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Infectivity of the Lung Fluke *Paragonimus westermani* in the Snail *Semisulcospira libertina* and Susceptibility of the Snail to the Fluke

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Abstract

S. libertina is known to be a snail host of *P. westermani* (triploid type). However, there are many problems concerning our understanding of intermediate host-parasite relationships in the infection and intramolluscan development of larvae. Therefore, the present study on host-parasite relationships was carried out to determine the infectivity of lung flukes in snails and the susceptibility of the snails to the flukes using experimental infection. Results revealed that penetration of the miracidia was seen only in snails, not in the other species of gastropod molluscs examined. Moreover, the number of miracidial penetrations was different between lung flukes obtained from two different localities, and between laboratory-raised 2nd generation juvenile snails derived from snails collected at three different localities. Furthermore, there was a significant difference in miracidial penetration depending on the size of snail. In the experimental infection using lung flukes, the sporocysts in a single infection were either surrounded or not surrounded by amebocytes in the snail, and were easily destroyed in the tissues about one week after miracidial exposure. In single infection, the snail showed resistance to intramolluscan development of the larvae of the lung fluke. Thus, neither the rediae nor the cercariae were found in laboratory-raised snails which were free of other species, trematodes and reared in aquaria. However, cercariae developed readily in younger female snails which had already been infected with other trematode larvae (prior natural and experimental infection). Thus, lung flukes have a high infectivity in younger female snails, and these snails have a high susceptibility to the flukes about 1 to 2 months after infection with other trematodes such as *Cercaria monostyloides*. Moreover, increasing the exposure to miracidia increased infection rates and the number of snail deaths during the prepatent period. Large and small forms of 2nd generation rediae were observed following prior infection with species of other trematodes. The morphological characteristics and sizes of the cercariae were similar to those of naturally infected snails collected from an endemic area.

Key words: *Paragonimus westermani, Semisulcospira libertina,* experimental infection, infectivity, susceptibility, cercariae

Introduction

Paragonimus westermani is recognized as an important lung fluke in human paragonimiasis in Japan and other countries in the Orient. The snail *Semisulcospira libertina* is known to be the intermediate host of the lung fluke (Nakagawa, 1915a, b). However, the incidence of cercariae of the fluke in the snail has been found to be extremely low, even in areas endemic for paragonimiasis (Ito *et al.*, 1959; Hamajima, 1975). Further, no cercariae have been obtained from snails experimentally infected with miracidia of the lung fluke, in spite of many years of efforts (Nakagawa, 1917; Miyairi, 1919, 1922; Ando, 1921a; Kobayashi, 1921).

Lung fluke sporocysts in the snails were found to be surrounded by amebocytes or encapsulated

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by fibroblasts, and to die about 7 days after exposure to the miracidia (Williams and Miyasaka, 1969; Endo and Suzuki, 1971). However, Komiya *et al.* (1961), Shimazu (1981) and Hamajima *et al.* (1981a, b) have reported cercariae in snail hosts collected from fields and experimentally exposed to the miracidia in laboratory aquaria.

Thus, many problems are involved in understanding larval development in the snails, e.g., host strain, size, sex, cellular reaction, interference and antagonism. In connection with this host-parasite relationship, the present study was carried out to investigate the infectivity of the lung flukes in the snails and the susceptibility of the snails to the flukes using experimental infection.

Materials and Methods

The eggs of *Paragonimus westermani* (triploid type) used in the present experiment were obtained from the uteri of adult worms removed from worm cysts in dog lungs 10 months after inoculation with the metacercariae, which were collected from naturally infected crabs, *Eriocheir japonicus,* captured at Takahama on Amakusa Islands in Kumamoto Prefecture, and Nita on Tsushima Island in Nagasaki Prefecture. The eggs were put in water in Petri dishes (diameter 9.0 cm, depth 2.0 cm) and incubated at 27°C. The water was changed every day, and after 16 days, the miracidia of the lung flukes were hatched by cooling at 4°C for 10 min.

Experiments I — III examined the effects of species of molluscs, and strain and size (age) of *S. libertina* snails on penetration by *P. westermani.* Molluscs used in Experiment I for miracidial penetration of the lung flukes were collected from several different localities: *Assiminea parasitologica* was obtained from Ozuki in Yamaguchi Prefecture; *Oncomelania nosophora* from Ryuo in Yamanashi; *Oncomelania minima* from Tashia on Sado Island in Niigata; *Bythinella nipponica akiyoshiensis* from Mt. Tachibana in Fukuoka; and *Semisulcospira libertina* from Tokigawa in Saitama. In Experiment II, the 1st generation snails of *S. libertina* were collected from Izuhara on Tsushima Island in Nagasaki, Iiyama in Nagano and Tokigawa in Saitama. The laboratory-raised 2nd generation juvenile *S. libertina* snails ranging from 0.5 to 0.8 mm in shell width were used for miracidial penetration. In Experiments I — II, each snail was exposed to 100 miracidia of lung flukes obtained from Amakusa and Tsushima in dechlorinated water filled-Petri dishes with a diameter of 1.0 cm. In Experiment III, the 1st generation snails of *S. libertina* were collected from Izuhara on Tsushima Island in Nagasaki. The laboratory-raised 2nd generation juvenile snails ranging from 1.5 to 7.0 mm in shell width were used for miracidial penetration. Each snail was exposed to 300 miracidia of lung flukes obtained from Amakusa in dechlorinated water-filled Petri dishes of 2.0 cm in diameter. At 24 hr after exposure to the miracidia, these snails were rinsed with water and crushed in water on a slide glass with a pincette, and the number of fluke sporocysts in the molluscs were carefully counted with a microscope.

