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Emerging Helminths Infection in Snails and Cyprinoid Fish in Sewage Treatment Wetlands Waters in Cambodia

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Abstract: The genesis of this study was to document the presence of infected freshwater snails and cyprinoid fish species that may serve as a source of trematode infection and to assess the prevalence of trematode infection in both snails and cyprinoid fish in the sewage treatment wetlands of Phnom Penh, Cambodia. The field component was conducted two times a year (dry and rainy season) from March 2007 to May 2008. Snails and cyprinoid fish samples were obtained from four sites within the Boeng Cheung Ek wetland. *Lymnaea (Radix) auricularia rubiginosa* snail was found to be infected with the trematode cercariae of Gymnocephalus cercariae (intestinal and liver parasites in herbivorous mammals). Among 15 species of infected fish, the metacercariae of the liver fluke, *Opisthorchis viverrini*, was found in this study. *Puntius orphoides* contained the highest number of metacercariae of *O. viverrini* (374.31/kg) followed by *Hampala dispar* (120.33/kg), and *Henicorhynchus* sp. (92.66/kg). The infection rate in both snails and fish exhibited a higher prevalence in the dry season than the rainy season. This investigation can help to characterize potential human health risks in the Boeng Cheung Ek wetlands of Phnom Penh.

Key words: *Opisthorchis viverrini*, *Bithynia* snails, Cyprinidae fish, wetland, Cambodia.

Introduction

Fish-borne parasitic zoonoses are mostly helminthic disease caused by trematodes, cestodes and nematodes (Chai et al., 2005). The World Health Organization (1995) has estimated that the number of people currently infected with fish-borne trematodes (FBT) exceeds 18 million, but worldwide the number of people at risk, including those in developed countries, is more than half a billion. Liver flukes, digenic trematodes dwelling in the bile duct of vertebrates, have long been known to cause serious disease in Southeast Asia. At least 13 species of liver flukes, which belong to family Opisthorchiidae, Fasciolidae and Dicrocoeliidae, have been recovered from humans. Three species that most commonly are found in humans are members of the family Opisthorchiidae including *Clonorchis sinensis*, *Opisthorchis viverrini* and *Opisthorchis felineus* (Kaewkes, 2003). *O. viverrini* is the most serious species of fish-borne zoonotic parasite and is highly prevalent in Thailand, Laos, and South Vietnam while *C. sinensis* is widely distributed and endemic in East Asia including South Korea, China (except northwestern parts), Taiwan and northern Vietnam. *O. felineus* is recognized as a natural parasite

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of dogs, cats, foxes, and pigs which can subsequently infect humans (Rim, 1982; Beaver et al., 1984).

The life cycle of *O. viverrini* is complex involving two intermediate hosts and one definitive host (Harinasuta and Vajrasthira, 1960; Wykoff et al., 1965). The first intermediate hosts are snails of the genus *Bithynia* spp., *Bithynia* (*Digoniostoma*) *funiculata*, *Bithynia* (*Digoniostoma*) *siamensis siamensis*, and *Bithynia* (*Digoniostoma*) *siamensis goniomphalos* (Lohachit, 1998). Numerous species of Cyprinidae freshwater fish can serve as second intermediate hosts. When humans consume raw or undercooked infected fish, the infective state of the liver fluke will excyst in the duodenum, and migrate through the common bile duct to the intrahepatic bile ducts and cause severe manifestations of opisthorchiasis disease associated with portal cirrhosis ascending cholangitis, bilary obstruction and cholagiocarcinoma (biliary cancer) (Jongsuksuntigul and Imsomboon, 1998; Sithithaworn and Haswell-Elkins, 2003).

Considering the climate and biomes, the geographic character of Cambodia is consistent with conditions observed in other Southeast Asian countries where liver fluke infection is well-documented and suitable as a breeding place for both bithyniid snails and cyprinoid fish. Compared with other well-studied FBT for Thailand, Laos, and Vietnam, there is relatively little data on these zoonoses and human health impact in Cambodia. Most studies have assessed the prevalence of other helminthic diseases such as schistosomiasis, fasciolosis and intestinal parasite infections in humans (Park et al., 2004; Chhakda et al., 2006; Sinuon et al., 2007; Tum et al., 2007). Therefore, an understanding about the existence of liver fluke's intermediate hosts in major water resource development schemes of Cambodia is required since the liver fluke infection can contribute to negative socioeconomic effects (e.g. lost work time due to illness) in Cambodian people, as it does elsewhere (Jongsuksontigul and Imsomboon, 1998).

