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博 士 論 文

Dynamics of *Centrocestus armatus* transmission in endemic river in Hyogo Prefecture, Japan
（兵庫県下の流行河川における *Centrocestus armatus* の感染動態）

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Dynamics of *Centrocestus armatus* transmission in endemic river in Hyogo Prefecture, Japan

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ABSTRACT

Centrocestus armatus is an intestinal parasite belonging to the family Heterophyidae. We developed an apparatus for recovering cercariae and clarified the infection dynamics of this parasite. To clarify the circadian rhythm of cercarial shedding in the summer season, we filtrated 30 l of river water every 2 h for 24 h. Cercariae were first detected between 06:00 and 08:00 h, increased over time to r a peak at 16:00 h and decreased thereafter, thus showing a single-peak pattern. In a survey of seasonal change, approximately 200 cercariae were contained in 1 l of river water during the summer season, while none were found during the winter. This cercarial shedding pattern appeared to be related to sunrise/sunset and water/atmosphere temperature. Therefore, we examined whether cercarial shedding was affected by light or temperature changes under laboratory conditions, and confirmed that both light and temperature were important factors for cercarial shedding. Light was a stronger factor than water temperature. Cercarial shedding of *C. armatus* occurred in response to temperature and light. The change in the number of juvenile metacercariae detected in fish brain corresponded with monthly detection rates of cercariae; however, the incidence of new infections decreased in August. This suggests that *Nipponocypris temminkii* contains a defense mechanism against new infections that may have hindered the increase in parasite infectivity. These results clarified the smooth infection from the first to the second intermediate host of *C. armatus* in the endemic river. Throughout the study period, fecal samples were collected from 19 kites, 114 herons, and three unidentified species. However, our results using *C. armatus* showed a low value of 1% in herons and 5% in kites. The infection dynamics of final host to first intermediate host need to be further investigated.

INTRODUCTION

Centrocestus armatus is a minute intestinal trematode belonging to the family Heterophyidae. *C. armatus* is widely distributed in Taiwan, the Korean Peninsula and Southeast Asia. The life cycle of this trematode requires two intermediate hosts; the first is a freshwater snail such as *Semisulcospira libertina*, and the second is a freshwater fish such as *Nipponocypris temminkii* or *Zacco platypus* [1]. The final host is a fish-eating bird such as *Nycticorax nycticorax*. Four species of *Centrocestus*, namely *C. armatus* [1], *C. formosanus* [2], *C. asadai* [3], and *C. nycticoracis* [4], have been reported in Japan. With the exception of *C. nycticoracis*, these species have been reported to infect humans [5, 6]. Although human infection by this trematode has not been reported in Japan, it has been reported in Korea after ingestion of improperly prepared infected raw fish, *Z. platypus* [5].

Epidemiological surveys of the genus *Centrocestus* in Japan are limited [7]. One such study by Kimura and Uga in 2003 [8] revealed that infection rates of the parasite in the snail host *S. libertina* were 13–49%. The authors suggested that the pattern of infection of snails infected with *C. formosanus* is related to environmental or biological factors, such as movements of final hosts, water density, and water temperature and/or water quality [7]. However, little is known about the circadian rhythms of cercarial shedding from infected snails, seasonal patterns, and the effects of different temperatures and light exposure. Many studies on cercarial shedding have been conducted by cercariometry using the genus *Schistosoma*. Cercarial shedding of *Schistosoma haematobium* and *S. mansoni* is known to exhibit a diurnal pattern in river water [9, 10], while *S. japonicum* shows nocturnal shedding [11]. This shows that the pattern of cercarial shedding is not necessarily uniform. Furthermore, these shedding patterns are reported to be light and temperature dependent [12]. Similarly, Lo and Lee [13] have reported that cercarial shedding of *C. formosanus* depends on light and temperature; that is, cercarial shedding increases under high water temperature and decreases under dark conditions. However, the infection dynamics of *Centrocestus* cercariae in the natural

environment have not been investigated. In addition, the temporal pattern of *C. armatus* cercarial shedding in endemic river water has not been reported. Knowledge of how cercariae infect the second intermediate host efficiently is essential to determine the distribution of this trematode in endemic areas. However, investigations of this subject are far from adequate.

Several authors have investigated the metacercariae of fish [14, 15]. Kimura and Uga [16] reported the prevalence of the second intermediate host, *N. temminkii*, almost 100% of which were positive for metacercariae in Japan. In Korea, where *C. armatus* is widely distributed, the infection rate in *Z. platypus* is approximately 87% [17]. Paller *et al*. [18] reported that, in the presence of *N. temminkii,* the second intermediate host, *C. armatus* cercariae have a high level of adherence and infectivity after 1 h and survive for approximately 30 h in laboratory conditions. However, the number of cercariae in endemic rivers and the rate of *N. temminkii* infection have not been determined.

