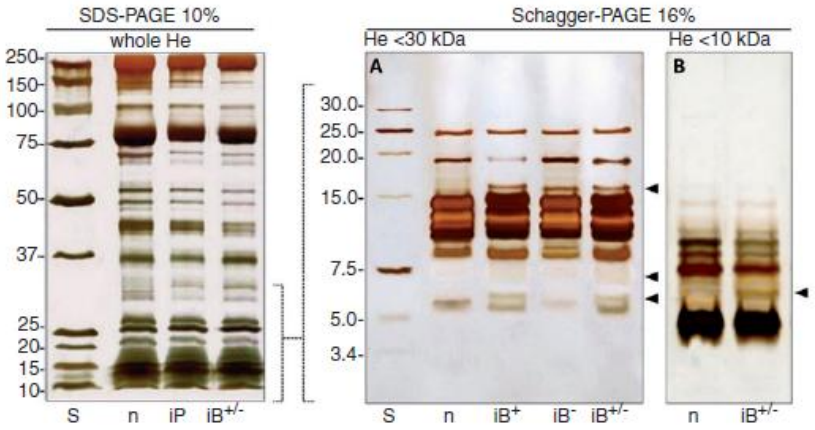
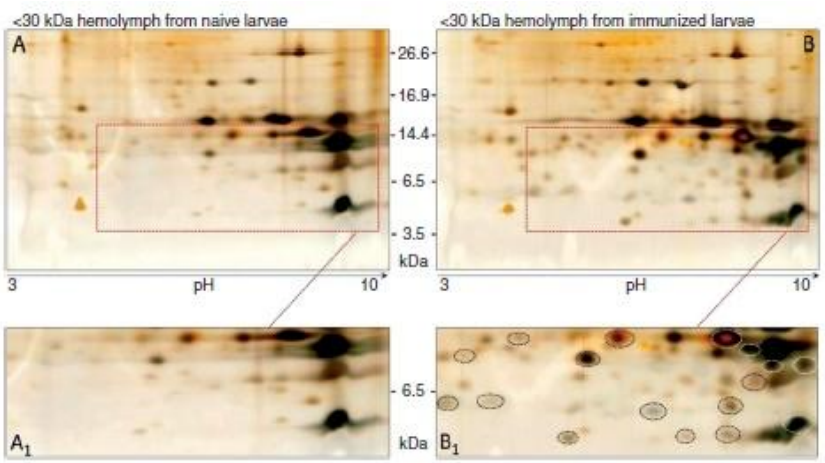


Figure 7. Monodimensional PAGE analysis of hemolymph samples from naïve and immunized RPW larvae. Whole and fractioned (<30 and <10 kDa) hemolymph from naïve, PAMP-infected and bacteria-infected larvae, were analysed by SDS-PAGE and Tricine-PAGE. Left: 10% SDS-PAGE of whole hemolymph. S: standard molecular mass; n: whole hemolymph from naïve larvae; iP: whole hemolymph from PAMP-injected larvae; $iB^{+/-}$: whole hemolymph from mixed (Gram-positive plus Gram-negative) bacteria immunized larvae. Right: 16% Tricine PAGE of fractioned hemolymph. (A) S: standard low molecular mass; n: <30 kDa hemolymph from naïve larvae; iB^{+}: <30 kDa hemolymph from Gram-positive-immunized larvae; iB^{-}: <30 kDa hemolymph from Gram-negative immunized larvae; $iB^{+/-}$: <30 kDa hemolymph from Gram-positive plus Gram-negative-immunized larvae. (B) n: <10 kDa hemolymph from naïve larvae; $iB^{+/-}$: <10 kDa hemolymph from Gram-positive plus Gram-negative-immunized larvae.

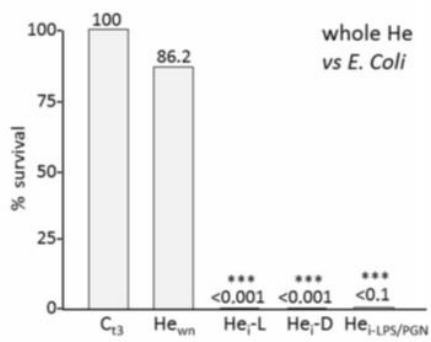
Figure 8. Bi-dimensional PAGE analysis. Two dimensional-PAGE analysis of proteins and peptides in fractions <30 kDa of hemolymph from (A) naïve and (B) bacteria-immunized larvae. IEF first dimension was run on a 3–10 linear pH gradient and the second dimension was carried out on 16% Tricine-PAGE. Dotted boxes refer to areas in which new or up-regulated spots were observed (B). Boxed areas are enlarged below the panels (A1 and B1); circled spots (panel B1) indicate newly appeared or up-regulated spots.



To further investigate the expression of new peptides (putative AMPs) we analysed the 2D-PAGE proteomes of <30 kDa hemolymph fractions (Figure 8). The first dimensions (isoelectric focusing gels) were carried out on linear 3-10 pH gradient gels, followed by a second dimension on Tricine-PAGE (16%). Two-dimensional analysis patterns of hemolymph samples are observable in Figure 8. More than 70 spots are counted and dotted boxes refer to areas in which new or up-regulated spots were observed in immunized samples, when compared to naïve sample.

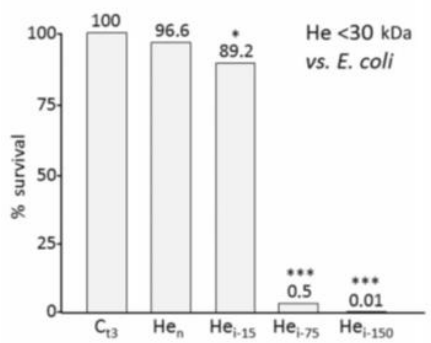
3.7 Antimicrobial activity assays versus Gram negative bacteria

The antimicrobial activity (AMPs) in RPW hemolymph was verified carrying out bacteria growth tests on solid agar after serial dilution of bacteria incubated with AMPs. Samples assayed were either the whole hemolymph (cell-free) or low molecular weight fractions (< 30 kDa and < 10 kDa). Figure 9A shows the effects of whole hemolymph from naïve larvae (He_{wn}), live and dead bacteria-infected larvae ($He_{i\text{-}L}$ and $He_{i\text{-}D}$) and PAMP immunized ($He_{i\text{-}LPS/PGN}$) larvae against Gram-negative bacteria (*E. coli*). In all assays with immunized larvae bacteria mortality exceeds 99.9% (compared with the control growth, C_{t3}) indicating a drastic antimicrobial activity. Moreover, in naïve larvae hemolymph (He_{wn}), low activity is present. The high mortality rate was confirmed even in fractioned hemolymph samples. Figure 9 (B, C) shows data obtained with <30 and <10 kDa hemolymph, respectively. In both cases, the activity of AMPs increased with total protein concentration ($He_{i\text{-}15}$<$He_{i\text{-}75}$ <$He_{i\text{-}150}$). The antimicrobial activity of the <30 and <10 kDa hemolymph fractions was also assayed on Pseudomonas sp., which seems to be highly susceptible to the action of RPW peptides.



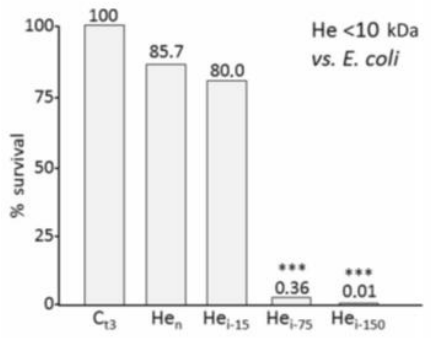
Panel A

Whole hemolymph *vs. E. coli*		
	CFU /ml mean ± SD n=5	% survival
C_{t3}	$6.73 \times 10^7 \pm 2.47$	100.0
He_{wn}	$5.80 \times 10^7 \pm 0.13$	86.2
He_i-L	$1.32 \times 10^2 \pm 0.25$	<0.001
He_i-D	$1.16 \times 10^2 \pm 0.29$	<0.001
$He_{i-LPS/PGN}$	$6.20 \times 10^4 \pm 2.09$	<0.1



Panel B

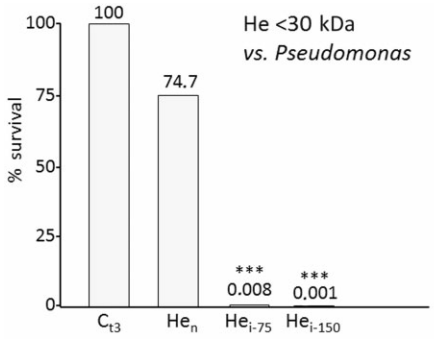
He <30 kDa *vs. E. coli*		
	CFU /ml mean ± SD n=5	% survival
C_{t3}	$6.70 \times 10^7 \pm 2.49$	100.0
He_n	$6.50 \times 10^7 \pm 1.23$	96.6
He_{i-15}	$6.00 \times 10^7 \pm 2.21$	89.2
He_{i-75}	$3.70 \times 10^5 \pm 1.51$	0.5
He_{i-150}	$8.30 \times 10^3 \pm 3.12$	0.01



