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Hemocyte classification of three mosquito vectors: *Aedes togoi*, *Anopheles lesteri* and *Culex quinquefasciatus*

Dedkhad, W.[1], Bartholomay, L.C.[2], Christensen, B.M.[2], Hempolchom, C.[3], Chaithong, U.[3] and Saeung, A.[3*]

[1]Graduate PhD's Degree Program in Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

[2]Department of Pathobiological Sciences, University of Wisconsin, 1656 Linden Drive, Madison, WI 53706

[3]Center of Insect Vector Study, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

[*]Corresponding author e-mail: atisaeung.noi@gmail.com

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Abstract. Insect blood cells or hemocytes play an important role in the defense against parasites and other pathogenic organisms. However, the hemocyte types of three mosquito vectors, *Aedes togoi*, *Anopheles lesteri* and *Culex quinquefasiatus* are not well known. Therefore, the aim of this study was to characterize the hemocytes of these three mosquito species based on morphology using light microscopy. The abdominal cutting and perfusion method was used in this study as it took the fewest steps, provided the largest number of hemocytes and yielded less contamination with fat body cells. Hemocyte typing, based on morphology, revealed three types of hemocytes (prohemocytes, oenocytoids and granulocytes) that were contained in the hemolymph of all three mosquito species. This study demonstrated that the use of distinct morphology with light microscopy provided sufficient criteria to characterize and differentiate mosquito hemocytes. This technique will be useful in terms of cost saving and for new researchers who begin to study in this field.

INTRODUCTION

Mosquitoes are medically important arthropods vectors of disease agents (Beerntsen *et al*., 2000). *Aedes togoi* is a vector of fila-riasis in China, Japan and Taiwan (Cheun *et al*., 2011; Ramachandran *et al*., 1963). In Thailand, *Ae. togoi* (Chanthaburi Province, eastern Thailand) was proven to be an efficient laboratory vector for a wide range of genera and species of filarial nematodes, including the nocturnally subperiodic *B. malayi* (Choochote *et al*., 1987). *Anopheles lesteri* has been reported as a primary vector of malaria in central China (Yang *et al*., 2011). *Culex quinquefasciatus* is an important vector that can transmit human diseases such as lymphatic filariasis, Japanese encephalitis, West Nile virus, and St. Louis encephalitis (Wang *et al*., 2011).

It has long been known that innate and adaptive immunity are the most important systems to protect a host from a pathogen. The effective innate response is divided into humoral and cellular compartments. The humoral responses include soluble factors, such as inducible anti-microbial peptides (AMPs), pattern recognition receptors (PRRs), the phenoloxidase cascade system that regulates coagulation or melanization of the hemolymph in insects, and reactive oxygen and nitrogen intermediates (Hillyer & Christensen, 2005). The cellular response consists of hemocytes or blood cells that are suspended in the hemolymph or attached to visceral tissues (King & Hillyer, 2013). Hemocytes are a key component of the

mosquito immune system that kill pathogens via phagocytic, lytic and melanization pathways (Hillyer & Strand, 2014). Insect hemocytes have been identified and classified using morphological, histochemical, functional characteristic, antigenic and molecular markers (Gupta, 1985; Hillyer & Christensen, 2002; Wang *et al*., 2011). Several investigators have reported different types of hemocytes in different insects. The most common types of hemocytes described in Diptera species are prohemocytes, granulocytes, plasmatocytes, adipohemocytes and oenocytoids (Brayner *et al*., 2005; Da Silva *et al*., 2000; Hernandez *et al*., 1999; Hillyer & Christensen, 2002).

Understanding the mechanisms underlying vector competence may allow the development of new tools in reducing transmission of vector-borne diseases (Araujo *et al*., 2008; Beaty, 2000). However, basic information on hemocytes of *Ae. togoi* and *An. lesteri* are lacking, whereas very few papers regarding *Cx. quinquefasciatus* hemocytes have been reported (Brayner *et al*., 2005; Wang *et al*., 2011). Herein, we described the morphological types of hemocytes of *Ae. togoi*, *An. lesteri* and *Cx. quinquefasciatus*.

MATERIALS AND METHODS

2.1 Mosquito species

The isoline colony of *An. lesteri* was kindly provided by Professor Gi-Sik Min, Department of Biological Sciences, Inha University, South Korea. Exact species identification was based on a combination of morphological characters and DNA-based assays (Joshi *et al*., 2010).

The *Ae. togoi* colony has been maintained continuously for several generations since 1983 in the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai (Choochote *et al*., 1987).

