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Morphological characterization of hemocyte types in some species belonging to Tettigoniidae and Pamphagidae (Insecta: Orthoptera)

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Abstract: In the present study, the species *Eupholidoptera smyrnensis* (Brunner von Wattenwyl, 1882) (Orthoptera: Tettigoniidae), *Decticus verrucivorus* (Linnaeus, 1758) (Orthoptera: Tettigoniidae), and *Glyphotmethis* spp. (Orthoptera: Pamphagidae) were studied in order to determine hemocyte types. To that end, hemolymph smear preparations were stained using Wright's stain. According to light microscopic examinations, five hemocyte types, prohemocytes, plasmatocytes, granulocytes, spherulocytes, and oenocytoids, were determined in each species. Prohemocytes are basic and the smallest cell type in the hemolymph. Plasmatocytes show polymorphism and they may have pseudopodia. Granulocytes are characterized by granules in their cytoplasm. Spherulocytes typically include spherical vacuoles. Oenocytoids are the largest cells among hemocyte types. These hemocyte types have similar characteristics in all examined species. Hemocyte measurements were also made. According to these measurements, *E. smyrnensis* hemocytes were smaller than those of *Decticus verrucivorus* and *Glyphotmethis* spp. Although included in different families, the two closest species in terms of hemocyte size were *D. verrucivorus* and *Glyphotmethis* spp.

Key words: Orthoptera, Tettigoniidae, Pamphagidae, hemolymph, hemocyte types

Although insects do not have an acquired immune system, they have a well-developed innate response (Lavine and Strand, 2002). The protection against infections first begins with some barriers including the integument, gut, and trachea (Tsakas and Marmaras, 2010; Stanley et al., 2012; Hillyer, 2016). On the other hand, hemocytes in the hemolymph are responsible for defense reactions to clear pathogens from circulation (Jalali and Salehi, 2008; Hillyer, 2015; League and Hillyer, 2016). Cellular components of immunity include phagocytosis, nodulation, and encapsulation. Humoral components of immunity include induced expression of genes encoding antimicrobial peptides and activation of complex enzymatic cascades that regulate coagulation and melanization of the hemolymph, pathogen recognition, and production of reactive oxygen and nitrogen species. Prostaglandins and other eicosanoids are of high importance in innate immune responses (Vilmos and Kurucz, 1998; Strand, 2008; Tsakas and Marmaras, 2010; Stanley et al., 2012; Silva et al., 2013). The first population of circulating hemocytes in insect development is composed of head or dorsal mesoderm during embryogenesis. On the other hand, a second population is composed of mesodermally derived hematopoietic organs during the larval or nymphal stages (Strand, 2008). However, Duressa et al. (2015) reported that the main sources of new hemocyte production are circulating hemocytes in adult locusts while hematopoietic tissue has primarily phagocytic activity rather than a hematopoietic nature. The fat body is a main site for the production and secretion of antimicrobial peptides (Hoffmann, 2003; Tsakas and Marmaras, 2010).

Because of both economic and ethical problems with the use of vertebrates in biomedical studies, insects are of great importance for toxicological preclinical studies. Hematology is a substantial part of preclinical studies (Berger et al., 2003; Berger and Slavickova, 2008). In the invertebrate immune system, hemocyte response is a very important component of the defense mechanism (Wu et al., 2016). According to the literature, prohemocytes, plasmatocytes, granulocytes, spherulocytes, adipocytes, and oenocytoids are the most common types of hemocytes in insects. Their properties may show slight variations in various insects (Berger and Slavickova, 2008). There are some studies recognizing hemocyte types in different insect orders. Considering this, the aim of this study was to determine the hemocyte types together with their measurements in some orthopteran species including *Eupholidoptera smyrnensis* (Brunner von Wattenwyl,

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1882) (Orthoptera: Tettigoniidae), *Decticus verrucivorus* (Linnaeus, 1758) (Orthoptera: Tettigoniidae), and *Glyphotmethis* spp. (Orthoptera: Pamphagidae).

