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Histology and ultrastructure of the midgut of the parasitoid *Eibesfeldtphora tonhascai* (Diptera Phoridae)

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Abstract

Eibesfeldtphora tonhascai (Brown) (Diptera Phoridae) is a parasitoid of leaf-cutting ants *Atta sexdens* (Forel) and *Atta laevigata* (Smith) (Hymenoptera Formicidae), which cause considerable economic damage to agriculture and forests. The larvae of this natural enemy feed on their host, but little is known about adult dietary habits. Thus, studies on midgut morphology may provide relevant information about adult feeding. The midgut morphological and functional characteristics of *E. tonhascai* were investigated in this work. The digestive cells presented a well-developed nucleus rich in decondensed chromatin and a well-developed brush border on the apical surface. The basal portion of the digestive cells presented a broad and long cell basal labyrinth. The perinuclear cytoplasm was rich in rough endoplasmic reticulum that extends towards the subapical portion of the cell, free ribosomes and autophagosomes. The apical region of the cells showed long and numerous microvilli, with the underlying cytoplasm rich in mitochondria and rough endoplasmic reticulum. In the midgut lumen, an evident peritrophic matrix was found. The characteristics of the digestive cells of adult *E. tonhascai* midgut indicate protein production and liquid food absorption in this organ. Therefore, the results reported here contribute to understanding biological aspects still unknown of these natural enemies.

Key words: digestive tract, leaf-cutting ants, microvilli, morphology, natural enemy.

Introduction

The phorid fly *Eibesfeldtphora tonhascai* (Brown) (Diptera Phoridae) is a parasitoid of the leaf-cutting ants *Atta sexdens* (Forel) and *Atta laevigata* (Smith) (Hymenoptera Formicidae) (Bragança *et al*., 2002; Farder-Gomes *et al*., 2016), which are dominant in natural and human-disturbed environments, causing economic damage to agriculture and forests (Fowler *et al*., 1989; Della Lucia *et al*., 2014).

The presence of phorids on the trails changes ant behaviour and reduces foraging, worker recruitment and the amount of plant material transported (Guillade and Folgarait, 2015). Thus, these natural enemies are potential biological control agents of leaf-cutting ants (Farder-Gomes *et al*., 2016; 2018; 2020). However, rearing these parasitoids under laboratory conditions requires data on their feeding habit in their natural habitat (Bragança *et al*., 2009). We know that *E. tonhascai* larvae feed on the cephalic content of their host (Tonhasca, 1996; Farder-Gomes *et al*., 2016), but there is little information about adult dietary habits. Nectar, sap and/or honeydew are suggested as likely food sources (Disney, 1994). From this perspective, studies on the midgut morphology of these insects may provide relevant information and shed light on this important issue.

The midgut of insects performs ion transport, digestion and nutrient absorption, and it is also the gateway for several pathogens (Billingsley and Lehane, 1996; Santos *et al*., 2017). Digestive (columnar) cells with developed microvilli and basal labyrinth, which are specialized in nutrient digestion and absorption (de Sousa *et al*., 2009; Santos *et al*., 2017), are the most abundant cell type in the midgut epithelium.

The midgut lumen of insects is covered by the peritrophic matrix (PM), an acellular structure composed of chitin, proteins and proteoglycans, that separates the food content from the gut epithelium (Hegedus *et al*., 2019). This compartmentalization of the midgut lumen into an endoperitrophic space and an ectoperitrophic space prevents non-specific binding of undigested particles to the epithelium and allows the digestive enzyme recycling in the midgut (Bolognesi *et al*., 2008). In addition, the peritrophic matrix protects the epithelium against mechanical damage, pathogens and toxins (Erlandson *et al*., 2019; Liu *et al*., 2019).

Despite the potential of *E. tonhascai* to control leaf-cutting ants, there is no data on the morphology of its digestive tract. Therefore, in this study, we describe the histology and ultrastructure of the midgut of this parasitoid.