Panel C

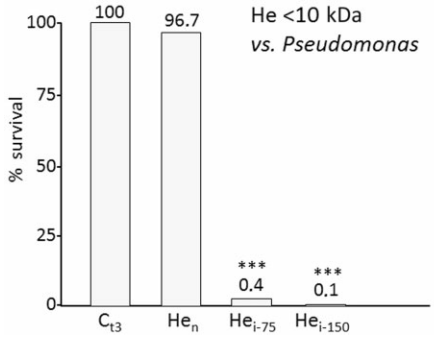
He <10 kDa *vs. E. coli*		
	CFU /ml mean ± SD n=5	% survival
C_{t3}	$7.00 \times 10^7 \pm 1.45$	100.0
He_n	$6.00 \times 10^7 \pm 1.79$	85.7
He_{i-15}	$5.60 \times 10^7 \pm 2.11$	80.0
He_{i-75}	$2.50 \times 10^5 \pm 1.58$	0.36
He_{i-150}	$5.70 \times 10^3 \pm 1.82$	0.01

Figure 9 (A,B,C). Effects of AMPs versus Gram-negative bacteria. Hemolymph (He) from RPW larvae, not fractioned and fractioned were assayed against Gram-negative bacteria (*E. coli*). (A) Whole He (500 mg/ml proteins) was incubated with 106 CFU/ml *E. coli*; after 3 h, bacterial survival was evaluated by CFU count. C_{t3}: control (bacteria); He_{wn}: bacteria + He cell-free fraction (CFF) from naïve larvae; He_i-L: bacteria + He from larvae immunized with live bacteria; He_i-D: bacteria + He from larvae immunized with dead bacteria; $He_{i-LPS/PGN}$: bacteria + He from larvae immunized with bacterial PAMPs. (B, C) He fractions (<30 kDa and <10 kDa) from RPW larvae were assayed against Gram-negative bacteria (*E. coli*). (B) Fractioned CFF samples (<30 kDa) were incubated with 10^6 CFU/ml *E. coli*; after 3 h, bacterial survival was evaluated by CFU count. C_{t3}: control (bacteria); He_n: bacteria + He fraction (<30 kDa; 150 mg/ml) from naïve larvae; He_{i-15}, He_{i-75}, He_{i-150}: bacteria + He fraction (<30 kDa, 15, 75, 150 mg/ml, respectively) from immunized larvae. (C) C_{t3}: control (bacteria); Hen: bacteria + He fraction (<10 kDa; 150 mg/ml) from naïve larvae; He_{i-15}, He_{i-75}, He_{i-150}: bacteria + He fraction (<10 kDa, 15, 75, 150 mg/ml, respectively) from immunized larvae. Results are expressed as percentage of survival compared with the control C_{t3} (100% survival). *p<0.05; ***p<0.001 vs naïve sample.



Panel D

He <30 kDa *vs. Pseudomonas sp.*		
	CFU /ml mean ± SD n=5	% survival
C_{t3}	$9.05 \times 10^8 \pm 1.24$	100.0
He_n	$6.76 \times 10^8 \pm 2.55$	74.7
He_{i-75}	$7.10 \times 10^4 \pm 2.70$	0.008
He_{i-150}	$1.32 \times 10^4 \pm 0.67$	0.001



Panel E

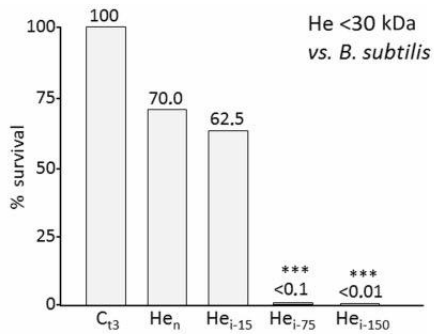
He <10 kDa *vs. Pseudomonas sp.*		
	CFU /ml mean ± SD n=5	% Survival
C_{t3}	$3.77 \times 10^8 \pm 1.04$	100.0
He_n	$3.65 \times 10^8 \pm 1.08$	96.7
He_{i-75}	$1.50 \times 10^6 \pm 1.05$	0.4
He_{i-150}	$3.70 \times 10^6 \pm 0.99$	0.1

Figure 9 (D,E). Effects of AMPs versus Gram-negative bacteria. He from RPW larvae was assayed against Gram-negative *Pseudomonas sp*. (D) C_{t3}: control (bacteria); He_n: bacteria + He fraction (<30 kDa; 150 mg/ml) from naïve larvae; He_{i-75}, He_{i-150}: bacteria + He fraction (<30 kDa; 75 and 150 mg/ml, respectively) from immunized larvae. (E) Assays as in (D) carried out with <10 kDa fractions. Results are expressed as percentage of survival compared with the control C_{t3} (100% survival). ***p<0.001 vs naïve sample.

3.8 Antimicrobial activity assays versus Gram positive bacteria

Hemolymph fractions were also assayed against Gram positive bacteria (*B. subtilis,* Figure 10 A,B and *M. luteus* Figure 10 C,D). Fractions <30 kDa possess a dose-dependent antibacterial activity either versus *B.subtilis* (Figure 10A) or versus *M. luteus* (Figure 10C). In addition, a rate of bacteria mortality was also observed in the sample from naïve larvae, particularly evident against *M. luteus* (Figure 10C, He_n). Assays assessed with <10 kDa

fractions, versus *B. subtilis* and *M. luteus*, show a reduced antibacterial activity either in samples from immunized larvae or from naïve larvae (Figure 10B, D).



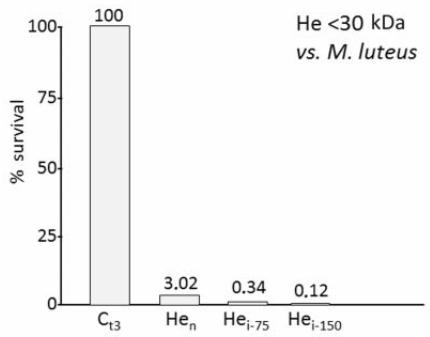
Panel A

He <30 kDa *vs. B. subtilis*		
	CFU /ml mean ± SD n=5	% Survival
C_{t3}	$4.00 \times 10^7 \pm 1.41$	100.0
He_n	$2.80 \times 10^7 \pm 0.17$	70.0
He_{i-15}	$2.50 \times 10^7 \pm 3.53$	62.5
He_{i-75}	$3.50 \times 10^4 \pm 0.66$	<0.1
He_{i-150}	$3.00 \times 10^3 \pm 2.19$	<0.01

Panel B

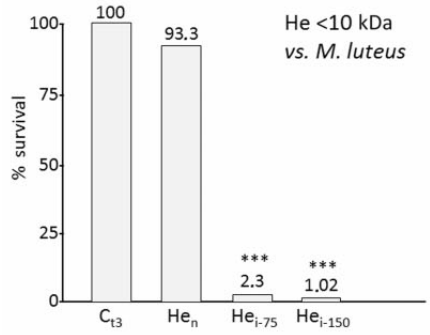
He <10 kDa *vs. B. subtilis*		
	CFU /ml mean ± SD n=5	% survival
C_{t3}	$5.00 \times 10^7 \pm 1.77$	100.0
He_n	$4.80 \times 10^7 \pm 2.65$	96.0
He_{i-75}	$8.30 \times 10^6 \pm 2.30$	16.6
He_{i-150}	$2.90 \times 10^6 \pm 1.95$	5.8

Figure 10 (A,B). Effects of AMPs versus Gram-positive bacteria (*B.subtilis*). Fractioned cell-free fraction (CFF) samples were incubated with 10^6 CFU/ml *B.subtilis*; after 3 h, bacterial survival was evaluated by CFU count. (A) C_{t3}: control (bacteria); He_n: bacteria + He fraction (<30 kDa;150 mg/ml) from naïve larvae; He_{i-15}, He_{i-75}, He_{i-150}: bacteria + He fraction (<30 kDa, 15, 75, 150 mg/ml, respectively) from immunized larvae. (B) Assays as in (A) carried out with <10 kDa fractions. Results are expressed as percentage of survival compared with the control C_{t3} (100% survival). ***P<0.001. Figure 10 (C,D). Effects of AMPs versus Gram-positive bacteria (*M.luteus*).He fractions from RPW larvae were assayed against *M. luteus*. (C) He fraction <30 kDa; C_{t3}: control (bacteria); He_n: bacteria + He fraction (150 mg/ml) from naïve larvae; He_{i-75} and He_{i-150}: bacteria + He fraction from immunized larvae (75 and 150 mg/ml, respectively). (D) Assays as in (C) carried out with <10 kDa fractions. Results are expressed as percentage of survival compared with the control C_{t3} (100% survival). ***p<0.001 vs naïve sample.



