CHAPTER 4

RESULTS

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

Cyprinoid fish were collected from Wangpan Dam, Chom Thong District and Mae Ngad Somboonchon Reservoir, Mae Tang District, Chiang Mai Province. Fish were monthly examined during November 2001 to October 2002. Six hundred and seventeen fish of 15 species were investigated for *H. taichui* metacercariae. The result showed that 540 of 617 fish were infected with *H. taichui* and other heterophyid metacercariae, Among the fish collected, *Henicorhynchus siamensis* was the most predominantly found (24.80%; 153/617) while 2 species of fish were found in the smallest number, *e.g. Amblyrhynchichthys truncatus* and *Cyclocheilichthys armatus* (0.30%; 2/617) (Table 1, 2).

Four species of heterophyid metacercariae were found in cyprinoid fishes from both locatities; *Centrocestus caninus*, *Haplorchoides* sp., *Haplorchis pumilio* and *Haplorchis taichui*. The most abundant species was *H. taichui*, which was found in all species of fishes examined. Moreover, the result showed that *Haplorchoides* sp. was infected in a large number and predominant in some species of fishes while *C. caninus* and *H. pumilio* presented in a small number (Table 1, 2). The distribution of metacercariae in the fish body from both localities, Chom Thong and Mae Tang showed *H. taichui* predominant in muscles, *Centrocestus caninus* in gills, *H. pumilio* in muscles and *Haplorchoides* sp. in scales of fish (Table 3).

The prevalence of infection of *H. taichui* metacercariae in Chom Thong and Mae Tang Districts for a year-round survey are shown in Figure 12. In Chom Thong District, 266 of 290 fish in 14 species harbored metacercariae of *H. taichui* (Table 1). The prevalence of *H. taichui* was 91.70% (266/290), and the monthly prevalence of infections in a year round were 56.00-100.00% with a mean intensity of 242.93 metacercariae/fish (Table 1 and Figure 12). The highest prevalence was found in January, February, May, July and August and the lowest in April (Figure 12). The prevalence of infections did not differ significantly between the three seasons (cool = November-February, summer = March-June, rainy = July-October) ($p > 0.05$).

In Mae Tang District, 274 of 327 fish in 7 species harbored *H. taichui* metacercariae. The infection rate for *H. taichui* was 83.80% (274/327), and the monthly prevalence of infections were 54.20-93.10% with a mean intensity of 107.44 metacercariae/fish (Table 2 and Figure 12). The highest prevalence was found in October and the lowest in May (Figure 12). The prevalence of infections were compared between the three seasons. Significant differences were found during the summer season (March-June) and rainy season (July-October) ($p < 0.05$).

The present study showed that, in both localities, the muscles harbored the highest number of *H. taichui* metacecaria, followed by the head, fins, scales and finally the gills (Figures 13A-B). The number of *H. taichui* metacercariae were significantly different in the muscles, head and fins of parasitised fish ($p < 0.05$), but in the scales and gills did not significantly different ($p > 0.05$).

Table 1. The distribution of *Haplorchis taichui* metacercaria and other heterophyid metacercariae were found in 14 species of fish from Chom Thong District.

Fish species	Number of fish Infected/ examined	Number of metacercariae, mean (range)			
		Centrocestus caninus	*Haplorchis pumilio*	*Haplorchis taichui*	*Haplorchoides* sp.
Amblyrhynchichthys truncatus	2/2	0	0	29.50 (23-36)	197.50 (140-255)
Barbodes gonionotus	25/25	2.44 (0-17)	0.16 (0-4)	192.72 (0-814)	184.56 (0-1629)
Barbodes schwanenfeldi	13/13	0	0	109.00 (0-613)	28.38 (0-122)
Cyclocheilichthys armatus	2/2	0	0	76.50 (34-119)	352.50 (261-444)
Cyclocheilichthys repasson	13/13	0	0	11.54 (0-26)	617.54 (70-2054)
Henicorhynchus siamensis	91/93	0.72 (0-15)	0.12 (0-4)	391.57 (0-2248)	12.19 (0-102)
Labiobarbus siamensis	33/34	0.18 (0-3)	0	68.97 (0-327)	41.03 (0-184)
Mystacoleucus marginatus	37/37	0.24 (0-5)	0	532.78 (8-3165)	1055.68 (52-2872)
Osteochilus hasselti	3/5	0	0	2.60 (0-9)	0
Paralaubuca barroni	8/8	0	0	16.75 (2-44)	
Puntioplites proctozysron	37/42	0.67 (0-8)	0	27.00 (0-180)	97.12 (0-644)
Raiamas guttatus	3/3	0	0	990.00 (648-1356)	
Rasbora tornieri	2/3	0	0	0.67 (0-2)	13.67 (0-23)
Systomus orphoides	10/10	1.60 (0-7)	0	112.70 (0-264)	227.60 (42-639)
Total	266/290	0.64 (0-17)	0.05 (0-4)	242.93 (0-3165)	214.12 (0-2872)

Table 2. The distribution of *Haplorchis taichui* metacercaria and other heterophyid metacercariae were found in 7 species of fish from Mae Tang District.

Fish species sp.	Number of fish Infected/ examined	Number of metacercariae, mean (range)			
		Centrocestus caninus	*Haplorchis pumilio*	*Haplorchis taichui*	*Haplorchoides*
Barbodes gonionotus	44/55	1.13 (0-17)	0	30.42 (0-496)	8.62 (0-127)
Hampala macrolepidota	37/60	0.08 (0-3)	0	23.20 (0-447)	12.18 (0-70)
Henicorhynchus siamensis	57/60	0.38 (0-7)	0.05 (0-2)	403.87 (0-1731)	9.23 (0-197)
Labiobarbus siamensis	46/48	0.14 (0-7)	0.02 (0-1)	29.65(0191)	23.48 (0-135)
Mystacoleucus marginatus	18/18	0.11 (0-2)	0	289.33 (2-2131)	387.72 (15-1492)
Puntioplites proctozysron	55/60	0.17 (0-6)	0	15.48 (0-126)	24.80 (0-148)
Systomus orphoides	17/26	0	0	10.58 (0-49)	28.80 (0-179)
Total	274/327	0.35 (0-17)	0.01 (0-2)	107.44 (0-2131)	37.01 (0-1492)

Table 3. Number of metacercariae in each part of fish body from Chom Thong and Mae Tang Districts.

Districts	Metacercariae species	Number of metacercariae in each part					Total
		Scales	Fins	Gills	Head	Muscle	
Chom Thong	*Centrocestus caninus*	0	0	185	2	0	187
	Haplorchis pumilio	0	0	0	7	8	15
	Haplorchis taichui	70	5,334	31	24,277	40,739	70,451
	Haplorchoides sp.	47,182	8,269	199	3,778	2,664	62,092
Total		47,252	13,603	415	28,064	43,411	132,745
Mae Tang	*Centrocestus caninus*	0	0	109	4	0	113
	Haplorchis pumilio	0	0	0	1	3	4
	Haplorchis taichui	34	3,525	10	11,284	20,279	35,132
	Haplorchoides sp.	7,968	2,619	20	1,021	480	12,108
Total		8,002	6,144	139	12,310	20,762	47,357

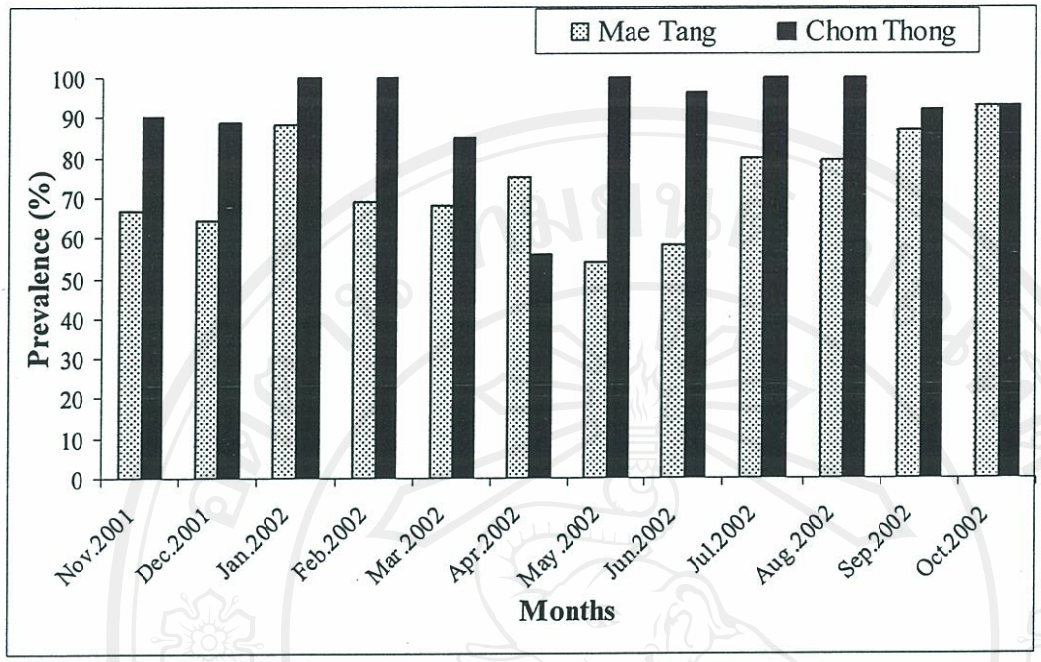


Figure 12. Monthly infection rates of *Haploschis taichui* metacercariae (for a year-round) in Chom Thong and Mae Tang Districts, Chiang Mai.

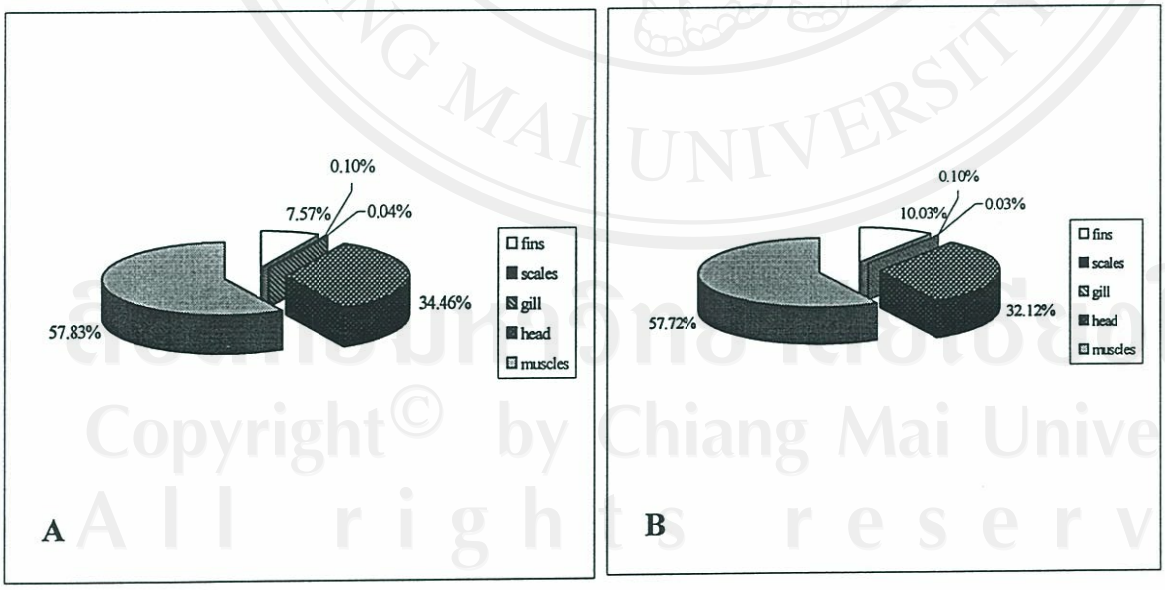


Figure 13. Occurrence of *Haploschis taichui* metacercariae in various parts of the fish body from both localities, A) Chom Thong District and B) Mae Tang District.

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

The recovery rate of *H. taichui* adult was performed in chicks and mice. Two hundred encysted metacercariae were forced fed to each chick and mouse. One chick was sacrificed daily at days 1-54 post-infection (PI). A mouse was sacrificed daily at days 1-15 PI. The worms were collected from the small intestines of chicks and mice.

Worm recovery in chicks

Metacercariae of *H. taichui* developed into mature adults in chicks by 3 days PI. Worms were recovered in the small intestines. On day 1 PI, one worm was found in the duodenum, but on day 2 PI and later the worms were predominantly recovered from the jejunum and ileum. After days 18 to 48 PI, the worms were found only in the ileum. The worm recovery was the highest at day 11 PI (29.5%), but decreased after day 16 PI (Figure 14). Moreover, one active metacercaria was observed in the ileum at day 11 PI. During days 1-54 PI, the majority of worms were collected from the ileum (85.57%); a few from the jejunum (14.33%), and the least in the duodenum (0.10%). The infection rate was 79.63% (43/54) and the mean intensity was 17.96 (970/54) worms/chick. The parasite was found to survive in chicks until day 48 PI.

Worm recovery in mice

H. taichui adults developed in mice after 3 days PI. On day 1 PI, juveniles were found in the jejunum and ileum. After days 7 to 12 PI, the worms were found only in the ileum. The worm recovery was the highest at day 2 (25%) and decreased after day 7 PI (Figure 15). The majority of parasites were collected from the ileum (63.36%), followed by the jejunum (36.64%), but adults could not be found in the duodenum.

The infection rate was 66.67 (10/15) worms/mouse and the mean intensity was 17.46% (262/15).

In this study, the worm recovery in chicks was higher than in mice, thus chicks can serve as a more susceptible definitive host of *H. taichui*.

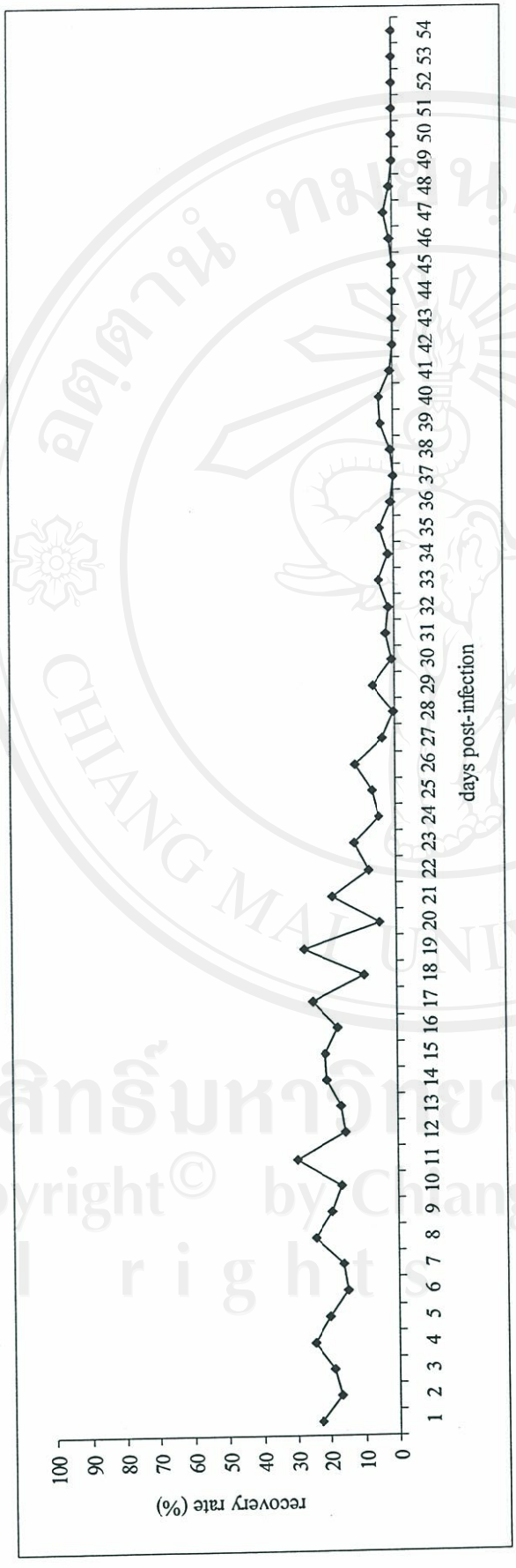


Figure 14. The recovery rates of *Haplorchis taichui* from chicks each experimentally infected with 200 metacercariae 1-54 days post-infection.

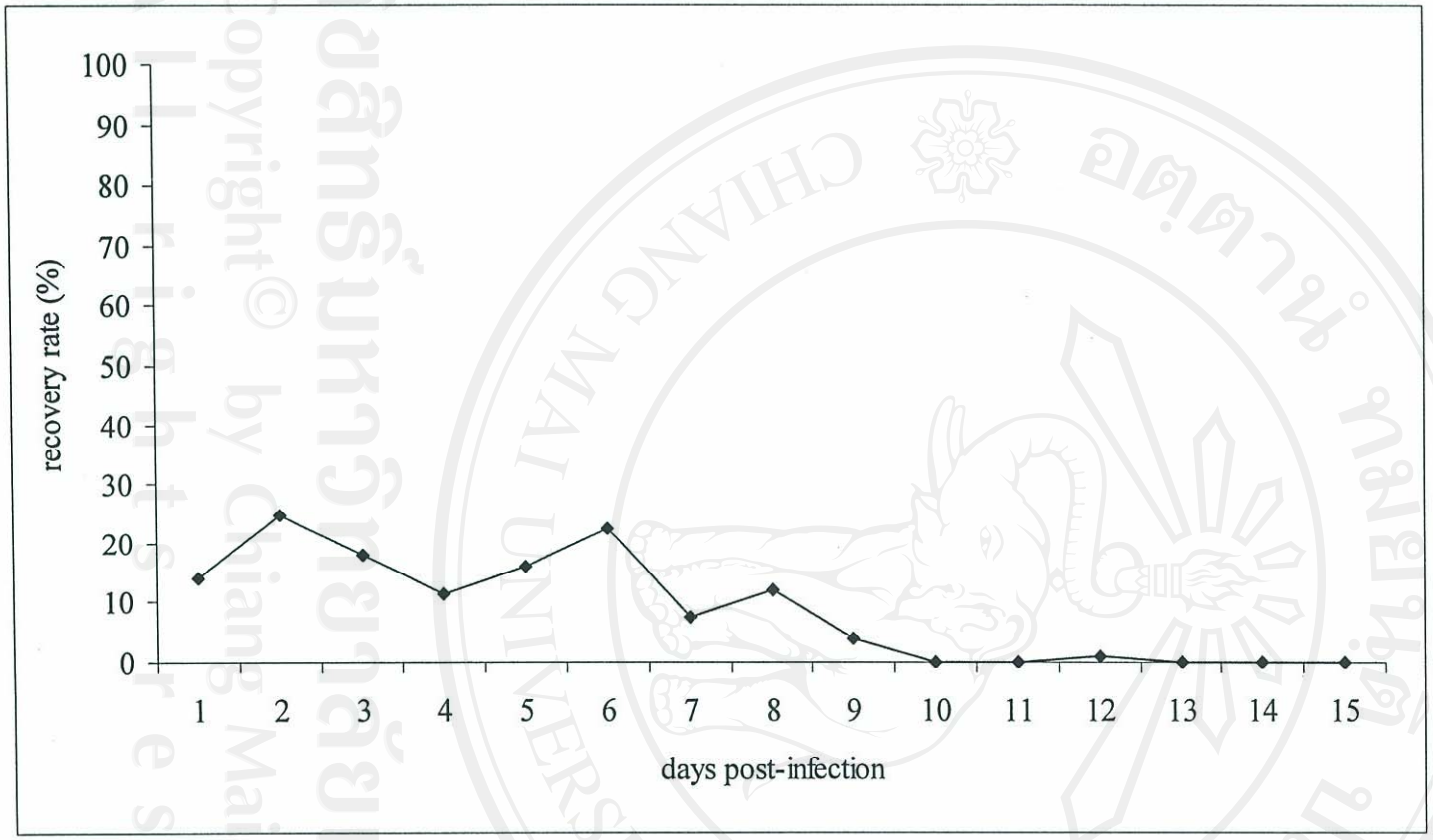


Figure 15. The recovery rates of *Haplorchis taichui* from mice each experimentally infected with 200 metacercariae 1-15 days post-infection.