Materials and methods

Parasitoid collection

Adult females of *E. tonhascai* were collected from nests of the leaf-cutting ant *A. sexdens* around the campus of the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil.

Light microscopy

Ten *E. tonhascai* females were immobilized by cold and dissected in saline solution for insects (0.1 M NaCl, 20 mM KH_2PO_4 and 20 mM Na_2HPO_4). Then, their whole midguts were transferred to Zamboni's fixative solution (Stefanini *et al*., 1967) for 24 hours. The samples were dehydrated in a graded ethanol series (70, 80, 90,

and 99%) and embedded in Historesin (Leica Microsystems, Buffalo Grove, IL, USA). Next, 3-µm-thick sections were obtained using a Leica RM2255 microtome. The sections were stained with different techniques: hematoxylin and eosin staining; for histochemistry, the periodic acid Schiff (PAS) reaction was carried out for the detection of neutral glycoproteins, carbohydrates and glycoconjugates (Layton and Bancrof, 2019); and bromophenol blue, for total protein detection (Wei, 1996). Subsequently, the slides were mounted under a glass coverslip in Entellan (Merck) and analysed with an Olympus BX-60 light microscope (Olympus Corporation, Tokyo, Japan).

WGA staining

The sections from five midguts were washed three times in 0.1 M PBS and incubated for 1 hour with FITC-conjugated Wheat Germ Agglutinin (WGA-FITC, Sigma Aldrich, # L4895, Israel) diluted in 0.1 M PBS, to detect the presence of polysaccharides containing β-1-4 *N*-acetyl-glucosamine residues in the peritrophic matrix. The sections were washed three times, mounted with a glass coverslip in 50% sucrose solution and analysed under a fluorescence microscope - Olympus BX-60 light microscope (Olympus Corporation, Tokyo, Japan).

Transmission electron microscopy

Five midguts were dissected in 0.1 M sodium cacodylate buffer (0.2 M sucrose; pH 7.2) and transferred to 2.5% glutaraldehyde, in the same buffer, for 24 hours. The samples were post-fixed for 2 hours, at 1% osmium tetroxide and dehydrated in a graded ethanol series (50-99%). Following dehydration, the samples were embedded in LR White resin (Sigma-Aldrich Co., St. Louis, MO, USA) and ultrathin sections (60 nm) were obtained using an RMC Power Tome-X ultramicrotome. Next, the ultrathin sections were stained with 1% aqueous uranyl acetate and 0.2% lead citrate and examined with a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Jena, Germany).

Results

The midgut of *E. tonhascai* had similar histology and ultrastructure along the whole length with a single-layered epithelium of digestive (columnar) cells, which have a well-developed nucleus rich in decondensed chromatin and dense brush border on the apical surface (figures 1A and 1B). The lumen was lined by a peritrophic matrix evidenced by PAS reaction and WGA staining. (figures 1B and 2).

The histochemical test showed a strong reaction to PAS in the brush border and cytoplasmic granules in the digestive cells, whereas the basal lamina presented a weak reaction (figure 1B). Furthermore, the bromophenol blue test for total protein showed a positive reaction throughout the cytoplasm of digestive cells (figure 1C).

The transmission electron microscopy analyses revealed that the apical surface of the digestive cells had long and numerous microvilli and the cytoplasm was rich in mitochondria and rough endoplasmic reticulum (figure 3A).

The median region of the digestive cells presented a well-developed nucleus with decondensed chromatin and evident nucleolus (figure 3B). The cytoplasm of the perinuclear region was rich in rough endoplasmic reticulum that extends towards the apical portion of the cell and autophagosomes (figure 3B).

The basal portion of the cells had enlarged and long plasma membrane infoldings, forming a well-developed basal labyrinth associated with mitochondria and with few openings to the hemocoel (figure 3C). Digestive cells are enclosed externally by muscle layers (figure 3C).

