สัญญาเลขที่ RMU4980007

รายงานวิจัยฉบับสมบูรณ์

โครงการ จุลกายวิภาคของระบบทางเดินอาหารและระบบสืบพันธุ์ของแมลงวันหัวเขียว *Chrysomya megacephala*

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สังกัด ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์
มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
สำนักงานคณะกรรมการการอุดมศึกษา

กิตติกรรมประกาศ

ขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย และสำนักงานคณะกรรมการการอุดมศึกษา ซึ่งเป็นผู้สนับสนุนการวิจัยในโครงการนี้ ขอขอบคุณ ศาสตราจารย์ ดร. พงษ์ศักดิ์ อังกสิทธิ์ อธิการบดี มหาวิทยาลัยเชียงใหม่, รองศาสตราจารย์ นายแพทย์นิเวศน์ นันทจิต คณบดี คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่, รองศาสตราจารย์ ดร. อุดม ชัยทอง หัวหน้าภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ที่อนุญาตและสนับสนุนการทำวิจัยครั้งนี้

ขอขอบคุณ รองศาสตราจารย์ นายแพทย์ คม สุคนธสรรพ์ ที่เป็นผู้สนับสนุนการวิจัย ทำให้งานวิจัยครั้งนี้สำเร็จลุล่วงไปด้วยดี, ขอขอบคุณคณาจารย์ ภาควิชานิติเวชศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ (ศาสตราจารย์ นายแพทย์ ไพฑูรย์ ณรงค์ชัย, รองศาสตราจารย์ นายแพทย์ พงษ์รักษ์ ศรีบัณฑิตมงคล, รองศาสตราจารย์ นายแพทย์ จาตุรงค์ กันชัย, รองศาสตราจารย์ แพทย์หญิง กานดา วิชัยรัตน์, ศาสตราจารย์ นายแพทย์ ธานินทร์ ภูพัฒน์, นายแพทย์ มาโนช โชคแจ่มใส) สำหรับความร่วมมือในงานวิจัยทางด้าน Forensic Entomology ในประเทศไทย ซึ่งเป็นส่วนหนึ่งของงานวิจัยในการศึกษาครั้งนี้

ขอขอบคุณคณะผู้ร่วมงานวิจัย: Prof. Jimmy K. Olson (Department of Entomology, Texas A&M University), Dr. Hiromu Kurahashi (Department of Medical Entomology, National Institute of Infectious Diseases, Japan), Dr. Roy C. Vogtsberger (Department of Biology, Midwestern State University, USA), ผศ.ดร. อุไร ไชยศรี (ภาควิชาพยาธิวิทยาคลินิค คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล), คุณบุษบง กันทะลือ (หน่วยจุลทรรศน์อิเล็กตรอน คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่), คุณสมศักดิ์ เปียงใจ, อ.ดร. นพวรรณ บุญชู, อ.ดร. ธารินี ไชยวงศ์, ดร. สิริสุดา สิริวัฒนารังษี, ดร. วรโชติ บุญศรีวงศ์, คุณรัชฎาวรรณ เงินกลั่น, คุณกิตติคุณ หมู่พยัฆค์, คุณสรวิชญ์ อุปคุตม์ และเจ้าหน้าที่ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

บทคัดย่อ

รหัสโครงการ RMU4980007

ชื่อโครงการ จุลกายวิภาคของระบบทางเดินอาหารและระบบสืบพันธุ์ของแมลงวันหัวเขียว *Chrysomya megacephala*

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เนื้อหางานวิจัย

แมลงวันหัวเขียว *Chrysomya megacephala* เป็นแมลงวันที่มีความสำคัญทางการแพทย์และเป็นแมลงวันหัวเขียวชนิดที่พบมากที่สุดในประเทศไทย ตัวเต็มวัยนอกจากจะเป็นพาหะนำเชื้อโรคต่าง ๆมาสู่มนุษย์ ยังก่อให้เกิดความรำคาญ นอกจากนี้ตัวอ่อนยังก่อให้เกิดโรคหนอนแมลงวันทั้งในมนุษย์และสัตว์ การหาวิธีควบคุมจำนวนประชากรแมลงวันจึงนับว่าเป็นสิ่งที่จำเป็น ซึ่งต้องอาศัยองค์ความรู้พื้นฐานทางชีววิทยาในด้านต่าง ๆของแมลงวันหัวเขียวชนิดนี้ การศึกษาครั้งนี้มีจุดประสงค์เพื่อศึกษาระบบทางเดินอาหาร (ในตัวอ่อนระยะที่ 3 และตัวเต็มวัยเพศผู้ เพศเมีย) และระบบสืบพันธุ์ (ตัวเต็มวัยเพศผู้ เพศเมีย) ของแมลงวันเขียว *C. megacephala* ในระดับจุลกายวิภาค โดยศึกษาภายใต้กล้องจุลทรรศน์แบบธรรมดา (Light microscopy), กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (Scanning electron microscopy) และกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน (Transmission electron microscopy) ท่อทางเดินอาหารของตัวอ่อนคล้ายคลึงกับของตัวเต็มวัยคือประกอบด้วยทางเดินอาหารส่วนต้น (ปาก, คอคอย, หลอดอาหาร, ต่อมน้ำลาย, กระเพาะพักอาหาร, ปากกระเพาะ) ทางเดินอาหารส่วนกลาง (ลำไส้) และทางเดินอาหารส่วนปลาย (ท่อมัลพิเจียน, ลำไส้เล็กส่วนต้น, ลำไส้เล็กส่วนปลาย, ไส้ใหญ่, ไส้ตรงและทวารหนัก รูปร่างลักษณะของต่อมน้ำลาย, กระเพาะพักอาหารและไส้ตรงของตัวเต็มวัยแตกต่างไปจากในตัวอ่อนระยะที่ 3 ความยาวของท่อทางเดินอาหาร (ไม่นับส่วนที่เป็นแขนงออกไป) ของตัวอ่อนระยะที่ 3 มีค่าเฉลี่ย 89.15 มิลลิเมตร ซึ่งยาวมากกว่าของเพศผู้ (ค่าเฉลี่ย 36.23 มิลลิเมตร) และเพศเมีย (ค่าเฉลี่ย 37.73 มิลลเมตร) ส่วนระบบสืบพันธุ์เพศผู้นั้น ประกอบด้วยอัณฑะ, ท่อนำอสุจิตอนต้น, ท่อนำอสุจิตอนปลาย, ท่อฉีดอสุจิ, sperm pump, ต่อม accessory ส่วนเพศเมียประกอบด้วยรังไข่, ท่อนำไข่ด้านข้าง, ท่อนำไข่หลัก, ช่องอวัยวะสืบพันธุ์, ต่อม accessory, ถุงเก็บอสุจิ การศึกษาระบบอวัยวะสืบพันธุ์ภายนอกของเพศผู้ พบว่าประกอบด้วย cercus, surstylus, epandrium, phallus, ejaculatory apodeme และ aedeagal apodeme มีอวัยวะรับความรู้สึกสองแบบบริเวณ cercus และ surstylus ส่วนอวัยวะสืบพันธุ์ภายนอกของแมลงวันเพศเมีย บริเวณ supra-anal plate, sub-anal plate และ cercus และมีอวัยวะรับความรู้สึกประมาณสี่หลายชนิด ซึ่งผลจากการศึกษาในระดับจุลกายวิภาคของทั้งสองระบบที่สำคัญของแมลงวันครั้งนี้นับเป็นข้อมูลพื้นฐานสำคัญที่จะนำไปสู่การพัฒนาวิธีการควบคุมประชากรแมลงวันหัวเขียวชนิดนี้ต่อไปในอนาคต

คำหลัก แมลงวันหัวเขียว *Chrysomya megacephala*, จุลกายวิภาค, ระบบทางเดินอาหาร, ระบบสืบพันธุ์, กล้องจุลทรรศน์อิเล็กตรอน

Abstract

Project Code: RMU4980007

Project Title: Ultramorphology of alimentary and reproductive systems of blow fly *Chrysomya megacephala*

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Content

Chrysomya megacephala (F.) (Diptera: Calliphoridae) is the most important blow fly species of medical concern in Thailand. The adults are mechanical vectors of various pathogens and nuisance pests, while the larvae can cause myiasis. Regarding this, a method to control fly population is mandatory, with all aspects of the basic research of this fly species being needed. The objective of this study was to investigate morphology of the alimentary and reproductive tract of *C. megacephala* at the ultrastructural level using light microscopy (LM), scanning (SEM) and transmission electron microscopy (TEM). In the third instar, males and females were similar in the alimentary canal, consisting of the foregut (mouth, esophagus, crop, salivary gland, cardia), midgut and hindgut (Malpighian tubules, pylorus, ilium, colon, rectum and anus) portions, with the midgut being the longest portion. The median of the entire gut length of the third instar measured 89.15 mm, which was significantly longer than in males (36.23 mm) and females (37.73 mm). Morphologies of the salivary gland, crop and rectum were similar in males and females, but much different from those of the third instar. The internal reproductive organs in males comprised a pair of testes, vas deferens and accessory glands, one ejaculatory duct and one sperm pump; while those of females consisted of 2 ovaries, 2 lateral oviducts, a common oviduct, 3 spermathecae, 2 accessory glands and a genital chamber or vagina. The external genitalia of males exhibited specific characters of the cercus, surstylus, epandrium, phallus, ejaculatory apodeme, and aedeagal apodeme. The cercus and surstylus were densely covered with two types of sensilla. Regarding females, several types of sensilla were found on the supra-anal plate, sub-anal plate and cercus. Information gained from this thorough study in both alimentary and reproductive systems established a basic database, which was useful in understanding their functional role and serving as knowledge to address a strategy to control this fly species in the future.

Keywords: *Chrysomya megacephala*, ultrastructure, alimentary canal, reproductive tract, electron microscopy

จุลกายวิภาคของระบบทางเดินอาหารและระบบสืบพันธุ์ของแมลงวันหัวเขียว *Chrysomya megacephala*

PROBLEMS AND RESEARCH RATIONALE

Chrysomya megacephala (F.) is a blow fly species that has a wide distribution covering many parts of the world. In Thailand, it is extremely abundant in urban areas and the most prevalent of blow fly species collected. *C. megacephala* has been proven as a mechanical transmitter of numerous disease pathogens to humans, as well as a source of annoyance. Problems arising from *C. megacephala* in the country result from its rapid development under warm temperatures as well as the available source of material filth as its breeding place. Regarding this, control of fly populations under the threshold of disease transmission is mandatory.

