Parasitology

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Cite this article: Kopolrat KY *et al* (2022). Population dynamics and diversity of trematode infections in *Bithynia siamensis goniomphalos* in an irrigated area in northeast Thailand. *Parasitology* **149**, 407–417. https://doi.org/10.1017/S0031182021001992

Received: 14 June 2021
Revised: 25 October 2021
Accepted: 2 November 2021
First published online: 12 November 2021

Key words:
Bithynia siamensis goniomphalos; land use practice; *Opisthorchis viverrini*; paddy field; population dynamics; trematode diversity; virgulate trematode; water irrigation schemes

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Population dynamics and diversity of trematode infections in *Bithynia siamensis goniomphalos* in an irrigated area in northeast Thailand

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Abstract

Several trematodes including *Opisthorchis viverrini* utilize *Bithynia siamensis goniomphalos* as a snail intermediate host in their life cycles. In order to capture a comprehensive range of host–parasite interactions and their transmission dynamic patterns, *B. s. goniomphalos* were sampled monthly over 4 consecutive years in an irrigated paddy-field habitat in northeast Thailand. Using a standard cercarial shedding method, a high diversity of trematodes (17 types) was recovered. Virgulate xiphidiocercariae were the most prevalent (7.84%) followed by *O. viverrini* (0.71%). In addition to seasonal and environmental factors, the quantity of irrigation water for rice cultivation correlated with transmission dynamics of trematodes in *B. s. goniomphalos*. The peak prevalence of all trematode infections combined in the snails shifted from the cool-dry season in 2010–2012 to the hot-dry season in 2013 associated with an increasing quantity of water irrigation. A low frequency of mixed trematode infections was found, indicating that the emergence of virgulate cercariae, but not of *O. viverrini*, was negatively impacted by the presence of other trematodes in the same snail. Taken together, the observed results suggest that interactions between host and parasite, and hence transmission dynamics, depend on specific characteristics of the parasite and environmental factors including irrigated water for rice cultivation.

Introduction

Parasites are a major problem, both in terms of their influence on human health and their economic significance in animal husbandry (Petney, 1997). Over 100 species of food-borne trematodes are known to parasitize humans, many of which also infect domestic animals (Keiser and Utzinger, 2009). At least 750 million people are at risk of infection by food-borne trematodes and more than 40 million people are infected (Hotez *et al*., 2008; Keiser and Utzinger, 2009). All these trematodes use gastropods as intermediate hosts in their life cycles and many employ freshwater fish or crustaceans as second intermediate hosts. Second intermediate host species are often used in aquaculture, a valuable source of nutrition, employment and export trade, especially in developing countries. Infection of these hosts by metacercariae of food-borne trematodes is a cause of considerable economic losses in aquaculture (Keiser and Utzinger, 2005; Clausen *et al*., 2012).

In order to incorporate the parasite ecology into the epidemiology of infections and to increase efficient control and prevention measures in the future, the correct identification of trematodes at all life-cycle stages is important. The transmission of digenetic trematodes from the snail to the next host in the life cycle depends largely on the proportion and density of snails that release cercariae, as well as the number of cercariae released from each infected snail (Anderson and May, 1991; Petney *et al*., 2012; Kiatsopit *et al*., 2015). In Thailand, the family Bithyniidae is represented by three genera. However, only snails of the genus *Bithynia* are of medical importance (Brandt, 1974). There are three currently recognized taxa in this genus that have been reported as intermediate hosts of *Opisthorchis viverrini*. These taxa are widely distributed in continental Southeast Asia and appear to be extremely susceptible to diverse trematode infections (Brandt, 1974; Lohachit, 2004–2005; Ngern-klun *et al*., 2006; Sri-Aroon *et al*., 2007; Chontananarth *et al*., 2013; Tesana *et al*., 2014). Our recent report revealed that as many as 20 different types of trematode cercariae are found in *Bithynia siamensis goniomphalos* (Kiatsopit *et al.*, 2015). In addition to *O. viverrini*, several groups of trematodes can be found concurrently in *B. s. goniomphalos*, including virgulate xiphidiocercariae. In endemic areas of *O. viverrini*, *B. s. goniomphalos* have varying trematode prevalence levels that are associated with seasonal and host factors (Kiatsopit *et al.*, 2012; Namsanor *et al.*, 2015). We recently reported the influence of water irrigation schemes and seasonality on transmission dynamics of *O. viverrini* in *B. s. goniomphalos* in the rice paddy fields in northeast Thailand (Kopolrat *et al.*, 2020). In addition to *O. viverrini*, this study also reported several other trematode cercariae found in *B. s. goniomphalos*, but how the environmental conditions, i.e. irrigation and seasonality affect the transmission of these trematodes is not clear. Furthermore, data on host–parasite interactions between *B. s. goniomphalos* and their multi-trematode parasites are limited, with most coming from cross-sectional surveys of the prevalence of infections. Long-term studies to capture yearly variation, the effect of seasonality and human land use have not been reported. We hypothesize that seasonality and land use for agriculture have a profound impact on transmission dynamics of trematode cercariae in *B. s. goniomphalos* similar to that observed in the case of *O. viverrini* cercariae previously reported (Kopolrat *et al.*, 2020).

To understand the transmission dynamics of trematode parasites in their snail hosts, we investigated the impact of season, year-to-year variation, amount of rainfall and snail host factors, as well as fluctuations in the release of irrigation water, on the prevalence and diversity of trematode infection in *B. s. goniomphalos*.

Materials and methods

Study area and sampling periods

The study area was a rice paddy-field habitat of *B. s. goniomphalos* in Sakon Nakhon Province, northeast Thailand, the same area as discussed in our previous report where detailed ecology and land-use practices in paddy rice cultivation were described (Kopolrat *et al.*, 2020). The area has a tropical monsoon climate with the year being divided into three seasons. The hot-dry season is from March to May, the rainy season and cool-dry season occur from June to October and November to February, respectively (Namsanor *et al.*, 2015).

Snail collection and cercarial shedding

To measure the prevalence of trematode infection in naturally occurring snail populations, monthly collections were made at ten sampling sites in an irrigated area of approximately 4879 m^2 from January 2010 to December 2013. Snails were collected by handpicking from objects and solid surfaces and by dredging the sediment with a scoop from shallow water for 10 min/site by two collectors. The snails were then cleaned, blotted dry and placed into plastic bags, and transported to the laboratory where they were identified using standard morphological criteria (Brandt, 1974; Upatham *et al.*, 1983; Chitramvong, 1992). We obtained monthly data on the rainfall (mm), and release of irrigation water for the study area ($\times 10^5\ m^3$) during the sampling period from the Thai Meteorological Office and the Royal Irrigation Department.

Trematode infection in *B. s. goniomphalos* was examined by cercarial shedding (Kiatsopit *et al.*, 2012). Individual snails were placed separately into a plastic container (3 cm in diameter by 2.5 cm high) filled with dechlorinated tap water. The containers were covered with a lid studded with pins to prevent the snail from escaping. The snails were then exposed to a light intensity of 1200 lux, with the lamp placed 30 cm above the container, for 5 hours during the daytime at room temperature (25 ± 2°C). The presence of cercariae in water was observed under a stereoscopic microscope. Trematode cercariae were identified morphologically under a high-magnification compound microscope. Cercariae were fixed with 1% iodine and were photographed using software DP2-BSW by Olympus (Olympus DP 25; Olympus, Tokyo, Japan) fitted to an Olympus BX 51 microscope. Cercariae were identified using keys or other information as in Ditrich *et al.* (1997); Ito *et al.* (1962); Schell (1970) and Yamaguti (1975).

