TEMPORAL VARIATIONS OF *OPISTHORCHIS VIVERRINI* AND OTHER TREMATODE INFECTION RATES IN *BITHYNIA SIAMENSIS SIAMENSIS* FROM *O. VIVERRINI*-ENDEMIC AREAS, CHACHOENGSAO PROVINCE,CENTRAL THAILAND

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Abstract. A field study was conducted to investigate monthly variations in cercarial trematode infections in *Bithynia siamensis siamensis* snails in an *Opisthorchis viverrini*-endemic area of Chachoengsao Province, Thailand. A total of 45, 511 snails collected each month from September 2010 to August 2011, were examined for trematode infection, resulting in overall 0.10, 4.94, 1.03, and 0.01% prevalence of *O. viverrini*, xiphidiocercaria, amphistome, and furcocercous cercaria, respectively in *B. s. siamensis*. Highest prevalence of *O. viverrini* (0.33%) and amphistome (2.13%) were observed during the dry season (mid-February to mid-May) when rice paddies were dry, while highest prevalence of xiphidiocercaria (7.92%) occurred in the rainy season (mid-May to mid-October) when rice paddies were flooded. Monthly snail density ranged from 8.40 snails/m^2 as observed in January 2011 to 32.39 snails/m^2 observed in November 2010. These observations indicated dynamics of *B. s. siamensis* snail intermediate host of liver flukes in central Thailand depended on population density, cercarial trematode prevalence and ecology.

Keywords: *Bithynia siamensis siamensis*, *Opisthorchis viverrini*, liver fluke, trematode, prevalence, central Thailand

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INTRODUCTION

Liver fluke infection caused by *Opisthorchis viverrini* is an endemic disease in Southeast Asia, especially in Cambodia, Lao People's Democratic Republic (PDR), Thailand, and Vietnam, where local people consume raw or undercooked cyprinoid fish that could be infected with metacercariae (Chanawong and Waikagul, 1991; Kaewpitoon *et al*, 2008). Infection with *O. viverrini* is believed to be an important risk factor for cholangiocarcinoma, cancer of the bile duct (Haswell-Elkins *et al*, 1992; Sripa *et al*, 2007). *O. viverrini* requires freshwater snails of genus *Bithynia* and certain species of cyprinoid fish as its first and second intermediate host respectively (Wykoff *et al*, 1965).

In Thailand, different taxa of *Bithynia* snails have exclusive geographic distributions, for example *Bithynia siamensis goniomphalos* is found in northeast Thailand, *B. funiculata* in the north, and *B.s.siamensis* in the central part of the country (Wykoff *et al*, 1965; Brandt, 1974; Harinasuta and Harinasuta, 1984). Although *O. viverrini* infection is widespread and prevalence are high in northern and northeastern regions, a lower prevalence was observed in the central part of the country (Jongsuksuntigul and Imsomboon, 2003). However, in 2009 Rangsin *et al* (2009) reported prevalence of opisthorchiasis as high as 21.6/100 person-years in Baan Na Yao Village, Sanamchaiyakate District, Chachoengsao Province, central Thailand. The population in this area originally migrated from the northeast more than 30 years ago and high prevalence of opisthorchiasis may be due to people having maintained their habit of eating raw or undercooked freshwater fish, which may be carrying *O. viverrini*.

Prevalence of *O. viverrini*-infected snail intermediate host is an important factor that affects its infection rate in humans (Nithiuthai *et al*, 2002). Several factors relating to snail biology, besides environmental factors, have been studied for their impact on this snail's prevalence, such as snail species, abundance, size, season, and habitat type (Kiatsopit *et al*, 2012; Kiatsopit *et al*, 2014; Namsanor *et al*, 2015). However, a single survey may not be able to determine the true prevalence due to the dynamic nature of these factors. Here, monthly variations in *O. viverrini* infection rates of *B. s. siamensis* snails in a fluke-endemic area of central Thailand were investigated to determine if this could be useful as an indicator of the risk of *O. viverrini* infection in humans. Variations in other trematode infections were also included because *B. s. goniomphalos* has been reported to be an intermediate host for many different species of trematodes (Nithiuthai *et al*, 2002; Phongsasakulchoti *et al*, 2005).

