

EFFECT OF TEMPERATURE ON THE INFECTIVITY OF METACERCARIAE OF *ZYGOCOTYLE LUNATA* (DIGENEA: PARAMPHISTOMIDAE)

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ABSTRACT: As a test of the energy limitation hypothesis (ELH), we predicted that temperature would have a significant influence on the infectivity of metacercariae of the digenetic trematode *Zygocotyle lunata*. Snails infected with *Z. lunata* were collected from ponds near Crawfordsville, Indiana, isolated at room temperature, and examined for the release of cercariae. Newly encysted metacercariae were collected and incubated 1–30 days at 1 of 5 temperatures (0, 3, 25, 31, 37 C). Twenty-five cysts were fed to each of 5 or 10 mice per treatment group (temperature). At 17 days postinfection, mice were killed and worms were recovered; data were collected on levels of infection in each group and the total body area of each worm. No worms were found in mice fed cysts that had been held at 0 C or 37 C (after 30 days). There were no differences in prevalence, infectivity, or mean intensity among the 3, 25, and 31 C treatments. Infectivity of metacercariae incubated at 37 C for 1 day was significantly greater than in all other treatments, while infectivity of metacercariae in the 37 C/15-day treatment was significantly lower than in all others. Mean body area of worms at 37 C/15 days was significantly greater than at other temperatures, suggesting density-dependent increases in growth. These results, particularly those from the 37 C treatments, are consistent with the ELH; infectivity was lower at high temperatures or when incubated for more time at 1 temperature (fewer energy reserves). It is suggested that microhabitat conditions experienced by metacercariae of *Z. lunata* could contribute to longer larval life, thus influencing this parasite's temporal dispersal.

Zygocotyle lunata is a digenetic trematode that naturally inhabits the cecum of various species of North American waterfowl but has also been found in mammals such as deer and cattle (Willey, 1941). Additionally, a variety of hosts, including rodents, ducks, and domestic chicks, have been used in a number of experimental studies (Willey, 1941; Fried, 1970; Fried and Nelson, 1978; Fried and Gainsburg, 1980; Fried et al., 1978; Huffman et al., 1991; Etges, 1992). Upon release from a snail intermediate host (*Helisoma* sp.), cercariae encyst on aquatic vegetation, snail shells, or other substrata that may be ingested by a suitable definitive host (Willey, 1941). It is likely that the amount of time metacercariae remain in the aquatic environment is highly variable; consequently, prolonged survival of cysts may be necessary. Willey (1941) reported successful infection in rats fed cysts that were 138 days old. Fried and Wilson (1981) reported that metacercariae stored at 4 C remained viable after 440 days. Although external environmental effects on *Zygocotyle* metacercariae are potentially substantial, relatively little is known in this respect.

Previous studies on trematode cercariae have demonstrated significant variability in infectivity and survival. Whereas a cercaria may be alive (viable), it is not necessarily infective (Shostak, 1993; Lowenberger and Rau, 1994; Pechenik and Fried, 1995; Wetzel and Esch, 1995). Successful transmission for cercariae depends on the duration of their infectivity, which is limited by the extent of motility and age. Motile cercariae are generally short-lived, limiting the time for successfully locating and infecting a host. These limitations are probably due to energy constraints considering that energy reserves are fixed upon cercarial release (Combes et al., 1994; Tinsley, 1996). Correlations between cercarial swimming time (in which energy stores are depleted) and survival/infectivity have been demonstrated (Rea and Irwin, 1992, 1995; Graczyk and Shiff, 1994; McCarthy, 1999), whereas others have substantiated a decline

in endogenous glycogen levels with age (Anderson and Whitfield, 1975; Lowenberger and Rau, 1994; Fried et al., 1998).

Related studies have suggested that exposure to higher environmental temperatures may be energetically equivalent to increases in cercarial age, i.e., both result in the depletion of energy stores (Shostak and Esch, 1990; Rea and Irwin, 1992, 1995; Shostak, 1993; Haas, 1994). Interestingly, McKindsey and McLaughlin (1994) demonstrated decreased survivorship and infectivity of *Cyclocoelum mutabile* miracidia at higher temperatures. Similar work on cercariae of *Echinostoma trivolvis* by Pechenik and Fried (1995) resulted in the proposal of the energy limitation hypothesis (ELH). This hypothesis suggests that (1) cercariae have finite energy stores, (2) the rate at which energy is used, i.e., the metabolic rate, limits absolute/functional life-spans, and (3) the metabolic rate of a trematode varies proportionally with environmental temperature.

