

## *Fasciola hepatica*: cercarial productivity of redial generations in long-surviving *Galba truncatula*

M. Belfaiza<sup>1</sup>, D. Rondelaud<sup>2\*</sup>, M. Moncef<sup>1</sup> and G. Dreyfuss<sup>2</sup>

<sup>1</sup>Laboratoire d'Hydrobiologie, Département de Biologie, Faculté des Sciences, BP 20, 24000 El Jadida, Morocco: <sup>2</sup>UPRES EA no. 3174, Facultés de Pharmacie et de Médecine, 2 rue du Docteur Raymond Marcland, 87025 Limoges, France

### Abstract

Bimiracidial infections of *Galba truncatula* with *Fasciola hepatica* were carried out to determine the effect of food quality on the frequency of 1- and 2-sporocyst infections, to analyse its impact on the developmental patterns (normal, or abnormal) of redial generations, and to verify its consequences on cercarial production. These investigations were performed in snails reared at 20°C and provided with cos lettuce and commercial fish food (Tetraphyll<sup>®</sup>) as a food source until their death. Double-sporocyst infections with normal development of redial generations were recorded in 43.9% of infected snails (out of 296). Single-sporocyst infections were noted in the other snails, with normal development of generations in 53.7% and abnormal development (the first mother redia early degenerated) in 2.4%. Four successive redial generations were found in long-surviving snails (more than 90 days). In both 1- and 2-sporocyst infections, showing normal development of generations, the daughter rediae, which exited from the first mother redia (R2a rediae), constituted the greater group of free rediae and produced the highest percentages of cercariae (46.2–48.2%). However, the development of these rediae inside the snail body was slower in 2-sporocyst infections than in 1-sporocyst infections. The numbers of rediae noted in subsequent generations (R2b/R3a and R3b/R4a rediae) were similar, whatever the number of full-grown sporocysts. The number of shed cercariae recorded in the 1- and 2-sporocyst infections did not significantly differ. When long-surviving snails died, 19.8–20.7% of cercariae produced by free rediae (essentially by R2b/R3a and R3b/R4a rediae) were still present in their bodies. The increased frequency of 2-sporocyst infections demonstrated that food quality had a significant effect on the redial burden of *F. hepatica* developing inside *G. truncatula*.

### Introduction

In single-miracidium infections of *Galba truncatula* with *Fasciola hepatica*, at least three redial generations normally develop within the snail and the quantity of rediae produced depends on the behaviour of the first mother

redia (R1a redia) after its exit from the sporocyst. If this redia remains alive throughout snail infection, normal development of redial generations (fig. 1) occurs (Augot *et al.*, 1998). By contrast, if this mother redia dies during weeks 2 or 3 of snail infection, the development of generations is abnormal (fig. 1) and results in a lower redial burden (Augot *et al.*, 1999). Both developmental patterns of infection have an effect upon cercarial productivity from these rediae. On day 63 post-exposure (p.e.) and at 20°C, the majority of cercariae are produced

\*Author for correspondence  
Fax: 33 555 435893  
E-mail: rondelaud@pharma.unilim.fr