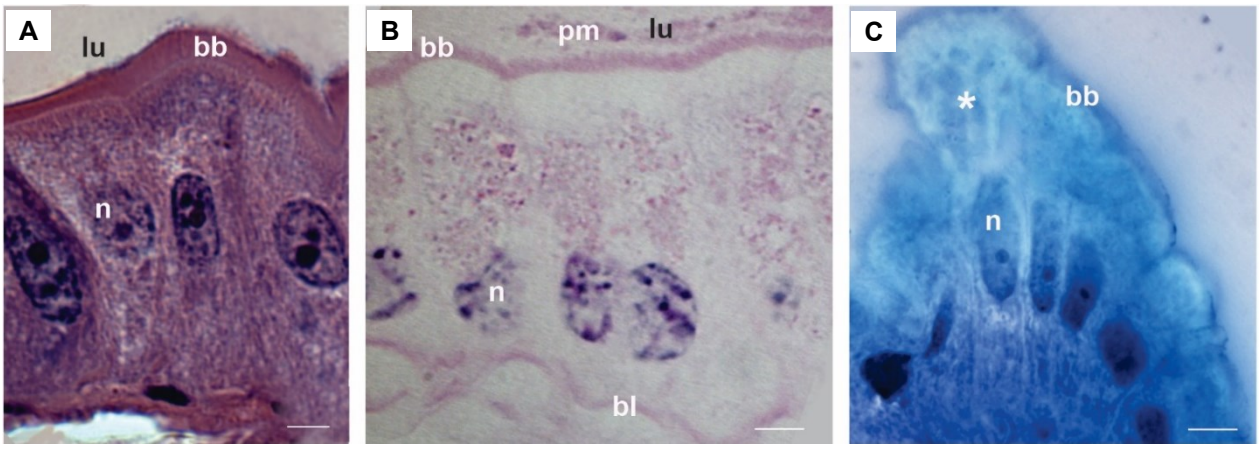

Figure 1. Histological sections of the midgut of adult *E. tonhascai* stained with hematoxylin and eosin **(A)**, periodic acid Schiff (PAS) **(B)** or bromophenol blue **(C)**. **A.** Simple layer of columnar cells showing oval nucleus (n) with decondensed chromatin and well-developed brush border (bb); lumen (lu); muscle (m). Scale bar: 2 µm. **B.** PAS-positive brush border (bb), peritrophic matrix (pm) and basal lamina (bl); nucleus (n); lumen (lu). Scale bar: 3 µm. **C.** Epithelium showing cytoplasm of the cell rich in proteins (asterisk); brush border (bb); nucleus (n). Scale bar: 3 µm. Slides were analysed with an Olympus BX-60 light microscope using magnifications of 400× and 1000×.

