Aspects of the Maintenance of the Life Cycle of *Fasciola hepatica* in *Lymnaea columella* in Minas Gerais, Brazil

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Fascioliasis is a parasitic disease of domestic ruminants that occurs worldwide. The lymnaeid intermediate hosts of Fasciola hepatica *include* Lymnaea columella, *which is widely distributed in Brazil. A colony of* L. columella *from Belo Horizonte, MG, was reared in our laboratory to be used in studies of the* F. hepatica *life cycle, the intermediate host-parasite relationship and development of an anti-helminthic vaccine. In the first experiment 1,180 snails were exposed to miracidia of* F. hepatica *eggs removed from the biliary tracts of cattle from the State of Rio Grande do Sul. In the second and third experiments the snails were exposed to miracidia that had emerged from* F. hepatica *eggs from Uruguay, maintained in rabbits. The rates of infection in the first, second and third experiments were 0, 42.1 and 0% respectively. Over 15,806 metacercariae were obtained and stored at 4ºC. Four rabbits weighing 1.5 kg each were infected with 32-44 metacercariae and two with 200. Three rabbits begin to eliminate eggs of the parasite in the feces from 84 days after infection onwards. The biological cycle of* F. hepatica *in* L. columella *and the rabbit was completed within 124 days.*

Key words: fascioliasis - *Lymnaea columella* - infection - *Fasciola hepatica* - Minas Gerais - Brazil

The trematode *Fasciola hepatica* (Linnaeus, 1758) is of great economic importance in areas dedicated to the rearing of cattle, buffalo, goats and sheep all over the world, producing considerable economic losses in dozens of countries. It is commonly found in sheep in Europe, North, South and Central America, Australia and New Zealand (Saleha 1991). Infestations may cause mortality or reductions in the growth rate and production of meat and milk.

Human cases of fascioliasis have been reported from Mexico, Cuba, Costa Rica, Puerto Rico, Uruguay, Argentina, Chile, Peru and Venezuela (Rey 1991), as well as the Brazilian states of Mato Grosso do Sul, Bahia, São Paulo and Paraná (Pessoa & Martins 1982, Rey 1991). The known agents of *F. hepatica* are pulmonate freshwater snails of the genus *Lymnaea* (Saleha 1991) that present a right-turning curvature of the shell, lack an operculum and are hermaphrodite. There are more than 20 species in the genus, many of which transmit *F. hepatica* and *F. gigantica* (Rey 1991). The geographical distribution of lymnaeid snails in Brazil is very wide, these mollusks are found in the states of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Rio de Janeiro, Minas Gerais, Goiás, Distrito Federal, Mato Grosso, Mato Grosso do Sul, Amazonas and Bahia (Paraense 1982a, b, 1983, 1986). Among the species that transmit *F. hepatica* in Minas Gerais is *L. columella* (Say, 1817), which occurs throughout the state (Souza et al. 1998). Snails from the municipalities of Sete Lagoas, Santa Rita do Sapucaí, Viçosa and Belo Horizonte have been found to be susceptible to experimental infection with miracidia of *F. hepatica* (Dacal et al. 1988). Silva et al. (1995) reported the first finding of *L. columella* naturally infected with *F. hepatica* in the municipality of Itajubá in southern Minas Gerais.

Studies are being conducted to develop a bivalent anti-helminthic vaccine against fascioliasis and schistosomiasis using the recombinant antigen Sm14, potentially capable of stimulating protective immunity against the two parasites (Tendler et al. 1995, 1996, Katz 1997).

In the present study *L. columella* from Minas Gerais was experimentally infected with *F. hepatica* and the life cycle maintained in the laboratory to obtain eggs, cercariae, metacercariae and adults for the study of the invertebrate host-parasite interaction and to assist in the development of an anti-helminthic vaccine.

MATERIALS AND METHODS

Snails - Snails measuring 3-8 mm, collected in Belo Horizonte and reared in the laboratory of Malacology of the Centro de Pesquisas René Rachou (CPqRR) (Souza & Magalhães 2000) were used for the infections.