From the initial shedding, those snails infected with either *O. viverrini* or with virgulate or those with mixed infections of the two were allowed to stay in the dark for 12 h under laboratory conditions before being exposed to light from 6.00 am to 6.00 pm, as above. The cercariae were counted under a stereomicroscope after staining with 1% Lugol's iodine solution.

To investigate the influence of snail size on prevalence, the shell length was measured using digital vernier calipers. The length of a snail shell was measured between the tip of the apex and the lower edge of the lip.

Diversity index calculation

The following parameters and indices were used to describe species abundance and diversity (Spellerberg and Fedor, 2003; Tabbabi *et al.*, 2011). Relative abundance, defined as the mean proportion of cercariae of each species contributed by a single snail individual, was calculated and expressed as a percentage to assess dominance. Species richness was defined as the total number of trematode species in sampled snails. Shannon's diversity index (H) was computed to evaluate the trematode diversity across infected snails with the following formula:

$$H = -\sum_{i=1}^{n} p_i \ln p_i$$

where p_i is the proportion of snails infected with the ith trematode species (i.e. a relative abundance of species i). A higher value indicates a large number of species with similar abundances, whereas a lower value indicates low diversity dominated by one or a few species (Hill, 2005). Species evenness (E), describing equality of the prevalence of each trematode species, was computed with the following formula:

$$E = H/\ln S$$

where S is the total number of species/types (i.e. species richness) and H refers to Shannon's diversity index. The values of E range from 0 to 1; values closer to zero represent communities that are dominated by one species, while values closer to 1 represent communities comprised of several species with similar abundances (Hillebrand, 2008).

Statistical analyses

Data were analysed using SPSS version 21.0 software (IBM Software Company, Chicago, IL) and GraphPad Prism 5 (La Jolla, CA). Environmental profile data and prevalence of all trematode infections combined in *B. s. goniomphalos* were obtained at monthly intervals. By using individual snails as a study unit, odds ratios (OR) with 95% confidence intervals (CI) from logistic regression analyses were calculated to investigate the association between environmental variables (rainfall and irrigation water) and the prevalence of all trematode infections combined. Factors associated with the prevalence of trematode infections were first analysed using univariate analysis, and covariates were considered for further analysis using a mixed-effects multivariable logistic regression

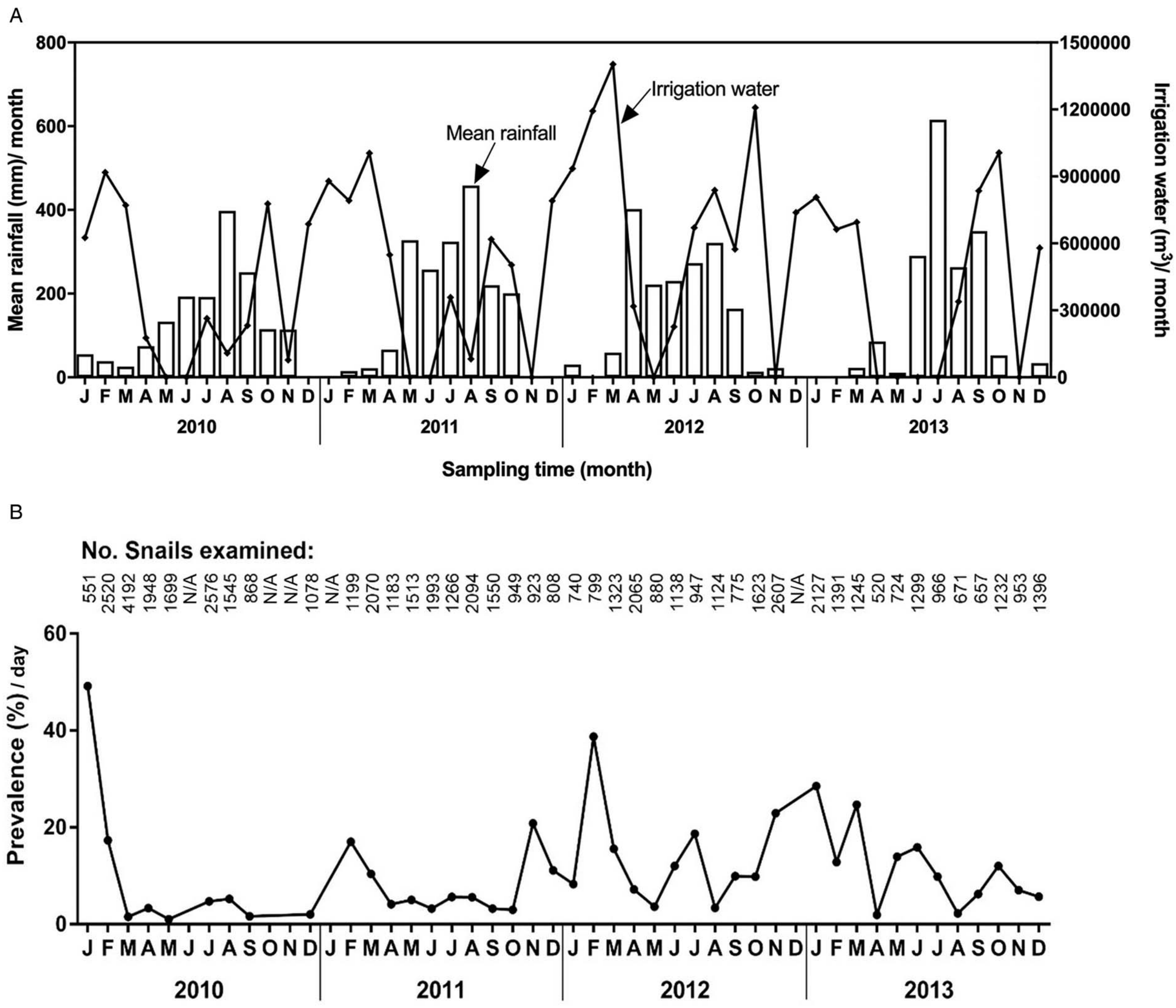

Fig. 1. Profiles of monthly prevalence of trematodes in *Bithynia siamensis goniomphalos* in relation to rainfall, irrigation water and number of snails sampled over the 4-year period from 2010 to 2013. (A) Mean rainfall and irrigation water (Kopolrat *et al*., 2020) and (B) prevalence of trematodes and number of snails examined.

model. To avoid collinearity in the multivariable models, the covariance of the selected variables was investigated pairwise to determine the correlation among the variables. Since the patency of trematodes takes around 4 weeks in a snail (Sorensen and Minchella, 2001; Mohammed *et al*., 2016), a lag period of 4 weeks is included in data analysis in the logistic regression analyses. The prevalence of all trematode cercariae in *B. s. goniomphalos* was calculated as a percentage for each cercarial type. χ^2 tests were performed to compare the prevalence of all trematode infections combined between seasons in different study years. Independent *t*-tests and analysis of variance (ANOVA) were used for normally distributed data to assess size differences between groups of infected and uninfected snails. The correlation of cercarial emergences/snail/day between single and mixed infections was evaluated by linear regression and Pearson's correlation coefficient. Kruskal–Wallis tests were used to compare cercarial emergences/snail/day between single and mixed infections. A *P* value of <0.05 was considered statistically significant.

