Morphology and Development of a Lung Fluke of the Genus *Paragonimus* (Trematoda : Paragonimidae) from Primor'e, USSR, in Snails, *Semisulcospira libertina*, in the Laboratory

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Abstract Experiments were made to infect Japanese snails, *Semisulcospira libertina*, with miracidia of a lung fluke, *Paragonimus* sp., from Primor'e, USSR, in the laboratory. Cercariae developed fully at above 25°C in about 9 weeks after infection. The miracidium, sporocyst, mother and daughter rediae, and cercaria are described. The development of the fluke in the snail is outlined. The fluke is considered most closely related to *P. westermani ichunensis* Chung, Hsu et Kao, 1978. Cercariae of three other species of natural infection also were found in the snails used. The fluke cercariae were recovered at much higher rates from the snails infected concurrently with the fluke and *Cercaria incerta* than from those infected with in other combinations of the trematodes. A positive intramolluscan interaction between the fluke and *C. incerta* is suggested.

A lung fluke of the genus *Paragonimus* ranges in the Far East of the USSR. Posokhov and Shabanova (1977) recorded trematodes as *P. westermani* (Kerbert, 1878) Braun, 1899, from domestic cats, *Nyctereutes procyonoides*, *Canis lupus*, *Vulpes vulpes* and *Felis bengalensis*, and *Paragonimus* eggs from feces of *Panthera tigris*, in Priamur'e and the Ussuri Valley, Khabarovsk region. Posokhov *et al.* (1979) studied the miracidial development in the laboratory and, exposing *Juga* sp. snails (formarly *Semisulcospira cancellata*, Pachychilidae) to miracidia, they obtained cercariae in a minimum of 61 days after exposure. They failed in experimental infection of *Cambaroides* spp. crayfishes with the cercariae, but they found that *C. dauricus dauricus* and *C. schrenckii* were naturally infected with *Paragonimus* metacercariae in Priamur'e and the Ussuri Valley. Posokhov *et al.* (1981) showed that the *Juga* snails served as the first intermediate host also in the field. Kurochkin and Sukhanova (1979), describing a lung disease, previously reported as Löffler syndrome, of man in Primor'e, suggested that it might be caused by infection with *Paragonimus* larvae. They obtained *Paragonimus* metacercariae from *C. dauricus wladiwostokiensis* taken in the areas where the patients had been catching crayfish to eat, and were able to produce experimental larval paragonimiasis in white rats. Later they (1980) confirmed this suggestion by detecting many *Paragonimus* juveniles in the intercostal and diaphragm muscles and lungs on postmortem examination of a patient who died several months after eating raw cryfish. They believed that the causative parasite was not *P. westermani*.

In view of studies of the taxonomy and life cycle of the fluke, we attempted several times to maintain the life cycle of the fluke in the laboratory. The purpose of this paper is to describe the morphology and development of the miracidium, sporocyst, mother and daughter rediae and cercaria of the fluke, which were obtained in experiments to infect

Table 1. Results of experimental infections of snails, *Semisulcospira libertina*, with miracidia of a lung fluke, *Paragonimus* sp., from Primor'e, USSR

Exp. No.	No. of snails used	No. of miracidia per snail	Weeks after infection	No. of snails examined	*P.**1
1	unknown	unknown	13	269	3 (1.1)
2	550	182	11	8	0
3	200	60	8-12	117	9 (7.7)
4	400	70	10-17	318	35 (11.0)
5	1000	50	4-20	512	16 (3.1)
6	764	30	23	241	1 (0.4)
Total			4-23	1465	64 (4.4)

*1 *Paragonimus* sp. larvae of experimental infection.
*2 *Cercaria incerta* of natural infection.
*3 *Notocotylus magniovatus* of natural infection.
*4 *Centrocestus armatus* of natural infection.

snails, *S. libertina*, with miracidia. The taxonomy of the trematode also is discussed.

