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Morphological Characterization of Hemocytes in *Ectemnaspis rorotaense* (Floch & Abonnenc) and *Ectemnaspis trombetense* (Hamada, Py-Daniel & Adler) (Diptera: Simuliidae)

Juciane Conceição da Silva[1], Felipe Arley Costa Pessoa[2], Cláudia Maria Ríos-Velásquez[2], Helena Rocha Côrrea de Araújo[3], Ana Paula Sampaio Feitosa[4], Luiz Carlos Alves[4], Fábio Andre Brayner[4] & Jansen Fernandes Medeiros[5]

1. Instituto Nacional de Pesquisa da Amazônia, Programa de Pós-graduação em Entomologia, e-mail: jucianeconceicao@gmail.com. 2. Instituto Leônidas & Maria Deane, Fiocruz Amazônia, e-mail: facpessoa@gmail.com, criosvelasquez@gmail.com. 3. Universidade de São Paulo-USP, e-mail: helenarca@gmail.com. 4. Centro de Pesquisa Aggeu Magalhães, Fiocruz Pernambuco (CPqAM), e-mail: sampaiofeitosa@hotmail.com, able.lcalves390@gmail.com, brayner.santos@gmail.com. 5. Fundação Instituto Oswaldo Cruz, Fiocruz Rondônia, e-mail: jmedeiro@gmail.com (Autor para correspondência✉).

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Abstract. Hemocytes are insect immune cells which are responsible for processes of phagocytosis, encapsulation, and coagulation. This study aim to characterize the hemocyte cells in two species amazon blackflies: *Ectemnaspis rorotaense* (Floch & Abonnenc) and *Ectemnaspis trombetense* (Hamada, Py-Daniel & Adler). Black fly larvae and pupae were collected from streams in Presidente Figueiredo Municipality, Amazonas State, Brazil. Hemolymph of 36 individuals of *E. rorotaense* (12 larvae, 12 pupae and 12 adults) and 38 of *E. trombetense* (12 larvae, 12 pupae and 14 adults) were collected and the cells were characterized by light microscopy; 200 adults of each species were used to transmission electron microscopy study. In this work were showed, by the first time, the hemocyte cells of black flies amazon. Four cell types were identified: prohemocytes, granulocytes, oenocytoids, and plasmatocytes. Prohemocytes were the smallest cells and they exhibited a high nuclear-cytoplasmic ratio. Granulocytes possessed large, eccentric nuclei, and they were characterized by the presence of granules that differed in size and shape. Oenocytoids presented poorly developed nucleus with localization in central region. Plasmatocytes showed more morphological variations and large projections in the cytoplasmic membrane. The prohemocytes were the most frequent in *E. rorotaense*, with nearby 45% of total cells, whereas plasmatocytes and granulocytes, each one with 38%, were the most abundant in *E. trombetense*. This study showed that prohemocytes, granulocytes, oenocytoids, and plasmatocytes were present in the hemolymph of *E. rorotaense* and *E. trombetense* during all stages.

Keywords: Black fly; cellular immunity; ultrastructure.

Caracterização Morfológica de Hemócitos em *Ectemnaspis rorotaense* (Floch & Abonnenc) e *Ectemnaspis trombetense* (Hamada, Py-Daniel & Adler) (Diptera: Simuliidae)