Table 1. Numbers and percent of *Bithynia siamensis goniomphalos* samples categorized by the status of trematode infection and sampling years and seasons

Factors	No snails (*n*)	%
Total no. of snails	59 727	100.00
Positive for trematodes	6134	10.27
Negative	53 593	89.73
Years		
2010	16 977	28.42
2011	15 548	26.03
2012	14 021	23.48
2013	13 181	22.07
Seasons		
Hot-dry	19 362	32.42
Rainy	23 273	38.97
Cool-dry	17 092	28.62

Results

Profiles of trematode infection, environmental and irrigation factors

The patterns of rainfall were consistent between years while the duration of irrigation and amount of irrigated water varied during the study period as previously reported (Kopolrat *et al*., 2020) (Fig. 1). Rainfall averages about 120–170 mm per month and falls mostly between April and the end of October. The amount

Table 2. Associations between prevalence of combined trematode infection and environmental (season, rainfall and irrigated water) and biological factors (size of snail)

Factors	No. of snails examined	No. of snails infected (%)	cOR	aOR	95% CI	*P* value
Year[a]						
2010	16 977	1182 (7.0)	Ref	Ref		
2011	15 548	1156 (7.4)	1.073	1.053	0.967–1.148	0.233
2012	14 021	1942 (13.9)	2.148***	1.903	1.761–2.057	**<0.001**
2013	13 181	1854 (14.1)	2.187***	1.728	1.595–1.871	**<0.001**
Season[b]						
Hot-dry	19 362	1289 (6.7)	Ref	Ref		
Rainy	23 273	1730 (7.4)	1.126**	0.954	0.884–1.030	0.229
Cool-dry	17 092	3115 (18.2)	3.125***	2.779	2.589–2.983	**<0.001**
Rainfall[c]						
Low (0–150 mm)	35 950	4386 (12.2)	Ref	Ref		
Medium (151–300 mm)	13 714	1231 (9.0)	0.710***	0.947	0.877–1.023	0.166
High (>300 mm)	10 063	517 (5.1)	0.390***	0.577	0.522–0.639	**<0.001**
Irrigation water[d]						
Low (0–500 000 m^3)	24 155	1621 (6.7)	Ref	Ref		
Medium (500 001–1 000 000 m^3)	27 441	3445 (12.6)	1.996***	1.579	1.475–1.692	**<0.001**
High (1 000 001–1 500 000 m^3)	8131	1068 (13.1)	2.102***	1.395	1.263–1.541	**<0.001**
Size of snail (shell length)[e]						
Small (<8.00 mm)	847	219 (25.9)	Ref	Ref		
Medium (8.01–10.00 mm)	3422	1388 (40.6)	1.957***	2.212	1.857–2.635	**<0.001**
Large (>10.01 mm)	1953	1207 (61.8)	4.640***	5.677	4.692–6.869	**<0.001**

Data presented were analysed by logistic regression model showing cOR and aOR with 95% CI and *P* values.
aOR by a: season, b: year, c: year, season and irrigated water, d: year, season and rainfall, e: year and season.
, * indicate cORs with a significance level of $P < 0.01$ and $P < 0.001$, respectively.

of irrigated water in the whole study area increased during 2011 and again at the start and towards the end of 2012 and remained high until mid-2013. The abundance of snails varied widely over the sampling period, but there was no relationship with the season. The total number of snails collected was 59 727 and the highest number collected on a single occasion was 4192 in March 2010.

As reported previously (Kopolrat *et al.*, 2020), 17 types of trematodes cercariae belonging to eight taxonomic groups were found in *B. s. goniomphalos* snails (Fig. S1). The details of snail samples categorized by infection status and sampling times by season and year are shown in Table 1. The monthly prevalence of all trematode cercariae was variable over season and study years (Fig. 1).

Association between the prevalence of trematode infection and environmental variables

The results by multivariable logistic regression model with a lag period of 4 weeks to allow cercarial development in the snail host shown as crude (cOR) and adjusted (aOR) odd ratios (i.e. by year, season, rainfall, irrigated water and snail size) are shown in Table 2. There was an association between the prevalence of all trematode infections combined in *B. s. goniomphalos* and with reference to 2010, the aOR was significant for 2012 and 2013 ($P < 0.001$). Considering the seasonal factor in using the hot-dry season as the reference, there was a significant association of all trematode prevalence with the cool-dry season (aOR = 2.779, $P < 0.001$). The prevalence of trematode infections was found to be negatively associated with the amount of rainfall, i.e. increased rainfall volume reduced the risk of trematode infection in *B. s. goniomphalos* ($P < 0.001$). There was a significant association between the prevalence of trematode infections and levels of irrigation water, in comparison with the baseline level, the aORs being 1.579 and 1.395 for medium and high irrigation water categories, respectively ($P < 0.001$). In addition, the prevalence of trematode infections was significantly associated with the snail size (shell length) when adjusted by year and season ($P < 0.001$), i.e. larger snail size had a higher risk of trematode infection.

Profiles of trematode infection and seasonality

The prevalence of all cercarial infections in *B. s. goniomphalos* for each year and season is shown in Fig. 2. Overall, the prevalence varied significantly between years ($P < 0.001$) and season ($P < 0.001$). In 2010, the highest prevalence occurred in the cool-dry season, followed by the rainy season and the lowest prevalence was in the hot-dry season ($P < 0.001$). In 2011, the prevalence peaked in the cool-dry season and was low in the hot-dry and rainy seasons ($P < 0.001$). In 2012, the greatest prevalence was in the cool-dry season being significantly higher than in the hot-dry and rainy seasons ($P < 0.001$). In 2013, peak prevalence occurred in hot-dry, followed by the rainy season and the lowest prevalence was in the cool-dry season ($P < 0.001$).

Only 80 snails (0.14%) were parasitized with more than one type of trematode larvae. Table 3 details the single infections with each trematode type. Almost all the multiple infections were double infections with virgulate cercariae being one of the groups involved (Table S1). Only in a single snail was

