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Experimental Infection of the Freshwater Crabs, *Geothelphusa dehaani,* with the Cercariae of *Paragonimus miyazakii*

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Introduction

In order to clarify the biological features of the lung fluke, *Paragonimus* sp., it is necessary to maintain its life cycle in a laboratory. The developmental experiment from the egg to adult worm, has already been successfully carried out for *P. kellicotti* (Ameel, 1934), *P. ohirai* (Yoshida, 1961a, b) and *P. westermani* (Shimazu, 1981; Shibahara, 1985). And Habe *et al.* (1983 a,b) made interspecific hybrids in *P. ohirai, P. iloktsuensis* and *P. sadoensis* by maintaining these life cycles in the laboratory and then taxonomically re-examined these lung flukes.

In *P. miyazakii,* the developmental experiment from the eggs to cercariae was first performed by Kawashima and Miyazaki (1964), and from the metacercariae to adult worms by Kamo *et al.* (1961), and both of the developmental processes have been observed by many investigators. The developmental experiment from the cercariae to metacercariae, intra-crab host stage, however, has not yet been carried out. Thus, in order to successfully replicate the complete life cycle of *P. miyazakii* in the laboratory, it is necessary to achieve its development in the crab host. The author attempted to infect the crab, *Geothelphusa dehaani,* with *P. miyazakii* cercariae and was succeeded in obtaining *P. miyazakii* metacercariae from the infected crabs.

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Materials and Methods

P. miyazakii metacercariae were harvested from freshwater crabs, *G. dehaani,* collected in Kuma-cho, Ehime Prefecture, and given to a dog, from lung cysts of which the eggs were collected. The miracidia were obtained by incubating the eggs in water (25°C) for 23 days.

The snail hosts, *Bythinella nipponica nipponica,* were collected in a small stream at Hoino in Tambara-cho, Ehime Prefecture.

The crabs, *G. dehaani,* were collected from a stream at Komenono in Matsuyama City, Ehime Prefecture. These crabs were considered to be free from natural infection of *Paragonimus* spp. metacercariae, because none of 150 crabs collected in the same station were infected.

The infection of the snails with the miracidia was performed by submerging approximately fifty snails for 18 hrs, in a petri dish (5 cm in diameter) containing approximately five hundred actively moving miracidia and water (5 mm deep). A total number of 612 snails were infected and raised in a polyethylene container (37 cm in length, 26 cm in width and 6 cm in depth) containing specially treated water with continual gentle air-stone aeration at a water temperature of 21°C. The snails were fed with a small mount of decomposing

leaves collected from the natural habitat of *B. n. nipponica*. The treated water was a special solution consisting of 1 part artificial spring water (Nihei, 1978) and 3 parts distilled water and changed every 10 days.

The developmental stages of *P. miyazakii* within the snails were observed every 10 days from 30 to 70 days after infection. After confirming that the development of miracidium into the cercaria, cercarial emergence from the snails into the water was assessed by maintaining 100 snails with the water in a petri dish (9 cm in diameter) under a stereoscopic microscope every day from days 72 to 82. These observations indicated that *P. miyazakii* cercariae emerged from the snails into the water. Three hundred ninety surviving snails were transfered into 3 petri dishes and cercariae emerging from these snails were collected for use in the experimental infection of the crabs every day from days 83 to 130. During this period, the diet and water in the petri dishes were changed once a week.

Eight crabs free of the *Paragonimus* spp. metacercariae were infected with the cercariae of *P. miyazakii* by maintaining a single crab for 3–5 hrs in a petri dish containing 20–92 cercariae together with the treated water (5 mm deep). These crabs were then raised in an aquarium for 60–75 days at 18°C. All were examined for metacercarial infection.

For the examination of the *Paragonimus* sp. metacercaria, the organs of the crabs were flattened between 2 plain glass slips and examined under a stereoscopic microscope. The remaining muscles were crushed, digested in artificial gastric juices at 37°C for 18 hrs and filtrated through a sieve of one-mm meshes. The sediment obtained was washed several times with tap water and examined for the metacercariae. The metacercariae were identified as *P. miyazakii* on the basis of the morphological characteristics.

