

SEATO MEDICAL RESEARCH STUDIES ON GNATHOSTOMIASIS IN THAILAND

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Objectives To determine the prevalence of Gnathostoma spinigerum in man and animals in Thailand and to carry out clinical and epidemiologic studies on diagnostic methods, pathology, treatment and preventive measures. Additional objectives are to further delineate the life cycle, mode of transmission and identify intermediate hosts, as well as compare G. spinigerum with G. hispidum, G. doloresi and other gnathostomes of animals, which are also present in Thailand.

General Methods

The stomachs of dogs killed at the Bangkok-Thonburi Municipality Rabies Control Unit were examined for the presence of gnathostomes. Periodically similar examinations were made of stomachs (and occasionally other tissue) from pigs, obtained from the Bangkok Slaughter house. As opportunity permitted, stool examinations for gnathostome ova were carried out on various animal species.

Each month, about 2 Kg. of fresh-caught snake-headed fish were purchased in the markets of Ayuthaya and Phetburi. These fish were examined for the presence of 3rd stage larvae of G. spinigerum. This data was used for monthly prevalence estimation. Poisonous snakes from the Thai National Red Cross Snake Farm which died spontaneously, were examined for G. spinigerum.

Experimental infection was attempted by feeding 2nd and 3rd stage larvae to various laboratory and wild-caught vertebrates, to determine potential second intermediate or paratenic hosts. Comparisons were made of G. spinigerum, G. hispidum and G. doloresi. Penetration of rodent skin by G. spinigerum was investigated.

Viability of G. spinigerum larvae in fresh water, with and without animal tissue was studied. Experimental studies began in previous years on the effects of G. spinigerum infections in various animal hosts were continued. These included: pathologic changes in white mice, infectivity rate, spontaneous cure and egg production in dogs and cats, infection in egg-laying chickens, and effects on skin sensitivity and peripheral hemogram in the monkey.

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Progress

Investigation of human gnathostomiasis as described in previous annual reports was suspended for this reporting year, in order to concentrate more fully on infections in animals.

Natural infections

Table 1 summarises the seasonal distributions of findings upon examination of dog stomachs from the Bangkok-Thonburi Municipality Rabies Control Unit, snake-headed fish purchased at fish markets in Ayuthaya and Phetburi, and snakes from the Thai National Red Cross snake farm.

Forty two (0.8%) of 5,372 dog stomachs were positive for G. spinigerum. Advanced 3rd stage larvae of G. spinigerum were found in 23.8% of snake-headed fish from Ayuthaya and Phetburi. One specimen contained 59 3rd stage larvae. An additional 495 young snake-headed fish were supplied by the Bureau of Fisheries. 127 (25.7%) were found to be infected. Among poisonous snakes listed on Table 1 G. spinigerum were found in 2 of 16 Naja hannah (king cobra) and 4 of 319 Naja naja (cobra) examined.

Additional species in which 3rd stage larvae were found include: Rana rugulosa (frog) with 3 of 5 positive, one common bittern (Ixobrychus cinnamneus).

Table 2 summarises species investigated which have not been found to harbor 3rd stage larvae.

The golden cat (Protilis temmincki) was shown to be a natural definitive host. Ova of G. spinigerum were found in the stool of one wild-caught specimen. These ova were hatched, and successfully passed through Cyclops spp and proved infective to white mice and to one adult dog (#8). However one cat (#68) fed 3rd stage larvae from these mice has not yet begun to pass gnathostome ova.

Stool examination of 11 domestic cats from Bangkok were negative.

A total of 959 adult gnathostomes were recovered from the stomachs of pigs from the Bangkok Slaughter House. None were G. spinigerum, 359 (80 male, 279 female) were identified as G. hispidum and 620 (86 male, 534 female) as G. doloresi.

These findings, both as to seasonal distributions and species involved are consistent with data from previous annual reports.

Experimental infection of certain vertebrate animals with known number of the third-stage larvae of the parasite was repeated on 3 more crab-eating monkeys (Macaca irus No. 14, 15, and 16) each of them was orally given 17, 14 and 15 third-stage larvae obtained from chickens and white mice. All monkeys showed infection with the unencysted larvae as follows: 9 in the flesh of all monkeys, 1 in each of two livers and 2 in the esophageal wall and subcutaneous tissue of the abdominal wall when sacrificed 10-34 days after the feeding experiment. It is worthy of note that the larvae seen in the livers of two monkeys show no cyst wall formation. Macroscopically only a few small yellowish gray irregular necrotic areas are seen on the upper surface of the right lobes of the livers otherwise normal. Also one white rat and one hamster sacrificed 180 days and 190 days respectively after each being fed with only 1 third-stage larvae of 250 days and 212 days old removed from the preceding experimentally infected rats and hamsters under the plan of experiment for determining the survival of the larvae shows still one living encysted third-stage larvae in the flesh of each animal.

One palm civet cat (Paradoxurus hermaphroditus canus) was sacrificed 584 days after being fed with 95 advanced third-stage larvae removed from infected snakes, white rats and white mice showed 23 encysted larvae in the subcutaneous tissue of the chest, abdomen, back and limbs. Each of these larvae was surrounded by rather thick fibrotic wall and their size was not markedly different from those of the originals.

An experimental feeding with various numbers of fully developed larvae in cyclops was repeated with 8 adult quails (Coturnix coturnix). Each quail was fed with from 20-100 fully developed larvae (10

Table 1. Result to study for 12 months on the incidence of infection of Bangkok Domestic dogs with adult G. spinigerum and infection of second intermediate or paratenic hosts (snake-headed fish and poisonous snakes) with the advanced third-stage larvae in few endemic areas nearby Bangkok.

	Total	Apr67	Ma	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Jan68	Feb	Mar
Dog's stomach (Infection with adult <u>G. spinigerum</u>) % positive	42/5372 0.8	0/408 0	2/378 0.53	6/410 1.46	12/456 1.63	10/560 1.78	1/395 0.25	7/527 1.32	1/570 0.17	1/389 0.25	1/474 0.21	0/501 0	1/304 0.33
<u>Ophicephalus striatus</u> (Snake-headed fish)													
(No. pos./No. exam)	8/19	—	—	0/2	4/6	—	—	—	1/2	1/3	1/4	0/1	1/1
large*	33/153	—	8/9	4/15	1/5	6/8	0/14	1/20	1/13	2/15	0/16	4/22	6/16
small*	41/172	—	8/9	4/17	5/11	6/8	0/14	1/20	2/15	3/18	1/20	4/23	7/17
Total	23.8	—	88.8	23.5	45.4	75.0	0	5.0	13.3	16.6	5.0	17.3	41.1
Infection with advanced third-stage larvae													
% positive	5/340	2/9	1/11	2/49	0/9	0/83	0/65	0/40	0/31	0/11	0/14	0/8	1/10
Poisonous snakes (No. pos./No. exam.)													
(Naja naja & hannah, Viperaruselli, Bungarus fasciatus) Infection with advanced third-stage larvae	1.5	22.2	9.1	4.1	0	0	0	0	0	0	0	0	10.0
% positive													

* Large Ophicephalus striatus = 500 grams and over

* Small Ophicephalus striatus = Less than 500 grams

Table 2. Animals found to be negative for G. spinigerum third-stage larvae.

<u>Class</u>	<u>Species</u>	<u>Number</u>
Pisces	Ophicephalus gachua	2
	Clarias macrocephalus	5
	Lambiobarbus sumatranus	2
	Trichopsis vittatus	16
	Trichogaster trichogaster	1
Amphibia	Bufo melanostictus	16
Reptilia	Bungarus fasciatus	1
	Vipera russelli	4
	Agkistrodon blomhoffi	1
	Boiga	1
	Homalopsis buccata	1
	Pyas mucosus	1
	Xenopeltis unicolor	1
	Calotes versicolor	4
	Gekko gekko	1
	Physignathus cocincinus	3
Aves	Coturnix coturnix	1
	Pycnonotus blanfordi	1
	Phragamaticola acdon	1
	Passer montanus	1
	Diceum cruentatus	1
	Aegithina tiphia	1
	Lonchula punctulata	1
	Merops orientalis	1
	Timalia pileata	1
	Centropus sinensis	1
	Passer goiavier	7
	Rhipidura javanica	14
	Cynopterus brachyotis	57
	Passer flaveolus	84
	Pycnonotus goiavier	97
Mammalia	Rhizomys pronosus	1
	Tupaia glis	1
	Rattus rajah	1
	Mus famulus	1
	Herpestes javanicus	1
	Callosciurus finlaysoni tachardi	1
	Rattus exulans	2
	R. rattus	3
	Aonyx cinerca	9

days old) in cyclops. Of 8 quails, 5 were sacrificed on 20-21 days and 3 were autopsied 27-33 days after the experiment; all were negative.

This experiment was also attempted with negative results using a tree lizard (Calotes versicolor) and a palm civet cat. However, one of 2 toads (Bufo melanostictus) was found positive when sacrificed after such an exposure.

The possibility of migration of larvae from chicken to egg was further examined. The 13 hens previously described (Annual Report SMRL 67) were carried forward; 430 eggs laid were found to be negative. During this year 7 of the 13 hens died (119-548 days after feeding) of unknown causes. 95 encysted larvae were found in the 7 hens.

Study on first intermediate host: A study was developed with Miss Varunee Sooksri, candidate for the Master Degree of Science (Parasitology), University of Medical Science, to determine species of cyclops capable of acting as the first intermediate host of the worm. The result has been accepted as a thesis entitled "Studies on species of cyclops in Bangkok and neighbouring areas with reference to those acting as first intermediate host of *Gnathostoma spinigerum*", and are summarised below. Cyclopoid copepods were collected from shallow ponds and fresh water ditches in Bangkok and Samutprakan areas. Specimens were tentatively identified.* They were fed with *G. spinigerum* first-stage larvae. The larvae were then allowed to develop at room temperature (28°-30°C). The fully developed stage, in the cyclops, were fed to laboratory bred white mice (*Mus musculus*) for further development to the advanced third-stage larvae. Results show 4 species of cyclops capable of acting as the first intermediate host of *G. spinigerum*: *Mesoocyclops leuckarti* Claus (1857), *Thermocyclops Kiefer* (1927) sp., *Eucyclops agilis* Koch 1838 and *Cyclops varicans* Sars (1863).

Skin penetration of *G. spinigerum* advanced third-stage larvae. The possibility exists that the vertebrate host is infected by *G. spinigerum* advanced third-stage larvae through direct skin penetration. To investigate this possibility, the following study was devised. The skin of white mice or rats was prepared by shaving. Some shaved areas were allowed to remain intact (healthy skin); others were scratched 20 times with the tip of a #17 needle (scratched skin) or had two 1.0 mm cuts made with a razor blade (incised skin). A total of 11 adult white rats and 14 adult white mice were so treated.

On each area of skin (about 4 cm²), 3-4 advanced 3rd stage larvae were placed, in a drop of tap water. Skin penetration was observed with a stereoscopic microscope. The results were as follows:

(1) Healthy Skin.

20 larvae successfully penetrated the healthy skin of white mice. The total time required from placing each larvae on the skin until completely penetrated through it was from 6 to 197 minutes. (9 larvae penetrated in 6-20 minutes, 11 larvae penetrated in 30-197 minutes). For each of 15 larvae the actual time required to complete the penetration (or penetrating time) varied between 1-10 minutes, while the other two took 15 minutes each and the rest finished in 12, 21 and 49 minutes.

Fifteen larvae penetrated the healthy skin of white rats. The penetrating time required by each larvae varied from 5-87 minutes. (5 larvae penetrated in 5-10 minutes, 4 larvae in 14-19 minutes, and 6 larvae in 37-87 minutes). The total time required by each of the 15 larvae was from placement to complete penetration (9 larvae required 14-31 minutes, 6 larvae took 71 to 180 minutes).

(2) Scratched Skin.

24 larvae were placed on the scratched skin of white mice. The total time required for each larva to completely penetrate the skin varied from 8-48 minutes. (3 larvae required 8-10 minutes, 11 larvae

* Final identification of cyclops was made by Dr. Harry C. Yeatman, Dept. of Biology, the University of the South, Sewanee, Tennessee.

required 11–19 minutes and the other 10 larvae required a total time of 20–48 minutes); the actual penetrating time required varied from 1–12 minutes (10 larvae required 1–10 minutes and 14 larvae required 11–21 minutes). 21 larvae were placed on the scratched skin of white rats. The total time required for penetration was in the range of 15–70 minutes and the actual penetrating time required was 10–50 minutes. For total time; 3 larvae required 15–20 minutes, 4 larvae required 21–30 minutes and 14 larvae required 31–70 minutes. For actual penetrating time; the 9 larvae required 10–20 minutes and 12 larvae required 23–50 minutes.

(3) Incised Skin.

18 larvae were placed in the incised skin of two mice. The total time required for the larvae to completely penetrate the skin into the subcutaneous tissue was 3–24 minutes, 5 larvae completed penetrating within 3–5 minutes. The actual penetrating time was 3–22 minutes, 6 larvae complete within 3–5 minutes. It is obvious that advanced third-stage larvae can completely penetrate healthy skin, scratched skin, or incised skin of the experimental animals within a short period of contact. Accordingly it may be reasonably assumed that other vertebrate hosts, including man, may be also naturally infected by skin penetration of the larvae.

The study on infectivity rate of the advanced third-stage larvae and the egg production per day per female *G. spinigerum* in the definite host.

Cat #47 was found positive with 2 adult male and 5 female *G. spinigerum* in one gastric tumor of about the size of 3.0×2.0 cm. The cat died 370 days after being fed with 33 *G. spinigerum* advanced third-stage larvae from the livers of white mice. The first positive stool was found 235 days after feeding; the cat died of unknown cause on day 135 of the positive period. The egg-count per day per female worm was found to be about 230, 750 and the infectivity rate was 21.0%.

Cat #50 was fed with 34 *G. spinigerum* advanced third-stage larvae 122 days after proved negative in the laboratory. The cat died of unknown cause 216 days after the experimental feeding and on examination there were 10 immature *G. spinigerum* (3 males measured at $5.6-14.1 \times 0.6-1.1$ mm. and 7 females measured at $5.9-12.6 \times 0.6-1.1$ mm.) Each of them had 8 rows of cephalic hooklets incompletely developed. They were found in the following organs; 3 under the serous layer of the stomach, 2 at greater omentum, 2 in diaphragm, 2 in chest muscles and 1 in the flesh of the upper part of the back. More-over 3 full-grown larvae, measuring $4.3-7.5 \times 0.6-0.7$ mm. were found in fatty tissue around the right kidney and the upper part of the chest, each had 4 rows of cephalic hooklets.

Cat #51 was kept in the laboratory for 328 days before its death. It was kept for 122 days to show lack of natural infection, then fed with 33 *G. spinigerum* advanced third-stage larvae. This cat also died of unknown cause 206 days after the experimental feeding. On examination there were 21 immature male and female *G. spinigerum* and 2 which could not be differentiated as to sex. All worms had 7–9 rows of cephalic hooklets. The distribution of the worm in various organs and their sizes are as follows:

1 in a small gastric nodule showing no opening and 3 in the stomach cavity (1 male at 13.0×0.8 mm, 2 females at $11.6 \times 0.8-0.9$ mm), 7 at the greater omentum and 2 in the submucous layer of the stomach (5 males at $9.3-14.9$ mm \times $0.6-0.8$ mm, 4 females at $12.6-14.9 \times 0.8-0.9$ mm), 1 in the diaphragm, 3 in the peritoneum attached to the lesser curvature of the stomach (2 males of equal size at 14.3×0.8 mm and 1 female) and 4 in the flesh of abdominal wall, hind leg and intercostal region (3 males at $9.0-13.3 \times 0.6-0.8$ mm, 1 female at 10.0×0.6 mm).

Recovery ratios for larvae fed these two cats were 38 and 64% respectively.

