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Research Report

Early suppression of immune response in *Heliothis virescens* larvae by the endophagous parasitoid *Toxoneuron nigriceps*

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Abstract
Toxoneuron nigriceps is an endophagous parasitoid of larval stages of the noctuid moth *Heliothis virescens*. As all parasitoids, this wasp avoid host immune reaction by a combination of several passive and active mechanisms. Secretions injected by ovipositing females, which contain venom, calyx fluid and polydnaviruses, are the most probably factors actively disrupting *Heliothis virescens* immune system. This paper describes the main alterations of the host immune response observed shortly after oviposition by *T. nigriceps*. A transient block of prophenoloxidase activity is registered along with changes in hemocyte number, adhesion and structure, which suggest the occurrence of apoptosis. In contrast, the host plasmatocytes appear structurally unaltered, but unable to produce a capsule *in vitro*.

Key words: insects; parasitoid; immune defenses

Introduction

Foreign objects entering insect hemocoel are recognized as non-self and elicit a variety of defense reactions, which are somewhat arbitrary divided into humoral and cellular responses (Hoffman, 1995; Gillespie *et al*., 1997; Lavine and Strand, 2002). Humoral responses include the production of antibacterial/antifungal peptides (Boman *et al*., 1991; Hoffmann *et al*., 1993; Hultmark, 1993; Cociancich *et al*., 1994; Lowenberger, 2001) and of reactive intermediates of oxygen or nitrogen (Bogdan *et al*., 2000; Vass and Nappi, 2001), as well as in the activation of enzymatic cascades regulating coagulation or melanization of hemolymph (Gillespie *et al*., 1997). Host cellular immune responses, like phagocytosis, nodulation and encapsulation (Schmidt et al., 2001; Lavine and Strand, 2002) are mediated by different types of hemocytes, but the regulatory molecular mechanisms involved are much less understood than those controlling humoral responses (Lavine and Strand, 2002).

Endophagous parasitoids, entering the host body, have to protect themselves from these defense barriers (Schmidt *et al*., 2001). Non-permissive hosts typically eliminate endoparasitoids by encapsulation, which usually involves the binding of overlapping layers of hemocytes to the surface of parasitoid. Larval endoparasitoids evade host immune defenses either passively, or by active suppression of the host immune system, or by a combination thereof (Schmidt *et al*., 2001; Lavine and Strand, 2002). Female secretions injected at the oviposition are the most common factors

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actively disrupting the host immune response. These secretions include venom and calyx fluid, which may contain different types of viruses and virus-like particles, often involved in the suppression of the immune response (Schmidt *et al.*, 2001). Among these viruses, the polydnaviruses are by far the most studied and their molecular characterization in different systems has allowed to shed new light on their role in the host regulation process (Kroemer and Webb, 2004; Webb and Strand, 2005).

Toxoneuron nigriceps (Hymenoptera, Braconidae) is an endophagous parasitoid of the larval stages of the tobacco budworm, *Heliothis virescens (*Lepidoptera, Noctuidae). The bracovirus associated with this wasp (*Tn*BV) is currently being studied and several genes expressed in parasitized host larvae have been isolated and their possible role partly elucidated (Varricchio *et al*., 1999; Pennacchio *et al.*, 2001; Falabella *et al.*, 2003; Malva *et al.*, 2004; Provost *et al.*, 2004; Lapointe *et al.*, 2005). Most of the isolated genes are actively expressed in the hemocytes of parasitized hosts and, then, probably involved in disruption and/or suppression. However, to better analyze the role played by these *Tn*BV genes, and by other female secretions injected at the oviposition along with the egg, we need a better understanding of the alterations of the immune responses occurring in naturally parasitized hosts. This information is largely lacking, while for the passive evasion of host immune response it appears that the egg fibrous layer may play an important role (Davies and Vinson, 1986).

The present paper aims at filling this gap by providing data on the most relevant precocious alterations associated with the suppression of host immune response observed in tobacco budworm larvae parasitized by *T. nigriceps*.

