Modulation of immune responses of *Rhynchophorus ferrugineus* (Insecta: Coleoptera) induced by the entomopathogenic nematode *Steinernema carpocapsae* (Nematoda: Rhabditida)

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**Abstract** Aim of this study was to investigate relationships between the red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) and the entomopathogenic nematode *Steinernema carpocapsae* (EPN); particularly, the work was focused on the immune response of the insect host in naive larvae and after infection with the EPN. Two main immunological processes have been addressed: the activity and modulation of host prophenoloxidase-phenoloxidase (proPO) system, involved in melanization of not-self and hemocytes recognition processes responsible for not-self encapsulation. Moreover, immune depressive and immune evasive strategies of the parasite have been investigated. Our results suggest that RPW possess an efficient immune system, however in the early phase of infection, *S. carpocapsae* induces a strong inhibition of the host proPO system. In addition, host cell-mediated mechanisms of encapsulation, are completely avoided by the parasite, the elusive strategies of *S. carpocapsae* seem to be related to the structure of its body-surface, since induced alterations of the parasite cuticle resulted in the loss of its mimetic properties. *S. carpocapsae* before the release of its symbiotic bacteria, depress and elude RPW immune defenses, with the aim to arrange a favorable environment for its bacteria responsible of the septicemic death of the insect target.

**Key words** encapsulation; immune depression; immune evasion; proPO system; *Rhynchophorus ferrugineus*; *Steinernema carpocapsae*

**Introduction**

The international trade of plants and goods around the world may cause the unintentional diffusion of various alien insect pests in new areas, this is the case of *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), also named red palm weevil (RPW). RPW is native to Southeast Asia but today it is distributed in almost all Mediterranean areas, China, the United States, Middle East, and Japan (Roda et al., 2011; Huang, 2013). As reported, the RPW is one of the most serious pests affecting at least 19 palm species (EPPO, 2008; Dembilio et al., 2009; Wang et al., 2013), including ornamental palms and species of economic interest such as coconut, date, and oil palms.

RPWs is wood-boring insect, often attack healthy palms and larvae feed on the soft fibers and on apical meristem of the palms creating massive damage to palm tissues, weakening the structure of the palm trunk and leading to the death of infested palms. The resulting cutting down of infested trees is very significant in countries where palm trees are ornamental, changing the landscape; also, the damage is economically considerable where palms are grown as crops.
In order to limit the uncontrolled diffusion of this insect pest, chemical, biological, and physical methods are being tested (Gitau et al., 2009). The efficacy of chemical treatments has been evaluated when applied to the soil, when injected into palm trunks or sprayed on the foliage, procedures currently used to control RPW are largely based on dispersal of large quantities of chemical pesticides, although these treatments cause great concern for the environment and health which is not advisable in the urban areas (Faleiro, 2006). However, the cryptic habitat of RPW during its life-cycle makes these treatments ineffective unless they are extensive and protracted over long periods (Ferry & Gomez, 2002).

An alternative to chemical treatments could be the use of biocontrol agents such as bacteria, fungi, or entomopathogenic nematodes. Biological control with Bacillus thuringiensis (Bt) is now common in many agricultural systems, besides the troubles related to the concealed habitat of the RPW, a major weakness in the use of Bt is the onset of resistance to the bacterial toxin in the host target (Toubarro et al., 2010, 2013; Castillo et al., 2011). Insects are able to maintain self-integrity discriminating self from not self by means of a powerful immune system (Schmid-Hempel, 2005); consequently, endoparasites have to overcome the host immune defenses to complete their life-cycles (Loker, 1994, Loker et al., 2004; Carton et al., 2008; Schmid-Hempel, 2012).