Results

(1) Collection of *P. miyazakii* cercariae

In order to determine when it is possible to start collecting the cercariae, 1-3 snails infected were dissected every 10 days from 30 to 70 days after infection. The developmental stages of *P. miyazakii* within the snail host are shown in Table 1. All the larvae detected were sporocysts at days 30. Rediae with germ balls and cercariae were detected at days 40 and 60 respectively. The cercariae were detected within the snail host at days 70. Then, the cercarial emergence from the snails occurred after days 74 (Figs. 1, 2). Therefore, the collection of the cercariae was performed for the experiment, during the period from days 83 to 130, as a result, a total of 630 emerging cercariae of *P. miyazakii* were obtained. During this period, the snails decreased in number from 390 to 108 for their decease and the cercarial emergence was not recognized after that time.

Table 1 Development of *Paragonimus miyazakii* within snail host, *Bythinella nipponica nipponica*.

Days after exposure	No. of snails examined	No. of snails infected	No. of			
			sporocyst	redia with germ balls	redia with cercariae	cercaria
30	1	1	3			
40	3	3	11	16		
50	2	2	3	10		
60	3	3	4	24	5 2 – 5*	
70	1	1			4 4 – 5*	7

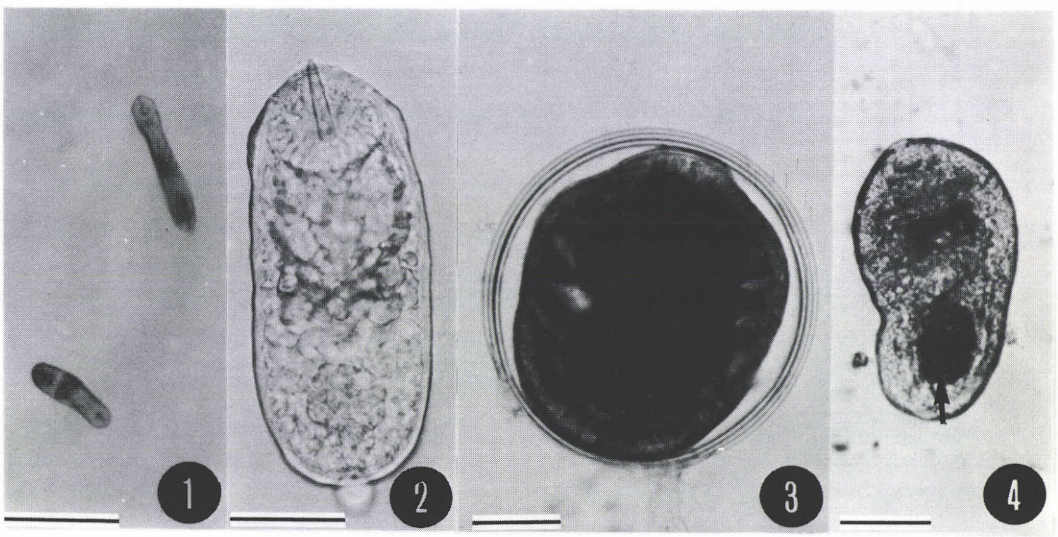
*: No. of cercariae in one redia.

(2) Experimental infection of the crabs with *P. miyazakii* cercariae.

Infection of 8 crabs were made as a trial by exposure to the emerging cercariae, but only 4 of them were infected with *Paragonimus* metacercariae (Table 2). The number of the metacercariae per infected crab was 1 to 5 (average, 3.0), a total of 12 metacercariae were obtained (recovery rate, 3.4%). Out of these metacercariae, 2 were found in the pericardial

Table 2 Experimental infection of freshwater crabs, *G. dehaani* with *P. miyazakii* cercariae

Crabs		Days after exposure	No. of cercariae exposed	No. (%) of metacercariae recovered	No. of metacercariae at			
Sex	Carapace size (mm)				pericardial cavity	liver	genital organ	muscle
Male	20 x 16	75	50	2 (4.0)				2
Male	19 x 16	75	50	0				
Female	26 x 22	60	50	4 (8.0)		3	1	
Female	25 x 20	60	25	5 (20.0)	1	3	1	
Female	24 x 21	75	20	0				
Female	23 x 19	68	92	1 (1.0)	1			
Female	23 x 18	60	27	0				
Female	18 x 14	60	43	0				
Total			357	12 (3.4)	2	6	2	2



Figs. 1–4 *Paragonimus miyazakii* cercariae used for the experimental infection of freshwater crabs, *Geothelphusa dehaani*, and its metacercariae detected from the infection.