Growth of *Haplorchis taichui* in chicks

Metacercariae of *H. taichui* were collected from a naturally infected fish, *Henicorhynchus siamensis*. Encysted metacercariae were 185-245 µm long and 200-300 µm wide. Cyst walls were 2.50-5.00 µm in thickness (Figure16). The newly excysted fluke was elongated and ovoid, 280-390 µm long and 110-175 µm wide. The ventrogenital sac was located ventrally in the middle of the body and armed with 16 sclerites. Several organs were rudimentary developed and only visible in some specimens. A single circular testis, 26-48 µm in diameter, was observed. The ovary was as a nearly circular or ovoid organ, situated posterior to the ventrogenital sac, approximately 10-30 µm in diameter (Figure 17).

On day 1 PI, the flukes differed from the metacercariae in the body size. The body was 350-470 µm long and 125-200 µm wide. The ovary was 10-45 µm in diameter. The testis was 30-50 µm in diameter (Figure 18).

On day 2 PI, visceral organs were more distinct. The body size was 400-580 µm long and 200-320 µm wide. The testis enlarged to 80-150 µm in diameter. The ovary was circular, 40-70 µm in diameter. The seminal vesicle was constricted in the middle and divided into two parts, while the uterus was developed in some specimens. Vitellariae could not be observed (Figure 19).

Mature worms were observed at day 3 PI, where the body size was 400-700 µm long and 200-360 µm wide. The testis enlarged to 80-165 µm in diameter. The ovary was circular, 40-85 µm diameter, and the seminal receptacle was greatly enlarged. Eggs were found in the uterus ranging from 1-300, only a few adults contained more than 100 eggs in their uteri. Eggs were 25.0-28.75 µm long and

12.5-16.25 μm wide. Vitellariae were distributed in the area from the anterior border of the ovary to the posterior extremity (Figure 20).

Definitive host infections

Adult worms were recovered in chicks (*Gallus gallus domesticus*). Modification of the formalin-ether concentration technique was used to examine *H. taichui* eggs from chicks. Egg of *H. taichui* was first found in feces at 9 days PI. Chicks were used for collecting adult worms, which contained embryonated eggs. The embryonated eggs were collected from adults 9 days PI which had miracidia developed inside. The embryonated eggs were 25.0-28.75 μm long and 12.5-16.25 μm wide (Figure 21). The miracidium is pyriform, 20.0- 25.0 μm long, and 7.0-10.0 μm wide (Figure 22).

First intermediate host infections

Embryonated eggs were collected from adults in chicks 9 days PI. Fluke's eggs were ingested by the snail, *Tarebia granifera*. Thirty nails were killed after 1, 2, 3, 4, 5, 6, 7, 10 and 14 days to find the larval stages of *H. taichui*. One of 270 snails was infected with a larval fluke. On the tenth day PI some sporocysts can be observed, but the details could not be studied because the specimens were damaged by crushing under a cover slip. Numerous young rediae developed in the digestive tract and tissue of the snail. The young mother redia is elongate, 47-65 μm long 16-27 μm wide and the pharynx diameter was 9-12 μm (Figure 23). The larval stage could not be observed by the shedding method. Ninety snails were crushed to observe the cercarial stage. One of ninety snails had two generations of rediae and cercariae developed 7 weeks (49 days) PI. The mother redia is elongate, 300-520 μm long, 70-140 μm wide, and the pharynx diameter was 20-35 μm (Figure 24). The

daugther redia is elongate, 330-680 μm long, 120-210 μm wide, and the pharynx diameter was 20-35 μm (Figure 25). The cercaria is a parapleurolophocercous. The body was 100-230 μm long and 50-120 μm wide. There are 7 penetration glands on each side of the body. The tail with a fin was elongate and it set in a socket of the body, 270-400 long, and 10-25 μm wide. It is provided with lateral fin folds in the anterior region and dorso-ventral fin folds posteriorly (Figure 26).

Second intermediate host infections

Freely swimming cercariae were obtained from experimentally infected snail hosts after 7 weeks PI. Thirty cercariae were given to each fish by keeping them together. Encysted metacercariae were found in the head and muscles of fish *Barbodes gonionotus*. Out of 30 fish, five were infected with *H. taichui* metacercariae. On the first day, five encysted metacercariae were found in two fish. The encysted metacercariae were 85-100 x 100-125 μm. The cyst walls were 2.5-4.0 μm thick (Figure 27). One metacercaria was found from the head of one fish on the third day PI. Immature metacercaria presented a fan-shaped, hollow spine in the middle region and the genital rudimentary organ could be observed. The encysted metacercariae were 158-162 x 175-183.5 μm (Figure 28). On day 6 PI, two metacercariae infected the head of the fish and one in the muscles. After six days metacercariae were fully developed and their rudimentary genital organ was distinctly observed (Figure 29). The encysted metacercariae were 210-232 x 275-295 μm, and cyst walls were 3-5 μm in thickness. One metacercaria was also found in the muscles of a fish on the ninth day PI. Metacercarial cysts were 180-185 x 250-252 μm and was similar 6 days PI in size and morphology.

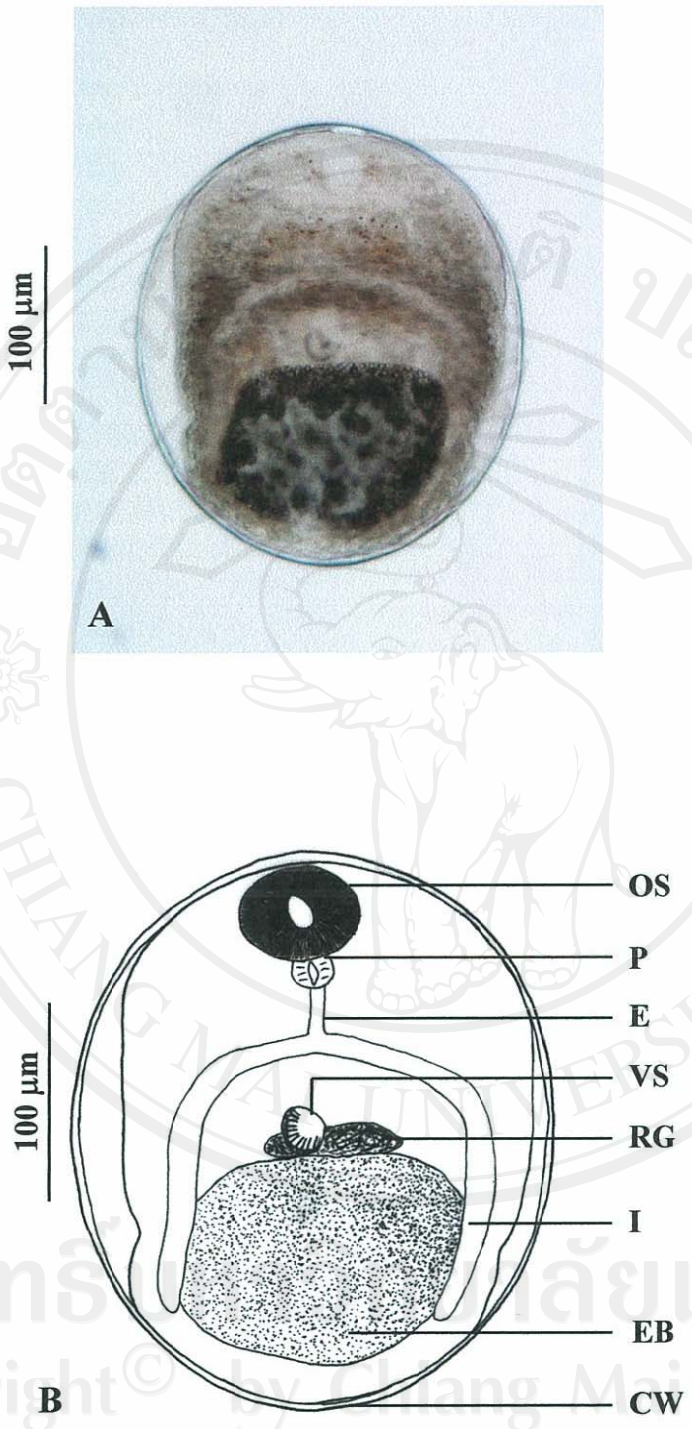


Figure 16. Encysted metacercaria of *Haplorchis taichui* from a naturally infected fish, *Henicorhynchus siamensis*. A. Photograph of an encysted metacercaria. B. Drawing of an encysted metacercaria.

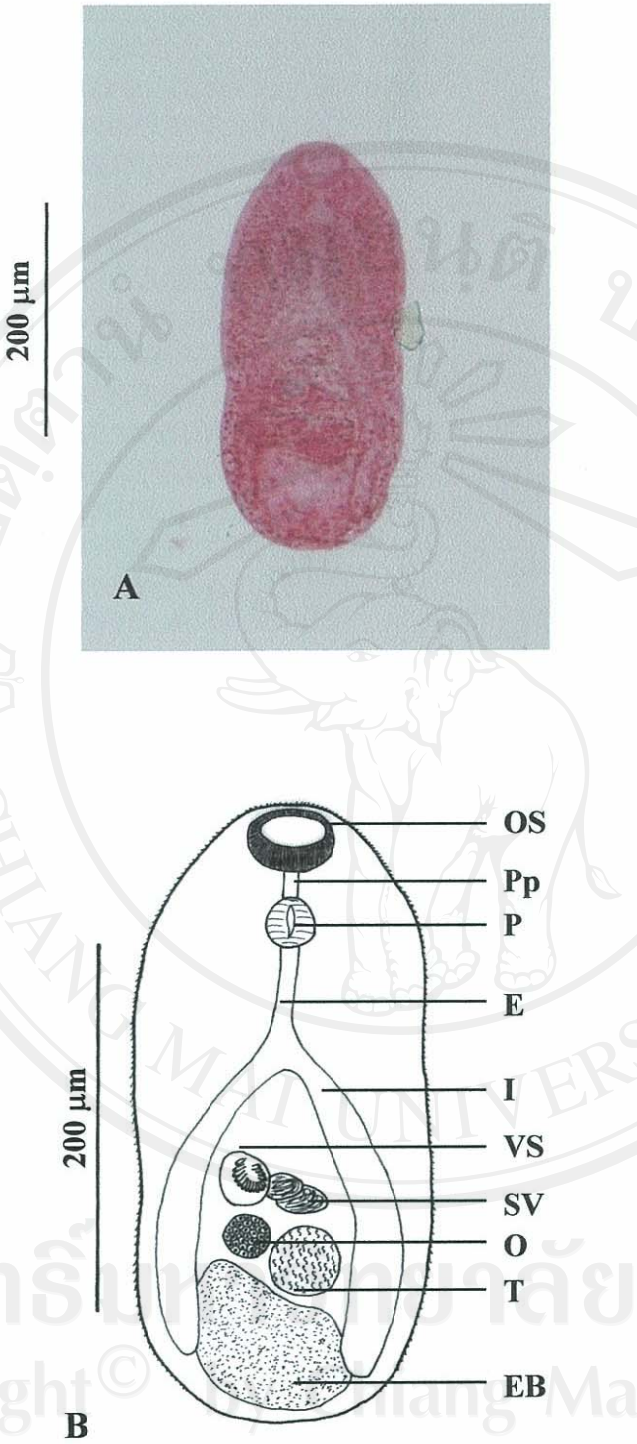


Figure 17. Excysted metacercaria of *Haplorchis taichui* from a naturally infected fish, *Henicorhynchus siamensis*. A. Photograph of an excysted metacercaria. B. Drawing of an excysted metacercaria.

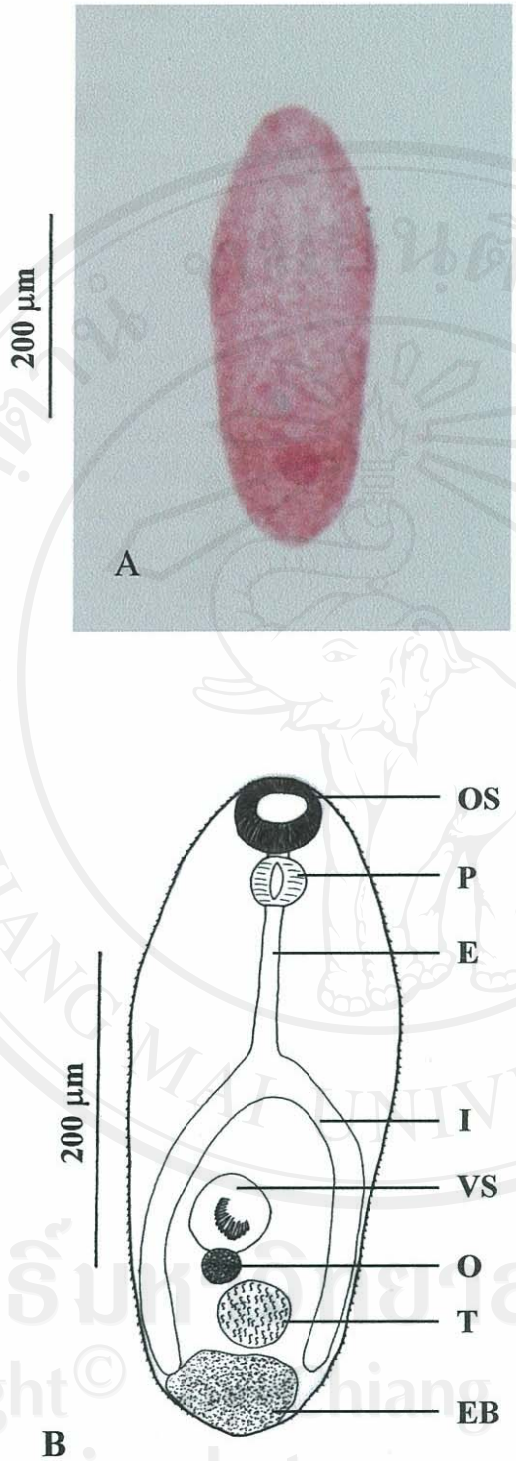


Figure 18. A juvenile of *Haplorchis taichui* 1 day PI in a chick. A. Photograph of a juvenile 1 day PI. B. Drawing of a juvenile 1 day PI.

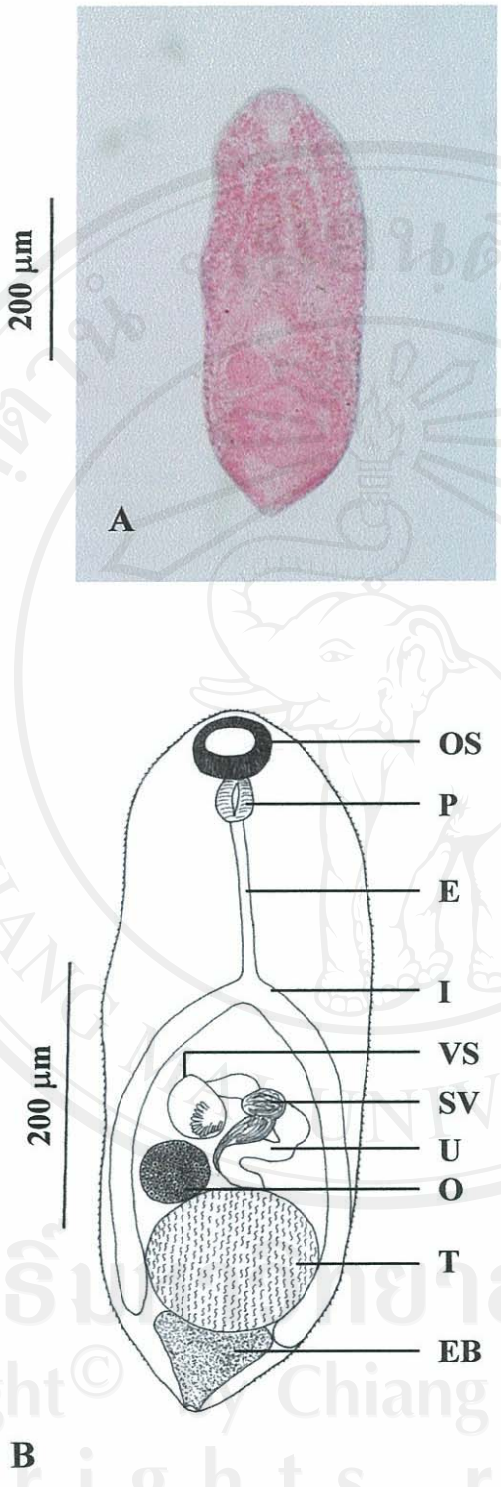


Figure 19. A juvenile of *Haplorchis taichui* 2 days PI in a chick. A. Photograph of a juvenile 2 days PI. B. Drawing of a juvenile 2 days PI.