The genesis of this study was to document the presence of infected freshwater snails and cyprinoid fish species that serve as a source of liver fluke infection and to assess the prevalence and intensity of trematode cercariae and metacercariae in both snails and cyprinoid fish in the Boeng Cheung Ek wetland of Phnom Penh, Cambodia. This investigation can help to characterize potential human health risks due to exposure to infected snails and fish in the treatment wetlands of Phnom Penh.

Materials and Methods

Study Areas

In view of the fact that the city of Phnom Penh, the capital city of Cambodia, does not have a conventional wastewater treatment plant, most domestic sanitary flow and stormwater runoff is discharged to the main natural wetlands—Boeng Trabek, Boeng Tumpun and Boeng Cheung Ek—located in the southern part of the city. There also is concern that sewage discharges to Boeng Poung Peay and Boeng Kak may result in trematode infection of the bithyniid snails and cyprinoid fish in these wetlands (Muong, 2003, 2004). The wetland of Boeng Cheung Ek is the largest basin. Its surface area is as large as the centre of Phnom Penh—about 2000 ha in the wet season and shrinks to 1300 ha in the dry season (Muong, 1999). Effluents from the wetlands of Boeng Trabek, Boeng Tumpun and surrounding areas are loaded into this wetland. Not only does the wetland serve a wastewater purification role, but it also is a natural and economic resource for the peri-urban community around this area (Muong, 2004).

Field Methods

The field component of this study was conducted twice a year from March 2007 to May 2008. In 2007, the samples were done in March and July and, for the year 2008, field data were obtained in March and May. These sample times represented the dry season and the early rainy season. Fish and snail samples were obtained from three sites within the Boeng Cheung Ek wetland and one site at the outlet channel from the wetland that leads to the Bassac River (Figure 1). All sites were georeferenced with a Garmin E-trex hand-held GPS receiver.

Snail samples were collected for 10 minutes by hand collection. Within each of the four general site locations, samples were collected in three different areas. All snails were put into paper bags marked with the relevant site codes and then brought back to the laboratory facilities at Resource Development International – Cambodia (RDIC) for analysis.

Samples of cyprinoid fish (as many species of fish as possible) were obtained from local fishermen near as many of the snail sample sites as possible, following the schedule for snail collection. These fish were placed in an ice-box and transported to the RDIC laboratory.

Laboratory Methods

In the lab, the snails were separated and identified, following the methods of Brandt (1974) and Upatham

