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ORIGINAL PAPER

Effect of temperature on the development of *Schistosoma japonicum* within *Oncomelania hupensis*, and hibernation of *O. hupensis*

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Abstract The objectives of this investigation were to assess the effect of temperature on the development of *Schistosoma japonicum* harboured in *Oncomelania hupensis* and to determine the lowest temperature threshold at which the hibernation of *O. hupensis* occurs. In the first experiment, adult infection-free *O. hupensis*, collected from Jiangsu province in eastern China, were infected with *S. japonicum* miracidia and raised at different temperatures under laboratory conditions. The development of miracidia until the release of cercariae was monitored employing the cercarial shedding method. In the second experiment, batches of *O. hupensis* were kept at temperatures below 13°C with the temperature gradually reduced. Snail activity was assessed by a pin puncture method. We found a positive relationship between the development of *S. japonicum* within *O. hupensis* and temperature. In snails kept at 15.3°C, *S. japonicum* arrested their development, while the fastest development occurred at 30°C. The temperature at which half of the snails were in hibernation (ET_{50}) was 6.4°C. Our results underscore the pivotal role temperature plays on the biological activity of *O. hupensis* and the development of *S. japonicum* within the intermediate host. These findings are likely to have implications for the transmission of schistosomiasis in a warmer future China.

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Introduction

Schistosomiasis is a snail-borne parasitic disease, which affects an estimated 207 million people in tropical and subtropical environments (Steinmann et al. 2006). In China, schistosomiasis has a documented history of over 2 millennia (Zhou et al. 2005). In the mid-1950s the disease was endemic in 12 provinces located along the Yangtze River and south of it with >10 million people infected with the causative agent, i.e. *Schistosoma japonicum*. Historically, the geographic distribution of the disease was restricted south to the 33°15′ N latitude, governed by the distribution of its intermediate host snail, i.e. *Oncomelania hupensis* (Mao 1990; Zhou et al. 2005). Control measures implemented and sustained over the past 50 years have brought down the number of human infection to <1 million and the disease was eliminated in five provinces (Utzinger et al. 2005; Zhou et al. 2005).

The life cycle of *S. japonicum* consists of a sexual generation in the vascular system of the definitive host and an asexual generation in *O. hupensis*. The stages of schistosomula and adult worms are confined to the warm-blooded definitive hosts, i.e. humans and >40 species of domestic and wild animals, particularly bovines, cattle and goats (Wang et al. 2005). The other stages, i.e. eggs, miracidia, sporocysts and cercariae, develop in the external environment or in the cold-blooded intermediate host snail. Therefore, the geographic distribution of *S. japonicum* is closely related to environmental factors (Mao 1990; Ross et al. 2001; Yang et al. 2005a). Previous research showed that the development of miracidia into sporocysts in the intermediate host snail is driven by environmental temperature (Shao and Xu 1956; Pesigan et al. 1958; Nagasaki 1960). The optimal temperature for *O. hupensis* ranges between 20°C and 30°C. Temperatures below or above this range result in delayed or arrested development and reproduction of *O. hupensis* (Mao 1990; Liu 1993). Lower environmental temperatures reduce the physiological functions of *O. hupensis* (Mao 1990). Field studies found that *O. hupensis* can withdraw deeply into their shell, coupled with arrested biological activities, such as feeding or movement. It was also documented that hibernation occurs in *O. hupensis* when temperature drops below a critical threshold (Mao 1990). However, simple measures to determine whether snails are in a state of hibernation remain to be developed. We have recently documented that China's average temperature in January has increased by almost 1°C over the past 30 years (Yang et al. 2005b). The plausibility of this finding is supported by global warming, which is particularly pronounced over land in the northern hemisphere and during winter months (Murphy et al. 2004). The significance of this finding for the distribution of *O. hupensis*, and hence the level and extent of schistosomiasis transmission in China, was stressed (Yang et al. 2005b).

In the present study, we carried out laboratory investigations to study the effect of temperature on the development of *S. japonicum* in their intermediate host snail, and to investigate the temperature at which *O. hupensis* show hibernation, hence to determine the lowest thermal limit for both parasite and snail. This work is part of a larger investigation of the potential impact of climate change on the transmission of *S. japonicum* in a warmer future China.

Materials and methods

Effect of temperature on the development of *S. japonicum* larvae within *O. hupensis*

Adult *O. hupensis* (age: ~6 months; presence of 7–8 whorls) were collected in November 2001 along the beaches of Xinba, Jiangsu province (geographical coordinates: 119°32′ E longitude, 32°17′ N latitude). The proportion of male to female *O. hupensis* was about 1:1. Snails were transferred to the laboratory and raised for 4 weeks at a temperature of 25°C. Each snail was tested twice for natural infection using the cercarial shedding method (MOH 1982). Since none of the snails had a natural infection, all were used for subsequent investigations.