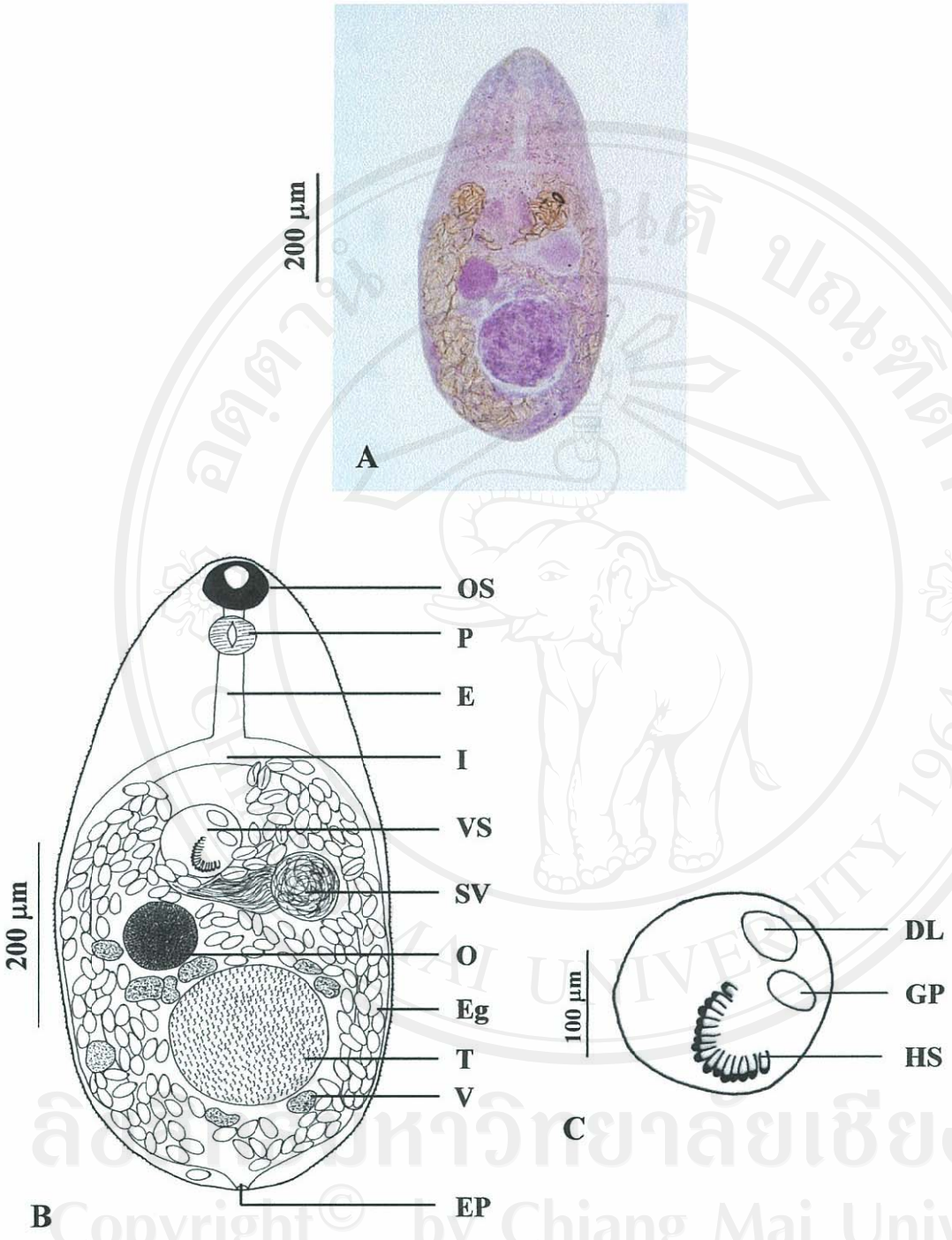


Figure 20. Mature adult of *Haplorchis taichui* 3 days PI in a chick. A. Photograph of a 3 days old mature adult. B. Drawing of a 3 days old mature adult. C. The ventrogenital sac with 16 hollow spines.

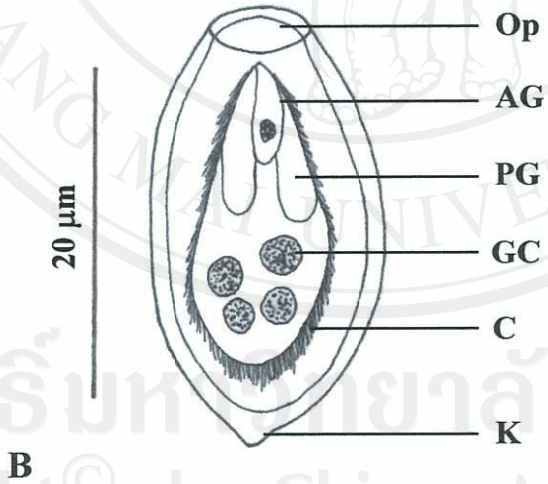
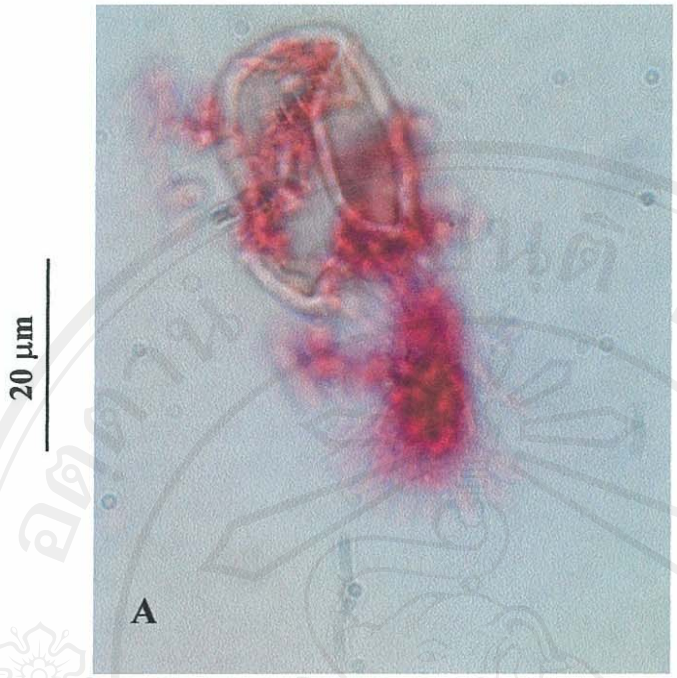


Figure 21. Egg of *Haplorchis taichui*, collected from chick feces 9 days PI. A. Photograph of an embryonated egg. B. Drawing of an embryonated egg.



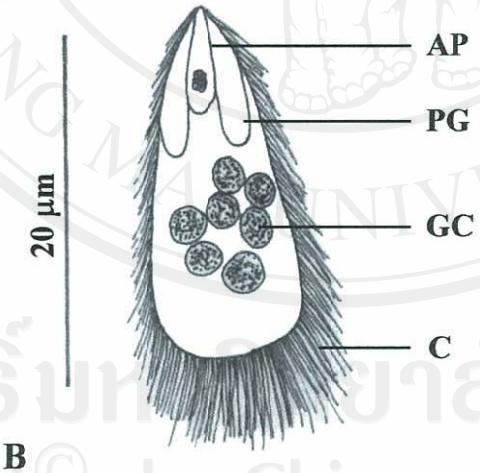


Figure 22. A miracidium escaped from an egg by pressure under a cover slip (adult 9 days PI in a chick). A. Photograph of a miracidium. B. Drawing of a miracidium of *Haplorchis taichui*.

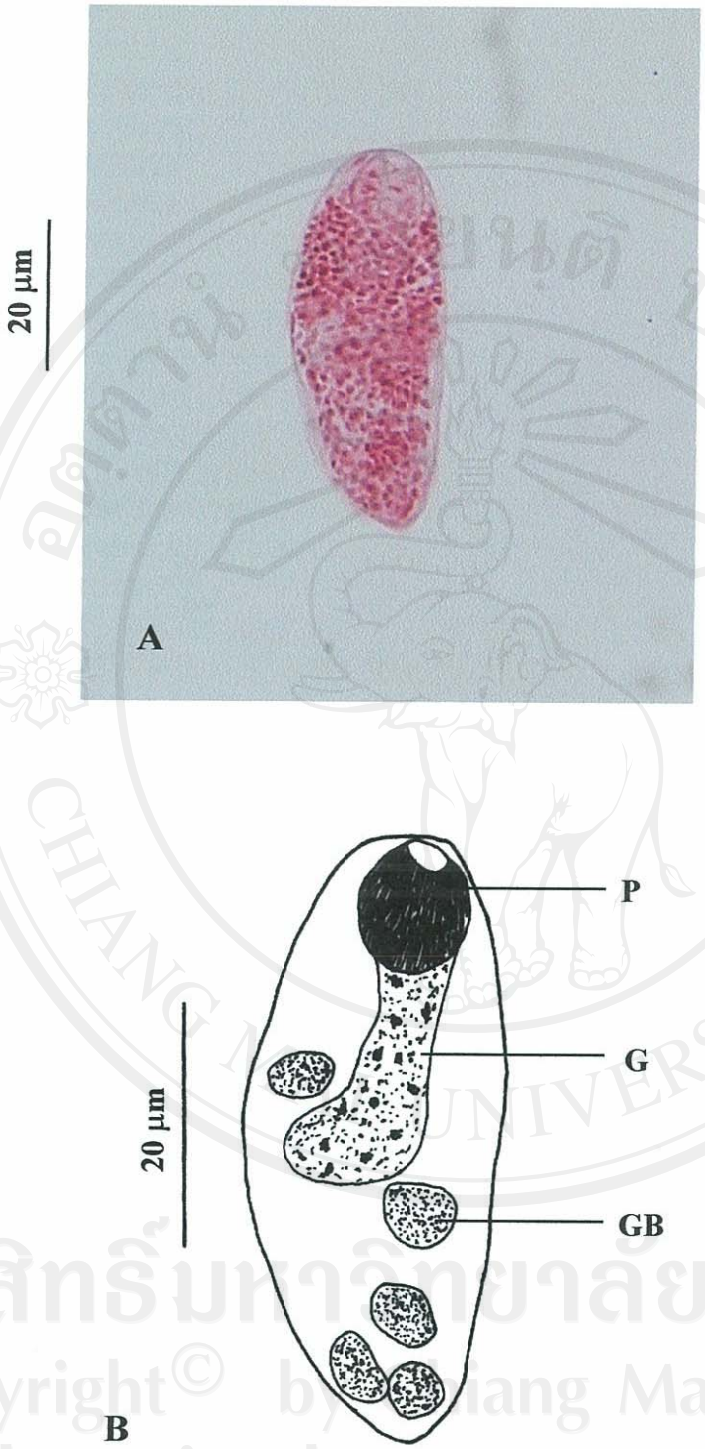


Figure 23. Young redia of *Haplorchis taichui* from a snail, *Tarebia granifera* (10 days PI). A. Photograph of a young redia 10 days PI. B. Drawing of a young redia 10 days PI.

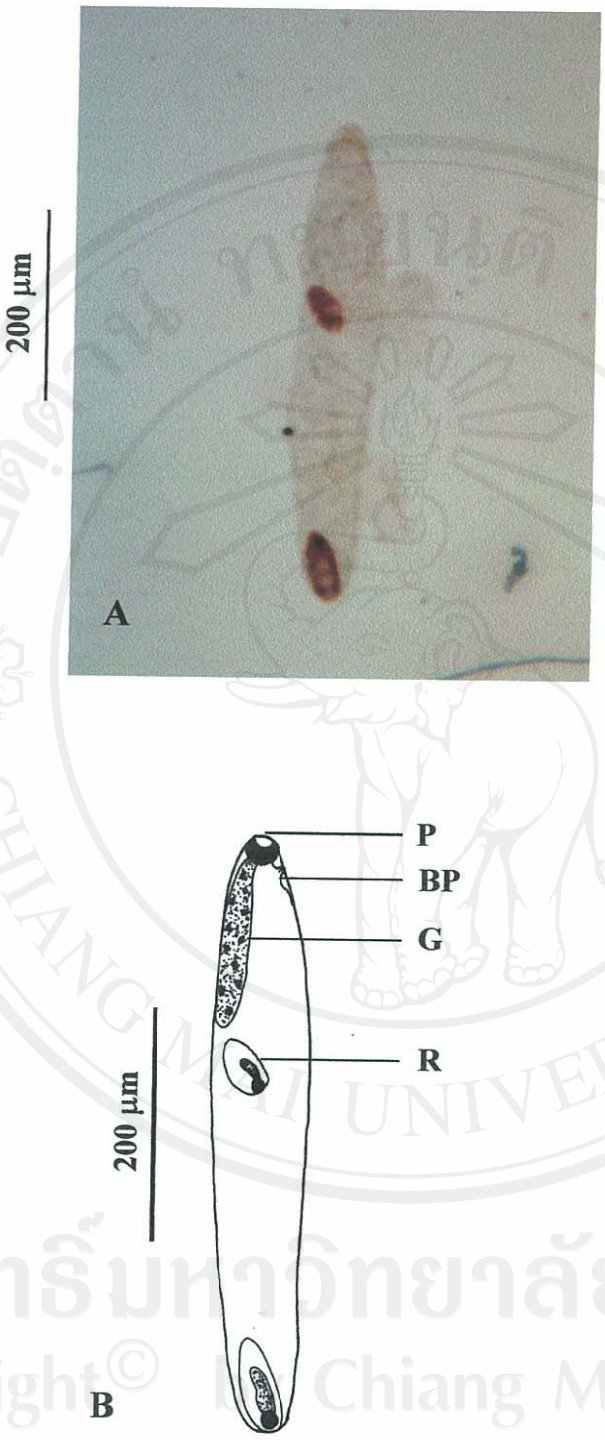


Figure 24. Mother redia of *Haplorchis taichui* from a snail, *Tarebia granifera* (49 days PI). A. Photograph of mother redia 49days PI. B. Drawing of mother redia 49days PI.

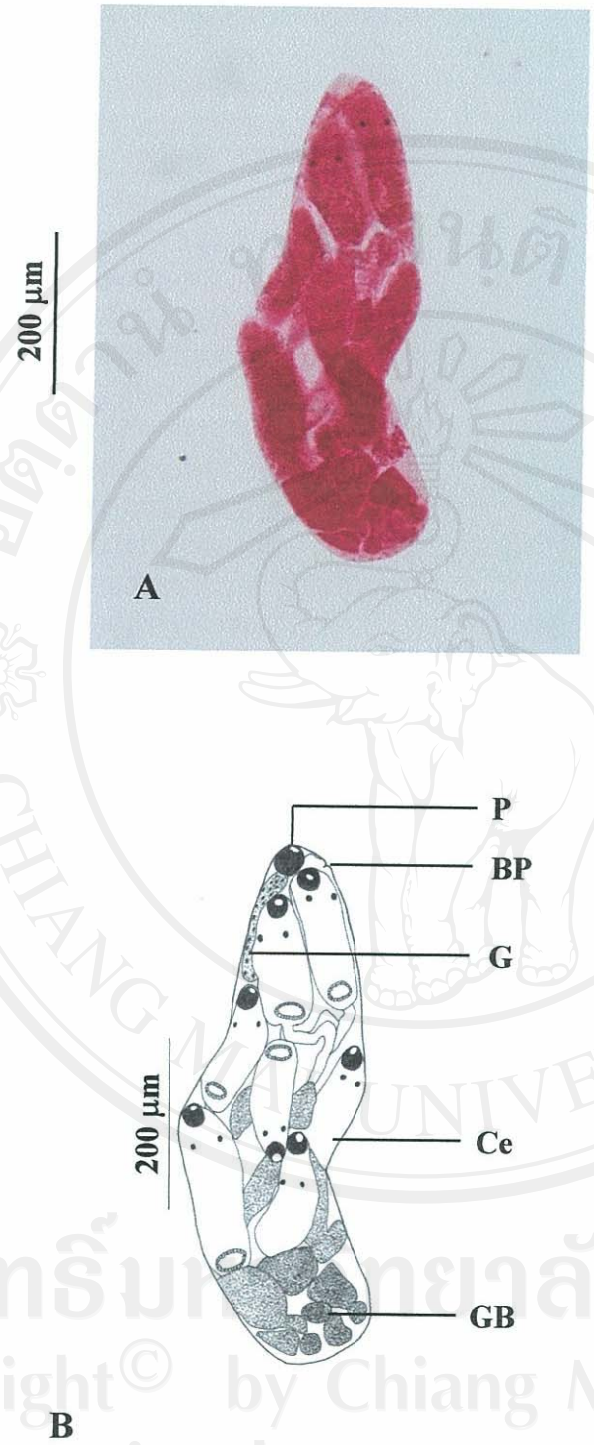


Figure 25. Daughter redia of *Haplorchis taichui* from a snail, *Tarebia granifera* (49 days PI). A. Photograph of a daughter redia 49 days PI. B. Drawing of a daughter redia 49 days PI.

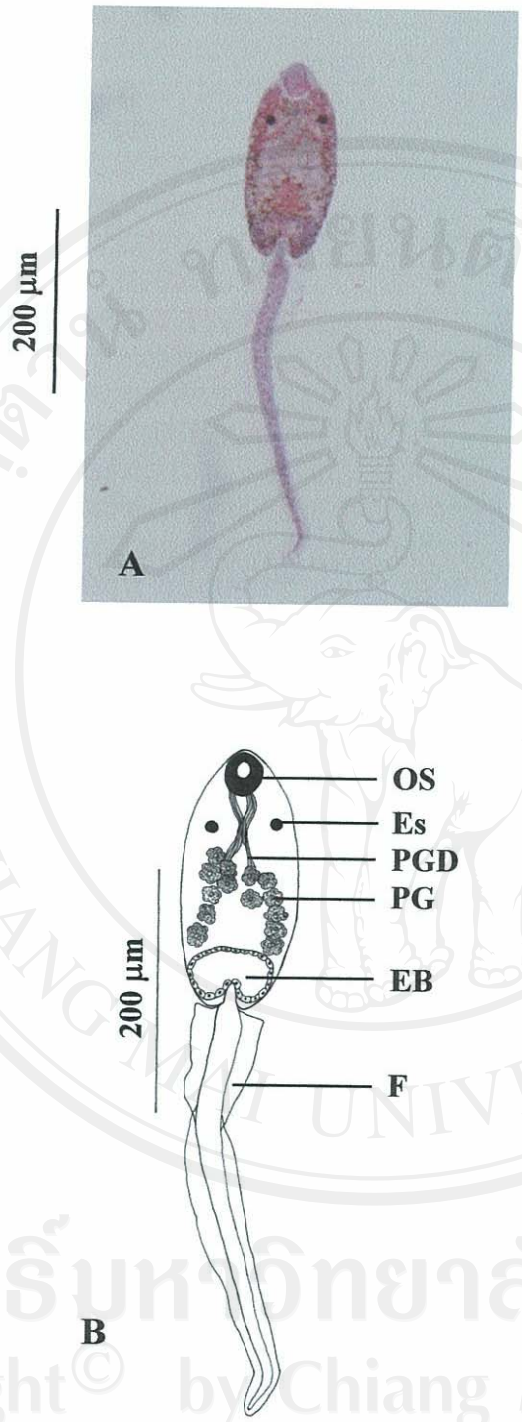


Figure 26. Cercarial stage of *Haplorchis taichui* from a snail, *Tarebia granifera* (49 days PI). A. Photograph of a cercaria 49 days PI. B. Drawing of a cercaria 49 days PI.

