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Review

Parasite-host relationship: a lesson from a professional killer

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Abstract

This paper outlines some of the main features of parasites immunoevasion/depression strategies. Insects humoral and cellular responses are briefly discussed and correlated to the active and passive strategies of insects parasites, with particular emphasis on nematocomplexes used as biological insecticides. We have reviewed data on the interaction, at immunological level, of the parasite *Steinernema feltiae* (Rhabditidae) with the host model *Galleria mellonella* (Lepidoptera, Pyralidae); the putative role of the parasite body-surface in active and passive evasion mechanisms has been evaluated and discussed.

Key words: insect; immunity; parasite; PRRs-PAMPs; immunodepression; immunoevasion

Introduction

Invertebrates, particularly insects, act as vectors of important diseases such as malaria, Chagas' disease, sleeping sickness, filariases, Dengue fever, yellow fever, etc. Moreover many insect species, usually named insect pests, have a strong impact on the environment since they are phytophagous and harmful for both crops and urban areas.

As a consequence of the widespread diffusion of insect species (reflecting the great success of this group) occupying almost all the habitats on Earth, many insects live in environmental conditions infested by parasites and pathogens. Insects can survive mainly because of the extreme efficacy of their immune system; any foreign parasite must then counteract these defenses to survive into its host.

Many parasites reproduce, develop and survive in invertebrate hosts that possess an immune system devoted to self-integrity and to discriminate self from not self.

Invertebrates lack finely tuned immunorecognition receptors but they possess instead useful pattern-recognition molecules (PRRs); these factors are able to interact specifically with a broad range of foreign antigenic surface compounds (commonly named PAMPs and defined as pathogen-associated molecular patterns). PAMPs-PRRs interaction is a key process among the discriminatory steps of innate immunity that usually precede the effectors-based mechanisms responsible for the elimination of not self (Medzhitov, 2001; 2002; Kanost *et al*., 2004). PAMPs consist of various compounds, including oligosaccharides, proteins, glycoproteins, lipids and distinct nucleic acid motifs that are unique to, and essential for, microorganism survival. An important feature of PAMPs is their strongly conserved structures, which are invariant among organisms of a given class (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002).

In insects, infections with different microorganisms or parasites selectively activate various defense reactions and effector-coding genes. The molecular basis of discrimination between different types of not self and the activation of immune responses is attributed to the specificity of PRRs toward PAMPs, such as LPS (lipopolysaccharide), PGLC (peptidoglycan) or various glucans (Medzhitov and Janeway, 2002; Dimopoulos, 2003). Several proteins both in insect hemolymph or on hemocytes plasma membrane seem to function as PRR, as they perform surveillance by binding to molecular patterns (Hoffmann *et al*., 1999; Hoffmann, 2003) (Fig. 1).

In *Galleria mellonella* naïve larvae, two LPS-binding proteins, named LBP-1 (17.2 kD) and LBP-2 (26 kD), have been described by Dunphy and Halwani (1997); these humoral factors can be considered as PRRs. These receptors bind the surface of bacteria and seem to act as detoxifiers, thus protecting

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° In memory of the late M Pagani

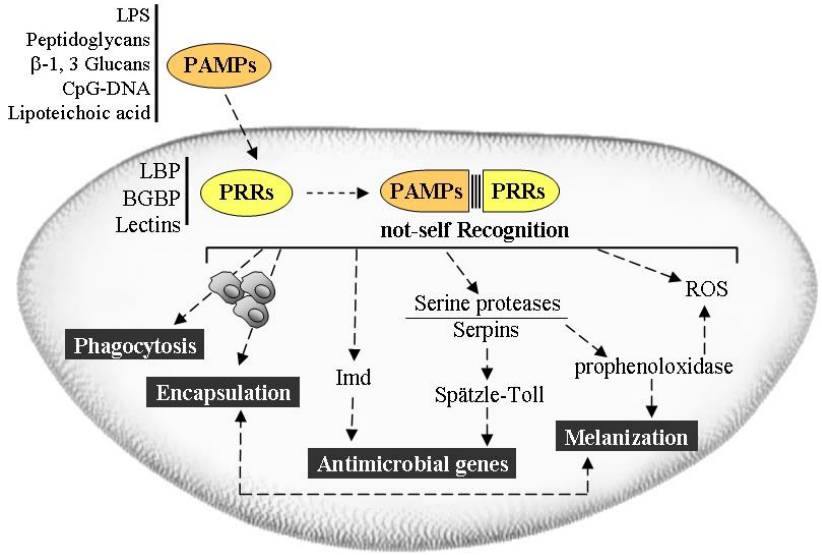

Fig. 1 Foreign stimula and receptors of insect immune system. Perceiving of not-self in invertebrate seems to be modulated by the presence of PAMPs (pathogen-associated molecular patterns) which interact with free or membrane-bound receptors called PRRs (pattern-recognizing receptors). These interactions lead to the activation of different cell-mediated and humoral effector immune processes.

hemocytes from damage. Both the LBPs are specific for the lipid A portion of LPS, in addition LBP-1 seem to act as an activator of *Galleria* pro-phenoloxidase (proPO) system. In the same year, Wiesner and colleagues (1997) isolated and described a similar protein, called Apolipophorin-III (ApoLp-III) of 17 kD of molecular mass; ApoLp-III has been identified as an immune-stimulating molecule and is an exchangeable apolipoprotein, abundant in lepidopteran insects. The immune-stimulating capacity of ApoLp-III came as a surprise, since this protein had been previously known to play a main role as lipid carrier in flying insects (Niere *et al.*, 1999).

On insects hemocytes, cell receptors responsible of Toll and Imd pathways activation can be considered as the main PRRs involved in cellular PAMPs sensing leading to antimicrobial peptides (AMPs) synthesis (De Gregorio *et a*l, 2002); their importance is not only restricted to the process of AMPs genes activation but, evolutionary, they also represent a further confirmation of the ancient origin of innate immunity, since they have been identified in several taxa from invertebrates to vertebrates (Hoffmann and Reichhart, 2002). After detection of not-self by PAMPs-PRRs interactions, the recognition machinery can stimulate defensive humoral and cellular responses: among them, an important humoral defensive process is the melanization (humoral encapsulation) of foreign bodies. The melanization reaction, which is a common response to not self entry in invertebrates, especially arthropods, is due to the activity of an oxidoreductase called PO. This enzyme, in the hemolymph, is a component of a complex system of proteases, proteases inhibitors (serpins) and humoral PRRs, constituting the so-called proPO-Activating system (proPO-As). proPO-As is proposed to be a not self recognition system, because conversion of proPO to the enzymatically active form can be induced by foreign PAMPs, particularly lipopolysaccharides and β-1,3-glucans. proPO-As, which is physiologically activated by invading micro-organisms or parasites, is a complex enzyme cascade in which the last active enzyme (phenoloxidase) can oxidize phenols into quinones, that in turn will convert into melanin autocatalytically (Nappi *et al.*, 2004). This system is a key element in the recognition of foreign bodies and in the production of opsonic factors; moreover, it is now considered to represent an integral component of the insect immune system (Ashida, 1990; Brivio *et al.*, 1996; Söderhall and Cerenius, 1998; Dimopoulous *et al.*, 2001; Cerenius and Söderhall, 2004). Several hemolymph 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 protease cascades that turn on prophenoloxidase enzymatic activity (Jomori and Natori, 1992; Söderhall, 1999).

