MIDGUT ULTRASTRUCTURE OF FOURTH INSTAR *OCHLEROTATUS TOGOI* (DIPTERA: CULICIDAE)

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Abstract. The ultrastructure of the midgut of fourth instar *Ochlerotatus togoi* was investigated by light, scanning and transmission electron microscopy. This study was performed to provide information to help devise future control efforts aimed at the larval stages of this vector of filariasis. The fourth instar midgut was approximately 2 mm in length and consisted of three morphologically distinct cell types: epithelial, regenerative, and endocrine cells. There was a monolayer of epithelial cells on the luminal surface of the midgut, with multiple folds of the plasma membrane where it adjoined the basement membrane. Regenerative cells were scattered throughout the basal portion of the epithelium, along with endocrine cells. No evidence of division or differentiation was seen in any of the cell types. Six layers of the peritrophic matrix were observed in the gut lumen which separated ingested food from the midgut epithelial cells. Cytoplasmic protrusions were seen in many areas of the luminal midgut surface and numerous autophagosomes were seen in the epithelial cells of both early and late fourth instar larvae, suggesting autophagy is involved in the degeneration process of the midgut in preparation for pupation. This study provides a basis for understanding normal *Oc. togoi* larval midgut development. Further studies are needed to determine the factors that control larval growth and the nutritional state. Such information could be used to reduce adult fecundity and develop biological control mechanisms.

Keywords: *Ochlerotatus togoi*, fourth instar, larva, midgut, mosquito, ultrastructure

INTRODUCTION

The midgut is the largest organ in the mosquito larval body, responsible for key physiological functions, such as ion transport and biomolecule absorption, and is also an entry site for several pathogens (Lord and Fukuda, 1988; Seif, 2000; Apte-Deshpande *et al*, 2012). Factors that influence the growth and nutritional status of mosquito larvae also affect the reproductive potential of adult mosquitoes (Soliman *et al*, 1995; Zhou *et al*, 2004). Small, poorly fed mosquito larvae develop into adults with reduced reproductive potential (Telang and Wells, 2004; Telang *et al*, 2006, 2007). Interfering with the normal development of the larval midgut might

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reduce its ability to absorb and store nutrients and reduce adult fecundity.

One method of controlling diseases transmitted by mosquitoes is larvicides. Larvicides used include temephos (Cetin *et al*, 2006; Tawatsin *et al*, 2007; Soltani *et al*, 2013), methoprene (McCarry, 1996), oils (Champakaew *et al*, 2007; Zhu and Tian, 2011; Ivoke *et al*, 2013), and monomolecular films (Wang *et al*, 2013). Biological insecticides used include *Bacillus thurigiensis* ssp *israelensis* (Bti) and *Lysinibacillus sphaericus (L. sphaericus*; formerly known as *Bacillus sphaericus,* Bs) (Berry, 2012; Guidi *et al*, 2013; Kroeger *et al*, 2013).

Bti is a naturally occurring soil bacterium found throughout the world. The Bti larvicide is composed of a dormant spore form of the bacterium (Poopathi and Archana, 2012). When mosquito larvae eat Bti toxin from the spores is released and disrupts the gut of the mosquito by binding to receptor cells present in insects, but absent from mammals (Lacey, 2007; Ben-Dov, 2014).

L. sphaericus is also a naturally occurring bacterium with a similar mode of action as Bti (Baumann *et al*, 1991; Lacey, 2007). The target spectrum of *L. sphaericus* is more limited than Bti (Berry, 2012). *Culex* species are highly sensitive to *L. sphaericus*, but among the genera *Aedes* and *Anopheles,* some species are sensitive and others are resistant to *L. sphaericus* (Davidson *et al*, 1984). Although the larval midgut is a target for controlling mosquitoes, relatively little is known about the anatomy and physiology of a normal mosquito larval midgut.

Ochlerotatus togoi is a vector of filariasis in Asia such as China, Japan and Taiwan (Ramachandran *et al*, 1963; Cheun *et al*, 2011). This mosquito species breeds year round, overwintering as fourth stage larvae or eggs, feeds on birds and mammals, and is a common biting nuisance (Kwon *et al*, 2011). In Thailand, *Oc. togoi* (Chanthaburi strain) can carry rural nocturnally subperiodic *Wuchereria bancrofti* (Tak and Kanchanaburi strains), nocturnally subperiodic *Brugia malayi* (Narathiwat strain), *B. pahangi* (Malaysia strain) and *Dirofilaria immitis* (Chiang Mai strain) (Choochote *et al*, 1983, 1987). Lymphatic filariasis is a tropical disease targeted by the World Health Organization for elimination by 2020; this has spurred research into vaccines, drugs and new methods of vector control (WHO, 2012).

In this study, the ultrastructure of the midgut epithelium during early and late fourth instar *Oc. togoi* was examined by light, scanning and transmission electron microscopy to provide a better understanding of normal mosquito larval midgut anatomy.

MATERIALS AND METHODS

Mosquitoes

Oc. togoi mosquitoes (Koh Nom Sao, Chantaburi Province, southeastern Thailand) were used in this study. This mosquito strain has been maintained in the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand, since 1983. It is susceptible to nocturnally subperiodic *B. malayi* (Choochote *et al*, 1987). The studied mosquitoes were reared as described by Choochote *et al* (1987). Early fourth instar larvae (8-12 hours after moulting) and late fourth instar larvae (92-96 hours after moulting) were processed for light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Ten samples were prepared from each time point for each microscopy technique.