Despite the control strategy of pest populations relying typically on insecticides, some problems have been encountered. These include environmental pollution, insecticide resistance and increased pesticide costs. With the current movement towards using decreased amounts of any kind of insecticide, alternative control methods that can be included in an integrated control program should be tested and applied. An interesting issue in controlling insect populations is the suppression of feeding and/or reproductive success, such as disruption of the progeny production or worsening metamorphosis during development. To encompass these purposes, ultramorphology of the alimentary tract and reproductive organs in arthropods of particular medical importance has been studied for either basic information or applied researches associated with alimentary tract and reproductive morphologies. Light and/or electron microscopy have been employed to investigate the alimentary and reproductive organs of many arthropods and flies, both in basic and applied researches.

In insects, the alimentary tract is a vital system for their life, and involves digestion and absorption of nutrients, regulation of hemolymph ionic composition and pH, detoxification, and production of semiochemical compounds such as pheromones. In recent years, some biological substances such as bacterial toxins and crude plant extracts or active compound extracted from plants have been reported as having some insecticidal property directly on the alimentary tract, or indirectly to other organs in insects. For example, application by feeding of azadirachtin extracted from the neem tree, affects the fecundity of the fly, *Ceratitis capitata*, or mosquitoes, *Culex tarsalis* and *Culex quinquefasciatus*; while such feeding in the fly larvae, *Musca domestica* or *Stomoxys calcitrans*, inhibits development. Recently, pathology of the midgut surface of the mosquito, *Aedes aegypti*, caused by the bacterium, *Bacillus thuringiensis*, was clearly shown using scanning electron microscopy (SEM). A large hole and blister were evident in the midgut

wall, according to the exposure of Cry-IVB toxin produced by this bacterium. So far, many researchers have reported the ultrastructural study of the alimentary tract in several groups of insects (e.g. mosquito, ant, bee, tick, bot fly, fruit fly, and sand fly) that are involved in pathogen transmission.

Regarding the reproductive organ, the examples of basic research include morphological observation in the reproductive system of the female blow fly, *Chrysomya bezziana* (Diptera: Calliphoridae), accessory glands of *Chrysomya putoria* (Diptera: Calliphoridae), female reproductive tract of the fly, *Curtonotum helvum* (Diptera: Curtonotidae), *Pseudacteon wasmanni* (Diptera: Phoridae) or Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). In males, studies have included the sperm structure of the fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae), and glandular cells at the terminalia of the reduvid bug, *Triatoma rubrofasciata* (Hemiptera: Reduviidae). Concerning applied researches, studies have been reported on the bacterium, *Rickettsiella,* in the ovary of the Oriental cockroach, *Blatta orientalis*, insect virus Hz-2V, in the reproductive organs of the female corn earworm moth, *Helicoverpa zea* (Lepidoptera), or infection of the endobacterium, *Wolbachia*, in the ovary of the sand flea, *Tunga penetrans*. Application of some substances induces alteration of the reproductive system in arthropods. For example, precocene II induces abnormality of the ovary and accessory glands of the female rich moth, *Corcyra cephalonica*; or the oil extracts from the plant, *Thevetia peruvine*, cause pathological alterations in the ovary of the mosquito, *Culex pipiens*.

Concerning such published information, it was the objective of this study to investigate thoroughly the alimentary and reproductive systems of *C. megacephala*, using light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). This information will establish a database of this species, which will be useful in understanding its functional role and serving as knowledge to address a strategy to control this fly species in the future.

OBJECTIVES

1. To determine the alimentary tract of *C. megacephala* in ultrastructural studies in larval (the third instar) and adult stage using LM, SEM and TEM.

2. To determine the reproductive organs of *C. megacephala* in ultrastructural studies in adults (both male and female) using LM, SEM and TEM.

MATERIALS AND METHOD

1. Rearing *C. megacephala* in the laboratory

Adult *C. megacephala* was obtained from a laboratory colony maintained for ~4 years under ambient temperature and natural conditions in the fly rearing room at the Department of Parasitology, Faculty of Medicine, Chiang Mai University. Fifty of each adult female and male *C. megacephala*, were reared in rearing cages (30×30×30 cm) screened with black cloth. Adults were reared on two kinds of food; (I) a mixture of 10% (w/v) sucrose solution at 985 ml and multivitamin syrup (Syn-O-Vits: Thailand) at 15 ml; and (II) fresh pork liver as both a food source (protein) and oviposition site. Small pieces of 40 g fresh pork liver was placed in a glass petri dish (9 cm in diameter) and changed daily. The dish with the liver was located at the bottom of the cage. A plastic cup (5×4 cm), with a hole centrally located in the lid, was used to contain the mixture of sucrose solution and multivitamin syrup. A wick (10 cm in length) was inserted through the hole in the lid and used as a feeding site for the adult flies, and it was changed on alternate days. Subsequently, the oviposition sites were observed daily for the presence of fly eggs; and if present, they were transferred to a 12×15×6 cm transparent plastic box, with 40 g of fresh pork liver provided as larval food. The lid of the box had a rectangular-shaped hole cut into 3/4 of its total area, which was covered with the finest silk screen cloth (100 meshes/mm^2) for ventilation as well as preventing other small insects from entering the rearing box to oviposite. The box was covered by the lid and sealed tightly with adhesive paper tape to prevent the larvae from crawling out. It was kept under room temperature (24-28 °C) in a cabinet at the rearing room of the Department of Parasitology. Liver was replaced daily until some third instars developed into prepupa, the nonfeeding period. The box with pupae was still covered and tightly sealed until some pupae emerge as adults. Then, it was placed into a rearing cage before the lid was taken off to release the adults into the cage.

2. Alimentary tract and reproductive organ dissections

All fly specimens were individually dissected in phosphate buffer (PB) at pH 7.4 under a binocular dissecting microscope to obtain the alimentary tract. These samples were primarily kept in the same buffer and prepared for the next light and electron microscopic study.

2.1 Larval dissection

Thirty individual dead larvae were transferred onto a centrical-well paraffin plate and arranged in a suitable position (to prevent sliding of the specimen) under the dissecting microscope. The larva was placed on its back in a vertical position and pierced with tiny insect pins on the lateral side of the prothorax. The pins were carefully inserted through the larval integument and fixed into the paraffin plate. A few drops of PB was added around the sample

and dissecting area. The larva was ventrally dissected to extract the entire alimentary canal using two fine forceps. At the first step, the integument of larva was ascendingly split in the midline and opened from the terminal end of the caudal to the cephalic segment. It was washed thoroughly with PB to remove fat body and other material contents in the hemocoel. The integument was opened until the entire alimentary tract was extracted. Care had to be taken, so as not to cut the internal organs. Larval integument was taken off and separated from the entire tract by a cut at the cephalic region. Thus, the alimentary tract was still attached to the cephalopharyngeal skeleton, an internal skeleton of the larval anterior region. This skeleton supported the fine tubular structures of the foregut such as the pharynx and median salivary duct. The entire tract was carefully transferred to a single-well glass slide (7.5×2.5 cm) containing PB to await further dissection. This tract, coiled in the ventral part, was detached from the Malpighian tubules, and many of the tracheoles around the gut by the assistance of insect needles. Care was taken to cut these parts as close as possible to their insertion without tearing the gut. Another drop of PB was added, if necessary, to prevent the sample from drying. When the gut was drawn out completely, it might be removed and transferred to a cleaner part of the slide. This entire gut sample was prepared for the next procedure; light and electron microscopic studies.

2.2 Adult dissection

The male and female *C. megacephala* used in this study were 3-7 day-old flies. They were sacrificed by keeping in a freezer set at 4º C for 15 mins. All dead fly samples were prepared before dissection by removing their wings and legs using fine forceps. Thirty flies of each sex were individually placed on their back in a vertical position on the paraffin plate. Fly specimens were arranged in a suitable position and pinned with tiny insect pins on the lateral side of the mesothorax. The pins were carefully pierced through the cuticle and fixed into the paraffin plate. A few drops of PB were added around the specimen and dissecting area. Dissection was performed using fine forceps under the dissecting microscope. The sample was ventrally dissected to obtain the entire alimentary tract by ascending dissection from the posterior to anterior end. First, the abdominal sclerites were split and taken off. When the gut was partially extracted and all the abdominal sclerites removed, the sample was washed thoroughly with PB to remove fat body and other contents. After that, it was carefully transferred for further dissection to a single-well glass slide (7.5×2.5 cm) containing PB.

The alimentary tract, positioned in the abdominal region, was coiled and passed straight through the highly muscular thorax. The thorax of the insects comprised hardened sclerites with dense musculatures, thus, the dissection was performed with more care in attempting to prevent tearing of alimentary organs such as the esophagus, salivary glands and

anterior part of the midgut. The sclerites and thoracic muscles were gently removed to extract the gut without tearing.

The head capsule was dissected next; and dissection in this region had to be performed carefully, since the head capsule is composed of the fine tubular organs of the foregut, such as pharynx and salivary ducts. During dissection, the sample was rinsed with a few drops of PB to remove unnecessary tissue residue. The exoskeleton of the head, including compound eyes, was removed, but not the mouthpart, which supports the fine tubular organs of the foregut. After the entire alimentary tract was completely dissected, it was kept in PB and prepared for the next study.

In conclusion, the alimentary tract of larva and the adult fly consisted of the following organs: **Foregut** (esophagus, salivary gland, crop, proventriculus), **Midgut** (gastric caeca, anterior midgut, mid midgut, posterior midgut) and **Hindgut** (pylorus, Malpighian tubule, ileum, colon, rectum, anus). All of these organs were studied further using LM, SEM and TEM.

Regarding the reproductive organ, adult flies were dissected to obtain the reproductive organs. For males, the testis, accessory gland and ejaculatory duct were examined; while in females, the ovary, common oviduct, spermathecae, accessory gland and vagina were investigated, using LM, SEM and TEM. The external morphology of male and female genitalia was also examined using SEM.