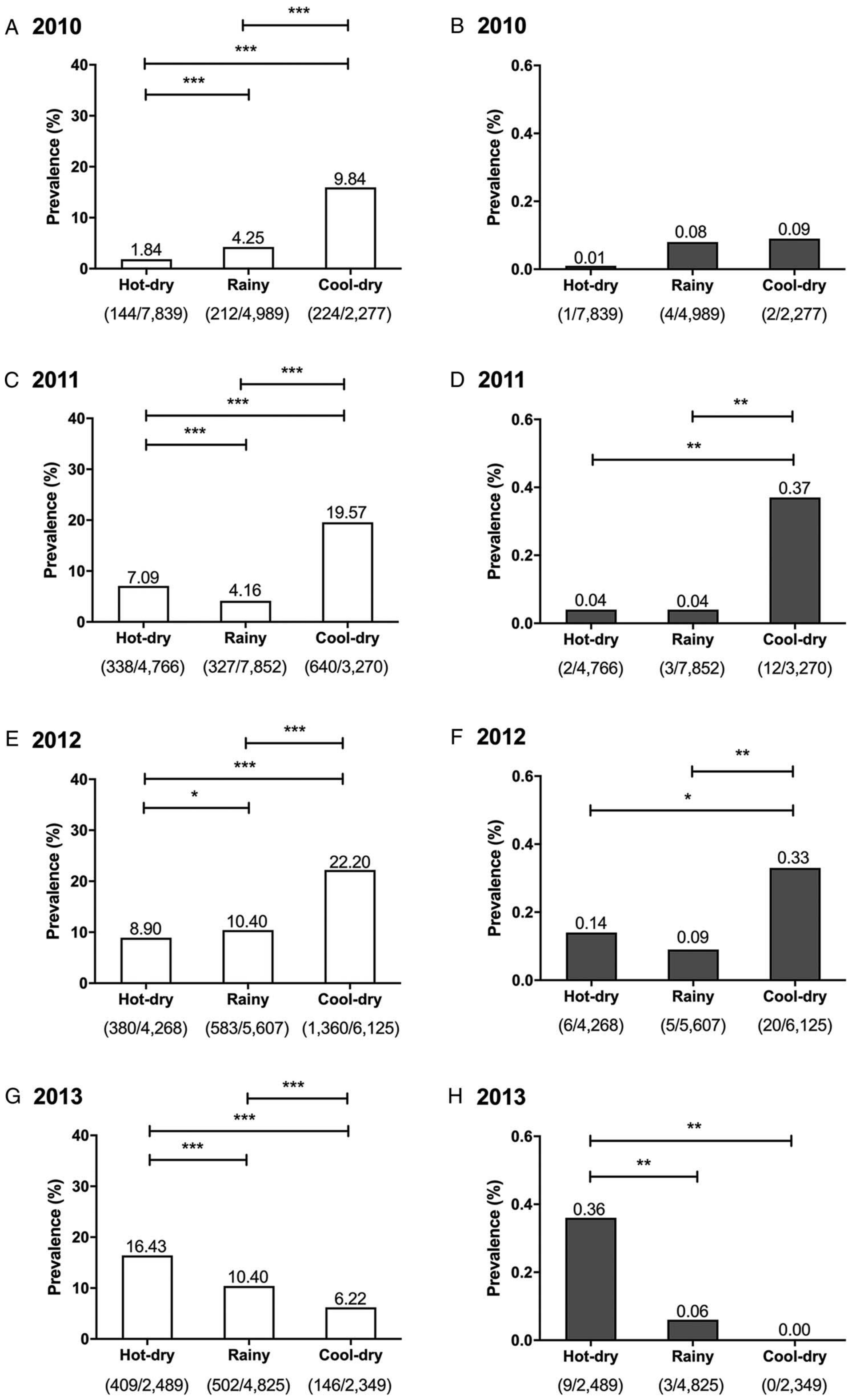

Fig. 2. Prevalence of single and multiple trematode infections in *B. s. goniomphalos* at different seasons and years. Single trematode infections (A, C, E and G). Data shown are percent positive of infection in the snails and number positive over the number of snails examined. Multiple trematode infections (B, D, F and H). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

echinostome cercariae 1 and 2 found together. Within six multiply infected snails, *O. viverrini* cercariae existed together with virgulate cercariae. As shown in Fig. 3, there was a significant correlation between the prevalence of single and multiple trematode infections ($R^2 = 0.77$, $P < 0.001$).

The diversity of trematodes across year and season

Details of species richness, abundance, diversity and evenness separated by year and season are shown in Table S2. The species richness peaked in the cool-dry season except in 2012–2013 when it peaked in the hot-dry season. Similarly, abundances were highest in the cool-dry season in 2010–2012 but the hot-dry season in 2013. Shannon index and species evenness are variable and inconsistent with no clear pattern.

Snail size distribution in different seasons

A total of 6222 snails were included for size comparisons from 2011 to 2013. Of these, 2433 snails were infected by virgulate

Table 3. Seasonal and yearly prevalence of trematode diversity in *B. s. goniomphalos* in Sakon Nakhon Province, Thailand

	2010			2011			2012			2013		
	Number of infections (%)			Number of infections (%)			Number of infections (%)			Number of infections (%)		
Type of cercariae	Hot-dry (N = 7,839)	Rainy (N = 4,989)	Cool-dry (N = 2,277)	Hot-dry (N = 4,766)	Rainy (N = 7,852)	Cool-dry (N = 3,270)	Hot-dry (N = 4,268)	Rainy (N = 5,607)	Cool-dry (N = 6,125)	Hot-dry (N = 2,489)	Rainy (N = 4,825)	Cool-dry (N = 2,349)
Xiphidiocercariae												
1. Virgulate 1	35 (0.45)	102 (2.04)	85 (3.73)	156 (3.27)	141 (1.80)	465 (14.22)	291 (6.82)	441 (7.87)	965 (15.76)	233 (9.36)	347 (7.19)	80 (3.41)
2. Virgulate 2	-	33 (0.66)	7 (0.31)	65 (1.36)	37 (0.47)	80 (2.45)	12 (0.28)	19 (0.34)	40 (0.65)	14 (0.56)	4 (0.08)	1 (0.04)
3. Virgulate 3	5 (0.06)	28 (0.56)	89 (3.91)	56 (1.17)	34 (0.43)	11 (0.34)	6 (0.14)	25 (0.45)	63 (1.03)	29 (1.17)	54 (1.12)	31 (1.32)
4. Virgulate 4	-	-	-	-	-	-	-	-	-	6 (0.24)	13 (0.27)	4 (0.17)
Pleurolophocercous cercariae												
5. *Opisthorchis viverrini* cercariae	-	4 (0.08)	3 (0.13)	6 (0.13)	52 (0.66)	19 (0.58)	7 (0.16)	44 (0.78)	149 (2.43)	53 (2.13)	42 (0.87)	10 (0.43)
6. Parapleurolophocercous cercariae	7 (0.09)	3 (0.06)	7 (0.31)	2 (0.04)	-	18 (0.55)	1 (0.02)	8 (0.14)	10 (0.16)	13 (0.52)	1 (0.02)	-
Cystophorous cercariae (Hemiuridae)												
7. Cystophorous cercariae 1	36 (0.46)	2 (0.04)	22 (0.97)	36 (0.76)	20 (0.25)	18 (0.55)	7 (0.16)	5 (0.09)	36 (0.59)	14 (0.56)	13 (0.27)	5 (0.21)
8. Cystophorous cercariae 2	-	-	-	-	1 (0.01)	1 (0.03)	2 (0.05)	1 (0.02)	-	-	-	-
Monostome cercariae												
9. Monostome	-	1 (0.02)	-	8 (0.17)	12 (0.15)	9 (0.28)	32 (0.75)	16 (0.29)	76 (1.24)	22 (0.88)	4 (0.08)	6 (0.26)
Furcocercouscercariae												
10. Furcocercous cercariae 1	1 (0.01)	-	3 (0.13)	1 (0.02)	5 (0.06)	4 (0.12)	3 (0.07)	3 (0.05)	-	2 (0.08)	-	3 (0.13)
11. Furcocercous cercariae 2	-	1 (0.02)	1 (0.04)	-	-	2 (0.06)	-	-	-	-	-	-
12. Longifurcate-pharyngeate cercariae 1	1 (0.01)	17 (0.34)	1 (0.04)	-	12 (0.15)	3 (0.09)	5 (0.12)	17 (0.30)	13 (0.21)	17 (0.68)	10 (0.21)	1 (0.04)
13. Longifurcate-pharyngeate cercariae 2	-	-	-	-	2 (0.03)	1 (0.03)	1 (0.02)	-	-	-	-	-
Mutabile cercariae												
14. Mutabile	45 (0.57)	-	4 (0.18)	2 (0.04)	-	2 (0.06)	11 (0.26)	-	2 (0.03)	4 (0.16)	-	-
Echinostome												
15. Echinostome cercariae1	-	6 (0.12)	2 (0.09)	5 (0.10)	9 (0.11)	5 (0.15)	1 (0.02)	3 (0.05)	6 (0.10)	1 (0.04)	10 (0.21)	5 (0.21)
16. Echinostome cercariae 2	-	-	-	1 (0.02)	1 (0.01)	1 (0.03)	-	1 (0.02)	-	1 (0.04)	4 (0.08)	-
Amphistome cercariae												
17. Amphistome	14 (0.18)	15 (0.30)	-	-	1 (0.01)	1 (0.03)	1 (0.02)	-	-	-	-	-

Data shown are number and percent positive snails.

