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The Effect of Cyclic Temperatures on the Growth of *Fasciola hepatica* and *Lymnaea viatrix*

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SUMMARY

The experiment aimed to measure the effect of constant and variable temperatures on the growth of *Lymnaea viatrix* snails, on the development of a Peruvian isolate of *Fasciola hepatica* eggs and on the development of *F. hepatica* in the snails. This was carried out by cultivating infected and uninfected snails and fluke eggs in artificial, temperature controlled chambers. *L. viatrix* snails were found to develop at a rate dependent on environmental temperature, but developed at least as well under conditions of varying temperature as at the same mean constant temperature. *F. hepatica* eggs held at constant or varying temperatures, developed at a rate comparable to other reports. However, eggs developing at varying temperatures appeared to have reduced hatchability. Parasite development within the snails was slow, though within the limits calculated from the literature, and varying temperature did not appear to reduce development compared to constant temperatures.

KEYWORDS: *Lymnaea viatrix*; fasciolosis; *Fasciola hepatica*; environment; temperature.

INTRODUCTION

Development of the intermediate-host snails of liver fluke and of the intra-molluscan and free-living stages of the parasite themselves, are known to depend on environmental temperature, with a minimum temperature for the development of the snail and parasite of about 9.5°C (Rowcliffe & Ollerenshaw, 1960). In temperate areas, seasons with low mean temperatures restrict the development of the snail and parasite, so that the development of fasciolosis may be seasonal. In tropical areas, mean temperatures may remain above 10°C consistently. This mean temperature, however, may result from wide daily variations including periods overnight well below the 10°C threshold.

Cajamarca is a city in an inter-Andean valley in northern Peru, situated at 2700 m above sea level. Hourly temperatures typically vary from 8.9°C in the morning to 21.5°C in the afternoon during January, and from 4.2 to 21.6°C during July, with a mean daily temperature of 14.2°C all year. Hourly ground temperatures have an even wider range, from −0.2 to 24.2°C in January and from −5.0 to 23.8°C in July (data from the Peruvian Servicio Nacional de Meteorología e Hidrología, SENAMHI, in Cajamarca).

The purpose of this investigation was to measure, under controlled conditions, the effect of variable temperatures on the development of *Lymnaea viatrix* snails and *Fasciola hepatica* parasites from Cajamarca.

MATERIALS AND METHODS

Four climatic chambers were set up to run at different temperatures controlled by an antifreeze/water mixture supplied from two water baths through a pair of heat exchangers in each chamber. The environment in three of the chambers (chambers 1–3) was set at a fixed temperature, and in the fourth chamber (chamber 4), the temperature varied on a 24-h cycle, using a time clock to alternate the cold and hot water flows (Fig. 1).

