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The Attachment of *Paragonimus miyazakii* Cercariae to Crab Hosts, *Geothelphusa dehaani*

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Abstract

The present study was designed to elucidate the behavior of *Paragonimus miyazakii* cercariae just prior to penetration of its crab host, *Geothelphusa dehaani*. To carry out the study, we developed a method of whole preparation for light microscopy using crab exoskeleton and gill. The method was applied to crabs experimentally exposed to *P. miyazakii* cercariae, post 1, 3 or 24 hrs of exposure, and gave satisfactory images of cercariae around the exoskeleton and gill. Three morphological types of cercariae were found, mainly on the exoskeleton of the legs. The first was completely enveloped in mucoid, had a rounded body and was attached to the outer surface of the exoskeleton. The second type was flat, and also enveloped in mucoid and located on the outer surface of the exoskeleton. The remaining type was flat, had lost most of its mucoid and was located on the inner surface of the exoskeleton. These 3 types are indicative of cercarial behavior around the exoskeleton of crabs. The bodies of cercariae become rounded and attach to the outer surface of the crab exoskeleton before penetration. This attachment helps cercariae to percutaneously penetrate their hosts. This is the first description of *Paragonimus* cercarial attachment to crustacean hosts.

Key words: *Paragonimus miyazakii*; cercarial attachment; crab; exoskeleton specimen.

Introduction

In previous studies, crustacean hosts free from *Paragonimus* larvae were fed snails with cercariae or submerged in water containing the cercariae. Several months later, the hosts were found to harbor *Paragonimus* metacercariae (Ando, 1920; Ameel, 1934; Wu, 1935; Chen, 1940; Tang, 1940; Yoshida, 1961; Glenn, 1963; Yohimura *et al.*, 1970; Shimazu, 1981; Gyoten, 1986; Shibahara, 1991; Yaemput *et al.*, 1994). Although, there is no commonly accepted explanation as to how *Paragonimus* cercariae enter crustacean hosts, two hypotheses prevail; peroral (Yokogawa, 1953) and percutaneous penetration (Yoshida, 1961). Neither have been fully proven, since the process has not been observed. In *Paragonimus* spp. employing percutaneous penetration, some of the cercariae in water are suggested to develop into metacercariae through contact with and percutaneous penetration of the crustacean hosts.

To expose the process involved, we used cercariae of *Paragonimus miyazakii*, which have been suggested to employ percutaneous penetration into crab hosts, *Geothelphusa dehaani* (Gyoten, 1986; 1995). The present study was designed to develop a method of whole preparation for time-effective microscopical examination and to reveal the process of contact of cercariae with crab hosts based on microscopical findings of cercariae around the exoskeleton and gill (the water-contact locations).

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Materials and Methods

Snails, *Bythinella nipponica*, were infected with *P. miyazakii* miracidia and the cercariae that emerged after 80 days were collected. Crabs, *G. dehaani*, were collected from a mountain stream at Komenono in Matsuyama, Ehime Prefecture, Japan. The crabs were considered to be free from *Paragonimus* larvae, because none of the 150 specimens previously collected at this site had been infected (Gyoten, 1986).

Eleven of the crabs (3 females and 8 males, carapaces measuring 6–13 × 5–11 mm) were ex-

posed to 86–147 cercariae in dishes (60 mm in diameter) containing water. After 1, 3 or 24 hrs, the crabs were placed in acetic alcohol (25 ml acetic acid in 75 ml absolute ethanol) for 3 days, to separate chelate and walking legs from the bodies, then in 70% alcohol. The specimens were decalcified and softened in 45% acetic acid for 5 hrs, after which their internal tissues were removed (Fig. 1). The remaining exoskeletons and gills were placed in 50% alcohol, and then pressed tightly between two slide glasses with thread. After 24 hrs, one slide glass was removed. The specimens were placed in new 50% alcohol for 3 hrs, followed by 25% alcohol for 1 hr, and then stained with 0.1% toluidine blue o (TBO) for 1 hr. They were then dehydrated in graded alcohol, cleared in xylene and mounted in Canada balsam.

To elucidate the internal morphology of the cercariae, a crab (female, carapace measuring 8 × 6 mm) exposed to 50 cercariae for 3 hrs was cut into serial sections (10 μm in thickness) and stained with 0.1% TBO using the method of Gyoten (1995).