MATERIALS AND METHODS

Study site

The study site is located at Ban Na Yao, Tha Kradan Sub-district, Sanam Chai Khet District, Chachoengsao Province, central Thailand, approximately 158 km from Bangkok.

This area is a plateau, with mountains and canals forming natural boundaries. Agriculture forms the majority of land use, particularly rice cultivation. Six sampling locations were selected lying between latitude 11°51′14.76″ N - 11°52′7.43″N and longitude 13°31′40.35″E - 13°32′57.37″E (Fig 1). Rainy season starts from mid-May to mid-October, followed by a cool dry (mid-October to mid-February) then a hot dry (mid-February to mid-May) season.

Snail sampling

B. s. siamensis and other co-habiting snails were collected each month from September 2010 to August 2011 by handpicking or using a wire mesh scoop and involved a one man-hour search of each sampling site by three surveyors. Typical *B. s. siamensis* and other co-habiting freshwater snails collected were brought to the laboratory at room temperature within 4 hours, stored at room temperature and examined within 24 hours for cercarial infection. Monthly *B. s. siamensis* density was measured by quadrat sampling using a 50×50 cm frame over a total of 20 quadrats per site (Lohachit, 2001). All *B. s. siamensis* and other snails found on the ground (or 3 cm below ground during the dry season) within the quadrats of each survey site were placed in separate plastic bags, counted

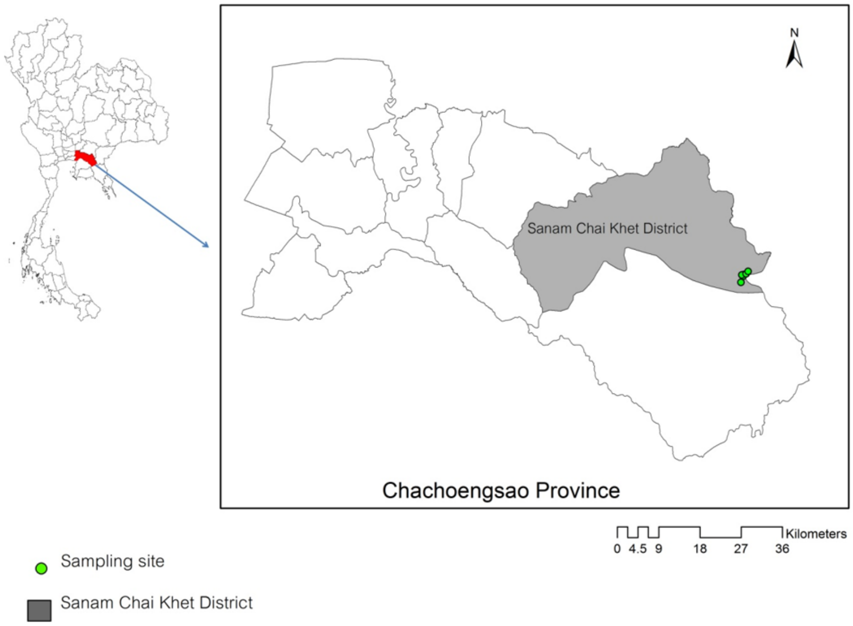

Fig 1 - Location of freshwater snail sampling sites in Chachoengsao Province, central Thailand

and 100 snails per site were randomly selected during each sampling period for identification and measurement of shell length and width. Snail identification was based on shell morphological characteristics as previous described (Chitramvong, 1992; Sripa *et al*, 2007).