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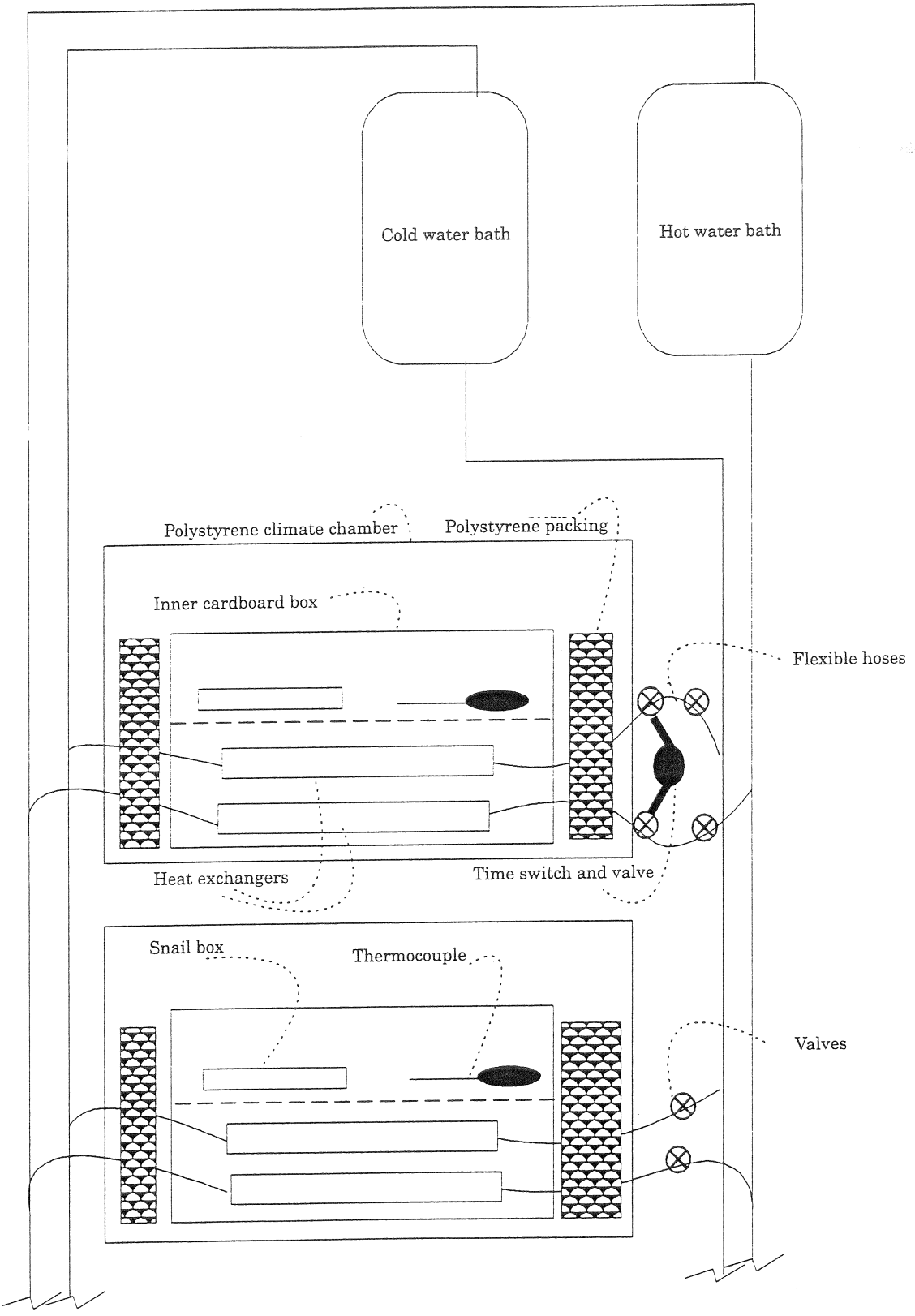

Fig. 1. Schematic diagram of the apparatus used to control temperature for the snails and parasite-development experiments. There were two further chambers connected, both with static temperature settings (identical to the bottom chamber in the diagram).

Temperature was recorded hourly using an electronic printing thermometer (Hanna instruments), and data were processed on Microsoft Excel spreadsheets and analysed using SPSS/PC+ and SPSS.

Development of the snails

L. viatrix snails, originally from Peru but maintained at the Liverpool School of Tropical Medicine, were hatched on algae (*Oscillatoria* spp.)-covered filter paper in Petri dishes, and then raised on algae-covered mud packs ('plates'). Groups of snails were allowed to develop from birth to 21 days on the plates in the four environment chambers, and their size was measured at the start and end of the period, and average daily growth was calculated. Following the first run, the experiment was repeated with a fresh set of newly-hatched snails. Each chamber was set at a different temperature during the second run than it was during the first, thus producing a set of eight results at different temperatures.

Development of Fasciola *eggs*

F. hepatica eggs were extracted from the faeces of an infected sheep by filtering the faeces through a sieve of mesh size 53 μm. Eggs were then stored in approximately 15–35 ml of water in 75 cm^2 tissue culture flasks in each of the four environment chambers. The eggs were washed each day for 5 days by allowing the eggs to sediment and pouring off the supernate, which was then replaced with clean water. Following the washing process, aliquots of 1 ml were dispensed into bijou bottles. Each bijou was wrapped in aluminium foil and returned to their respective environment chambers. The eggs were left to develop, and individual bijous were removed at intervals to determine if the eggs could hatch. This was carried out by removing the foil from the bijou, adding 4 ml of fresh water to each tube and then examining the sample under a stereo microscope for the presence of free-swimming miracidia. The egg development experiment was carried out twice, producing eight results.