Preparation of samples for light microscopy

The midguts of the larvae were dissected in phosphate buffer solution (PBS) (pH 7.4) and allowed to settle onto slides without drying. Photographs of the glands were taken using a digital camera (Cannon, Tokyo, Japan) attached to a light microscope.

Preparation of samples for scanning electron microscopy

Dissected larval midguts were fixed with 2.5% glutaraldehyde solution prepared in PBS and kept at 4°C for 24 hours. The fixed samples were then rinsed twice in PBS ten minutes apart and post-fixed in a solution of 1% osmium tetroxide for 2 hours. They were then dehydrated in a series of acetone solutions. Finally, the specimens were critical point dried, attached with double-sided tape to aluminum stubs, and coated with gold in a sputter-coating apparatus before being viewed with a JEOL JSM-5910 scanning electron microscope (JEOL, Tokyo, Japan). To observe the interface between the larval midgut surface and the blood meal, some fixed samples were fractured before coating with gold, while others were gently opened and the contents washed out with PBS before fixation.

Preparation of samples for transmission electron microscopy

Dissected midguts were fixed overnight in 2.5% glutaraldehyde prepared in PBS at 4°C. They were then rinsed twice with PBS ten minutes apart and post-fixed in a solution of 1% osmium tetroxide for 2 hours. Post-fixation was followed by rinsing twice with PBS and dehydrating with alcohol. The specimens were dehydrated by washing with increasing concentrations of alcohol (30%, 50%, 70%, 80%, 90%, and 95% v/v). The specimens were then placed in absolute alcohol for two 12 hour periods to ensure complete dehydration, and then placed in acetone for 2 hours before being placed in resin/acetone solutions with increasing concentrations of Spurr's resin (1:3 for 24 hours, 1:1 for 24 hours, and 3:1 for 24 hours). Finally they were placed in pure resin twice for 3 hours each time to ensure removal of any trace acetone. Each sample was then embedded by placing it in a plastic block in resin and incubating at 70°C for 24 hours. Sections (0.5 μm) of each sample were made with a glass knife using an Ultramicrotome (Boeckeler®, Tucson, AZ). The sections were then stained with 1% methylene blue and 1% Azure II (1:1) and viewed under a light microscope (Olympus®, Tokyo, Japan). Ninety nanometer sections were prepared, stained with uranyl acetate and lead citrate and observed using a ZEISS EM 10 transmission electron microscope (Oberkochen, Germany).

RESULTS

Ultrastructure of midgut epithelium in fourth instar *Ochlerotatus togoi*

The larval midgut was approximately 2 mm in length with a monolayer of epithelial cells exposed on the luminal surface (Fig 1). The plasma membrane had multiple folds where it adjoined the basement membrane. The midgut consisted of at least three morphologically distinct cell types: epithelial, regenerative, and endocrine cells (Fig 2, 3).

Epithelial cells were the major cellular component of the midgut epithelium. These cells had morphological characteristics of absorptive cells. TEM analysis revealed the cytoplasm of the perinuclear region was rich in cisternae of rough (RER) and smooth (SER) endoplasmic reticulum and Golgi complex. In the apical region of the cytoplasm there was an abundance

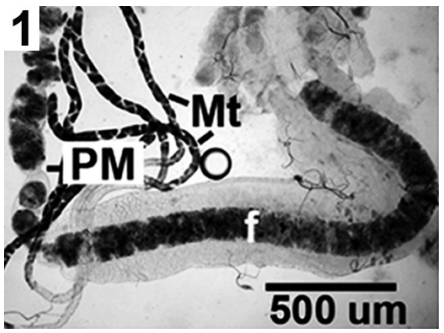

Fig 1–A representative midgut of early fourth instar before defecation showing larval food (f) covered by a peritrophic matrix (PM) in the midgut lumen and Malpighian tubules (Mt).

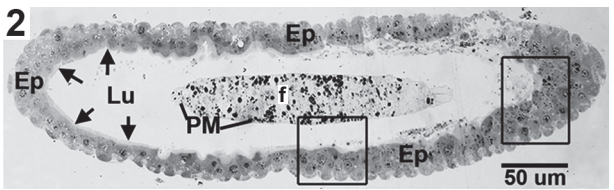

Fig 2–Transverse section through the middle region of a representative midgut of early fourth instar showing midgut epithelium composed of columnar epithelial cells (Ep), larval food (f), midgut lumen (Lu), and peritrophic matrix (PM). Arrows indicate microvilli.

of mitochondria, cisternae of RER and SER, free ribosomes, and lamellar bodies. The apical membrane was formed by numerous dense microvilli (Fig 3, 4). The basal regions of epithelial cells were rich in mitochondria (Fig 5, 6). Within the epithelial cells the nuclei were positioned closer to the basement membrane than to the apical surface, situated about one third of the total cell length from the basement membrane. Septate junctions were seen between midgut epithelial cells (Fig 5, 7). SEM analysis showed two morphological features of the luminal surface of epithelial cells. Some cells were covered by a thin membrane that obscured the microvilli