One rabbit was infected with 2000 *S. japonicum* cercariae (Wuxi isolate) through the shaved abdominal skin. Forty-five days post-infection, the rabbit was killed. The liver was removed, ground and screened through a wire (mesh size: 50 μm) to collect eggs of *S. japonicum*. Eggs were stored in de-chlorinated water at a temperature of 25°C and exposed to artificial light for miracidia hatching.

Approximately 15,000 freshly hatched miracidia were placed in a container with 200 ml of de-chlorinated water. Next, 750 snails were added, hence resulting in a snail to miracidia ratio of ~1:20. The container was covered with a fine-meshed gauze to prevent the snails from escaping. Snails were exposed for 4 h at 25°C under illuminated conditions. Snails were then removed and placed into 20×30 cm trays and kept on culture paper made of dried grass and bamboo materials, which served as food and maintained a certain moisture level (Jiang et al. 1997). De-chlorinated water was added daily to the culture trays to keep the moisture at a relative humidity of 85%. Snails were divided into five equally sized groups (n=150) and raised at different temperatures, i.e. 18°C, 21°C, 24°C, 27°C and 30°C (accuracy of temperature: ±1°C), in culture boxes. Boxes were checked daily and dead snails were removed with forceps and counted. Culture paper was changed weekly to provide sufficient food and moisture for the snails throughout the experiment (Jiang et al. 1997).

Since the development of miracidia into sporocysts and cercariae within intermediate host snails is closely related to environmental temperature, the first time point of checking snails for cercarial shedding in groups kept either at 30°C, 27°C, 24°C, 21°C or 18°C was carried out at days 30, 40, 50, 60 and 70 post-infection, respectively. The number of snails that shed cercariae was counted and recorded. Snails that shed cercariae were removed and the remaining snails were again tested for cercarial shedding 5 days later. This procedure was repeated at 5-day intervals until no cercariae were released for three successive sheddings. Snails that survived until the end of the experiment but failed to shed cercariae, were dissected to check for the presence of sporocysts and cercariae. The shortest, longest and average time from infection to cercarial shedding was noted for the different groups.

Effect of temperature on the hibernation of *O. hupensis*

Active adult *O. hupensis* (age: ~6 months; presence of 7–8 whorls, n=780) were divided into 26 groups of 30

snails each and placed in Petri dishes (diameter: 9 cm). Snails were kept at 13°C on culture paper and de-chlorinated water was added daily as described before. Hibernation was investigated by gradually reducing the temperature. For snails belonging to groups 1–13, the temperature was reduced from 13°C to 1°C at a rate of 1°C per day, while the temperature in groups 14–26 was reduced at a rate of 1°C every other day.

Snail activity and hibernation status were observed as follows. Snails with a closed operculum and/or lack of movement were pinched by a pin on the operculum and the foot-head. Snails, which showed no reaction when pinched were placed in de-chlorinated water at a temperature of 13°C for several hours. Snails were tested again and in case normal activity resumed (i.e. response after pinching), they were considered in hibernation state before. The temperature at which hibernation occurred was recorded. Snails in the control group were raised at a constant temperature of 13°C. All experiments were repeated two to three times.

Statistical analyses

All data were entered in Excel (Microsoft Corporation; Redmond, WA, USA) and statistical analyses were done with STATA (version 8.0; Stata Corporation; College Station, TX, USA).

The shortest, longest and average pre-patent period of *S. japonicum* in groups of snails kept at different temperatures were summarized by arithmetic mean and standard deviation (SD). The development from miracidia to cercariae is the reverse of the pre-patent period (N), i.e. $V_P=1/N$ where V_P is the development rate (Zou 1983). The Wilcoxon rank–sum test was used to compare the difference between the hibernation of *O. hupensis* in groups 1–13 and groups 14–26. The Kruskal–Wallis test was applied to compare the pre-patent period in the different temperature groups.

Linear regression analysis was used to investigate the effect of temperature on the development of *S. japonicum* within *O. hupensis*. A logarithmic transformation was applied on V_P to transform to normality. In case V_P equals to 0, the logarithmic transformation V_P+1 was used (i.e. ln (V_P+1)). Logistic regression was carried out to assess the relationship between the probability of hibernation and temperature (T).

Results

Effect of temperature on the development of *S. japonicum* within *O. hupensis*

From the 750 snails kept at various temperatures, 710 (94.7%) were still alive when the first cercarial shedding was carried out, and over the course of the experiment, 171 snails (24.1%) released cercariae (Table 1). Snails still alive at the end of the experiment that failed to shed cercariae were indeed infection-free upon dissection. The effect of temperature on the pre-patent periods was highly statistically significant (Kruskal–Wallis test, $\chi^2=22.7$, $df=3$, $p<0.001$).