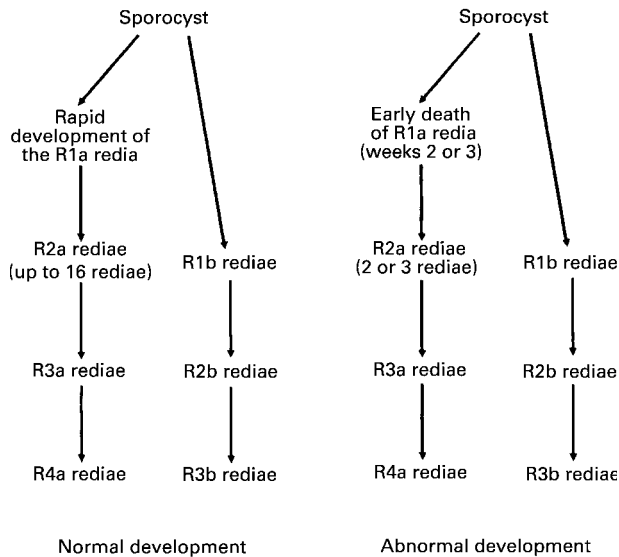


Fig. 1. Two developmental patterns of redial generations in the case of single-sporocyst infections of *Fasciola hepatica* in *Galba truncatula*. See fig. 2 for the chronology in the succession of generations. The normal development of rediae in the case of 2-sporocyst infections is similar to that shown for 1-sporocyst infections.

by daughter rediae originating from the R1a rediae when the redial development is normal, whereas cercariae are formed by other mother rediae in a case of abnormal development (Augot & Rondelaud, 2001).

In bimiracidial infections of *G. truncatula*, originating from the department of Haute Vienne (central France), single-sporocyst infections of *F. hepatica* developed in 77.8% of infected snails, with normal development of redial generations in 72.2% and abnormal development in 5.6%. Infections derived from the development of both sporocysts and a normal pattern of redial generations were observed in the other 22.2% (Vignoles *et al.*, 2003). However, one particular issue raised by these first findings was the life span of these experimentally-infected *G. truncatula*, which did not exceed 90 days (at 20°C) for the highest survival values occurring when lettuce only was provided as food for snails (Abrous *et al.*, 1998). Under these conditions, it is difficult to determine the precise role of the third and subsequent redial generations in cercarial production. As high survival (up to 130 days at 20°C) was noted for cercariae-shedding snails fed on a mixture of lettuce and commercial fish food (Rondelaud *et al.*, 2003), it is of interest to study cercarial productivity of redial generations in these long-surviving snails by addressing the following three questions: (i) may better food increase the frequency of 2-sporocyst infections due to the higher likelihood of snail survival; (ii) how may food quality influence the developmental patterns of redial generations; and (iii) what are the consequences of the 1- and 2-sporocyst infections on the production of *F. hepatica* cercariae? In an attempt to answer these questions, snails infected with *F. hepatica* were observed until their death to follow up cercarial shedding over time and compare shedding with redial burdens in snail cadavers.

## Materials and methods

The population of *G. truncatula* originated from a ditch along the D 128 road, at La Châtre, commune of Saint Jouvent, department of Haute Vienne in central France. A total of 327 snails, measuring 4 mm in height and belonging to the spring generation, were collected from this site from March to June 2002. Eggs of *F. hepatica*, which originated from heavily infected cattle, were washed several times with spring water and incubated at 20°C for 20 days in the dark (Ollerenshaw, 1971).

Each snail was subjected to a routine bimiracidial exposure for 4 h and individually reared in a 35-mm diameter Petri dish, with 2–3 ml of spring water. A mixed diet was given as food for snails and this comprised: (i) leaves of cos lettuce, originating from a private garden, used after soaking for five days in stagnant spring water, and provided *ad libitum*; and (ii) commercial fish food: Tetraphyll® (Tetrawerke, Melle, Germany) provided twice per week on the basis of 0.20–0.25 cm<sup>2</sup> of Tetraphyll® flake per snail for 10 h (Rondelaud *et al.*, 2003). The water was changed daily. These dishes were placed in an air-conditioned room at 20°C, with a diurnal photophase of 3000–4000 lux light intensity.

