Hemocytes of *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae) larvae: morphological and quantitative studies

Fábio Goulart de Andrade[1]; Maria Cláudia Cordeiro de Negreiro[1]; Elisa A. Gregório[2]; Flávio Moscardi[3]; Ângela Maria Ferreira Falleiros[1]

[1]*Programa de Pós-graduação em Agronomia, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Caixa postal 6001, CEP 86051-990, Londrina-PR, E-mail: angefal@uel.br*

[2]*Centro de Microscopia Eletrônica, UNESP, Botucatu-SP*

[3] *Embrapa Soja, Londrina-PR*

Abstract

The aim of this study was to characterize and quantify the hemocytes types of *A. gemmatalis* (Lepidoptera: Noctuidae) larvae in the light and the scanning electron microscopy. Six hemocytes types were identified: prohemocytes (PR), plasmatocytes (PL), granulocytes (GR), spherulocytes (SP), oenocytoids (OE) and vermicytes (VE). The PR (0.9%) were the smallest cells, with voluminous nucleus, scarce cytoplasm and smooth surface. The PL (49.7%) were polymorphic cells with cytoplasmic projections in cellular surface. The GR (23%) presented cytoplasmic granules and phylopodial projections in the cellular surface. The SP (20.9%) presented the cytoplasm full of spherules and smooth surface. The OE were the largest cellular type with homogeneous cytoplasm and smooth surface. The VE (0.5%) were fusiforms cells with elongated extremities and smooth cellular surface.

Keywords: Hemocytes, morphology, Lepidoptera, *Anticarsia gemmatalis*.

Introduction

The hemocytes are the circulating cells in the hemolymph of the insects, responsible for the defense reactions, against strange agents that penetrate its hemocele [1,2,3]. They present variable morphology and different functions, as recognition, phagocytosis, formation of nodules, encapsulation, coagulation and citotoxicity [4,5]

The velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae) is a key plague of the soy which attacks the crop with voracity and if not controlled, can cause a great economic damage to the producers countries. Despite from the economic importance and the wide literature about the biological aspects, the reactions of defense of this insect are almost unknown, mainly those related to the hemocytes.

The aim of this study was to characterize the hemocytes in *A. gemmatalis* larvae, in the light and scanning electron microscope, as well as to quantify these cells through the total (THC) and differential (DHC) counts, once that the success of the immune response depends the number and the types of hemocytes involved in this mechanisms [6].

Materials and Methods

The larvae of *A. gemmatalis* were obtained from the Embrapa Soja/Londrina-PR. The larvae were created in a acclimatized room (temp. 25-27°C; 80% relative humidity) using 14h light/10h dark cycle and fed up with artificial diet, according to [7]. The experimental procedures were accomplished in the Laboratory of Histology/Universidade Estadual de Londrina.

Larvae from 4th to 6th instars (8-16 days of development post-eclosion), were anesthetized by cold (0°C, 5 min), cleaned in 70% alcohol, delicately punctured with needle in the abdominal region and the hemolymph collected with the aid of Pasteur pipette. An hemolymph drop was placed on a glass slide for the smears which were slightly spread along the glass slide with the aid of a glass coverslip, air-dried for cellular adhesion and stained with solution of Seller (1% basic fuchsin in methanol P.A and 1% methylene blue in methanol P.A., 1:2) for 20 seconds. For the observation in fresh, the hemolymph was

collected in glass slide, recovered with glass coverslips and immediately observed on phase contrast.

For analysis in the scanning electronic microscopy (SEM), the hemolymph was dropped directly on coverslips covered by 0.1% poly-l-lysine for 5 min in a moist chamber. After the adhesion of the hemocytes, the monolayer was fixed in cacodylate buffer (0.1 M, pH 7.3) with glutaraldehyde solution (2.5%), post-fixed in osmium tetroxide, dehydrated in graded acetone and dried at the critical point. The preparations were mounted on stubs, gold coated in a sputter coater and analyzed by SEM.

For the total hemocytes count (THC), the hemolymph of 30 larvae per day of development was collected (not diluted) and analyzed in Neubauer chamber (0.1 mm in depth).

For the differential hemocytes count, (DHC), 15 larvae were used per day of development. For this procedure, the hemolymph of each larva was collected and diluted in a drop of anticoagulant solution for insect [8], dropped in on glass slide, covered with glass coverslips and analyzed in phase contrast.

The data obtained to the total and differential count swere analyzed through ANOVA and Tukey's test ($p<0.05$).

