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Exposure to *Fasciola hepatica* Miracidia Increases the Sensitivity of *Lymnaea* (*Fossaria*) *humilis* to High and Low pH

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ABSTRACT: Humidity and temperature have been considered important factors affecting the infectivity of *Fasciola hepatica* to its molluscan host. One hundred and thirty laboratory-reared *Lymnaea humilis* were exposed for 4 hr to the miracidia of *F. hepatica* over a pH range from 4.0 to 10.0, and their rates of survival were compared with 130 similarly treated but unexposed control snails. All control snails died within 24 hr at pH 4.0, but they showed better survival at pH 5.0–10.0. Their sensitivity to solutions with high and low pH, however, was increased if kept in the presence of *F. hepatica* miracidia. Snails exposed at pH 5.0 died within 24 hr, whereas most other pHs also affected survival such that by day 18 only those snails exposed at pH 7.2 remained alive. The increased sensitivity of the snails to pH could be explained by a damage-mediated release of parasite enzymes, because infectivity was highest at pHs associated with the lowest host mortality.

Fasciola hepatica is considered to be one of the most important parasitic diseases of domestic animals worldwide (Dreyfuss and Rondelaud, 1997; Mas-Coma et al., 2001). Although the life cycle of this fluke is generally well known, we do not currently have an adequate understanding of the miracidial invasion of the snail host, especially in terms of the effects of environmental variables on this process. Christensen (1980) demonstrated that the swimming miracidium is attracted to the snail by chemotactic factors released by the host. The parasite attaches to the epithelial cell surface of the mantle, which it invades by secreting digestive enzymes (Kendall, 1965). Several field studies have demonstrated that the prevalence and intensity of infection in snail hosts change seasonally (Craig and Bell, 1978; Boyce and Courtney, 1990; Cruz-Mendoza et al., 2002, 2004, 2005), and although humidity and temperature are thought to be the most important factors determining miracidial infectivity (Kendall, 1965; Boray, 1969), comparative studies on the effects of other environmental parameters on snail and miracidial survival are scarce. Abrous et al. (2001) reported that experimentally induced cold, fasting, and detergent treatment of *L. truncatula* increased the intensity and prevalence of infection by *F. hepatica* miracidia.

It also has been shown that a pH range between 7.0 and 9.0 is optimal for miracidial activity as well as salinity up to 5%, whereas pH conditions below 5.0 or above 10.0 kill the parasites in vitro as does a salt concentration above 7% (Cruz-Reyes, 1986).

The purpose of our study was to add to the understanding of the potential influence of environmental factors on infection of *Lymnaea* (*Fossaria*) *humilis* snails with *Fasciola hepatica* miracidia, by experimentally determining the effects of different pHs on parasite infectivity and host snail survival. Unexpected findings of these experiments are presented below.

The eggs of *F. hepatica* used as the source of miracidia for infection of the experimental host *L. humilis* were extracted from the gall-bladder of an infected bovine by filtering the bile, letting the sediment settle, pouring off the supernatant, and replacing it with clean water. Eggs were then incubated at 27–29 C for 13–16 days, to induce hatching, i.e., release of miracidia (Cruz-Mendoza et al., 2002). *Lymnaea humilis* snails were collected at the ranch of the Autonomous University of Hidalgo, located in the State of Hidalgo, central México. In the laboratory, egg masses collected from snails were allowed to hatch in Petri dishes with aerated water. Hatchlings were raised on mud with the blue-green alga *Oscillatoria* sp.

In total, 260 F_1 *L. humilis* 3.0–4.0 mm in length (20 to 22 days old) were used. Snails were kept individually for 4 hr at 20–22 C in 96 culture wells with 0.35 ml/well of 0.01 M phosphate-buffered solution at different pH values from 4.0 to 10.0 and in the presence (experimental) or absence (controls) of 3 miracidia/snail. Within each group, 10 snails were incubated at each pH (Cruz-Mendoza et al., 2002). Snails were then transferred to petri dishes on mud as described above and observed after 24 hr and then at 3-day intervals. Dead snails were dissected and examined to determine infection by *F. hepatica*. At day 18, the surviving snails were dissected and analyzed. Voucher shells of snails used in the experiment were deposited in the "Colección Nacional de Moluscos" at the National Autonomous University of México with the number CNMO-1657. Significant differences in proportion of viable snails between control and experimental groups were obtained by chi-square or Fisher exact test.