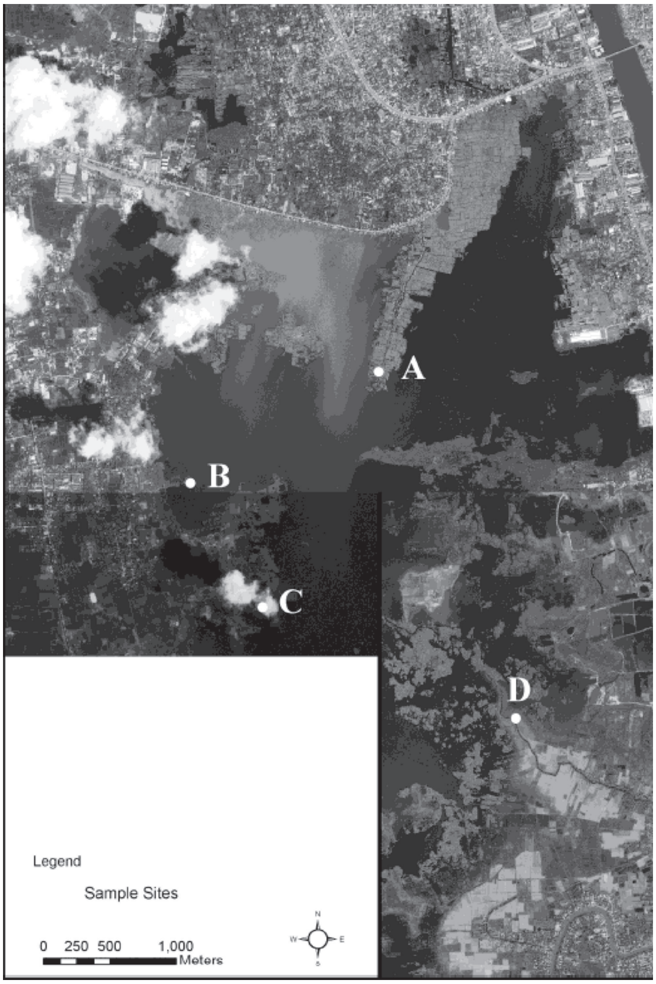


Figure 1: Ikonos satellite image displaying the snail and fish sampling sites at Boeng Cheung Ek wetland; A: middle site, B and C: western sites, and D: outlet site.

et al. (1983). All fish were identified morphologically to the species level according to the illustrations distributed by the Department of Fisheries, Ministry of Agriculture and Cooperatives, Cambodia.

Observation of Natural Emerging Trematode Cercariae from Bithynid Snails

Snails from each site were individually separated in transparent plastic cups containing the appropriate amount of tap water and exposed to the illumination of a 25-W light source at a distance of 30 cm at room temperature. The possible emergence of cercairae must be checked at regular short intervals because emerged cercariae may encyst on external substrate or re-enter the snails rather quickly. After 4-6 hours, the water in each of the cups was separately decanted into petri dishes to determine the presence of shedding cercariae under a dissecting microscope. The shed cercariae were transfered in a drop of water onto a glass slide and identified to a mature type level according to Frandsen and Christensen (1984) under a stereomicroscope at 10-40 times magnification. The number of infected snail specimens and the cercarial prevalence was recorded separately. Snails that did not shed cercariae on the first exposure were reexposed on the second day.

The Occurence of Metacercaria

The examination of metacercariae in fish was performed using the pepsin digestion method as previously described (Sukontason et al., 1999). This approach allows processing of fish in large quantity to examine the presence of metacercariae. In brief, trematode metacercariae were immediately collected using acid pepsin solution (100% hydrochloric acid 1 ml : porchine pepsin (Sigma) 1 g : 0.85% sodium chloride solution 99 ml) in a mixer blender at the ratio of 1 gram of fish : 10 ml acid pepsin solution. Firstly, fish scale, fin and bone were removed as much as possible and fish were weighed in the wet (g) to assess the exact ratio of acid pepsin solution. After blending, the digested material was transferred into a beaker and put in a water bath at 37°C for 90 minutes, then it was passed through two layers of gauze to be fractionated in a conical flask. After 30 minutes, the supernatant was discharged and the sediment was rinsed with 0.85% sodium chloride solution. This process was done twice. The occurrence of metacercariae was examined from the sediment with the stereomicroscope. The identification of metacercaria was carried out by morphological examination based on Scholz et al. (1991). Finally, the total number of metacercariae was counted for each species of fish and calculated to the number of metacercaraie per kilogram of fish.

Results and Discussion

Out of eight snails genus, only *Lymnaea* (*Radix*) *auricularia rubiginosa* obtained from site B in July, 2007, was found infected with gymnocephalus cercariae with the prevalence of 1.26% (Table 1; Figure 2). The gymnocephalous cercariae are produced by species of the family Fasciolidae, an intestinal and liver fluke in herbivorous mammals (Frandsen and Christensen, 1984). It was not possible to determine if the gymnocephalous cercariae belonged to *Fasciola hepatica* or *Fasciola gigantic*, but they both can cause Fascioliasis in humans (Aroonroch et al., 2006; Le et al., 2008). Common symptoms are epigastric pain, upper abdominal pain and malaise while fever and arthralgia are common in acute

Table 1: List of snails found in the sampling sites and observation of natural emerging trematode cercariae from snails