Every day, metacercariae present in the dishes were counted and removed until snail death. A routine post-mortem dissection of snail cadavers was then performed to determine the number of R1a rediae (1 or 2) and verify if these R1a rediae had not died since the first weeks of infection (these larvae can be easily recognized by the complete degeneration of the redia body, with nuclear pycnosis: Rondelaud & Barthe, 1980). The remainder of the rediae, still containing intraredial germinal embryos, were counted and their redial category identified on the gross morphology of the pharynx and the age of infection (Augot *et al.*, 1998, 1999). They were classified into the following four categories: (i) second-appearing mother rediae from the sporocyst (R1b); (ii) daughter rediae from R1a (R2a); (iii) daughter rediae from R1b and grand-daughter rediae from R1a (R2b/R3a); and (iv) grand-daughter rediae from R1b and great-granddaughter rediae from R1a (R3b/R4a). As the pharynxes of R2b/R3a and R3b/R4a rediae did not permit separation of these categories, the identification of R3b/R4a rediae was only performed using the age of the snail infection and the presence of daughter rediae in the body of R2b/R3a rediae (after day 63 p.e.).

The first seven parameters of the characterization of snail infections were the frequency of infected snails for each developmental pattern of redial generations, the number of infected snails which died during the first, second, third, or fourth months of snail infection, the increase of shell growth during experiment, the length of time between exposure and the first cercarial shedding, the duration of shedding period, the total number of cercariae shed by each infected snail, and that of cercariae which stayed in the snail haemocoel. Two other parameters were the number of free rediae containing germinal embryos, and the total number of these intraredial germinal embryos (morulae, pro-cercarial embryos, pro-cercariae, and cercariae), whatever their type, and these last parameters were only determined in the R1b, R2a, R2b/R3a, and R3b/R4a categories, as the

R1a rediae only produced daughter rediae. A Chi-square test and a one-way analysis of variance (Stat-Itcf, 1988) were used to establish levels of significance.

A further two parameters were the cercarial productivity in each redial group (Augot & Rondelaud, 2001) and the number of cercariae which remained in the bodies of snails at death. They were only determined in a total of 21 snails which died between days 102 and 118 of the experiment. Cercarial productivity was determined by calculating, for each redial category, the following ratio: (highest mean number of germinal embryos in young rediae) – [(number of germinal embryos remaining in rediae at snail death) + (number of morulae which differentiated into daughter rediae)]. Multiplication of this productivity by the mean number of living and free rediae in each category gave the total number of cercariae this redial category might produce. The final parameter was calculated by the difference between the total number of cercariae produced by all rediae and that of cercariae which exited from the snail.

## Results

### *Principal characteristics of snail infections*

A total of 296 snails harboured larval forms of *F. hepatica* at their death and 271 snails had shed cercariae. Table 1 gives their distribution in relation to the three developmental patterns of redial generations. A total of 159 *G. truncatula* (53.7%) showed single-sporocyst infections and normal development of redial generations (with a single live R1a redia throughout snail infection), while both sporocysts and, consequently, two live R1a rediae developed in each of another 130 snails (43.9%). The remaining seven snails (2.3%) showed 1-sporocyst infections and abnormal development of redial generations (with a degenerated R1a redia per snail) and this was significantly lower ( $P < 0.01$ ) than the percentages recorded in the other two snail groups. A significant difference ( $P < 0.05$ ) between the frequency of 1- and 2-sporocyst infections (with normal development of rediae) was also noted. Of these 296 snails, 45 had died before day 31 p.e., 114 between days 31 and 60 p.e., 93 between days

61 and 90 p.e., and the remaining 44 between days 91 and 127. The length of time between exposure and the first cercarial shedding, the duration of cercarial shedding, the total number of cercariae shed by each infected snail, and that of cercariae which stayed in the snail haemocoel are also indicated in table 1 for the three groups of snails. No significant differences between these mean values were noted, whatever parameter was considered.

The growth of infected snails was determined only for long-surviving snails (their death occurred after day 90 p.e.). Under these conditions, the mean increases in shell height ranged from 3.8 to 4.1 mm and no significant difference was noted (data not shown).

### *Redial burden in snails following mortality*

The count of free rediae containing germinal embryos and that of intraredial contents were only performed in the case of normal development of redial generations in snails. Rediae resulting from abnormal development were not considered in the present study due to the low number of snails (7 only).