Materials and Methods

Metacercariae of the fluke were collected from naturally infected *C. dauricus* taken at Ussuriysk, Primor'e, USSR, in June 1980. Part of a lot of specimens brought to Japan were fed to several dogs in this study. Other parts of the lot were used in their studies by Oshima *et al.* (1981) and Yokogawa *et al.* (1981, 1982).

Fluke eggs in worm cysts in the lungs, and in feces, of the dogs were incubated at 25°C for 4 to 7 weeks according to the method of Shimazu (1981). Hatched miracidia were obtained by giving the incubated eggs strong light. *S. libertina* (5 to 20 mm high) were collected about 1 to 6 weeks before use for experimentation at Matsuoka, Matsumoto, where no *Paragonimus* species were distributed (Shimazu, unpublished data). They were exposed to miracidia by placing them in water about 5 mm deep in a 27×34-cm pan and adding both hatched and unhatched miracidia. After 5 to 21 hours all the contents of the pan were transferred to the aquarium. The snails were reared at above 25°C according to the method of Shimazu (1976) and crushed for *Paragonimus* larvae at irregular intervals after exposure.

Intramolluscan larvae recovered were slightly flattened, fixed in 70% ethyl alcohol, stained with alum carmine and mounted in Canada balsam. Some mature daughter rediae were fixed in Schaudinn's solution, stained with 0.05% thionine in 30% ethyl alcohol and mounted in balsam. The cell boundaries of the ciliated epithelial cells of miracidia were demonstrated by the silver impregnation method (Lynch, 1933). Some internal details of larvae were observed in living specimens. Miracidia, daughter rediae and cercariae (ten each) which were fixed in hot 5% formalin, living sporocysts, and stained whole-mounted mother rediae were measured. Most of the specimens studied are deposited in the collection of the National Science Museum (Natural History), Tokyo (Coll. Nos. NSMT-Pl 2594-2613).

Results

Results of infection experiments

From March 1981 to April 1983, six experiments to infect the snails with the miracidia were conducted. Results of them are summarized in Table 1. Those obtained when snails were dissected in 1 week after exposure to observe the sporocyst development are omitted from the table. In the experiments 1 and 3 to 6, many of the snails used survived, and mature cercariae of the fluke were recovered. The experiment 2 failed because of the early death of most of the snails used probably from poor management of the aquarium. Besides *Paragonimus* larvae of experimental infection, cercariae of three other species of natural

Table 1. (continued)

No. (%) of snails infected with						
[*P.* only	*P.*+*Ce.**2	*P.*+*N.**3	*P.*+*Ce.*+*N.*]	*Ce.* only	*N.* only	*Cen.**4
[2 (0.7)	1 (0.4)	0	0]	4 (1.5)	8 (3.0)	2 (0.7)
[0	0	0	0]	0	1 (12.5)	0
[1 (0.8)	8 (6.8)	0	0]	2 (1.7)	0	0
[0	33 (10.4)	1 (0.3)	1 (0.3)]	4 (1.2)	2 (0.6)	0
[2 (0.4)	13 (2.5)	1 (0.2)	0]	4 (0.8)	12 (2.3)	0
[1 (0.4)	0	0	0]	0	1 (0.4)	0
[6 (0.4)	55 (3.7)	2 (0.1)	1 (0.1)]	14 (1.0)	24 (1.6)	2 (0.1)

infection were detected in the snails examined. They were: *Cercaria incerta* (Kobayashi, 1922) Faust, 1924; *Notocotylus magniovatus* Yamaguti, 1934; and *Centrocestus armatus* (Tanabe, 1922) Price, 1932. As will be described later, sporocysts and mother rediae of the present fluke developed in the stomach region, and anterior to it, of the snail, and daughter rediae mainly in the midgut gland. Cercariae in sporocysts of *Ce. incerta* and cercariae in rediae of *N. magniovatus* were found in the stomach and intestinal walls and vicinity. Sporocysts containing cercariae of *Cen. armatus* occurred in the midgut gland.