Besides, cell-mediated defenses are performed by cellular elements represented by several types of hemocytes that are commonly identified using morphological, histochemical and functional features (Gupta, 1985, Brehelin and Zachary, 1986). Hemocytes are immunoreactive cells playing a central role in maintaining host integrity; they are involved in various defense mechanisms such as phagocytosis, nodule formation, encapsulation, melanization, and synthesis of antimicrobial peptides (Bulet *et al.*, 1999; Kanost *et al.*, 2004). Both cellular and humoral factors seem to be involved in the stimulation of cellular defenses, specifically in the early recognition and binding to PAMPs. Several researches have demonstrated that humoral recognition receptors are also needed to stimulate hemocytes aggregation on the target surface during encapsulation processes (Bulet *et al.*, 1999, Schmidt *et al.*, 2001).

The complex relationships between hosts and parasites can be clarified only considering the host

defense mechanisms and parasites evasion stategies altogether; the purpose of this paper is to outline some of the main parasite evasion strategies; in particular, the immunological interaction between entomopathogenic nematodes (*Steinernema feltiae*) and the lepidopteran model insect *G. mellonella* will be described. The chosen examples are focused on insect hosts because of their economical and medical importance and considering their susceptibility to the symbiontic complex *Steinernema-Xenorhabdus* commercially available as biological insecticide.

The convergence of parasitological and immunological studies also provides valuable knowledge in understanding the evolution of both parasitism and host immune system.

Parasite evasion strategies: general considerations

Parasites may successfully colonize their hosts by evading recognition, thus preventing immune defenses; circumvention of the host immune system can be achieved by molecular mimicry (or disguise) strategies or by colonization of young hosts, or host tissues, with low immunocompetence. Alternatively (or concurrently) many parasites are able to depress either cell-mediated or humoral effectors mechanisms in a process usually called *interference* (Lie and Heyneman, 1976).

As pointed out by Götz and Boman (1985), in order to survive, a parasite must reach an equilibrium with its host; a too efficient parasite may exterminate its hosts, whereas a too permissive parasite could have a low fitness and reproduction efficiency too low to guarantee its survival.

In many cases, evolution and selection have finely-tuned host-parasite relationships leading to a long survival of parasitized invertebrate hosts; this process resulted in the production of vector species responsible of the transmission to human and animals of important diseases (Richman and Kafatos, 1995; Ratcliffe and Whitten, 2004).

Molecular mimicry is a strategy by which parasites became antigenically closely related to the host and thus avoid to evoke host immune responses. True molecular mimicry can be defined as the endogenous production of mimicking molecules that are usually exposed on the body-surface (or cell surface) of the parasite.

Despite the intuitive appeal of molecular mimicry as a mechanism of avoidance, few studies with any invertebrate parasites demonstrate a functionally protective effect of shared antigens (Bayne *et al.*, 1987; Weston and Kemp, 1993). However, antigens such as: α-macroglobulin, immunoglobulin receptors, tropomyosin, MHC I and II antigens, blood group glycolipids and oligosaccharides, related to both hosts and parasites have been identified and characterized (Smithers *et al.,* 1969; Damian, 1991; Vellupilai and Harn, 1994).

The molecular disguise, another form of mimicry, is described as the acquisition (sequestering) of molecular components from the host (Ratcliffe *et al.*, 1985; Loker, 1994; Strand and Pech, 1995). In *Biomphalaria glabrata*, several studies have demonstrated the ability of *Schistosoma mansoni* to acquire host plasma proteins (e.g. hemoglobin, hemagglutinins, etc.) to form a coat of host factors (Yoshino and Bayne, 1983; Dunn and Yoshino, 1991; Johnston and Yoshino, 1996).

More recently, Kathirithamby and co-workers (2003) have described an alternative disguise mechanism carried out by a Strepsiptera (*Stichotrema dallatorreanum*) that is able to manipulate host (*Segestidea novaeguineae* and *S. d. defoliaria,* Orthoptera) epidermal tissues and wraps itself within it. This sort of bag acts thus as a camouflage for the endoparasite which is recognized as self by the host.

The stage of development of the host is also essential in determining the outcome of parasitization (Khafagi and Hegazi, 2004). In general, early instars larvae of insects show a reduced immune activity often due to a lower hemocytes number or to a different array of cell populations (Gardiner and Strand, 2000; Beetz *et al.*, 2004). Due to this, parasites penetrating young hosts can find a more favorable environment to overcome host defenses.

Finally, as a passive strategy, some parasites are able to colonize low-reactivity tissues of the host. A good example is represented by insect parasitoids that lay their eggs, with surgical precision, in nerve ganglia of their hosts into which the hemocytes do not normally circulate, so parasite embryos can develop unmolested within the host (Götz and Poinar, 1968; Salt, 1971).

As mentioned above, alternative active strategies are referred as *interference*; in this case, parasites show an aggressive suppression or alteration of the host immune system defenses. *Interference* can be directed toward host humoral factors that are neutralized by the parasite or, as more commonly proposed, immunocompetent cells could be targeted instead (Loker, 1994).

Another important aspect of host-parasite relationships is host humoral depression. With respect to this, proPO system is one of the main target for many parasites or microorganisms; this is probably due to the need to neutralize its drastic and rapid effect when a host is in the presence of not self infections.