Figure 2 shows the results found in the single-sporocyst infections of *F. hepatica*. The highest mean number of R2a rediae was greater than those recorded for R1b, R2b/R3a, and R3b/R4a rediae. Compared to R1b rediae, the decrease in the numbers of R2a and R2b/R3a rediae was longer in time (8 and 7 weeks, respectively, instead of 6 weeks). Conversely, the development of R3b/R4a rediae was slower (5 weeks instead of 4 for R2b/R3a rediae, for example). These findings were also noted in the dynamics of intraredial contents, as the exit of daughter rediae and cercariae from mother rediae occurred from weeks 4 to 11 in the case of R1b rediae, and from weeks 4 to 14 in the case of R2a rediae. In the other two redial categories, this exit was later, i.e. from weeks 8 to 14 for R2b/R3a rediae, and from week 13 for R3b/R4a rediae.

The results recorded in the 2-sporocyst infections are shown in fig. 3. In these *G. truncatula*, the development of the redial burden was slower than that in 1-sporocyst infections, as the highest mean number of R1b and R2a rediae, for example, was found on weeks 8 and 9, respectively (instead of weeks 6 and 7: see fig. 2a). The

Table 1. Distribution of *Galba truncatula* infected with *Fasciola hepatica* relative to the developmental patterns of redial generations and the characteristics of snail infections.

Parameters	1 sporocyst, normal development	1 sporocyst, abnormal development*	2 sporocysts, normal development
No. of snails (%)	159 (53.7)	7 (2.4)	130 (43.9)
No. of infected snails died:			
- before day 31 p.e.	25	1	19
- between days 31 and 60	64	1	49
- between days 61 and 90	44	3	46
- after day 90	26	2	16
Length of time (days) between exposure and first cercarial shedding†	42.7 ± 5.6	41.3 ± 8.2	43.2 ± 6.0
Shedding period† (days)	23.4 ± 18.3	21.4 ± 13.9	25.5 ± 17.3
Shed cercariae†	355.2 ± 124.8	214.3 ± 135.3	395.2 ± 173.9
Cercariae in the snail haemocoel†	24.3 ± 17.6	14.7 ± 11.2	27.6 ± 19.3

\* R1a rediae were degenerated after weeks 2 or 3 of snail infection.

† Mean values and SD originated from cercariae-shedding snails.