In Japan, three reports [1, 2, 7] have provided information on the definitive host of the genus *Centrocestus*. In one of these [2], *Nyc. nycticorax* infection by *C. formosanus* was seen in two out of three birds (67%), and in another [7], all six birds were positive for parasitic infection. Tanabe meanwhile [1] showed that, in Okayama Prefecture, only one of 12 types of heron (8%) was found to be positive for *C. armatus* infection. However, the current conditions for definitive host infection are not clear because of study limitations such as outdated information and limited sample sizes.

Even though several studies have been conducted on specific aspects of the life cycle of this trematode, the epidemiological features of the entire life cycle remain unknown. Comprehensive studies on the long-term trematode infection dynamics have not been conducted within one given endemic area. Therefore, in this study, we conducted an epidemiological survey of *C. armatus* infection from an endemic river located in Hyogo Prefecture, Japan.

MATERIALS AND METHODS

1. Survey area

This study was carried out from July 2007 to September 2013 in Chikusa River, Hyogo Prefecture, Japan. The river runs for approximately 68 km, with a basin area of approximately 730 km^2. The snail, fish, and fecal samples were collected at the midstream of the river, which was 30 m wide and 0.1–1.5 m deep. In some experiments, fecal collection was done in the entire basin of the river.

2. Cercariometry and cercarial shedding

1) Development of filtration apparatus and detection of cercariae

A filtration device for collection and observation of cercariae was prepared for this experiment. The filtration device was 1500 mm high and 400 mm wide (Fig. 1). It consisted of three parts: a tank for keeping river water (Fig. 1A), a valve for regulation of water flow (Fig. 1B), and a filter (30-μm pore size plankton net attached to the filtration device; KS-142; Advantec, Tokyo, Japan), for filtration of cercariae (Fig. 1C).

For the water filtration, 30 l of river water was collected at a depth of 200 mm and filtrated at a flow rate of 1.2 l/min. The plankton net (30-μm pore size) attached to the filtration device was removed after filtration, kept in a Petri dish, suspended in 3 ml 3% formalin, and brought back to the laboratory for further examination. In the laboratory, the plankton net was washed with normal saline solution and debris particles were removed and suspended in normal saline. The plankton net was sandwiched between two transparent plastic boards (150×150 mm), stained with 2 ml of 0.1% acridine orange, and placed in a transparent plastic bag with an air-tight zipper (180×250 mm). Microscopic observation was performed directly through the transparent plastic bag using a stereomicroscope. The removed suspension was also observed for the presence or absence of cercariae in the Petri dish.

2) Temporal and seasonal changes in the number of cercariae in river water

To clarify temporal changes in the number of cercaria, cercariometry was performed in the endemic river, and the number of *C. armatus* cercariae in the water was determined every 2 h for 24 h. To clarify seasonal changes, the number of *C. armatus* cercariae in the river water was determined (between 12:00 and 14:00 h) every month from January to December 2008. The water temperature was also recorded during cercariometry.

3) Shedding of *C. armatus* cercariae from *S. libertina*

In the laboratory, *S. libertina* were individually placed in a beaker (30 mm diameter, 30 mm high) with 15 ml river water, exposed to fluorescent lighting (FPL27PG-W3, 27W, NISSO, Tokyo, Japan) at a height of 300 mm, left at room temperature (24±1°C, water temperature 24°C) for 24 h, and finally observed for any sign of cercarial shedding. Observations were made with a stereoscopic microscope (15–30× magnification), and snails were considered infected when signs of emergence were detected. The cercariae from infected snails were collected and used for cercariometry and the cercarial shedding experiment.

4) Effect of light on cercarial shedding

Four cercaria-positive snails were placed in an aquarium (305×185×330 mm) kept at 20°C and exposed to bright light (2,000–10,000 lux) for 12 h. The snails were transferred into darkness (0 lux) for a further 12 h. This experiment was repeated on four consecutive days. The total number of shedding cercariae was counted for each condition using the filtration apparatus.

5) Effect of water temperature on cercarial shedding

To determine the effects of water temperature on *C. armatus* shedding from infected *S. libertina*, nine cercaria-positive snails were maintained at 19°C in an aquarium containing 3 l dechlorinated water, under bright light (10,000 lux) for 12 h. The snails were transferred at varying water temperatures of 19, 21, 23, 25, 23, 21 and 19°C, for 2 h each. These experiments were carried out on each container (diameter×height; 260×120 mm).