Fasciola hepatica eggs - (a) obtained from cattle: preliminary studies involved miracidia that were obtained from *F. hepatica* eggs removed from the biliary tracts of cattle from Rio Grande do Sul, where the life cycle of the parasite is maintained in *L. viatrix* and cattle. The eggs were aged 97-131 days when used; (b) obtained from rabbits: eggs obtained from the feces of five New Zealand white rabbits, reared on the Experimental Farm of the School of Veterinary Medicine, Universidade Federal de Minas Gerais, were used in the second and third experiments. After infection with 30-41 Uruguay strain metacercariae the rabbits were maintained in the CPqRR animal house.

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About 2.6 g of feces were collected daily from each animal after detection of eggs. These samples were macerated, diluted in 1 l of distilled water, passed through a double layer of gauze six times and placed in glass jars during 30 min for sedimentation. Dilutions and sedimentations were repeated until the supernatant remained clear. The clear sediment was then transferred to Petri dishes. Eggs were collected by micropipette under a stereomicroscope, transferred to Petri dishes of diameter 150 mm containing distilled water and stored at 4ºC (Gomes 1985).

Ten months after infection the rabbits were sacrificed and their livers and gall bladders removed to collect adult flukes of *F. hepatica* and the eggs they had liberated. The eggs were obtained in large quantities, washed three times with distilled water, placed in glass flasks and stored at 4ºC.

Production of miracidia for infection of snails - Eggs between 14-131 days old were transferred to the Petri dishes with distilled water and incubated at 27ºC for 14-30 days. They were subsequently exposed to light for about 2 h, at a temperature of 27 ± 1ºC, to stimulate eclosion of the miracidia.

Experiment of snails - (a) individual exposure: for the individual exposures the miracidia were collected by micropipette under a stereomicroscope and transferred to transparent plastic cell culture dishes with 24 conical compartments, each with a capacity of 2.5 ml. One snail was placed in each compartment in the presence of 1-7 recently eclosed miracidia and the volume was completed with dechlorinated water. The dishes were covered with a lid and the snails remained totally immersed during the entire exposure period; (b) *en masse* exposure: for the mass exposure experiments, the eggs were placed in a round-bottomed volumetric flask (1,000 ml) partially covered with aluminum foil, the upper extremity of the neck being left uncovered and exposed to a beam of light. The recently hatched miracidia attracted to light were collected from the neck of the flask using a 1 ml automatic pipette and counted under a stereomicroscope (Chaia 1956). Aliquots of a suspension containing 25 miracidia were placed in 150 mm diameter Petri dishes in the presence of five snails (5 miracidia per snail). The volume was completed with dechlorinated water and the assembly sealed with a lid; (c) age of the eggs in the experiments*:* in the first experiment 1,180 snails were exposed individually to 1-5 miracidia originating from 97-131 day-old eggs of a *F. hepatica* strain from Rio Grande do Sul. In the second experiment 121 snails were used: 87 were exposed individually to 1-7 miracidia and 34 snails were exposed *en masse* to 5 miracidia per snail, originating from Uruguayan strain eggs aged up to 45 days. In the third experiment, 123 snails were exposed individually to miracidia from eggs of the same strain as those used in experiment 2, but aged 46-90 days.

The exposure time of the snails to miracidia in the light was about 2-4 h at temperatures of 26-28ºC.

Post-exposure management of snails - After exposure the snails were maintained in plastic aquaria containing about 4 l of dechlorinated water in a running water bath switched on for 8 h per day at 23-25ºC room temperature. Each aquarium contained a substrate consisting of sterile laeterite earth plus 10% of calcium carbonate. The exposed snails were fed on lettuce and ground rodent chow supplemented with 10% of calcium carbonate.

Observation of snails for presence for rediae and production of cercariae - Snails were observed under the stereomicroscope to detect the presence of larvae from the 35th day post-exposure onwards.

Collection of cercariae - They were placed in Petri dishes lined with transparent cellophane and exposed to light for up to 2 h. The individuals that had eliminated cercariae spontaneously or which presented larvae internally were separated and dissected. The negative snails were examined weekly up to 60 days post-infection, after which they were dissected.