Materials and methods

Insect rearing

Toxoneuron nigriceps was reared in the laboratory according to the methodology described by Vinson *et al.* (1973). *Heliothis virescens* larvae were maintained on a modified artificial diet developed by Vanderzant *et al.* (1962) (Corn Earworm Diet, BioServe, Frenchtown, NJ, USA). Rearing temperature was 29±1° C for both the host and parasitoid, whereas *T. nigriceps* adults were kept at 25±1 °C. In both cases a 16 h light photoperiodic regime was adopted and the relative humidity was 70±5 %. Insect hosts *H. virescens* last instar larvae were staged according to Webb and Dahlman (1985) and synchronized as reported by Pennacchio *et al.* (1992).

Enzymatic test for phenoloxidase (PO) activity

Hemolymph was obtained by puncturing with a needle a proleg of last instar larvae cold anesthetized. All bleedings were done on ice-cold Petri dishes and the hemolymph was transferred with a micropipette into ice-cold Eppendorf tubes. Hemolymph samples were then processed by low-speed centrifugation (1200 rpm for 3 min at 4 °C), to eliminate hemocytes and tissue debris. The supernatant (plasma) was immediately used or stored at –80 °C. Time course analysis of PO relative activity of plasma samples from parasitized and nonparasitized *H. virescens* larvae was carried out spectrophotometrically, by recording the formation of dopachrome from the L-DOPA (dihydroxyphenylalanine) (Sigma Chemicals, St. Louis, MO, USA) substrate. Changes in absorbance were recorded at 490 nm (µA 490 nm/10 min) at 20 °C by a double-beam Jasco V-560 spectrophotometer (Jasco Int. Co., Tokyo, Japan). All assays were performed by adding 5 µl of plasma to 1 ml of L-dopa buffer (4 mM L-dopa dissolved in 10 mM Tris-HCl, pH 7.2). The L-DOPA buffer was used as a blank. To assess the effect of protease (EC 3.4.21.4) treatment on phenoloxidase activation, trypsin (15 IU) (Sigma) was added to the substrate.

In vitro *encapsulation*

H. virescens hemocytes were obtained from last instar larvae 2 h after parasitization by *T. nigriceps* and from synchronous nonparasitized controls. Insects were surface sterilized by rapid immersion in 70 % ethanol, washed in sterile distilled water, dried on sterile filter paper and bled by proleg amputation. Samples of 40-60 µl hemolymph per larva were collected onto ice-cold Petri-dishes, lined with 100 % ethanol washed parafilm. Whole hemolymph samples were transferred in Eppendorf tubes containing an equal volume of anticoagulant buffer MEAD (98 mM NaOH, 145 mM NaCl, 17 mM EDTA, 41 mM citric acid, pH 4.5). Hemocytes were pelletted by centrifugation (1200 RPM for 10 min at 4 °C, in a Sorvall RMC-14 refrigerated microcentrifuge) and twice washed in Grace's insect Medium (Sigma) containing 10 % fetal bovine serum (FBS), 1 % glutamine and 1 % antibiotic-antimycotic solution (Sigma). The hemocytes were resuspended in 1 ml of the same medium and seeded at a final density of 2×10^5 per well and finally cultured in micro-wells (24-well culture plates, Flat Bottom, Corning Incorporated, COSTAR, NY, USA). *In vitro* encapsulation assays were carried out immediately by adding chromatographic beads (Dowex 1X2 mesh 100-400) to cultured hemocytes, as described by Lavine and Strand (2001).

Hemocyte numbers and adhesion

Hemocytes were obtained as described in the above section from measured volumes of hemolymph extracted from nonparasitized and parasitized *H. virescens* larvae. Cells were counted by using a Burker chamber and subsequently cultured in micro-wells (24-well culture plates), at 25 °C. After 16 h from cell seeding, the culture medium was removed and the non-adhering cells were counted.

Transmission and scanning electron microscopy

Hemocytes, obtained as described above, were fixed for 30 min in 0.1 M cacodylate buffer, pH 7.2, containing 2 % glutaraldehyde. Cells were then washed in the same buffer and postfixed for 20 min with 1 % osmic acid in 0.1 M cacodylate buffer, pH 7.2. After a

standard step of serial ethanol dehydratation, cells were pelletted and embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica, Wien, Austria). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol, Tokyo, Japan). Hemocytes for scanning electron microscopy (SEM) were fixed and dehydrated as described above, treated with hexamethildisilazane and mounted on polylysinated slides. Samples were then air dried and covered with a 9 nm gold film by flash evaporation of carbon in an Emitech K 250 sputter coater (Emitech, Baltimore, MD, USA). Specimens were then examined with a SEM-FEG Philips XL-30 microscope (Philips, Eindhoven, Netherlands).