Snails	*Number of infected snails/ number of snails*	*Emergence of trematode cercariae (% infection rate)*
Bithynia sp.	0/27	not found
Camtoceros sp.	0/2	not found
Filopaludina sp.	0/1	not found
Gyraulus sp.	0/17	not found
Indoplanorbis sp.	0/22	not found
Lymnaea (*Radix*) *auricularia rubiginosa*	0/79	Gymnocephalus (1.26%)
Melanoides sp.	0/5	not found
Pomacae canaliculata	0/108	not found

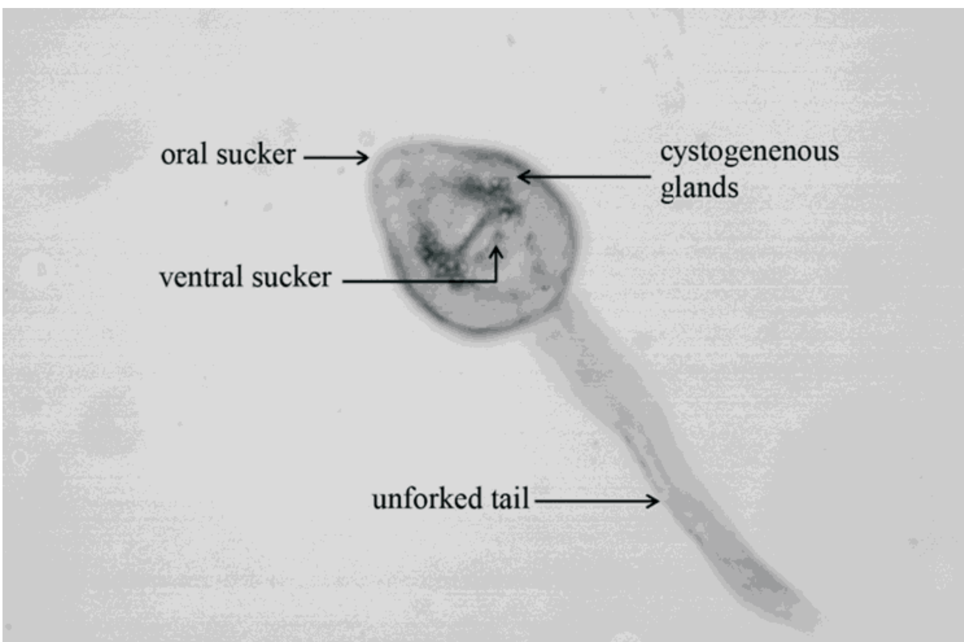


Figure 2: Gymnocephalous cercariae: tail unforked, ventral sucker on mid-ventral surface of body, neither stylet nor spiny collar present, no eyespot and numerous cystogenenous glands in body.

manifestations of the disease (Saba et al., 2004). In Cambodia, Fascioliasis caused by infection with *Fasciola gigantica* is the most serious problem in cattle and buffaloes (Tum et al., 2004; Suon et al., 2006; Tum et al., 2007). People living around the wetland should be aware of these parasites and avoid drinking water directly from the basin or eating improperly cleaned aquatic vegetables such as Morning glory and water mimosa since the fasciolid metacercarial cyst (an infective state) can float in water and become encysted on leaves of water plants (Theresa et al., 2000). In Thailand, there was evidence that this snail species had been infected with trematodes; echinostomes (an intestinal trematode) and schistosomes (a blood fluke) (Charoenchai et al., 1997).

Pomacae canaliculata or apple snails were the predominant species at every site since its pink egg clutches were frequently observed above the water line, usually on the aquatic plants. In particular, the Morning glory fields (in the dry season) and water mimosa fields (in the wet season) supported the breeding habitat of this snail. In this study, the special technique using enzyme digestion (Tesana et al., 2008) was not used to investigate the shedding cercariae in apple snails, so we did not assess the occurrence of trematode in these snails. However, it should be noted that *P. canaliculata* species have been identified as a possible intermediate host for *Angiostrongylus cantonensis*, a rat lung nematode or roundworm, that can cause eosinophilic meningitis or

Table 2: The occurrence of metacercariae in cyprinoid fish from both dry and rainy season sampling in 2007