Panel C

He <30 kDa *vs. M. luteus*		
	CFU /ml mean ± SD n=5	% survival
C_{t3}	1.12 x 10^8 ± 0.75	100.0
He_n	3.87 x 10^6 ± 2.16	3.45
He_{i-75}	3.85 x 10^5 ± 3.53	0.34
He_{i-150}	1.30 x 10^5 ± 0.15	0.12



Panel D

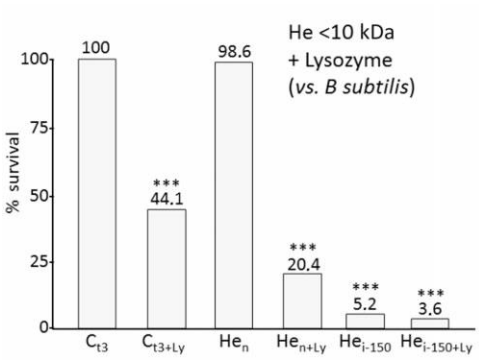
He <10 kDa *vs. M. luteus*		
	CFU /ml mean ± SD n=5	% Survival
C_{t3}	6.43 x 10^8 ± 5.04	100.0
He_n	6.00 x 10^8 ± 3.39	93.3
He_{i-75}	1.48 x 10^7 ± 3.12	2.3
He_{i-150}	6.58 x 10^6 ± 4.16	1.02

Figure 10 (C,D). Effects of AMPs versus Gram-positive bacteria (*M.luteus*).He fractions from RPW larvae were assayed against *M. luteus*. (C) He fraction <30 kDa; C_{t3}: control (bacteria); He_n: bacteria + He fraction (150 mg/ml) from naïve larvae; He_{i-75} and He_{i-150}: bacteria + He fraction from immunized larvae (75 and 150 mg/ml, respectively). (D) Assays as in (C) carried out with <10 kDa fractions. Results are expressed as percentage of survival compared with the control C_{t3} (100% survival). ***p<0.001 vs naïve sample.

3.9 Role of lysozyme on Gram positive bacteria

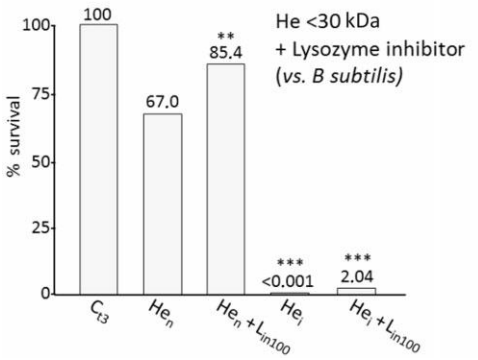
As the antimicrobial activity in the <10 kDa sample was not sustained by endogenous lysozyme, we carried out assays adding exogenous lysozyme to the fractioned hemolymph. Figure 11 A shows the strong effect of the pure enzyme when added to <10 kDa naïve larvae hemolymph (He_n). Likewise, when in the presence of AMPs (He_{i-150}+Ly), a further increase in bacteria mortality is observable compared with the <10 kDa fraction from immunized larvae (He_{i-150}).To establish if the mortality observed in samples from naïve

larvae could be attributed to the presence of constitutive lysozyme, we repeated the assay adding the specific lysozyme inhibitor, LHMED (Figure 11 B). LHMED induced a substantial neutralization of the antibacterial effects, clearly evident in samples from naïve larvae (He_n+L_{in100}). Also, the effects were less evident when the inhibitor was added to samples from immunized larvae (He_i+L_{in100}).



Panel A

Lysozyme effects in He <10 kDa		
	CFU /ml mean ± SD n=5	% Survival
C_{t3}	$2.12 \times 10^7 \pm 0.13$	100.0
C_{t3+Ly}	$9.35x\ 10^6 \pm 2.04$	44.1
He_n	$2.09 \times 10^7 \pm 0.31$	98.6
He_{n+Ly}	$4.32 \times 10^6 \pm 0.37$	20.4
He_{i-150}	$1.10 \times 10^6 \pm 1.8$	5.2
$He_{i-150+Ly}$	$7.64 \times 10^5 \pm 2.86$	3.6



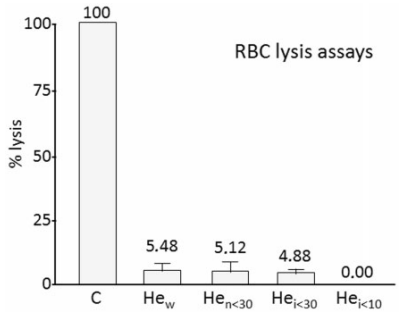
Panel B

Lysozyme inhibitor (LHMED) in He <30 kDa		
	CFU /ml mean ± SD n=5	% survival
C_{t3}	$4.10 \times 10^7 \pm 2.47$	100.0
He_n	$2.75 \times 10^7 \pm 0.62$	67.0
$He_n + L_{in100}$	$3.50 \times 10^7 \pm 2.35$	85.4
He_i	$2.93 \times 10^3 \pm 3.23$	<0.001
$He_i + L_{in100}$	$8.38 \times 10^5 \pm 0.15$	2.04

Figure 11. Effects of lysozyme and LHMED added to AMP fractions. Hemolymph (He) fractions from RPW larvae were assayed against Gram-positive bacteria (*B. subtilis*). (A) He samples (150 mg/ml of <10 kDa fraction) were incubated with 10^6 CFU/ml *B. subtilis* in the presence of lysozyme (Ly; 2 mg/ml); after 3 h, bacteria survival was evaluated by CFU count. (B) <30 kDa fractioned He samples (150 mg/ml) were incubated with 10^6 CFU/ml *B. subtilis* in the presence of 100mM LHMED. After 3 h, bacteria survival was evaluated by CFU count. C_{t3}: control (bacteria); He_n: bacteria + He fraction from naïve larvae; He_n+L_{in100}: bacteria + He fraction from naïve larvae + 100mM LHMED; He_i: bacteria + He fraction from immunized larvae; He_i+L_{in100}: bacteria + He fraction from immunized larvae + 100mM LHMED. Bacterial growth was not affected by the presence of 100mM LHMED (data not shown). C_{t3}: control (bacteria); C_{t3} + Ly: control bacteria + lysozyme; He_n: bacteria + He fraction from naïve larvae; He_n+Ly: bacteria + He fraction from naïve larvae + lysozyme; He_{i-150}: bacteria + He fraction from immunized larvae; He_{i-150} + Ly: bacteria + He fraction from immunized larvae + lysozyme. Results are expressed as percentage of survival compared with the control C_{t3} (100% survival). **p<0.01; ***p<0.001 vs naïve sample.

3.10 Hemolytic activity of RPW hemolymph

Human erythrocytes were used to test the hemolytic activity in RPW hemolymph samples. Hemolymph from naïve and immunized larvae were incubated with human Red blood cells (RBCs), and cell lysis was assessed as release of hemoglobin. Figure 12 shows the results of the lysis tests: whole and fractioned samples did not possess hemolytic activity (He_w, $He_{n<30}$, $He_{i<30}$, $He_{i<10}$) compared with Triton X-100 treatment as positive control.



RBC lysis assays	
sample	% lysis
C	100.0
He_w	5.48
$He_{n<30}$	5.12
$He_{i<30}$	4.88
$He_{i<10}$	0.00

Figure 12. RBC lysis assays. RBC lysis was checked in presence of whole or fractioned hemolymph (He) samples (150 mg/ml). Assays were performed by recording the absorbance at λ=404 nm to measure the release of hemoglobin. The graph shows the percentage of lysis referred to a 100% lysis positive control (C) obtained by RBC incubation with 0.02% Triton X-100. C: control; He_w: RBC + whole He from naïve larvae; $He_{n<30}$, RBC +<30 kDa He fraction from naïve larvae; $He_{n<10}$: RBC +<10 kDa hemolymph fraction from naïve larvae; $He_{i<30}$: RBC +<30 kDa hemolymph fraction from immunized larvae; $He_{i<10}$: RBC +<10 kDa hemolymph fraction from immunized larvae.

3.11 Bacterial cell wall damage induced by AMPs

The damage of bacterial walls was evaluated detecting the ability of propidium iodide to cross a damaged wall only: bacteria were treated with hemolymph extracted from naïve and immunized larvae, and the fluorescence in the damaged cells was visualized. Micrographs show the effects of AMPs against *E. coli* (Figure 13) and *B. subtilis* (Figure 14) at t=0 and 3 h after treatment (t=3). We can see a large number of fluorescent cells for both Gram negative and Gram positive bacteria after 3h incubation only with hemolymph extracted from immunized larvae.