Experiments IV and V were conducted to examine histopathological changes in the snail tissues and cercaria formation in the snail digestive gland. In Experiment IV, *S. libertina* snails ranging from 4.0 to 6.0 mm in shell width were collected from Tokigawa in August. They were divided into two groups. One group was exposed in mass to 300 miracidia (per snail) of *P. westermani* obtained from Tushima Island by immersing snails in dechlorinated water-filled Petri dishes (diameter 9.0 cm, depth 2.0 cm). The other group was not exposed to miracidia and served as the control. Three hours after exposure, the snails were transferred to aquaria (45 × 25 × 30 cm) and reared at 25°C. Some of the infected and control snails were separated from the shells during the first 2 weeks after exposure under a dissecting microscope, and fixed in Bovin's fixative, embedded in paraffin, sectioned serially, stained with hematoxylin-eosin and studied histologically. In Experiment V, living cercariae from the remaining snails of the experimental groups used in Experiment IV were examined with a dissecting microscope 16 weeks after ex-

posure. Lung flukes and other trematodes were identified according to the characteristics and development of intramolluscan larvae described by Ito (1964).

Experiment VI was undertaken to elucidate the effect of different numbers of miracidia on the infection rates in the snails. In Experiment VI, *S. libertina* snails ranging from 4.0 to 6.0 mm in shell width were collected from Tokigawa in August. They were divided into five groups. Four groups were individually exposed in mass to various numbers (10, 20, 40 and 80 per snail) of *P. westermani* miracidia obtained from Tsushima Island by immersing 100 snails in the same Petri dishes used in Experiments IV and V. The remaining group was not exposed to miracidia and served as the control (100 snails). Three hours after exposure, these snails were transferred to the separate aquaria mentioned above and reared in aerated water at 25°C. These snails were then crushed at 16 weeks after exposure. The living cercariae in the snails were examined with a dissecting microscope.

The influence of rearing the snails in the aquaria and prior infection with other species of trematode larvae in *S. libertina* snails on the cercaria formation of *P. westermani* in the snails was examined in Experiments VII and VIII. In Experiment VII, *S. libertina* snails were collected from Tokigawa in August. They were reared at 25°C in the aquaria mentioned above. They were divided into five groups for experimental infection of the snails with *P. westermani* obtained from Tsushima Island. One group was not exposed to the miracidia and served as the control. The others were exposed in mass to 300 miracidia (per snail) of the lung fluke by immersing the snails in the aquaria after 0, 1, 2 and 3 months of rearing in the laboratory. In Experiment VIII, the laboratory-raised 2nd generation snails ranging from 1.5 to 6.4 mm in shell width from the 1st generation *S. libertina* collected from Tokigawa in May were divided into four groups. Two were previously exposed in the aquaria to 300 eggs (per snail) of monorchid worms (*Cercaria monostyloides*) obtained from the digestive organs of a loach, *Cobitis biwae*, collected from Tokigawa (Hamajima *et al.*, 1982). The other two groups were not exposed to the eggs and served as controls. In these experimental infections, 2 months after exposure to the eggs, each group of the infected and control snails was exposed in the aquaria to 100 miracidia (per snail) of *P. westermani* obtained from Tsushima Island by the same method mentioned in Experiment VII, and then reared at 25°C in separate aerated aquaria. All groups of snails were carefully examined under the dissecting microscope during the period from 12 to 16 weeks after exposure for the presence of intramolluscan larvae.

Water in the aquaria was changed occasionally. Powdered commercially available pellet food for rats and dry leaves of maple (for breeding the snails) and oriental elm (for developing larval worms) were given once a day as food.

Morphological observations of each larval form were made using fresh materials. All the measurements were made by placing the larvae in water on a slide glass with a cover slip.

The data from Experiments I — III and IX — X were expressed as the mean ± SD. Student's t-test and the chi-square test were used to determine the statistical significance of data. Differences between rates of penetration, infection with the lung fluke in the snails and sizes of the redia were considered to be significant at $P<0.05$.

Results

The results of miracidial penetration of five gastropod molluscs by *P. westermani* in Experiment I are shown in Table 1. All snails of *A. parasitologica, O. nosophora, O. minima, B. n. akiyoshiensis* and *S. libertina* collected from the field were proved not to be infected with *Paragonimus* sporocysts before the miracidial penetration. Miracidial penetration was seen only in *S. libertina* snails, and sporocysts were found in all *S. libertina* examined. However, no sporocysts were found in any of the other gastropod molluscs examined.

In Experiment II, no sporocysts of *Paragonimus* were found in any of the control snails before the miracidial penetration. The

Table 1. Miracidial penetration of some gastropod molluscs at 24 hr after exposure to miracidia of *Paragonimus westermani* obtained from Amakusa

Species of snails	No. of snails examined	No. of snails with sporocysts (%)	Total no. of sporocysts recovered	No. of sporocysts per snail Mean±SD
Assiminea parasitologica	10	0 (0)	0	0
Oncomelania nosophora	10	0 (0)	0	0
Oncomelania minima	10	0 (0)	0	0
Bythinella n. akiyoshiensis	10	0 (0)	0	0
*Semisulcospira libertina**	10	10 (100)	35	3.5±7.0

* The shell width ranged from 4 to 5 mm.

laboratory-raised 2nd generation juvenile snails bred from *S. libertina* collected from Izuhara, Iiyama and Tokigawa were experimentally penetrated by the miracidia of lung flukes obtained from Tsushima Island (Table 2). Sporocysts were found in all snails 24 hr after exposure to 100 miracidia per snail. However, there was a difference in the number of sporocysts recovered according to the localities where the 1st generation snails were collected ($P<0.01$ or $P<0.001$). The highest mean number of larvae per snail was in the 2nd generation juveniles of snails collected from Iiyama ($P<0.001$), followed by those from Tokigawa ($P<0.01$) and then those from Izuhara.

On the other hand, in *P. westermani* obtained from Amakusa, the highest mean number of sporocysts per snail was in the 2nd generation juveniles of snails collected from Tokigawa ($P<0.001$). In addition, the mean number from Izuhara was higher than that from Iiyama ($P<0.01$).

The mean number of sporocysts for snails with different shell widths (in mm) in Experiment III is given in Table 3. No sporocysts of *Paragonimus* were found in any of the control snails before miracidial penetration. Sporocysts were found in all of the snails. The highest mean number of sporocysts per snail was in snails with a shell width of 2.0 mm ($P<0.001$). The mean number decreased with increased shell width except for the shell width of 1.5 mm.

