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Identification and Quantification of the Most Abondant Hemocytes in the Pine Processionary Caterpillar;ThaumetopoeaPityocampa (Notodontidae)

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Abstract

Thaumetopoeapityocampa is an important pine pest in the Mediterranean basin and central Europe. The insecte is a butterfly, whose larvae are called pine processionary caterpillars. To understand the resistance mechanism of the insect in order to proceed of its control and to diminuate their effect in nature, it was necessary to study of the immune system and reactions of the larva in different stages. The aim of our work is to identify the hemocytes formula of the caterpillar during the larval stages L2, L3, and L4, as well as the quantification of the different cells during each stage. After extraction of the hemolymph by centrifugation, the cells were placed in culture medium and then incubated. Microscopic observation has shown that prohemocytes population appear early in hemolymph, they differentiate into plasmatocytes and granulocytes during the advanced stages. However, the quantification process (THC), carried out in a Malassez counting chamber has shown that granulocytes are the most abundant cell population in the hemolymph of the insect larvae. To investigate the role of hemocytes in immune responses, we have co-incubate T. pityocampa cells with bacteria, entomopathogenic nematodes, and synthetic beads. Both humoral and cellular encapsulation processes have been observed early in larval stages, all hemocytes seem to be involved in the formation of nodules and capsules against bacteria and microbeads. At the opposite, entomopathogenic nematodes (Steinernemafeltiae) were not recognized and encapsulated, but their presence can strongly damage host hemocytes.

Key words:Thaumetopoeapityocampa, prohemocytes, plasmatocytes, granulocytes, encapsulation.

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1. Introduction :

Insect's immunity consists of both, humoral and cellular reactions[4]. The humoral reactions include the production of antibacterial, peptides and enzymatic complex of coagulation or melanization in hemolumph[21] and [19]. In contrast, cellular reactions consist of hemocytes-mediated immune responses, representing by the activity of circulating cells in different forms such a phagocytosis, nodule formation and encapsulation [16].

The phagocytosis is a process aimed to eliminate foreign particles, usually single cells, as bacteria or protozoa; the process follows few steps: recognition, attachement, pseudopodia formation, engulfing and finally intracellular lysis of foreign body [3]. If not-self invasions carried out by larger organisms, encapsulation takes place, thus the formation of a multilayered capsule of hemocytes that surround the foreign organism occasionally. Host hemocytes can also form nodules to entrap groups of bacteria; this reaction is conventionallynamed nodulation [11].

Thaumetopoeapityocampa, also known as pine processionary, is a moth of the Notodontidaefamily;this insect is an important pine forest pest in Europe and in all Mediterranean area[20].The Lepidopteran insect is also characterized by the presence of different populations of hemocytes freely circulating in the hemolymph, they mediate all the cellular reactions, comprising phagocytosis of small microorganisms and formation of nodules and capsule around foreign particles. Considering the remarkable development of experimental methods to collect cells and to classify the different hemocytestypes [6] and in order to understand the immune system of T. pityocampas, an accurate identification of hemocytes sub-populations is an essential starting point to study of immune processes.

Our main goal is to study the hemocytes formula of Thaumetopoeapityocampa, firstly by proceding the cells quantification in the hemolymph during the larval life, then by the identification of cells populations in each instar. Moreover, we have started to investigate some humoral and cellular reactions of the insect studying different immunological and cellular responses in larval stage when in absence and presence of entomoparasitic nematode (Steinernema), (Escherichia coli) as a bacteria or synthetic microbeads.

2. Material and methods

2.1. Hemolymph collection

Processionary moth larvae were been checked to select the healthy and bigger ones; we used about 10 to 15 larvae in each instar. Before proceed to the centrifugation, larvae were washed with ethanol extensively (several times) to sterilize them. Small larvae were been injured, by cutting the lower and the tail regions, and were placed inside a *double Eppendorf system* built with two different Eppendorf tubes (the big outside and the small inside). The small was previously perforated at the bottom to allow the hemolymph to flow out in the bigger tube, but retaining the fragments of the larvae body.

Centrifugations were been made at 1200 rpm in a refrigerated eppendorf centrifugemodel (5804R centrifuge, for 10 minutes at 10°C). From late instar larvae, we extracted the haemolymph by puncturing the ventral side of the insect. After centrifugation, hemocytes from 2nd, 3rd and 4th instars, cells were been collected and separated from the humoral fraction of the haemolymph. Hemocytes were cultured in Grace Medium, and then observed by phase contrast light microscopy.

