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# The Development of the Host-Response in Juvenile *Lymnaea palustris* to Invasion by *Fasciola hepatica*

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**Abstract.** Laboratory studies indicate that miracidia of *Fasciola hepatica* can penetrate several species of lymnaeids in Europe, other than the natural host *Lymnaea truncatula*. However, the production of cercariae from infections in abnormal hosts is limited to a small percentage of juvenile snails infected before a species-specific age.

Juvenile *Lymnaea palustris*, of known ages, were exposed individually to *F. hepatica* miracidia and killed at 24 h intervals up to 14 days post exposure, and then processed for histological examination to ascertain reasons for the failure of *F. hepatica* infections to develop successfully in this abnormal host.

The results indicate that the course of infection is rapidly halted by a cellular encapsulation response against the sporocyst. The response may be divided into two stages: first, the development of a cellular capsule and the concomitant degeneration of the sporocyst; second, the removal of the remains of the parasite and dispersal of the capsule. The efficiency of the response appears to increase with increasing age of the snail; statistical manipulation of the data obtained from histological investigations suggest that the defence mechanisms develop quickly in the first 6 days post hatching with little subsequent development during the experimental period. However, as sporocysts were encapsulated and killed regardless of age of the snail at exposure to infection, it is unlikely that in the host-parasite system used any of the infections would have survived.

## Introduction

Larval digeneans tend to exhibit a high degree of first intermediate host specificity. The natural host for *Fasciola hepatica* in Great Britain, Europe, and parts of Africa, is *Lymnaea truncatula;* however, it has been established for a number

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of years that lymnaeids other than *L. truncatula* are susceptible to invasion by *F. hepatica* miracidia (Kendall 1950; Boray 1966; Sazanov 1972; Nansen et al. 1976). Infections in such "abnormal" hosts were found to be largely unsuccessful, cercariae being produced by only a small percentage of juvenile snails, exposed to miracidia, below a certain species-specific age (Kendall 1950; Boray 1966).

Since these reports, little research has been carried out to determine the basis for the failure of *F. hepatica* infections in abnormal snail hosts, apart from a histological study by Sazanov (1972) who found that infections with Russian strains of *F. hepatica* in *L. ovata, L. stagnalis* and *L. palustris* were rapidly halted by a tissue response against the sporocyst. Similar responses have been observed in incompatible relationships between certain strains of *Biomphalaria glabrata* and *Schistosoma mansoni* by Newton (1952), Sudds (1960) and Cheng and Garrabrant (1977) and between *B. glabrata* and *Echinostoma lindoense* by Lie and Heyneman (1976). It is possible that a similar host defence mechanism is in effect against *F. hepatica* sporocysts in abnormal lymnaeid hosts; reports of infections surviving in juvenile abnormal hosts (Kendall 1950; Boray 1966) may suggest that the defence mechanism which operates against *F. hepatica* infection is insufficiently developed in some snails below a certain age when exposed to the infection.

The aim of the present investigation is to follow the course of infection of *F. hepatica* in *L. palustris* of known ages in order to determine the fate of sporocysts resulting from miracidia penetrating these snails and to describe in a quantitative manner the development of the host defence mechanism.

## Materials and Methods

Adult *L. palustris* for breeding purposes were collected from Aldsworth Pond, Sussex, and from drainage ditches at Pevensey Levels, Sussex. Tap water was conditioned to make it suitable for the culture of this snail species by keeping it in a 45 l tank with several dozen *Lebistes reticulatus* for at least 6 weeks. Breeding snails were maintained in approximately 2 l conditioned water in covered $30 \times 24 \times 5$ cm polypropylene trays and were fed ad libitum with blanched, dried lettuce and dried *Oscillatoria* spp. Newly laid egg masses were removed from the trays of breeding snails and maintained in $20 \times 15 \times 5$ cm polypropylene trays, with approximately 1 l of conditioned water. The newly hatched snails were removed from the egg cultures every third day, so that the following age classes of snails were obtained: 0–3 day old (d.o.); 3–6 d.o.; 6–9 d.o.; 9–12 d.o.; 12–15 d.o.; 15–18 d.o.; 18–21 d.o. The juvenile snails were maintained in polypropylene trays in a similar manner to that described for breeding snails.