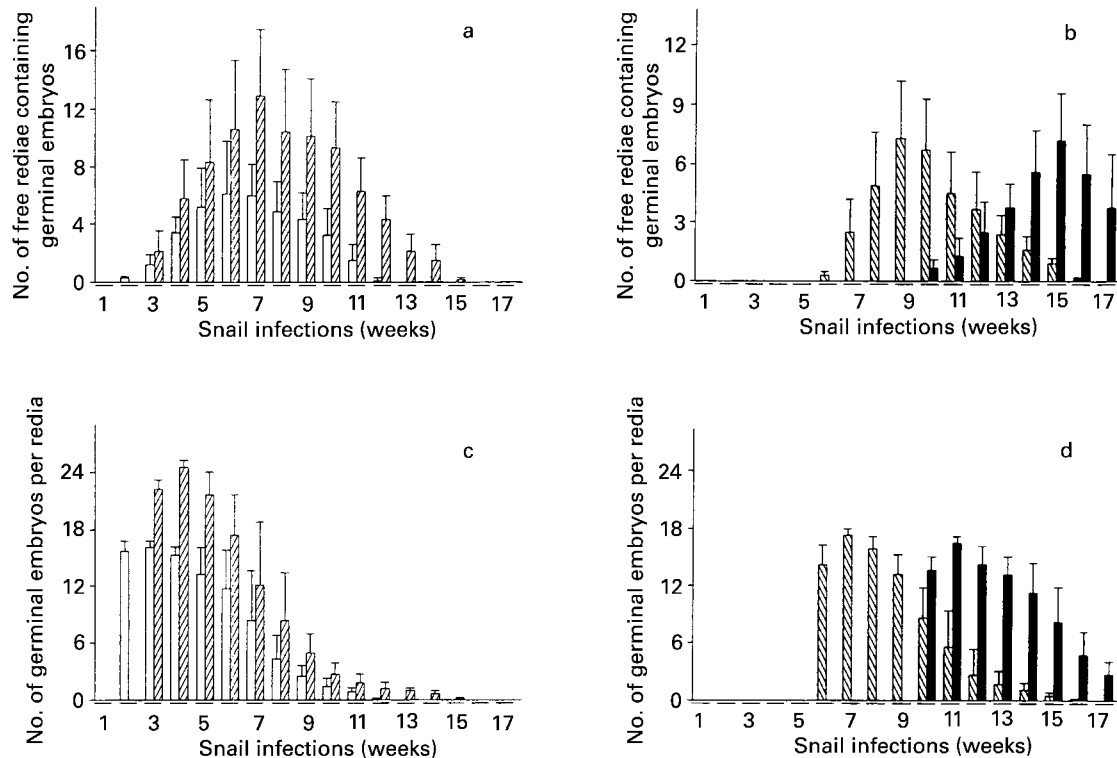


Fig. 2. Mean values and SD for the redial burden of *Fasciola hepatica* and its contents in *Galba truncatula* dissected just after snail death: the number of free rediae containing germinal embryos (a, b) and the number of intraredial germinal embryos per free redia (c, d) in relation to the R1b (□), R2a (▨), R2b/R3a (▩), or R3b/R4a (■) redial groups. The results originated from snails with 1-sporocyst infections and normal development of redial generations.

number of free rediae found in each category was also higher in the case of 2-sporocyst infections and this finding is particularly marked in the case of R2a rediae (a mean of 20.2 at week 9, for example, instead of 12.1 rediae at week 7: see fig. 2a). Despite this increase in redial burden in the 2-sporocyst infections, the highest number of intraredial germinal embryos recorded in R1b rediae did not significantly differ, whatever the number of sporocysts. Similar findings could also be noted for the other three redial categories (R2a, R2b/R3a, and R3b/R4a).

#### *Cercarial productivity of rediae and cercarial shedding in long-surviving snails*

Table 2 gives the results found in a total of 21 long-surviving *G. truncatula*, characterized by 1- or 2-sporocyst infections and a normal developmental pattern of redial generations. The highest percentages of cercariae (46.2–48.2%) were produced by R2a rediae and there was no significant difference. Despite some variation in percentages found for R1b rediae, differences between the values recorded in the 1-sporocyst and 2-sporocyst infections were not significant. Each of the other two redial categories (R2b/R3a, and R3b/R4a) produced less than 20% of cercariae and no significant differences between these percentages were found, whatever the mode of comparison. If the number of cercariae produced

by the four redial categories in each snail are added, a total of 629.4 and 1105.1 cercariae was noted in the case of 1-sporocyst and 2-sporocyst infections per *G. truncatula*, respectively. A comparison of these figures with the number of cercariae which were shed from infected snails demonstrated that 19.8–20.7% of cercariae produced by free rediae (essentially by R2b/R3a and R3b/R4a rediae) were still present in the bodies of snails at their death.

All cercariae found in these long-surviving snails still had their tail and no internal metacercariae were found.

#### Discussion

In *G. truncatula* infected with cattle-derived miracidia and reared with a mixed diet (cos lettuce + Tetraphyll®) as food, normal development of redial generations was found in the case of single- (53.7% of snails) or 2-sporocyst (43.9%) infections, while abnormal development was only noted in the remaining 2.3%. These values disagreed with those found in snails infected with the same isolate of miracidia and reared with cos lettuce only, as the corresponding percentages of snail infections were 72.2%, 5.5% and 22.2%, respectively, for the *G. truncatula* originating from Berneuil (department of Haute Vienne, central France), or 67.8%, 10.7% and 21.4% for the snails from Migné (department of Indre) (Vignoles *et al.*, 2003). As the frequency of 2-sporocyst infections noted in the present study was significantly ( $P < 0.05$ ) greater than