Detection of cercariae

Cercarial infection in *B. s. siamensis* was determined using a light shedding method (Upatham and Sukhapanth, 1980). Snails from each sampling site were pooled and groups of 20 snails were placed in a petri dish containing 20 ml of dechlorinated water, exposed to a light source (20 W light bulb) for 2 hours (to stimulate cercarial shedding) and examined under a stereomicroscope (10x magnification). Any petri dish with no observable cercariae was stored at room temperature for 24 hours and re-examined. Snails in petri dishes with observable cercariae were removed, individually placed in wells of 96-well plates and exposed to light source for a further 2 hours. Any snail not shedding cercariae was crushed between two glass plates and digestive glands were examined for parasite sporocyst stages of under a stereomicroscope (20x magnification).

Cercaria identification

Cercariae from infected snails were identified based morphology (Schell, 1970). *O. viverrini* was confirmed by a PCR method using primers OV-6F (5′-CTGAATCTCTCGT-TTGTTCA-3′) and OV-6R (5′-GTTCCAGGTGAGTCTCTCTA-3′) (Wongratanacheewin *et al*, 2001). Genomic DNA from eight samples of fresh cercariae shed by *B. s. siamensis* snails was extracted using a Genomic DNA Tissue Mini Kit (Geneaid, New Taipei City, Taiwan ROC). Reaction mixture (25 μl) contained PCR buffer (10 mM Tris HCl pH 9.0 containing 50 mM KCl), 1.5 mM MgCl2, 100 μM of each dNTP, 400 μM of each primer, 3 μl of DNA sample, and 1.5 U Taq DNA polymerase (Invitrogen, Carlsbad, CA). Thermocycling, conducted in an Eppendorf Mastercycler (Eppendorf, Westbury, NY), was carried out as follows: 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 45 seconds; and a final heating at 72°C for 5 minutes. Amplicons (330 bp) were detected by 1.5% agarose gel-electrophoresis and staining with GelStarTM dye (Lonza, Basel, Switzerland). *O. viverrini* adult worm was used as positive control.

Statistical analysis

Descriptive statistics were used for comparing snail densities and cercarial prevalence. ANOVA test at significance level of p-value <0.05 was used to compare mean shell lengths and widths employing Statistical Package for the Social Sciences (SPSS) version 15.0 (SPSS Inc, Chicago, IL).

RESULTS

In September 2010, paddy fields at the sample sites were filled with water and contained newly planted rice, with water levels in the paddy fields decreasing from September to October, and a new round of rice planting began in April 2011 (Table 1). A typical sampling site

Table 1

Season and rice planting cycle of the sampling sites, Ban Na Yao, Tha Kradan Sub-district, Sanam Chai Khet District, Chachoengsao Province, central Thailand (September 2010 - August 2011)

Month	Season	Planting cycle	Ecosystem condition
September 2010	Wet	Planting	Irrigated, water level >50 cm
October 2010	Wet	Planting	Irrigated
November 2010	Dry	Harvesting	Water drainage
December 2010	Dry	Post-harvesting	Dry
January 2011	Dry	Land preparation	Completely dry
February 2011	Dry	Land preparation	Dry with some puddles from precipitation
March 2011	Dry	Land preparation	Dry
April 2011	Wet	Start planting	Irrigated
May 2011	Wet	Planting	Irrigated
June 2011	Wet	Planting	Irrigated, water level increased
July 2011	Wet	Planting	Irrigated
August 2011	Wet	Planting	Irrigated, water level >30 cm

cm: centimeter

paddy field ecosystem in November 2010 (cool dry season) and in May 2011 (start of rainy season) are shown in Fig 2. There were two major peaks in *B. s. siamensis* density (Fig 3), in November 2010 (32.39 snails/m^2) when the rice crop was almost ready to be harvested, with the majority of snails accumulating at paddy field edges where water was available, and in May 2011 (22.95 snails/m^2), the early part of the rainy season when rice shoots began to appear. Following harvesting of rice in December 2010 (with lowest snail density), snail habitat became drier and was completely dry by January 2011, during which snails were found underground and estivating. Snail sizes ranged 8.3-9.3 mm in length and 4.9-5.5 mm in width (Table 2). Average snail shell length and width are significantly greatest in March 2011 compared with other months observed during the study (p-value <0.001, ANOVA test). Other freshwater mollusk species co-habiting with *B. s. siamensis* were classified into eight families, 17 genera and 23 species using Brandt's key (Brandt, 1974) (Table 3).