Immunocytochemistry

Hemocytes were collected as described above and plated on glass coverslips cultured in micro-wells (24-well culture plates). Cells were washed with PBS and then fixed for 10 min in PBS buffer, pH 7.6, containing 2 % sucrose and 3 % paraformaldehyde. Cells were treated for 10 min at 4 °C with a permeabilizing solution (HEPES 20 mM, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl2, 0.5 % Triton X-100), then washed with PBS buffer containing 2 % bovine serum albumine (BSA) and finally incubated 1.5 h at 37 °C with tetramethylrhodamine (TRITC)-labeled phalloidin (Sigma) (diluted 50 µg/ml) in PBS buffer containing 2 % BSA. Coverslips were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA, USA); slides were examined with a confocal laser microscope (laser 568 nm; MRC 1024, Bio-Rad Laboratories, Hemel, Hempstead, UK) and images were recorded with a Delta Vision microscope (Deltavision Real Time System (Applied precision, Elcomind, Italy).

Results

Prophenoloxidase(proPO)-PO cascade

T. nigriceps oviposition determined a rapid inhibition of the proPO cascade. The PO activity was nearly abolished by 15 min after parasitization and then 4 h later gradually resumed, reaching levels recorded in nonparasitized controls (Fig. 1). In order to assess if the inhibited PO was still potentially functioning, a trypsin treatment of the host plasma samples was carried out. The restored PO activity in plasma samples of parasitized larvae, previously found to be inactive, indicated that the host PO was not damaged, since the pro-enzyme was converted into its active form by trypsin mediated cleavage (Fig. 2).

Hemocyte number morphology and behaviour

The total number of hemocytes in parasitized larvae started to decrease shortly after *T. nigriceps* oviposition. The lowest number of hemocytes was registered after 4 h and reached the 60 % of the value measured in synchronous nonparasitized controls (Fig. 3). After 40 h from parasitization the hemocyte number returned to the control condition (Fig. 3). SEM observations of hemocytes extracted from nonparasitized host larvae allowed to easily discriminate between granulocytes and plasmatocytes. Granulocytes were roundish and showed prominent nuclei while plasmatocytes were larger, with a highly ruffled surface and pseudopodia of varying size (Fig. 4). Both cell types displayed attachment and spreading behaviours (only 5 % of the total amount of cells did not adhere under *in vitro* condition). Adhering cells showed bundles of actin filaments located close to the cell membrane and in the cytoskeleton of the pseudopodia (Figs 5, 6). Hemocyte morphology in *H. virescens* larvae rapidly changed after parasitisation. SEM observation showed that granulocytes were evidently damaged, while plasmatocytes were apparently unaltered (Figs 7-9). The major morphological alterations in granulocytes consisted of cytoplasmic vacuolization, which became more pronounced over time, membrane blebbing, chromatin condensation and nuclear envelope breakdown (Figs 7-14). Under *in vitro* condition after two hours from oviposition, about 33 % of the total cell number was unable to adhere to the substrate (Fig. 15). Non-adhesive hemocytes showed cytoskeleton disruption, with broken filaments visible close to the cell membrane (Fig. 16), where actin oligomers were detected (Figs 17, 18).

Encapsulation assay

Encapsulation and subsequent melanization of Dowex beads rapidly occurred in presence of hemocytes extracted from non parasitized *H. virescens* larvae (Figs 19-22). Different types of haemocytes were involved in host defense and both plasmatocytes and granulocytes were recruited to form a developing capsule onto the surface of the Dowex beads. When the beads were added to the culture of hemocytes extracted from parasitized *H. virescens* larvae, the capsule did not develop and just a few granulocytes were visible close to the beads, while plasmatocytes

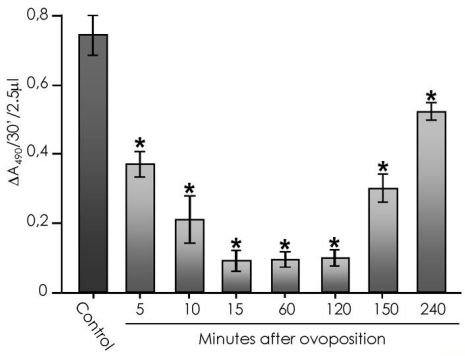


Fig. 1 After parasitization instead of the expected durable activation of proPo system, an inhibition of melanization and a reduction in the enzymatic activity was observed from 15 min to 4 h. Data represent mean ± SD, **p*< 0,01.

