รายงานวิจัยฉบับสมบูรณ์

การเกิดลูกผสมของหอยที่เป็นโฮสต์กลางของพยาธิใบไม้ตับ *Opisthorchis viverrini* และความไวต่อการติดเชื้อของหอยลูกผสม

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สัญญาเลขที่ TRG 5880212

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และต้นสังกัด

บทคัดย่อ

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หอยไซเป็นหอยที่มีความสำคัญทางการแพทย์เนื่องจากเป็นตัวกลางของพยาธิใบไม้ตับชนิด *Opisthorchis viverrini* ในการส่งต่อสู่คน ในปี ค.ศ. 2011 ประเทศไทยประสบอุทกภัยครั้งใหญ่ อาจทำให้เกิดหอยไซลูกผสมได้โดยเฉพาะในพื้นที่ระหว่างภูมิภาค เนื่องจากในปัจจุบันการระบุชนิดของหอยไซยังคงใช้สัณฐานวิทยาของเปลือกหอยร่วมกับพื้นที่อาศัยของหอย ดังนั้นจากเหตุการณ์อุทกภัยดังกล่าว การเกิดลูกผสมของหอยจึงเป็นที่น่ากังวล ดังนั้นในการศึกษาครั้งนี้ผู้วิจัยได้ทำการเก็บตัวอย่างหอยในสกุล *Bithynia* ตามภูมิภาคต่างๆ ในประเทศไทย เพื่อศึกษาการเกิดหอยลูกผสม นอกจากนี้ยังได้ศึกษาความไวต่อการติดเชื้อพยาธิใบไม้ตับของหอยในสกุล *Bithynia* ที่พบในประเทศไทยด้วย โดยศึกษาความไวต่อการติดเชื้อ ณ ช่วงเวลา 1 7 14 28 และ 56 วัน หลังการติดเชื้อ จากผลการการสำรวจและเก็บตัวอย่างหอยในสกุล *Bithynia* ในประเทศไทยพบว่า ไม่มีการเกิดหอยลูกผสมตามธรรมชาติ สำหรับการติดเชื้อหอยด้วยพยาธิใบไม้ตับ *O. viverrini* เพื่อทดสอบความไวต่อการติดเชื้อของหอยในสกุล *Bithynia* ในห้องปฏิบัติการพบว่า หอยไซทั้ง 2 ชนิด คือ *Bithynia funiculata* และ *B. siamensis siamensis* มีความไวต่อการติดเชื้อสูงมากในช่วงวันแรกๆ ของการติดเชื้อ (1 และ 7 วันหลังการติดเชื้อ) จากนั้นความไวต่อการติดเชื้อลดลงอย่างเห็นชัด นอกจากนี้ ณ วันที่ 56 หลังการติดเชื้อ พบว่าหอยไซชนิด *B. funiculata* มีความไวต่อการติดเชื้อสูงถึง 25% ในขณะที่ *B. siamensis siamensis* ไม่พบการติดเชื้อเลย จากผลการศึกษาสามารถสรุปในเบื้องต้นได้ว่าถึงแม้จะไม่พบหอยลูกผสมเกิดขึ้นตามธรรมชาติ อย่างไรก็ตามหอยในสกุล *Bithynia* ที่พบในประเทศไทยมีความไวที่เพียงพอต่อการติดเชื้อและส่งต่อพยาธิใบไม้ตับไปสู่คนโดยเฉพาะหอยไซชนิด *B. funiculata* ดังนั้นการเฝ้าระวังการติดเชื้อพยาธิใบไม้ตับในหอยจึงเป็นสิ่งที่ไม่ควรละเลย

คำหลัก: พยาธิใบไม้ตับ, หอยไซ, ลูกผสม, ความไวต่อการติดเชื้อ, มะเร็งท่อน้ำดี

Abstract

Project Code: TRG 5880212

Project Title: Hybridism of Snail Intermediate Hosts of the Carcinogenic Liver Fluke, *Opisthorchis viverrini* and Their Susceptibility to Infection

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Project Period: 2 Years

Among snail species acting as hosts for parasites, three taxa of *Bithynia* are responsible for transmission of the carcinogenic liver fluke *Opisthorchis viverrini* to humans in Thailand. Due to overlapping areas of geographic distribution of the snails and flooding event in 2011, occurrence of hybrid snails is now considered. To indicate a risk of increasing prevalence of *O. viverrini* infection and introducing *O. viverrini* into new areas, this study aimed to 1) investigate the hybridism between snails of genus *Bithynia* and 2) to verify the susceptibility of the snails to *O. viverrini* infection. In order to investigate the snail hybridisms, *Bithynia* snails were sampling from different parts of Thailand especially border area between the regions of Thailand including central-northeastern area. In addition, susceptibility to *O. viverrini* infection among *Bithynia* taxa was investigated throughout time course of 1, 7, 14, 28 and 56 day post infection (dpi). Based on field survey, there was no occurrence of *Bithynia* snail hybridisms found in Thailand. The susceptibility to *O. viverrini* infection in both *B. siamensis siamensis* and *B. funiculata* was high at early period of infection (1 and 7 day dpi) thereafter dramatically declined at extra period of dpi. Interestingly, at 56 dpi, the susceptibility to *O. viverrini* infection in *B. funiculata* (25%) was shown higher than in *B. siamensis siamensis* where the infection was not observed. In conclusion, there was no occurrence of hybridism in *Bithynia* snail taxa in Thailand. However, this is solely based on field survey study. The experimental study should be further investigated in order to find an evidence of hybridism in *Bithynia* snails. However, the surveillance of *O. viverrini* infection in snail hosts should not be ignored as the high susceptibility to *O. viverrini* infection in *B. funiculata* was observed.

Keywords: *Opisthorchis viverrini*, *Bithynia*, hybridism, susceptibility, choloangiocarcinoma

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CHAPTER I

INTRODUCTION

1. Introduction to the research problem and its significance

Floods are the most common natural disasters in both developed and developing countries (Ohl, Tapsell, 2000; Coker et al., 2011). The severe flood in Thailand occurred in 2011 devastated the large part of Thailand. In total, 65 of 77 provinces of Thailand were impacted particularly in the north, northeast and central Thailand. The flood resulted in 815 deaths and millions of residents were either left homeless or displaced (Ngaosuwankul et al., 2013). Floods are associated with many outbreaks of infectious diseases especially water-borne diseases such as typhoid fever, hepatitis, cholera and diarrheal diseases (Kondo et al., 2002; Harris et al., 2008; Carrel et al., 2010). The pattern of prevalence of not only water-borne diseases but also vector-borne diseases appears to have changed after the floods (Cardenas et al., 2011; Harrison et al., 2009). Snail-borne infections, is one of the vector-borne diseases, have not been studied yet although fresh water snails serve as hosts/intermediate hosts for numerous species of parasites. Among snail species acting as hosts for parasites, *Bithynia* species are responsible for transmission of the carcinogenic liver fluke *Opisthorchis viverrini* to humans. More than 10 million people worldwide are infected by *O. viverrini*, which is classified as a group 1 carcinogen (IARC 1994, 2011).

In Thailand, three taxa of *Bithynia* have been reported as natural first intermediate host of *O. viverrini* in different geographical habitats; *Bithynia funiculata* in the north, *B. siamensis siamensis* in the central and the north and *B. siamensis* *goniomphalos* in the northeast region (Wykoff et al., 1965; Brandt, 1974). The life cycle of the parasite requires contamination of a water body with feces containing parasite eggs of an infected definitive host. The snails become infected by ingesting the fully developed eggs. Miracidia hatch from the ingested eggs and then penetrate snail tissue to develop into sporocysts. Asexually reproduction and development generate large numbers of rediae and cercariae the latter of which are released from the snail host. The released cercariae become metacercariae in fish and adult stage in fish-eating mammals, respectively. Seasonal variation was influenced by environmental conditions, especially duration and quantity of rainfall, played important roles in a complex interplay between host and parasite (Brockelman et al., 1986). Additionally, the population dynamics of *Bithynia* snails fluctuated according to rainfall, with *O. viverrini* infection occurring almost throughout the year (Upatham, Sukhapanth, 1980). The natural infection rates of *O. viverrini* in *Bithynia* snails were varied from 0.083 to 3.04% (Wykoff et al., 1965; Upatham, Sukhapanth, 1980; Brockelman et al., 1986; Sri-aroon et al., 2005; Kiatsopit et al., 2012; Prasopdee et al., 2013). In laboratory infection, *B. funiculata* and *B. siamensis siamensis* were 4-7 times more susceptible to *O. viverrini* than *B. siamensis goniomphalos* (Chanawong, Waikagul, 1991).

Species identification of *Bithynia* is basically based on anatomical and morphological characteristics together with geographic distribution (Wykoff et al., 1965; Brandt, 1974). However, the geographic distribution alone was often used for identification of closely related species of the snails (Kulsantiwong et al., 2013). Due to overlapping of geographic distribution areas of the *Bithynia* snails and furthermore the flooding event in 2011, possibility of hybridization of them and occurrence of hybrid snails is now considered. Particularly, hybrids between high susceptible species of *B. siamensis siamensis* and *B. funiculata* will be focused. Previously, hybridism between snail species was proved that can be occurred naturally and experimentally (Chi et al., 1971; Yousif et al., 1998; Facon et al., 2005; Teodoro et al., 2011). Moreover, susceptibility of hybrids to parasite infections can be considered potential hosts of parasites (Chi et al., 1971; Teodoro et al., 2011). This project aims to investigate the hybridism between snails of genus *Bithynia* and to verify the susceptibility of hybrids to *O. viverrini* infection. Moreover, infectivity of *O. viverrini* infection to parents and hybrids is also compared. The project will result in a fundamental aspect of the biology of snails acting as intermediate hosts of a medically important parasite. The data generated from this project will be of great benefit for parasitologist and malacologist to indicate a risk of increasing prevalence of *O. viverrini* infection and introducing *O. viverrini* into new areas.

2. Objectives

2.1 To investigate the hybridism between *B. siamensis siamensis*, *B. siamensis goniomphalos* and *B. funiculata*

2.2 To verify susceptibility of *Bithynia* snails to *O. viverrini* infection

3. Research plan

Study	Work	2015-2016												2016-2017											
		July	August	September	October	November	December	January	February	March	April	May	June	July	August	September	October	November	December	January	February	March	April	May	June
1. Occurrence of *Bithynia* snail hybridisms	- Field survey and sampling of hybrid snails - Cercarial shedding and snail dissection - DNA extraction and species specific primers PCR - Producing a laboratory hybrid snails - Data analysis																								
2. Susceptibility of *Bithynia* snails to *O. viverrini* infection	- Preparation of *Bithynia* snails - Preparation of *O. viverrini* eggs - Testing of susceptibility - Data analysis - Preparation of manuscripts																								
3. Data analysis and Preparation of full report and manuscripts	- Data analysis and preparation of full report and manuscripts																								

4. Expected benefits

4.1 Providing, for the first time, a hybridism of *Bithynia* snails in Thailand.

4.2 Providing a great benefit for parasitologist and malacologist to indicate a risk of increasing prevalence of *Bithynia*-borne *O. viverrini* infection.

CHAPTER II

LITERATURE REVIEWS

5.1 Morphology, life cycle and epidemiology of *O. viverrini*

The adult *O. viverrini* parasite is monoecious, elongated, dorso-ventrally flattened and lancet-shaped with an average body size of 7.0 × 1.5 mm. The oral sucker is situated subterminal end, while the ventral sucker is on the ventral side at approximately one-fourth of the body length from the anterior end. The deeply lobed testes situated diagonally, located near the posterior extremity. The multilobulated ovary is situated in front of the anterior testis. The long, tightly packed coiled and eggs filled uterus is located between ventral sucker and ovary. The genital orifice is opened directly in front of the ventral sucker. The digestive tract comprises of the muscular pharynx behind the oral sucker and a short esophagus followed by bifurcated intestinal ceca which run nearly to the posterior end. The vitellaria posed in the lateral fields between ventral sucker and testes and consist of numerous follicles organized as several columns. The long excretory bladder runs S-shaped between the two testes (Sadun, 1955).

The adult stage of *O. viverrini* resides in the secondary bile ducts and may be found in common bile duct, cystic duct and gall bladder in case of heavy infection. Embryonated eggs are laid from mature adult worms into the biliary system and excreted with the feces. *Bithynia* snails may find and ingest the parasite eggs if the contaminated feces pollute natural water reservoirs and miracidia will hatch from the ingested embryonated eggs in the snail digestive tract. The hatched miracidia penetrate through the snail tissue and develop into sporocysts. The sporocysts asexually reproduce and develop to rediae which then produce cercariae. Free swimming cercariae are released from infected snails passing with expiration water from the mantle cavity. The cercariae attach to and penetrate the flesh of the second intermediate host, cyprinid fish (about 18 species are susceptible), they shed their tails and encyst to become metacercariae (Harinasuta C, Harinasuta T, 1984; WHO, 1995; Waikagul, 1998). The Metacercaria is an infective stage to humans and other fish-eating mammals such as cats and dogs.

Opisthorchiasis due to *O. viverrini* infection is mainly endemic to South East Asia. The number of infected people was approximately 10 million of people (WHO, 1995; Jongsuksuntigul, Imsomboon, 2003). In Thailand, the high endemic country for *O. viverrini* infection, it was estimated that 6 million people were infected (Jongsuksunsigul, Imsomboon, 2003). It was estimated that 1.7 million people in Laos were infected with *O. viverrini* (WHO, 1995). Moreover, Cambodia and the southern region of Vietnam have also been reported to be endemic for *O. viverrini* (Lee et al., 2002; De et al., 2003).

5.2 Correlation of opisthorchiasis and cholangiocarcinoma

The majority of persons infected with *O. viverrini* are symptomless. Nevertheless, a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, periportal fibrosis, cholecystitis and cholelithiasis were associated with heavy and chronic infection (Harinasuta C, Harinasuta T, 1984; Osman et al., 1998; Mairiang E, Mairiang P, 2003; Sripa et al., 2005, 2007). Moreover, the infection was found to be a significant risk factor for development of cholangiocarcinoma (CCA) in humans (Haswell-Elkins et al., 1992) and was classified into carcinogenic group 1 (IARC, 1994, 2011). Chronic inflammation as a result of chronic infection is accepted to be involved in carcinogenesis of CCA (Holzinger et al., 1999; Sirica, 2005; Kawanishi, Hiraku, 2006). The occurrence of CCA in Thailand had a strongly positive correlation with the prevalence of *O. viverrini* infection (Srivatanakul et al., 1991; Sriamporn et al., 2004). The incidence of CCA in Udornthani and Khon Kaen Provinces, northeast Thailand is the highest in the world (Vatanasapt et al., 1990; Parkin et al., 2002; Sriamporn et al., 2004; Khuhaprema, Srivatanakul, 2007), where favorite dishes of raw, fermented or undercooked cyprinid fish are the sources of infection.

5.3 Classification, ecology and geographic distribution of *Bithynia* snails in Thailand

Bithynia snails are classified in phylum Molluska, class Gastropoda, subclass Prosobranchia, order Mesogastropoda, family Bithyniidae and genus *Bithynia*. In Thailand, three taxa of *Bithynia* have been reported as natural first intermediate hosts for *O. viverrini* are *B. siamensis goniomphalos*, *B. siamensis siamnesis* and *B. funiculata*. The *B. siamensis goniomphalos* were found in northeast Thailand (Brandt, 1974). It lives in various habitats ranging from shallow, mud, rocks, temporary pond and mashed to permanent reservoirs, rice paddy field and the edge of water reservoirs with the depth of water less than 30 cm (Papasarathorn et al., 1980; Brockelman et al., 1986;

Chitramvong, 1992), some of them were found at depths up to 3 m (Suwannatrai et al., 2011). The *B. siamensis siamensis* were found in artificial ponds and preferred to attach to artificial pond plants such as grasses, weeds, sticks and the beneath of lotus leaves. The snails populations were larger recovered on a substrate in the level of water surface than on the mud bottom of the pond (Chitramvong, 1992; Upatham, Sukhapanth, 1980). The *B. funiculata* were widely spread in a rice field especially on a mud substrate in the north of Thailand (Brandt, 1974; Kulsantiwong et al., 2013). The sexes of snails can be distinguished by the presence of a penis or verge on the right side of the neck of the males (Kruatrachue et al., 1982).