Many parasitic wasps inject maternal factors into the host's hemocoel to suppress the host immune system and to ensure successful development of their progeny; Asgari and colleagues (2003) isolated a 50 kD protein from *Cotesia rubecula* (Hymenoptera, Braconidae) that blocked melanization in the hemolymph of its host *Pieris rapae* (Lepidoptera). The protein, named Vn50, is a serine proteinase homolog containing an amino-terminal clip domain; recently the authors also demonstrated that Vn50 is stable in the host hemolymph for at least 72 hrs after parasitization (Zhang *et al*, 2004). Using *M. sexta* as a model system, they found that Vn50 efficiently down-regulated proPO system, by significantly reducing its proteolytic activation. This occurred without directly inhibiting and/or damaging the active phenoloxidase. According to the above description, we have obtained similar results in the tobacco budworm *Heliothis virescens* (Lepidoptera) larvae infected by *Toxoneuron nigriceps* (Hymenoptera).

Gregorio and Ratcliffe (1991) demonstrated that the presence of *Tripanosoma rangeli* in the hemolymph of two insects (*Rhodnius prolixus* and

Triatoma infestans) significantly reduced the level of proPO activation. In their paper, the authors suggested that the susceptibility to *Tripanosoma* infection of both the hosts is strongly dependent on the proPO activation intensity.

Finally, in a recent study, we observed a drastic reduction of PO activity in the hemolymph of *G. mellonella* induced by heat-killed entomoparasite nematodes (*S. feltiae*) or purified parasites cuticles (Brivio *et al.*, 2002).

Considering that cellular encapsulation is one of the most effective processes directed toward large parasites, it is reasonable to discuss *interference* mechanisms acting against host immunocompetent cells. With regard to this, parasites might reduce the recognition capability of hemocytes, by damaging surface PRRs, by down-regulation of their synthesis, or simply rejecting active cells by means of noxious secretions or refractive body-surface; alternatively, direct damage of host hemocytes could be achieved.

A great number of evidences suggest that parasitoid wasps inject factors suppressing host immune system; well-described suppressive factors are venom glands secretions (in Braconid wasps), polyDNA virus and co-injected teratocytes cells (Vinson, 1990; Summers and Dib-Hajj, 1995). Infection with wasps is known to affect host hemocytes (particularly plasmatocytes) ability to attach to substrates, to aggregate and to spread correctly. Moreover, virus-containing calyx fluid from *Campoletis sonorensis* induces a significant reduction in the number of circulating hemocytes, when injected in *H. virescens* larvae (Davies and Vinson, 1988).

Effects on host cells have been broadly described in insects infected (or in *in vitro* assays) with nematode symbiontic bacteria. The nematocomplex *Steinernema carpocapsae*/-*Xenorhabdus nematophilus* seems to be responsible of almost two effects on Lepidoptera hemocytes; Ribeiro *et al*. (1999) demonstrated unsticking and cytotoxic effects of two factors released by nematocomplexes in *in vitro* experiments. However, from this paper is not clear if bacteria themselves, nematodes, or both, produced the above factors.

An interesting aspect of the immunodepressive action of *Xenorhabdus* was described by Park *et al*. (2004) in *M. sexta*; inhibitor(s) released by live bacteria seems to block eicosanoid biosynthesis (that are crucial mediators of insects cellular defense reactions) by reducing phospholipase A2 intracellular activity.

Finally, processes such as the release of toxic factors from hemocytes, phagocytosis, encapsulation and nodulation are also probably to be targets of parasite-derived *interference* factors. However, it is reasonable to expect different parasites to adopt different strategies of immunoevasion and that any particular parasite species would employ a variety of evasive tactics.

A short *profile* of the *killer* (entomopathogen nematocomplex)

As described by Nathan Cobb (1915), nematodes are one of the most abundant type of animals on Earth. Nematodes, thanks to their small size, to the resistant cuticle and to the ability to adapt to severe environmental changes, have colonized a wide range of habitats including vertebrate and invertebrate bodies. Nematodes may be free-living or parasitic; the latter are usually considered pests because they cause important diseases in animals, humans, and for their economic impact on many agricultural products.

A small but significant number of parasitic nematodes, called entomopathogenic, are of considerable interest because they possess various features as biological control agents for pest insects (Gaugler and Kaya, 1990; Georgis and Manweiler, 1994; Poinar, 1998). Entomopathogenic nematodes must meet some criteria to be considered good candidates for an overall biological control: they should neutralize agriculture insect pests and, possibly, insect vectors responsible of human and animal diseases; practically, they should be able to kill, sterilize, or hamper the development of their insect targets.

Insect-parasitic nematodes that possess optimal features as bioinsecticides belong to the families Steinernematidae and Heterorhabditidae (Nematoda, Rhabditida). Steinernematidae and Heterorhabditidae are not closely related phylogenetically but, through convergent evolution, they share similar life histories (Poinar, 1993); the main difference between them is the reproductive strategy (Steinernematidae are gonochoric, Heterorhabditidae are hermaphroditic). These families differ from other Rhabditids by having a species-specific mutualistic relationship with bacteria of the genus *Xenorhabdus* (Enterobacteriaceae) (Poinar, 1979; Kaya and Gaugler, 1993). *X. nematophilus* is associated with Steinernematidae and *Photorhabdus luminescens* with Heterorhabditidae in a specie-specific manner (Forst and Nealson, 1996; Forst *et al.,* 1997; Forst and Clarke, 2001; Silva *et al.*, 2002). The symbiontic 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 microorganisms. At the same time the nematode acts as a vector for the symbiontic 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).

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 (Fig. 2). In general, nematodes moult four times during each life cycle with a moult occurring at the end of each larval stage. 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 up to the size limit of its new cuticle.

The third juvenile stage (IJ3) of nematodes is known as the “infective juvenile” or “dauer” stage 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 (Poinar, 1990; Akhurst and Dunphy, 1993).

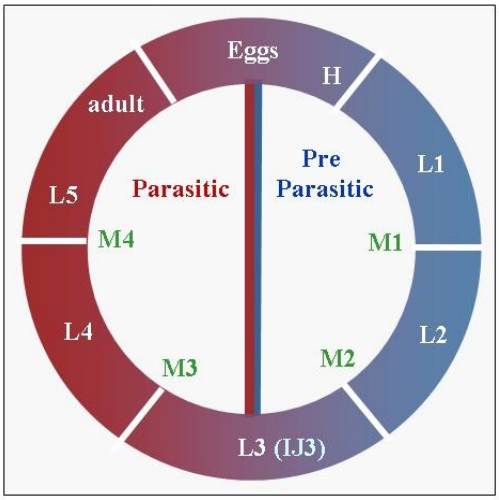

Fig. 2 A (L1) develops inside the egg, hatches (H), grows rapidly, then moults (M1) to L2. The second stage larva also shows a rapid growth followed by a second moult (M2) to third stage larva (L3), the infective juvenile stage 3 for many nematode species (also named IJ3). This (L3) grows, then moults (M3) inside the host to a L4 larvae. The final larval stage grows and undertakes a final moult (M4) to an immature adult (L5).