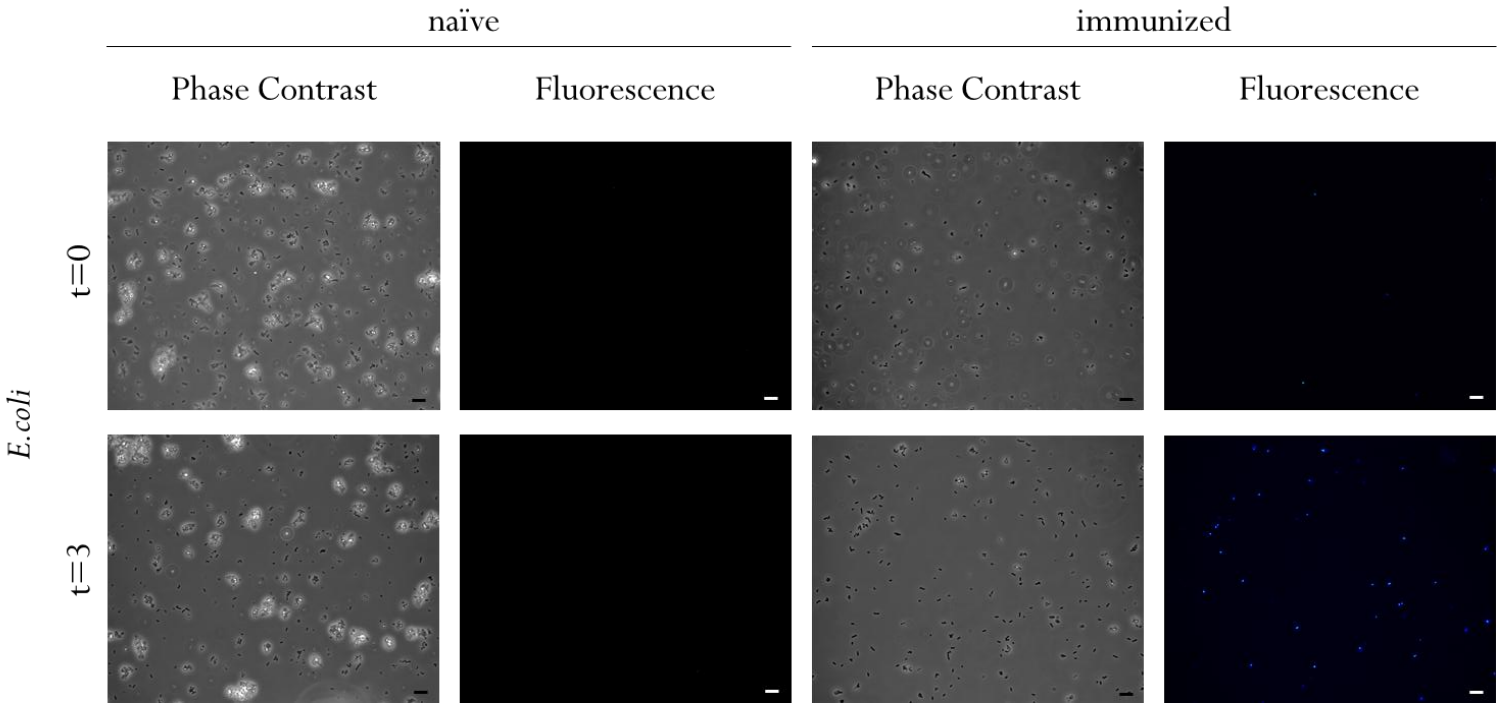


Figure 13. PI uptake after AMP treatments. Gram negative (*E. coli*) bacteria were treated with fractioned (<30 kDa) hemolymph from naïve and immunized larvae. t=0: start of treatment; t=3: 3 h after start of treatment; Fluorescent cells in micrographs at right (Immunized, Fluorescence, t=3) indicate propidium uptake as result of the damage of cell surface. Bar 10µm

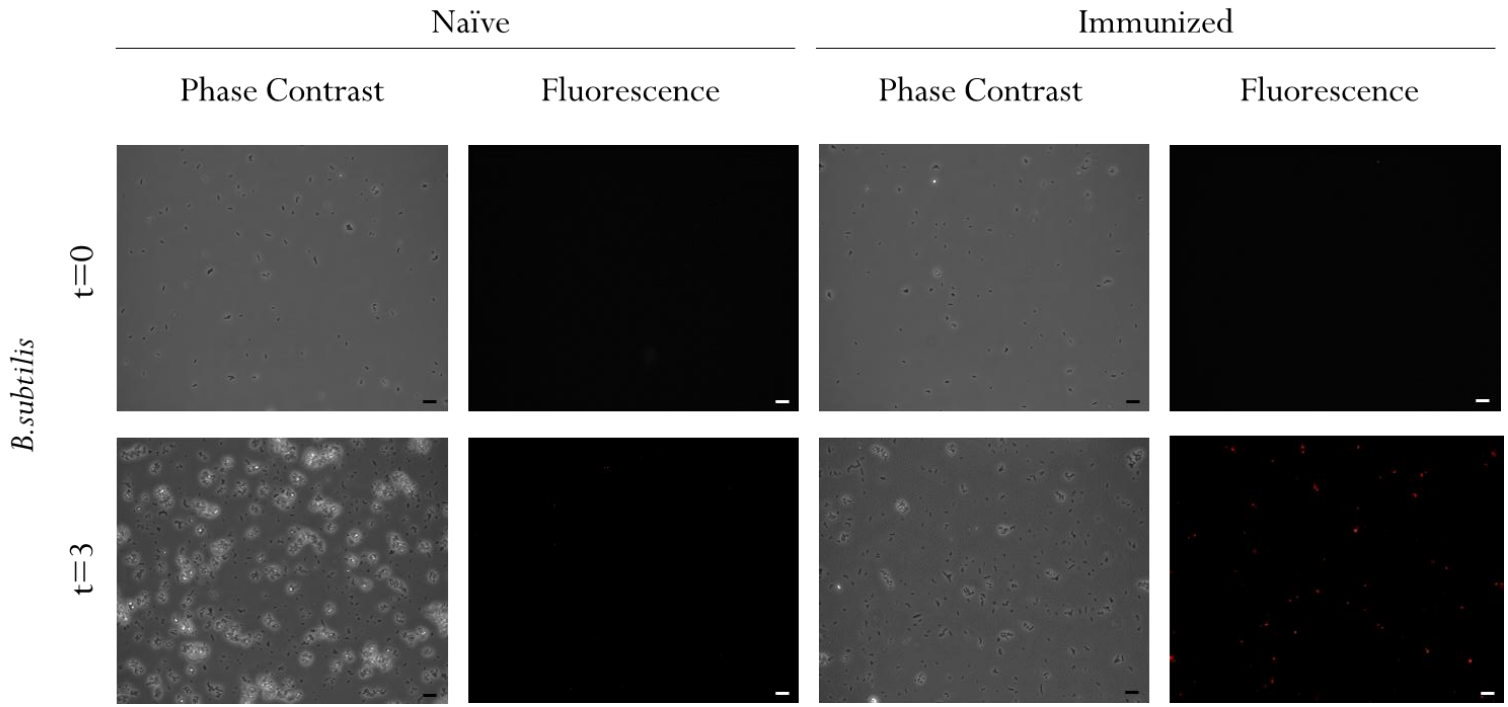


Figure 14: PI uptake after AMP treatments. Gram negative (*E. coli*) bacteria were treated with fractioned (<30 kDa) hemolymph from naïve and immunized larvae. t=0: start of treatment; t=3: 3 h after start of treatment; Fluorescent cells in micrographs at right (Immunized, Fluorescence, t=3) indicate propidium uptake as result of the damage of cell surface. Bar 10 µm

We have also verified the damage of the bacterial cell wall by scanning electron microscopy after treatments with AMPs (Figure 15): the changes are visible on both Gram negative and Gram positive bacteria. On the *E.coli* surface many blebs are evident (arrowheads), whereas *B. subtilis* cells appear burst and flattened.

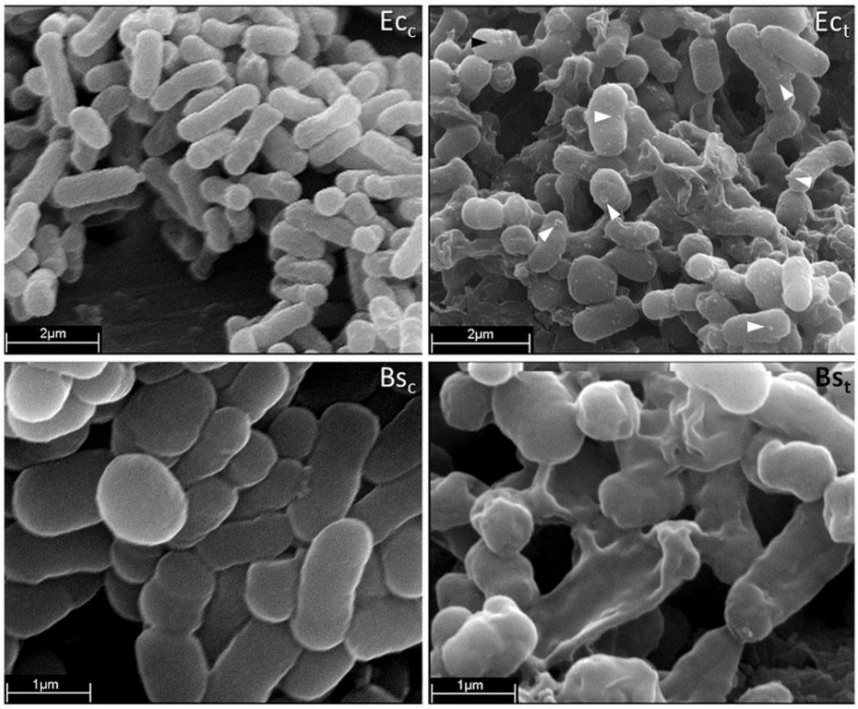
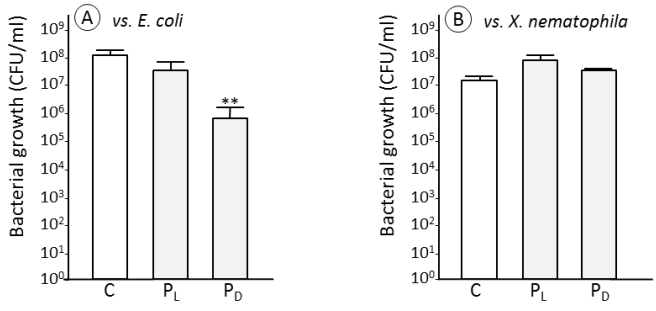


Figure 15. SEM of bacterial wall damage. Micrographs show the alteration of the bacterial wall after AMP treatments (<30 kDa fraction). Left panels are controls of untreated *E. coli* (Ec_c) and *B. subtilis* (Bs_c), right panels are results after 3 h of AMPs treatment against *E. coli* (Ec_t) and *B. subtilis* (Bs_t).