3. Light microscopic (LM) observation

The entire alimentary tract of all fly samples (30 third-instar, males and females) was morphologically investigated and morphometrically analyzed, using a vernier caliper, under dissecting microscope (Olympus®, Japan). The samples were photographed using an Olympus C4040Z digital camera (Olympus®, Japan). The photographs of all samples were individually measured for the length of each gut region.

4. Scanning electron microscopy (SEM) observation

The alimentary tract and reproductive organ (~20 third-instar, males and females) were prepared for SEM investigation. Specimens were carefully transferred from PB and separately placed in 2.5% glutaraldehyde in phosphate buffer solution at pH 7.4 at 4°C for 24 hr. After that, they were rinsed with PB 2 times at 10-min intervals. The samples were post-fixed in 1% osmium tetroxide at room temperature for 2-3 hr. Then, they were rinsed twice with PB and gradually dehydrated with different concentrations of 10%, 30%, 50%, 70%, 80%, 90%, 95% and twice with 100% alcohol. Acetone was subsequently applied twice. Critical point drying (CPD) was performed thereafter. The specimens were attached to the double-stick tape of

aluminum stubs, and coated with gold (Au) in sputter-coating apparatus before being viewed with a JEOL JSM-5910LV scanning electron microscope (JEOL®, Japan).

5. Transmission electron microscopy (TEM) observation

The methodology for the TEM process was the same as that for the SEM procedure until the specimens were placed in absolute alcohol for two 12 hr periods. After that, they were placed in acetone for 2 hr before transferring to a ratio of resin : acetone of 1:3 for 24 hr, 1:1 for 24 hr and 3:1 for 24 hr, subsequently. Each placement was carried out twice in only resin for 3 hr. Each sample was embedded in Spurr's resin by placing them into a plastic block, and incubating later at 70°C for 24 hr. A thick section (0.5 μm) of each sample was cut with a glass knife on an Ultramicrotome (Boeckeler®, USA). Ultrathin sections (90 nm) were prepared from these re-embedded blocks, with serial sections collected from copper slot grids. The sections were post-stained with uranyl acetate and lead citrate before examination by a JEOL JEM-2010 transmission electron microscope (JEOL®, Japan).

RESULT

1. Alimentary tract of the third instar

1.1 LM observation

The entire alimentary tract of the third instar *C. megacephala* is displayed in Figure 1. Morphometric analyses of laboratory-reared flies are shown in Table 1. When comparing between the two sexes, no significant difference was observed between the median body weight of females (0.052 g) and males (0.041 g) (Mann-Whitney *U* test; *P* = 0). However, a significant difference was detected between the wing length of males (7.48 mm) and females (7.43 mm) (Mann-Whitney *U* test; *P* = 0.529).

Results of the morphometric analyses of the alimentary canal of *C. megacephala* are shown in Table 2. The esophagus of the third instar (median = 5.05 mm) was significantly longer than those of males (median = 2.63 mm) and females (median = 2.65 mm) (Mann-Whitney *U* test; *P* = 0). On the other hand, the crop length of males (median = 4.70 mm) and females (median = 4.70 mm) was significantly longer than that in the third instar (median = 1.50 mm) (Mann-Whitney *U* test; *P* = 0). The midgut length of the third instar (median = 46.35 mm) was much longer than that in the males (median = 25.00 mm) and females (median = 25.75 mm) (Mann-Whitney *U* test; *P* = 0). Likewise, the median length of ilium, colon and rectum of the third instar was significantly longer than that in the males and females (Mann-Whitney *U* test; *P* = 0). Overall, the entire gut length from esophagus to anus (excluding the branches of salivary gland, crop tube, gastric caeca and Malpighian tubule) of the third instar (median = 89.15 mm) was significantly longer than that in the males (median = 36.23 mm) and females (median = 37.73 mm) (Mann-Whitney *U* test; *P* = 0), but no significant difference was found between males and females (Mann-Whitney *U* test; *P* = 0.109) (Table 2).

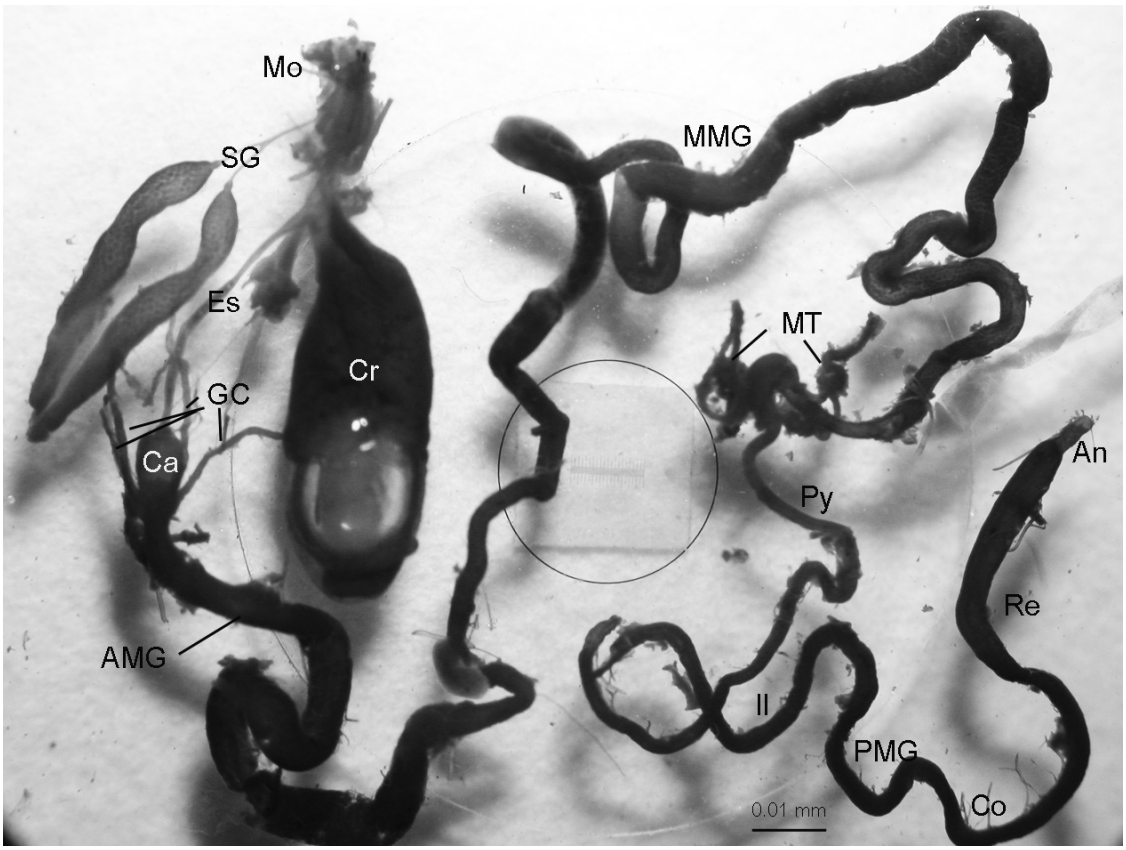

Figure 1. Alimentary canal of the third instar, *Chrysomya megacephala*, indicating the foregut consisting of mouth (Mo), salivary glands (SG), esophagus (Es), and crop (Cr). The cardia (Ca) represents the junction of the foregut and midgut. The midgut consists of the gastric caeca (GC), anterior midgut (AMG), middle midgut (MMG), and posterior midgut (PMG). The hindgut begins with the pylorus (Py), the Malpighian tubules (MT), ileum (Il), colon (Co), rectum (Re) and anus (An).

Table 1. Morphometric evaluation of *Chrysomya megacephala*

Morphometric evaluation[1]	Median (range)[2]			*P value*
	Third instar	Male	Female	
Body weight (g)	0.068 (0.060-0.076)	0.041 (0.038-0.052)	0.052 (0.042-0.062)	0.00
Larval length (mm)	16.10 (15.50-16.85)	-	-	
Adult wing length (mm)	-	7.48 (6.85-8.25)[a]	7.43 (6.68-8.20)[b]	0.529

[1] n = 30

[2]Medians within rows were significantly different when followed by different letters (Mann-Whitney *U* test).

Males and females examined were 7-days-old.

Table 2. Morphometric analyses of the internal organ of *Chrysomya megacephala*

Organ in length (mm)	Median (range)*			*P value* L3/M	*P value* L3/F	*P value* M/F
	Third instar (L3)	Male (M)	Female (F)			
Esophagus	5.05 (4.80-5.60)[a]	2.63 (2.50-2.85)[b]	2.65 (2.50-2.85)[b]	0	0	0.476
Salivary duct (main)	1.40 (1.30-1.55)[a]	1.23 (1.20-1.30)[b]	1.25 (1.20-1.30)[b]	0	0	0.701
Salivary duct (left)	0.80 (0.65-1.05)[a]	1.00 (0.90-1.10)[b]	1.00 (0.90-1.10)[b]	0	0	0.811
Salivary duct (right)	0.78 (0.65-1.05)[a]	1.00 (0.90-1.10)[b]	1.00 (0.90-1.10)[b]	0	0	0.811
Salivary gland (left)	5.10 (4.40-5.70)[a]	0.5 (0.45-0.50)[b]	0.5 (0.45-0.50)[b]	0	0	0.601
Salivary gland (right)	5.10 (4.30-5.70)[a]	0.5 (0.45-0.50)[b]	0.5 (0.45-0.50)[b]	0	0	0.605
Crop tube	1.50 (1.30-1.75)[a]	4.70 (4.20-5.10)[b]	4.70 (4.20-5.10)[b]	0	0	0.726
Cardia	0.90 (0.75-0.95)[a]	0.45 (0.40-0.55)[b]	0.45 (0.40-0.55)[b]	0	0	0.660
Gastric caeca	3.49 (3.00-4.65)[a]	-	-	-	-	-
Midgut	46.35 (40.00-52.00)[a]	25.00 (22.00-28.50)[b]	25.75 (22.00-29.00)[b]	0	0	0.132
Pylorus	0.38 (0.30-0.45)[a]	0.43 (0.35-0.50)[b]	0.43 (0.35-0.50)[b]	0	0	0.817
Malphigian tubule	1.04 (0.80-1.20)[a]	2.61 (2.30-3.20)[b]	2.70 (2.30-3.25)[b]	0	0	0.215
Ilium	25.00 (20.90-28.10)[a]	3.75 (3.40-4.50)[b]	4.00 (3.40-4.50)[b]	0	0	0.092
Colon	9.25 (7.40-11.00)[a]	2.30 (2.00-2.60)[b]	2.38 (2.00-2.60)[b]	0	0	0.255
Rectum	2.20 (1.80-2.80)[a]	1.20 (1.00-1.50)[b]	1.23 (1.00-1.55)[b]	0	0	0.195
Anus	0.91 (0.80-1.25)[a]	1.08 (0.90-1.35)[b]	1.15 (0.90-1.35)[b]	0	0	0.173
Entire gut	89.15 (81.40-99.70)[a]	36.23 (32.60-41.20)[b]	37.73 (32.70-42.15)[b]	0	0	0.109

*Medians within a row followed by the same letter were not significantly different (Mann-Whitney *U* test).