Host infection consists of various steps (Fig. 3); (A) the IJ3 parasites find hosts by chemotaxis towards chemical concentration gradients of carbon dioxide, and/or host excretory products. Infective juvenile stage enters the host through natural body openings (mouth, anus, spiracles), it reaches the hemocoel of the host, and later on (B) it releases bacterial spores by defecation or regurgitation. Symbiontic bacteria live in a monoxenically area or in differentiated vesicles of the anterior part of the infective juvenile intestine modified as a bacterial chamber. After release, bacteria quickly multiply in the hemolymph (C); they are mainly responsible for the host mortality because they produce and release exo- and endotoxins to which the insect succumbs by septicemia (D) within 24-48 h of infection; furthermore, bacteria secrete antibiotics that prevent multiplication of the other microflora (Khandelwal and Banerjee-Bhatnagar, 2003). Bacterial cells also express and release proteases and lipases that degrade insect host tissues to be utilized by the parasite as a food source. As reported by several authors (Wouts, 1984, Tanada and Kaya, 1993), the parasite itself produces toxins that are lethal to the host, even without its associated bacteria but, in this case, it is unable to reproduce; moreover, without the nematode, bacteria cannot reach and invade the host hemocoel.

After mating, the females lay the eggs that hatch as first-stage juveniles that moult successively to second, third and fourth-stage juveniles and then to males and females of the second generation (E). 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 (F), and moults to the infective juvenile stage (IJ3). When the host has been consumed, the infective juveniles (G) emerge from the exoskeleton of the host, move into the soil and begin the search for a new host (H). In nature, under ideal conditions, Steinernematidae and Heterorhabditidae emerge 6-11 and 12-14 days after the initial infection respectively (Kaya and Koppenhöfer, 1999).

Since entomopathogenic nematodes represent an alternative to chemicals for insect pest control, it is fundamental to understand the basis of the infectivity of nemato-bacterial complexes and the interaction with the insect host immune systems. Although the immune depressive and lethal effects induced by bacteria (long-term infection phase) are well known (ffrench-Constant *et al.*, 2000), the short-term infection phase, particularly the role of the parasite itself, are not clearly understood (Brivio *et al.*, 2002).

Gun and *bullets*: a lecture from the *killer*

The efficacy of the nematode *S. feltiae* in killing its hosts is mainly attributable to the severe effects of its symbiontic bacteria (*X. nematophilus*) that, by means of multiple factors, cause the death of the host in the later phase of infection.

Symbiontic bacteria could be viewed as bullets of a gun (the parasite): as it is well known, after a murder the effect and characteristics of the bullets found on the crime scene are easily assessed; but detectives are in trouble when trying to investigate on the missing gun.

Given that, the bacterial *bullets* have been well studied; a lot of papers provide in depth descriptions of the immunodepressive effects of bacteria-released factors. Host physiological disorder caused by the release and proliferation of the symbiontic bacteria have been investigated (Chattopadhyay *et al.*, 2004); particularly, these microorganisms seem to be able to arrange the environment (host's body) in such a manner to allow the parasite to survive and spawn unmolested.

X. nematophilus, upon release into the hemolymph of *G. mellonella* host, adhere to the surface of hemocytes, proliferate and damage the cells that became vacuolated, unable to adhere to surfaces and finally show positivity to trypan blue (Dunphy and Webster, 1984, 1986). At the same time, *Xenorhabdus* synthesizes and releases antibiotic compounds within the insect hemocoel that suppress competing microorganisms; in this way they acquire the optimal condition to proliferate, allowing the parasites to complete its development (Gaugler and Kaya, 1990).

A growing number of *Xenorhabdus*-produced factors have been described; recently a cytotoxic pilin subunit (17 kD) of *X. nematophilus* has been isolated: the protein is expressed on the bacterial surface and also secreted in the extracellular medium; it binds to the surface of larval hemocytes and shows cytotoxic properties against immunocompetent cells of *Helicoverpa armigera*, finally causing agglutination of the cells (Khandelwal *et al.*, 2004).

Ribeiro and colleagues (2003) reported the purification of a cytotoxin of 10.7 kD of molecular weight from *X. nematophilus*, named

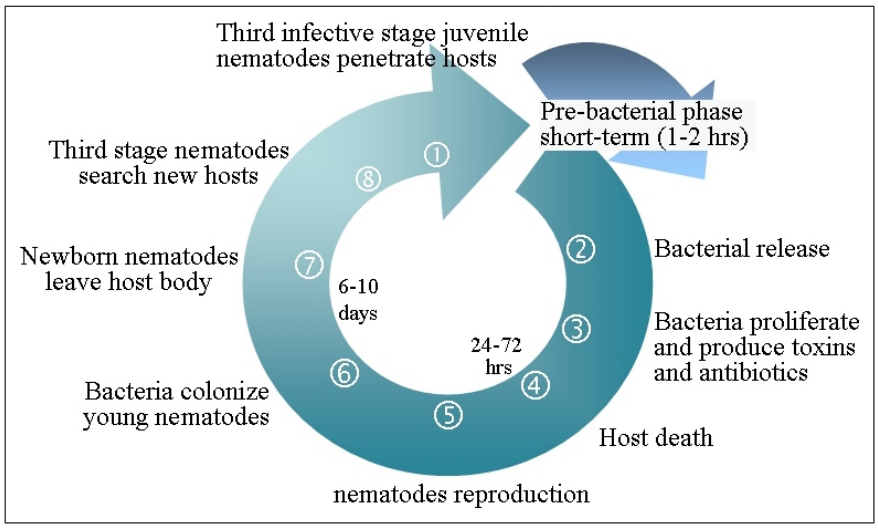

Fig. 3 Life cycle of entomopathogenic nematodes.

alpha-Xenorhabdolysin (alphaX) peptide; the plasma membrane of *Spodoptera littoralis* hemocytes seems to be the main target of the peptide. AlphaX peptide induces an increase of monovalent cations permeability that is sensitive to potassium channel blockers, even on mammal macrophages or erythrocytes. As a consequence of AlphaX binding to the plasma membrane, several events occur intracellularly, such as selective vacuolation of the endoplasmic reticulum, cell swelling and cell death.