The *Cx. quinquefasciatus* colony was kindly provided by Saowanee Chamnanya, Department of Parasitology Faculty of Medicine, Chiang Mai University, Chiang Mai.

All species were maintained in an insectary at 27±2°C, 70-80% relative humidity, and illumination from a combination of natural daylight from a glass window and fluorescent lighting was provided for approximately 12 hours a day (Saeung & Choochote, 2013). Female pupae were separated from males and kept in a cup half-full with water. One-day-old adult females were collected into cartons and prepared for hemolymph collection.

2.2 Hemolymph collection using abdominal cutting and perfusion (volume displacement) method

Five adult female mosquitoes (1-day old) of each species were cold-anesthetized and placed on a glass slide. Wings and legs were removed and placed on 70% alcohol cleaned slide. Hemolymph diluent that consisted of 60% Schneider's medium, 10% fetal bovine serum (FBS), and 30% citrate buffer (anticoagulant; 98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 4.5) was used.

Mosquitoes were placed on a glass slide and a tear was made at the last two segments of the abdomen. An inoculation needle was inserted into the thorax and 15-20 µl of hemolymph diluent was injected so that diluent/hemolymph was forced out of the abdominal tear. The diluted hemolymph was collected and allowed to dry on the slide.

2.3 Hemocyte characterization

After air-drying, the slides were fixed with methanol for 5 minutes, stained with Giemsa (1:9 in phosphate buffer pH 7.2) for 5 minutes and washed with distilled water. Hemocytes types were identified using previously reported characteristics (Hillyer & Strand, 2014) and with the aid of a compound microscope (BX53, Olympus®, Japan). In order to estimate the percentage of each type of hemocyte, at least 500 hemocytes collected from five mosquitoes were counted.

RESULTS

Three types of hemocytes were identified, prohemocytes, oenocytoids and granulocytes, from the hemolymph of *Ae. togoi*, *An. lesteri* and *Cx. quinquefasciatus*, based on microscopic observation, details of morphology, cell size, nucleus and cytoplasm characteristics. Prohemocytes are the smallest cells with oval or rounded shapes (Figures 1A-1C), and represented 6%, 6.36% and 2.76% of the total hemocyte population in *Ae. togoi*, *An. lesteri* and *Cx. quinquefasciatus*, respectively (Figure 2). Prohemocyte diameters were measured at 2.3-7.86 µm in *Ae. togoi*, 3.44-5.71 µm in *An. lesteri* and 2.86-6.25 µm in *Cx. quinquefasciatus*. A large nucleus occupied the majority of the cell so that only a narrow band of cytoplasm surrounded the nucleus.

Oenocytoids are round and larger than prohemocytes with an eccentric nucleus contained in a homogenous cytoplasm (Figures 1D-1F). The smallest oenocytoid was 4.23 µm in diameter, found in *Ae. togoi*, and the largest was 11 µm, found in *Cx. quinquefasciatus*. This cell was very rare in hemocyte populations from all three