3.12 *Steinernema carpocapsae* modulates the host antibacterial activity

The inhibitory properties of the parasite nematocomplex on the antimicrobial activity of *R. ferrugineus* were investigated evaluating the bacterial growth of both *E. coli* and *X. nematophila* treated with hemolymph (<30 kDa) from insect larvae infected with live or dead parasites. Fig. 16, panel A, shows that live parasite (P_L) interferes with AMPs synthesis, as evidenced by microbiological assays. A marked growth *of E. coli* is observable. Instead, when in the presence of dead parasites (P_D), RPW larvae show a slight AMPs activity probably due to either inflammatory effects of necrotic materials released from nematodes corpses or to the inability of dead parasites to interfere with host AMPs synthesis. Since the injection of dead parasites results in a slight production of AMPs, microbiological assays were also carried out on *X. nematophila* (Fig. 16, panel B). Host AMPs (P_D) seem to be ineffective towards symbionts bacteria. As expected, a marked grown is observable after incubation with samples from live parasites-treated larvae (P_L).



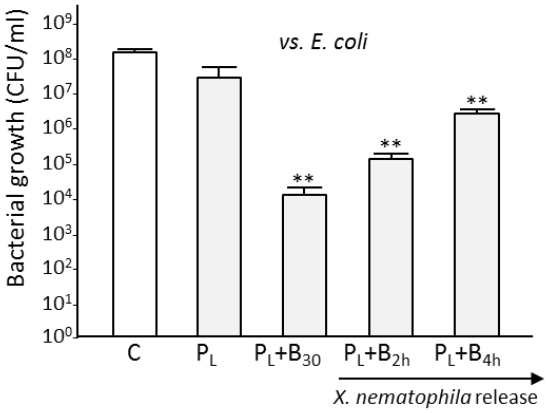
Assay *vs* Ec	mean ± SD
C	$1,52x10^8±1,40$
P_L	$4,04x10^7±3,74$
P_D	$8,6x10^5±3,80$

Assay *vs* Xn	mean ± SD
C	$1,65x10^7±1,21$
P_L	$8,42x10^7±2,74$
P_D	$4,52x10^7±0,47$

Figure 16. Interference of *S. carpocapsae* complexes on host antimicrobial activity. Cell-free fractions (<30 kDa) of hemolymph from RPW larvae infected with live (P_L) or dead parasites (P_D), have been tested against *E. coli* panel A) and *X. nematophila* (panel B). As controls of bacterial growth, three hours cultures of *E. coli* and *X. nematophila* were compared (panels A and B, bars C). **p <0.01 vs C.

3.13 RPW Antibacterial activity after double infections (parasite/exogenous bacteria)

The ability of live parasites and their symbiotic bacteria to inhibit larvae antimicrobial activity has been investigated after a further stimulation with exogenous bacteria (mix of *E. coli/B. subtilis*). Briefly, larvae have been infected with *S. carpocapsae* and with bacteria mix after 30 min, 2 h and 4 h (Fig. 17; P_L+B_{30}, P_L+B_{2h}, and P_L+B_{4h} respectively). Twenty-four hours after treatments, fractioned hemolymph samples have been tested. As observable, the inhibitory effect of parasites on antimicrobial response seems to be time-dependent (Fig. 17, from B_{30} to B_{4h}) and concurrent with the release and proliferation of symbionts bacteria which occurs between 1-2 hours after parasites injection.

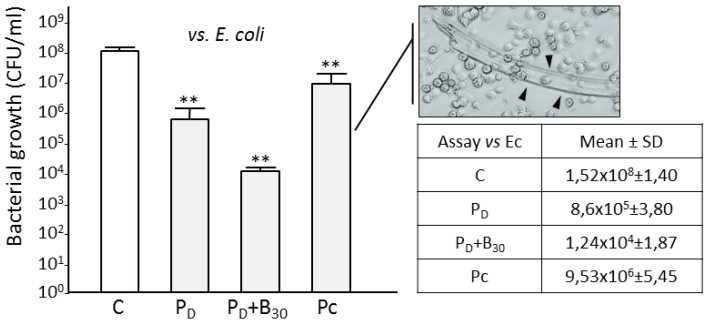


Assay *vs* Ec	Mean ± SD
C	$1,35x10^8±0,39$
P_L	$4,04x10^7±3,74$
P_L+B_{30}	$1,90x10^4±2,86$
P_L+B_{2h}	$1,74x10^5±1,72$
P_L+B_{4h}	$4,02x10^6±0,71$

Figure 17. RPW antimicrobial activity after sequential infections with live *S. carpocapsae* and exogenous bacteria. Antimicrobial activity of hemolymph fractions (<30 kDa) from RPW larvae injected with parasite complexes followed by infections with bacteria mix (*E. coli/B. subtilis*) at different times: 30 min, 2 h and 4 h. Hemolymph samples were collected 24 h after parasites infections and tested vs. *E. coli*. C: *E. coli* growth control; P_L: living parasites; P_L+B_{30}: double treatment with parasites at t=0 and bacteria t=30 min; P_L+B_{2h}: double treatment with parasites at t=0 and bacteria t=2 h; P_L+B_{4h}: double treatment with parasites at t=0 and bacteria t=4 h. ** $p < 0.01$ vs C.

3.14 Effects of dead parasites and their isolated cuticles on host AMPs

We have valued the interference of dead *S. carpocapsae* and their isolated cuticles on the host antimicrobial activity. As showed in Fig. 18 P_D, dead parasites did not completely inhibit the immune response. This is further confirmed by a second infection with exogenous bacteria that drastically activates the AMPs synthesis (Fig. 18, P_D+B_{30}). The injection of *S. carpocapsae* purified cuticles (inset) appears to be neither immunogenic nor inhibitory (Fig. 18, P_C). The inset shows an injected cuticle (arrowheads) recovered from the host hemocoel and some hemocytes are observable nearby the cuticle.



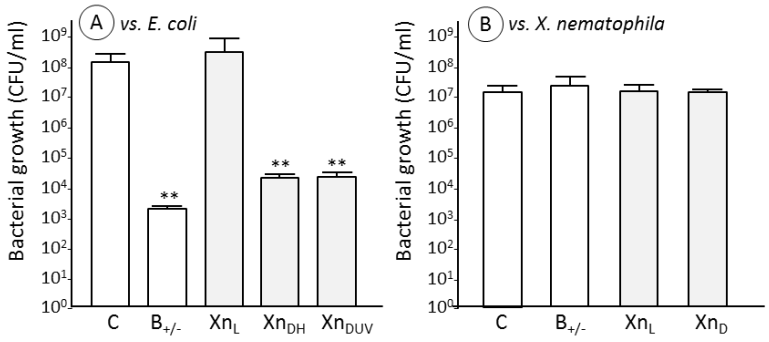
Assay *vs* Ec	Mean ± SD
C	$1,52x10^8±1,40$
P_D	$8,6x10^5±3,80$
P_D+B_{30}	$1,24x10^4±1,87$
Pc	$9,53x10^6±5,45$

Figure 18. Antimicrobial activity after injection of dead *S. carpocapsae* or isolated cuticles. Cell-free hemolymph fractions (<30 kDa) from RPW larvae infected, with cold-killed *S. carpocapsae* or isolated cuticles, have been assayed against *E. coli*. C: *E. coli* growth control; P_D: cold-killed parasites; P_D+B_{30}: double infection with parasites (t=0) and bacteria (after 30 min); P_C: isolated parasites cuticles. **p <0.01 vs C.

3.15 Host AMPs activity after injection of isolated parasite symbionts *X. nematophila*

X. nematophila has been isolated from parasites and tested for their inhibition properties on the host antimicrobial activity (Fig. 19, panels A and B). Panel A (test vs. *E. coli*) shows a strong immune stimulation after infection with exogenous bacteria mix, used as

positive control ($B_{+/-}$). Comparable results have been obtained by the injection of *X. nematophila* both heat-killed (Xn_{DH}) and UV-killed (Xn_{DUV}). Antibacterial activity is strongly stimulated by exogenous bacteria or killed *X. nematophila*, but is completely lacking when larvae were infected with live *X. nematophila* (Xn_L). Figure 19 panel B (test *vs. X. nematophila*) shows the same experimental approach with microbiological assays carried out with symbionts bacteria. All assays confirm the resistance of *X. nematophila* to RPW antimicrobial peptides (Fig. 19, panel B, $B_{+/-}$, Xn_D); furthermore, symbionts seem to possess inhibitory properties against the antimicrobial response, as observable in Figure 19 panel A (Xn_L) and in Figure 20 (Xn_L+B_{5h}).



