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Morpho-Cytometric Investigations on Haemolymph Collected from Honeybees Originated from South of Romania

Agripina ŞAPCALIU[1], Crenguța PAVEL[1], Eliza CĂUIA[1], Ion RĂDOI[2], Nicolae TUDOR[2], Poliana TUDOR[2], Florin MILEA[2]

[1]Beekeeping Research and Development Institute, 42 Ficus Bv., Bucharest, Romania ; agripinasapcaliu@yahoo.com
[2]University of Agronomical Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, 105 Spl. Independentei Street, Bucharest, Romania

Abstract. The studies were carried out in 2007-2009, in the frame of the Pathology Department belonging to the Beekeeping Research and Development Institute-Bucharest. There were performed complex morpho-cytological investigations on haemolymph samples collected from honeybees originated from honeybee colonies located in the southern part of Romania. These researches mainly aimed to identify and classify the haemocytes according to the literature, to study the morphological characters of haemocytes, to establish an optimal staining method for morphologic studies, to study the stage transformation of haemocytes, to highlight the phagocytosis process in natural bacterial infection. The haemolymph samples were collected from healthy and natural infected honeybees and slides were prepared using the following staining methods: Giemsa, May Grunwald-Giemsa and May Grunwald-Giemsa modified and adapted for the haemocytes sensitivity testing.

The following parameters of the haemocytes from centrifuged and non-centrifuged haemolymph were investigated and described: the density and their type, percentage of the phagocytes and non-phagocytes in honeybees from healthy and natural infected colonies, stages of haemocytes formation by morphometrical analyses using a specific measurement software.

Keywords: honeybee, haemolymph, haemocyte, morpho-cytometry

INTRODUCTION

Honey bee haemocytes which circulates in haemolymph are categorized into several types depending on authors' system [1-5]. Their primary functions, phagocytosis, encapsulation, detoxification, and storage and distribution of nutritive materials.

Our researches mainly aimed the follow: to establish the optimal staining method for morphologic studies of haemocytes; to study the morphological characters of haemocytes; to identify and classify the haemocytes according to the literature; to count the percentage of the different cell types from haemolymph; to study the stage transformation of haemocytes and their possibilities of multiplication.

The study was carried out as a part of a research contract named: "The surveillance of honey bee colonies health by complex diagnosis exams performed on haemolymph and optimization of diet and metabolic profile in correlation with immunologic status in honey bees – *Apis mellifera*"

MATERIALS AND METHODS

The studies were carried out in 2007-2009, in the frame of the Pathology Department from Beekeeping Research and Development Institute - Bucharest.

The biological material was provided from healthy honeybee colonies located in the southern part of Romania (Băneasa - zonal station of ICDA; private honeybee colonies breed in ecologic system and kept under medical observation).

From these honeybees there were performed morpho-cytological investigations on haemolymph samples and haemolymph sediment using different staining methods: Giemsa, May Grünwald-Giemsa and May Grünwald-Giemsa modified and adapted for the haemocytes sensitivity testing.

The haemolymph slides were examined on optic microscope with immersion objective (100x) for morpho-cytologic studies that aimed: intensity of staining, the differentiation of cells types, the differentiation grade of cell membrane, cytoplasm, nucleus and different and specific intra-haemocyte or intra-nuclear elements.

RESULTS AND DISCUSSION

Among the three staining method, the optimal one we consider being Giemsa (inalterably), but applied on slides from haemolymph sediment. The slides obtained from haemolymph were extremely scarce in cells.

We observed on haemolymph sediment slides Giemsa stained the following cells: small cells with dense nucleus and ringshaped cytoplasm; small oval-shaped cells with dense round-oval nucleus, with larger cytoplasm as the first, small fusiform cells with granular nucleus and cytoplasm, large cells with round marginal nucleus, more dark stained cytoplasm as the previous ones with small, medium or large vacuoles and cells with decay cytoplasm similar to the previous ones.

According to Van Steenkiste's (1988) classification, which divide haemocytes in two big classes based on their functions: non-phagocyte and phagocyte cells, we placed this founded cells types in the next proportion (see Tab. 1, 2):

Tab. 1

Non-phagocyte cells types and their rate (%) in haemolymph of adult honey bee (7-45 days)

Biologic material: adult bees	*Nonfagocyte* cell types						
	Proleucocyte	Eosinophil	Basophile	Neutrophil	Oenocyte	Picnocyte	Sferulocyte
Personal results %	0.20	73.63	0.40	5.47	3.95	0.20	0.30

Tab. 2

Phagocyte cells and other cell types and their rate (%) in haemolymph of adult honey bee (7-45 days)

Biologic material: adult bees	*Fagocyte* cell types					Other cells
	Granulocyte	Macrocyte	Microcyte	Plasmatocyte	Fusiform cell	
Personal results %	0.50	1.85	7.00	0.61	0.20	5.70 (fat cell)

Our results are medium values obtained from the 48 haemolymph samples. By the side of this classification we found differences like: a significant rise of eosinophile percent and lower neutrophil, oenocyte and microcyte percents while the percents of proleucocyte, basophile, picnocyte, sferulocyte granulocyte, macrocyte, plasmatocyte and fusiform cell had proximate values.