The study on spontaneous cure of the infection with adult *G. spinigerum* on the definite host shows as follows:

Cat #37, adult male from Bangkok was first found naturally positive with the presence of ova of *G. spinigerum* in its stool for 107 days; the estimated output by Stoll's egg-count method was 258, 440

eggs for the ova per day initially, falling gradually to 103,20 eggs per day. Subsequently, weekly stool examinations done by the concentration method were negative for a period of 202 days. Forty three 3rd stage larvae were fed to this cat, but ova were not detected by the concentration method until 181 days after the experimental feeding.

The second patent period lasted about 155 days. The animal was sacrificed 90 days after being found stool negative; it had a normal gastro-intestinal tract without G. spinigerum. This experimental infection lasted for 336 days counting from the day on which the feeding experiment (about 5 months) was undertaken and the egg-positive period (patent period) was only 155 days. Spontaneous cure apparently occurred.

Cat #67, a female from Bangkok, was negative on stool examination for 54 days, after which it showed ova of G. spinigerum with great variations of numbers of eggs per day as counted weekly as shown by the Stoll's egg-count technique, from 334, 800 eggs to 45,080 eggs per day. Patent period was 134 days. This animal died of unknown cause after having been found stool negative for a period of 125 days. Examination showed no G. spinigerum, with a normal gastro-intestinal tract.

Skin sensitivity test on monkeys and rabbits. The plan for the determination of skin sensitivity on monkeys experimentally infected with third-stage larvae obtained from vertebrates, and fully developed larvae in cyclops, was continued from last year and appeared in the last Annual Progress Report. Skin testing of 4 monkeys was done as follows.

Monkey #9 (Macaca irus), intraspinally inoculated with 4 larvae obtained from a rat, was skin test positive up to 409-425 days later thereafter, it was negative. It was sacrificed 501 days after the experiment, and had one encysted advanced third-stage larvae in the flesh of a hind leg on examination.

Monkey #10 (Macaca irus), intraspinally inoculated with 6 larvae obtained from a snake, was skin test negative for 277 1 days after the experiment. It was then fed with 85 larvae obtained also from a snake, and was positive when skin tested on 105 days and 216 days after the feeding. Thereafter the test was negative until the date of sacrifice which was 353 days after the experimental feeding (day 620 of observation). At the autopsy the monkey showed 23 encysted advanced third-stage larvae in the flesh of body and legs, each being surrounded by a fibrous wall about 0.1 mm. to 0.3 mm. thick.

Monkey #13 (Macaca irus), infected orally with 200 fully developed larvae in cyclops (early third-stage), was skin test positive between days 159-258 of the experiment. On examination at autopsy (day 272) this monkey had 1 encysted advanced third-stage larvae (5.0 mm. x 4.0 mm.) in the flesh of its right hind leg.

Monkey #16, (Macaca irus) was skin test negative on 7 and 10 days after being fed with 15 larvae removed from white mice and on examination after autopsy done 10 days after the feeding shows 1 larvae in the liver and another in the esophageal wall.

Monkey #19, (Macaca irus) was added this year to the experiment and fed with 17 advanced third-stage larvae removed from the flesh of white rats and white mice. Skin test was done weekly for 3 weeks and 1 test for the week before writing this report shows still negative. Additionally, the study on peripheral white blood cells change being done at the same time, shows up to now no significant change. This study is to be continued.

In summary the result of skin tests done on five monkey showed during this period 1 monkey (#9) became skin test positive from 158 days as long as 425 days after being intraspinally given the advanced third-stage larvae. Another monkey (#10) which was skin test negative as long as 277 days after being also given the larvae intraspinally became positive beginning day 105 and lasting to day 216 after being fed with 85 larvae obtained from a snake. The third monkey (#13) became skin test positive as early as 21 days, and continues to be positive as long as 258 days after being fed with fully developed larvae in cyclops, the fourth (#16) was negative when skin tested on 7 and 10 days after

being fed with advanced third-stage larvae. The fifth (#19) has been studied only recently for skin sensitivity after being fed with the larvae and the test done about one month after the infection is negative. The animal is still being studied.

Two skin-tested laboratory rabbits (Oryctolagus cuniculus L.) (#1 and #4) died of unknown causes 522 days and 397 days respectively after a feeding experiment with larvae. Ten encysted living G. spinigerum advanced third-stage larvae were found in the flesh of the hind legs and the costal region of one rabbit (#1) the other (#4) had 4 encysted larvae in its hind legs. Rabbit #1 became skin test positive 13 days and became negative 500 days after being fed with the larvae; on the other hand rabbit (#4) was found first skin test positive 53 days and became negative 327 days after the experiment.

Peripheral white blood cells changes. The study on the effect of infection with G. spinigerum advanced third-stage larvae on changes of peripheral white blood cells was continued on 3 infected crab-eating monkeys (Macaca irus #9, #10 and #13) and one monkey (Macaca irus #5) being used as the control. Peripheral white blood cells counts of these monkeys made during the period covered by this Annual Progress Report showed neither significant changes in total white blood cells nor in eosinophiles. In order to determine the presence of G. spinigerum larvae in these monkeys, they were sacrificed and every organ was examined for the larvae, the results of which have been presented.

Viability of G. spinigerum advanced third-stage larvae. A study to determine the viability of G. spinigerum advanced third-stage larvae after being kept for various days in fresh water and in animal flesh room temperature (29°C—31°C) has been completed, the result, summarised on Table 3 shows that a few larvae are viable as long as 17 days after being left in fresh water with a small piece of infected flesh. One of 59 encysted larvae was viable on day 11 and of 289 larvae dissected from the flesh and cyst walls only 1 was found viable on day 15. In this attempt 1 of 2 viable larvae found on day 17 was fed to a white mouse for testing its infectivity, the living larva was located in the flesh of the animal sacrificed 21 days after the experiment. This experiment shows that very few larvae could be viable and become infective to vertebrate host after two weeks exposure to water.

Pathological Study.

White mice (Mus musculus musculus) are fed advanced 3rd stage larvae and sacrificed at intervals. This study was originally planned to last 6 months, but has been extended as a result of the findings of persistent lesions at 6 months.

The first 12 days of observation were included in the 1967 Annual Report (q.v.). The study was continued as follows:

Pairs (usually) of mice were sacrificed on days 14, 16, 19, 23, 26, 30, 40, 52, 70, 90, 110, 130, 150, 175, 200, 225, 250, and 300 after being fed five larvae each. By 14 days larvae were found 1 in the liver and peripheral tissues, with slight tissue reactions and no cyst formations. As early as 16 days there was diffuse acute degeneration of liver cells. Liver cellular reaction was more intense, with PMN and lymphocytes but few eosinophils.

At 26 days, thin fibrotic walls were found around most larvae except in the liver; by day 30 cysts were seen in all organs.

By day 40, foreign body giant cells were seen in the liver. Cyst walls were more prominent. Some evidence of healing with scar formation was seen. Between 50 and 90 days few changes were seen except for thickening of cyst walls. Cyst with degenerating larvae and attendant cellular reaction were seen occasionally after 120 days. Necrotizing pneumonitis was noted after 130 days. After day 150 all livers looked grossly normal, no intact larvae were found. Encysted apparently intact larvae were found in peripheral tissue, and degenerated larvae in liver as late as day 300.

It may be concluded that after being fed to mice, larvae quickly penetrate the stomach wall and migrate into the liver, peritoneal cavity, subcutaneous tissue and skeletal muscles. Larval encystment begins

Table 3. An experimental study on viability of the advanced third-stage larvae of *G. spinigerum* after being kept in water and in the animal's flesh at room temperature (29°C-31°C) on various days.

No. of day	59 viable encysted larvae		289 viable freed larvae		66 viable larvae in the flesh		Total experimented larvae 414	
	No. viable	%	No. viable	%	No. viable	%	No. viable	%
1	59	100	268	93	66	100	393	95
5	14	28	47	16	54	82	115	28
7	7	12	10	4	44	67	61	15
9	3	5	7	2	40	61	50	12
11	1	2	5	2	29	4	35	9
13	0	0	1	0.4	15	23	16	4
15	0	0	1	0.4	5	8	6	2
17	0	0	0	0	3	5	3	0.7
21	0	0	0	0	0	0	0	0

in muscle as early as 19 days, with young fibroblasts and monocytes involved. Encystment in the liver is common before 40 days. The inflammatory reaction of the liver is similar to an allergic response, with polymorphonuclear leucocytes, monocytes and eosinophiles. Encystment in the muscles is often accompanied by hyaline degeneration of adjacent tissue.

Liver reactions become less intense with time; after 175 days tissues appear virtually normal.

Comparative study on the size and morphology of *G. spinigerum*, *G. hispidum* and *G. doloresi*.

To further determination of various species of vertebrate host acting as definitive host, in which the adult *G. spinigerum* is located in the stomach, and as second intermediate and paratenic hosts, in which the advanced third-stage larvae of *G. spinigerum* is involved, a comparative study of significant morphological differences was undertaken to seek additional characters for the specific identification of the three species of *Gnathostoma*, *G. spinigerum*, *G. hispidum* and *G. doloresi*. In this connection, the number of cephalic hooklets in each row, and the number of rows formed by them on the head bulb of the adult worm, the size and morphology of the body spines for the three species of the parasite were investigated. The resulting description of the morphologic features tentatively suggested for identification is as follows:

Size: All adult *G. spinigerum* removed from the stomachs of naturally infected domestic dogs and all adult *G. hispidum* and *G. doloresi* collected from stomachs of naturally infected domestic pigs, were measured, after being fixed in warm 70% ethanol. The results of such measurements on 9-189 sex and species groups is summarised in Table 4.

Table 4. Measurements of mature adult Gnathostoma spinigerum, G. hispidum, and G. doloresi obtained from stomach walls of naturally infected domestic dogs and pigs.

Measurements		<u>G. spinigerum</u> in mm.	<u>G. hispidum</u> in mm.	<u>G. doloresi</u> in mm.
<u>Head</u> <u>and</u> <u>body</u>	Males: Total	39	18	60
	width range	1.0-3.0	1.0-2.0	1.5-2.5
	average	1.7	1.6	2.2
	Length range	21.0-40.0	13.0-35.0	13.0-30.0
	average	28.2	22.0	22.6
	Females: Total	87	85	189
width range	1.0-3.0	1.5-3.0	1.0-4.5	
average	2.3	2.0	2.7	
Length range	13.0-55.0	16.0-42.0	15.0-63.0	
average	34.7	26.2	34.0	
<u>Head</u>	Males: Total	20	17	9
	width range	0.3-0.8	0.6-0.8	0.7-0.9
	average	0.6	0.7	0.8
	Length range	0.2-0.6	0.3-0.6	0.3-0.6
	average	0.4	0.5	0.5
	Females: Total	20	79	48
width range	0.9-1.2	0.7-1.0	0.8-1.3	
average	1.0	0.8	1.0	
Length range	0.5-0.7	0.3-0.6	0.3-0.8	
average	0.6	0.5	0.5	

General appearance reveals significant differences as follows:

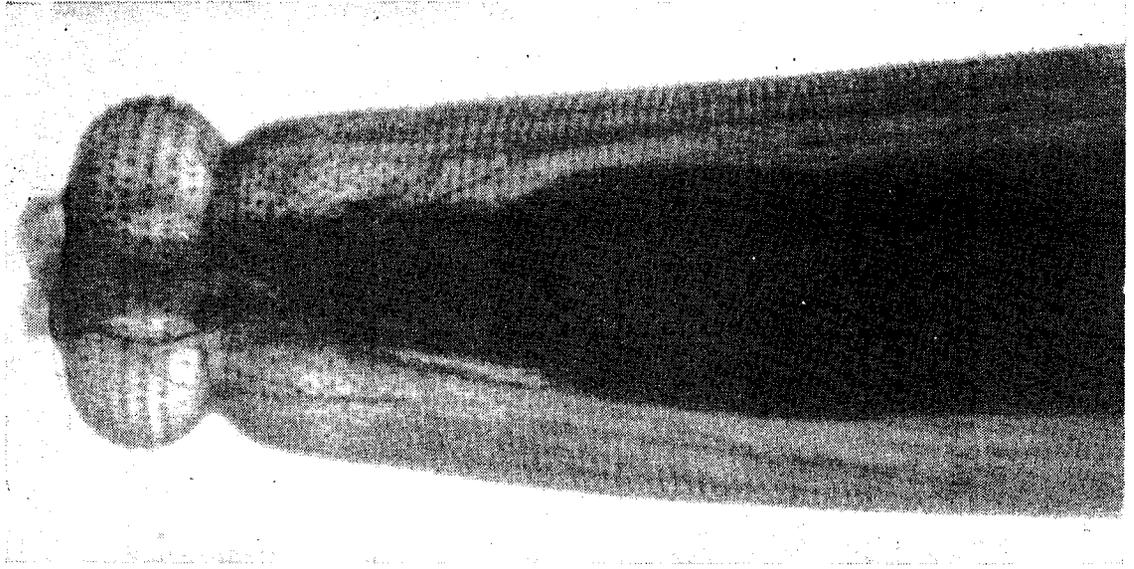
The adult G. spinigerum has tapering anterior and posterior parts with a graceful curve of the whole body, the maximum width is normally found at about the middle part of the worm. (Figure 1). The adult G. hispidum shows, immediately behind the disc-shaped cephalic bulb, the body circumference increasing considerably and rather suddenly and retaining the same diameter for a short distance. Thereafter diameter diminishes suddenly, then increases; consequently the maximum width is located at the beginning of the posterior half of the body. The remaining part of the body becomes smaller and rather cylindrical to the posterior end (Figure 2). The adult G. doloresi has a small diameter for about the anterior 2/5 to 1/2 of the body; the remaining part is considerably bigger and covered with swollen cuticle (Figure 3).



A

Figure 1 Adult G. spinigerum

A. A close up photograph showing of male and female with the general appearance tapering anterior and posterior parts as well as graceful curve of the worm. The maximum width of each is at its middle part.



B

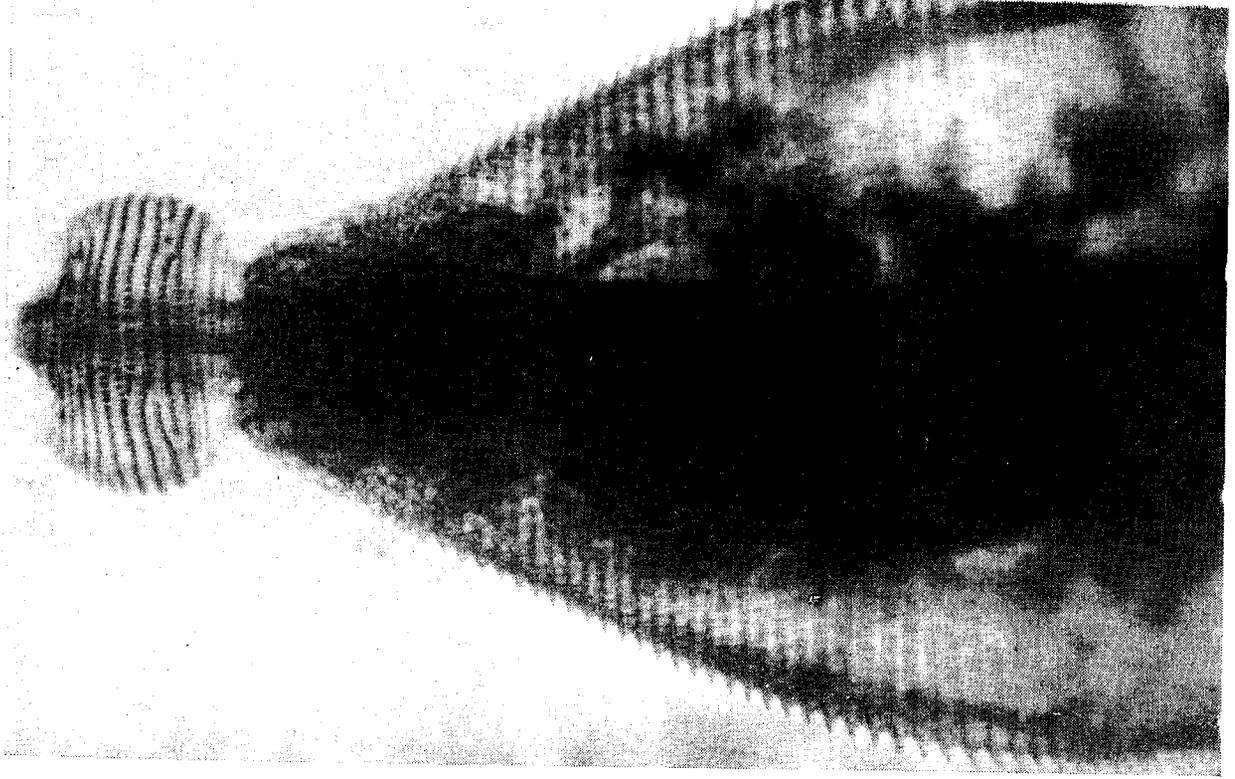
B. A photomicrograph shows the general appearance of the head bulb with 8 parallel rows of cephalic hooklets connecting with the anterior end.



