CHAPTER II

MATERIALS AND METHODS

1. Rearing *Chrysomya megacephala* in the laboratory

Adult *Chrysomya megacephala* were obtained from a laboratory colony maintained for approximately four years under ambient temperature and natural conditions in the fly rearing room at the Department of Parasitology, Faculty of Medicine, Chiang Mai University. Adult fly colonies consisted of approximately 50 adults of each sex maintained in black screened rearing cages (30×30×30 cm). Adults were maintained on two kinds of food; (I) mixture of 10% (w/v) sucrose solution at 985 ml to 15 ml of multivitamin syrup (Syn-O-Vits: Thailand); and (II) fresh pork liver as both a food source (protein) and oviposition substrate. Small pieces of 40 g of fresh pork liver were provided in a glass petri dish (9 cm in diameter) located at the bottom of the cage and were replenished daily. 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 cotton wick (10 cm in length) was inserted through the hole in the lid of the cup to provide a feeding site for the adult flies, and this was replenished on alternate days.

As the liver in the adult cages was changed daily it was observed daily for the presence of fly eggs; and if present, they were transferred into a 12×15×6 cm^3 transparent plastic box, with 40 g of fresh pork liver provided as larval food. To reduce intense larval competition, each box was house 30-40 larvae. The lid of the

box has a rectangular shaped hole cut in to 3/4 of its total area, covered with the fine silk screen cloth (100 meshes/mm^2) for ventilation as well to aid in preventing other small insects from entering the rearing box to oviposit. The boxes were covered by the lid and sealed tightly with adhesive paper tape to prevent larvae from crawling out. Both the adults and larvae were kept under ambient temperature conditions (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 prepupae, the nonfeeding - wandering stage. The box with pupae was still covered and tightly sealed until some pupae emerged to the adult stage. Then, the entire box was placed into a rearing cage before the lid was taken off and the adults released into the cage.

For observations involving age-sensitive dissections flies were reared in small groups and monitored more closely to ensure that they were of the correct age.

2. Light microscopic study and measurements of the reproductive organs of *Chrysomya megacephala*

2.1 Male reproductive organs

2.1.1 Testes

To document the changes in the development of the testes light microscopy (LM) was selected as a rapid technique for assessing daily changes. Five males that had been reared from the same egg mass were killed by transferring them from the rearing cages by mouth aspiration and placing them in a freezer set at 4ºC for 15 min.

This procedure was repeated daily for male flies that were newly emerged flies (0-day-old) until they were 29-days-old. Males were dissected on the glass slide containing phosphate buffered saline (pH 7.4) under a dissecting microscope (Olympus, Japan) at 30× magnification to obtain their testes. The shape of the testes was also illustrated under the dissecting microscope (Olympus, Japan) at 30× magnification. Measurements were made along the long axis of each testis and at the widest point of each testis (Figure 6). The left and right testes were measured individually under the compound microscope (40×) by using ocular micrometer and the results were compared using a paired t-test (SPSS for Windows, Release 14.0.1. Chicago, IL, USA). They were then photographed using a mounted digital camera (Nikon®, Tokyo, Japan) to document changes in color and further changes in general shape. Observations on changes in the color and shape of the testes were made every day for flies aged zero to 29 days.

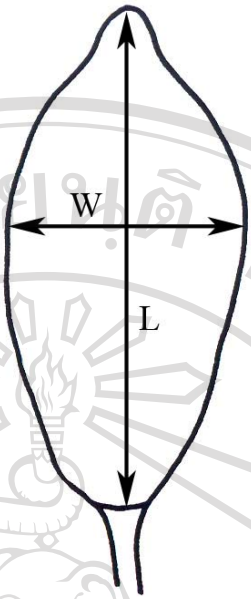

Figure 6 Illustration of a testis of *C. megacephala* showing where daily measurements were made along the long axis and the widest point of each testis