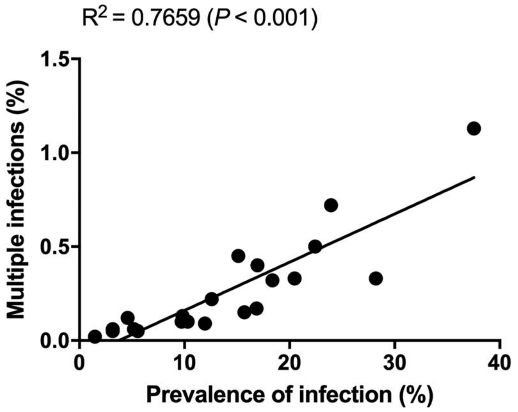

Fig. 3. Relationship between monthly prevalence of single and multiple (double) trematode infections in *B. s. goniomphalos* (dots) during the 4-year study period.

cercariae combined, and 381 snails were infected by *O. viverrini* cercariae. The frequency distributions of snail size (shell length) between uninfected compared with infected snails by virgulate and *O. viverrini* cercariae during the different seasons of each year are shown in Fig. 4. The shell length was significantly influenced by season and infection status (ANOVA, $P < 0.001$). Over 3 years, snails infected with virgulate and *O. viverrini* cercariae were significantly larger than uninfected snails in all seasons (*t*-test, $P < 0.001$) but were similar in the cool-dry season in 2012. The average shell length ranged from 8.76 to 10.69 mm. Small snails (<8 mm) made up 6.12% of those measured, 48.34% were pre-reproductive and of medium size (8–10 mm) and 45.54% were reproductive, large snails (>10 mm).

Cercarial emergence of single and double trematode infection

In the March 2013 sample, there were 51 snails infected with *O. viverrini* cercariae and 188 virgulate cercariae-infected snails. There were five snails with mixed infections which were used for cercarial emergence examinations (Fig. 5). *Opisthorchis viverrini* cercarial emergence was similar in single and in double infections. Significantly more virgulate cercarial emerged in single infections compared with double infections ($P < 0.05$).

Discussion

One of the main findings in this study is the discovery of up to 17 types of cercariae, including *O. viverrini*, in *B. s. goniomphalos* from a single locality compared with 20 types previously reported across several localities (Kiatsopit *et al*., 2015), indicating the rich trematode fauna in the rice-paddy habitat which receives regular irrigation water. Our data also confirm the high trematode diversity and susceptibility to infection in *B. s. goniomphalos*. All trematode cercariae were found in 9.95% of the snails sampled, with the highest prevalence (49.18%) in the cool-dry season in January 2010. The 17 morphologically identified larval trematodes included types similar to those found in other studies (Ito *et al*., 1962; Wykoff *et al*., 1965; Ditrich *et al*., 1990, 1992; Giboda *et al*., 1991; Adam *et al*., 1993; Nithiuthai *et al*., 2002; Lohachit, 2004–2005; Sri-Aroon *et al*., 2005, 2007; Tesana *et al*., 2014; Kiatsopit *et al*., 2015). Previous studies have also shown that individual species of snails can act as intermediate hosts for several trematode species (Sousa, 1993; Esch *et al*., 2001; Loy and Haas, 2001).

Natural infections of *O. viverrini* in *B. s. goniomphalos* were few. The monthly infection rates in our study ranged from 0.01 to 4.10%, and the average infection rate was 0.71% (Kopolrat *et al*., 2020). These results were similar to Brockelman *et al.* (1986) and Lohachit (2004,–2005) who reported infection rates of 0.10–0.36%. In contrast, the virgulate group was present in 7.84% of all snails that we examined. Virgulate cercaria is a stage in the life-cycle of a very complex group of trematodes with many different subgroups (Schell, 1970). Kiatsopit *et al.* (2015) and Namsanor *et al.* (2015) recently reported that *B. s. goniomphalos* shows particularly high susceptibility to infection by virgulate cercariae, making them the dominant cercarial fauna. Moreover, this particular group of cercariae can be found in other snails, such as *Melanoides tuberculata* (Krailas *et al*., 2014), *Thiara scabra* (Ukong *et al*., 2007) in Thailand and *Biomphalaria tenagophila* from South America (Moraes *et al*., 2009).

The strongest factor relating to high all trematode prevalence rates and double infection was the season, with a high prevalence in the cool-dry season in 2010–2012. However, this is not consistent as prevalence peaked in the hot-dry season in 2013. Seasonal variations are the major extrinsic factor associated with prevalence, particularly the amount of rainfall plays an important role in the complex interplay between host and parasite (Brockelman *et al*., 1986). Seasonal changes in rainfall cause marked fluctuations in the transmission rates of diseases and parasites (Mouritsen and Poulin, 2002; Cattadori *et al*., 2005; Kim *et al*., 2005; Altizer *et al*., 2006).

In addition to environmental factors, our study found that irrigation intensity in 2012 also influenced all trematode prevalence rates in the snails. In this study, we showed an increase in the prevalence of all trematode infections combined in *B. s. goniomphalos* with an increasing quantity of irrigation water (45.36–64.67%) in 2012 compared with 2011 and 2013. Irrigation in paddy-field agriculture makes a second crop of rice in the cool-dry season possible. This practice can alter the natural cycle of snails by increasing the snail population density in rice fields, resulting in a higher prevalence in the hot-dry season in 2013 as opposed to the conventional peak prevalence in the cool-dry seasons in 2010–2012. The changes in the seasonal pattern are probably due to irrigation ditches being functionally connected to domestic and wild animals, human and snail hosts through the transport of feces from households and villages. In areas where sanitary facilities are underdeveloped, human or animal reservoirs host feces containing trematode eggs that can be flushed into water bodies directly or through irrigation ditches in the monsoon season. Trematode eggs from animal and human hosts are likely to sediment quickly and may not disperse over a large distance unless substantial water flow occurs, however, infected snails may travel with water flow to the paddy fields. Thus, the biological characteristics of the cercariae, the snail host population together with the environmental conditions, play important roles in trematode transmission (Haas, 1994; Petney *et al*., 2012; Ziegler *et al*., 2013; Wang *et al*., 2015).

Interestingly, the virgulate type, being the most common class of cercariae in *B. s. goniomphalos*, was a major type found in double infections. Nevertheless, only 0.14% of the infected snails harboured more than one larval trematode species, which is not unexpected based on the low percentages reported in previous studies (Sousa, 1990, 1993; Lafferty *et al*., 1994). This finding may represent inter-specific competition among trematode parasites, including *O. viverrini*, occurring in the natural environment (Bayne and Loker, 1987; Jourdane and Theron, 1987). Some trematode parasites, particularly the virgulate group, may have evolved their competitive ability by becoming more efficient resource users and by developing interference mechanisms that keep competing species from using scarce resources. Thus, virgulate cercariae are the most successful group parasitizing the field population of *B. s. goniomphalos*, whereas cercariae of *O. viverrini* are only of moderate prevalence in the paddy-field habitat.

