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EVOLUTION OF TISSUE LESIONS IN *LYMNAEA TRUNCATULA* INFECTED BY *MUELLERIUS CAPILLARIS* AND BY *NEOSTRONGYLUS LINEARIS* *(NEMATODA: PROTOSTRONGYLIDAE)*

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SUMMARY

Juvenile and adult *Lymnaea truncatula,* 1 and 4 mm in height, were exposed to L1 of *Muellerius capillaris* and of *Neostrongylus linearis.* They were raised at 23° C until day 35 post-exposure and batches of five survivors were killed at periodic intervals for histological studies. Different lesions were observed in all the snails with living or abortive larvae. An inflammatory reaction developed in the foot and in the tentacles. Epithelial necrosis, often followed by reconstitution, occurred in the four studied organs (albumen gland, digestive gland, gonad, kidney). The frequency of the different lesion types varied in relation to the protostrongylid species and to snail size at the time of exposure. Principal components analysis showed *i*) the association of the presence of *N. linearis* larvae with lesions of the digestive glands, and *ii*) the association of the presence of *M. capillaris* larvae with foot lesions.

RÉSUMÉ : **Évolution des lésions viscérales chez *Lymnaea truncatula* infesté par *Muellerius capillaris* et *Neostrongylus linearis* (Nematodes, *Protostrongylidae*).**

Des Limnées tronquées de 1 et 4 mm de hauteur ont été exposées à des L1 de *Muellerius capillaris* et de *Neostrongylus linearis* avant d'être élevées à 23° C jusqu'au 35e jour postexposition. Des échantillons de cinq survivants par groupe ont été sacrifiés à intervalles réguliers pour des études histologiques. Différentes lésions se rencontrent chez tous les mollusques, que l'infestation soit évolutive ou abortive. Une réaction inflammatoire se développe dans le pied et les tentacules. Une nécrose épithéliale, souvent suivie par une reconstitution, touche les quatre organes étudiés (glande de l'albumine, glande digestive, gonade, rein). La fréquence des différents types de lésions présente des variations en fonction de l'espèce du protostrongle et de la taille du mollusque au moment de l'exposition. L'emploi de l'analyse en composantes principales montre : *i*) une association entre la présence des larves de *N. linearis* et les lésions de la glande digestive et *ii*) une association entre la présence des larves de *M. capillaris* et les lésions du pied.

INTRODUCTION

Several tissue lesions were described in adult *Lymnaea truncatula* infected by *Fasciola hepatica* (Rondelaud and Barthe, 1983). Epithelial necrosis developed in four viscerae: albumen gland, digestive gland, gonad and kidney; it was followed by epithelial reconstitution with cell hyperplasia. This pathology occurred, with some differences, in the juveniles of six other lymnaeid species exposed to fasciolid miracidia two hours post-hatching (Bouix-Busson *et al.,* 1985; Sindou *et al.,* 1990).

Amoebocyte proliferation was also noted in the hemolymphatic spaces of infected snails. Most cells originated from amoebocyte-producing tissue located in the hind part of the kindey (Rondelaud and Barthe, 1980, 1981).

However, the influence of another parasite species on the development of tissue lesions and on amoebocyte proliferation has not yet been tested in *L. truncatula.* A previous note reported the developmental rate of larvae of two protostrongylid species *(Muellerius capillaris, Neostrongylus linearis)* and the variation of their numbers in the foot of *L. truncatula* (Hourdin *et al.,* 1990). The aim of the present study was to record the evolution of tissue lesions related to the course of infection in the snail.

MATERIALS AND METHODS

1. Snails and parasites

Snails originated from a population living in a road ditch at Chezeau-Chrétien near Migné (Indre) and were assumed to be free of infection by protostrongylid larvae since snails collected in this

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habitat regularly had negative histological examinations. The animals were 1 or 4 mm high and formed part of the first annual generation. They were brought to the laboratory in isothermal conditions. Prior to infection, the molluscs were maintained 48 hours in standard breeding containers.

First-stage larvae (L1) were extracted from faeces of an experimentally infected goat *(M. capillaris)* or naturally infected sheep *(N. linearis)*.