A

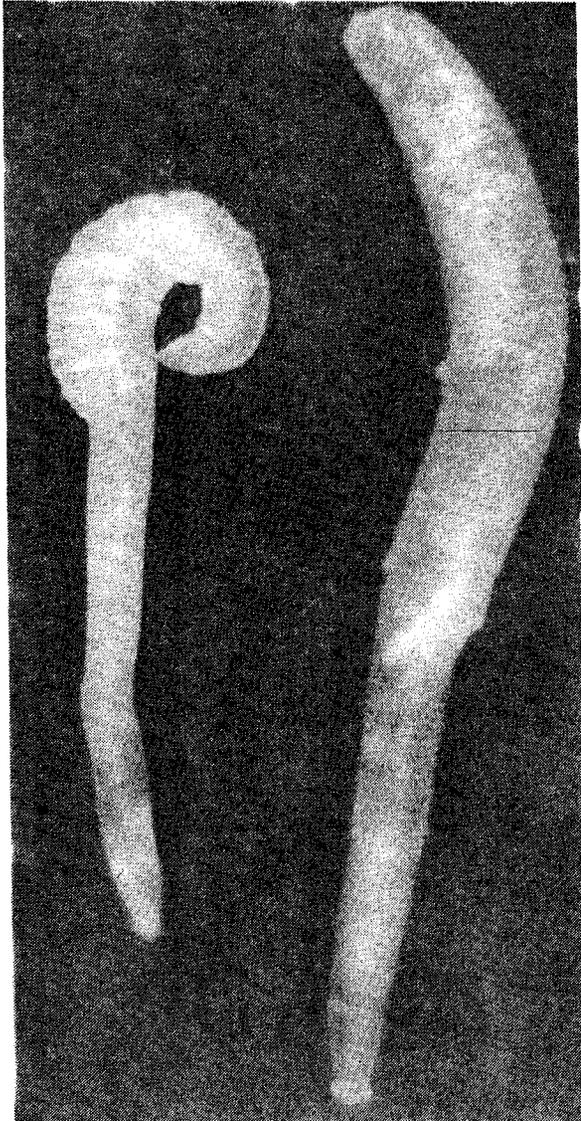
Figure 2 Adult G. hispidum

A. A close up photograph showing the general appearance of male and female with abrupt increase of anterior body circumference immediately behind the disc-shaped head bulb.



B

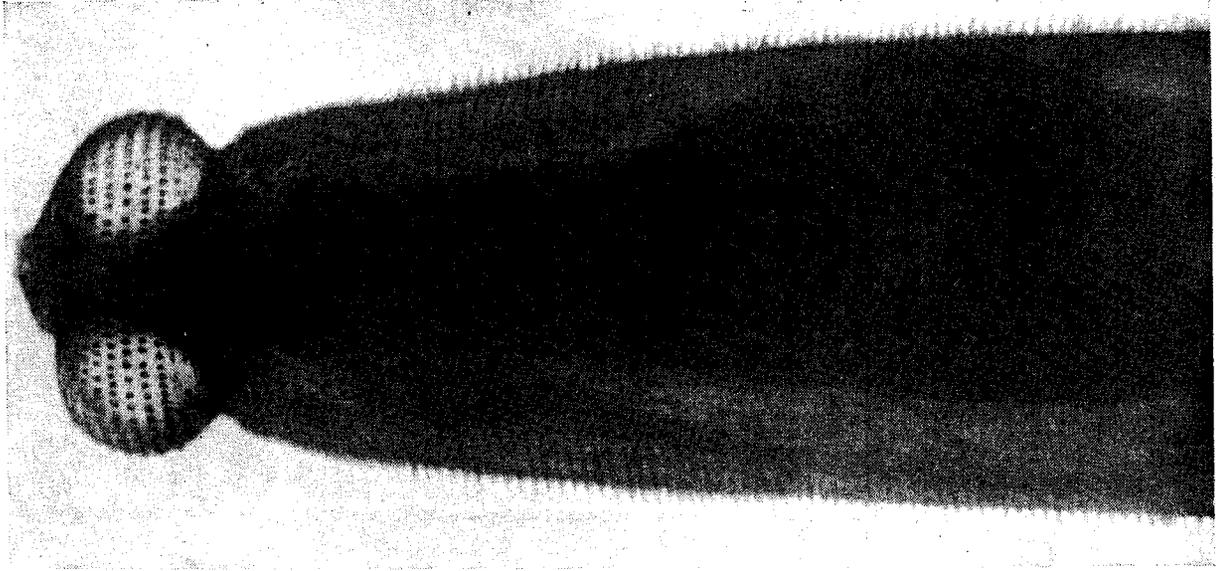
B. A photomicrograph showing the disc-shaped cephalic bulb with 12 rows of hooklets and conical anterior end.



A

Figure 3. Adult *G. doloresi*

A. A close up photograph showing the general appearance of male and female with smaller diameter of the anterior than the posterior parts which are bigger with swollen cuticle.



B

B. A photomicrograph showing the general appearance of the head bulb with 10 parallel rows of cephalic hooklets connecting with the anterior end.

Head: Cephalic hooklets in each row and the number of rows formed by them on the head of different species of the adult worm is as follows: 19 adult G. spinigerum have 7 to 9 rows of cephalic hooklets (2 with 7 rows, 2 with 9 rows and 15 with 8 rows), all hooklet rows parallel. The number of cephalic hooklets of one adult worm is in the range of 50–80 hooklets per row; the first row has only 20 hooklets of about equal size (8.0×13.0 microns) (Figure 4). 12 adult G. hispidum have 11 to 12 rows of hooklets (8 had 11 rows and 4 had 12 rows) and most of these rows are parallel, a few diverging here and there. (Figure 5). The first and last rows of hooklets are smaller, 15×10 microns and 10×5 microns respectively. The tips of the last row are less pointed in comparison with the other hooklets. The numbers of hooklets increase from 12 in row 1 to 155 in row 11 (Table 5). 30 adult G. doloresi under study show 9–12 rows of cephalic hooklets (1 has 12 rows of cephalic hooklets, 10 have 11 rows, 14 have 10 rows and 5 have 9 rows) (Figure 6). All rows are parallel and all hooklets are more or less the same size (15×11 microns) but increasing in number from 5 in row 1 to 147 in row 8 (Table 6).

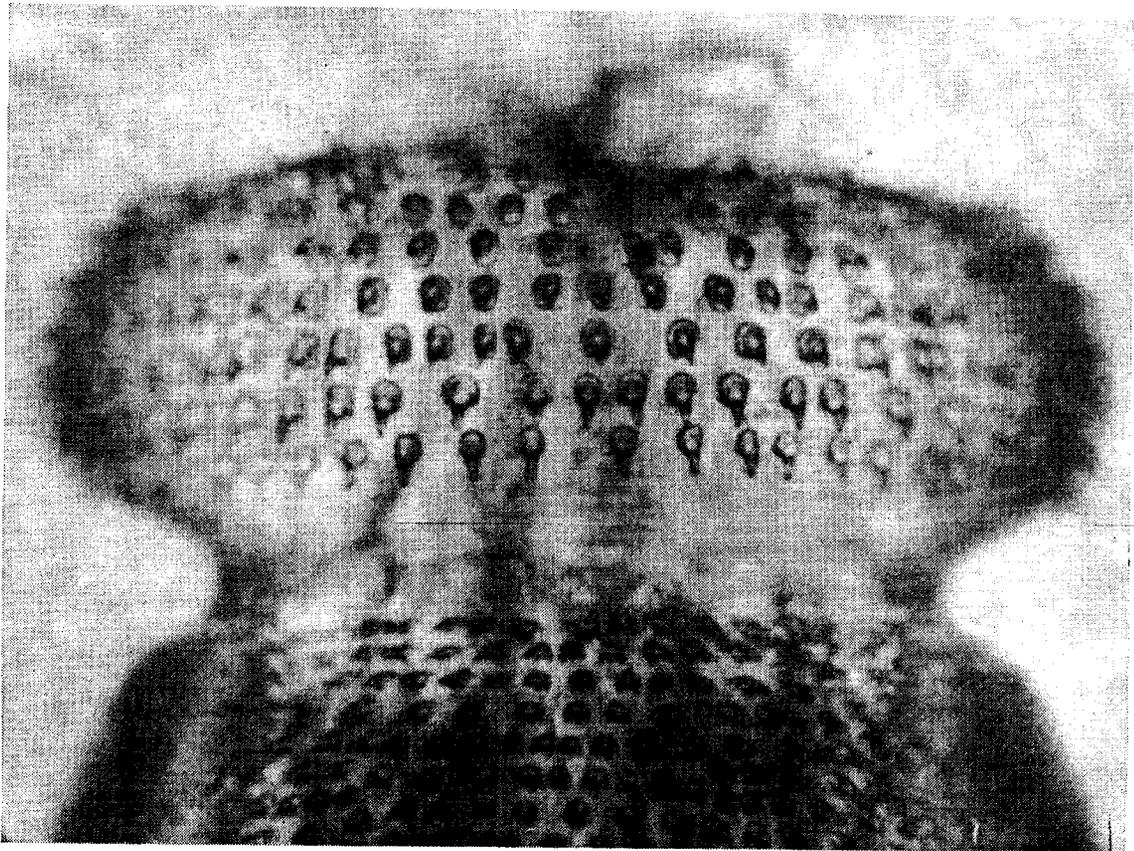


Figure 4. Photomicrograph of adult G. spinigerum showing 8 parallel rows of cephalic hooklets. All hooklets are about equal size (8×13 microns).



Figure 5. Photomicrograph of cephalic hooklets of G. hispidum. Most rows are parallel. Hooklets of the first row are 15×10 microns and of the last row are 10×5 microns.

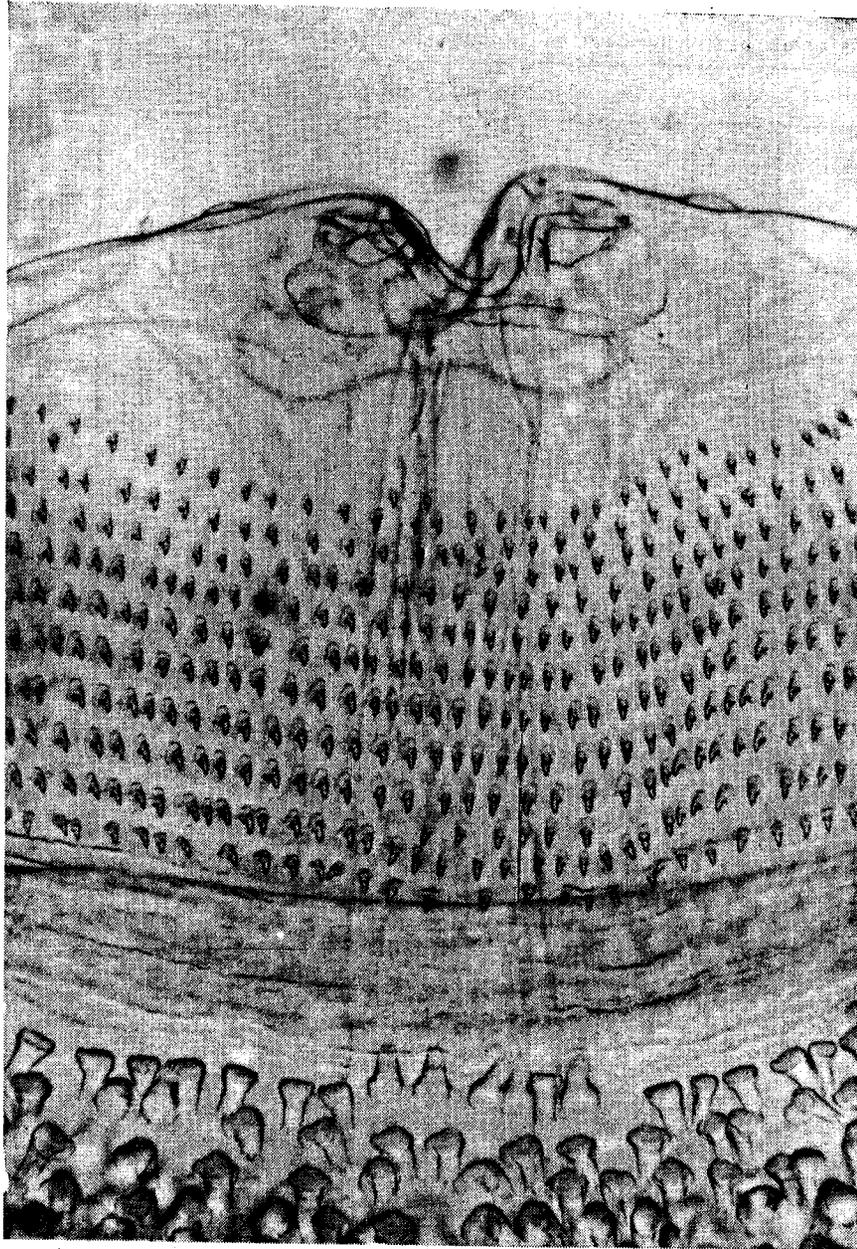


Figure 6. Photomicrograph of head bulb of adult *G. doloresi* armed with 11 parallel rows of cephalic hooklets of 15×11 microns increasing in number posteriorly from row 1 to row 8.

Table 5. Cephalic bulbs of 12 adult male and female G. hispidum from stomachs of naturally infected pigs; numbers of cephalic hooklets by row.

		Nos. of adult <u>G. hispidum</u>											
Nos. of rows	Nos. cephalic hooklet in groups.	1	2	3	4	5	6	7	8	9	10	11	12
		12-19	1	0	1	0	0	0	0	1	0	0	0
20-39	2	1	0	0	0	0	0	0	0	0	1	0	0
40-59	3	2	0	0	0	0	0	0	0	0	0	1	2
60-79	3	4	2	2	3	1	0	0	0	0	0	0	0
80-99	2	4	6	9	5	6	2	3	1	1	1	1	0
100-119	0	0	2	1	4	5	9	8	6	4	0	0	1
120-139	0	1	0	0	0	0	0	1	5	5	7	7	1
140-155	1	0	1	0	0	0	0	0	0	1	2	2	0

Table 6. Cephalic bulbs of 30 adult male and female G. doloresi from stomachs of naturally infected domestic pigs; numbers of cephalic hooklets by row.