*F. hepatica* eggs were obtained from flukes raised in laboratory rats and mice, as well as from sheep and cattle. The eggs were embryonated in filtered conditioned water at 25–27° C for 10–14 days and maintained in darkness; hatching of the miracidia was initiated by placing a sample of eggs in a watch glass with conditioned water, in natural light. Miracidia were used for infection experiments within 1 h of hatching.

The juvenile snails were exposed individually in watch glasses containing approximately 1.5 ml conditioned water, and 10 miracidia. Eight snails from each age class were sacrificed at each of the following times post infection (termed "infection times" herewith): 1 day post exposure (d.p.e.); 2 d.p.e.; 3 d.p.e.; 4 d.p.e.; 5 d.p.e.; 7 d.p.e.; such that 48 snails from each age class were examined. In addition a further eight snails were exposed to infection at 0–3 d.o. and sacrificed at 14 d.p.e.

The snails were fixed in Bouin's fluid for at least 24 h and, once fixed, the shells were gently removed. After dehydration in alcohol, the snails were double embedded in celloidin and paraffin wax and serial sections (6 μm) were stained with Ehrlich's haematoxylin and eosin.

In addition to the number of sporocysts in each snail, the appearance of the parasite was noted and also the intensity of any host reaction. The statistical techniques used are described in the appropriate section of the results.

## Results

Most of the sporocysts were located in the subepithelial tissues of exposed parts of the body, i.e. the mantle and the lateral and dorsal surfaces of the head and foot. Sporocysts were less frequently seen in the visceral mass. The histological investigation indicates that the course of infection of *F. hepatica* in *L. palustris* is usually halted by a cellular encapsulating response against the sporocysts. This encapsulation response, which follows a reaction-free period, may be divided into two stages: (1) capsule formation, resulting in the death of the sporocyst; (2) removal of the remains of the parasite and dispersal of the capsule. As the results also indicate that the onset and speed of the response are age dependent, the results will be presented in two parts: (1) the histology of the encapsulation response and (2) the development of the host defence mechanism in juvenile snails.

### *The Histology of the Encapsulation Response*

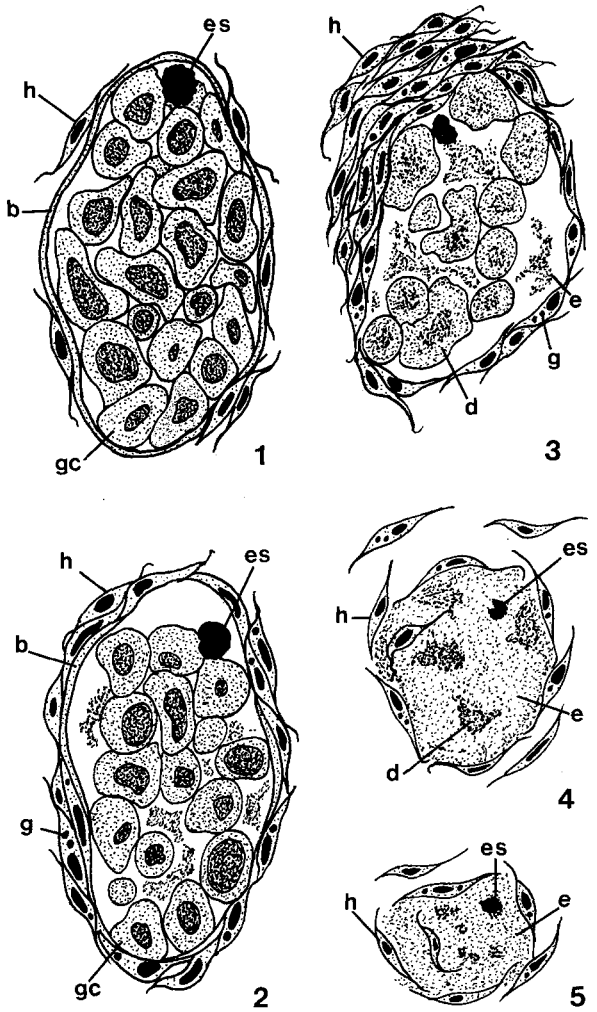
*Stage 1 of the Encapsulation Response.* The intact sporocyst appears as a compact mass of basophilic cells, surrounded by a thin body wall. As the eyespots may persist for several days after the miracidium/sporocyst metamorphosis, their presence is a useful criterion for identifying sporocysts in the later stages of encapsulation.