2. Experimental groups

Three groups of 75 snails were constituted from the 1 mm individuals: the first was used as a control; the two others were exposed to *M. capillaris* or *N. linearis*. Three other groups were similarly formed for the 4 mm snails. Snails were infected according to the method of Cabaret and Dakkak (1979): each group was exposed to 100 L1 for 4 hours at 20° C.

After exposure, the snails were maintained in covered, closed-circuit aquaria for a maximum of 35 days with 10 individuals /dm^3 of water. The sediment consisted of gravel lying under an oxygenated eucalcic water sheet, 10 cm thick. The aquaria were kept at a constant temperature of 23° C with a balanced photoperiod (L-D 12-12). The snails were fed with lettuce *ad libitum*.

Batches of five snails were randomly collected among the surviving *L. truncatula* of the six groups for every date: 3.5, 7, 14, 21 and 28 days post-infection (p. i.). At day 35, five batches were each constituted of 5 snails from the controls, from the infected juvenile snails, and from the adults infected by *N. linearis*; the sixth batch included the two last survivors from the adults infected by *M. capillaris*. Snails were dipped in the fixative of Bouin and their shell was immediately broken under the stereomicroscope. Serial sections 5 μm thick were obtained and subsequently stained by Harris' haematoxylin-modified Gabe's trichrome.

3. Organs and morphological states

Previous studies on visceral pathology in *L. truncatula* infected by *F. hepatica* reported the presence of marked lesions in four internal organs: albumen land, digestive gland, gonad, and kidney (Rondelaud and Barthe, 1983). Our observations were carried out mostly in these four organs and we have recognized three morphological states in these organs: normal development, epithelial necrosis, and epithelial reconstitution with cell hyperplasia. The processes of necrosis and reconstitution have been previously described (Rondelaud and Barthe, 1983).

The foot and the tentacles of *L. truncatula* can be normal. An inflammatory reaction was often present with hyperactivity of the mucous glands, intraepithelial vacuolization, lysis of subepithelial areas, and the presence of tunnel-shaped lacunae.

The lesions in each organ were coded as follows: 0 (absence of lesion); 1, 2, 3 (epithelial necrosis or inflammatory reaction in increasing intensivy); 4 (reconstitution).

4. Processing of data

Principal components analysis was performed on reduced and centered data with a statistical package (Stat-Itcf, 1987). For the entire analysis, the data structure was as follows: the columns were the parameters (type of lesions on each organ or tissue) and the rows were the values obtained in snails (scores of lesions) arranged in increasing date p. i. The first analysis was performed on average scores (5 snails per period) of lesions for the 1 mm and the 4 mm *L. truncatula* infected with *M. capillaris* or *N. linearis*. The second analysis was done on individual values for the 4 mm snails infected with *M. capillaris:* the larvae recovered from the snails were included as supplementary variables. The last analysis was done similarly on *N. linearis*. The aim of the different analyses was respectively to: *i*) compare the chronology of the onset of lesions in 1 and 4 mm snails infected either with *M. capillaris* or *N. linearis*; *ii*) evaluate the timing of the relation between infection and lesions of the various organs in 4 mm high snails infected with each protostrongylid.

The interpretation (see Dagnelie, 1975) of each graphical representation is as follows:

i) the circle of correlation of variables in the right upper corner indicates which variables are important (near the circle) or not (near the center); each of the four quadrants (I to IV) determined by the two axes may be related to one or several variables and thus simplify the interpretation of the next graphical representation;

ii) the projection of individual values on the two axis plane. Mean or individual scores per period also have four quadrants I, II, III and IV. Each quadrant is oriented according to the findings in the circle of correlation of variables. For example, if scores of period 1 are in quadrant I and those of period 2 in quadrant III, it might be concluded that variables related to quadrant I are important in period 1 and those related to quadrant III in period 2.

RESULTS

The survival rate of controls at day 35 was 91 % in the 4 mm group and 89 % in the juvenile group, the fixed snails being excluded. Survival at day 35 p. i. was nil in adult snails infected with *M. capillaris* and 40 % in adult snails infected with *N. linearis,* the fixed individuals being excluded. It was 33.3 % and 20 % in juvenile snails infected with *M. capillaris* and *N. linearis* respectively.

