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Development of *Echinostoma hortense* in Rats, with Special Reference to the Genital Organs

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Key words: *Echinostoma hortense*, experimental infection, rat, growth curve, genital organs, gametogenesis

Introduction

Since a human infection with *Echinostoma hortense* was reported by Tani *et al.* (1974), this fluke has attracted the attention of many parasitologists. It is supposed that the infection is mainly caused by eating raw loach. The genital organs of the metacercaria of this fluke develop very poorly and are only recognized as two masses of germ cells in front of and behind the ventral sucker (Saito and Tani, 1982). In the final host, the rat, however, the genital organs grow rapidly in size, and the eggs are detected in feces of rats about 10 days after infection (Ono, 1930; Asada, 1939; Tani, 1978). Investigations on the development of this worm in rats have been already done by Asada (1939) and Tani (1978). However, precise investigations on the development of this worm remain to be performed because it grows very rapidly in rats and past observations were carried out in intervals of several days during the course of development. The present paper will demonstrate the daily development of *E. hortense* from 1 to 10 days and further development 15, 38, and 50 days after infection.

Materials and Methods

Metacercariae of *E. hortense* used for the present experiments were obtained by digesting minced loach (*Misgurnus anguillicaudatus*) from Yamagata City for 1 hour at 37°C in artificial gastric juice consisting of 100 μg/ml pepsin (1 : 10,000) and 0.08 N HCl.

Wister rats raised under conventional conditions were given orally 30 to 50 metacercariae, and 3 to 5 rats each were sacrificed daily for 10 days, and at 15, 38, and 50 days postinfection. The small intestines of infected rats were vertically opened by bud blunt point scissors, and rinsed in 0.9% NaCl. The flukes were well washed in saline, fixed in Schaudinn's solution under cover glass pressure, stained with Haidenhain's iron-hematoxylin or borax carmine, and mounted in balsam. All measurements were recorded in μm with the aid of a camera lucida.

Part of the flukes obtained was used for observation of gametogenesis. Living worms were left in the culture fluid (TC MEDIUM 199 (Difco)) containing 0.2 μg/ml Colcemid for 2 to 3 hours at 37°C. Cultured worms were further treated with either of the following two methods. First, the worms were fixed in Carnoy's solution (ratio of acetic acid to methanol, 1 : 3), stained with alcoholic hydrochloric acid-carmine and kept in 70% ethanol. At the time of observation, the worms were decolored with 45% acetic acid solution, and the testes and the ovary were removed with micropins under a dissection microscope. Then each of the testes and ovaries was put on a glass slide with a few drops of 45% acetic acid solution and crushed under cover glass pressure for observation (Snow's modified methods, 1963). Secondly, the air-dry method was used. Briefly, the testes and the ovary excised from

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Table 1 Measurements of *Echinostoma*

Days after infection	No. of specimens measured	$\bar{X} \pm SD$ long × Body	Head collar	Oral sucker	Pharynx
1	10	374.6± 85.4 × 140.4± 19.1	118.1±14.6	61.5± 6.4 × 69.9± 7.6	29.5± 3.2 × 26.9± 3.6
2	19	554.1± 80.8 × 157.6± 10.6	132.9±11.5	70.9± 5.4 × 78.7± 6.2	38.2± 3.1 × 34.8± 4.3
3	32	951.2± 81.2 × 205.2± 16.5	156.8±10.5	81.1± 5.1 × 88.9± 4.2	54.6± 4.8 × 48.9± 4.9
4	19	1080.5±230.0 × 249.5± 28.0	171.9±17.8	88.4± 8.8 × 91.8± 8.6	65.2± 7.9 × 59.2± 9.2
5	17	1522.3±274.9 × 282.8± 34.3	193.1±17.5	98.7± 9.4 ×105.4± 9.6	78.0±10.3 × 76.2±12.3
6	18	1931.5±405.4 × 367.9± 30.3	219.3±13.5	112.6±10.5 ×117.9± 7.8	103.3±11.6 × 99.1± 9.9
7	19	3111.8±410.3 × 470.4± 47.6	261.4±21.7	132.4±11.5 ×138.7±10.0	123.4± 9.6 ×119.7±13.5
8	32	3457.3±543.2 × 545.9± 61.4	282.9±27.9	143.4±13.7 ×153.7±13.2	143.8±10.1 ×140.0±14.5
9	25	4180.6±736.0 × 666.2±103.6	303.4±28.8	152.4±21.8 ×161.4±13.7	152.5±14.0 ×143.9±11.7
10	24	4883.5±565.3 × 787.0±123.2	332.8±21.0	174.0±12.9 ×179.4±14.2	168.6±15.9 ×159.2±14.9
15	12	6130.6±721.4 ×1026.1±100.8	318.0±22.8	161.8± 9.6 ×189.4±14.6	186.5±11.8 ×150.6±11.0
38	8	8010.8±813.2 ×1393.4± 86.3	415.3±23.6	222.3±19.3 ×243.3±12.5	212.9±14.3 ×189.4±17.6
50	23	9309.9±838.0 ×1703.4±105.0	438.6±27.5	233.7±20.5 ×263.8±10.6	224.8±15.0 ×239.7±20.7