2.2. Culture medium conditions

Aftercentrifugation, humoral fraction was collected and discarded; pelletted cells were washed with grace insect medium. The procedure was repeated twice to avoid contamination of tissues or cells debris. Hemocytes were suspended in a complete culture medium (10% fetal calf serum, 1% antimycotic antibiotic, 1% glutamine in Grace Medium). The cells were cultured in 96 Micro well plates (cluster cell cultures, flat bottom, Iwaki). And kept at 25-26 °C in a moistened incubator (Cellstar) without CO_2. As reported in literature the most common populations of insectshemocytes are pro-hemocytes, granulocytes, plasmatocytes,spherulocytes, and oenocytoids[16].

2.3. Immune reactions and Light microscopy

For the observation, we used an inverted phase contrast light microscopy (Olympus IX51, Olympus INC), to investigate the cell-mediated responses and immunity reactions of *T. pityocmapa*hemocytes against not-self reactions. Different responses have been monitored such as, the ability to encapsulate insects parasite, the bacteria nodulation focusing on the ability of the cells to recognize and encapsulate abiotic material (synthetic microbeads).

2.4. Total haemocytes count

The total haemocytes count (THC) is a measure of the concentration and abundance of haemocytes inside the hemolymph (cells/ml). For observation, we used light microscopy to identify and quantify the cells in hemolymph. An estimation of cells number from 2nd, 3rd and 4thlarval instar, was calculated. The granulocytescellnumber (THCg) can be estimated from the THC (cells/ml). Aliquots of hemocytes were used to determinate the granulocytes percentage in larval hemolymph; observation was realized on counting grids by adding a small quantity of hemolymph to ahemacytometer slider (Malassez). The cell formular in the 2nd instar was less abundante, this is why the dilluation of the hemocytes was not necessary. The number of cells from the three instars was estimated in about 100 cubics. The percentage of hemocytes types were calculates, we also estimated the (THC) of all cells and for each hemocyte. A comparison between the (THC) of different stages was assessed.

2.5. Statistical analysis

We useanalysis of variance (ANOVA) on XLStat, to investigate the differences between the cells quantification in each stage in order to identify the rhythm of their development along the larval stage. A comparison of the (THC) of different cells in each *Thaumetopoeapityocampa* instar has been also estimated.

3. Results

Since the first microscopic observations, many forms and cell aggregation were observed in *Thaumetopoeapityocampa*hemolymph. In early stages, cells abundance was less developed. Those forms increased in size and number along larval development of the larvae(Fig 1).