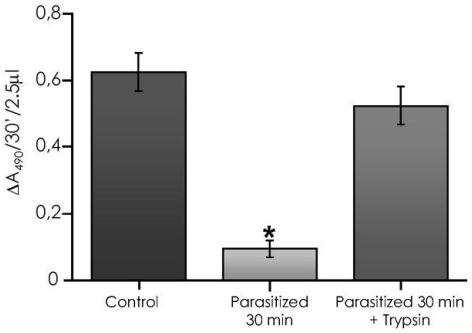


Fig. 2 Tryptic enzyme treatment restores proPO activity (Trypsin is usually used to check proPO integrity). Data represent mean ± SD, *p< 0,01.

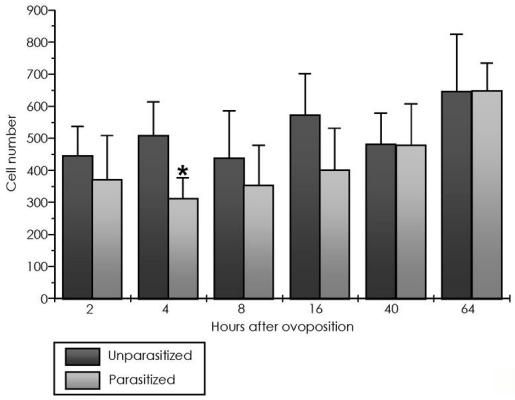


Fig. 3 The number of hemocytes decreases in hemolymph of parasitized *H. virescens.* Data represent mean ± SD, *p< 0,01.