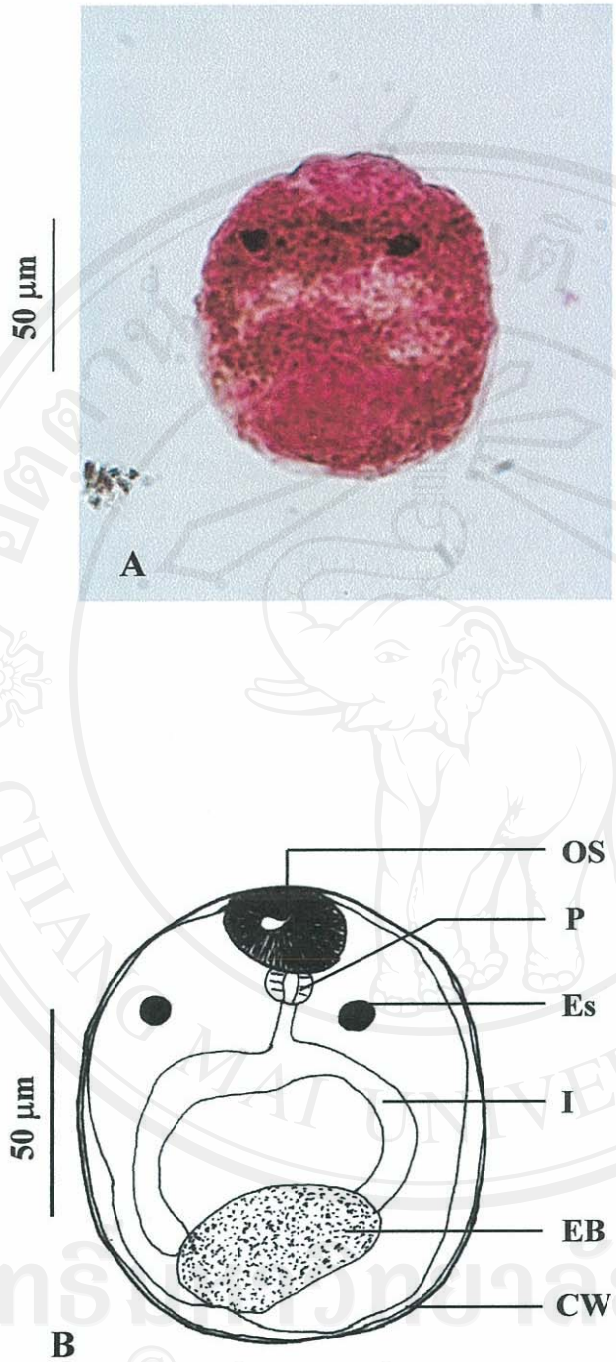


Figure 27. Metacercarial cyst of *Haplorchis taichui* from a fish, *Barbodes gonionotus* (1 day PI). A. Photograph of an encysted metacecaria 1 day PI. B. Drawing of an encysted metacercaria 1 day PI.

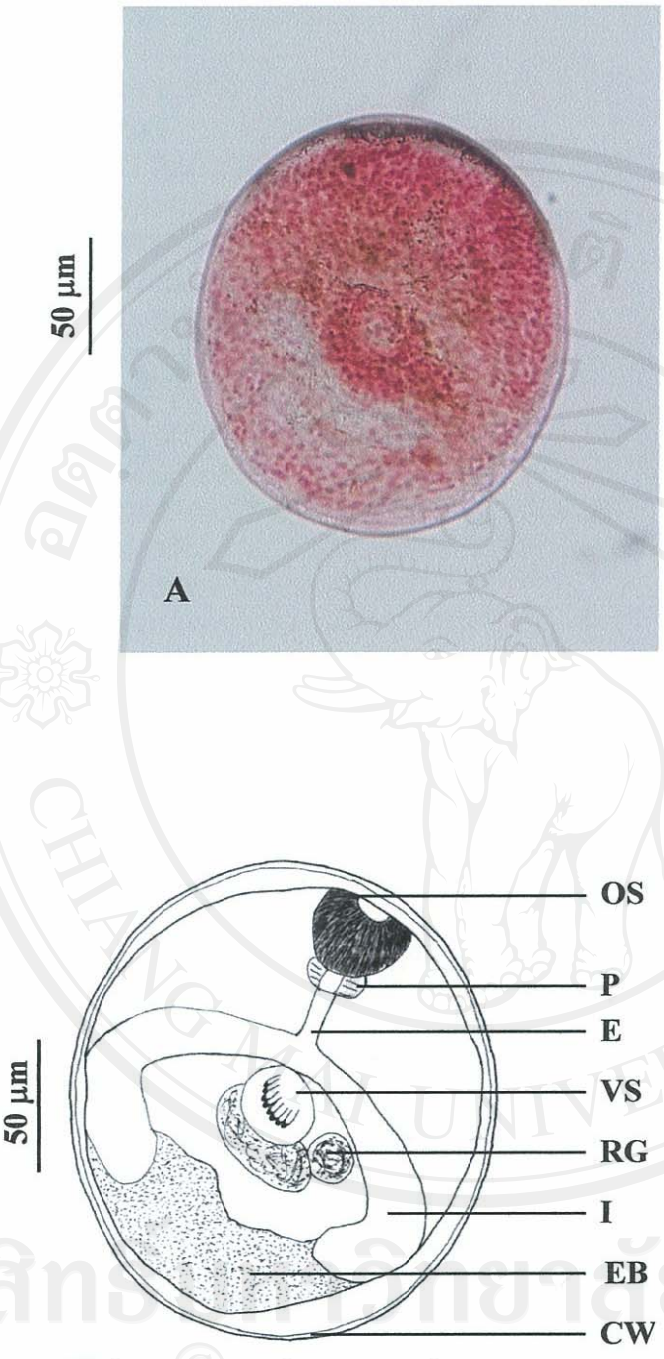


Figure 28. Metacercarial cyst of *Haplorchis taichui* from a fish, *Barbodes gonionotus* (3 days PI). A. Photograph of an encysted metacercaria 3 days PI. B. Drawing of an encysted metacercaria 3 days PI.

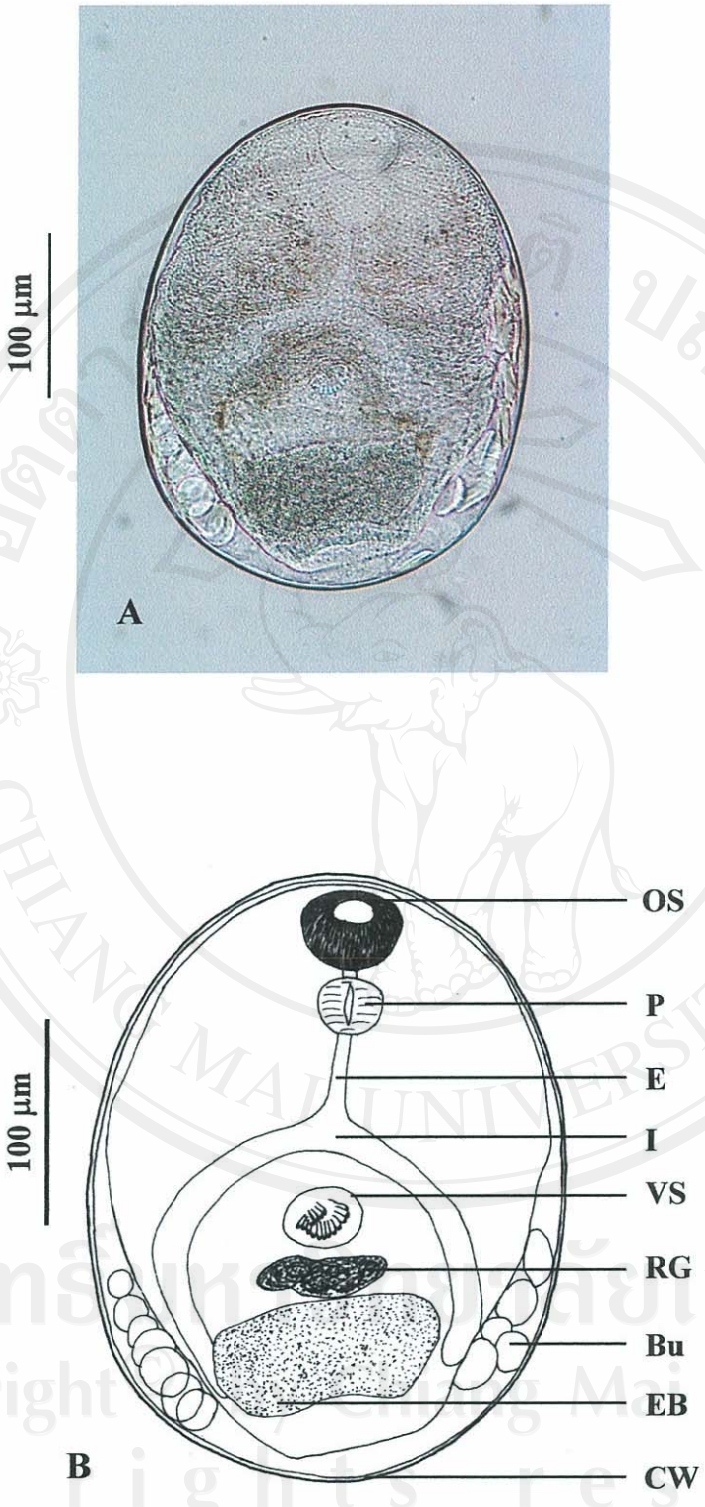


Figure 29. Metacercarial cyst of *Haplorchis taichui* from a fish, *Barbodes gonionotus* (6 days PI). A. Photograph of an encysted metacercaria 6 days old. B. Drawing of an encysted metacercaria 6 days old.

3. Surface ultrastructure of *Haplorchis taichui* infective stage and adults

Newly excysted metacercariae

Metacercariae were isolated from a naturally infected fish, *Henicorhynchus siamensis*. The body is pyriform and concave ventrally. The surface of the surface texture has cobblestone-like cytoplasmic processes and covered with scale-like multi-pointed tegumental spines. The arrangement of spines on the body is similar on both the ventral (Figure 30A) and dorsal surfaces (Figure 30B). Tegumental spines around the oral sucker in 1-3 rows covered with 3-7 points, followed by 7-9 points (Figure 30C). Dorsally, the spines in the anterior part were arranged in 5-8 points. The anterior 2/5 were digitated into 8-11 points (Figure 30D). The middle surface has 6-9 points, except around the ventrogenital sac which is in 1-5 rows and covered with 3-7 points (Figure 30E). The posterior 4/5 were covered with 6-8 points (Figure 30F) and on the dorsal side Laurer's canal opening was observed (Figure 30G). Posteriorly, the spines have 6 points followed by 5 points, 4 and decreased into 3 points and the spaces become wider (Figure 30H). The surface of the posterior end of the body has cobblestone-like cytoplasmic processes and was without spines around the excretory pore (Figures 30I-J).

One week old adults

Adult worms were collected from experimentally infected chicks 7 PI. The body is similar to metacercariae in shape, but became much enlarged and covered with more cobblestone-like and tegumental spines in a larger numbers on the anterior surface (Figures 31A-B). The anterior 2/5 on the ventral and dorsal sides were digitated into 9-12 points (Figures 31C). The digitated spines were occurred in 6-9 points around the ventrogenital sac (Figure 31D). In the posterior part, the spines

continuously decreased from 9 into 3 points (Figures 31E-F). The surface around the excretory pore was irregularly wrinkled and has more cobblestone-like processes which more prominent in the posterior region (Figure 31F). The dorsal surface has a prominent Laurer's canal with sperm entering which was located in the anterior 4/5 on the left side of the body (Figures 31G-H). The outer surface of the eggshells of *H. taichui* had flat, thread-like curly ridges, and the operculum which difficult to seen under a light microscope (Figures 31I-J).

Sensory papillae were presented on the body, consisting of four different types: type I, round swellings of the tegument (Figure 32A); type II, ciliated dome-shaped, solitary, groups of 2, 3, or 5 and 6 sensory papillae (Figures 32B-G); type III, non-ciliated dome-shaped, and always found together with a group of type II, papillae (Figures 32B and 32E); and type IV, button-shaped papillae, solitary or mixed with type II papillae (Figure 32H). The distribution of sensory papillae on the ventral side has 2 type I papillae on the lower lip. Twelve to fifteen pairs of type II sensory papillae are arranged around the lip of the oral sucker and on the inner side of the lip. Five small type II sensory papillae were presented on each side (Figure 33A). The distribution of sensory papillae on the ventral side is shown in Figure 33B. The single type II papillae were bilateral symmetrically arranged in 9 pairs, while groups of five to six of type II and III sensory papillae in 3 to 4 pairs were above the ventrogenital sac. Level with the ventrogenital sac is a group of 3 type II or type II mixed with type III papillae on each side. Two groups of only 2 type II or type II and III are found below the ventrogenital sac. On each side of the excretory pore appeared 3 type II or type III sensory papillae. Fewer sensory papillae are present on the dorsal side than the ventral side (Figure 33C). Anteriorly most of the body has

one pair in a group of type II papillae. Three pairs in a group of five or six type II and III papillae lined the lateral tracts and 2 pairs of single type II papillae were presented between three groups of a group of five or six papillae. In the middle region, types III and IV papillae in a group of three are on each side of the body and these groups of papillae are in the anterior 4/5 of the body, level with Laurer's canal. The distribution of the sensory papillae of adult worms is similar to the metacercarial stage, but differs in only some individuals where the dorsal side level with Laurer's canal had a single type IV papillae (Figure 31H).

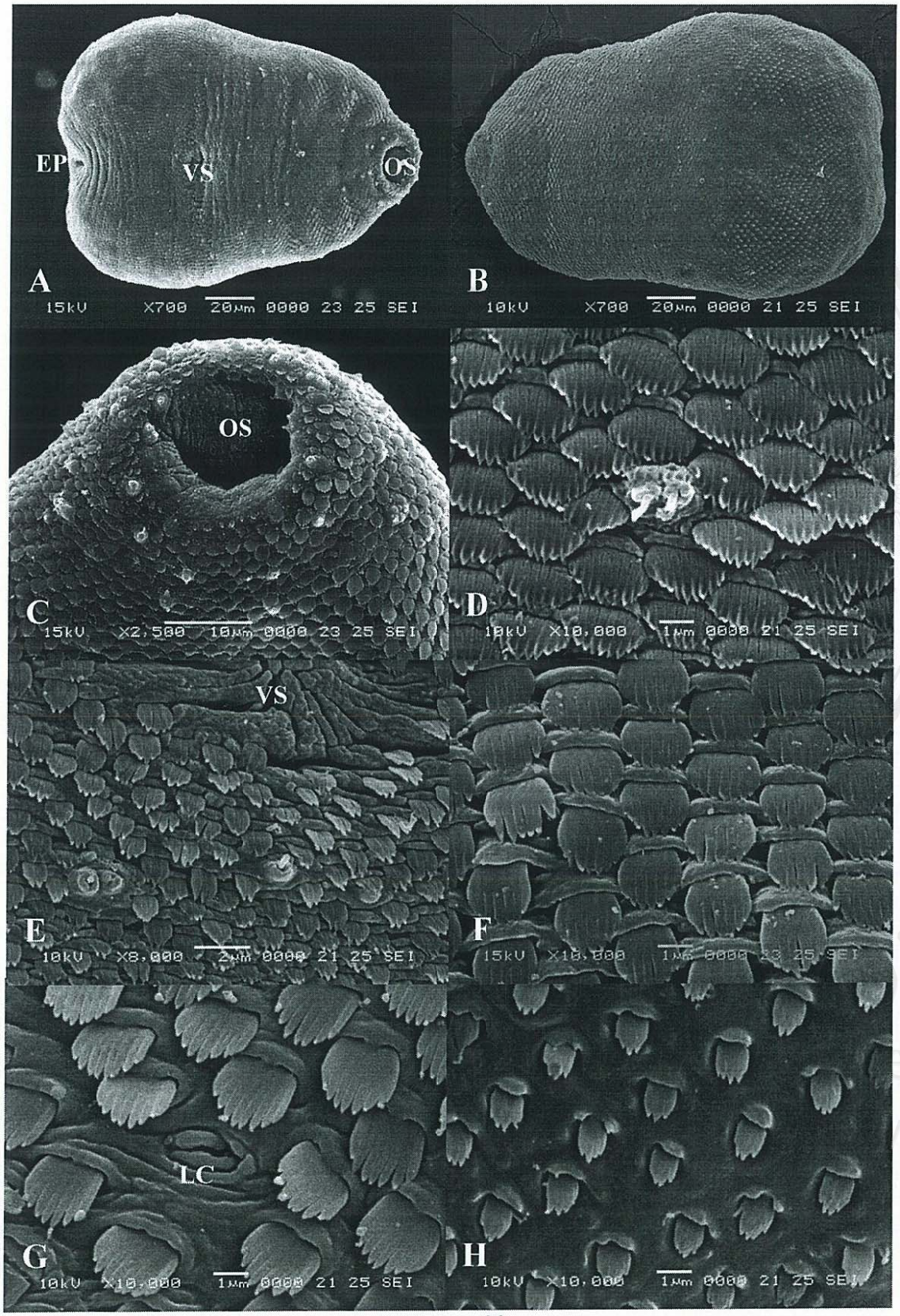

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Figure 30

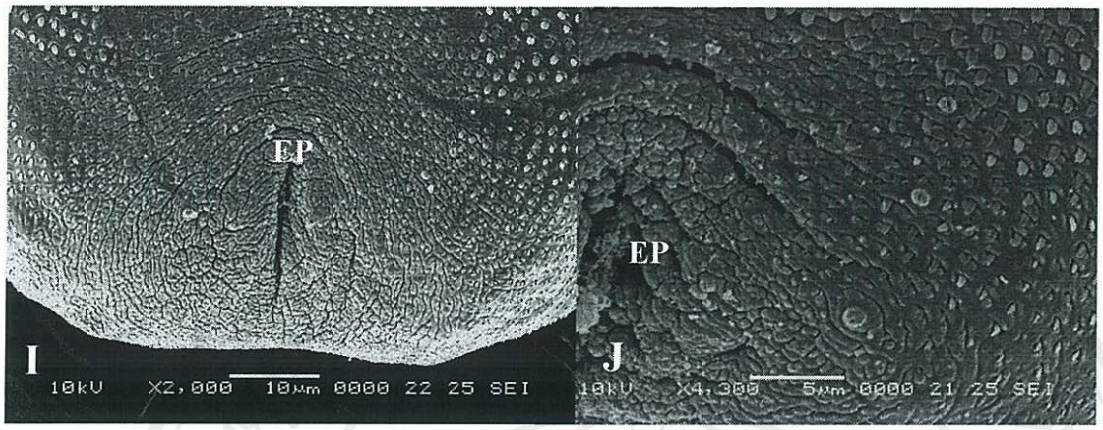

Figure 30. Tegumental surface of *Haplorchis taichui* metacercaria.