This study was carried out in the Invertebrate Culture and Research Laboratory at Ege University, Turkey (temperature: 26 ± 2 °C; relative humidity: 45 ± 5%; photoperiod: natural). All specimens (*E. smyrnensis*, *D. verrucivorus*, and *Glyphotmethis* spp.) were collected around İzmir in spring and summer of 2013 and 2014. Each species was put into different plastic containers and fed on grass and chicken grain. Their water needs were supplied using small covers filled with water. They remained in the laboratory until the experimental procedure.

Hemolymph smear preparations were made to determine the hemocyte types of *E. smyrnensis*, *D. verrucivorus*, and *Glyphotmethis* spp. To that end, hemolymph samples of all insects were obtained from the junction of the femur and thorax of living specimens via heparinized hematocrit capillary tubes. For light microscope analyses, smears were immediately made, air-dried, and then stained using Wright's stain. Photomicrographs of hemocytes were taken with an Axio Scope.A1 photomicroscope.

A total of 100 hemocytes were measured with a MOB-1-15x micrometric ocular tool for each species and data are given in Tables 1–3.

Table 1. Hemocyte measurements of *Decticus verrucivorus.*

Decticus verrucivorus	Min. length (μm)	Max. length (μm)	Mean length (μm)	Min. width (μm)	Max. width (μm)	Mean width (μm)	Min. size (μm^2)	Max. size (μm^2)	Mean size (μm^2)
Prohemocytes	10	15	13.38 ± 1.34	-	-	-	78.50	176.63	141.90 ± 26.48
Plasmatocytes (spherical)	14.25	21.75	16.97	-	-	-	159.40	371.35	229.75 ± 64.79
Plasmatocytes (elliptic)	11.5	24.25	17.71 ± 3.11	9.75	28	17.5 ± 3.95	122.46	434.105	242.44 ± 67.72
Granulocytes	13.25	21	16.74 ± 1.62	-	-	-	137.82	346.18	222.02 ± 43.44
Spherulocytes	14.5	18	16.30 ± 1.26	-	-	-	165.05	254.34	210.46 ± 32.21
Oenocytoids	21	28.5	23.54 ± 2.83	-	-	-	346.18	637.62	440.29 ± 109.71

Table 2. Hemocyte measurements of *Eupholidoptera smyrnensis*.

Eupholidoptera smyrnensis	Min. length (μm)	Max. length (μm)	Mean length (μm)	Min. width (μm)	Max. width (μm)	Mean width (μm)	Min. size (μm^2)	Max. size (μm^2)	Mean size (μm^2)
Prohemocytes	8.75	11.75	10.29 ± 0.88	-	-	-	60.10	108.38	83.70 ± 14.10
Plasmatocytes (spherical)	10.25	16	12.7 ± 2.48	-	-	-	82.47	200.96	139.49 ± 50.32
Plasmatocytes (elliptic)	8.75	18.75	14.04 ± 2.85	8	18.75	11.77 ± 2.69	64.37	212.93	130.44 ± 41.94
Granulocytes	10	16.25	13.13 ± 1.65	-	-	-	78.5	207.29	137.39 ± 34.30
Spherulocytes	10.5	15.5	13.8 ± 1.83	-	-	-	86.55	188.60	152.42 ± 37.55
Oenocytoids	17.25	22.5	20 ± 1.89	-	-	-	233.59	397.41	316.40 ± 58.82

Table 3. Hemocyte measurements of *Glyphotmethis* spp.

Glyphotmethis spp.	Min. length (μm)	Max. length (μm)	Mean length (μm)	Min. width (μm)	Max. width (μm)	Mean width (μm)	Min. size (μm^2)	Max. size (μm^2)	Mean size (μm^2)
Prohemocytes	11.25	13.75	12.25 ± 0.69	-	-	-	99.35	148.41	118.43 ± 13.05
Plasmatocytes (spherical)	12.50	20	17.80 ± 2.56	-	-	-	122.66	314	253.69 ± 65.20
Plasmatocytes (elliptic)	13.50	22.75	18.59 ± 2.23	9.5	24	16.65 ± 3.45	100.68	372.09	239.34 ± 66.31
Granulocytes	12.50	19.50	16.50 ± 1.52	-	-	-	118.60	298.50	213.94 ± 40.92
Spherulocytes	13	16	15.04 ± 1.14	-	-	-	132.67	200.96	178.47 ± 26.11
Oenocytoids	21.50	24.75	23.31 ± 1.40	-	-	-	362.87	480.86	427.79 ± 50.93