The presence of individual host reaction cells in the vicinity of the sporocysts or in contact with the body wall indicates the initiation of the host reaction against *F. hepatica* sporocysts in *L. palustris* (Fig. 1).

The response appears to involve only one cell type, this being referred to as the "host-reaction cell". The cell (overall length approximately 8 μm) is crescent-shaped, has a central, oval, basophilic nucleus and is drawn out into long cytoplasmic processes. Small basophilic granules are often seen in the cytoplasmic processes (Fig. 3), especially in the later stages of encapsulation.

Capsule formation occurs as more host reaction cells appear at the locus of infection and overlie those cells already in contact with the parasite (Fig. 2). The sporocyst retains its compact appearance in the early stages of encapsulation, although the body wall may be difficult to discern in areas overlaid with host reaction cells (Figs. 1 and 2). However, once the capsule is greater than 2–3 cell layers thick, a dramatic change occurs in the nature of the sporocyst. The body wall is no longer definable and the germ cells begin to dissociate from one another, and to degenerate (Fig. 3); as these processes continue the degenerating sporocyst fills with a flocculent, eosinophilic material (Fig. 3). At this stage the capsule reaches its maximum thickness of 6–7 cell layers, although the capsule is rarely consistent in thickness (Fig. 3).

*Stage 2 of the Encapsulation Response.* The degenerated sporocyst appears as a dense eosinophilic mass containing isolated areas of basophilic material (Fig. 4)




**Figs. 1–5.** Diagrams of the encapsulation process of *Fasciola hepatica* sporocysts in *Lymnaea palustris*, based on histological observations. (approx. ×1,900) *b* body wall; *d* degenerating germ cells; *es* miracidial eyespots; *e* eosinophilic mass; *g* granules; *gc* germ cells; *h* host reaction cells

**Fig. 1.** Slight encapsulation: body wall difficult to discern where host reaction cells are in contact with the sporocyst

**Fig. 2.** Between two and three cell layer capsule: body wall often indefinable, but germ cells still well organized

**Fig. 3.** Between five and six cell layer capsule, inconsistent in thickness. Note the degeneration of the germ cells

**Fig. 4.** Degenerated sporocyst: germ cells are no longer distinct and the capsule is beginning to disperse

**Fig. 5.** Eosinophilic 'scar' representing the remains of the sporocyst. Only a few host reaction cells remain

which probably represent the remains of the germ cells. The capsule appears to be less thick than previously observed, perhaps caused by the migration of host reaction cells away from the locus of infection. Both the size of the capsule and the area occupied by the degenerated sporocyst continue to decrease in size until the only visible remains of the parasite and its capsule are a small eosinophilic scar and a small number of host reaction cells (Fig. 5). At this stage, but not before, host reaction cells appear inside the remains of the sporocyst. Snails examined at infection times after those in which the terminal dispersal of the capsule is observed often contained no recognizable traces of sporocysts or of encapsulation. Therefore, it is thought that the host reaction is capable of removing all remains of the parasite from the snails' tissues.

*The Development of the host Encapsulation Response*

*Susceptibility to Invasion.* The histological evidence suggests that up to and including 15–18 d.o., snails are capable of completely removing few, if any sporocysts at 1 d.p.e., and it is possible that the mean number of sporocysts found at 1 d.p.e. in these age classes represents the susceptibility to invasion of each age class. A comparison of means suggests that there is no significant difference between the mean number of sporocysts found at 1 d.p.e. in snails exposed at 0–3 d.o., and snails exposed at 15–18 d.o., and therefore no change in the susceptibility to invasion. The significant ($P=0.01$) fall in the mean number of sporocysts at 1 d.p.e. in snails exposed at 18–21 d.o. is thought to be caused by the removal of sporocysts from the system – a supposition borne out by the histological evidence – and not by a decrease in the susceptibility to invasion in snails of this age class.