2.1.2 Male accessory glands, vas deferens and ejaculatory duct

To assess the changes in the development (length and width) of the accessory glands, vas deferens and ejaculatory duct under light microscopy, five males that had been reared from the same egg mass were killed by transferring them from the rearing cages by aspiration and placing them in a freezer set at 4ºC for 15 min on a daily basis. This procedure was repeated daily for newly emerged males (0-day-old) until they were 9-days-old. Males were dissected on a glass microscope slide containing phosphate buffered saline (pH 7.4) under a dissecting microscope (Olympus, Japan) at 30× magnification to obtain their accessory glands, vas deferens and ejaculatory duct. The glass slide containing each organ was transferred to be examined under a compound microscope (Olympus, Japan) and photographed using a mounted digital

camera (Nikon®, Tokyo, Japan). Measurements of each organ were made along the long axis and at the widest point of each organ as in the testes (see Figure 1). The left and right of each organ of each fly were measured under a compound microscope (40×) by using ocular micrometer. Data analyses were carried out using SPSS version 14.0.1 for Windows (SPSS Inc., Chicago, IL, U.S.A.). A paired *t*-test was used to examine the potential for differences between the length and width of the left and right sides of each paired organ.

2.2 Female reproductive organs (ovaries, ovarioles, female accessory glands and spermathecae)

The procedures for the examination of female reproductive structures using compound microscopy and measurements of each female reproductive organ were the same as previously described for the male reproductive organs as 2.1.2. This procedure was also repeated daily for newly emerged females (0-day-old) until they were 9-days-old.

3. SEM investigation of the reproductive system of *Chrysomya megacephala*

The ultrastructure of male and female reproductive organs were studied using scanning electron microscopy (SEM). Thirty, 3-5 day-old male and female flies, were sacrificed by placing them in a freezer set at 4ºC for 15 mins. The dead flies were dissected in normal saline under a dissecting microscope to obtain their reproductive organs. The organs were fixed in 2.5% glutaraldehyde in phosphate buffer (PB) at pH 7.4 at 4°C for 24 hr. After primary fixation, samples were rinsed with PB two times at

15-min intervals. The rinsed samples will then be place in 1% osmium tetroxide as a post-fixative at room temperature for 2-3 hr. Subsequently, they were rinsed twice with PB and dehydrated gradually at 30 min intervals with increasing concentrations of ethanol 10%, 30%, 50%, 70%, 80%, 90%, 95% and twice with 100% ethanol, to replace water with ethanol. Following water displacement the specimens were soaked in acetone twice for 30 mins. Critical point drying (CPD) was performed. Specimens were then attached to aluminum stubs with double-stick tape and coated with gold (Au) in a sputter-coating apparatus before being viewed with a JEOL JSM-5910LV scanning electron microscope (JEOL: Japan).

SEM analysis of selected male reproductive organs was observed according to the previous literature (Clift and McDonald, 1973; Spradbery and Sands, 1976; Fausto et al., 2001; Marchini et al., 2001; Fernandes et al., 2004). Female reproductive organ analysis was conducted according to that described in previous publications (Nenon et al., 1995; Dallai et al., 1996; Fausto et al., 1997; Brown and Anderson, 1998; Qui et al., 1998; Fritz and Turner, 2002; Mukhopadhyay et al., 2003; Zacaro and Porter, 2003), as recorded in the literature review.

4. TEM investigation of the reproductive system of *Chrysomya megacephala*

The procedures for dissection and fixation of organs for the TEM study were the same as previously described for the SEM study. After post-fixation, the internal reproductive organs of males (testes, vas deferens, accessory glands and ejaculatory duct) and females (ovaries, spermathecae, oviducts and accessory glands) were cut into separate units using a razor-blade. Subsequently, they were dehydrated and

placed in acetone for 2 hr before transferring to a solution of resin:acetone in a ratio of of 1:3 for 24 hr, 1:1 for 24 hr and 3:1 for 24 hr. Each specimen was then soaked in a solution of in resin only for 3 hr twice. Specimens were then embedded in Spurr's resin by placing them into a plastic block, and incubating at 70°C for 24 hr. A thick section (0.5 μm) of each organ was cut with a glass knife on an Ultramicrotome (Becthai, USA). Ultrathin sections (90 nm) were prepared from these re-embedded blocks, with serial sections collected from copper slot grids. Sections were post-stained with uranyl acetate and lead citrate before examination by a Hitachi H700 transmission electron microscope (Japan) operated at 100 kV.