Resumo. Os hemócitos são células do sistema imune dos insetos responsáveis pelos processos de fagocitose, encapsulação e coagulação. O objetivo desse trabalho foi caracterizar os hemócitos nos simulídeos amazônicos, *Ectemnaspis rorotaense* (Floch & Abonnenc) e *Ectemnaspis trombetense* (Hamada, Py-Daniel & Adler). Larvas e pupas de simulídeos foram coletadas em igarapés no município de Presidente Figueiredo, Amazonas, Brasil. Para caracterização celular através da microscopia óptica, foi coletada a hemolinfa de 36 espécimes (12 larvas, 12 pupas e 12 adultos) de *E. rorotaense* e 38 espécimes (12 larvas, 12 pupas e 14 adultos) de *E. trombetense*. Para o estudo com microscopia eletrônica de transmissão, 200 adultos de cada espécie foram utilizados. Neste trabalho foram descritos, pela primeira vez, os hemócitos de simulídeos amazônicos. Foram identificados quatro tipos celulares em larvas, pupas e adultos: prohemócitos, plasmatócitos, granulócitos e oenocitóides. Os prohemócitos, com um núcleo volumoso em relação ao citoplasma, se mostraram as menores células. Os granulócitos foram caracterizados pela presença de grânulos de diferentes tamanhos e formas e um núcleo grande e excêntrico. Os oenocitóides revelaram núcleo pouco desenvolvido, geralmente localizado na região central. Os plasmatócitos apresentaram grandes projeções da membrana citoplasmática e maior variação morfológica. Os prohemócitos foram as células mais frequentes em *E. rorotaense* com 45% do total das células, enquanto os plasmatócitos e granulócitos, ambas com 38% cada, foram as mais abundantes em *E. trombetense*. Esse estudo mostrou que prohemócitos, granulócitos, oenocitóides e plasmatócitos são presentes na hemolinfa de *E. rorotaense* and *E. trombetense* durante todos os estágios.

Palavras-Chave: Imunidade celular; simulídeos; ultra estrutura.

In general, insects are protected from invading agents by an efficient defense system. The first barriers of defense include: the external cuticle, the chitin lining of the trachea, the cibarial armature, and the peritrophic matrix in the midgut (Lemaitre & Hoffmann 2007). When these physical barriers are penetrated, invading agents come into contact with the hemolymph and activate the innate immune system. One defense mechanism is the humoral immune response, which produces antimicrobial peptides and triggers a cascade of chemical reactions that regulate coagulation and melanization (Bulet *et al.* 1999). Another pathway of defense is the cellular immune response. This pathway is mediated by hemocytes which participate in the processes of phagocytosis, melanization, and encapsulation (Lavine & Stand 2002; Hillyer *et al.* 2003).

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The population and number of circulating hemocytes in the hemolymph of insects is important information to gather because it contributes to our understanding of the host-parasite relationship (Hernández *et al.* 1999; Silva *et al.* 2002). Gupta (1985) grouped hemocyte cells into seven types: prohemocytes, plasmatocytes, granulocytes, spherulocytes, adipohemocytes, oenocytoids, and coagulocytes. Several studies have aimed to characterize hemocytes in some invertebrates as Arachnidae (Kuhn & Haug 1994; Carneiro & Daemon 1996) and dipterian such as: Sarcophagidae - *Sarcophaga falculata* Pandelle, *Sarcophaga bullata* (Parker); Muscidae - *Glossina morsitans* (Wiedemann), *Stomoxys calcitrans* (Linnaeus); Calliphoridae - *Calliphora erythrocephala* (Meigen) and *Lucilia sericata* (Meigen); and Culicidae - *Culex quinquefasciatus* Say, *Anopheles gambiae* Giles, *Aedes aegypti* (Linnaeus), and *Aedes albopictus* (Skuse) (Brayner *et al.* 2005; Castillo *et al.* 2006; Araújo *et al.* 2008; Siddiqui & Al-Khalifa 2012).

Black flies are insects of medical and veterinary importance because some species are involved in the transmission of pathogens that infect humans and other vertebrates; these pathogens include protozoans such as *Leucocytozoon,* which parasitizes turkeys, ducks and birds, in the USA and New Zealand and filarial worms as *Onchocerca* which affects humans, cattle, primates and birds, in USA, and some countries in Africa, Europe and Americas and *Mansonella ozzardi* (Manson) that affects humans in some countries in the Americas (Coscarón & Coscarón-Arias 2007). In Brazil, black flies are involved in the transmission of the filarial worms *Onchocerca volvulus* (Leuckart) and *M. ozzardi* (Cerqueira 1959; Shelley *et al.* 1997; Pessoa *et al.* 2008).

