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Comparative studies on *Paragonimus sadoensis* and *P. ohirai*. IV. Comparison of adult worms obtained from experimental infections

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Introduction

Yoshimura *et al.* (1970b) experimentally exposed *Oncomelania minima* (abbreviated *O. m*) and *Assiminea parasitologica* (abbreviated *A. p*) snails to *Paragonimus sadoensis* (abbreviated *P. s*) and *P. ohirai* (abbreviated *P. o*) miracidia, obtaining the cercariae of the respective lung flukes from these snails. Further, Yoshimura *et al.* (1970c) experimentally exposed *Potamon dehaani* (abbreviated *P. d*) and *Sesarma dehaani* (abbreviated *S. d*) crabs to the aforementioned cercariae of the two lung flukes, reporting that *P. s* and *P. o* cercariae could develop to the metacercarial stage in either of *P. d* and *S. d* crabs.

The purpose of this study is to determine whether the metacercariae developed through the experimental first and second intermediate hosts have infectivity against the definitive host (white laboratory rat=*Rattus norvegicus*); rats were experimentally exposed to the metacercariae of the two lung flukes and then, morphological and electrophoretic comparisons were conducted to determine whether significant differences were found between the adult worms originating from the experimental intermediate hosts and those originating from their own intermediate hosts.

Materials and Methods

The derivation of *P. s* and *P. o* metacercariae used was previously described in detail in Part III of this study (Yoshimura *et al.*, 1970c). Adult rats were exposed *per os* to *P. s* and *P. o* metacercariae which were developed through various combinations of intermediate hosts as shown in Table 1. The number of metacercariae fed per rat varied from 5 to 33.

Hereafter, *P. s* worms originating from *O. m* and *P. d* are abbreviated as *P. s-O-P.* Similarly, abbreviations *P. s-O-S*, *P. s-A-P* and *P. s-A-S* are given to *P. s* worms originating from the respective combinations of *O. m* and *S. d*, *A. p* and *P. d*, and of *A. p* and *S. d*. Likewise, abbreviations *P. o-A-S*, *P. o-A-P* and *P. o-O-S* refer to *P. o* worms originating from the combinations of *A. p* and *S. d*, *A. p* and *P. d*, and of *O. m* and *S. d* respectively.

Stool examinations of exposed rats were made every five days as a rule during the period of 25–55 days after exposure by means of AMS III technique. The prepatent period used in this study refers to the period of time from the lst day after exposure to the lst day eggs were found in the stools.

Exposed rats were necropsied to determine worm recovery rates 50–60 days after exposure. Worms collected were incubated in physiological saline solution for 5–6 hours at 28°C. Sixty eggs shed from each group of adult worms were measured. The remaining eggs were incubated and artificially hatched by the procedures previously described by Yoshimura *et al.* (1970b).

A variable number of adult worms (Table

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Table 1 Experimental infection of albino rats with *Paragonimus sadoensis* and *P. ohirai* metacercariae

Species	Snail	Crab	Rat No.	Days after exposure	Metacercariae fed/rat	Worms recovered (%)	Location			Prepatent period (date of eggs found in stool)
							Peritoneal cavity	Pleural cavity	Lungs	
P. s	*O. m*	*P. d*	23	50	6	6(100)	0	2	4	40
			12	59	9	2(22.2)	0	0	2	36
			13	59	9	7(77.8)	0	0	7	39
		S. d	8	56	10	4(40)	0	0	4	41
			9	60	12	2(16.7)	0	0	2	40
	A. p	*P. d*	11	32*	16	0	0	0	0	—
			24	55	6	3(50)	0	3	0	†
			10	60	12	2(16.7)	0	0	2	55
		S. d	7	56	19	5(26.3)	0	0	5	35
P. o	*A. p*	*S. d*	27	42*	30	12(40)	1	11	0	39
			25	55	22	10(45.5)	0	1	9	39
			26	55	33	6(18.1)	0	0	6	45
			28	55	30	25(83.3)	0	4	21	39
			29	55	30	22(73.3)	0	1	21	35
			16	58	12	1(8.3)	0	0	1	30
			17	58	22	13(59.1)	0	6	7	35
			22	59	22	14(63.6)	0	0	14	35
		P. d	20	58	8	3(37.5)	0	3	0	50
			21	58	5	3(60)	0	0	3	45
	O. m	*P. d*	18	0*	5	0	—	—	—	—
		S. d	14	59	6	3(50)	0	1	2	40
			15	59	7	3(42.9)	0	0	3	50

P. s=Paragonimus sadoensis. P. o=P. ohirai. O. m=Oncomelania minima. A. p=Assiminea parasitologica. P. d=Potamon dehaani. S. d=Sesarma dehaani. * Died. † Eggs were not found until 55 days after infection.

2) was flattened under slide glass pressure by inserting 2 pieces of bristol board between the edges of two slides, fixed with Schaudinn's fixative and stained with Delafield's hematoxylin. The whole mount specimens were used for measurements and morphological observations.

Worm materials used for electrophoretic experiments are shown in Table 4. Whole worm saline extracts were prepared according to the procedures described by Yoshimura (1969b). However, 20 mg of lyophilized worms were extracted in 0.8 ml of 0.9% saline solution since only a small amount of worm material was available for this study (protein content of the present extract was approximately one half of the extracts made by Yoshimura (1969 a, b)).

Electrophoresis was performed according to the methods described by Ornstein & Davis (1962) and Davis (1964) using an electrophoretic apparatus modified by Davis & Lindsay (1967). Samples employed in electrophoresis were made by diluting the aforementioned extracts with 5% (concentrated) spacer gel at a ratio of 1 : 3. Electrophoretic data were analyzed by the methods described by Yoshimura (1968) using good gel columns with a front migration between 34.0 and 35.5 mm. The bands separated were numbered with reference to the results reported by Yoshimura (1969 a, b).