There was 15 snails with an evolutive infection (with living *parthenitae*) in the *M. capillaris* 4 mm group. The number of these snails was lower in the other groups: 1-4 snails per group. All the other snails had an abortive infection.

All the larvae were found in the foot of the snails. The majority of living larvae (34 out of 45) were surrounded with granulomas; conversely, abortive larvae in granulomas were rare (2 cases out of 14). Abortive larvae were found in higher proportions in the *N. linearis* 4 mm group (9 out of 13 *versus* 5 out of 39 larvae in the *M. capillaris* 4 mm group).

1. Histological results

The controls did not show lesions in their organs. The foot and the tentacles had a normal structure (data not shown).

Tissue lesions were observed in each group, in molluscs with living *parthenitae* as well as in snails with abortive infection. Figures 1 and 2 indicate the schematic development of the morphological states in the organs of the four snail groups.

A. *Digestive gland and gonad* (fig. 1)

Epithelial necrosis of the digestive gland occurred in the *M. capillaris* 1 mm group from days 3.5 to 21 p. i.; the

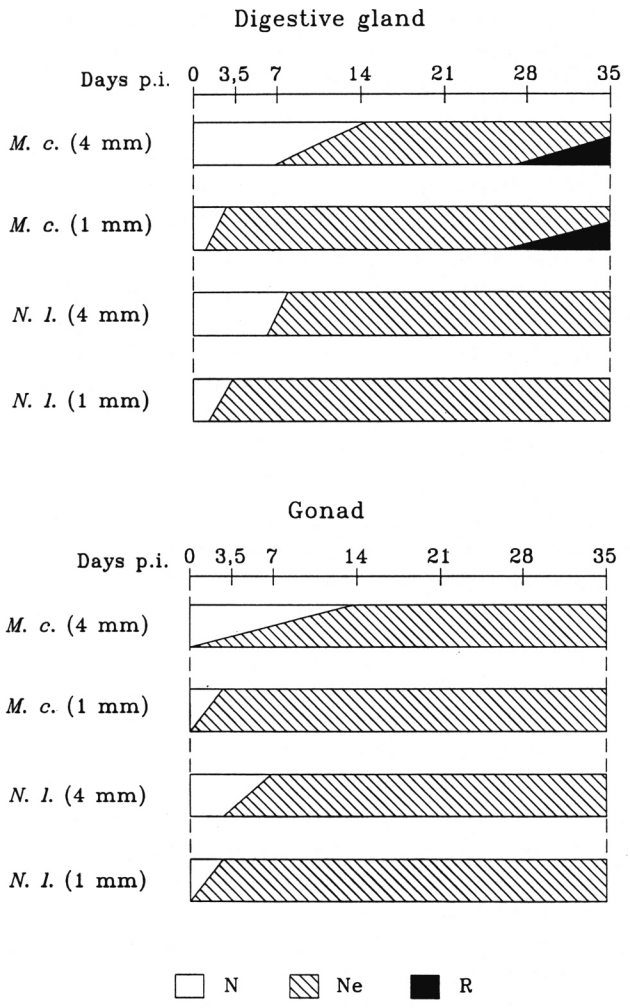

FIG. 1. — Schematic development of tissue lesions in the digestive gland (1*a*) and in the gonad (1*b*) of snail groups infected with *M. capillaris (M. c.)* or with *N. linearis (N. l.)*. Captions. N: normal organ structure; Ne: epithelial necrosis; R: reconstitution.