living worms were left in 1% sodium citrate for 30 minutes. The treated genital organs were placed on a glass slide with a few drops of Carnoy's solution, broken with micropins under a dissecting microscope, dried at room temperature, and stained with 10% Giemsa's solution for 10 to 30 minutes.

Results

The development of *E. hortense* was observed in the specimens obtained from rats at each of the designated days as described above.

1. Measurement of worms

Measurements of the entire worm, suckers, pharynx, head collar, cirrus pouch, testes and ovary were performed on 8 to 32 specimens obtained on each indicated day (Table 1). Growth curves were expressed as percentages of the values obtained 50 days after infection. As shown in Fig. 1, growth curves of the entire body, suckers, pharynx, and head collar which had already developed fairly well in the metacercarial stage, were steep up to 10 days after infection ; thereafter the curves changed to a gentle slope. On the other hand, growth curves of the genital organs such as the testes, ovary and cirrus pouch, which had been recognized only as rudiments in the metacercarial stage, showed on S-shape, that is they grew slowly for 5 days, then the growth curves became steep up to 10 days, and again the growth slowed down (Fig. 2).

2. Position of the center of body length

The center of body length of metacercaria existed in the anterior end of the ventral sucker, and that of the worm was located on the middle or somewhat anterior region of the ventral sucker 2 days after infection, on

hortense recovered from rats

$\bar{X} \pm$ SD wide, μm in

Ventral sucker	Cirrus pouch	Anterior testis	Posterior testis	Ovary
73.9±10.8 × 77.6± 7.9	26.2± 5.2 × 16.8± 4.1	9.6± 3.3 × 10.9± 2.8	10.9± 3.9 × 10.9± 3.4	11.0± 2.4 × 11.8± 3.0
98.5±11.6 × 96.9± 8.5	35.6± 8.5 × 21.2± 6.7	15.4± 2.2 × 18.7± 3.0	17.7± 3.2 × 19.7± 3.7	15.5± 3.2 × 15.9± 3.5
131.7±24.0 ×132.4±11.4	64.2±12.3 × 38.7± 6.4	26.3± 3.5 × 29.4± 4.3	30.0± 3.7 × 29.8± 5.0	23.1± 3.0 × 24.8± 4.1
154.2±24.0 ×153.7±22.6	67.6±14.6 × 45.1±13.4	30.1± 8.5 × 40.6± 10.1	40.9± 12.4 × 39.9± 9.6	24.3± 4.3 × 29.8± 8.5
193.9±27.7 ×188.3±25.8	91.8±17.9 × 57.7±10.8	45.1± 11.1 × 52.9± 11.0	60.8± 10.3 × 55.8± 10.7	33.9± 7.7 × 34.2± 6.7
240.0±34.4 ×235.8±31.0	151.9±22.9 × 89.1±16.0	74.2± 31.0 × 90.9± 29.3	102.4± 42.1 × 89.1± 23.8	45.3±10.6 × 51.7±12.8
316.9±28.7 ×309.3±29.1	202.8±38.3 ×107.7±16.7	190.5± 49.6 ×179.7± 36.8	245.8± 54.6 ×164.5± 36.0	67.1±12.3 × 72.6±14.5
366.8±35.4 ×354.8±31.2	262.4±64.3 ×114.9±15.9	289.0± 54.7 ×269.3± 47.7	358.8± 59.9 ×236.9± 46.0	99.2±19.9 ×106.1±19.9
407.6±31.5 ×398.2±31.9	315.6±59.8 ×127.6±15.9	373.2± 82.7 ×345.4± 70.0	470.5±102.1 ×314.2± 69.7	138.6±35.1 ×137.6±28.2
451.1±35.0 ×434.5±35.7	344.0±49.4 ×147.0±19.0	448.2± 78.1 ×429.0± 74.0	541.9± 80.7 ×382.9± 75.0	179.6±34.6 ×175.6±34.8
521.0±36.8 ×517.9±33.1	434.4±59.0 ×201.8±22.7	611.8± 83.3 ×580.8± 71.1	739.3± 88.5 ×474.4± 43.1	234.1±43.7 ×243.8±34.1
741.8±30.8 ×685.1±45.0	664.0±106.6 ×242.8±31.3	707.0±128.9 ×632.3± 98.2	799.6±132.5 ×512.6±108.7	305.4±26.5 ×296.8±18.5
816.9±44.6 ×769.2±35.8	897.3±120.4 ×345.8±20.8	845.8±113.1 ×854.2±120.0	1071.3±113.8 ×706.9± 85.9	359.7±24.9 ×349.1±25.4