Miracidium (Figs. 1 and 2)

Body elongate-oval or -oboval or pyriform, 0.060–0.066 mm long by 0.026–0.034 mm wide, variable in shape when alive. Ciliated epithelial cells covering all over body, totaling 17, arranged in four transverse rows of 6: 7: 3: 1 starting from anterior or first row. Gland cells in one or two groups occupying anterior third of body, opening to terebratorium. Nerve center immediately posterior to gland cells. A mass of large, probably germinal cells in posterior third of body. Flame cells two, one on each side of body, level with second row of ciliated epithelial cells, each with a long, tightly convoluted tubule and lateral pore on border between second and third rows of epithelial cells. Other details not worked out.

Newly-hatched miracidia penetrated snails readily. Upon coming in contact with the mucus secreted by snails, some miracidia shed the ciliated coat.

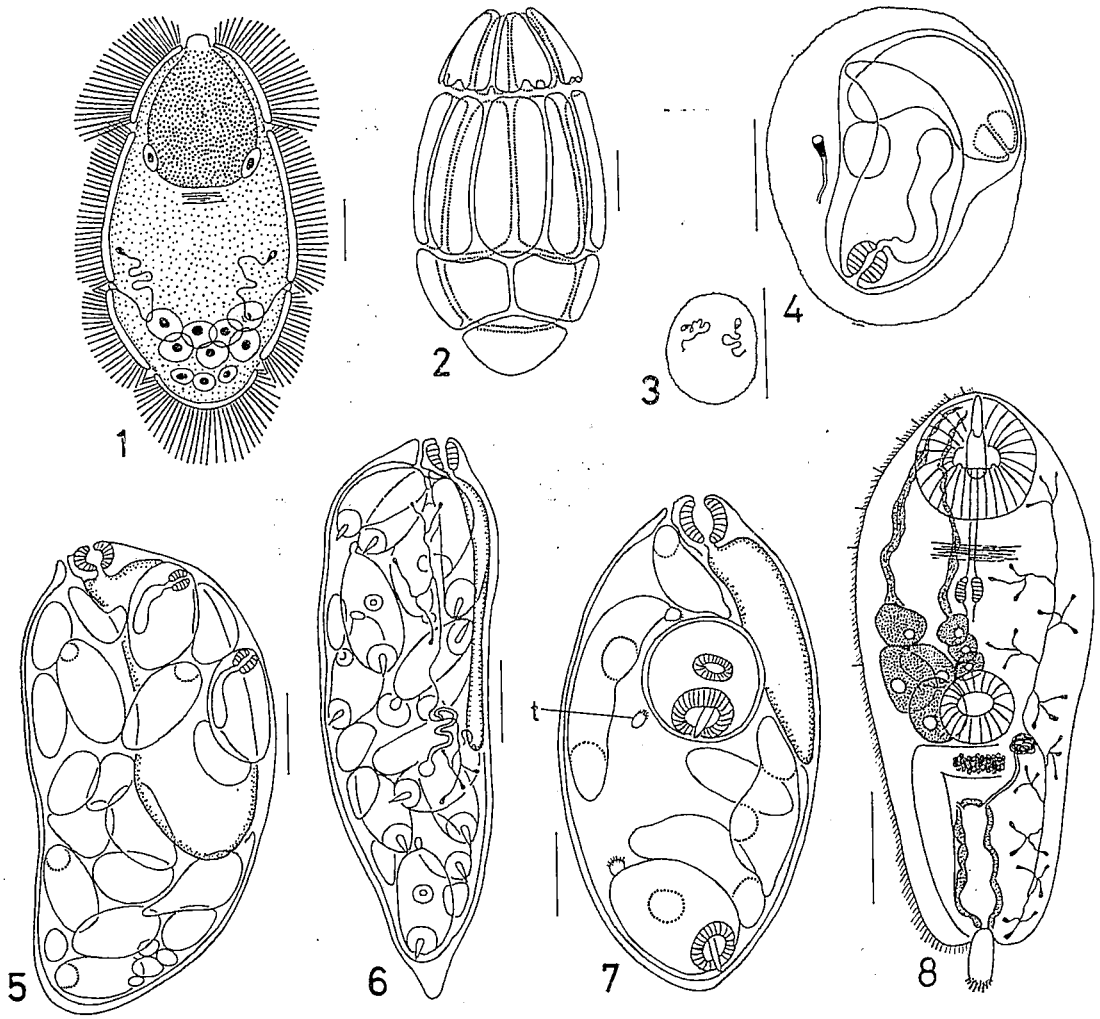
Sporocyst (Figs. 3 and 4)