Table 2. Miracidial penetration of the 2nd generation juvenile snails of *S. libertina* collected from three different localities at 24 hr after exposure to miracidia of *P. westermani* obtained from two different districts

Localities of the fluke collection	Localities of the 1st generation snails collected	No. of snails examined	No. of snails penetrated (%)	No. of sporocysts	No. of sporocysts per snail Mean±SD	P*	
Tsushima	Izuhara	12	12(100)	120	10.0± 2.8	<0.01	<0.001
	Tokigawa	12	12(100)	199	16.6± 6.5		
	Iiyama	12	12(100)	308	25.7±10.2	<0.001	
Amakusa	Iiyama	12	11(92)	126	10.5± 6.7	<0.01	<0.001
	Izuhara	12	12(100)	205	16.7± 4.3		
	Tokigawa	12	12(100)	299	25.8±11.8	<0.001	

* P-values refer to the results of pairwise t-tests comparing the mean number of sporocysts in 2nd generation snails from three different localities with that of the other snails.

Table 3. Influence of snail host size on miracidial penetration of the 2nd generation snails of *S. libertina* at 24 hr after exposure to miracidia of *P. westermani* obtained from Amakusa

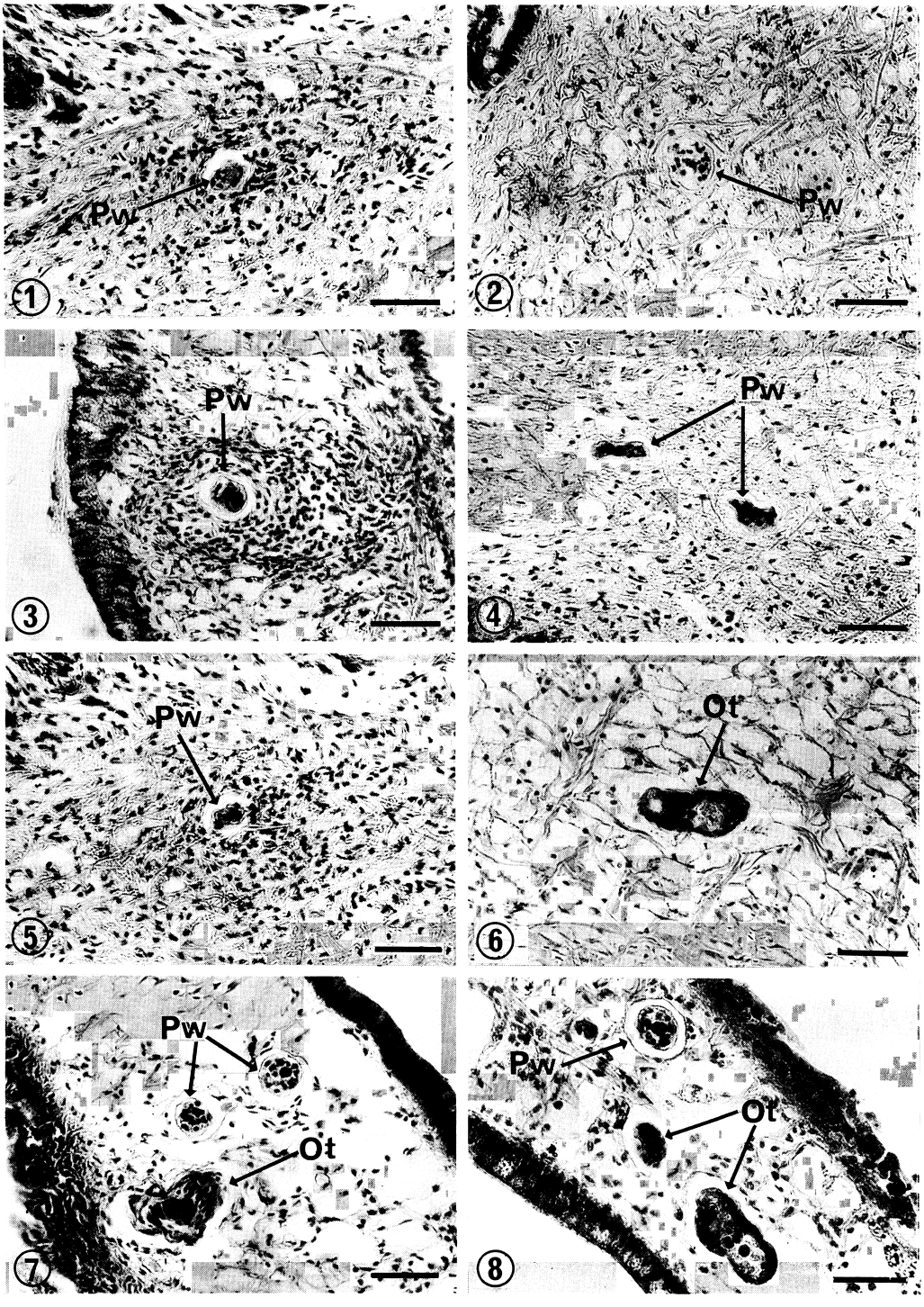
Shell width in mm (Day age)	No. of snails examined	No. of snails with sporocysts (%)	No. of sporocysts	No. of sporocysts per snail Mean±SD	P*
1.5 (15)	10	10 (100)	93	9.3± 7.4	NS
2.0 (30)	10	10 (100)	547	54.7±25.8	<0.001
2.5 (60)	10	10 (100)	260	26.0±13.6	<0.001
3.0 (90)	10	10 (100)	178	17.8±12.0	<0.01
4.0 (120)	10	10 (100)	127	12.7± 9.5	<0.05
5.0 (150)	10	10 (100)	100	10.0± 5.9	<0.05
6.0 (180)	10	10 (100)	67	6.7± 2.6	NS
7.0 (210)	10	10 (100)	49	4.9± 2.2	—

* P-values refer to the results of pairwise t-tests comparing the mean number of sporocysts in snails 7.0 mm in shell width with that in other snails ranging from 1.5 to 6.0 mm in shell width. NS = not significant.

