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Research article

Morphological characterization and percentage of circulating haemocytes of European hornet adults *Vespa crabro* L. (Hymenoptera: Vespidae)

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Abstract: This study represents the first data for size, morphology, surface area and a number of haemocytes in European hornet (*Vespa crabro*). Circulating haemocytes were microscopically characterized and their surface was determined by software for image analysis. Three types of haemocytes have been identified: prohaemocytes, basophilic haemocytes and plasmatocytes. Prohaemocytes are the dominant type of haemocytes with smallest surface area (54.10±2.42; A=25.79 μm^2), plasmatocytes are the largest circulating haematocytes (41.70±2.41; A=123.56 μm^2), and basophilic haemocytes are the least represented (4.20±1.03; A=49.35 μm^2). In addition to circulating haemocytes, we recorded fourth type, oenocytes. These cells undergo various transformation stages and mature into large cells. Oentocytes"type I" have eosinophilic cytoplasm and have a surface area A=3664.62 μm2. Oentocytes "type II" are significantly larger and have basophilic cytoplasm with surface area A=8411.66 μm^2. Oenocytes are poorly represented, but with 100% incidence. The size of these haemocytes represents the largest oenocytes in insects. The developmental stages of giant haemocytes are presumably result of specific physiological processes. Future studies should provide more information about types, developmental stages of oenocytes including larval hemocytes which will contribute to the understanding of many physiological and metabolic processes in these insects.

Keywords: European hornet, haemocytes, plasmatocytes, haemolymph, *Vespa crabro*.

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Introduction

Characterization of haemocytes in recent literature looks discouraging due to a series of confusing terminology in the attempt to classify them (Gupta, 1985). Many data have been published including ultrastructural and functional analyzes of haemocytes. However, the classification of cells that were already controversial is not simplified, but even more complicated (Manfredini et al., 2008). It has been known that the right methodological approach requires combined techniques from the ultrastructural level to gene markers (Riberio and Brehélin, 2006). Therefore as a result of demanding research methods recent data of haemocytes types are known only for certain insect families and species. Currently, haemocytes have been described in several insect species such as *Drosophila melanogaster* (Williams, 2007), mosquito *Aedes aegypti* (Oliver et al., 2011), silkworm *Bombyx mori* (Nakahara et al., 2009) and also in some shells, crustaceans and molluscs (Suljević et al., 2018). The haemocytes of bees (Hymenoptera) are observed as a component of innate immune system, therefore they are not responsible only for cell immunity (Richardson et al. 2018). Haemocytes participate in the humoral defence by secreting antimicrobial peptides (AMPs), lectins and lysozymes (Sumathipala and Jiang, 2010). The most frequent type of insect haemocytes that have been described in the literature are prohaemocytes, granulocytes, plasmatocytes, spherulocytes, and

oenocytes (Lavine and Strand, 2002). Khosravi et al. (2016) have reported four types of haemocytes in rose sawfly *Arge ochropus,* plasmatocytes are the most typical haemocytes during the larval stage, while granulocytes are most abundant in imaginal stage. The insect haemocytes differ in regards to other insects. However, the most common type of haemocytes in the animal world are granulocytes. Granulocytes undergo various developmental stages, changing their numbers but always being present in circulation (Suljevic et al., 2019).

Haemocytes have many roles. Circulating haemocytes are mediators of cellular immunity which are responsible for phagocytosis and pathogen nodulation (Strand, 2008). Haemocytes synthesize the enzyme phenoloxidase and promote the formation of melanin which is used to produce nodules against foreign substances (Marmaras and Lampropoulou, 2009), phagocytosis and encapsulation as a defence mechanism; synthesis and transport of nutrients and hormones for proper growth and wound healing by way of connective tissue formation (Pandey et al., 2010). Maringa et al. (2014) consider that the number and type of haemocytes in the honey bee depend on the type of infectious agents, xenobiotics, eating habits, migration, seasonal influences and age. They found that understanding the profile of haemocytes provides insight into physiological responses. Haemocyte populations are dynamic and they change depending on different conditions. The formation and origins of haemocytes are also unexplored. Yamashita and Iwabuchi (2001) have assumed that haemocytes differentiate in haematopoietic organs or from circulating prohemocytes from which they subsequently differentiate into other cell types (Beaulaton, 1979). The number of haemocytes is regulated by the mitosis of circulating haemocytes (Saito and Iwabuchi, 2003). Although there is confusion in the classification of haemocytes, morphological and functional characterization is now accepted in Diptera and Lepidoptera while only a few data is available for Hymenoptera (Giglio et al., 2008).