Despite the importance of these insects in transmission of pathogens to humans little is currently known about the hemocytes of black flies in the Neotropical region. Hemocyte studies conducted to date have dealt only with species from the Nearctic and Palearctic regions. Rubtsov (1959) used an old classification system to study some Palearctic species, and was the first to describe hemocytes in larvae, pupae, and adults, identifying: oenocytes, oenocytoids, granular hemocytes, proleucocyts, macronucleocytes, micronucleocytes, fusiform hemocytes, phagocytes, and adipocytes. Luckhart *et al.* (1992) studied cell types in the hemolymph of the adult female *Psilozia vittata* [= *Simulium vittatum*] (Zetterstedt), identifying: prohemocytes, plasmatocytes, granulocytes, and spherulocytes. Cupp *et al.* (1997) described changes in the number and morphology of hemocytes following the experimental infection of *P. vittata* by *Onchocerca linealis* Stiles worm.

In this study, two species of black fly were used to analyze and describe hemocyte cell types: *Ectemnaspis rorotaense* (Floch & Abonnenc) and *Ectemnaspis trombetense* (Hamada & Py-Daniel Adler). This study had two aims: first, to characterize hemocyte cells morphologically using optical microscopy and transmission electron microscopy; and second, to perform the differential counting of cell types.

MATERIAL AND METHODS

Black fly collection. Black flies (pupae and larvae) were collected between June until December of 2013, in the following localities: Corredeira da Naza, (Km 06) (02°02'57"S 59°58'29"W) and Sossêgo da Pantera, (Km 20) (02°02'34"S 59°51'08"W), Presidente Figueiredo Municipality, Amazonas State, Brazil. Larvae and pupae were collected from submerged substrates (leaves, branches, and roots), stored in plastic bags containing water from the stream, and transported to the Laboratório de Ecologia e Doenças Transmissíveis da Amazônia (EDTA), Manaus Municipality, Amazonas State, Brazil. The mature pupae were separated, placed individually in vials, and kept at 27°C to allow for adult emergence. The remaining specimens (12 pupae of each species) were kept for hemolymph collection. After the adults (426 specimens) had emerged, hemolymph collection was performed. Immature and newly emerged adults were identified using the keys of Hamada & Adler (2001). The taxonomic nomenclature used to Simullidae here followed Py-Daniel & Moreira-Sampaio (1994) proposal.

Hemolymph Collection. The larvae, pupae, and newly emerged adults were placed on ice (1–2 minutes) for immobilization. Perfusion was performed by injecting the thoracic region with 4µl of anticoagulant citrate buffer (98 mM ClOH, 145 mM NaCl, 1.7 mM EDTA and 41 mM citric acid). Injections were made with a micro siliconized glass capillary attached to a Hamilton syringe. The hemolymph was collected from a small incision in the abdomen with the aid of a pipette (Araújo *et al.* 2008). For this procedure was used hemolymph of 474 blackfly specimens, corresponding to 24 larvae, 24 pupae and 426 adults.

Hemocytes Characterization

Optical Microscopy (OM). For each specimen (larvae, pupae, and adult), approximately 10µl of hemolymph was placed on separate glass slides, left to dry for 30 minutes at room temperature, and stained with Panotic Rápido Laborclin ® (Brayner *et al.* 2005). The hemocytes were examined with a Leica DMLB optical microscope and photographed with a JCV KYF55-BE digital camera, using Auto Montage 4.0 (Syncroscopy). Cell types were identified by analyzing morphological characteristics such as cell size, nucleus size, projections of the cytoplasmic membrane, and the presence of granules. The percentage of each cell type was then calculated in relation to the total number of cells. In all, 36 slides held hemolymph from *E. rorotaense* (12 larvae, 12 pupae and 12 adults), and 38 slides held hemolymph from *E. trombetense* (12 larvae, 12 pupae e 14 adults).

Transmission electron microscopy (TEM). Hemolymph was collected from 200 *E. rorotaense* adults, and 200 *E. trombetense* adults. Sample pools were placed in plastic tubes previously treated with Sigmacote® (Sigma) and were fixed with Karnovsky's fixative (2.5% glutaraldehyde, 4.0% formaldehyde, and sodium cacodylate buffer 0.1 M pH 7.2). The hemolymph was centrifuged at 6000 rpm for 5 minutes to obtain the hemocytes and these were washed with sodium cacodylate buffer (0.1M, pH 7.4). The samples were post-fixed with 1% osmium tetroxide in phosphate buffer (0.1 M, pH 7.2), and dehydrated in a crescent series of ethanol. The samples were then infiltrated and embedded in Epon 812/Araldite (Electron Microscopy Sciences). Ultrathin sections were contrasted with lead citrate and 5% uranyl acetate, and examined using a transmission electron microscope (Tecnai Spirit Twin G1 Bio of Fei Company). The images were obtained using Microscope User Interface (Fey Company) and TEM Imagine and Analysis (Brayner *et al.* 2005).