6) Relationship between the effect of light and water temperature on cercarial shedding

Five cercaria-positive snails were maintained at 23°C in an aquarium, containing 3 l dechlorinated water, under bright light (2,000 lux) for 1 h. The snails were transferred to water at 25, 27 and 29°C for 2 h each. The number of cercariae was counted at each water temperature setting. The same experimental procedure was performed under dark conditions.

3. Conditions of metacecariae infection within the *N. temminkii* brain

1) Infection of *N. temminkii* located in endemic river beds

A portion of *N. temminkii* brain was pressed between two microscope slides and examined under a stereoscopic microscope (15–30× magnification). In a previous study, we were able to distinguish between juvenile and mature metacercariae located in the brain, based on their shape, and we recorded the same information during the present investigation. Specifically, metacercariae less than 120 μm along their major axis, lacking an X-shaped excretory bladder and bearing an eyespot, were classified as juvenile, while metacercariae with an X-shaped excretory bladder were classified as mature. The results of a preliminary experiment revealed that metacercariae required up to three weeks to mature after host infection.

2) Sentinel fish

To determine the frequency of cercarial infection in the second intermediate host, *N. temminkii* collected from non-endemic areas were placed in the *C. armatus* endemic river, cultivated for 6 h, and 7, 12, 20, and 27 days, and the number of juvenile metacercariae formed in the brain was recorded. *N. temminkii* were reared by keeping the specimens at a depth of 600 mm and encased in wire netting each at a range of 450 mm. This was conducted in June.

4. Infection of the definitive host

Fecal examination was conducted using the birds inhabiting the area surrounding the endemic river. Feces were collected by observing rocks located along the banks for signs of bird droppings (black substance within white uric acid). Speciation of birds was not performed. All samples were brought back to the laboratory, suspended in 1 ml water, microscopically examined, and checked for *C. armatus* eggs.

RESULTS

1. Cercariometry and cercarial shedding

The preliminary experiment on cercarial harvest using the filtration apparatus showed a 97% recovery rate of *C. armatus* cercariae when river water was filtrated at a rate of 1.2 l/min. *C. armatus* cercariae appeared to be microcercous with a pair of eyespots, a body length of 108±8 μm and an overall length of 200±11 μm. Four species of cercariae other than *C. armatus* were detected from *S. libertina* during the survey, but were not identified. They were also microcercous cercariae, but unlike *C. armatus* cercariae, they had no eyespots. In this filtration experiment, contamination with debris was inevitable, making identification of *C. armatus* cercariae was difficult. However, staining with acridine orange emphasized the presence of tails and eyespots, which helped in the identification.

1) Temporal change in cercarial shedding

Fig. 2 shows the number of cercariae shed in the endemic river every 2 h for 24 h. The number of cercariae detected in September 2007 showed a single-peak pattern; that is, the number of cercariae increased from 08:00 h (1.4 cercariae/l) in the morning, peaked at 16:00 h (7.2/l), and then decreased thereafter. The average number of cercariae between 22:00 and 06:00 h was 0.6/l, one-twelfth the number at 16:00 h. The number of cercariae in the same river in September 2008 was slightly lower than in September 2007, but the pattern of cercarial shedding was the same (Fig. 2). These patterns of cercarial shedding may have been

related to atmosphere and/or water temperature. Cercarial shedding increased and decreased with water temperature. Two-hour time lags were confirmed between the peak water temperature and the number of cercariae. In addition, the patterns of cercarial shedding seemed to be related to the strength of the light; that is, there were many in the daytime, but few at night.

2) Seasonal changes in cercarial shedding

Experiments on the seasonal change in cercarial shedding revealed a peak from July to September (average 2.6/l, Fig. 3). Cercariae were not detected from January to April when water temperature was below 11°C (Fig. 3). The cercariae began to appear again from May when the average water temperature rose above 16°C. The seasonal pattern of cercarial shedding was also associated with changes in water and atmospheric temperature, which were recorded simultaneously (Fig. 2).

3) Effect of light on cercarial shedding

The effects of light and/or dark conditions on cercarial shedding were examined in the laboratory (Fig. 4). The average number of cercariae shed from infected snails under bright conditions for 12 h was found to be 1,200/l. However, the number decreased by only one-ninth (130/l) when snails were transferred to dark conditions. These results were reproducible over the following four consecutive days. On average, the number of cercariae obtained under bright conditions was more than seven times that under dark conditions. Furthermore, light stimulation was also confirmed at less than 2,000 lux. We confirmed that cercarial shedding was directly affected by light.

4) Effect of water temperature on cercarial shedding

The effect of changing water temperature on cercarial shedding was examined (Fig. 5). The average cercarial shedding from infected snails kept in water at 19°C was 1,538/l, but this increased to 7,551/l when the snails were transferred to water at 25°C. In contrast, cercarial shedding decreased when snails were transferred to the colder water temperature. Cercarial

shedding was affected by both light and temperature.