5.4 Species identification of *Bithynia* snails in Thailand

Species of *Bithynia* snails is practically identified based on shell morphology. The *Bithynia* snail is different from other genera with the presence of a more or less strong carina around the umbilicus and an angled base of the peristome. *B. siamensis goniomphalos* differs from *B. funiculata* by a narrower umbilicus with a much weaker carina and slender shape. The shell is dull, normally reddish-brown colored and a subovated conic. The whorls are a little rounded with horizontal and indented sutures. The surface sculpture consists of thick transverse raised lines and fine spiral incised lines. The apex of the shell is eroded when old, relatively wide and deep. The outer part of last whorl is quite straight. The basal lip of aperture is appeared sharply angled on the left side. The average shell size has been reported at 10.54 and 6.48 mm of length and width, respectively. In the past, it was often identified as *B. siamensis siamensis*, which is distributed in central Thailand. Later the snails were identified as a single species but with the subspecies *B. siamensis goniomphalos* and *B. siamensis siamensis* (Brandt, 1974). Recently, molecular based identification was introduced by Kulsantiwong et al. (2013) who distinguished three taxa of *Bithynia* snails by specific primers designed from RAPD.

5.5 Genetic variation of *Bithynia* snails

Shell morphology is currently used to identify species of *Bithynia* snails. Genetic variation among *Bithynia* snails has been investigated by various molecular methods. Previous work revealed only EST was different within the two subspecies of *B. siamensis* whereas four enzymes (LDH, PGM, GPI and EST) differed between *B. funiculata* and *B. siamensis*, (Viyanant et al., 1985). Random amplified polymorphic DNA polymerase chain reaction (RAPD) based on two primers has also shown that different genotypes of snails in family Bithyniidae in Thailand may be correlated to specific wetlands (Duangprompo, 2007). Recently, multilocus enzyme electrophoresis (MEE) was used to investigate the systematics and population genetics of the different species and subspecies of *Bithynia* snails in Thailand, revealed fixed genetic differences at 67-73% of these taxa. The fixed genetic differences at 73% were found between the species *B. funiculata* and *B. siamensis* whereas 67% fixed genetic differences were detected between subspecies *B. s. siamensis* and *B. s. goniomphalos* (Kiatsopit et al., 2011). Furthermore, MME results demonstrated correlations between genetic clusters of *O. viverrini* and *B. s.*

goniomphalos in different wetlands which suggesting possible co-evolution (Saijuntha et al., 2007).

5.6 *O. viverrini* infection in snail hosts

The overall of natural infection rates of *O. viverrini* in *Bithynia* snails varied from 0.083 to 1.6% (Wykoff et al., 1965; Upatham, Sukhapanth, 1980; Brockelman et al., 1986). Prasopdee et al. (2013) reported the prevalence of *O. viverrini* infection in *B. siamensis goniomphalos* that was 0.45% which is in an agreement with Sri-aroon et al. (2005) who reported that the natural infection rate varied from 0.61 to 1.3%. Nevertheless, Kiatsopit et al. (2012) showed a higher prevalence of infection than any report with an averaged 3.04% and a hot spot of 6.93% in Sakon Nakhon Province. In laboratory infection, *B. funiculata* and *B. siamensis siamensis* were 4-7 times more susceptible to *O. viverrini* than the most medically important *B. siamensis goniomphalos* which is widely distributed in the endemic areas for opisthorchiasis. In experimental trials, the highest infection rate was obtained with an optimum dose of 50 fully developed *O. viverrini* eggs per snail. The increment number of eggs ingested was proportional to the mortality rate of the infected snails (Chanawong, Waikagul, 1991). In the same endemic area of opisthorchiasis, the prevalence of *O. viverrini* infection was high in humans and fish intermediate hosts up to 90% and 97%, respectively compared to a low infection rate of 0.11% in the snail intermediate host (Vichasri et al., 1982; Upatham et al., 1984; Brockelman et al., 1986).

5.7 Hybridism of parasite-harboring snails

The possibility of hybrids between species of parasite-harboring snails has been studied (Chi et al., 1971; Yousif et al., 1998; Facon et al., 2005; Lotfy et al., 2005; Teodoro et al., 2011). Experimentally, hybridism between *Biomphalaria cousini* and *B. amazonica* has shown that is possible and hybrids of them were susceptible to *Schistosoma mansoni* infection (Teodoro et al., 2011). In addition, laboratory hybrids of four subspecies of *Oncomelania hupensis* served as intermediate hosts of both human and zoophilic strains of *S. japonicum,* while parents *O. hupensis hupensis* and *O. hupensis formosana* was susceptible to only human and zoophilic strains, respectively (Chi et al., 1971). Natural survey in Egypt also showed that a hybrid of *B. glabrata* and *B. alexandrina* has invaded irrigation and drainage systems in Nile Valley and found naturally infected with *S. mansoni* thus indicating that it is already participating in transmission of *S. mansoni* in Egypt (Yousif et al., 1998).

5.8 Detection of trematodes infection in snail hosts

The detection of trematode infected snails by exposure of snails to artificial light and observation of cercarial shedding is commonly used (Upatham, Sukhapanth, 1980; Adam et al., 1993). Alternatively, snails are crushed between glass slides and examined for the presence of intramolluscan stages of trematodes. Low parasite burden, death of snails prior to crushing and pre-patent infections are the limitations of this technique (Barbosa, 1992; Hanelt et al., 1997). These conventional techniques are usually performed for identification of infected snails because of ease and low material costs, but time consuming and personal experience are necessary. In 1985, the amplification of target DNA sequences via in vitro replication by PCR was first described (Saiki et al., 1985). Presently, molecular techniques have been extensively used as diagnosis tools. In the past, pre-patent *S. mansoni* infection in snails was approached by a PCR assay based on a highly repeated tandemly arranged DNA sequence (Hamburger et al., 1998). Recently, a PCR method was used to detect *Fasciola gigantica* infection in snail intermediate host, *Lymnaea auricularia* with high sensitivity and specificity (Velusamy et al., 2004). In another study, a PCR assay was developed for detection of *Clonorchis sinensis* infected snails (Muller et al., 2007). A multiplex PCR targeting the internal transcribed spacer (ITS) region of rDNA was alternatively used for identification of infected *L. columella* by *F. hepatica* (Magalhaes et al., 2004). In addition, a nested PCR amplifying the 18S rDNA of *S. mansoni* was also developed for detection of infection in snails (Hanelt et al., 1997). Particularly, detection of intramolluscan *O. viverrini* using specific primers PCR was used for examination of infection status (Prasopdee et al., 2013).

CHAPTER III

MATERIALS AND METHODS

6. Occurrence of *Bithynia* snail hybridism:

6.1 Field survey and sampling of hybrid snails

Adult snails in genus *Bithynia* were collected with wire-mesh scoop or by hand from three regions of Thailand (north, northeast and central) based on the information of previous reports (Brandt, 1974; Kiatsopit et al., 2011; Kulsantiwong et al., 2013). Based on geographic distribution of snail species, the overlapping area especially region borders were surveyed for occurrence of hybrid snails. Each sampling site was recorded and its GPS coordinates was determined. Each sampled locality, the snails were randomly selected for species identification using available protocols based on shell morphology (Brandt, 1974; Upatham et al., 1983; Chitramvong, 1992), and molecular identity (Duangprompo, 2007; Kulsantiwong, 2013; Kulsantiwong et al., 2013).

6.2 Cercarial shedding and snail dissection

The snail samples were examined the trematode infection based on cercarial shedding method. Prior to the cercarial shedding, the snails were cleaned with de-chlorinated tap water several times. The snails were placed individually into plastic container filled with 5 ml de-chlorinated tap water. Releasing of cercaria was induced by exposing the snails to 8 W electric light for at least 2 hours following with covering the snails with black plastic sheet for overnight. After the cercarial induction, the water of each snail was examined for cercariae under stereomicroscope. Uninfected snails were dissected to remove soft body from their shell. The snail's soft bodies were stored in -20 °C until DNA extraction.

6.3 DNA extraction and species specific primers PCR

DNA extraction of the snails followed the protocol described by Prasopdee et al (2015). Briefly, The soft bodies were homogenized in CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v beta-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl, pH 8 .0,0.2 mg/ml proteinase K) (Winnepenninckx et al.,1993), and then incubated at 55 °C for 6 hours. Snail homogenate proteins were precipitated with phenol/chloroform, centrifuged at 12,000 × g for 10min at 4 °C. Protein was doubly precipitated with phenol/chloroform/isoamyl alcohol, centrifuged at 12,000 × g for 10 min at 4 °C, and DNA precipitated with isopropanol then washed twice with 70 % ethanol followed by absolute ethanol. The DNA pellet was air-dried, re-dissolved with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), diluted to 10 ng/ul and used as template for PCR.

Species specific primers PCR for molecular identity of *B. siamensis siamensis, B. siamensis goniomphalos and B. funiculata* followed protocol described by Kulsantiwong (2013). Briefly, designed species specific primer sets for *B. funiculata* was forward primer (BF2F) 5'-GGG ATG CTC GAT TGA AAG TG-3' and reverse primer (BF2R) 5'-GAC CTT CCG TGA AAG TCC TG-3', for *B. siamensis siamensis* was forward primer (BS1F) 5'-GCG AAG GAC AGA CCT GGA T-3' and reverse primer (BS1R) 5'-GGG GAC TCA CAG CAT AAT GG-3', and for *B. siamensis goniomphalos* was forward primer (BG1F) 5'-GGC TCA ATG ACA GAC ATT CG-3' and reverse primer (BG1R) CGG GGG AAG GAA TTG ATC AG-3'. Each PCR was carried out with total volume of 25 ul containing 1 Ready-To-Go bead, 1 ul of 10 ng DNA template and 19 ul of distilled water. Themal cycle was programmed by 45 cycles of 95 °C for 1 min, 62 °C for1 min, 72 °C for 2 min, and a final extension at 72 °C for 5 min. The PCR products were separated by 1.5% TBE agarose gel electrophoresis. Specific amplicons of 314 bp, 516 bp and 502 bp were revealed to identify *B. siamensis siamensis, B. siamensis goniomphalos and B. funiculata,* respectively.

6.4 Producing of laboratory hybrid snails

Parent snails, male and female were prepared by observing presence of the verge for male and absence of the verge for female. The parent species were chosen from the collected sites which already approved the species based on both shell morphology and specific primers PCR (as shown in Table 3). The snails were allowed to breed freely in 15 x 20 cm glass containers with the ratio of 5 female and 5 male per container. First offspring were observed every day.

7. Susceptibility of *Bithynia* snails to *O. viverrini* infection:

7.1 Preparation of *Bithynia* snails

Each sampling site was recorded and its GPS coordinates was determined. The snails were sorted and identified based on shell morphology following available protocols (Brandt, 1974; Upatham et al., 1983; Chitramvong, 1992). In addition, two subspecies (*B. siamensis siamensis* and *B. siamensis goniomphalos*) were categorized by geographic distribution. Each species of *Bithynia* snails was randomly selected and confirmed using specific primers (Duangprompo, 2007; Kulsantiwong, 2013; Kulsantiwong et al., 2013).

7.2 Preparation of *O. viverrini* eggs

Golden syrian hamsters (*Mesocricetus auratus*) were experimentally infected with 50 *O. viverrini* metacercariae per hamster. The infected animals were euthanized 6 weeks post infection. *O. viverrini* adults were obtained from biliary tracts and gall bladders of hamsters and then washed with 0.85% sodium chloride solution. Mature eggs were dissected from the distal portion of the uterus of adult flukes under a stereoscope (Khampoosa et al., 2012). The eggs were washed several times with distilled water and kept at room temperature for 2 weeks to undergo full maturation for further experimental infection (Chanawong, Waikagul, 1991).

7.3 Testing of susceptibility

All snails were used for infection were full grown. These snails were placed individually in transparent plastic containers with 6 ml of de-chlorinated tap water and exposed to 50 embryonated *O. viverrini* eggs (Chanawong, Waikagul, 1991; Prasopdee et al., 2013). The snails were placed into plastic containers covered with porous lids and activated by exposure to electric light. Let the snails ingested parasite eggs freely under these conditions for 24 h. After that, the snails were washed and reared in 15 x 20 cm glass container, with no more than 50 snails in each container. These snails were raised at room temperature with a dark and light cycle as in natural conditions and fed on synthetic snail food (Sumethanurungkul, 1970). The containers were checked daily for mortality and dead snails were removed and recorded. All exposed snails were checked weekly for shedding of cercariae for a total of 80 days (Teodoro et al., 2011). The first eight weeks post infections, the snails were randomly selected and pre-patent examined using specific primers at 1, 7, 14, 28, and 56 day post infection. The snails that survive for 80 days without shedding of cercariae were examined for intramolluscan stages by crushing method.

DNA extraction and specific primer PCR was applied to examine susceptibility of snails to *O. viverrini* infection as described by Prasopdee et al (2015). Briefly, The soft bodies were homogenized in CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v beta-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl, pH 8 .0,0.2 mg/ml proteinase K) (Winnepenninckx et al.,1993), and then incubated at 55 °C for 6 hours. Snail homogenate proteins were precipitated with phenol/chloroform, centrifuged at 12,000 × g for 10min at 4 °C. Protein was doubly precipitated with phenol/chloroform/isoamyl alcohol, centrifuged at 12,000 × g for 10 min at 4 °C, and DNA precipitated with isopropanol then washed twice with 70 % ethanol followed by absolute ethanol. The DNA pellet was air-dried, re-dissolved with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), diluted to 10 ng/ul and used as template for PCR.

The specific primers, OV-6F (5′-CTG AAT CTC TCG TTT GTT CA-3′) and OV-6R (5′ -GTT CCA GGT GAG TCT CTC TA-3′) (Wongratanacheewin et al., 2001) were used to amplify the pOV-A6 specific region of 330 bp. The PCR reaction was performed using a DNA Thermal cycler, performed in a final volume of 10 ul with 0.04 ul TaKaRa Ex Taq 250 U, 1 ul dNTP mixture, 1 ul 10x Ex Taq buffer, 3 ul DNA sample, 3 ul distilled water, and 5 pmol of each primer. The PCR carried out with cycling conditions of initial denaturation at 94 ° C for 5 min followed by 35 cycles of 1 min denaturation at 94 ° C, 1 min annealing at 55 °C, and 1 min extension at 72 °C, followed by a final extension for 7 min at 72 ° C. PCR products were analyzed by 1.5% TBE agarose gel electrophoresis.

7.4 Statistical analysis

Infection rate of *O. viverrini* in natural field *B. funiculata*, *B. siamensis goniomphalos* and *B. siamensis siamensis* were reported as % prevalence of infection. The association between the laboratory *O. viverrini* infection (binary outcomes) of *B. siamensis siamensis*, and *B. funiculata* and day post infection (predictor) was gauged by crude odds ratios obtained from binary logistic regression analysis.

CHAPTER IV

RESULTS

8.1 Localities of snail samples and *O. vivierrini* infection

Bithynia snails were sampled at different parts of Thailand (see Table 1). *B. siamensis siamensis* were sampled from provinces in central and north parts of Thailand, *B. siamensis goniomphalos* were sampled from provinces in northeast part of Thailand, and *B. funiculata* were solely sampled from Chiang Mai province in northern Thailand. Sampling habitats of *Bithynia* spp. are not strictly to rice field even they can be found mostly. Puddle, dam and ditch were also included in the habitats of sample collection (Table 1 and Figure 1). Based on morphology, in each region, there was no report of *Bithynia* species found apart from described in previous studies (Brandt, 1974; Kiatsopit et al., 2011; Kulsantiwong et al., 2013).

