SEVERAL OBSERVATIONS CONCERNING CERCARIAL SHEDDINGS OF *FASCIOLA GIGANTICA* FROM *LYMNAEA NATALENSIS*

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Summary :
The shedding of cercariae from *Lymnaea natalensis* and their transformation into metacarcariae occurred mainly at night, and 70.7 % of the cysts were counted between 7 p.m. and 1 a.m. There is no infradian rhythm in the numerical distribution of the daily mean values as related to experiment duration or patent period duration. One quarter of the snails shed their cercariae in a single wave and the others in 2 to 13 waves. Floating cysts represent 35 % of the total number of metacercariae and their number decreased during the two first waves of shedding. Aberrant metacercarial localizations were noted on the inner surface of the shell when the snail died.

KEY WORDS : Cercaria. Cercarial shedding. *Fasciola gigantica*. *Lymnaea natalensis*. Metacercaria.
MOTS CLES : *Cercaire. Emission cercarienne.* Fasciola gigantica. Lymnaea natalensis. *Métacercaire.*

RÉSUMÉ : LES ÉMISSIONS CERCARIENNES DE *FASCIOLA GIGANTICA* CHEZ *LYMNAEA NATALENSIS*. A PROPOS DE QUELQUES OBSERVATIONS.

La production des cercaires par Lymnaea natalensis *et leur transformation en métacercaires sont paur la plupart nocturnes, avec 70,7 % des kystes dénombrés entre 19 heures et 1 heure. Il n'y a pas de rythme infradien dans la distribution numérique des moyennes journalières par rapport à la durée de l'expérience ou de la période patente. Un quart des limnées émettent leurs cercaires en une seule vague et les autres les produisent en 2 à 13 temps. Les kystes flottants représentent 35 % du nombre total des métacercaires et leur nombre diminue au cours des jours qui constituent les deux premières vagues d'émission. Des localisations aberrantes de métacercaires ont été notées à l'intérieur de la coquille lorsque le mollusque meurt.*

INTRODUCTION

There are two rhythms of cercarial shedding of *Fasciola hepatica* from *Lymnaea truncatula* : a circadian rhythm of parasite shedding during the night and an infradian rhythm with a periodicity of 7 days (Audousset *et al.*, 1989). The latter rhythm does not occur with cercarial sheddings from *F. gigantica* when the parasites are shed from *L. truncatula*, an abnormal intermediate host of this trematode (Rakotondravao and Rondelaud, 1991).

In view of these results, the question arises whether this infradian rhythm occurs with cercarial sheddings of *F. gigantica* from its natural intermediate host in Africa , *L. natalensis*. Cercariae of this trematode are already known for their shedding at night (Kwo *et al.*, 1970 ; Albaret *et al.*, 1980) ; however, there are no other data in the literature concerning the existence of another rhythm. The purpose of the present study was to answer this question by reporting results of an experimental infection of *L. natalensis* by *F. gigantica* under laboratory conditions.

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MATERIALS AND METHODS

SNAILS AND PARASITE

The colony of *L. natalensis* originated from natural watercress beds located at Ambanidia and at Vohisarika in the province of Tananarive (Madagascar). The adults were transported to France and placed in covered aquaria (at 23° C) where they laid eggs. These eggs gave rise to the 4-mm snails that were used in this experiment.

The *F. gigantica* eggs were regularly collected from heavily infected cattle gallbladders at the main slaughterhouse in Tananarive (Madagascar). They were filtered through several sieves, washed, and placed in total darkness for 20 days at 20° C.

EXPERIMENTAL PROTOCOL

The 483 snails were each exposed for 4 hours to 2 *F. gigantica* miracidia in a 35-mm diameter petri dish (Falcon) with 2-3 ml of water from the breeding container. Twenty-five snails that were not subjected to the parasites were used as controls. The snails were subsequently raised in covered closed-circuit aquaria, 5 snails per liter of water. The aquaria were placed in an air-conditioned room at 23° C. For 12 hours per day (7 a.m. to 7 p.m.), an electric light provided light of 3,000 lux intensity at the surface of the containers. The snails were fed fresh lettuce *ad libitum*.