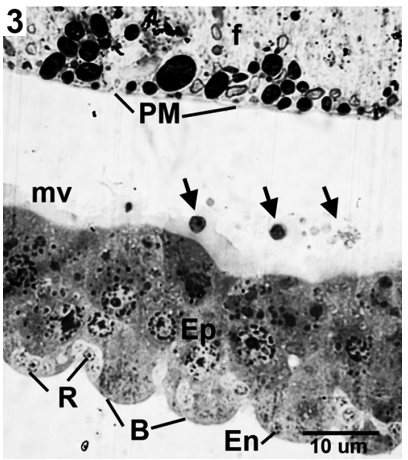

Fig 3–Higher magnification of square boxed region in (Fig 2) displaying basement membrane (B), endocrine cells (En), epithelial cells (Ep), larval food (f), microvilli (mv), peritrophic matrix (PM), and groups of regenerative cells (R). Arrows indicate degenerated organelles and nuclei discharged into the midgut lumen.

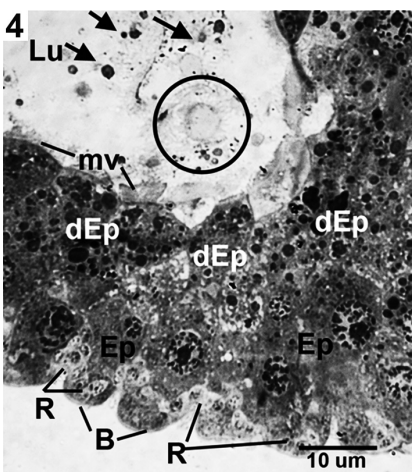

Fig 4–Higher magnification of rectangular boxed region in (Fig 2) displaying basement membrane (B), degenerated epithelial cells (dEp), epithelial cells (Ep), microvilli (mv), and groups of regenerative cells (R). Arrows indicate degenerated organelles and nuclei discharged into the midgut lumen (Lu). Circle indicates a transverse section of an apical membrane of a degenerated epithelial cell protruding into the midgut lumen.

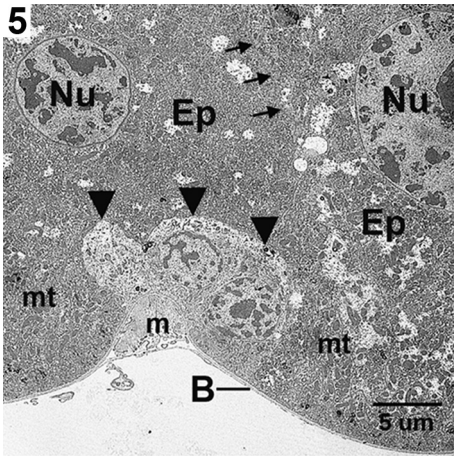

Fig 5–TEM micrograph of a region of the midgut epithelium of early fourth instar showing epithelial cells (Ep), a group of regenerative cells (arrowheads), septate junctions (arrows), basement membrane (B), muscle (m), mitochondria (mt), and nuclei of epithelial cells (Nu).

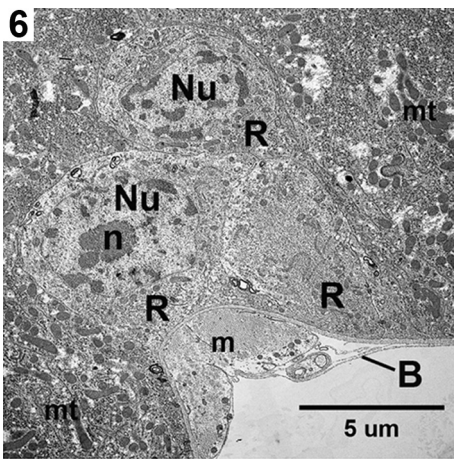

Fig 6–TEM micrograph of a region of the midgut epithelium of early fourth instar displaying a group of regenerative cells (R), basement membrane (B), muscle (m), mitochondria (mt), nucleus of regenerative cell (n), and nucleus of epithelial cell (Nu).

underneath (Fig 8, 9), whereas others had their microvilli exposed (Fig 10). The thin membrane appears to be shed as the cells mature, as shown in Fig 11, which shows

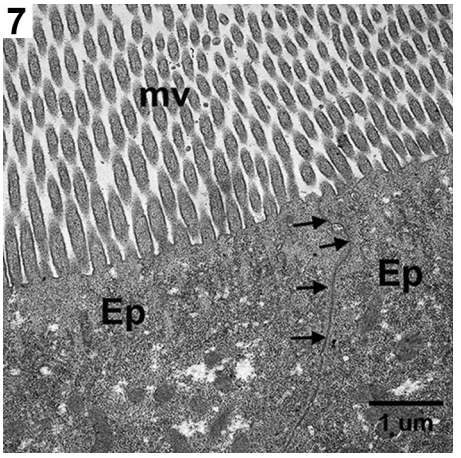

Fig 7–TEM micrograph of an apical part of a midgut epithelial cell of early fourth instar (Ep), microvilli (mv), and septate junctions (arrows).