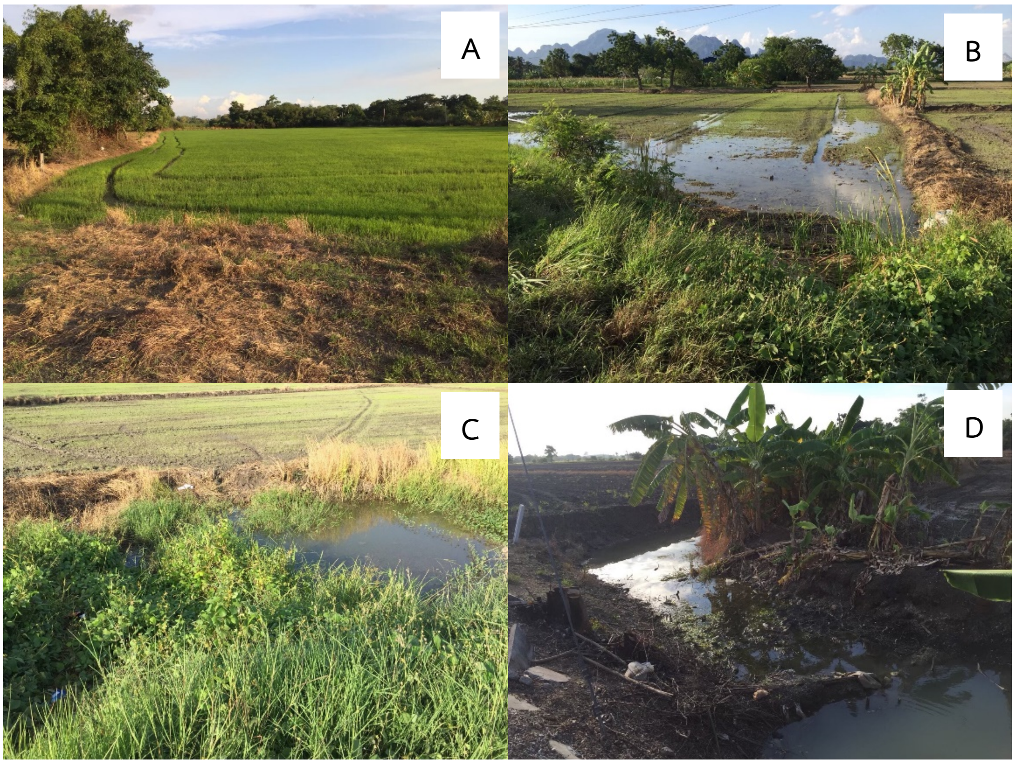

Figure 1 Sampling habitats of *Bithynia* spp. in Thailand.

(A, B) rice field, (C) pond, (D) ditch.

Table 1 Collection sites for *Bithynia* spp. from Thailand with GPS coordinates.

Species	Province	District	Habitat	Latitude	Longitude
		Central			
Bithynia siamensis siamensis (Morelet, 1866)	Bangkok (BK)	Kasetsat University	Puddle	13.85270023	100.5699997
	Bangkok (BK)	Chiang Rak Noi	Rice field	14.62575	100.341119
	Phra Nakhon Si Ayutthaya (AU)	Bang Sai	Rice field	14.112994	100.282822
	Suphan Buri (SP)	Bang Pla Ma	Rice field	14.242701	100.64638
	Suphan Buri (SP)	Doembang	Rice field	14.532525	100.52342
	Chai Nat (CN)	Sapphaya	Rice field	15.94546	100.143996
	Nakhon Sawan (NS)	Nakhon Sawan	Ditch	15.431085	100.104614
	Chachoengsao (ChS)	Ban Pho	Dich	13.362	101.452
	Chon Buri (CB)	Phanat Nikom	Rice field	13.51933	101.15396
	Sara Buri (SB)	Ban Mo	Rice field	14.58466	100.74742
	Sara Buri (SB)	Phra Phutthabat	Ditch	14.3626	100.4842
		Northeast			
Bithynia siamensis goniomphalos (Walker, 1927)	Udon Thani (UD)	Kut Chap	Dam	17.202962	102.335197
	Udon Thani (UD)	Nong Wu So	Dam	17.211346	102355616
	Udon Thani (UD)	Nong Han	Ditch	17.151296	103.6974
	Udon Thani (UD)	Udon Thani	Ditch	17.211426	102.355546
	Khon Kaen (KK)	Khon Kaen	Rice field/ Ditch	16.447400	102.902918
	Kalasin (KL)	Yang Talat	Dam	16.352191	103.24407
	Kalasin (KL)	Yang Talat	Puddle	16.35459	103.243435

Table 1 Collection sites for *Bithynia* spp. from Thailand with GPS coordinates (Cont).

Species	Province	District	Habitat	Latitude	Longitude
North					
Bithynis siamensis siamensis (Morelet, 1866)	Chiang Mai (CM)	San Kamphaeng	Ditch	18.51104	99.24301
	Chiang Mai (CM)	Hang Dong	Ditch	18.404659	98.55740
	Chiang Mai (CM)	Mae Rim	Rice field	18.552205	98.573477
	Chiang Mai (CM)	Saraphi	Rice field	18.423527	99.14597
Bithynia funiculata (Walker, 1927)	Chiang Mai (CM)	San Kamphaeng	Ditch	18.51104	99.24301
	Chiang Mai (CM)	Mae Rim	Rice field	18.552205	98.573477
	Chiang Mai (CM)	San Sai	Rice field	18.5246	99.318

The infection of *O. viverrini* in *Bithynia* snails was solely observed in *B. siamensis goniomphalos* sampled from Khon Kaen Province. The prevalence of *O. viverrini* infection the snail was 1.55% (12/772) with a shell size of greater than 0.6 mm.

8.2 Molecular species identification of *Bithynia* snails in Thailand

Specific primers PCR amplicons sized 314 bp, 516 bp and 502 bp were used to identify *B. siamensis siamensis, B. siamensis goniomphalos* and *B. funiculata*, respectively. The molecular based species identification was used along with shell morphology and sampling locations. There was no hybrids observed by showing the specific amplicons corresponding to morphologic based species identification and sampling locations (see Table 2).

Table 2 Presence of specific amplicons of *Bithynia* snails in Thailand.

Species	Province	District	Specific bands (bp)
		Central	
Bithynia siamensis siamensis (Morelet, 1866)	Bangkok (BK)	Kasetsat University	314
	Bangkok (BK)	Chiang Rak Noi	314
	Phra Nakhon Si Ayutthaya (AU)	Bang Sai	314
	Suphan Buri (SP)	Bang Pla Ma	314
	Suphan Buri (SP)	Doembang	314
	Chai Nat (CN)	Sapphaya	314
	Nakhon Sawan (NS)	Nakhon Sawan	314
	Chachoengsao (ChS)	Ban Pho	314
	Chon Buri (CB)	Phanat Nikom	314
	Sara Buri (SB)	Ban Mo	314
	Sara Buri (SB)	Phra Phutthabat	314
		Northeast	
Bithynia siamensis goniomphalos (Walker, 1927)	Udon Thani (UD)	Kut Chap	516
	Udon Thani (UD)	Nong Wu So	516
	Udon Thani (UD)	Nong Han	516
	Udon Thani (UD)	Udon Thani	516
	Khon Kaen (KK)	Khon Kaen	516
	Kalasin (KL)	Yang Talat	516
	Kalasin (KL)	Yang Talat	516

Table 2 Presence of specific amplicons of *Bithynia* snails in Thailand (Cont).

Species	Province	District	Specific bands (bp)
North			
Bithynis siamensis siamensis (Morelet, 1866)	Chiang Mai (CM)	San Kamphaeng	314
	Chiang Mai (CM)	Hang Dong	314
	Chiang Mai (CM)	Mae Rim	314
	Chiang Mai (CM)	Saraphi	314
Bithynia funiculata (Walker, 1927)	Chiang Mai (CM)	San Kamphaeng	502
	Chiang Mai (CM)	Mae Rim	502
	Chiang Mai (CM)	San Sai	502

8.3 Laboratory production of hybrid *Bithynia* snails

Hybrid offspring was only produced from cross breeding between male *B. siamensis siamensis* and female *B. siamensis goniomphalos* (Table 3). Unfortunately, the laboratory produced hybrids were all dead after 2 weeks.

Table 3 Occurrence of laboratory hybrids of *Bithynia* snails.

Male	Female	Offspring
BSG	BF	Not found
BF	BSG	Not found
BSG	BSS	Not found
BSS	BSG	Found
BSS	BF	Not found
BF	BSS	Not found

BSG: *Bithynia siamensis goniomphalos*; BSS: *Bithynia siamensis siamensis*; BF: *Bithynia funiculata*

8.4 Susceptibility of *Bithynia* snails to *O. viverrini* infection

Susceptibility of *Bithynia* snails to *O. viverrini* infection were examined based on cercarial shedding and specific primers PCR by presenting of *O. viverrini* cercaria and 330 bp specific band of PCR product, respectively (Figure 2). Based on cercarial shedding, there was no released *O. viverrini* cercariae were detected throughout time course of laboratory infection and there was no *Bithynia* snails survived until 80 dpi. However, based on molecular based-detection, *O. viverrini* infection was detected by revealing the specific band (Table 4). The odds ratio (OR) for association between susceptibility to *O. viverrini* infection of *Bithynia* snails and day post infection (dpi) are presented (see Table 5 and Table 6). There was evidence of an association between dpi and susceptibility to infection of *B. siamensis siamensis* that at least two groups differed (T_{wald}=11.309, df=4, P=0.023). Relative to day 1 (as baseline), there was a decrease in odds of infection for 14 dpi (P = 0.03), where at 14 dpi was associated with a 3% decrease in the odds of infection (P = 0.003). At 7 dpi showed susceptibility to infection was similar to 1 dpi (P = 0.2). Interestingly, there was no infection detected at 28 and 56 dpi. There was evidence of an association between dpi and susceptibility to infection of *B. funiculata* that at least two groups differed (T_{wald}=16.065, df=4, P=0.03). Relative to day 1 (as baseline), there was a decrease in odds of infection for 7, 28 and 56 dpi (P < 0.05). However, at 14 dpi was not showed statistical evidence of a decrease in odds of infection, there was no infection detected. There was evidence that at 7 and 56 dpi were associated with a 7.7% decrease in the odds of infection (P = 0.003). At 28 dpi, 1.5% decrease in the odds of infection was shown associated (P = 0.001).

Figure 2 Detections of *O. viverrini* infection in snails. 330 bp specific band of PCR product for *O. viverrini;* lane M = 100bp DNA ladder, lane 1 = Negative control, lane 2-3 = positive to *O. viverrini* infection.

Table 4 Percentage of susceptibility to *O. viverrini* infection of *B. siamensis siamensis* and *B. funiculata* categorized by post infection (dpi)

% positive snails / dpi	*B. siamensis siamensis* (positive/total)	*B. funiculata* (positive/total)
1	94 (15/16)	82 (13/16)
7	75 (12/16)	25 (4/16)
14	32 (5/16)	0 (0/16)
28	0 (0/16)	7 (1/16)
56	0 (0/16)	25 (4/16)
overall	40 (32/80)	28 (22/80)

Table 5 Association between infection and day post infection of *B. siamensis siamensis*[i]

Day post infection	OR	95% CI
1 (reference)	T_{wald}=11.309, df=4, P=0.023	
7	0.200	0.020-2.033
14	0.030*	0.003-0.297
28	0.000	-
56	0.000	-

* Indicate significance at P< 0.05, [i] Results of association are shown as odds ratio (OR).

Table 6 Association between infection and day post infection of *B. funiculata*[i]

Day post infection	OR	95% CI
1 (reference)	T_{wald}=16.065, df=4, P=0.03	
7	0.077*	0.014-0.417
14	0.000	-
28	0.015*	0.001-0.167
56	0.077*	0.014-0.417

* Indicate significance at $P< 0.05$, [i] Results of association are shown as odds ratio (OR).

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

Among three taxa of *Bithynia* snails, laboratory hybrid offspring can only be produced from male *B. siamensis siamensis* and female *B. siamensis goniomphalos*. It is possible that these snails are very closely in morphology and genetic as classified as the same species unlike *B. funiculata*. This may suggest possibility of occurrence the natural hybrid *Bithynia* snails especially in central, north and northeast regions of Thailand where the *B. siamensis* were found (Kulsantiwong et al., 2013). However, the produced offspring were all dead after 2 weeks of laboratory rearing. Previous studies demonstrated the laboratory reared snail particularly juvenile is highly susceptible to be infected with pathogen (Chanawong, Waikagul, 1991; Prasopdee et al., 2015). In the other words, defense system is not competent. This could be a leading cause of the laboratory offspring's death after 2 weeks. However, as Thailand floods spread in 2011, northeast region where the *B. siamensis goniomphalos* distributed in was not affected (Mhuantong et al., 2015). Therefore, no snail hybridism concern is raised. In addition, likewise this study, there was no survey-based hybridism report of *Bithynia* snails in Thailand. Although laboratory hybrid offspring can be produced from male and female of *B. siamensis siamensis* and *B. siamensis goniomphalos,* respectively, the border area of sample collection including central-northeast regions showed no report of hybridism. This is possible that there are natural boundaries between central and northeast regions which is difficult to spread across. Although *B. funiculata* and *B. siamensis siamensis* are found in the same area (central and north), there was no report of hybridism. This might be the morphologic and genetic background differences as mentioned above.

Infectivity of *O. viverrini* in *Bithynia* snail was proved temperature dependent (Prasopdee et al., 2015). The chosen temperature of 28 $\pm$ 2 $^{\circ}$C (room temperature) was applied to test the infectivity of *O. viverrini* in *B. siamensis siamensis* and *B. funiculata* due to it was demonstrated as optimum temperature (ranging from 22 to 34 $^{\circ}$C) of *O. viverrini* infection in the *B. siamensis goniomphalos* to generate high infection rates (Prasopdee et al., 2015). In addition, as reported by the Thai Meteorological Department, this temperature is comparable with average minimum and maximum weather temperatures in northeast Thailand in the hot weather period (March to October) between 1971 to 2000 were 23 – 35 $^{\circ}$C. Previous study showed the percentage of natural *O. viverrini* infection in field *B. siamensis goniomphalos* snails was higher in the cold season (December to February) than other seasons (Brockelman et al., 1986). Moreover, metacercarial load in fish intermediate host was found most abundant in the late rainy and cold seasons (July to January) (Sithithaworn et al., 1997). This implies that the snails get infected with the parasites during the hot weather period and require about 2 months to liberate free-swimming cercariae (Harinasuta, Harinasuta, 1984; Upatham, Viyanant, 2003). The released cercariae encounter then infect the fish intermediate host and develop metacercariae within 6 weeks (Harinasuta, Harinasuta, 1984). Despite the *Bithynia* snail has two sexes male and female, the sex was not considered as independent factor in the experimental infection setting since there was no significantly difference of *O. viverrini* infection (Prasopdee et al., 2015). In cross sectional study, among three taxa of *Bithynia* in Thailand, *B. siamensis goniomphalos* was reported highest prevalence than other species (Kulsantiwong et al., 2015). Nevertheless, in experimental infection, *B. funiculata* and *B. siamensis siamensis* were demonstrated more susceptible to *O. viverrini* than *B. siamensis goniomphalos* (Chanawong, Waikagul, 1991). However, of previous studies, the *O. viverrini* infection detection in *Bithynia* was solely based on cercarial shedding. Data present herein revealed the infection status of *O. viverrini* were detected by both observing elicited cercariae using cercarial shedding and observing specific band (330 bp) using specific primers PCR to *O. viverrini.* The latter step of *O. viverrini* detection was used to find whether throughout the time course of infection (1, 7, 14, 28 and 56 dpi), the *Bithynia* infected with intramolluskan stages or not (Prasopdee et al., 2015). Surprisingly, the susceptibility to *O. viverrini* infection in both *B. siamensis siamensis* and *B. funiculata* was high at 1 dpi (94% and 81.3%, respectively) thereafter dramatically declined at extra period of dpi for *B. funiculata*. In case of *B. siamensis siamensis*, the susceptibility at 7 dpi (75%) of was similar to 1 dpi. However, there was dramatically declined at 14 dpi and thereafter unsusceptible for later period of dpi. This possibly due to 2 distinct phenomena as proposed in previous study (Prasopdee et al., 2015) – 1) immune responses and 2) nutritional status of snails. Initial parasite invasion trigger immune responses of snails, which led to diminish and complete elimination of the parasites, respectively, hence the number of infected snails over time were decreased. Malnutrition of infected snails rearing under laboratory conditions resulted in poor condition for development of intramolluskan larval stages. In addition, snail death due to the harm arising from parasite infection are suggested, however this point remains murky and should be investigated further. In addition, we can observe that at the end of laboratory time course of infection (56 dpi) there was no liberated cercaria. However, specific PCR showed evidence of developed intramolluskan stages. At this point, it might be the poor nutrition of laboratory rearing snail slowdowns the development of *O. viverrini* cercaria. Furthermore, at 56 dpi, the susceptibility to *O. viverrini* infection in *B. funiculata* (25%) was shown higher than in *B. siamensis siamensis* where the infection was not observed. This signifies that *O. viverrini* cercaria development is prone to be achieving higher in *B. funiculata* host.