Light microscope examinations showed that five hemocyte types including prohemocytes, plasmatocytes, granulocytes, spherulocytes, and oenocytoids were determined in all examined species including *E. smyrnensis*, *D. verrucivorus*, and *Glyphotmethis* spp. Because each hemocyte type had similar characteristics in the examined three species, we show all hemocyte types in Figure 1.

Prohemocytes are the basic cell type in the hemolymph. They are small cells characterized by a very large nucleus nearly covering the whole cell and a small cytoplasmic region (Figure 1a). Plasmatocytes are the most frequently seen cell type in this study. These cells have several forms. In other words, they show polymorphism. Plasmatocytes may have a spherical shape (Figure 1b), or they may have an oval shape with thin and long cytoplasmic expansions (pseudopodia) (Figure 1c). They are found individually or form groups in the hemolymph. Similarly, granulocytes are one of the cell types mostly seen in the hemolymph preparations when compared to other cell types. These cells are round in shape and characterized by granular cytoplasm (Figure 1d). Spherulocytes have spherical vacuoles in their cytoplasm (Figure 1e). On the other hand, oenocytoids are the largest cells among hemocyte cells (Figure 1f). In addition, the general appearance of hemolymph smear preparations of *D. verrucivorus*, *E.*

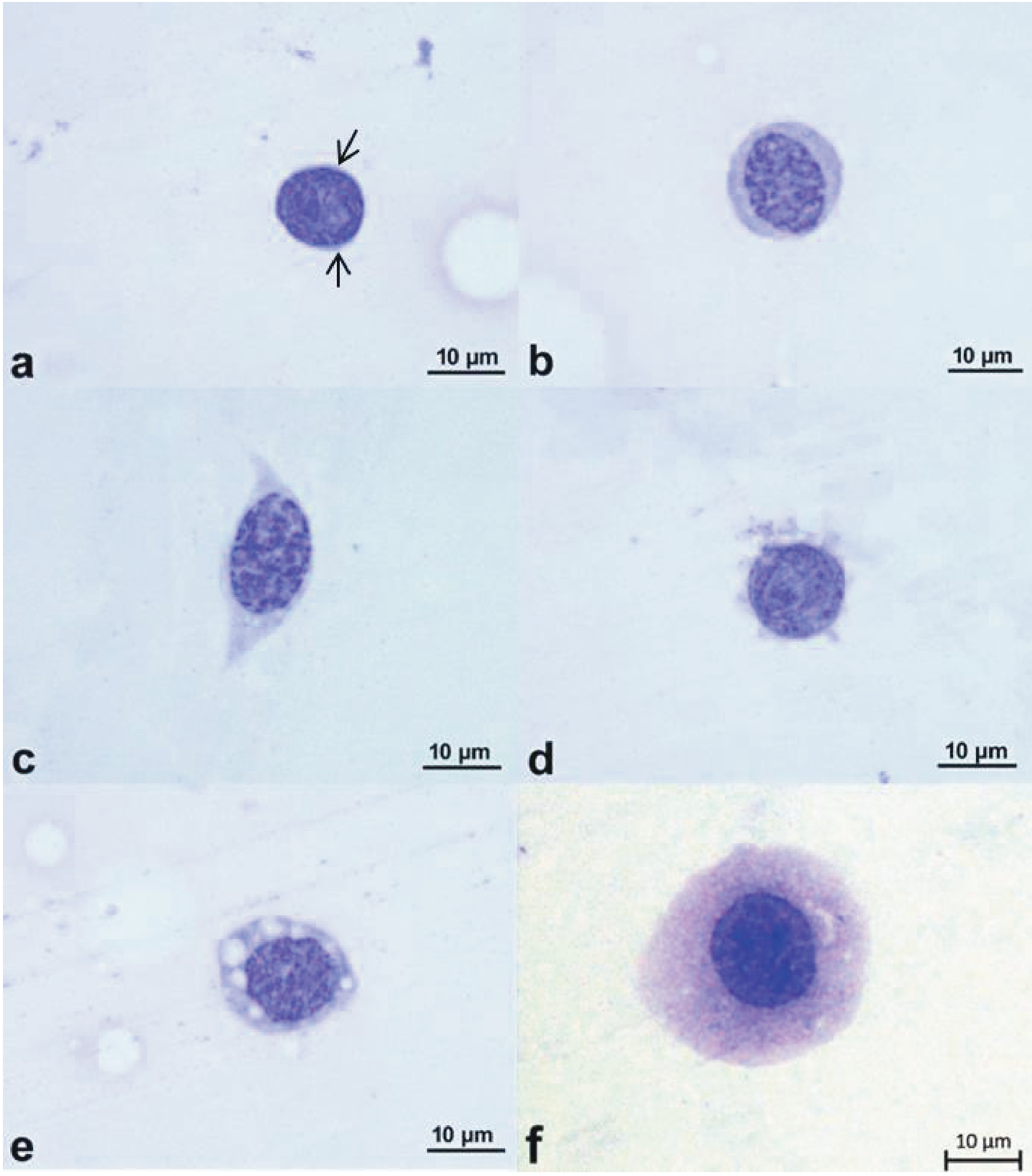

Figure 1. a) Prohemocyte: large nucleus covering whole cell and small cytoplasmic area (arrows). b) Spherical plasmatocyte with oval nucleus. c) Oval plasmatocyte with thin and long pseudopodia. d) Granulocyte. e) Spherulocyte. f) Oenocytoid.

