CHAPTER II

MATERIALS AND METHODS

1. Maintenance of the *Chrysomya megacephala* colony

Laboratory-reared blow fly colonies were used in this study. The colonies were maintained under ambient temperature and condition at the fly rearing room of the Department of Parasitology, Faculty of Medicine, Chiang Mai University. The rearing room was open-air on the 6th floor of the building. The temperature was 25 to 31°C and the relative humidity was 70 to 80%. The colony of *C. megacephala* was originated from numerous adult flies collected since 2000 from two fresh markets in Muang district, Chiang Mai province, Thailand, by using a sweeping net. The procedure of fly rearing was described by Sukontason et al. (2004). Briefly, 50 of each adult females and males, was reared in a standard cage (30×30×30 cm) screened with black net cloth (25 meshes/mm^2). They were fed with two kinds of food including (I) a mixture of 10% (w/v) sugar solution and 1.5% multivitamin syrup (Syn-O-Vits®, Thailand) (carbohydrate source) and (II) fresh pork liver (protein source). Small pieces of fresh pork liver were placed in a glass petri dish (9 cm in diameter) at the bottom of the cage and changed daily. A plastic cup (5 cm in diameter), with a hole centrally located in the lid, was used as a container for the

mixture of sugar solution and multivitamin syrup. A wick (10 cm in length) was inserted in the hole of the lid and used as a feeding site for the adults. This solution mixture was changed on alternate days. Fresh pork liver was used both a food source and oviposition site. Subsequently, the oviposition sites were observed daily for the presence of fly eggs. If eggs were present, they were transferred into a 12×15×6 cm transparent plastic box by using a camel haired paintbrush (No. 4), and 40 g of fresh pork liver was served as larval food. To prevent over population, each box should not contain more than one hundred larvae. The lid of the box had a rectanglular hole punched into approximately three-fourths of its total area, covered with the finest silkscreen cloth (100 meshes/mm^2) for ventilation as well as prevention of other small insects entering to oviposit. The box was covered by the lid and both parts were sealed tightly with adhesive paper tape to prevent the larvae from crawling out. These rearing boxes were kept under ambient temperature and natural conditions in a cabinet at the fly rearing room. Fresh pork liver was substituted daily to prevent decomposition and strong odor. When all of the larvae pupate, the liver was removed. The box containing the pupae was covered and tightly sealed again until some of the pupae emerge as adults. When the pupae emerged, the box containing adult flies was placed in the rearing cage and then the lid of the box was taken off to release the adult flies inside the cage. Then, the adults were reared in the same manner as previously described.

2. Fly specimens and external morphometric evaluation

Third instar and 7-day-old adult male and female *C. megacephala* were used as fly specimens in a morphological study. The preparation of these specimens and their external morphometric evaluation were described as follows:

2.1 Third instar

The third instar of *C. megacephala* used in this experiment was obtained from a laboratory-reared colony. Once the first-instar hatched from the same batch of eggs, it was reared with fresh pork liver as previously described until reaching the third instar (~3-days after hatching from the egg). Thirty of the third instar were randomized from the rearing box and washed several times with normal saline solution to remove any pork liver tissue residue. These larvae were transferred to filter paper and placed on the bottom of another rearing box. They were anesthetized by keeping this box in a freezer set at 0ºC for 5 mins. Subsequently, each larva was individually weighed (using digital decimal scales; ViBTRA® model AJ-420 E, Japan) and recorded. Then, all larvae were sacrificed by immersion in near boiling water for a few minutes and dried on the filter paper. Each larva was transferred by using a fine forcep, strained on a glass slide, and the length from the apical of the anterior part to the terminal end of the posterior part was measured using a vernier caliper under a dissecting microscope (Olympus®, Japan). Based on the parameters of weight and length, the larval size was obtained. After being measured, these larvae were individually transferred onto a paraffin plate and prepared for alimentary tract dissection.

