CHAPTER 3

MATERIALS AND METHODS

1. Study on the prevalence of *Haplorchis taichui* infections in cyprinoid fish in Chiang Mai Province

1.1 Equipment and Materials

1. Compound and stereo microscope with ocular micrometer
2. Shaking water bath
3. Staining jar
4. Glass slides and cover slips
5. Scissors, needle, forceps and brush
6. Blender
7. Sieve tubes
8. Beakers and Petri dishes
9. 100% pineapple juice
10. 0.85% normal saline solution
11. 1% acid pepsin powder (Sigma)
12. Ethyl alcohol serial solution: 10%, 20%,30%,50%,70%,85%,95%, and absolute
13. Xylene
14. Permount
15. Staining dyes: borax carmine, haematoxylin, eosin and fast green

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1.2 Studying for the prevalence of *Haplorchis taichui* infections in cyprinoid fish in Chiang Mai Province

1.2.1 Cyprinoid fish, the common intermediate host of *H. taichui*, were collected from Wangpan Dam (Figure 4), Chom Thong District and Mae Ngad Somboonchon Reservoir (Figure 5), Mae Tang District, Chiang Mai Province. A year-round examination for metacercaria-parasitizing fish was monthly performed from November 2001 to October 2002.

1.2.2 Fish were obtained from the local fishermen and placed in ice boxes, then transported to the laboratory. All fish were identified by examination of fresh specimens using the illustrations in Smith (1945), Department of Fisheries, Ministry of Agriculture and Co-operative (2003), and Wittayanon (2003).

1.2.3 Each fish was separated into 5 parts: head, gills, scales, fins separated muscles (all of fins: dorsal, pectoral, pelvic, anal, and caudal) and muscles of the body. Fish were digested by 1% acid pepsin solution (1g of fish : 10 ml of pepsin solution) at 37 °C for 2 hours. The digested material was passed through graded sieves and rinsed with 0.85% normal saline solution, then examined for metacercariae under a stereo microscope.

1.2.4 The number of metacercariae were recorded. The monthly data of prevalence was compared between the three seasons (November 2001 - February 2002 = cool, March 2002 - June 2002 = summer, and July 2002 – October 2002 = rainy) and the intensity of metacercariae infections on each part of the fish body were analyzed by ANOVA.

1.2.5 Some specimens of encysted metacercariae were excysted by 100% pineapple juice for 3 hours at 37°C.

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1.2.6 Specimens were fixed in 5% formalin under a cover slip pressure, then stained with borax carmine or haematoxylin, counterstained with eosine and fast-green, dehydrated in alcohol series (10%, 20%, 30%, 50%, 70%, 85%, 95% and Absolute ethanol), cleared in xylol, and mounted in permount. The metacercariae were morphologically identified based on Yamaguti (1958), Pearson (1964) and Pearson and Ow-Yang (1982).

2. Study of the life history of *Haplorchis taichui* by experimental infection to various hosts

2.1 Equipment and Materials

1. Bearmann's apparatus
2. Aquarium, tanks and air pumps
3. Autoclave
4. Centrifuge
5. Incubator
6. Passture pipete
7. Gauze
8. 0.05% neutral red
9. 5%, 10% formalin
10. Ether
11. 1% iodine solution
12. Animals serving as experimental hosts: mice (*Mus musculus*, chicks (*Gallus gallus domesticus*), cyprinoid fish (*Barbodes gonionotus*), snail (*Tarebia granifera*)

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2.2 Life history studies

Definitive host infections:

2.2.1 Metacerariae of *H. taichui* were collected from naturally infected fish (*Henicorhynchus siamensis*) which are common widely spread in Chiang Mai (Figure 6). Fish were digested by 1% acid pepsin solution. The digested material was passed through graded sieves, rinsed in 0.85% saline solution, then collected for the metacercarial stage under a stereo microscope.

2.2.2 Chick (*Gallus gallus domesticus*) (Figure 7) and ICR mice (*Mus musculus*) (Figure 8) were used as the experimental definitive hosts. Two week-old chicks, 54 in number, weighing between 30-45 g were used as the experimental definitive host. Chicks were purchased from P. Asiapasusat, a merchant in Chiang Mai. Mice were purchased from Mahidol University, Salaya, Nakornpathom. Six to eight week-old mice, 15 in number, weighing between 23-30 g. Control animals for each experiment were autopsied and found to be negative. Two hundred encysted metacercariae, actively moving inside, were forced fed to each chick and mouse.