*Qualitative Observations. Reaction-free Period.* The length of the reaction-free period decreases as the age of the snail increases (Table 1), ranging from 3 to 7 d.p.e. in snails exposed at 0–3 d.o. to less than 1 d.p.e. in snails exposed at 18–21 d.o.; within the format of this investigation, it is not possible to determine the minimum reaction-free period in snails exposed at this age. During the reaction-free period, the sporocyst maintains its integrity, and in younger snails where this period is relatively long, germ cell division appears to occur.

*Stage 1 of the Encapsulation Response.* The minimum time for snails of different age classes to encapsulate 50% of the sporocysts is given in Table 1. In snails exposed at 0–3 d.o., 50% encapsulation has not been achieved by 7 d.p.e. In snails exposed at 3–6 d.o., 6–9 d.o., 9–12 d.o., or 12–15 d.o., the time period for 50% encapsulation is reduced to 3 or 4 d.p.e. This time period decreases even further to 2 d.p.e. in snails exposed at 15–18 d.o. and to just 1 d.p.e. in snails exposed at 18–21 d.o.

*Stage 2 of the Encapsulation Response.* The minimum times to remove the infection (i.e. when no sporocysts are found in any of the snails of that age class) are given in Table 1. The minimum time to remove the infection is 14 d.p.e. in snails exposed at 0–3 d.o. and drops to 5 d.p.e. in snails exposed at 9–12 d.o., after which there is no further improvement.

**Table 1.** Encapsulation data of *Fasciola hepatica* sporocysts in *Lymnaea palustris*

| Age class at exposure (days old) | Range of reaction-free period (days post exposure) | Minimum time for 50% encapsulation (days post exposure) | Minimum time to remove all sporocysts (days post exposure) |
|---|---|---|---|
| 0–3 | 3–7 | * | 14 |
| 3–6 | 2–7 | 3 | * |
| 6–9 | 2–5 | 4 | 7 |
| 9–12 | 1–4 | 3 | 5 |
| 12–15 | 1–4 | 3 | 5 |
| 15–18 | 1–3 | 2 | 5 |
| 18–21 | < 1 | 1 | 5 |

* Result not obtained within experimental parameters

**Table 2.** Mean number of sporocysts of *Fasciola hepatica* per snail found in each group of infected *Lymnaea palustris* together with variance and 95% confidence limits. There are eight snails in each group and a total of 42 groups. Each snail was exposed to ten miracidia of *F. hepatica*

| Age class of snail at exposure to infection (days old) | | Infection time (days post exposure) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 0–3 | $\bar{x}$ | 8.3 ± 2.1 | 6.3 ± 2.2 | 4.6 ± 2.2 | 3.4 ± 2.7 | 6.4 ± 2.5 | 4.3 ± 1.8 |
| | $s^2$ | 6.2 | 12.5 | 7.1 | 10.5 | 9.1 | 4.5 |
| 3–6 | $\bar{x}$ | 8.1 ± 2.3 | 5.5 ± 2.0 | 3.5 ± 1.5 | 3.3 ± 2.7 | 1.4 ± 1.09 | 0.9 ± 1.2 |
| | $s^2$ | 7.3 | 5.4 | 3.1 | 10.6 | 1.7 | 2.2 |
| 6–9 | $\bar{x}$ | 7.4 ± 2.8 | 6.3 ± 3.2 | 2.4 ± 2.1 | 2.0 ± 1.8 | 0.7 ± 0.8 | 0.0 |
| | $s^2$ | 12.0 | 14.8 | 6.6 | 4.6 | 0.8 | 0.0 |
| 9–12 | $\bar{x}$ | 2.4 ± 1.7 | 4.5 ± 2.2 | 3.2 ± 1.9 | 2.0 ± 1.7 | 0.0 | 0.4 ± 0.6 |
| | $s^2$ | 4.3 | 7.1 | 5.3 | 4.0 | 0.0 | 0.6 |
| 12–15 | $\bar{x}$ | 7.2 ± 2.9 | 1.0 ± 1.0 | 1.6 ± 1.5 | 2.3 ± 2.5 | 0.3 ± 0.6 | 0.0 |
| | $s^2$ | 12.1 | 1.4 | 3.4 | 9.1 | 0.5 | 0.0 |
| 15–18 | $\bar{x}$ | 6.7 ± 2.3 | 4.6 ± 3.0 | 2.0 ± 2.4 | 0.8 ± 0.6 | 0.0 | 0.5 ± 1.2 |
| | $s^2$ | 7.4 | 13.1 | 8.0 | 0.5 | 0.0 | 2.0 |
| 18–21 | $\bar{x}$ | 3.1 ± 2.8 | 2.4 ± 1.3 | 2.4 ± 1.5 | 0.9 ± 0.7 | 0.0 | 0.0 |
| | $s^2$ | 11.0 | 6.1 | 3.4 | 0.7 | 0.0 | 0.0 |