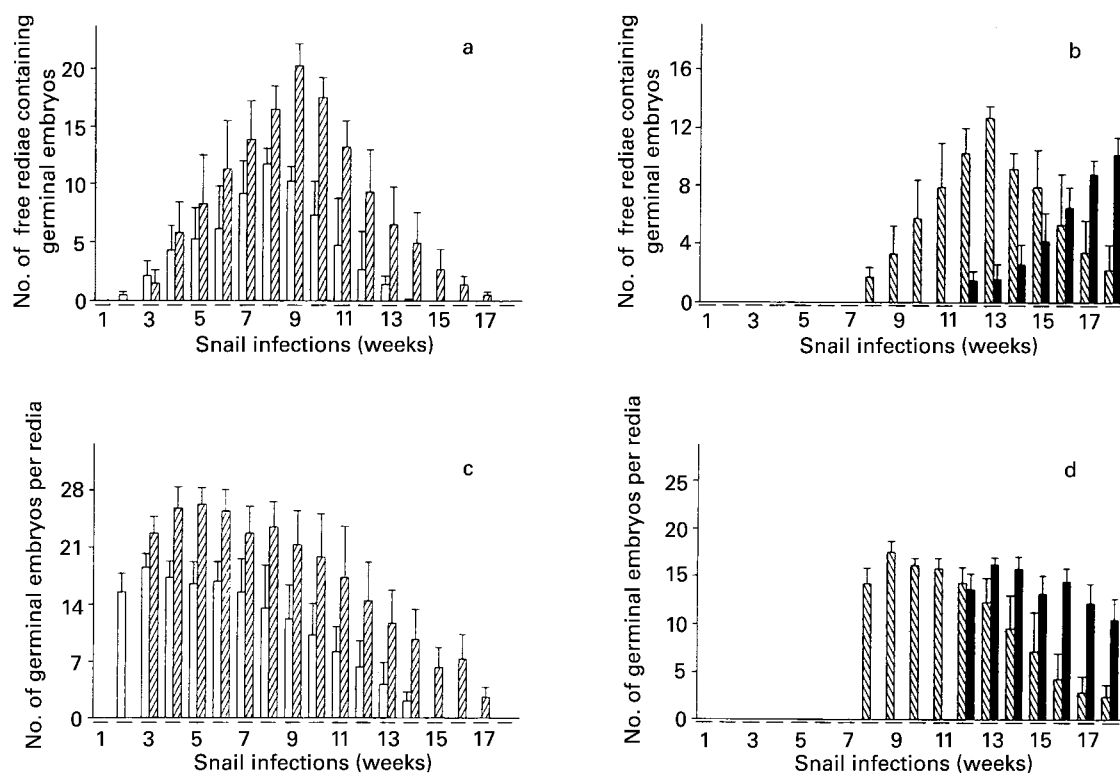


Fig. 3. Mean values and SD for the redial burden of *Fasciola hepatica* and its contents in *Galba truncatula* dissected just after snail death: the number of free rediae still containing germinal embryos (a, b) and the number of intraredial germinal embryos per free redia (c, d) in relation to the R1b (□), R2a (▨), R2b/R3a (▩), or R3b/R4a (■) redial groups. The results originated from snails with 2-sporocyst infections and normal development of redial generations.

those found in snails reared with only lettuce as food source, this finding can only be explained by the more rapid growth of infected snails when they fed on the aforementioned mixed diet (Rondelaud *et al.*, 2003). Such rapid growth would permit the simultaneous

development of both sporocysts in the snail and, consequently, would limit the frequency of degenerated R1a rediae in the first weeks of infection. An argument in support of this hypothesis was strengthened by the higher production of *F. hepatica* cercariae noted in the

Table 2. Cercarial productivity of free rediae and cercarial shedding of *Fasciola hepatica* in *Galba truncatula* in relation to the number of full-grown sporocysts per snail and redial category. These figures originated from long-surviving snails (which died between days 102 and 118).