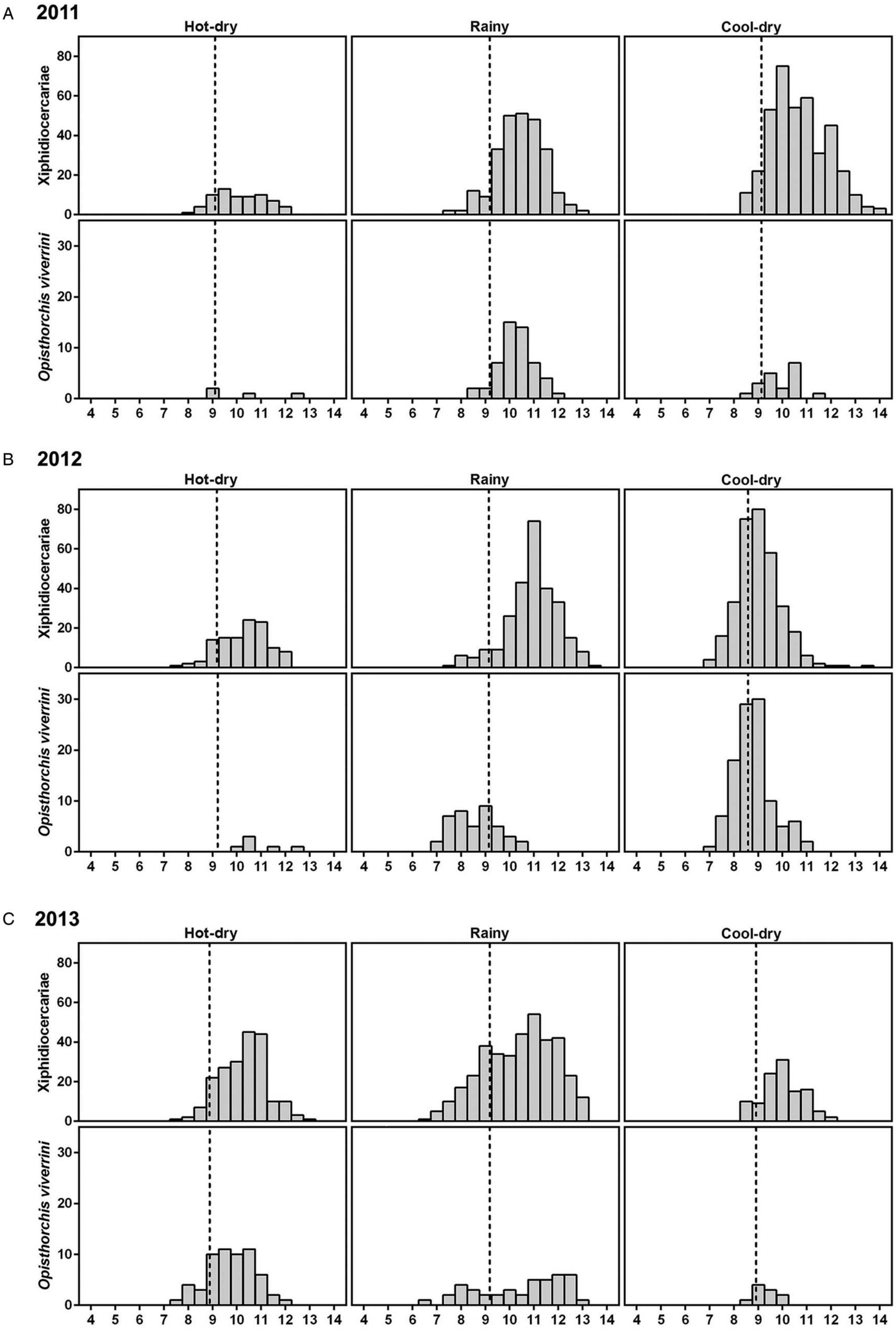

Fig. 4. Comparison of snail size distributions in different seasons between the snails with virgulate (xiphidiocercaria) and *Opisthorchis viverrini* cercariae in comparison with uninfected snails (vertical dotted line represents the mean shell length of uninfected snails). (A) 2011, (B) 2012 and (C) 2013.

How the virgulate trematode interacts with *O. viverrini* is not known. Nevertheless, the fact that they are smaller in body size compared with *O. viverrini* (Schell, 1970) and appear to have lower rates of cercarial emergence in mixed infections, suggests that they are unlikely to exert a substantial negative effect on *O. viverrini*. Laboratory studies of freshwater snail–trematode associations have demonstrated the presence of strong antagonistic interactions between the intra-molluscan of redia and sporocyst stages of species that infect the same host individual and that double infections are more pathogenic to the snails when compared

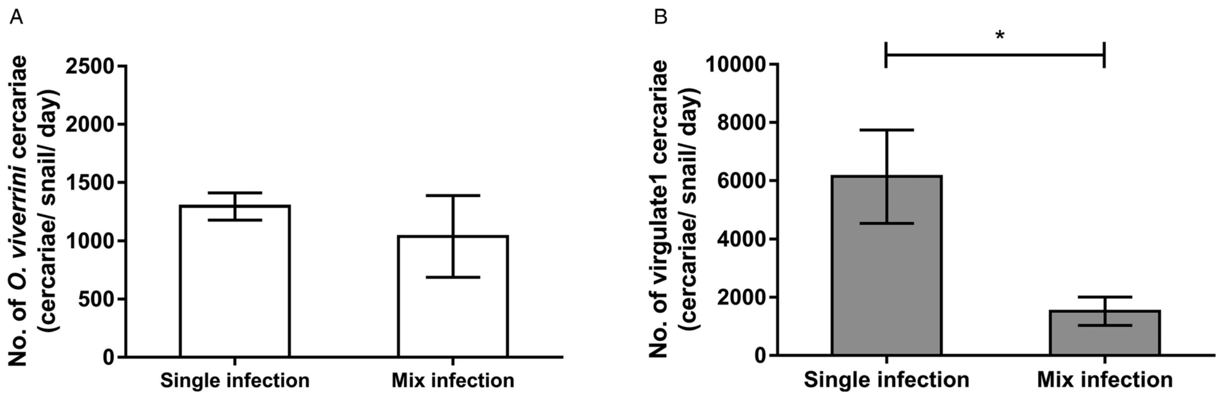

Fig. 5. Effects of mixed infection on the cercarial emergence of *O. viverrini* (A) and virgulate 1 (B) in *B. s. goniomphalos*.

to infections of only one trematode species (Lim and Heyneman, 1972; Sousa, 1992; Lafferty *et al.*, 1994). The competition among larvae from different trematode species inside mollusks can reduce both parasite number and the snail population (Basch *et al.*, 1969; Lim and Heyneman, 1972; Frandsen, 1987). Larvae of *Schistosoma mansoni* do not develop in *B. tenagophila* previously infected by longifurcate cercariae with or without an eyespot (Machado *et al.*, 1988). These authors observed that mollusks infected by echinostome and other cercariae were protected from *S. mansoni* infection at about 73 to 87%, respectively. The time of the first infection may have some impact on additional cercarial infections, and internal mechanisms repel further cercarial infections in an already infected snail. Fluke development in intermediate hosts depends on genetic factors as well as environmental factors, which might facilitate eggs and miracidia dispersion (Williams and Esch, 1991; Fernandez and Esch, 1991*a*, 1991*b*). Since the trematode infection is usually harmful to the mollusk host, the impact of double or even triple infections on the snail host should be investigated in the future. In addition, a similar study in a paddy rice field that received no irrigation is needed for comparison.

The current study revealed that the virgulate species and *O. viverrini* tended to infect larger snails. This is consistent with the results of Namsanor *et al.* (2015) who showed that larger snails were more susceptible to virgulate infection than were small snails. For snail–trematode interactions, gigantism is often postulated to benefit the parasite as a larger host provides a greater volume for parasite occupation and reproduction (Sandland and Minchella, 2003; McCarthy *et al.*, 2004). In the case of *O. viverrini*, a possible explanation involves the irrigation water. Our previous report shows that the prevalence of *O. viverrini* was positively associated with the size of *B. s. goniomphalos* in 2010, 2011 and 2013, but the relationship was reversed in 2012 (Kopolrat *et al.*, 2020). This may be partially explained by the fact that in 2012 there were more snails available as a result of more irrigated water, hence more small snails (pre-reproductive size) were infected compared with larger snails in the rainy and cool-dry seasons.