Minimum thermal limit for *S. japonicum* development within *O. hupensis*

The rate at which development of *S. japonicum* within *O. hupensis* occurred showed a positive association with temperature. The average development rate per day of the snails kept at 21°C, 24°C, 27°C and 30°C was 0.008, 0.011, 0.014 and 0.016, respectively. The following regression describes the relationship between V_P and

Table 1 *S. japonicum* cercarial shedding and pre-patent period of *S. japonicum* within *O. hupensis* kept at different temperatures

Temperature (°C)	Initial no. of snails	First test by shedding		Final test by shedding			Parasite pre-patent period (days)		
		Day	No. of snails	Day	No. of snails	No. of snails shedding cercariae	Minimum	Maximum	Average (SD)
24*	150	50	147	195	0	–	–	–	–
18	150	70	138	210	0	–	–	–	–
21	150	60	135	180	20	18	110	165	128.9 (16.1)
24	150	50	145	160	45	34	65	145	95.0 (21.0)
27	150	40	149	115	50	57	50	100	71.9 (12.7)
30	150	30	143	115	45	62	40	100	62.7 (14.2)

*Control group

temperature: $\ln(V_P + 1) = 0.025 \ln(T) - 0.067$ (r^2=0.53; F test=187.52; p<0.001). The minimum thermal limit derived from this regression is 15.3°C at which the development of *S. japonicum* is arrested (Fig. 1).

Effect of temperature on the hibernation of *O. hupensis*

Employing the Wilcoxon rank–sum test, no statistically significant difference was observed for the temperature at which *O. hupensis* hibernated, as assessed by two different temperature decline rates (z=−0.128, $p>|z|$=0.90). At temperatures below 13°C, the lower the temperature, the less active the snail were, and the higher the proportion of snails with closed operculum. The snails began to hibernate at a temperature of 11°C. The proportion of snails, which hibernated at 6°C, 3°C and 1°C were 56.7%, 91.7% and 100%, respectively. The logistic regression between the probability of hibernation (H) and temperature (T) is given by the following equation: $\log it(H) = 3.93 - 0.61 \times T$. The temperature at which half of the snails were hibernating (ET_{50}) was 6.4°C (Fig. 2).

Discussion

Based on the report of the Intergovernmental Panel on Climate Change (IPCC 2001), the Earth's surface temperature is likely to increase, on average, by 1.4°C to 5.8°C over the period 1990 to 2100. This increase is about two to tenfold higher than the average temperature increase already observed during the 20th century. Climate change shows strong spatial and temporal heterogeneity. For instance, warming at high latitudes of the northern

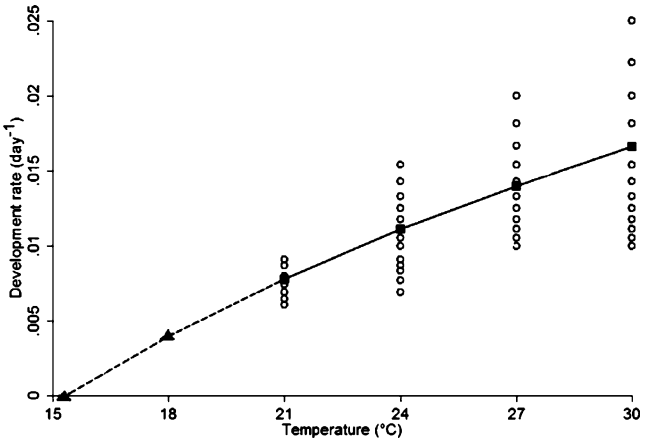

Fig. 1 Relationship between temperature and *S. japonicum* development within *O. hupensis* (*open circles* indicate the experimentally obtained data; *solid squares* show the averaged development rate at a given temperature; *triangles* represent the extrapolated values at 18°C and 15.3°C; *solid line* denotes the regression between temperature and development rate between 21°C and 30°C; *dashed line* indicates the extrapolated values according to the regression equation)

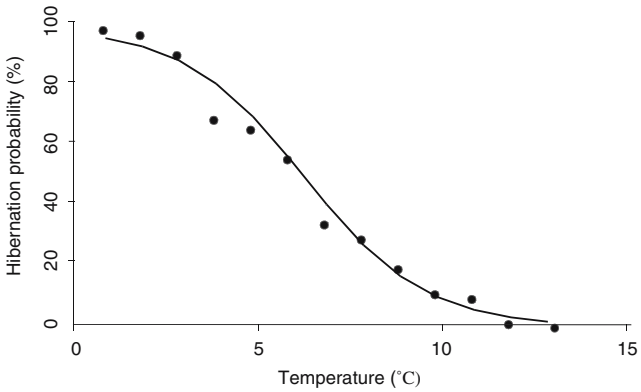

Fig. 2 Relationship between temperature and probability of hibernation of *O. hupensis*

hemisphere is more significant than elsewhere (Murphy et al. 2004), and changes of average maximum and minimum temperatures in winter months are more pronounced than in the summer (Easterling et al. 2000).