Invertebrates (i.e., insects) lack finely tuned immunorecognition receptors (antibodies and B and T cells are absent) but they possess a pool of cellular and humoral Pattern-Recognition Receptors (PRRs) able to interact specifically with a broad range of foreign antigenic surface compounds, commonly named PAMPS (pathogen-associated molecular patterns). PAMPS-PRRs interaction is a key process of the discriminatory steps of innate immunity that usually lead to effectors mechanisms responsible for the elimination of not self (Medzhitov, 2001; Janeway & Medzhitov, 2002; Kanost et al., 2004). Insect immune responses involve both humoral and cellular defenses mechanisms (Hoffmann et al., 1996; Janeway & Medzhitov, 2002; Hoffmann, 2003); humoral defenses include processes such as, antimicrobial peptides synthesis, lectin-mediated recognition and melanization (also called humoral encapsulation), the latter is consequent to the activation of the prophenoloxidase-phenoloxidase (proPO) system (Cerenius & Söderhäll, 2004; Wang & Jiang, 2004; Xu et al., 2006; Cytryńska et al., 2007). Moreover, main cellular processes such as phagocytosis and encapsulation are carried out by immunocompetent cells called hemocytes (Ribeiro et al., 1999; Ribeiro & Brehelin, 2006; Tojo et al., 2000; Lavine & Strand, 2002).

Though, both proPO system activity and cellular encapsulation are the more effective defenses processes against foreign multicellular organisms, in previous works we described how the entomoparasite Steinernema feltiae is able to escape from immunological detection of the host Galleria mellonella (Lepidoptera: Pyralidae), avoiding hemocytes encapsulation and inhibiting the activity of the host proPO system (Brivio et al., 2002, 2004; Ma store & Brivio, 2008). The majority of studies regarding the interaction between EPNs and immune host system, are focused on lepidopteran and dipteran species (Li et al., 2007; Castillo et al., 2013), few data are available.
regarding the huge order of Coleoptera. Although studies on the distribution of RPWs (Rugman-Jones et al., 2013) and on the damage caused to palms are available in literature (Gitau et al., 2009), data about the immune defenses of this species are scarce (Manachini et al., 2011, 2013), thus, we believe that it is important to study this aspect of the physiology of *R. ferrugineus* with the aim to improve control strategies against this insect pest.

In this paper, we have examined some aspects of the immune response of *R. ferrugineus* larvae, considering the effects induced by the presence of *S. carpocapsae*. The potential interference of the EPNs in host cellular and humoral immunological processes has been assessed; particularly, we have evaluated the activity of the host proPO system and the efficiency of hemocytes to recognize and encapsulate parasites. From the obtained data, we have ascertained the ability of *S. carpocapsae* nematocomplexes to modulate proPO system activity of RPW, thus interfering with insect melanization processes. Moreover, we have also confirmed the mimetic role of the nematode body-surface to circumvent host cell recognition and encapsulation processes, as a result of immune-depressive/elusive strategies; the parasite seems to be able to overcome successfully the immune responses also in this insect species.

Materials and methods

Reagents and instruments

Instruments and reagents were purchased from Bio-Rad 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. All assays were performed at 4°C under sterile conditions when required.

Insects and parasites

*R. ferrugineus* later instars larvae were used to study relationships between insect hosts and the parasites. Larvae were collected from Canary palm trees (*Phoenix canariensis*), in 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. Entomopathogenic nematodes, *S. carpocapsae* 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 g 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×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.

**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: 10 mmol/L Tris-HCl, 10 mmol/L EDTA, 1 mmol/L PMSF, pH 7.2) and subjected to 2 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.

**Microorganisms**

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 = 600 nm). Subsequently, the cultures were centrifuged at 1700×g for 15 min, the bacterial pellet was washed several times with sterile PBS (138 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4/KH2PO4, pH 7.4), cells were killed (20 min 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 overnight at 37°C.
Cultures were centrifuged at 1700 × g for 10 min 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 10^5 cells/mL. Finally, yeast cells were heat-killed (at 95 °C for 30 min), centrifuged and washed several times in sterile buffer, aliquots of all microorganisms were immediately used or stored at −20 °C.