On the 30th day of the experiment, the 348 survivors and 25 controls were isolated in 35-mm petri dishes

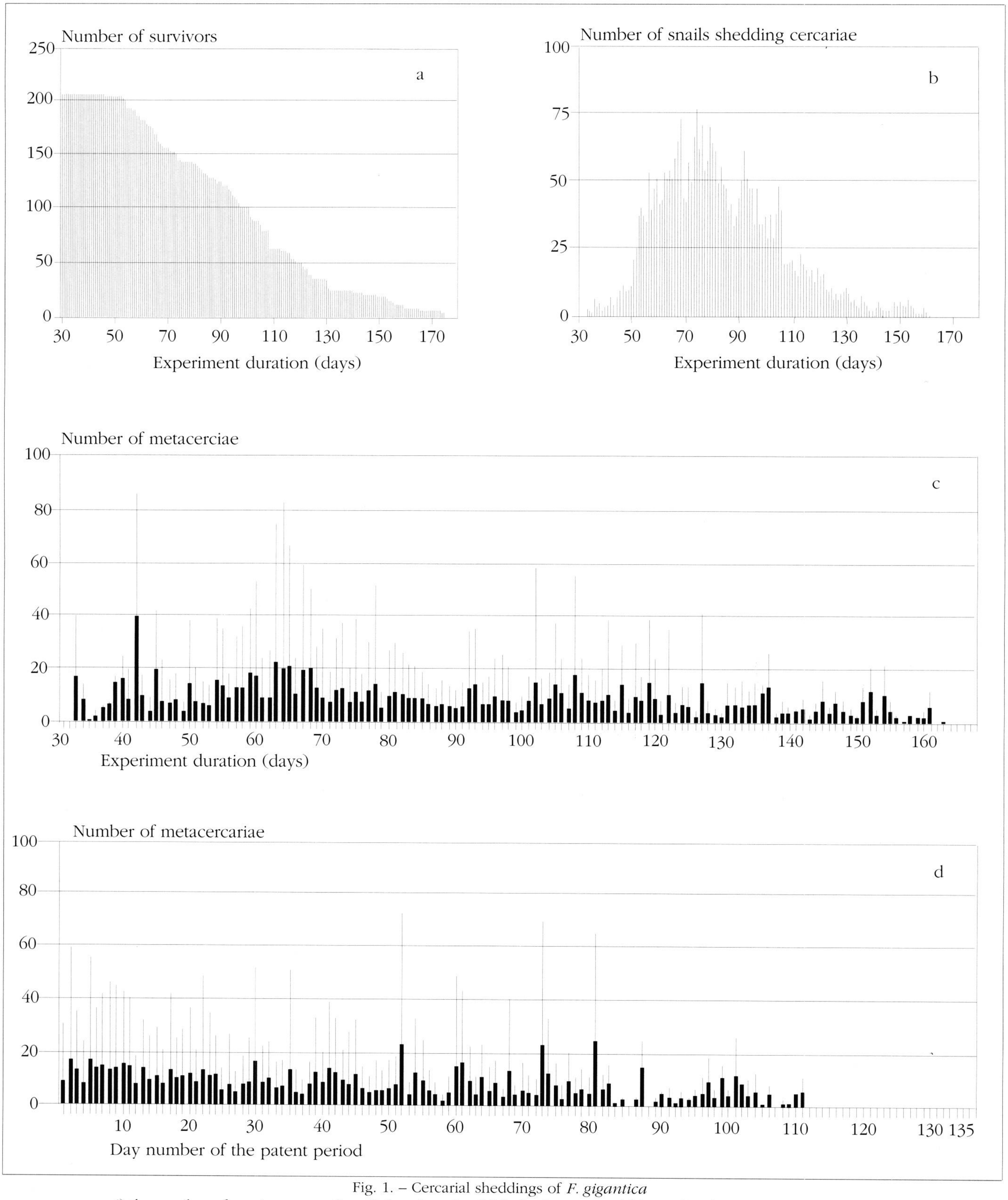

Fig. 1. – Cercarial sheddings of *F. gigantica*
i) the number of survivors over time (1a) and snails which shed cercariae (1b) as related to experiment duration ;
ii) daily distribution of cercariae from the beginning of the experiment (1c) and that of the patent period (1d).

with 2-3 ml of water and a piece of lettuce (0,5 cm^2) per recipient. The dishes were placed in the same air-conditioned room where the original colony was raised. Each day the snails were monitored from 2 p.m. to 4 p.m., during which time the water was changed and maintained at the same temperature, the lettuce replaced, and the fixed or floating metacercariae counted until snail death. At this time the shell height was measured.

A special count was performed every six hours (1 a.m., 7 a.m., 1 p.m., 7 p.m.) for 15 days to determine the time of maximal shedding.