Table 4. Tissue reactions to *P. westermani* sporocysts in mantle, ctenidium, tentacle, foot and proboscis of *S. libertina* snails at various days after exposure to the miracidia

Days after miracidia exposure	No. of snails examined	No. of snails positive for						No. of snails negative for trematodes
		P. westermani only	Amebocyte aggregation	*P. westermani* and other trematodes	Amebocyte aggregation	Other trematodes only	Amebocyte aggregation	
1	6	4	4	2	0	0	0	0
Controls	6	0	0	0	0	2	0	4
2	6	5	4	1	0	0	0	0
Controls	6	0	0	0	0	2	9	4
3	3	0	0	3	0	0	0	0
Controls	3	0	0	0	0	1	0	2
4	3	3	2	0	0	0	0	0
No controls	—	—	—	—	—	—	—	—
5	3	2	2	1	0	0	0	0
Controls	3	0	0	0	0	2	0	1
6	3	2	2	1	0	0	0	0
No controls	—	—	—	—	—	—	—	—
7	6	2	2	2	0	0	0	2
Controls	6	0	0	0	0	3	0	3
14	6	0	0	2	0	0	0	4
Controls	6	0	0	0	0	1	0	5
Total Subtotal	36	18	16(89)	12	0(0)	0	0	6
Total Controls	30	0	0	0	0	11	0(0)	19

Number in parentheses is the percentage of the snails positive for amebocyte aggregation.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In Experiment IV, the sections of *S. libertina* snails fixed on various early days during the first 2 weeks after exposure to miracidia of *P. westermani* revealed single infection with the lung fluke or other trematodes and mixed infection with the lung fluke and other trematodes (Table 4). In single infection with *P. westermani,* the sporocysts were surrounded by host amebocytes in 16 of 18 snails. The amebocytes had infiltrated around the parasite (Figs. 1, 3 and 5). The sporocysts were surrounded by numerous amebocytes (Fig. 3). No host cellular response was observed around the sporocysts in the other two snails. The amebocytes did not produce aggregates around the parasite (Figs. 2 and 4). However, most of the sporocysts were destroyed within the first week of infection. In contrast, such amebocyte aggregation in snail tissue was not seen in 11 cases (controls) with natural single infection with other trematodes (Fig. 6) or 12 cases of mixed infection with *P. westermani* and other trematodes. The lung flukes appeared to be developing without any reaction of the snail tissues in snails with mixed infection (Figs. 7 and 8).

In Experiment V, the cercariae of the lung fluke were detected in some snails of the same experimental groups used in Experiment IV at 16 weeks after exposure (Table 5). There was single infection with other trematodes (38%) in the control snails and double infection with the lung fluke and other trematodes (35%) in the experimental snails. Single infection with lung fluke cercariae was not found in the snails (0%).

The results of Experiment VI are shown in Table 6. In Experiment VI, living cercariae of the lung fluke were detected as mixed infection in snails of all groups exposed to the miracidia except for the control snails. However, single infection with *P. westermani* was not detected. Moreover, by increasing the exposure dosage from 10 to 80 miracidia, infection rates in the snails with lung fluke and other trematodes also increased slightly. Furthermore, the percentage

Table 5. Cercaria formation in *S. libertina* snails exposed to miracidia of *P. westermani*

Weeks after miracidia exposure	No. of snails examined	No. of snails positive for				
		P. westermani only (%)	*P. westermani* and other trematodes (%)	P*	Other trematodes only (%)	P*
16	97	0 (0)	34 (35)	<0.001	7 (7)	—
Controls	95	0 (0)	0 (0)	—	36 (38)	<0.001

* P-values refer to the results of pairwise χ^2 tests comparing the percentage of snails as controls and snails infected with *P. westermani* with that of snails infected with *P. westermani* cercariae and other trematodes, and other trematodes only.

Explanation of Figures

Scale = 50 μm for Figs. 1—8; Scale = 100 μm for Figs. 9—14. Pw: *P. westermani*; Ot: Other trematode

Fig. 1. A sporocyst (arrow) of *Paragonimus westermani* surrounded by some amebocytes in the foot of a *Semisulcospira libertina* 24 hr after exposure to miracidia.

Fig. 2. A hypertrophic sporocyst (arrow) degenerating in the foot of a host snail 2 days after exposure to miracidia.

Fig. 3. An injured sporocyst (arrow) completely surrounded by a dense clump of amebocytes in the mantle of a host snail 4 days after exposure to miracidia.

Fig. 4. Shrunk sporocysts (arrow) degenerating in the foot of a host snail 4 days after exposure to miracidia.

Fig. 5. Necrotic sporocyst (arrow) surrounded by thin amebocytes in the foot of a host snail 7 days after exposure to miracidia.

Fig. 6. Other trematode redia (arrow) in the foot of a host snail.

Fig. 7. Developing sporocysts (arrow) in the foot of a host snail harboring another trematode (arrow) 5 days after exposure to miracidia.

Fig. 8. A developing sporocyst (arrow) in the mantle of a host snail harboring other trematodes 5 days after exposure to miracidia.

Table 6. Experimental infection of *S. libertina* snails with varing number of miracidia of *P. westermani*

No. of miracidia exposed per snail	No. of snail used	No. of snail deaths during prepatent period (%)	P*	No. of snail examined	No. of snails positive for				
					P. westermani only (%)	*P. westermani* and other trematodes (%)	P*	Other trematodes only (%)	P*
Controls	100	13(13)	—	87	0(0)	0(0)	—	26(30)	—
10	100	16(16)	NS	84	0(0)	22(26)	<0.001	5(6)	<0.001
20	100	18(18)	NS	82	0(0)	24(29)	<0.001	5(6)	<0.001
40	100	22(22)	NS	78	0(0)	26(33)	<0.001	4(5)	<0.001
80	100	32(32)	<0.01	68	0(0)	27(40)	<0.001	3(4)	<0.001

* P-values refer to the results of pairwise χ^2 tests comparing the percentages of snails as controls and other snails with those of snail deaths during the prepatent period, snails infected with *P. westermani* cercariae and other trematodes, and snails infected with other trematodes only.

of snail deaths during the prepatent period was higher in snails exposed to 80 miracidia than in those exposed to 10 miracidia ($P<0.01$).