Fish species (number of fish)	*Common name*	*Number of metacercariae/ weight of fish examined (g)*	*Number of metacercariae/kg*
Dry Season: March 2007			
(***infected fish species: 87.50%)			
Cirrhinus jullieni (N/A)	Trey krawlang	3/50	60.00
Cirrhinus microlepis (N/A)	Kralang	2/420	4.76
Osteochilus hasseltii (N/A)	Trey kros	56/683	81.99
Osteochilus melanopleurus (N/A)	Trey krom	0/125	0.00
Puntius orphoides (N/A)	Ampil tum	171/430	397.67
Puntioplites proctozystron (N/A)	Chra keng	2/30	66.67
Barbonymus gonionotus (N/A)	Chppin	5/162	30.86
Trichogaster microlepis (N/A)	Trey kamhalaenh	1/18	55.56
Rainy Season: July 2007			
(***infected fish species: 75.00%)			
Barbonymus altus (5)	Kahe	0/278	0.00
Barbonymus gonionotus (4)	Chhpin	3/211	14.2
Cyclocheilichthys furcatus (3)	Trey chhkok ploeung	9/155	58.06
Henicorhynchus sp. (5)	Trey riel	4/20	200.00
Leptobarbus hoevenii (2)	Pror loung	0/55	0.00
Osteochilus hasseltii (3)	Trey kros	3/245	12.24
Osteochilus schlegelii (7)	Lolokror	7/21	333.33
Puntius rhombeus (6)	–	9/300	30.00

Note: *** Percentage of infected fish was calculated from the number of infected fish species from the total number of sampled fish species; N/A: No available data.

Table 3: The occurrence of metacercariae in cyprinoid fish from both dry and rainy season sampling in 2008

Fish species (number of fish)	*Common name*	*Number of metacercariae/ weight of fish examined (g)*	*Number of metacercariae/kg*
Dry Season: March 2008			
(***infected fish species: 100.00%)			
Barbonymus altus (13)	Kahe	253/412	614.08
Barbonymus gonionotus (4)	Chhpin	18/299	60.02
Cyclocheilichthys furcatus (1)	Trey chhkok ploeung	4/132	30.30
Hampala dispar (3)	Trey khmann	29/171	169.59
Henicorhynchus sp. (18)	Trey riel	18/184	97.83
Osteochilus hasseltii (29)	Trey kros	54/293	184.30
Osteochilus melanopluerus (7)	Trey krom	3/115	26.00
Rainy Season: May 2008			
(***infected fish species: 87.71%)			
Barbonymus altus (7)	Kahe	27/285	94.73
Barbonymus gonionotus (7)	Chhpin	4/282	67.37
Puntioplites proctozystron (6)	Chra keng	5/80	62.50
Hampala dispar (3)	Trey khmann	0/70	0.00
Henicorhynchus sp. (5)	Trey riel	2/55	36.36
Osteochilus hasseltii (10)	Trey kros	6/196	30.61
Puntius orphoides (9)	Ampil tum	33/115	286.95

Note: *** Percentage of infected fish was calculated from the number of infected fish species from the total number of sampled fish species; N/A: No available data.