2.2.3 A chick was sacrificed daily at days 1-54 post-infection (PI) for examination of the parasite, while a mouse was sacrificed daily at days 1-15 PI. The small intestine, roughly divided into the duodenum, jejunum, and ileum, and then opened in 0.85% NaCl solution. In chicks, duodenum was cut in the area of the duodenal loop, jejunum was cut at level with the remnant of the yolk sac, and ileum was cut at the caeca. In mice, duodenum were cut in the area of the duodenal loop, the jejunum and ileum was divided into two parts in equal length.

2.2.4 Worms were collected in a modified Baermann's apparatus (Beaver *et al.*, 1984) (Figure 9), then examined under a stereo microscope.

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2.2.5 The number of worms was recorded. The collected flukes were fixed in 5% formalin under a cover slip pressure and made into permanent slides. The development and the morphological details of the worms were observed from living specimens and permanent slides under a light microscope.

Stool examination

Modification of the formalin-ether concentration technique was used for examine *H. taichui* eggs from chicks and mice. To know the best time to get embryonated eggs from fluke. Preliminary study was observed after infecting 200 metacercariae in chicks and mice, eggs in feces could not be seen because sometimes only few flukes infected and released a few eggs which difficult were detected.

Three chicks and three mice were infected with approximately 1,000 metacercariae. Feces was collected from chicks and mice during 5-14 days PI. The formalin-ether concentration technique involves filtering a small sample of host feces through two layers of gauze, washed, and centrifuged with formalin and ether (7 ml of 10% formalin : 3 ml of ether). The feces was mixed with 1% iodine solution on a glass slide, then the eggs of worm were observed under a light microscope.

Collection of the embryonated eggs:

Metacerariae of *H. taichui* were isolated from natural infections. One thousand metacercariae were forced fed to each chick. These chicks were sacrificed after 9 days PI after the worm developed and produced mature eggs, when the embryonated eggs were fully developed. The small intestine was opened in 0.85% saline solution and adults were collected by the Baermann's apparatus. Active worms were dissected by using needle and crushed under a cover slip, then collected eggs of worms under a stereo microscope. The embryonated eggs and miracidium already developed were

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seen in yellow-brown color. The eggs were washed in distilled water to use for infecting snails.

First intermediate host infection:

Freshwater snails, *Tarebia granifera* (Figure 10) were obtained from the Department of Fisheries Technology, Maejo University, Chiang Mai. One hundred snails were found negative. All snails used in the experimental infections were laboratory reared in aerated tanks and fed with alginate food, *Spirogyra* sp. and *Cladophora* sp. They were kept in an aquarium about six months before being used. Snails 1.0-2.1 cm in length were used for parasitic infections. A total of 360 snails were used for experimental infections with embryonated eggs, 30 individauls were use in each experiment. Embryonated eggs were collected from adults 9 days PI and washed with distilled water in a small Petri dish. Snails were kept for 12 hours in these small Petri dishes which contained numerous fluke' eggs which were ingested. Thirty nails were killed after 1, 2, 3, 4, 5, 6, 7, 10 and 14 days to find the larval stage of *H. taichui*. A total of 270 snails were crushed, the head region was removed, and the body examined under a cover slip. The natural shedding of mature cercariae were examined by keeping them in a small bottle overnight and observing them in the morning every two days after 5, 6, and 7 weeks PI, all of 90 snails were crushed to observe the cercarial stage. Some specimens of larval stage were observed under a stereo microscope and stained with 0.05% neutral red for the fresh samples and some worms were fixed with 5% formalin and stained with borax carmine or haematoxylin.

Second intermediate host infection:

Young fish (*Barbodes gonionotus*) 3-5 cm in length (Figure 11) were obtained from the Department of Fisheries Technology, Maejo University, Chiang Mai. Thirty

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fish were used for cercarial infections. Freely swimming cercariae were obtained from experimentally infected snail hosts 7 weeks PI. Thirty cercariae were experimentally infected to each fish by keeping them together in a small bottle for 6 hours and then transferred to an aerated aquarium. Three infected fish were observed daily for 10 days after infection to examine encysted metacercariae. Developmental changes were recorded. Some specimens were permanently mounted after staining in borax carmine or haematoxylin.