$\bar{x}$ = mean 95% confidence limits; $s^2$ = variance

*Quantitative Analysis. Mean Number of Sporocysts.* The mean number of sporocysts in each class falls with increasing infection time (Table 2) and the drop usually coincides with the observation of encapsulated and dying sporocysts in the host's tissues. The fall in the mean number of sporocysts with time is less marked in snails exposed at 0–3 d.o. than in the other age classes.

*Two-way Analysis of Variance.* A two-way analysis of variance, the results of which are shown in Table 3, indicate that if the effect of age class at exposure is ignored, the length of infection time exerts a significant ($P = 0.01$) effect

**Table 3.** Two-way analysis of variance on the number of *Fasciola hepatica* sporocysts found in 336 *Lymnaea palustris* of known ages

| Source of variation | DF | SS | MS | VR (F-ratio)[a] |
|---|---|---|---|---|
| Row (infection time) | 5 | 1,131.6 | 226.3 | 41.9 |
| Columns (age class) | 6 | 548.2 | 91.4 | 16.9 |
| Interaction | 30 | 393.9 | 13.1 | 2.4 |
| Residual | 294 | 1,586.0 | 5.4 | |
| Total | 335 | 3,659.7 | | |

DF = Degrees of freedom; SS = Sums of squares; MS = Means of squares; VR = Variance ratio

[a] Significant at $P = 0.01$

**Table 4.** Overall mean number of *Fasciola hepatica* sporocysts found in *Lymnaea palustris* of known ages

| Age class at exposure (days old) | 0–3 | 3–6 | 6–9 | 9–12 | 12–15 | 15–18 | 18–21 |
|---|---|---|---|---|---|---|---|
| Mean number of sporocysts per snail | 5.5 | 3.8 | 3.1 | 2.1 | 2.0 | 2.4 | 1.5 |

on the number of sporocysts found. Additionally, if the effect of length of infection is ignored, the age class at exposure also exerts a significant ($P = 0.01$) effect on the number of sporocysts.

*Student-Newman-Keuls Test.* This analysis allows an *a posteriori* comparison of the overall mean number of sporocysts in each age class (Table 4), using a constant sample size and indicates that at the 5% probability level, there is no significant difference between the mean number of sporocysts found at 3–6 d.o. and in age classes up to 18–21 d.o. A significant ($P = 0.01$) difference, however, does exist between snails exposed at 0–3 d.o. and 3–6 d.o., suggesting that the major development of the host defence mechanism occurs between 0 and 6 days post hatching.

## Discussion

The histological investigation described in this paper indicates that although juvenile *L. palustris* are susceptible to invasion by *F. hepatica* miracidia, the host-parasite relationship is generally incompatible and the infection is halted by a cellular encapsulating response against the sporocyst. During encapsulation the sporocyst is killed and subsequently removed completely from the host's tissues. Quantitative examination of the data obtained during the investigation indicates that the host defence mechanism increases in efficiency with age, and that the development is at its most rapid in the first 6 days after hatching.

Only one cell type appeared to be involved in encapsulation, the process of which could be divided into two stages: (1) the formation of the capsule and degeneration of the sporocyst and (2) the complete removal of the parasite from the host's tissue and the dispersal of the capsule. Encapsulation in two stages similar to that described in the present study has been observed by Cheng and Garrabrant (1977) in incompatible relationships between certain strains of *B. glabrata* and *S. mansoni.*

The moment of infiltration of host reaction cells into the sporocyst brood chamber is often difficult to ascertain at the light microscopical level. The present study indicates that such infiltration probably does not occur until after the degeneration of the sporocyst. However, Loverde (1979), in a study of the reactions of snails against schistosomes, found that once the parasite tegument is breached then filopodia of host reaction cells are able to enter the sporocyst brood chamber and in turn encapsulate smaller fragments of the parasite until the parasite is destroyed.