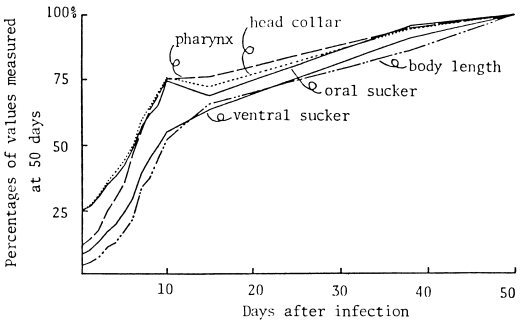

Fig. 1 Growth curves of *Echinostoma hortense* in rats [I].

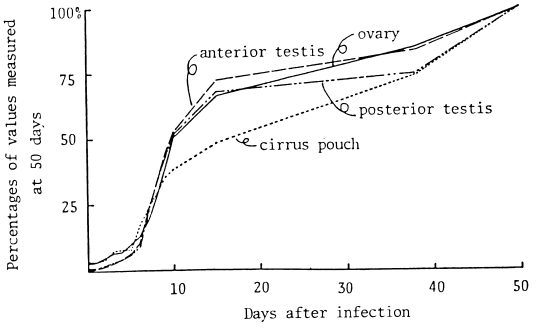

Fig. 2 Growth curves of *Echinostoma hortense* in rats [II].

the posterior end of the sucker at 3 days, in the middle region between the sucker and the ootype at 4 days, on the ootype at 5-6 days, on the anterior testis at 7-8 days, between both testes at 9-10 days, and on and after 15 days the center settled on the posterior testis (Photo. A-J). As the genital organs, except for the cirrus pouch, were located in the area from the posterior end of the ventral sucker to the posterior extremity of the adult worm, the growth rate of the posterior parts of the worm was higher than that of the anterior parts in the final host.