Uninfected *L. humilis* survived over a wide pH range (Fig. 1). At pH 4.0, all control snails died within 24 hr. In contrast, snails exposed to *F. hepatica* miracidia and kept in the different test solutions were more sensitive to solutions with low, and to a lesser extent, high pH. Snails exposed to pH 4.0 and 5.0 died within the first day after exposure; all snails incubated with the parasite, except some snails exposed at pH 7.2, died by day 18. In general, low and high pHs affected the infected snails, particularly the low pHs, but as the pH approached neutrality (i.e., pH 7.2), the effect was less marked. Interestingly, snails survived better at basic pHs (8–10), and the infected snails kept at pH 7.2 seemed not to be affected by the parasite. Miracidia as well as snails might have been affected by the various test solutions because rate of infection was lower at low and high pHs. Half of the experimental molluscs were infected with *F. hepatica* at pH 6.0, 7.0, and 7.5 compared with 80% at pH 7.2 (Fig. 2). A positive correlation was observed between snail viability and infection frequency ($R^2 = 0.62$, $P < 0.05$ at day 15). During this experiment, the optimal pH for survival and infectivity was 7.2 (Fig. 2), which does not support Cruz-Reyes' (1986) conclusion that the optimal pH range for miracidial activity was between 7 and 9. To confirm these results it will be necessary to design extended experiments, to obtain the second free-swimming stage (cercaria) so as to be able to accurately establish the parasite's optimal pH range.

In this study, we observed the dual effect on host snail survival of changing pH and miracidial infectivity. Other researchers have studied stress factors on infection by trematodes. The number of challenging parasites can be life-threatening, because the proportion of living *L. truncatula* snails dramatically decreased when infected with 10 or 20 miracidia, whereas the proportion of snails infected with 5 or fewer miracidia was not decreased (Dreyfuss et al., 1999). *Lymnaea truncatula* was more susceptible to infection by *F. hepatica* after being exposed to stressors such as fasting, detergent, or cold pretreatment. The pH also affected the host-finding capacity of miracidia as was found for the infectivity of *Schistosoma mansoni* to *Biomphalaria glabrata* (Dreyfus et al., 1999).

Kendall (1965) described the process of invasion, which may shed some light on what is probably damaging the snail at low and high pHs in the presence of the parasite. The miracidium first attaches to the host mantle epithelium, causing local damage as it does so. As noted by Graczyk and Fried (1999), "the host tissue is lysed by the gland secretions only in direct proximity of the apical region of the migrating miracidium." This process is probably mediated by proteolytic enzymes delivered by the apical gland of the parasite (Graczyk and Fried, 1999). Apparently, no protease has been reported to be synthesized by miracidia, although the presence of these enzymes has been documented in adults (Dalton et al., 2003). Nevertheless, Wilson (1969) described 3 types of vesiculated cells in the tegument of the miracidium of *F. hepatica*, some present near the surface. Because the snail epithelium is