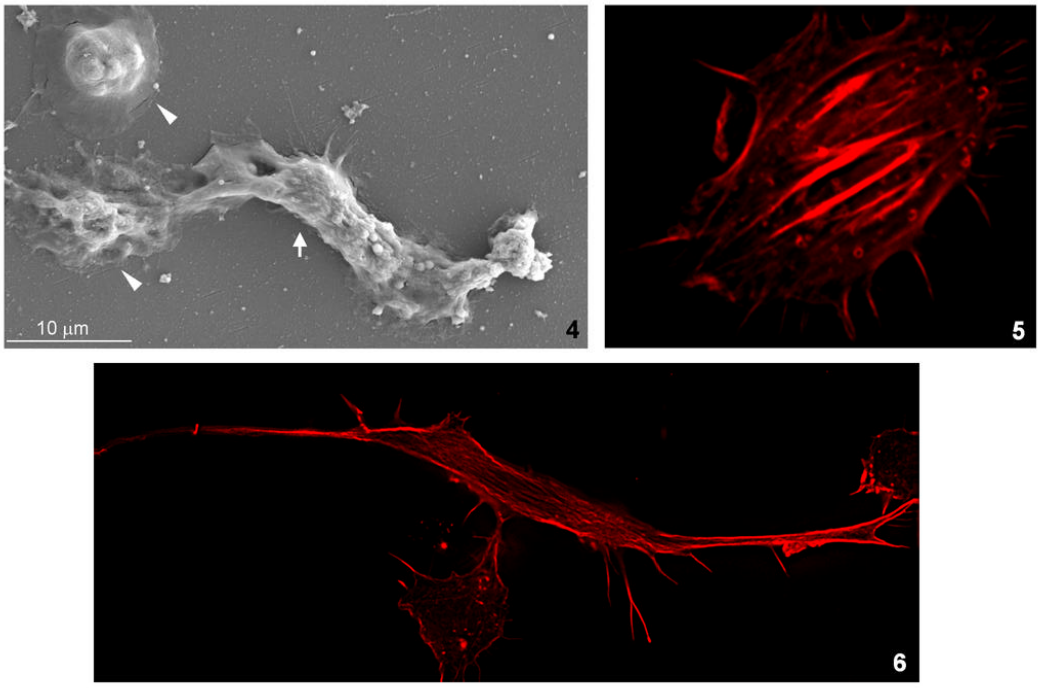
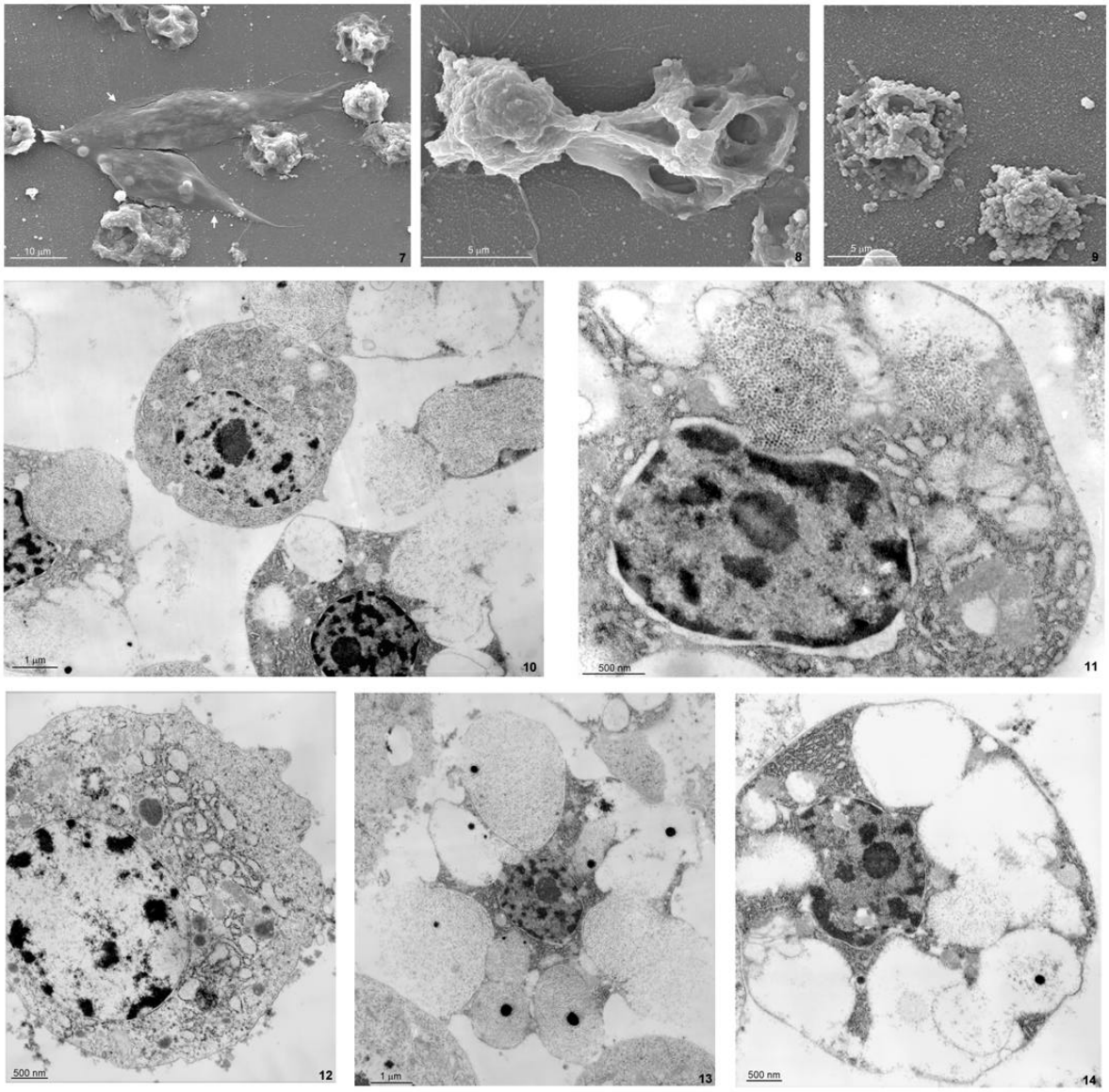


Fig. 4 Nonparasitized *H. virescens*: SEM observations of collected hemocytes show different cell types: granulocytes (arrowheads) and plasmatocytes (arrows).

Figs 5, 6 Nonparasitized *H. virescens*: in hemocytes, actin filaments, detected with phalloidin (arrowheads), are grouped in bundles under the membrane and in the pseudopodia.