Whereas previous studies used cercariae to infect a second intermediate host, in the present study metacercariae were used to infect experimental definitive hosts. Although multiple factors may influence the successful dispersion and survival of digenetic trematodes throughout their life cycles, the transmission of metacercariae to the definitive host enables the development of an ovigerous adult capable of reproduction, thereby making it an important link to consider. In this study, it was assumed that the metabolic rate of metacercariae varied with environmental temperature and that the amount of energy stored within the metacercariae was finite, as suggested by Pechenik and Fried (1995). Thus, as a test of the ELH we predicted that increasing temperature would cause a significant decrease in the infectivity of metacercariae of *Z. lunata*.

MATERIALS AND METHODS

Snails (*Helisoma trivolvis*) were collected from Pennington Lake (PL), a small farm pond near Crawfordsville, and from the Purdue Wildlife Area (PWA), 8 mi west of W. Lafayette, Indiana. Individuals with *Z. lunata* infections were isolated and maintained in small glass jars on which cercariae encysted following their release from the snails. Metacercariae <1 day old were collected and pooled into small plastic petri dishes and maintained in fresh artificial pond water (APW) at 1 of 5 different temperatures (0, 3, 25, 31, 37 C) for 30 days, as well as for 1 day and 15 days at 37 C (hereafter denoted as 37(1) C and 37(15) C,

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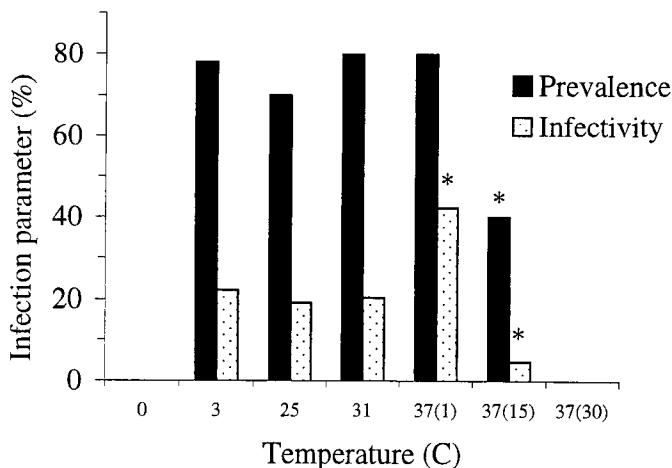


FIGURE 1. The effect of incubation temperature on prevalence of infection and infectivity of *Zygocotyle lunata* metacercariae. Infectivity is defined as the proportion of cysts recovered as worms. Unless otherwise noted, incubations were for 30 days. Note that no worms were recovered in the 0 and 37 C (30 day) treatment groups (* $P \leq 0.05$).

respectively). Inclusion of the 0 C treatment was done to confirm earlier observations (Wiley, 1941) that metacercarial cysts cannot tolerate freezing stress. The APW in each dish was replaced every 3–4 days. Metacercariae incubated at each temperature represent a treatment group (thus, there were 7 groups). Following incubation, all metacercariae appeared normal, i.e., undamaged, upon inspection with a dissecting microscope. While not done for all treatments, flame cell activity was confirmed before infection for some metacercariae used in the 37(1) C and 37(15) C treatments. Twenty-five cysts were given per os to each of 10 male ICR mice (Harlan Sprague Dawley, Inc., Indianapolis, Indiana) per treatment group with the exception of the 0 C, 37(1) C, and 37(15) C groups, each of which only included 5 mice. At 17 days postinfection, mice were killed and worms recovered; 1 mouse in the 3 C group died before 17 days. Prevalence (proportion of hosts infected), infectivity (proportion of cysts recovered as worms), and mean intensity (average number of worms per infected host) were then calculated for each treatment group (Bush et al., 1997).