		Nos. of adult <u>G. doloresi</u>											
Nos. of rows.	Nos. cephalic hooklet in groups.	1	2	3	4	5	6	7	8	9	10	11	12
		5-19	5	0	0	0	0	0	0	0	0	0	1
20-39	13	1	0	1	0	0	0	0	0	0	3	1	0
40-59	4	4	2	2	0	0	0	0	0	0	1	2	0
60-79	4	9	6	4	2	1	1	2	1	3	3	3	1
80-99	4	13	11	10	11	11	9	5	13	5	2	2	0
100-119	0	3	8	10	15	15	11	14	12	11	3	3	0
120-139	0	0	3	3	1	3	9	8	4	1	0	0	0
140-147	0	0	0	0	1	0	0	1	0	0	0	0	0

This study will be concluded when 50 adults of each species have been completely described.

Body cuticular spines. G. spinigerum: Cuticular spines are conspicuous, and cover the area from immediately behind the head-bulb to the middle of the body, the cuticle of the posterior half being irregularly scattered with a very few minute spines almost to the terminal part of the body. In the female, dense minute spines are seen in transverse rows on the terminal end and especially clearly seen on the ventral surface. In the male, the ventral surface of the terminal end is also covered with numerous minute spines in which a Y- or fork-shaped naked area is seen around the cloacal aperture, behind which some minute spines are seen arranged in two or three short arched lines.

The shape and size of cuticular spines varies with their position on the body, the first few rows behind the head-bulb showing 3 large teeth of which the middle is slightly longer than the lateral teeth; usually there are also one or two smaller and shorter teeth present. The size of each spine is about 25.0×15.0 microns (Figure 7). There are altogether about 30 rows of such spines, followed by many rows of densely arranged longer 3-tooth spines of which the middle tooth is clearly seen to be longer than the lateral teeth. Small, short accessory teeth are rarely seen; each spine measures at 62×16 microns (Figure 8). Spines end at about the junction of the middle third and the last third of the anterior half of the body. Subsequently the cuticular spines are slightly wider and shorter (56.0×24.0 microns) with 2 to 3 equal teeth occupying the last third of the anterior half of the body (Figure 9). The number of cuticular spines decreases posteriorly and the spines become smaller and shorter with mostly single-pointed or with heart-shaped appearance, measuring at 28×14 microns, to about the middle of the body (Figure 10).

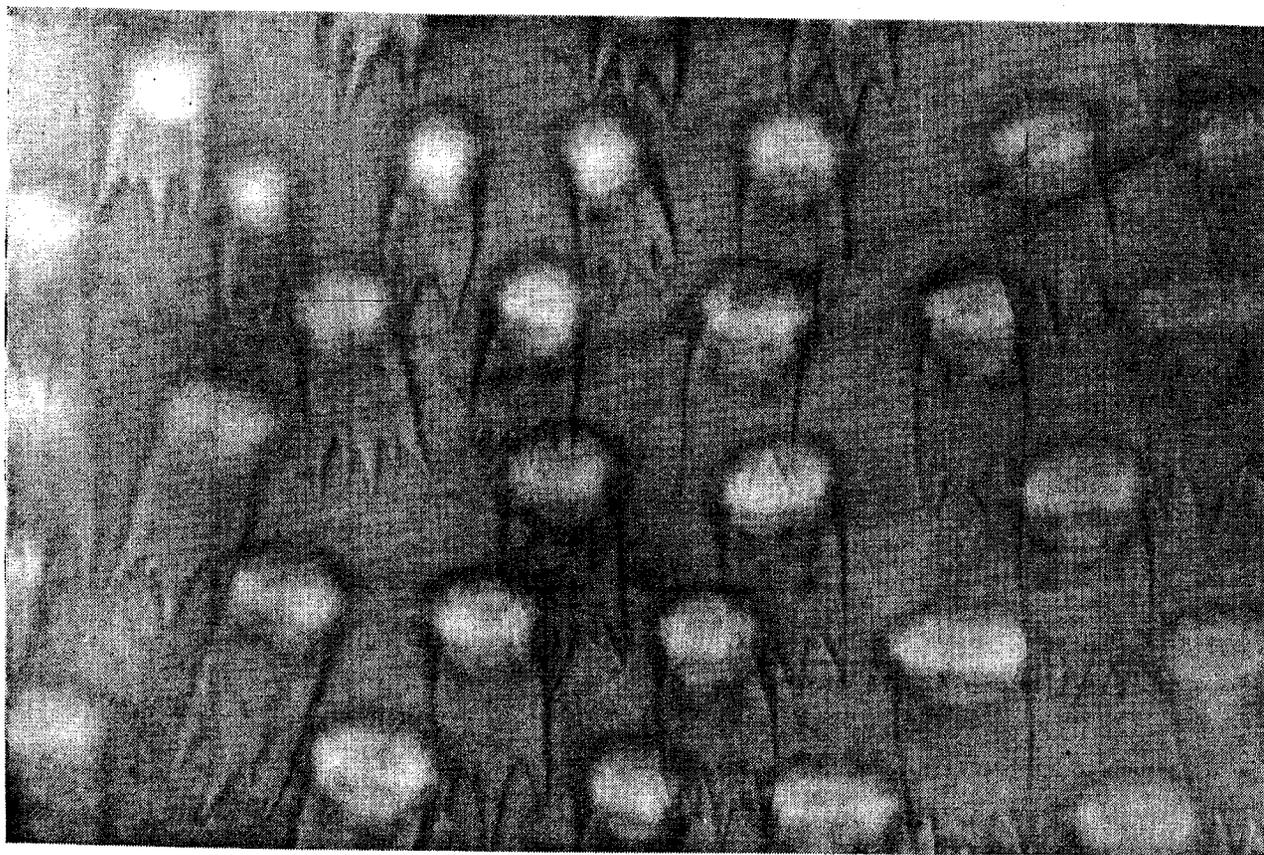


Figure 7 Photomicrograph. G. spinigerum. Cuticular spines of the area posterior to head-bulb. Each measures 25×15 microns.

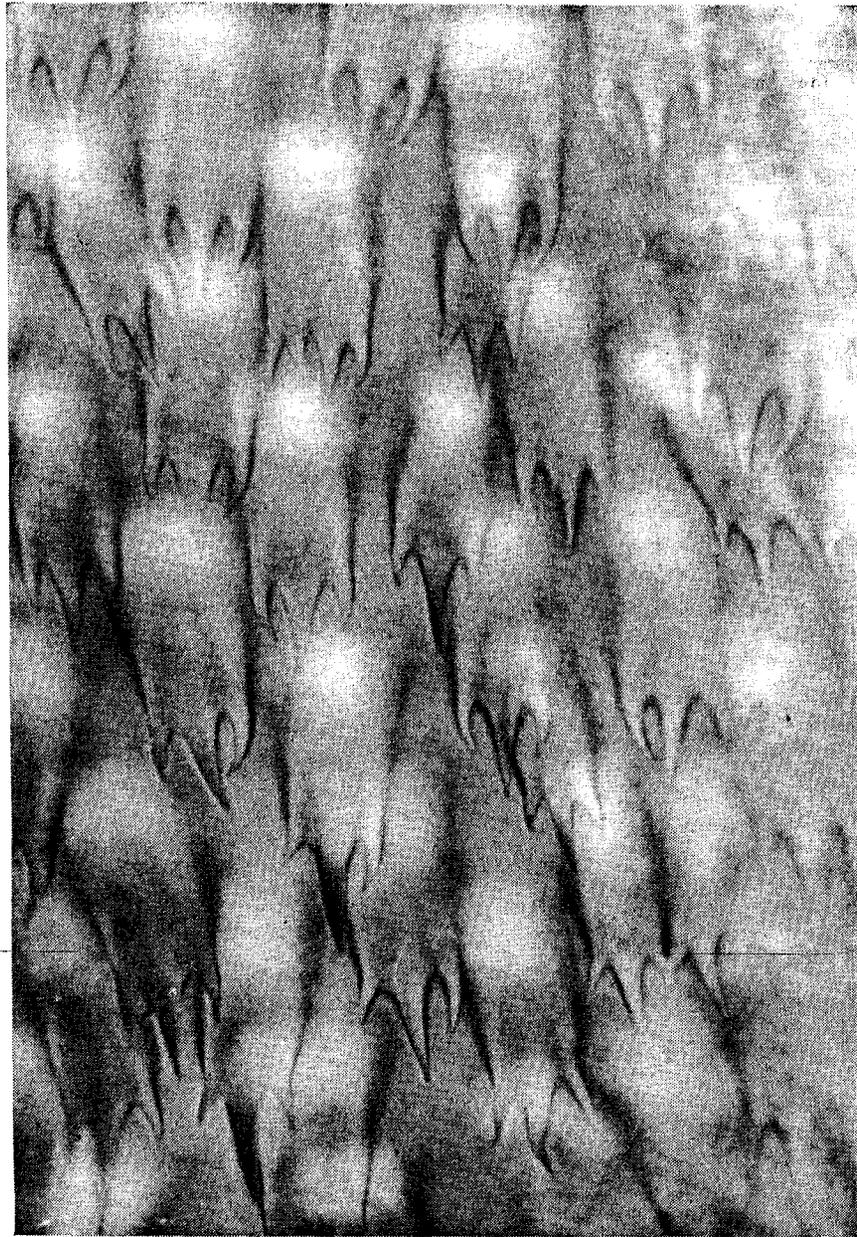


Figure 8 Photomicrograph G. spinigerum. Cuticular spines of middle third of anterior half of body. Each measures 62 x 16 microns.

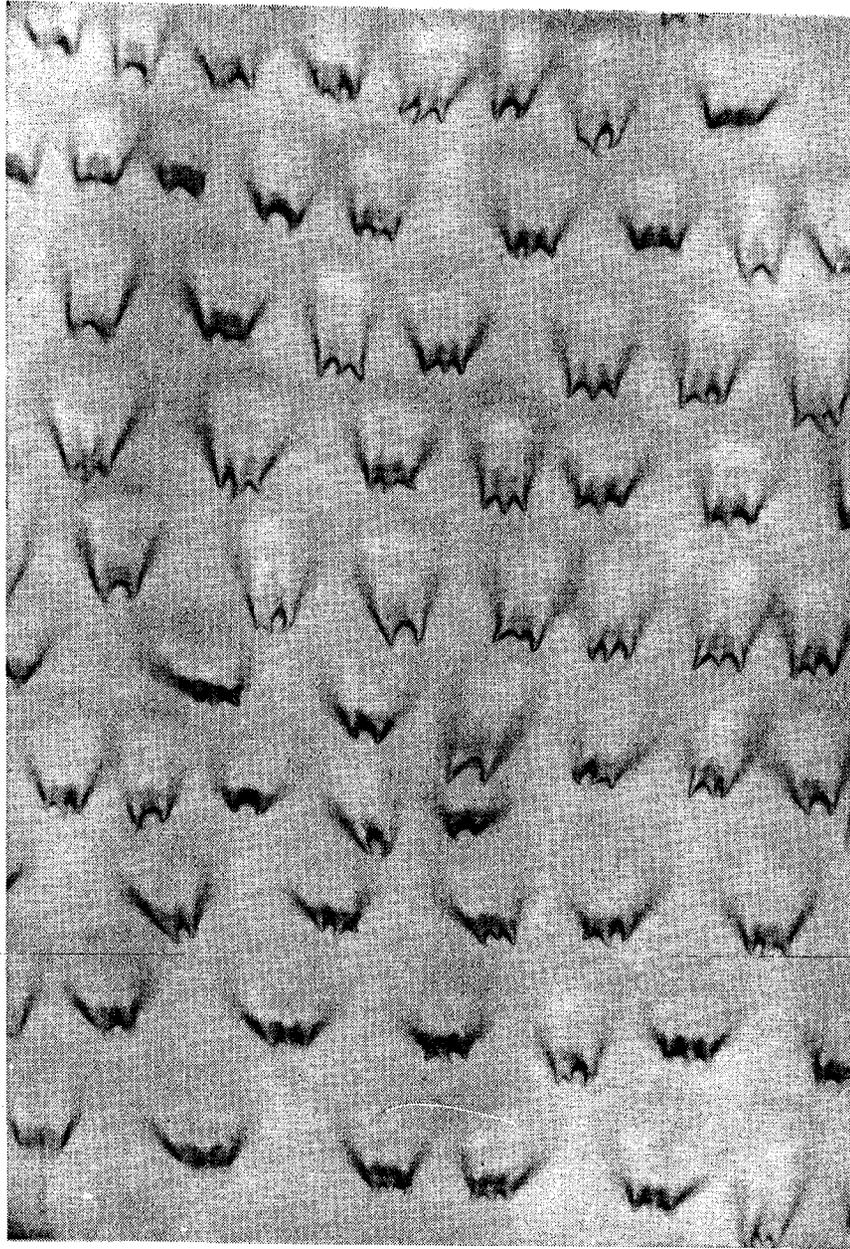


Figure 9 Photomicrograph. G. spinigerum. Cuticular spines of last third of anterior half of body. Each measures 56 x 24 microns.

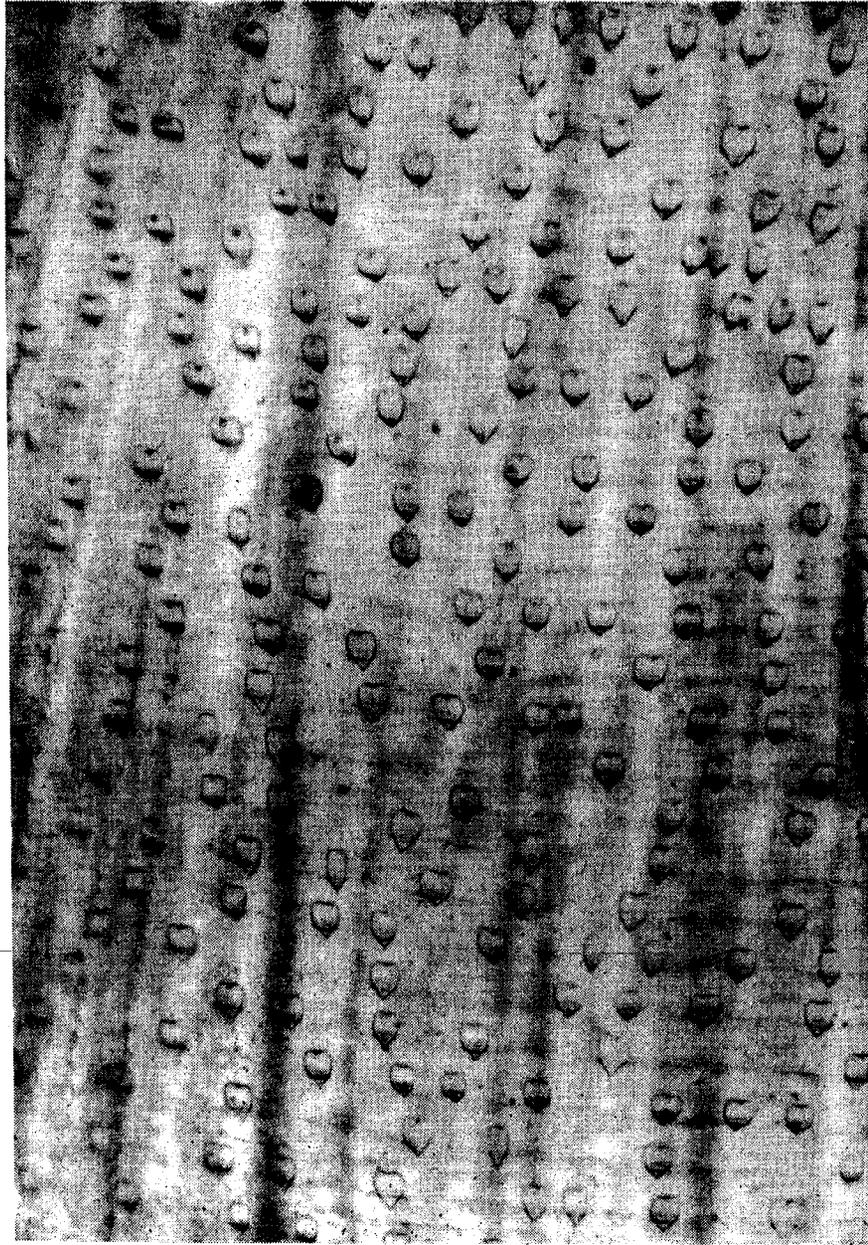


Figure 10 Photomicrograph. G. spinigerum. Cuticular spines of mid portion of body. Each measures 28 x 14 microns.