Results

1. Experimental infection of rats with *P. sadoensis* and *P. ohirai* metacercariae

Results of the experimental infection of rats with the metacercariae of the two lung flukes are summarized in Table 1. The data indicate that both *P. s* and *P. o* metacercariae which were developed through the various combinations of intermediate hosts have infectivity against the rat. Mean *P. s* worm recovery rates were 63% (15/24) in *P. s-O-P*, 27% (6/22) in *P. s-O-S*, 15% (5/34) in *P. s-A-P*, and 26% (5/19) in *P. s-A-S* respectively. *P. o* worm recovery rates were 51% (103/201) in *P. o-A-S*, and 46% (6/13) in both *P. o-A-P* and *P. o-O-S*. It was uncertain whether *P. o* metacercariae which were developed through the intermediate hosts (*O. m* & *P. d*) of *P. s*, have infectivity against the rat, since a single rat exposed to the *P. o-O-P* metacercariae died immediately after exposure (Table 1).

As shown in Table 1, worm recovery rates of the two lung flukes were generally higher in rats exposed to the metacercariae originating from their own intermediate hosts than in rats exposed to the metacercariae originating from various combinations of intermediate hosts, although the worm recovery rates were of course variable.

The shortest prepatent period was 35 and

Table 2 Measurements of *Paragonimus sadoensis* and *P. ohirai* adult worms (in mm)

Species		*P. sadoensis*				*P. ohirai*		
Snail		*O. minima*		*A. parasitologica*		*A. parasitologica*		*O. minima*
Crab		*P. dehaani*	*S. dehaani*	*P. dehaani*	*S. dehaani*	*S. dehaani*	*P. dehaani*	*S. dehaani*
No. of worms measured		3	2	1	2	9	2	3
Body	L	8.4 -9.7 (8.9)	10.1*	9.5	6.5*	6.8 -10.2 (8.2)	7.9 -8.6 (8.3)	8.5 -9.2 (8.9)
	W	4.0 -4.5 (4.3)	5.5*	4.5	2.9*	3.5 -4.5 (4.1)	4.0 -4.2 (4.1)	3.9 -4.6 (4.4)
Ratio of L/W		1.98-2.16 (2.08)	1.84	2.11	2.24	1.58-2.33 (2.00)	1.98-2.05 (2.02)	1.96-2.18 (2.05)
Oral sucker	L	0.50-0.58 (0.530)	0.40-0.43 (0.415)	0.52	0.26*	0.40-0.54 (0.468)	0.43-0.47 (0.450)	0.48-0.62 (0.553)
	W	0.58-0.67 (0.620)	0.59-0.71 (0.650)	0.68	0.45*	0.56-0.68 (0.627)	0.58-0.61 (0.595)	0.63-0.71 (0.667)
Acetabulum	L	0.74-0.78 (0.757)	0.67-0.73 (0.700)	0.68	0.67-0.73 (0.700)	0.67-0.85 (0.730)	0.75-0.78 (0.765)	0.73-0.81 (0.767)
	W	0.78-0.86 (0.813)	0.74-0.82 (0.780)	0.77	0.63-0.76 (0.695)	0.73-0.83 (0.768)	0.89 (0.890)	0.80-0.83 (0.813)
Ovary	L	0.89-1.19 (1.073)	0.78-1.40 (1.090)	1.10	0.78 (0.780)	0.70-1.21 (0.984)	1.09-1.34 (1.215)	0.89-1.01 (0.970)
	W	0.70-1.02 (0.903)	0.89-1.24 (1.065)	1.06	0.66-0.68 (0.670)	0.66-1.32 (0.949)	0.87-0.92 (0.895)	0.86-1.14 (0.987)
Right testis	L	1.90-1.93 (1.920)	1.21-1.61 (1.410)	1.74	1.50**	1.16-1.95 (1.476)	1.16-1.48 (1.320)	1.06-1.67 (1.280)
	W	0.81-0.95 (0.863)	0.83-0.96 (0.895)	0.91	0.68**	0.56-1.19 (0.859)	0.80-1.04 (0.920)	0.76-1.10 (0.940)
Left testis	L	1.19-1.61 (1.347)	1.51-1.71 (1.610)	1.45	1.50**	1.01-1.95 (1.386)	1.35-1.44 (1.395)	1.16-1.24 (1.213)
	W	0.99-1.24 (1.143)	0.81-0.89 (0.850)	0.76	0.71**	0.54-1.32 (0.897)	0.96 (0.960)	0.77-1.19 (0.973)

Figures in parentheses show averages. * Only one specimen could be measured. ** The testes of one specimen were not stained due to degeneration. L=length. W=width.

30 days for *P. s* and *P. o* respectively. In both species of lung flukes, no appreciable differences in prepatent period were found between rats exposed to the metacercariae originating from their own intermediate hosts and those exposed to the metacercariae originating from various combinations of intermediate hosts.

2. Morphological observations of *P. sadoensis* and *P. ohirai* adult worms

Cuticular spines of *P. s* adult worms are arranged in groups. The ovary is delicately branched just coralloid with secondary and tertiary branchings. The testes are slightly lobed. The oral sucker is smaller than the acetabulum. Body measurements of the adult worms originating from various combinations of intermediate hosts were very similar to those of the worms originating from their own intermediate hosts (Table 2). In 2 whole mount specimens of *P. s-A-S*, however, the worms were slightly small and degenerated. Cuticular layers of the two worms were almost lost, thus, some groups of the cuticular spines were rarely seen. The number of intrauterine eggs was also few in these worms. The testes of one of the 2 worms were not stained with hematoxylin due to degeneration.

The ovaries of 2 (25%) of 8 adult worms presented in Table 2 were located on the right side and the ovaries of the remaining 6 worms (75 %) were located on the left side.

The egg is oval and usually symmetrical with an operculum. The egg shell is about 1.5 μ thick and uniform throughout. The maximum width of the egg is usually located slightly posterior to the midline. Measurements of the eggs are presented in Table 3.

The aforementioned characteristics were common to all of the *P. s* worms (*P. s-O-P*, *P. s-A-P*, *P. s-O-S* and *P. s-A-S*) obtained in this study.

The morphology of *P. o* adult worms, including eggs, was identical with that of *P. s* worms. As noted in various combinations of *P. s*, all of the *P. o* worms (*P. o-A-S*, *P. o-A-P* and *P. o-O-S*) obtained in this study had the same morphological features. The ovaries of 9 (64%) of 14 *P. o* adult worms shown in Table 2 were located on the right side, and those of the remaining 5 worms (36%) were located on the left side. Measurements of adult worms as well as eggs are presented in Tables 2 and 3 respectively.