5) Comparison of the effect of light and water temperature on cercarial shedding

Table 1 compares the effect of light and water temperature on cercarial shedding. The average number of cercariae shed from snails was increased (6.3 times) from 90 to 564/l when the water temperature was increased from 23 to 29°C. In contrast, the number of cercariae obtained under bright conditions (996/l) was more than 20 times that under dark conditions (44/l). These results indicate that light had a greater effect on cercarial shedding than temperature did.

2. Relationship of *N. temminkii* infection and number of cercariae in water

Fig. 6 shows the number of cercariae found in river water (sample collected at 14:00 h) and monthly changes in juvenile metacercariae numbers detected in the brain of *N. temminkii.* Cercariae were not detected during March and April when the water was at its coldest; however, cercariae became detectable in May (0.4 parasites/l) when the water reached 18°C. From May, the number continued to increase, with a peak in August (26°C, 2.4 parasites/l), and continued to be detectable until the end of collection in September (18°C, 1.8 parasites/l). Similarly, the change in the number of juvenile metacercariae detected corresponded with monthly detection rates of cercariae; that is, an average of 0.5 parasites in May, 1.6 in June, 18.2 in July, 12.6 in August, and 10.6 in September.

3. Sentinel fish

Negative *N. temminkii* rose in an endemic river for seven days had three juvenile metacercariae detected in the brain. This number increased with time; that is, six juveniles within 12 days and 15 within 20 days (Fig. 7). With regard to the daily infection rate, there were 0.4 juveniles within seven days, 0.5 within 12 days, 0.8 within 20 days, and 0.6 within 27 days, which clearly showed a consistent rate of increase in metacercarial numbers.

4. *C. armatus* infection in definitive host

Fecal samples were collected from 19 kites, 114 herons, and three unidentified species. *C. armatus* eggs were detected in samples from one kite (5%) and one heron (1%) (Fig. 8).

DISCUSSION

Cercariometry has been studied extensively in *Schistosoma* spp. [9, 10, 12, 19], but little attention has been paid to other trematode species. Prentice [20] reported that the recovery rate of *Schistosoma* cercariae using a multi-nylon filter was over 80%. In the present study, we investigated the shedding of *C. armatus* cercariae under natural and laboratory conditions. Since *C. armatus* cercaria is smaller than those of *Schistosoma* spp., we developed a new filtration method. A 30-μm pore size plankton net was used for filtration; we stained the cercariae, and then removed debris particles from the plankton net by washing with normal saline. The recovery efficiency of *C. armatus* cercariae was 97% using this method. Which might also be effective for cercariae of other trematodes because it was possible to filter 30 l of water. Several different species of cercariae were detected throughout this survey, but they were easily distinguishable from *C. armatus* based on the size and presence of eyespots and tails.

The cercarial shedding patterns of trematodes are known to vary with light stimulus; for example, *Ichtyyocotylurus erraticus* cercariae are detected in the while those of *Apatemon gracilis* are detected under in darkness [21]. Furthermore, reports on *Haplorchis pumilio* have revealed that the shedding pattern is not affected by light [13]. *S. mansoni* and *S. haematobium* cercariae are abundant at noon and sparse at night [10, 12]. In contrast, more *S. japonicum* are detected at night than during the day [11]. These studies have revealed that even cercariae of the same genus, but different species, might show different responses to light [10–12]. Our survey demonstrated a diurnal shedding pattern for *C. armatus* cercariae, with a peak at 16:00 h, which was similar to that for *S. mansoni* and *S. haematobium* cercariae.

Therefore, we suggest that *C. armatus* cercariae are mainly transmitted to *N. temminkii*, the second intermediate fish host, during the day. There was an increase in the number of *C. armatus* cercariae observed in the endemic river at 08:00 h, when the water temperature had not risen. It is reasonable to assume that cercarial shedding was started not by the stimulus of temperature but by increased light at sunrise.

Cercarial shedding is affected by both temperature and light, and increases with water temperature [13, 22]. In our study, the pattern of *C. armatus* cercarial shedding in relation to temporal and seasonal changes showed a trend similar to that of changing water temperature. According to Lo and Lee [13], the number of *C. formosanus* cercariae detected reached a maximum at a water temperature of 35°C, but no shedding was observed below 10°C [13]. Seasonal changes in cercarial shedding of *S. haematobium* have been reported by Muhoho *et al*. [19]. Cercariae of *S. haematobium* were detected between May and April (high-temperature seasons) but not at a water temperature below 25°C. These reports indicate that cercarial shedding is strongly affected by temperature. The present study was conducted throughout the year and revealed that *C. armatus* cercariae decreased with water temperature from October to December and that there was low if any detection at water temperatures below 11°C from January to April. Cercarial shedding increased again from May to September when water temperature was high. The change in water temperature may therefore have affected cercarial shedding from the first intermediate host.