We can conclude that there was no occurrence of hybridism in *Bithynia* snail taxa in Thailand. However, this is solely based on field survey study. The experimental study should be further investigated in order to find an evidence of hybridism in *Bithynia* snails. However, the surveillance of *O. viverrini* infection in snail hosts should not be ignored as we can observe that the susceptibility to *O. viverrini* infection especially in *B. funiculata* was high.

REFERENCES

Adam R, Arnold H, Pipitgool V, Sithithaworn P, Hinz E, Storch V. Studies on lophocercous cercariae from *Bithynia siamensis goniomphalus* (Prosobranchia: Bithyniidae). **Southeast Asian J Trop Med Public Health** 1993; 24: 697-700.

Barbosa CS. Methods for malacological work in schistosomiasis. **Mem Inst Oswaldo Cruz** 1992; 87 Suppl 4: 311- 312.

Brandt RAM. The non-marine aquatic Mollusca of Thailand. **Arch Mollusk** 1974; 105: 1-423.

Brockelman WY, Upatham ES, Viyanant V, Ardsungnoen S, Chantanawat R. Field studies on the transmission of the human liver fluke, *Opisthorchis viverrini*, in northeast Thailand: population changes of the snail intermediate host. **Int J Parasitol** 1986; 16: 545-552.

Cardenas VM, Jaime J, Ford PB, et al. Yard flooding by irrigation canals increased the risk of West Nile disease in El Paso, Texas. **Ann Epidemiol** 2011; 21: 922-929.

Carrel M, Voss P, Streatfield PK, et al. Protection from annual flooding is correlated with increased cholera prevalence in Bangladesh: a zero-inflated regression analysis. **Environ Health** 2010; 9: 13.

Chanawong A, Waikagul J. Laboratory studies on host-parasite relationship of *Bithynia* snails and the liver fluke, *Opisthorchis viverrini*. **Southeast Asian J Trop Med Public Health** 1991; 22: 235-239.

Chi LW, Wagner ED, Wold N. Susceptibility of *Oncomelania* hybrid snails to various geographic strains of *Schistosoma japonicum*. **Am J Trop Med Hyg** 1971; 20: 89-94.

Chitramvong YP. The Bithyniidae (Gastropoda: Prosobanchia) of Thailand: comparative external morphology. **Malacol Rev** 1992; 25: 21-38.

Coker RJ, Hunter BM, Rudge JW, Liverani M, Hanvoravongchai P. Emerging infectious diseases in southeast Asia: regional challenges to control. **Lancet** 2011; 377: 599-609.

De NV, Murrell KD, Cong le D, Cam PD, Chau le V, Toan ND, et al. The food-borne trematode zoonoses of Vietnam. **Southeast Asian J Trop Med Public Health** 2003; 34: 112-134.

Duangprompo W. **Genetic variation of snails in the family Bithyniidae in Thailand and identification of *Bithynia siamensis goniomphalos* by PCR** [M.Sc Thesis]. Khon Kaen: Faculty of Medicine, Khon Kaen University; 2007.

Facon B, Jarne P, Pointier JP, David P. Hybridization and invasiveness in the freshwater snail *Melanoides tuberculata*: hybrid vigour is more important than increase in genetic variance. **J Evol Biol** 2005; 18: 524-535.

Harinasuta C, Harinasuta T. *Opisthorchis viverrini*: life cycle, intermediate hosts, transmission to man and geographical distribution in Thailand. **Arznei-Forschung** 1984; 34: 1164-1167.

Harris AM, Chowdhury F, Begum YA, et al. Shifting prevalence of major diarrheal pathogens in patients seeking hospital care during floods in 1998, 2004, and 2007 in Dhaka, Bangladesh. **Am J Trop Med Hyg** 2008; 79: 708-714.

Harrison BA, Whitt PB, Roberts LF, et al. Rapid assessment of mosquitoes and arbovirus activity after floods in Southeastern Kansas, 2007. **J Am Mosq Control Assoc** 2009; 25: 265-271.

Haswell-Elkins MR, Sithithaworn P, Elkins D. *Opithorchis viverrini* and cholangiocarcinoma in northeast Thailand. **Parasitol Today** 1992; 8: 86-89.

Hamburger J, He N, Xin XY, Ramzy RM, Jourdane J, Ruppel A. A polymerase chain reaction assay for detecting snails infected with bilharzia parasites (*Schistosoma mansoni*) from very early prepatency. **Am J Trop Med Hyg** 1998; 59: 872-876.

Hanelt B, Adema CM, Mansour MH, Loker ES. Detection of *Schistosoma mansoni* in *Biomphalaria* using nested PCR. **J Parasitol** 1997; 83: 387-394.

Holzinger F, Z'graggen K, Buchler MW. Mechanisms of biliary carcinogenesis: a pathogenetic multi-stage cascade towards cholangiocarcinoma. **Ann Oncol** 1999; 4: 122-126.

International Agency for Research on Cancer. Infection with liver flukes (*Opisthorchis viverrini*, *Opisthorchis felineus* and *Clonorchis sinensis*). **IARC Monogr Eval Carcinog Risk Hum** 1994; 61: 121-175.

______. A review of human carcinogens (biological agens: *Opisthorchis viverrini* and *Clonorchis sianensis*). **IARC Monogr Eval Carcinog Risk Hum** 2011; 100B: 351-376.

Jongsuksuntigul P, Imsomboon T. Opisthorchiasis control in Thailand. **Acta Trop** 2003; 88: 229-232.

Kawanishi S, Hiraku Y. Oxidative and nitrative DNA damage as biomarker for carcinogenesis with special reference to inflammation. **Antioxid Redox Signal** 2006; 8: 1047-1058.

Khampoosa P, Jones MK, Lovas EM, Srisawangwong T, Laha T, Piratae S, et al. Light and electron microscopy observations of embryogenesis and egg development in the human liver fluke, *Opisthorchis viverrini* (Platyhelminthes, Digenea). **Parasitol Res** 2012; 110: 799-808.

Khuhaprema T, Srivatanakul P. Liver and bile duct. In: Khuhaprema T, Srivatanakul P, Wiangnon S, Sumitsawan Y, Attasara P, editors. **Cancer in Thailand Vol IV, 1998-2000.** Bangkok: Bangkok Medical Publisher; 2007. p. 36-38.

Kiatsopit N Sithithaworn P, Boonmars T, Tesana S, Chanawong A, Saijuntha W, Petney TN, Andrews RH. Genetic markers for studies on the systematics and population genetics of snails, *Bithynia* spp., the first intermediate hosts of *Opisthorchis viverrini* in Thailand. **Acta Trop** 2011; 118: 136–141.

______, Sithithaworn P, Saijuntha W, Boonmars T, Tesana S, Sithithaworn J, et al. Exceptionally high prevalence of infection of *Bithynia siamensis goniomphalos* with *Opisthorchis viverrini* cercariae in different wetlands in Thailand and Lao PDR. **Am J Trop Med Hyg** 2012; 86: 464-469.

Kondo H, Seo N, Yasuda T, et al. Post-flood-infectious diseases in Mozambique. **Prehosp Disaster Med** 2002; 17: 126-133.

Kruatrachue M, Jantataeme S, Ratanatham S, Vichasri S, Upatham ES. A culture method for *Bithynia* (Prosobranchia: Bithyniidae), snail hosts for the trematode *Opisthorchis viverrini*. **Malacol Rev** 1982; 15: 63-67.

Kulsantiwong J. **Identification of snail species of family Bithyniidae in Thailand by DNA barcoding and specific primer PCR** [PhD Thesis in Parasitology]. Khon Kaen: Faculty of Medicine, Khon Kaen University; 2013.

______, Prasopdee S, Piratae S, Khampoosa P, Suwannatrai A, Duangprompo W, et al. Species-specific primers designed from RAPD products for *Bithynia funiculata*, the first intermediate host of liver fluke, *Opisthorchis viverrini*, in North Thailand. **J Parasitol** 2013; 99: 433-437.

_______, Prasopdee S, Ruangsittichai J, Ruangjirachuporn W, Boonmars T, Viyanant V, et al. DNA barcode identification of freshwater snails in the family Bithyniidae from Thailand. **PLoS One** 2013; 8: e79144.

Lee KJ, Bae YT, Kim DH, Deung YK, Ryang YS, Kim HJ, et al. Status of intestinal parasites infection among primary school children in Kampongcham, Cambodia. **Korean J Parasitol** 2002; 40: 153-155.

Lotfy WM, DeJong RJ, Black BS, Loker ES. Specific identification of Egyptian *Biomphalaria* species and possible hybrids using the polymerase chain reaction based on nuclear and mitochondrial loci. **Mol Cell Probes** 2005; 19: 212-5.

Magalhães KG, Passos LKJ, Carvalho OS. Detection of *Lymnaea columella* Infection by *Fasciola hepatica* through Multiplex-PCR. **Mem Inst Oswaldo Cruz** 2004; 99: 421-424.

Mairiang E, Mairiang P. Clinical manifestation of opisthorchiasis and treatment. **Acta Trop** 2003; 88: 221-227.

Mhuantong W, Wongwilaiwalin S, Laothanachareon T, et al. Survey of Microbial Diversity in Flood Areas during Thailand 2011 Flood Crisis Using High-Throughput Tagged Amplicon Pyrosequencing. **PLoS One**. 2015; 10(5): e0128043.

Müller B, Schmidt J, Mehlhorn H. Sensitive and species specific detection of *Clonorchis sinensis* by PCR in infected snails and fishes. **Parasitol Res** 2007; 100: 911-914.

Ngaosuwankul N, Thippornchai N, Yamashita A, Vargas RE, Tunyong W, Mahakunkijchareon Y, et al. Detection and characterization of enteric viruses in flood water from the 2011 thai flood. **Jpn J Infect Dis** 2013; 66: 398-403.

Ohl CA, Tapsell S. Flooding and human health: the dangers posed are not always obvious**. BMJ** 2000; 321: 1167-1168.

Osman M, Lausten SB, El-Sefi T, Boghdadi I, Rashed MY, Jensen SL. Biliary parasites. **Dig Surg** 1998; 15: 287-296.

Papasarathorn T, Sumethanurugkul P, Viboolyavatana J, Temcharoen P, Tongkoom B. Studies on life history and ecological requirements of *Bithynia* (Digoniostoma) *siamensis goniomphalos*, the snail intermediate host of *Opisthorchis viverrini*. **J Parasitol Trop Med Assoc Thailand** 1980; 3: 21-28.

Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DBE. Cancer incidence in five continents. **IARC Sci Publ** 2002; 8: 1-781.

Prasopdee S, Kulsantiwong J, Piratae S, Khampoosa P, Thammasiri C, Suwannatrai A, et al. Temperature dependence of *Opisthorchis viverrini* infection in first intermediate host snail, *Bithynia siamensis goniomphalos*. **Acta Trop** 2015; 141(Pt A): 112-117.

Sadun EH. Studies on *Opisthorchis viverrini* in Thailand. **Am J Hyg** 1955; 62: 81-115.

Saijuntha W, Sithithaworn P, Wongkham S, Laha T, Pipitgool V, Tesana S, Chilton NB, Petney TN, Andrews RH. Evidence of a species complex within the food-borne trematode *Opisthorchis viverrini* and possible coevolution with their first intermediate hosts. **Int J Parasito**l 2007; 37: 695–703.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for the diagnosis of sickle cell anemia. **Science** 1985; 230: 1350-1354.

Sirica AE. Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. **Hepatology** 2005; 41: 5-15.

Sriamporn S, Pisani P, Pipitgoo V, Suwanrungruang K, Kamsa-ard S, Parkin DM. Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, northeast Thailand. **Trop Med Int Health** 2004; 9: 588-594.

Sri-aroon P, Butraporn P, Limsomboon J, Kerdpuech Y, Kaewpoolsri M, Kiatsiri S. Freshwater mollusks of medical importance in Kalasin Province, northeast Thailand. **Southeast Asian J Trop Med Public Health** 2005; 36: 653-657.

Sripa B, Kaewkes S, Sithithaworn P, Mairiang E, Laha T, Smout T, et al. Liver fluke induces cholangiocarcinoma. **Plos Negl Trop Dis** 2007; 7: 1148-1155.

______, Leungwattanawanit S, Nitta T, Wongkham C, Bhudhisawasdi V, Puapairoj A, et al. Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). **World J Gastroenterol** 2005; 11: 3392-3397.

Srivatanakul P, Ohshima H, Khlat M, Parkin M, Sukarayodhin S. Endogenous nitrosamines and liver fluke as risk factors for cholangiocarcinoma in Thailand. **IARC Sci Publ** 1991: 88-95.

Sumethanurungkul P. **Studies on physical effects on snail intermediate hosts of a liver fluke (*Opisthorchis viverrini*)** [Master Thesis in Parasitology]. Bangkok: Faculty of Graduates Studies, Mahidol University; 1970.

Suwannatrai A, Suwannatrai K, Haruay S, Piratae S, Thammasiri C, Khampoosa P, et al. Effect of soil surface salt on the density and distribution of the snail *Bithynia siamensis goniomphalos* in northeast Thailand. **Geospat Health** 2011; 5: 183-190.

Teodoro TM, Jannotti-Passos LK, Carvalho Odos S, Grijalva MJ, Baús EG, Caldeira RL. Hybridism between *Biomphalaria cousini* and *Biomphalaria amazonica* and its susceptibility to Schistosoma mansoni. **Mem Inst Oswaldo Cruz** 2011; 106: 851-855.

Upatham ES, Sornmani S, Kitikoon V, Lohachit C, Burch JB. Identification key for the fresh-and brackish-water snails of Thailand. **Malacol Rev** 1983; 16: 107-132.

_______, Sukhapanth N. Field studies on the bionomics of *Bithynia siamensis siamensis* and the transmission of *Opisthorchis viverrini* in Bangna, Bangkok, Thailand. **Southeast Asian J Trop Med Public Health** 1980; 11: 355-358.

_______, Viyanant V, Kurathong S, Rojborwonwitaya J, Brockelman WY, Ardsungnoen S, et al. Relationship between prevalence and intensity of *Opisthorchis viverrini* infection and clinical symptoms and signs in a rural community in northeast Thailand. **Bull WHO** 1984; 62: 451-461.

Vatanasapt V, Uttaravichien T, Mairiang EO, Pairojkul C, Chartbanchachai W. Cholangiocarcinoma in north-east Thailand. **Lancet** 1990; 335: 116-117.

Velusamy R, Singh BP, Raina OK. Detection of *Fasciola gigantica* infection in snails by polymerase chain reaction. **Vet Parasitol** 2004; 120: 85-90.

Vichasri S, Viyanant V, Upatham ES. *Opisthorchis viverrini*: intensity and rates of infection in cyprinoid fish from an endemic focus in northeast Thailand. **Southeast Asian J Trop Med Public Health** 1982; 13: 138-141.

Viyanant V, Upatham ES, Siriteramongkol S. Enzyme analysis of *Bithynia* (Mollusca: Bithyniidae) by isoelectricfocusing. **Malacol Rev** 1985; 18: 15–20.

Waikagul J. *Opisthorchis viverrini* metacercaria in Thai fresh water fish. **Southeast Asian J Trop Med Public Health** 1998; 29: 324-326.

World Health Organization. Control of food borne trematode infections. **World Health Organ Tech Rep Ser** 1995; 849: 1-157.

Wykoff DE, Harinasuta C, Juttijudata P, Winn MM. *Opisthorchis viverrini* in Thailand the life cycle and comparison with *O. felineus*. **J Parasitol** 1965; 51: 207-214.

Yousif F, Ibrahim A, Abdel Kader A, el-Bardicy S. Invasion of the Nile Valley in Egypt by a hybrid of *Biomphalaria glabrata* and *Biomphalaria alexandrina*, snail vectors of *Schistosoma mansoni*. **J Egypt Soc Parasitol** 1998; 28: 569-582.