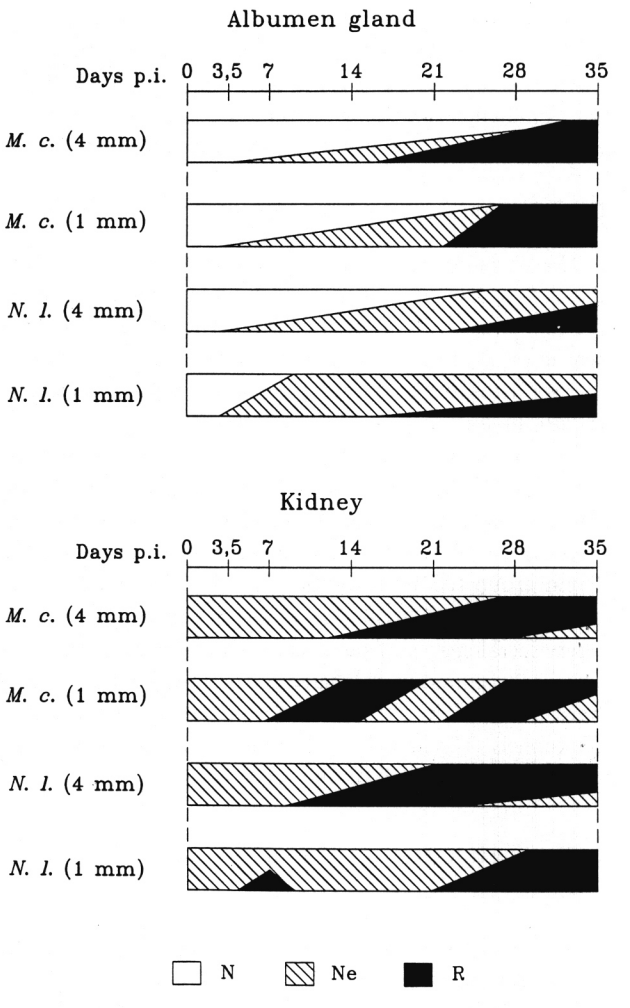

FIG. 2. — Schematic development of tissue lesions in the albumen gland (2*a*) and in the kidney (2*b*) of snail groups infected with *M. capillaris (M. c.)* or with *N. linearis (N. l.)*. Captions. N: normal organ structure; Ne: epithelial necrosis; R: reconstitution.

lesion appeared at day 3.5 in the 4 mm group and affected all glands on days 14 and 21 p. i. Afterwards, the frequency of the lesion decreased and reconstitution gradually developed in the glands.

Necrosis was the only lesion in the *M. capillaris* groups: it was multifocal and often entailed atrophy of the organ. The lesion affected all the 1 mm snails; it was more progressive in the 4 mm group and was observed in all the gonads after day 7 p. i.

Only multifocal necrosis of the digestive gland and of the gonad occurred in adult and juvenile snails in the *N. linearis* groups. The lesion was present in all glands of the 1 mm group; it occurred less frequently in the 4 mm group and was observed in the digestive gland after day 7 p. i., and in the gonad after day 3.5.

B. *Albumen gland and kidney* (fig. 2)

Necrosis appeared on day 3.5 p. i. in the albumen gland of the four groups and its frequency rose in later weeks up to the development of reconstitution (on days 21 or 28).

Two successive cycles of necrosis-reconstitution and the development of a third episode of necrosis were noted in the kidney of the *M. capillaris* 1 mm group: the first reconstitution was observed on days 14 and 21 p. i.; the second was seen on days 28 and 35. In the 4 mm group, there was a single cycle with gradual reconstitution from days 14 to 35 p. i., and the beginning of a second necrosis phase at day 35.

There were two successive cycles of necrosis-reconstitution in the kidney of the *N. linearis* 1 mm group: the first

reconstitution began at day 7 p. i.; the second developed at day 21 and affected all the snails on days 28 and 35. There was a single complete cycle in the 4 mm snail kidney with gradual reconstitution from day 7 p. i. to the end of experiment; another necrosis phase appeared at day 28.

C. *Other lesions (data not shown)*

The inflammatory reaction appeared in the foot at day 3.5 p. i. and was observed within both goups of *M. capillaris* snails after this date. This lesions was present in the foot of the *N. linearis* 4 mm snails from days 3.5 to 35 p. i.; in the 1 mm group, its frequency was maximum at day 14 and afterwards decreased.

The inflammatory reaction was observed in the snail tentacles of all the 4 mm groups. This lesion affected only 20-40 % of snails in the *M. capillaris* 1 mm group from days 3.5 to 28 p. i.; it was uncommon in the *N. linearis* 1 mm group (20 % of molluscs on days 14 and 21 p. i.).

There was no circulating amoebocytic reaction in the intervisceral spaces of these snails. Amoebocytes were present in the granulomas located in the foot, and a few cells were observed in the areas of subepithelial lysis and in the tunnels. The amoebocyte-producing tissue enlarged but without proliferation of the stem cells.