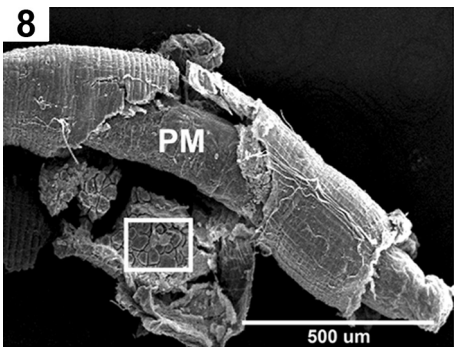

Fig 8–SEM micrograph of a posterior part of a larval midgut peritrophic matrix (PM) and a group of epithelial cells (rectangle).

examples of the loss of membranes from cellular apical surfaces. Fully formed epithelial cells with long microvilli were seen in only a few regions of the midgut in early fourth instar larval midguts (Fig 10). In late fourth instar larvae, a mixture of fully formed epithelial cells and epithelial cells with cytoplasmic protrusions were observed (Fig 12).

Regenerative cells are similar to stem cells, responsible for maintaining the

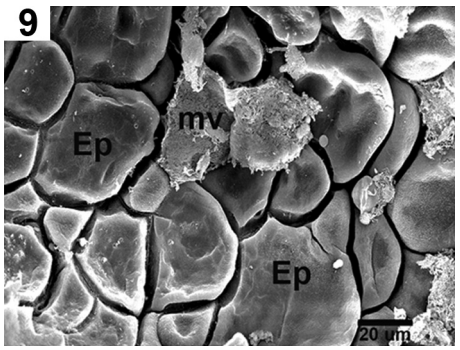

Fig 9–Higher magnification of boxed region in (Fig 8) displaying a group of epithelial cells (Ep) with microvilli (mv) covered by a thin membrane.

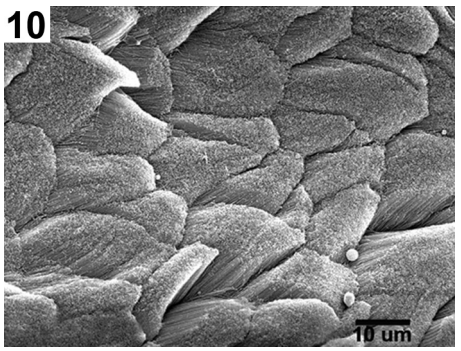

Fig 10–SEM micrograph of a region in the midgut of early fourth instar with fully formed epithelial cells.

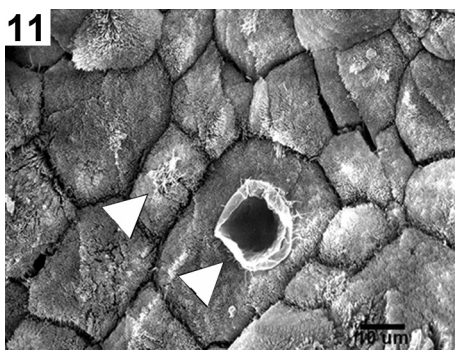

Fig 11–SEM micrograph of a group of midgut epithelial cells (Ep) of early fourth instar where the thin membrane is being gradually shed from cellular apexes (arrowheads).

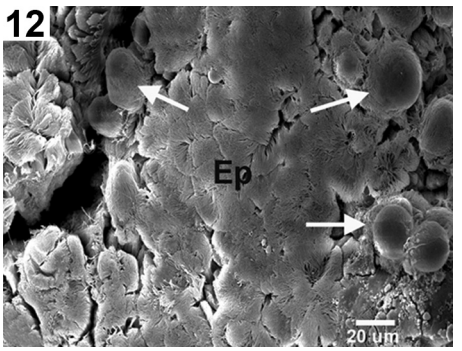

Fig 12–SEM micrograph of a region in the midgut of late fourth instar showing fully formed epithelial cells (Ep) and epithelial cells with cytoplasmic protrusions (arrows).

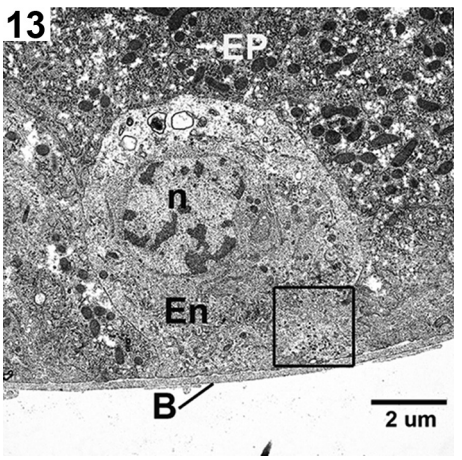

Fig 13–TEM micrograph of an endocrine cell with many secretory granules in the basal region (square).

homeostatic balance of a healthy midgut. They were scattered among the epithelial cells, throughout the basal portion of the epithelium, never reaching the lumen (Fig 2, 3). Approximately 70 to 80 groups of regenerative cells were found in a complete transverse section through the midgut (Fig 2), each group being composed of three to four cells (Fig 3, 4). As expected for a stem cell population, regenerative cells contained relatively few organelles

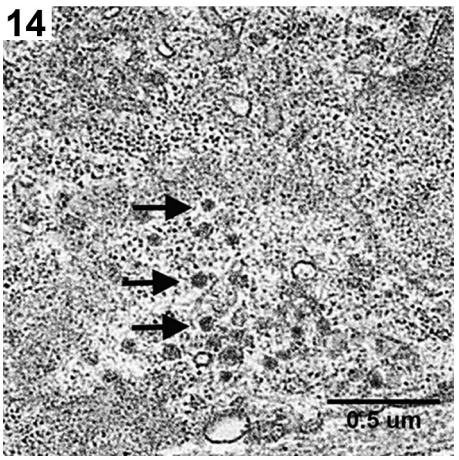

Fig 14–Higher magnification of square boxed region in (Fig 13) displaying the secretory granules (arrows).