Figs 7-14 Parasitized *H. virescens*: SEM observations of collected hemocytes (Figs 7-9) show that the granulocyte subpopulation is particularly damaged while plasmatocytes appear morphologically unaltered (arrows). Several ultrastructural features typical of apoptotic cell death are visible in the granulocytes: surface blebbing, chromatin aggregation, broken nuclear envelope, altered cytoplasm with swollen vacuoles. Granulocytes examined by TEM show numerous and large vacuoles in the cytoplasm (Figs 10, 14).

Discussion

The "host regulation" by insect parasitoids, as defined by Vinson and Iwantsch (1980), is the final result of the evolution of host-parasitoid relationship towards host control for the benefit of the parasitoid progeny. Parasitoid has to colonize and use the host, that provides food and shelter for the progeny of parasitoid (Vinson *et al.*, 2001). The tactics for circumventing the host defense and for redirecting its physiology, growth and reproduction, to support the development of the parasitoid juvenile stages, are key-factors in successful parasitism.

The study of the molecular details of the physiological mechanisms underlying host-parasitoid interactions in insects has generated a fairly large amount of information (Vinson *et al.*, 2001; Schmidt *et al.*, 2001; Beckage and Gelman, 2004), in particular relatively to the major categories of host regulation factors produced by parasitic Hymenoptera, such as polydnaviruses (Kroemer and Webb, 2004; Webb and Strand, 2005) and venom (Weaver *et al.*, 2001; Asgari *et al.*, 2003; Zhang *et al.*, 2004a, 2004b) suppressing the host immune responses. The suppression seems to be largely associated with inhibition of serine protease cascades (Brehelin *et al.*, 1975; Beckage *et al.*, 1990; Beck *et al.*, 2000) and hemocyte disruption and/or death (Strand and Pech, 1995; Schimdt *et al.*, 2001).

This article is focused on the *in vivo* and *in vitro* study of survival strategies adopted by *T. nigriceps* during the early time of parasitization (i.e. the most vulnerable period of development). *T. nigriceps* avoids the host immune reaction by a combination of both passive and active mechanisms. The outer fibrous layer of the egg seems to be the first protecting barrier (Davies and Vinson, 1986), which is effectively complemented by a very precocious and transient suppression of the PO activity. This transient block could be interpreted as a mechanism to disrupt the early steps activated by the recognition of an invading organism. The observed reactivation of the enzyme in parasitized plasma samples by trypsin treatment indicates that the PO retains its functionality. We can differently interpret this result: a parasitoid-derived molecule could bind the PO, protecting the cleavage site, thus inhibiting its activation or a parasitoid factor involved in host regulation likely hit the protease cascade rather than the enzyme itself. This may inhibit both the melanization response and the possible production of signal molecules that would in turn activate the cellular immune reaction. In fact, it is reasonable to speculate that the proPO-activating system (proPO-AS) plays a key-role in the regulation of these important mechanisms, as suggested for Crustacea and Lepidoptera by Soderhall and Cerenius (1998). The source of the host regulatory factors involved in the PO inactivation is still unknown, but based on preliminary experimental data and on the fact that this alteration is triggered within minutes after parasitization, we can predict that venom and/or ovarian secretes in the calyx fluid may play an important role.

As evidenced by several authors (Lavine and Beckage, 1995; Doucet and Cusson, 1996; Hu *et al.*, 2003), many parasitoids are able to suppress host immune defenses by altering the number and/or the behaviour of the circulating hemocytes. There are a number of studies available on this type of alteration, which, for example, can be induced by parasitoid-associated PDVs, as in *Choristoneura fumiferana-Trasonema rostrale* (Doucet and Cusson, 1996) and *Pseudoplusia includens-Microplitis demolitor* (Strand and Pech, 1995) associations, or by the combined action of PDVs and ovarian proteins, as in *Heliothis virescens-Campoletis sonorensis* (Luckhart and Webb, 1996), or by the venom alone, such as in *Lacanobia oleracea-Pimpla hypochondriaca* (Richards and Parkinson, 2000). In all these model systems, the numerical variation of hemocytes is always paired with an altered morphology and/or functionality of the cells.