Eggs shed from various groups of *P. s* and *P. o* adult worms were incubated for 15–17 days at 28°C and artificially hatched. All of these eggs were found to release miracidia.

3. Electrophoresis

Table 3 Measurements of *Paragonimus sadoensis* and *P. ohirai* eggs (in microns)

Species	Snail	Crab	No. of eggs measured	Length	Width
P. sadoensis	*O. minima*	*P. dehaani*	60	68.9–88.4 (79.70±5.39)*	45.5–54.1 (49.87±1.67)
		S. dehaani	60	70.2–91.0 (80.74±4.44)	41.6–52.0 (48.50±1.73)
	A. parasitologica	*P. dehaani*	60	71.0–85.8 (80.69±3.49)	47.3–53.3 (50.08±1.26)
		S. dehaani	60	72.3–98.8 (83.43±5.03)	46.8–57.2 (50.24±2.26)
P. ohirai	*A. parasitologica*	*S. dehaani*	60	75.1–96.2 (85.58±5.10)	46.5–55.1 (50.11±2.20)
		P. dehaani	60	70.5–96.2 (86.43±6.03)	44.2–65.8 (51.24±4.03)
	O. minima	*S. dehaani*	60	72.8–96.2 (86.54±5.63)	47.1–58.5 (52.62±2.38)

* Mean±Standard deviation.

Table 4 Source of whole worm extract for electrophoretic experiments

Worm source			Total No. of lyophilized worms used	No. of experiments	Total No. of gels	No. of gels analyzed
Species	Snail	Crab				
P. sadoensis	*O. minima*	*P. dehaani*	12	9	36	15
		S. dehaani	3	3	11	7
	A. parasitologica	*P. dehaani*	4	2	8	7
		S. dehaani	3	2	7	7
P. ohirai	*A. parasitologica*	*S. dehaani*	23	12	46	10
		P. dehaani	4	4	16	7
	O. minima	*S. dehaani*	3	4	16	7

Table 5 Mean Rf values of protein bands identified from adult *Paragonimus sadoensis* originating from *Potamon dehaani* and *Sesarma dehaani* crabs, both of which had been experimentally exposed to the cercariae originating from *Oncomelania minima* snails

Protein bands	From *P. dehaani*			From *S. dehaani*		
	Mean Rf values†	SD	Frequency of band (%)	Mean Rf values†	SD	Frequency of band (%)
1	0.016		100	0.016		100
1a	0.036		27	+		14
* 2(A)	0.043	0.010	100	0.045	0.011	100
4	0.077		100	0.077		100
5	+***		+	0.087		57
6	0.119		100	0.128		100
7	0.203		100	0.194		86
* 9(C)	0.232	0.030	100	0.225	0.016	100
9a	0.262		33	0.251		14
10	0.289		100	0.269		100
* 12(E)	0.362	0.032	100	0.356	0.029	100
13	0.422	0.035**	80	0.426		100
14	0.472		93	0.472		71
* 16(G)	0.512	0.022	100	0.509	0.021	100
* 17(H)	0.589	0.034	100	0.572	0.032**	100
18	0.607		80	0.590		71
19	0.634		87	0.635		100
20	0.674		100	0.671		100
21	0.703		13	0.680		14
* 22(J)	0.779	0.024	100	0.778	0.020	100
* 25(K)	0.870	0.024	100	0.867	0.021	100
26	0.935		93	0.950		100
27	1.000		100	1.000		100

† There is no significant difference in mean Rf value of each band separated from worms originating from *P. dehaani* and *S. dehaani* crabs. * Prominent band. ** Greatest deviation. *** See text. Letters in parentheses coorrespond to prominent peaks in the densitometric tracings.

Table 6 Mean Rf values of protein bands identified from adult *Paragonimus sadoensis* originating from *Potamon dehaani* and *Sesarma dehaani* crabs, both of which had been experimentally exposed to the cercariae originating from *Assiminea parasitologica* snails

Protein bands	From *P. dehaani*			From *S. dehaani*			Significant difference
	Mean Rf values	SD	Frequency of band (%)	Mean Rf values	SD	Frequency of band (%)	
1	0.015		100	0.014		100	
1a	0.030		57	0.028		14	
* 2(A)	0.044	0.004	100	0.037	0.005	100	
4	0.077		100	0.064		100	
5	—		—	0.077		71	
6	0.122		100	0.113		100	
7	0.201		86	0.188		100	
* 9(C)	0.232	0.010	100	0.214	0.015	100	Yes, $p<0.05$
9a	0.274		57	0.252		57	
10	0.287		100	0.271		100	
* 12(E)	0.353	0.025	100	0.346	0.025	100	
13	0.432		100	0.404		100	
14	0.472		100	0.447		71	Yes, $p<0.02$
* 16(G)	0.515	0.014	100	0.494	0.018	100	Yes, $p<0.05$
* 17(H)	0.591	0.019	100	0.587	0.026	100	
18	0.624		71	0.621		29	
19	0.651	0.027**	100	0.642	0.040**	100	
20	0.692	0.027**	100	0.676		100	
21	—		—	—		—	
* 22(J)	0.788	0.022	100	0.771	0.018	100	
* 25(K)	0.876	0.019	100	0.858	0.024	100	
26	0.950		100	0.936		100	
27	1.000		100	1.000		100	

* Prominent band. ** Greatest deviation. Letters in parentheses correspond to prominent peaks in the densitometric tracings.

The reproducible bands identified from *P. s* (*P. s-O-P*, *P. s-O-S*, *P. s-A-P* & *P. s-A-S*) are presented in tabular form for Rf values (Tables 5 & 6) while the corresponding bands separated from *P. o* (*P. o-A-S*, *P. o-A-P* & *P. o-O-S*) are seen in Table 7. Infrequently resolved bands are also listed in Table 8 for both *P. s* and *P. o* worms. Densitometric tracings from electrophoretic patterns of the above described worms are presented in Figs. 1 & 2 respectively. Electrophoretic patterns of both *P. s* and *P. o* worms always yielded 7 major prominent bands (peaks A, C, E, G, H, J & K; Figs. 1 & 2).