On the posterior part of the body, the cuticular striations are clearly seen with very few minute spines irregularly scattered on it (Figure 11).

G. hispidum The body is entirely covered with transverse rows of cuticular spines of different size and shape densely arranged in a regular manner. Each of these spines shows mostly 5 teeth of different size but a few have 6 teeth. These spines increase in size and number posteriorly to be 7–10 teeth being measured at about 43.0×26.0 microns (Figure 12). Afterward the teeth per spine rapidly decrease, but increasing in length, to three, two and finally one-toothed spines at about the junction of the anterior third and the middle third of the body (Figure 13). These one-toothed spines are seen for a short distance being measured at the size of 110×8 microns and then become longer and narrower transforming into hair-like or fishbone-like spines covering the rest of the posterior part of the worm (Figure 14). At the terminal part, these hair-like spines are shorter, diminish in number and point anteriorly. On the ventral posterior end of the male worm there are a few small naked areas nearby the cloaca (Figure 15).

G. doloresi This nematode is also covered with cuticular spines all over the body. The anterior half is covered with transverse rows of spines each provided with 2 to 6 teeth of different size (mostly 3 to 5 teeth); the posterior half is densely covered with long single-pointed cuticular spines which gradually decrease in length and number posteriorly. For about 30 transverse rows of spines from the neck of the worm rows have mostly 3–5 teeth of different size (Figure 16); a few may be seen with 2 or 6 teeth being measured at 41×27 microns and usually the middle tooth is slightly longer and larger than the others. Afterwards the teeth of these spines gradually reduce in number to only 3, of which the middle one is conspicuously longer than the short lateral ones (Figure 17). These three toothed spines covering as far as the middle part of the body, are measured at 69×30 microns. Each of the spines covering the posterior end of the body has 1 tooth, they are shorter and smaller (Figure 18). The cuticle bearing the single-pointed spines is swollen. At the posterior end of the worm the spines are pointed anteriorly. In the male, the body terminal has a roundish or oval spineless area on its ventral surface around the cloaca and the tail end (Figure 19).

The study to determine the significant morphological difference and size of the advanced third-stage larvae of the three species of *Gnathostoma* during this period is as follows:

50, 30 and 7 advanced third-stage larvae of G. spinigerum, G. hispidum and G. doloresi respectively were removed from experimentally infected mice, rats and one crab-eating monkey (Macaca irus) for this present study. Morphologically on macroscopic appearance of the living specimen, G. spinigerum larvae is reddish in color and bigger as well as longer than the other two species. Microscopically it has a reddish body cavity probably due to the presence of hemoglobin taken in from the warm-blooded host. The head bulb shows 4 transverse rows of oblongated cephalic hooklets of practically the same size and morphology except that those of the first row are somewhat smaller than the others and all hooklets are slightly longer than those of the other two species. (Figure 20 A). G. hispidum larvae shows on gross examination of the living specimen a somewhat whitish color but microscopically the body cavity has a pinkish color perhaps due to the presence of hemoglobin from the host, and on its cephalic bulb there are 4 transverse rows of many somewhat rectangular-shaped hooklets. These are conspicuously narrow near the middle (Figure 21A). Of the 4 rows the cephalic hooklets of the first row are obviously smaller than the others. The living G. doloresi larvae appears somewhat white by naked eye appearance and each larvae also has 4 transverse rows of cephalic hooklets each with an irregular square base being pieced together to form one (Figure 22A). The tip of these hooklets are not as clearly seen as those of G. spinigerum and G. hispidum.

The study was also undertaken to determine the numbers of cephalic hooklets being developed in each of 4 rows on the head bulbs of which the result shows as follows: Each G. spinigerum larvae had 40 or more cephalic hooklets in each row except row I of 10 larvae and row IV of 1 larva had less than 40 hooklets. (Figure 20B). Each G. hispidum larva had less than 40 cephalic hooklets in every row except row II of 1 larva, row III of 4 larvae and row IV of 14 larvae had 40 to 46 hooklets. (Figure 21 B). Each of 7 G. doloresi larvae studied had comparatively more hooklets formed on row I than row IV except one.

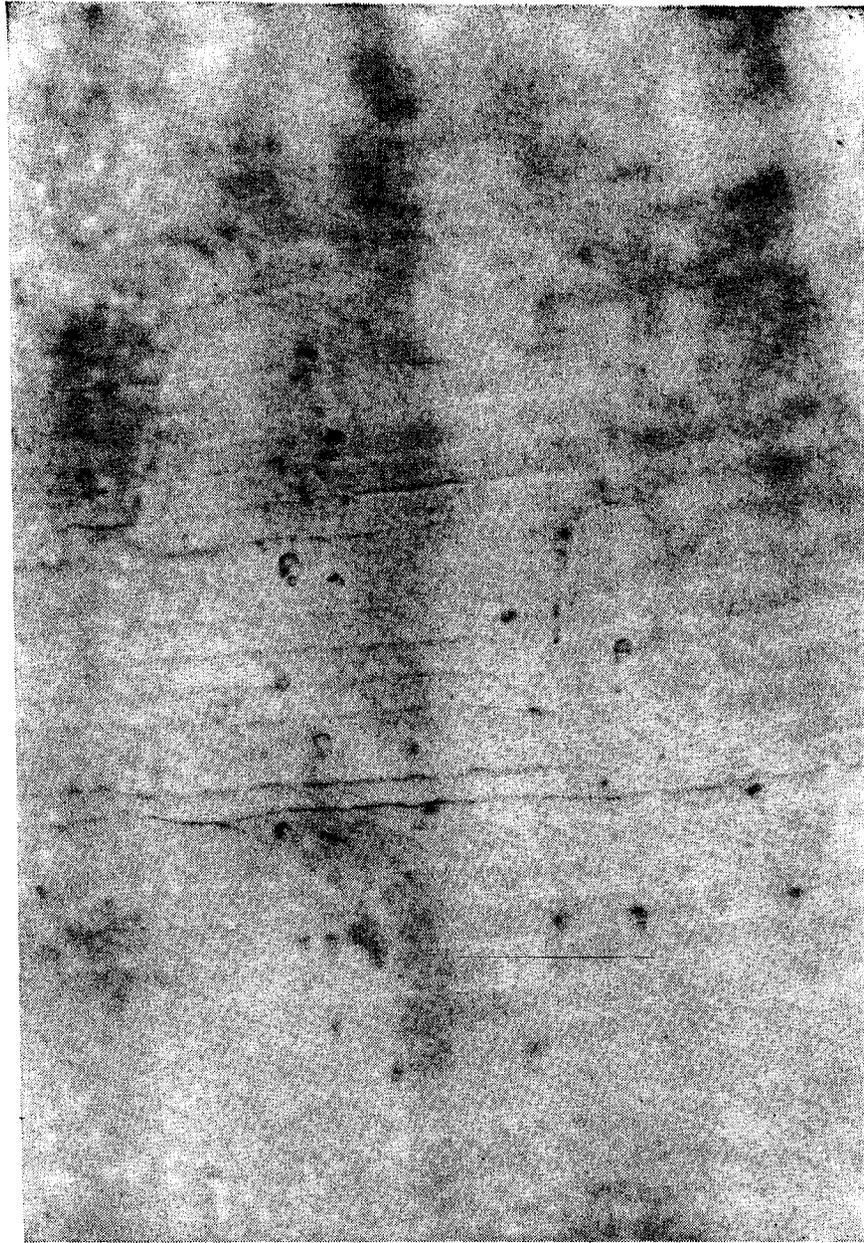


Figure 11 Photomicrograph. G. spinigerum. Cuticular spines of posterior half of body. A few minute spines are seen.

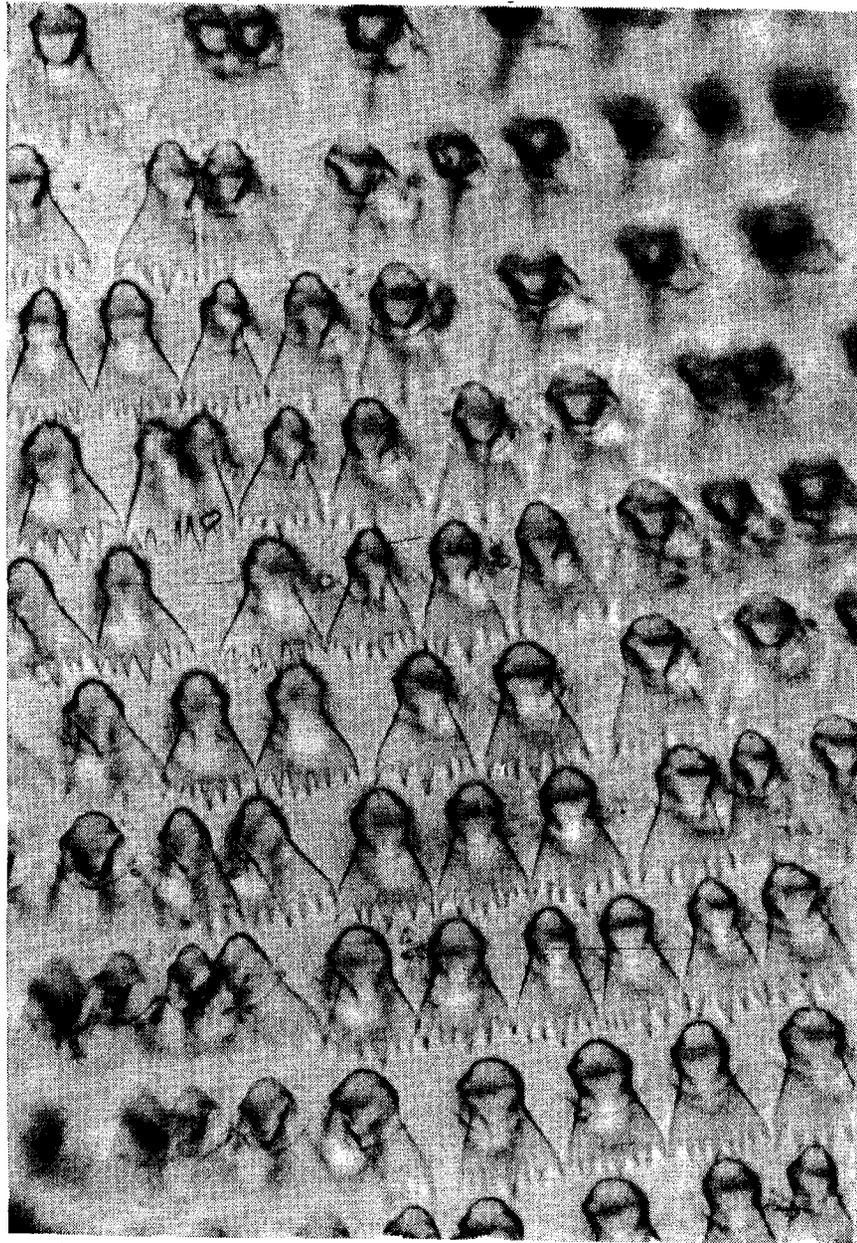


Figure 12 Photomicrograph. G. hispidum. Cuticular spines of the area posterior to head bulb. Each measures 43 x 26 microns. Compare Fig. 7.

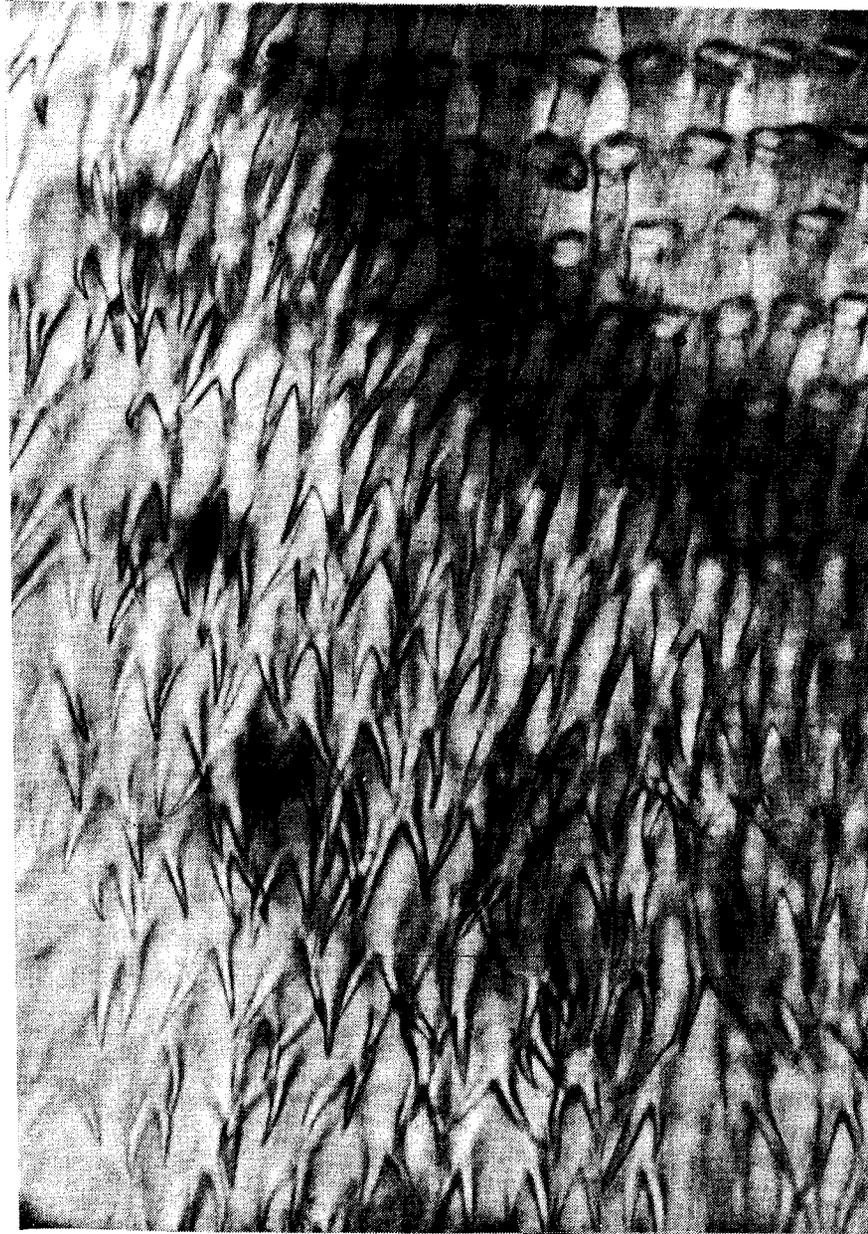


Figure 13 Photomicrograph. G. hispidum. Cuticular spines of middle third of anterior half of body. Each measures about 110 x 8 microns. Compare Figs 8-10.