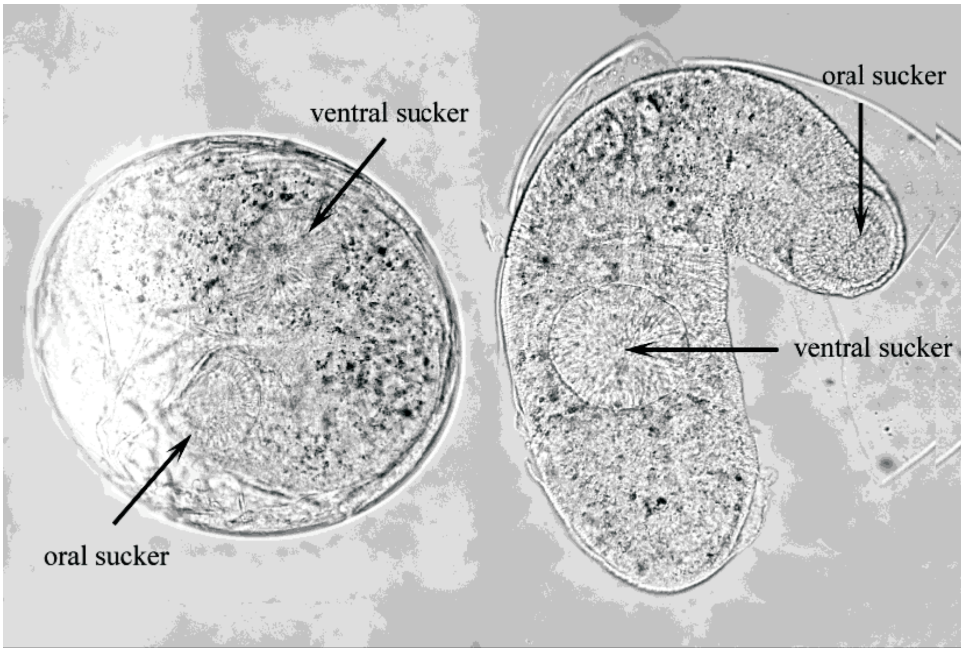
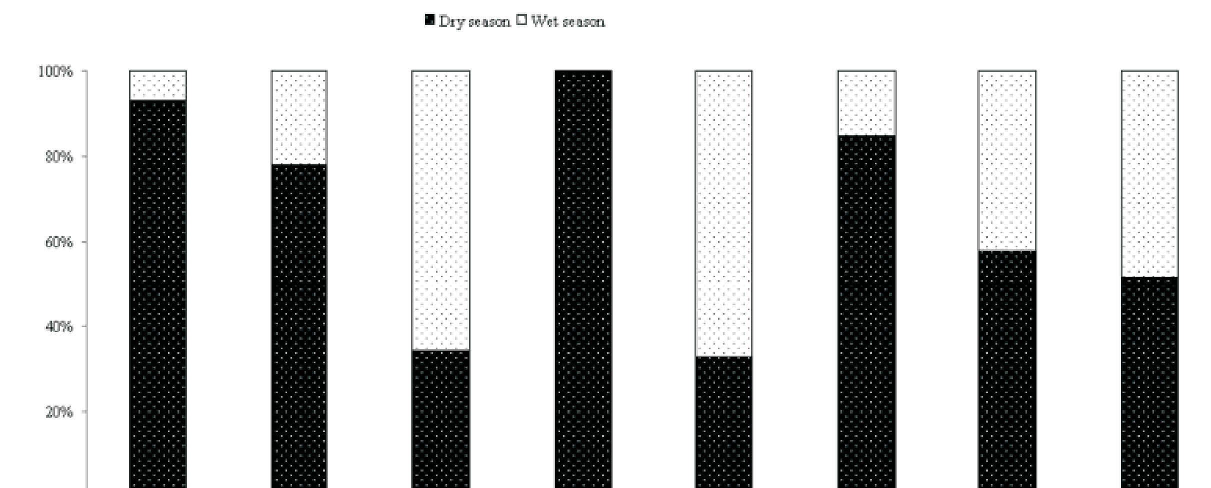


Figure 3: Metacercaria and excyst of *O. viverrini* trematode examined from cyprinoid fish; a cyst of metacercariae showing a double-walled cyat, oval in shape, oral sucker and ventral sucker are clearly seen in 50 magnification.



	B. altus	*B. gonicnotus*	*C. furcatus*	*H. dispar*	*Henicorhynchus sp.*	*O. hasseltii*	*P. orphoides*	*P. proctozystron*
Wet season	47.96	14.2	58.06	0	200	20.41	286.95	62.5
Dry season	641.08	49.19	30.3	169.59	97.83	112.7	397.67	66.67

Figure 4: Proportion of metacercarial load per kilogram found in cyprinoid fish between wet and dry seasons during 2007 and 2008. The seasonal load per kilogram is shown numerically along the bottom of the figure.

meningoencephalitis in humans, particularly in Pacific and Southeast Asian countries including Cambodia (Kliks and Palumbo, 1992; Radomyos et al., 1994; Hollingsworth and Cowie, 2006; Hochberg et al., 2007; Baheti et al., 2008; Wang et al., 2008). In Thailand, many cases of human infection with *A. cantonensis* have a history of eating *P. canalicuta* (Tesana et al., 2008). Therefore, Cambodian people should avoid eating raw or improperly cooked apple snails, as they traditionally consume this type of snail.