3. Study on the surface ultrastructure of *Haplorchis taichui* infective stage and adult.

3.1 Equipment and Materials

1. Scanning electron microscopes (SEM) model JEOL-JSM 840A and JEOL-JSM2001LV
2. Sputtering coater
3. Critical Point Dryer
4. Stubs and carbon sticky tape
5. 2.5% glutaraldehyde
6. 1% osmium tetroxide
7. 0.1 M phosphate buffer (pH 7.3)
8. 0.85% normal saline
9. Ethyl alcohol serial solution: 10%, 20%,30%,50%,70%,85%,95%, and absolute
10. Acetone

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3.2 Studying the tegumental surface of *Haplorchis taichui* infective stage and adult

3.2.1 Metacercariae of *H. taichui* were collected from the muscles of the cyprinoid fish (*Henicorhynchus siamensis*) by an artificial digestion technique (1% acid pepsin solution) and some worms were excysted in 100% pineapple juice at 37 °C for 3 hours. Adult flukes were collected from infected chicks 7 days PI.

3.2.2 The excysted metacercariae and adult flukes were washed in 0.1 M phosphate buffer (pH 7.3) for several times. For scanning electron microscopic observations, worms were fixed in 2.5% glutaraldehyde at 4°C for 24 hours and post fixed with 1% osmium tetroxide for 2 hours. They were dehydrated in a graded series of ethanol (10%, 20%, 30%, 50%, 70%, 85%, 95%, and absolute), then transferred into acetone, and finally dried in a critical-point dryer. The flukes were mounted on stubs, gold-coated and observed with JEOL-JSM840A and JEOL-JSM2001LV scanning electron microscope at accelerating voltage of 10-15 KV.

4. Study on anthelmintic drug *in vitro*

4.1 Equipments and Materials

1. Scanning Electron Microscope (SEM) model JEOL-JSM2001LV and Daul Stage DS-130C
2. Transmission Electron Microscope (TEM) model JEOL JEM-2010
3. Ultramicrotome
4. Glass knife maker
5. Glass knife
6. Mold block

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7. Storage grid
8. Copper grid (200 mesh)
9. Tyrode's solution
10. Niclosamide tablet (Hexin®, Thailand)
11. Ethyl alcohol serial solution: 10%, 20%,30%,50%,70%,85%,95%, and absolute
12. Acetone
13. Xylene
14. Spurr's kit
15. 1% Tuloidine blue
16. Uranyl acetate
17. Lead citrate

4.2 Studying the effect of niclosamide on *Haplorchis taichui* (*in vitro*)

4.2.1 Encysted metacercariae of *H. taichui* used to infecting chicks were obtained from naturally infected cyprinoid fish, *Henicorhynchus siamensis*. Encysted metacercariae were isolated by 1% acid pepsin solution. Three days old chicks (*Gallus gallus domesticus*) were fed with approximately 1,000 metacercariae. At the end of the seventh days PI, adult worms were collected from their small intestines by the Bearmann's technique.

4.2.2 Niclosamide (Hexin,® Thailand) ; 1 tablet included with 500 mg niclosamide and 75 mg phenolphthalein were made to various concentrations. The stock of niclosamide was prepared by dissolving 500 mg niclosamide in 5 ml ethanol and diluted with distilled water into 50 µg/ml. Niclosamide was mixed with Tyrode's solution in various concentrations; 0.01, 0.1, 1.0 and 10.0 µg/ml.

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4.2.3 The adult worms were washed in Tyrode's solution and immediately used for the experiment. In each experiment, 5 ml of solution and one hundred active worms were put in each Petri dish and incubated at 37°C, including control group which was incubated in Tyrode's solution for 30 minutes, 1, 6, 12 and 24 hours.

4.2.4 Worms were washed three times in 0.1M phosphate buffer (pH 7.3). The actively moving and dead worms were observed under a stereo microscope and recorded.

4.2.5 For scanning electron microscopy, specimens were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated in a graded ethanol series and acetone, transferred to acetone, mounted on stubs, and coated with gold. Coated specimens were observed with a Daul stage DS-130C and JEOL-JSM2001LV scanning electron microscope at 10-15 KV.