3. Development of the genital organs

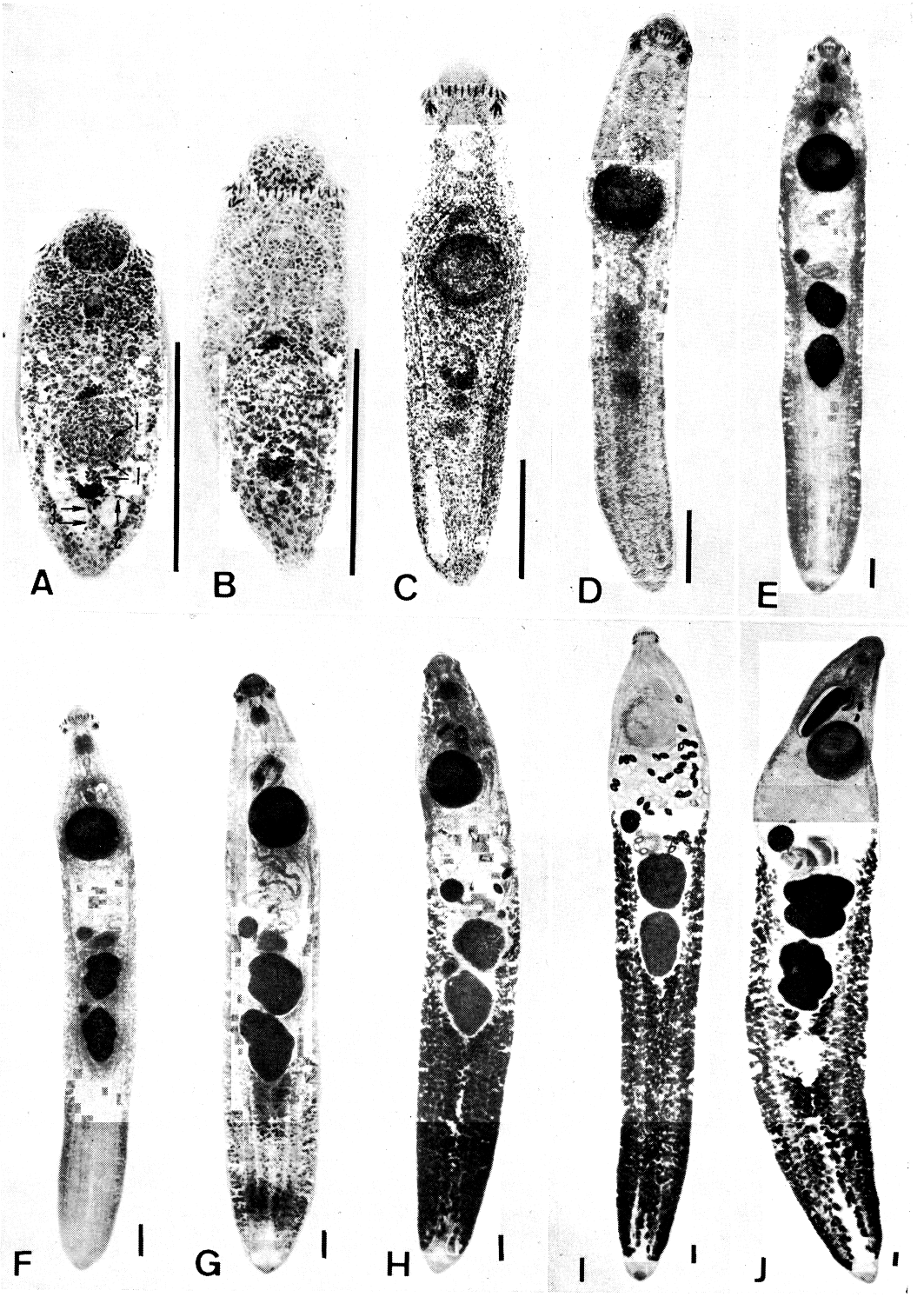

Photo. A Metacercaria of *E. hortense* excysted by using trypsin solution. Fixed in Schaudinn's solution and stained with Haidenhain's iron-hematoxylin. Bar indicates 200 μm.
Photo. B-J *E. hortense* at 1 (B), 3 (C), 5 (D), 7 (E), 8 (F), 9 (G), 10 (H), 15 (I), and 50 (J) days after infection in rats. All the specimens were treated the same as mentioned above. Bars indicate 200 μm.