In conclusion, this study demonstrated that *B. s. goniomphalos* in paddy-field habitats supports a high diversity of trematode fauna led by virgulate cercariae and followed by *O. viverrini*. Irrigation water for rice cultivation has a strong influence on the transmission dynamics of the trematodes by supporting the abundance of snails and hence shifting the peak of all trematode prevalence in the snails from the cool-dry to the hot-dry season. The findings of very low levels of mixed trematode infections and potential interference of cercarial emergence in virgulate cercariae, but not *O. viverrini*, indicates the importance of trematodes developing in the snail, which may contribute to host gigantism and parasite-induced mortality. Taken together, the observed results suggest that interactions between host and parasite depend not only on a specific characteristic of the parasite but also the environmental factors including irrigation practices for rice cultivation, which affects the transmission dynamics. In addition, this 4-year study shows that variation between years can be considerable and that short-term studies are unlikely to unravel the complexity of factors influencing trematode population biology.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021001992.

Acknowledgements. We would like to acknowledge Prof David Blair for editing the manuscript *via* Publication Clinic KKU, Thailand. We thank the Provincial Meteorological Office of Sakon Nakhon Province, Thailand, for providing climate and environmental data. We are also grateful to the chief of Phang Khon District Agriculture Department, Sakon Nakhon Province, Thailand, for data for the rice production area and rice production. Data for the irrigation water were supplied by the Royal Irrigation Department, Sakon Nakhon Province, Thailand.

Author contribution. Conceived and designed the experiments: PS, ST and KYK. Performed the experiments: KYK, NK, JN, OP, PY and PS. Analysed and interpreted the data: KYK and PS. Contributed reagents/materials/analysis tools: PS, ST and WS. Writing-original draft: KYK. Writing-review and editing: PS, RHA and TNP

Financial support. This study described here was supported by the Cholangiocarcinoma Research Institute, Khon Kaen University, and Fluke-Free Thailand, National Research Council of Thailand.

Conflict of interest. None.

References

Adam R, Arnold H, Pipitgool V, Sithithaworn P, Hinz E and Storch V (1993) Studies on lophocercous cercariae from *Bithynia siamensis goniomphalos* (Prosobranchia: Bithyniidae). *The Southeast Asian Journal of Tropical Medicine and Public Health* **24**, 697–700.

Altizer S, Dobson A, Hosseini P, Hudson P, Pascual M and Rohani P (2006) Seasonality and the dynamics of infectious diseases. *Ecology Letters* **9**, 467–484.

Anderson RM and May RM (1991) *Infectious Diseases of Humans, Dynamics and Control.* New York, USA: Oxford University Press.

Basch PF, Lie KJ and Heyneman D (1969) Antagonistic interaction between strigeid and schistosome sporocysts within a snail host. *Journal of Parasitology* **55**, 753–758.

Bayne CJ and Loker ES (1987) Survival within the host. In Rollinson D and Simpson AJG (eds), *The Biology of Schistosomes from Genes to Latrines.* London, UK: Academic Press, pp. 321–346.

Brandt RAM (1974) The non-marine aquatic mollusca of Thailand. *Archiv für Molluskenkunde* **105**, 1–423.

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.

Cattadori IM, Haydon DT and Hudson PJ (2005) Parasites and climate synchronize red grouse populations. *Nature* **433**, 737–741.

Chitramvong YP (1992) The Bithyniidae (Gastropoda: Prosobranchia) of Thailand: comparative external morphology. *Malacological Review* **25**, 21–38.

Chontananarth T, Wongsawad C and Chai JY (2013) Epidemiology of cercarial stage of trematodes in freshwater snails from Chiang Mai Province, Thailand. *Asian Pacific Journal of Tropical Biomedicine* **3**, 237–243.

Clausen JH, Madsen H, Murrell KD, Phan Thi V, Nguyen Manh H, Viet KN and Dalsgaard A (2012) Relationship between snail population density and infection status of snails and fish with zoonotic trematodes in Vietnamese carp nurseries. *PLoS Neglected Tropical Diseases* **6**, e1945.

Ditrich O, Scholz T and Giboda M (1990) Occurrence of some medically important flukes (Trematoda: Opisthorchiidae and Heterophyidae) in Nam Ngum water reservoir, Laos. *The Southeast Asian Journal of Tropical Medicine and Public Health* **21**, 482–488.

Ditrich O, Nasincova V, Scholz T and Giboda M (1992) Larval stages of medically important flukes (Trematoda) from Vientiane Province, Laos. Part II. Cercariae. *Annales de Parasitologie Humaine et Comparee* **67**, 75–81.

Ditrich O, Schoiz T, Aguiree-Macedo L and Vargas-Vazquez J (1997) Larval stages of trematodes from freshwater molluscs of the Yucatan Peninsula, Mexico. *Folia Parasitlogica* **44**, 109–127.

Esch GW, Curtis LA and Barger MA (2001) A perspective on the ecology of trematode communities in snails. *Parasitology* **123**(suppl), S57–S75.

Fernandez J and Esch GW (1991a) Guild structure of larval trematodes in the snail *Helisoma anceps*: patterns and processes at the individual host level. *Journal of Parasitology* **77**, 528–539.

Fernandez J and Esch GW (1991b) The component community structure of larval trematodes in the pulmonate snail *Helisoma anceps*. *Journal of Parasitology* **77**, 540–550.

Frandsen F (1987) Control of schistosomiasis by use of biological control of snail hosts with special reference to competition. *Memorias do Instituto Oswaldo Cruz* **82**, 129–133.

Giboda M, Ditrich O, Scholz T, Viengsay T and Bouaphanh S (1991) Human *Opisthorchis* and *Haplorchis* infections in Laos. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85**, 538–540.

Haas W (1994) Physiological analyses of host-finding behaviour in trematode cercariae: adaptations for transmission success. *Parasitology* **109**(suppl), S15–S29.

Hill D (2005) *Handbook of Biodiversity Methods : Survey, Evaluation and Monitoring*. Cambridge, UK: Cambridge University Press.

Hillebrand H (2008) Dominance. In Fath SEJD (ed.), *Encyclopedia of Ecology*, Oxford, UK: Academic Press, pp. 938–944.

Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ and Jacobson J (2008) Helminth infections: the great neglected tropical diseases. *The Journal of Clinical Investigation* **118**, 1311–1321.

Ito J, Papasarathorn T and Tongkoom B (1962) Studies on cercariae from fresh water snails in Thailand. *Japanese Journal of Medical Science and Biology* **15**, 249–270.

Jourdane J and Theron A (1987) Larval development: eggs to cercariae. In Rollinson D and Simpson AJG (eds), *The Biology of Schistosomes from Genes to Latrines*. London, UK: Academic Press, pp. 83–106.

Keiser J and Utzinger J (2005) Emerging foodborne trematodiasis. *Emerging Infectious Diseases* **11**, 1507–1514.

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, Namsanor J, Andrews RH and Petney TN (2015) Trematode diversity in the freshwater snail *Bithynia siamensis goniomphalos* sensu lato from Thailand and Lao PDR. *Journal of Helminthology* **90**, 312–320. doi: 10.1017/S0022149X15000292

Kim K, Dobson AP, Gulland FMD and Harvell CD (2005) Diseases and the conservation of marine biodiversity. In Norse EA and Crowde LB (eds), *The Science of Maintaining the Sea's Biodiversity*. Washington, DC: Island Press, pp. 149–166.

Kopolrat K, Sithithaworn P, Kiatsopit N, Namsanor J, Laoprom N, Tesana S, Andrews RH and Petney TN (2020) Influence of water irrigation schemes and seasonality on transmission dynamics of *Opisthorchis viverrini* in the snail intermediate host, *Bithynia siamensis goniomphalos* in rice paddy fields in Northeast Thailand. *American Journal of Tropical Medicine and Hygiene* **103**, 276–286.