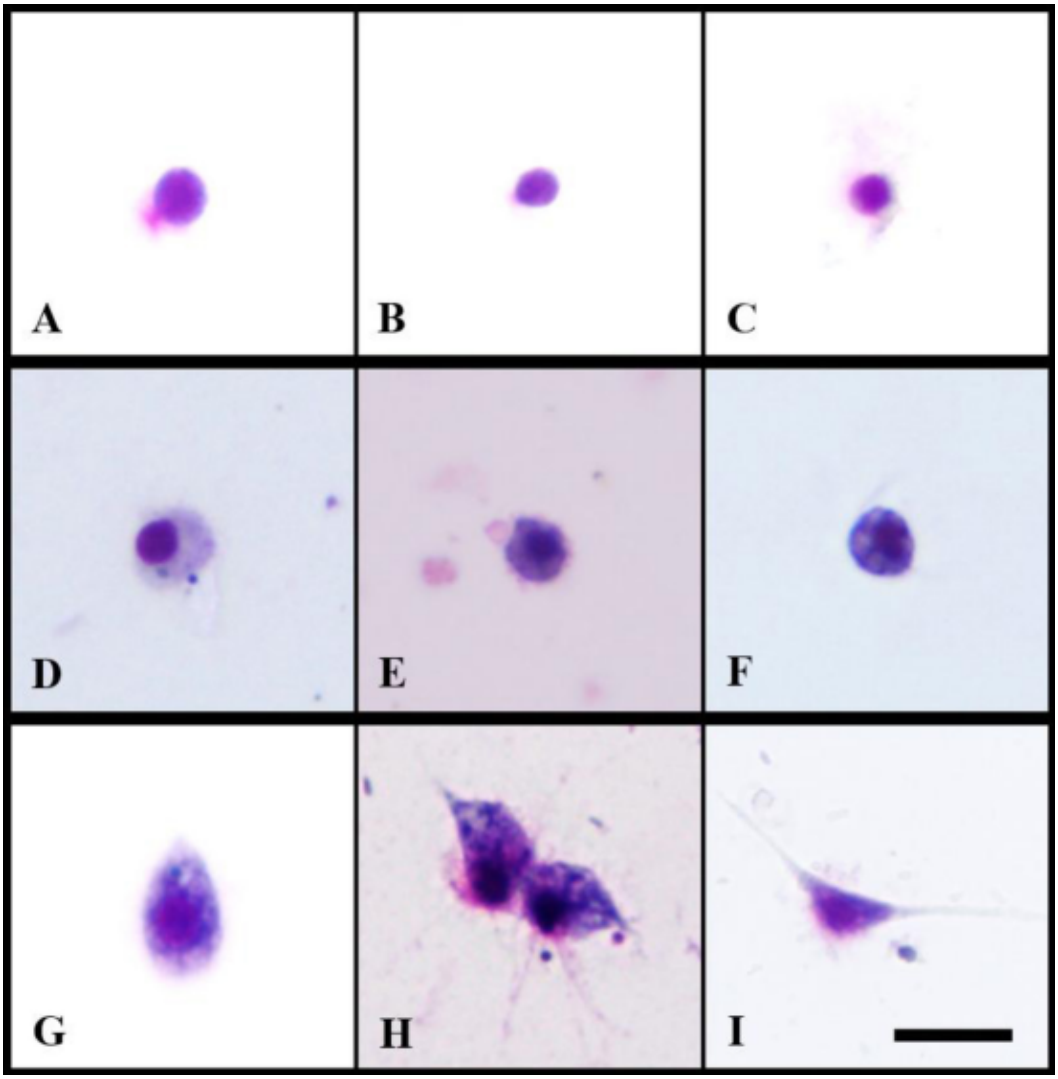

Figure 1. Light microscopy of Giemsa-stained hemocytes collected from *Ae. togoi*, *An. lesteri* and *Cx. quinquefasciatus* adult females. Panels A-C showed prohemocytes, panels D-F showed oenocytoids, and panels G-I showed granulocytes. Scale bar: 10 µm.

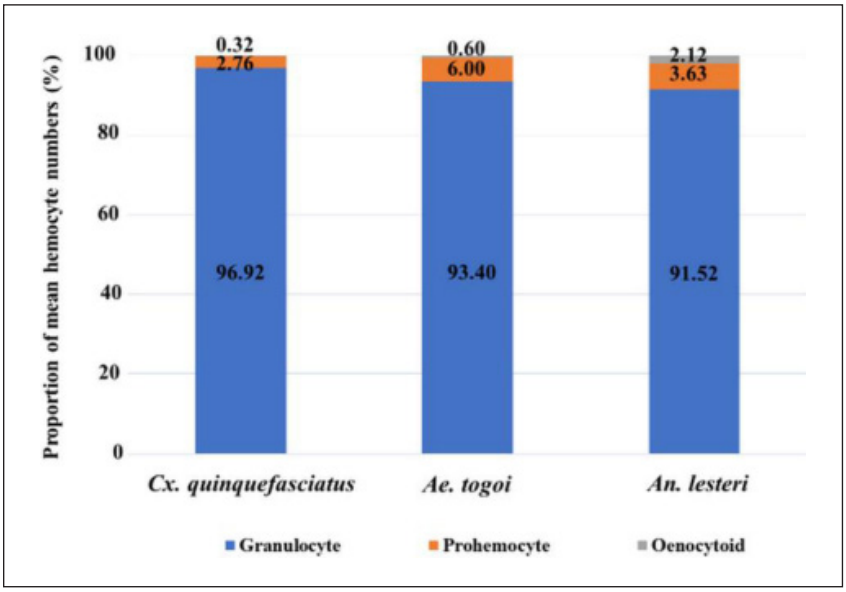

Figure 2. Circulating hemocyte composition derived from the abdominal cutting and perfusion method. Percentage of different hemocyte types obtained from hemolymph of adult female *Cx. quinquefasciatus*, *Ae. togoi* and *An. lesteri*.

species. Oenocytoids represented less than 1% of the hemocyte population in *Ae. togoi* and *Cx. quinquefasciatus*, and 2.12% in *An. lesteri* (Figure 2).