Results

Detection of cercariae in crabs

Exoskeletons and gills of 11 crabs were examined for *P. miyazakii* cercariae under a light microscope. A total of 74 *P. miyazakii* cercariae were detected, corresponding to 6.2% of the number to which the crabs were exposed (Table). Most (87%) of the detected cercariae were located on the exoskeleton of the legs. The percentage of detection was approximately 10% within 3 hrs of exposure. At

Table Detection of *P. miyazakii* cercariae in the water contact locations on crab hosts, *G. dehaani*, previously exposed to cercariae experimentally

Hours of exposure	Crabs		No. of cercariae				
	Sex	Size of carapace (mm)	Exposed	Detected in/from			
				Total (%)	Exoskeletons of		Gills
					Bodies	Legs*	
1	M	6 × 5	100	7 (7.0)	1	5	1
1	F	10 × 7	118	19 (16.1)		19	
1	M	10 × 8	100	6 (6.0)	1	5	
1	M	11 × 9	100	4 (4.0)		4	
Subtotal			418	36 (8.6)	2	33	1
3	M	10 × 9	103	18 (17.5)	5	13	
3	F	11 × 9	86	8 (9.3)	1	7	
3	F	12 × 10	95	8 (8.4)		8	
Subtotal			284	34 (12.0)	6	28	
24	M	11 × 10	126	1 (0.8)		1 (1†)	
24	M	10 × 9	101	0			
24	M	13 × 11	117	1 (0.9)	1		
24	M	12 × 10	147	2 (1.4)		2 (1†)	
Subtotal			491	4 (0.8)		4 (2†)	
Grand total			1193	74 (6.2)	8	65 (2†)	1

F, Female; M, Male.

*Legs including chelae and walking legs, †Cercariae located on the internal surfaces of exoskeletons.

post 24 hrs, however, this had declined to less than 1%, and of the detected cercariae, 2 had penetrated the inside of the exoskeleton at the leg.

Morphological findings of detected Paragonimus miyazakii *cercariae*

All of the 74 cercariae detected were more or less covered in mucoid which stained purplish red with TBO (Figs. 2 and 5–8). Along with them, a number of small clumps of the substances without cercariae were observed (Figs. 3 and 4). Some of them seemed to be exuviae, from which cercariae had emerged. Of the detected cercariae, 48 had rounded bodies (average size 84 × 70 μm) and were enveloped in mucoid (Figs. 2 and 5). In these cercariae, blue-stained cells were detectable in the interior of the body, however, internal organs peculiar to *Paragonimus* cercariae were not visible. As the rounded specimens were too thick to give a satisfactory image under a light microscope, serial sections of the additional crab specimen were made to elucidate their morphological characteristics. In the sagittal sections, rounded specimens had elliptical out lines and transparent teguments with purplish red-stained mucoid (Fig. 6). The sections contained fragments of blue, as well as transparent cells and internal organs, the latter with features peculiar to the *Paragonimus* cercariae; a large stylet, a muscular oral sucker, a ventral sucker, transparent penetration gland cells and an excretory bladder. The rounded *P. miyazakii* cercariae were located on the outer surfaces of the exoskeletons and gill.

Another 24 cercariae were flat in shape. Their bodies were also enveloped in mucoid (Fig. 7). Their internal structures were clearly visible under a light microscope and they were identified as *Paragonimus miyazakii* cercariae with the morphological features of the free swimming-phase of this parasite. These cercariae were also located around the outer surface of the exoskeleton.

The remaining 2 cercariae, located on the inner surfaces of the exoskeletons, were flat and had the same unique internal structures as the *P. miyazakii* cercariae did. However, they had less mucoid than those located on the outer surfaces (Fig. 8).