Vespa crabro is an eusocial insect that inhabits Europe, Asia, Africa and America. There is not enough information about its physiology in the literature. So the aim of this study is to make a contribution about this issue and provide information about morphological characteristics, percentage ratio and analysis of haemocyte sizes in *V. crabro.*

Materials and Methods

Collecting of animals on localities

Ten adult hornets were collected in the wider area of Sarajevo Canton (Kremeš, 43º92', 18º33') during June, 2019. Animals were collected separately in large plastic tubes with perforated lid. Animals were transported to the laboratory after assemblage and were analyzed the same day. All procedures with animals were conducted in accordance with the standards prescribed by the Declaration on the Rights of Animals, UNESCO, 1978, the Universal Declaration on Animal Welfare, WSPA, 2000, Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Haemolymph sampling

Haemolymph contamination is a common occurrence during sampling. The reason is that the insects are very active furthermore have the ability to sting so haemolymph quickly melanises in contact with air. Sampling was performed according to the protocol of Borsuk et al. (2016). The method involves the detachment of the antenna and light pressure on the animal's thorax and/or abdomen region. A large drop of haemolymph (cca. 50 µl) appears at the base of hornet's detached antenna and it was aspirated by using automatic pipette. Horn is aggressive insect if disturbed, so this method should be obtained very carefully. It is necessary that animal is immobilized with pincette, pre-cooled on ice for 10 minutes.

Haemocyte analysis

A drop of haemolymph was transferred to a microscope slide and fresh smear was made. Three microscopic slides were prepared from each animal. After 10 minutes, 1 ml of methanol was used for fixation. Fixation lasted 5 minutes followed by Papenheim staining by using Giemsa solution (Semikem, Bosnia and Herzegovina) for 30 minutes. Haemocytes analysis included determination of cells based on their morphology and size, followed by percentage ratio and surface area of 100 cells in total per slide. Analyzes were performed using Olympus BX41 light microscope. Haemocyte identification was achieved by usage of Olympus DP12 camera. Image analysis and processing were done in the licensed software (Olympus DP12 Soft DP12-CB Ver.01.01.01.42. ® Olympus Corp.). Statistical analysis was performed using the IBM SPSS Statistics (v.20; SPSS, Inc., Chicago, IL, USA).

Results

Ten adult hornets were used for detailed analysis and three types of haemocytes have been identified: prohaemocytes, basophilic haemocytes and plasmatocytes in haemolymph of hornets. Their characteristics are presented in Table 1.

Table 2 presents the surface area of haemocytes cytoplasm, nuclei and nuclear-cytoplasmic ratio (N:C). The largest cells and nuclei are plasmatocytes followed by basophils. Prohamemocytes are characterized by the smallest surface and nuclei area. Nuclear: cytoplasmic ratio was greatest in prohaemocytes and smallest in plasmatocytes.

Three types of circulating haemocytes in hornet are presented in Fig. 1 (scale bar 20 μm, 100× magnification).

Table 1. Morphological characteristics of haemocytes

Haemocytes	Description
Plasmatocytes	Largest haemocytes. Their shape is irregular, round and prolated. The cytoplasm is mildly blue, often completely light, without granulation, usually with at least one small vacuole. The nucleus is large, round, often eccentric, with a larger ratio of nuclei in comparison to cytoplasm. The nucleus is made of condensed chromatin wich resembles to granules.
Basophilic haemocytes	Cytoplasm is dark blue with small granules. Cell is round or oblong, nucleus is round or elliptical and is centrally positioned. This type of cell is very rare.
Granulocytes	They are very different in shape and size. Whole cell is covered with small red granules. The nucleus is invisible. The shape of the cells is round/elongated.

Table 2. Surface area of cytoplasm, nuclei and N:C ratio of haemocytes

Surface of haemocyte cytoplasm (out of 100 cells)	**Plasmatocytes**	**Prohaemocytes**	**Basophilic haemocytes**
Mean (%)±SD	123.56±5.64	25.79±4.01	49.35±3.16
Range (%)	115.62-133.49	20.29-32.51	43.09-52.65
Surface of haemocyte nuclei (out of 100 cells)	**Plasmatocytes**	**Prohaemocytes**	**Basophilic haemocytes**
Mean (%)±SD	52.70±2.54	21.70±3.15	22.80±2.78
Range (%)	46.80-57.90	19.40-22.53	18.76-26.31
N:C ratio	**0.43**	**0.84**	**0.46**