smyrnensis, and *Glyphotmethis* spp. are demonstrated in Figure 2. As shown in Figure 2, *E. smyrnensis* hemocytes were smaller than those of *D. verrucivorus* and *Glyphotmethis* spp. Hemocyte measurements for *D. verrucivorus*, *E. smyrnensis*, and *Glyphotmethis spp.* are demonstrated in Tables 1, 2, and 3, respectively. As seen in these tables, *E. smyrnensis* hemocytes were smaller than those of *D. verrucivorus* and *Glyphotmethis* spp. Although included in different families, the two closest species in terms of hemocyte size were *D. verrucivorus* and *Glyphotmethis* spp.

Hemocytes have been classified by some criteria such as morphology, function, and molecular markers and a scheme universally accepted is not defined for this classification (Chapman, 2013). Although all hemocyte types identified until now are not present in all insects, the six common types are known as adipohemocytes, granulocytes, oenocytoids, plasmatocytes, prohemocytes, and spherule cells (Lawrence, 2008). In addition to these cells, the cytocyte was also determined (Gupta, 1985). There were five types of hemocytes classified as prohemocytes, granulocytes, plasmatocytes, spherulocytes, and oenocytoids in *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) (Liu et al., 2013). The same hemocyte types were detected in this study. Each hemocyte type showed similar characteristics in the examined species. In *Gryllus bimaculatus* De Geer, 1773 (Orthoptera: Gryllidae), the five hemocyte types detected were the prohemocyte, plasmatocyte, granulocyte, coagulocyte, and spherulocyte (Sokolova et al., 2000). On the other hand, different cell types are found in some insects. For example, there were three main types of hemocytes, which are plasmatocytes, crystal cells, and lamellocytes, in *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) (Anderl et al., 2016).