Development of infection in the snails

Miracidia, produced from eggs recovered from infected sheep, were used to infect individual snails. Single miracidia were placed with single snails in 2 ml of water in a tissue culture plate and left for 3.5 h. During this period, snails that crawled out of the water were gently pushed back in. Snails were then put onto algae plates (as described above) into each of the four temperature chambers. Twenty exposed snails were put on each plate and 10 non-infected (control) snails were placed on a second plate in each chamber. Plates were changed regularly to ensure that there was always algae available to the snails.

Twice every week, from 18 days after infection, snails were placed individually in 3 ml of distilled water and examined for shedding of cercariae. Each snail was left in water for 1 h at room temperature and then returned to the plates in their respective environment chambers. The water was examined for cercariae and metacercariae.

RESULTS

As an example, hourly temperature curves for the first run of the first experiment are shown in Fig. 2. Temperature varied in chamber 4 between −1.7 and 23.9°C during each 24-h period. Temperature curves for the second run of the snail growth experiment and all the other experiments were similar to Fig. 2.

Growth of the snails is shown in Fig. 3. All the snails died in one of the eight chambers, so that there are only seven points on the graph. The line of best fit, calculated from the constant temperature chambers only, and excluding the data from the hottest chamber, is also shown. Although the relationship is almost linear, the line of best fit was found to be quadratic. Of the two points

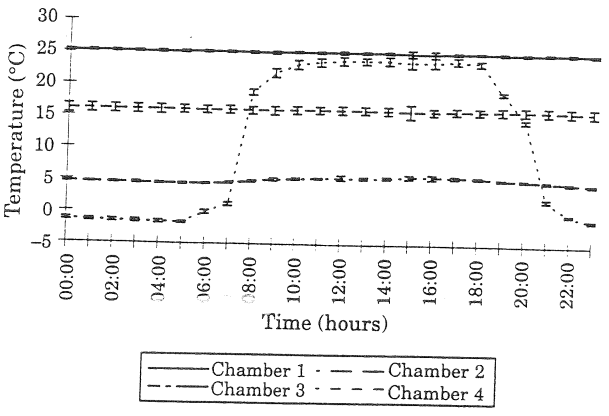

Fig. 2. Average hourly temperature patterns for the four chambers during the first experiment [k-type thermocouple-temperatures recorded on a printing thermometer (Hanna instruments)].

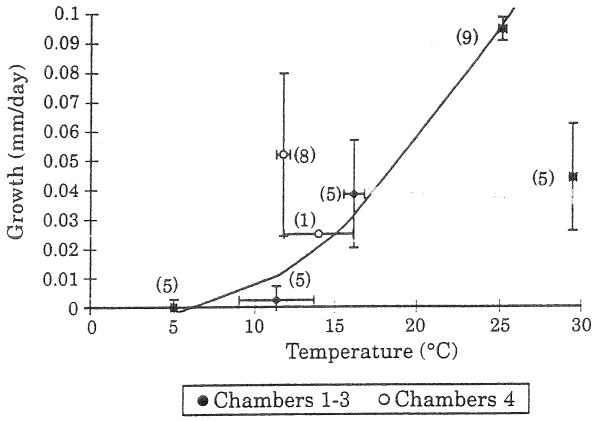

Fig. 3. Growth of snails with temperature. The line represents the best fit generated from the stable temperature chambers, the point on the far right (hottest) being ignored. The open circles represent the data from chamber 4 (variable temperature), numbers in parenthesis record the number of snails at the end of each experiment. The formula of the line is: Growth (mm) $=0.000208(T°C)^2 - 0.00137(T°C) - 0.00056$. Bars are standard deviations, those for growth are estimated from total deviation in final snail size divided by 21 (days of growth).