The results of Experiment VII are shown in Table 7. In Experiment VII, *S. libertina* snails collected from Tokigawa were infected with *P. westermani* and *C. monostyloides*, or *Metagonimus yokogawai* in experimental infection with *P. westermani*. When the snails were exposed to miracidia of the lung flukes at 0 and 1 month of laboratory rearing of snails collected from Tokigawa, 17 and 8 of the 33 and 29 snails infected with other trematode larvae were infected with cercariae of other trematodes alone, respectively. The remaining 16 and 21 cases were doubly infected with cercariae of the lung flukes. However, at 2 months after rearing, single infection with the cercariae of *C. monostyloides* was not detected in the snails, whereas those of *P. westermani* were detected in all 31 snails harboring *C. monostyloides* larvae. On the other hand, 3 months after rearing, the larvae of other trematodes were detected in 32 snails, 17 of which were harboring developmentally retarded sporocysts or 1st generation rediae of the lung flukes. However, 4 snails harboring other trematode larvae were infected with mature cercariae of *P. westermani*. In the snails ranging from 7.4 mm and below, the infection rates (33%, 20/61 and 45%, 29/64) of cercariae of the lung flukes in the snails at 1 and 2 months of rearing were higher than those (5%, 3/60 and 0%, 0/62) at 3 months of rearing and in the controls ($P<0.001$), respectively. On the other hand, after 1 and 2 months' rearing, the infection rates (55%, 26/47 and 30%, 23/78) of cercariae of the lung flukes in the snails ranging from 6.4 mm and below, and from 6.5 to 7.4 mm in shell width were higher than those (6%, 3/47 and 0%, 0/23) in snails ranging from 7.5 to 8.4 mm and 8.5 mm and above in shell width ($P<0.001$), respectively. After 1 and 2 months of rearing, the infection rate (55%, 42/76) of cercariae of the lung fluke in female snails ranging from 7.4 mm or below was higher than that (9%, 4/46) of males ($P<0.001$).

In Experiment VIII, when the snails were exposed to miracidia of the lung fluke after exposure to the eggs of *C. monostyloides*, 4 of 103 infected snails were infected with cercariae of *C. monostyloides* alone (Table 8). The other 99 snails were doubly infected with cercariae of the lung fluke and *C. monostyloides*. However, cercariae of the lung fluke were not detected in any of 260 control snails exposed only to lung fluke miracidia. On the other hand, the infection rate (49%, 83/168) of cercariae of lung flukes in snails ranging from 3.5 to 6.4 mm in shell width (about

Table 7. Influence of intervals after laboratory-reared of the snails on experimental infection of *S. libertina* snails exposed to miracidia of *P. westermani*

Snail examined: Shell width in mm	Sex of host	Cercariae found	No. of snails infected (%): Experiment: Month intervals after rearing of sanils 0	P†	1	P†	2	P†	3	Controls
6.4 and below	♀	*P. w.**+*C. m.**	4 (24)		9 (39)		10 (41)		0 (0)	0 (0)
		C. m.	5 (28)		2 (9)		0 (0)		11*(50)	12 (54)
		*C. nip.**	0 (0)		0 (0)		0 (0)		0 (0)	1 (5)
		Negative*	2 (12)		1 (4)		3 (13)		3 (14)	0 (0)
	♂	*P. w.*+*C. m.*	0 (0)		0 (0)		4 (17)		0 (0)	0 (0)
		C. m.	3 (18)		2 (9)		0 (0)		2 (9)	2 (9)
		Negative	3 (18)		8 (35)		5 (21)		6 (27)	7 (32)
	unknown	*P. w.*+*C. m.*	0 (0)		1 (4)		2 (8)		0 (0)	0 (0)
	Total		17		23		24		22	22
		P†		NS		<0.001		<0.001		NS —
6.5—7.4	♀	*P. w.*+*C. m.*	6 (29)		10 (26)		13 (33)		2 (5)	0 (0)
		C. m.	6 (29)		3 (8)		0 (0)		12‡(32)	12 (30)
		P. w.+*M. y.**	0 (0)		0 (0)		0 (0)		1 (3)	0 (0)
		M. y.	0 (0)		0 (0)		0 (0)		0 (0)	2 (5)
		Negative	3 (14)		12 (32)		13 (33)		8 (21)	14 (35)
	♂	*P. w.*+*C. m.*	1 (5)		0 (0)		0 (0)		0 (0)	0 (0)
		Negative	5 (23)		13 (34)		14 (34)		15 (39)	12 (30)
	Total		21		38		40		38	40
		P†		NS		<0.001		<0.001		NS —
7.5—8.4	♀	*P. w.*+*C. m.*	5 (19)		1 (4)		2 (10)		0 (0)	0 (0)
		C. m.	1 (4)		1 (4)		0 (0)		3§(12)	2 (8)
		M. y.	1 (4)		0 (0)		0 (0)		0 (0)	0 (0)
		Negative	11 (40)		16 (61)		10 (47)		14 (53)	13 (52)
	♂	Negative	9 (33)		8 (31)		9 (43)		9 (35)	10 (40)
	Total		27		26		21		26	25
		P†		NS		NS		NS		NS —
8.5 and above	♀	*P. w.*+*C. m.*	0 (0)		0 (0)		0 (0)		1 (9)	0 (0)
		C. m.	1 (8)		0 (0)		0 (0)		0 (0)	0 (0)
		Negative	10 (77)		8 (80)		12 (92)		9 (82)	7 (87)
	♂	Negative	2 (15)		2 (20)		1 (8)		1 (9)	1 (13)
	Total		13		10		13		11	8
				—		—		—		

* *P.w.* = *Paragonimus westermani, C.m.* = *Cercaria monostyloides, C.nip.* = *Cercaria nipponensis, M.y.* = *Metagonimus yokogawai,* Negative = Trematode-free, + = 8/11, ‡ = 8/12, § = 1/3; Numerator is the no. of snails harboring the retarded development of sporocysts or the 1st generation rediae of *P. westermani.*

† P-values refer to the results of pairwise χ^2 tests comparing infection with cercariae of *P. westermani* in control snails or snails ranging from 8.5 mm and above in shell width with those of snails of comparable shell width at other intervals after rearing or snails of comparable intervals after rearing but with other shell widths. NS is the same as in Table 3.