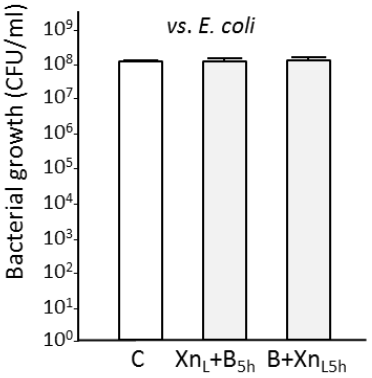
Assay *vs* Ec	mean ± SD
C	1,83x10^8±2,08
$B_{+/-}$	3,00x10^3±0,45
Xn_L	4,44x10^8±4,43
Xn_{DH}	3,42x10^4±0,60
Xn_{DUV}	3,98x10^4±0,92

Assay *vs* Xn	mean ± SD
C	1,65x10^7±1,21
$B_{+/-}$	3,52x10^7±2,33
Xn_L	1,66x10^7±1,01
Xn_D	1,41x10^7±0,45

Figure 19. Panels A and B. Antimicrobial activity in hemolymph from RPW larvae infected with live or dead *X. nematophila*: assays vs. *E. coli* and *X. nematophila*. Panel A: hemolymph fraction (<30 kDa) from RPW larvae infected with live or dead *X. nematophila* assayed vs. *E. coli*. C: *E. coli* control growth; $B_{+/-}$: Gram positive/negative dead bacteria; Xn_L: live *X. nematophila*-treated larvae; Xn_{DH}: heat-killed *X. nematophila*-treated larvae; Xn_{DUV}: UV-killed *X. nematophila*-treated larvae. Panel B: Assays as in (Panel A) carried out vs. *X.nematophila*. Xn_D: dead *X. nematophila*-treated larvae. **p<0.01 vs C.

3.16 Effects of sequential infections of RPW larvae with parasite symbionts (*X. nematophila*) and exogenous bacteria (*E. coli*/*B. subtilis*)

We have examined whether sequential infections (*X. nematophila* and exogenous bacteria, or the opposite) could affect the antibacterial response in the RPW larvae. Results (Fig. 20) demonstrate that, in both cases, the presence of live symbionts (Xn_L) strongly interferes with the host antimicrobial activity; consequently, bacterial growth is unaffected (Fig. 20, Xn_L+B_{5h} and $B+Xn_{L5h}$).



Assay *vs* Ec	mean ± SD
C	$1,56x10^8±0,08$
Xn_L+B_{5h}	$1,52x10^8±0,41$
$B+Xn_{L5h}$	$2,25x10^8±0,43$

Figure 20. Effects of sequential infections with live symbionts *X. nematophila* and exogenous bacteria. Hemolymph fraction (<30 kDa) from larvae infected with *X. nematophila* and exogenous bacteria mix (*E. coli*/*B. subtilis*). Assays vs. *E. coli*. C: *E. coli* growth control; Xn_L+B_{5h}: live *X. nematophila* at t=0 plus Gram -/+ mix at t=5 h; $B+Xn_{L5h}$: Gram -/+ mix at t=0 plus live *X. nematophila* at t=5 h.

3.17 Monodimensional PAGE patterns of RPW antimicrobial peptides

Electrophoretic assays (Tricine-PAGE method of Schagger and Von Jagow) have been carried out to visualize proteins and peptides of infected and naïve RPW larvae. Briefly,

hemolymph samples have been collected from naïve larvae (Fig. 21, He), bacteria mixture-infected larvae (Fig. 21, $He_{B+/-}$), Gram negative-infected larvae (Fig. 21, He_{B-}) and dead or live *X. nematophila*-infected larvae (Fig. 21, He_{XnD} and He_{XnL}, respectively). The figure shows that immunizations with both exogenous ($He_{B+/-}$ and He_{B-}) and dead *X. nematophila* (He_{XnD}) stimulate the synthesis of several peptides in the range from 4 to 17 KDa (empty arrowheads). These molecules are not observable in naïve larvae (He). Furthermore, peptides are also lacking in larvae immunized with live symbionts (He_{XnL}) together with other proteins which decreased in concentration (arrowheads).

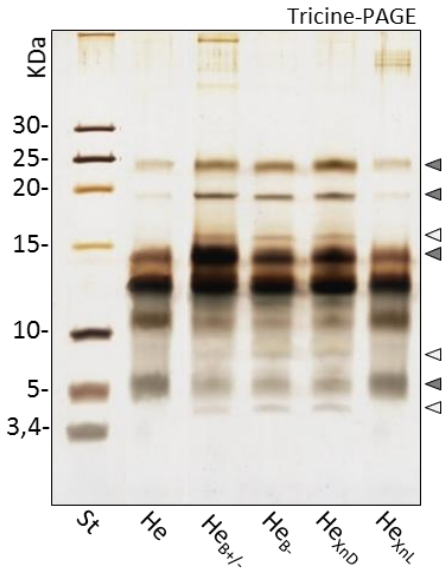
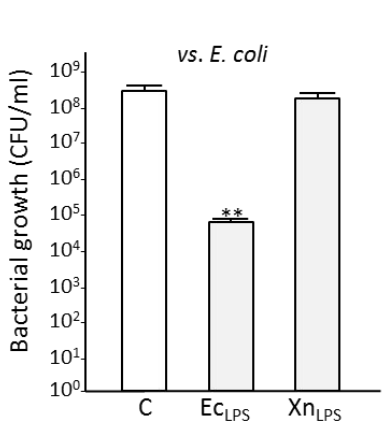
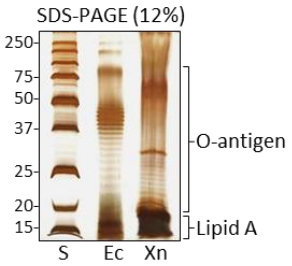


Figure 21. Tricine-PAGE of hemolymph (<30 KDa) immunized with exogenous or parasites symbiotic bacteria. St: Standard molecular weights marker; He: hemolymph from *naive* larvae; $He_{B+/-}$: hemolymph from *E. coli/B. subtilis*-immunized larvae; He_{B-}: hemolymph from *E. coli*-immunized larvae; He_{XnD}: hemolymph from dead *X. nematophila*-immunized larvae; He_{XnL}: hemolymph from live *X.nematophila*-immunized larvae.

3.18 Effects of the injection of LPS purified from *E. coli* or *X. nematophila* on RPW larvae: assays of antimicrobial activity

The immunogenic properties of LPS purified from symbionts bacteria has been compared with LPS from Gram negative exogenous bacteria. As shown in the graphs (Fig.22), the *X. nematophila* endotoxins do not trigger the AMPs synthesis while *E. coli* LPS stimulates synthesis at a level comparable to that obtained by bacteria mix infections (Fig. 19, $B_{+/-}$). A silver silver-stained SDS-PAGE separation of LPS samples from *E. coli* (inset, Ec) and *X. nematophila* (inset, Xn) is visualized at right.





Assay *vs* Ec	mean± SD
C	$2,92x10^8$±1,24
Ec_{LPS}	$8,26x10^4$±0,46
Xn_{LPS}	$1,42x10^8$±1,02

Figure 22. RPW infection with LPS purified from *E. coli* or *X. nematophila*: effects on host antibacterial response. Assays vs. *E. coli*. C: *E. coli* bacterial growth; Ec_{LPS}: hemolymph from RPW larvae injected with *E. coli* LPS; Xn_{LPS}: hemolymph from RPW larvae injected with *X. nematophila* LPS. At right the electrophoretic separation of injected LPS. S: standard Precision plus (Bio-Rad); Ec: LPS from E. coli (Serotype 055:B5, 5µg); Xn: LPS from X. nematophila (about 5µg). ** $p < 0.01$ vs C.

3.19 Effects of exogenous and symbionts bacteria on hemocytes phagocytosis activity

Finally, to investigate the effect of *X. nematophila* on the phagocytic competence of the host hemocytes, we examined their ability to perform phagocytosis *in vitro* on dead exogenous bacteria (*E.coli, M.luteus and S.aureus*) or dead *X.nematophila*. We used, as a model, *G. mellonella* hemocytes. Hemocytes, in particular granulocytes cells, show the ability to detect the presence of exogenous bacteria (Fig. 23 A, C, D) which when co-incubated are rapidly engulfed. One hour after the addition of exogenous bacteria is clearly visible a cellular response. Cells that have ingested bacteria are identified by fluorescence issued by FITC or TRITC fluorophores linked to the bacterial wall.