While entering snails, miracidia took off the epithelial overing and transformed into sporocysts. Sporocysts were found in the tissues (mainly muscular) shallow under the epidermis of the foot, mantle and tentacles of the snails. Five living ones detected 1 week after infection were ellipsoidal and 0.047 to 0.062 mm long by 0.035 to 0.057 mm wide, with finely wrinkled body surface (Fig. 3). The germinal cells and paired excretory organs were seen in them. A 4-week-old sporocyst, 0.125 by 0.112 mm, contained three developing germinal balls measuring 0.038 to 0.070 mm long by 0.023 to 0.041 mm wide in the body cavity. There were recovered three living sporocysts, 0.100 to 0.150 mm long by 0.125 mm wide, which had been producing mother rediae, 17 weeks after infection (Fig. 4). They were surrounded by a thin capsule of host origin.

Mother redia (Fig. 5)

Only once in the course of this study, four larvae which were definitely determined as mother rediae were found in the tissues between the head-foot and midgut gland of one of ten snails examined 4 weeks after infection.

Body elongate-oval, 0.52–0.72 mm long by 0.24–0.32 mm wide. Pharynx 0.040–0.048 mm long by 0.048 mm wide. Intestine 61–78% of body langth. Birth pore lateral to pharynx. Daughter rediae developing in body cavity 16–21 in number; ones with well-differentiated pharynx and intestine measuring about 0.15 by 0.08 mm. Excretory system similar to that of daughter redia.



Figs. 1-8. Miracidium and intramolluscan stages obtained in experimental infections of snails, *Semisulcospira libertina*, with miracida of a lung fluke of the genus *Paragonimus* from Primor'e USSR.

Fig. 1. Miracidium, living.
Fig. 2. Miracidium, showing the arrangement of the ciliated epithelial cells.
Fig. 3. Sporocyst, living, 1 week after infecton.
Fig. 4. Sporocyst, living, 17 weeks after infection.
Fig. 5. Mother redia, slightly flattened whole-mount, 4 weeks after infection.
Fig. 6. Daughter redia, living, 15 weeks after infection.
Fig. 7. Daughter redia, flattened whole-mount, 16 weeks after infection, containing an encysted cercaria and its discarded tail (t) after being preserved in saline at 5°C for 24 hours.
Fig. 8. Cercaria, living, ventral view, 15 weeks after infection, showing the penetration glands and ventral sulcus on the right side of the body and the excretory organs on the left side.
Scale bars. 1, 2=0.01 mm; 3, 4, 8=0.05 mm; 5, 6, 7=0.1 mm.

Daughter redia (Figs. 6 and 7)

Daughter rediae were found mostly in the midgut gland of snails and some in the tissues between the head-foot and this organ. Larger ones with fully-formed cercariae in them, obtained 15 weeks after infection were:

Body elongate, egg-shaped, finely annulated, 0.48-0.72 mm long by 0.16-0.24 mm wide.

Pharynx wider than long, 0.032-0.036 mm long by 0.036-0.052 mm wide. Esophagus thick-walled, very short. Intestine 40-60% of body length. Birth pore lateral to pharynx. Developing cercariae in body cavity many; about 25 mature cercariae in a redia 0.72 by 0.16 mm. Excretory organs present on each side of body; flame cells arranged in a formula of 2 (4+4)=16; main collecting tube convoluted; pore slightly postequatorial.

Smaller daughter rediae containing fewer cercariae also were found together with the above-described larger ones in the same snails. Some were much smaller, with only a single well-developed cercaria and a few germinal balls in them. Twenty whole-mounts of daughter rediae obtained 8 weeks after infection were 0.21 to 0.76 mm in body length, and the intestine was 43 to 61% of the body length. It did not seem that the intestinal length was proportional to the body length. *Paragonimus* daughter rediae and *Ce. incerta* sporocysts numbered 2,082 and 103 in a snail 17 mm high and 480 and 65 in another snail 14 mm high, respectively.