Twenty floating cysts were immersed in Bouin's fixative for histological study. Serial sections, 5 µm thick, were obtained and stained using Harris' hematoxylin and modified Gabe's trichrome.

PROCESSING OF DATA

The values obtained from the total metacercarial counts were averaged and standard deviations determined for each observation date, specifying the colony utilized and the particular day of the patent period. The mean daily values were then subjected to the autocorrelation test (Broom, 1979) to determine whether there was periodicity in cercarial shedding.

A wave of cercarial shedding was defined as a period where at least 10 cercariae were shed over one or several successive days without interruption. The characteristics of these waves of shedding were studied by Anova (Stat-Itcf, 1988).

RESULTS

GENERAL CHARACTERISTICS OF INFECTION

206 snails shed cercariae and the other 142 snails died without emitting parasites. In snails that shed parasites, the life span was 98.9 ± 30.7 days ; in snails without cercarial shedding it was 66.7 ± 23.4 days, and 132.4 ± 31.5 days in controls. The post-mortem shell height was respectively 8.6 ± 1.5 mm, 8 ± 2 mm, and 12.4 ± 2.7 mm.

In snails that shed cercariae, the mean day of onset of the patent period was day 57 of the experiment, with individual values ranging from day 32 to day 105. The duration of the patent period was 41 ± 30.9 days, with extremes of 1 and 135 days. A total number of 35,456 cercariae was obtained of which 64.9 % of the cysts were fixed on substrate, 35 % were floating, and 0.059 % were found free on the floor of the recipient.

PERIODICITY OF SHEDDING

There were 206 survivors until day 46 of the experiment, after which the number of survivors gradually decreased until day 131. The numbers leveled off from day 131 to day 143 (at 22-24 snails) ; the last snails died on day 175 (Fig. 1a). Until day 45, there were no more than 10 snails that shed cercariae ; this number then increased to a maximum between days 68 and 79 (up to 77 snails on day 74). Subsequently, the number dropped below 10 snails per day from day 122 and ceased on day 165 when shedding stopped (Fig. 1b).

The distribution of cercariae throughout the experiment was irregular with daily peaks which did not exceed 40 parasites per snail with sometimes very wide standard deviations. Mean maximal values occured between days 62 and 67 of the experiment (18-23 parasites per snail per day) and subsequently decreased progressively until shedding stopped on day 162 (Fig. 1c).

Examination of the mean daily values in relation to the patent period (Fig. 1d) revealed that the highest mean values occured between day 2 and day 11 (with 9 to 17 cysts per snail per day) ; they subsequently decreased progressively until they ceased on day 112 of the patent period despite the survival of several snails until day 135.

Analysis of these mean daily values using the autocorrelation test did not demonstrate an infradian rhythm in the distribution of mean values as related to experiment duration or duration of the patent period (results not shown).

The majority of the cercariae were shed throughout the night (70.7 % during the first half, 7 p.m. to 1 a.m. ; 20.4 % during the second half). During the day the percentages were respectively 1 and 7.9 %.

WAVES OF SHEDDING

Twenty-five percent of *L. natalensis* shed their cercariae in a single wave. Most of these snails died a few days after the onset of the patent period, while the others died after longer periods (after day 115 and more).

Second, third, and fourth waves occurred respectively in 18.2 %, 16.5 % and 10.6 % of the snails. The remaining snails (29.4 %) shed their parasite in 5 to 13 waves (results not shown).

Mean wave duration was 2.7 to 4.9 days. The first interwave period was longer than subsequent ones : 4.6 to 10.9 days instead of 2.3 to 7.1 days (Fig. 2a). The number of cysts per wave did not exceed one hundred and often ranged between 50 and 70 metacercariae (Fig. 2b). Fluctuation of the mean values were observed according to the numerical wave order. Comparison of these mean values using Anova did not demonstrate any significant difference between the durations of waves, those of interwaves, as well as the cercariae production per wave.

The chronology of these waves was studied in snails that shed cercariae in 3 and 4 waves (Fig. 3).

Cercarial sheddings were irregular over time. The waves followed each other to one-day intervals or greater lengths of time.

Floating cysts

180 snails (87.3 %) shed cercariae which became floating cysts. These cysts represented 35 % of the total number of metacercariae ; however, there was substantial individual variation, and if only snails that shed 500 parasites or more are considered, the minimum and maximum percentages were respectively 4.5 % and 81 % (with 827 floating cysts out of a total of 1,014).