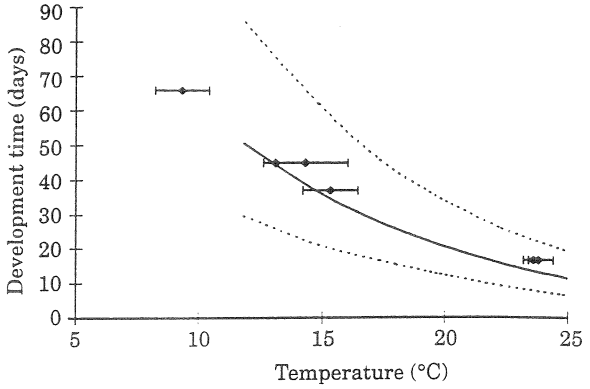

Fig. 4. Egg development time to hatching with temperature. The solid line is derived from the literature (Ollerenshaw, 1971; Over, 1982; Gettinby & Byrom, 1991) and the dotted lines are the 95% confidence limits from those data. The points marked represent the data from the experiment. Bars = 1 standard deviation. All data points are well within the expected range of development times.

representing the snails from the variable temperature chambers, one, at 11.7°C, was significantly different from the line of best fit ($P<0.05$), indicating that the snails appeared to have grown faster than would have been predicted from the mean temperature of the chamber. The second point (at 13.6°C) was not significantly different from the line of best fit.

Development times for the eggs are shown in Fig. 4, superimposed on a graph of development times derived from the literature (see the legend of Fig.4). Of the eight sets of eggs incubated, one set from the variable chamber did not hatch (see below) and the experiment had to be discontinued before one set from the coldest chamber were expected to hatch. There are, therefore, only six points on the graph. The eggs developed within the 95% confidence limits predicted from the literature. Those developing at a variable temperature also developed at a rate predictable from the mean temperature of the chamber. Hatching of the eggs from this chamber, however, appeared to be affected; hatching from one set did not occur at all, and from another was not regular once hatching had started. Hatching in all eggs developing at a constant temperature was consistent once those in one bijou were observed to have hatched.

Development times for the intra-molluscan stages are shown in Fig. 5, superimposed on growth rates derived from the literature (see legend of Fig. 5). Two sets of snails did not produce cercariae during the experiment and development times for those that did produce cercariae were long but still within the 95% confidence limits for the literature-derived data.

DISCUSSION

The equipment used to simulate the effects of variable temperature generally worked well, although presetting precise temperatures was difficult and, in addition, there were occasional problems with leaks or air locks, especially in the hot circuit. Any resultant variation in temperature, however, was recorded by the electronic thermometer and, through its impact on variance of the mean temperature, was taken into account in the statistical analysis.

Snail growth

The growth of snails remained very slow at temperatures below 10°C, and then increased rapidly to around 25°C, after which it fell again. The increase in growth rate with temperature was almost linear over the range 10–25°C, but the line of best fit was found to be quadratic. Despite the temperature of the variable chamber (chamber 4) remaining below 10°C for 11 h a day, the growth rate of the

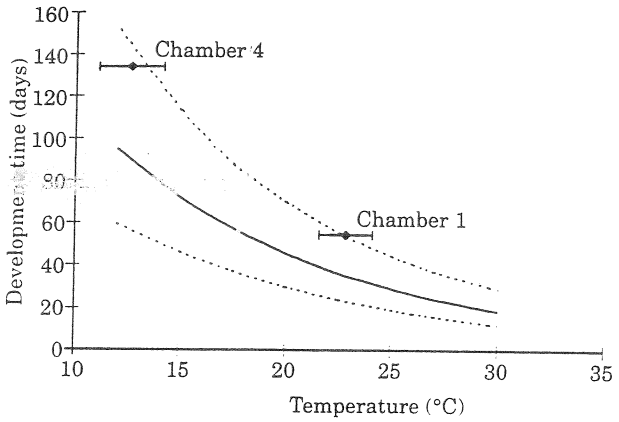

Fig. 5. Development time to cercarial shedding with temperature. The solid line is calculated from the literature (Ollerenshaw 1971; Over, 1982; Gettinby & Byrom, 1991), and the dotted lines are the 95% confidence limits from those data. The two points represent the first snails shedding in chambers 1 and 4 and are located at the limit of the range of expected development times. Bars = 1 standard deviation.