Propagation of S. carpocapsae in R. ferrugineus

To evaluate the ability of penetration of S. carpocapsae inside R. ferrugineus larvae, the method of Dutky et al. (1964) was used. Ten to fifteen milliliters of an aqueous solution of S. carpocapsae (approximately 2 × 10^3 nematodes/mL) was layered on a sterile filter paper placed in a Petri dish (90 mm diameter), larvae were incubated up to 48 h at 20 °C. After incubation, dead or living larvae were dissected to assess the presence of parasites in the hemocoelic cavity. Host body cavity was washed with Mead buffer (98 mmol/L NaOH, 145 mmol/L NaCl, 17 mmol/L EDTA, 41 mmol/L citric acid, pH 4.5), the buffer was harvested and the number of nematodes evaluated under a stereomicroscope.

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 2 low-speed centrifugations (200 × g, for 10 min, at 4 °C) of whole hemolymph, previously flushed out from healthy larvae. Bacteria (2 × 10^2), yeast cells (2 × 10^5), lipopolysaccharides (10 μg), 10 μL of a saturated solution of β-Glucans, or peptidoglycans (10 μg), were added to 200 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 490 nm 5 min^-1, at 20 °C), by evaluating the dopachrome formation from L-Dopa substrate. As control, basal activity of proPO system was analyzed in extracted hemolymph samples without activators.

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 coincubation 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 microsyringes 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 “proPO system activity of R. ferrugineus” section. As controls in all in vivo assays, larvae were injected with sterile PBS.

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), hemolymph 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 × 10^5 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.

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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 coincubations 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 2 and 8 h after the start of incubation, observations were made under an inverted microscope (Olympus IX51) 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 2 h, after hosts infection, larvae were bled and extracted nematodes and hemocytes were observed by microscopy.

Data processing and statistical analysis

Statistical analysis were performed using the Student’s unpaired t-test, differences between mean values were analyzed and considered significant when \( P < 0.05 \) or considered extremely significant when \( P < 0.0001 \) with respect to control values. All experiments were replicated at least 10 times. Data were processed with GraphPad Prism 4 and GraphPad InStat 4 (GraphPad Software, Inc., CA, USA).

Results

RPW infestation

The capability of S. carpocapsae to penetrate and kill RPW larvae was assessed, data outlined in Table 1 show the number of penetrated parasites at various times within 48 h and the R. ferrugineus mortality.

The proPO system of RPW

Relative activity of host phenoloxidase was evaluated by means of spectrophotometric analyses either in naive CFF samples or in pretreated hemolymph samples, in the presence of PAMPs or whole microorganisms. As shown in Fig. 1A, the presence of lipopolysaccharides (from E. coli), peptidoglycans (from B. subtilis), or β-glucans (from S. cerevisiae), resulted in a strong increase of activity (Fig. 1A, LPS, PGN, GLU); absorbance recorded values were, on average, more than twice as compared to the control (Figs. 1A and B).

Table 1 Propagation test, white trap infestation of R. ferrugineus (RPW) with S. carpocapsae (EPN). Data refer to the number of parasites inside host hemocoel cavity and to mortality of RPW (means ± SEM, \( n = 20 \)).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>No. EPNs (means ± SEM)</th>
<th>RPW mortality(%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2 ± 0.55</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>3 ± 0.70</td>
<td>0</td>
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<tr>
<td>24</td>
<td>5 ± 0.91</td>
<td>10</td>
</tr>
<tr>
<td>48</td>
<td>5 ± 0.74</td>
<td>80</td>
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The proPO system modulation induced by parasites

The effects of S. carpocapsae on the activity of host proPO system were assessed by in vitro and in vivo assays (Fig. 2). Fig. 2A shows the phenoloxidase relative activity when live parasites, or isolated parasites cuticles, were coincubated with CFF in vitro; the presence of whole parasites (Nem) resulted in a marked decrease of the enzyme relative activity, instead, isolated parasite cuticles (Cut) lacked inhibiting properties. When parasites were injected into the host hemocoel cavity in the early phase of infection (Fig. 2B, Nem10), a level of inhibition comparable to that recorded in the in vitro assay (Fig. 2A, Nem) was observed. In the late phases after injection (60 and 120 min) a marked activation of proPO system was observed (Fig. 2B, Nem60, Nem120).