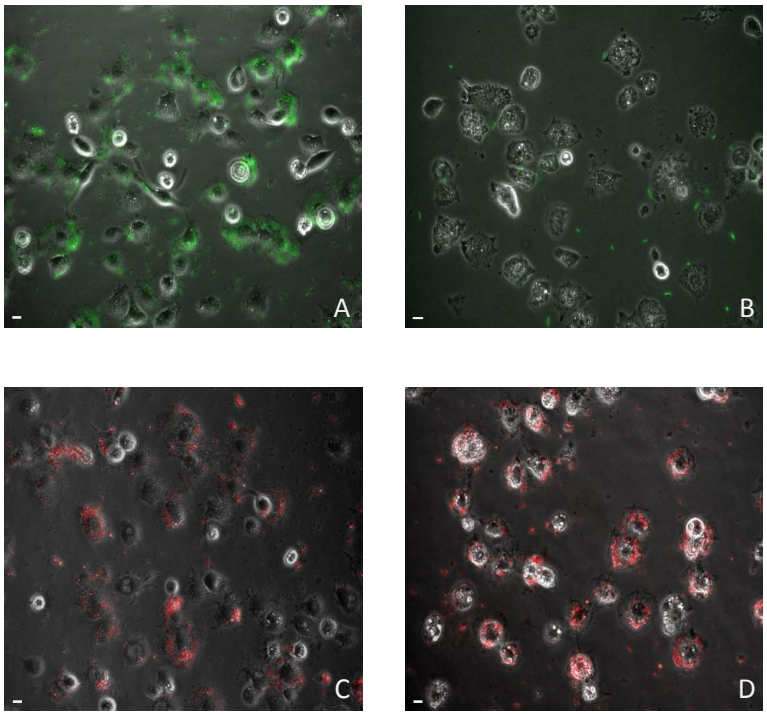


Figure 23: *G. mellonella* hemocytes culture. Epifluorescence micrographs show phagocytosis by hemocytes at 1 hour after challenge. (A) FITC- conjugated dead *E.coli*; (B) FITC- conjugated dead *X. nematophila*; pHrodo red dye conjugated- *S. aureus*; (D) TRITC- conjugated dead *M. luteus.* Bar=10 µm

Preliminary experiments (data not shown) have monitored a time course *E.coli*-FITC phagocytosis showing that cells migrate toward *not-self* early and subsequently starts to phagocyte. Conversely, *X. nematophila* is not phagocytosed rapidly (Fig. 23 B).

Moreover, experiments with double infection (Fig. 24 A, B) have shown that exogenous bacteria (green) are quickly engulfed whereas *X. nematophila* (red) is not recognized. Meanwhile, *X. nematophila* does not prevent the engulfment of the other bacteria. Therefore, the symbiotic bacterium seems not directly inhibit phagocytosis in general but it seems that some membrane components could not be recognized by immunocompetent cells in a short time.

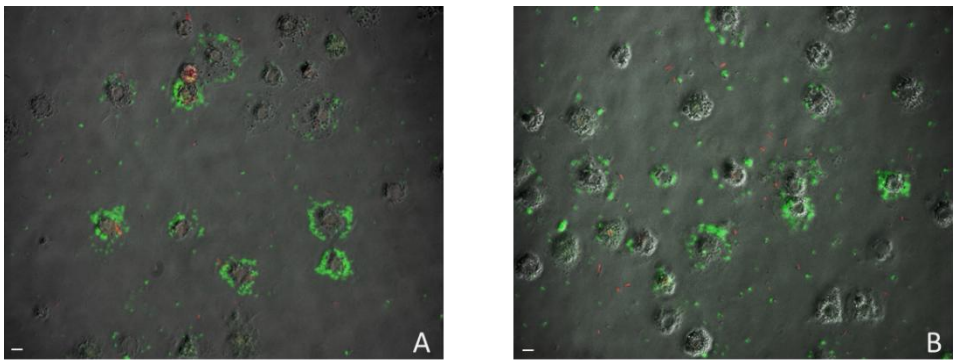


Figure 24: *G. mellonella* hemocytes culture. Epifluorescence micrographs show phagocytosis by hemocytes at 1 hour after challenge. (A) FITC- conjugated dead *E.coli* and TRITC-conjugated dead *X. nematophila*; (B) FITC- conjugated dead *M. luteus* and TRITC-conjugated dead *X. nematophila*. Bar=10 µm

Afterwards, we have monitored the phagocytosis and we have observed that about 12 hours after challenge with dead *X. nematophila,* immunocompetent cells start to engulf symbiotic bacteria (Fig.25 B, arrowhead). In Figure 25 A, granulocytes have phagocytosed so much exogenous bacteria that it is impossible to discriminate single entities inside the cytoplasm. Now, it is not clear which is the mechanism that slows down this immune process.

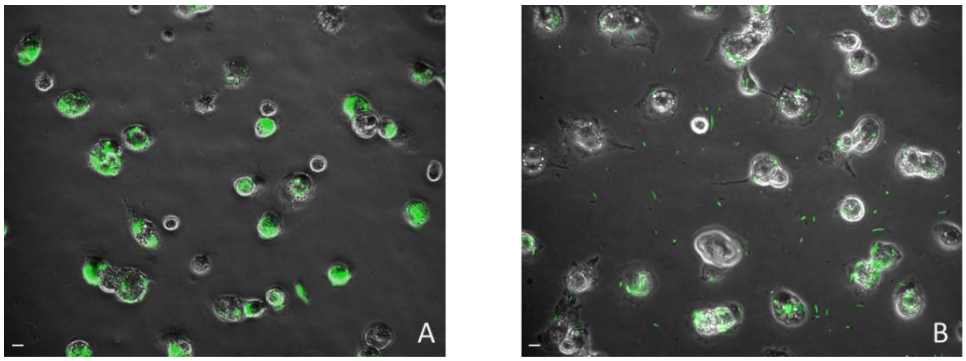


Figure 25: *G. mellonella* hemocytes culture. Epifluorescence micrographs show phagocytosis by hemocytes at 12 hour after challenge. (A) FITC- conjugated dead *E.coli* (B) FITC- conjugated dead *X. nematophila*. Arrowhead shows cells that have phagocytosed *X. nematophila* Bar=10 µm

4 DISCUSSION

In our work we have focused on the study of the relationship between entomoparasite *S. carpocapsae* and insect host (*R. ferrugineus* and *G. mellonella*), we have examined both host defenses processes and the evasive/depressive strategies of the parasites. To date, few studies have been published about both host immunological responses and evasive strategies of *S. carpocapsae* (Manachini *et al.*, 2013; Mazza *et al.*, 2011; Thomas and Nair, 2010). Most of the published articles are based on the study of insectides to control the insect pest (Güerri-Agulló *et al.*, 2011;Dembilio *et al.*, 2010 , Llacer *et al.*, 2009). From previous results obtained in our laboratory, we had obtained that parasite cuticle of a different nematode (*Steinernema feltiae*) was responsible of molecular disguise strategies and cause host immune suppression. Lipids of *S. feltiae* interacted and removed humoral components from the host hemolymph causing the observed disguise, resulting from the covering of the parasite with host antigens (Mastore and Brivio, 2008). Moreover the removal of host factors down- regulated antimicrobial peptides synthesis, inhibited proPO system and affected encapsulation and phagocytosis of host hemocytes (Brivio *et al.*, 2004; 2010; Mastore and Brivio, 2008).

Our results show that interaction between *R. ferrugineus* and *S. carpocapsae* is in part different compared to *S. feltiae- G. mellonella*. We have observed as Balasubramanian *et al.*, 2009 and Toubarro *et al.*, 2009; 2010 that immune depressive strategies of living *S. carpocapsae* culminating in rapid inhibition of host proPO system. This phenomenon was evident in the early phase of parasitation (30 min). The delayed activation of proPO system could be to release of toxins by symbionts (*X. nematophila*) into the hemocoel. (Song *et al.*, 2009). Our results confirmed that immediately after entry, parasites are able to neutralize different immune responses carried out by the host (Dunphy and Webster 1987; Brivio *et al.*, 2005; 2010).

As described above with *S. feltiae*, the early inhibitory effect of *S. carpocapsae* did not seem be mediated by cuticles because only living parasites show inhibitory effects. These effects are probably induced by parasite secretions as serine proteases (Toubarro *et al.*, 2013;

Balasubramanian *et al.*, 2010; 2009) and not to the cuticle properties. According to the literature (Gaugler and Kaya, 1990) the variety of the effects observed depends on different parasites and different hosts that are infected. This could be the reason why we had observed the variety of effects when *S. feltiae* and *S. carpocapsae* infected different hosts. Furthermore, cold- killed parasites do not inhibit host proPO system demonstrating that active secretions are involved in inhibition processes that are carried out by living parasites.

Once verified the depressive ability of parasites on host proPO system, we had analyzed proPO system both in physiological conditions and in presence of exogenous microorganism (*E. coli*, *B. subtilis* and *S. cerevisiae*) and their PAMPs (LPS, PGNs and β-Glucans). Our data indicate that naïve larvae possess a basal activity of phenoloxidase but at the same time the proPO system is strongly triggered by microorganisms and their PAMPs confirming that this enzymatic system is reactive against not self targets. On the contrary, parasites can modulate the phenoloxidase activity, surviving and assuring its symbiont the suitable environment for their growth.

A further immune response involved in the not- self recognition is carried out by immunocompetent cells as granulocytes and plasmatocytes. These hemocytes usually participate in capsule formation around multicellular organisms or apoptotic cells (Strand, 2008; Castillo *et al.*, 2012) When abiotic beads were co-incubated with RPW hemocytes, beads were surrounded by a multilayered cellular structure and melanin intrapped inside the targets.

Even if the body surface of *S. carpocapsae* did not seem to interfere with humoral response of RPW , a main role of the cuticle has been ascertained to avoid cellular recognition. Alive and cold-killed parasites were not recognized by immunocompetent cells. Recognition and cellular encapsulation are completely lacking against *S. carpocapsae*. Cuticles isolated from nematodes are not identified confirming the involvement of the body surface in mimetic strategies. Furthermore, the absence of recognition is not due to a cellular damage by an active release by nematodes because when were co-incubated with agarose beads, abiotic

materials were usually encapsulated. This mimetic role of body surface is particularly evident when heat-treated *S. carpocapsae* were injected *in vivo* into RPW larvae. The cellular response was strongly elicited and melanin formation was very rapid and strong.