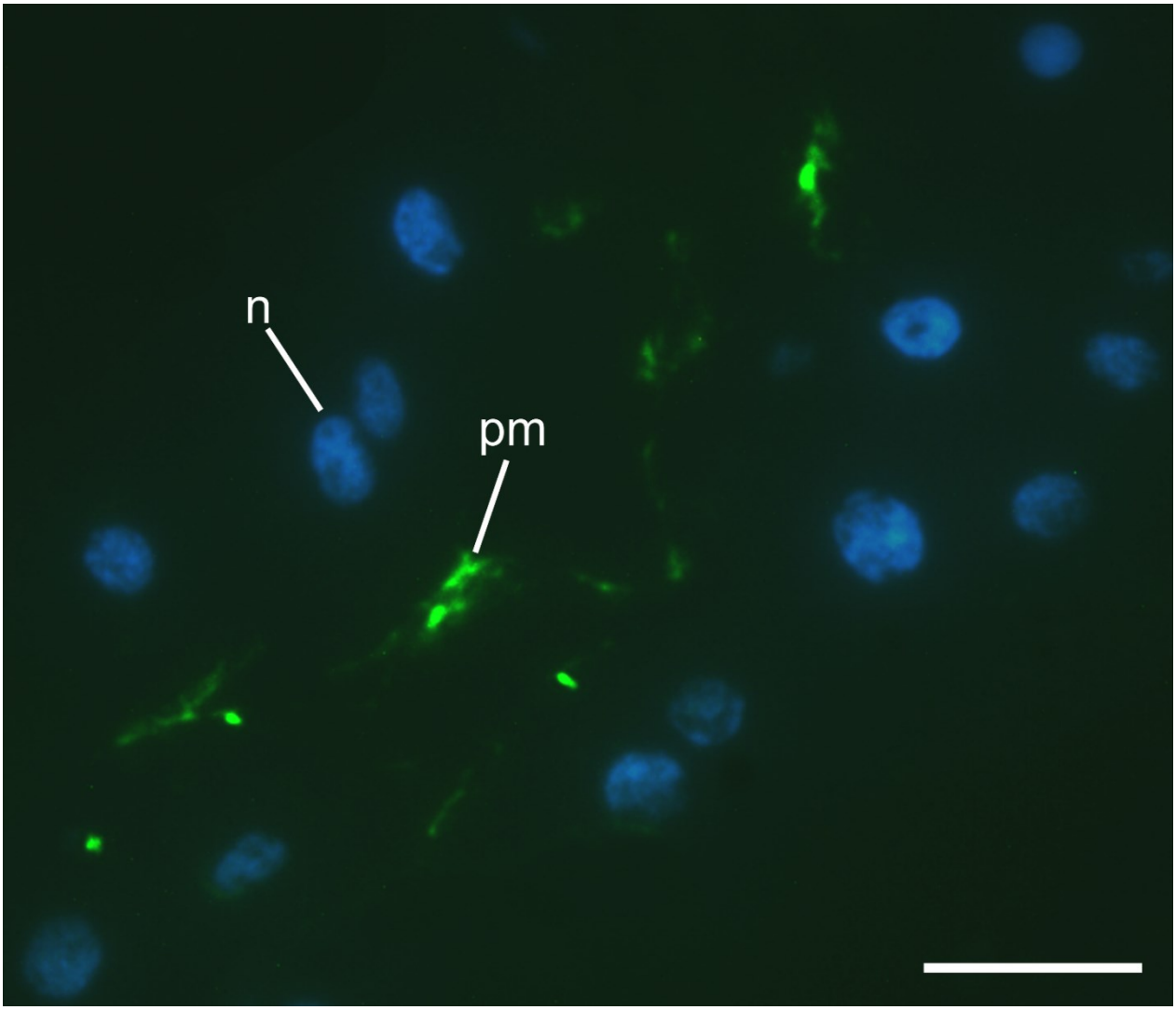

Figure 2. Histological sections of the midgut of adult *E. tonhascai* labeled with WGA. Epithelium covered by the WGA-labeled peritrophic matrix (pm). Cell nuclei (n) were stained with DAPI (blue). Scale bar: 20 µm. Slides were analysed under a fluorescence microscope - Olympus BX-60 light microscope using magnification of 400×.

Discussion

This is the first description of the midgut morphology of a phorid parasitoid. The midgut of *E. tonhascai* is composed of a single-layered epithelium of columnar digestive cells with a well-developed brush border surrounded by muscles, similar to other dipterans (Hecker, 1977; Andrade-Coêlho *et al*., 2001; Okuda *et al*., 2002; Godoy *et al*., 2015).

The PAS-positive reaction in the brush border and cytoplasmic granules of digestive cells indicate the presence of glycoconjugates released along the entire midgut, which may be digestive enzymes and components of the PM, similar to that reported for the hymenopteran parasitoid *Campoletis flavicincta* (Ashmead) (Hymenoptera Ichneumonidae) (Gonçalves *et al*., 2013).

The positive reaction of the bromophenol blue test and richness in rough endoplasmic reticulum in the digestive cells indicate intense protein synthesis by the epithelium. Similar characteristics have been reported in the digestive cell of the sugary-diet feeding mosquito *Toxorhynchites theobaldi* (Dyar et Knab) (Diptera Culicidae), suggesting that sugar-rich meals might stimulate protein secretion (Godoy *et al*., 2015).