Granulocytes were the most abundant hemocyte, representing 93.4%, 91.51% and 96.92% of the total population in *Ae. togoi*, *An. lesteri* and *Cx. quinquefasciatus*, respectively (Figure 2). Granulocytes have various morphological shapes, including oval, tear drop, fibroblast-liked cell, fusiform and fan-like cells (Figures 1G-1I). Cell sizes ranged in diameter from 5.17 µm in *Ae. togoi* to 66.43 µm in *An. lesteri*. Granulocytes presented with relatively large nucleus when compared to other hemocyte types. Multinucleated granulocytes were commonly seen and many particles were scattered in the cytoplasm, with some cells showing accumulated particles around the nucleus.

The thoracic tissue of *An. lesteri* was stained and this revealed cells that resemble hemocytes in this tissue (Figure 3). Interestingly, hemocyte morphology was sometimes different when compared with hemocytes derived from perfused hemolymph. Prohemocytes showed similar characteristics whether from thoracic tissue or perfused hemolymph (Figures 3A and 3B). In contrast to oenocytoids found in hemolymph, tissue-associated oenocytoids presented a round shaped with a round eccentric nucleus and granules were evident in the cytoplasm (Figures 3C and 3D). Granulocytes from the thorax showed a similar morphology to those in perfused hemolymph (Figures 3E and 3F). Notably, a few of the large size cells with large round nuclei and perinuclear area seemed to fit the description of thrombocytoids (Figure 3G). Additionally, a few thorax-derived cells had lipid droplets and granules scattered in the cytoplasm which is characteristic of adipohemocytes (Figure 3H).

DISCUSSION

Insects have a well-developed innate immune system (i.e. cell cellular and humoral components), which can rapidly respond to invading pathogens (Christensen *et al.*, 2005). The cellular response consists of hemocytes that are suspended in the hemolymph (called circulating hemocytes)