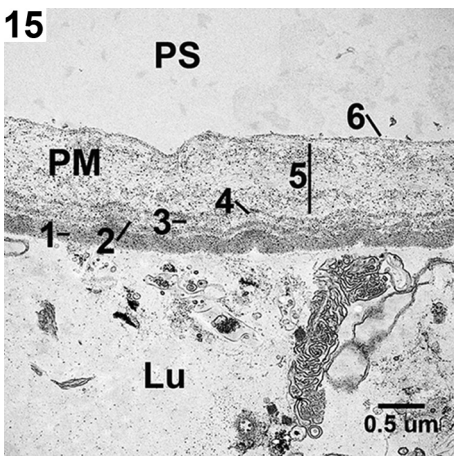

Fig 15–TEM micrograph of a peritrophic matrix (PM) consisting of at least six layers, peritrophic space (PS), and ingested food and food debris in the lumen of a larval midgut (Lu).

compared to the differentiated epithelial and endocrine cells, with occasional mitochondria and cisternae of RER. The regenerative cell nucleus was oval with a centrally located nucleolus (Fig 5, 6).

Endocrine cells were identified by the presence of numerous electron-dense secretory granules (Fig 13, 14). They

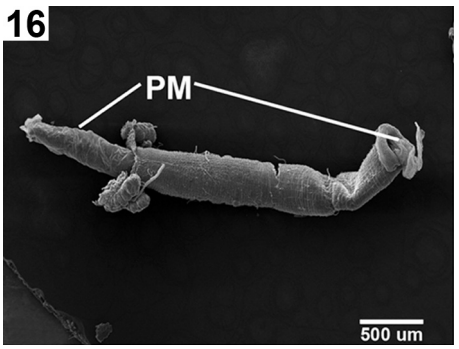

Fig 16–SEM micrograph of a representative midgut of an early fourth instar with a peritrophic matrix (PM).

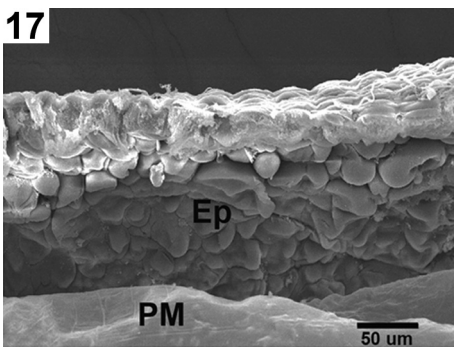

Fig 17–SEM micrograph of a middle part of a midgut of an early fourth instar showing epithelial cells (Ep) and a peritrophic matrix (PM) separated from the midgut epithelium.

were cone-shaped and located basally in the midgut epithelium as single cells. Approximately, 30 to 40 endocrine cells were distributed in a complete transverse section through the midgut (Fig 2). Midgut endocrine cells were smaller than epithelial cells. These cells displayed weakly staining cytoplasm and nuclei, contrasting with the more electron dense epithelial cells. No visible folding of the basal membranes of the endocrine cells was observed. Numerous round secretory granules were observed along the lateral and basal plasma membrane endocrine

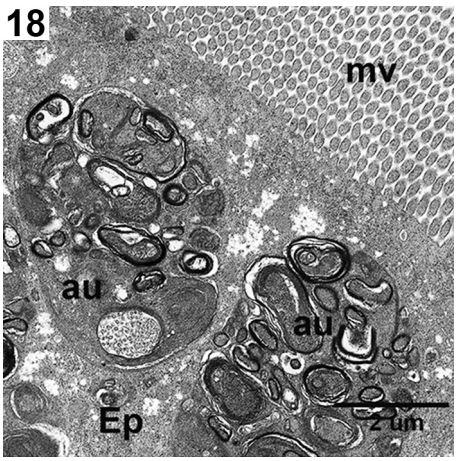

Fig 18–TEM micrograph of a midgut epithelium of late fourth instar showing epithelial cells (Ep) with apical cytoplasm rich in autophagosomes (au) and microvilli (mv).

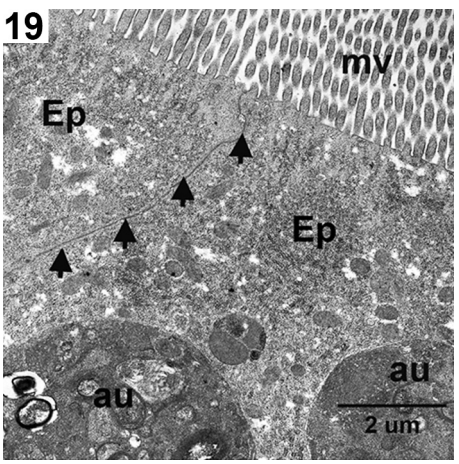

Fig 19–TEM micrograph of a midgut epithelium of late fourth instar showing epithelial cells (Ep) with apical cytoplasm rich in autophagosomes (au) and microvilli (mv). Arrows indicate septate junctions.