A. Ventral surface showing the oral sucker, ventrogenital sac, and excretory pore.

B. Dorsal surface covered with multi-pointed spines.

C. Enlarged surface surround the oral sucker.

D. Tegumental spines on the anterior region.

E. The ventrogenital sac surrounded with 3-7 pointed spines.

F. Tegumental spines on the middle part of the body.

G. Laurer's canal located on the anterior 4/5 dorsally.

H. Tegumental spines on the posterior region are smaller in size and number.

I. The surface surround the excretory pore is covered with coble stone-like processes and lacks spines.

J. Posterior surface covered with small spines with 3-5 points.

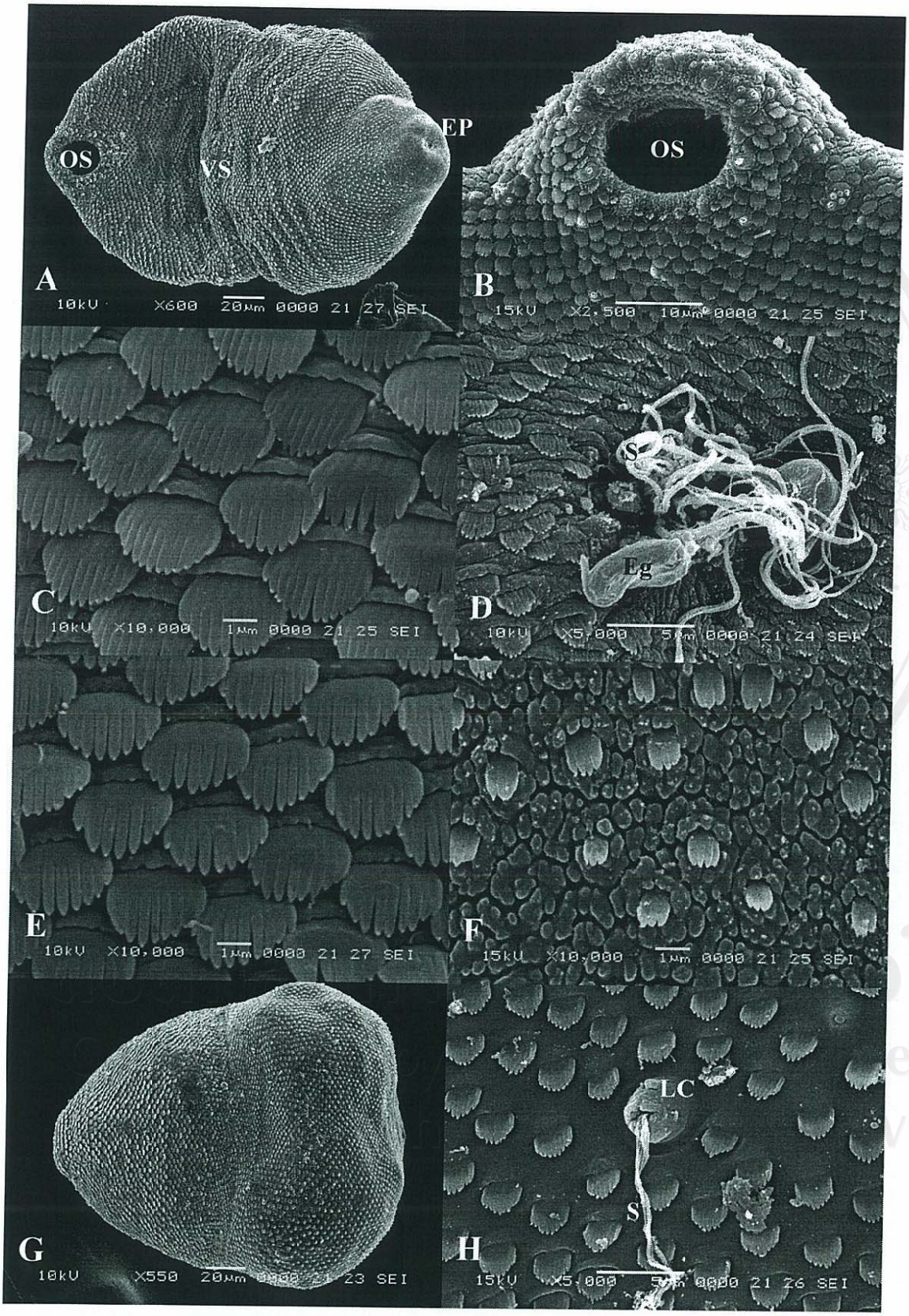
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Figure 31

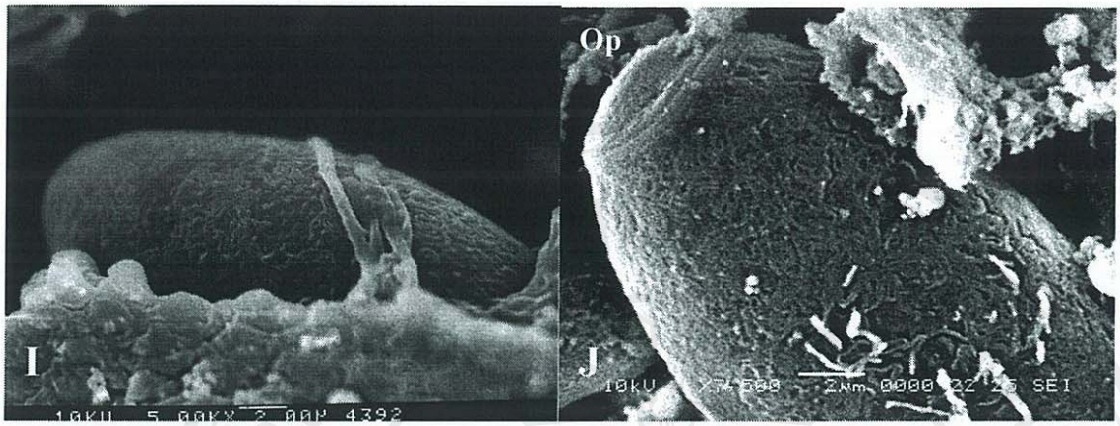


Figure 31. Adult surface of *Haplorchis taichui*, 7 days post-infection.

A. Ventral side is slightly concave.

B. The anterior end of the body is densely covered with spines.

C. The large digitated spine on the anterior 2/5 of the body.

D. Ventrogenital sac covered with 6-9 pointed spines and sperm released from the genital pore.

E. The posterior part of the body with digitately 7-9 pointed spines.

F. The areas adjacent to excretory pore has few spines and prominently cobblestone-like processes.

G. Dorsal surface with Laurer's canal.

H. Posterior part with Laurer's canal with sperm entering and type IV button-shaped sensory papillae.

I. Adult worm which an egg inside.

J. Egg showing the operculum and the surface covered with flat, thread-like curly ridges.

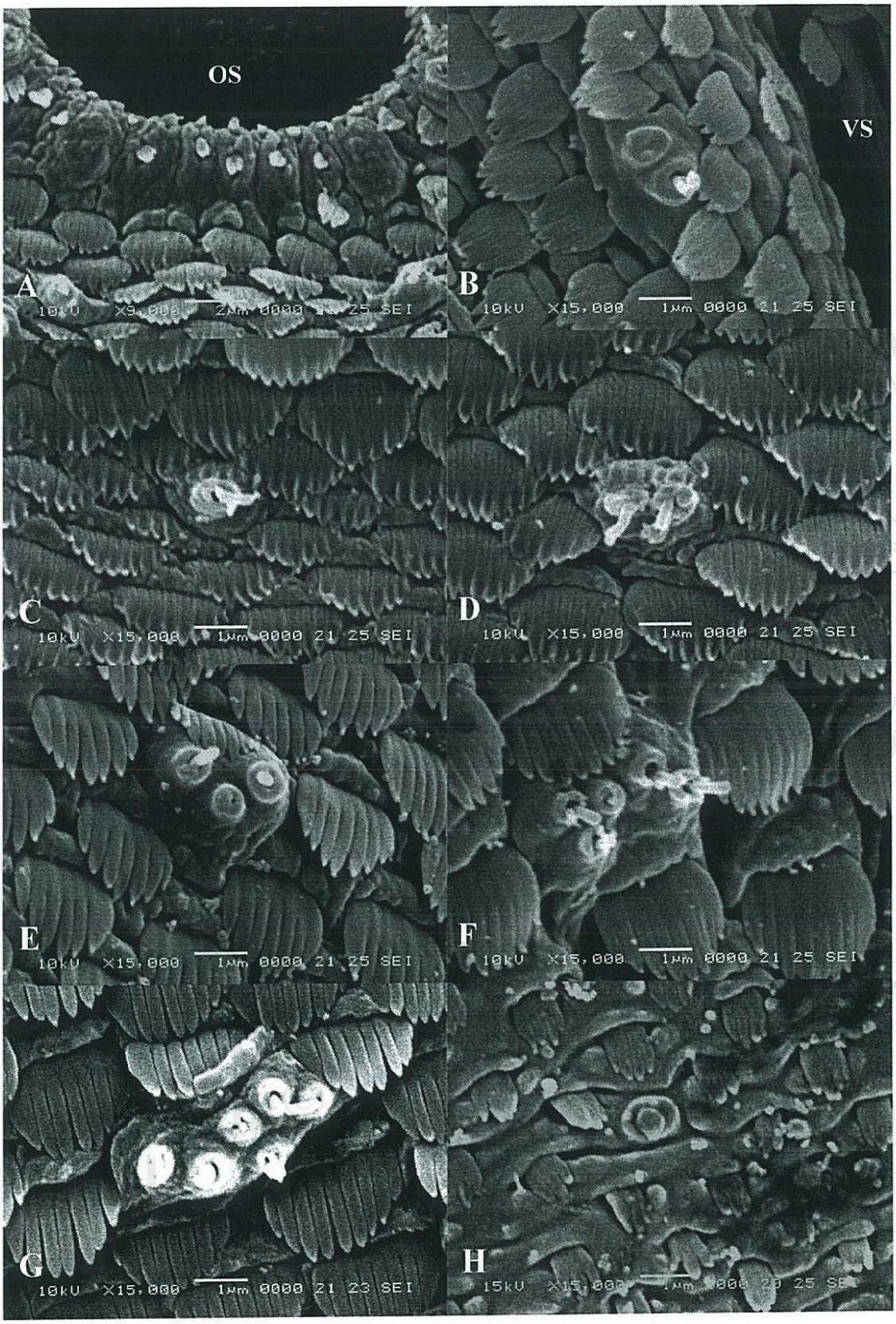


Figure 32

Figure 32. Four types of sensory papillae on metacercariae and adults of *Haplorchis taichui*.

A. Type I, round swellings of the tegument papillae.

B. Type II, ciliated dome-shaped together with type III, non-ciliated dome-shaped papillae.

C. Single ciliated dome-shaped papillae.

D. A group of 2 ciliated dome-shaped papillae.

E. A group of 3 type II ciliated dome-shaped papillae and type III non-ciliated dome-shaped papillae

F. A group of 5 type II ciliated dome-shaped papillae.

G. A group of 6 type II ciliated dome-shaped papillae.

H. Type IV, button-shaped sensory papillae.

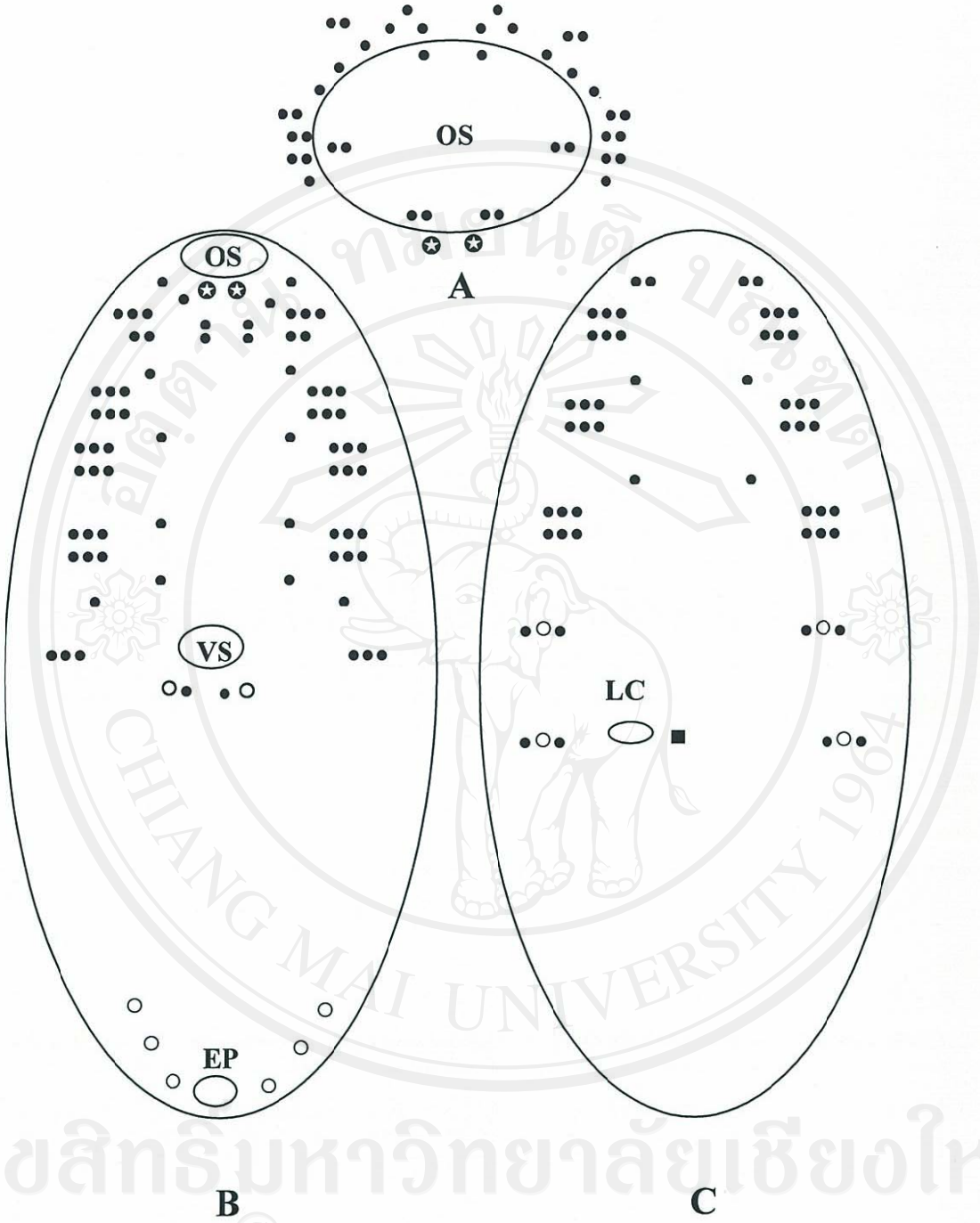


Figure 33. The distribution of sensory papillae on the tegumental surface of *Haplorchis taichui*. A) oral sucker B) ventral view and C) dorsal view, ✪ = type I round swellings of the tegument papillae ; ● = type II ciliated dome-shaped papillae; ○ = type III non-ciliated dome-shaped papillae and ■ = type IV button-shaped papillae.

4. Study on an anthelmintic drug *in vitro*

Niclosamide was effective against *H. taichui* after *in vitro* treatment. One hundred active worms were treated in each experiment. In 0.01 μg/ml of niclosamide, the result showed 3 active worms, 62 slightly active movement, and 35 dead at 30 minutes. One hour later, 7 worms had slightly active movement and 93 worms died. In 0.1μg/ml of niclosamide solution, only 5 of 100 worms showed slightly active movements after 30 minutes and all worms died after 1 hour. In 1.0 and 10.0 μg/ml of niclosamide all worms died at 30 minutes. Control groups showed active and slightly active worms until 24 hours in Tyrode's solution (Table 4).

Table 6. Activity of *Haplorchis taichui* after incubation with various concentrations of niclosamide from 100 active worms in each experiment.

Drug Concentration (μg/ml)	Incubation time				
	30 min	1 hr	6 hrs	12 hrs	24 hrs
10.0	-	-	-	-	-
1.0	-	-	-	-	-
0.1	5*/95-	-	-	-	-
0.01	3**/62*/35-	7*/93-	-	-	-
Control	100**	100**	100**	88**/12*	74**/26*

** active movement (1-5 sec/time) * slightly active movement (6 -10 sec/time)

- dead (>10 seconds no movement)

Investigations of worms by scanning and transmission electron microscopy showed that niclosamide caused irreversible alterations in the covering tegument. Niclosamide was effective against *H. taichui* after *in vitro* treatment. The grade of damage was correlated with the concentration of drugs and the exposure time. The posterior region showed more severe damaged than the anterior part.

Scanning electron microscopic findings

Control groups : Tyrode's solution

The tegument surface morphology of parasites after various incubation times appeared normal. The tegumental surface is covered with scale-like, multi-pointed spines and numerous sensory papillae. The anterior region is covered with large and dense spines, which decrease in size and number posteriorly (Figures 34A-F).

SEM observations on the tegumental change after treatment with niclosamide 0.01 μg/ml

Flukes were observed after 30 minutes to 1 hour, most of the tegument and sensory papillae on both surfaces still retained their normal topography (Figures 35A-B). At 6 hours after treatment the tegumental surface of some specimens became swollen and some small blebs developed on the body surface. In some areas the spines were sunken due to the swelling of the tegument (Figure 35C). Treated flukes at 12 hours showed surface damage by swelling of tegument and some small blebbing. In some specimens, the tegument surrounding the oral sucker displayed cracks and lesions (Figure 35D). Spines lost their morphology with the margins becoming irregular and more space between spines and some lesions occurred (Figure 35E). After 24 hours, the tegumental change was characterized by formation of numerous blebs, followed ruptured to form lesions and some spines became eroded (Figure 35F).

SEM observations on the tegumental change after treated with niclosamide 0.1 μg/ml

At 30 minutes the surface is rougher than normal while the sensory papillae are unaffected. In some specimens appeared swollen on the tegument surrounding the spines (Figure 36A). After 1 hour, variously sized blebs were present on some specimens in the area above the ventrogenital sac which was more severe damaged than other parts. Some blebs collapsed and shriveled also spine sinking occurred (Figures 36B-C). After 6 hours, variously sized blebs developed in larger numbers and some of these blebs ruptured (Figure 36C, inset). After 12 hours, treated worms had numerous blebs on the body surface. In some cases, the spines around the ventrogenital sac and mid-body to posterior region were eroded (Figure 36D). After 24 hours numerous blebs occurred and the tegumental surface became swollen (Figure 36E). Some flukes lost the apical plasma membrane in the middle to posterior regions on both their ventral and dorsal surfaces, exposing the empty spine sockets in the underlying tegumental syncytium. The tegument on both surfaces showed similar changes where numerous blebs formed, spines lost, while spine sockets and some lesions occurred after longer exposure (Figures 36E-F).