Observations of the semi-thin sections and TEM morphology of male reproductive organs was conducted following the previous descriptions (Snodgrass, 1993; Dallai et al., 1997; Carpupino et al., 1998; Dallai et al., 2000; Marchini et al., 2001; Alberti and Meyer-Rochow, 2002; Dallacqua and Cruz-Landim, 2003; Dallai et al., 2003; Dallai et al., 2005). Axoneme type of the spermatozoa was also examined. As for females, the reproductive organ analysis followed the previous descriptions (Adams and Reinecke, 1979; Snodgrass, 1993; Nenon et al., 1995; Dallai et al., 1996; Fausto et al., 1997; Tirone and Avancini, 1997; Bene et al., 1998; Stys et al., 1998; Szklarzewicz, 1998; Winterton et al., 1999; Zelazowska and Bilinski, 2001; Fritz and Turner, 2002; Koteja et al., 2003; Zacaro and Porter, 2003; Tanaka and Hartfelder, 2004).

5. Effect of a human contraceptive on adult male and female *Chrysomya megacephala*

5.1 Preparation of chemicals

This study examined the effects of the human contraceptive: Microgest® Thailand (HC), on the reproductive biology of *C. megacephala*. Each beige tablet contained levonorgestrel at 0.15 mg and ethinylestradiol at 0.03 mg.

Two different doses of human contraceptive were examined and the concentrations were prepared as follows:

0.036 mg/ml HC: 10 beige tablets were homogenized with 50 ml of 10% (w/v) sucrose solution by using a magnetic stirrer.

0.072 mg/ml HC: 20 beige tablets were homogenized with 50 ml of 10% (w/v) sucrose solution by using a magnetic stirrer.

Control: The control solution consisted of a 10% (w/v) sucrose solution only.

The overall study design consisted of a series of three experiments which examined the effects of feeding the human contraceptive on males only (Experiment I), females only (experiment II), and to both males and females (Experiment III).

5.2 Experiment I: Effect of human contraceptive fed to male *C. megacephala*

On the first day of eclosion adult flies were moved from rearing cages and placed into treatment cages (30×30×30 cm^3) and divided into six groups (100 flies/group) which were subsequently provided the food treatments, as shown in Table 1. This food was provided for 7 days. After 7 days, ten flies of each group were randomly collected and dissected in the same manner as previously described in part 2.1.1 in order to obtain the reproductive organs for SEM and TEM investigations. In addition, ten flies from each female group were collected and dissected to examine the number of eggs in the ovaries. The reason that the dissection and enumeration of ovarioles was chosen to determine fecundity this was considered a more accurate method when compared to enumerating eggs laid due to the difficulty involved in defining a blowfly egg mass.

In addition to determining direct effects of the human contraceptive on the flies matings were conducted between the treatment groups to determine female fecundity. Three groups of males and females were created by pairing them as follows: males (group 1) + females (group 4), treated males with 0.036 mg/ml (group 2) + untreated females (group 5), and treated males with 0.072 mg/ml (group 3) + untreated females (group 6). Each combined group was provided with 50 ml of sucrose solution and a piece of fresh pork liver (≈10 g), until eggs were found. After that, the eggs of each combined group were reared separately as previously described in part 1.

Additionally, developmental changes in each combined group were observed for assessing the possible effects of human contraceptive on this fly. Observations

included failure of larvae to molt normally, failure of larvae to form pupae, and inability of flies to complete adult eclosion. Any developmental changes observed in each stage were morphologically examined using SEM.

Following adult eclosion, the first progeny of each combined group were provided with sucrose solution and fresh pork liver, as previously described. Subsequently, 30 of 5 to 7-day-old male and female flies (F_1 progeny of each combined group) were dissected for observation of their reproductive organs using SEM, as described in part 3. Likewise, the reproductive organs of males (testes, seminal ducts, accessory glands and ejaculatory duct) and females (ovaries, spermathecae, oviducts and accessory glands) of the F_1 progeny were examined using TEM, as described in part 4. The criteria for cellular changes in each reproductive organ were described and analyzed following the descriptions of the authors in the literature review. In addition, the ultrastructural changes of the treated groups were compared with controls using SEM and TEM. Treatment with human contraceptive was conducted only with the parental or F_0 generation but observations on fecundity, SEM, and TEM were made for the three subsequent generations (F_1 to F_3).