Based on the above results, *C. armatus* cercarial shedding seems to be affected by light and temperature. Hence, laboratory experiments were performed to clarify the strength of the effect of these factors. The number of cercariae shed from the infected snails was more than nine times higher in the light (10,000 lux) than in darkness (0 lux). In addition, cercarial shedding was detected in light of 2,000 lux. Therefore, we concluded that the light stimulus had a strong effect on cercarial shedding. This agreed with our previous survey on the natural environment; that is, the number of cercariae increased from early morning to twilight.

Moreover, cercarial shedding was also affected by changes in temperature. The number of cercariae shed from the snails changed with a difference of 2°C in water temperature. Our results revealed that *C. armatus* was highly sensitive to temperature changes. Therefore, we compared the strength of the effect of temperature and light on cercarial shedding. Cercarial shedding from the intermediate snails responds to changes in water temperature and light [13, 22]. However, the relationship of these two factors has not yet been reported. The present study revealed that light was a stronger factor than temperature in triggering cercarial shedding.

Therefore, to determine how cercariae infect the second intermediate host, *N. temminkii*, we examined brains with a high rate of *C. armatus* metacercariae infection. In new infections of *N. temminkii* inhabiting endemic rivers, cercarial shedding began in May and rapidly increased to early July. However, in spite of the increase in cercariae, the incidence of infection decreased. This suggests that *N. temminkii* contains a defense mechanism against new infection, which may have hindered the increase in parasite infectivity. In contrast, the opportunity for infection was greater with a linear increase in negative sentinel fish (conducted in June). This shows that, based on the levels of infection within one month, there was no evidence of any defense mechanism. *Nyc. nycticorax* and other birds have been reported as the definitive host for *Centrocestus* [1–3, 7, 23]. According to past reports of *Nyc. nycticorax* in Japan, these birds had an infection rate of 67–100%, but our results for *C. armatus* showed low infection rates of 1% in herons and 5% in kites. This may have been due to the difference in parasite detection methods used; the previous studies examined hosts by autopsy, while we used only fecal examination (which was chosen because capturing wild birds is now generally prohibited). Although we cannot overlook the possibility that parasite eggs expelled in feces can disappear or become deformed, the eggs found in the two positive samples did not show any marked deformities, which suggests that the rate of parasitic infection is decreasing in recent bird populations. *C. armatus* does infect other mammals such

as hamsters [24], Hong *et al.* [5] also reported human infection. These findings show that mammals as well as birds have the potential to become the primary definitive host. However, Yanohara [7] reported that all mice collected along an endemic river were negative for *C. formosanus* infection.

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Figures and Table

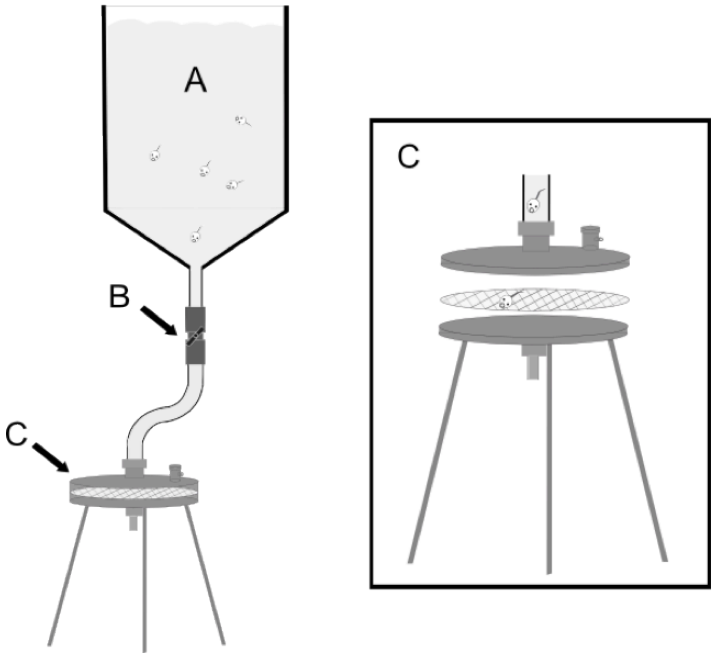

Fig. 1. Apparatus for cercariometry. It consists of three parts: A; a tank for keeping water, B; a valve, and C; a filter.