Results

Six of hemocytes types were characterized in the hemolymph of the larvae of *Anticarsia gemmatalis*: prohemocytes (PR), plasmatocytes (PL), granulocytes (GR), spherulocytes (SP), oenocytoids (OE) and vermicytes (VE).

The PR were the smallest cells observed and characterized by the presence of the big, voluminous and central nucleus, scarce cytoplasm and smooth cellular surface (Figs. 1a, 1c, 2e, 3d, 3f); represented 0.9% of the total of circulating hemocytes (Fig 4).

The PL were polymorphic cells, with form varying from oval to round, central nucleus, homogeneous cytoplasm and abundant cytoplasmic projections (Fig. 1d, 1f,2a, 3a, 3d, 3f); they were the most cellular types frequently observed, representing 49.7% of the total of the hemocytes (Fig 4).

The GR were round cells, with round and central nucleus, cytoplasmic granules and plasmatic membrane with projections, mainly the philopodial type (Fig. 1b, 1c, 1f, 2b, 3a); they presented a percent of 23% (Fig 4). The SP were spherical cells with varied sizes, easily identified by the presence of cytoplasmic spherules; the cellular surface was smooth, although it showed prominences correspond to the spherules (Fig 1b, 1c, 1f, 2b, 3a). The SP represented 20.9% of the total hemocytes (Fig 4).

The OE was the largest cellular types observed. They were round, with small and eccentric nucleus, homogeneous cytoplasm and plasmatic membrane without projections (Figs.1a, 1f, 2c, 3a); presented a percent of 5% (Fig. 4). The VE were fusiforms cells, with elongated extremities, prolonged and central nucleus, sometimes with slightly granular cytoplasm and smooth cellular surface. They were the less frequent hemocytes, with 0.5% (Fig. 4).

The values obtained for the total of hemocytes count (Figure 5) showed a decrease in the number of the cells, during the larval development, varying from 11537 (initial), to 9327 cells per hemolymph, in the end of the period.

Discussion

The cellular types observed in the hemolymph of *A. gemmatalis* larvae were similar to the ones described by several authors for others species [9-16], in spite of the existent controversies among the authors, about the classification of the hemocytes in the light microscopy.

The controversies can occur in reason of the variable form, functions and fragility of the hemocytes, as well as the stages of the insect development, the environmental conditions, beyond the study methodologies employed. In this research, the identification and the morphologic characterization of the hemocytes types present in the hemolymph of *A. gemmatalis* larvae were accomplished in light microscopy, in stained hemolymph smears as in fresh hemolymph in phase contrast.

Some authors reported the difficulty on obtaining good results with hemolymph smears, in larvae of Lepidoptera, as [10] in study with *Diatrea saccharalis* in stained smears and identified less hemocytes types than [9], when they analyzed the hemolymph of the same insect later, in phase contrast and credited such result to the cellular damage which may occur mainly in the most fragile types, during the preparation of the smear, implying in a reduction in the number of the types observed by those authors.

Our difficulty to identify the hemocytes in smears of the *A. gemmatalis* hemolymph was due to the attempts of adaptation of staining techniques of blood of mammals, for the insect in subject. After the time standardization and the stain used, the results were satisfactory. Our observations in the phase contrast were similar to those described by [11-13].

Although six hemocytes types in *A. gemmatalis* larvae were described and characterized, it was possible to observe a seventh cellular type in most of the smears and in some fresh observations. These cells were big, squamous type, similar to the exfoliative cells found in stained smears of vertebrates mucous. Such cells are rarely described in insects, being necessary other techniques for better characterization.

The identification of the VE, as a different cellular type is not a consensus among the authors. Some consider this cellular type as a differentiated stage of PL [17,18].

In the present study, the VE were characterized by presenting fusiform aspect and cytoplasmic granules. Besides that, they did not adhere to the glass slide and they did not emit pseudopodes, like the PL. These results were similar to the ones described by other authors who studied larvae of different species of Lepidoptera [9,13-15]

The THC described a decreasing tendency in the number of hemocytes along the period studied, occurring variations statistically significant only in the 10° and 14° days of development. These variations can be attributed to the ecdise, because in the 10^{th} and 14^{th} days occur the changes from 4^{th} to 5^{th} instars and from 5^{th} to 6^{th} instars, respectively, when the hemocytes are possibly mobilized in other inherent functions to the ecdise, in conformity with [19,20]

In general, DHC presented similar results to the other author as [9,10,14,16,21], where the PR presented a very low frequency (0.9%) and the PL (49.7%) and the GR (23%) presented the highest frequencies.