A comparative study of *P. s* and *P. o* was made using the data of *P. s-O-P* and *P. o-A-S*, both of which had been developed through their own natural first and second intermediate hosts. As seen in Table 4, adequate worm material was available for a reliable comparison between *P. s-O-P* and *P. o-A-S* (12 and 23 worms respectively). The electrophoretic pattern of *P. s-O-P* was found to be essentially identical with that of *P. o-A-S* (I of Figs. 1 & 2). Comparisons of both major and minor bands separated from *P. s-O-P* and *P. o-A-S* indicated that a statistically significant difference in Rf value was found

Table 7 Mean Rf values of protein bands identified from adult *Paragonimus ohirai* originating from *Sesarma dehaani* and *Potamon dehaani* crabs, which had been experimentally exposed to the cercariae originating from *Assiminea parasitologica* or *Oncomelania minima* snails

Snail	*A. parasitologica*							*O. minima*		
Crab	*S. dehaani*			*P. dehaani*				*S. dehaani*		
Protein bands	Mean Rf values	SD	Frequency of band (%)	Mean Rf values	SD	Frequency of band (%)	Significant difference	Mean Rf values	SD	Frequency of band (%)
1	0.016		100	0.014		100		0.013		100
1a	0.032		60	0.033		86		0.026		100
* 2(A)	0.041	0.009	100	0.042	0.006	100		0.040	0.005	100
4	0.075		100	0.073		100		0.071		100
5	0.079		80	—		—		0.086		100
6	0.114		100	0.120		100		0.114		100
7	0.188		100	0.188	0.019†	100		0.191		71
* 9(C)	0.223	0.019	100	0.224	0.009	100		0.221	0.010	100
9a	0.255		60	0.251		100		0.247		29
10	0.280		100	0.277		100		0.281		100
* 12(E)	0.338	0.026	100	0.320	0.017	100		0.348	0.024†	100
13	0.409		90	0.403		100		0.426		100
14	0.469		100	0.440		100	Yes, $p<0.02$	0.469		100
* 16(G)	0.504	0.022	100	0.505	0.012	100		0.506	0.013	100
* 17(H)	0.555	0.026	100	0.555	0.011	100		0.551	0.011	100
18	0.587		100	0.585		90		0.578		57
19	0.612		80	0.612		71		0.609		100
20	0.647	0.036†	80	0.667		14		0.643		29
21	0.682		100	0.685		100		0.685		100
* 22(J)	0.775	0.022	100	0.771	0.013	100		0.769	0.011	100
* 25(K)	0.863	0.024	100	0.854	0.014	100		0.842	0.014	100
26	0.930		90	0.934		100		0.931		100
27	1.000		100	1.000		100		1.000		100

* Prominent band. † Greatest deviation. Letters in parentheses correspond to prominent peaks in the densitometric tracings.

in only one major band 17 (peak H) ($p<0.02$; Tables 5 & 7, Figs. 3 & 4); this was due to a shift of Band 17 to the anodic end in *P. s-O-P.* A sporadic band 16a resolved from *P. s* but not from *P. o* (Table 8).

The influences of differences in intermediate hosts (snail and crab) on the electrophoretic pattern of whole body protein were examined, although the worm materials available for this study were not enough for a detailed analysis (Table 4). Compounding the problem of comparison was the death of a single rat infected with metacercariae of the *P. o-O-P* so that no comparison could be made with this combination. Comparisons were made between *P. s-O-P* and *P. s-A-P* as well as *P. s-O-P* and *P. s-O-S.* No significant difference was found in electrophoretic pattern, except for a difference in density of peak J between *P. s-O-P* and *P. s-A-P* (Fig. 1; and see below). No significant difference in Rf value was found in all 7 major bands between the aforementioned two combinations (Fig. 3). In minor but reproducible

Table 8 Comparisons of the occurrence of four seldom resolved bands separated from *Paragonimus sadoensis* and *P. ohirai* worms originating from the various combinations of the first and second intermediate hosts

Species	*P. sadoensis*				*P. ohirai*		
Snail	*O. minima*		*A. parasitologica*		*A. parasitologica*		*O. minima*
Crab	*P. dehaani*	*S. dehaani*	*P. dehaani*	*S. dehaani*	*S. dehaani*	*P. dehaani*	*S. dehaani*
Protein bands	Mean Rf	Mean Rf	Mean Rf	Mean Rf	Mean Rf	Mean Rf	Mean Rf
11	+*	+*	+*	−	+(20)**	−	0.315(29)
12a	0.386(20)	0.393(29)	−	0.377(14)	+*	+*	−
16a	0.568(47)	0.568(29)	0.550(29)	0.529(43)	−	−	−
21a	−	−	−	−	−	−	0.710(14)

* See text. ** Figures in parentheses show the occurrence frequency (%) of band.