Fig 2 - Sampling site ecosystem in November 2010 (left) and May 2011 (right) at Ban Na Yao, Tha Kradan Sub-district, Sanam Chai Khet District, Chachoengsao Province, central Thailand
Inset shows *Bithynia siamensis siamensis* on the rice leaf.

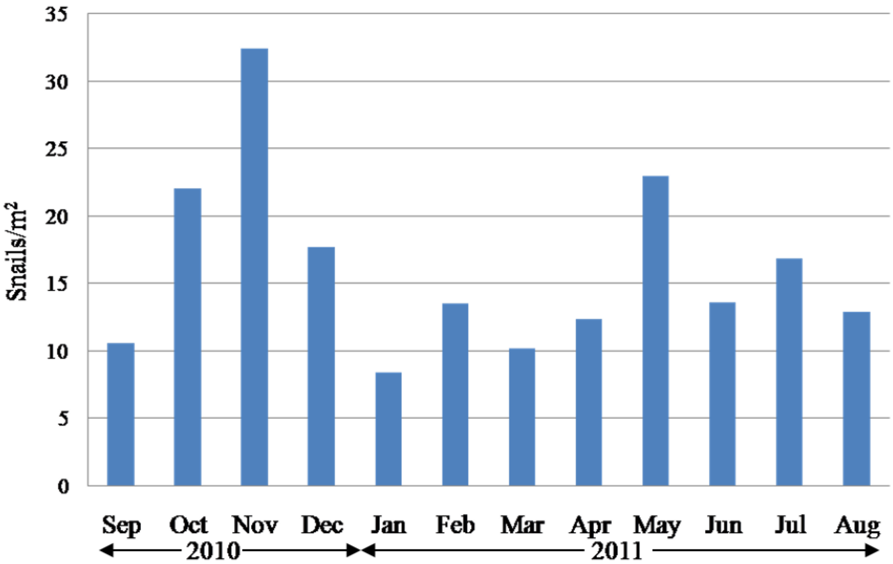

Fig 3 - Monthly *Bithynia siamensis siamensis* density at sampling sites at Ban Na Yao, Tha Kradan Sub-district, Sanam Chai Khet District, Chachoengsao Province, central Thailand (September 2010 – August 2011)

Table 2

Bithynia siamensis siamensis shell length and width collected during each sampling period at Ban Na Yao, Tha Kradan Sub-district, Sanam Chai Khet District, Chachoengsao Province, central Thailand (September 2010 – August 2011)

Month	Shell width (mm)		Shell length (mm)	
	Mean±SD*	Range	Mean±SD*	Range
September 2010	4.9±0.7	2.0-6.5	8.3±1.3	3.1-12.1
October 2010	5.1±0.5	3.1-6.4	8.6±1.1	4.9-11.2
November 2010	5.0±0.6	2.1-6.2	8.4±1.3	2.6-11.4
December 2010	5.1±0.6	2.9-6.5	8.6±1.1	4.3-11.3
January 2011	5.3±0.7	3.0-7.1	8.9±1.4	4.5-13.0
February 2011	5.1±0.7	2.7-7.1	8.7±1.3	4.6-12.1
March 2011	5.4±0.6	3.5-7.5	9.3±1.1	5.5-12.5
April 2011	5.0±0.8	2.4-6.9	8.4±1.6	3.9-12.1
May 2011	5.2±0.6	2.7-7.2	8.8±1.1	4.1-13.0
June 2011	5.2±0.6	2.7-7.1	8.5±1.3	3.8-11.5
July 2011	5.1±0.6	3.2-7.4	8.6±1.0	4.4-12.0
August 2011	4.9±0.7	2.6-7.1	8.2±1.3	3.7-12.3
Total	5.1±0.7	2.0-7.5	8.6±1.3	2.6-13.0