However, our results shown in total disagreement with the ones presented by [22] as for the frequency observed for PR (37%) and the GR (4%). The authors as [4,5,21] reported a percent medium under 5% of the total of the hemocytes for PR. The different results obtained by [22], probably were due to the technique maid (stained smears), a example to occurred with [9] and [10], already referenced at the beginning of this discussion, besides the difficulty of differentiating, in smears, the PR of the young or small PL. Our results are in agreement with [4,5,21,23], which described high frequency in the initial stages of larval development to the PR, therefore they are considered "stem cells". In work with larvae of *Euxoa* (Lepidoptera: Noctuidae), [20] indicated a frequency of 6% for the PR, results contested later by [24] who affirmed that were included in this index also young PL, due to its great likeness to the PR.

As to the other cellular types (SP, OE and VE), our results are similar to the ones found by [9,13-16].

Acknowledgment

The authors are grateful to Nivalde Basso, Maria H. Moreno and Maria Euleda Perez, for the technical support and to Embrapa Soja, for supplying *A. gemmatalis* larvae. This research was supported by CPG/UEL, CNPq and PRONEX.

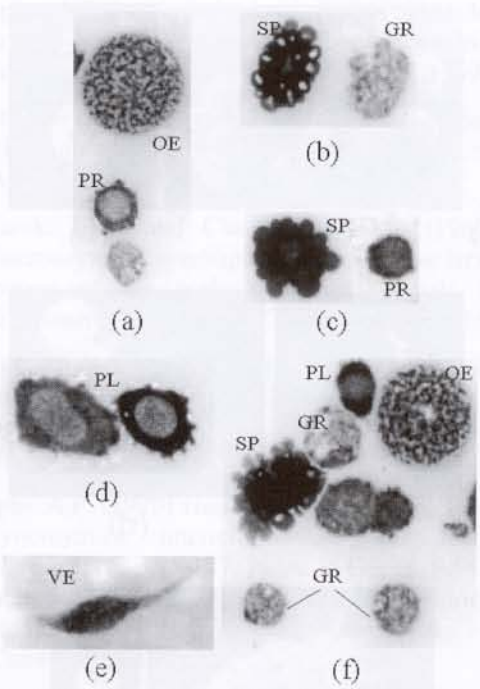

Figure1 – Light micrograph of hemolymph smears, stained with mixture Seller showing different cells types: (a) prohemocyte (PR), oenocytoid (OE) X1260; (b) spherulocyte (SP), granulocyte (GR) X1260; (c) spherulocyte (SP), prohemocyte (PR) X1260; (d) plamatocyte (PL) X1260; (e) vermicyte (VE) X1008; (f) plasmatocyte (PL), oenocytoid (OE); spherulocyte (SP), granulocyte (GR) X1260.

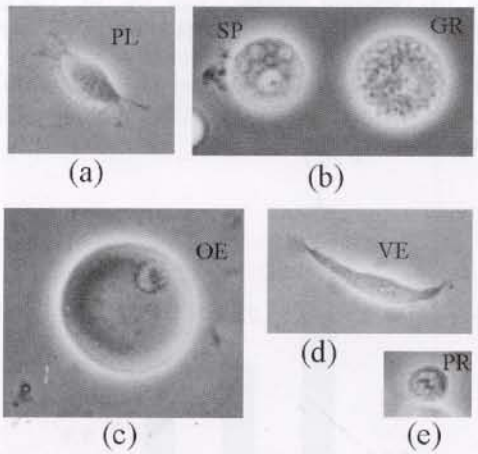

Figure 2 – Phase contrast micrograph showing cellular types: (a) plasmatocyte X640; (b) spherulocyte and granulocyte X800; c) oenocytoid X800; (d) vermicyte X800; (e) prohemocyte X800.