We also performed in vivo assays with cold-killed nematodes, Fig. 2C shows the proPO activity after injection of cold-killed parasites; the strong inhibition effects observed at 30 min with live nematodes (Fig. 2B) seem to be lost; for all the analyzed time periods (Fig. 2C, dNem30, dNem60, dNem120) a weak inhibition was evident when compared to the control (Fig. 2C).
Host hemocytes and encapsulation of abiotic not self

Main hemocytes populations from late stage larvae of *R. ferrugineus* are showed in Fig. 3A (left and right), granulocytes (Gr), plasmatocytes (Pl), 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 and however, they can be clearly distinguished by their spindle-shaped appearance. Membrane extensions, which originate from the cell surface can be seen in culture (after 30 min), which later develop into motile pseudopodia that move and spread on the surface of culture wells (not shown). 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 observable in Fig. 3B, RPW cells are able to react against *not self*. Agarose beads were coincubated with hemocytes, after 2 h (left), both plasmatocytes and granulocytes move toward and surround the bead, in panel right (8 h), beads are enclosed by several cellular layers and melanin formation within the capsule is evident.

Host cells reaction vs. parasites (*in vitro*)

We tested the host cellular response against live and dead parasites. Fig. 4A shows *in vitro* incubations of host hemocytes with live and dead *S. carpocapsae* (left and middle). After 8 h of incubation, both living and...
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, Nem30, Nem60, Nem120, 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; dNem30, dNem60, dNem120, dead S. carpocapsae-injected larvae, 30, 60, and 120 min after infection. Mean ± SEM, n = 5, *P < 0.05.

cold-killed parasites were not recognized and not encapsulated, hemocytes seem to be healthy, no esocytosis process was observable and melanin formation was absent. In order to verify if the presence of parasites affected cell health and functions, nematodes and microbeads were incubated concurrently with host hemocytes (Fig. 4A, right); the presence of cold-killed parasites did not interfere with hemocytes encapsulation properties since host cells were able to encapsulate beads (arrow head). Given that hemocytes did not recognize S. carpocapsae as not self, the parasite itself seems to possess mimetic properties. The elusive role of the parasite body-surface was ascertained by encapsulation assays against isolated cuticles, as shown in Fig. 4B, cuticles were not detected by host cells, no migration and encapsulation were observed (panels left and middle, 2 and 8 h postincubation, respectively). However, when parasite body-surfaces were damaged by heat-treatment, S. carpocapsae seems to lose its mimetic features; as shown in Fig. 4B (right), cuticle-damaged parasites are recognized and surrounded by cellular multilayered capsules. Cell migration processes toward parasites were usually preceded by the formation of several stretched motile pseudopodia (Fig. 4B, right, arrowheads).
Rhynchophorus ferrugineus immune responses

Fig. 3 In vitro assay with Rhynchophorus ferrugineus hemocytes. (A) micrographs show main cell populations from last instar larvae. Gr, granulocytes; Pl, plasmatocytes; Oe, oenocyteoids (400×). (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 (200×).

Host immune reaction versus parasites (in vivo)

The host–parasite interaction assays were carried out in vivo by microinjection of nematodes into the host hemocoel (Fig. 4C). When injected into the host, cold-killed parasites show mimetic properties (Fig. 4C, left); the lack of cell responses was similar to that observed when the assays were carried out in vitro (Fig. 4A, middle). Instead, when heat-killed parasites were injected, cellular and humoral reactions were faster and stronger with respect to in vitro assays. Fig. 4C (middle) shows the formation of dark melanin aggregates around a parasite after 30 min and after 2 h (Fig. 4C, right) melanin masses fully engulf parasites (arrowheads) and hemocytes are barely visible since they were enclosed inside the dark capsule.