2 to 4 mm at time of exposure) was higher than that (16%, 4/25) in snails ranging from 7.5 to 8.4 mm in shell width ($P<0.05$—0.001). Cercariae of the lung fluke or *C. monostyloides* were seen only in female snails ($P<0.001$).

The sizes of the body, pharynx and intestine and the number of germ balls and mature cercariae in 2nd generation rediae obtained experi-

Table 8. Influence of experimental prior infection with the eggs of a monorchid worms (*C. monostyloides*) in the snails on cercarial formation of the 2nd generation snails of *S. libertina* exposed with *P. westermani* miracidia

Snail examined		Cercariae found	No. of snails examined				P†
			No prior infection with *C. monostyloides*		Prior infection with *C. monostyloides*		
Shell width in mm	Sex of snail		Non-exposed to *P. westermani* (%)	Exposed to *P. westermani* (%)	Non-exposed to *P. westermani* (%)	Exposed to *P. westermani* (%)	
3.5—4.4	unknown	*P. w.**+*C. m.**	0 (0)	0 (0)	0 (0)	11 (52)	<0.02
		C. m.	0 (0)	0 (0)	12 (48)	0 (0)	
		Negative*	30 (100)	25 (100)	13 (52)	10 (48)	
	Total		30	25	25	21	
4.5—5.4	♀	*P. w.*+*C. m.*	0 (0)	0 (0)	0 (0)	42 (63)	<0.001
		C. m.	0 (0)	0 (0)	45 (61)	0 (0)	
		Negative	65 (90)	63 (91)	21 (28)	19 (28)	
	♂	Negative	7 (10)	6 (9)	8 (11)	6 (9)	
	Total		72	69	74	67	
5.5—6.4	♀	*P. w.*+*C. m.*	0 (0)	0 (0)	0 (0)	30 (38)	<0.05
		C. m.	0 (0)	0 (0)	32 (41)	4 (5)	
		Negative	62 (75)	64 (77)	24 (31)	25 (31)	
	♂	Negative	21 (25)	19 (23)	22 (28)	21 (26)	
	Total		83	83	78	80	
6.5—7.4	♀	*P. w.*+*C. m.*	0 (0)	0 (0)	0 (0)	12 (25)	NS
		C. m.	0 (0)	0 (0)	11 (26)	0 (0)	
		Negative	28 (51)	30 (54)	12 (28)	14 (30)	
	♂	Negative	27 (49)	26 (46)	20 (46)	21 (45)	
	Total		55	56	43	47	
7.5—8.4	♀	*P. w.*+*C. m.*	0 (0)	0 (0)	0 (0)	4 (16)	—
		C. m.	0 (0)	0 (0)	3 (11)	0 (0)	
		Negative	23 (79)	22 (81)	19 (70)	17 (68)	
	♂	Negative	6 (21)	5 (19)	5 (19)	4 (16)	
	Total		29	27	27	25	

* *P.w.*, *C.m.* and Negative are the same as in Table 7.

† P-values refer to the results of pairwise χ^2 tests comparing infection with cercariae of *P. westermani* in the snails ranging from 7.5 to 8.4 mm in shell width with those of snails of other shell widths. NS is the same as in Table 3.

mentally are shown in Table 9. In the experimental infection of *S. libertina* with *P. westermani,* rediae were divided into small and large forms (Figs. 9, 10, 11 and 12). The former was found in snails of the mixed infection with *P. westermani* and *C. monostyloides.* The latter was found in snails of the mixed infection with *P. westermani* and *M. yokogawai.* Differences in body size and number of germ balls and cercariae in the rediae between the two forms were statistically significant ($P<0.05$—0.001).

Cercariae of the microcercous type, which has a very short tail, were recovered from the experimentally infected snails (Figs. 13 and 14). Sizes of the body, oral sucker, acetabulum, stylet and tail of the cercariae are shown in Table 10. Each of these measurements was similar to those of *P. westermani* from naturally infected snails except for those of body length and body width. The body size of the cercariae was slightly bigger than that of cercariae obtained from naturally infected snails.

Table 9. Mean sizes of the 2nd generation rediae of *P. westermani* in experimentally infected snail (Living specimen, in micron)

Form	No. of rediae measured	Body Length×Width Mean±SD		Pharynx Length×Width Mean±SD		Intestine Length×Width Mean±SD		No. of germ balls and cercariae per redia Mean±SD
Small	17	544.1±120.6×	263.5±39.4	68.5±9.0×	67.7±10.0	362.9±104.8×	73.2±34.3	1.5±0.6
Large	15	974.7±167.9×	390.7±65.9	78.7±9.6×	74.0± 4.9	679.3±133.8×	123.3±29.4	16.5±5.0
P*		<0.001	<0.001	<0.01	<0.05	<0.001	<0.001	<0.001

* P-values refer to the results of pairwise t-tests comparing the mean size and number of germ balls and cercariae in small rediae with those of large rediae. Small and large forms were obtained from snails harboring *C. monostyloides* and *M. yokogawai* larvae, respectively.

Table 10. Mean size of cercariae obtained from *S. libertina* experimentally infected with *P. westermani* (Living specimen, in micron)

No. of cercariae measured	Body Length×Width Mean±SD		Oral sucker Length×Width Mean±SD		Acetabulum Length×Width Mean±SD		Stylet Length Mean±SD	Tail Length Mean±SD
15	319.2±40.4×	137.1±15.7	69.9±7.2×	69.1±5.6	34.6±4.7×	43.5±4.3	37.5±7.6	22.5±2.9

Discussion

The snail *S. libertina* is known to be a molluscan host of *P. westermani* (Nakagawa, 1915a, b). There are two main processes in infection with the lung fluke in gastropod molluscs: penetration of the miracidia into the snails and development of the larvae in the snails. However, there are still many problems involved in understanding the intermediate host-parasite relationships in the penetration of the miracidia into the snails and the intramolluscan development of larvae of the fluke. Therefore, in this experiment the infectivity of the fluke in various snails and the susceptibility of these snails against the fluke were investigated.