Cercaria (Fig. 8)

Chaetomicrocercous cercaria. Body obovate, elongate, 0.23-0.26 mm long by 0.08-0.09 mm wide, covered with fine spines; forebody 0.13-0.16 mm long; tail knoblike, densely spinous posteriorly, 0.020-0.023 mm long. Brain lying across prepharynx. Ventral sulcus present in hindbody. Oral sucker round, 0.050-0.055 mm in diameter. Stylet 0.032-0.035 mm long by 0.007 mm wide. Prepharynx long, 0.02-0.03 mm long. Pharynx midway between two suckers, 0.010-0.011 mm in diameter. Esophagus long. Intestinal bifurcation just anterior to ventral sucker; ceca not traced. Ventral sucker ellipsoidal, approximately equatorial, 0.024-0.030 mm long by 0.030-0.035 mm wide; sucker width ratio 1: 0.60-0.66. Penetration glands divided into two groups on each side of body; inner group consisting of three smaller cells with finely granular contents located between pharynx and ventral sucker, with processes (or ducts) passing anteriad in a bundle and opening lateral to stylet; outer group composed of four larger cells with coarsely granular contents lying anterolateral to ventral sucker and laterally overlapping it, with processes running forward in a bundle and opening lateral to those of inner glands. Genital primordium behind ventral sucker. Excretory vesicle saccular, thick-walled, extending to genital primordium; flame cell formula $2\ [(3+3+3+3+3)+(3+3+3+3+3)]=60$; main collecting tubes much coiled; pore not seen. Other details not worked out.

Cercariae obtained 8 weeks after infection were immature, but those obtained 10 weeks after infection were fully mature. After isolated daughter rediae were preserved in 0.4% saline at 5°C for 12 to 24 hours, some mature cercariae in them were found densely covered up with the mucoid substance secreted by the mucoid glands of the cercariae themselves, and some others were found enclosed in a cyst (Fig. 7). Such encysted cercariae, 0.090 to 0.120 mm in cyst size, did not have their tails, which were found left out of their cysts. The cysts, about 0.007 mm thick, were globular, single-layered and transparent. The mucoid covering and cyst wall similarly stained metachromatically purplish red with thionine. This encystment may correspond to the encapsulation that *Acanthatrium oregonensis* cercariae do on the gill of caddis fly larvae before penetrating it (Burns, 1961). Its biological significance remains to be explained.

Several attemps were made to infect crabs, *Geothelphusa dehaani*, and crayfish, *Procambarus clarki*, with the cercariae without success. No metacercariae were recovered from them 8 to 12 weeks later.

Discussion

In this study fully-formed cercariae of the fluke were obtained from experimentally exposed *S. libertina*, but the life cycle could not be completed in the laboratory. The development of the fluke in the snail at above 25°C is outlined as follows. While penetrating through the epidermis of the foot, mantle and tentacles of snails, miracidia cast off the ciliated epithelial cells and transform into sporocysts. After penetrating, sporocysts establish themselves in the tissues shallow under the points where they have just entered.

As they grow, germinal cells within them develop into mother rediae, usually two to three at the same time. They then continue producing mother rediae for up to 17 weeks after exposure. By 4 weeks after infection, mother rediae begin to emerge out of sporocysts. While or after migrating to the midgut gland, they grow up and eventually lay daughter rediae. Daughter rediae grow mainly in the midgut gland, and cercariae in them develop. In about 9 weeks after infection, cercariae become well differentiated, but they scarcely escape from daughter rediae.