No. of sporocysts (no. of snails)	Redial categories*	No. of rediae per snail† (no. of germinal embryos per redia which differentiated into cercariae)‡	No. of cercariae produced by living rediae (%)	No. of shed cercariae: mean ± SD	No. of cercariae in the snail at death (%)
1 (11)	R1b	6.1 (15.1)	92.1 (14.6)	504.3 ± 121.9	125.1 (19.8)
	R2a	12.9 (23.5)	303.1 (48.2)		
	R2b/R3a	7.3 (16.8)	122.6 (19.5)		
	R3b/R4a	7.2 (15.5)	111.6 (17.7)§		
2 (10)	R1b	11.7 (21.7)	253.8 (23.0)	876.2 ± 279.5	228.9 (20.7)
	R2a	20.2 (25.3)	511.0 (46.2)		
	R2b/R3a	12.7 (15.4)	195.5 (17.7)		
	R3b/R4a	10.2 (14.2)	144.8 (13.1)§		

\* R1a rediae produced only daughter rediae.

† The highest mean number of rediae recorded in snail cadavers.

‡ Calculation: (highest mean number of germinal embryos in young rediae) – [(number of germinal embryos remaining in rediae at snail death) + (number of morulae which differentiated into daughter rediae)].

§ Total number of cercariae produced by free rediae: 629.4 (1-sporocyst infections) and 1105.1 (2-sporocyst infections).



present study (a mean of 876.2 cercariae instead of 504.3 in 1-sporocyst infections, see table 2), or by that reported by Rondelaud *et al.* (2003) in the case of snails reared with a mixed diet (a mean of 358.9 cercariae instead of 112.4 per snail for lettuce only-reared snails).

In long-surviving snails, there was a succession of four redial generations in the course of snail infections. As little information on the third and fourth redial generations of *F. hepatica* in the snail was available in the literature, it is interesting to note that the number of R3b/R4a rediae was similar to that of R2b/R3a, whatever the number of sporocysts (1 or 2) in the snail. The slower development of redial burden in the case of 2-sporocyst-derived infections, particularly in the case of R2b/R3a and R2b/R4a rediae, was more difficult to interpret and two hypotheses are proposed to comment upon this finding. The first was to consider the volume of the snail body and, consequently, the quantity of glycogen and fat available which would only permit the simultaneous differentiation of a limited number of rediae during the same length of time. This assumption was supported by the fact that the size of the redial burden was related to that of infected snails (Rondelaud & Barthe, 1987). Another hypothesis was to envisage a production of free rediae, particularly in the case of R2a, R2b/R3a, and R3b/R4a categories, which would be higher than that noted in the present study (see figs 2 and 3) and would be more prolonged over time.

In long-surviving snails, the productivity of each redial category in terms of cercariae agreed with those reported by Augot & Rondelaud (2001) or by Vignoles *et al.* (2003). If the developmental pattern of redial generations was normal, the majority of cercariae were produced by R2a rediae. The percentages of cercariae formed by R3b/R4a rediae (less than 20%) pose the question of whether this production was useful, as 19.8–20.7% of cercariae (or germinal embryos differentiating into cercariae) remained in the bodies of snails at their death. As old rediae did not exhibit the formation of new morulae, the only valid explanation was that this cercarial production of R3b/R4a rediae corresponded to a normal continuation of parasite production in the course of snail infections and was thus not disturbed by the presence of old rediae belonging to previous generations. This fact suggested that the final phase of intraredial differentiation for cercariae, their glycogen accumulation in the snail haemocoel, and cercarial shedding would continue over time without any control by the intermediate host, at least if the snail was able to sustain parasite development.

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(Accepted 27 August 2003)

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