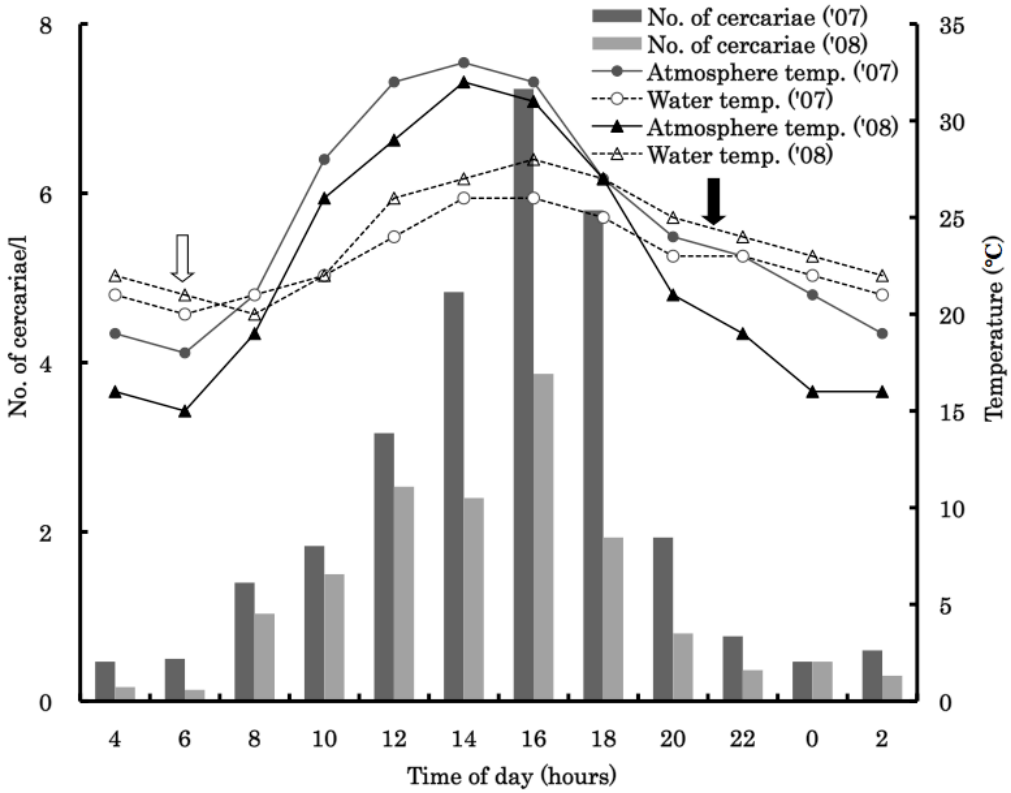

Fig. 2. Daily change in the number of *C. armatus* cercariae in the endemic river.
White arrow = Sunrise; Black arrow = Sunset.

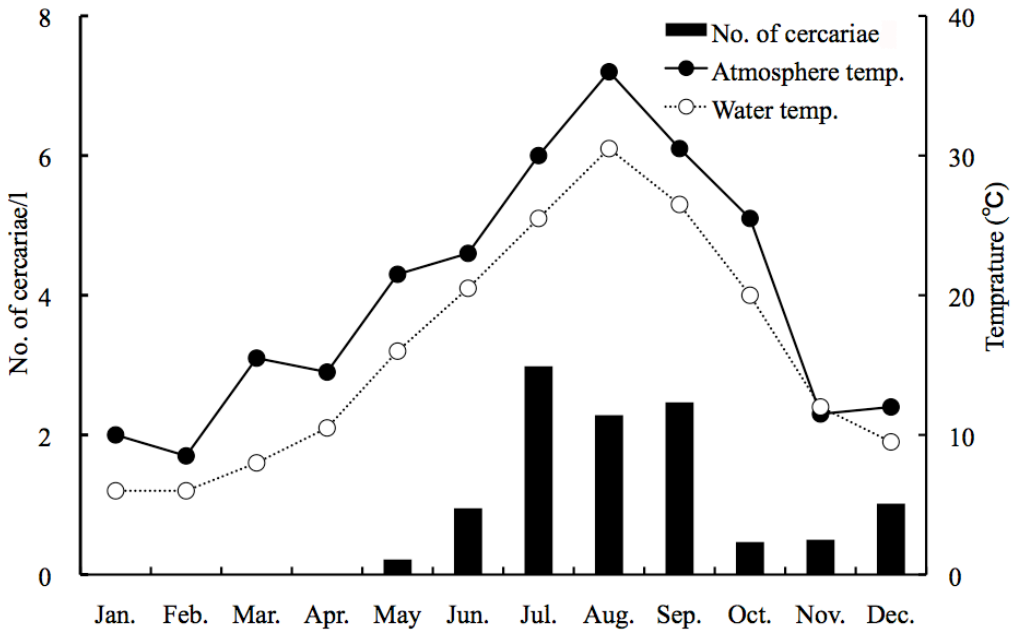

Fig. 3. Relationship between temperature and number of *C. armatus* cercariae.