Upon recovery, worms were fixed in alcohol–formalin–acetic acid (AFA) on a slide (with coverslip) and refrigerated overnight (with the entire slide preparation in AFA), then stored in 70% ethanol. For most treatments, an outline of each worm (magnified 25 \times) was traced using a drawing tube, and the images scanned into a computer; for some, a digital picture was taken of the worm. SigmaScan Pro 4 software (SPSS Inc., Chicago, Illinois) was used to measure the body length, maximum width, and total body area of each worm. Because they were found essentially to summarize the related data, only data on total body area are presented here. Data were analyzed with analysis of variance tests using SigmaStat (version 2.0, SPSS Inc., Chicago, Illinois) and JMP 3.2.2 (SAS Inst., 1997, Cary, North Carolina) unless assumptions were violated in which case a Mann–Whitney rank sum test (when comparing just 2 groups) was applied. Prevalence and infectivity data were compared using χ^2 analysis. Differences were considered to be significant at $P \leq 0.06$.

RESULTS

No worms were recovered in the 0 C or 37 C (30 days) treatment groups (Fig. 1). Thus, incubation temperature influenced *Z. lunata* infectivity in these treatments dramatically. Low (0 C) and high (37 C for 30 days) incubation temperatures rendered metacercariae completely uninfected. In contrast, 22.2%, 19.2%, and 20.4% infectivity were observed in the 3, 25, and 31 C treatment groups, respectively; there was no significant difference in infectivity among these 3 groups ($P <$

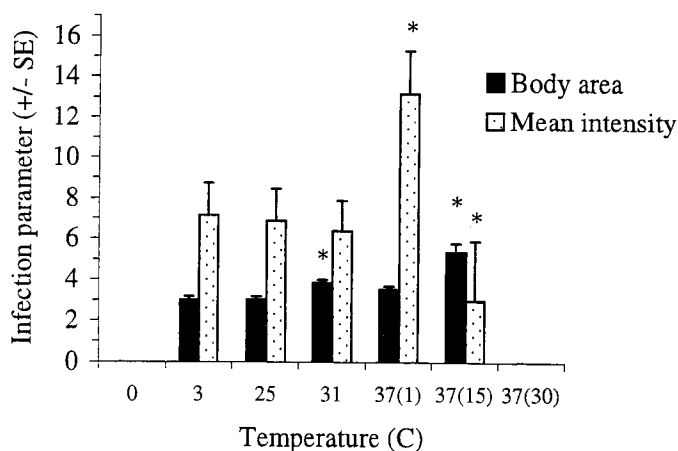


FIGURE 2. The mean intensity and body area (mm²) of *Zygocotyle lunata* adults recovered from mice. Bars represent mean values (\pm SE) (* $P \leq 0.056$).

0.05; Fig. 1). Moreover, there were no significant differences when comparing among the 3, 25, and 31 C treatments for the prevalence (78% vs. 70% vs. 80%; Fig. 1) and the mean intensity of infection (7.1 ± 1.6 vs. 6.9 ± 1.7 vs. 6.4 ± 1.5 ; $P = 0.75$; Fig. 2). In contrast to the 30-day treatment at 37 C in which no worms were recovered, infectivity of metacercariae incubated at 37 C for 1 day was significantly greater than at all other treatments (42.4%; $P < 0.01$), although the prevalence of infection was similar to the 3–31 C treatment groups (Fig. 1). Likewise, mice infected with cysts from the 37(1) C group had a significantly higher mean intensity of infection (13.2 ± 2.1 worms) than in any other group ($P = 0.056$). Infectivity in the 37(15) C group was significantly lower than in all other groups in which worms were recovered ($P < 0.05$). Despite a lower average (3.0 ± 2.9), the mean intensity of the 37(15) C group did not differ significantly from those in the 3–31 C treatments (Fig. 2; $P > 0.06$). However, worms from this treatment were significantly larger (in body area) than worms from other treatments (Fig. 2).