*Number of snails per sampling period = 600
mm: millimeter; SD: standard deviation

A total of 45,511 live *B .s. siamensis* were examined for natural cercarial infection, consisting of *O. viverrini*, *Amphistome* sp, xiphidiocercariae, and furcocercous (Table 4). Overall cercarial infection rate of *B. s. siamensis* was 6.07%, with highest infection rate observed in June 2011 and lowest in September 2010. The most common type of cercariae was xiphidiocercaria, with infection rates ranging from 1.24% in September 2010 to 7.92% in June 2011. *Amphistome* cercariae were observed at every sampling period, lowest (0.17%) in October 2010 and highest (2.13%) in January 2021. *O. viverrini* infection rate was low throughout the year, ranging from 0% in September 2010 to 0.33% in March 2011. Only three snails shed furcocercous cercariae, one infected snail each collected in January, May and July 2011 (overall infection rate of 0.01%). No co-infection with different cercaria species were observed.

Table 3

Freshwater mollusks co-habiting with *Bithynia siamensis siamensis* collected from rice paddy fields at Ban Na Yao, Tha Kradan Sub-district, Sanam Chai Khet District, Chachoengsao Province, central Thailand (September 2010 - August 2011)

Family	Scientific name
Amblemidae	*Pilsbryoconcha* sp
	Scabies sp
	Uniandra sp
Ampullariidae	*Pila ampullacea*
	Pila pesmei
	Pila polita
	Pomacea canaliculata
Bithyniidae	*Bithynia siamensis siamensis*
	Wattebledia sp
Buccinidae	*Clea helena*
	Gabbia sp
Lymnaeidae	*Radix rubiginosa*
Planorbidae	*Indoplanorbis exustus*
	Gyraulus convexiusculus
Thiaridae	*Melanoides tuberculata*
	Tarebia granifera
	Thiara scabra
Viviparidae	*Filopaludina (Siamopaludina) martensi martensi*
	Filopaludina (Siamopaludina) martensi cambodjensis
	Filopaludina (Filopaludina) sumatrensis polygramma
	Filopaludina (Filopaludina) sumatrensis speciosa
	Idiopoma umbilicata
	Trochotaia trochoides

Table 4

Infection rate of *Bithynia siamensis siamensis* according to cercaria species collected at sampling sites at Ban Na Yao, Tha Kradan Sub-district, Sanam Chai Khet District, Chachoengsao Province, central Thailand (September 2010 – August 2011)

Month	Number of snails	*Opisthorchis viverrini* cercaria		Xiphidiocercaria		*Amphistome* sp		Furcocercous		All cercaria species	
		Number of infected snails	Infection rate (%)	Number of infected snails	Infection rate (%)	Number of infected snails	Infection rate (%)	Number of infected snails	Infection rate (%)	Number of infected snails	Infection rate (%)
September 2010	2,784	0	0.0	34	1.2	7	0.2	0	0.0	41	15
October 2010	5,954	2	<0.1	106	1.8	10	0.2	0	0.0	118	2.0
November 2010	5,036	6	0.1	376	7.5	60	1.2	0	0.0	442	8.8
December 2010	4,891	5	0.1	240	4.9	93	1.9	0	0.0	338	6.9
January 2011	2,677	3	0.1	163	6.1	57	2.1	1	<0.1	224	8.4
February 2011	3,281	1	<0.1	143	4.4	21	0.6	0	0.0	165	5.0
March 2011	2,438	8	0.3	157	6.4	44	1.8	0	0.0	209	8.6
April 2011	3,888	2	<0.1	186	4.8	31	0.8	0	0.0	219	5.6
May 2011	4,690	6	0.1	307	6.5	65	1.4	1	<0.1	379	8.1
June 2011	3,548	6	0.2	281	7.9	44	1.2	0	0.0	331	9.3
July 2011	3,851	4	0.1	157	4.1	24	0.6	1	<0.1	186	4.8
August 2011	2,509	1	<0.1	96	3.8	14	0.6	0	0.0	111	4.4
Total	45,511	44	0.1	2,246	4.9	470	1.0	3	0.01	2,763	6.1