TITLE: Study on susceptibility of *Bithynia siamensis siamensis* and *B. funiculata* to *Opisthorchis viverrini* infection: a risk indication of *O. viverrini* infection outside endemic area of Thailand

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ABSTRACT

Among snail species acting as hosts for parasites, three taxa of *Bithynia* are responsible for transmission of the carcinogenic liver fluke *Opisthorchis viverrini* to humans in Thailand. Despite *Bithynia siamensis goniomphalos* is a major species responsible for *O. viverrini* transmission in endemic area of Thailand, the rest two species *B. siamensis siamensis* and *B. funucilata* should not be ignored. To indicate a risk of *O. viverrini* infection outside the endemic area of Thailand, this study aimed to investigate the susceptibility to *O .viverrini* infection of *B. siamensis siamensis* and *B. funiculata*. The snails were infected with *O. viverrini* eggs and investigated throughout time course of 1, 7, 14, 28 and 56 day post infection (dpi). The susceptibility to *O. viverrini* infection in both *B. siamensis siamensis* and *B. funiculata* was high at early period of infection (1 and 7 day dpi) thereafter dramatically declined at extra period of dpi. Interestingly, at 56 dpi, the susceptibility to *O. viverrini* infection in *B. funiculata* (25%) was shown higher than in *B. siamensis siamensis* where the infection was not observed. In conclusion, surveillance of *O. viverrini* infection in snail hosts should be paid more attention as the high susceptibility to *O. viverrini* infection in *B. funiculata* was observed.

Keywords: *Bithynia siamensis siamensis, Bithynia funiculata, Opisthorchis viverrini,* Cholangiocarcinoma, Thailand

INTRODUCTION

In Thailand, three taxa of *Bithynia* have been reported as natural first intermediate host of *O. viverrini* in different geographical habitats; *Bithynia funiculata* in the north, *B. siamensis siamensis* in the central and the north and *B. siamensis goniomphalos* in the northeast region (Wykoff et al., 1965; Brandt, 1974). The life cycle of the parasite requires contamination of a water body with feces containing parasite eggs of an infected definitive host. The snails become infected by ingesting the fully developed eggs. Miracidia hatch from the ingested eggs and then penetrate snail tissue to develop into sporocysts. Asexually reproduction and development generate large numbers of rediae and cercariae, the latter of which are released from the snail host. The released cercariae become metacercariae in fish and adult stage in fish-eating mammals, respectively. The natural infection rates of *O. viverrini* in *Bithynia* snails were varied from 0.083 to 3.04% (Wykoff et al., 1965; Upatham, Sukhapanth, 1980; Brockelman et al., 1986; Sri-aroon et al., 2005; Kiatsopit et al., 2012; Prasopdee et al., 2015). In laboratory infection, *B. funiculata* and *B. siamensis siamensis* were 4-7 times more susceptible to *O. viverrini* than *B. siamensis goniomphalos* (Chanawong, Waikagul, 1991). However, research on *B. funiculata* and *B. siamensis siamensis* are scant. Among three taxa of *Bithynia* in Thailand, Research on *B. siamensis goniomphalos* has been widely published in many reputable journals (Suwannatrai et al., 2011; Tesana et al., 2012; Cantacessi et al 2013; Prasopdee et al., 2014; Prasopdee et al., 2015, Prasopdee et al., 2015). The research on *B. funiculata* and *B. siamensis siamensis* should be more paid attention as they also play major role in *O. viverrini* transmission to humans outside the endemic area in Thailand. This study aimed to update investigation on the susceptibility of *B. siamensis siamensis* and *B. funiculata* to *O .viverrini* infection with the conventional cercarial shedding along with PCR assay detection. This would allow parasitologist and malacologist to raise awareness of *O. viverrini* infection outside Northeast region of Thailand.

MATERIALS AND METHODS

Preparation of *Bithynia* snails

Bithynia siamensis siamensis and *B. funiculata* were collected from natural field. The snails were sorted and identified based on shell morphology following available protocols (Brandt, 1974; Upatham et al., 1983; Chitramvong, 1992). Each species of *Bithynia* snails was randomly selected and confirmed using specific primers (Duangprompo, 2007; Kulsantiwong, 2013; Kulsantiwong et al., 2013).

The snail samples were examined for trematode infection based on cercarial shedding method. The snails were placed individually into plastic container filled with 5 ml de-chlorinated tap water. Releasing of cercaria was induced by exposing the snails to 8 W electric light for at least 2 hours following with covering the snails with black plastic sheet for overnight. Uninfected snails of each species were used for further experiment.

Preparation of *O. viverrini* eggs

Golden syrian hamsters (*Mesocricetus auratus*) were experimentally infected with 50 *O. viverrini* metacercariae per hamster. The infected animals were euthanized 6 weeks post infection. *O. viverrini* adults were obtained from biliary tracts and gall bladders of hamsters and then washed with 0.85% sodium chloride solution. Mature eggs were dissected from the distal portion of the uterus of adult flukes under a stereoscope (Khampoosa et al., 2012). The eggs were washed several times with distilled water and kept at room temperature for 2 weeks to undergo full maturation for further experimental infection (Chanawong, Waikagul, 1991).

Testing of susceptibility

All snails were used for infection were full grown. A hundred snails per species were placed individually in transparent plastic containers with 6 ml of de-chlorinated tap water and exposed to 50 embryonated *O. viverrini* eggs (Chanawong, Waikagul, 1991; Prasopdee et al., 2015). The snails were placed into plastic containers covered with porous lids and activated by exposure to electric light. Let the snails ingested parasite eggs freely under these conditions for 24 h. After that, the snails were washed and reared in same 15 x 20 cm glass container according to their species, with no more than 50 snails in each container. These snails were raised at room temperature with a dark and light cycle mimics the actual natural light conditions and fed on synthetic snail food (Sumethanurungkul, 1970). The containers were checked daily for mortality and dead snails were removed. All exposed snails were checked weekly for shedding of cercariae for a total of 80 days (Teodoro et al., 2011). The first eight weeks post infections, 16 snails per time point were randomly selected and pre-patent examined using specific primers at 1, 7, 14, 28, and 56 day post infection. The snails that survive for 80 days without shedding of cercariae were examined for intramolluskan stages by crushing method.

DNA extraction and specific primer PCR was applied to examine susceptibility of snails to *O. viverrini* infection as described by Prasopdee et al (2015). Briefly, The soft bodies were homogenized in CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v beta-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl, pH 8 .0,0.2 mg/ml proteinase K) (Winnepenninckx et al.,1993), and then incubated at 55 °C for 6 hours. Snail homogenate proteins were precipitated with phenol/chloroform, centrifuged at 12,000 × g for 10min at 4 °C. Protein was doubly precipitated with phenol/chloroform/isoamyl alcohol, centrifuged at 12,000 × g for 10 min at 4 °C, and DNA precipitated with isopropanol then washed twice with 70 % ethanol followed by absolute ethanol. The DNA pellet was air-dried, re-dissolved with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), diluted to 10 ng/ul and used as template for PCR.

The specific primers, OV-6F (5′-CTG AAT CTC TCG TTT GTT CA-3′) and OV-6R (5′ -GTT CCA GGT GAG TCT CTC TA-3′) (Wongratanacheewin et al., 2001) were used to amplify the pOV-A6 specific region of 330 bp. The PCR reaction was performed using a DNA Thermal cycler, performed in a final volume of 10 ul with 0.04 ul TaKaRa Ex Taq 250 U, 1 ul dNTP mixture, 1 ul 10x Ex Taq buffer, 3 ul DNA sample, 3 ul distilled water, and 5 pmol of each primer. The PCR carried out with cycling conditions of initial denaturation at 94 ° C for 5 min followed by 35 cycles of 1 min denaturation at 94 ° C, 1 min annealing at 55 °C, and 1 min extension at 72 °C, followed by a final extension for 7 min at 72 ° C. PCR products were analyzed by 1.5% TBE agarose gel electrophoresis.

Statistical analysis

Infection rate of *O. viverrini* in natural field *B. funiculata*, *B. siamensis goniomphalos* and *B. siamensis siamensis* were reported as % prevalence of infection. The association between the laboratory *O. viverrini* infection (binary outcomes) of *B. siamensis siamensis*, and *B. funiculata* and day post infection (predictor) was gauged by crude odds ratios obtained from binary logistic regression analysis.

RESULTS

Susceptibility of *Bithynia* snails to *O. viverrini* infection

Susceptibility of *Bithynia* snails to *O. viverrini* infection were examined based on cercarial shedding and specific primers PCR by presenting of *O. viverrini* cercaria and 330 bp specific band of PCR product, respectively (Figure 1). Based on cercarial shedding, there was no released *O. viverrini* cercariae were detected throughout time course of laboratory infection and there was no *Bithynia* snails survived until 80 dpi. However, based on molecular based-detection, *O. viverrini* infection was detected by revealing the specific band (Table 1). The odds ratio (OR) for association between susceptibility to *O. viverrini* infection of *Bithynia* snails and day post infection (dpi) are presented (see Table 2 and Table 3). There was evidence of an association between dpi and susceptibility to infection of *B. siamensis siamensis* that at least two groups differed (T_{wald}=11.309, df=4, P=0.023). Relative to day 1 (as baseline), there was a decrease in odds of infection for 14 dpi (P = 0.03), where at 14 dpi was associated with a 3% decrease in the odds of infection (P = 0.003). At 7 dpi showed susceptibility to infection was similar to 1 dpi (P = 0.2). Interestingly, there was no infection detected at 28 and 56 dpi. There was evidence of an association between dpi and susceptibility to infection of *B. funiculata* that at least two groups differed (T_{wald}=16.065, df=4, P=0.03). Relative to day 1 (as baseline), there was a decrease in odds of infection for 7, 28 and 56 dpi (P < 0.05). However, at 14 dpi was not showed statistical evidence of a decrease in odds of infection, there was no infection detected. There was evidence that at 7 and 56 dpi were associated with a 7.7% decrease in the odds of infection (P = 0.003). At 28 dpi, 1.5% decrease in the odds of infection was shown associated (P = 0.001).

DISCUSSIONS AND CONCLUSION

Infectivity of *O. viverrini* in *Bithynia* snail was proved temperature dependent (Prasopdee et al., 2015). The chosen temperature of 28 $\pm$ 2 $^{\circ}$C (room temperature) was applied to test the infectivity of *O. viverrini* in *B. siamensis siamensis* and *B. funiculata* due to it was demonstrated as optimum temperature (ranging from 22 to 34 $^{\circ}$C) of *O. viverrini* infection in the *B. siamensis goniomphalos* to generate high infection rates (Prasopdee et al., 2015). In addition, as reported by the Thai Meteorological Department, this temperature is comparable with average minimum and maximum weather temperatures in northeast Thailand in the hot weather period (March to October) between 1971 to 2000 were 23 – 35 $^{\circ}$C. Previous study showed the percentage of natural *O. viverrini* infection in field *B. siamensis goniomphalos* snails was higher in the cold season (December to February) than other seasons (Brockelman et al., 1986). Moreover, metacercarial load in fish intermediate host was found most abundant in the late rainy and cold seasons (July to January) (Sithithaworn et al., 1997). This implies that the snails get infected with the parasites during the hot weather period and require about 2 months to liberate free-swimming cercariae (Harinasuta, Harinasuta, 1984; Upatham, Viyanant, 2003). The released cercariae encounter then infect the fish intermediate host and develop metacercariae within 6 weeks (Harinasuta, Harinasuta, 1984). Despite the *Bithynia* snail has two sexes male and female, the sex was not considered as independent factor in the experimental infection setting since there was no significantly difference of *O. viverrini* infection (Prasopdee et al., 2015). In cross sectional study, among three taxa of *Bithynia* in Thailand, *B. siamensis goniomphalos* was reported highest prevalence than other species (Kulsantiwong et al., 2015). Nevertheless, in experimental infection, *B. funiculata* and *B. siamensis siamensis* were demonstrated more susceptible to *O. viverrini* than *B. siamensis goniomphalos* (Chanawong, Waikagul, 1991). However, of previous studies, the *O. viverrini* infection detection in *Bithynia* was solely based on cercarial shedding. Data present herein revealed the infection status of *O. viverrini* were detected by both observing elicited cercariae using cercarial shedding and observing specific band (330 bp) using specific primers PCR to *O. viverrini.* The latter step of *O. viverrini* detection was used to find whether throughout the time course of infection (1, 7, 14, 28 and 56 dpi), the *Bithynia* infected with intramolluskan stages or not (Prasopdee et al., 2015). Surprisingly, the susceptibility to *O. viverrini* infection in both *B. siamensis siamensis* and *B. funiculata* was high at 1 dpi (94% and 81.3%, respectively) thereafter dramatically declined at extra period of dpi for *B. funiculata*. In case of *B. siamensis siamensis*, the susceptibility at 7 dpi (75%) of was similar to 1 dpi. However, there was dramatically declined at 14 dpi and thereafter unsusceptible for later period of dpi. This possibly due to 2 distinct phenomena as proposed in previous study (Prasopdee et al., 2015) – 1) immune responses and 2) nutritional status of snails. Initial parasite invasion trigger immune responses of snails, which led to diminish and complete elimination of the parasites, respectively, hence the number of infected snails over time were decreased. Malnutrition of infected snails rearing under laboratory conditions resulted in poor condition for development of intramolluskan larval stages. In addition, snail death due to the harm arising from parasite infection are suggested, however this point remains murky and should be investigated further. In addition, we can observe that at the end of laboratory time course of infection (56 dpi) there was no liberated cercaria. However, specific PCR showed evidence of developed intramolluskan stages. At this point, it might be the poor nutrition of laboratory rearing snail slowdowns the development of *O. viverrini* cercaria. Furthermore, at 56 dpi, the susceptibility to *O. viverrini* infection in *B. funiculata* (25%) was shown higher than in *B. siamensis siamensis* where the infection was not observed. This signifies that *O. viverrini* cercaria development is prone to be achieving higher in *B. funiculata* host.

We can conclude the surveillance of *O. viverrini* infection in snail hosts should not be ignored as we can observe the susceptibility to *O. viverrini* infection especially in *B. funiculata* was high.

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REFERENCES

Brandt RAM. The non-marine aquatic Mollusca of Thailand. **Arch Mollusk** 1974; 105: 1-423.

Brockelman WY, Upatham ES, Viyanant V, Ardsungnoen S, Chantanawat R. Field studies on the transmission of the human liver fluke, *Opisthorchis viverrini*, in northeast Thailand: population changes of the snail intermediate host. **Int J Parasitol** 1986; 16: 545-552.

Cantacessi C, Prasopdee S, Sotillo J, Mulvenna J, Tesana S, Loukas A. Coming out of the shell: building the molecular infrastructure for research on parasite-harbouring snails. **PLoS Negl Trop Dis**. 2013;7(9):e2284. Published 2013 Sep 12. doi:10.1371/journal.pntd.0002284

Chanawong A, Waikagul J. Laboratory studies on host-parasite relationship of *Bithynia* snails and the liver fluke, *Opisthorchis viverrini*. **Southeast Asian J Trop Med Public Health** 1991; 22: 235-239.

Chitramvong YP. The Bithyniidae (Gastropoda: Prosobanchia) of Thailand: comparative external morphology. **Malacol Rev** 1992; 25: 21-38.

Duangprompo W. **Genetic variation of snails in the family Bithyniidae in Thailand and identification of *Bithynia siamensis goniomphalos* by PCR** [M.Sc Thesis]. Khon Kaen: Faculty of Medicine, Khon Kaen University; 2007.

Harinasuta C, Harinasuta T. *Opisthorchis viverrini*: life cycle, intermediate hosts, transmission to man and geographical distribution in Thailand. **Arznei-Forschung** 1984; 34: 1164-1167.

Khampoosa P, Jones MK, Lovas EM, Srisawangwong T, Laha T, Piratae S, et al. Light and electron microscopy observations of embryogenesis and egg development in the human liver fluke, *Opisthorchis viverrini* (Platyhelminthes, Digenea). **Parasitol Res** 2012; 110: 799-808.

Kiatsopit N, Sithithaworn P, Saijuntha W, Boonmars T, Tesana S, Sithithaworn J, et al. Exceptionally high prevalence of infection of *Bithynia siamensis goniomphalos* with *Opisthorchis viverrini* cercariae in different wetlands in Thailand and Lao PDR. **Am J Trop Med Hyg** 2012; 86: 464-469.