Table 1 Conditions for treatment of adult *C. megacephala* in Experiment I: the effect of feeding human contraceptive to male flies only.

Group #	Caged flies	Treatment	Adult diet
1	100 males	Untreated	Sucrose + fresh pork liver
2	100 males	Treated	0.036 mg/ml HC+ sucrose + fresh pork liver
3	100 males	Treated	0.072 mg/ml HC+ sucrose + fresh pork liver
4	100 females	Untreated	Sucrose + fresh pork liver
5	100 females	Untreated	Sucrose + fresh pork liver
6	100 females	Untreated	Sucrose + fresh pork liver

5.3 Experiment II: Effect of human contraceptive fed to female *C. megacephala*

The procedure for rearing and treatment was the same as previously described in part 5.2, however, the six groups of flies were supplied food as shown in Table 2. Only female flies were fed with the human contraceptive treatment solutions. This resulted in the formation of combined treatment groups as follows: males (group 1) + females (group 4), untreated males (group 2) + treated females with 0.036 mg/ml HC (group 5), and untreated males (group 3) + treated females with 0.072 mg/ml HC (group 6). SEM and TEM investigations of each treated group and their F_1 progeny were also observed as previously described in part 5.2 with observations continued through the F_3 generation.

Table 2 Conditions for treatment of adult *C. megacephala* in Experiment II: the effect of feeding human contraceptive to female flies only.

Group #	Caged flies	Treatment	Adult diet
1	100 males	Untreated	Sucrose + fresh pork liver
2	100 males	Untreated	Sucrose + fresh pork liver
3	100 males	Untreated	Sucrose + fresh pork liver
4	100 females	Untreated	Sucrose + fresh pork liver
5	100 females	Treated	0.036 mg/ml HC+ sucrose + fresh pork liver
6	100 females	Treated	0.072 mg/ml HC+ sucrose + fresh pork liver

5.3 Experiment III: Effect of human contraceptive fed to male and female *C. megacephala*

The methods were the same as those previously described in part 5.2, but the six groups of flies were offered food as shown in Table 3. The resulting fly groups consisted of the following: untreated males (group 1) + untreated females (group 4), treated males with 0.036 mg/ml HC (group 2) + treated females with 0.036 mg/ml HC (group 5), and treated males with 0.072 mg/ml HC (group 3) + treated females with 0.072 mg/ml HC (group 6). SEM and TEM studies of each treated group and their F_1 progeny were performed as previously described in part 5.2 and replicated for the following two generations (F_2 and F_3).

Table 3 Conditions for treatment of adult *C. megacephala* in Experiment III: the effect of feeding human contraceptive to both female and male flies**.**

Group #	Caged flies	Treatment	Adult diet
1	100 males	Untreated	Sucrose + fresh pork liver
2	100 males	Treated	0.036 mg/ml HC+ sucrose + fresh pork liver
3	100 males	Treated	0.072 mg/ml HC+ sucrose + fresh pork liver
4	100 females	Untreated	Sucrose + fresh pork liver
5	100 females	Treated	0.036 mg/ml HC+ sucrose + fresh pork liver
6	100 females	Treated	0.072 mg/ml HC+ sucrose + fresh pork liver

In conclusion, Experiment I, II and III of efficacy of human contraceptive on *C. megacephala* are diagrammatically shown in Figures 7, 8 and 9, respectively.