Prohemocytes are also called stem cells because other hemocyte types are derived from these cells (Yamashita and Iwabuchi, 2001; Chapman, 2013). Prohemocytes were the smallest cells with large nuclei in the hemolymph of the examined three species of this study. Prohemocytes, which have similar characteristics, were determined in adult *Pyrrhocoris apterus* (Linnaeus, 1758) (Hemiptera:

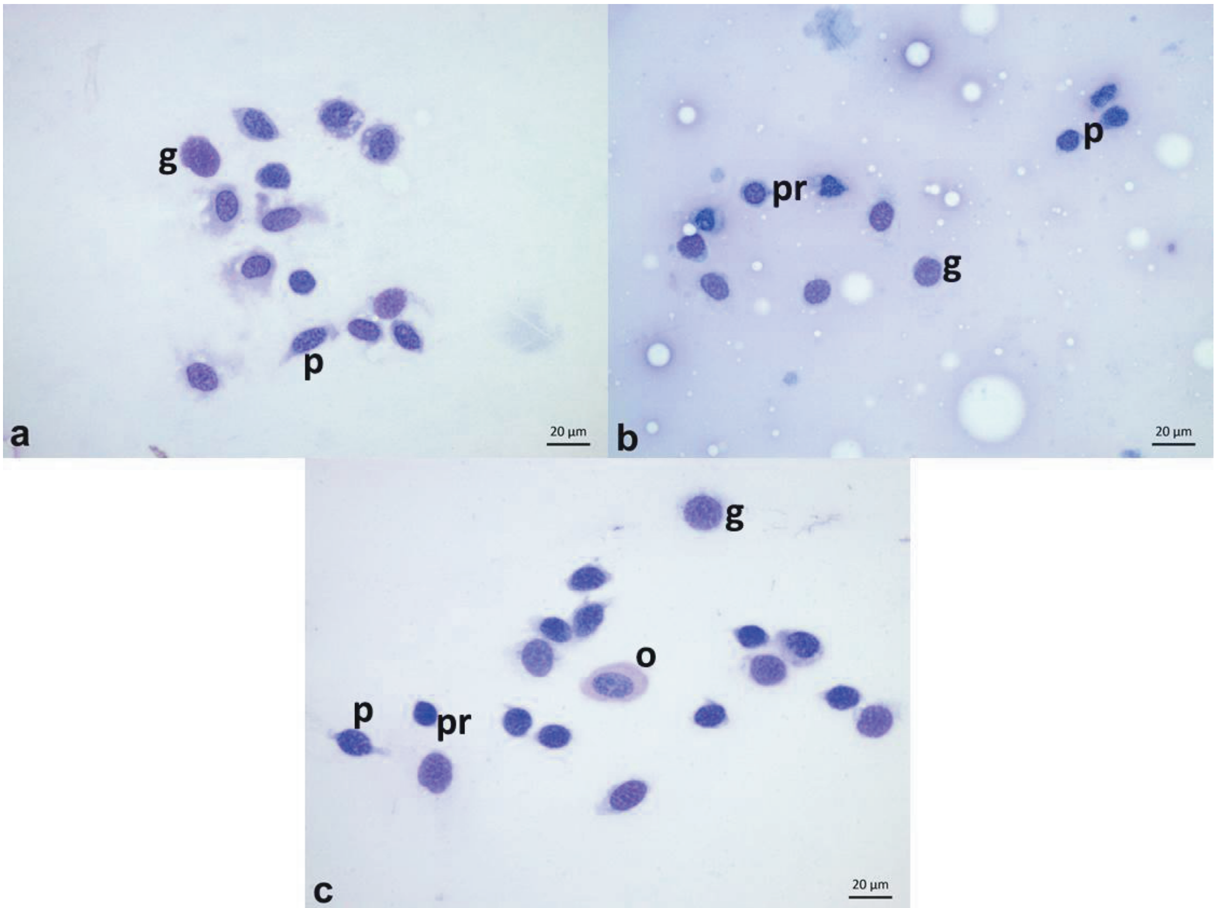

Figure 2. General appearance of hemocytes in hemolymph preparations: a) *Decticus verrucivorus*, b) *Eupholidoptera smyrnensis*, and c) *Glyphotmethis* spp. pr: Prohemocyte, p: plasmatocyte, g: granulocyte, o: oenocytoid.