Kulsantiwong J. **Identification of snail species of family Bithyniidae in Thailand by DNA barcoding and specific primer PCR** [PhD Thesis in Parasitology]. Khon Kaen: Faculty of Medicine, Khon Kaen University; 2013.

Kulsantiwong J, Prasopdee S, Piratae S, Khampoosa P, Suwannatrai A, Duangprompo W, et al. Species-specific primers designed from RAPD products for *Bithynia funiculata*, the first intermediate host of liver fluke, *Opisthorchis viverrini*, in North Thailand. **J Parasitol** 2013; 99: 433-437.

Kulsantiwong J, Prasopdee S, Ruangsittichai J, Ruangjirachuporn W, Boonmars T, Viyanant V, et al. DNA barcode identification of freshwater snails in the family Bithyniidae from Thailand. **PLoS One** 2013; 8: e79144.

Prasopdee S, Sotillo J, Tesana S, et al. RNA-Seq reveals infection-induced gene expression changes in the snail intermediate host of the carcinogenic liver fluke, *Opisthorchis viverrini*. **PLoS Negl Trop Dis**. 2014;8(3):e2765. Published 2014 Mar 27. doi:10.1371/journal.pntd.0002765

Prasopdee S, Tesana S, Cantacessi C, et al. Proteomic profile of *Bithynia siamensis goniomphalos* snails upon infection with the carcinogenic liver fluke *Opisthorchis viverrini*. **J Proteomics**. 2015;113:281-291. doi:10.1016/j.jprot.2014.09.018

Prasopdee S, Kulsantiwong J, Piratae S, Khampoosa P, Thammasiri C, Suwannatrai A, et al. Temperature dependence of *Opisthorchis viverrini* infection in first intermediate host snail, *Bithynia siamensis goniomphalos*. **Acta Trop** 2015; 141(Pt A): 112-117.

Sithithaworn P, Pipitgool V, Srisawangwong T, Elkins DB, Haswell-Elkins MR. Seasonal variation of *Opisthorchis viverrini* infection in cyprinoid fish in north-east Thailand: implications for parasite control and food safety. **Bull World Health Organ**. 1997; 75(2): 125-131.

Sri-aroon P, Butraporn P, Limsomboon J, Kerdpuech Y, Kaewpoolsri M, Kiatsiri S. Freshwater mollusks of medical importance in Kalasin Province, northeast Thailand. **Southeast Asian J Trop Med Public Health** 2005; 36: 653-657.

Sumethanurungkul P. **Studies on physical effects on snail intermediate hosts of a liver fluke (*Opisthorchis viverrini*)** [Master Thesis in Parasitology]. Bangkok: Faculty of Graduates Studies, Mahidol University; 1970.

Suwannatrai A, Suwannatrai K, Haruay S, Piratae S, Thammasiri C, Khampoosa P, et al. Effect of soil surface salt on the density and distribution of the snail *Bithynia siamensis goniomphalos* in northeast Thailand. **Geospat Health** 2011; 5: 183-190.

Tesana S, Thapsripair P, Thammasiri C, et al. Effects of Bayluscide on *Bithynia siamensis goniomphalos*, the first intermediate host of the human liver fluke, *Opisthorchis viverrini*, in laboratory and field trials. **Parasitol Int**. 2012;61(1):52-55. doi:10.1016/j.parint.2011.08.003

Upatham ES, Sornmani S, Kitikoon V, Lohachit C, Burch JB. Identification key for the fresh-and brackish-water snails of Thailand. **Malacol Rev** 1983; 16: 107-132.

Upatham ES, Sukhapanth N. Field studies on the bionomics of *Bithynia siamensis siamensis* and the transmission of *Opisthorchis viverrini* in Bangna, Bangkok, Thailand. **Southeast Asian J Trop Med Public Health** 1980; 11: 355-358.

Upatham ES, Viyanant V, Kurathong S, Rojborwonwitaya J, Brockelman WY, Ardsungnoen S, et al. Relationship between prevalence and intensity of *Opisthorchis viverrini* infection and clinical symptoms and signs in a rural community in northeast Thailand. **Bull WHO** 1984; 62: 451-461.

Winnepenninckx B, Backeljau T, De-Wachter R. Extraction of high molecular weight DNA from mollusks. **Trends Genet** 1993; 9: 407.

Wongratanacheewin S, Pumidonming W, Sermswan RW, Maleewong W. Development of a PCR-based method for the detection of *Opisthorchis viverrini* in experimentally infected hamsters. **Parasitology**. 2001; 122(Pt 2): 175-180. doi:10.1017/s0031182001007235

Wykoff DE, Harinasuta C, Juttijudata P, Winn MM. *Opisthorchis viverrini* in Thailand the life cycle and comparison with *O. felineus*. **J Parasitol** 1965; 51: 207-214.

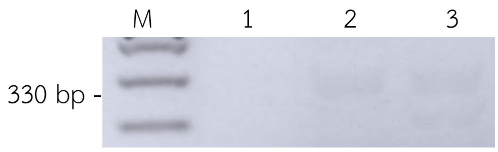

Figure 1 Detections of *O. viverrini* infection in snails. 330 bp specific band of PCR product for *O. viverrini;* lane M = 100bp DNA ladder, lane 1 = Negative control, lane 2-3 = positive to *O. viverrini* infection.

Table 1 Percentage of susceptibility to *O. viverrini* infection of *B. siamensis siamensis* and *B. funiculata* categorized by post infection (dpi)

% positive snails / dpi	*B. siamensis siamensis* (positive/total)	*B. funiculata* (positive/total)
1	94 (15/16)	82 (13/16)
7	75 (12/16)	25 (4/16)
14	32 (5/16)	0 (0/16)
28	0 (0/16)	7 (1/16)
56	0 (0/16)	25 (4/16)
overall	40 (32/80)	28 (22/80)

Table 2 Association between infection and day post infection of *B. siamensis siamensis*[i]

Day post infection	OR	95% CI
1 (reference)	T_{wald}=11.309, df=4, P=0.023	
7	0.200	0.020-2.033
14	0.030*	0.003-0.297
28	0.000	-
56	0.000	-

* Indicate significance at P< 0.05, [i] Results of association are shown as odds ratio (OR).

Table 3 Association between infection and day post infection of *B. funiculata*[i]

Day post infection	OR	95% CI
1 (reference)	T_{wald}=16.065, df=4, P=0.03	
7	0.077*	0.014-0.417
14	0.000	-
28	0.015*	0.001-0.167
56	0.077*	0.014-0.417

* Indicate significance at $P < 0.05$, [i] Results of association are shown as odds ratio (OR).

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The effects of temperature and salinity on the longevity of *Opisthorchis viverrini* cercariae: a climate change concern

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Abstract

Research on the effects of environmental factors influenced by climate change on parasite transmissibility is an area garnering recent attention worldwide. However, there is still a lack of studies on the life cycle of *Opisthorchis viverrini*, a carcinogenic trematode found in countries of the Lower Mekong subregion of Lao PDR, Cambodia, Myanmar, Vietnam and Thailand. To evaluate the influences of environmental factors water temperature and salinity on the transmissibility of the liver fluke *O. viverrini* through cercarial stage, longevity of *O. viverrini* cercaria was examined at different experimental temperatures (22°C, 30°C and 38°C) and salinities (2.5 parts per thousand (PPT), 3.75 PPT and 5 PPT). The results reveal that different temperatures have statistically significant effects on cercarial longevity. The cercariae exhibited a thermostability zone ranging between 22°C and 30°C. Cercarial longevity was significantly shortened when water temperatures reached 38°C. Salinity also plays a key role in cercarial longevity, with cercarial survival significantly shorter at a salinity of 3.75 PPT than at 2.5 PPT and 5 PPT. A combined analysis of salinity and temperature revealed unique trends in cercarial longevity. At all experimental salinities, cercarial longevity was lowest when incubated in 38°C, but statistically significant from cercarial longevity at temperatures of 22°C and 30°C, and salinities of 2.5 PPT and 5 PPT. The results suggest that higher temperatures negatively impact parasite longevity. This reflects that *O. viverrini* transmission patterns may be impacted by changes in water temperature and salinity resulting from climate change.

Introduction

Opisthorchis viverrini is a highly endemic liver fluke found in the Lower Mekong subregion countries Lao PDR, Cambodia, Myanmar, Vietnam and Thailand (Sithithaworn *et al.*, 2012; Aung *et al.*, 2017). At least ten million people worldwide are infected with this parasite, and more than 67 million people are at risk of being infected (Andrews *et al.*, 2008; Keiser & Utzinger, 2009; Traub *et al.*, 2009). Chronic *O. viverrini* infections have proven to be a precipitating factor in the development of cholangiocarcinoma (CCA), a bile duct cancer (IARC, 2002; Bouvard *et al.*, 2009). In order for the parasite to infect humans, freshwater snails from the genus *Bithynia* and freshwater fish from the family Cyprinidae must first become the parasite's first and second intermediate hosts, respectively (Wykoff *et al.*, 1965). Piscivorous mammals including cats and dogs also act as reservoir hosts for the parasite. In the first intermediate host, the *Bithynia* snail, *O. viverrini* undergoes asexual reproduction through several intramolluscan stages of sporocysts, rediae and cercariae. Thereafter, these cercariae excyst and become free-swimming, finding and encysting under the scales and in the flesh of the second intermediate host, Cyprinid fish, as metacercariae (Wykoff *et al.*, 1965; WHO, 1995; Sithithaworn & Haswell-Elkins, 2003). Humans and reservoir hosts become infected by consuming freshwater fish containing metacercariae.

Due to its complex transmission cycle, *O. viverrini* infections remain an unresolved problem (Vonghachack *et al.*, 2017). In the endemic areas of north-eastern Thailand and southern Laos, the prevalence of *O. viverrini* was found to be high in fish, at 26.9%–97% (Vichasri *et al.*, 1982; Vonghachack *et al.*, 2017), and low in snails, at 0.3%–3.04% (Sri-Aroon *et al.*, 2005; Kiatsopit *et al.*, 2012; Prasopdee *et al.*, 2015; Vonghachack *et al.*, 2017). The rate of infection in cats, one of the parasite's reservoir hosts, was found to be slightly high, at 35.5%–53.1% (Enes *et al.*, 2010; Aunpromma *et al.*, 2012; Vonghachack *et al.*, 2017). This indicates that cercariae play a key role in host-to-host transmission. As the Fifth Assessment Report of The United Nations Intergovernmental Panel on Climate Change (IPCC) indicates, climate change ultimately results in rising temperatures and changes in intensity and frequency of rainfall (Stocker *et al.*, 2013), thus affecting both the temperature and salinity of water sources.

Presently, the effect of climate change on parasitic transmission is an area of interest currently attracting attention (Poulin, 2006; Morley, 2011; Barber et al., 2016). The rising global temperatures may impact the parasitic transmission to humans due to the temperature-dependent metabolism of non-feeding free-swimming cercariae (Mouritsen, 2002; Thieltges & Rick, 2006; Koprivnikar et al., 2010). As the longevity of the cercariae increases, the chances that the parasites will be exposed to a host increase. However, it is important to recognize that the longevity of temperature-mediated cercariae is likely a character specific to the parasite's species. Previous reports have demonstrated that the cercariae of *Transversotrema patialensis* could survive for approximately 44 h at water temperatures of 24°C (Anderson & Whitfield, 1974), while the cercariae of *Plagiorchis elegans* could only survive in such temperatures for 30 h (Lowenberger & Rau, 1994). However, an increase in water temperatures is more likely to decrease the overall cercarial survival times (Mouritsen, 2002; Thieltges & Rick, 2006; Studer & Poulin, 2013). Despite scant reports on *O. viverrini* infectivity in host snail increasing as water temperature increases (Prasopdee et al., 2015), little is known regarding the influence of temperature on *O. viverrini* cercariae. Nevertheless, temperatures continue to rise; past reports from the Thai meteorological department reports average air temperatures during the cool and dry seasons of the years 1981 to 2010 to be 24.2°C, with mean minimum and mean maximum air temperatures of 18°C and 30°C. In addition to temperature, salinity has also been recognized as one of the most important environmental influences on parasite biology (Zander, 1998; Koprivnikar et al., 2010; Studer & Poulin, 2013). Based on the distribution and density of *Bithynia* snails in north-east Thailand, an area endemic with opisthorchiasis and opisthorchiasis-induced CCA, it was found that *Bithynia* snails prefer brackish water over fresh water, with the highest snail population densities found in areas where the water salinity ranges from 2.5 parts per thousand (PPT) to 5 PPT (Suwannatrai et al., 2011). Despite these reports, the effects of these levels of salinity on *O. viverrini* cercariae are unknown. All in all, there are also a lack of experimental studies on the effects of global warming and salinity on the longevity of *O. viverrini* cercariae.

The aim of the present study is to investigate the effects of temperature and salinity on the overall survival of *O. viverrini* cercariae in order to develop a better understanding of *O. viverrini* cercarial transmission and, thus, establish strategies for the surveillance and control of this snail-borne parasitic disease in the context of climate change.

Materials and methods

Procurement of snail samples

Opisthorchis viverrini cercariae were obtained by collecting *Bithynia siamensis goniomphalos* snails, the parasite's first intermediate hosts, from Bueng Niam, Mueang Khon Kaen District, Khon Kaen Province, Thailand (16°26′50.64″N, 102°54′10.5048″E).

Assessment of snail infection status

The sample snails were then screened for infection by *O. viverrini* cercariae using the cercarial shedding method. The snails were first exposed to 3 h of constant light from an 8-W LED bulb during the daytime. The shed cercariae were then identified under a stereomicroscope according to the parasite's morphologic features published in the available literature (Frandsen & Christensen, 1984). Morphologically similar cercariae were then confirmed to be *O. viverrini* through the use of a polymerase chain reaction (PCR) protocol described by Wongratanacheewin et al. (2001) targeting the pOV-A6 gene. The presence of specific amplicons of approximately 330 bp in size was considered to denote *O. viverrini* cercariae. Snails which were found to shed the cercariae of *O. viverrini*, and, thus, infected with the parasite, were selected for the experiment.

Experimental procedure

The selected snails were randomly assigned to each salinity group. Four snails were placed into plastic cups 4 cm in diameter along with 15 ml of saline solutions at the salinity level corresponding to each experimental group. The saline solutions of differing salinity levels, defined as the amount of salt dissolved in a certain volume of water and measured in PPT, was prepared by mixing salt sodium chloride (NaCl) (VWR BDH Prolabo, Leuven, Belgium) with distilled water. The saline solutions were prepared at the experimental salinities of 2.5 PPT, 3.75 PPT and 5 PPT. The snails were once again exposed to 1 h of constant light from an 8-W LED bulb at room temperature, which stimulated the cercariae to emerge from the snail. The snail was then removed from the cups. The plastic cups containing the cercariae were then submerged in a water bath set to different experimental temperatures (22°C, 30°C, 38°C – chosen based on published reports from the Thai Meteorological Department) while facing light sources that matched the daily light–dark cycle. Every 2 h the number of dead cercariae in each container was measured. The plastic cups were transferred to a smaller water bath under a stereomicroscope consisting of a petri dish filled with water of the same temperature as the water bath. Under the stereomicroscope, all cercariae were observed for motion. All motionless cercariae were stimulated with a 25-gauge needle (McCarthy, 1999; Koprivnikar et al., 2010). If the cercariae remained motionless after stimulation, it was declared dead, removed from the plastic cup and tallied. The plastic cup was then returned to the large water bath of the same temperature for another 2 h before another round of counting. This counting process was continued until all the cercariae in each plastic cup died.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp, Armonk, NY, USA). Parametric assumptions, normal distributions and homogeneity of variance were tested beforehand in order to confirm whether the data met the assumptions. The Kolmogorov–Smirnov 'Goodness-of-Fit' test was first used in conjunction with a normal probability plot to determine if the survival data was congruent with that of a normal distribution. If $P > 0.05$, then the data are parametric, and the data would then be tested for homogeneity of variance. However, if $P < 0.05$, then the data are compared with the normal Q–Q plot in order to check for normality. The data are normally distributed if they follow the diagonal line closely and do not appear to have a non-linear pattern. The Levene test was then used to determine the homogeneity of variance; if $P > 0.05$, then the data have homogeneity of variance. If the survival data proved to be normally distributed and their homogeneity of variance is true, a one-way analysis of variance

(ANOVA) and subsequent Scheffe post-hoc analysis would then be performed to determine if there were statistically significant differences between the survival times of each group. However, if the survival data proved to be non-parametric, the non-parametric Kruskal–Wallis test would be used instead to analyse the data, followed by a pairwise comparison (Dunn test) to determine if there were statistically significant differences between the survival times of each group.