SEM observations on the tegumental change after treatment with niclosamide 1. 0 μg/ml

At 30 minutes, some specimens exhibited numerous spherical blebs on the tegument, mostly in the area between the oral sucker and ventrogenital sac (Figure 37A). One hour later, numerous blebs developed on the body surface (Figure 37B). However, most of the tegumental surface was very swollen and the spines appeared sunken. Six hours after exposure, the surface blebs became more numerous and more widespread, especially on the surface between spines. Very severe blebbing was also observed in some specimens (Figure 37C). Some blebs ruptured to form tegumental lesion spots. The damaged tegument began to slough off in some regions (Figure 37D). After 12 hours, the number of variously sized blebs and lesions increased and most of the tegumental surface appeared partly damaged (Figure 37E). In a number of specimens, loss of tegument and empty spine sockets was observed in the mid-body region into the posterior (Figure 37F). Twenty-four hours after exposure, severely damaged tegument occurred in some specimens. Flukes lost the apical plasma membrane from both their ventral and dorsal surfaces, showing empty spine sockets in the underlying tegumental syncytium (Figure 37F). In the posterior region, more severe damage occurred with numerous small holes forming on the basal lamina in areas where the tegument had been removed (Figure 37G) and most sensory papillae were affected (Figure 37H).

SEM observations on the tegumental change after treatment with niclosamide 10. 0 μg/ml

At 30 minutes, most of worms exhibited swelling of the tegument surrounding the spines. One hour after incubation, numerous variously sized blebs were scattered over the inter-spine areas and some specimens had burst blebs, causing lesions (Figures 38A-B). After 6 hours, treated worms showed more pronounced blebs ruptured and the surface had a more flaky texture on the ventral and dorsal sides (Figure 38C). In some specimens, the tegument was covered with numerous small blebs and the surface had a flaky appearance, causing lesions and spine were removed. After 12 hours, the loss of spines and increasing extent of lesions appeared on the dorsal and ventral surfaces (Figures 38D-E). In some cases, numerous small holes were widespread throughout the tegument, especially in the mid-body to posterior region. Some treated worms had lesions on the basal lamina. After 24 hours the tegumental changes were similar to those after 12 hours where the apical plasma membrane was completely lost from both their ventral and dorsal surfaces while empty spine sockets appeared. The posterior surface is fined and spine sockets indistinct, round holes and lesions occurred due to the loss of spines (Figure 38F). Overall, the tegumental surfaces of flukes were similarly affected on both surfaces, but the anterior region appeared less damaged than the posterior region.

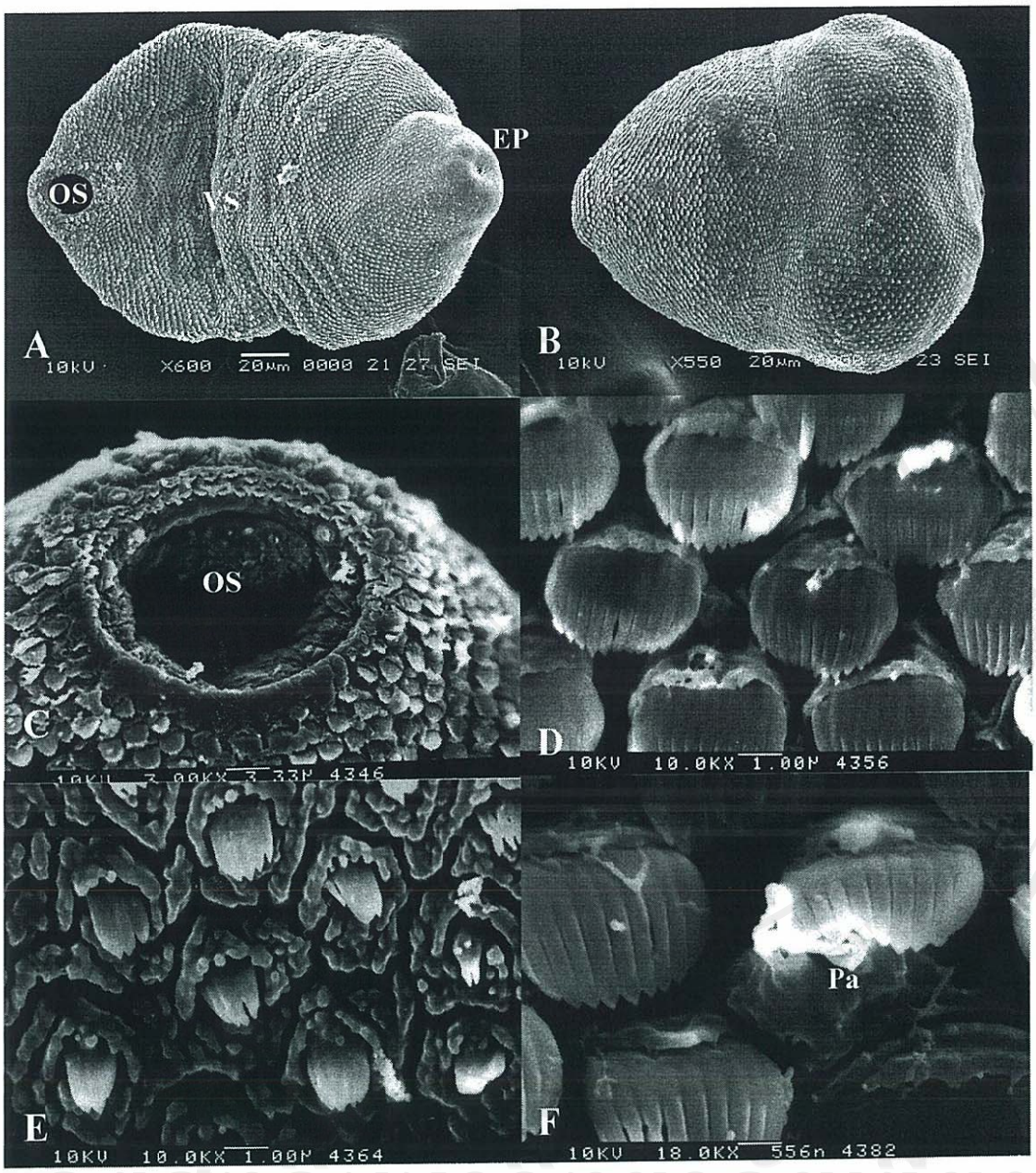


Figure 34

Figure 34. The adult surface of *Haplorchis taichui* in the control group, 24 hours after incubation in Tyrode's solution.

A. Ventral surface of worm, 1 hour after incubation.

B. Dorsal surface of worm, 6 hours after incubation.

C. Tegumental surface around the oral sucker of worm, 12 hours after incubation.

D. Anterior part covered with densely large spines, 12 hours after incubation.

E. Posterior part of the body showing smaller size and number of spines, 24 hours after incubation.

F. The sensory papillae are still normal photography, 24 hours after incubation.

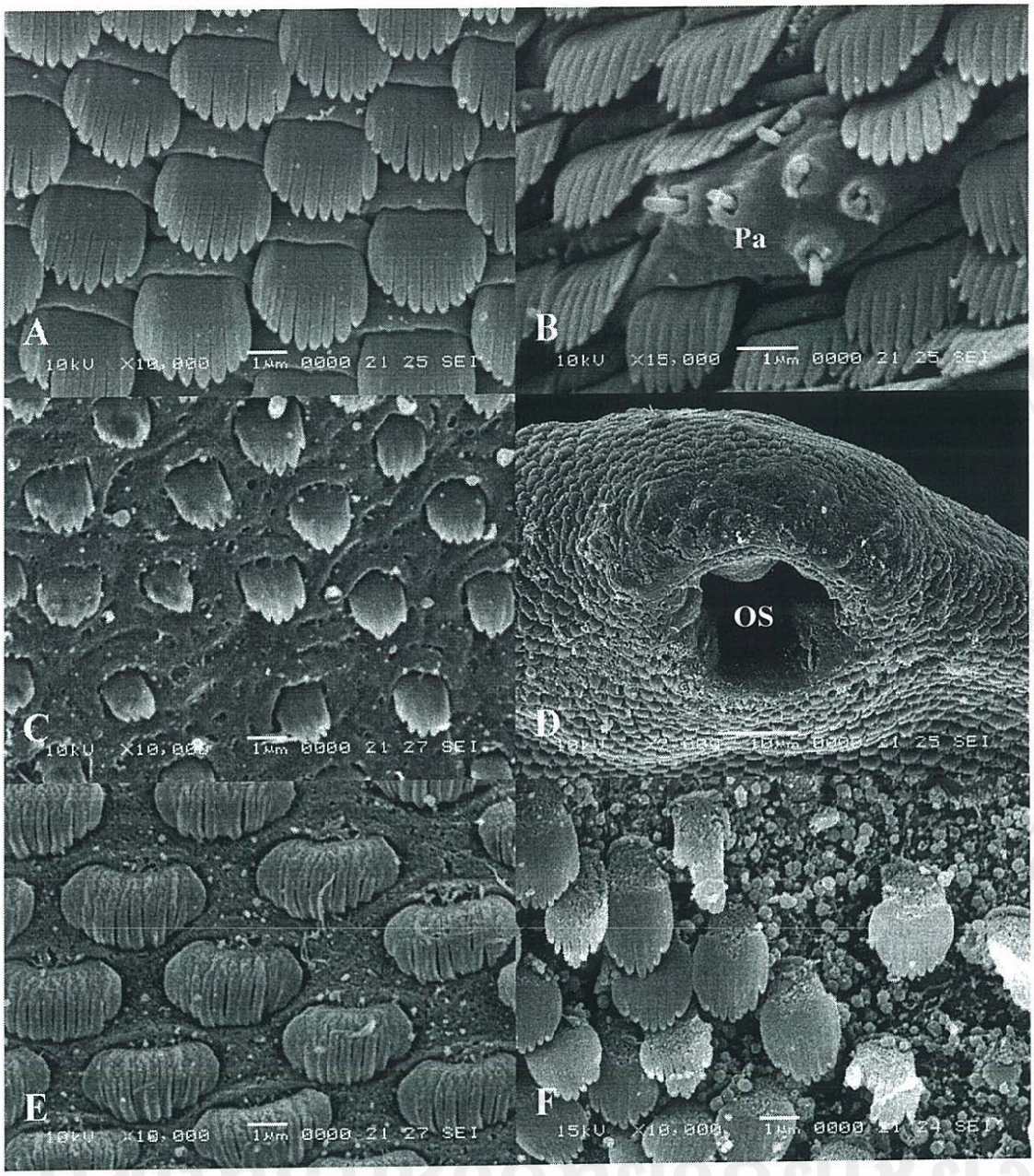


Figure 35

Figure 35. The tegumental changes after incubation in 0.01 μg/ml niclosamide solution for 24 hours.

A. The body surface is normal photography after 30 minutes.

B. Sensory papillae still unaffected after 1 hour.

C. The body surface is swollen and covered with some small blebs after 6 hours.

D. The surface around the oral sucker was destroyed and lesions occurred at 12 hours.

E. The tegument showed spines damage and some lesions after 24 hours.

F. Numerous small blebs appeared after 24 hours, causing some lesions and spines loss.

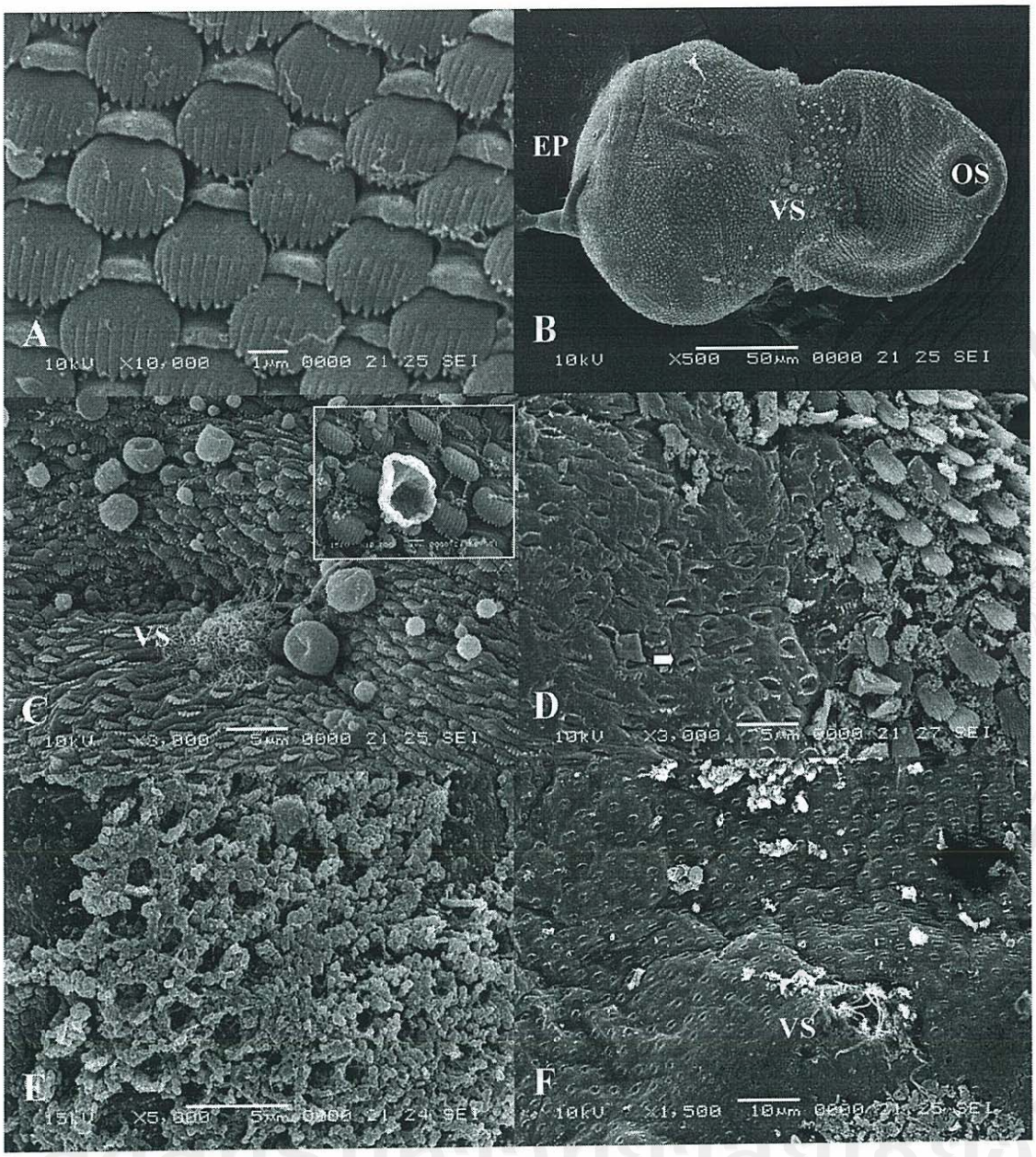


Figure 36

Figure 36. Tegumental changes after treatment with 0.1 μg/ml niclosamide solution for 24 hours.

A. Anterior region showed the swelling of tegument between spines after 30 minutes.

B. Spherical blebs appeared more prominent above the ventrogenital sac after 1 hour.

C. Enlarged blebs and sunken spines appeared after 1 hour, (inset) ruptured blebs occurred 6 hours after exposure.

D. Tegumental spines on the midbody surface were removed and spine sockets (⇨) appeared after 12 hours.

E. Numerous blebs formed on the tegumental surface after 12 hours.

F. Spine loss and spine sockets presented around the ventrogenital sac after 24 hours.

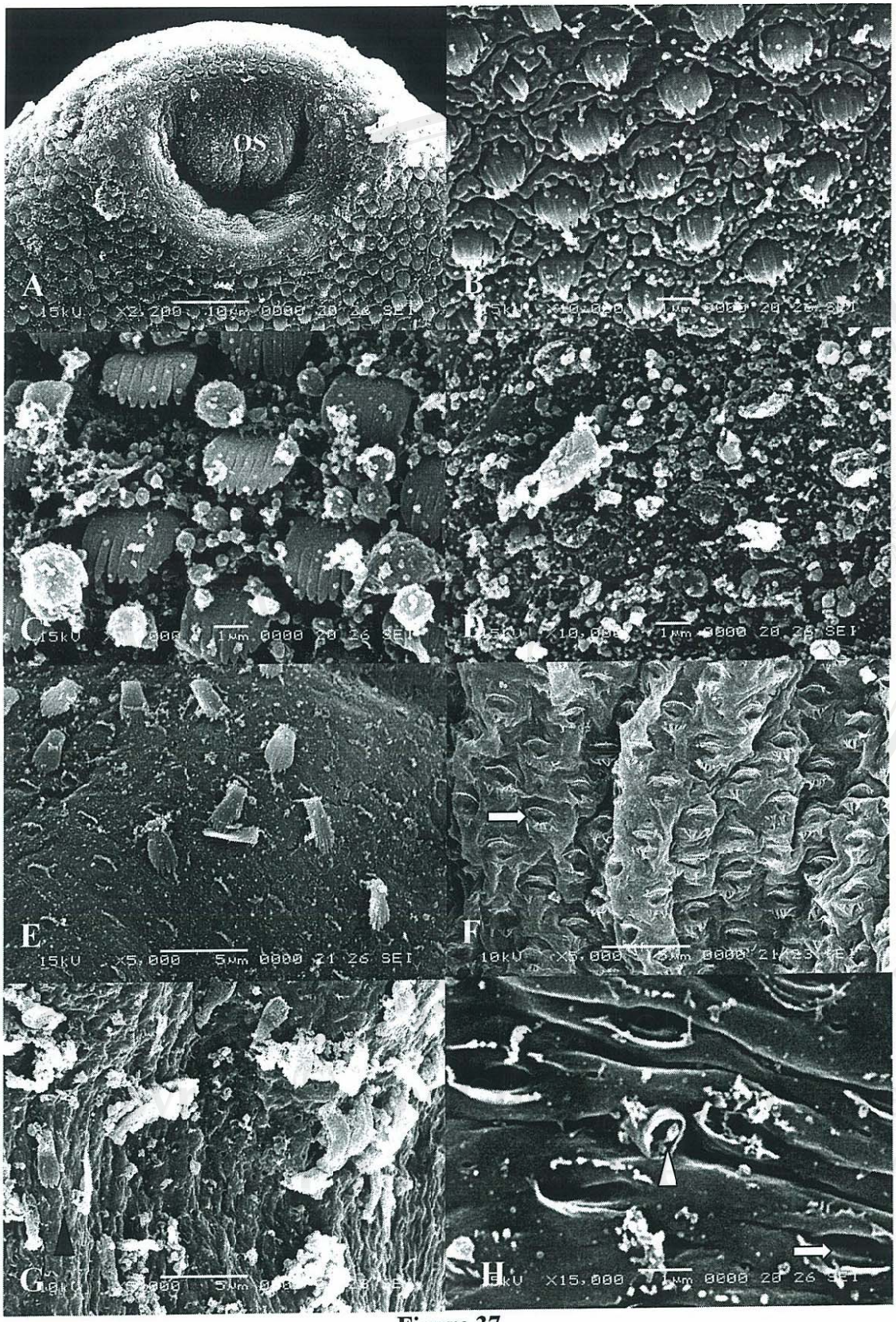


Figure 37

Figure 37. Tegumental changes after treatment with 1.0 μg/ml niclosamide solution for 24 hours.