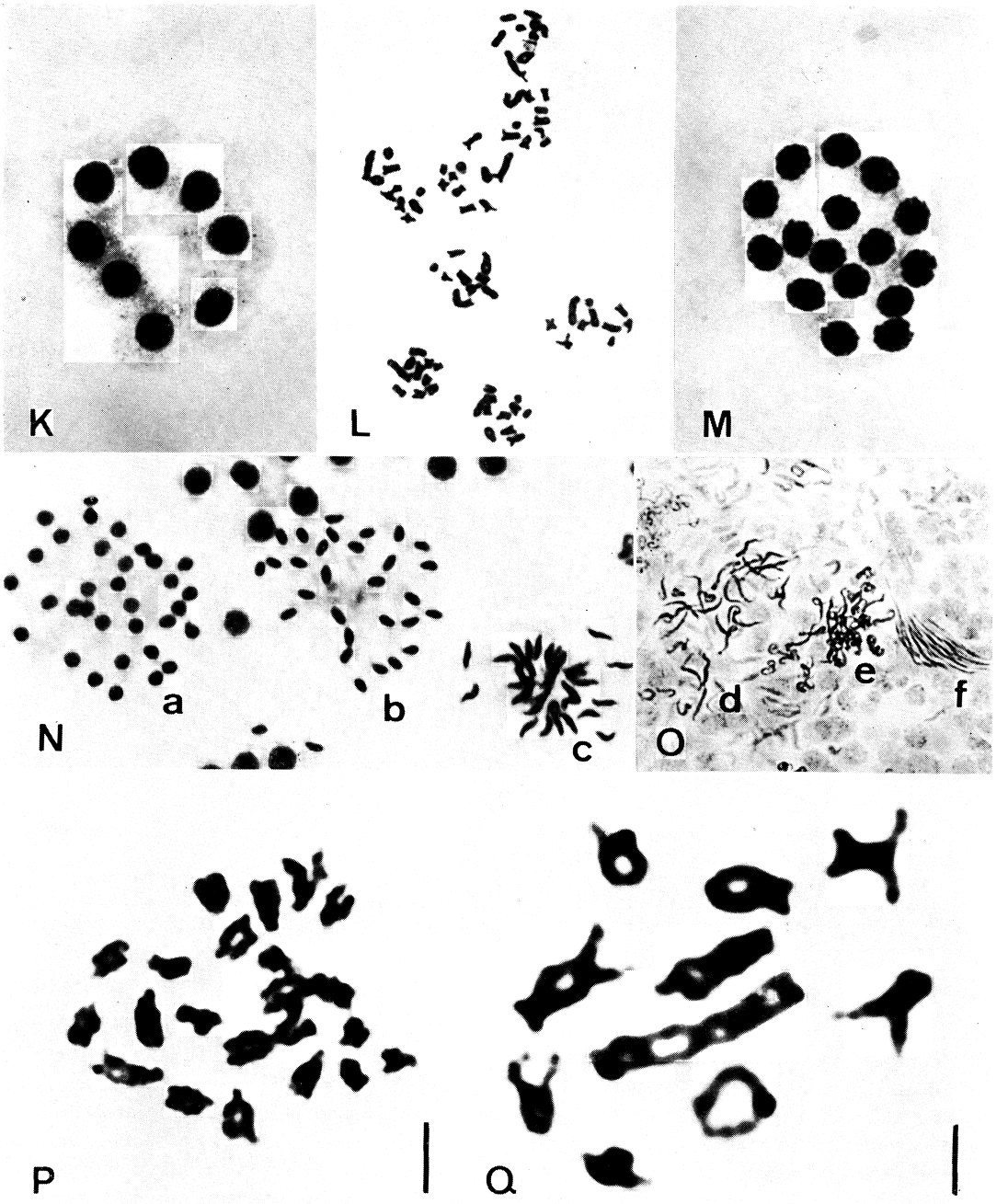

Photo. K-O Spermatogenesis in testes of *E. hortense*.
Photo. P Mitotic metaphase with 20 chromosomes.
Photo. Q Meiotic metaphase with 10 chromosomes.
Bars of Photo. P and Q indicate 3 μm.
K : Eight primary spermatocytes in resting stage.
L : Metaphase of first meiotic division of primary spermatocyte.
M : Sixteen secondary spermatocytes in resting stage.
N-O : Various stages of spermiogenesis from 32 spermatids (a) to bundle of sperm (f).