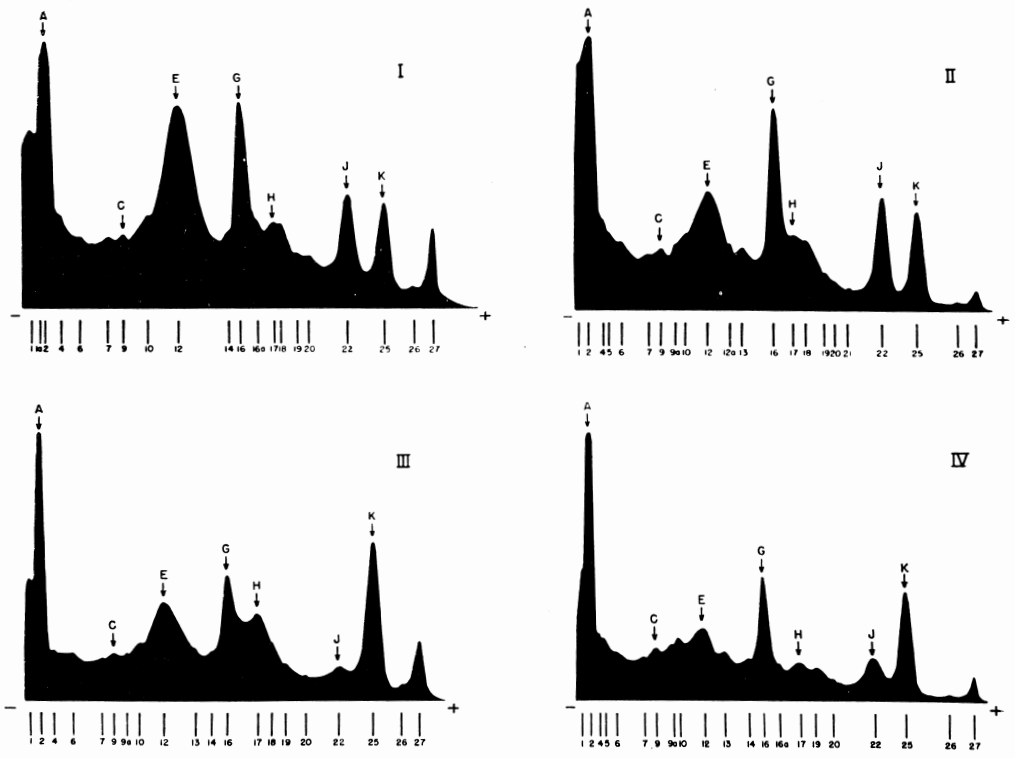

Fig. 1 Densitometric tracings of disc electrophoretic patterns from *Paragonimus sadoensis* adult worms. The bands are numbered below the densitometric tracings and the most characteristic bands for the species are lettered. I: *P. s-O-P*; II: *P. s-O-S*; III: *P. s-A-P*; IV: *P. s-A-S* (see text for abbreviations)

bands (Fig. 4), however, Bands 5 and 21 were infrequently resolved from *P. s-O-P* and not resolved from *P. s-A-P*. Similar comparisons were also made between *P. o-A-S* and *P. o-O-S* as well as between *P. o-A-S* and *P. o-A-P*. It was noted that the density of peak K (Band 25) was always low in all gels examined for *P. o-A-P* and *P. o-O-S*, conversely,

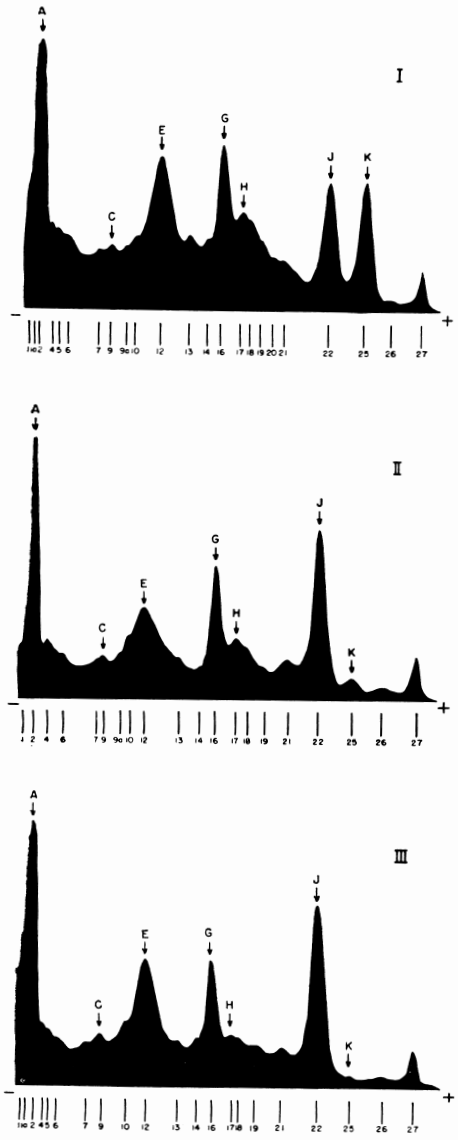

Fig. 2 Densitometric tracings of disc electrophoretic patterns from *Paragonimus ohirai* adult worms. The bands are numbered below the densitometric tracings and the most characteristic bands for the species are lettered.
I: *P. o-A-S;* II: *P. o-A-P;* III: *P. o-O-S* (see text for abbreviations)

	P. s-O-P	*P. s-O-S*	*P. s-A-P*	*P. s-A-S*	*P. o-A-S*	*P. o-A-P*
P. s-O-S *	–					
P. s-A-P	–	–				
P. s-A-S	–	–	9 & 16**			
P. o-A-S	17	–	17	17		
P. o-A-P	12 & 17	12	12, 17 & 25	12 & 17	–	
P. o-O-S	17 & 25	25	17 & 25	17	25	12

Fig. 3 Divergence in Rf value of seven major prominent bands separated from *Paragonimus sadoensis* and *P. ohirai* worms originating from various combinations of the first and second intermediate hosts.
* See text for abbreviations. ** Protein band number in which a significant difference was found.

	P. s-O-P	*P. s-O-S*	*P. s-A-P*	*P. s-A-S*	*P. o-A-S*	*P. o-A-P*
P. s-O-S *	–					
P. s-A-P	5 & 21**	5 & 21				
P. s-A-S	21	6 & 21	5 & 14			
P. o-A-S	–	26	5, 9a, 13, 18, 19, 20, 21 & 26	21		
P. o-A-P	5 & 14	5 & 14	9a, 13, 14, 18, 19 & 21	5, 18 & 21	5 & 14	
P. o-O-S	–	19	5, 9a, 18, 19, 20, 21 & 26	14, 18 & 21	–	5, 13 & 14

Fig. 4 Divergence in the occurrence and Rf value of fifteen minor but reproducible bands separated from *Paragonimus sadoensis* and *P. ohirai* worms originating from various combinations of the first and second intermediate hosts.
* See text for abbreviations. ** Protein band number in which a significant difference was found.

the peak K of *P. o-A-S* was consistently high in density (Fig. 2). Statistically significant difference in Rf values of major bands was found only in Band 25 (peak K) between *P. o-A-S* and *P. o-O-S* while differences in the occurrence of minor bands and their Rf values were found in Bands 5 and 14 between *P. o-A-S* and *P. o-A-P* (Figs. 3 & 4).