The negative effects of climate change on public health were reviewed recently (Beggs 2004; Knowlton et al. 2004; Patz et al. 2005; Haines et al. 2006; McMichael et al. 2006). The potential impact of global warming on the transmission of vector-borne diseases received particular attention, most notably malaria (Rogers and Randolph 2000; Reiter 2001; Hay et al. 2002; Hunter 2003; Sutherst 2004; Pascual et al. 2006). With regard to schistosomiasis, some studies have addressed the potential impact due to climate change (Mao 1990; Martens et al. 1995; Yang et al. 2005b). It was agreed that global warming may affect transmission of schistosomiasis in two ways, namely, to enhance transmission intensity and to expand areas where transmission occurs (Zhou et al. 2002a). In our recent work, using geographic information system and remote sensing technologies, coupled with spatial statistics, we showed that the 0–1°C January isotherm in China has shifted ~26′ northward, which corresponds to 40 km. As a consequence, an area of about 41,000 km^2 was created where transmission of *S. japonicum* could theoretically occur (Zhou et al. 2002b; Yang et al. 2005b). To further our understanding of the regional effects of global warming on the transmission of *S. japonicum* in China, temperature thresholds for parasite development within *O. hupensis* and the intermediate host snail itself must be taken into account.

The development of cold-blooded animals is positively related with temperature. Development will arrest when temperature drops below a critical threshold, which is considered the lowest developing temperature or "biological zero" (Yiteng 1986). The development of *S. japonicum* within *O. hupensis* follows this rule. The temperature at which snails hibernate is considered the lowest developing temperature because the metabolic rate of the snail will drop dramatically to "biological zero" when it reaches the stage of hibernation (Storey and Storey 1990).

Our results confirm earlier findings; within a certain temperature range, the higher the temperature the shorter the pre-patent period of *S. japonicum* within *O. hupensis*. Under our laboratory conditions, we found 15.3°C as the theoretical lowest developing temperature. This temperature is considerably higher than that documented previously, i.e. 10°C (MOH 1980). The difference may ensue as the previous study examined each developing stage in the intermediate host snail rather than observing the whole development process from miracidia to cercarial shedding. The latter approach seems more meaningful when studying the transmission of schistosomiasis.

Previous studies have investigated parasite–intermediate host snail interaction with an experimental focus on *S. mansoni* and *S. haematobium*. It was found that the lowest developing temperature was higher than the theoretical minimum temperature threshold. For instance, the theoretical minimum temperature threshold of *S. mansoni* within *Biomphalaria pfeifferi* and *S. haematobium* in *Bulinus truncatus* are 14.2°C and 15.3°C, respectively. However, no cercariae were shed when *B. pfeifferi* were kept at a temperature below 16°C and *B. truncatus* below 17°C. In our study, *O. hupensis* failed to shed cercariae when snails were kept at 18°C, which is almost 3°C higher than the observed theoretical lowest developing temperature. Our findings thus confirm previous observations made with other human schistosome species. Another possible explanation is that the theoretical pre-patent period of *S. japonicum* within *O. hupensis*, which was calculated to be 256.9 days at a temperature of 18°C, is considerably longer than our observation period of 210 days.

Not only low, but also high temperature influences the development of the parasite in the intermediate host snail. Previous investigations showed that intermediate host snails failed to shed *S. mansoni* and *S. haematobium* when they were kept at temperatures above 35 and 33°C, respectively (Pflüger 1980; Pflüger et al. 1983). It remains to be investigated at what maximum temperature the development of *S. japonicum* will cease.

Metabolic rate depression is a common adaptive strategy of hibernation (Storey and Storey 1990). We found that a simple pin pinch method applied to *O. hupensis* kept at cold temperatures, followed by the transfer of snails into warmer water, is relatively straightforward to test whether snails are in a state of hibernation.

The acquired information on the lowest developing temperature for *S. japonicum* within *O. hupensis*, and the intermediate host snail itself, can be used to estimate the growing degree–days. In turn, this information will facilitate the assessment of the impact of temperature on the development of the parasite and intermediate host snail, and to better understand their interaction (Malone and Zukowski 1992; Zhou et al. 1999; Yang et al. 2006).

S. japonicum found on the Chinese mainland comprises a strain complex consisting of different sub-strains, which differ in their geographic distribution (He 1993). Distinct genetic diversity is also detected between different subspecies of *O. hupensis* (Davis et al. 1995, 1999). Whether the lowest developing temperature varies between different parasite strains and between different regions is among our current research emphases. Taken together, this line of investigation will enhance our understanding and prediction capabilities with regard to the level and extent of schistosomiasis transmission in a warmer future China.

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