Differential hemocyte counts. The count was performed using optical microscopy (1000x) to examine hemolymph from adults of both species. Ten slides per species were examined, and each slide held 200 stained cells. The percentages of each cell type were calculated. This experiment was performed in triplicate.

RESULTS

Four morphological types were identified in hemolymph from larvae, pupae, and adults of *E. rorotaense* and *E. trombetense*. The types identified were: prohemocyte, granulocyte, oenocytoid, and plasmatocyte.

Prohemocytes. Prohemocytes were the smallest cells found in the hemolymph. They are round or oval in shape, and 5–10 µm in diameter. They have a nucleus that is large in relation to the cytoplasm, and some cells exhibit small projections of the plasma membrane (Figures 1A and 2A). Few organelles can be seen in the cytoplasm other than the mitochondria and endoplasmic reticulum (Figures 3A and 4A). In *E. rorotaense*, this was the most abundant cell type, comprising 45% of the total; while in *E. trombetense*, this was the second least abundant cell type,

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comprising only 13% of the total.

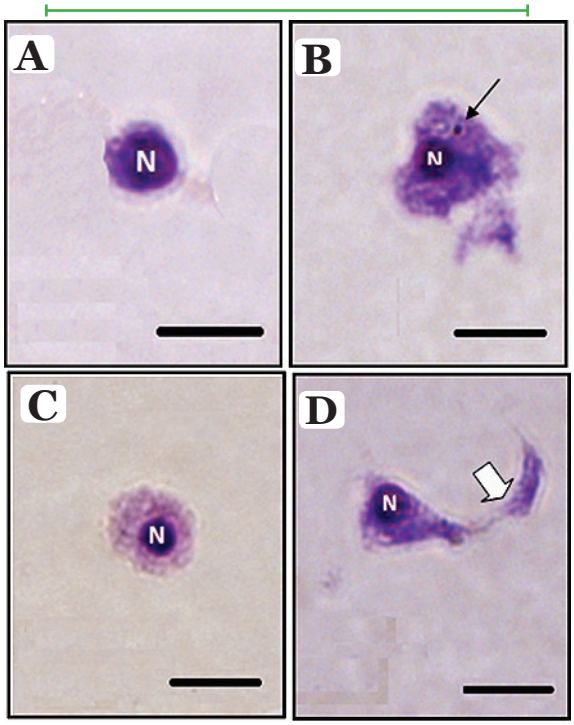

Figure 1. Hemocyte characterization of *Ectemnaspis rorotaense* in optical microscopy. (A) Prohemocyte with a large nucleus occupying almost the entire cytoplasm. (B) Granulocyte with an irregular shape and granules (thin arrow) in the cytoplasm. (C) Oenocytoid with a small centrally located nucleus. (D) Plasmatocyte with visible extensions of the cytoplasmic membrane (large arrow) and a central nucleus. N = nucleus; Bars = 5 µm.