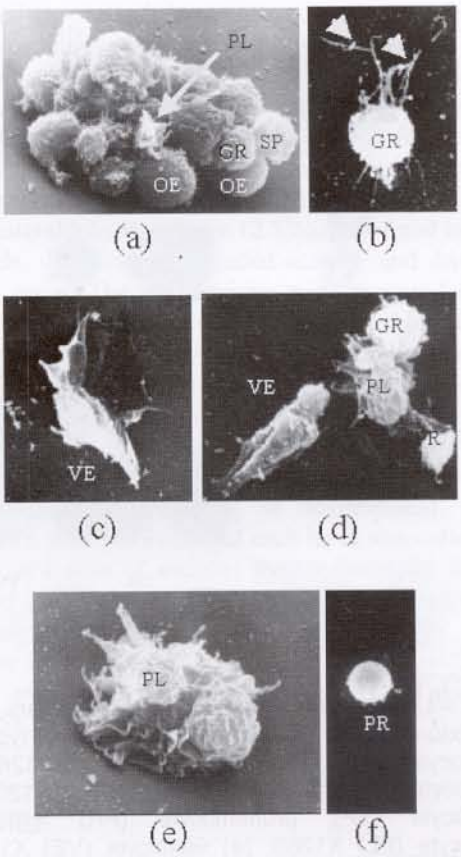

Figure 3 - Scanning electron micrograph of the hemocytes types: (a) aggregate of hemocytes showing OE, GR, SP and PL (X926); (b) Granulocyte with longs philopodial cytoplasmatic process (arrows) (X2536); (c) Vermicyte (X1415); (d) Different cellular types, VE, PL, GR and PR (X1415); (e) Plasmatocytes (X2403); (f) Prohemocyte (X2866).

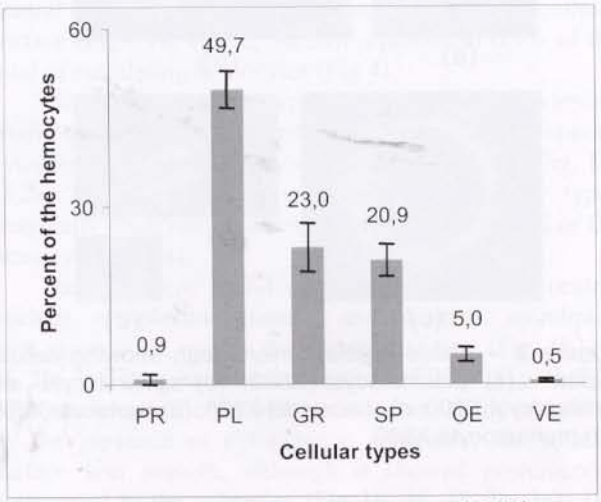

Figure 4 – Differential of hemocytes count (DHC) present in hemolymph of the *A. gemmatalis*, during the larval development. Values represent the means ± SD of 15 larvae/age.

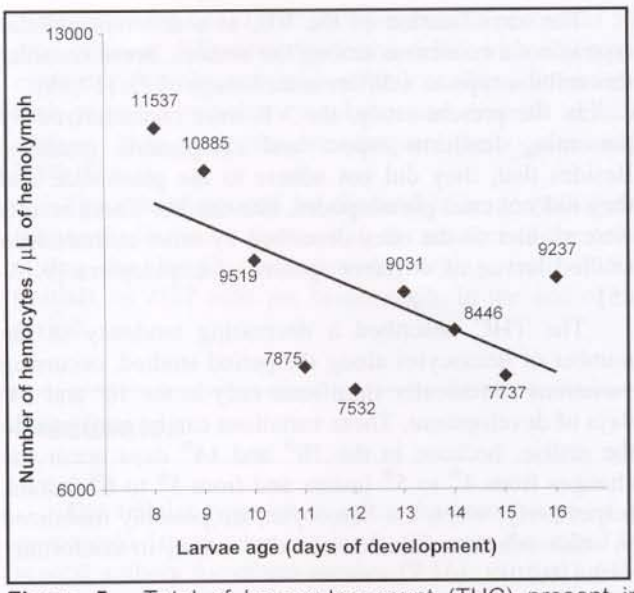

Figure 5 - Total of hemocytes count (THC) present in hemolymph of the *A. gemmatalis*, during the larval development.

References

1. Tepass, U., Fessler, L.I., Aziz, A. and Hartensein, V. (1994) Embrionic origin of hemocytes and their relationship to cell death in Drosophila. Development. 120:1829-1837.

2. Falleiros, A.M.F. and Gregório, E.A. (1995) Hemócitos fagocitários em larvas de Diatraea saccharalis (Fabricius) (Lepidoptera, Pyralidae). Revista Brasileira de Zoologia. 12(4): 751-758.

3. Inoue, N., Hanada, K., Naotoshi, T., Igarashi, I., Nagasawa, H., Mikami, T. Fujisaki, K. (2001) Characterization of phagocytic hemocytes in Ornithodoros moubata (Acari: Ixodidade). Journal of Medical Entomology. 38(4):514-519.

4. Gupta, A.P. (1985) Cellular elements in the hemolymph, p.402-444. In: A.P. Gupta (Ed.). Compreensive insect physiology, biochemistry and pharmacology. Pergamon Press, vol. 3., Oxford, 625p.

5. Ratcliffe, N.A., Rowley, A.F., Fitzgerald, S.W. and Rhodes, C.P. (1985) Invertebrate immunity: basic concepts and recents advances. International Review of Cytology.97:183-349.