Fig. 1 Actively moving cercariae in water (Scale: 200 μm).

Fig. 2 A magnification of the cercaria, showing morphological characteristics of the *P. miyazakii* cercaria (Scale: 50 μm).

Fig. 3 A metacercaria harvested from the crab 60–75 days after infection, showing the morphorogical characteristics of an early stage-mature metacercaria of *P. miyazakii* (Scale: 100 μm).

Fig. 4 An excysted immature metacercaria of *P. miyazakii*, from the crab, showing narrow intestinal ceca and a small mount of granules (arrow) at the posterior region of the excretory bladder (Scale: 100 μm).

cavity of the crabs, 6 in the liver, 2 in the genital organ and 2 in the muscle. Seven of these metacercariae had a spherical outer wall measuring 2–5 μm in thickness and an inner wall 358–400 by 358–406 μm (average, 382 by 388 μm) in diameter and 3–4 μm in thickness. The contracted larvae within the inner wall had a large I-shaped excretory bladder filled with highly refractive excretory granules and large convoluted intestinal ceca (Fig. 3). These morphorogical characteristics agree with those of an early stage-mature metacercaria of *P. miyazakii* observed in the field survey by Gyoten (1983). The remaining 5 metacercariae had thin cyst walls. Out of these, the cyst walls of 2 metacercariae measured 295–300 by 302–324 μm in diameter and 3–5 μm in thickness. The larvae removed from cyst wall were ellipsoidal, having an oral sucker at the anterior extremity and an acetabulum larger than the oral sucker at the front of the middle region. The intestinal ceca of these larvae were narrow, showing three large curves each and terminating at the posterior extremity. The excretory bladder was large, filling up almost the entire intercecal field between the intestinal bifurcation and the posterior extremity. The interior of the bladder, however, was not filled up with the excretory granules, containing a small amount of granules at the posterior region of the bladder (Fig. 4). These morphological characteristics agree with those of an immature metacercariae of *P. miyazakii* observed in the field survey by Gyoten (1983).

Discussion

In order to successfully carry out experimental infection of crabs with *P. miyazakii*, it is necessary to determine the most efficient method for collecting the cercariae. Minute snails, *Bythinella nipponica nipponica*, *B. n. akiyoshiensis*, and *Saganoa* sp., have been reported by Kamo *et al.* (1967), Hatsushika *et al.* (1966) and Sano *et al.* (1979), as the natural snail hosts for *P. miyazakii*. However, because of the quite low incidence and parasite burden of *P. miyazakii* in the field, it has been difficult to obtain a sufficient number of the cercariae from these naturally infected snails.

The cercariae have been obtained from snails experimentally infected with *P. miyazakii* miracidia in the laboratory. Kawashima and Miyazaki (1964) and Kamo *et al.* (1967) attempted the infection of *Oncomellania nosophora*, a snail host of *Schistosoma japonicum*, with *P. miyazakii* miracidia and observed that *P. miyazakii* developed into cercariae within this snail, although the infection rate of the snails was quite low. Saitoh *et al.* (1980) performed the similar experiment, reporting that 31–95% of the snails were infected and a large number of cercarie were produced within the snails. They, however, stated that the cercariae did not emerge from the snails into the water.

An experimental infection of *B. n. akiyoshiensis*, with *P. miyazakii* miracidia was performed by Hashiguchi and Miyazaki (1968) and Kawanaka *et al.* (1979). In these experiment, the infection rate of the snails was 66% in the former and 100% in the latter, and both groups of investigators observed that the miracidia could develop into cercariae. In addition, Kawanaka *et al.* (1979) demonstrated that the cercariae spontaneously emerged from the snails into water.

The mode of the cercarial infection of *P. miyazakii* has been still unknown. However, if it starts from the invasion of the emerging cercaria into the crab, such as *P. ohirai* (Yoshida, 1961 a), *B. n. akiyoshiensis* shall be chosen as a snail host.