In *H. virescens* larvae parasitized by *T. nigriceps*, the total number of circulating hemocytes transiently decreases, with a minimum peak registered within few hours, followed by a slow recovery towards values normally registered in synchronous nonparasitized controls, which were attained by 40 h after parasitoid oviposition. During this interval, the hemocytes show different structural damages, evident actin cytoskeleton disruption and lost of adhesion properties, with general morphological changes, which suggest the occurrence of apoptosis. These hemocyte alterations seem to be selectively induced in granulocytes, while plasmatocytes appear to be morphologically unaltered. However, the apparently unaltered plasmatocytes do not start any encapsulation process of Dowex beads *in vitro*. It remains to be studied if this is a consequence of granulocytes degeneration, which may prevents the plasmatocyte recruitment they regulate during encapsulation (Lavine and Strand, 2002), or if a more subtle functional alteration of plasmatocytes occurs. All these changes in hemocyte structure and function could be induced by venom and calyx fluid right after oviposition, and, after few hours, reinforced by the expression of *Tn*BV genes.

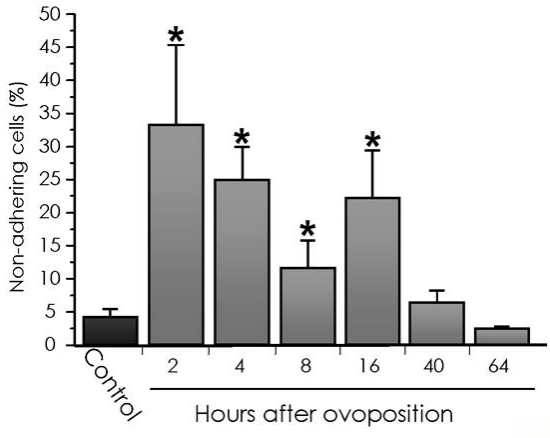
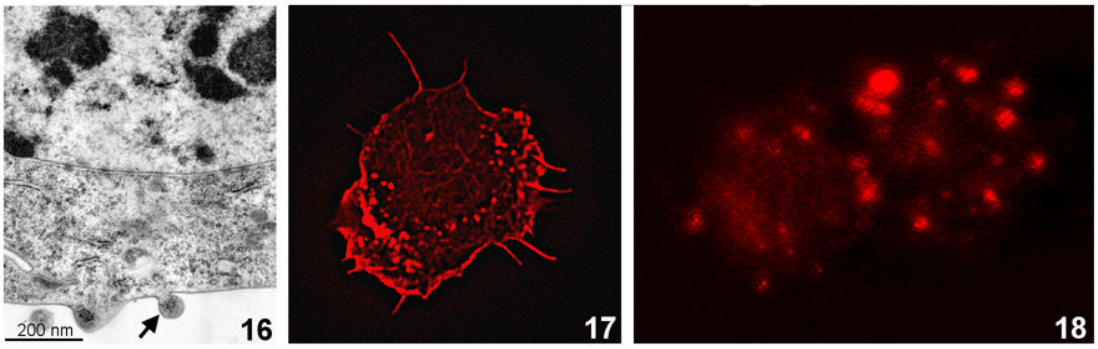
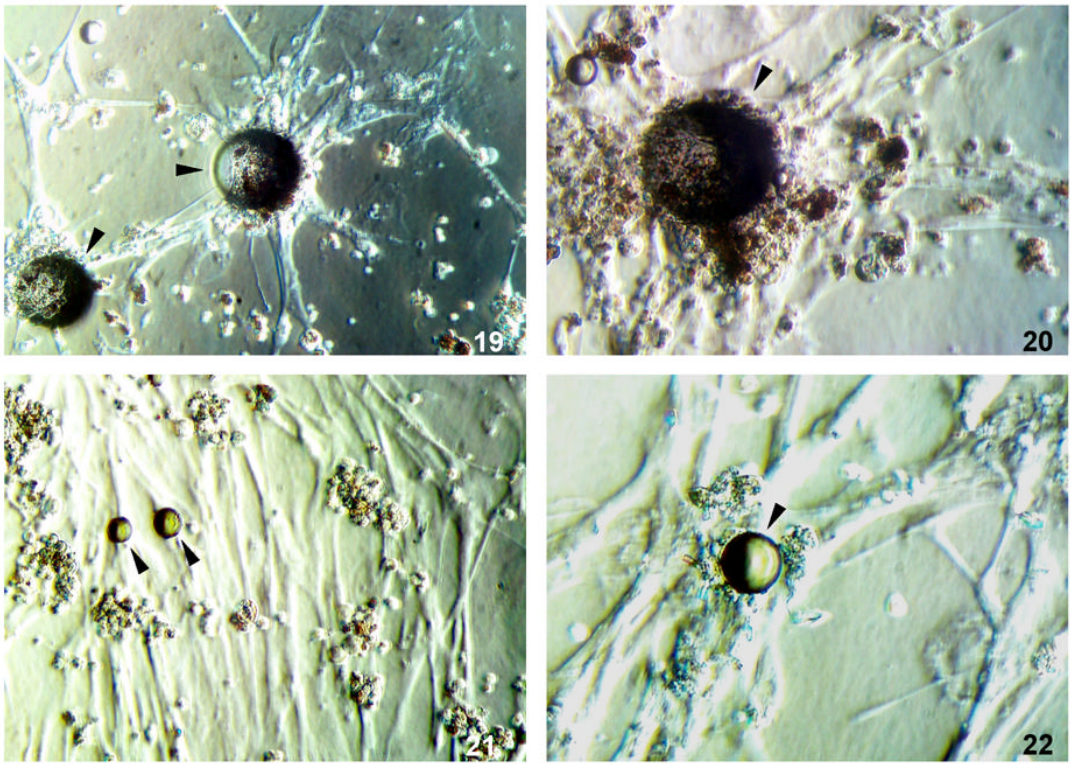