Discussion

In this work, we investigated the interaction of S. carpocapsae with R. ferrugineus; in particular, some immunological features of the host and elusive/depressive strategies of the parasite have been elucidated, the obtained data could be of value to improve actions to control the insect pest. Some works on RPW have been performed in field (Dembilio et al., 2011; Triggiani & Tarasco, 2011), but only a few papers address both host immunological responses and evasive strategies of the S. carpocapsae (Manachini et al., 2011, 2013).

From the data previously obtained with S. feltiae and G. mellonella, we ascertained the primary role of the parasite cuticle, the epicuticular zone seems to be the key element responsible of molecular disguise strategies and at the same time, able to cause host immune suppression. The dual role of the cuticle was evident when we assayed the biochemical properties of its lipidic moiety; lipids of S. feltiae epicuticle interacted and removed 3 main humoral components from the host hemolymph (named HIPs). The subtraction consequent to cuticle adhesion of specific host proteins was the main mechanism responsible of the observed disguise, resulting from the covering of the parasite with host antigens, (Mastore & Brivio, 2008).

The removal of hemolymph host factors downregulated antimicrobial peptide synthesis (Brivio et al., 2006), inhibited proPO system activation (Brivio et al., 2004) and finally affected encapsulation and phagocytosis capability of host hemocytes (Brivio et al., 2010).

Interactions between S. carpocapsae and R. ferrugineus seem to be only partly similar to that described for S. feltiae–G. mellonella (for a review see: Brivio et al., 2005); according to literature (Balasubramanian et al., 2009; Hao et al., 2009; Toubarro et al., 2009, 2010) we observed immune depressive effects of living S. carpocapsae culminating in the fast inhibition of host proPO system. The decrease of phenoloxidase activity was evident only in the early phase of infection (30 min), since after 1 h the parasite did not induce activation of proPO system. As described by Shi et al. (2012), the delayed activation of phenoloxidase could be due to the release of Xenorhabdus toxins into the hemocoelic cavity, though, inhibitory effects of symbionts bacteria have been ascertained in other insect species (de Silva et al., 2000; Song et al., 2011). Even if the efficacy of EPNs is based on the lethal action of symbiotic bacteria in the late phase of infection, results confirmed that, immediately after the entry, parasites must neutralize a complex series of adverse immune reactions performed by the host (Dunphy & Webster, 1987; Brivio et al., 2005; Brivio et al., 2010).

The early inhibitory effects did not seem be mediated by processes comparable to that observed with S. feltiae, because only living S. carpocapsae possess inhibitor effects, probably induced by their secretions, for example, serine proteases (Toubarro et al., 2013) and not to cuticle properties. Moreover, when EPNs are used against a range of insect targets, the behavior of parasites and host responses are often inhomogeneous (Gaugler & Kaya,
Fig. 4  (A) *S. carpocapsae* living (left) and cold-killed (middle) were incubated in vitro with host hemocytes (8 h). In both cases parasites were not recognized and encapsulation process was not observed (100×). Right, nematodes and microbeads were co-incubated, even if in the presence of parasites cells are able to encapsulate agarose beads (arrowhead) (200×).  
(B) Immune evasion properties of the parasite body surface. Isolated cuticles were incubated with host hemocytes for 2 h (left) and 8 h (middle), either at short or long in vitro incubations, cuticles seem to avoid cell recognition. Right, heat-induced cuticle damage resulted in the loss of the mimetic properties of the parasite (n), thus a strong encapsulation was observed (200×).  
(C) 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 bleded, hemocytes and parasites were placed in microwells and observed under a light microscope. Left, cold-killed parasites after 2 h from injection were not encapsulated. Panels middle and right, show assays carried out with heat-killed nematodes 30 min and 2 h after injection, respectively, conspicuous formation of melanin was evident; in right panel, parasites (arrowheads) are completely surrounded by dark melanin masses (100×).