We also associated our data with another Van Steenkiste's (1988) classification which morphological describes the cell types as follow:

PL1 cells (round shaped plasmatocyit and prohaemocyte) are small (4-11 µ diameter), rotunde or ovale with dense, homogeneous, large (3.5-6 µ diameter), round, dark red and central placed nucleus. The cytoplasm is transparent, omogeneous, uncolloured or light pink, rarely violet and exceptional blue stained; in the majority of cases is fine, ring-like placed aroud the nucleus (0.5-2 µ distanse between the nucleus and cell membrane). Cell membrane can be more or less obviously.

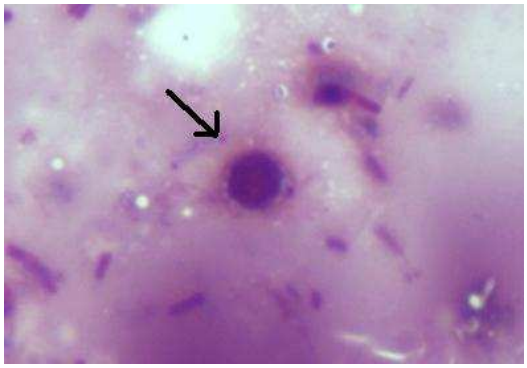

Fig. 1. Plasmatocyit (PL1). Giemsa x 100

PL2 cell (intermediate plasmatocyte) has generally an intermediate shape among PL1 and PL3, larger size than PL1 cells (6-16/5-13 µ), round or oval dense nucleus, central or marginal placed, smaller than PL1 cells recording to the cell size, having approximately the same size as these cells. The cytoplasm has the same features like PL1 cells, but it is larger than their. The cell cytoplasm can be more or less obviously.

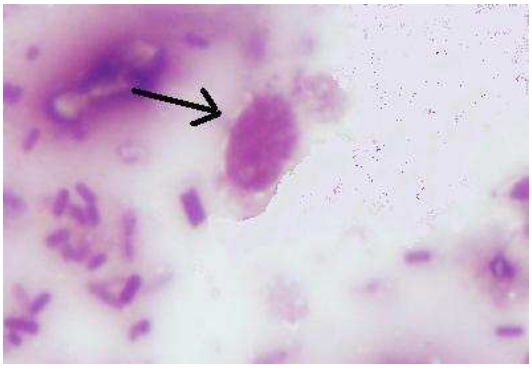

Fig. 2. Intermediate plasmatocyte (PL2). Giemsa x 100

PL3 cell (intermediate/oval plasmatocyte) has a large size (6-18/5-14 µ) and oval or, rarely, round shape. The nucleus is dense or granular (polyhedric grains of chromatin, of 0,5-1/0,1-0,5 µ, dark red or violet stained), round, oval or very oblong, of 4-9/4-7 µ size, central placed. The quantity of cytoplasm can varyes. The cytoplasm is transparent omogeneous, dark pink, violet or rarely blue stained. Generally, the cell cytoplasm is in evidence.

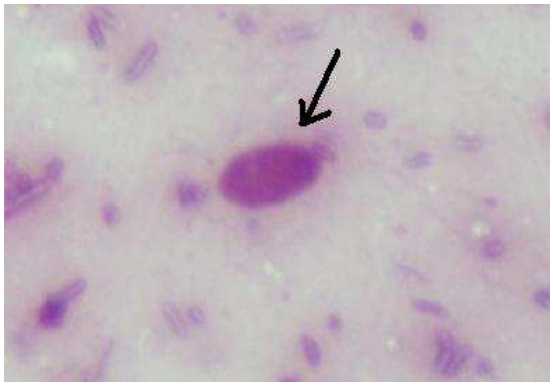

Fig. 3. Intermediate plasmatocyte (PL3). Giemsa x 100

PL4 cell (fusiform plasmatocyte) has oblong shape, often duble pointed, 11-19/3.5-7 µ size. The nucleus is allways granular and oval and the cytoplasm has allways round or polyhedric, dark red, violet or rarely blue grains (0,2-0,7/0,1-5 µ). In the rest the cytoplasm is transparent and light pink or violet stained. Generally the cell cytoplasm is in evidence.

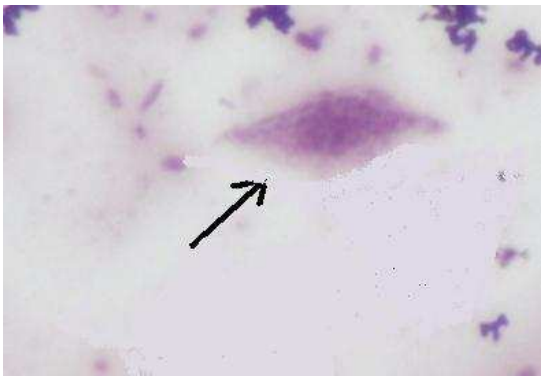

Fig. 4. Plasmatocyte (PL4). Giemsa x 100

GR and OE (granulocyte and oenocyte) are commonly large cells (7-20/6-20 µ, exceptional bigger sizes up to 26 µ), round or slight oval, relative small (5-8 µ), round, marginal placed, dense, dark red stained nucleus. The cytoplasm is dark violet stained (much more intense stained than plasmocyte's one), opaque, dense, without or with more (which have the tendency to fill the cytoplasm) or with a few (large (2-5 µ) or small (1-2 µ)) vacuoles.