Comparisons of the occurrence of both major and minor bands as well as of their Rf values were made for all combinations of *P. s* and *P. o* worms (Figs. 3 & 4) although the worm material available was not adequate for a detailed analysis (Table 4). The greatest number of significant differences in Rf values for major bands (Fig. 3) involved a comparison of *P. s-A-P* and *P. o-A-P*. The greatest number of differences in reproducible minor bands was 8 (Fig. 4) involving a comparison of *P. s-A-P* and *P. o-A-S*. This was

followed by 7 differences (*P. s-A-P* and *P. o-O-S*) ; 6 differences (*P. s-A-P* and *P. o-A-P*). It was noted that the principal differences in Rf values of the 7 major, prominent bands were found in Bands 12 (peak E), 17 (H) and 25 (K), especially in Band 17, between the various groups of *P. s* and *P. o* worms and that only a few divergence was found within the various groups of *P. s* and *P. o* (Fig. 3). A minor band, No. 5, frequently did not resolve from both *P. s* and *P. o* worms. Divergence in Rf values of minor bands was usually found in Bands 13 and 14. Band 21 was characteristic for *P. o* worms. Large differences between *P. s-A-P* and *P. o* worms were found in Rf values of Bands 9a, 18, 19 and 20 (Fig. 4).

Characteristics of electrophoretic profiles and separated bands are described below for each individual case.

P. s-O-P: A total of 26 bands was separated, 19 of which were highly reproducible (Tables 5 & 8). It was noted that standard deviations of the Rf values were generally large. Peak J (Band 22) was frequently low in density. A faint band 5 sometimes appeared to resolve between Bands 4 and 6, especially at a position close to Band 4.

P. s-O-S: A total of 26 bands was identified, 19 of which were highly reproducible (Tables 5 & 8).

P. s-A-P: A total of 23 bands was separated, 19 of which were highly reproducible (Tables 6 & 8). It was of interest to note that prominent Band 22 (peak J) was very low in density, conversely, the density of Band 25 (peak K) was relatively high.

P. s-A-S: A total of 24 bands was separated, of which 19 were highly reproducible (Tables 6 & 8). As recognized in the electrophoretic pattern of *P. s-A-P*, the density of Band 22 (peak J) was always lower than that of Band 25 (peak K).

In addition to the aforementioned bands, a faint band resolved between Bands 6 and 7 (Rf : 0.169 & 0.165) in 47% (7/15) of *P. s-O-P* gels and 14 % (1/7) of *P. s-O-S* gels analyzed respectively. A faint band resolved between Bands 10 and 12 (Rf : 0.344, 0.336 & 0.340) in *P. s-O-P*, *P. s-O-S* and *P. s-A-P* in the respective frequencies of 13 % (2/15), 29 % (2/7) and 29% (2/7). In *P. s-O-S* and *P. s-A-S*, a very faint band resolved between Bands 25 and 26 (Rf : 0.909 & 0.898) in 29% (2/7) and 43% (3/7) of gels analyzed.

P. o-A-S: A total of 25 bands was separated, 21 of which were highly reproducible (Tables 7 & 8). A faint band resolved between Bands 12 and 13 (Rf : 0.363) in 20 % (2/10) of the gels analyzed ; this band appeared to correspond to Band 12a separated from *P. s.* The density of Band 22 (peak J) was consistently high in *P. o-A-S* in contrast with the Band 22 separated from *P. s.*

P. o-A-P: A total of 23 bands was recovered, 21 of which were highly reproducible (Tables 7 & 8). It was of interest to note that peak J (Band 22) was high in density whereas peak K (Band 25) was relatively low. A very faint, fuzzy band resolved between Bands 12 and 13 (Rf : 0.374) in 14% (1/7) of the gels analyzed.

P. o-O-S: A total of 25 bands was separated, of which 20 were highly reproducible (Tables 7 & 8). As described in *P. o-A-P*, it was noted that Band 25 (peak K) was very low in density. Density of Band 25 separated from *P. o-O-S* appeared to be lower than that of the same band separated from *P. o-A-P.*

Discussion

In either species of *P. s* and *P. o*, the metacercariae which were developed through various combinations of intermediate hosts (*P. s-O-S*, *P. s-A-P*, *P. s-A-S*, *P. o-A-P* & *P. o-O-S*) were found to have almost the same infectivity against the definitive host (rat) as the metacercariae which were developed through their own intermediate hosts (*P. s-O-P* & *P.o-A-S*). Prepatent periods of *P. s* and *P. o* were common to the rats exposed to metacercariae originating from their own intermediate hosts as well as to the rats exposed to metacercariae originating from experimental intermediate hosts. The shortest prepatent period (35 days) of *P. s* is almost identical with the results previously reported by Miyazaki *et al.* (1968) and Yoshimura (1969a). The

corresponding prepatent period (30 days) of *P. o* worms in this study resembles the data of Miyazaki (1939a) who recovered eggs from *P. o* infected cats approximately 40 days after exposure. *P. s* and *P. o* eggs shed from the worms originating from the experimental intermediate hosts released miracidia. All of the above data suggest that the normal development of *P. s* and *P. o* larvae can be made in various combinations of the first and second intermediate hosts of both lung flukes. In either species of the two lung flukes, however, worm recovery rate was generally higher in the rats exposed to metacercariae originating from their own intermediate hosts than in the rats exposed to metacercariae originating from experimental intermediate hosts. This fact implies that differences in intermediate hosts may have some influence on the infection and development of worms in definitive hosts.

In both lung flukes, no significant differences in morphology of adult worms and their measurements were observed between the worms originating from experimental intermediate hosts and the worms originating from their own intermediate hosts. This indicates that *P. s* can undergo normal larval development even through the first and second intermediate hosts (*A. p & S. d*) of *P. o*. However, it is uncertain whether *P. o* metacercariae which were developed through the first and second intermediate hosts (*O. m & P. d*) of *P. s*, can develop to adult stage in a definitive host, since the single exposed rat died.