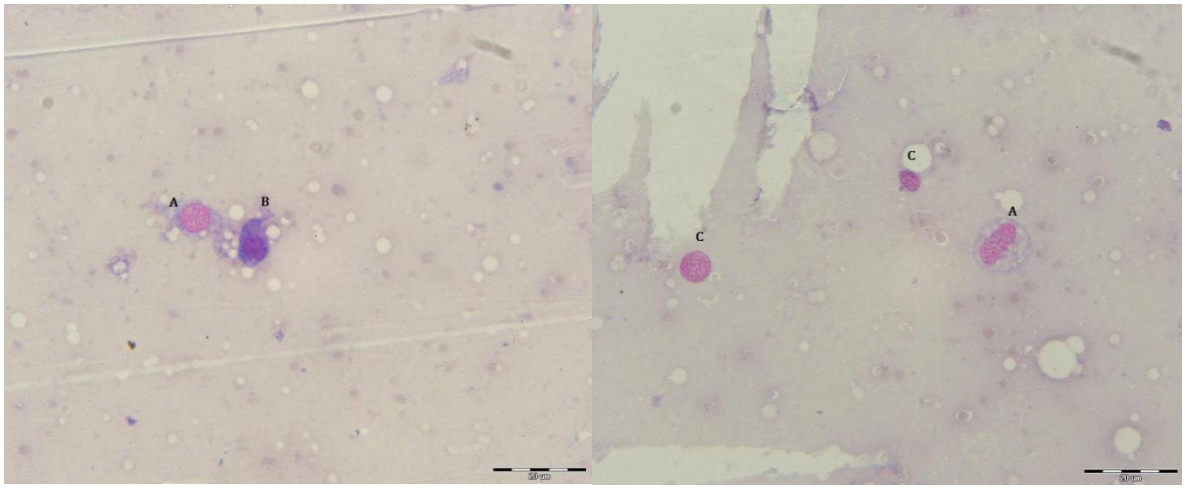

Figure 1. Types of haemocytes: A) plasmatocytes, B) basophilic haemocyte, C) prohaemocyte

Fig. 2 and 3 represent very large cells (giant oenocytes). They are very rare in hornet's haemolymph. These cells vary in size, shape and especially in colour. We believe that staining could be an important character for their identification. Type I (Fig. 2) are coloured red and three stages of development have been identified as it follows: cells A and B are morphologically similar to plasmatocytes. We believe that oenocytes could undergo different stages of development and result as a giant oenocyte (C).

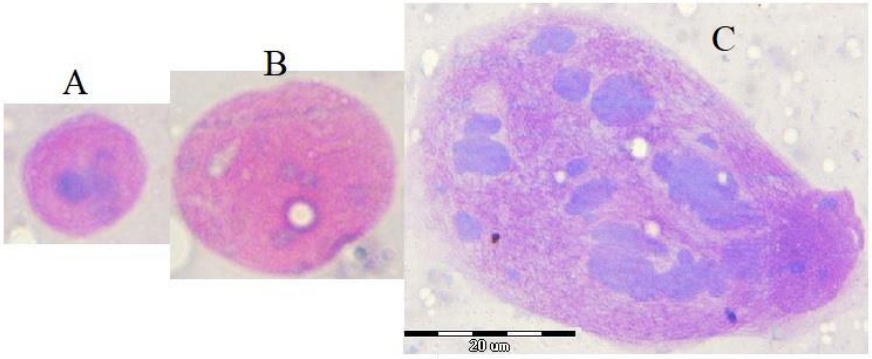

Figure 2. Oenocytes type I. Transformational/development stages of oenocytes: A) first stage (surface area=461.85 μm^2); B) intermediate stage (surface area=2787.67 μm^2); C) final/last stage (surface area=3664.62 μm^2).

Figure 3 may represent the transformation and/or developmental forms of basophilic oenocyte, according to its morphological characteristics. Cell type II (C) is almost twice larger (8411.66 μm^2) than the giant cell presented in Fig. 2C. The structure within the cell clearly shows a heterogeneous composition.