1.2 SEM and TEM observations

The external surface of the esophagus was longitudinally folded with faint muscular fibers. Posterior to the tubular esophagus was a bulb-like cardia, with smooth surface, similar to that of the salivary gland (Figure 2A). The wall of the salivary gland consisted of closely-packed simple cuboidal cells (Figure 2B).

The larval midgut of *C. megacephala* began with the posterior midgut tissue of the cardia just anterior to the gastric caeca, where the external surfaces were smooth. Investigations of the cardia revealed that this organ comprised two compartments – the anterior foregut tissue and the posterior midgut tissue (Figure 3A). The anterior foregut tissue consisted of shortened columnar epithelial cells containing numerous dark-stained secretory granules (Figure 3A). Concerning the posterior midgut tissue, epithelial cells more or less flattened and with a large nucleus were evident (Figure 3A). Also, an internal folded thread-like structure, most likely the peritrophic membrane (PM), was seen (Figure 3A).

The semi-thin sections of the gastric caecum revealed that the inner walls of its lumen comprised layers of cuboidal epithelial cells (Figure 3B). These cells contained large nuclei. TEM studies revealed that the inner margin of these gastric caeca cells consisted of short microvilli to increase surface area for absorption of nutrients in this region.

The midgut was the longest region of the third instar alimentary canal. The external surface of the midgut was smooth, but lined with longitudinal muscle (Figure 3C). In semi-thin sections, the midgut tissue contained cuboidal epithelial cells interspersed with fat body cells. A considerable number of dark-stained secretory granules was found inside the cells (Figure 3D).

The Malpighian tubules emerged from the midgut-hindgut junction and were proximally paired (Figure 1). SEM investigation revealed that this organ consisted of a chained globular structure (Figure 4A). Each cell contained a large nucleus (Figure 4B) and numerous minute granules as well as abundant microvilli at their apical surface, which was in contact with the lumen. For the rectum, it was evident that its wall was formed primarily by a monolayer of cuboidal epithelial cells with large, oval nuclei (Figure 4C). In the anal part, myo-epithelial cells comprised the largest component. Peritrophic membrane was still obvious within the anal tube (Figure 4D).

2. Alimentary tract of males

2.1 LM observation

The alimentary canal of the male *C. megacephala* looked similar to that in the third instar, consisting of the anterior (foregut), median (midgut) and posterior (hindgut) portions, with the midgut being the longest portion (Figure 5). However, some organs appeared different from

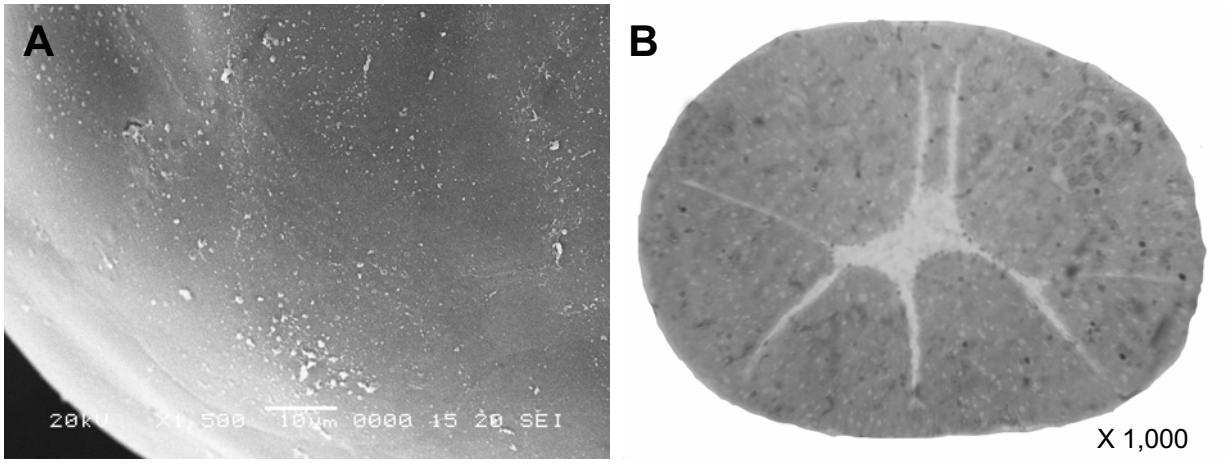

Figure 2. Micrographs of the third instar, *Chrysomya megacephala*. (A) SEM micrograph of the salivary gland with smooth surface. (B) Thick section of the salivary gland showing closely-packed simple cuboidal cells.

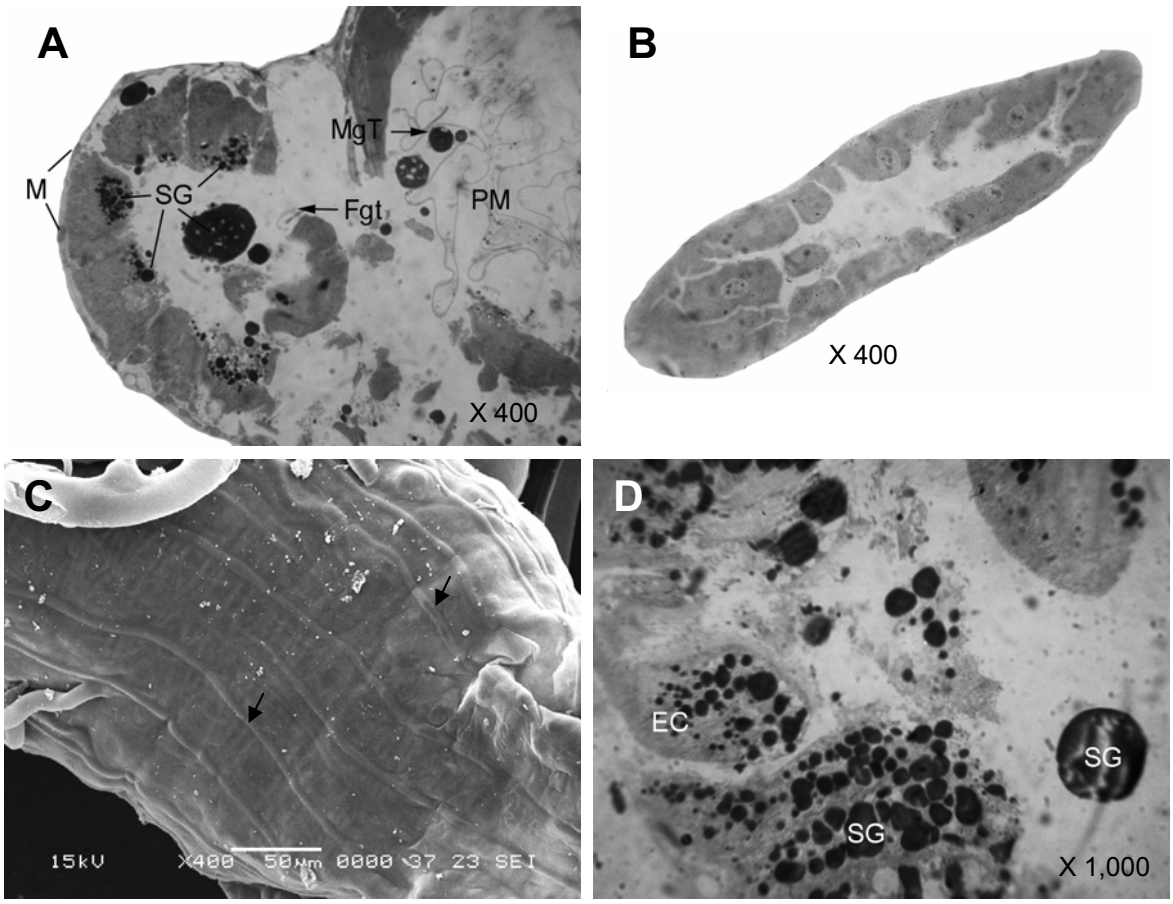

Figure 3. Micrographs of the third instar, *Chrysomya megacephala*. (A) Thick section of cardia presenting the anterior foregut tissue (Fgt), posterior midgut tissue (MgT), peritrophic membrane (PM) and muscle (M). (B) Thick section of gastric caeca showing smooth surface and closely-packed simple cuboidal cells. (C) SEM micrograph of midgut showing smooth surface with longitudinal muscle (arrows). (D) Thick section of midgut showing epithelium cells (EC) containing numerous secretory granule (SG).