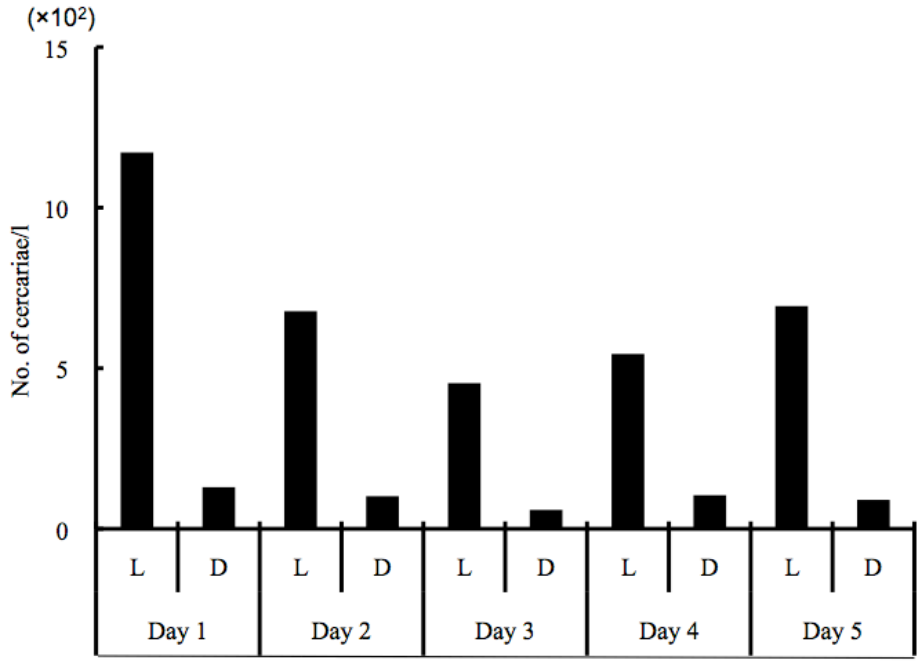

Fig. 4. Effect of light on cercarial shedding in a 5-day observation period. All experiments were performed at a water temperature of 20°C.

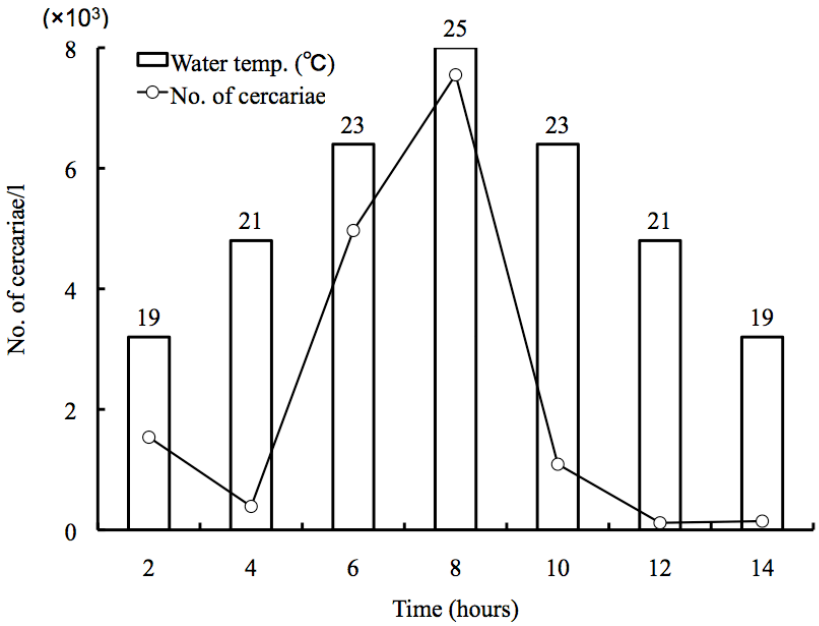

Fig. 5. Effect of temperature on cercarial shedding in a 2-h observation. All experiments were performed under light conditions (10,000 lux).