These cysts float due to the presence of an outer ring that is formed by the outer layer of the external wall of the cyst and contains air-filled lacunae which dissociate the fibrils of this layer. The three other layers are not involved in this ring formation. The total diameter was 346 ± 42.5 µm and the height was 128.4 ± 28.6 µm on the serial sections. The ring's radius was 60.3 ± 28.4 µm and its thickness was 16.3 ± 4.8 µm at the base of the cyst.

If the number of floating and fixed cysts that were collected during the first two waves of shedding are considered (Fig. 4), it was found, despite several divergent figures in the percentages over time, that the floating cysts became less and less numerous during the first wave : from 49.8 % on the first day to 7.1 % on the tenth (Fig. 4a). Inversely, the percentage of fixed cysts increased in a corresponding manner. There were similar findings for the second wave of shedding (Fig. 4b) : a similar decrease occurred, with only 12 % of floating cysts on the tenth day of the wave. Examination of the other waves also showed a numerical decrease in floating cysts, however, there was greater variation of the results beginning with the fourth wave of shedding (results not shown).

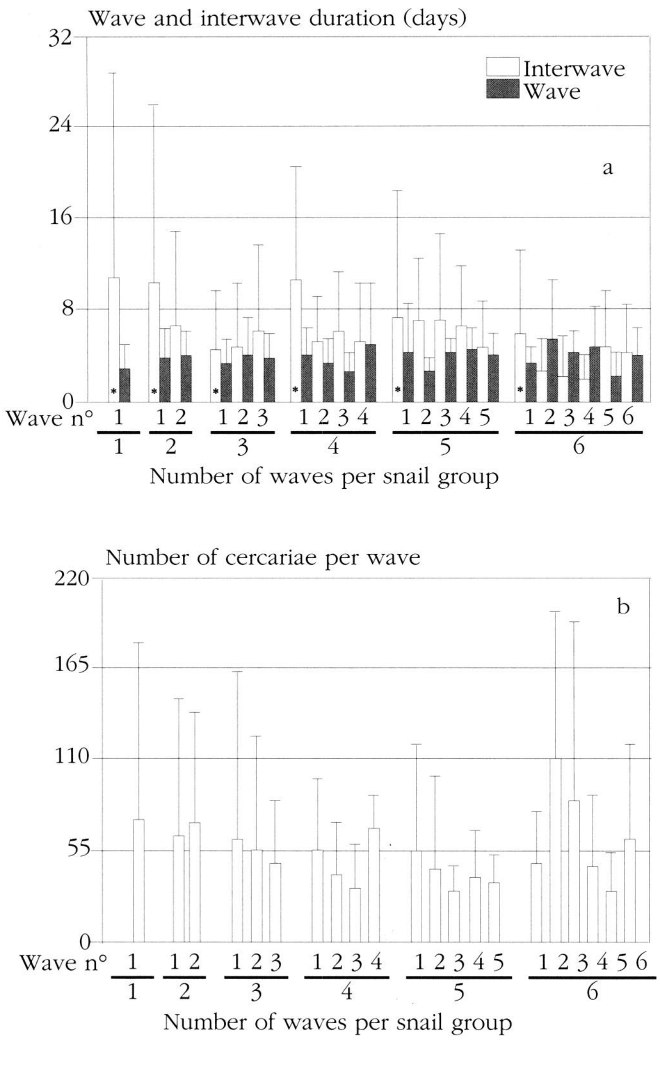

Fig. 2. – Characteristics of waves of shedding :
i) wave and interwave duration in numerical order (2a) ;
ii) the number of metacercariae per wave (2b). The first interwave (*) corresponds to the period from day 1 of the patent period to the onset of the first wave of shedding. The results are presented in relation with a) the wave number per snail group, and b) the numerical wave order.