DISCUSSION

At the highest temperature treatment (37 C), the infectivity of *Z. lunata* metacercariae was significantly reduced with increasing incubation time that presumably corresponds to fewer energy reserves; after 30 days of incubation at 37 C, metacercariae were no longer infective. Given the significant declines in prevalence and infectivity both within the 37 C treatments as well as between the 31 C and 37(30) C treatments, we suggest that a threshold of energy exists for infection of the experimental hosts by metacercariae of *Z. lunata*; we suggest these patterns of infection support the ELH of Pechenik and Fried (1995). Interestingly, along with the significant decline in infectivity and mean intensity of worms from the 37(1) C to the 37(15) C treatments, there was a significant increase in the body size of worms from those infections. This increase in mean body size is most likely due to reduced competition for resources, given the lower number of worms in the host’s cecum; on average, with a greater number of worms, worm body size declined. Although not tested in this study, it is not unlikely that other life history characteristics, such as reproduction and

development, may be affected as well; food availability, i.e., energy, influences life history characteristics in a number of animals (Jokela and Mutikainen, 1995; Stadler, 1995; Giuliano et al., 1996; Rollo et al., 1997; Doughty and Shine, 1998; Koskela et al., 1998).

The absence of infectivity in the 0 C treatment group was not surprising, as it has previously been shown that *Z. lunata* cysts cannot withstand the process of freezing (Willey, 1941). The infective capacity of metacercariae to the experimental definitive host also was lost in the highest temperature treatment (after 30 days), a result similar to previous work involving cercarial infectivity to second intermediate hosts (Shostak, 1993; Lowenberger and Rau, 1994; Pechenik and Fried, 1995; Rea and Irwin, 1995). There were no differences in prevalence or mean intensity of infection among the 3, 25, or 31 C (all 30-day) treatments; thus, over a 30-day period, there is not a substantial effect of temperature on metacercarial infectivity. These results were similar to those of Huffman et al. (1991) in which 20% infectivity was obtained using 30 cysts per mouse. Given the disparity in temperatures used here, there clearly is a large amount of plasticity in the response of *Z. lunata* metacercariae to changes in environmental conditions (at least over this time scale).

It is notable that, despite the similarity to those from the 3, 25, or 37(1) C groups in terms of prevalence, infectivity, or mean intensity, worms from the 31 C treatment were significantly larger than worms from these 3 groups. Because of the availability of infected snails and the scheduling of experiments, all snails shedding cercariae used in the 31 C, 37(1), and 37(15) C treatments were collected from a different location (PWA) than snails used in the other treatments (collected from PL). Thus, we suggest that this interesting density/size interaction may represent geographical variation in body size of this parasite. As noted above, there were significantly fewer worms harvested from mice after 15 days at 37 C; not surprisingly, these worms were significantly larger (in body area) than worms from all other treatments. Using this logic, one might have predicted that worms from the 37(1) C treatment, in which there was a significantly higher mean intensity of infection, should have been smaller than in other treatments; however, there was no significant difference in body size among these worms and those from the 3 and 25 C treatments. Thus, we suggest that if metacercariae originating from PWA snails give rise to worms that are, on average, larger than those from PL, this may have dampened the density-dependent effect one might have predicted based on the observed differences in mean intensity. When applied to the 37(15) C data, the significantly greater body area of worms from this treatment would thus be due not only to the increased level of resources available because of a lower mean intensity, but also because PWA worms might simply be larger than those from PL. Of course, we cannot at this time separate these latter effects, but we feel these data raise interesting questions regarding potential habitat effects on the fitness of this parasite (at least as indicated here by size).

Microhabitat choice by cercariae that encyst in the external environment could influence survivorship and infectivity of the subsequent metacercariae. As mentioned previously, metacercariae of *Z. lunata* stored at 4 C remained viable for more than 1 yr (Fried and Wilson, 1981). Cercariae encysting in micro-

habitats that may experience cooler temperatures, e.g., shaded locations, greater water depths, or both, may remain infective longer, thus extending the opportunity for transmission. Unpublished results from related studies in our laboratory indicate a wide range of variation in encystment rates for *Z. lunata* cercariae. Therefore, we suggest that natural selection will favor those cercariae that encyst on substrata with physical characteristics correlated with slightly reduced temperatures, e.g., in shaded conditions on the undersides of vegetation. These microhabitat conditions could be expected to contribute to longer larval life, thereby enhancing the parasite's temporal dispersal. Additional studies of cercariae of *Z. lunata* could shed light on whether specific encystment behaviors increase transmission success and fitness.

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