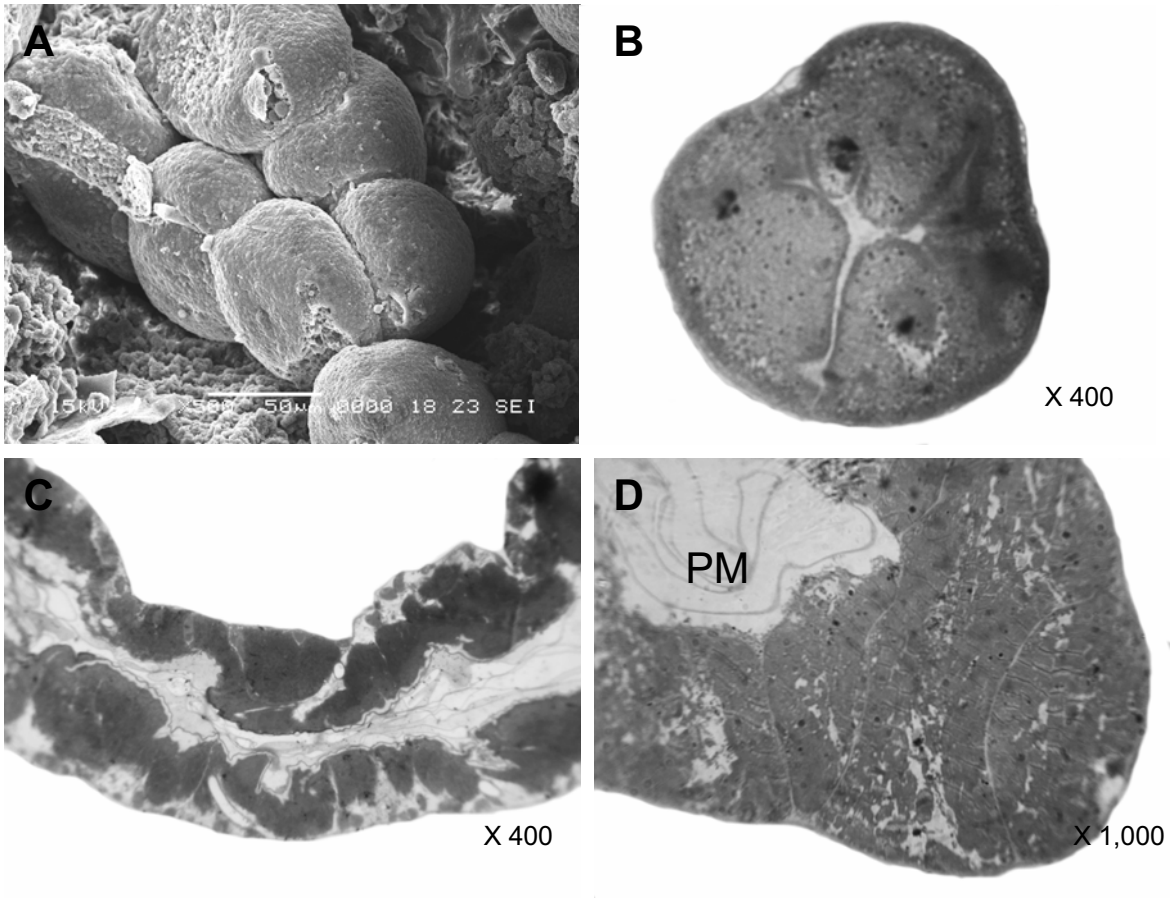

Figure 4. Micrographs of the third instar, *Chrysomya megacephala*. (A) SEM micrograph of the Malpighian tubule. (B) Thick section of the Malpighian tubule. (C) Thick section of the rectum showing the monolayer of cuboidal epithelial cells. (D) Thick section of the anal region revealing the myo-epithelial cell. Peritrophic membrane (PM) is obvious.

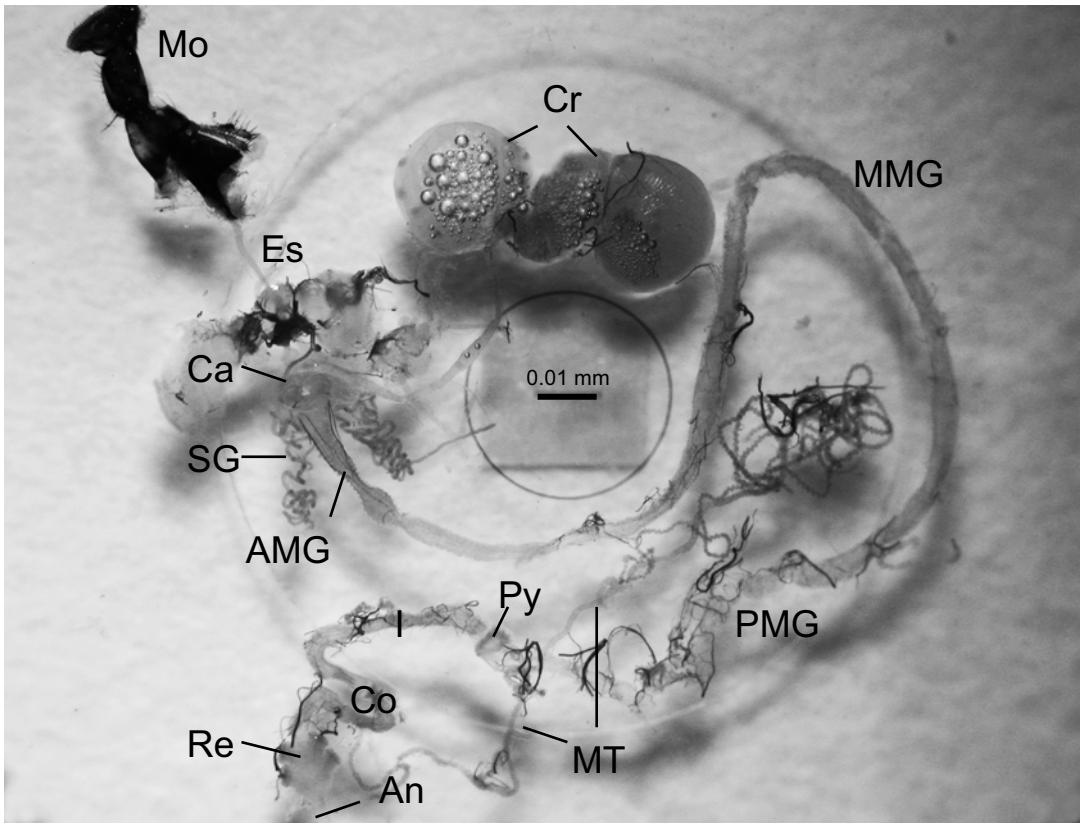

Figure 5. Alimentary canal of male *Chrysomya megacephala* indicating the foregut consisting of mouth (Mo), salivary glands (SG), esophagus (Es), and crop (Cr). The cardia (Ca) represents the junction of the foregut and midgut. The midgut consists of anterior midgut (AMG), middle midgut (MMG), and posterior midgut (PMG). The hindgut begins with the pylorus (Py), the Malpighian tubules (MT), ileum (Il), colon (Co), rectum (Re) and anus (An).

those found in the third instar. These organs were the salivary gland, crop and rectum. The crop appeared as the bi-lobed crop connected to the straight, slender tube. The crop was a distensible and contractile sac that occupied the antero-ventral region of the abdomen. Cardia appeared as a globular structure lying between the end of the esophagus and anterior midgut. The midgut was a cylindrical structure, occupying the main part of the body. The Malpighian tubules emerged from the midgut-hindgut junction. Each tube diverged into two long tubes. The junction between the ilium and colon was noticeable. The rectum of the male flies was the most distinctive organ of the hindgut, appearing as long a bulb shape.

2.2 SEM and TEM observations

Salivary gland

The salivary glands were paired organs lying in the thorax on either side of the esophagus. Each gland presented a coil-shaped long tubular gland (Figure 5). In male *C. megacephala*, each gland was a highly coiled tube apically, and formed a big, short straight unpaired duct proximally (Figure 6A). In the coiled part, the tubules coiled only in the anterior direction and then looped back in a relatively straight line toward the posterior end of the coil. Using higher magnification of TEM, the basal lamina of the salivary gland had a smooth surface (Figures 6B, 6C). Variable size of secretory vesicles was observed (Figure 6D). The membrane network was evident (Figure 6D). In the central lumen, higher magnification showed that the outer most layer, adjacent to the lumen, was double-layered (Figure 6E). In some parts, the area of double-layered, lumen showed the secretion of materials from the cells. Variable size of dark-stained secretory materials and vesicles was observed inside the cytoplasm of the cells. In addition, mitochondria with packed cristae were observed (Figure 6F).

Crop

The crop appeared as a thin sac (Figures 5, 7A). The external surface of this organ was covered with muscle (Figure 7B). The semi-thin cross section displayed a covering of muscle and convoluted cuticle centrally adjacent to the lumen containing food particle (Figure 7C). Using higher magnification of TEM, epithelial cells, with a large nucleus, were found lying on the basal lamina. The cuticle was clearly evident. Unknown structure was observed in the epithelial cells (Figure 7D).

Cardia

SEM investigation revealed that the cardia was the bi-lobe globular structure, of which the posterior portion connected to the anterior midgut (Figure 8A). The thick section clearly indicated two compartments; the anterior and posterior portion (Figure 8B). The anterior portion was located centrally, and received food content directly from the esophagus. The epithelial cells of the anterior portion were loose in distribution (Figure 8C). As for the posterior portion,