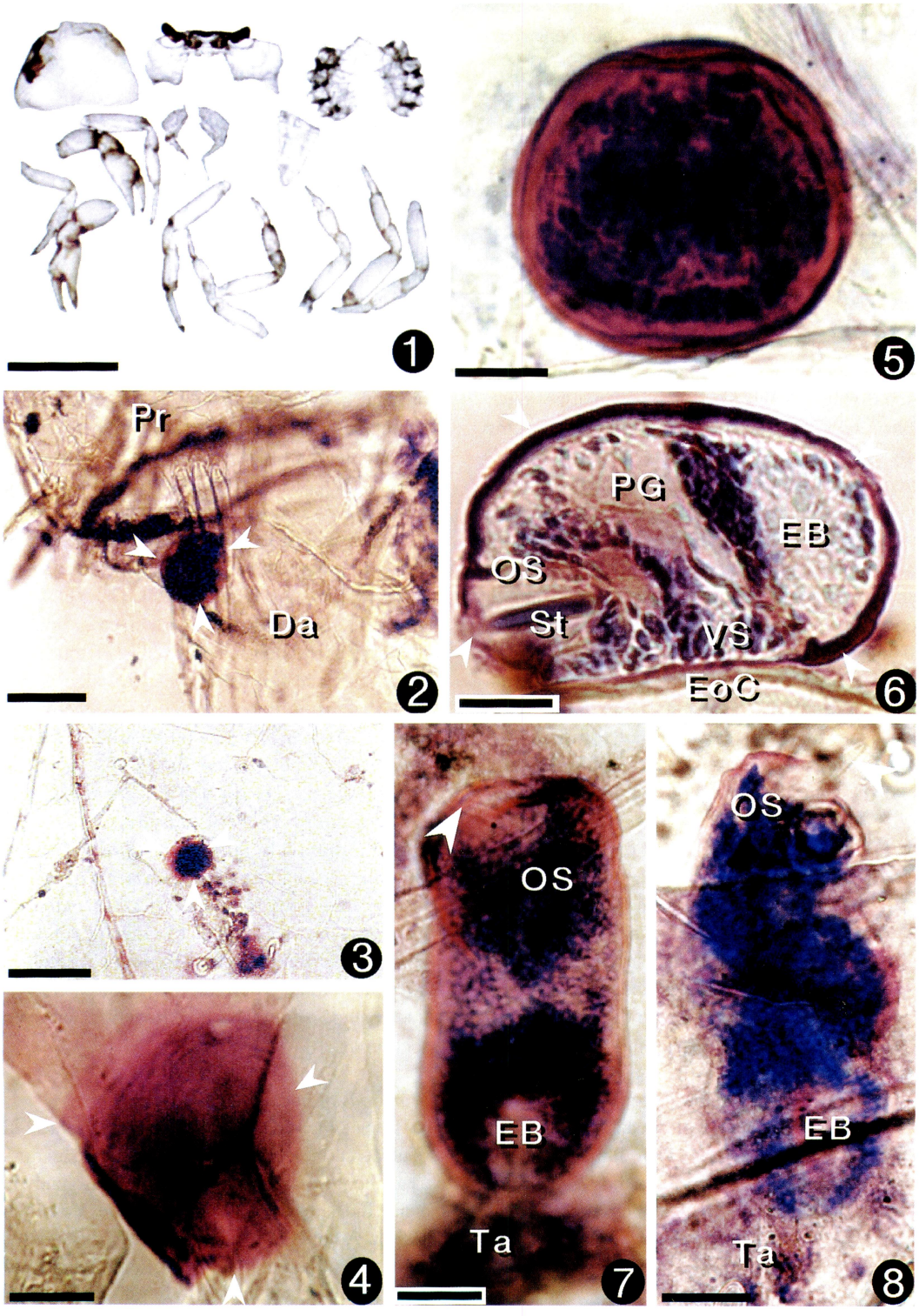
Discussion

Development of a method of preparing whole specimens of exoskeletons and gills

To study the process of contact and penetration between cercariae and crustacean hosts, a light microscopic examination must include those parts of the crustacean exposed directly to cercariae in water; the exoskeleton and the gill. In the past, serial sectioning of the crab hosts has been used (Gyoten, 1995). The technique, however, is time-consuming, with a single crab requiring approximately 450 serial sections, all of which must be examined thoroughly in order to determine the number of cercariae present. In the present study, we have developed a technique for light microscopy involving whole preparation of exoskeletons and gills combined with TBO staining (Yokogawa and Yoshimura, 1956), which stains cercarial mucoid purplish red. This technique was applied to crabs exposed to cercariae and was found to have substantial advantages over serial sectioning (Gyoten, 1995). The technique is time-effective, and provided satisfactory images of cercariae as well as of the crab exoskeleton and gill. Mucoid stained purplish red was particularly prominent, making for improved detection of cercariae in specimens of the exoskeleton and gill. A disadvantage of the technique, however, is that only specimens of the exoskeleton and gill, can be examined, resulting in low recovery rates in specimens 24 hrs after exposure, as by this time *P. miyazakii* cercariae have already left the exoskeleton and penetrated the inside of the body (Gyoten, 1995). Also, the technique can not be applied to crabs administrated cercariae perorally, because the internal organs can not be examined. Therefore, we recommended that this method should be used together with serial sectioning.