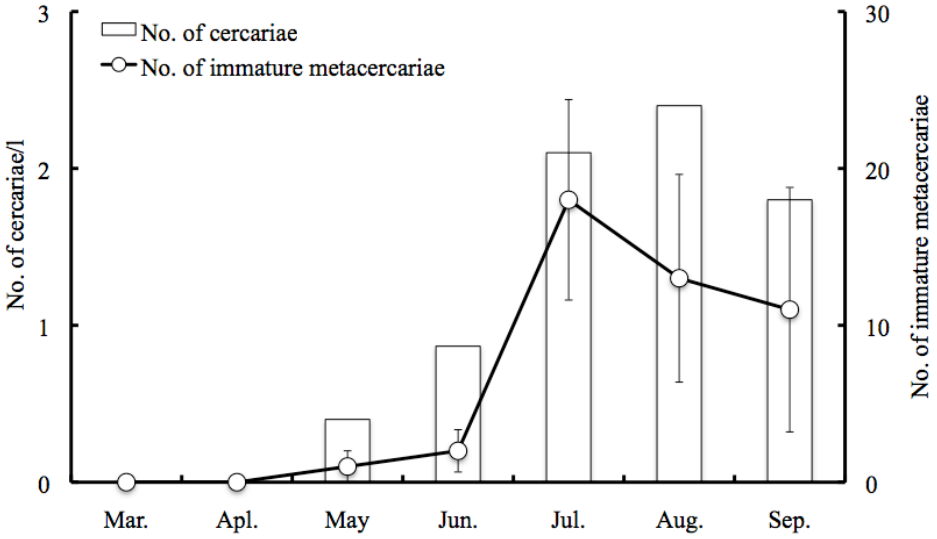

Fig. 6. New infection of metacercariae in brain of fish and the number of *C. armatus* cercariae in the endemic river. All values are mean of 10 of fishes. This experiment was carried out from March to September 2013.

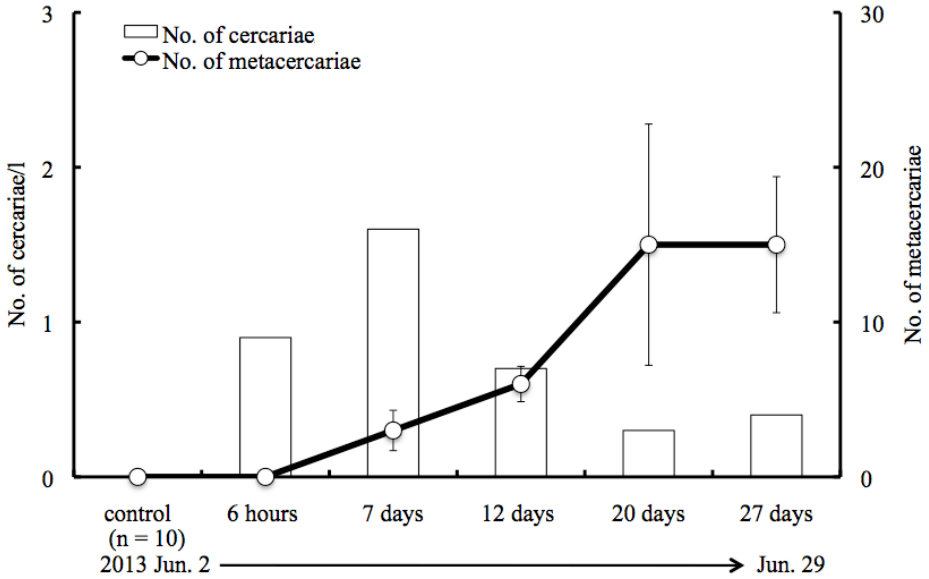

Fig. 7. Infection of negative fish in the endemic river and the number of *C. armatus* cercariae. 6 h to 27 days values are mean of five fishes.

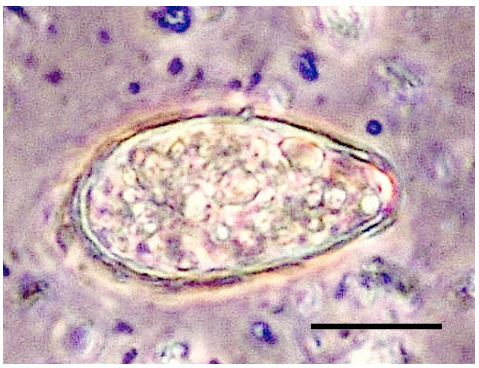

Fig. 8. An egg of *C. armatus* detected from a heron. Scale bar = 10 μm.

Table 1. Relationship between the effect of light and water temperature on cercarial shedding

		Light		Total no. of cercariae/l
		−	+	
Water temp. (°C)	23	8	82	90
	25	10	167	177
	27	12	197	209
	29	14	550	564
Total no. of cercariae/l		44	996	1,040

All values are mean of five of fishes. Bright conditions are 2,000 lux.