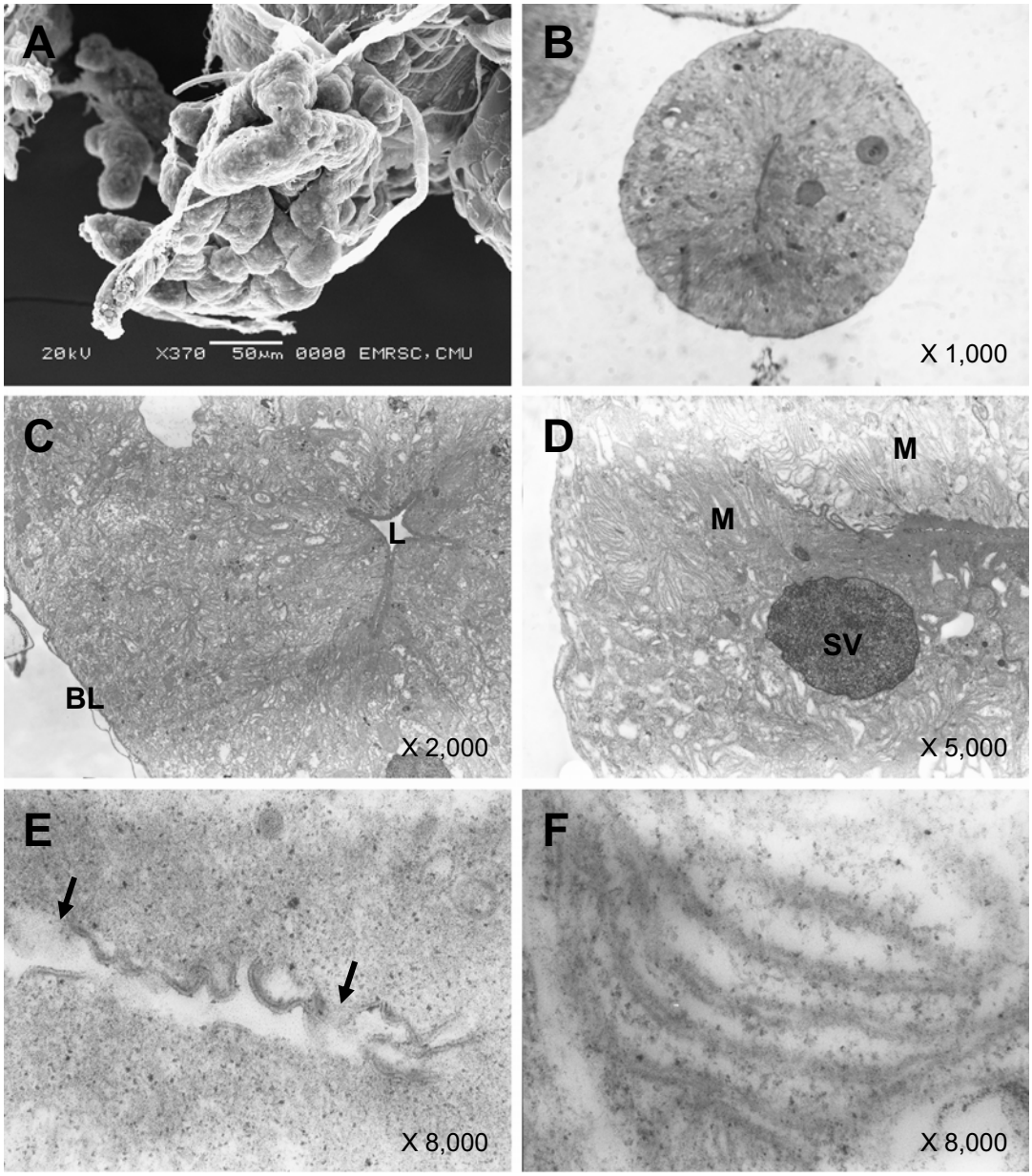

Figure 6. Salivary gland of male *C. megacephala*. (A) SEM micrograph. (B) Thick section showing the whole gland. (C-F, TEM micrograph) (C) Smooth basal lamina (BL) and central lumen (L). (D) Dark-stained secretory vesicles (SV) and membrane network (M). (E) The central lumen showing double-layer and some secretion of materials from the cells (arrows). (F) Mitochondria with packed cristae.

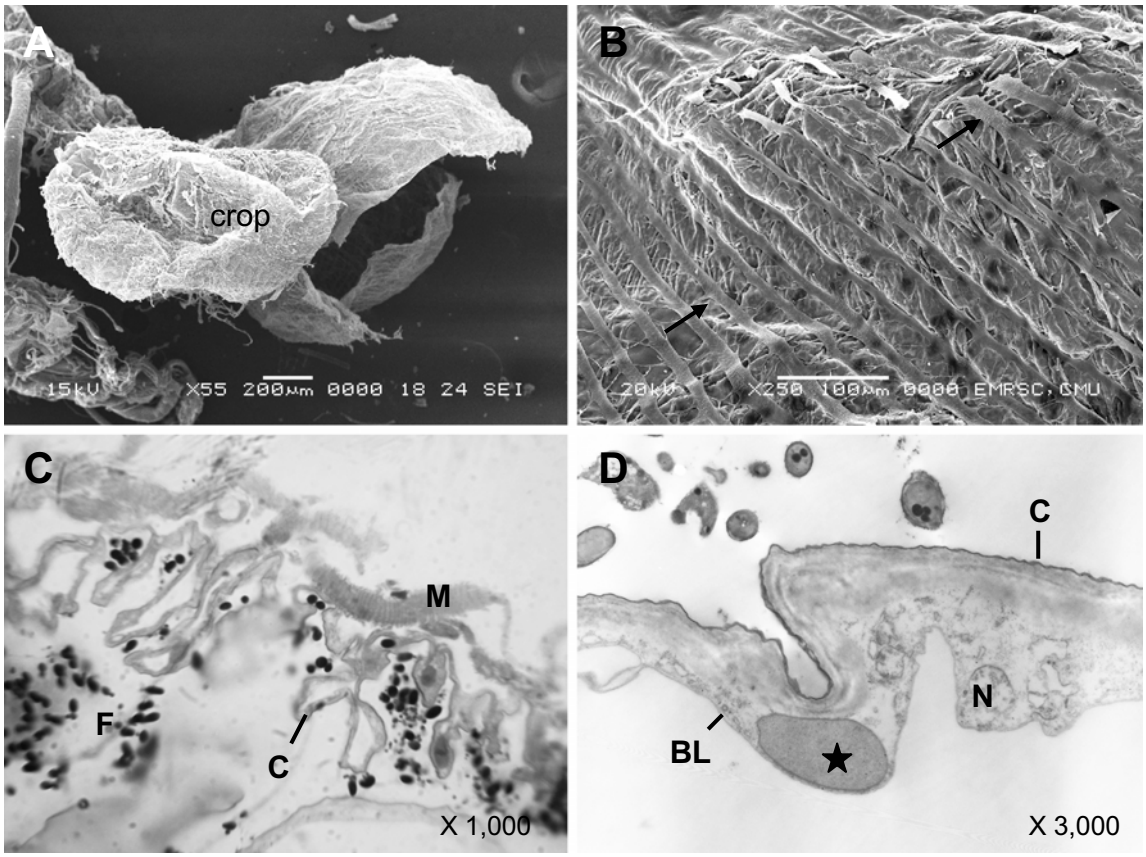

Figure 7. Crop of male *C. megacephala*. (A) SEM micrograph showing the thin, bi lobed sac. (B) SEM micrograph showing numerous muscles (arrows) covering the crop. (C) Thick section presenting the muscle (M), cuticle (C) and food particle (F) inside. (D) TEM micrograph displaying the large nucleus (N) of the epithelial cell lying on the basal lamina (BL), cuticle (C) and unknown content (star).

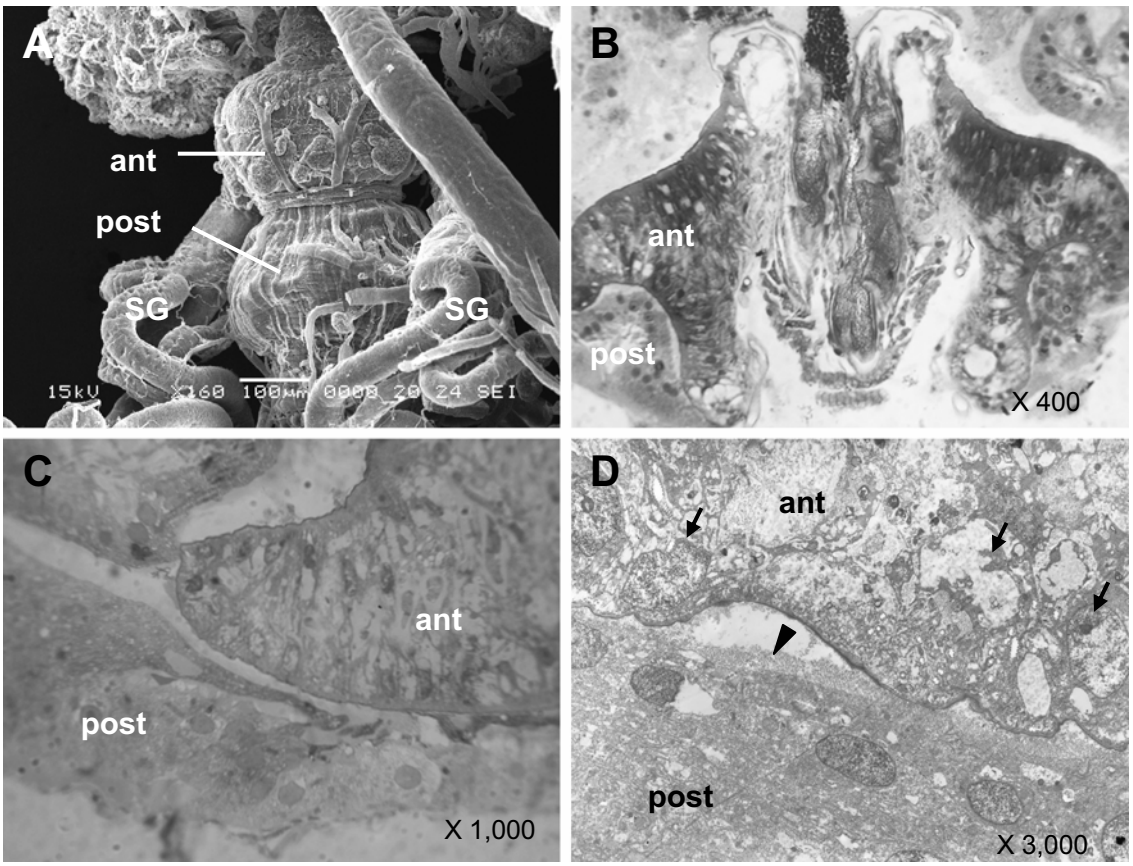

Figure 8. Cardia of male *C. megacephala*. (A) SEM image revealing the bi lobe globular structure; anterior portion (ant), posterior portion (post), salivary gland (SG). (B) Longitudinal section indicating the anterior (ant) and posterior portions (post). (C) Thick section showing the anterior (ant) and posterior portion (post). (D) TEM image showing nucleus of cells in the anterior portion (arrows). Microvilli (arrowhead) lying along epithelial cells of the posterior portion.

microvilli were obvious along the epithelial cells (Figure 8D). Peritrophic membrane was evident in this portion.

Midgut

Upon SEM, the pouch-like protrusions of the circular muscle occurred on the external surface of the anterior midgut; however, longitudinal fine muscle tended to distribute quite uniformly along the outer surface of the mid midgut (Figure 9A). Rupture of the midgut presented the compact midgut epithelium cells and central peritrophic membrane (Figure 9B). The midgut epithelium lay on a thin basal lamina, consisting of a single layer of columnar microvillated cells (Figure 9C). These cells contained a large nucleus. Long microvilli presented at the rim of the cells (Figure 9D).