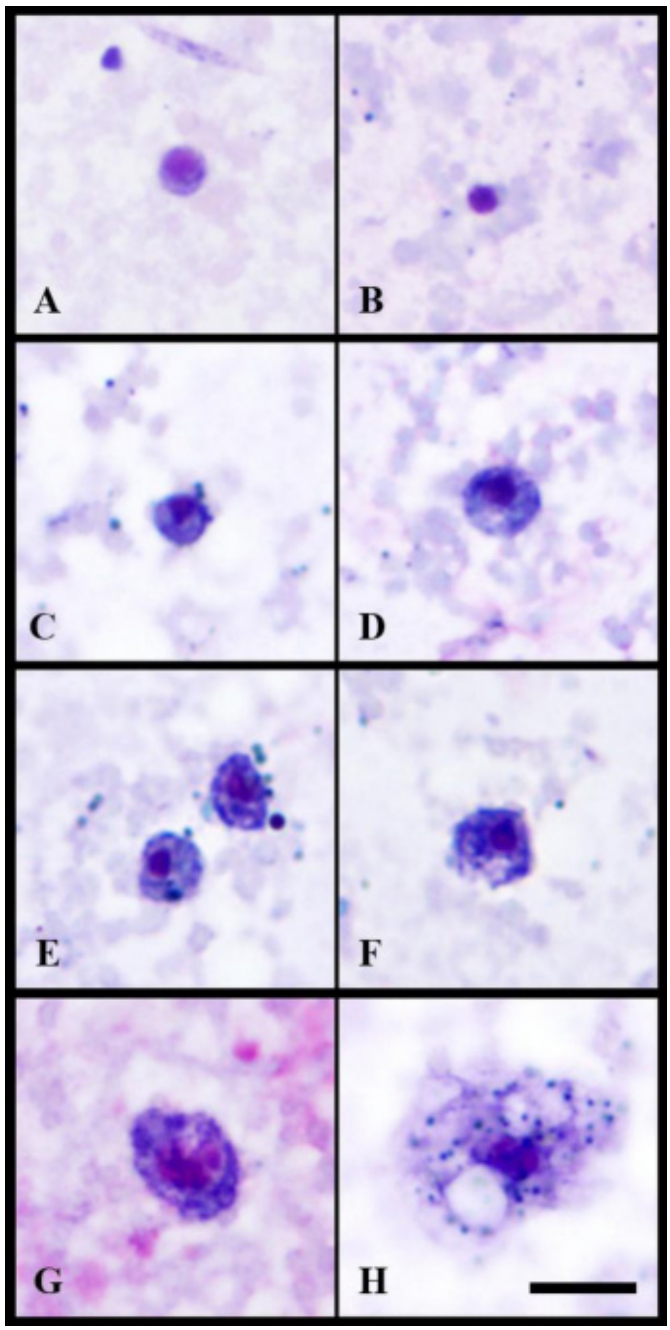

Figure 3. Light microscopy of cells that resemble hemocytes collected from thoracic tissue of *An. lesteri* adult females by using Giemsa staining. Prohemocyte (A and B). Oenocytoid (C and D). Granulocyte (E and F). Thrombocytoid (G). Adipohemocyte (H). Scale bar: 10 µm.

and those attached to visceral tissue in the mosquito (called sessile hemocytes) (Hillyer & Strand, 2014). The ability to collect and identify hemocytes is important for studies in mosquito cellular immunity (Castillo *et al.*, 2006). Thus, numerous studies have been done on hemocyte collection and identification in various

mosquito species (Brayner *et al*., 2005; Da Silva *et al*., 2000; Hernandez *et al*., 1999; Hillyer & Christensen, 2002; Hillyer *et al*., 2003; Wang *et al*., 2011). For example, Castillo *et al*. (2006) collected hemocytes from *Anopheles gambiae* and *Aedes aegypti* using a low and high injection/recovery method. They injected hemolymph diluent between the last two abdominal sclerites and the lateral wall of the mesothorax and collected the diluted hemolymph by capillary from the original injection site in the abdomen. They found that the high injection/recovery method yielded the largest number of hemocytes. However, the disadvantage of this method was using many steps of injection and collecting. Subsequently, Qayum and Telang (2011) demonstrated the modified method for collecting hemocytes from *Ae. aegypti* hemolymph. They combined perfusion and anticoagulant injection, and demonstrated high recovery of hemocytes with fewer injection and collecting steps. In our preliminary study, we evaluated the three hemocytes collection methods; proboscis cutting and perfusion, modified tear and release and abdominal cutting and perfusion method. We found that proboscis cutting and perfusion provided the lowest number of hemocytes but produced the least contamination from fat body cells. Modified tear and release yielded a fair number of hemocytes but most of them were accumulated and trapped in the tissues and carcasses. Moreover, only two types of hemocytes were found by using this method. Therefore, measuring and characterizing each cell were quite difficult to perform. The abdominal cutting and perfusion method yielded the largest number of hemocytes overall and revealed three types of hemoctyes. Although some fat body cells were observed using this method, they could be easily differentiated from hemocytes by sizes and morphology. Therefore, we used the abdominal cutting and perfusion method for collecting hemocytes throughout this experiment. We used anticoagulant buffers which yield a population of hemocytes that properly reflects the numbers *in vivo* followed the modified methods as previously described (Qayum & Telang, 2011). This method required simple steps, produced high recovery of hemocytes and provided less contamination with fat body cells.

Mosquito hemocytes have been identified and classified using morphological, histochemical, and functional characteristics, as well as antigenic and molecular markers (Gupta, 1985; Hillyer & Christensen, 2002; Jung *et al*., 2005; Lanot *et al*., 2001; Wang *et al*., 2011; Willot *et al*., 1994). Hillyer and Christensen (2002) used light microscopy, electron microscopy, enzyme activity assays, and lectin binding assays to classify *Aedes aegypti* hemocytes into granulocytes, oenocytoids, adipohemocytes and thrombocytoids. Hemocytes derived from hemolymph of *Anopheles gambiae* and *Ae. aegypti* were classified into granulocytes, oenocytoids and prohemocytes using a combination of morphological and functional markers (Castillo *et al*., 2006). Araujo *et al*. (2008), however, reported six types of hemocytes from the hemolymph of *Ae. aegypti*, i.e., prohemocytes, adipohemocytes, granulocytes, plasmatocytes, oenocytoids and thrombocytoids by using light and transmission electron microscopy (TEM). In this present study, *Ae. togoi*, *An. lesteri* and *Cx. quinquefasciatus* adult mosquitoes possess three different types of hemolymph-derived hemocytes, which vary in their morphology and size, namely: prohemocytes, oenocytoids and granulocytes. On the other hand, Brayner *et al*. (2005) reported six hemocytes types from *Cx. quinquefasciatus*, i.e. prohemocytes, spherulocytes, adipohemocytes, oenocytoids, plasmatocytes and granulocytes based on light and transmission electron microscopy. Wang *et al*. (2011) identified only four hemocytes types, i.e., prohemocytes, oenocytoids, plasmatocytes and granulocytes from *Cx. quinquefasciatus*. However, adipohemocytes, thrombocytoids and plasmatocyte were subsequently identified as fat body, pericardial cells and granulocytes, respectively, based on functional and comparative studies (Castillo *et al*., 2006; Hillyer, 2010; Hillyer & Christensen, 2005).