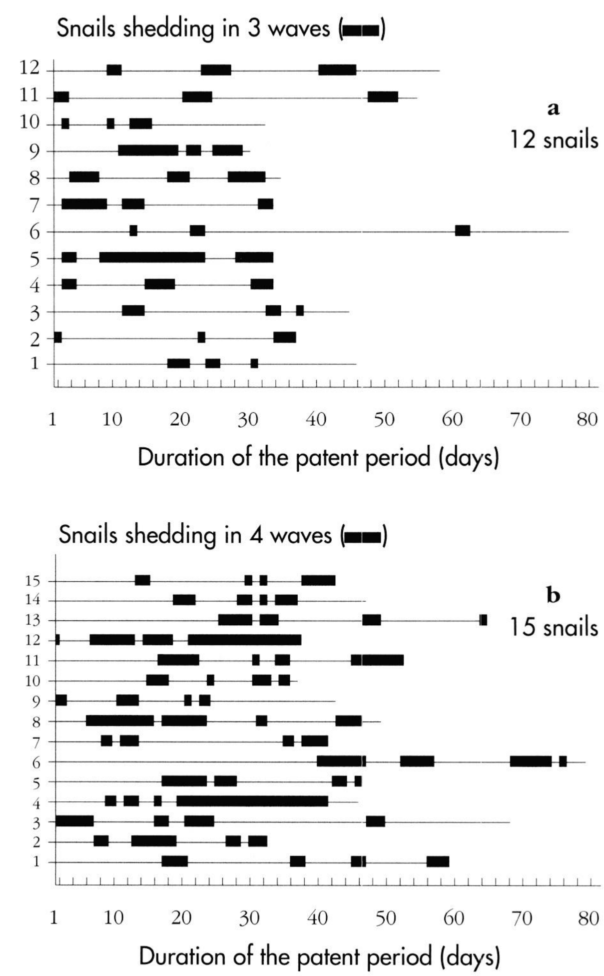

Fig. 3. – Distribution of the waves of shedding and their duration in snails which shed cercariae in 3 waves (3a) or 4 waves (4b).

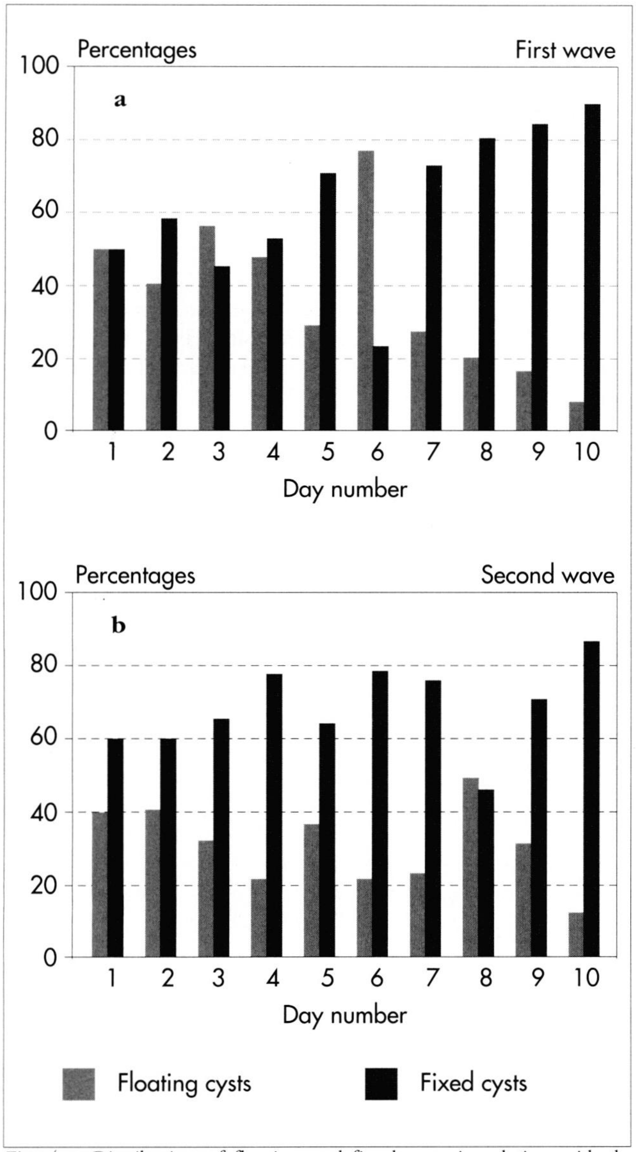

Fig. 4. – Distribution of floating and fixed cysts in relation with the shedding day for the first wave of shedding (4a) and the second (4b).

ABNORMAL LOCALIZATIONS OF CYSTS

With snail death, abnormalities in the formation and localization of the metacercariae were observed. Many fixed cysts were found on the inner surface of the shell near the peristome. Rarely, other metacercariae had implanted on the bottom of the petri dish, in the immediate vicinity of the dead snail. Occasionally, dead cercariae were found.