Hindgut

The hind gut started at the pylorus (Figure 10A), and connected to the ilium, colon, rectum and anus, consecutively (Figure 10B). The pylorus comprised epithelial cells with a large nucleus (Figure 10C). The basal lamina was relatively smooth. The cuticle was presented in the internal lumen of the pylorus (Figure 10D).

Upon SEM view, the junction between the ilium and colon was noticeable (Figure 11A). The external surface of the ilium was covered with muscle (Figure 11B). The epithelium cells contained a large nucleus, which was more or less ovoid to elongated (Figures 11B,C). Epithelium cells were covered by the cuticle, which was the barrier between cells and lumen (Figure 11D).

As for the colon, the bundle of muscle was evident (Figure 12A) by covering the epithelium cells. The cuticle was also observed (Figure 12B). Food particles were found along the lumen.

The rectum of the males was noticeable, appearing as a muscular cone-shaped sac-like structure, with the anterior region being enlarged (Figure 13A). The anal tube was located at the posterior end. Four protruding muscle-free rectal pads were obvious at the anterior end (Figure 13A). No evidence of swollen rectal sac was found at the posterior end. The rectal pad, forming the bulge-like structure, was heavily supplied with tracheae that penetrated into the center of the pad (Figure 13B). Only thick circular muscle heavily surrounded the male rectal gland (Figures 13A,B). Sections of the anterior end of the rectum displayed dark-staining of four rectal pads (Figure 13C). Columnar cells of the rectum contained a large oval nucleus (Figures 13D,E) and other organelles. Mitochondria and stacked Golgi apparatus were distributed within the cytoplasm. The cuticle was evident (Figure 13F). The anus was the last part of the alimentary canal. It appeared as a large lumen with numerous particles, which were most likely waste particles (Figure 14A). The cells of the anus contained a large nucleus. The cuticle was still presented within this organ (Figure 14B).

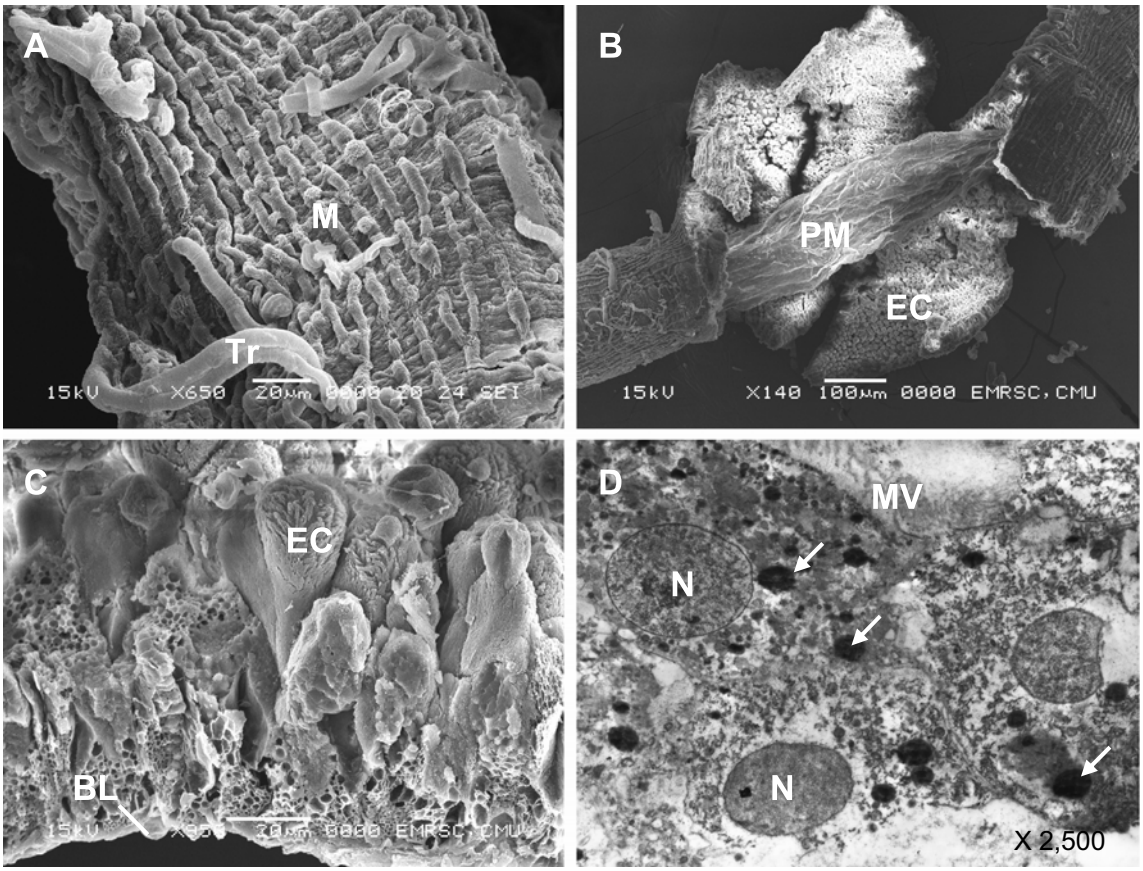

Figure 9. Midgut of male *C. megacephala*. (A,B,C; SEM) (A) External surface of the midgut revealing longitudinal fine muscle (M). Tracheole (Tr) inserted into the midgut. (B) Ruptured midgut presented the compact epithelium cells (EC) and central peritrophic membrane (PM). (C) Single layer columnar microvillated cells lying on thin basal lamina (BL). (D) TEM micrograph showing epithelium cells with a large nucleus (N) and peripheral microvilli (MV).

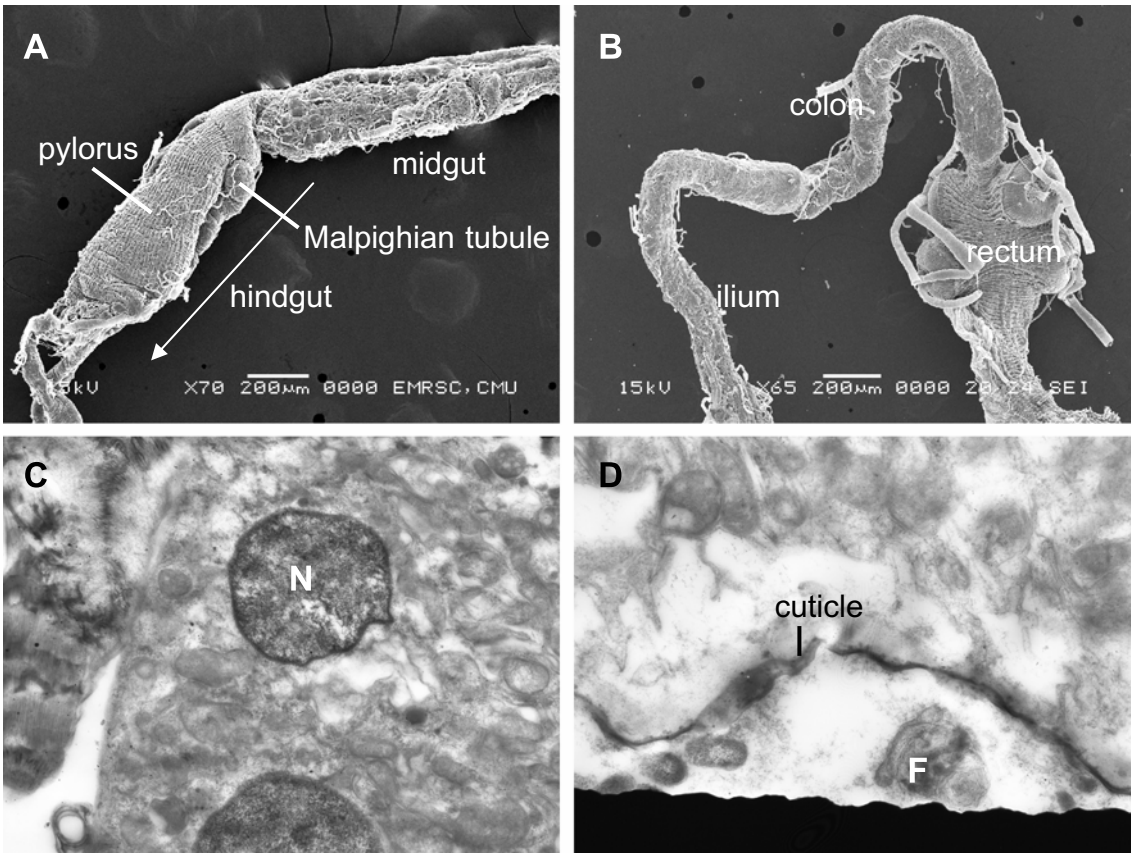

Figure 10. Hindgut of male *C. megacephala*. (A,B: SEM; C,D: TEM) (A) Junction between the midgut and hindgut. (B) Ilium, colon and rectum. (C) Epithelium cells of the pylorus with nucleus (N). (D) Cuticle of pylorus. Food contents (F).