Therefore, it is now generally accepted that mosquitoes contain three populations of hemocytes including granulocytes, oenocytoids, and prohemocytes (Hillyer & Strand, 2014).

The present study has classified hemocytes of all three mosquito species using the morphological criteria set up by Hillyer and Strand (2014). Prohemocytes showed oval or round shape with large nuclear-cytoplasmic ratios. These cells were in agreement with those prohemocytes described by several studies (Araujo *et al.*, 2008; Brayner *et al.*, 2005; Castillo *et al.*, 2006; Hillyer & Strand, 2014; Kaaya & Ratcliffe, 1982). We found small populations of oenocytoids, which displayed a round shape, with small and eccentric nucleus, in all species. Their characteristics are compatible with those previously reported in various mosquitoes (Araujo *et al.*, 2008; Brayner *et al.*, 2005; Christensen *et al.*, 2005; Hillyer & Christensen, 2005). Prohemocytes and oenocytoids are similar in having an oval or round shape. Normally, prohemocytes are smaller than oenocytoids but sometimes large prohemocytes are difficult to differentiate from small oenocytoids. Nonetheless, there are some specific characters that can be used to differentiate both of them. Prohemocytes are oval cells without pseudopodia and contain a large nucleus and very little cytoplasm. Oenocytoids are round or oval cells without pseudopodia and nucleus is round and eccentric.

Granulocyte morphologies reported in the literature include spherical, circular, slightly tear-shaped, fusiform, fan-like, star-like and elongated fashion cells (Araujo *et al.*, 2008; Brayner *et al.*, 2005; Castillo *et al.*, 2006; Hillyer & Christensen, 2002; Lavine & Strand, 2002; Wang *et al.*, 2011). Giulianini *et al.* (2003) and Da Silva *et al.* (2002) suggested that the granulocytes are easily identified by their size and cytoplasm characteristically filled with basophilic granules in Giemsa stained smears. In the present work, we observed all shapes in all three mosquito species. Likewise, pseudopodia were often present and their cytoplasm contained granules. Previous studies also reported granulocytes as spindle-shaped or fibroblast-like and having filopodia and pseudopodia, similar to plasmatocytes (Araujo *et al.*, 2008; Brayner *et al.*, 2005; Wang *et al.*, 2011). We rarely found spindle shaped or fibroblast-like morphology in hemocytes that did not also contain granules. Thus, the granules inside cytoplasm seem to be an important point to differentiate granulocyte from those of plasmatocytes (Lavine & Strand, 2002). In the present study, the thrombocytoids, which presented with a perinuclear area around the nucleus and large irregularly shapes, are not seen in the hemolymph of all three mosquito species. This cell possesses numerous cytoplasmic invaginations and homogeneous cytoplasm similar to oenocytoids but appears larger in size (approximately 30 µm in diameter) (Araujo *et al.*, 2008; Hillyer & Christensen, 2002).

We stained the thorax of *An. lesteri* during our studies and some hemocytes were observed, but their morphology was different, and they were difficult to identify. Only prohemocytes in the thorax showed similar characteristics to those of cells found in hemolymph. We also observed hemocytes which showed morphology similar to thrombocytoids and adipohemocytes. Our results are in agreement with Hillyer and Christensen (Hillyer & Christensen, 2002), who stated that these cells are not circulating hemocytes, and are likely attached to fixed tissues.

We concluded that hemolymph from *Ae. togoi*, *An. lesteri* and *Cx. quinquefasiatus* contains three hemocyte types (prohemocytes, oenocytoids and granulocytes) as revealed using a simple and reliable hemocyte collection method. The hemocyte cell types of *Ae. togoi* and *An. lesteri* are described for the first time. Granulocytes are the most abundant, more than 90% of total population, followed by prohemocytes and oenocytoids, which is congruent with observations in other mosquito species (Castillo *et al.*, 2006; Hillyer & Christensen, 2002). Our results provide important information for further

studies of host-parasite interactions in these mosquitoes and the role of cellular immune responses might play in vector competence.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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