Tissue responses, involving capsule formation by flattened host cells, have been observed by other authors in various incompatible host-parasite relationships, for example in *B. glabrata* against *S. mansoni* sporocysts (Newton 1952; Cheng and Garrabrant 1977), in *Stagnicola emarginata augulata* against *Trichobilharzia acline* sporocysts (Sudds 1960) and in *B. glabrata* against *E. lindoense* sporocysts (Lie and Heyneman 1976). However, not only parasites in incompatible host-parasite relationships evoke the host response, often larval stages in a compatible relationship with their host, resulting in the production of viable cercariae, may evoke a host response. A thin layer of amoebocytes was found to surround sporocysts of compatible strains of *S. mansoni* in *B. glabrata* by Carter and Bogitsh (1975) and in *Biomphalaria pfeifferi* by Meuleman et al. (1980), with no apparent invasion of the parasites integrity. The sporocyst is not the only larval stage to evoke a response; Pan (1965) found that although mother sporocysts of *S. mansoni* in a certain strain of *Biomphalaria glabrata* elicited only a slight response and were not damaged by the capsule, approximately 2 weeks after the emergence of the first cercariae from the daughter sporocysts, a massive tissue response was evoked. This response included hyperactivity in the cellular elements of the connective tissue and amoebocytic capsules forming around both live and dead cercariae trapped in the connective tissue. Daughter sporocysts, previously unaffected by the host response, became encapsulated and died. A general tissue response involving an increase in the number of amoebocytes in the tissues was observed by Rondelaud and Barthe (1980) in *L. truncatula* one to 7 weeks after infection with *F. hepatica.*

Although the events leading to the death and removal of *F. hepatica* sporocysts in *L. palustris* have been elucidated at the light microscope level in the present study, it is not certain how the sporocyst is being killed. One explanation is that lysis of the constituent layers of the sporocysts occurs through the release of enzymes released from the host reaction cells. Increases in the levels of acid phosphatase activity in cells encapsulating *S. mansoni* sporocysts (Cheng and Garrabrant 1977) and *Angiostronglyus cantonensis* (Harris and Cheng 1975) in *B. glabrata* have been reported. In the case of *S. mansoni,* the sporocysts were killed by the encapsulation response and Cheng and Garrabrant (1977)

believe that this could be caused by the release of enzymes, such as acid phosphatase, from the encapsulating cells, but the validity of this hypothesis is yet to be tested. Certainly once the tegument has been lysed the way is then open for filopodia of the host reaction cells to ramify into the brood chamber contents to continue the process of destruction in a manner similar to that described by Loverde (1979). The possibility that the capsule kills the sporocyst by depriving the parasite of nutrient is unlikely in this study, as the capsule is rarely consistent in thickness and while some areas may be highly encapsulated, other areas of the same parasite may remain reaction free.

Quantitative examination of the data obtained in the present study indicates that in each age class of snail the mean number of sporocysts falls with increasing length of infection, and in general this drop coincides with the presence of encapsulating and dying sporocysts in the host tissues. Consideration of the overall mean number of sporocysts found in each age class indicates that the major development of the defence mechanism occurs during the first 6 days after hatching with no significant improvement after this within the experimental parameters. It is interesting to note that Kendall (1950) and Boray (1966) were unable to obtain successful infections in snails over 7 d.o., which, according to our results is approximately the same age at which the defence mechanism reaches full efficiency. However, unlike the findings of Kendall (1950) and Boray (1966), the results of the present study indicate that it is unlikely that any infection will survive, regardless of the age of snail at exposure. This difference could be due to the use of different strains of host and parasite compared with those used in the present study and/or the level of infection administered to each snail. Both Kendall (1950) and Boray (1966) exposed the snails to at least twice as many miracidia as used in this study, and little is known of the effects on the host response of *L. palustris* against higher levels of infection of *F. hepatica.*

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