Obtention and storage of metacercariae - The metacercariae were stored at 4ºC and later used to infect rabbits.

Determination of infectivity of metacercariae - To test the viability of the metacercariae, pieces of cellophane containing 32-44 and 200 cysts were wrapped in cabbage leaves and fed to groups of four and two rabbits, respectively. Each rabbit weighed about 1,5 kg.

Determination of infection of rabbits fed metacercariae - Examinations of rabbit feces to verify the presence of parasite eggs were initiated 30 days after infection, using the water sedimentation method (Lutz 1919).

RESULTS

Miracidia and infection of snails - The miracidia hatched after a period of the 14-30 days of incubation: (a) in the first experiment, in which 1,180 snails maintained at 24-25.5°C were infected with miracidia from 97-131 day-old eggs, the infection rate was 0% and mortality was 100% up to 50 days after exposure. The miracidia hatched but did not infect the snails; (b) in the second experiment, in which 121 snails (Tables I, II) maintained at 23-25°C were infected with miracidia from 14-45 day-old eggs, the mean infection rate was 42.1% and the mean mortality rate was 38.8% up to 60 days after exposure; (c) in the third experiment, in which 123 snails maintained at 24-25.5°C were infected with miracidia from 46-90 day-old eggs, the infection rate was 0% and mortality 60 days after exposure was 73.1%.

Observation of snails for rediae and production of cercariae - The presence of rediae and cercariae in the snails was observed 35-60 days after exposure in the second experiment.

Observation on cercariae - Some of the snails eliminated cercariae spontaneously while others were dissected to liberate the larvae.

Observation on metacercariae - The cercariae eliminated took 30-120 min to encyst, changing into metacercariae that adhered to the cellophane. The total number of metacercariae obtained for the 35 snails exposed individually was 12,379 (Table I), a mean of 353.7 metacercariae per mollusk. The total number of metacercariae obtained from the snails infected *en masse* was 3,427 (Table II), a mean of 214.1 metacercariae per mollusk. In the majority of the infected dissected snails the observed number of sporocysts and rediae was very high. The lowest number

TABLE I

Results of individual infection of *Lymnaea columella* from Belo Horizonte, MG, with miracidia of *Fasciola hepatica*, Uruguay strain, originating from eggs obtained from rabbit feces or from the uteri of adult worms

Snail						
No. exposed	Length in mm	No. miracidia	No. infected	Infection (%)	Mortality (%)	No. metacercariae
4	5-8	1-2	2	50	25	2,111
14	5-6	3-5	3	35.7	28.5	434
38	5-6	5-6	21	58.3	44.7	5,248
16	5-8	5-6	4	25	56.2	2,157
15	3-6	5-7	5	33.5	13.2	2,429
87	3-8	1-7	35	40.2	33.52	12,379

Infection and mortality rates, 60 days after exposure to miracidia

TABLE II

Results of mass infection of *Lymnaea columella* from Belo Horizonte, MG, with miracidia of *Fasciola hepatica*, Uruguay strain, originating from eggs of the uteri of adult worms

Snail						
No. exposed	Length in mm	No. miracidia	No. infected	Infection (%)	Mortality (%)	No. metacercariae
34	4-5	5	16	47.0	41.1	3,427

Rates of infection and mortality, 60 days after exposure to miracidia

of cercariae obtained per snail was 24 and the highest 1,530. No rediae or sporocysts were observed in the snail that produced 1,530 cercariae.

Observation on rabbits - All the rabbits survived, but only one (25%) of the first group (treated with 32-44 metacercariae) and two (100%) of the second (treated with 200 metacercariae) became infected. The elimination of parasite eggs in the feces of the animals was observed from 84 days after ingestion of the metacercariae onwards. The life cycle of the parasite in the snail and rabbit was completed within 124 days.