It was not surprising that no emerging trematode cercariae were found in the bithynid snails. In general, the natural infection rate of *O. viverrini* cercariae in *Bithynia* sp. is very low: 1.60% for *B. siamensis*; 0.005%

for *B. goniomphalos* and 0.57% for *B. funiculata* (Upatham and Sukhapanth, 1980; Chanawong and Waikagul, 1991; Ngern-klun et al., 2006). In this examination, a very small number of *Bithynia* species was found. The snails were only found in a man-made pond at site C from the first survey in March 2007. In July 2007, this site was flooded in the late dry season and no bithyniid snail could be found because of unsuitable breeding habitat. In March and May 2008, no bithyniid snails were found in these particular areas because of road construction surrounding site C and the landscape had been completely changed. However, the metacercariae of *O. viverrini* was investigated in many species of cyprinoid fish.

Among fifteen species of cyprinoid fish examined, fourteen fish species harboured the metacercariae of *O. viverrini* (Tables 2 and 3; Figure 3). Considering the fish species which were found in both dry and wet season, *Puntius orphoides* contained the highest number of metacercariae of *O. viverrini* (374.31/kg) followed by *Hampala dispar* (120.33/kg), *Henicorhynchus* sp. (92.66/kg), *Osteochilus hasseltii* (84.00/kg), *Puntioplites protozysron* (63.64/kg), *Barbonymus altus* (54.36/kg), *Cyclocheilichthys furcatus* (45.61/kg) and *Barbonymus gonionotus* (31.45/kg) (Figure 4). From this result, the highest intensity of metacercaraie of *O. viverrini* in *P. orphoides* was consistent with the findings of Touch et al. (2009) in Kandal and Takeo provinces, Southern Cambodia and Sukontason et al. (1999) in Ban Pao district, Chiang Mai Province, northern Thailand. In our discovery, the metacercariae were identified by morphology. Touch et al. (2009) confirmed the speciation of liver fluke metacercariae obtained from cyprinoid fish as *O. viverrini* by experimental infection in hamster and partial COI sequencing of the metacercairae.

The prevalence of cyprinoid fish infected with the parasite was highest in the dry season for both field surveys in 2007 and 2008 (Tables 2 and 3). This result correspond with previous reports which established that metacecarial low burden occurred in the rainy season (Vichasri et al., 1982; Sukontason et al., 1999). Wetlands have a capacity to reduce trace metals, toxic organic compounds, total dissolved solids, enteric pathogens and parasites naturally (DeBusk, 1999; Ayres et al., 1992; Stott et al., 2003). Natural wetlands can remove parasite eggs throughout the year and removal efficiency is influenced by flow-rate (Jimenez, 2007; Reinoso et al., 2008; Shalaby et al., 2008). The lower infection in the rainy season could occur as a result of the high flow-rate, freshwater pulse, into Boeng Chung Ek during the rainy season (see Visoth et al., 2010) which would both dilute the concentration of parasite eggs and may bring uninfected fish into the wetland from the Bassac River system.

Conclusion

In Cambodia, fish is the main source of protein in the Mekong basin, with an estimated average per capita consumption of 30 to 40 kg/person/year or 40-60% of the protein intake of the population (Nam and Thuok, 1999; Nam et al., 2007). This indicates the value of fish as a primary source of nutrition for Cambodian people and it is important for national food security. The results suggested that people living around the treatment wetlands, especially Boeng Cheung Ek, have a high risk of liver fluke infection. Of the three species of cyprinoid fish, *Puntius orphoides*, *H. dispar* and *Henicorhychus* sp. that had the highest intensity of *O. viverrini* metacercariae infection in this study, *Henicorhychus* sp. or Trey riel has been used to make fermented fish paste as a traditional Cambodian dish. Thorough cooking is the best way to ensure that fish are safe to consume. Fermentation for several months or a high concentration of salt and spices may not kill the parasite, as there is a report of metacercariae remaining active in fermented fish (Chuboon et al., 2005). Providing knowledge about the parasite transmission to the community along with good sanitation is also important to prevent the spread of disease for people living around the wetland. However, more study about the prevalence of liver fluke disease in humans is still needed to complete and support this exploration.

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