The WGA staining and PAS reaction corroborated the presence of the peritrophic matrix lining the midgut surface of *E. tonhascai*, suggesting that the peritrophic matrix produced by this species is of type I, which is synthesized throughout the midgut epithelium (Hegedus *et al*., 2019). The peritrophic matrix of *E. tonhascai* allows the spatial separation of the digestive process, which increases the efficiency of digestion (Bolognesi *et al*., 2008), in addition to protect the epithelial cells, especially against microorganisms present in sugar- rich diets, such as nectar and honeydew (Olaitan *et al*., 2007; Leroy *et al*., 2011).

The well-developed basal labyrinth associated with mitochondria in the digestive cell suggests active transport of ions and water in the basal region, such as reported for fluid-feeding insects (Gonçalves *et al*., 2014; Urbanek

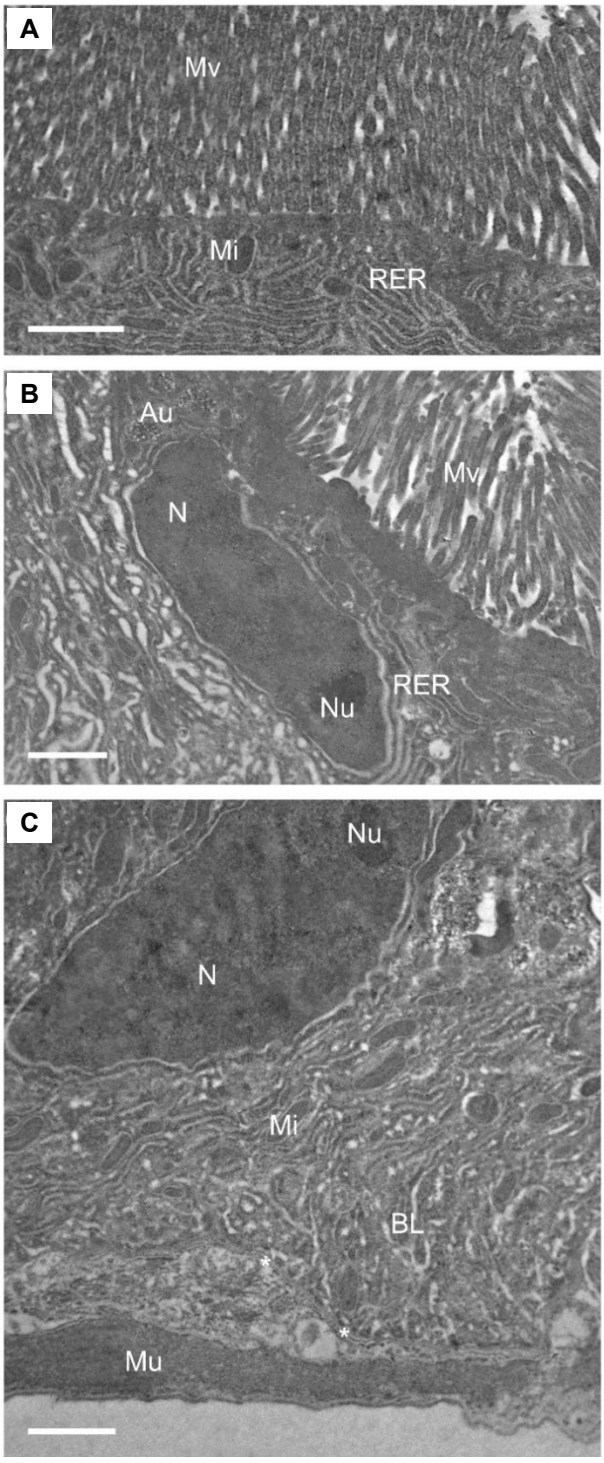

Figure 3. Transmission electron micrographs of the midgut digestive cell of *E. tonhascai* adults. **A.** Apical portion of the digestive cell with long microvilli (Mv), rich in mitochondria (Mi) and rough endoplasmic reticulum lamellae (RER). Scale bar: 1 µm. **B.** Perinuclear portion showing the abundant rough endoplasmic reticulum lamellae (RER) and nucleus (N) with prominent nucleolus (Nu); autophagosomes (Au); and microvilli (Mv). Scale bar: 1 µm. **C.** Basal portion of digestive cells with the basal lamina (asterisk); a well-developed basal labyrinth (Bl) in association with mitochondria (Mi); nucleus (N); nucleolus (Nu); muscle (Mu). Scale bar: 1 µm. Sections were examined with a Zeiss EM 109 transmission electron microscope.