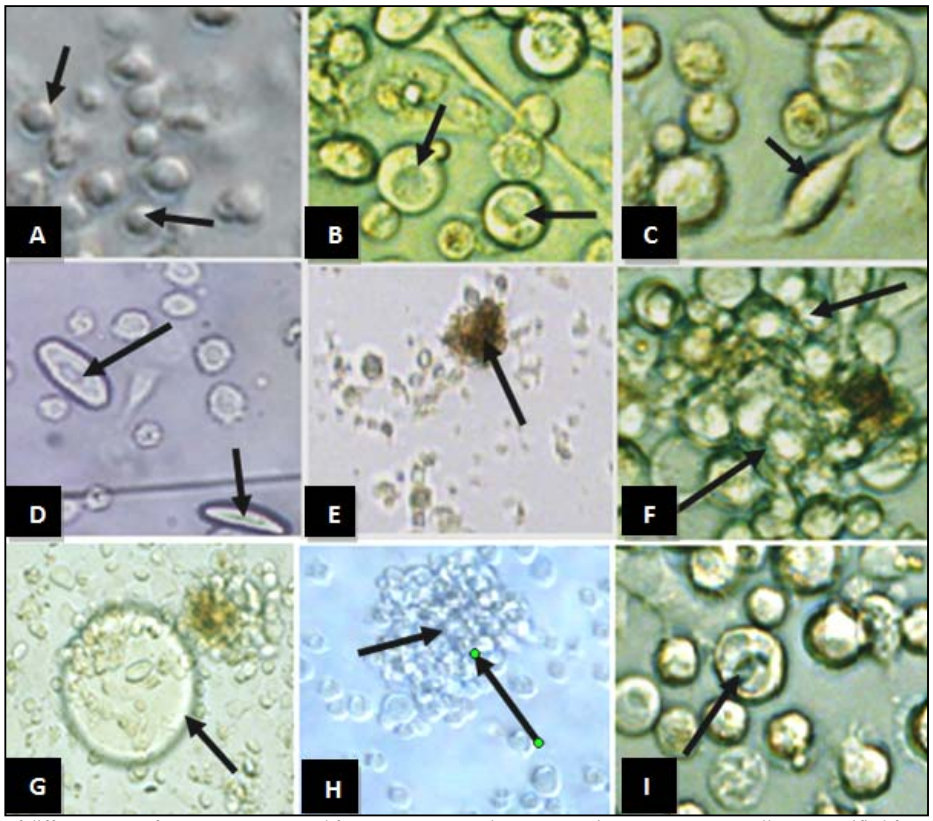

Fig. 1. Morphology of different types of Hemocytes, extracted from *T. pityocampa* larvae. Insect immunocompetent cells were purified from larvae hemolymph. (A) *Prohematocytes,* (B) *Granulocytes,* (C) Plasmatocyte, (D) *Oenocytoids,* (E) cellular debris, (F) *cellular aggregation* (G: 40x10). (G) Microbeaud, (H) Nodulation forms (G: 10x10) (I) Cell in mitotic features (G: 40x10)

3.1. Identification and quantification of cells

The total count of haemocytes in haemolymph of L_2 stage larvae was approximately of (75×10^2)cells/ml, the number increased constantly to (250×10^3)cells/ml in L_3 and to (63×10^4) cells/ml in the L_4 stage. Granulatocytes were the most abundant hemocytes observed in*T. pityocampa*hemolymph, from the 2nd to the 4th instar **(Tab)**.

Table : Total cells abandance in different instars of *Thaumetopoeapityocampa*

Instars	2nd instar	3rd instar	4th instar
Cells/ml of hemolymph	75×10^2	250×10^3	63×10^4

In the last instar, The most abundant cells are granulocytes, their HTC was about 208×10^3 t cells / ml of hemolymph, followed by plasmatocytes that recorded an HTC equal to (113 x 10) cells / ml. The cells that recorded less abundance in the last development larval stage of *T. pityocampa* are prohemtocytes with an HTC estimated at to (130 x 10) cells / ml of hemolumph. (fig. 2). We have demonstrated that the most important cells identified are Pro-hemocytes, plasmatocytes and granulocytes.
Oenocytoidswere observed in 2nd instar, (fig. 1), their form was large, round and often contain granules. Their number was instable in the first instars. They were observed only in the first instars, they completely disappeared in the last instars(fig. 2). Cells evolution is estimated along the larval stage and compared between each one (fig. 2). The variance analyse of each value showed that all cells quantification increase significantly along the larval stage development (ANOVA, $p < 0.05$)

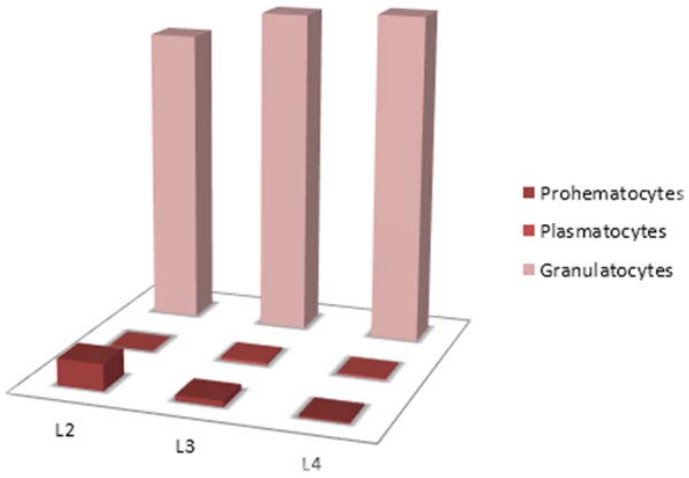

Fig. 2.Proportional counts Ratio of *Plasmatocyte*, *Granulocyte* and *Prohemocyte* and comparaison of each instar level of each hemocyte in hemolymph of *thaumetopoeapityocampa* larvae.