Pyrrhocoridae) (Berger and Slavickova, 2008), larvae of *Anastrepha obliqua* (Macquart, 1835) (Diptera: Tephritidae) (Silva et al., 2002), *Papilio demoleus* Linnaeus, 1758 (Lepidoptera: Papilionidae) (Jalali and Salehi, 2008), and *B. mori* (Yamashita and Iwabuchi, 2001). Plasmatocytes are small to large polymorphic cells with variable sizes. Plasmatocytes are round or oval in the hemolymph, but when they come into contact with foreign surfaces or during encapsulation they become attached and flattened cells, by sending out filopodia (Levin et al., 2005). In *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae), plasmatocytes were small round or spindle-shaped cells with round or lobulated nuclei that have well-developed nucleoli. The plasma membrane had philopodia and pseudopodia (Wu et al., 2016). As stated previously, plasmatocytes may have various shapes. In our study, oval-shaped plasmatocytes were the most frequently seen type. However, other forms of plasmatocytes were also determined. As with the shape of cells, nucleus forms show diversity. Plasmatocytes originate from prohemocytes. In the examined specimens, transition forms between these two cell types were observed. In *Zonocerus variegatus* (Linnaeus, 1758) (Orthoptera: Pyrgomorphidae), plasmatocytes had the highest total mean count while oenocytoids had the lowest total number of the six hemocytes (Ademolu et al., 2010). In *Melanoplus sanguinipes* (Fabricius, 1789) (Orthoptera: Acrididae), two major types of hemocytes were seen. Granulocytes covered 95% of the total hemocyte population and the rest included plasmatocytes (Miranpuri et al., 1991). As said previously, five hemocyte types were determined in our study. Plasmatocytes were more abundant when compared to granulocytes. Granulocytes differ in size and shape and include uniform acidophilic cytoplasmic inclusions (Lawrence, 2008). In this study, they were noticed as spherical cells with granules. The hemolymph of *Tettigonia cantans* (Fuessly, 1775) (Orthoptera: Tettigoniidae) was studied at the preimago and imago stages by using electron and light microscopes. PAS-negative granules were determined in histochemical reactions. According to electronograms, one type of hemocyte known as granulocyte was identified in the hemolymph (Silina, 2003a). In another study, hemolymph samples of larval and imago stages of *D. verrucivorus* were examined with electron and light microscopic methods. This study determined the PAS-positive and PAS-negative granules in hemocytes. Based on the electronograms, granulocytes were detected as the only type of hemocytes (Silina, 2003b). Spherulocytes are the least seen hemocyte type among hemocyte types. They were determined as rounded or ovoid in shape in *P. demoleus* (Jalali and Salehi, 2008). In the larvae of *A. obliqua*, the spherulocytes had various shapes and there were highly basophilic or acidophilic spherules or spherical vacuoles in cytoplasm (Silva et al., 2002). In this work, many spherical vacuoles were determined in the cytoplasm of these cells. Although oenocytoids are rarely found in the hemolymph, they are determined in many insect species. These cells were spherical or ovoid in the larvae of *A. obliqua* (Silva et al., 2002), rounded in *P. demoleus* (Jalali and Salehi, 2008), and rounded with small eccentric nuclei in the larvae of *G. mellonella* (Wu et al., 2016). As in these species, these cells were determined as the largest cells in our study. Brehelin and Zachery (1986) reported that even though the function of oenocytoids is unknown, the presence of phenol oxidase activity shows their roles in the metabolism of melanin and tanning agents. Wu et al. (2016) also reported that oenocytoids could have a phagocytosis function in *G. mellonella*.

In conclusion, five hemocyte types called prohemocytes, plasmatocytes, granulocytes, spherulocytes, and oenocytoids were determined for each species and these detected cell types had similar characteristics among three species. According to hemocyte measurements, *E. smyrnensis* hemocytes were smaller than those of *D. verrucivorus* and *Glyphotmethis* spp. On the other hand, the two closest species in terms of hemocyte size were *D. verrucivorus* and *Glyphotmethis* spp. These findings revealed that in spite of belonging to different families hemocyte sizes can be similar between different species. We hope that this study will be the basis for more comprehensive investigations in determination of hemocyte types and their functions.

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