Subsequently, we have investigated the inducible immune response of RPW larvae to evaluate the presence of antimicrobial peptides using either biochemical analyses or microbiological assays. The principal purpose is to investigate physiological responses in inducible immunity by the synthesis ex novo of antimicrobial molecules. RPW larvae, when immunized with microorganisms, up-regulate the expression of peptides into the hemolymph. AMPs act in synergy against both Gram positive and Gram negative bacteria. Viable and killed microorganisms and even PAMPs (e.g. LPS, PGN) stimulate the onset of AMPs. We carried out electrophoretical analyses of hemolymph to verify the inducibility of *ex novo* synthesis of peptides. We detected almost 14 spots in the range between 5 and 10 kDa and in the basic area. These are typical characteristics of antimicrobial peptides (Epand and Vogel, 1999). About microbiological assays, we have detected a strong activity of whole hemolymph against *E. coli* from challenged larvae. To determine the range of AMPs activity we performed microbiological assays on both Gram negative (*E. coli*, *Pseudomonas sp*.) and Gram positive (*B. subtilis, M. luteus*). Hemolymph < 30 kDa revealed a marked dose-dependent efficacy against both Gram negative and Gram positive bacteria. The activity of fraction < 30 kDa could be due to the presence of lysozyme whose activity acts in a synergic way with antimicrobial peptides (Hultmark, 1996; Kalfa and Brogden, 1999; Zdybicka-Barabas *et al.*, 2012). Meanwhile, hemolymph fraction < 10 kDa (no presence of lysozyme) showed a lower activity against *B.subtilis* compared to Gram negative bacteria and *M. luteus* . Gram negative bacteria are less sensitive to lysozyme activity due to their bacterial wall structure that does not present the multilayered peptidoglycans. It is well known that lysozyme is present constitutively in naïve larvae (Stanley, 2014) and it is confirmed by our data against *M. luteus* in hemolymph from unchallenged larvae. The fraction < 30 kDa, in which lysozyme is present only constitutively, showed a strong activity against *M. luteus.* The synergyc role of lysozyme to improve the efficacy of AMPs was confirmed by our results.

Indeed, the additional of purified lysozyme in <10 kDa fraction strongly increased the mortality of *B.subtilis* and conversely, when we added lysozyme inhibitor (LHMED) to the <30 kDa fraction, AMPs activity was reduced and bacterial survival increased. Antimicrobial peptides are composed of 10–50 amino-acid residues, and arranged in different groups depending on the amino-acid composition, size, and conformation. The classic action mechanism of AMPs involves their ability to cause cell membrane damage. AMPs can interact with microorganisms by electrostatic forces between their positive amino acid residues and negative charges exposed on cell surfaces. More recently, it has been proposed that AMP driven microbial death can be caused by others mechanisms in addition to membrane disruption, followed by cell lysis. Many evidences indicate that some AMPs can interact with intracellular targets inducing cell damages, such as the inhibition of cell wall, DNA, RNA, and protein synthesis (Brogden, 2005; Straus and Hancock, 2006). We have confirmed, by our results, the action of RPW peptides on bacterial walls evaluating the uptake of propidium iodide. As observed by fluorescence microscopy, DNA intercalating agent inside bacteria confirmed the presence of damages on the bacterial surfaces. Furthermore, SEM observations suggest that RPW AMPs trigger the formation of breaches and blebs on the bacterial cell walls.

Later, we have concentrated on the modulation of the antimicrobial activity by *S. carpocapsae* and its symbionts. Some papers describe a down regulation of the humoral response due to the presence of parasites and symbionts (Ji and Kim, 2004; Duvic *et al.*,2012; Bisch *et al.*, 2015). *S. carpocapsae* is not only considered as a vector of *X. nematophila* since after the entry into hemocoelic cavity, parasite implements series of strategies intended to halt the immune system to establish the environment for the bacterial growth. We have previously demonstrated that *S. carpocapsae* is able to elude cellular encapsulation and inactivate proPO system. As known, the presence of exogenous targets into the hemocoelic cavity can trigger the AMPs synthesis. Our data show in larvae infected with living *S. carpocapsae*, antimicrobial activity was strongly reduced. As, antimicrobial activity was detected after infection with dead parasites, we supposed that inhibition of antimicrobial activity could due

to the release of symbionts starting from 5 hours after entry into host. In addition, the lack of interference of the nematode is clearly evident when the larvae were injected with isolated cuticles. This result suggests that body surface of parasite does not activate AMPs pathways. The cuticle seems to behave as a disguising structure that lets the nematode disguises in the early infection stage; effectively neither proPO system activation nor cellular encapsulation have been observed. So we have focused on the study of the effects induced by isolated symbionts. Live *X. nematophila* injected into RPW larvae do not stimulate the AMPs synthesis, as observed after sequential injections with exogenous bacteria. A further evidence is given by analytical electrophoresis and the absence of low molecular bands in hemolymph sample infected by *X. nematophila* confirming a possible interference with Toll and IMD pathways. Bacteria, damaged by heat treatments or by UV irradiation, lack the interference with the antimicrobial peptides synthesis. The host, in this case, is able to produce efficiently AMPs to counteract and survive to the infection Also, the presence of AMPs is confirmed by electrophoretic patterns. *X. nematophila* is a Gram negative bacteria and it is well known that LPS of the outer membrane is a potent immunogenic complex either in invertebrates or in vertebrates (Medzhitov and Janeway, 2002; Kanost *et al.*, 2004). LPS acting as a PAMPs interacts with host PRRs to trigger all the immune processes. LPS isolated from *X. nematophila* is thought to be cytotoxic to hemocytes of the *G. mellonella* and *S. exigua* insects. Live *X. nematophila* suppress nodulation in *S. exigua* and *M. sexta*. *X. nematophila* suppress antimicrobial induction in *S. exigua* (Ji and Kim, 2004) and has suppressive qualities to proPO system (Dunphy and Webster, 1988; 1991). The virulence repertoire of *X. nematophila* include factors that kill and unstick hemocytes, as well as protease that destroy insect antimicrobial peptides and a secreted factors that inhibit insect phospholipase A2 activity (Shrestha and Kim, 2007). In addition, *X. nematophila* produces a large number of protein insecticidal toxins that are active against a wide range of insects (Eom *et al.*, 2007). In this work, we observed that LPS did not stimulate the humoral defences since antimicrobial activity is completely missing. Instead, LPS from *E. coli* strongly elicited the AMPs pathways.

Symbionts seem, moreover, to be resistant to antimicrobial peptides. AMPs assayed against *X. nematophila* do not interfere with bacterial growth as already described by Duvic *et al*. (2012) in *S. frugiperda*.

Now, the present work is focused on the study of a possible modulation, by *X. nematophila*, of phagocytosis. As an infection model, we used *G. mellonella* because the wax moth has several advantages over other invertebrates. The low cost and ease of maintenance of the larvae also allow large experimental groups to be used. We have identified four types of hemocytes in the last larval instar of *G. mellonella*: Plasmatocytes, Granulocytes, Oenocytoids and Spherulocytes. Granulocytes are the main cells able to phagocyte and the first cells to come into contact, in small numbers, with a foreign body at the beginning of capsule/nodule formation. When in contact with the foreign body, they release their granular content (Ratcliffe and Gagen, 1977 and Schmit and Ratcliffe, 1977). According to most authors, this exocytosis of typical inclusions by granular hemocytes serves to attract plasmatocytes (Gillespie *et al*., 1997) or at least helps plasmatocytes to build the capsule or nodule (Pech and Strand, 1996). This exocytosis of opsonin-like material is another main function of granular haemocytes. The *in vitro* phagocytosis assay used here was suitable for the demonstration of variation in phagocytic capacity of *G. mellonella* hemocytes when incubated with dead exogenous bacteria (*E. coli*, *M. luteus* and *S. aureus*) and dead *X. nematophila*. To optimize incubation time for in vitro phagocytosis, time courses were determined. Starting from 30 minutes from incubation, it is possible to observe some granulocytes engulfed exogenous bacteria achieving the maximal efficiency in 2 hours. *X. nematophila,* instead, was slightly engulfed after about 12 hours from incubation. Assuming that dead *X. nematophila* is not able to release toxins, we supposed that this result could be due to the presence of structures (e.g. LPS) on the cellular membranes that prevent their engulfment by hemocytes. Also, live *X . nematophila* do not preclude phagocytosis of bacteria exogenous. Experiments in order to understand this evidence are ongoing in our laboratory.

5 CONCLUSIONS

The obtained data, altogether, confirm the efficacy of the parasite complex to control insect pests. The nematode strategy, in the early phase, is based on sequential actions achieved by the evasive properties of the parasites (by means of released of proteases and the disguising properties of the cuticle) followed by, in the late infection phase, the immune depression induced by symbionts leading to septicemia and host death.

This data provide further knowledge to understand approaches carried out by the parasite to elude and inhibit the insect immune system. This knowledge of host-parasite relationship could provide a basis to optimize pest control with the aim to eliminate or reduce the use of chemical pesticides in agriculture and urban environment.

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