Functions of mucoid

In specimens post 1, 3 hrs of exposure, the bodies of all cercariae were enveloped in mucoid identified as a mucoid coat (Kruidenier, 1953); also known as a mucoid substance (Yokogawa and Yoshimura, 1956). A layer of mucoid existed between the tegument of rounded cercariae and crab exoskeleton. As mucoid is known to be adhesive in nature (Kruidenier, 1953), this layer was considered to play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a role in binding cercariae to crabs. In post 24 hrs specimens, mucoid containing no cercariae was present on the outer surfaces of the exoskeleton. The amount of mucoid on cercariae located inside the exoskeletons, was less than that on cercariae from the outer surface, suggesting that mucoid had peeled off during penetration of the crab exoskeleton. The finding that cercariae that have penetrated the inside of crabs no longer seem to require mucoid supports the hypothesis of Yokogawa and Yoshimura (1956), suggesting that mucoid isn't a constitute element of the larva in the intracrustacean phase, but plays a role in the phase prior to cercarial penetration of crabs. The only role afforded mucoid to date, has been the protection of cercariae from adverse environmental conditions in water, during the free-swimming phase of the life cycle. Furthermore, mucoid also forms mucoid strands, which result in the entanglement of cercariae with crustacean hosts, aiding infection (Kruidenier, 1953; Gyoten, 1991). The present study revealed that another mucoid function, it makes cercariae attach to crab hosts directly, a process that occurs just prior to cercarial penetration. Thus, mucoid is an important substance for the percutaneous penetration of cercariae into crab hosts.

Cercarial attachment

In most of the *Paragonimus* spp. cercariae in which percutaneous penetration of crustacean host has been suggested, the organisms are released into water from snails, after which they creep along the bottom and/or float on the surface (Ando, 1920; Ameel, 1934; Wu, 1935; Tang, 1940; Yoshida, 1961; Gyoten, 1986; Yaemput *et al.*, 1994). Once they make contact with crustacean hosts, mucoid enveloping the cercariae forms mucoid strands ensuring entanglement and eventual penetration. However, the behavior of cercariae during entanglement and penetration, is not well understood. Therefore, we exposed crab hosts, *G. dehaani*, to *P. miyazakii* cercariae to elucidate this unknown behavior. We found that the cercariae became rounded and attached mainly to the leg exoskeleton of the crabs, following entanglement. To date, this is the first report on the attachment of the genus *Paragonimus* to crustacean hosts. We conclude, based on the following findings, that such a process is indispensable to penetration: Most of the cercariae detected from preparations in the early stage of exposure (post 1–3 hrs), had rounded bodies and were attached to the outer surface of the exoskeletons. In preparations post 24 hrs, the number of attached cercariae was reduced, with many clumps of mucoid without cercariae appearing on the outer surface, and a few cercariae having penetrated into the interior of the exoskeleton. This indicated that attachment occurred prior to, and helped with cercarial penetration of crab hosts. Also, cercarial exposure

Fig. 1 The body and legs of a crab, *Geothelphusa dehaani*. The exoskeleton was decalcified and softened with 45% acetic acid (Scale bar = 10 mm).

Figs. 2–8 *Paragonimus miyazakii* cercariae and mucoid found from exoskeletons of crabs stained with 0.1% toluidine blue o.

Fig. 2 A cercaria of the first type. A cercaria (arrows) with purplish red-stained mucoid and the rounded body attached to a walking leg of a crab (Da, Dactylus; Pr, Propodus; Scale bar = 100 μm).

Fig. 3 A clump (arrows) of mucoid on the exoskeleton of the abdomen of a crab (Scale bar = 100 μm).

Fig. 4 High-power magnification of a clump (arrows) that seemed to be a exuviae, from which a cercaria had probably already emerged (Scale bar = 25 μm).

Fig. 5 High-power magnification of a cercaria of the first type, with blue stained cells and hardly visible cercarial organs (Scale bar = 20 μm).

Fig. 6 A sectioned specimen of a rounded cercaria produced satisfactory images of cercarial organs. A stylet (arrow), an oral sucker (OS), penetration glands (PG), a ventral sucker (VS) and an excretory bladder (EB) are visible. The specimen was located on the exoskeleton of a crab (EoC) (Scale bar = 15 μm).

Fig. 7 A cercaria of the second type. This is flat and similar to cercariae in the free-swimming phase, with visual organs unique to *Paragonimus* cercariae; a stylet (arrow), an oral sucker (OS), an excretory bladder (EB) and a tail (Ta), and a lot of mucoid on the body surface, especially around the oral sucker and excretory bladder. The specimen was located on the outer surface of the exoskeleton (Scale bar = 25 μm).

Fig. 8 The third type, a flat cercaria located on the interior surface of the exoskeleton, had only a small amount of mucoid as compared with the cercaria in Fig. 7. It did, however, have *P. miyazakii* cercarial organs (Scale bar = 25 μm).

was shown to precede attachment in the present study. Exposure is thought to complete the life cycle of *P. miyazakii* (Gyoten, 1986), thus, attachment is actually another process of the cycle.

In summary, the cercariae of *P. miyazakii* are released from snail hosts into water, creep along the bottom and/or float along the surface, and become entangled in the legs of those crab hosts with which they make contact. Their bodies become rounded and attach to the hosts, then they penetrate the interior of the legs through the exoskeleton. The penetration process, however, has yet to be observed, and is the subject of further study.

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