In order to find associations between salinity and cercarial survival, data of different temperature treatments with the same experimental salinity were pooled and subsequently analysed. In order to find associations between temperature and cercarial survival times, survival data of different salinity levels at the same experimental temperatures were pooled and analysed. The correlation between temperature–salinity combinations and survival of cercariae incubated at 22°C, 30°C and 38°C were analysed according to experimental salinity. This was to determine whether cercarial survival was temperature–salinity dependent.

Results

Infection rate

Of all the collected snails, 12 out of 772 were positive for *O. viverrini* infection. Thus, the infection rate was 1.55% in snails with a shell length of greater than 0.6 mm.

Temperature

Survival data of different salinity levels at the same experimental temperatures were pooled and analysed, revealing mean survival times ± standard deviation (SD) (along with median and the interquartile range (IQR)) for *O. viverrini* cercariae incubated at 22°C, 30°C and 38°C to be 34.71 ± 18.44 h (24, 26), 29.68 ± 8.7 h (28, 10) and 19.91 ± 1.87 h (20, 2), respectively. At the observed time points of 22 h (43.5%; 232/533), 30 h (17%; 63/371) and 20 h (44%; 121/275), the largest percentages of cercariae found to be dead were at the respective temperatures of 22°C, 30°C and 38°C. Thus, cercarial longevity decreases with increasing temperatures (fig. 1). The Kolmogorov–Smirnov test and Levene test confirmed that the temperature–cercarial survival data set was non-parametric. Median survival times were used, which resulted in the optimum incubation temperature being 30°C (28), rather than at 22°C (24) or 38°C (20). In order to find associations between temperature and cercarial survival times, the Kruskal–Wallis test was applied, revealing a significant correlation between temperature and cercarial survival times (χ^2 (2) > 393.5, $P < 0.001$). Median survival times of cercariae incubated at 22°C, 30°C and 38°C were found to be 24 h, 28 h and 20 h, respectively. Survival times of cercariae incubated at 22°C at 30°C were found to not be significantly different from each other ($P > 0.05$). However, at the highest experimental temperature of 38°C, there were significantly different cercarial survival times compared to survival times at 30°C ($P < 0.01$) and 22°C ($P < 0.01$), with a two-fold decrease in survival times.

Salinity

The Kolmogorov–Smirnov test with Q–Q plot analysis and Levene test confirmed that the salinity–cercarial survival data set was parametric. In order to analyse the effects of salinity on cercarial survival, data of different temperature treatments with the same experimental salinity were pooled and subsequently analysed using a one-way ANOVA followed by a Scheffe post-hoc analysis. Mean survival times ± SD were found to be 30.94 ± 13.79 h at 2.5 PPT, 27.27 ± 14.01 h at 3.75 PPT and 30.40 ± 15.5 h at 5 PPT. Mean survival was significantly greater at 2.5 PPT than at 3.75 PPT ($P < 0.05$; mean difference: 3.67; 95% confidence interval (CI): 1.08, 6.25) and at 5 PPT versus at 3.75 PPT ($P < 0.05$; mean difference: 3.13; 95% CI: 0.55, 5.70). However, there was no difference between survival at 2.5 PPT and at 5 PPT ($P = 0.86$; mean difference: 0.53; 95% CI: −1.92, 3.00) (fig. 2). The highest percentage of dead cercariae was observed at 22 h, with 21.7% (91/419) of all cercariae dead at 5 PPT, 27.1% (94/347) dead at 3.75 PPT and 21.8% (90/413) dead at 2.5 PPT.

Salinity–temperature combinations

The survival of cercariae incubated at 22°C, 30°C and 38°C were analysed according to experimental salinity. Temperature–salinity combinations were found to be non-parametric, according to the Kolmogorov–Smirnov and Levene tests. In terms of correlates between temperature–salinity combinations and cercarial survival, the Kruskal–Wallis test was used, revealing evidence indicating survival of cercaria was temperature–salinity dependent. At 2.5 PPT, a total of 413 cercariae were analysed, revealing significant differences in cercarial survival at different temperatures (χ^2 (2) > 118.19; $P < 0.001$). The mean ± SD (along with median, IQR) survival of cercariae incubated at 22°C, 30°C and 38°C was 33.83 ± 16.74 (24, 26), 32.39 ± 9.26 (30, 16) and 20.40 ± 2.22 (20, 4), respectively. Pairwise comparisons demonstrated no significant differences between cercarial survival at 22°C and 30°C. However, cercarial survival at 38°C was statistically significant compared to the lower experimental temperatures of 22°C and 30°C ($P < 0.001$). A total number of 347 cercariae at 3.75 PPT were analysed, and revealed a significant difference of survival at different temperatures (χ^2 (2) > 79.47, $P < 0.001$), with mean ± SD (along with median, IQR) cercarial survival at 22°C, 30°C and 38°C of 32.59 ± 18.7 (22, 6), 28.75 ± 11.49 (26, 26) and 20.34 ± 1.78 (20, 1), respectively. Thus, at 3.75 PPT, cercarial survival was significantly different at all temperatures. At 5 PPT, a total of 419 cercariae were analysed. Significant differences of survival in different degrees of temperature was revealed (χ^2 (2) > 184.10; $P < 0.001$), with mean ± SD (along with median, IQR) of cercariae incubated at 22°C, 30°C and 38°C of 36.95 ± 19.72 (24, 42), 27.60 ± 3.98 (29, 4.5) and 18.77 ± 0.98 (18, 2), respectively. There was no evidence indicating the difference between survival times of cercariae incubated in 22°C and 30°C. However, at the high temperature of 38°C, cercarial survival was significantly different from cercariae incubated at lower experimental temperatures ($P < 0.001$) (see table 1).

Discussion

As a foodborne trematode, *O. viverrini* must first mature from free-swimming cercariae into infective metacercariae. However, it is during this cercarial stage that the parasite is exposed to many environmental factors, making it a critical part of *O. viverrini*'s life cycle. As previous studies have reported that *O. viverrini* infections in snails, its first intermediate host, are temperature dependent (Prasopdee *et al.*, 2015), one of the objectives of this study is to investigate the effects of temperature on the survival of cercariae. The incubation temperatures 22°C, 30°C and 38°C were chosen as they represent the air temperatures of the endemic

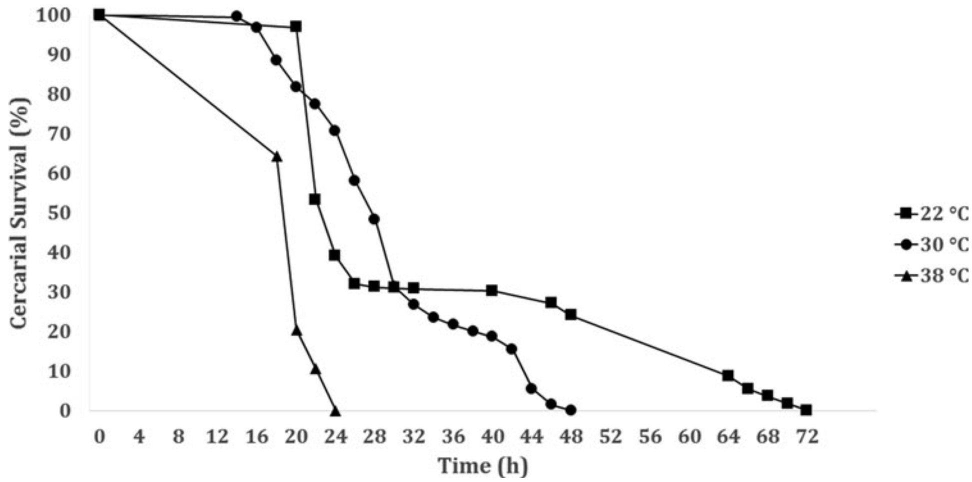

Fig. 1. Cercarial survival at different experimental temperatures. Percentage of surviving *O. viverrini* cercariae at the incubation temperatures of 22°C, 30°C and 38°C (obtained from 533 cercariae, 371 cercariae and 275 cercariae, respectively).

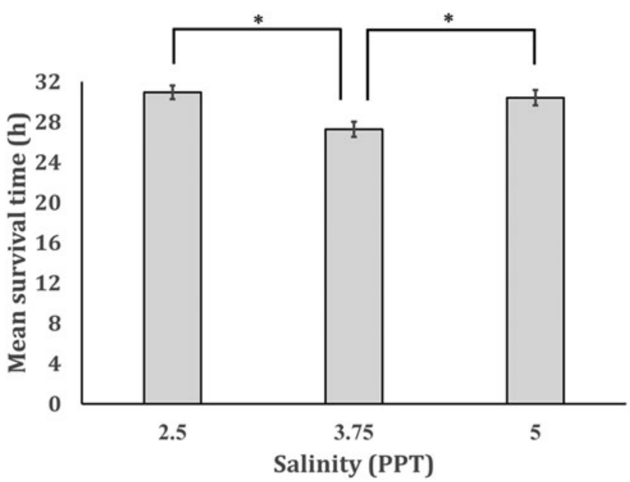

Fig. 2. Mean cercarial survival at different experimental salinities. Mean (±SD) survival time of *O. viverrini* cercariae at the saline solutions of 2 PPT, 3.75 PPT and 5 PPT. *Indicates statistical significance ($P < 0.05$) analysed using a one-way ANOVA test followed by Scheffe post-hoc analysis ($N = 413$, 347 and 419 for 2 PPT, 3.75 PPT and 5 PPT, respectively).

Table 1. Pairwise comparisons of salinity–temperature combinations with cercarial survival.

Salinity (PPT) × temperature (°C)		*N*	Median	IQR	Mean (±SD)
2.5 PPT		413			
	22°C	201	24	26	33.83 ± 16.74[a]
	30°C	138	30	16	32.39 ± 9.26[a]
	38 °C	74	20	4	20.40 ± 2.22[b]
3.75 PPT		347			
	22°C	131	22	6	32.59 ± 18.70[1]
	30°C	95	26	26	28.75 ± 11.49[2]
	38°C	121	20	1	20.34 ± 1.78[3]
5 PPT		419			
	22°C	201	24	42	36.95 ± 19.72[i]
	30°C	138	29	4.5	27.60 ± 3.98[i]
	38°C	80	18	2	18.77 ± 0.98[ii]

Statistically significant differences were analysed using the Kruskal–Wallis test followed by a pairwise comparison.
[a,b]Significant differences at 2.5 PPT ($P < 0.01$).
[1,2,3]Significant differences at 3.75 PPT ($P < 0.01$).
[i,ii]Significant differences at 5 PPT ($P < 0.01$).

north-eastern Thailand (Harinasuta & Harinasuta, 1984; Haswell-Elkins *et al.*, 1994; Jongsuksuntigul & Imsomboon, 2003). According to latest Thai Meteorological Department reports of air temperatures during the cool and dry seasons of the years 1981 to 2010, the mean air temperature was reported to be 24.2°C, and the mean minimum and maximum air temperatures were reported to be 18°C and 30°C, respectively. Due to reports indicating an increase in *O. viverrini* infections in field snails during the cool-dry season in north-eastern Thailand (Brockelman *et al.*, 1986) and the geographically similar Laos (Kiatsopit *et al.*, 2014), the experimental temperatures of 22°C and 30°C were selected to represent these weather conditions. The highest experimental temperature of 38°C was chosen to represent the effects of global warming, which caused a 0.2°C increase in global temperatures per decade during the late 19th century, until the first half-decade of the 20th century (Hansen *et al.*, 2006). As Thai Meteorological Department reports of mean air temperatures in north-eastern Thailand during 1981–2010 have a mean maximum temperature of 35.2°C, and even reached a record high of 43.9°C in 1960, the experimental temperature is not out of the realm of possibility, especially when previous studies have demonstrated a considerably low 3°C deviation between air temperature and water temperature (Prasopdee, 2013). The results of this experiment support the hypothesis that temperature affects the survival of *O. viverrini* cercariae, with high temperatures decreasing the survival of the cercariae, similarly to the results of studies in other trematodes (McCarthy, 1999; Mouritsen, 2002; Thieltges & Rick, 2006; Koprivnikar *et al.*, 2010). This may be explained by the fact that cercariae are non-feeding and must use energy from non-renewable glycogen stores (Ginetsinskaya, 1988), which, at higher temperatures, may require the increased usage of glycogen, ultimately shortening the parasite's lifespan (Ginetsinskaya, 1960). Interestingly, statistical analysis demonstrates that only survival times of the cercariae studied at 38°C were statistically significant from 22°C and 30°C; however, cercariae studied between 22°C and 30°C were not found to be statistically significant from each other, which indicates that this temperature range is the thermostability zone specific to *O. viverrini* cercariae. This phenomenon is also observed in the Tanzanian strain of *Schistosoma mansoni*, where glycogen utilization remains constant between 18°C and 27°C, but dramatically rises when the temperature exceeds 27°C (Purnell, 1966; Morley, 2011).This is consistent with reports on wild snails infected with *O. viverrini*, where it was found that cercarial emerge peaks between 8 AM and 10 AM during the hot season, and between 12 PM and 2 PM during the cool-dry and rainy seasons (Kiatsopit *et al*., 2014). Measurements of water temperatures during these time frames were found to be consistent with the zone of thermostability derived from the results of this study. Although the rise in average water temperatures due to global warming may appear to shorten cercarial lifespans, the effects of rising water temperatures on other aquatic animals, including *O. viverrini's* second intermediate host, the fishes, remains uncertain. As such, further studies on cercarial infectivity and metacercarial burden are required.

Another factor important to cercarial survival and longevity is the salinity of water. The salinity values chosen for this experiment, at 2.5 PPT, 3.75 PPT and 5 PPT, were chosen to represent zones with the highest density of *Bithynia* snails found in the endemic north-eastern Thailand (Suwannatrai *et al*., 2011). From the results above, cercarial survival at salinities of 2.5 PPT and 5 PPT were significantly higher than the cercarial survival at 3.75 PPT, suggesting that salinity plays a role in cercarial survival, despite the results of past reports (Rees, 1948; Mouritsen, 2002). However, the mechanisms behind this trend remain unclear, and, thus, require further study.

When data from both salinity and temperature were analysed together, it was found that *O. viverrini* cercarial survival was highest at extremes of salinity such as at 2.5 PPT and 5 PPT, rather than at a moderate salinity level of 3.75 PPT. However, when cercarial survival at these extremes of salinity were studied at a temperature of 38°C, cercarial survival at both salinities significantly shortened, correlating to cercarial survival when only temperature is concerned. Although cercarial survival at 38°C was shortest when salinity was at 3.75 PPT, survival times were statistically significant compared to temperatures of 22°C and 30°C, unlike survival times between temperatures studied at the other two levels of salinity. This suggests that cercariae become more sensitive to changes and extremes of temperatures when the water salinity is moderate, at a level of 3.75 PPT. Thus, it can be inferred that seasonal temperature changes play a significant role in overall cercarial survival. During the hot-dry and cool-dry seasons, water salinity levels increase due to increased evaporation from sun and wind exposure. Conversely, during the rainy seasons, water salinity levels decrease due to dilution from increased rainfall. As demonstrated by the results above, variations in salinity levels may play a role in *O. viverrini* transmission patterns. In addition to influencing cercarial survival, salinity is positively correlated with the first intermediate *Bithynia* host populations, as it is with second intermediate cyprinid host populations (Kim *et al*., 2016). This may create conditions ideal for *O. viverrini* transmission during the hot-dry and cool-dry seasons. However, from past studies, metacercarial burden in the cyprinid fishes was highest during the late rainy season and throughout the cool-dry season, but lowest during the hot-dry season (Sithithaworn *et al*., 1997). Since *Bithynia* snails prefer to stay in shallower, warmer waters (Suwannatrai *et al*., 2011), *O. viverrini* cercariae will be exposed to temperatures higher than their thermostability zone, resulting in shortened longevity that may lead to shorter metacercarial longevity and, ultimately, an overall decrease in the number of infected fish during the hot-dry season. In addition to salinity and temperature, water contaminants such as fertilizers influence the local fish and snail population, thus affecting the transmissibility of *O. viverrini* (Kim *et al*., 2016).