In insects, bacterial infections usually evoke the activation of the Toll/Imd pathways that culminate in the synthesis of an array of antibacterial peptides; a well-known property of the parasite symbionts is to interfere with antibacterial responses. *Xenorhabdus* affects antimicrobial activity of Lepidoptera by means of two distinct released proteases; Caldas and colleagues (2002) demonstrated that one of the two above mentioned proteases (Protease II) destroyed antibacterial activity in the hemolymph of insect larvae (*G. mellonella* and *P. unipuncta*) challenged with inoculated bacteria; particularly, the bacteriolytic activity of the inducible antibacterial peptide cecropin A was drastically reduced. Furthermore, Protease II did not show toxicity to host hemocytes.

Finally, symbionts of entomopathogenic nematodes showed inhibitory effects on host proPO activating system (Yokoo *et al.*, 1992; Dunphy *et al.*, 1998).

The presence of a large number of studies on bacterial symbionts are mainly due to the economical interests that have led researchers to focus on bacteria-derived patentable molecules valuable in integrated pests management.

On the other hand, in order to study the role of the parasite itself, it would be necessary to work with axenic nematodes; unfortunately, it is difficult to obtain nematodes completely deprived of their symbionts and, in this case, parasites might not be in a physiological condition, increasing the risk of obtaining artifacts.

It is thus probably that experimental troubles and economical interests could be responsible for the scarcity of literature available on this topic.

With the aim of clarifying the involvement of the parasite itself in the relationships with the host, at first we have carried out our experiments in a short period (0-30 min) following *S. feltiae* infection, when bacteria are not yet released into the *G. mellonella* hemolymph; besides, we carried out many studies utilizing the isolated body surface (i.e. cuticle and epicuticle) of the parasite. Cuticles of *Steinernema* have been obtained without bacterial contamination to a good level of purity (Fig. 4) by developing a technique based on sonication, washes and sterilization of parasites.

Parasite immunoevasion strategies often involve the parasite body surface, which seems to play a key role in the interaction with the host environment (Blaxter *et al.*, 1992). Nematodes moult several times throughout their developmental cycle, each time changing their body surface with the formation of a new cuticle (Cox *et al.*, 1981a, 1981b); although a common model of nematode cuticle has been proposed (Maizels *et al.*, 1993), single species may have significant differences in molecular organization and surface properties. This is particularly true for parasitic species (i.e. *S. feltiae*) that must interact with an unfavorable host environment. Furthermore, parasitic nematodes may easily elaborate the composition and organization of the epicuticular external layer, depending upon the particular environment of each species (Maizels *et al.*, 1993). Together with other surface and secreted molecules (Politz and Philipp, 1992), the cuticle of parasitic nematodes seems to be involved in immunoevasion and suppression of host's defenses, as suggested also by Akhurst and Dunphy (1993); thus, it is likely that nematode body surface plays a crucial role in parasite success.

The hypothesis of a key role of the body-surface of parasites was proposed early by Vinson (1977); Vinson suggested that in absence of active suppression mechanisms the prevention from encapsulation could be achieved by means of: a) the acquisition of a coat composed of host proteins (molecular disguise); b) the possession of heterophilic antigens; c) the presence of a non reactive body-surface, or molecular mimicry.

In 1987 Dunphy and Webster presented preliminary evidences in favor of a possible role for the epicuticle layer of *S. feltiae* in cellular immuno-depression. The paper pointed out interactions of the body surface of the entomopathogen with *G. mellonella* hemocytes and suggested its involvement in avoiding cellular encapsulation. The authors described a partial characterization of cuticle sugars by means of lectin specificity but, more interestingly, they assessed the role of the lipidic moiety of the epicuticle of *S. feltiae*. A simple assay based on lipase treatments determined that surface lipids played a role in escaping from hemocytes recognition; on this basis, they supposed that modifications of the lipidic surface resulted in a changed molecular architecture of the epicuticle, thus exposing discriminable antigens.

Primarily inspired by these suggestions, we have focused the research on the role of *S. feltiae* cuticle, with the aim to exclude any contribution from symbiontic bacteria and/or active secretions of the parasite. Our preliminary observations (Brivio *et al*., 2002) showed host proPO system inhibition in *G. mellonella* larvae infected with heat-killed nematocomplexes; although these results suggested that factors released from the parasite were not responsible of proPO inhibition, they did not completely exclude the involvement of bacteria. However, these data supported the attractive hypothesis that the parasite, after entry into the host hemocoel, exploits its body-surface to immunoevade and/or immunodepress host defenses. A strong confirmation of the above hypothesis came from the assays carried out with isolated cuticles. The suppression of the hemolymph phenoloxidase activity, observed after either *in vivo* cuticle injection or *in vitro* co-incubation (cuticles *plus* cell-free hemolymph), was comparable to that obtained in the experiments performed with killed whole parasites.

The integrity of the molecular architecture of the cuticle seems to be essential to retain its immunodepressive properties, since chemical alterations of the structure result in a marked loss of inhibition of the host proPO system. Moreover, confirming Dunphy's suggestions, the main effect was observable after damage or removal of the lipidic layer obtained with lipase and methanol-chloroform treatments (Fig. 5).

These data confirmed the first assumption of a key role of the lipids in the host-parasite interaction, although no information concerning the mechanisms by which cuticular lipids may affect the activation of the proPO system was provided (Brivio *et al*., 2004).

The process by means of which *S. feltiae* cuticle lipids showed inhibitory effects on the host proPO system was further investigated hypothesizing that these molecules might interact with hemolymph factor involved in the activation pathway of host phenoloxidase. A set of experiments based on *in vitro* interaction of purified parasite cuticle with cell-free host hemolymph (Fig. 6), demonstrated a specific binding property of the cuticle: particularly, the lipidic moiety interacts and sequesters three hemolymph proteins (17, 26, 35 kD), named HIPs (Host-Interacting Proteins), possibly involved in the proPO activation cascade.

Concerning the identity and functions of HIPs, on the basis of preliminary characterization based on molecular mass and according to the literature (Dettloff *et al*., 2001), we supposed that, firstly, the 17 kD HIP could be identified as the insect lipid-carrier Apolipophorin III. Besides reports on its well-known functions in the lipid metabolism (Ryan and Van der Horst, 2000), exhaustive studies have been carried out on the involvement of this protein in immunological responses (Wiesner *et al*., 1997; Halwani and Dunphy, 1999; Zakarian *et al*., 2002). HIP26 has a molecular weight comparable to that of LBP-2 (a lipopolysaccharide-binding protein) described by Dunphy and Halwani (1997) in *G. mellonella*. This protein shows a specific affinity for endotoxin lipid-A and seems to be involved both in hemocytes activation and proPO system regulation. The third factor of 35 kD has a molecular weight similar to the protease-like molecule scolexin. Insect scolexin is well characterized at molecular level, although its biological function is not yet understood (Finnerty *et al*., 1999).