The morphology of *P. s* adult worms is identical with the descriptions made by Otsuru *et al.* (1957), Miyazaki *et al.* (1968) and Yoshimura (1969a). Miyazaki *et al.* reported that the body of *P. s* adult worms was stouter than that of *P. o*, i. e., the ratio of body length to width was smaller in *P. s* than in *P. o*, and that this characteristic was considered to be useful for differentiation between *P. s* and *P. o*. They reported that the ratio of 60 day old worms was 1.51–2.10 (mean : 1.70±0.17) in *P. s* and 1.69–2.25 (1.96 ±0.20) in *P. o* and that the difference in the above ratios was significant between the two lung flukes. Mean ratios of the adult worms obtained in this study were 2.08 and 2.00 for *P. s* and *P. o* respectively. No appreciable differences in ratios of body length to width were found between the two lung flukes although only three adult *P. s* worms could be measured. Except for the ratio of body length to width, our measurements of *P. s* worms were similar to the data previously reported by Miyazaki *et al.* (1968). Similarly, our measurements of *P. o* resemble the results described by Miyazaki (1939b) and Tomimura (1959).

The average length of *P. o* eggs was longer than that of *P. s* eggs, however, it would be difficult to distinguish the eggs of *P. o* from those of *P. s* because of the large variation. Our measurements of *P. s* eggs were slightly larger, especially in width, than those described by Miyazaki *et al.* (1968) but were close to the data reported by Otsuru *et al.* (1957) and Yoshimura (1969a). Mean size of *P. o* eggs obtained in this study was slightly larger than that reported by Miyazaki (1939a, b).

Electrophoretic and densitometric patterns of *P. s* and *P. o* adult worms are the same and correspond to those previously reported by Yoshimura (1969a, b) ; all 7 prominent bands which characterized both *P. s* and *P. o*, were always recognized. As previously described by Yoshimura (1969a), the electrophoretic pattern of *P. s-O-P* was found to be essentially identical with that of *P. o-A-S*. The shift of Band 17 to the anodic end and a sporadic band 16a appeared to occur in *P. s-O-P*. The shift of the Band 17 in *P. s* may be correlated with the appearance of a very faint, sporadic band (16a) (Table 8), which was not described by Yoshimura (1969a). This significant difference in location of a prominent band, No. 17, between *P. s-O-P* and *P. o-A-S* appears to be the only difference that may be of genetic origin. Relative to the differences in densitometric pattern when other species of *Paragonimus* were compared (Yoshimura, 1969a, b ; Yoshimura *et al.*, 1969, 1970a), this difference in band position is

minor and perhaps indicative of an intraspecific variation. Future research is needed to clarify this problem. As previously reported by Yoshimura (1969a), the occurrence of Band 21 appears to be characteristic for *P. o* worms.

Yoshimura (1969a) reported that "the Rf value of each band separated from *P. s* seemed to be slightly smaller (but not, for the most part, significantly different) when compared with *P. o.*" Based on the present electrophoresis of 12 lyophilized worms, the Rf values of the bands separated from *P. s* were slightly larger than those of *P. o* but again not significantly different. This can be surmised due to the large variation in band position (great deviation in Rf value) as was found in most of the bands separated from *P. s-O-P* (Table 5); the positions of individual bands being generally shifted towards the anode.

The data of *P. o-A-S* are analogous to those of *P. o* reported by Yoshimura (1969b) in both aspects of electrophoretic pattern as well as mean Rf values of individual bands. However, the mean Rf values of the bands separated from *P. s-O-P*, *P. s-O-S* and *P. s-A-P* are significantly larger than those of *P. s* reported by Yoshimura (1969a); the corresponding values of 35% of the bands separated from the present *P. s-A-S* are significantly different from those of *P. s* reported by Yoshimura (1969a).

The following two hypotheses may account for the above dissimilarities in the mean Rf values of the bands separated from *P. s* worms: (1) The individual protein bands of *P. s* worms obtained from experimental infections (even bands from those worms developed through their own intermediate hosts) shift to the anodic end in comparison with bands of the worms obtained from natural infection, i. e., the occurrence of the large Rf values is due to the "artificial treatment" of the experimental infection, and (2) differences in Rf values are ascribed to the complex of inevitable technical errors. The former hypothesis, however, does not have good support since the mean Rf values of bands from *P. o-A-S* in this study resembled those of *P. o* obtained from natural infecion (Yoshimura, 1969b). Thus, the aforementioned differences in Rf values of *P. s* worms are probably due to the complex of various ungovernable technical errors. Considerable factors can account for the technical errors: (1) Gel conditions may not be identical between individual runs, (2) variation in band position frequently occurs even in some gels of the same run, (3) variation occurs in the total length of protein migration (distance from origin to front band), e. g., the protein migrations of the gels examined varied from 35.0 to 35.5 mm in the previous *P. s* (Yoshimura, 1969a) and ranged from 34.0 to 35.5 mm in the present *P. s* worms, and (4) differences in protein constitution may exist between the lyophilized worm materials. However, no definite conclusion pertaining to the difference in Rf values can be drawn since the worm materials available for this study were not enough for a detailed analysis. Since electrophoretic patterns are consistently identical, however, future research is needed to clarify the factor(s) responsible for less reproducibility of band Rf values of *P. s* worms.

In analyses of electrophoretic data involving relatively few experiments, therefore, special attention should be given to the taxon specific electrophoretic pattern (or its densitometric tracing), since variations of band position or the occurrence of sporadic bands are possibly influenced by the above described technical errors. For example, Band 5 was detected in 80% of the gels examined for *P. o-A-S* while the same band was detected in only 30% of the gels of the previous *P. o* (Yoshimura, 1969b). Thus, the total number of reproducible bands is 20 in the previous data (Yoshimura, 1969b) and 21 in the present *P. o-A-S*. As seen in this case, a faint band is resolved from some gels and not from others. Similarly, faint bands resolved in one experiment are lacking or appear as only blurs of diffuse protein in others. Therefore, differences in Band 5 and most of the seldom resolved, sporadic bands listed in Table 8 can not be used as characteristics of the taxon

specific electrophoretic pattern. Similar type of results and discussion can be seen in the report of Davis & Takada (1969).