Krailas D, Namchote S, Koonchornboon T, Dechruksa W and Boonmekam D (2014) Trematodes obtained from the thiarid freshwater snail *Melanoides tuberculata* (Müller, 1774) as vector of human infections in Thailand. *Zoosystematics and Evolution* **90**, 57–86.

Lafferty KD, Sammond DT and Kuris AM (1994) Analysis of larval trematode communities. *Ecology* **75**, 2275–2285.

Lim HK and Heyneman D (1972) Intramolluscan inter-trematode antagonism: a review of factors influencing the host–parasite system and its possible role in biological control. *Advances in Parasitology* **10**, 191–268.

Lohachit C (2004–2005) Ecological studies of *Bithynia siamensis goniomphalos*, a snail intermediate host of *Opisthorchis viverrini*, in Khon Kaen Province, Northeast Thailand. *Malacological Review* **37/38**, 1–26.

Loy C and Haas W (2001) Prevalence of cercariae from *Lymnaea stagnalis* snails in a pond system in Southern Germany. *Parasitology Research* **87**, 878–882.

Machado SM, Magalhaes LA and de Artigas PT, da Cordeiro NS and de Carvalho JF (1988) Verification of antagonism between larvae of *Schistosoma mansoni* and other Digenea in *Biomphalaria tenagophila*, a planorbid mollusc from a natural breeding in the region of Campinas, SP, Brazil. *Revista de Saúde Pública* **22**, 484–488.

McCarthy HO, Fitzpatrick SM and Irwin SW (2004) Parasite alteration of host shape: a quantitative approach to gigantism helps elucidate evolutionary advantages. *Parasitology* **128**, 7–14.

Mohammed NA, Madsen H and Ahmed AA (2016) Types of trematodes infecting freshwater snails found in irrigation canals in the East Nile locality, Khartoum, Sudan. *Infectious Diseases of Poverty* **5**, 16–26. doi: 10.1186/s40249-016-0108-y

Moraes J, Silva MP, Ohlweiler FP and Kawano T (2009) *Schistosoma mansoni* and other larval trematodes in *Biomphalaria tenagophila* (Planorbidae) from Guarulhos, Sao Paulo State, Brazil. *The Revista do Instituto de Medicina Tropical de São Paulo* **51**, 77–82.

Mouritsen KN and Poulin R (2002) Parasitism, climate oscillations and the structure of natural communities. *Oikos* **97**, 462–468.

Namsanor J, Sithithaworn P, Kopolrat K, Kiatsopit N, Pitaksakulrat O, Tesana S, Andrews RH and Petney TN (2015) Seasonal transmission of *Opisthorchis viverrini* sensu lato and a lecithodendriid trematode species in *Bithynia siamensis goniomphalos* snails in Northeast Thailand. *American Journal of Tropical Medicine and Hygiene* **93**, 87–93. doi: 10.4269/ajtmh.14-0639

Ngern-klun R, Sukontason KL, Tesana S, Sripakdee D, Irvine KN and Sukontason K (2006) Field investigation of *Bithynia funiculata*, intermediate host of *Opisthorchis viverrini* in northern Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health* **37**, 662–672.

Nithiuthai S, Wiwanitkit V, Suwansaksri J and Chaengphukeaw P (2002) A survey of trematode cercariae in *Bithynia goniomphalos* in northeast Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health* **33**(suppl. 3), 106–109.

Petney TN (1997) Ecological implications of control strategies: arthropods of domestic and production animals. *International Journal for Parasitology* **27**, 155–165.

Petney TN, Sithithaworn P, Andrews R, Kiatsopit N, Tesana S, Grundy-Warr C and Ziegler A (2012) The ecology of the *Bithynia* first intermediate hosts of *Opisthorchis viverrini*. *Parasitology International* **61**, 38–45.

Sandland GJ and Minchella DJ (2003) Effects of diet and *Echinostoma revolutum* infection on energy allocation patterns in juvenile *Lymnaea elodes* snails. *Oecologia* **134**, 479–486.

Schell SC (1970) *How to Know the Trematode*. Dubuque, IA: WMC Brown Company Publishers.

Sorensen RE and Minchella DJ (2001) Snail–trematode life history interactions: past trends and future directions. *Parasitology* **123**, S3–S18.

Sousa WP (1990) Spatial scale and the processes structuring a guild of larval trematode parasites. In Esch G, Bush A and Aho J (eds), *Parasite Communities: Patterns and Processes*. Chaman and Hall, London, UK: Springer Netherlands, pp. 41–67.

Sousa WP (1992) Interspecific interactions among larval trematode parasites of freshwater and marine snails. *American Zoologist* **32**, 583–592.

Sousa WP (1993) Interspecific antagonism and species coexistence in a diverse guild of larval trematode parasites. *Ecological Monographs* **63**, 103–128.

Spellerberg IF and Fedor PJ (2003) A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon–Wiener' index. *Global Ecology and Biogeography* **12**, 177–183.

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. *The Southeast Asian Journal of Tropical Medicine and Public Health* **36**, 653–657.

Sri-Aroon P, Butraporn P, Limsoomboon J, Kaewpoolsri M, Chusongsang Y, Charoenjai P, Chusongsang P, Numnuan S and Kiatsiri S (2007) Freshwater mollusks at designated areas in eleven provinces of Thailand according to the water resource development projects. *The Southeast Asian Journal of Tropical Medicine and Public Health* **38**, 294–301.

Tabbabi A, Ghrab J, Aoun K, Ready PD and Bouratbine A (2011) Habitats of the sandfly vectors of *Leishmania tropica* and *L. major* in a mixed focus of cutaneous leishmaniasis in southeast Tunisia. *Acta Tropica* **119**, 131–137.

Tesana S, Thapsripair P, Suwannatrai A, Harauy S, Piratae S, Khampoosa P, Thammasiri C, Prasopdee S, Kulsantiwong J, Chalorkpunrut P and Jones MK (2014) Parasite surveys and environmental management for prevention of parasitic infection in cultivated *Barbonymus gonionotus* (Cyprinidae) in fishponds, in an opisthorchiasis endemic area of northeast Thailand. *Aquaculture* **428-429**, 54–60.

Ukong S, Krailas D, Dangprasert T and Channgarm P (2007) Studies on the morphology of cercariae obtained from freshwater snails at Erawan Waterfall, Erawan National Park, Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health* **38**, 302–312.

Upatham ES, Sornmani S, Kitikoon V, Lohachit C and Bruch JB (1983) Identification key for fresh-brackish water snails of Thailand. *Malacological Review* **16**, 107–132.

Wang YC, Ho RC, Feng CC, Namsanor J and Sithithaworn P (2015) An ecological study of *Bithynia* snails, the first intermediate host of *Opisthorchis viverrini* in northeast Thailand. *Acta Tropica* **141**, 244–252.

Williams JA and Esch GW (1991) Infra- and component community dynamics in the pulmonate snail *Helisoma anceps*, with special emphasis on the hemiurid trematode *Halipegus occidualis*. *Journal of Parasitology* **77**, 246–253.

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.

Yamaguti S (1975) *A Synoptical Review of Life Histories of Digenetic trematodes of Vertebrates*. Tokyo, Japan: Keiguku Publication Co.

Ziegler AD, Petney TN, Grundy-Warr C, Andrews RH, Baird IG, Wasson RJ and Sithithaworn P (2013) Dams and disease triggers on the lower Mekong River. *PLoS Neglected Tropical Diseases* **7**, e2166.