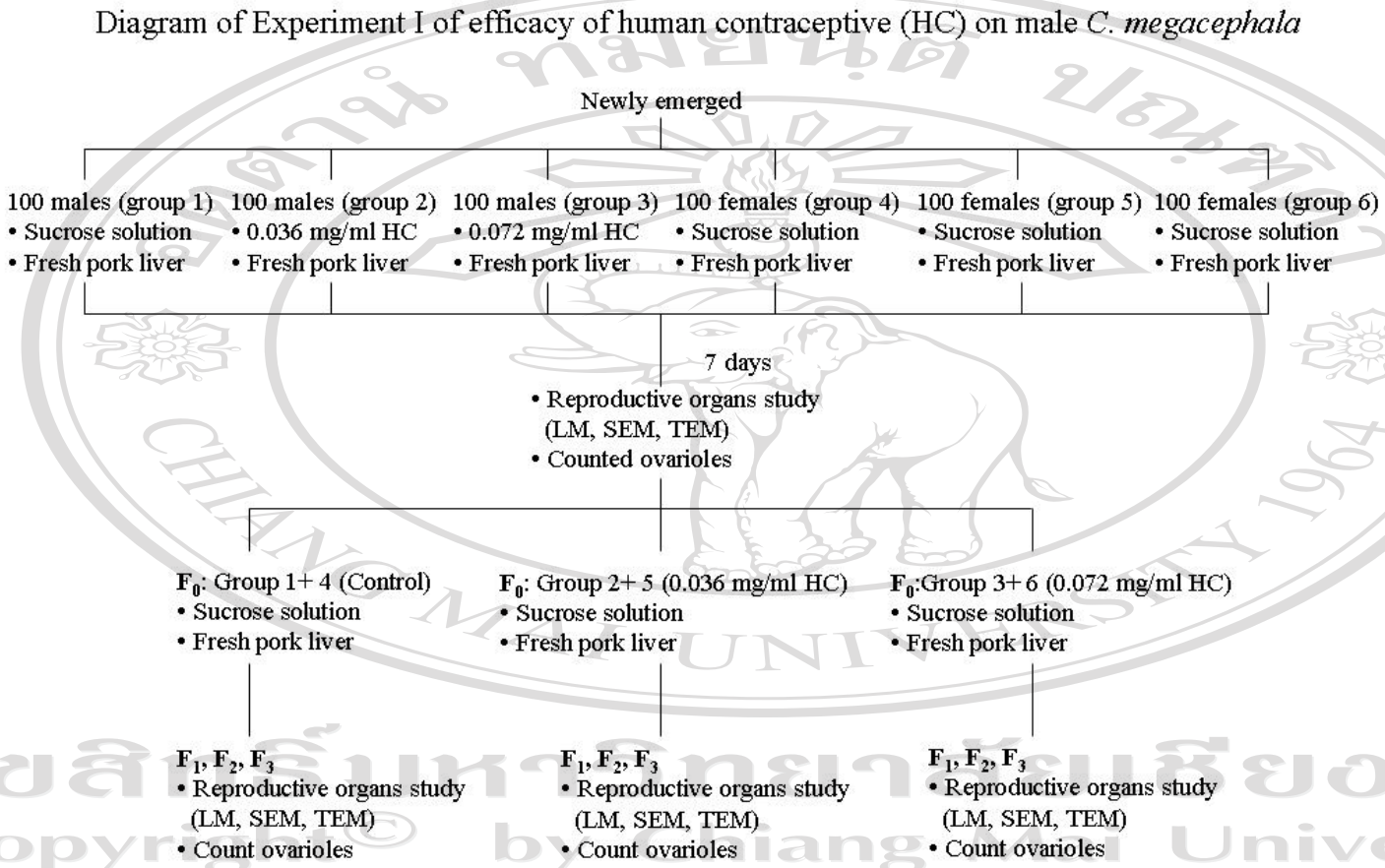

Figure 7 Diagram of Experiment I of efficacy of human contraceptive on male *C. megacephala*.