The miracidial penetration of the lung flukes into *A. parasitologica* and *O. nosophora*, amphibious molluscs, and into *O. minima, B. n. akiyoshiensis* and *S. libertina,* aquatic molluscs, was observed, but data from the experimental infections indicated that no sporocysts were recovered in the molluscs of species other than *S. libertina.* This result is in agreement with results that experimental infection of the lung fluke to molluscs was negative for the larvae (Yoshida, 1960; Kawashima and Miyazaki, 1964; Hashiguchi and Miyazaki, 1968). Recently, Yamakami and Hamajima (1987) reported that proteases which hydrolyze collagen have been found in the miracidia of the lung fluke. The above-mentioned fact indicates that the difference in susceptibility of the molluscs to the lung fluke may be due to a difference in the biochemical composition and inhibitors in tissues of these snails to the "penetration" protease of the miracidia.

In Experiment II on miracidial penetration, the mean number of lung fluke sporocysts per *S. libertina* snail was different between flukes obtained from two different localities and among laboratory-raised 2nd generation juveniles of snails collected from three different localities. Terasaki (1980) clarified the morphological differences in spermatozoa between flukes obtained from Tsushima and those from other regions. In addition, Davis (1969) found vari-

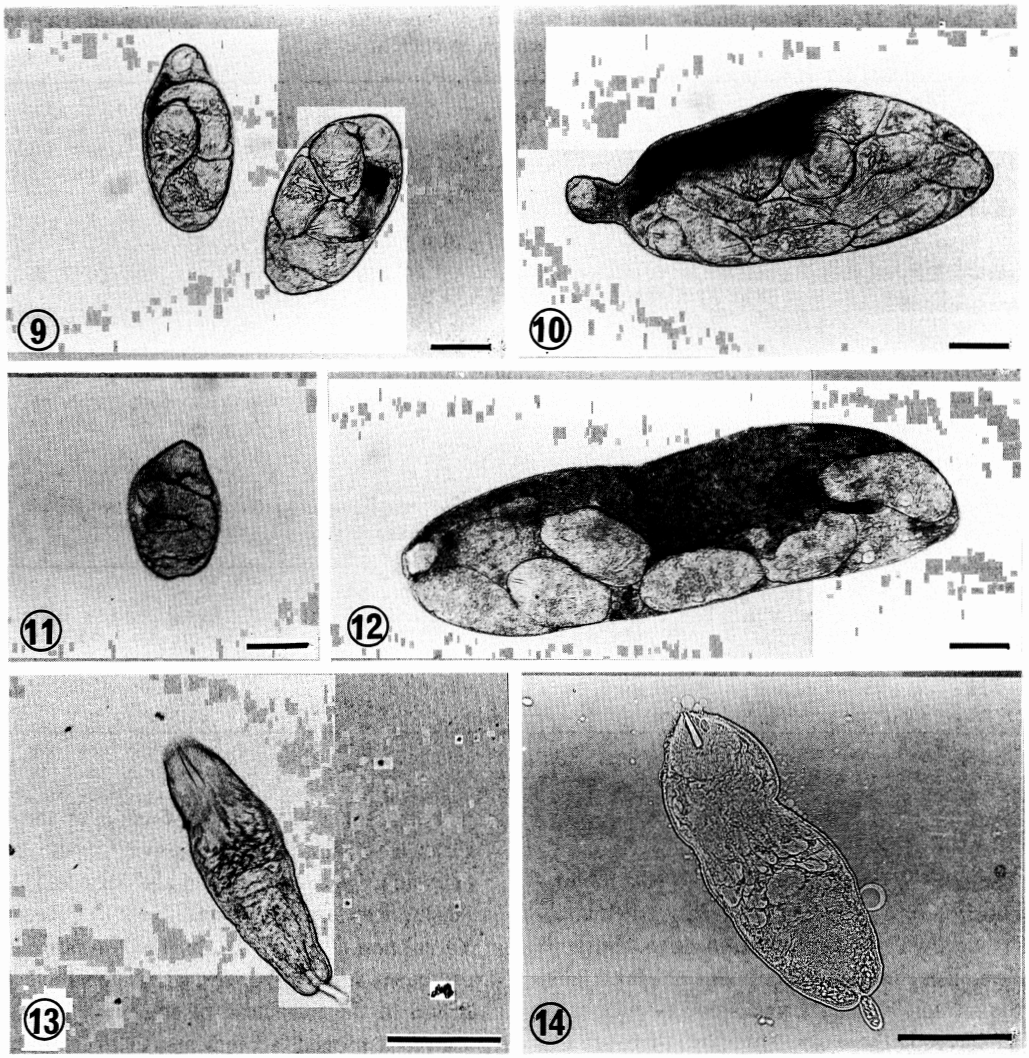

Fig. 9. Small *P. westermani* second generation rediae removed from an experimentally infected host snail harboring *Cercaria monostyloides* larvae.
Fig. 10. Large *P. westermani* second generation redia removed from an experimentally infected host snail harboring *Metagonimus yokogawai* larvae.
Fig. 11. Smaller *P. westermani* second generation redia removed from an experimentally infected host snail harboring *C. monostyloides* larvae.
Fig. 12. Larger *P. westermani* second generation redia removed from an experimentally infected host snail harboring *M. yokogawai* larvae.
Fig. 13. Active cercaria removed from *P. westermani* second generation redia.
Fig. 14. Extended cercaria removed from *P. westermani* second generation redia.

ability (inter-population) within species in comparing *S. libertina* from Shimoda and Nahari. From these findings, it seems that the differences in the infectivity of the lung flukes in the snails and the susceptibility of the snails to the flukes may be influenced not only by the amount of genome and heterogeneity in allele frequencies among the flukes but also by variability in snail

strains.