A. The anterior region presented numerous small blebs at 30 minutes.

B. The posterior region is covered with numerous blebs and sunken spines after 1 hour.

C. Various size blebs appeared in the anterior region after 6 hours.

D. The posterior surface region was destroyed, some spines fell off and the surface is covered with blebs after 6 hours.

E. Tegumental spines were removed after 12 hours incubation and spines sockets occurred.

F. Spine sockets (⇨) appeared after spines lost and some small holes formed on the basal lamina.

G. Numerous small holes (▲) appeared on the basal lamina after 24 hours.

H. Some sensory papillae ruptured (△) and spine sockets (⇨) occurred after 24 hours.

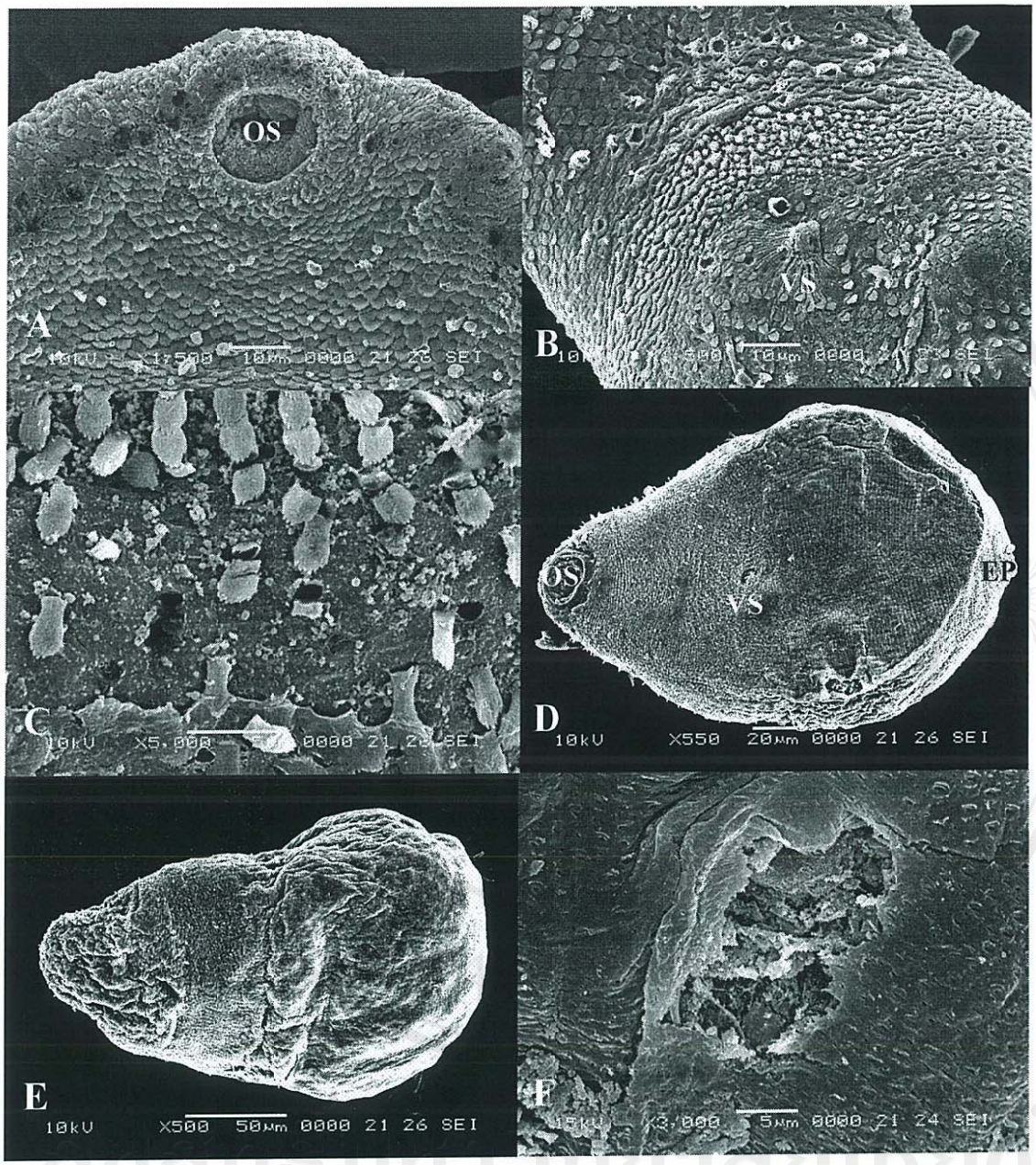


Figure 38

Figure 38. Tegumental changes after treatment with 10.0 μg/ml niclosamide solution for 24 hours.

A. Some blebs ruptured and lesion occurred in the anterior region after 1 hour.

B. Numerous blebs ruptured causing lesions after 1 hour.

C. Numerous blebs scattered on the tegument and loss of the plasma membrane after 6 hours.

D. The ventral surface of worm showing the lost of spines after 12 hours.

E. The tegumental spines on the dorsal surface were removed at 12 hours after exposure.

F. Completely loss of the apical plasma membrane and some lesions occurred after 24 hours.

Transmission electron microscopic observations

TEM observations on the tegument of control groups were found to be normal. Sections from the posterior region of worms were examined. Mitochondria and secretory bodies were present throughout the syncytium. There was abundant tegumental musculature and complete organelles 24 hours after treatment (Figures 39A-D).

TEM observations on the tegumental ultrastructure after treatment with 0.01 μg/ml niclosamide

The tegument still retained a normal morphology. The tegumental changes after 30 minutes to 1 hour treatment were still normal (Figure 40A). Sections through the tegument of parasites exposed to niclosamide at 0.01 μg/ml for 6 hours had some specimens with swollen mitochondria while the muscle layers were unaffected (Figure 40B). After 12 hours, the subtegumental muscle layers appeared normal, but some vacuoles occurred in the parenchymal cells (Figures 40C-D). However, by 24 hours the tegumental cells showed damage with more severe vacuolization and swelling of the basal infolds in some areas (Figures 40E-F).

TEM observations on the tegumental ultrastructure after treatment with 0.1 μg/ml niclosamide

Tegument sections after 30 minutes were normal (Figure 41A). After 1 hour, there was some surface blebbing and the apical surface was more irregular than normal (Figure 41B). After 6 hours, the tegument was damaged by some vacuolizations. The mitochondria in the tegumental syncytium were more swollen and rounded than normal (Figure 41C). Vacuolization became more pronounced after 6 hours of exposure (Figure 41D). After 12 hours, the tegument showed swelling of the

basal infolds and severe distortion with disorganization of the tegumental musculature (Figure 41E). In some areas, the inner plasma membrane was swollen and partially detached from the underlying basal lamina after 24 hours (Figure 41F).

TEM observations on the tegumental ultrastructure after treatment with 1.0 μg/ml niclosamide

After 30 minutes to 1 hour, blebbing on the apical surface was more pronounced than in lower concentrations (Figure 42A). In some areas, the tegument had open bodies and swelling of the basal infolds after 1 hour (Figure 42B). In some specimens, the inner plasma membrane was swollen and detached from the underlying basal lamina after 6 hours (Figure 42C). After 12 hours, the tegument became more vacuolized and the subtegumental muscle cells also exhibited depolymerization (Figures 42D-E). After 24 hours, there was disorganization and damage of the surface layer. The tegument of treated worms became swollen on the basal infolds. Some of the connections between the basal infolds and the rest of the basal plasma membrane were broken, so that in places the entire syncytium became detached, exposing the basal lamina beneath (Figure 42F).

TEM observations on the tegumental ultrastructure after treatment with 10.0 μg/ml niclosamide

After 30 minutes, mitochondria were variously swollen and rounded with distinct cristae (Figure 43A). After 1 hour, basal infolds occurred resulting in the detachment of the apical plasma membrane (Figure 43B). Mitochondria in the tegument were swollen and became rounder than normal and some of them were vacuolized after 6 hours (Figure 43C). After 12 hours, the tegument was disrupted and the syncytium appeared vacuolated. The apical region of the tegument became

detached due to the presence of basal infolds (Figure 43D). The tegument showed extensive blebbing and vacuolization in the deep layers (Figure 43E). The subtegumental muscles and all other tissue were extensively depolimeried after 24 hours. Numerous vacuoles formed throughout the tegument. The surface of the tegument showed disrupted areas on the basal lamina and muscles underneath (Figures 43F-G). The tegument mostly became disrupted and detached, while erosion exposed the basal lamina and the muscle layer. These tegument changes were similar to lower exposure dosages.

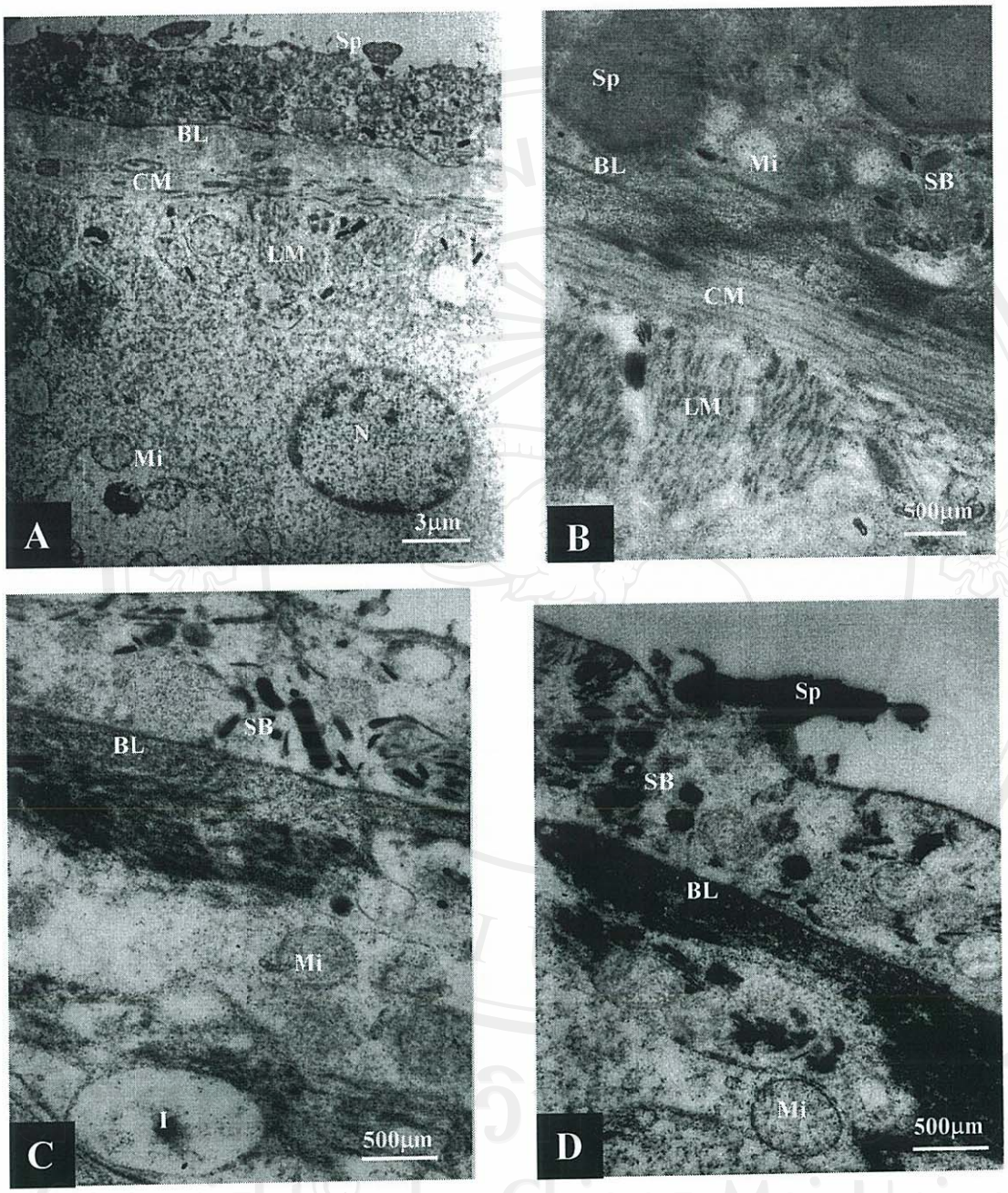


Figure 39

Figure 39. Tegument of *Haplorchis taichui* in the control group, 24 hours after exposure to Tyrode's solution.

A. The control group tegument of worm is still normal after 1 hour.

B. The tegumental cell are still normal after 6 hours.

C. The mitochondria and secretory bodies are still present throughout the tegument after 12 hours.

D. The mitochondria and subtegumental musculature retain a relatively normal appearance after 24 hours.

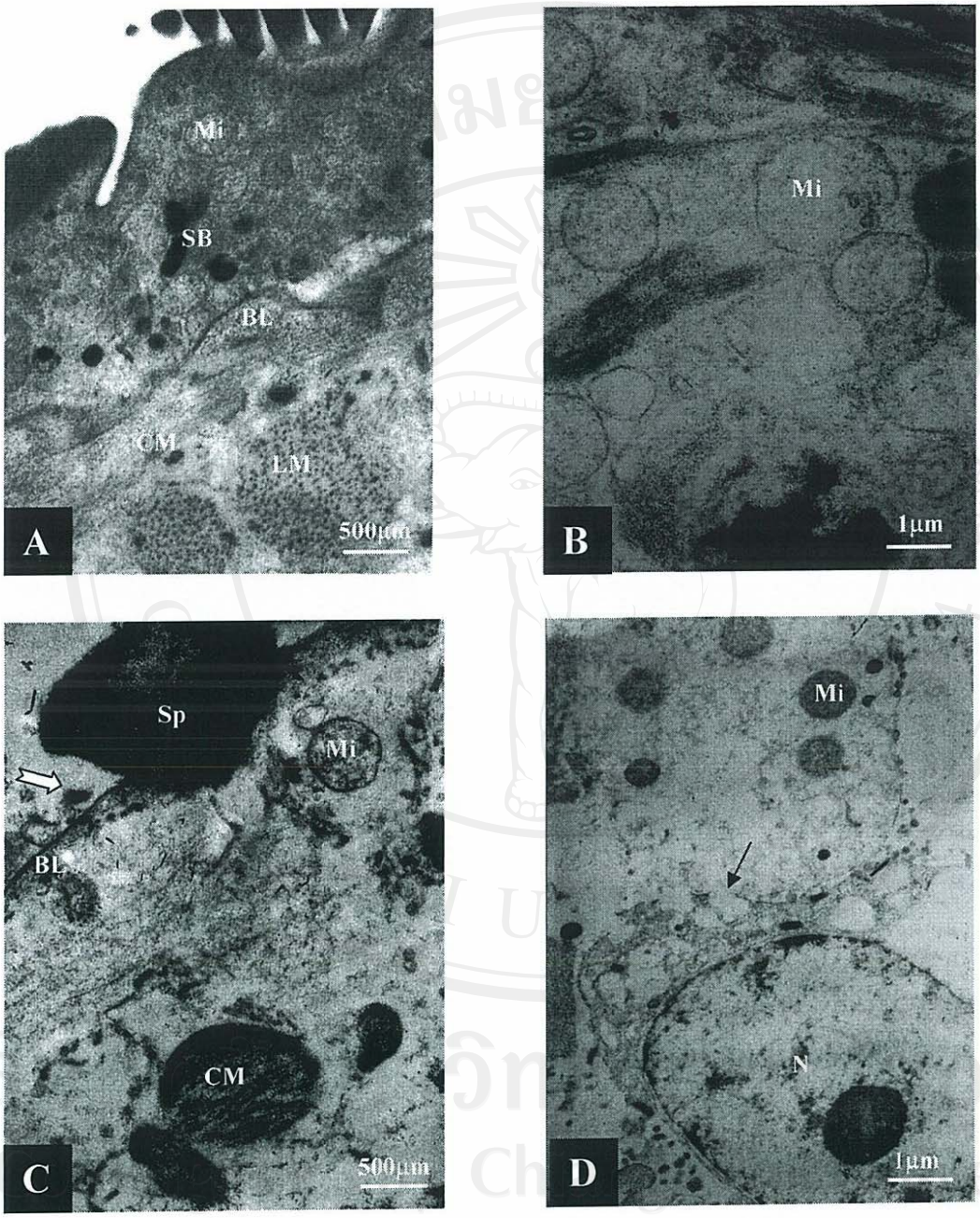


Figure 40

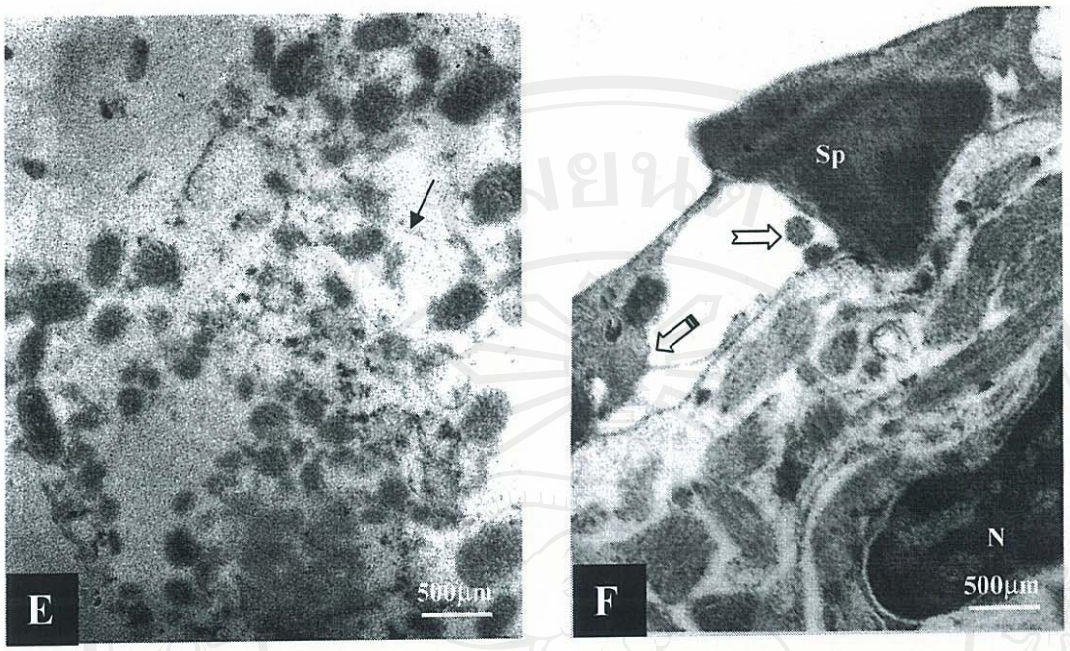


Figure 40. Transmission electron micrographs of *Haplorchis taichui* adult following incubation in 0.01 µg/ml niclosamide solution for 24 hours.