Although the results of this study imply that rising global temperatures impair the transmissibility of *O. viverrini* during the cercariae–metacercariae transition period as a result of shortened cercarial longevity, conclusions cannot yet be drawn. This is due to a lack of studies on the effects of salinity and temperature on the infectivity of *O. viverrini* cercariae, as it is unknown whether these factors are favourable to parasite infectivity. This is also true concerning cercarial emergence, as more studies are required to determine how variations in temperature affect cercarial emergence. Overall, although the results of this study suggest that *O. viverrini* is negatively affected by rising global temperatures and salinities, further studies on the different aspects of the parasitic life cycle – namely, longevity, infectivity and cercarial emergence – are required before any accurate predictions can be made.

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Conflicts of interest. None

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards stated in the Animals for Scientific Purposes Act 2015 of Thailand and were approved by the Institutional Ethics Committee of Thammasat University (Animal Ethics clearance number 014/2559).

References

Anderson RM and Whitfield PJ (1974) Proceedings: a model for the survival rate of non-feeding larval parasites. *Parasitology* **69**, 7.

Andrews RH, Sithithaworn P and Petney TN (2008) *Opisthorchis viverrini*: an underestimated parasite in world health. *Trends in Parasitology* **24**, 497–501.

Aung WPP, Htoon TT, Tin HH, *et al*. (2017) First report and molecular identification of *Opisthorchis viverrini* infection in human communities from Lower Myanmar. *PLOS ONE* **12**, e0177130.

Aunpromma S, Tangkawattana P, Papirom P, Kanjampa P, Tesana S, Sripa B and Tangkawattana S (2012) High prevalence of *Opisthorchis viverrini* infection in reservoir hosts in four districts of Khon Kaen Province, an opisthorchiasis endemic area of Thailand. *Parasitology International* **61**, 60–64.

Barber I, Berkhout BW and Ismail Z (2016) Thermal change and the dynamics of multi-host parasite life cycles in aquatic ecosystems. *Integrative and Comparative Biology* **56**, 561–572.

Bouvard V, Baan R, Straif K, *et al*. (2009) A review of human carcinogens–Part B: biological agents. *Lancet Oncology* **10**, 321–322.

Brockelman WY, Upatham ES, Viyanant V, Ardsungnoen S and Chantanawat R (1986) Field studies on the transmission of the human liver fluke, *Opisthorchis viverrini*, in northeast Thailand: population changes of the snail intermediate host. *International Journal for Parasitology* **16**, 545–552.

Enes JE, Wages AJ, Malone JB and Tesana S (2010) Prevalence of *Opisthorchis viverrini* infection in the canine and feline hosts in three villages, Khon Kaen Province, northeastern Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **41**, 36–42.

Frandsen F and Christensen NO (1984) An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. *Acta Tropica* **41**, 181–202.

Ginetsinskaya T (1960) The relationship between the distribution of glycogen in the bodies of different cercariae and their biological peculiarities. *Doklady Biological Sciences* **135**, 949–951.

Ginetsinskaya T (1988) *Trematodes, their life cycles, biology and evolution*. 559 pp. New Delhi, Amerind Publishing Company.

Hansen J, Sato M, Ruedy R, Lo K, Lea WW and Medina-Elizade M (2006) Global temperature change. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 14288–14293.

Harinasuta C and Harinasuta T (1984) *Opisthorchis viverrini*: life cycle, intermediate hosts, transmission to man and geographical distribution in Thailand. *Arzneimittelforschung* **34**, 1164–1167.

Haswell-Elkins MR, Mairiang E, Mairiang P, Chaiyakum J, Chamadol N, Loapaiboon V, Sithithaworn P and Elkins DB (1994) Cross-sectional study of *Opisthorchis viverrini* infection and cholangiocarcinoma in communities within a high-risk area in northeast Thailand. *International Journal of Cancer* **59**, 505–509.

IARC (2002) *Cancer incidence in five continents*. Vol. 8. Lyon, France, IARC Scientific Publication, 1–781.

Jongsuksuntigul P and Imsomboon T (2003) Opisthorchiasis control in Thailand. *Acta Tropica* **88**, 229–232.

Keiser J and Utzinger J (2009) Food-borne trematodiases. *Clinical Microbiology Reviews* **22**, 466–483.

Kiatsopit N, Sithithaworn P, Saijuntha W, Boonmars T, Tesana S, Sithithaworn J, Petney TN and Andrews RH (2012) Exceptionally high prevalence of infection of *Bithynia siamensis goniomphalos* with *Opisthorchis viverrini* cercariae in different wetlands in Thailand and Lao PDR. *American Journal of Tropical Medicine and Hygiene* **86**, 464–469.

Kiatsopit N, Sithithaworn P, Kopolrat K, Andrews RH and Petney TN (2014) Seasonal cercarial emergence patterns of *Opisthorchis viverrini* infecting *Bithynia siamensis goniomphalos* from Vientiane Province, Lao PDR. *Parasites & Vectors* **7**, 551.

Kim CS, Echaubard P, Suwannatrai A, Kaewkes S, Wilcox BA and Sripa B (2016) Seasonal and spatial environmental influence on *Opisthorchis viverrini* intermediate hosts, abundance, and distribution: insights on transmission dynamics and sustainable control. *PLOS Neglected Tropical Diseases* **10**, e0005121.

Koprivnikar J, Lim D, Fu C and Brack SH (2010) Effects of temperature, salinity, and pH on the survival and activity of marine cercariae. *Parasitology Research* **106**, 1167–1177.

Lowenberger CA and Rau ME (1994) *Plagiorchis elegans*: emergence, longevity and infectivity of cercariae, and host behavioural modifications during cercarial emergence. *Parasitology* **109**(Pt. 1), 65–72.

McCarthy AM (1999) The influence of temperature on the survival and infectivity of the cercariae of *Echinoparyphium recurvatum* (Digenea: Echinostomatidae). *Parasitology* **118**(Pt. 4), 383–388.

Morley NJ (2011) Thermodynamics of cercarial survival and metabolism in a changing climate. *Parasitology* **138**, 1442–1452.

Mouritsen KN (2002) The Hydrobia ulvae-Maritrema subdolum association: influence of temperature, salinity, light, water-pressure and secondary host exudates on cercarial emergence and longevity. *Journal of Helminthology* **76**, 341–347.

Poulin R (2006) Global warming and temperature-mediated increases in cercarial emergence in trematode parasites. *Parasitology* **132**, 143–151.

Prasopdee S (2013) Differential protein expression of Bithynia siamensis goniomphalos snail upon infection with liver fluke, Opisthorchis viverrini, a thesis for the degree of doctor of philosophy. p. 174. Khon Kaen University, Khon Kaen.

Prasopdee S, Kulsantiwong J, Piratae S, *et al.* (2015) Temperature dependence of *Opisthorchis viverrini* infection in first intermediate host snail, *Bithynia siamensis goniomphalos*. *Acta Tropica* **141**, 112–117.

Purnell RE (1966) Host-parasite relationships in schistosomiasis. 3. The effect of temperature on the survival of *Schistosoma mansoni* miracidia and on the survival and infectivity of *Schistosoma mansoni* cercariae. *Annals of Tropical Medicine and Parasitology* **60**, 182–186.

Rees G (1948) A study of the effect of light, temperature and salinity on the emergence of *Cercaria purpurae Lebour* from *Nucella lapillus* (L). *Parasitology* **38**, 228–242.

Sithithaworn P and Haswell-Elkins M (2003) Epidemiology of *Opisthorchis viverrini*. *Acta Tropica* **88**, 187–194.

Sithithaworn P, Pipitgool V, Srisawangwong T, Elkins DB and Haswell-Elkins MR (1997) Seasonal variation of *Opisthorchis viverrini* infection in cyprinoid fish in north-east Thailand: implications for parasite control and food safety. *Bulletin of the World Health Organization* **75**, 125–131.

Sithithaworn P, Andrews RH, Nguyen VD, *et al.* (2012) The current status of opisthorchiasis and clonorchiasis in the Mekong Basin. *Parasitology International* **61**, 10–16.

Sri-Aroon P, Butraporn P, Limsomboon J, Kerdpuech Y, Kaewpoolsri M and Kiatsiri S (2005) Freshwater mollusks of medical importance in Kalasin Province, northeast Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **36**, 653–657.

Stocker TF, Qin D and Plattner GK (2013) *Climate change 2013: the physical science basis*. Working Group I contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, United Kingdom and New York, NY, USA.

Studer A and Poulin R (2013) Cercarial survival in an intertidal trematode: a multifactorial experiment with temperature, salinity and ultraviolet radiation. *Parasitology Research* **112**, 243–249.

Suwannatrai A, Suwannatrai K, Haruay S, *et al.* (2011) Effect of soil surface salt on the density and distribution of the snail *Bithynia siamensis goniomphalos* in northeast Thailand. *Geospatial Health* **5**, 183–190.

Thieltges DW and Rick J (2006) Effect of temperature on emergence, survival and infectivity of cercariae of the marine trematode *Renicola roscovita* (Digenea: Renicolidae). *Diseases of Aquatic Organisms* **73**, 63–68.

Traub RJ, Macaranas J, Mungthin M, Leelayoova S, Cribb T, Murrell KD and Thompson RC (2009) A new PCR-based approach indicates the range of *Clonorchis sinensis* now extends to Central Thailand. *PLoS Neglected Tropical Diseases* **3**, e367.

Vichasri S, Viyanant V and Upatham ES (1982) *Opisthorchis viverrini*: intensity and rates of infection in cyprinoid fish from an endemic focus in Northeast Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **13**, 138–141.

Vonghachack Y, Odermatt P, Taisayyavong K, Phounsavath S, Akkhavong K and Sayasone S (2017) Transmission of *Opisthorchis viverrini*, *Schistosoma mekongi* and soil-transmitted helminthes on the Mekong Islands, Southern Lao PDR. *Infectious Diseases of Poverty* **6**, 131.

WHO (1995) Control of foodborne trematode infections. Report of a WHO Study Group. *World Health Organization technical report series* **849**, 1–157.

Wongratanacheewin S, Pumidonming W, Sermswan RW and Maleewong W (2001) Development of a PCR-based method for the detection of *Opisthorchis viverrini* in experimentally infected hamsters. *Parasitology* **122**, 175–180.

Wykoff DE, Harinasuta C, Juttijudata P and Winn MM (1965) *Opisthorchis viverrini* in Thailand–the life cycle and comparison with *O. Felineus*. *Journal of Parasitology* **51**, 207–214.

Zander CD (1998) Ecology of host parasite relationships in the Baltic Sea. *Naturwissenschaften* **85**, 426–436.

APPENDICES

APPENDIX A

Reagents and procedures of metacercaria preparation

Reagents and procedures of metacercaria preparation

1. 0.25% pepsin solution

Pepsin A powder	2.5	g
HCl	1.0	ml
NaCl	8.5	g
Distilled water to a final volume of	1,000	ml

2. 0.85% NaCl

NaCl	8.5	g
Filtered water to a final volume of	1,000	ml

3. Procedures

3.1. Crushed and spun fish in 0.25% pepsin until incorporated.

3.2. Incubated in shaking water bath at 37 °C for 1 h.

3.3. Filtered samples with grating at 1,000 and 300, respectively.

3.4. Filtered pass-through fraction with grating at 106 μm.

3.5. Discarded the pass-through fraction and washed the rest by several times with 0.85% NSS in sedimentation jar until the supernatant clear.

3.6. Filtered by grating at 250 μm and examined sediment for metacercariae.

3.7. Collected *O. viverrini* metacercariae under dissecting microscope.

APPENDIX B

Reagents for DNA extraction

Reagents for DNA extraction

1. 2X CTAB buffer

CTAB (Cetyltri-ammonium bromide, $C_{12}H_{42}NBr=$)	2.00	g
1.4 M NaCl	8.18	g
20 mM EDTA, pH 8.0	0.75	g
100 mM Tris-HCl	1.21	g

Add distilled water to a final volume of 100 ml

Autoclave before used

CTAB buffer

Add 0.2 % of 2-mercaptoethanol 200 μl into 2X CTAB (adjust before used).

2. TE buffer, pH 8.0

10 mM Tris-HCl, pH 8.0	1.00	ml of 1 M
1 mM EDTA, pH 8.0	200.00	μl of 0.5 M

Add distilled water to a final volume of 100 ml

Autoclave before used

3. Phenol

Phenol (Merk)	25.00	g
1 M Tris-HCL (pH 8.0) (Amresco)	25.00	ml
8-Quinolinol (Sigma)	0.025	g

Mixed immediately and then centrifuge at 3,000 rpm 10 min. Discard the supernatant and then added 0.1 M Tris-HCL (pH 8.0) 25.0 ml and centrifuged at 3,000 rpm 10 min. Discard the supernatant again and then repeated with 0.1 M Tris-HCL (pH 8.0) 25.0 ml and 2-mercaptoethanol 0.125 ml and then centrifuged at 3,000 rpm 10 min. Discard the supernatant and kept saturated phenol at 4 °C until used.

4. Chloroform ($CHCl_3$)

5. 1M Tris-HCl (pH 8.0)

Tris base ($C4H_{11}NO_3$=121.1 g/mole) (Amresco)	121.01	g
HCl (Merk)	42.00	ml
Add distilled water to a final volume of	1,000.00	ml

Adjust to pH 8.0 with HCl

Autoclave before used

6. 0.5 M EDTA (Ethylene diamine tetraacetic acid) (pH 8.0) (Vivantis)

EDTA ($C_{10}H_{14}O_8N_2Na_2.2H_2O$) (MW=372.2g/mole)	186.10	g
NaOH (MW=40g/mole)	20.00	g
Distilled water	800.00	ml

Adjust to pH 8.0 with NaOH

Add distilled water to a final volume of	1,000.00	ml

7. 70% ethanol (Merk)

Absolute ethanol	80.00	ml
Add distilled water to a final volume of	100.00	ml

8. 0.5 M NaCl (Sodium chloride) (BDH)

NaCl (MW=58.44g/mole)	292.20	g
Add distilled water to a final volume of	1,000.00	ml

Autoclave before used

9. Proteinase K 0.4 mg/ml (Invitrogen)

Proteinase K 20 mg/ml	20.00	μl
Distilled water	980.00	μl

Aliquots before use and Stored at -20 °C

10. 0.5 M NaOH (BDH)

NaOH	20.00	g
Add distilled water to a final volume of	100.00	ml

11. TE buffer

10 mM Tris-HCL (pH 8.0)	10.00	ml of 1 M
1 mM EDTA (pH 8.0)	200.00	µl of 0.5 M
Add distilled water to a final volume of	1,000.00	ml

Autoclave before used.

APPENDIX C

Reagents for DNA electrophoresis and staining solution

Reagents for DNA electrophoresis and staining solution

1. 10X TBE (Tris/Borate/EDTA) buffer

Trisma base	107.80	g
Boric acid	55.00	g
0.5 M EDTA, pH 8.0	40.00	ml
Add distilled water to a final volume of	1,000.00	ml

Autoclave before used.

2. 1X TBE buffer

10xTBE buffer	100.00	ml
Add distilled water to a final volume of	1,000.00	ml

3. 6X tracking buffer

Bromophenol blue	0.125	g
Glycerol	30.00	ml
20 mM Tris-HCl	2.00	ml of 1 M
Add distilled water to a final volume of	100.00	ml

4. Staining solution

Distilled water	200.00	ml
Ethidium bromide (10mg/ml)	10.00	μl

5. 1.5 % Agarose gel

Agarose	1.50	g
1xTBE buffer	100.00	ml

APPENDIX D

Research publications

Research publications

1. **Sattrachai Prasopdee**, Jutharat Kulsantiwong, Thanakrit Sathavornmanee and Veerachai Thitapakorn. The effects of temperature and salinity on *Opisthorchis viverrini* cercariae: a climate change concern. ***Journal of Helminthology*.** 2020; 94, e165, 1-6.

2. **Sattrachai Prasopdee**, Jutharat Kulsantiwong, Veerachai Thitapakorn and Smarn Tesana. Study on susceptibility of *Bithynia siamensis siamensis* and *B. funiculata* to *Opisthorchis viverrini* infection: a risk indication of *O. viverrini* infection outside endemic area of Thailand. ***(Manuscript in preparation)***

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