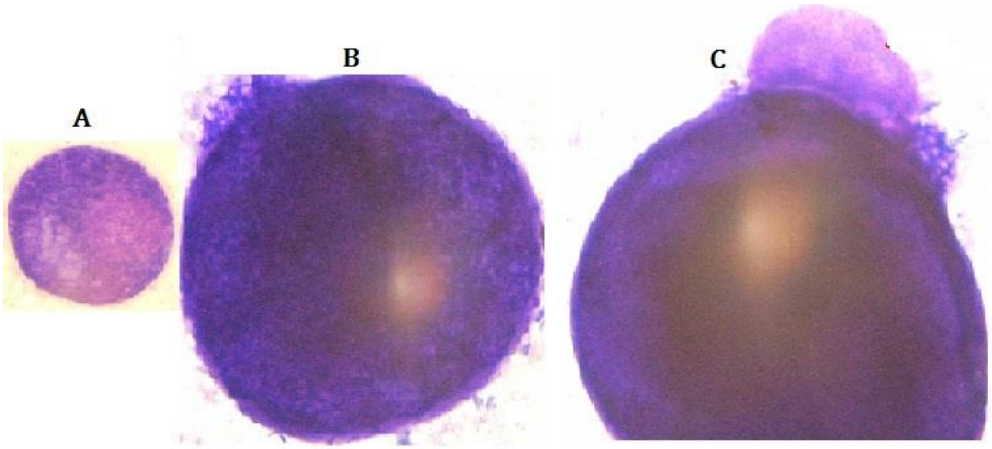

Figure 3. Oenocytes type II. Transformational/development stages of oenocyte: A) first stage (surface area=652,83 μm^2); B) intermediate stage (surface area=7676.22 μm^2); C) final stage (area=8411.66 μm^2).

Discussion

Most data about the European hornet has been linked to the effect of its poison in recent studies. Hornet poison can cause several symptoms in mammals such as: prolonged pain, local erythema and allergic reactions connected with lethal effects sometimes. This is result of release of catecholamines and endogenous histamines from granulocytes. The poison can provoke cytolysis and hemolysis (Anderson et al., 2011). Therefore, it is understandable that research is mainly focused on the analysis of its poison. One study confirmed that poisonous animals are not resistant to their own poison, and it was confirmed that lethal dose is 4mg/kg (Nadolski, 2013). However, other Hymenopterans have much more potent poison (Quistab et al., 1994). Wasps (and hornets) often build hives among human habitats so researches are mainly focused on their behaviour and ecology (Dehghani et al., 2019). Research on haemolymph has been highly controversial due to a different methodological approach (Ribeiro and Brehélin, 2006). Most studies have used morphological characterization as a determination character. Information about the morphological characterization of haemocytes is obtained from Lepidoptera, Drosophila and mosquitoes (Strand, 2008) so far. A detailed characterization of haemocytes among Hymenoptera is present only for honey bee *Apis melifera* (Richardson et al. 2018). Various techniques such as attachment to slide, spreading, presence of granules and development of pseudopodia were used (Negri et al., 2014). Our results are the first study on European hornet's haemocytes. We compared our results to previous reports, based on comprehensive analyze, SEM microscopy and flow cytometry that enabled the identification of haemocytes in bees.

We identified three types of haemocytes in adult hornets: plasmatocytes, prohaemocytes and basophilic

haematocytes. These haemocytes are morphologically similar to honey bees haemocytes where Richardson et al. (2018) also described two dominant types of haemocytes: plasmatocytes and granulocytes (prohaemocytes). Lavine and Strand (2002) reported the presence of prohaemocytes and spherulocytes in the insect haemolymph in addition. The haemocytes of the hornet are very similar to the honey bees' haemocytes in imaginal stage. Same study observed differences in the size of haemocytes and their numbers when comparing imago to larvae. Granulocytes are predominant cells during the larval stage (Richardson et al., 2018; Inoue et al., 2001; Kadota et al., 2003). Our data also indicate that prohaemocytes (possibly granulocytes) are the dominant haemocytes in adult individuals (imaginal stage). Granulocytes are small-sized in comparison to plasmatocytes. In *Drosophila sp.* (Holz et al., 2003) plasmatocytes are the dominant type of cells which have a role in phagocytosis, and granulocytes in mosquitoes (King and Hillyer, 2013).