and Rost-Roszkowska, 2015; Santos *et al*., 2017). This adaptation may be useful for the rapid water absorption from diluted foods to avoid the dilution of digestive enzymes along the midgut (Serrão *et al*., 2008; Gonçalves *et al*., 2014). In addition, when the basal labyrinth has few openings to the hemocoel, as observed in the *E. tonhascai*, it constitutes an extracellular compartment with restricted access to the circulating hemolymph, which results in an osmotic gradient between the cell cytoplasm and the midgut lumen, thus promoting water absorption from the lumen (Ribeiro *et al*., 1990; Biagio *et al*., 2009; Fialho *et al*., 2013; Santos *et al*., 2017).

The autophagosomes found in the digestive cells are related to the metabolism of proteins, lipids and polysaccharides during digestion, as well as the degradation and turnover of cellular components (Xu and Ren, 2015; Li *et al*., 2018), which corroborates the intense metabolic activity of these cells in *E. tonhascai*.

The digestive cells of *E. tonhascai* have a well-developed nucleus with decondensed chromatin and nucleolus, which suggests high metabolic activity, mainly for protein synthesis (Billingsley and Lehane, 1996). Highly metabolic cells have large nuclei, which facilitates the DNA transcription rate (Roma *et al*., 2003; Webster *et al*., 2009).

The apical cytoplasm of digestive cells is rich in mitochondria, reinforcing the function of these cells in the active transport of substances (Gonçalves *et al*., 2014). Moreover, the long and numerous microvilli in the digestive cells of *E. tonhascai* increase the cell surface for secretion and absorption of substances (Fialho *et al*., 2009; Godoy *et al*., 2015).

Adult feeding habits of leaf-cutting ant parasitoids are unknown. However, the features of the *E. tonhascai* midgut epithelium reported here, such as PAS and bromophenol blue positive reaction, well-developed brush border, a nucleus with decondensed chromatin and abundant rough endoplasmic reticulum, suggest high secretory activity of proteins and nutrient absorption. In addition, digestive cells have a basal labyrinth with few openings for hemocoel, which is characteristic of cells that absorb water from the midgut lumen to the hemolymph (Biagio *et al*., 2009; Fialho *et al*., 2013; Santos *et al*., 2017), suggesting that *E. tonhascai* diet may be a diluted-liquid food, such as nectar and honeydew. It has been demonstrated that hymenopteran parasitoids (Benelli *et al*., 2017; Lahiri *et al*., 2017; Picciau *et al*., 2019) and phorid flies *Pseudacteon* spp. (Chen and Fadamiro, 2006; Ajayi and Fadamiro, 2016) use sugar sources to increase their longevity. Considering that *E. tonhascai* can mature eggs during their lifetime (Farder-Gomes *et al*., 2019), they are likely to increase their fecundity and longevity by feeding on sugar-rich diets.

Our results contribute to understanding the feeding habit of adult phorid parasitoids, revealing that the midgut digestive cells of this insect are highly active in the production of glycoproteins, peritrophic matrix components, food digestion and absorption. The success of biological control programs depends on maintaining laboratory populations to improve rearing techniques and protocols (Parra and Coelho, 2022). For this, studies on the basic biology of parasitoids, such as the analysis of the

midgut morphology, are essential to elucidate life history traits still unknown. Therefore, this study represents a first step in developing future mass-rearing approaches of this natural enemy for the biological control of leaf-cutting ants.

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