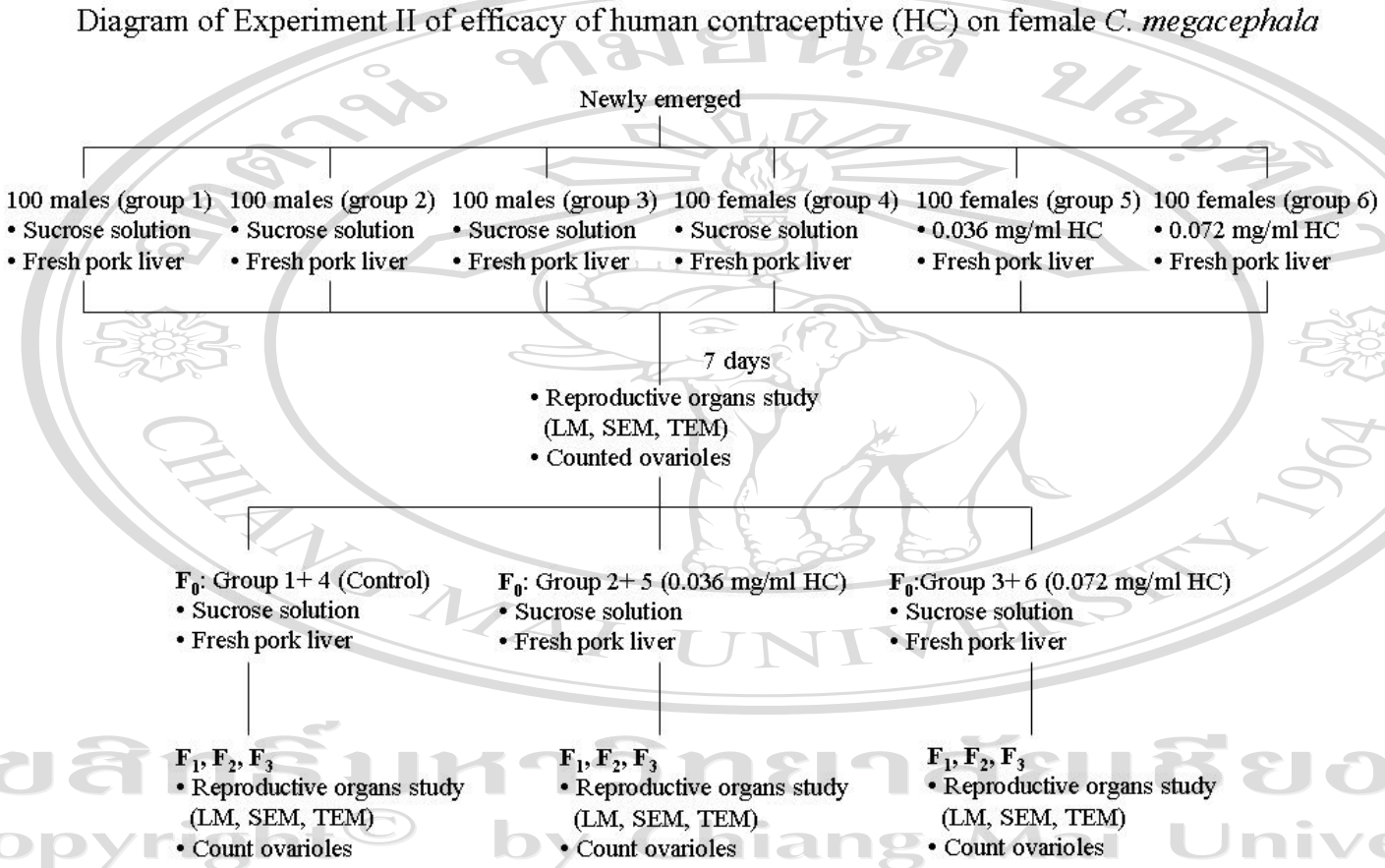

Figure 8 Diagram of Experiment II of efficacy of human contraceptive on female *C. megacephala*.