A. Micrograph showing the basal region of the tegumental syncytium, where the muscle blocks remain unchanged after 1 hour.

B. Mitochondria showing some swelling after 6 hours.

C. Some blebs (⇦) developed in syncytium after 12 hours.

D. Vacuolization (←) appeared in the deep layer of the tegumental cells after12 hours.

E. Tegumental cell showing widespread swelling and vacuolization (←) forming after 24 hours.

F. Some blebs (⇦) formed in the apical syncytium and swelling of the basal infolds (⇦) developed after 24 hours.

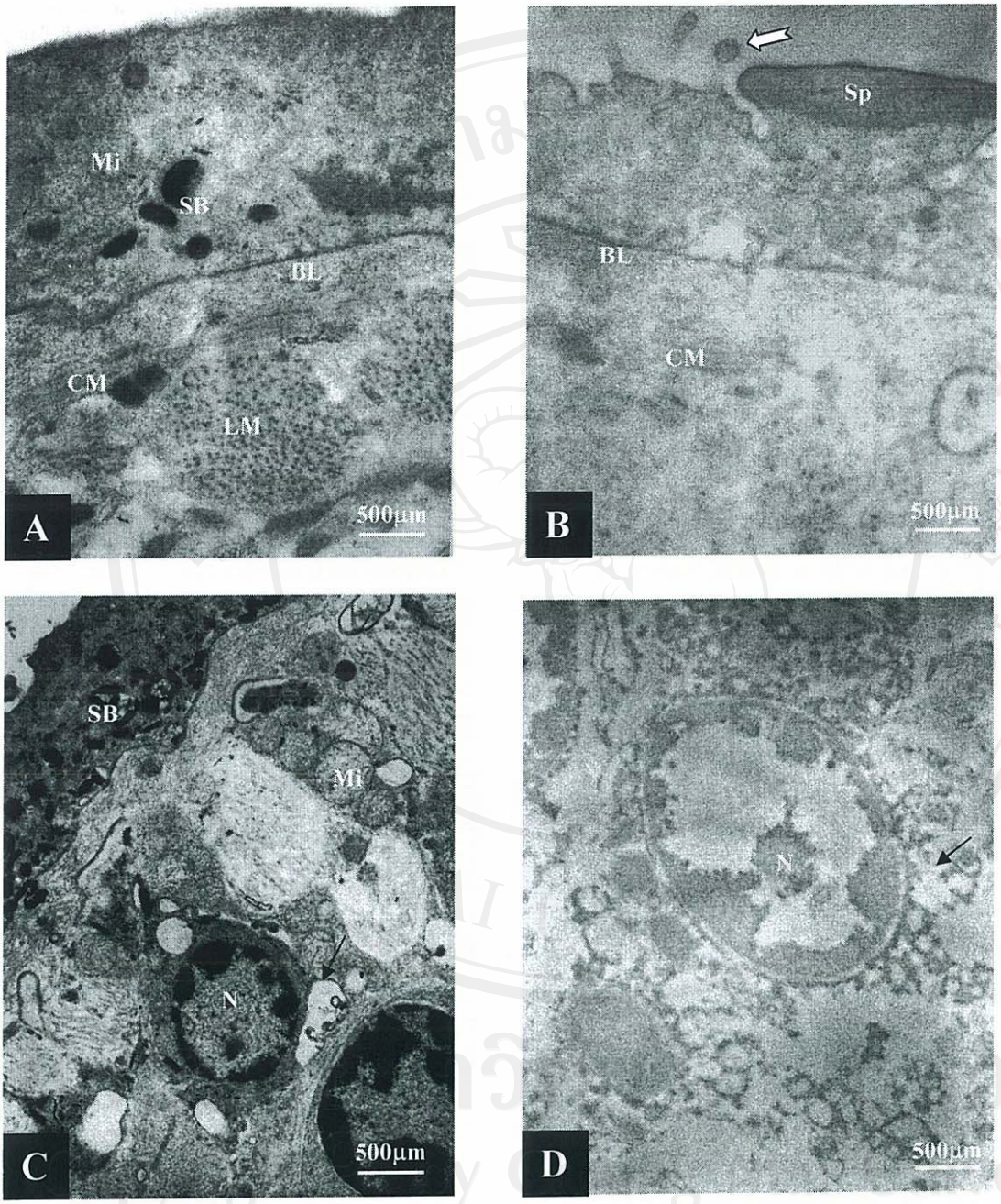


Figure 41

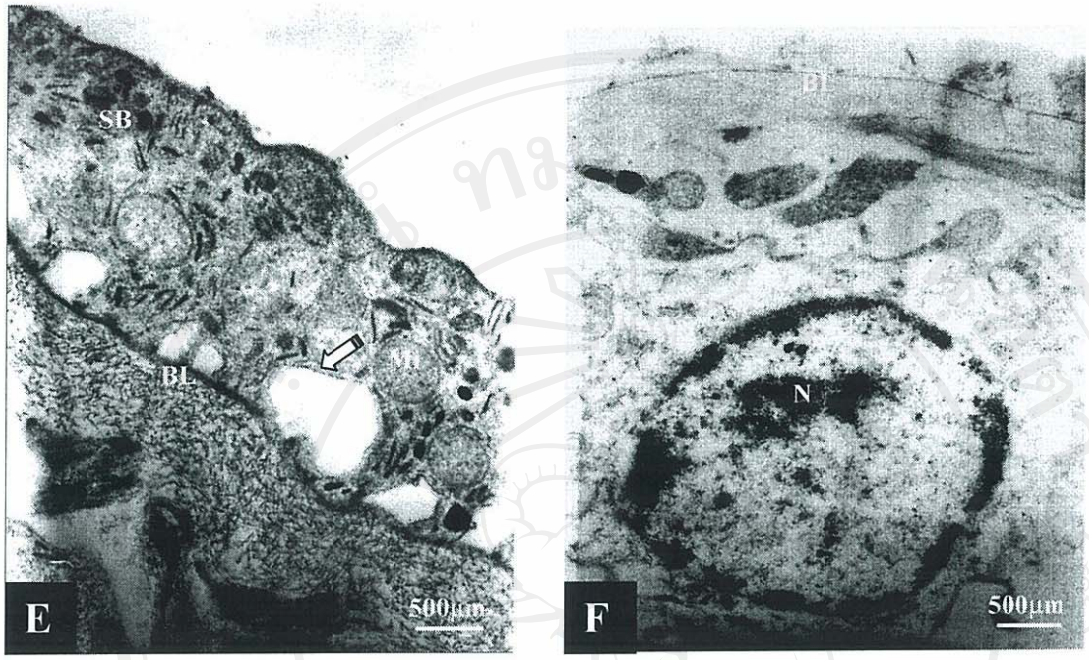


Figure 41. Transmission electron micrographs of *Haplorchis taichui* adult following incubation in 0.1 µg/ml niclosamide solution for 24 hours.

A. The tegument of worm is still normal after 30 minutes.

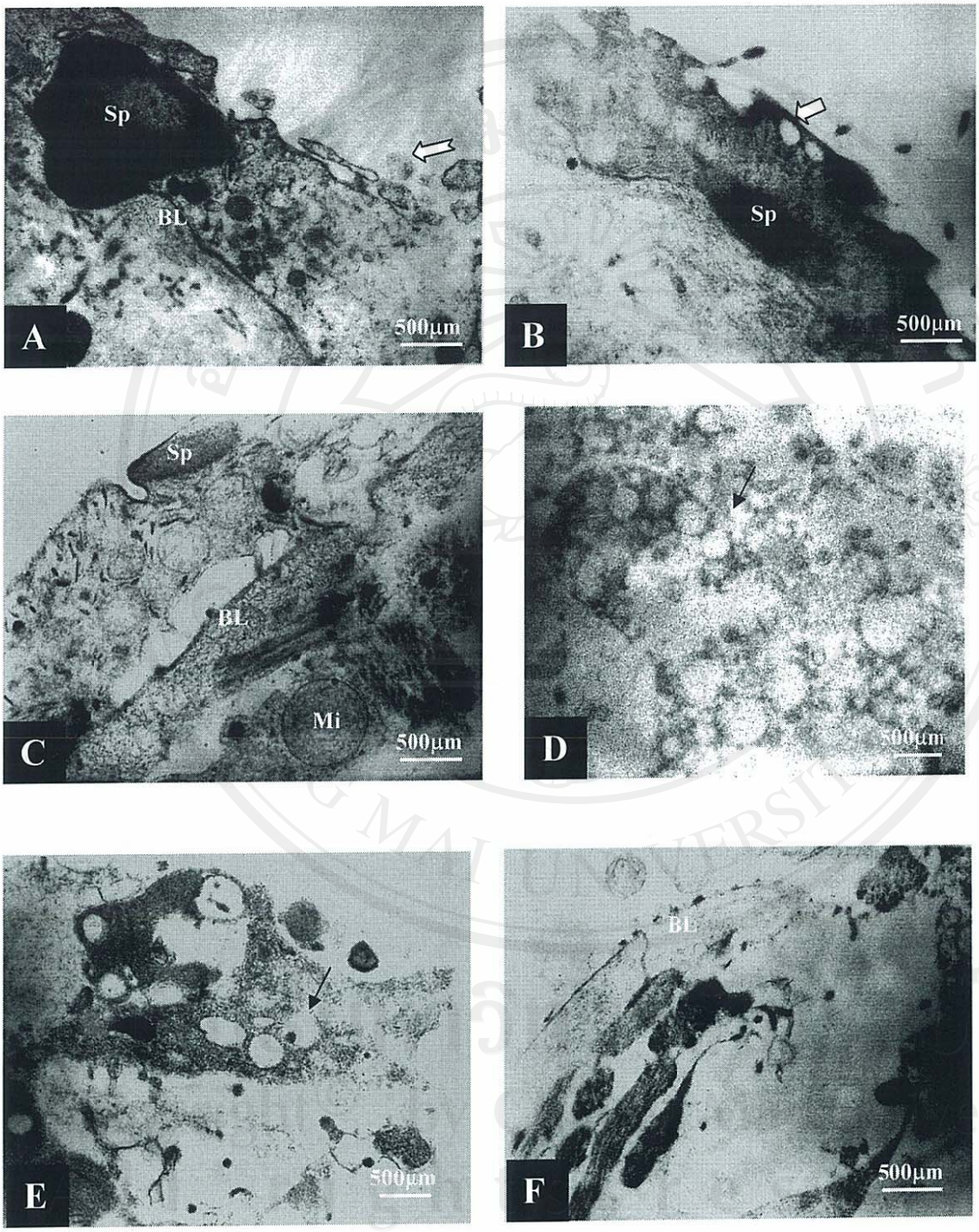
B. Tegument apex showing localized blebbing (⇦) after 1 hour.

C. Tegument syncytium showing vacuoles (←) after 6 hours.

D. The cytoplasm of the blebs is highly vacuolated in the tegument (←) after 6 hours.

E. Tegument showing swelling of the basal infolds (⇦) after 12 hours.

F. Some areas of tegument show detachment of the inner plasma membrane from the underlying basal lamina after 24 hours.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D
BL
500µm
E
500µm
F

Figure 42

Figure 42. Transmission electron micrographs of *Haplorchis taichui* adult following incubation in 1.0 μg/ml niclosamide solution for 24 hours.

A. Some blebs (⇦) can be seen on the apical surface after 1 hour.

B. Open bodies (⇦) are present at the apex of tegument after 1 hour.

C. The plasma membrane is swollen and detached from the basal lamina after 6 hours.

D. The large number of vacuoles (←) have formed in the tegumental cell after 12 hours.

E. Micrograph showing a badly disrupted parenchymal cell and vacuoles (←) are apparent after 12 hours.

F. The tegument is disrupted and detached from the body exposing the basal lamina and muscle after 24 hours.

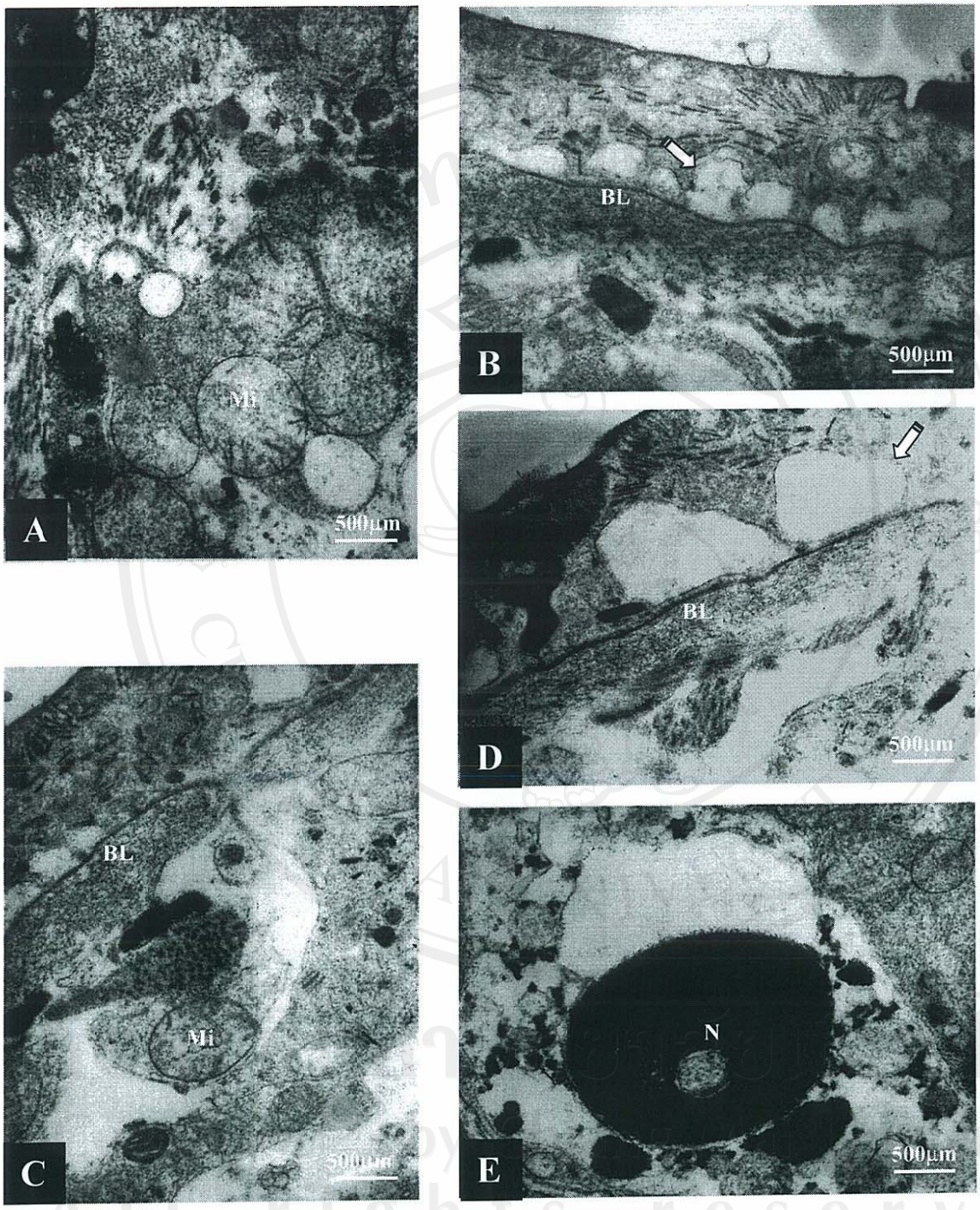

Figure 43

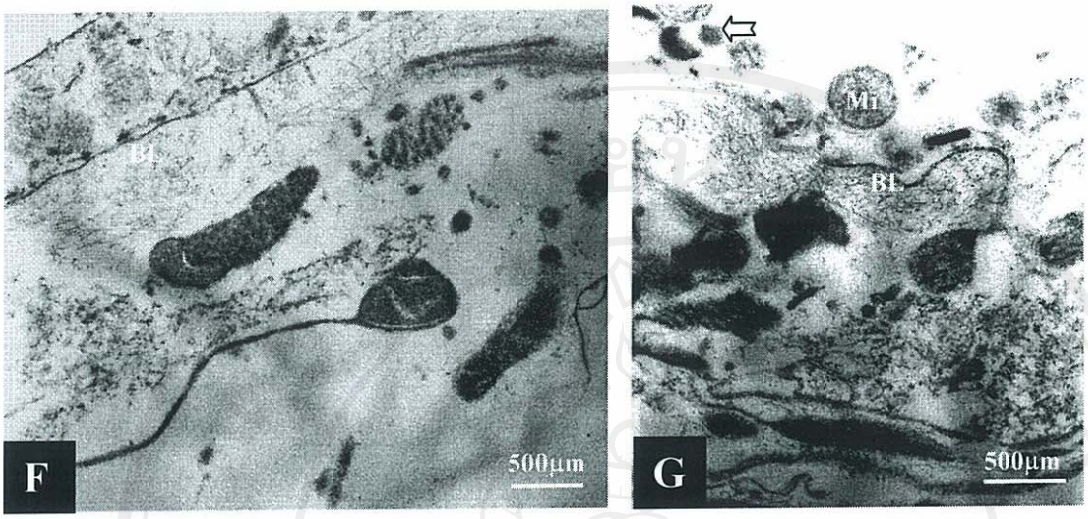

Figure 43. Transmission electron micrographs of *Haplorchis taichui* adult following incubation in10.0 µg/ml niclosamide for 24 hours.

A. The mitochondria become swollen and rounded in appearance, with defined cristae after 30 minutes.

B. The swellings of basal infolds (⇦) appeared normal after 1 hour.

C. Some vacuoles occurred resulting tegument damage after 6 hours.

D. Basal infolds (⇦) are more pronounced and the muscle layer become depolimerized after 12 hours.

E. The tegument becomes more vacuolized and the nucleus is damaged after 12 hours.

F. The subtegumental muscles are extensively depolymerized after 24 hours.

G. The apical surface is lost with some blebs (⇦) and disrupted tegument after 24 hours.