In Experiment III, the penetration rate of lung fluke miracidia was 100% in *S. libertina* snails ranging from 1.5 to 7.0 mm in shell width. The mean number of sporocysts per snails of 2 mm in shell width was greater than that of snails of other shell widths. Miyairi (1934) and Komiya *et al.* (1961) reported that young snails are infected with lung flukes. Consequently, it seems that differences in miracidial penetration rate according to snail size may be due to differences in the physiological and biochemical characteristics of the snails.

Histopathological observations of host tissue response to sporocysts of the lung fluke revealed that the sporocysts were either surrounded or not surrounded by host amebocytes in single infection and that most of the sporocysts were destroyed about 1 week after infection. Thus, the snails showed resistance to single infection by intramolluscan larvae of the lung fluke, and it seems that the larvae are destroyed by necrosis or hypertrophiclysis in the host tissue, the result of some unknown factors. Williams and Miyasaka (1969) and Endo and Suzuki (1971) reported that the young sporocyst sometimes became necrotic during the first week after exposure to the miracidia, though fluke miracidia readily invaded *S. libertina* snails. From the above-mentioned results, it was concluded that the fluke could not easily develop into 2nd generation rediae and cercariae in the snails.

In contrast, the early development of sporocysts and rediae of the lung fluke has been found in snails collected from various localities and exposed to miracidia (Miyairi, 1919; 1922; Kobayashi, 1921; Ando, 1921a, b). Moreover, Komiya *et al.* (1961) observed cercariae in experimentally infected snails collected from an area endemic for paragonimiasis. Furthermore, fully developed cercariae of the lung fluke have been obtained from experimentally exposed snails collected from various localities (Shimazu, 1981; Hamajima *et al.*, 1981a, b). However, in the present work and in previous reports (Hamajima *et al.*, 1981a, b), the host cellular response against the lung fluke was not seen when other trematodes were present in the snails. The cercariae were found in mixed infection with the lung fluke and the larvae of other trematode species in the snails collected from Tokigawa in experimental infection with the lung flukes. It seems that the snails became susceptible to the lung fluke following infection with other larval trematode species. Identical results have been reported by Lie and Heyneman (1976, 1977a, b) for interference by *Echinostome* larvae with natural resistance to *Schistosoma mansoni* in *Biomphalaria glabrata.*

Moreover, similar multiparasitism with *P. westermani* and other species of trematodes in snails collected from areas endemic for paragonimiasis has been reported (Yoshida, 1917; Yokogawa, 1952; Hamajima *et al.*, 1975). Multiparasitism with the lung fluke and another parasite was demonstrated by experimental infection of snails with *Paragonimus* flukes (Hamajima *et al.*, 1981a, b; Shimazu and Oshima, 1983). Thus, these results suggest that the lung fluke has high infectivity in the snails and the snails have high susceptibility to the fluke when they have been previously infected with other trematode larvae like *C. monostyloides, Pseudexorchis major, M. yokogawai* and *Cercaria yoshidae* (Hamajima *et al.*, 1981a, b). Furthermore, it seems that changes in the host cellular response and chemical composition of the snails caused by infection with other trematode larvae may be a possible factor in determining the development of *P. westermani* cercariae in the snails. Shimazu and Oshima (1983) reported that *P. westermani* (3n) rarely developed to the cercarial stage; this was true even in laboratory-raised snails 28 weeks after exposure to the miracidia.

Increasing the exposure dosage of miracidia increased the rates of infection and death during the prepatent period. Similar results were achieved in infection with *Schistosomatium douthitti* by increasing the miracidial exposure dosage (Kagan *et al.*, 1954; Loker, 1978).

In the experimental infection with *P. westermani* lung flukes at 0 and 1 month after laboratory rearing of the snails collected from Tokigawa, 17 and 8 of the snails were infected

with other trematode larvae alone at 12 to 16 weeks after exposure to miracidia of the lung flukes, respectively. These results show that the natural resistance against lung flukes of snails harboring other trematode larvae is stronger yet. However, in the infection by lung flukes at 1 and 2 months after rearing, 21 and all the snails harboring *C. monostyloides* larvae developed to the cercariae of the lung flukes, respectively. This result was demonstrated in this experiment when the snails became highly susceptible to the lung fluke following prior experimental infection with *C. monostyloides*. Therefore, the results indicate that *C. monostyloides* protects lung flukes in the snail by interfering with the resistance of the host. On the other hand, in the experimental infection with lung flukes in this study, at 3 months after rearing, most of the snails infected with other trematode larvae were infected with a few developmentally retarded sporocysts or the 1st generation rediae of the lung flukes. The results show that the development of the larvae of the lung flukes was arrested by other trematode larvae. This suggests that the antagonism of other trematode larvae against the lung flukes is more potent when the interval after rearing is more than 3 months. There is some indication that other trematode larvae may produce a substance, such as a protease, that can interfere with the snail's resistance and retards the development of lung fluke larvae. Similar interactions between *S. mansoni* and other trematode species in *Biomphalaria glabrata* have been reviewed by Lie *et al.* (1981) and Lie (1982), who reported that the parasite entering the snail first may interfere with the snail's low-grade resistance to the second parasite.

In this experiment, young snails showed a high rate of infection by the lung flukes. Similar results have been obtained by Miyairi (1934) and Komiya *et al.* (1961) for experimental infection of snails by lung flukes, and the snails showed a decreasing susceptibility to trematode larvae with age. Female snails were highly susceptible to the lung flukes in this experiment. The same result was reported by Hamajima *et al.* (1963, 1975) for infection of snails by lung flukes in a field survey.

The specimens in this experiment were ellipsoids, but there were two types of rediae, one small, the other large. The former was found in snails harboring *C. monostyloides* larvae and the latter in those previously infected with *M. yokogawai*. The size of the large rediae was similar to that reported for rediae from natural infections (Wu, 1935; Yamaguti, 1943; Komiya and Ito, 1950). Thus, it is possible that prior infection with other trematode larvae may interfere with the snail's resistance to the second parasite and with the development of larvae. The morphological characteristics and size of the cercariae were similar to those of the known *P. westermani* of the microcercous type (Wu, 1935; Yamaguti, 1943; Komiya and Ito, 1950).

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