3.2. Pro-hemocytes

They were thesmallest cells found in hemolymph of *Thaumetopeapityocampa*. They are described as precursor of all the immunocompetent cells in the early developmental stages, (fig. 1). These cells develop to plasmatocytesorgranulocytes in late instars and their shape varied between oval or elongated profiles. Prohemocytes represent the 10% of all theHemocytes of the first larval instar of *T. pityocampa,* and their number decrease in the last instars (fig. 3)

3.3. Granulotocytes

They were rounded, or irregularly shaped cells. The nucleus was also generally a central position in the cytoplasm(fig. 1. a). The plasma emitted pseudopodia and filopodia in order to encapsulate foreign bodies. Granulocytes were the main cells in haemolymph of *T. pityocampa*, they represented 80-90% of total hemocytes population (fig. 3)

3.4. Plasmatocytes

were large spindle-shaped cells, which when adherent in culture plates, showed cell protrusions, such as pseudopodia and filopodia (fig. 1. c).

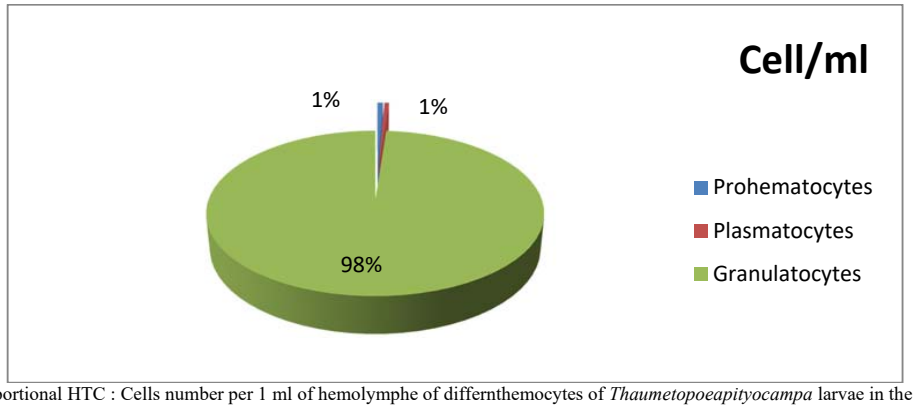

Fig. 3. Proportional HTC : Cells number per 1 ml of hemolymphe of differnthemocytes of *Thaumetopoeapityocampa* larvae in the last instar.

3.5. Encapsulation and phagocytosis

The hemocytes were examined by microscopy. Plasmatocytes, followed by pro-hemocytes were the most common cells in this insect. Pro-hemocytes were identified by their small size (fig. 1). When in culture medium, most of cell population identified participated with granulocytes in encapsulation processes,(fig. 4. b) forming a multilayered capsule around foreign bodies. Plasmatocytes were also involved in phagocytosis of bacteria. In *T. Pityocampa*, plasmatocytes represented 10-20% of total hemocytes (fig. 2).