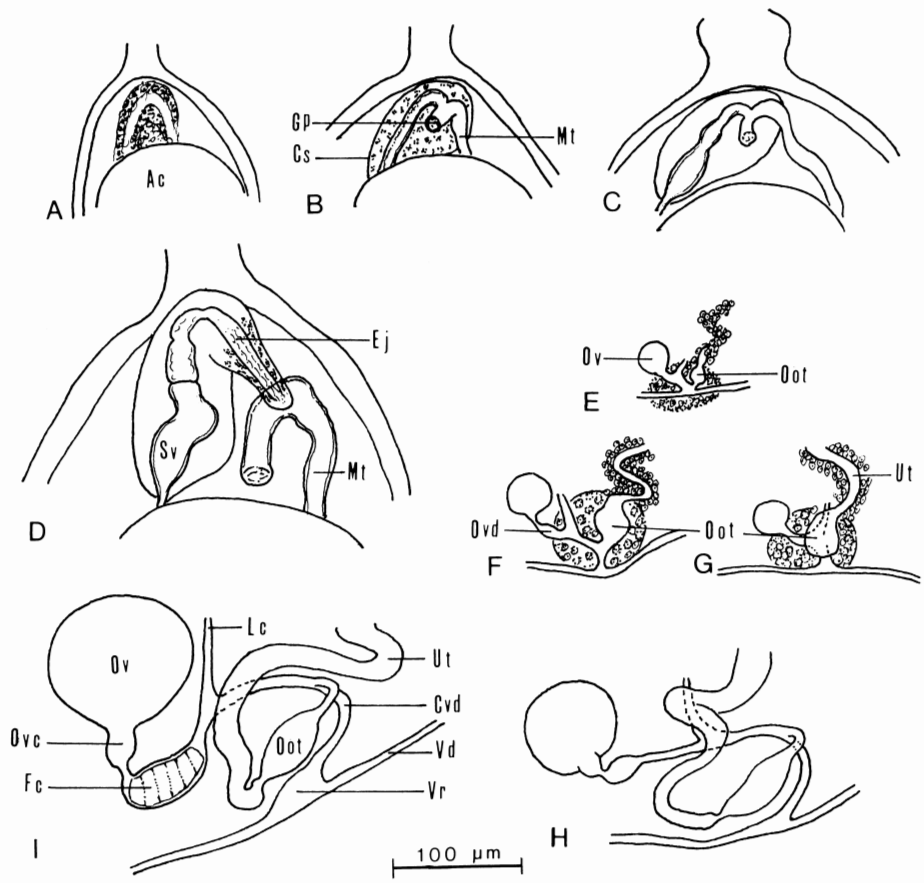

Fig. 3 Development of terminal genitalia 3 (A), 5 (B), 6 (C) and 8 (D) days after infection, and the ovarian complex 3 (E), 6 (F, G), 7 (H) and 8 (I) days after infection.
Ac: Acetablum, Cs: Cirrus sac, Cvd: Common vitelline duct, Ej: Ejaculatory duct, Fc: Fertilization chamber, Gp: Genital pore, Lc: Laurer's canal, Mt: Metraterm, Oot: Ootype, Ov: Ovary, Ovc: Ovicapt, Ovd: Oviduct, Ut: Uterus, Vd: Vitelline duct, Vr: Vitelline reservoir.

As observed by Saito and Tani (1982), the genital primordia of metacercaria were detected as two masses of germ cells lying in front of and behind the ventral sucker. A line (Photo. A ↓ 1) of germinal cells running zigzag between the two masses was detected as the primordium of the uterus, and a horizontal line (Photo. A ↓ 2) of cells deriving from the posterior end of the mass behind the ventral sucker was detected as the primordium of the vitelline duct. The anlagen of the testes (Photo. A ↓ 3) were each composed of a mass of 3-5 cells, lying one behind the other between the mass behind the ventral sucker and the division point of the V-shaped excretory sac.

The development of the terminal genitalia is shown in Fig. 3 A-D. Inside the mass of cells in front of the ventral sucker, a horseshoe-shaped clear zone appeared 2-3 days after infection. The cirrus sac was recognized at 3-4 days. At 5 days the margins of the clear zone were thickened and it was recognized as a duct. At the middle part of the horseshoe-shaped zone the genital pore was observed. The duct of the part left of the pore is the metraterm, and the cirrus opened directly into the lumen of the metraterm. At 6 to 7 days the duct of the part to the right of the genital pore swelled

into an 8 shape. The anterior swelling grew into the ejaculatory duct, the cirrus and prostatic gland and the posterior swelling became the seminal vesicle.

The development of the ovarian complex is shown in Fig. 3 E-I. In contact with the right border of the mass behind the ventral sucker, the small spherical ovary developed 3 days after infection. The oviduct, Laurer's canal and the ootype were also recognized at 3 days. The short narrow oviduct near the ovary enlarged abruptly and formed a organ called the fertilization chamber 6-7 days after infection. At the same time the ootype was located on the right of the commmon vitelline duct like the ootype of the adult worms, while at 5 to 6 days the ootype was located on the ventral side or somewhat left of the common vitelline duct. After 8-9 days five to seven circular ridges were formed on the inner surface of the chamber. The ovicapt projected from the postero-dorsal margin of the ovary. A line of cells running zigzag from the posterior end of the ventral sucker to the mass of germ cells behind the ventral sucker in the metacercarial stage grew into many lines, in which the very thin walled uterus appeared at 4 days. Winding of the uterus at 8 days became very complicated. The vitelline reservoir was detected at 3 days.

4. Gametogenesis

One day after infection the size of the testis was about 10 μm in diameter. Up to 5 days the testes, 50-60 μm in diameter, were composed only of the primary spermatogonia. In the testes at 6 days each cluster of 2 secondary spermatogonia, 4 tertiary spermatogonia, and 8 primary spermatocytes (Photo. K) was first observed at the same time. At 7 days clusters of the meiotic division of the primary spermatocytes (Photo. L) and the spermatids (Photo. N) were recognized. At this time, it was easily recognized that the chromosome number was $2n=20$ and $n=10$ (Photo. P, Q).

The nucleus of the spermatid morphologically changed from spherical to pear-shaped, then became spindled (Photo. N-a, b, c).

Table 2 Transfer of sperm to the genital organs of *Echinostoma hortense* in rats

Days after infection	7	8	9	10
No. of worms observed	27	32	22	24
Testes	0	31*	22	23
Vasa efferentia	0	1	17	21
Seminal vesicle	0	0	14	21
Metraterm	0	0	2	18
Uterus	0	0	0	15
Fertilization chamber	0	0	0	14

* No. of worms bearing sperm

After that it became gradually narrower, and then curly (Photo. O-d, e). Subsequently, the 32 curled nuclei forming a cluster straightened simultaneously, and resulted in a bundle of 32 parallel needle-shaped or filiform sperm at 8 days (Photo. O-f).