2. Principal components analysis of lesion scores

Analysis of the chronology of the lesions from days 3.5 to 28 p. i. (average scores) is in *figure 3*.

Axis 1 and axis 2 represent respectively 48 and 19 % of the total variance. The analysis took into account all the parameters of each period of snail examination; it includes thus all the information presented in *figures 1 and 2*. We can conclude that:

i) three groups of parameters can be distinguished on the correlation circle: lesions of the digestive gland (quadrant I), lesions of foot and tentacles (quadrant III), and those of gonads, albumen gland, and kidney (quadrant IV);

ii) the chronology of the lesions is different in 1 and 4 mm high snails, depending on the protostrongylid used. Most of the sampling periods of the 1 mm snails are in quadrant I: this means that digestive gland lesions were characteristic of these snails. The lesions of the 4 mm snails evolved from lesions of the foot and tentacles at the beginning of infection to lesions of the gonad, albumen gland, and kidney, 15 days p. i.;

iii) the location of snail groups in the first period of examination (day 3.5 p. i.) is dispersed; this is probably due to the influence of parasites from days 0 to 3.5 p. i.

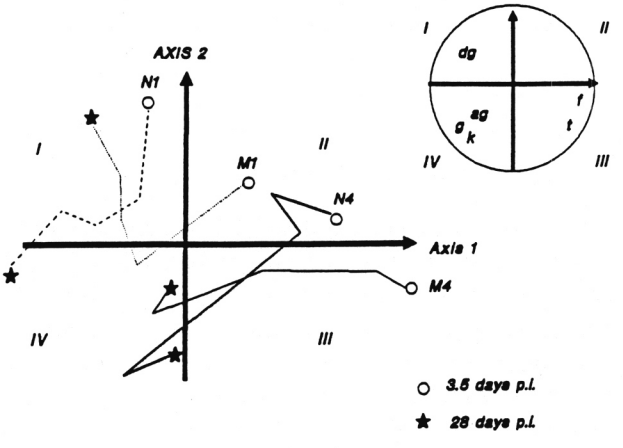

FIG. 3. — Principal components analysis of lesion scores in *L. truncatula* (1 mm and 4 mm height) infected with *M. capillaris* and with *N. linearis:* evolution from days 3.5 to 28 post-infection. Captions. N1: *N. linearis* infection, *L. truncatula* 1 mm height; N4: *N. linearis* infection, *L. truncatula* 4 mm height; M1, M4: *idem* for *M. capillaris*; ag: albumen gland; dg: digestive gland; f: foot; g: gonad; k: kidney; t: tentacles.

3. Relationship between infection and lesions

Only 4 mm high snails were used in the principal components analysis because they harboured more larvae (34 living larvae and 5 abortive L1 in the *M. capillaris* group, 4 and 9 respectively in the *N. linearis* group). The number of larvae (of any stage) was added as a supplementary variable. Individual values recorded in snails were used and separate analysis for each species of protostrongylid was performed. The correlation circles are in *figure 4*. For *N. linearis* infection, the presence of larvae was associated with lesions of the digestive gland; for *M. capillaris,* they were associated with foot lesions.

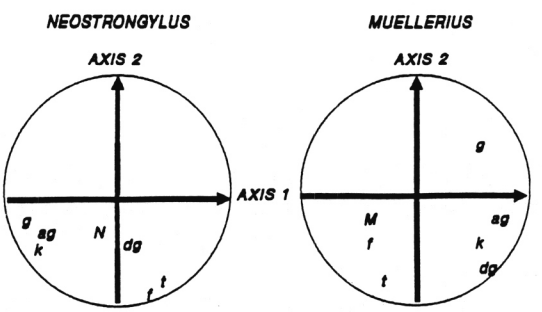

FIG. 4. — Principal components analysis on infection and associated lesions of *L. truncatula,* 4 mm height: correlation circles. Captions. N: number of larvae of *N. linearis*; M: number of larvae of *M. capillaris*; ag: albumen gland; dg: digestive gland; f: foot; g: gonad; k: kidney; t: tentacles.