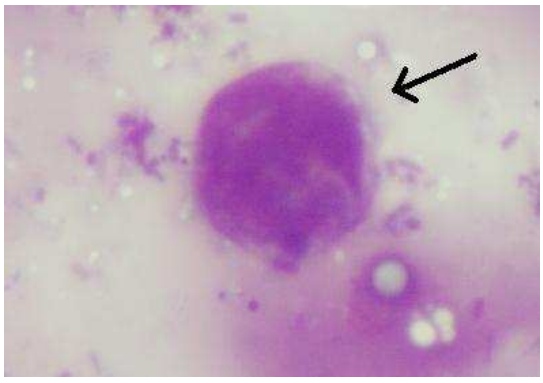

Fig. 5. Granulocyte. Giemsa x 100

CO (coagulocyte) are very labile staining cell, having generally the cytoplasm in different stages of disintegration or dispersion up to the lack of it, in which case it can be observed just the nucleus (small, round) without any change of cytoplasm. This cytoplasm (loaded with many large vacuoles) is lighter stained than the GR+OE one.

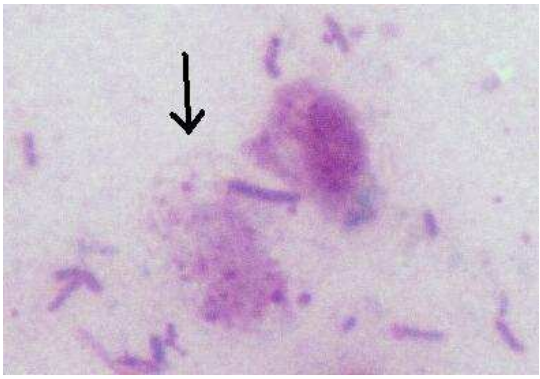

Fig. 6. Coagulocyte. Giemsa x 100

We consider Van Steenkiste's (1988) classifications more proper than other ones for our case and for *Apis mellifera carpatica* specia (spreads in the South-East of Europe).

The observation of Giemsa stained slides under the microscope emphasizes the presence of some haemocyte types (especialy PL1 and PL3) and elements of fat body. In the structure of these fragments of fat body (probably train in haemolymph duding the sampling procedure) we observed round large (30-50µ diameter), red or light violet structures, with dense and intense stained „islands" inside of them. These looked like the nucleus of fat cells from the fat body, but there were free in cytoplasm and presented „islands" accordindg to Sorescu who have word the hypothesis of „haemocyte forming centers".

We also noted that GR+OE are multiplying by mitosis (too) – there were observed cells with two nucleus, placed on the two pole of the cell, but also two very close cells with complementary semicircle shape and those two nucleus – while PL3 seems to multiply by amitosis (medial „strangulation").

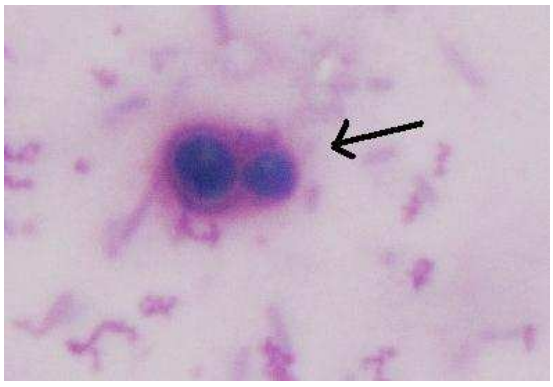

Fig. 7. Haemocyte division. Giemsa x 100

This study is a preliminary one, the haemocytes are actual in study and the haemocyte profile will be explore in the next researches.

CONCLUSIONS

1. The optimal stain method of haemocytes for morphological characters of haemocytes we establish to be Giemsa stain especially applied on slides of haemolymph sediment.
2. In hemocyte profile of healthy bees which divide haemocytes in non-phagocyte and phagocyte our comparative results with the literature showed a significant rise of eosinophile percent and lower neutrophil, oenocyte and microcyte percents while the percent of proleucocyte, basophile, picnocyte, sferulocyte granulocyte, macrocyte, plasmatocyte and fusiform cell had proximate values.
3. We identifyed and characterized in haemolymph of healthy bees all the cellular types: PL1 (round shaped plasmatocyit and prohaemocyte), PL2 (intermediate plasmatocyte), PL3 (intermediate/ oval plasmatocyte), PL4 (fusiform plasmatocyte), GR and OE (granulocyte and oenocyte) and CO (coagulocyte).
4. We observed in Giemsa stained slides of haemolymph cell divisions wich showed that GR and OE are multiplying by mitosis (too) while PL3 seems to multiply by amitosis.

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