The sperm moved from the vasa efferentia to the vas deferens, and were collected in the seminal vesicule. They were transferred through the cirrus to the metraterm at 9 days. After 10 days they traveled down the uterus and then reached the fertilization chamber through the ootype (Table 2). Although the ejaculatory duct opened into the metraterm near the genital pore, the sperm which were transferred from the ejaculatory duct to the metraterm traveled down in the direction of the proximal uterus. In most of the specimens, moreover, the cirrus was also inserted into its own metraterm in the direction of the proximal uterus.

The primary oocytes first appeared in the ovary, about 100 μm in diameter, 8 days after infection, and were liberated singly through the ovicapt into the fertilization chamber at 10 days.

The yolk cells were recognized in the posterior half of the body at 7 days, transferred to the vitelline duct at 9 days, and reached the vitelline reservoir at 10 days.

As described above, 10 days after infection was the first time that the sperm met the primary oocyte in the fertilization chamber,

and on the same day the yolk cells were transferred to the vitelline reservoir, and eggs appeared in the uterus.

Discussion

This paper mainly demonstrates the development of the genital organs of *Echinostoma hortense* based on the specimens obtained from rats daily for 10 days, and at 15, 30, and 50 days after metacercarial infection. From the measurements of the whole specimen, development of the entire worm, suckers, pharynx and head collar was expressed by straight lines up to 10 days, but on the other hand, growth of the testes, ovary and cirrus pouch showed S-shaped curves. It is well known that growth curves of individuals and organs in organisms commonly show on S-shape. This curve expresses the process of growth through the entire life span. The digenetic trematodes have a complicated life cycle especially in the larval stage that entirely differs in appearance from the miracidium to the cercaria. The metacercaria, however, is morphologically fairly similar to the adult worm except for the genital organs. As the metacercariae grow fairly well in the cyst, measurement should be made from the metacercaria immediately after encystment. It is likely that most of the metacercariae used for the present measurements were already fully matured. Viewed from this angle, growth of the entire worm, suckers, pharynx and head collar also seem to show S-shaped curves. On the other hand, the genital organs of the metacercaria grow poorly. In the final host, therefore, the growth of genital organs may show S-shaped curves. Growth curves changed from steep to shallow about 10 days after infection, when the worms began to lay eggs.

Asada (1939) stated that the testes of the adult *E. hortense* are located in the middle region of the body. Arizono *et al.* (1976) confirmed the results of Asada with their own observations and with reference to text figures in the reports of Asada (1. c.) and Kamiya and Ishigaki (1972). They suggested that localization of the posterior testis on the center line of the body might be one of the characteristic features of adult worms of *E. hortense*. This idea was supported by Saito and Tani (1982). In the present study, the same result was obtained in all adult worms on and after 15 th day of infection, while the center within 10 days was located between the ventral sucker and the anterior testis.

From the present observation the spermatogonia began to grow into primary spermatocytes 6 days after infection in the rats, and then grew into sperm at 8 days, i. e., 1 to 2 days were required for growing from young primary spermatocytes to sperm in *E. hortense*. This time required in the trematode *E. hortense* was very short as compared with that in some vertebrates and insects such as the mouse (26–28 days by Hertwing, 1938; Fogg and Cowing, 1951; 35 days by Oakberg, 1956), rat (26 days by Shaver and Mason, 1950; 27 days by Shaver, 1953), ram (40 days by Ortavant, 1954), man (about 10 weeks, generally), Japanese tree frog (35–40 days in 25°C by Toyoshima, 1982), guppy (21 days by Felice and Rasch, 1968; 36 days in 25°C by Billard, 1969), *Oryzias latipes* (10 days in 25°C and 20 days in 15°C by Egami and Hyodo-Taguchi, 1967), *Drosophila* sp. (9–10 days by Sobels and Vogel, 1976) and silkworm (about 15 days by Tazima, 1961). On the other hand, the primary oocyte developed in the ovary at 8 days and was released to the fertilization chamber at 10 days. The first primary oocyte may possibly be penetrated by the sperm, because the sperm has already been transferred to the fertilization chamber when the primary oocyte is first released from the ovary.