6. Russo, J., Brehélin, M. and Carton, Y. (2001) Haemocyte changes in resistant and susceptible strains of D. melanogaster caused by virulent and avirulent strains of the parasitic wasp Leptopilina bouladi. Journal of Insect Physiology. 47:167-172.

7. Hoffmann-Campo, C.B., Oliveira, E.B. and Moscardi, F. (1985) Criação massal da lagarta-da-soja (Anticarsia gemmatalis). EMBRAPA/CNPSo, Londrina, 23p.

8. Leonard, C., Söderhäll, K. and Ratcliffe, N.A. (1985) Studies on prophenoloxidase and protease activity of Blaberus craniifea haemocytes. Insect Biochemisytry. 6:803-810.

9. Bombonato, M.T.S. and Gregório, E.A. (1995) Estudo morfológico e quantitativo dos hemócitos em larvas de Diatraea saccharalis (Fabricius) (Lepidoptera, Pyralidae).Revista Brasileira de Zoologia. 12(4):867-879.

10. Barduco, M.C., Gregório, E.A. and Toledo, L.A. (1988) Hemócitos de Diatraea saccharalis (Lepidoptera: Pyralidae) no período larval: estudo morfológico e quantitativo. Revista Brasileira de Biologia. 48(4):925-932.

11. Chiang, A.S., Gupta, A.P., Han, S.S., (1988) Arthropod immune system: I. Comparative light and electron microscopic accounts of immunocytes and other hemocytes of Blattela germanica (Dictioptera: Blattellidade). Journal of Morphology. 198:257-267.

12. Azambuja, P., Garcia, E.S. and Ratcliffe, N.A. (1991) Aspects of classification of hemiptera hemocytes from six triatomine species. Memórias do Instituto Oswaldo Cruz. 86(1):1-10.

13. Kurihara, Y., Shimazu, T. and Wago, H. (1992) Classification of hemocytes in the common cutworm Spodoptera litura (Lepidoptera: Noctuidae). I. Phase microscopic study. Applied Entomology and Zoology. 27(2):225-235.

14. Saxena, B.P., Sharma, P.R. and Tikku, K. (1988) Scanning electron microscopical studies of the haemocytes of Spodoptera litura Fabr. Cytologia. 53:385-391.

15. Ribeiro, C., Simões, N. and Brehélin, M. (1996) Insect immunity: the haemocytes of the armyworm Mythimna unipuncta (Lepidoptera: Noctuidae) and their role in defense reactions in vivo and in vitro studies. Journal of Insect Physiology. 42(9):815-822.

16. Falleiros, Â.M.F., Bombonato, M.T.S. and Gregório, E.A. (2003) Ultrastructural and quantitative studies of hemocytes in the sugarcane borer Diatraea saccharalis (Fabricius) (Lepidoptera: Pyralidae). Brazilian Archives of Biology and Technology. 46(2):287-294.

17. Gupta, A.P., and Sutherland, D.J. (1966) In vitro transformations of the insect plasmatocyte in certain insect. Journal of Insect Physiology. 12:1369-1375.

18. Lea, M.S. and Gilbert, L.I. (1966) The hemocytes of Hyalophora cecropia (Lepidoptera). Journal of Morphology. 118(2):197-216.

19. Clarck, E.W. and Chadbourne, D.S. (1960) The haemocytes of nondiapause and diapause larvae and pupae of the pink bollworm. Annals of the Entomological Society of America. 53:682-685.

20. Arnold, J.W. and Hinks, C.F. (1976) Haemopoiesis in Lepidoptera. I. The multiplication of circulating haemocytes. Canadian Journal of Zoology. 54:1003-1012

21. Gupta, A.P. (1979) Hemocytes types: their structures, synomymies, interrelationships and taxonomic significance, p.85-127. In: A. P. Gupta (Ed.). Insect hemocytes: development, forms, functions and techniques. Cambridge University Press, Cambridge, 614p.

22. Silva, J.E.B., Boleli, I.C. and Simões, Z.L.P. (2002) Hemocyte types and total and differential counts in unparasitized and parasitized Anastrepha obliqua (Diptera, Tephritidae) larvae. Brazilian Journal of Biology. 62(4):689-699.

23. Yamashita, M. and Iwabuchi, K. (2001) Bombyx mori prohemocyte division and differentiation in individual microcultures. Journal of Insect Physiology. 47:325-331.

24. Kaaya, G.P. and Ratcliffe, N.A. (1982) A comparative study of hemocytes and associated cells of some medically important dipterans. Journal of Morphology. 173: 351-365