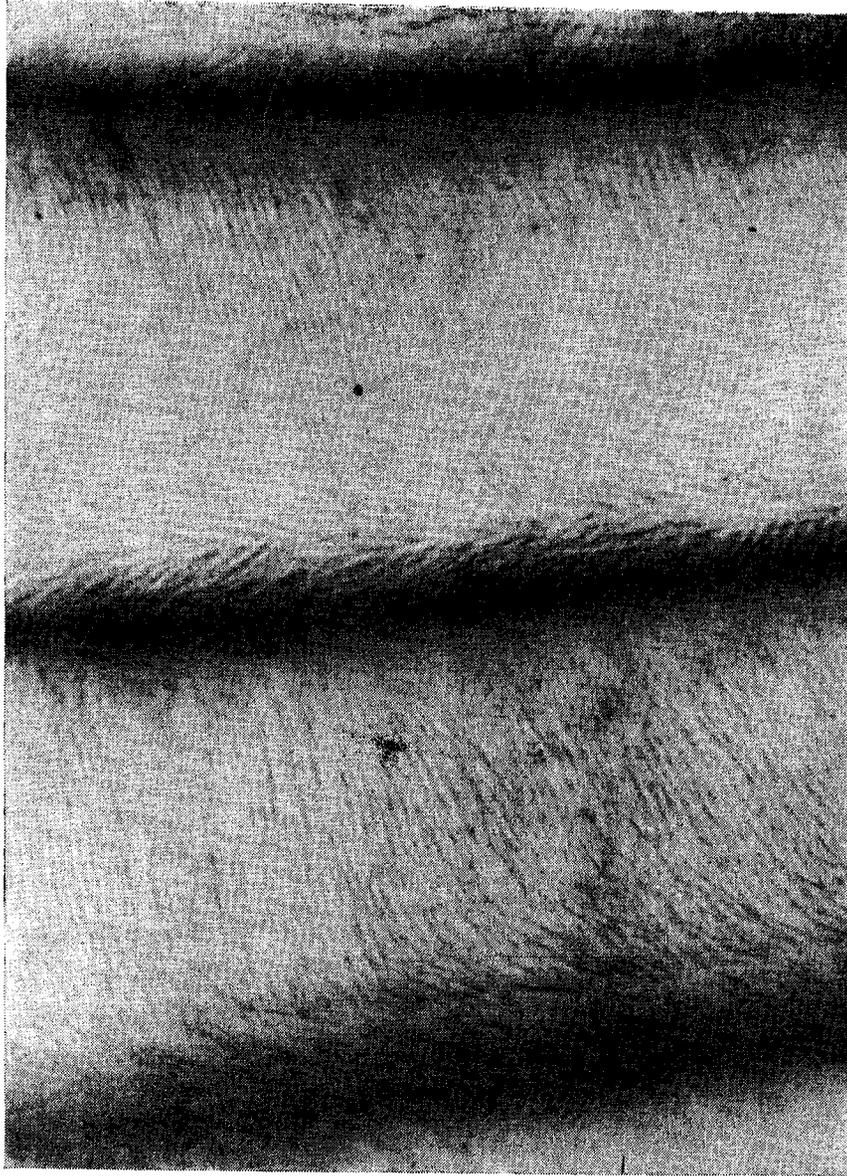


Figure 14 Photomicrograph. G. Hispidum. Hair-like spines of posterior half of body. Compare Fig. 11.

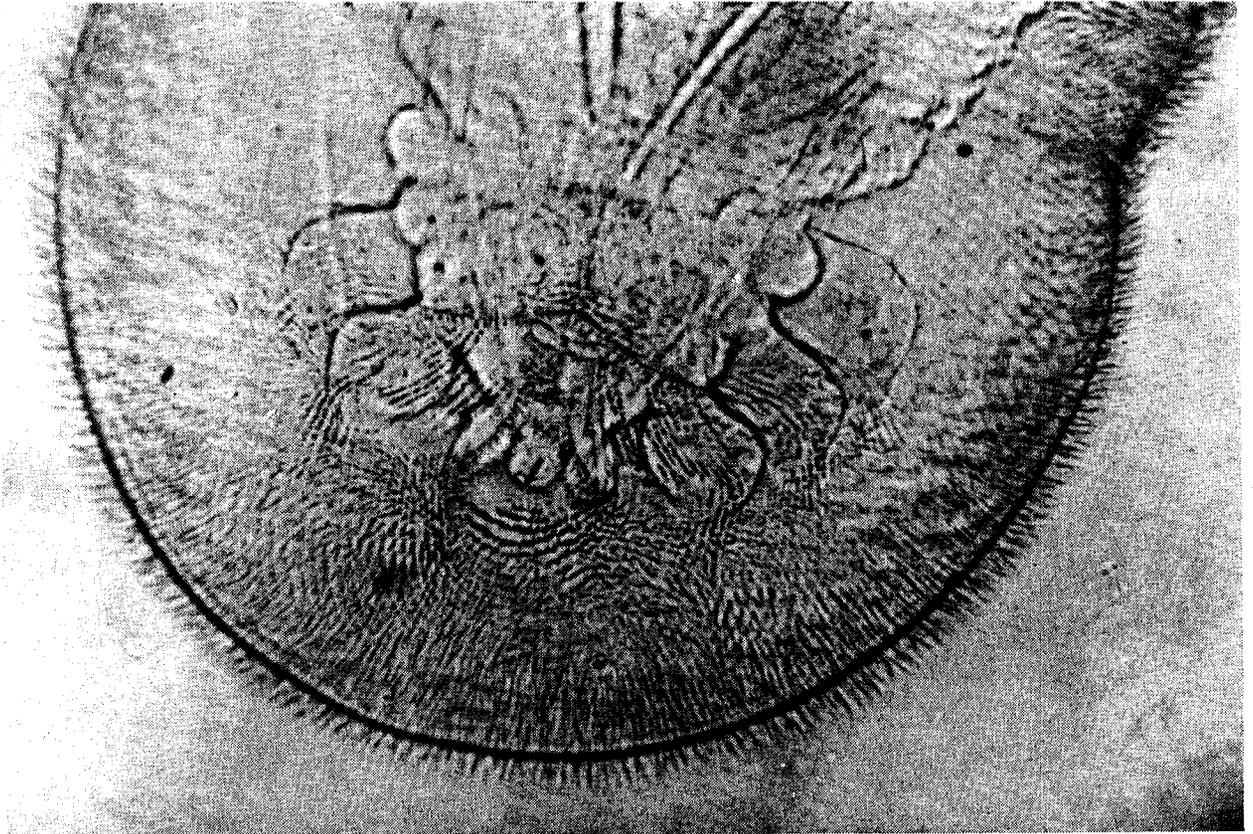


Figure 15 Photomicrograph. G. hispidum, male ventral surface of posterior end.

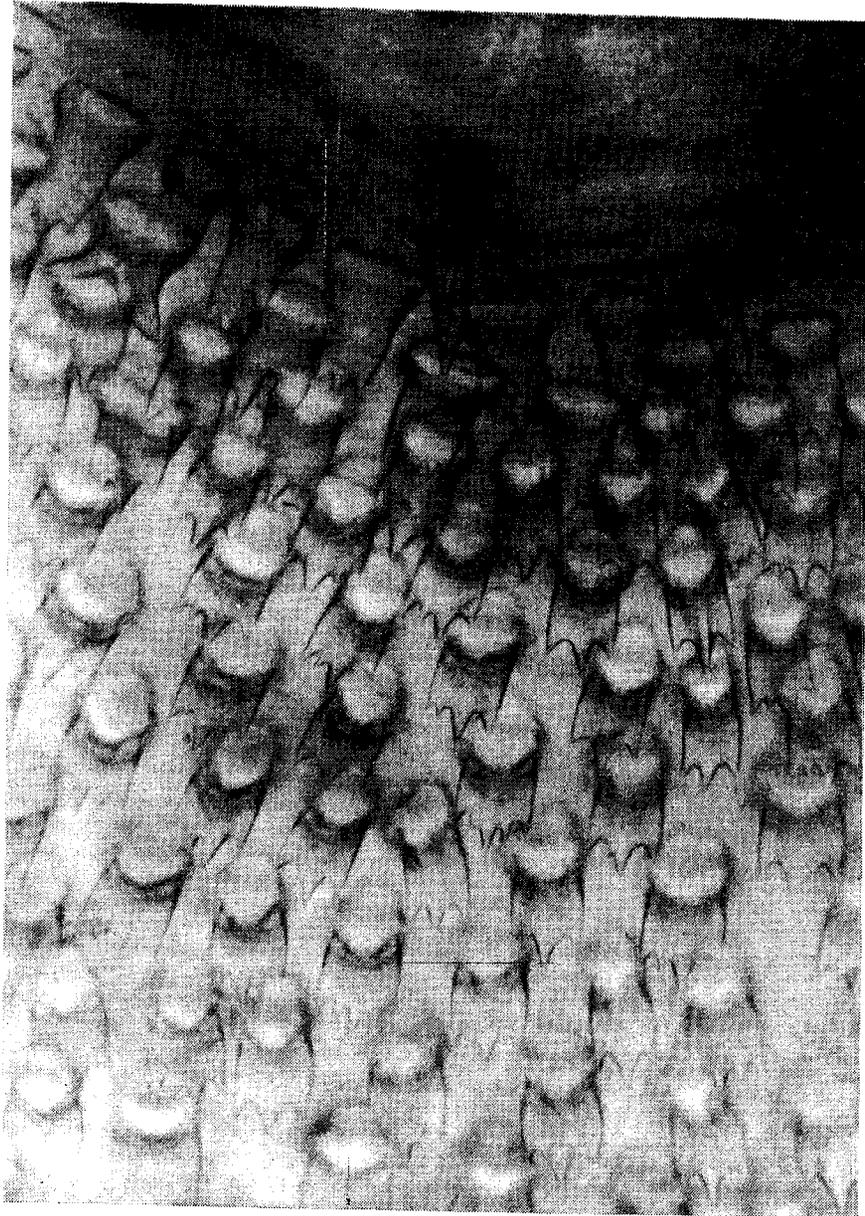


Figure 16 Photomicrograph. G. doloresi. Cuticular spines of the area posterior to head bulb. Compare Figs 7,12.

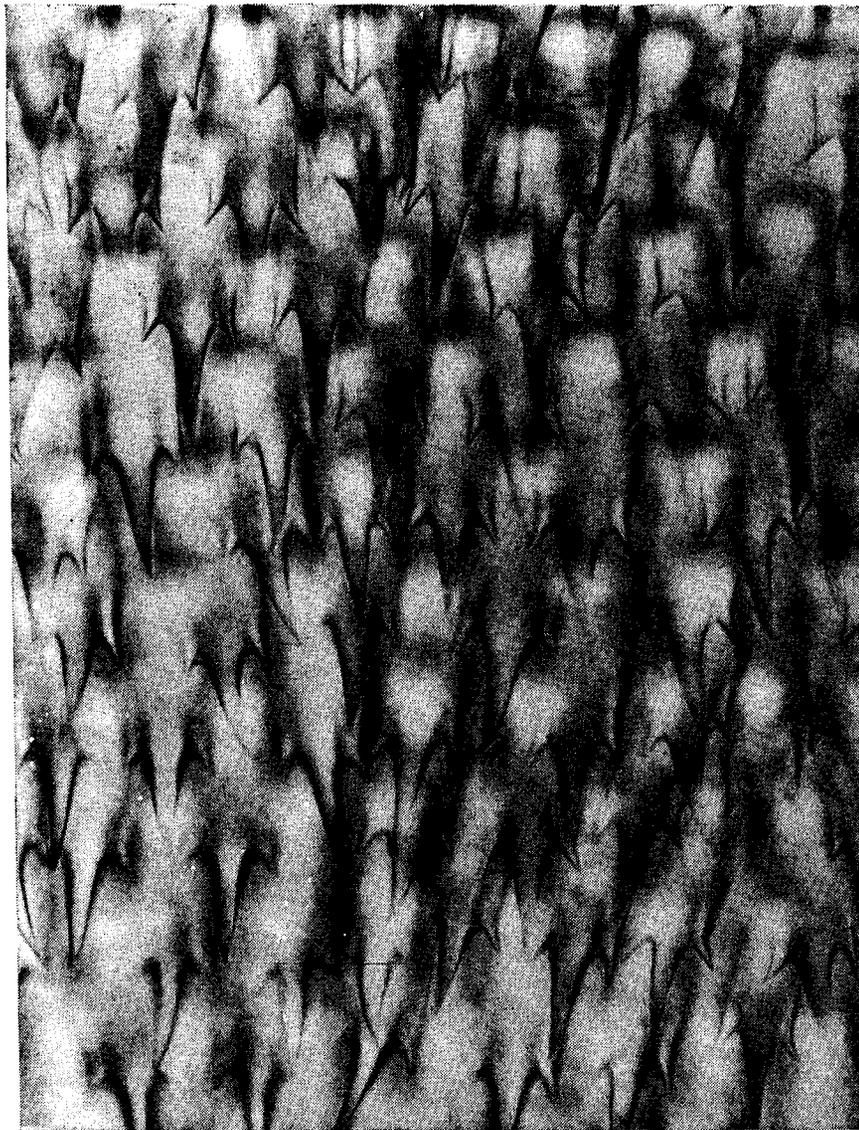


Figure 17 Photomicrograph. G. doloresi. Cuticular spines of anterior half of body. Each measures 30 x 69 microns. Compare Figs 8—10, 13.



Figure 18 Potomicrograph. G. doloresi. Cuticular spines of posterior half of body. Each measures 5 x 15 microns. Note that the posterior spines point anteriorly. Compare Figs 11, 14.

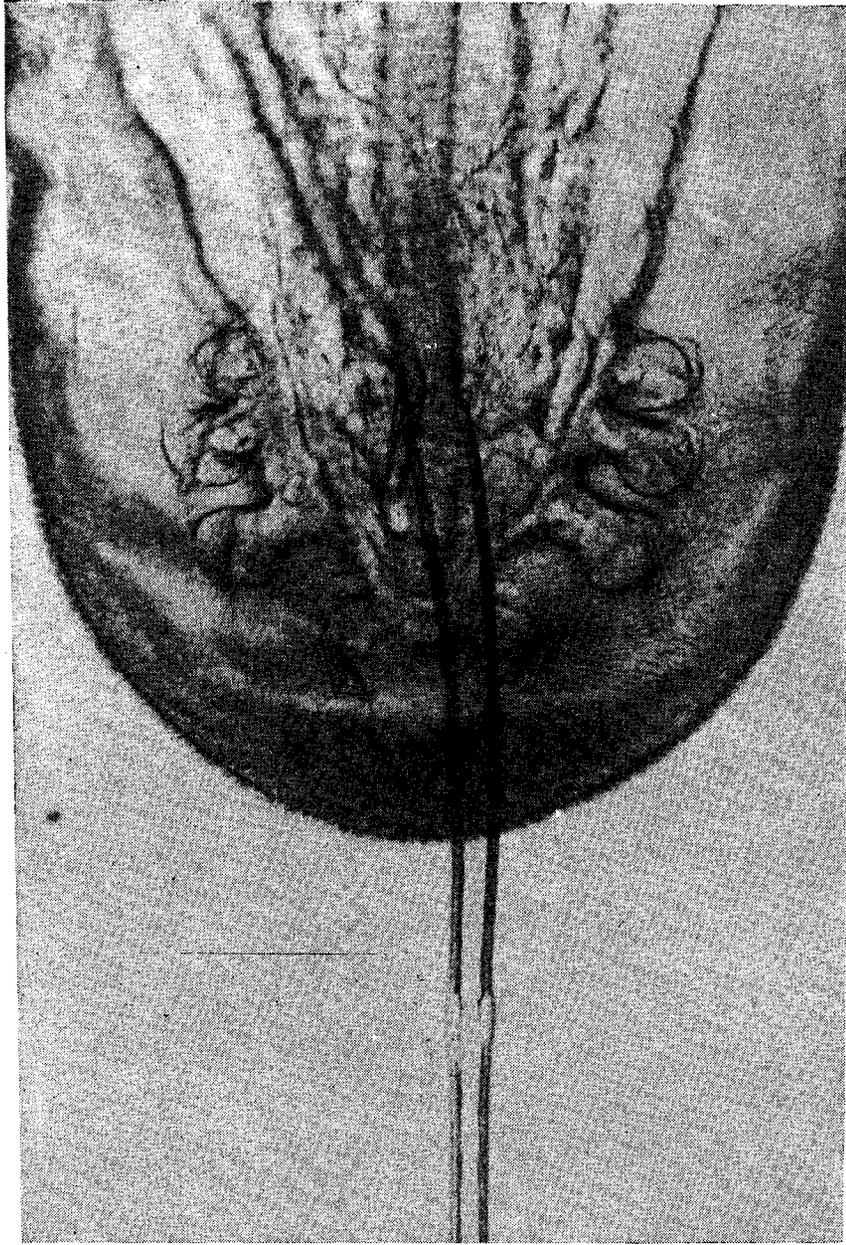
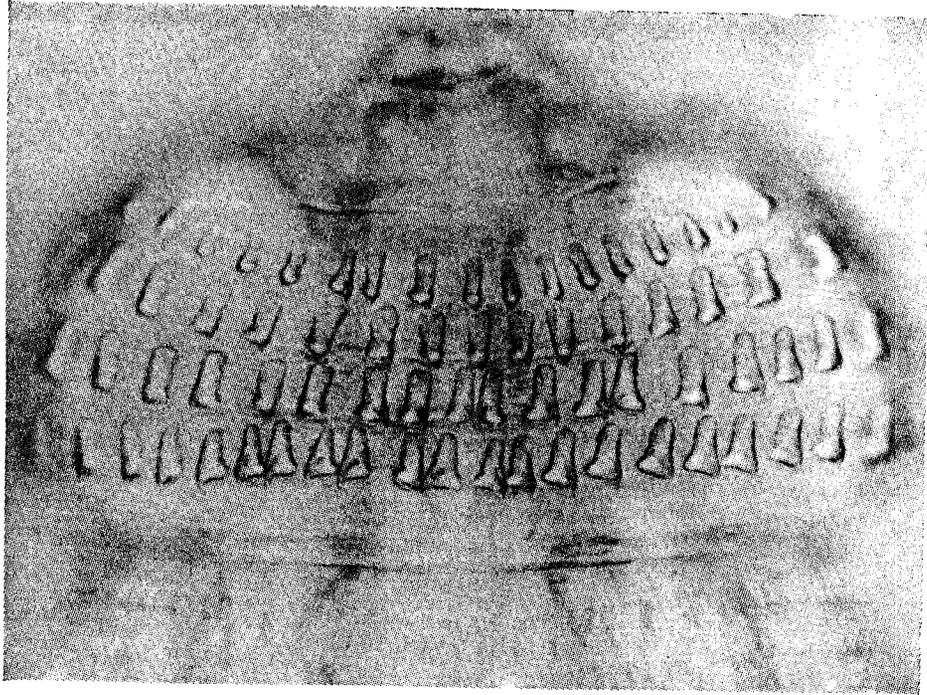