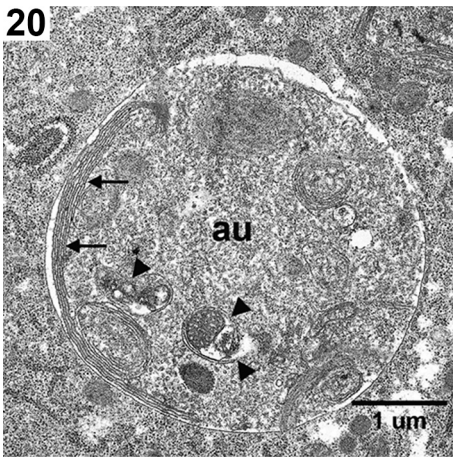

Fig 20–TEM micrograph of a midgut epithelium of late fourth instar showing an autophagosome (au) with degenerated organelles and lamellae of rough endoplasmic reticulum (arrows). Two small autophagosomes (arrowheads) are forming inside the large autophagosome (au).

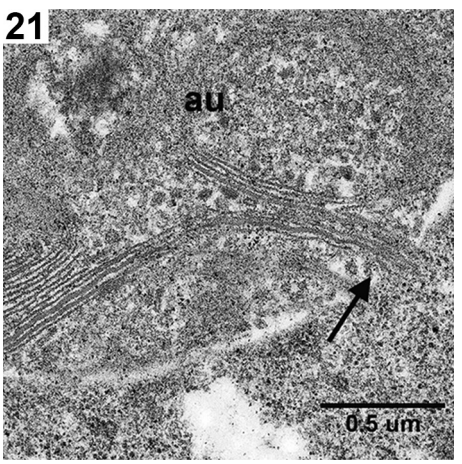

Fig 21–Higher magnification of an autophagosome (au) remaining in contact with the cytoplasm (arrow) via lamellae.

cells, ranging in size from 60 to 120 nm (Fig 13, 14).

The larval PM was Type II and formed a hollow posteriorly moving cylinder that forms from material secreted by a discrete ring of cells located in the larval cardia. The cardia is a distinctive organ in Diptera that surrounds the posterior end of the foregut and anterior end of the midgut. At least six distinct layers of peritrophic matrix (PM) were observed (Fig 15) in the gut lumen separating ingested food from

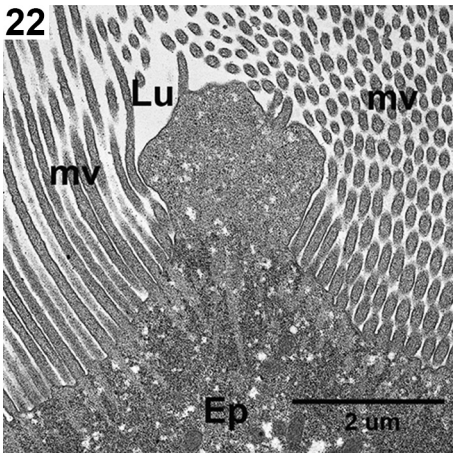

Fig 22–TEM micrograph of a midgut epithelium of late fourth instar showing protrusion of an apical membrane of a degenerated epithelial cell (Ep) into the midgut lumen (Lu) with microvilli (mv).

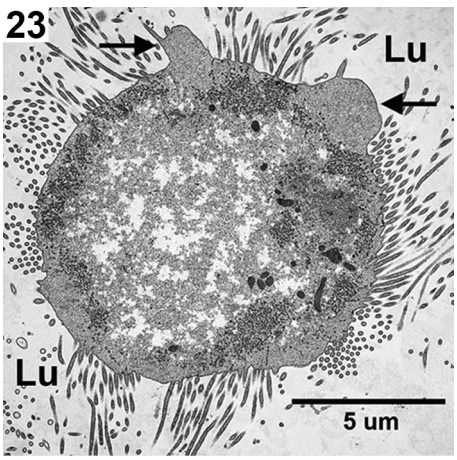

Fig 23–TEM micrograph of a midgut epithelium of late fourth instar showing a transverse section of the apical membrane of a degenerated epithelial cell protruding into the midgut lumen (Lu), showing the accumulation of degenerated organelles inside the cells and two regions of the apical membrane evaginated into the lumen (arrows).

the midgut epithelial cells (Fig 16, 17). The first layer on the luminal side was composed of electron-dense granules and was in close contact with the second layer.

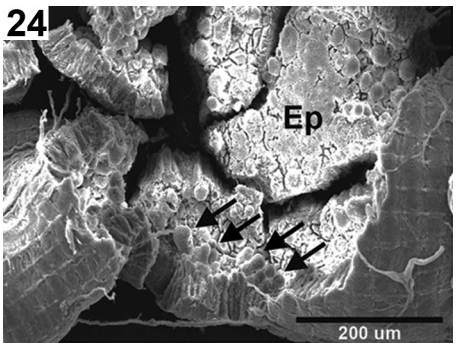

Fig 24–SEM micrograph of a midgut epithelium of late fourth instar showing numerous degenerated epithelial cells (Ep) with cytoplasmic protrusions (arrows) in the midgut lumen.