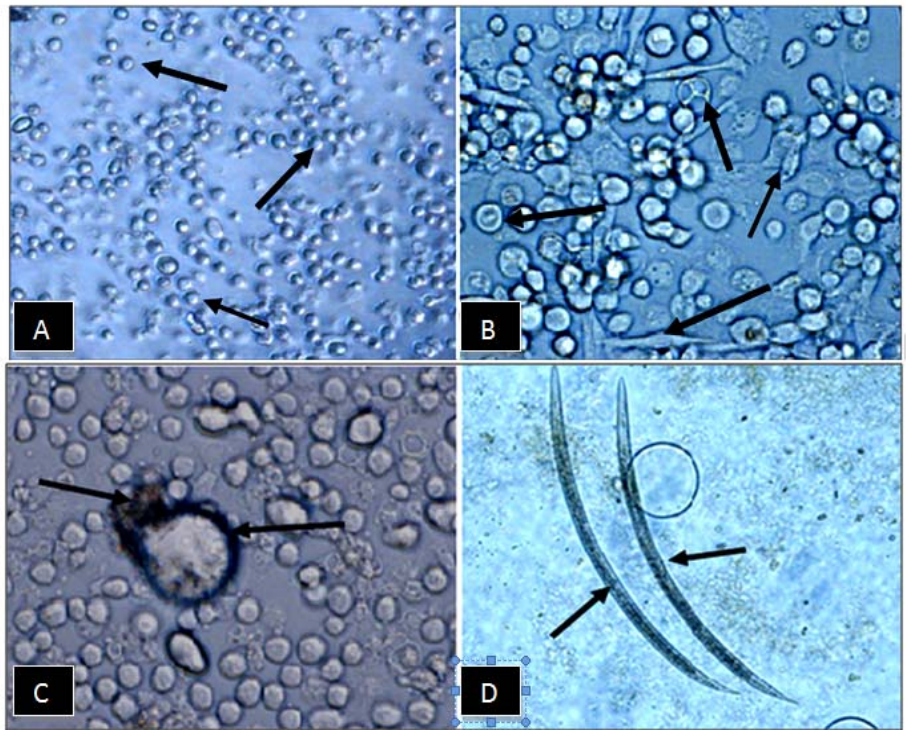

Fig.4. Phase-contrast microscopy showing different immunity reactions of cells of *Thaumetopoeapityocampa*. (A)Hemocytes morphology before being adding to the culture medium (G: 10x10); (B) Cells morphology in grace medium(C) Nodulation: Hemocytes forming nodules and encapsulation around Bacteria Incubation (G: 40x10) (D)Parasites "*Steinernimacarpocapsae"* added to the medium, (G: 10x10).

In the assays with bacterial cells, used as not-self target, granulocytes contacted rapidly the microorganisms then they participated to phagocytosis processus in the medium (fig. 4). To investigate in deep the cellular encapsulation responses, parasites and free living nematodes were added at various times to cells medium culture of *T. pityocampa*. The ability of hemocytes populations to recognize and encapsulate worms was investigated. A considerable number of granulocytes reach the body- surface of free-living nematodes and bacteria; plasmatocytes also participate and completed the capsule. The profile of pro- hemocytes and plasmatocytes significantly changed after immune challenges (fig. 5. c).

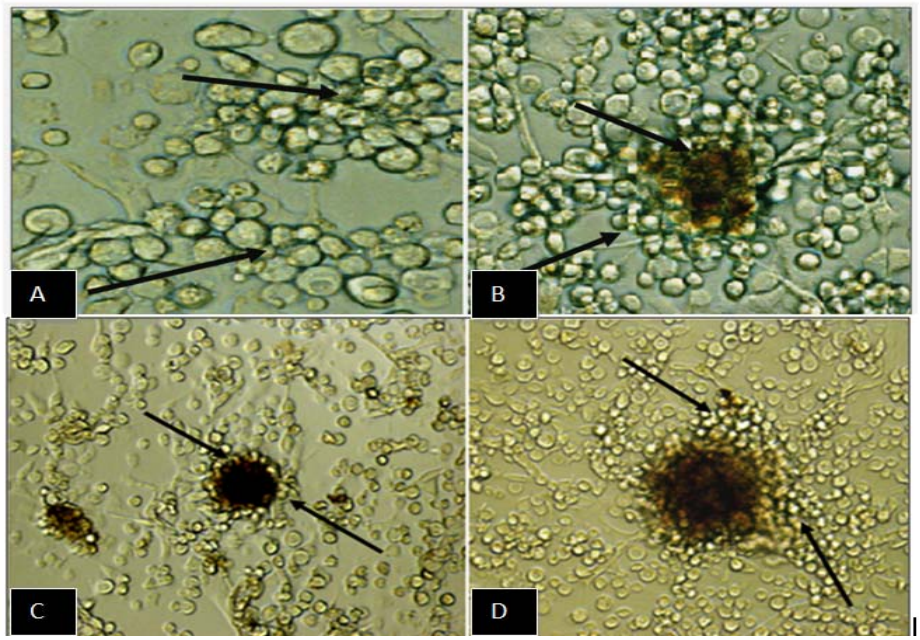

Fig.5. Phase-contrast microscopy showing different encapsulation reactions of in hemolymphe of *Thaumetopoeapityocampa*. (A) Cells aggregation (B) start of debris aggregation forming a simple humoral encapsulation (G:40x10). (C) humoral encapsulation (D)Different cells aggregate around humoral fraction forming cellular encapsulation. (G: 10x10).