The significant quantitative reduction of HIPs induced by the parasite is responsible for the blockage of the activation pathway of the proPO system; when these components, eluted from the parasite body surface, are added during *in vitro* assays, the normal hemolymph phenoloxidase activity of the host is restored. Furthermore, a function of the HIPs seems to be related to the activation of hemolymph serine proteases, given that their properties of reactivation (Fig. 7) are lost if they are assayed in the presence of protease inhibitors.

The precise reactivation mechanism of the proPO system mediated by HIPs still has to be determined; to this goal we carried out Far Western blot experiments by which these proteins showed LPS-binding properties (Fig. 8). This affinity has been confirmed by their ability to bind to the wall of Gram(-) bacteria (Fig. 6, panel C, lane B).

In addition, cuticle lipids seem to cross-react with anti-LPS antibodies suggesting a structural correlation between the parasite lipids and the bacterial LPS lipid-A domain (Fig. 9).

An intriguing hypothesis could be that surface lipids may act as pathogen-associated molecular patterns-like (PAMPs-like) but, in this case, their interaction with the host HIPs (comparable to PRRs) would result in the removal of the latter from the insect hemolymph, thus preventing the activation of hemolymph serine proteases required for proPO activation and melanotic encapsulation.

The *interference* of the parasite with cell-mediated defenses of the host was investigated by Ribeiro and co-workers (1999). The observed hemocyte damages were suggested to be related to factors released in the medium from nematocomplexes; these compounds (not identified) showed unsticking and cytotoxic effects on *G. mellonella* and *M. unipunctata* immunocompetent cells. The data presented seem to indicate an active interference process carried up by *S. carpocapsae*.

Moreover, the interaction between the parasite body-surface molecules and host hemolymph components could result in a coating effect of the nematode. This coat, composed of host self-proteins, could induce molecular disguise processes. To ascertain the above assumption we have performed various assays clearly showing that *Galleria* hemocytes are unable to recognize the parasites as

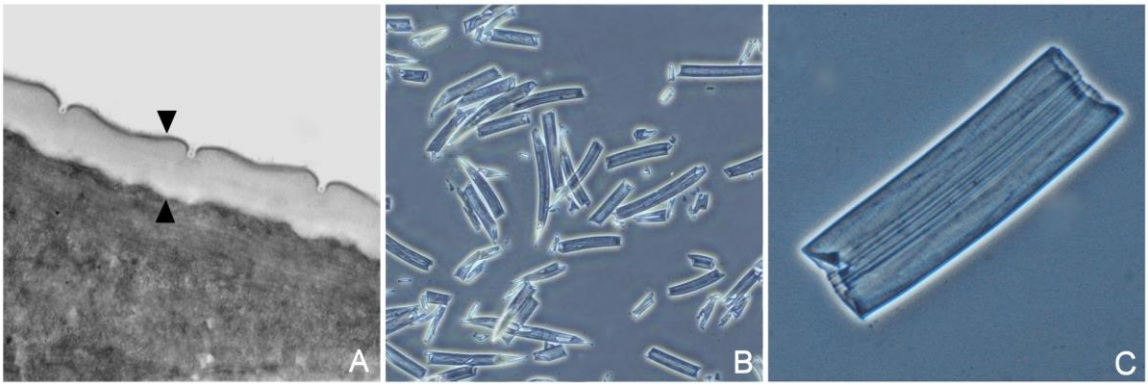

Fig. 4 A: longitudinal section of the parasite body (at TEM level) showing the cuticle and epicuticular layer of *S. feltiae*; in B and C purified cuticular fragments (phase contrast microscopy).

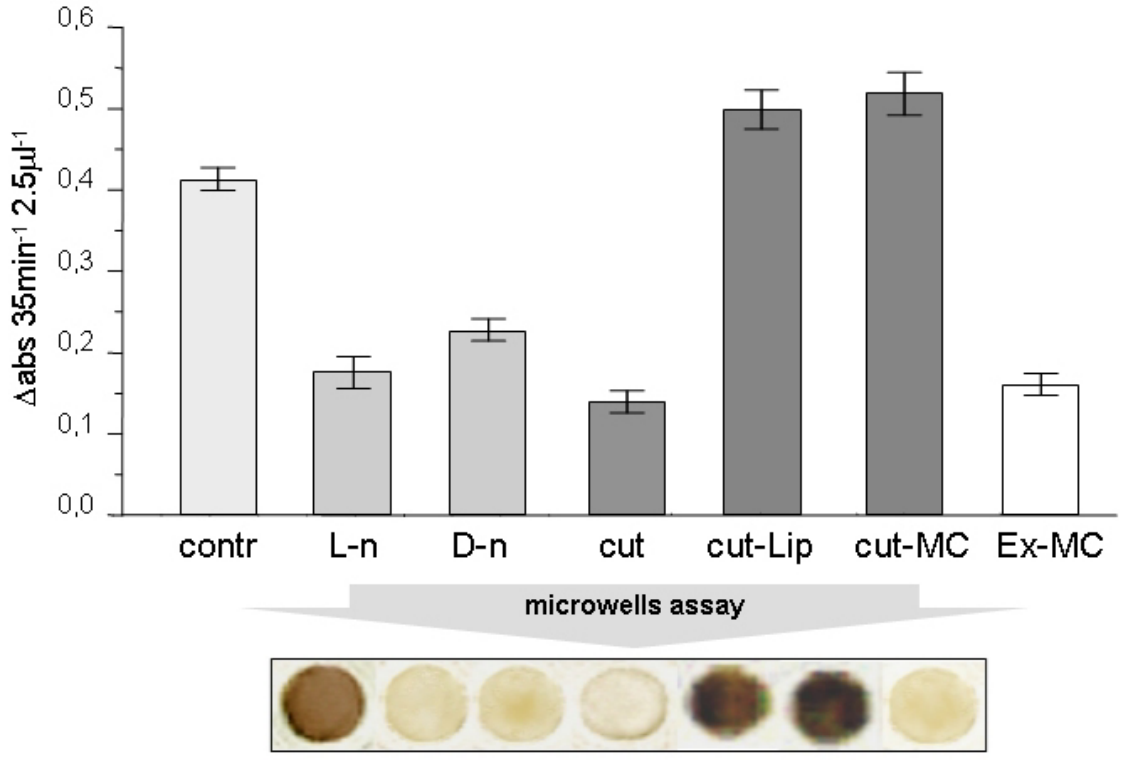

Fig. 5 Effects of the presence of *S. feltiae* whole individuals or isolated cuticles on proPO activity of *G. mellonella* larvae. In the upper area is shown relative phenoloxidase activity as modulated by: L-n, living parasites; D-n, heat-killed parasites; cut, isolated cuticles; cut-Lip, lipase-treated cuticles; cut-MC, methanol-chloroform-treated cuticles; Extr-MC, lipidic extracts from cuticles. Visualized below is the melanin production in microwells from the above assays.