DISCUSSION

Several researchers have maintained the *F. hepatica* life cycle for studies of fascioliasis. Boray (1996) obtained infection rates that varied from 0-100% in studies involving *L. stagnalis*, *L. palustris*, *L. peregra*, *L. truncatula*, *L. auricularia*, *L. tomentosa* and *L. lessoni*, originating from Germany, Austria, Kenya and Australia and infected with *F. hepatica* and *F. gigantica*. The mortality of snails exposed to 5 miracidia varied from 17-44%. The proportion that produced viable metacercariae of *F. gigantica* varied from 0-54%. The minimum time taken for development of the parasite in the snails up to the appearance of cercariae varied from 28-52 days at temperatures of 22-24ºC.

In Brazil, Gomes (1985) obtained mean of infection rates of 27.6% when *L. columella* from Rio de Janeiro were exposed to three miracidia and 11% when each snail were exposed to 5 miracidia, at room temperatures of 27-29ºC. The mean mortality was 60.8%. Cercariae were obtained 56 days after exposure. Dacal et al. (1988) carried out mass infections of 6 samples of *L. columella* (4 from Minas Gerais, 1 from Rio de Janeiro and 1 from Rio Grande do Sul) with 6, 8 and 10 miracidia per snail. They obtained infection rates that varied between 25-80%. The infection rates of the Belo Horizonte sample exposed to 6, 8 and 10 miracidia were 32.5, 46.6 and 58% respectively, although these differences were not statistically significant. The mortality rates 45 days after exposure were 85.1, 68.3 and 75.6% respectively. Mortality of unexposed control groups varied from 63.9-89.9%. Luz et al. (1996) carried out infections of *L. columella*, *Physa cubensis* and *P. marmorata* with 2 and 60 miracidia per snail, obtaining infection rates of 66.6 and 25% for the lymnaeid and 0% for the physids respectively.

In the present study the life cycle of *F. hepatica* (Uruguayan strain) in *L. columella* from Belo Horizonte was successfully obtained in the laboratory during the May-August 2000 period, at room temperatures of 23-25ºC. The age of *F. hepatica* eggs that gave rise to infective miracidia varied from 14-45 days. After 46-90 days the miracidia hatched but did not infect the snails. Thus the age of the eggs, which exceeded 46 days both in the first (1,180 snails) and the third experiments (123 snails), negatively influenced the capacity of the miracidia to infect *L. columella* from Belo Horizonte.

Cercariae were obtained 40-60 days after exposure of snails to miracidia. The infection rates of 40.2 and 47% obtained were similar to those of Dacal et al. (1988) for Belo Horizonte snails. The mean mortality rates up to 60 days (33.5% and 41.1%) were similar to those obtained by other authors (Gomes 1985, Dacal et al. 1988). High mor-

tality (100%) in the first experiment was observed after 44 days in the exposed snails and after only 34 days in the control group. This high mortality therefore was not due to the presence of the parasite but rather to other factors, or predatory organisms such as oligochaetes in the aquaria.

The mean numbers of metacercariae obtained from *L. columella* in the individual (353) and mass (214) infections were similar to the results obtained by Abrous et al. (1998) of 56.5-107 in the infection of *L. truncatula* but inferior to the numbers recorded by Kendall (1949), who obtained 800-2,300 larvae when snails of this species were fed on algae. The maximum number of cercariae eliminated per snail in this experiment was 1,530. The greatest number of metacercariae obtained was through the dissection of the snails, large numbers of cercariae being seen around the digestive gland, as described by Kendall (1949) in his study of developmental effects produced by nutritional factors. After 60 days of infection some metacercariae were found encysted on the internal surface of the shell.

The viability of the metacercariae to infect the rabbits was demonstrated by the presence of eggs in three (50%) of six infected animals, 84 days after ingestion of the larvae. The life cycle of *F. hepatica* in snails and rabbits was completed from 124 days onwards.

The techniques used demonstrated that it is possible to maintain the life cycle of the parasite in the laboratory, using miracidia originating from eggs that are up to 45 days old.

Since no references were found in the literature on the age of eggs used to provide infectious miracidia, the life cycle of *F. hepatica* in *L.columella* from Belo Horizonte will be maintained using miracidia obtained from eggs up to 45 days old.

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