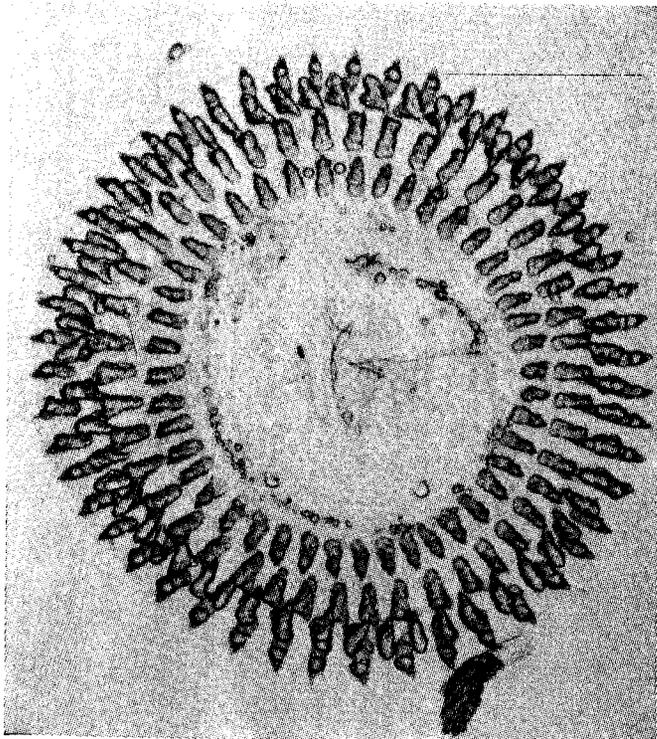
Figure 19 Photomicrograph. *G. doloresi*. Ventral surface of posterior end. Compare Fig. 15.



A.

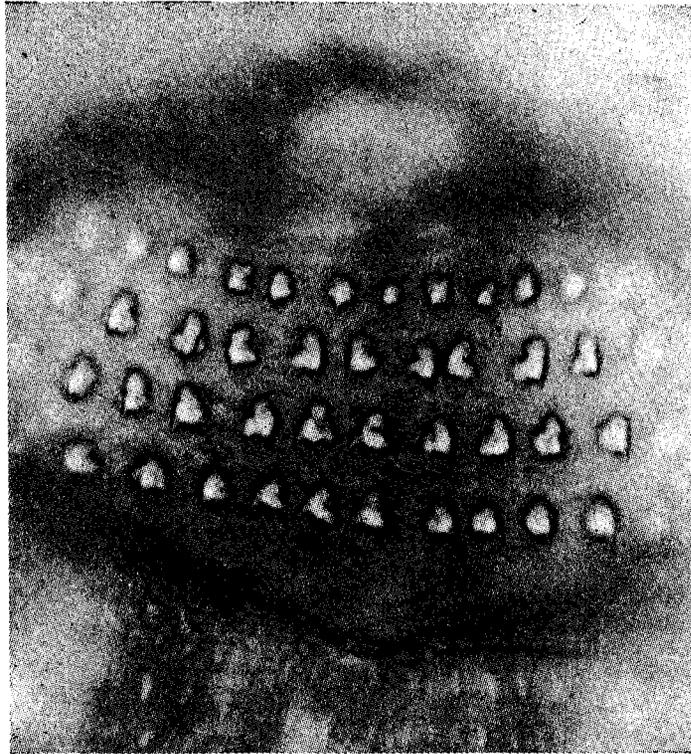
Figure 20.

Photomicrograph G. spinigerum advanced 3rd stage larva, cephalic bulb. A. Lateral view.



B.

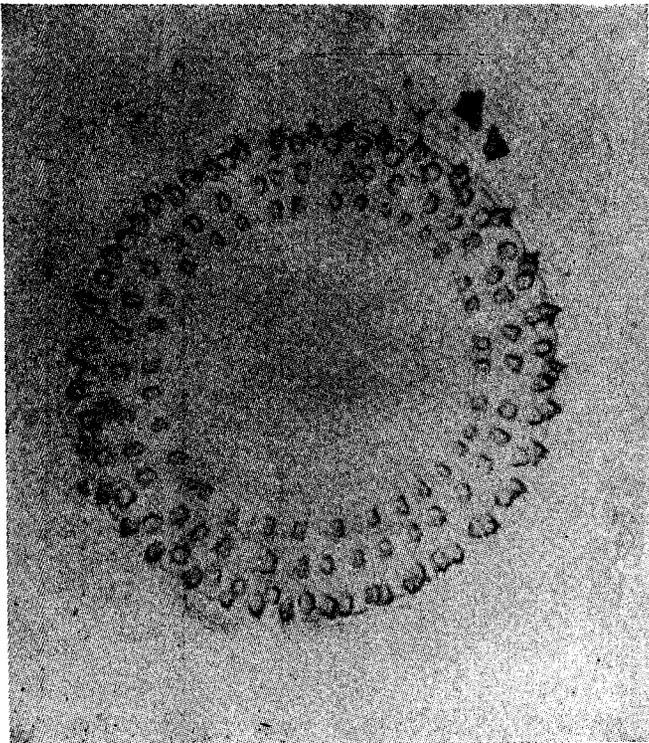
Anterior view.



A.

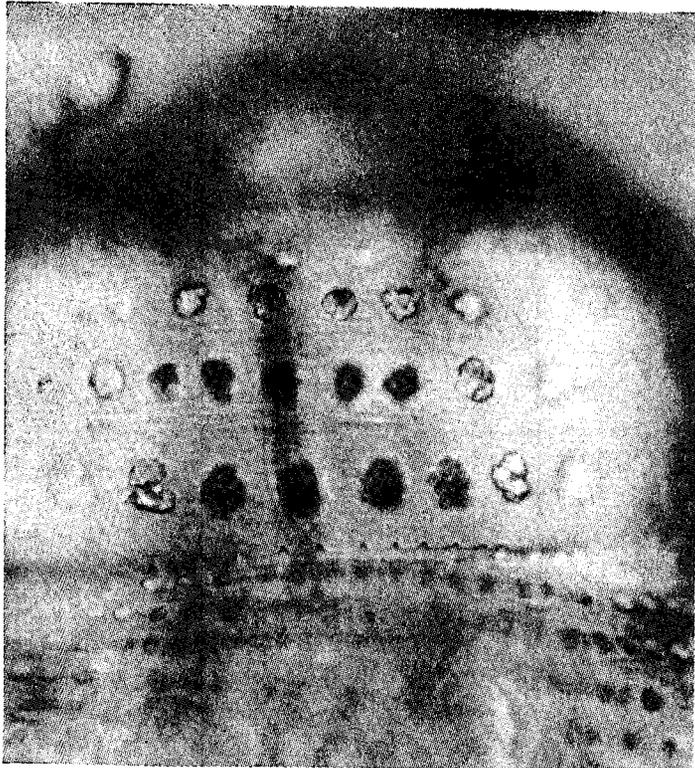
Figure 21.

Photomicrograph. G. hispidum advanced 3rd stage larva, cephalic bulb. A. Lateral view



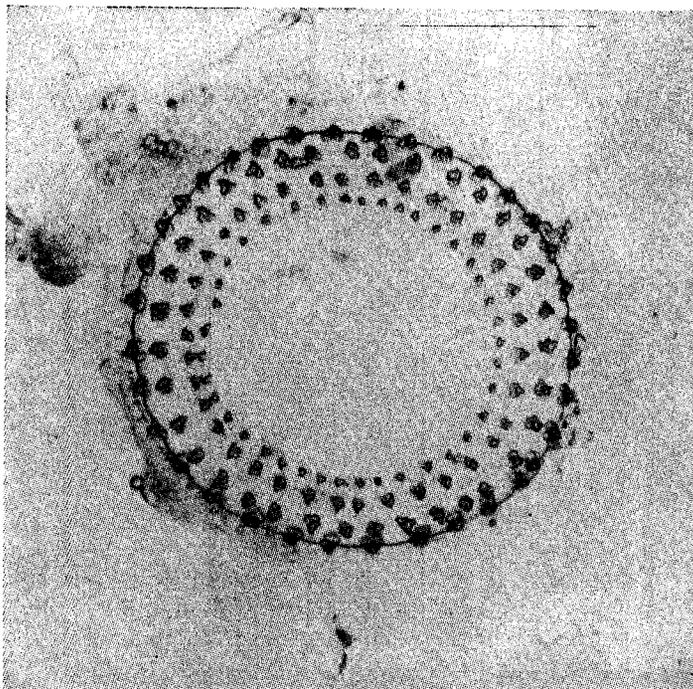
B.

Anterior view. Compare Fig. 20.



A.
Figure 22.

Photomicrograph. G. doloresi, advanced
3rd stage larva, cephalic bulb. A. Lateral view



B.
Anterior view. Compare Fig. 20, 21.

(Figure 22B). The average size of 50 *G. spinigerum* advanced third-stage larvae was more than that of *G. hispidum* or *G. doloresi* (Table 7, Table 8 and Table 9). This study will be continued until 50 advanced third-stage larvae each of *G. hispidum* and *G. doloresi* are measured and investigated.

Table 7. Distribution of cephalic hooklets of advanced third-stage larvae of *G. spinigerum*

Nos. of rows. Nos. of larvae						age (days)	size (mm.)
	I	II	III	IV	IV-I		
1.	37	42	45	50	13	50	—
2.	42	44	43	48	6	50	—
3.	41	45	43	48	7	50	—
4.	41	47	49	51	10	50	—
5.	43	45	51	51	8	50	—
6.	43	44	54	50	7	50	—
7.	43	45	48	52	9	50	—
8.	46	47	50	52	6	50	—
9.	41	42	43	46	5	78	3.0 x 0.4
10.	41	42	44	49	8	78	3.0 x 0.3
11.	41	44	45	49	8	78	3.0 x 0.4
12.	44	48	51	56	12	80	—
13.	42	41	49	48	6	80	—
14.	41	44	49	48	7	80	—
15.	44	43	45	49	5	80	—
16.	44	44	49	53	9	80	—
17.	37	42	43	48	11	80	—
18.	39	41	45	48	9	81	—
19.	46	47	52	51	5	81	—
20.	39	43	46	44	5	81	—
21.	48	47	50	53	5	81	—
22.	40	43	45	47	7	81	—
23.	45	47	48	51	6	85	—
24.	45	48	48	53	8	85	5.0 x 0.5
25.	46	44	47	50	4	85	5.1 x 0.5
26.	38	41	45	45	7	85	4.3 x 0.4
27.	40	45	49	50	10	85	5.2 x 0.5
28.	39	44	44	51	12	86	4.6 x x 0.45
29.	43	47	49	49	6	86	—
30.	41	41	42	45	4	86	—
31.	41	41	44	47	6	86	3.25 x 0.4
32.	41	44	47	51	10	86	4.8 x 0.4
33.	46	45	49	52	6	88	4.0 x 0.5
34.	39	42	49	47	8	88	—
35.	42	43	49	51	9	89	4.5 x 0.4
36.	39	43	45	47	8	89	4.5 x 0.4
37.	44	43	47	54	10	89	4.3 x 0.4
38.	45	46	48	52	7	355	4.0 x 0.4
39.	38	42	43	49	11	355	4.3 x 0.4
40.	44	42	47	45	1	355	4.2 x 0.4

(Table 7 continued).

Nos. of rows Nos. of larvae	I	II	III	IV	IV-1	age (days)	size (mm.)
41.	40	42	44	50	10	742	4.6 x 0.5
42.	43	48	48	59	16	742	4.6 x 0.4
43.	41	43	48	48	7	742	4.5 x 0.4
44.	41	44	49	49	8	724	3.1 x 0.3
45.	42	45	47	50	8	742	3.4 x 0.4
46.	43	46	48	50	7	742	3.8 x 0.4
47.	40	45	49	38	-2	742	2.9 x 0.4
48.	40	44	45	49	9	742	2.8 x 0.8
49.	37	41	41	46	9	742	3.4 x 0.4
50.	40	40	41	46	6	742	3.5 x 0.4
Average	41.72	43.92	46.78	49.3	7.59	226.04	3.99 x 0.4

Note All the larvae are obtained from 12 experimentally infected white mice.