Kusaura (1966) reported in detail on the development of the primary oocyte and the sperm inside the egg-shell of *E. hortense*. According to him, the primary oocyte liberated from the ovary is penetrated by the sperm inside the fertilization chamber, and is wrapped up with the yolk cells in the egg-shell at the ootype. Then, the oocyte extrudes the primary and secondary polar bodies, and grows into the secondary oocyte and then the egg cell. Subsequently, the

fusion of the nucleus of the sperm and that of the secondary oocyte takes place, and the egg cell is divided into four cells at the time of egg-laying. Kusaura (l. c.) also reported that the chromosome number of *E. hortense* was 2n=20 and n=10, and Terasaki *et al.* (1982) clarified the karyotype of the same species. In the present observation in rats, the chromosomes were detectable in the worms on and after 6 to 7 days after infection. The testes in rats from 1 to 5 days after infection were composed of only the spermatogonia in which chromosomes were undetectable. Kusaura (l.c.) also stated that the observation of chromosomes in the spermatogonia was very difficult.

Laurer's canal is recognized 3 days after infection. Until this time, there have been two opinions on the function of Laurer's canal. Some investigators presumed that Laurer's canal might be a vestigial duct which corresponds to the vaginal canal in tapeworms, and others thought that this duct might function to exclude the excess of egg-shell forming materials. Erasmus (1972) stated that the entry of sperm into the female reproductive system may take place via Laurer's canal or through the genital pore and the uterus. According to the observation of *Philophthalmus megalurus* (Nollen, 1968), the entry of sperm into the female reproductive system did not take place via Laurer's canal. In the present observation, the sperm was never detected in the fertilization chamber of the worm which had no sperm in its uterus, even if the worm harbored together with other worms bearing sperm. This result may support the idea that the sperm travel through the uterus to the fertilization chamber and not through Laurer's canal. Though the opening of the ejaculatory duct is connected with the metraterm near the genital pore, it was observed in a few specimens that the cirrus projected from the genital pore. In most of the specimens the cirrus was inserted in the proximal part of its own uterus and most of the sperms which were emitted from the ejaculatory duct descended through the uterus. From the above-mentioned results self-fertilization is possibly always occurring in *E. hortense*.

Summary

On the basis of the specimens obtained from rats every day for 10 days, and at 15, 38, and 50 days postinfection, the growth of *E. hortense* was observed. The results are summarized as follows:

1. Each growth curve of the testes, ovary and cirrus pouch showed S-shaped lines, while that of the entire worm, suckers, pharynx and head collar was given as straight lines until 10 days after infection.
2. In all the worms 15, 38, and 50 days after infection, the center of the body length was located on the posterior testis, while the center within 10 days was located between the ventral sucker and the anterior testis.
3. All the genital organs indistinctly appeared 2-3 days after infection, and were morphologically accomplished in 6-7 days.
4. Up to 5 days the testes were composed only of the primary spermatogonia. At 7 days clusters of the meiotic division of the primary spermatocytes and the spermatids were recognized. At this time, it was easily recognized that the chromosome number was 2n=20 and n=10. The sperm developed in the testes at 8 days.
5. The primary oocytes developed in the ovary at 8 days, and the eggs in the uterus at 10 days.
6. In the worms 3 days after infection, the ootype was located on the ventral or left side of the common vitelline duct. The ootype on and after 6-7 days, however, moved from the above-mentioned position to the right side.
7. In this species the entry of sperm into the female reproductive system may take place via the genital pore and the uterus, and not via Laurer's canal.

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ラット体内における *Echinostoma hortense* の発育 特に生殖器官のそれについて

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Echinostoma hortense のメタセルカリアをラットへ投与し，10日後までは毎日，その後は15，38および50日後の虫体について発育状況を観察した．体長，体幅，口吸盤，腹吸盤，咽頭，頭冠幅などは感染後10日まで直線的に成長したが，睾丸，卵巣，陰茎嚢など生殖器官はS字状の成長曲線を示した．本種成虫の特徴の一つは下睾丸が体の中央にあることである．メタセルカリアの体の中央は腹吸盤前端付近であるが，感染後15日以降の虫体から上記の特徴が備わつた．各生殖器官は感染2～3日後から，その一部が形成され始め，6～7日後には形態的にほぼ完成した．睾丸は5日後まで精原細胞だけで構成され，6日後には第一精母細胞，7日後には精細胞まで観察され，8日後には精子束を形成した．なお雄性生殖細胞の染色体数は2n＝20，n＝10であつた．一方，第一卵母細胞は8日後に卵巣内に現われ，子宮内に虫卵が出現したのは10日後であつた．感染3日後に認められた卵形成腔は総卵黄輸管の腹側に重なるか，あるいは，そのやや左側に存在していたが，6～7日後には成虫本来の位置である総卵黄輸管の右側を占めた．精子の雌性生殖器官への侵入はすべて子宮経由で，ラウレル管にはその役目を肯定する事実は認められなかつた．