The projection of individual snails (not shown here) on the plane delimited by axes 1 and 2 revealed other interactions between infection and lesions. In *N. linearis* infec-

tion, the lesions in the first two weeks p. i. were mostly in the digestive gland, foot, and tentacles, and they were found late in the gonad, kidney, and albumen gland. In *M. capillaris* infection, the first two weeks p. i. are characterized by progressive lesions of the foot and tentacles, and later gonadal lesions.

DISCUSSION

Reports on the histopathology of molluscs infected by protostrongylids are few in number (Zmoray *et al.*, 1969; Marcos-Martinez, 1975; Sauerländer, 1979; Cabaret and Weber, 1987). In addition, these observations always concern the development of larvae in the snail foot and on the characteristics of granulomas.

The inflammatory reaction observed in the foot and the tentacles must be reported in relation to the penetration of either L1 or of substances present in the larval suspension; the variations in the frequency of the reaction cannot be explained at that time. The role of granulomas in the development of larvae within the feet of snails is open to question: granulomas were found around abortive or living larvae, and were absent around several living L2 and L3. Further studies will be required to characterize the factors involved in the formation of granulomas around protostrongylid larvae in the foot of the snail.

This is the first report of tissue lesions in the internal organs of infected snails with protostrongylids. As the parasite is located in the foot, previous researchers had investigated foot lesions and did not record any other lesions. Only Samson and Holmes (1985) reported lesions in the intestine of *Vallonia pulchella* infected with *Protostrongylus* spp.; they were related to *per os* route of infection. Two explanations might be thought of for these lesions in organs where the parasites are not located. The first refered to the production of a toxic and the second may involve the side-effect of bacteriae which were conveyed with the L1 larvae, after penetration in the foot of snails. Thus our results are to be compared with what is known in *F. hepatica* or in *Schistosoma* infection.

Epithelial necrosis and reconstitution developed in the internal organs of *L. truncatula* whereas larvae were found in the foot. The characteristics of these processes described in the four viscerae were partly identical to the reports of Pan (1965) in *Biomphalaria glabrata* infected by *S. mansoni,* or to observations of several authors (Rondelaud and Barthe, 1963; Sindou *et al.*, 1990) in several lymnaeid snails infected by *F. hepatica.* Some differences can be observed on the timing of lesions appearance and duration as well as on the prevalence of lesion type:

i) the development of epithelial reconstitution in the digestive gland of the snails infected by *M. capillaris* and the lack of this process in those with *N. linearis* are open to question. The reconstitution of the albumen gland also was earlier and had a higher frequency in the snails harbouring *M. capillaris.* In contrast, the tissue lesions were roughly the same in the gonad and the kidney, regardless of the protostrongylid species. The disease process induced by *M. capillaris* might be milder in the internal organs in the lymnaeid snail. It may account for the wide range of susceptible species (Urban, 1980; Cabaret, 1984);

ii) the development of necrosis and of reconstitution in the kidney of the 4 mm group is in accordance with the observations of Rondelaud and Barthe (1983) in adult snails infected by *F. hepatica.* Conversely, the presence of two cycles in our juvenile kidneys during the experiment differs from the single cycle reported by Bouix-Busson *et al.* (1985) in young *L. glabra.* This difference may be partly due to the snail species chosen for the infection. Another explanation may also be proposed in relation with the snail size at the time of exposure: the number of epithelial cells could be insufficient to eliminate the putative toxic products in the juveniles infected by protostrongylids, which would oblige the kidney to develop short, successive cycles of necrosis-reconstitution;

iii) the lack of a circulating amoebocytic reaction in the intervisceral spaces of the snail and the normal structure of the amoebocyte-producing tissue are not so clear. The granulomas present in the foot developed during the first hours of the infection and were constituted by the snail amoebocytes (Marcos-Martinez, 1975; Cabaret and Weber, 1987). As the amoebocytic reaction is present in the adult *L. truncatula* infected by *F. hepatica* from day 7 to day 70 at 20° C (Rondelaud and Barthe, 1960), the role of this cellular reaction is open to speculation. In spite of its non specificity (Rondelaud *et al.*, 1984), this process does not develop in all the snails infected by any particular parasite species.

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