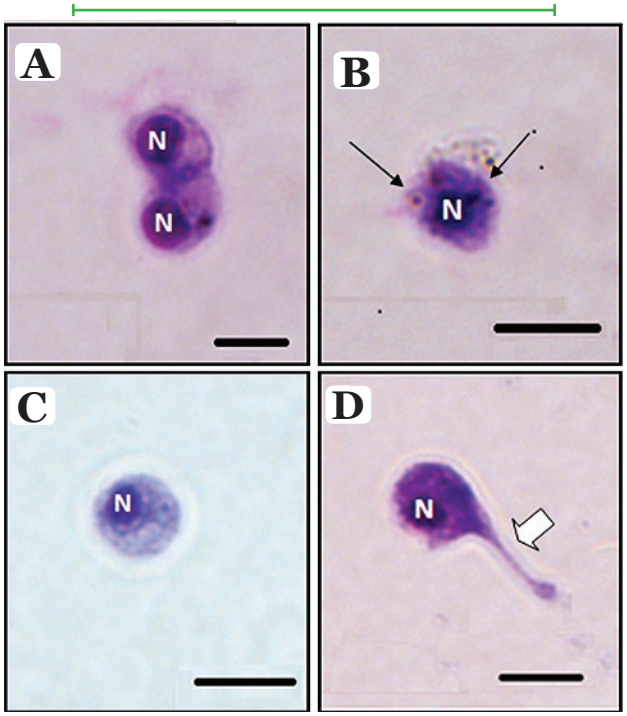

Figure 2. Hemocyte characterization of *Ectemnaspis trombetense* in optical microscopy. (A) Two prohemocytes in the process of cell division, both characterized by large nuclei occupying almost the entire cytoplasm. (B) Granulocyte with an irregular shape and several granules (thin arrows) in the cytoplasm. (C) Oenocytoid with a poorly developed nucleus located eccentrically. (D) Plasmatocyte with a long projection of the cytoplasmic membrane (large arrow). N = nucleus; Bars = 5 µm.

Granulocytes. Granulocytes are round or oval in shape. They range from 20–25µm in diameter, and may exhibit projections of the plasma membrane (Figures 1B and 2B). These cells were characterized by the presence of granules in the cytoplasm that differed in size and shape. In ultrastructure could be identified in the cytoplasm; the endoplasmic reticulum, Golgi complex, and some vesicles. Granulocytes possessed a large, generally eccentric nucleus exhibiting heterochromatin islands (Figures 3B and 4B). In *E. trombetense*, this cell type was one of the most abundant, comprising 38% of the total and only 20% of *E. rorotaense* cells.

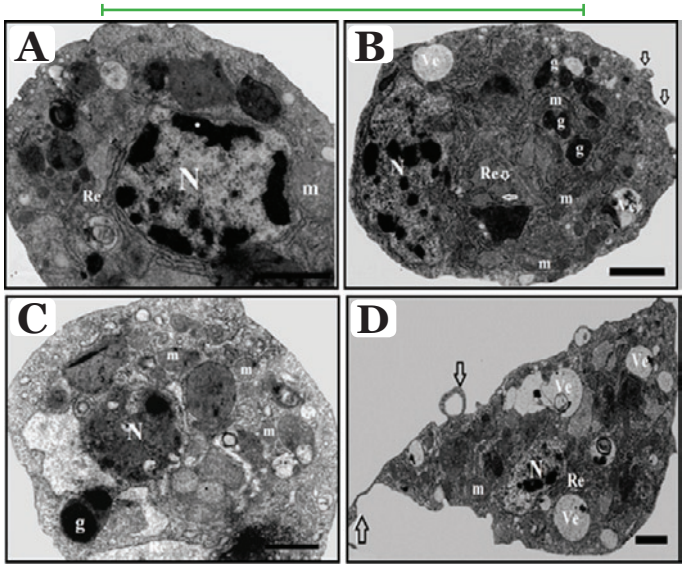

Figure 3. Hemocyte characterization of *Ectemnaspis rorotaense* in transmission electron microscopy. (A) Prohemocyte with a large nucleus (N). Cytoplasm with few organelles; only the mitochondria (m) and endoplasmic reticulum (Re) are in evidence. (B) Granulocyte with heterochromatin present in the nucleus (N), a cytoplasmic membrane with projections (large arrow), the endoplasmic reticulum (ER), granules (g) and vesicles (Ve). (C) Oenocytoid with a small nucleus (N), and mitochondria (m), vesicles (Ve) and the endoplasmic reticulum (ER) present in the cytoplasm. (D) Plasmatocyte exhibiting elongated shapes and projections of the cytoplasmic membrane (large arrow); mitochondria (m), vesicles (Ve) and the endoplasmic reticulum (ER) are present in the cytoplasm. Bars = 0,5 µm.