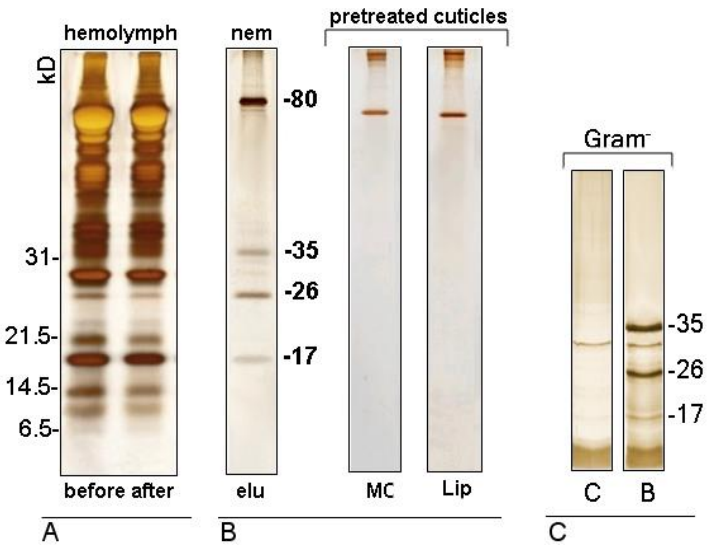

Fig. 6 To identify putative specific parasite-bound HIPs, high-salt elutions from parasite body surface (previously incubated with host hemolymph) were carried out. The supernatants of the elution were analyzed by SDS–PAGE (panel B). The parasites were thoroughly washed to eliminate all non-specific hemolymph proteins before elution. The electrophoretic pattern (panel B, lane "elu") revealed the presence of four main bands with molecular masses of about 80, 35, 26, and 17 kD, respectively. When the assay was carried out with methanol–chloroform or lipase-treated parasites, the lower bands (35, 26, and 17 kD) disappeared (panel B, lanes "MC" and "Lip") and only a reduced amount of the 80 kD band was observed by SDS–PAGE. Panel C: further interaction assays were carried out with hemolymph (<50 kD) incubated with Gram(-) bacteria (*E. cloacae*). *Enterobacter* was incubated with host hemolymph and 1M NaCl eluted proteins were analyzed by SDS–PAGE (lane B). Three main bands with molecular masses corresponding to the described HIPs were observed . As a control, high salt eluted bacteria (without prior hemolymph incubation) were analyzed (lane C).

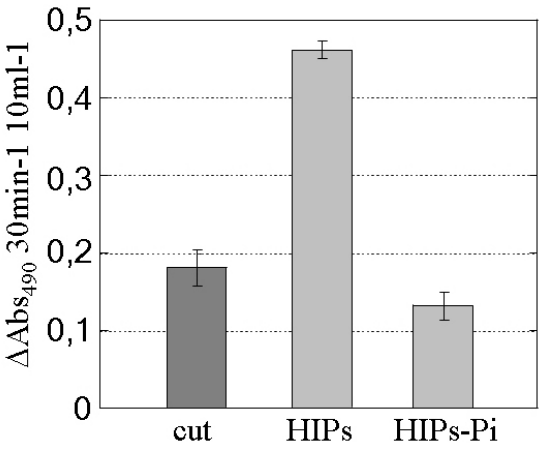

Fig. 7 Reactivation properties of HIPs directed against parasite-inhibited host proPO system are shown (HIPs). HIPs activating properties were also assayed in the presence of protease inhibitors: under these conditions host proPO system activity was not restored (HIPs-Pi).

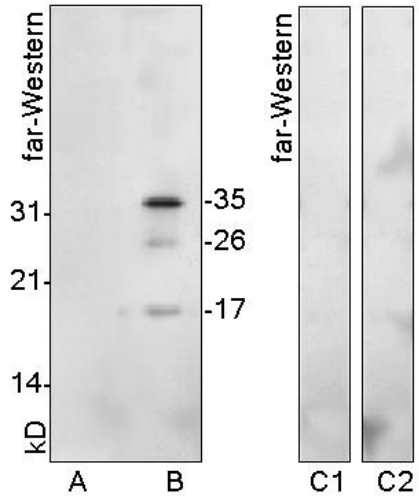

Fig. 8 Far-Western assay for LPS-binding activity of HIPs. Blotted HIPs were renatured *in situ* onto nitrocellulose sheets and incubated with lipopolysaccharides. Anti-LPS was used as primary antibody; the LPS-binding to HIPs was revealed by anti-IgG peroxidase-conjugated antibody and luminol. All HIPs (17, 26, and 35 kD) were positive to the assay (lane B); as a control, the assay was performed without HIPs *in situ* renaturation (lane A), without LPS incubation (lane C1) or omitting the secondary antibody (lane C2).

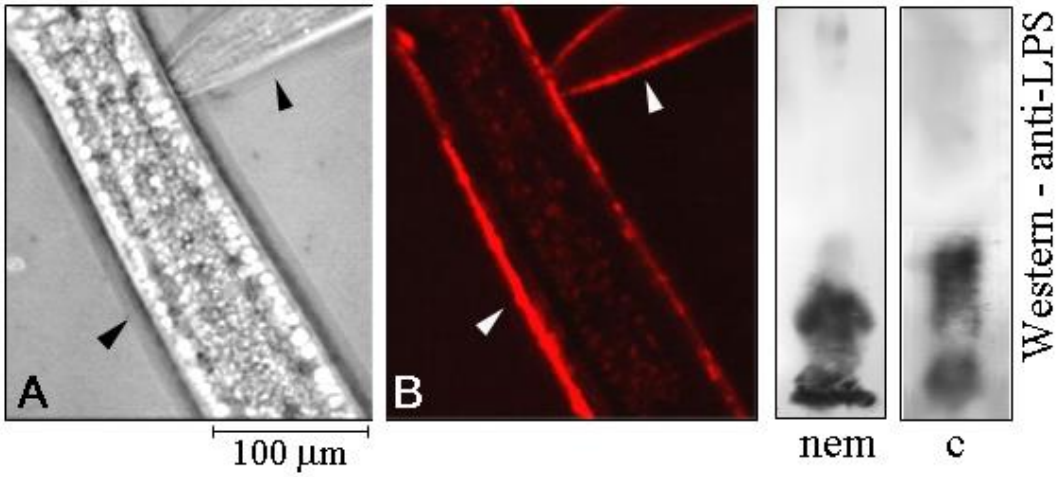

Fig. 9 Cross-reactivity of parasite cuticle compounds with anti-LPS antibodies. Parasites were immunostained with the primary antibody and then with anti-IgG TRITC-conjugated; as shown, a strong signal is localized at the parasite surface (panel B). Panel A shows light micrographs of parasite body section (arrowheads indicate cuticle and epicuticle zone). Anti-LPS Western blot, carried out with samples from methanol–chloroform cuticle extracts (lane nem) shows a positive smeared band observable at the bottom of the gel. The assay was carried out also with bacterial LPS, as a positive control (lane C).