snails in chamber 4 was at least as good as would have been expected if they had been at a constant temperature equal to the chamber mean and, in one case, the growth rate of the snails in the variable chamber was significantly greater than would have been expected. The hottest data point was excluded from the calculation of the line of best fit, as it did not appear to fit a curve. However, including the point would be expected to reduce the slope of the line of best fit, in which case the growth of snails in chamber four would have been even greater compared to those of chambers 1–3. Alternatively, if this point represents a fall in growth at high temperatures, the initial part of the curve would still be expected to fit with the calculated line of best fit. Al Habbib (1974) found that growth rates of *L. truncatula* at variable temperatures were predictable by summing the period of growth at each temperature. In the current experiment, with the growth rates almost linear except at low temperatures, it would be expected that the growth in chamber 4 would be the same as, or higher than, that predicted from the mean temperature, which was the case.

Growth of snails in the environment, therefore, can be expected to be at least as high as that predicted from the mean environmental temperature, and may even be significantly higher. Despite the low temperatures, including sub-zero temperatures, experienced in the early morning in Cajamarca, *L. viatrix* can be expected to grow throughout the year if there is sufficient moisture, since temperature is not limiting.

Development of Fasciola *eggs*

The development of *Fasciola* eggs at different temperatures was well within the 95% confidence limits for normal development times obtained from the literature, which includes the development of eggs kept at a variable temperature. There was some evidence, however, that the eggs developing in chamber 4 were inhibited from hatching since one set, though fully developed, did not hatch, and the hatching in the other set was not uniform, unlike the uniform hatching in all sets in the constant temperature chambers. Therefore, although the variable temperature did not appear to affect the time taken for the development of *Fasciola* eggs significantly, it may have reduced their viability, thus lessening the risk of infection. How important this reduction might be, if it occurs in the natural environment, has not been quantified.

Development of parasites in the snails

The infection parameters for the snails by miracidia were based on the work of Rondelaud and Barthe (1987) and Hourdin *et al.* (1993). It was not possible to be sure how many snails of each set were infected by the infection protocol; all sets dissected, however, showed several snails infected except those from chamber 3. The snails in chamber 3, however, had exhibited a high mortality, significantly greater than its control, so that only four of the 20 snails were left for dissection at the end of the experiment. This chamber was run at low mean temperature (9.9°C, chamber 3), and it is possible that the high mortality was due to the low temperature killing infected snails preferentially. Excluding chamber 3 and assuming that deaths were not infected, the minimum successful infection rates possible from the data were 10 and 25%, and the maximum rate, assuming all deaths were infected, was 70%. These figures compare to the published data of 60% (Preveraud Sindou & Rondelaud, 1995) and 4–75% (Preveraud Sindou *et al.*, 1991).

Development time of the monomiracidial infections was measured as time to cercarial shedding. This was believed to be more relevant than dissecting snails at regular intervals. The time taken for the development of parasites in the snails was within the 95% confidence limits for chambers 1 (hot) and 4 (variable), although in both cases

development was at the high extreme of the normal range. The development of parasites in chamber 2, though apparently complete, appeared to be longer than the normal range because no shedding occurred during the experiment.

Once shedding had commenced, groups of snails continued to shed only intermittently. This poor shedding rate may have been due to the method of shedding or the conditions of growing the snails (without light). Equally, it might suggest that the development of the parasite in *L. viatrix* is more sluggish than in *L. truncatula*. Though development of infection does vary between species of *Lymnaea* (Boray, 1966), there appears to be no report that development in *L. viatrix* is retarded.

In conclusion, daily variation in temperature does not adversely affect the growth of *L. viatrix* snails, even where the temperature falls below the normal minimum required for growth, or even below freezing. Indeed, growth may even be greater than predicted from the average recorded temperature. Similarly, temperature variation does not affect the rate of development of *Fasciola* eggs, though it may affect adversely the ability of those developed eggs to hatch. Development of infection in snails does not appear to be affected by variation in temperature, though in this experiment all development rates were slower than predicted. This may have been a result of the experimental technique used or possibly of the species, or strain, of snail, *L. viatrix*, a possibility which requires further investigation. The daily variation in temperatures found in Cajamarca, do not, therefore, appear to slow down development of the intermediate snail host or of the parasite.

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