We have not yet examined in detail the metacercaria and adult of the fluke. The metacercarial cyst size was reported as 0.294 to 0.350 mm in diameter (Posokhov *et al.*, 1979), 0.29 to 0.34 mm in diameter (Kurochkin and Sukhanova, 1979), 0.298 to 0.347 mm in diameter (Oshima *et al.*, 1981), and 0.320 to 0.330 (mean, 0.325) by 0.300 to 0.330 (mean, 0.315) mm (Yokogawa *et al.*, 1982). The adult worms described by Posokhov and Shabanova (1977) from naturally infected carnivores in Priamur'e were: body 9–12 mm long by 3.5–6.5 mm wide; cuticular spines single; oral sucker 0.75–1.32 mm in diameter; ventral sucker 0.60–0.96 mm in diameter; ovary 6-lobed; testes 5- to 6-lobed; eggs 74–93 by 42–66 μm. According to Yokogawa *et al.* (1981, 1982), 18 whole-mounted adults recovered from dogs 3 to 4 months after experimental feeding with the metacercariae were: body averaging 11 mm long by 6.7 mm wide; cuticular spines arranged mostly singly but occasionally in groups of two to four; oral sucker averaging 0.647 mm long dy 0.950 mm wide, slightly larger than ventral sucker averaging 0.775 mm long by 0.778 mm wide; ovary branching irregularly [sic] or into five or six (mostly six) lobes; spermatozoa filling up both seminal vesicle and seminal receptacle; eggs laterally symmetrical, 70–83 (mean, 75.3) by 46–54 (mean, 48.6) μm, with eggshell being uniform in thickness. In the present study, 200 eggs preserved in 5% formalin after being taken out of worm cysts in the dog's lungs about 59 months after infection measured 72 to 88 by 40 to 52 μm. In 187 or 93.5% of them, the smooth eggshell was uniform all over in thickness, and in the rest it was thickened on the anopercular pole. Yokogawa *et al.* (1982) reported the testes as usually 3- to 4-lobed. This is questionable and needs reexamination. Oshima *et al.* (1981) showed that the chromosome number was 2n=22 and n=11 in the spermatogonial cells. The present fluke does not attain sexual maturity in rats (Kurochkin and Sukhanova, 1979; Yokogawa *et al.*, 1981, 1982). When metacercariae were experimentally fed to rats, worms migrated into the body muscles, where they remained infective for long months without growing at all. Few were found in the lungs. Those recovered from there 4 months after feeding had grown a little with undeveloped genital organs (Kurochkin and Sukhanova, 1979).

In the above-mentioned morphological features of the abult and metacercarial stages, the present fluke resembles *P. westermani ichunensis* Chung, Hsu et Kao, 1978. This subspecies occurs in Ichun, Hokiang and Mutankiang, Heilungkiang province, China, adjacent to Priamur'e and Primor'e of the USSR. The first intermediate host there is *S. amurensis*. The second intermediate hosts are *C. dauricus* and *C. schrenckii*. Dogs and cats are heavily infected with adult worms (Chung *et al.*, 1978). The daughter rediae and cercariae described briefly by Chung *et al.* (1978) from naturally infected *S. amurensis* are somewhat smaller than the present specimens. They placed some importance on the size of the daughter redia and the number of cercariae in it in separating species or subspecies. However, it is apparent from the present study that, varying largely from specimen to specimen and from host to host, the size and number are of no taxonomic importance. Human paragonimiasis due to the subspecies is endemic in the areas. Inhabitants there acquire infection with the parasite by eating raw crayfish harboring metacercariae. A few days later, abdominal pain and diarrhea appear. The main clinical manifestations are general weakness, loss of appetite, fever, urticaria and cough with or without sputa. Usually there is marked eosinophilia in the peripheral blood. Pleural effusion is sometimes seen. Nonmigratory subcutaneous nodules without juvenile or adult worms or ova in them occasionally occur, most frequently during the first 2 months of the disease. Worms barely become sexually mature in the lungs, and ova are rare in sputa. Cerebral involvement is rare (Chung *et al.*, 1978). These clinical characteristics agree well with those observed by Kurochkin and Sukhanova (1979) in Russian cases of human paragonimiasis caused by the

present fluke. From the previous discussion, we conclude that the present trematode is most closely related to *P. westermani ichunensis*.