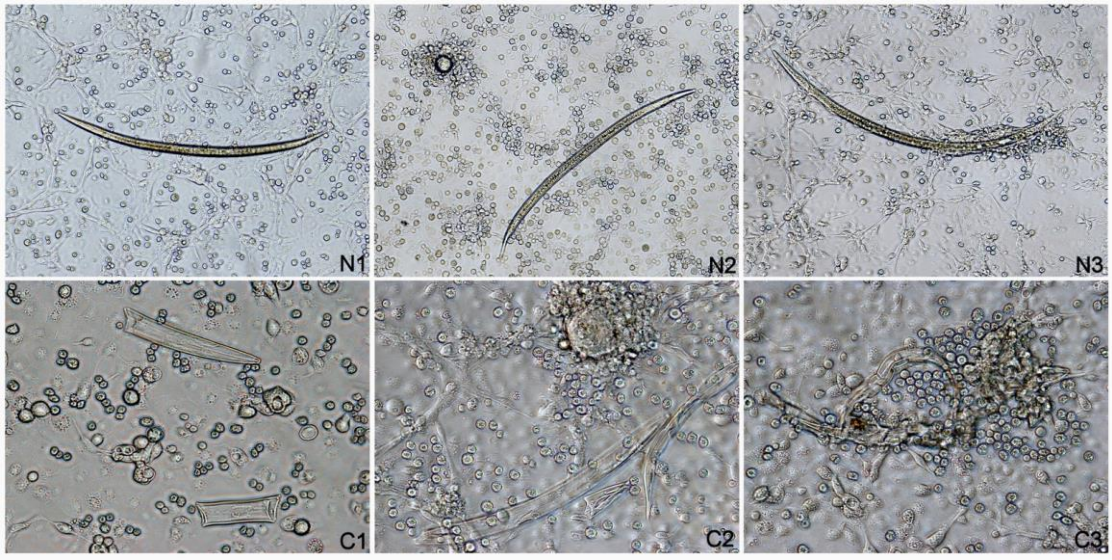

Fig. 10 Mimetic properties of *S. feltiae*. Parasites (N1, N2, N3) or cuticles (C1, C2, C3) were incubated with cultured hemocytes to assay for escaping from host cell-mediated encapsulation. N1 and C1 micrographs show the lack of encapsulation of whole parasite and cuticle fragments. In N3 and C3 is seen the formation of capsules on lipase-treated parasites and cuticles. N2 and C2 show the healthy state of hemocytes that, even in the presence of *S. feltiae* or cuticles, are able to encapsulate beads.

not self. The co-incubation experiments with parasites (or isolated cuticles) and abiotic materials provided further evidence that the host cells were healthy and capable of encapsulation (Fig. 10).

It can thus be supposed that this immunoevasion mechanism may be attributable to achievement of mimetic properties of the body-surface of *Steinernema* rather than to the cells having been damaged by the parasite. Finally, as observed in proPO system inhibition assays carried out with pre-treated cuticle, upon removal of lipidic compounds parasites become unable to evade cellular encapsulation of the host.

Based on the data obtained from the relationships between *S. feltiae* and *G. mellonella* we propose a possible schematic model (Fig. 11) describing some features of host-parasite (*Galleria-Steinernema*) immunological interaction.

In our lab we have recently undertaken a study to

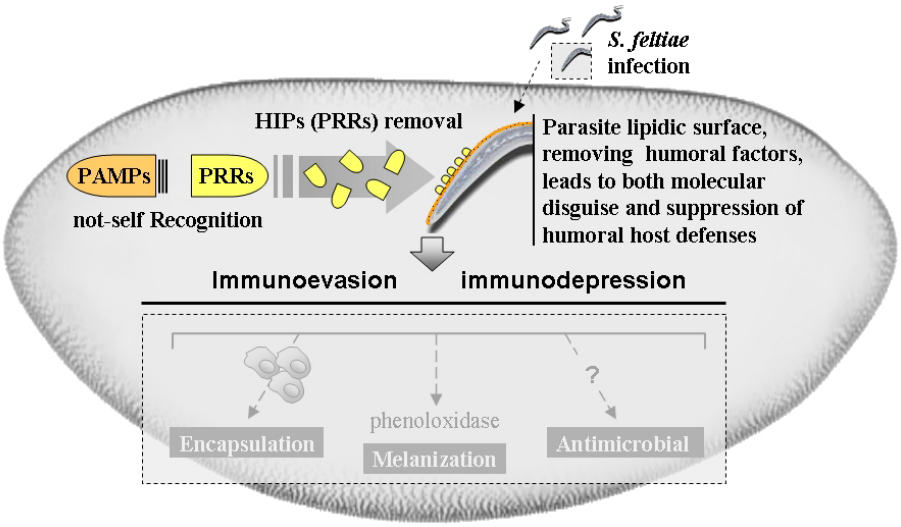

Fig. 11 A scheme delineating some parasite immunoevasion/depression processes related to hemolymph PRRs removal.

ascertain the *interference* of the parasite with the inducible antibacterial response of *G. mellonella*; this topic is particularly relevant in the studied model since these parasites, besides escaping host immune defenses, seem to be able to down-regulate AMPs genes reducing the synthesis of molecules potentially harmful for their symbiontic bacteria (manuscript in preparation).

Concluding remarks

Our society increasingly demands alternatives to chemicals for managing insect pests and insect vectors of diseases; even though biological control by means of bioinsecticides holds great promises, no more than 1.5 % of commercial pesticides are represented by biologicals. Researches directed towards a better knowledge of nematocomplexes-insects relationships could provide a valuable starting point for the improvement of integrated pest management techniques, with the aim of drastically reducing the use of chemicals.

Moreover, from studies of model insects immune system is clearly emerging a fascinating picture of the evolution of immune responses common to both invertebrates and vertebrates.

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