DISCUSSION

The abundance of intermediate hosts is an important factor that facilitates trematode life cycle (Wang *et al*, 2015; Kim *et al*, 2016). Several environmental factors can have an impact on intermediate host population, such as season, water quality and water level. Seasonal variations in snail abundance can also vary based on sampling location and habitat type. Although our 1-year study found two peaks of snail density, namely, in the cool dry (November 2010) and hot dry (May 2011) seasons, highest abundance of *B. funiculata* in northern Thailand and *B. s. goniomphalos* in northeastern Thailand were reported during the rainy season (July-October) (Ngern-klun *et al*, 2006; Kim *et al* 2016). Similar to our study, where sampling sites are irrigated, highest numbers of *B. s. goniomphalos* are found, eg during cool dry season (Namsanor *et al*, 2015) and hot season (Chaiyasaeng *et al*, 2019). Snail population is related to a water level suitable for their preferred habitat, either after rice planting or before harvesting (Wang *et al*, 2015). Water quality, such as salinity and nitrate content, is positively correlated with *Bithynia* spp abundance (Kim *et al*, 2016).

The lowest snail density was observed in January 2011, during a dry season, when snails burrow underground to a depth of up to 10 cm, close their operculum and enter estivation to protect themselves from the unfavorable conditions (Brockelman *et al*, 1986; Chaiyasaeng *et al*, 2019). This behavior might explain the small numbers of snails detected during this period of the year.

Our study demonstrates a diversity of mollusks co-habiting with *B. s. goniomphalos* in the rice paddy sampling habitat. In northeastern Thailand, while *B. s. goniomphalos* is the most abundant species, other snails found in the same habitat include *Pomacea canaliculata* and *Filopaludina martensi martensi* (Wang *et al*, 2015).

Factors related to *O. viverrini* infection in snails include season, water temperature, light intensity, habitat, and size and age of snails (Chanawong and Waikagul, 1991; Kaewkes *et al*, 2012; Piratae, 2015; Prasopdee *et al*, 2015). We found shell width and length are significantly greater during March 2011 observation, which coincided with the highest infection rate and support the notion that larger snails play a more important role in disease transmission (Brockelman *et al*, 1986). On the other hand, in an experimental study, smaller size snails are more susceptible to *O. viverrini* infection than their larger counterparts (Prasopdee *et al*, 2015). A higher percentage of other trematode infections was also reported in smaller or younger *Bithynia* spp (Chanawong and Waikagul, 1991; Kulsantiwong *et al*, 2017). Different in prevalence may result from various factors such as collection time, season, trematode types and water quality (Kulsantiwong *et al*, 2017). The effect of habitat on prevalence of *O. viverrini* infection in snail host is manifested in a study in Udon Thani Province, upper northeastern Thailand that revealed highest prevalence of trematode cercariae is present in samples from a reservoir, followed by samples from swamp, then canal, rice paddy field and river (Kulsantiwong *et al*, 2017). However, the highest recorded *O. viverrini* prevalence (6.93%) was in a rice paddy field in Sakon Nakhon Province, northeastern Thailand (Kiatsopit *et al*, 2012).

Monthly rates of trematode infections in *B. s. siamensis* depended on trematode species. The highest infection rate of xiphidiocercaria occurred in June 2011, when rain had already begun, whereas highest prevalence of other trematodes occurred during the dry season, which could be related to decreased volume of water, together with an increasing number of snail hosts, thereby promoting infection. On the other hand, eggs may be dispersed and washed into bodies of water more efficiently during the rainy season (Namsanor *et al*, 2015).