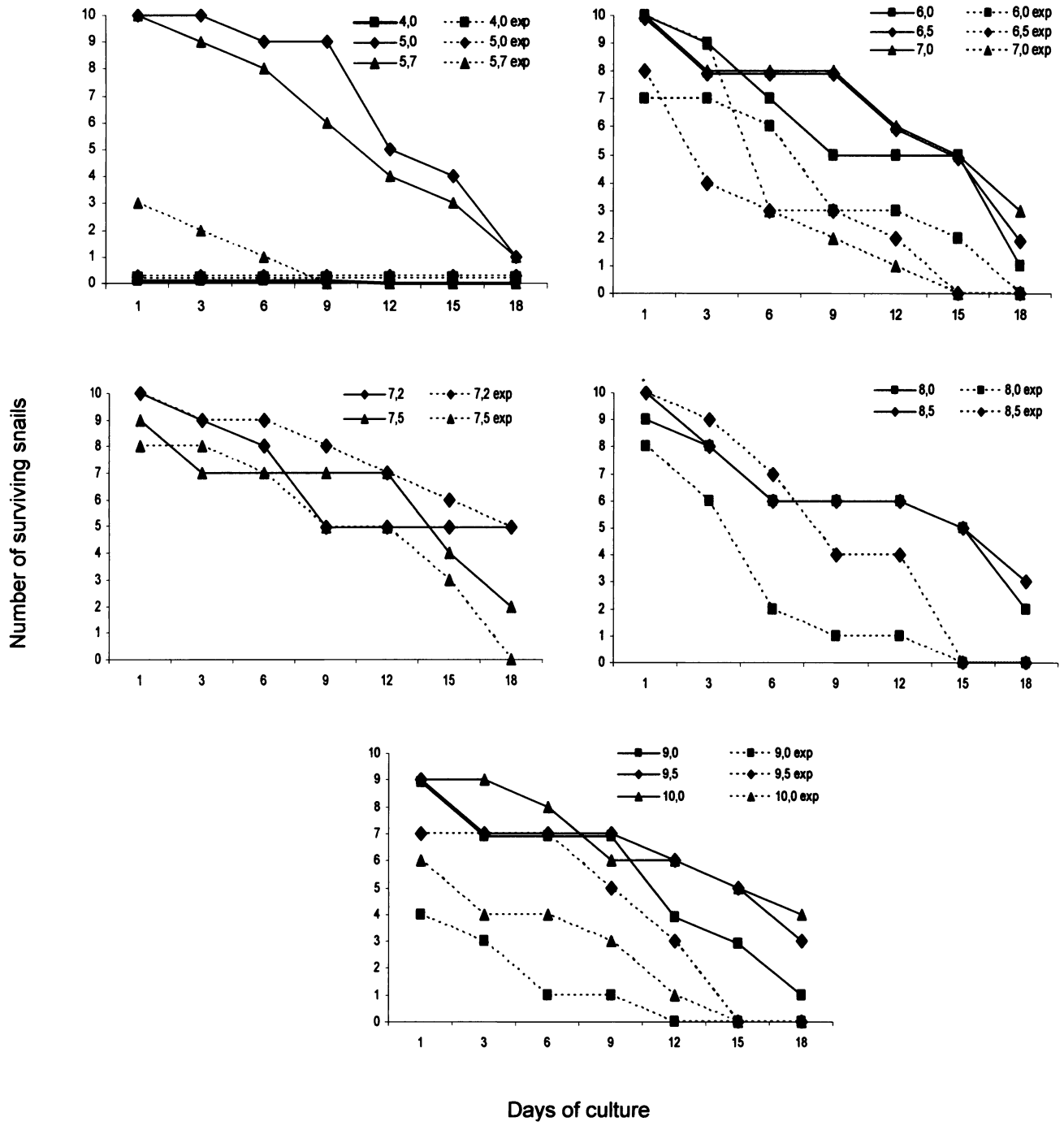

FIGURE 1. Effect of different pHs on survival of *L.* (*Fossaria*) *humilis*. Continuous lines depict control snails, whereas dashed lines correspond to molluscs coincubated with *F. hepatica* miracidia (exp). The numbers in the insets correspond to the pHs of the solutions. Statistical significance of differences between control and experimental groups was $P < 0.0001$ for pHs 5.0 and 5.7 and $P < 0.05$ for pHs 6.5, 7.0, 8.0, 9.0, 9.5, and 10.0.

covered by a mucous material, the parasite must digest it, probably via proteolytic/glycolytic enzymes liberated from the vesicles, as documented for other trematodes (Kendall, 1965).

However, the pH of the medium also affects *S. mansoni* miracidial infectivity, with low pHs producing less movement and less efficient penetration by the parasites in vitro (Upatham, 1972), although pH values lower than 5.0 were not tested in this report. Thus, low or high pH could damage the parasite in such a way that the contents of the vesicles are released into the medium, provoking generalized (as opposed to localized) damage of the snail epithelium. In agreement with this hypothesis, Cruz-Reyes (1986) reported that pH values below 5.0 or above 10.0 kill *F. hepatica* miracidia in vitro.

Besides the interest for optimal conditions of laboratory infection, this phenomenon might have implications for the natural transmission of the parasite, because the pH of the water may change in some areas where *F. hepatica* is present. Seasonal transmission of *F. hepatica* has been reported under natural conditions by us and others (Craig and Bell, 1978; Boyce and Courtney, 1990; Cruz-Mendoza et al., 2002, 2004,

FIGURE 2. Infection of *L. (Fossaria) humilis* with *F. hepatica* at different pHs. Every 24 hr, dead snails were dissected and examined to determine infection by *F. hepatica*. The experiment was stopped at day 18, when the remaining molluscs were examined.

2005). Humidity and temperature have been observed to correlate with peaks of infection in snails. Changing pH of a water body also could modify the infectivity of this parasite for its intermediate host, but this hypothesis has not yet been addressed under natural conditions.

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Lack of *Sarcocystis neurona* Antibody Response in Virginia Opossums (*Didelphis virginiana*) Fed *Sarcocystis neurona*–Infected Muscle Tissue

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ABSTRACT: Serum was collected from laboratory-reared Virginia opossums (*Didelphis virginiana*) to determine whether experimentally infected opossums shedding *Sarcocystis neurona* sporocysts develop serum antibodies to *S. neurona* merozoite antigens. Three opossums were fed muscles from nine-banded armadillos (*Dasypus novemcinctus*), and 5 were fed muscles from striped skunks (*Mephitis mephitis*). Serum was also collected from 26 automobile-killed opossums to determine whether antibodies to *S. neurona* were present in these opossums. Serum was analyzed using the *S. neurona* direct agglutination test (SAT). The SAT was modified for use with a filter paper collection system. Antibodies to *S. neurona* were not detected in any of the serum samples from opossums, indicating that infection in the opossum is localized in the small intestine. Antibodies to *S. neurona* were detected in filter-paper–processed serum samples from 2 armadillos naturally infected with *S. neurona*.

Sarcocystis neurona is a causative agent of the neuromuscular disease equine protozoal myeloencephalitis (EPM) (Dubey et al., 1991; 2001) and has been isolated from horses in North and South America (Dubey