Humoral melanisation is a humoralimmune response, which, in this case, cooperates with immuno-competent cells, by melanin synthesis and deposition, in the isolation of foreign elements from the host body. Insects use also melanisation to limit parasites trophic exchanges, enclosing the foreign target inside a hardened proteinaceous capsule. The microscopic observation of cells challenged with either bacteria or latex microbeads demonstrated that mainly plasmatocytes were able to phagocytise both beads and bacteria (fig. 4. c).

Aggregation of latex particles were not observed at any experimental time. Humoral encapsulation was the usually observed before cellular encapsulation, the debris in the hemolymph surround the nematode and the bacteria, cellular encapsulation appears later.

4. Discussion

In insects, immune resistance to diseases and infections is characterized by different immune reactions such as: phagocytosis, nodulation, encapsulation and melanisation [26]. Hemocytes are the main elements of cell-mediated immunity in insects [25]. They are able to phagocyte and eliminate both biotic particles, such as bacteria, and abiotic targets, such as synthetic beads [16].In literature, haemolymph in many insects' species such coleopteran and lepidopteran are characterised by the presence of several cell types: prohemocytes, plasmatocytes, granulocytes, oenocytoids and adipo-hemocytes[24], [10], [1] and[14]. Many of these cells types have been also identified in *Thaumetopeapityocampa*haemolymph. The most represented cells identified are prohemocytesplasmatocytes and granulocytes.

The observation from 2nd to the 4th larval instar instar was successful and showed that pro-hemocytes and granulocytes were the most abundant cells. The plasmatocytes were present in the later stages(fig. 1. c). In the 2nd instar, pro-haemocytes were recognized by their small round shape; they represented only 8% of the cells observed, their number increased remarkably in the 3rd instar (fig. 2)and was unchanged in the last larval instar; which confirms that the pro-haemocytes differentiated to plasmatocytes and granulatocytes after 2 days of incubation. The regular microscopic observation demonstrated that most of pro-haemocytes of the larvae, developed and transformed into plasmatocytes and granulatocytes(fig. 1).

In *T. pityocampa*, plasmatocyteswere recognizable by their elongated shape and pseudopodia (fig. 1. c), and the cell number increasesduring the larval stages. These cells represent the main cells in hematopoietic tissues in larvae and adults of *Carabus Lefebvre* (*Coleoptera, Carabidae)*[7]. Functionally, as cells, they could be compared to macrophages of vertebrates.In our study, granulocytes were detected by their round shape(fig. 1. b). With 90%, they are the most abundant cells observed in each stage. Phagocytosis is the process used by immunocompetent cells to engulf particles and microorganisms [13] to be eliminated.

In invertebrate, granulations are known to be the main cell type responsible for phagositosis process and nodulation of foreign targets [5] and [12]. In the 4th instar, the main cells types and their capability to aggregation have been identified; the haemocytes retained their ability to react and encapsulated either synthetic microbeads or free-living nematodes (fig. 4. c). At this stage the larva reached immunity maturity.

So, at this instar humoral and cellular encapsulation, phagocytosis and nodules, have been observed in the presence of target (fig. 5) In other insects, different types of haemocytes could also perform phagocytosis, such as pro-haemocytes of *B. Mori larvae*[17] and oenocytoids of the grub *Cetonischemaaeruginosa*[8]. The granulocytes of *P. Xylostella*are the only cells responsible of encapsulation.

Another study suggests that in mammals, macrophages, neutrophils and monocyte were the cells involved in phagocytosis [15] and [22].While in *Manducasexta*and *Blattellagermanica*, encapsulation was also realised by granulocytes [9] and [18]. In some cases, the bacteria clearance involved both phagocytosis and process of nodulation. Concerning molluscs, all hemocytes participate to realisephagocytose of different particles, such as bacteria, protozoan parasites, and latex beads. All hemocytes participate to realisephagocytose of different particles, such as bacteria, protozoan parasites, and latex beads. [23] and [2].

5. Conclusion and Acknowledgements

Knowledge of the cell population in T. pityocampa is important for analyzing immune processes and to develop strategies for fight this pest insect that causes both damage to plants and for human health. The most cells identified are there participated of all immunity reactions processus. After adding parasites and nematode, humoral and cellular reactions were more observed in all stages. Immunity of thametopoeapityocampa is develloped in the last instars then in the primary ones. The work was supported by the immune and parasitology laboratory in italy.

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