Fig. 15 Parasitized *H. virescens*: hemocytes loose their adhesion capability and the minimum was observed at two hour mark. Data represent mean ± SD, *p< 0,01.



Figs 16-18 Parasitized *H. virescens*: in hemocyte actin filaments are disassembled and are visible, under transmission electron microscope, at the periphery of the cell (arrows). Phalloidin evidences the oligomers of actin (red spots).



Figs 19, 20 Hemocytes of nonparasitized *H. virescens*: both plasmatocytes and granulocytes are recruited onto the surface of beads (arrowheads) forming a capsule.

Figs. 21, 22 Hemocytes of parasitized *H. virescens*: few granulocytes are visible near the beads and plasmatocytes, parallel disposed, are unable to adhere to Dowex beads (arrowheads).

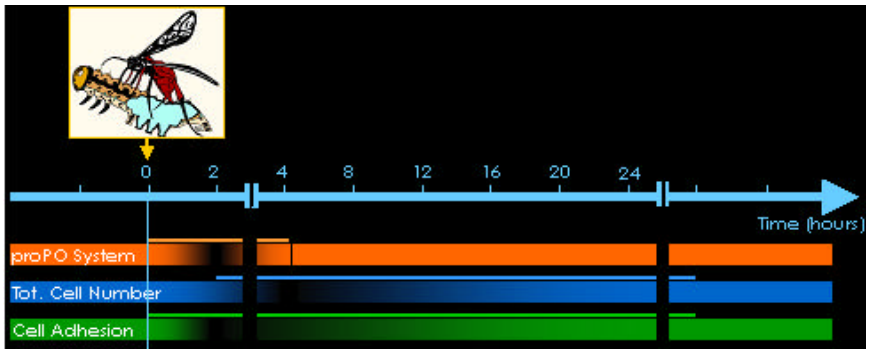


Fig. 23 Summary of different events transiently disabling host immune defenses. The thin lines indicate the interval in which a particular alteration occurs; the broad lines are darker where the inhibition is at the highest level.

A few genes expressed in host hemocytes have been isolated and, then, are considered to be putatively involved in the host immune disguise (Varricchio *et al.*, 1999; Pennacchio *et al.*, 2001; Falabella *et al.*, 2003; Malva *et al.*, 2004; Provost *et al.*, 2004). At the present, only for the viral gene *Tn*BV1, it has been demonstrated that an apoptosis-like degeneration is induced in insect cells (Lapointe *et al.*, 2005). Detailed functional analyses are required to establish the role of other *Tn*BV genes in the induction of the multifaceted immune syndrome recorded in parasitized *H. virescens*. However, we can predict that these genes, along with female secretion injected at the oviposition, coordinately induce a set of partially overlapping mechanisms, disabling cellular and humoral responses (Fig. 23).

Acknowledgements

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