21 cysts were found on the bottom of the dishes. They did not adhere to the wall (or to faeces), and did not possess a ring.

DISCUSSION

The mean number of metacercariae obtained for each *L. natalensis* (172.1) corresponds to the range of values reported by Cheruiyot and Wamae (1990) with a Kenyan colony of the same snail when it was infected by *F. gigantica* (68.4 per snail infected in the laboratory, 103 to 385.5 when it is naturally infected). On the other hand, there was a discrepancy with our results concerning the number of floating cysts. Our percentage (35 %) corresponds with that reported by Rakotondravao and Rondelaud (1991) in *L. truncatula* that was infected by the same trematode (31.6 % floating cysts) ; however, it was definitively higher than the figures cited by Albaret *et al.* (1980), and Cheruiyot and Wamae (1990) who reported respectively 25 and 1.6 %. Other authors such as Alicata (1953) or Hammond (1974) did not comment on any wide variation. Two probably complementary hypotheses can explain this discrepancy. The first confirms the approximative percentage value obtained by Rakotondravao and Rondelaud (1991) and suggests that these experimental conditions are particularly favourable for the formation of floating cysts. The second emphasizes the individual variability between the snails employed or even colonies as demonstrated by the percentages obtained by Esclaire *et al.* (1989) in three colonies of *L. truncatula* infected by *F. hepatica* (1.4 to 8.2 % floating cysts).

The majority of sheddings occurs at night, with most parasites being shed between 7 p.m. and 1 a.m. This first result concords with the observations of Kwo *et al.* (1970), Albaret *et al.* (1980), and Salimov and Azimov (1983). On the other hand, the time of shedding differs from that reported by Rakotondravao and Rondelaud (1991), where the latter stated that shedding predominantly occurred around 1 or 2 a.m. This discordance can not be explained by experimental conditions since they were identical, neither by the malagasy strain of the trematode which was also identical. It is thus logical to seek another explanation. Two hypotheses have been provided by authors studying schistosomes and their snail hosts. The first hypothesis involved the role played by the species of pulmonate snail utilized, suggesting that the latter may control to a degree the shedding of its parasite (Asch, 1972 ; Anderson *et al.*, 1976). The second hypothesis states that shedding is only parasite dependent, at least under normal environmental conditions. A tremadote's cercariae retain their own rhythm of shedding, regardless of the compatible snail host that it parasitizes (Mouahid and Theron, 1986 : Mouahid *et al.*, 1991). Additional studies are therefore necessary to determine the role played by the snail in the shedding of its cercariae.

The absence of an infradian rhythm in daily sheddings differs from that described by Audousset *et al.* (1989) concerning shedding of *F. hepatica* from *L. truncatula.* This discrepancy must be interpreted cautiously. It is indeed possible to regard this as a specific result of our trematode. However, another working hypothesis can not be dismissed. *L. natalen-*

sis has, in fact, been recognized by Boray (1978) as a species that has a normal parasite-host relationship. It should be remembered, however, that *F. gigantica* has only recently been detected into Madagascar (Daynès, 1966), and that infection due to the parasite has spread over the entire island and has become a major problem to the livestock industry (Touratier, 1988). The question arises whether the absence of rhythm represents an, as yet, incomplete compatibility between *F. gigantica* and the snail populations, despite the present frequency of this epizootic disease. This hypothesis is supported by Rondelaud (1993) when he observed that the characteristics of *Fasciola* infection are modified in populations of *L. truncatula* when the frequency of natural encounters with *F. hepatica* is not the same.

The presence of many floating cysts at the beginning of each wave was a new finding. No hypothesis can be put forth to explain this finding especially since most of the experimental factors did not influence floating cyst formation (Vareille-Morel and Rondelaud, 1991). Throughout our study, no dead larvae were observed after leaving a live snail or during cyst formation. In view of these observations, the hypothesis put forth by the latter authors concerning lesions that arise in cercariae which form these cysts during passage through the perianal region of the snail does not appear to apply to the results obtained with *F. gigantica*.

The finding of cysts fixed to the inside of the shell when the snail died requires another explanation. The presence of dead cercariae at that moment and/or rare cysts in the immediate vicinity of the snail suggests that the larvae have "expended" all their energy migrating through the snail's tissues which partially retract at the time of death, and no longer have the strength to swim and attach to their usual substrate. The presence of many cercariae below the mantle wall at the time of the snail's death also supports this hypothesis.

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