In both species of *P. s* and *P. o*, electrophoretic patterns of the adult worms originating from experimental intermediate hosts corresponded essentially to the patterns of the adult worms originating from their own intermediate hosts (Figs. 1 & 2). In *P. s*, the density of peak J (Band 22) was especially low in both *P. s-A-P* and *P. s-A-S*. However, the density of peak J was also low in some gels of *P. s-O-P* worms. Similar variation in density of peak J of *P. s* can be seen in Figures previously presented by Yoshimura (1969 a). In *P. o* worms, the peak K of *P. o-A-S* was consistently high in density in spite of low density being found in the same band separated from *P. o-A-P* and *P. o-O-S*. These facts imply that variation in density of peak K of *P. o* worms may be caused by differences in intermediate hosts. However, it is of course uncertain whether difference in density of peak K is a definite characteristic, since only 3-4 worms of *P. o-A-P* and *P. o-O-S* were available for electrophoretic experiments. It can be surmised that such variations may occur due to the component being chemically unstable (i. e., denaturization or destruction of the component during the periods of worm incubation, worm lyophilization, and/or preservation of the lyophilized vials). Nevertheless, it is of interest to note that variation in density of peak J (Band 22) was found in only *P. s* whereas the same type of variation in density of peak K (Band 25) was found in only *P. o* worms.

In both *P. s* and *P. o* worms, no consistent alteration due to differences in snail or crab hosts was found in divergence of either electrophoretic pattern or of the occurrence and location of the protein bands, i. e., no definite findings suggesting the influence of differences in intermediate hosts on morphology and electrophoretic pattern could be obtained.

The greatest significant difference in Rf values of both major and minor bands was found in combinations of *P. s-A-P* and 3 *P. o* worms, however, it is still uncertain whether this difference was due merely to a single factor of combination of intermediate hosts, i. e., *A. parasitologica* snail and *P. dehaani* crab. It was a fact that Rf values of Bands 17-26 were largest in *P s-A-P*, i. e., being strikingly shifted to the anodic end. In *P. s* worms, however, a major band 17 was usually shifted to the anode, consequently, the following bands Nos. 18, 19, 20, 22, 25 and 26 were inclined to be generally shifted to the anodic end. Thus, future research using an adequate number of worms is also needed to clarify these problems.

Summary

1. In both *Paragonimus sadoensis* and *P. ohirai*, the metacercariae which were developed through the various combinations of the first and second intermediate hosts were found to have almost the same infectivity against the rat as the metacercariae which were developed through their own intermediate hosts. However, worm recovery rates were generally higher in the latter situation than in the former.

2. The prepatent period was 35 days in rats exposed to *P. sadoensis* and 30 days in those exposed to *P. ohirai*. In both lung flukes, no appreciable differences in prepatent periods were found between rats exposed to metacercariae originating from their own intermediate hosts and those exposed to metacercariae originating from the experimental intermediate hosts.

3. Morphology of *P. sadoensis* adult worms, including eggs, was identical with that of *P. ohirai*. Their measurements were also very similar to each other. No significant differences were found in the above characters between the worms originating from their own intermediate hosts and those originating from the expeimental intermediate hosts.

4. In both *P. sadoensis* and *P. ohirai*, the worms originating from the various combinations of experimental intermediate hosts layed the same fertile eggs as the worms originating from their own intermediate hosts, since active miracidia were released from these

eggs by artificial incubation and hatching tests.

5. The electrophoretic pattern derived from whole worm saline extract of *P. sadoensis* was essentially identical with that of *P. ohirai*. Electrophoretic patterns of *P. sadoensis* and *P. ohirai* worms originating from the experimental first and second intermediate hosts were also essentially identical with those of the worms originating from their own intermediate hosts, except for minor differences in densities of peaks J and K being found in *P. sadoensis* and *P. ohirai* respectively, thus, suggesting no definite influence of differences in snail and crab hosts on the electrophoretic patterns of whole worm proteins.

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佐渡肺吸虫ならびに大平肺吸虫の比較に関する研究.
IV. 実験感染によつて得られた成虫の比較

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第1，第2中間宿主のいろいろな組合せを通つて発育した（以下，実験中間宿主内発育メタセルカリア，または虫体と呼び，固有中間宿主内発育のものと区別する）佐渡肺吸虫と大平肺吸虫メタセルカリアがラットに対して感染力を有するかどうかを検討するため本実験を行なつた．得られた成虫の形態，大きさ，ならびに体蛋白質抽出液の泳動像を調べ，固有中間宿主内発育虫体と実験中間宿主内発育虫体との間に差異がみられるかどうかを検討した．得られた成績は次の通りである．

1．2種肺吸虫のいずれにおいても，実験中間宿主内発育メタセルカリアがラットによく感染し，成虫にまで発育することが解かつた．しかし虫体回収率は固有中間宿主内発育メタセルカリアを感染させたラットの方が実験中間宿主内発育メタセルカリアを感染させたラットよりも一般に高かつた．

2．佐渡肺吸虫感染ラットではメタセルカリア試食後35日に，また大平肺吸虫感染ラットでは30日に初めて糞便内に虫卵の排出を認めた．2種肺吸虫のいずれにおいても，固有中間宿主内発育メタセルカリアを感染させたラットと実験中間宿主内発育メタセルカリアを感染させたラットとの間に，虫卵排出までに要する日数に有意な差異を認めなかつた．

3．佐渡肺吸虫成虫の形態は虫卵をも含めて大平肺吸虫のそれに一致し，また計測値も互いに類似している．2種肺吸虫のいずれにおいても，固有中間宿主内発育虫体と実験中間宿主内発育虫体との間に形態学上の差異は認められなかつた．

4．2種肺吸虫のいずれにおいても，固有中間宿主内発育虫体から産出された虫卵は勿論のこと，実験中間宿主内発育虫体から産出された虫卵からも，培養の結果ミラシジアの遊出が認められた．すなわちこれらの虫卵は総て正常な受精卵であつた．

5．佐渡肺吸虫のディスク電気泳動像は本質的には大平肺吸虫のそれに一致する．いずれの肺吸虫の場合も，実験中間宿主内発育虫体の泳動像は固有中間宿主内発育虫体のそれに一致した．すなわち，中間宿主（貝とカニ）が異なると虫体蛋白質の泳動像にも著明な差異が出てくるというような所見は得られなかつたが，実験中間宿主内発育大平肺吸虫の場合，ピークKのdensityが常に低く，一方佐渡肺吸虫ではピークJのdensityが一般に低いようであつた．