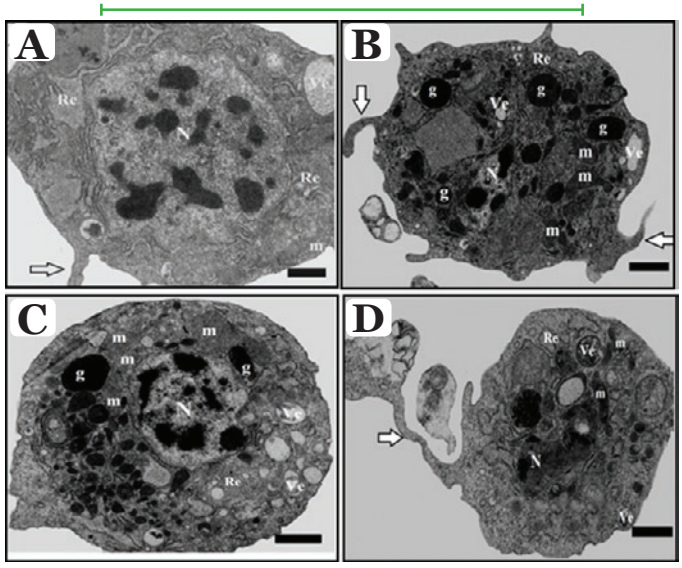

Figure 4. Hemocyte characterization of *Ectemnaspis trombetense* in transmission electron microscopy. (A) Prohemocyte with a large nucleus (N). Cytoplasmic membrane with small projections (large arrow) end with few organelles; only the mitochondria (m) and endoplasmic reticulum (Re) are in evidence. (B) Granulocyte with heterochromatin present in the nucleus (N), a cytoplasmic membrane with projections (large arrow), the endoplasmic reticulum (ER), granules (g) and vesicles (Ve). (C) Oenocytoid with a small nucleus (N), and mitochondria (m), granules (g) end vesicles (Ve) present in the cytoplasm. (D) Plasmatocyte exhibiting elongated shapes and projections of the cytoplasmic membrane (large arrow); mitochondria (m), vesicles (Ve) and the endoplasmic reticulum (Re) are present in the cytoplasm. Bars = 0,5 µm

Oenocytoids. Oenocytoids are round or oval in shape. They range from 10–15µm in diameter, and some cells exhibit projections of the plasma membrane. The nucleus is poorly developed and located in the central region (Figures 1C and

2C). Electron microscopy revealed a homogeneous cytoplasm exhibiting mitochondria, vesicles, ribosomes, and rough endoplasmic reticulum (Figures 3C and 4C). In both species, this cell type was the least abundant, comprising 11% of the total in *E. trombetense*, and 14% of the total in *E. rorotaense*.

Plasmatocytes. Plasmatocytes exhibit greater morphological variation, and range from 45–55µm in diameter. These cells were characterized by large projections of the cytoplasmic membrane (Figures 1D and 2D). Ultrastructural images allowed to be identified in the cytoplasm; the rough endoplasmic reticulum, Golgi complex, and vesicles (which were either empty or filled with amorphous substances). The nucleus of these cells is usually small and eccentric (Figures 3D and 4D). In *E. trombetense*, this cell type was the most abundant, comprising 38% of the total. Whereas in *E. rorotaense* was represented by 20% of the cells.

DISCUSSION

The larvae, pupae, and adults of *E. rorotaense* and *E. trombetense* possess four different types of circulating hemocytes called prohemocytes, granulocytes, oenocytoids, and plasmatocytes. In a study of black flies in the Nearctic region, Luckhart *et al.* (1992) used light microscopy and scanning to describe four types of hemocytes in the adult female *P. vittata* [=*S. vittatum*]. These types were classified as prohemocytes, plasmatocytes, granulocytes, and spherulocytes. In this study we identified oenocytoid, which is a cell type that was not characterized in *P. vittata*; notwithstanding we did not found spherulocyte, which was characterized in *P. vittata*. However, the types of hemocytes identified in this study have been described in other species of Diptera, including *Anastrepha obliqua* Macquart, *C. quinquefasciatus*, *A. gambiae* and *A. aegypti* (Silva *et al.* 2002; Brayner *et al.* 2005; Castillo *et al.* 2006; Araújo *et al.* 2008). According to Siddiqui & Al-Khalifa (2012), up to seven hemocyte cell types can be found in Diptera: prohemocytes, granulocytes, oenocytoids, plasmatocytes, spherulocytes, coagulocytes, and adipohemocytes.