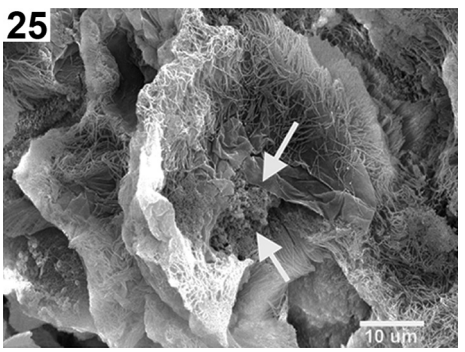

Fig 25–SEM micrograph of a region in the midgut of late fourth instar showing a broken apical membrane of a degenerated epithelial cell and organelle debris (arrows) discharged into the midgut lumen.

The second and fourth layers were very similar in appearance in having relatively electron-dense zones laterally alternating with less dense zones. The second layer was somewhat thicker than the fourth layer. The third and the fifth layers were also similar to each other in consisting of loosely woven, granular strands, although both varied in thickness. The fifth layer was the thickest and most of the variation in overall thickness of the larval PM was due to variation in the fifth layer. The sixth layer appeared as a dark, solid line

of varying electron density (Fig 15).

Morphological features of epithelial cell degeneration

Preparation for the major tissue reorganization that occurs upon pupation could be seen in the midgut of early fourth instar larvae. In LM midgut sections, evidence was found that epithelial cells started to prepare for degeneration while maintaining their functions (Fig 3, 4). Morphological features attributable to the degenerative process were also observed using the TEM and SEM (Figs 18-25). In the early larval midgut, autophagic compartments (autophagosomes) were clearly seen in some epithelial cells. Autophagosomes containing organelle debris were visible in the cytoplasm of cells undergoing degeneration (Figs 18-21). In addition, lamellar bodies, which represent the result of autophagic degradation of membranous cellular components, were observed (Fig 20, 21). Autophagy increased in the midgut cells of the late fourth instar larvae (Figs 18-23). A few organelles, such as mitochondria and vesicles, were observed near the apical membrane of the degenerating cells (Fig 22, 23). SEM analysis revealed cytoplasmic protrusions on the apical surface of epithelial cells undergoing degeneration (Fig 24). Such cytoplasmic protrusions were round and had a smooth surface (Fig 24). Fig 25 shows breakage of the apical membrane and discharge of organelle debris into the midgut lumen. In the late larval midgut sections, both new epithelial cells and epithelial cells with cytoplasmic protrusions were found on the luminal surface of the midgut (Fig 12).

DISCUSSION

This present study is the first description of the midgut of *Oc. togoi* fourth instar mosquito larvae and morphological features of epithelial cell degeneration at the ultrastructural level. In Diptera, Lepidoptera and Ephemeroptera, the midgut epithelium of the adult stage is always formed from two main types of cells: epithelial and regenerative cells, however, in different stages of development in some insect species, endocrine and goblet cells are also found (Billingsley and Lehane, 1996; Fialho *et al*, 2009). In fourth instar *Oc. togoi*, three types of cells were found, epithelial, regenerative and endocrine cells, but no goblet cells were observed.

Epithelial cells were the predominant cell type in the epithelium of the midgut wall of fourth instar *Oc. togoi* and showed morphological similarity to the equivalent cell type in *Aedes aegypti* (Zhuang *et al*, 1999). According to Richards and Davies (1994) and Jordao *et al* (1999), the midgut epithelial cells of insects present numerous long microvilli and large quantities of mitochondria in their apical portions. The well-developed rough endoplasmic reticulum and Golgi complex in the middle portions of the cells, and the basal plasma membrane infoldings with associated mitochondria in the basal portions, indicate that the columnar epithelial cells serve in nutrient absorption, protein synthesis related to digestive enzyme production and ion and water transport.

Regenerative cells are an insect midgut cell population that are able to proliferate and differentiate; therefore, they might be considered as stem cells, which depending on requirements could differentiate into epithelial, endocrine or goblet cells (Tettamanti *et al*, 2007a). Regenerative cells are either distributed as isolated cells among epithelial cells, or can form regenerative groups which, depending on their shape, are called regenerative nests or crypts (Garcia *et al*, 2001; Rost, 2006a,b;

Rost-Roszkowska *et al*, 2010a,b). In fourth instar *Oc. togoi* small groups of regenerative cells were found, but no evidence for their proliferation and/or differentiation was observed.