2.2 Adult male and female flies

The male and female *C. megacephala* used in this study were 7-day-old flies, obtained from the laboratory colony previously described. Flies of each sex were randomly caught using a transparent 10 ml glass tube. Thirty of each sex was randomly removed into small rearing cages (16×16×16 cm). They were sacrificed by keeping the cages in a freezer set at 0ºC for 15 mins. Regarding adult size, the body mass of the dead flies was individually weighed and the wing length measured. These parameters have been used as indicator of insect growth (Kitthawee et al. 1992, Armbruster and Hutchinson 2002). For the adult weighing procedure, dead flies were individually transferred onto the digital decimal scales. After the body mass measurements were taken, only the right wing of the flies was dissected, using fine forceps, and transferred to a glass slide. Another glass slide was placed to brace the wing. Wing length was individually measured to the nearest 0.1 mm as the distance from the axial incision to the R_{4+5} vein excluding the fringe setae (Figure 1) (Landry et al. 1988, Packer and Corbet 1989), using a vernier caliper under the dissecting microscope (Armbruster and Hutchinson 2002). The remainder of dead fly bodies was dissected later to determine the alimentary tract. The specimen preparation of this study was summarized and showed in Figure 2.

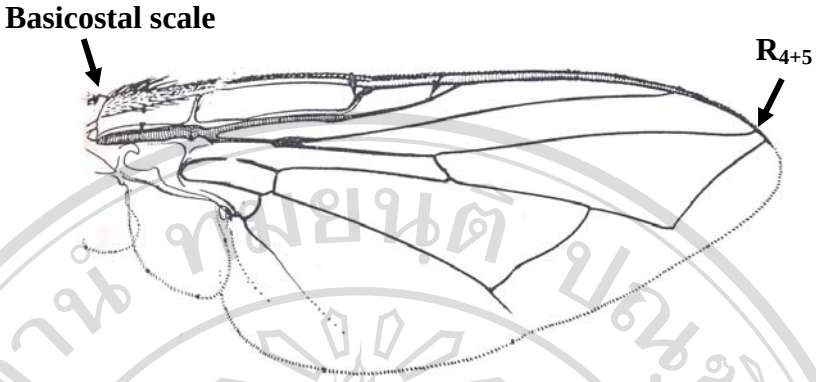

Figure 1 Wing veins of the adult fly (Greenberg 1971).