4.2.6 For transmission electron microscopy, specimens were dehydrated in a graded ethanol series and acetone, transferred to acetone: Spurr's resin 2:1, 1:1, 1:2 and complete Spurr's resin. Specimens were embedded with pure Spurr's resin in a mold block, then cured in a incubator at 60°C for 48 hours. The posterior part (posterior to the ventrogenital sac) of worms were cut on a Reichert Ultracut ultramicrotome. Thick sectioning, the roughly trimed blocks were mounted into the ultramicrotome and 0.5 μm sections were cut with a glass knife, adhesived on slide glass, and stained with a drop of 1% tuloidine blue. Ultrathin sections were cut at 90 nm, mounted on 200 mesh copper grids, and double-stained with uranyl acetate and lead citrate. The sections were examined under a JEOL JEM-2010 electron microscope at an accelerating voltage of 80 KV.

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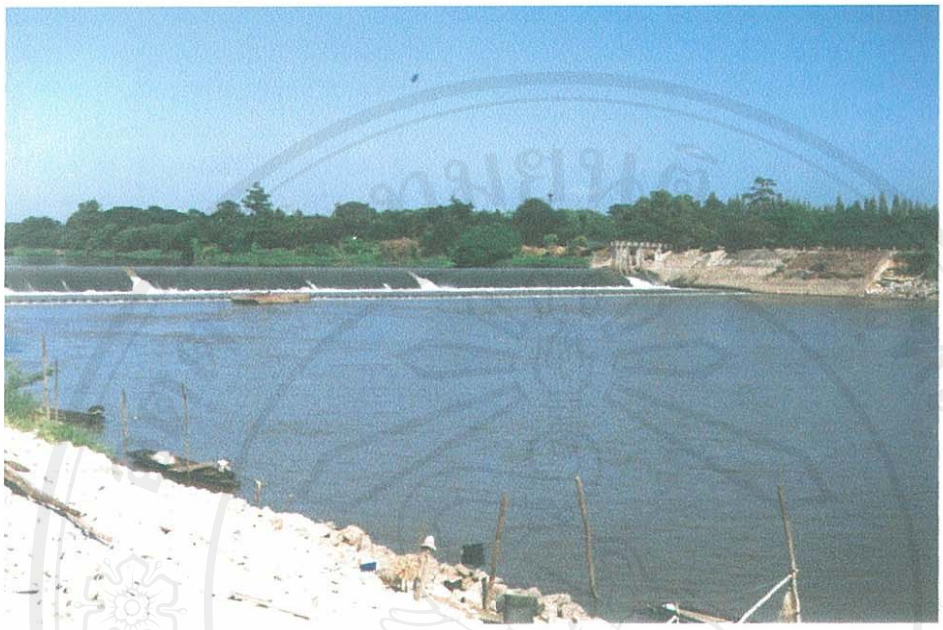

Figure 4. Wangpan Dam, Chom Thong District, Chiang Mai Province.

Figure 5. Mae Ngad Somboonchon Reservoir, Mae Tang District, Chiang Mai Province.

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Figure 6. The naturally infected fish, *Henicorhynchus siamensis*.

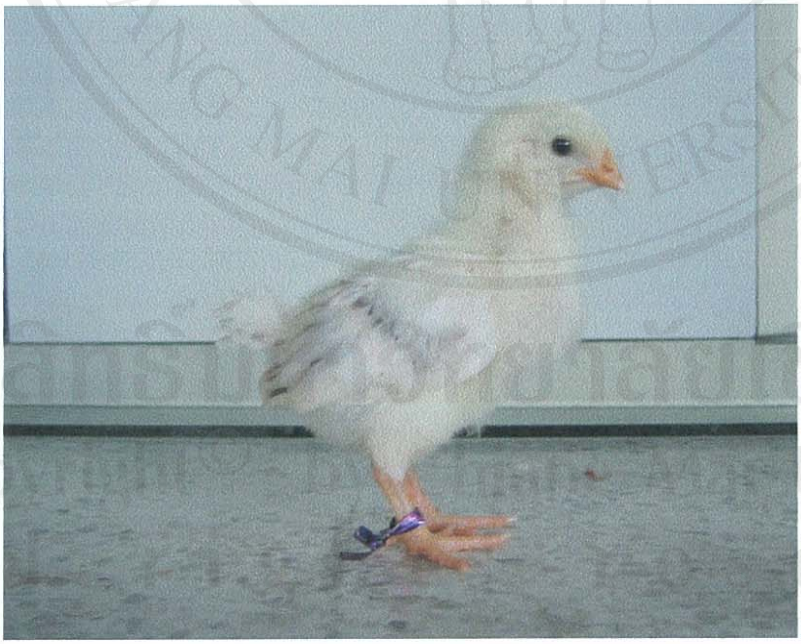

Figure 7. The experimental definitive host, chick (*Gallus gallus domesticus*).