Monthly *O. viverrini* infection rate in *B. s. siamensis* was low, similar to studies in other endemic areas of Thailand (0.03-2.0%) (Upatham and Sukhapanth, 1980; Brockelman *et al*, 1986; Adam *et al*, 1993; Lohachit, 2001; Sri-aroon *et al*, 2005; Ngern-klun *et al*, 2006). Although trematode infection rates of *B. s. siamensis* were low, their susceptibility to *O. viverrini* was 4-7 folds higher than that of *B. s. goniomphalos*. Thus, transmission of *O. viverrini* in non-endemic areas in central Thailand, where residing northeastern people still maintain their traditional eating habits, should not be overlooked (Chanawong and Waikagul, 1991). Snails infected with *O. viverrini* were detected in almost every months during the study period except in September 2010. The prevalence of *O. viverrini* and other trematodes detected in the present study may have been underestimated because of the classical method used for cercariae detection (Born-Torrijos *et al*, 2014).

Bithyniidae has been reported to have the ability to harbor different species of trematode cercaria: in northeastern Thailand, 3-10 types of cercariae (amphistome, cystophorous, echinostome, furcocercous, monostome, mutabile, parapleurolophocercous, pleurolophocercous, and virgulate ophthalmoxiphidiocercariae) were reported to infect *B. s. goniomphalos* (Nithiuthai *et al*, 2002; Kiatsopit *et al*, 2012; Kiatsopit *et al*, 2014; Kiatsopit *et al*, 2015); in northeastern Thailand and PDR Lao, the most common cercaria in *B. s. goniomphalos* is virgulate ophthalmoxiphidiocercariae, with average prevalence of 10.90 and 6.58% respectively (Kiatsopit *et al*, 2015); and central Thailand, our study found four different types of cercariae present in *B. s. siamensis*. Diversity of trematodes in *B. s. siamensis* was reported to be lower than in *B. s. goniomphalos*, six types of cercariae infecting *B. s. goniomphalos* compared to only two types, monostome (1.10% prevalence) and virgulate (0.55% prevalence), in *B. s. siamensis* from northern and central Thailand (Kulsantiwong *et al*, 2015). However, a higher diversity of trematode cercariae was observed in *B. s. siamensis* collected from the Chao Phraya Basin, central Thailand, including cercariae, furcocercous, monostome, parapleurolophocercous, pleurolophocercous, virgulate, and xiphidiocercaria (most common), with a total prevalence of 8.13% (Anucherngchai *et al*, 2016), similar to our study. Different types of cercariae in snail intermediate hosts indicate possible different interactions with animal hosts. A high diversity of trematodes may reflect the diversity of freshwater ecosystems in these two latter surveys.

Co-infection of cercariae in a single snail is rare. For example, Lymnaeid snails, *Biomphalaria glabrata* and *Indoplanorbis exustus* have been found to harbor two species of cercariae (Sandland *et al*, 2007; Martin and Cabrera, 2018). There has been no evidence of trematode co-infection in individual *Bithynia* sp snails (Chontananarth and Wongsawad, 2013; Kiatsopit *et al*, 2015; Kulsantiwong *et al*, 2015; Namsanor *et al*, 2015), including our study, although *Bithynia* sp is known to be susceptible to infection by various types of cercariae (Nithiuthai *et al*, 2002; Kiatsopit *et al*, 2015). Parasite competition and increased mortality of snail hosts following multiple infections may adversely affect the number of co-infected snails in the natural setting (Thiele and Minchella, 2013; Martin and Cabrera, 2018).

In conclusion, the study provides a year-long set of data on *O. viverrini* and other trematode infections of *B. s. siamensis* in Chachoengsao Province, central Thailand. Monthly prevalence and infection rates in relationship with the rice planting cycle were highlighted. These findings should be of use in developing public health policies on control and prevention of trematode infection in humans living in the rice growing regions of the central region of the country.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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