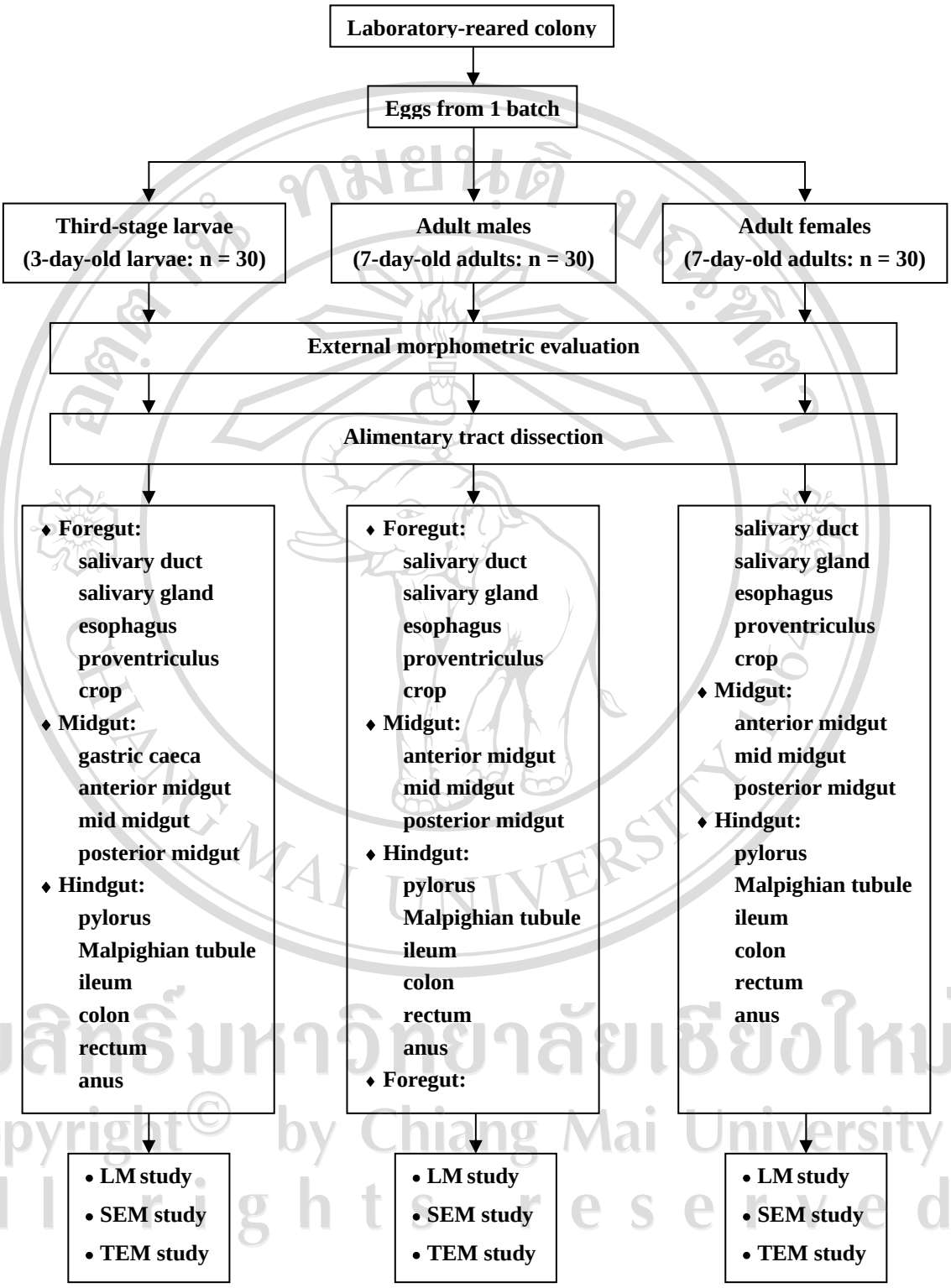

Figure 2 Flow chart showing summarized experiment design.

3. Alimentary tract dissection

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.

3.1 Larval dissection

Thirty individual dead larvae were transferred onto a centrical-well paraffin plate and arranged in a suitable position (to prevent gliding 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 must 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 was support the fine tubular structures of the foregut such as the pharynx and median salivary duct. The entire tract was carefully transferred onto a single-well glass slide (7.5×2.5 cm) containing PB to await further

dissection. This tract, which was coiled in the ventral part, was detached from the Malpighian tubules and many of tracheoles around the gut by the assistance of insect needles. Care should be taken to cut them 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 may 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.

3.2 Adult dissection

All dead fly samples were prepared before the 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 onto 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 was splited and taken off. When the gut was partially extracted and all the abdominal sclerites were removed, the sample was washed thoroughly with PB to remove fat body and other contents. After that, it was carefully transferred onto a single-well glass slide (7.5×2.5 cm) containing PB for further dissection.

The alimentary tract, which was positioned in the abdominal region, was coiled and passes straight through the highly muscular thorax. The thorax of insects

was comprised of hardened sclerites with dense musculatures, thus, the dissection should be 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 next dissection was at the head capsule; and dissection in this region should be carefully performed, since the head capsule was 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 consists 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.

4. Light microscopic study (LM)

The entire alimentary tract of all fly samples (30 third-instar, adult males and females) was morphologically investigated under dissecting microscope (Olympus®, Japan). They 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.

5. Scanning electron microscopy (SEM)

The entire alimentary tract of all samples (30 third-instar, adult males and females) was prepared for SEM investigation. They were dissected into three regions; foregut, midgut and hindgut before the initial chemical operation. This enabled an easy process as well as determination under SEM. Each gut region was 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 gut 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).

6. Transmission electron microscopy (TEM)

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) was 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).

The ultramorphology of the alimentary organs of the third-instar and adults were examined. Photographs were taken according to the noticeable morphology of each organ as scientific evidence.

7. Statistical analysis

The ultramorphology of each gut region of the third-instar and adult males and females of *C. megacephala* were described in the text. The data that had quantitative or qualitative characteristics were analyzed as previously mentioned.

Comparative analysis was executed according to the characteristics of data. For analysis and comparisons of morphological data (length and width data), the Kruskal-Wallis ANOVA was utilized in the datum sets of more than two groups due to the lower sample sizes used in the experiment. If significant differences were observed a Mann-Whitney *U* test was used to determine which groups differed from each other. Medians and ranges were used to illustrate the differences in length and width between sides or sexes of blow fly alimentary organs if observed. The α-error was set at 0.05, and statistical tests were performed using the computer program, SPSS version 14.0.1.