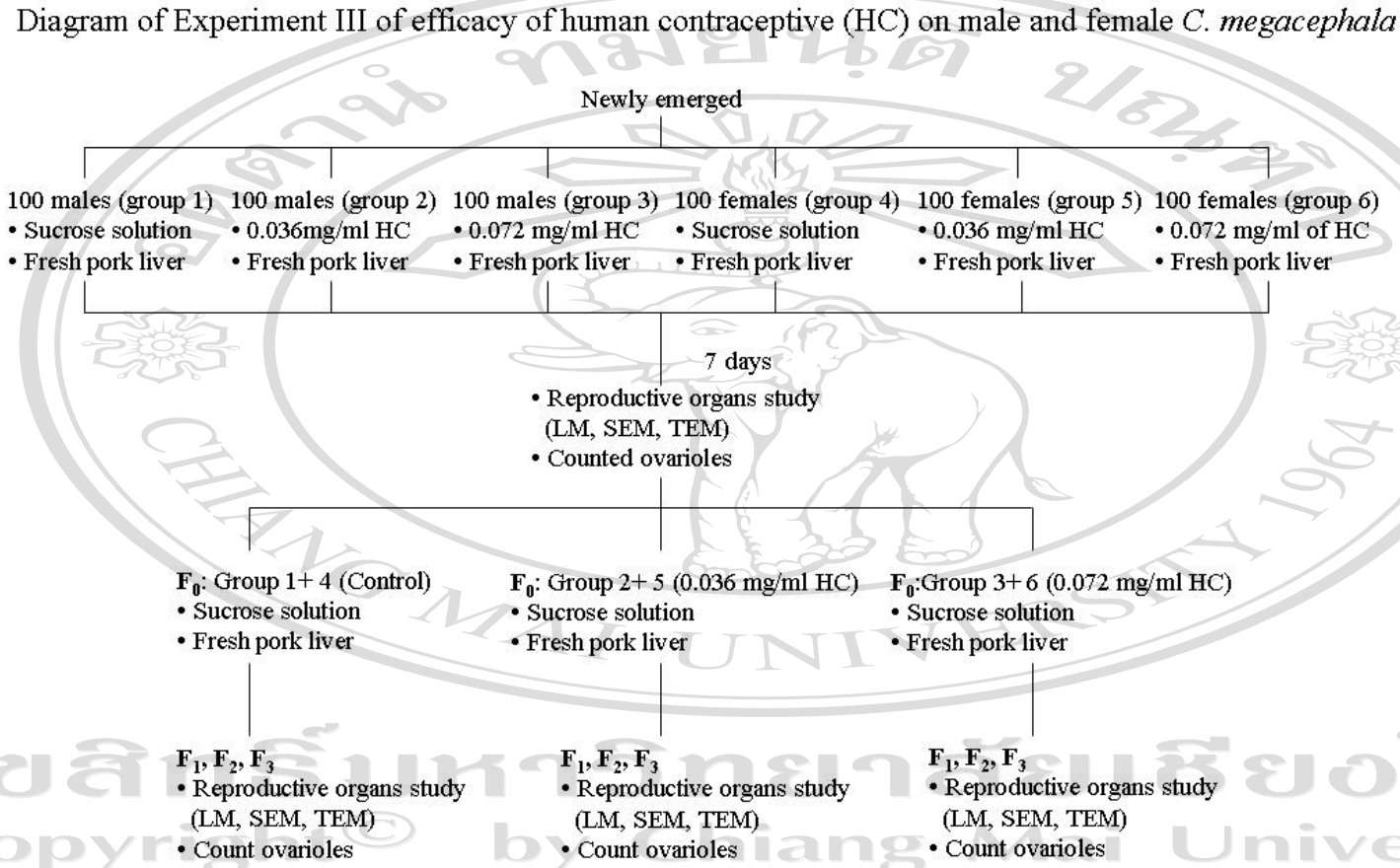

Figure 9 Diagram of Experiment III of efficacy of human contraceptive on male and female *C. megacephala*.

6. STATISTICAL ANALYSIS

The ultrastructural changes of the fly larvae, pupae, and adults under SEM and TEM were mostly qualitative and thus were described in the text.

For analysis and comparisons of morphological data (length and width data) a paired *t*-test was utilized. Plots depicting the change in morphometric characters over time were generated with the use of SigmaPlot version (9.0). Medians were plotted and range bars were used to illustrate differences in the development between sides if observed.

Analysis of fecundity data for the experiments involving the effects of human contraceptive were conducted using the Kruskal-Wallis analysis of variance. This approach was utilized 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.

For all experiments the α-error was set at 0.05, and statistical tests were performed using SPSS version 14.0.1.