1990), this could explain the variety of effects observed when *S. feltiae* or *S. carpocapsae* infected different hosts. In addition, our data show that cold-killed parasites do not inhibit the RPW proPO system, confirming that this mechanism implies active secretion processes and it is achieved early by living parasites.

After we verified the ability of parasites to depress host proPO system, we analyzed the system activity either in physiological conditions or in the presence of not entomopathogenic microorganisms; we observed that naïve larvae showed a basal activity, which is strongly elicited in the presence of foreign bodies (*E. coli*, *B. subtilis*, *S. cerevisiae*) or their cell wall compounds (LPS, PGNs, and β-glucans). These data seem to confirm that, although the RPW proPO system is very responsive to *not self*, the parasite is able to modulate temporarily the phenoloxidase activity, ensuring its survival and creating a favorable environment for its symbionts.

A further interesting aspect of the immune response of RPW is related to recognizing processes carried out
by circulating hemocytes; usually, insect immunocompetent cells, such as granulocytes and plasmatocytes are both involved in the recognition often followed by cellular covering surrounding not self bodies (Strand, 2008; Castillo et al., 2011). Considering our previous observations of immune evasion strategies of S. feltiae (Mastore & Brivio, 2008), we investigated the ability of S. carpocapsae to elude the RPW cell surveillance and avoid encapsulation processes. RPW hemocytes show a typical morphology and they actively participate in capsule formation also against abiotic targets; when agarose beads were coincubated with hemocytes, cellular encapsulation was evident, beads were surrounded by several cell layers and melanin enclosed targets inside the capsule.

Parasites may have sharp differences in molecular organization and surface properties, this is particularly true for EPNs, which once entered, must interact with a potentially harmful insects host environment. Even though the body surface of S. carpocapsae did not seem to be involved in depression of R. ferrugineus humoral responses, a key role of the cuticle has been ascertained to avoid host cell surveillance. Recognition and cell encapsulation are completely lacking against S. carpocapsae, either alive or cold-killed nematodes were not recognized by host cells; also, isolated cuticles are identified as self by RPW hemocytes confirming the involvement of the parasite body surface in elusive mimetic strategies. Lacking of cellular recognition against parasites was not due to damage of hemocytes, because when coincubated with EPNs, synthetic beads were normally encapsulated.

According to the data obtained with S. feltiae (Brivio et al., 2002, 2004), the mimetic role of S. carpocapsae cuticle was lost when the structure was damaged by heat treatments, any structural and chemical modifications caused the disappearance of the parasite elusive strategies culminating in the stimulation of cellular processes. Effects of damages of parasite body surface were particularly evident when treated S. carpocapsae was injected into larvae for in vivo assays, immune responses were strongly enhanced and in particular, melanin formation was very quick and intense.

The hypothesis of a central function of the body surface of parasites was proposed early by Vinson (1977, 1990), and further works confirmed the involvement of the cuticle in immune evasion and suppression of host defenses, (Blaxter et al., 1992; Politz & Philipp, 1992; Akhurst & Dunphy, 1993).

Although somewhat different, S. carpocapsae and S. feltiae share an overall strategy aimed to arrange a favorable environment for their symbiotic bacteria, by means of the elusion from cell recognition and depression of host humoral defenses. Our data can provide a useful starting point, however, we have to consider that the complete picture of the immune response of R. ferrugineus and its interactions with S. carpocapsae nematocomplexes is not yet exhaustive, further studies are needed to increase the knowledge of the physiology of this insect pest.

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Disclosures

Authors declare that there are no interest conflicts and no financial interests concerning the subject presented in the manuscript.

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