The present fluke is different from *P. westermani filipinus* Miyazaki, 1978, in not developing to sexual maturity in experimental rats. The latter matures in rats (Miyazaki, 1981). The present fluke also is distinguished from the triploid form of *P. westermani* or *P. pulmonalis* (Baelz, 1880) Miyazaki, 1978 (Miyazaki, 1978) in having smaller metacercarial cysts and eggs and fewer chromosomes (2n=22 instead of 2n=33) and in barely maturing sexually in the lungs in man. However, the present daughter rediae and cercariae resemble in morphology those of the triploid form obtained in Shimazu's (1981) experiment and those probably of the same form described by Komiya and Ito (1950) except that the intestine may be a little shorter in the daughter redia. According to Shimazu's unpublished data, 50 larger ones of mature daughter rediae recovered 13 to 28 weeks after infection were 0.19 to 1.20 mm in body length, and the intestine was 33 to 83% of the body length. Miyazaki (1982) has some doubt about the validity of *P. westermani ichunensis*. Examining adult specimens found in naturally infected *Panthera tigris* and *Nyctereutes procyonoides* from Khabarovsk and those raised experimentally from metacercariae found in *C. dauricus* from Vladivostok, he identified them as *P. westermani* as defined by him (1978).

The present infection experiments were designed primarily to obtain mature cercariae of the present fluke, so that the initial snail infection rate with each of the trematode species detected in the snails used had not been determined. The results (Table 1) indicate that the snail infection rate with the present fluke cercariae rose with increased number of miracidia exposed per snail, increased proportionately with the infection rate with *Ce. incerta* cercariae, and was much higher in snails infected with both the present fluke and *Ce. incerta* than in those infected with the present fluke only and in the combinations of the present fluke and *N. magniovatus*, and of the present fluke, *Ce. incerta* and *N. magniovatus*, and that the infection rate with the both the present fluke and *Ce. incerta* was higher than that with *Ce. incerta* only. It seems likely from this that a positive interaction worked at least between the present fluke and *Ce. incerta* in the snails infected simultaneously with the two species, of which cercariae generally occupied the different microhabitats in them. No other experimental evidence is available at present to enable to discuss this phenomenon further. Similar interactions between *Schistosoma mansoni* and other trematode species in the snail *Biomphalaria glabrata* have been reviewed by Lie (1982). Hamajima *et al.* (1981) also observed similar phenomena between the triploid form of *P. westermani* and other species trematodes in *S. libertina*. In their experimental exposures of snails to miracidia, cercariae of the triploid form were found only, and at fairly high rates, in snails which had prior infections with one or two other trematode species. The concurrent infection rate with the triploid form cercariae and *Ce. monostyloides* Ito, 1960, reached as high as 39.5%. They considered that changes of immune response and chemical composition of the snails caused by previous infections with other species cercariae might be a possible factor in determining the development of the triploid form cercariae of subsequent infection. The triploid form rarely develops to the cercarial stage even in laboratory-raised snails. In an experiment, 2 or 0.6% of 326 such snails (14 to 28 mm high) harbored fully-formed cercariae 28 weeks after exposure (Shimazu, unpublished observation). Although easily entering snails, sporocysts rapidly become enveloped by a cellular capsule of host origin within about 1 week after infection, and then most of them degenerate and finally die (Endo and Suzuki, 1971). Most presumably, few or none of sporocysts can survive the host tissue encapsulation to produce mother rediae. This host reactions seems to be restricted to sporocysts since mother and daughter rediae proved to grow and multiply with no apparent cellular capsule. As showed by Hamajima *et al.* (1981), on the other hand, cercariae can be formed at higher rates when snails have previously been infected with other species trematodes than when uninfected. Therefore, it is possible that prior infections with certain other trematode species increase the survival rate of the triploid form sporocysts of following infection by reducing, or making them to evade, the snail tissue response to them by unknown means. In other words, the parasite entering the snail first may interfere with the snail's low-grade resistance to the second parasite (Lie,

1982). This possibility may apply also to the case of the present fluke. Further critical studies are needed of the intertrematode relationships between the present fluke and other species in the snail host.

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