Billingsley and Lehane (1996) and Levy *et al* (2004) proposed that insect midgut endocrine cells might have functions similar to neurosecretory cells of the vertebrate alimentary tract. A large variety of polypeptide hormones are synthesized in the endocrine cells, which are responsible for secretion of appropriate concentrations of specific enzymes after feeding and which control the proliferation and differentiation of the regenerative cells (Zudaire *et al*, 1998). Endocrine cells can be categorized by the electron-density of their granules (Raes and Verbeke, 1994; Billingsley and Lehane, 1996; Jordao *et al*, 1999). In most insects, secretory vacuoles and granules are observed in the basal cytoplasm (Raes and Verbeke, 1994; Billingsley and Lehane, 1996; Levy *et al*, 2004). The structure of the endocrine cells in fourth instar *Oc. togoi* is similar to that described for many insect species: granular structures were observed predominantly in the basal cytoplasm. The fourth instar in *Oc. togoi* is the final stage before pupa formation; the organism is preparing for many changes associated with pupation. The secretory functions of the endocrine cells will likely increase as new hormones are synthesized.

The absence of goblet cells in the midgut of fourth instars *Oc. togoi* is similar to *Ae. aegypti* larvae (Zhuang *et al*, 1999). In the midgut of Lepidoptera larvae, goblet cells possess a goblet chamber formed by an apical infolding of the plasma membrane (Levy *et al*, 2004). Cell surface basal and lateral projections into this cavity extend from the cell surface, similar to microvilli, but the goblet chamber projections are filled with mitochondria (Levy *et al*, 2004). The presence of mitochondria is related to the active transport of potassium ions from the hemolymph to the midgut lumen and calcium ions from adjacent columnar cells into the goblet cells (Koch and Moffett, 1995; Moffett *et al*, 1995). The presence of such goblet cells may be responsible for alkalization (pH 8.0-12.0) of the midgut of Lepidoptera (Dow, 1984). However, alkalization in the midgut lumen of mosquito larvae apparently occurs in the absence of goblet cells. Basolateral V-ATPases drive strong luminal alkalinization in the anterior midgut of larval *Ae. aegypti* (Zhuang *et al*, 1999). The midgut epithelium of *Ae. aegypti* larvae generates a lumen negative transepithelial voltage instead of the lumen positive voltage observed in *Manduca sexta* larvae under comparable conditions (Clark *et al*, 1999).

Our findings of the PM in fourth instar *Oc. togoi* are similar to those reported for *Ae. aegypti*, in that they consist of at least six layers (Moncayo *et al*, 2005). The PM occurs continuously along the alimentary canal from the cardia to the anus. The larval PM serves two important functions: protection of the midgut epithelium from damage by food particles and in protection against pathogens (Peters, 1992; Lehane, 1997).

Another key feature of the larval midgut is the ability to renew itself, balancing new growth with mechanisms to remove damaged or redundant cells and organelles, and also to prepare for major tissue remodeling when pupation is initiated. Autophagy is a key cellular homeostatic mechanism that counteracts and controls cell growth that we examined. It is also a key process in apoptosis that enables degradation of organelles no longer needed (Lockshin and Zakeri,

2004; Levine and Yuan 2005; Tettamanti *et al*, 2007b). Two important features of autophagy can be detected at the ultrastructural level, autophagosomes and autolysosomes (Mizushima *et al*, 2008; Tettamanti *et al*, 2011). The autophagic process begins with the formation of a double-membrane structure, called a phagophore, which progressively expands and grows to engulf a portion of cytoplasm (Mizushima *et al*, 2008). This membrane structure wraps itself around the cellular components targeted for degradation and closes to become an autophagosome (Mizushima *et al*, 2008). The autophagosome membrane then fuses with lysosomes, small membrane-bound organelles containing digestive enzymes (Mizushima *et al*, 2008). After fusion the contents are then degraded and the resulting macromolecules are assimilated back into the cytosol (Mizushima *et al*, 2008). Such degeneration of midgut epithelial cells can occur during digestion as part of ongoing midgut renewal during the life of the insect depending on stress and external factors, such as harmful or toxic chemical compounds (Rost, 2006b; Rost-Roszkowska *et al*, 2008).

In our study, degeneration of midgut epithelial cells by autophagosomes was observed in both early and late fourth instar *Oc. togoi*. Autophagy proceeded intensively in the midgut epithelium of late fourth instar *Oc. togoi*, which might be a process for elimination of harmful or toxic substances from the organism. The process of degeneration and the following regeneration of the midgut epithelium can proceed in a cyclic manner closely associated with molting periods (Garcia *et al*, 2001). In this study, no mitotic activity in regenerative cells or cellular renewal due to the growing digestive tube at each ecdysis was observed. An explanation for this could be the larvae analyzed were not yet in the pre-pupal stage. A study of the proliferation and differentiation of regenerative cells in the pre-pupal and pupal stages is currently in progress in our laboratory.

In conclusion, this study describes the ultrastructure of the midgut of fourth instar *Oc. togoi* for the first time. Although the cell types found in the *Oc. togoi* midgut epithelium are similar to those described for other species of *Aedes* and *Ochlerotatus*, further studies of the factors that control growth and nutrition in *Oc. togoi* larvae are needed. These studies should help us better understand the physiology of the larval midgut and its interaction with biological control methods, such as Bti and *L. sphaericus*. They may also inform strategies on reducing adult fecundity.

ACKNOWLEDGEMENTS

This work was supported financially by the Thailand Research Fund (TRF Senior Research Scholar: RTA5480006 to WC, subproject to NJ) and the Medical Research Fund, Faculty of Medicine, Chiang Mai University (to NJ).

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