Prohemocytes exhibit characteristics that make them easy to recognize, like their small size and high nuclear-cytoplasmic ratio. These characteristics have been described in studies examining other insects (Silva *et al.* 2002; Falleiros *et al.* 2003; Berger & Slavíčková 2008). Prohemocytes were found with high frequency in the hemolymph of adult *E. rorotaense*. Brayner *et al.* (2007) observed a significant increase in the population of prohemocytes in *C. quinquefasciatus* following infection by filaria *Wuchereria bancrofti* (Cobbold). Prohemocytes are precursor stem cells which can differentiate into other types of hemocytes (Lemaitre & Hoffman 2007). Some prohemocytes observed in this study were evidently in the process of cell division. The reason that prohemocytes were found in greater numbers in *E. rorotaense*, is probably because adult emergence occurs more quickly in this species and this leaves prohemocytes unchallenged by the antigens that initiate cell differentiation.

Granulocytes also exhibit characteristics that make them easy to recognize, such as the presence of granules and small projections of the plasma membrane. This cell type was one of the most abundant found in the hemolymph of *E. trombetense* adults. Several studies suggest that granulocytes are one of the main cells involved in the process of phagocytosis. Cupp *et al.* (1997) reported that in the experimental infection of *P. vittata* by *O. linealis*, granulocytes increased within 10 hours of infection, indicating an intense process of phagocytosis. Araújo *et al.* (2008) and Hillyer *et al.* (2003) observed that granulocytes were the main cells involved in phagocytosis in *A. aegypti* challenged with latex particles.

Oenocytoids were less abundant in both species. They were characterized by a poorly developed, eccentric nucleus, and by cytoplasm that is homogeneous and has few organelles. Similar descriptions have been made of oenocytoids found in Cuterebridae larvae (Lello *et al.* 1987), in adults of *C. quinquefasciatus* (Brayner *et al.* 2005) in adults of *A. aegypti* (Araújo *et al.* 2008), and in larvae of *Mythimna unipuncta* (Haworth) (Ribeiro *et al.* 1996). Oenocytoids are also known as crystal cells (Joshi & Lambdin 1996), and were characterized in the hemolymph of *Dactyolopius confusus* (Cockerell) by the presence of many crystals in the cytoplasm. Borges *et al.* (2008) related an increase of oenocytoids in the hemolymph of *Rhodnius prolixus* Stal nymphs inoculated with bacteria. This suggests that oenocytoids are the main cells involved in the defense of this insect.

Plasmatocytes exhibited greater morphological variation, being round or elongated in shape, and exhibiting long cytoplasmic projections. Similar descriptions have been made of plasmatocytes found in other insect species (Han & Gupta 1988; Joshi & Lambdin 1996; Silva *et al.* 2002). Brayner *et al.* (2005) described granular and agranular plasmatocytes found in the hemolymph of *C. quinquefasciatus*. In the present study, plasmatocytes and granulocytes were the most abundant hemocytes in *E. trombetense*. In the study of *P. vittata,* plasmatocytes were the most abundant, followed by granulocytes (Luckhart *et al.* 1992). Several studies have confirmed that plasmatocytes are the main cells involved in the process of phagocytosis (Mclaughlin & Allen 1965; Falleiros & Gregório 1995; Joshi & Lambdin 1996; Falleiros *et al.* 2003; Borges *et al.* 2008).

This study showed that prohemocytes, granulocytes, oenocytoids, and plasmatocytes are present in the hemolymph of *E. rorotaense* and *E. trombetense* during the larval, pupal, and adult life stages. This study also showed that cell type percentages vary between species. Nevertheless, this variation must be carefully studied because we did not know if the immune system of the immature specimens were challenged by any invader agent in their breeding sites before the collections. Future studies in order to understand the effect of invader organisms on the cellular immune system of these black fly species must be done.

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