

Research Paper

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Author for correspondence:

S. Prasopdee, E-mail: sattra.vet16@gmail.com

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

S. Prasopdee¹ , J. Kulsantiwong², T. Sathavornmanee¹  and V. Thitapakorn¹

¹Research Group in Development of Diagnosis and Targeted Therapeutics for *Opisthorchis viverrini* and Cholangiocarcinoma, Chulabhorn International College of Medicine, Thammasat University, Klong Luang, Pathum Thani 12120, Thailand and ²Department of Biology, Faculty of Science, Udon Thani Rajabhat University, Udon Thani 41000, Thailand

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 \pm 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 ($\chi^2(2) > 393.5$, $P < 0.001$). 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 \pm 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 cercariae was temperature–salinity dependent. At 2.5 PPT, a total of 413 cercariae were analysed, revealing significant differences in cercarial survival at different temperatures ($\chi^2(2) > 118.19$; $P < 0.001$). The mean \pm 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 ($\chi^2(2) > 79.47$, $P < 0.001$), with mean \pm 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 ($\chi^2(2) > 184.10$; $P < 0.001$), with mean \pm 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

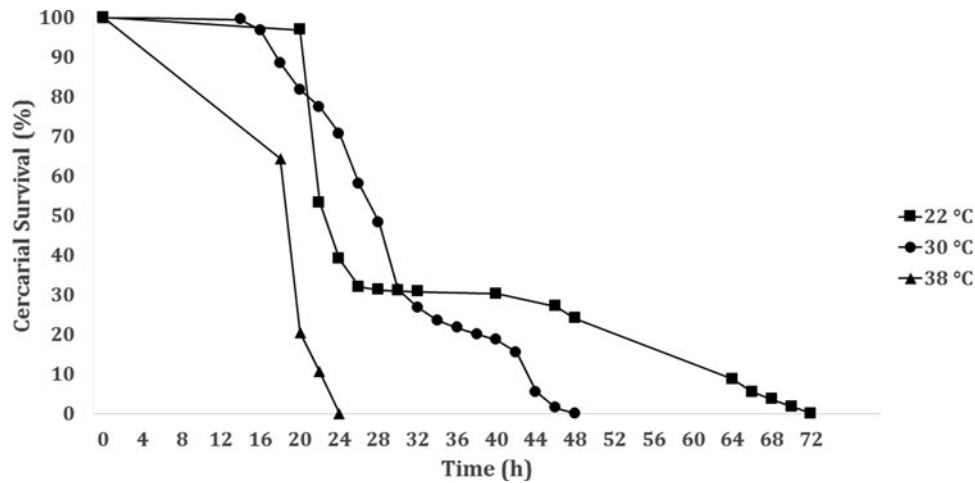


Fig. 1. Cercarial survival at different experimental temperatures. Percentage of surviving *O. viverrini* cercariae at the incubation temperatures of 22°C, 30°C and 38°C (obtained from 533 cercariae, 371 cercariae and 275 cercariae, respectively).

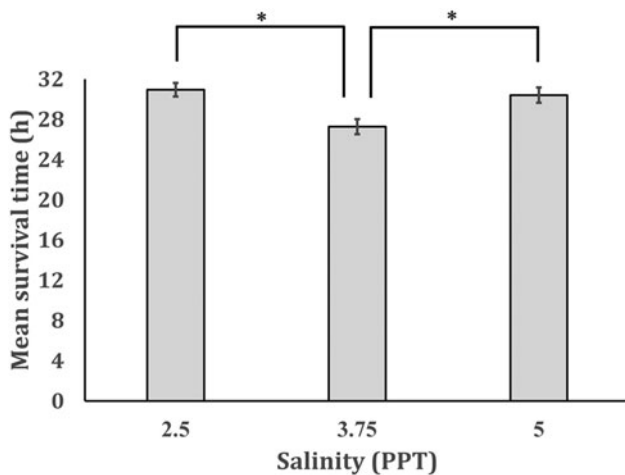


Fig. 2. Mean cercarial survival at different experimental salinities. Mean (\pm SD) survival time of *O. viverrini* cercariae at the saline solutions of 2 PPT, 3.75 PPT and 5 PPT. *Indicates statistical significance ($P < 0.05$) analysed using a one-way ANOVA test followed by Scheffe post-hoc analysis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

north-eastern Thailand (Harinasuta & Harinasuta, 1984; Haswell-Elkins *et al.*, 1994; Jongsuksuntigul & Insomboon, 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–

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

Salinity (PPT) \times temperature (°C)	<i>N</i>	Median	IQR	Mean (\pm SD)
2.5 PPT	413			
22°C	201	24	26	33.83 \pm 16.74 ^a
30°C	138	30	16	32.39 \pm 9.26 ^a
38 °C	74	20	4	20.40 \pm 2.22 ^b
3.75 PPT	347			
22°C	131	22	6	32.59 \pm 18.70 ¹
30°C	95	26	26	28.75 \pm 11.49 ²
38°C	121	20	1	20.34 \pm 1.78 ³
5 PPT	419			
22°C	201	24	42	36.95 \pm 19.72 ⁱ
30°C	138	29	4.5	27.60 \pm 3.98 ⁱ
38°C	80	18	2	18.77 \pm 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 ($P < 0.01$).

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).

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