In the present study, *B. n. nipponica*, was infected with *P. miyazakii* miracidia and it was observed that the developmental changes of *P. miyazakii* in *B. n. nipponica* showed a similar tendency to those in *B. n. akiyoshiensis* and the cercariae also emerged from *B. n. nipponica*. Then the emerging cercariae became metacercariae within the crabs. Thus it became clear that *B. n. nipponica* was a suitable snail host for completing the life cycle of *P. miya-*

zakii in the laboratory.

Various experimental infections of 2nd intermediate hosts with *Paragonimus* spp. cercariae have been attempted in order to obtain detailed information on the biological characteristics of *Paragonimus* spp. In these experiments, the methods of successful infection which have so far been used are as follows, (1) feeding the snails with the cercariae to the 2nd intermediate hosts (Yokogawa, 1953; Glenn, 1963; Araki and Yokogawa, 1975; Shibahara, 1985) (2) administering orally the cercariae to the 2nd intermediate host (Chen, 1940). (3) submerging the crabs in a stream or a small pool where snail hosts with the cercariae have lived (Ando, 1920; Tang, 1940). and (4) submerging the crabs in water containing the cercariae (Wu, 1935; Yoshida, 1961a, b; Yoshimura *et al.*, 1970; Shimazu, 1981; Shibahara, 1985).

The experimental infection of crabs with *P. miyazakii* cercariae has been attempted by oral feeding, percutaneous contact and by injection (Saitoh *et al.*, 1979). These experimental infection, however, were unsuccessful, because the metacercariae obtained were different from those of *P. miyazakii* in the morphorogical characteristics. A successful method had not so far been developed. The present study, however, proved that the successful infection could occur when *G. dehaani* submerged in water containing *P. miyazakii* cercaria, for 3–5 hrs. This made it possible to raise the complete life cycle of *P. miyazakii* in the laboratory.

The developmental period from the cercaria to mature metacercaria of *Paragonimus* sp. required a long time and this period varied with the temperature of the water where the infected hosts were raised (Ando, 1920). Shimazu (1981) obtained mature metacercariae of *P. westermani* 8 weeks after infection at water temperature of 25°C. Yoshimura *et al.* (1970) detected *P. ohirai* at days 57 at 20–30°C. In *P. kellicotti,* they were obtained at days 46 at 20°C by Ameel (1934) and at days 53 at 22–25°C by Yoshida and Nishimura (1968). In the present study, the crab hosts infected with *P. miyazakii* cercariae were raised in the period of 60–75 days at water temperature of 18°C, with reference to the above mentioned information. From these crabs, the early stage-mature metacercariae of *P. miyazakii* were detected, which have the same infectivity in a mammalian host as the mature metacercaria (Gyoten, 1983). This fact contributes to the studies on the maintenance of the complete life cycle of *P. miyazakii.*

Summary

Experimental infections of freshwater crabs, *Geothelphusa dehaani,* with *Paragonimus miyazakii* cercariae were carried out to maintain the life cycle of the lung fluke in the laboratory. *Bythinella nipponica nipponica* snails were infected with *P. miyazakii* miracidia for havesting the cercariae. The larvae fully developed into cercariae in the snails and the cercariae emerged from the snails into water after 74 days of infection. These emerging cercariae were used for the experimental infection of the crabs.

Eight *G. dehaani,* crabs free from *Paragonimus* spp. metacercariae, were exposed to 20–92 cercariae in water. Immature and early stage-mature metacercariae of *P. miyazakii* were obtained from 4 out of 8 infected crabs 60–75 days after infection.

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宮崎肺吸虫 *Paragonimus miyazakii* セルカリアのサワガニ *Geothelphusa dehaani* への感染実験

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実験室内において，宮崎肺吸虫の生活環を維持する目的でセルカリアのサワガニへの感染実験を行なった．まず感染に供するセルカリアを得るため，宿主貝であるホラアナミジンニナ *Bythinella nipponica nipponica* に宮崎肺吸虫ミラシジウムを感染させた．この幼虫は十分発育し，感染74日以降セルカリアが貝より水中へ遊出した．サワガニへの感染実験はこの遊出セルカリアを用いて行なった．

肺吸虫メタセルカリアの感染していない８匹のサワガニに，１匹あたり20～92隻の遊出セルカリアを水中にて暴露することにより，感染を行なった．感染60～75日後これらのカニを剖検し，４匹のカニから宮崎肺吸虫未熟および初期成熟メタセルカリアを得た．