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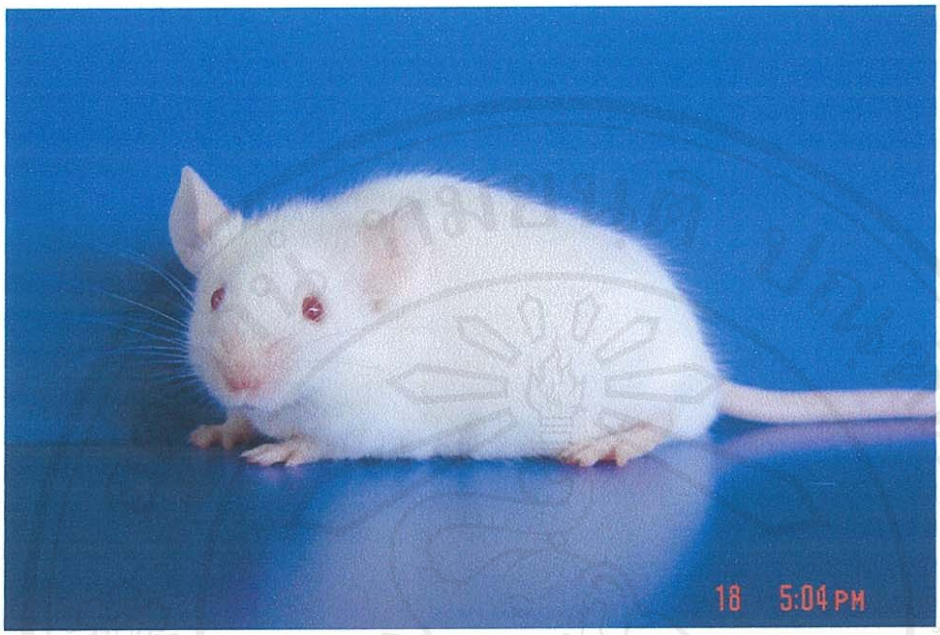

Figure 8. The experimental definitive host, ICR mouse (*Mus musculus*).

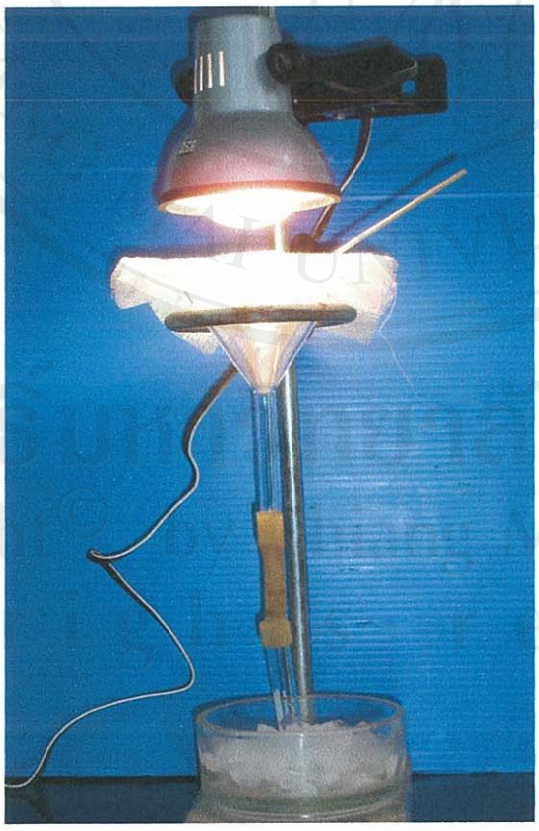

Figure 9. Bearmann's apparatus.

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Figure 10. The experimental first intermediate host, snail (*Tarebia granifera*).

Figure 11. The experimental second intermediate host, cyprinoid fish (*Barbodes gonionotus*).

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