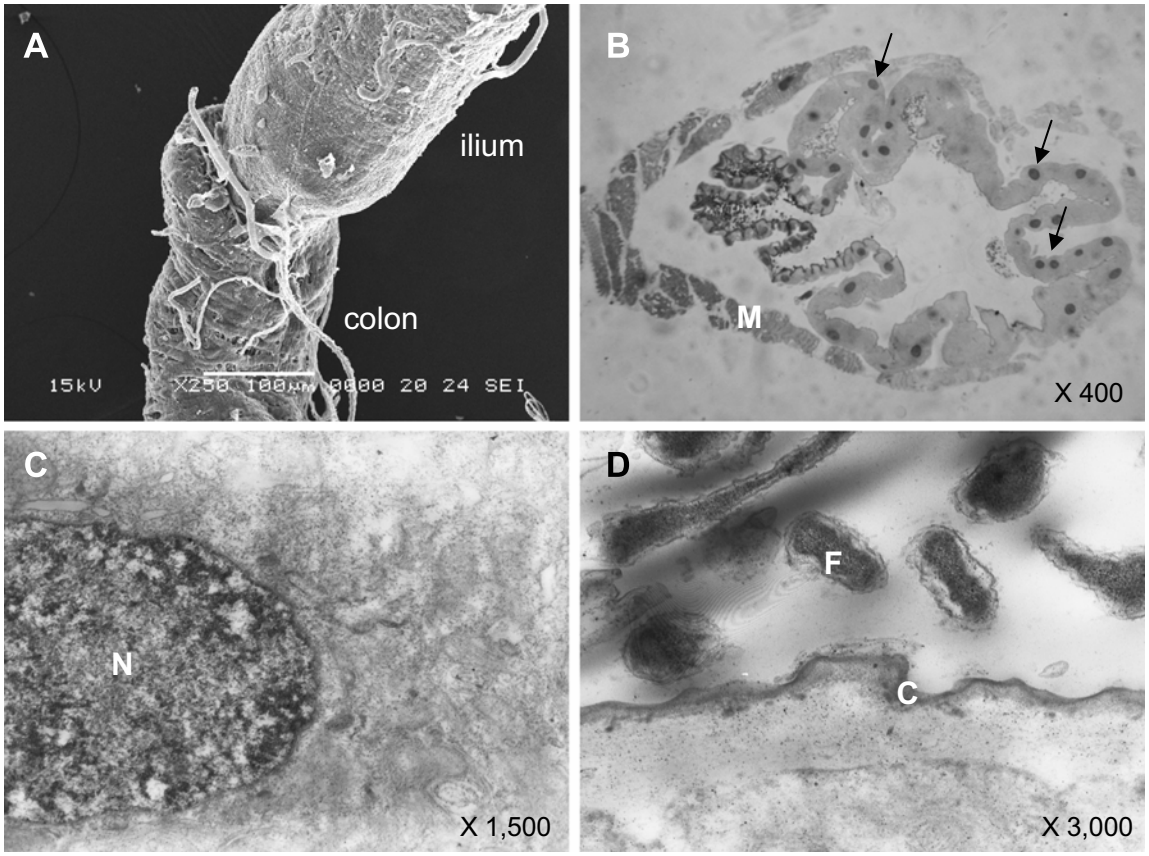

Figure 11. Ilium of male *C. megacephala*. (A) SEM micrograph viewing the ilium and colon. (B) Thick section of ilium displaying epithelium cells containing a large nucleus (arrows) and external covering of muscle (M). (C) TEM micrograph of epithelium cells with a large nucleus (N). (D) TEM micrograph of epithelium cells covered by the cuticle (C). Food particle (F).

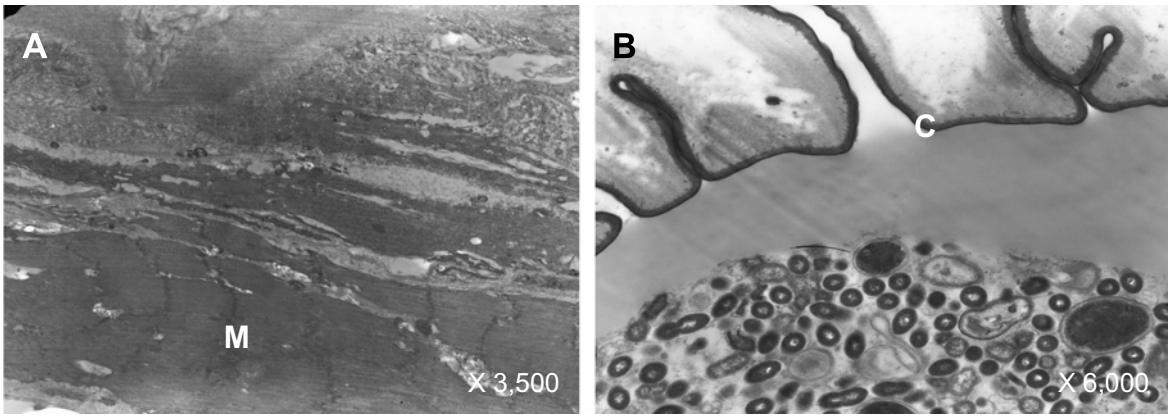

Figure 12. TEM micrographs of colon of male *C. megacephala*. (A) Bundle of muscle (M) covering the epithelium cells. (B) Cuticle.

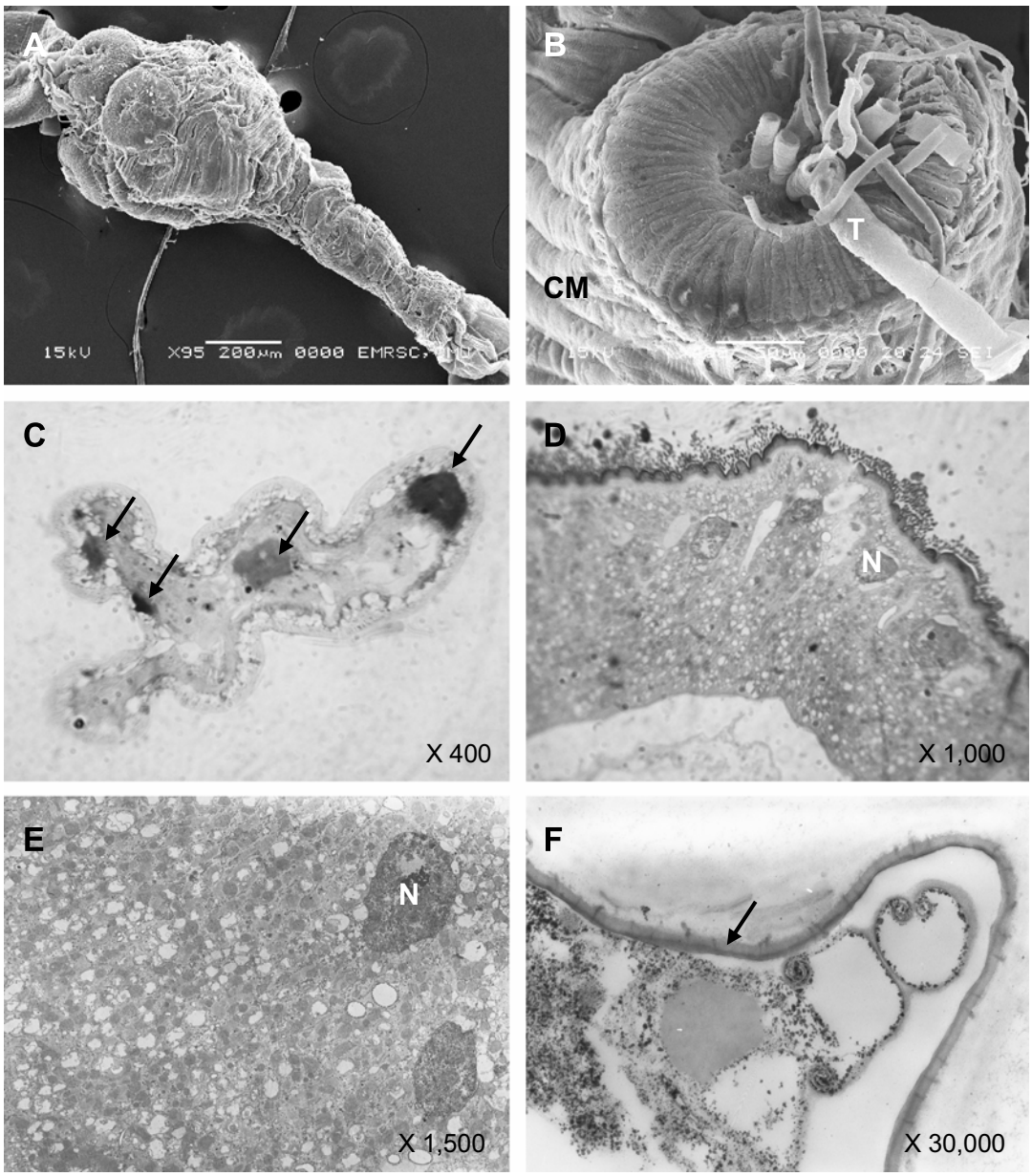

Figure 13. Rectum of male *C. megacephala*. (A,B: SEM; C,D: Thick section; E,F: TEM) (A) Muscular cone-shaped sac-like rectum, with the anterior region being enlarged. (B) Protruding muscle-free rectal pad forming bulge-like structure and heavily supplied with tracheae (T). CM, circular muscle. (C) Dark-staining of four rectal pads (arrows). (D) Columnar cells of contained large nucleus (N). (E) Cells with large nucleus and organelles. (F) Cuticle (arrow).

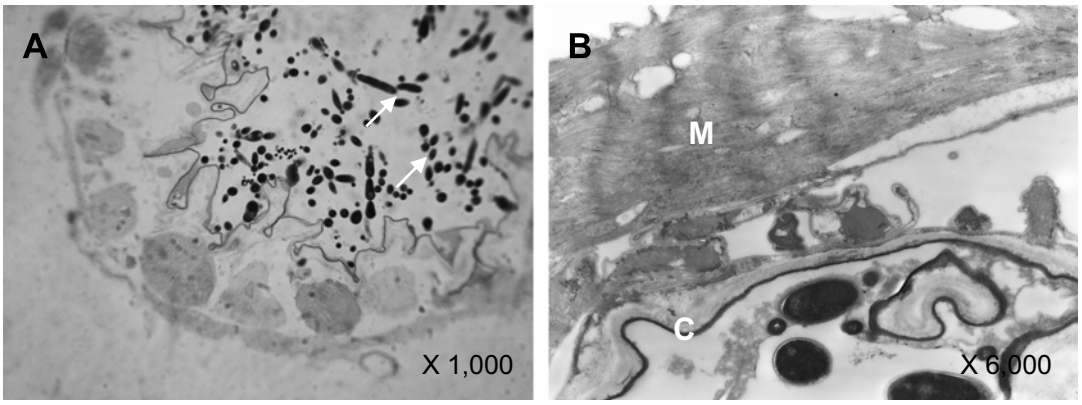

Figure 14. Anus of male *C. megacephala*. (A) Thick section of anus showing large lumen with numerous particles (arrows). (B) TEM micrograph of anus showing thick muscle (M) covering the anal tube. C, cuticle.