Table 8. Distribution of cephalic hooklets of advanced third-stage larvae of G. hispidum

Nos. of rows Nos. of larvae						age (days)	size (mm.)
	I	II	III	IV-	IV-I		
1.	39	39	40	44	5	29	—
2.	34	37	34	38	4	32	2.56 x 0.29
3.	35	36	38	28	-7	35	2.0 x 0.2
4.	29	34	36	41	12	35	2.0 x 0.2
5.	30	32	34	38	8	35	1.9 x 0.23
6.	38	36	39	46	8	35	2.4 x 0.23
7.	33	36	36	40	7	35	2.3 x 0.26
8.	32	32	36	38	6	35	2.3 x 0.23
9.	33	35	33	38	5	35	2.5 x 0.25
10.	27	30	31	32	5	35	2.1 x 0.22
11.	35	34	34	34	-1	35	2.17 x 0.26
12.	38	36	40	41	3	49	2.0 x 0.28
13.	33	36	37	39	6	49	2.12 x 0.27
14.	39	37	38	44	5	60	2.7 x 0.26
15.	36	34	37	40	4	60	2.7 x 0.27
16.	35	35	34	41	6	60	2.6 x 0.26
17.	32	34	34	38	6	60	2.17 x 0.22
18.	27	27	27	30	3	60	1.66 x 0.26
19.	35	34	33	39	4	60	2.6 x 0.26
20.	32	32	33	37	5	60	2.6 x 0.26
21.	33	30	33	37	4	62	2.5 x 0.26
22.	37	36	40	43	6	66	2.6 x 0.29
23.	30	33	34	40	10	66	—
24.	31	37	39	43	12	66	2.16 x 0.23
25.	37	39	37	42	5	79	1.8 x 0.26
26.	36	40	41	44	8	79	1.99 x 0.23
27.	33	36	37	39	6	81	2.62 x 0.29
28.	35	34	36	39	4	81	2.51 x 0.26
29.	33	37	34	37	4	81	2.57 x 0.26
30.	33	33	37	42	9	81	3.31 x 0.34
Average	33.67	34.70	35.70	39.07	5.4	54.54	2.34 x 0.25

Note All the larvae are obtained from 7 experimentally infected white mice except two from Rattus exulans and Macaca irus.

Table 9. Distribution of cephalic hoklets of advanced thirdstage larvae of G. doloresi

Nos. of rows Nos. of larvae	I	II	III	IV	IV-I	age (days)	size (mm.)
	1.	38	36	36	33	-5	66
2.	38	37	36	29	-9	33	2.50 x 0.30
3.	39	36	35	35	-4	66	2.99 x 0.30
4.	39	36	35	36	-3	33	2.80 x 0.34
5.	40	37	34	36	-4	33	2.70 x 0.26
6.	41	46	41	43	2	33	2.70 x 0.32
7.	42	39	33	40	-2	33	2.30 x 0.30
Average	39.5	38.1	35.7	36.0	-3.5	42.4	2.62 x 0.30

Note All the larvae are obtained from 2 experimentally infected white mice.

The study on the life cycle of G. hispidum and G. doloresi.

A. Development of the ova in water and the larvae in vertebrate hosts.

To determine the possibility of man acquiring G. hispidum and G. doloresi infection from the domestic pig, a study was initiated on the life cycle of the two species of the worm. The newly passed ova of the two species, of 2- to 8- cell stage, can be developed in the petri dish 9.0 cm. in diameter, containing small amount of fresh water at room temperature of 29°-31°C. Embryonic stage is reached at the earliest on 4 days; loosely ensheathed first-stage larvae with characteristic rapid movement in water are seen at 8 days for G. hispidum, while G. doloresi ova become embryonic on day 6 and a few embryos loosely ensheathed first-stage larvae with similar active movement are seen on day 10.

The total time required for hatching of all embryonic ova in this experiment was about 18 days for both G. hispidum and G. doloresi. These newly hatched or first-stage larvae of the two species, on examination, show almost same size, with voluminous sheath, morphology and activity as those of G. spinigerum. Cyclops (Mesocyclops leuckarti, Claus) acts as the first intermediate host. Experimentally the first-stage larvae of both species are ingested into the stomach of the cyclops. The larvae after being swallowed by cyclops loose their voluminous sheaths immediately, within a few minutes pierce through the gastric wall of the crustacean and enter the body cavity to develop into second-stage and early third-stage larvae in 9 to 10 days at the room temperature of 29°C to 31°C. 20 G. hispidum larvae and 10 G. doloresi larvae measured in the range of 342-380 microns x 34-45 microns and 285-300 microns x 34-40 microns respectively.

Subsequently the fully developed larvae of the two species in cyclops were fed to laboratory white mice with the following result. Of 34 white mice, 24 were positive (70.6%) each was infected with 1 to 13 G. hispidum advanced third-stage larvae. These living larvae measured in the range of $0.5-1.0 \times .08-1$ mm. and were found in the stomach and intestinal walls of all mice and the livers of 4 mice, 3-4 days after the experiment. Thereafter they were found to migrate into the livers, muscles and subcutaneous tissue of body and legs with an increase in size ranging at about $1.2-1.8 \times 0.16-0.25$ mm. for the 34 larvae found 10-15 days after the experiment. Of 150 advanced third-stage larvae found in this experiment most were in muscles and subcutaneous tissue of body and legs, very few were in 2 livers and only 1 in the lung of experimental mouse. The range of their size was $1.6-2.85 \times 0.2-0.3$ mm. measured 19 to 90 days after the experiment. Some larvae were found to be encysted as early as one month after the experiment. Of 939 fully developed larvae in infected cyclops fed to mice 188 (20.0%) were discovered to develop into the advanced third-stage larvae.

Of 7 adult toads (Bufo melanostictus) each fed with 30 fully developed larvae in cyclops 2 (28.5%) were found positive with 11 G. hispidum living third-stage larvae in the stomach walls. Size of these larvae was in the range of $0.2-0.4 \times .05-.06$. Two frogs, one fed with 15 and the other with 14 fully developed larvae, were negative. This experiment is to be repeated.

Regarding the development of G. doloresi, advanced third-stage larvae were fed to 25 white mice: 12 (48.0%) were found positive, each infected with 1 to 6 larvae. These larvae were living and measured at about $1.3-1.7 \times 0.2$ mm. for the 3 larvae found in 2 livers and about $1.8-2.0 \times .2-.3$ mm. for the larvae found in the flesh of the chest 14-16 days after the feeding experiment. Thereafter on 21-56 days of the experiment most were found in the flesh of legs and body of the mice except one larvae was discovered in the liver. The measurements of these 50 larvae ranged from $1.9-3.0 \times .2-.3$ mm. of the 597 fully developed larvae in cyclops fed to 25 white mice, 56 (9.4%) were found to be developed to advanced third-stage from 14-56 days after the experiment.

Of 30 toads (Bufo melanostictus) fed with total 878 fully developed larvae in cyclops, 4 (13.0%) were found positive with 15 (1.7%) G. doloresi advanced third-stage larvae (4, 9, 1 and 1) in total being measured at the range of $0.7-1.5 \times .08-0.12$ mm. They were seen in the stomach and intestinal walls and 4 larvae in the body flesh of one toad, 15-23 days after the feeding experiment.

Of 16 experimented frogs (Rana rugulosa) fed with a total of 442 G. doloresi fully developed larvae in cyclops only 1 frog (6.2%) sacrificed 17 days after being fed with the 30 larvae was proved to be infected, with 4 (0.9%) unencysted advanced third-stage larvae (2 in the liver and 2 in the flesh).

B. The experimental investigation on development of the advanced third-stage larvae of G. hispidum and G. doloresi in domestic pigs.

Two negative piglets were kindly made available by the Kasetsart University. One was fed 15 times from 31 March to 13 October 1967 with varying number (1-14) G. doloresi advanced third-stage larvae at each feeding for a total of 66 larva. These larvae were removed from the flesh of experimentally infected vertebrates, namely 2 toads, 1 frog, 11 white mice and 1 Rattus exulans and measured at 1.5 to 3.6 mm. \times 0.1 to 0.3 mm. This pig was sacrificed 290 days after the first feeding with 7 advanced third-stage larvae or 94 days after the last feeding with 4 larvae obtained from two white mice and showed 8 (12%) G. doloresi larvae located in the stomach wall. 4 of the larvae were measured at 11 to 12 mm. \times 1.0 to 2.0 mm. and each cephalic bulb had 4 rows of fully developed cephalic hooklets. An adult G. doloresi may have developed from the 8 larvae being found in the stomach, had this pig been sacrificed few weeks after the present date; this experiment is to be undertaken on more pigs before making the final conclusion on the problem. However it may be presumed that the life cycle of this worm can be completed by having two intermediate hosts namely cyclops as the first intermediate host, and white mice as the second intermediate host in which different stages of the larvae are developed up to the advanced

third-stage, subsequently the adult worm is developed when the advanced third-stage is fed to the domestic pig which is now thought to be the only definitive host of the worm.

The second piglet was fed 12 times between 20 March to 17 October 1967 with varying number of 1 to 18 G. hispidum advanced third-stage larvae at each feeding for a total of 93 larvae. The larvae were removed from the flesh of experimentally infected white mice and 1 Rattus exulans, the size varied from 1.2–4.2 mm × 0.2–0.3 mm. This pig was sacrificed 246 days after the first feeding or 35 days after the last feeding of which the result 1 shows 3 and 6 G. hispidum larvae (each being armed with 4 rows of fully developed cephalic hooklets), located in the liver and in the stomach wall respectively. These larvae had grown to bigger size than the originals, measuring about 10.6 × 0.8 mm and 14.0 × 1.2 mm for the two larvae in the liver and 11.0 × 0.9 mm to 16.3 × 1.0 mm for the larvae in the stomach wall. More-over 2 mature adult female G. hispidum were found in the stomach wall, each being armed with 11 rows of cephalic hooklets, and the uterine cavity and vagina of each worm were seen containing many 2 to 4-cell fertilized, and some undeveloped, ova. Their measurements were found to be 19.0 × 2.4 mm and 17.0 × 21.4 mm. In the stomach cavity one mature adult male G. hispidum was found in the contents. This worm measured 22.0 × 1.8 mm and was armed with 11 rows of cephalic hooklets. This result therefore, clearly shows that adult G. hispidum can be experimentally developed in the stomach wall of the domestic pig 8 months after the advanced third-stage larvae obtained from rats and mice were fed to the pig.

It is now reasonable to assume that natural infection of domestic pig with the adult G. hispidum may similarly occur. To determine the development of the early and advanced third-stage larvae of G. hispidum in other vertebrates which is closer to man than pig, three monkeys (Macaca irus) were fed 3rd stage larvae. One was sacrificed 3 days after being fed with 12 early third-stage larvae in 5 cyclops; it was negative. Each of the other two monkeys was fed with 8 advanced third-stage larvae obtained from infected flesh and liver of 2 experimentally infected white mice. They measured at 1.7–2.4 mm. × 0.2–0.3 mm. G. hispidum advanced third-stage larva (measuring at 2.5 mm × 0.3 mm with no change in morphology) in the stomach wall of one monkey when sacrificed 3 day after the experiment. The other monkey showed 2 encysted larvae of the worm in the flesh of the back of the thoracic region when sacrificed 69 days after the experiment. Each cyst was oval in shape and measured at about 1.5 mm × 1.8 mm in diameter surrounded by a rather tough fibrotic wall of about 0.4 mm thick.

The larva showed no morphological change and was measured at 2.7 × 0.3 mm. This experiment showed that the monkey can be infected if fed with the advanced third-stage larvae obtained from infected mice. In this vertebrate, the larva penetrated the stomach before reaching the muscles and would not migrate further, being walled off by a thick fibrous tissue. A longer period of such a feeding experiment will be developed further in monkeys before a conclusion can be definitely drawn on this problem.

G. vietnamicum and Gnathostoma, n. sp. During the investigation on the adult gnathostomes that might be found in wild animals other than reported in this country, 6 dead young and adult river otters, (a flesh-eating mammal feeding mostly on fish etc., frequently seen living and swimming in fresh-water canal and river), identified by Dr. Joe T. Marshall, Jr., of SEATO Medical Research Laboratory, as Aonyx cinerca, were obtained from southern Thailand. (1 from Songkhla Province and 8 from Nakornsrihamarat). These otters, weighing about 1.5 to 2.0 kilograms and measuring 21 to 34 inches in length, showed on examination the following: 4 (44%) otters (1 from Songkhla and 3 from Nakornsrihamarat) were found to be infected in tissue at the pelvis of the kidney with 17 (9 females and 8 males) adult gnathostomes tentatively identified as G. vietnamicum, measuring in the range of 14.0–35.0 mm × 1.4–2.3 mm for males and 17.5–57.0 mm × 1.2–2.7 mm for females. The eggs were ovoidal, colorless, superficially pitted with a mucoid plug or knob at one end and were at 2-to many-cell stage when newly laid by the female. These ova were 75.0 microns long on the average, (61–82 microns × 35–43 microns) and few developed into the embryonic stage at 7 days in fresh water at room temperature (29°–31°C). The study on this gnathostome is to be continued.

One more dead adult liver otter obtained from Nakornsrihamarat shows on examination one adult female and one probably fully grown larva of unidentified species of gnathostome from the tissue at the pelvis of the right kidney being measured at 32.2×1.3 mm and 6.3×0.2 mm respectively. The fertilized ovoidal ova have yellowish superficially pitted shells and mucoid plugs or knobs at both ends (one knob at each end) and are 2-to 4-cell stage. 4 eggs are measured at the average of 64×32 microns and the range is $60-68$ microns \times $31-34$ microns. Few ova become embryonic stage on 6 days and 2 larvae are first found hatching 18 and 19 days after being in small amount of fresh water under the room temperature. This adult gnathostome is morphologically different from that of *G. vietnamicum* and the eggs have 2 mucoid plugs or knobs, one knob at each end. The specimen is tentatively considered as new species of *Gnathostoma* pending further information to be available from the continuing study of the parasite.

References

- Anderson, Roy (c) 1964. *Gnathostoma miyazki* n.sp. from the otter (*Lutra C. Canadensis*) with comments on *G. sociale* (Leidy, 1858) of mink (*Mustela vison*). Canadian J. Zool. 42:249-254.
- Chitanondh, H., and Rosen, L., 1967. Fatal eosinophilic encephalomyelitis caused by the nematode *Gnathostoma spinigerum*. Am. J. Trop. Med. and Hyg. 16:639-645.
- Csokor, J, 1882. *Gnathostoma hispidum* Suis S. *Cheiracanthus* Diesing. Oester. Vtljschr. wissenschaft. Veterinark. 57:1-22. (German translated into English).
- Daengsvang, S., 1949. Human gnathostomiasis in Siam with reference to the method of prevention. The J. Parasitol. 35:116-121.
- Daengsvang, S., Papasarathorn, T., Chulalerk, U., and Tongkoom, B. 1964. Epidemiological Observation on *Gnathostoma spinigerum* in Thailand. J. Trop. Med. and Hyg. 67:144-147.
- Daengsvang, S., Thienprasithi, P., Chomcherngpat, P. 1966 Further investigation on Natural and Experimental Hosts of Larvae of *Gnathostoma spinigerum* in Thailand. Am. J. Trop. Med. and Hyg. 15:727-729.
- Faust, E.C. and Russel, P.F. 1964. Craig and Faust's Clinical Parasitology. Seventh Edition. 441-444.
- Golovin, O.V., 1956. Biology of the Nematode *Gnathostoma hispidum*. Doklady Akad. Nauk S.S.S.R. 1956, III. (1), 242-244 (Russian) and Helminthological Abstracts 25:265-266.
- Ho, Le-Yan 1965. A new gnathostome *G. vietnamicum* n.sp. from an otter, *Lutra elioti* in Viet-Nam. Bull. Soc. Patho. 58:228-235. (French text-English summary).
- Ishii, Yoich. 1956. Studies on the Life History of *Gnathostoma doloresi* Tubangui, 1925 in Japan. Fukuoka Acta Medica, 47:1474-1494. (Japanese text. English summary)
- Miyazaki, Ichiro 1960. On the Genus *Gnathostoma* and human Gnathostomiasis, with special reference to Japan. Exper. Parasitol. 9, 338-370.
- Miyazaki, Ichiro and Dunn L. Frederick, 1965. *Gnathostoma malaysiae* sp. n. from rats on Tioman island, Malaysia (Nematoda: Gnathostomidae) J. Parasitol. 51:382-384.
- Morishita, K.O.R. 1924. A pig nematode, *Gnathostoma hispidum*, Fedchenko, as a human parasite. Ann. Trop. Med. and Parasitol, 18:23-26.
- Prommas, C., and Daengsvang, S., 1936. Further report of a study on the life cycle of *Gnathostoma spinigerum*. J. Parasitol. 22:180-186.