Most data about the European hornet has been linked to the effect of its poison in recent studies. Hornet poison can cause several symptoms in mammals such as: prolonged pain, local erythema and allergic reactions connected with lethal effects sometimes. This is result of release of catecholamines and endogenous histamines from granulocytes. The poison can provoke cytolysis and hemolysis (Anderson et al., 2011). Therefore, it is understandable that research is mainly focused on the analysis of its poison. One study confirmed that poisonous animals are not resistant to their own poison, and it was confirmed that lethal dose is 4mg/kg (Nadolski, 2013). However, other Hymenopterans have much more potent poison (Quistab et al., 1994). Wasps (and hornets) often build hives among human habitats so researches are mainly focused on their behaviour and ecology (Dehghani et al., 2019). Research on haemolymph has been highly controversial due to a different methodological approach (Ribeiro and Brehélin, 2006). Most studies have used morphological characterization as a determination character. Information about the morphological characterization of haemocytes is obtained from Lepidoptera, Drosophila and mosquitoes (Strand, 2008) so far. A detailed characterization of haemocytes among Hymenoptera is present only for honey bee *A. melifera* (Richardson et al. 2018). Various techniques such as attachment to slide, spreading, presence of granules and development of pseudopodia were used (Negri et al., 2014). Our results are the first study on European hornet's haemocytes. We compared our results to previous reports, based on comprehensive analyze, SEM microscopy and flow cytometry that enabled the identification of haemocytes in bees.

Studies have shown that plasmatocytes are responsible for many cellular immune responses in insects (Ling and Yu, 2006). The number of haemocytes in circulation can change rapidly in response to environmental stress, injuries and infections. Diet can directly alter the number of haemocytes too (Gillespie et al., 1997). Li et al. (2019) reported that granulocytes in the *Bombix mori* participate in the aggregation of cells in the early and late immune stages, where plasmatocytes are responsible for prolonged agglomeration and melanization of haemocytes. Obviously, plasmatocytes play multiple roles in phagocytosis and accomplish this function with different mechanisms. Plasmatocytes are generally recognized as phagocytotic cells (macrophages) involved in the removal of apoptotic cells during development as well as in pathogen ingestion or encapsulation (Hartenstein, 2006). Girardin et al. (2002) reported that plasmatocytes in *Drosophila* are similar to the mammalian monocyte/macrophage lineage. The N:C ratio indicates the maturity of a cell, because as a cell matures the size of its nucleus generally decreases. In the research of Cousin et al. (2013) the variation of oenocytes N:C ratio was between 0.18 and 0.26 respectively.

The focus of our research will be on the analysis of the “novel types of cells” (oenocytes type I i II). Type I (eosinophil oenocytes) are large cells, red coloured and type II (basophil oenocytes) which are rare cells with basophilic cytoplasm and larger in comparison to type I. Type I are more frequent cells. Malfredini et al. (2008) identified large phagolysozymes in *Polistes dominulus* which morphologically resemble to our haemocytes. However, oenocytes in our study are much larger in comparison to the same in *P. dominulus*. These are membrane-limited inclusions of highly irregularly shaped oenocytes and coated with heterogeneous material. Type II of a giant oenocytes are rare but much larger.

Different color of the cytoplasm can be associated with different metabolic and physiological processes. Gould et al. (2001) reported different cytoplasmic colours that range from brown, yellow, green, or red and sometimes even colorless. Oenocytes are insect cells responsible for lipid processing and detoxification (Martins and Ramalho-

Ortigão, 2012). Lycett et al. (2006) showed that oenocytes play an important role in detoxification, protecting the body from toxic and potentially lethal compounds such as insecticides. A small number of oenocytes may be the result of their different localization in the body (Locke, 1969). In their study, three types of oentocytes were identified, differing morphologically and in size. The size depends on the developmental stage and their localization in the body (Johnson and Butterworth, 1985).

The observed type on Figure 3A is reminiscent of an oenocyte that is an integral part of circulating haemocytes in *Melipona scutellaris* (Amaral et al., 2010). However, oenocyte is a very small haemocyte compared to our detected cell and cannot be classified as a third type of haemocyte. As an insect that feeds on fruits and other insects, hornet is very agile and aggressive species. Probably this may be one of the reasons for the formation of such oversized cells due to the demanding metabolic processes. Future research should deal with this phenomenon.

Conclusion

Three types of circulating heamocytes were identified in hornet haemolymph. Two types of oenocytes (eosinophilic and basophilic) with different stages of development have been reported as the fourth type of very rare hemocytes. Some oenocytes appear to be able to undergo different stages of transformation and development into giant cells, so their basic function may be physiologically altered and enhanced. Such transformations and the presence of over-sized cells can be a physiological phenomenon. Studies that would deal with the characterization of these "novel" cells and examine their role in different physiological and metabolic processes from larval (if present) to imaginal stages are needed. Analysis of these processes in the future will provide many answers and shed light on the phenomenon of giant oenocytes and their types, which would be an important step towards in understanding the basis of the immune system in insects.

Ethical Approval

The authors don't declare ethical approval.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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