

STUDIES ON PARAMPHISTOMIASIS. II. THE MASS PRODUCTION OF  
METACERCARIAE OF *PARAMPHISTOMUM MICROBOTHRIUM*  
FISCHÖEDER 1901

P. J. SWART and R. K. REINECKE, Veterinary Research Institute, Onderstepoort

INTRODUCTION

Preliminary observations on infected sheep in the field indicated that, for an infection of paramphistomes to be fatal the load of young worms in the duodenum must be very heavy. Laboratory trials showed that of metacercariae dosed to a sheep less than 50 per cent survive and develop, and that a dose of a minimum of 90,000 metacercariae is required to produce clinical symptoms, and occasionally death in sheep. Therefore, the mass production of viable metacercariae is the next step in any research project on experimental paramphistomiasis.

EXPERIMENTAL

*The collection and hatching of paramphistome eggs*

Eggs were collected as follows: Fifty grams of infested faeces were thoroughly mixed with 500 ml of water and the suspension washed through a sieve (80 mesh to the linear inch) to remove the coarser faecal particles. The finer faecal material was separated from the faecal suspension as follows:—

A small (3 inch diameter) funnel was attached to a glass tube with the aid of a cork with a hole through the centre. The funnel with glass tube attachment was placed upside down in a 2 litre measuring cylinder, the funnel fitting loosely in the bottom of the cylinder while the glass tube extended an inch above the rim. Water was allowed to flow slowly, at the rate of 100 ml per minute, into the glass tube. At the same time the filtered faecal emulsion was rapidly poured into the measuring cylinder. The cylinder filled slowly, the finer particles of faeces rose and overflowed at the top, while the eggs only rose part of the way before sinking and rising again. Water flowed through the apparatus overnight, removing practically all the faecal debris; the water flow was stopped allowing the eggs to settle at the bottom. Later the method of Ritchie & Berrios-Duran (1961) was used. This was somewhat more successful than the above method.

Rowcliffe & Ollerenshaw (1960) stated that eggs of *Fasciola hepatica* would not develop unless freed from faecal material. The eggs of *P. microbothrium* separated from faeces, as already described, and mixed with a little water in a petri-dish, hatched after 12 days when incubated at 27 °C. The small amount of faecal material still present apparently did not influence the process of development and hatching.

Received for publication on 13 March, 1962.—Editor

## STUDIES ON PARAMPHISTOMIASIS II

A fair number of trials were carried out to establish whether eggs would hatch in the dung itself. Infested dung pads were incubated at 27 °C, kept immersed in water and examined every seven days. No development within the egg was noted, even after a period of four weeks. The eggs were then separated from the faecal mass and incubated at 27 °C for a period of 12 to 14 days. On exposure to light, miracidiae started to emerge. It was concluded that as long as the temperature and moisture conditions were adequate, development of the embryo within the egg could only start after it had been freed from the faecal mass. In this respect *P. microbothrium* resembles *F. hepatica*.

Another source of eggs was from live worms collected at abattoirs: these were placed in Tyrode solution at 38 °C for five hours. Large numbers of eggs were produced, and separated from the worms by pouring the Tyrode solution through a coarse sieve. However, as abattoir material was not consistently available this source had to be abandoned.

### *Infestation of Bulinus tropicus with P. microbothrium*

Preliminary attempts to infest *B. tropicus* snails were most disappointing. Adult snails varying in length from 4·7 to 10·7 mm were placed in water in petri-dishes to which newly hatched miracidiae were added. A total of 800 snails was examined every week from the 48th to the 114th day after infestation and only 99, or 12·4 per cent, were found to be infested.

On the other hand young snails were infested with the greatest of ease and a trial with newly hatched 7, 14 and 21 day old snails was completely successful. All snails became infested.

A snail infested at hatching and examined 11 days later harboured 14 sporocysts.

Two methods of infestation may be used:—

- (a) Snail eggs are hatched by the partial submersion technique (Swart & Reinecke, 1962). After seven days, when the eggs have hatched, the container is lifted out of the aquarium and placed in water in a petri-dish containing newly hatched miracidiae. Although the snails cannot go through the fine nylon gauze, miracidiae can easily penetrate the gauze and infest the snails. After two hours the plastic container is removed from the petri-dish and the newly infested snails washed into the aquarium by turning the container upside down and spraying water with a wash bottle through the gauze.
- (b) Swart & Reinecke (1962) have found that the best method of hatching snail egg masses is by the aeration technique. A simple method of infesting newly hatched snails is to add egg masses to the aeration apparatus every seven days over a period of four weeks. In the same container washed paramphistome eggs are added to the water at the same intervals. As the young snails hatch they move off the aeration apparatus into the surrounding water where they become infested.

Both these methods of infestation have proved successful. While the former is undoubtedly better for precise observations, the latter is more useful when dealing with large numbers of snails.

*Shedding of cercariae*

Lengy (1960) found that light is the only stimulus required for the emergence of paramphistome cercariae. The plastic aquaria containing the infested snails were illuminated by ordinary 40 watt electric light bulbs placed 15 inches above water level. These were switched on for eight hours every day. The aquaria were examined daily. Usually, seven weeks after infestation cercariae started to be shed and were easily seen, being black against the white background of the plastic aquaria. The snails were examined separately to establish which were shedding cercariae.

Each snail was removed from the aquarium and transferred to a separate test tube containing water. These test tubes were placed in racks arranged in a steplike fashion. They were then exposed to illumination from a 300 watt lamp 20 cm from the lower rack for a few hours and each tube examined. The snails shedding cercariae were transferred to a common aquarium painted black and surrounded by blinds to exclude light. These precautions were to prevent shedding until stimulated to do so by yellow artificial light (*vide infra*).

Snails not shedding cercariae upon stimulation were returned to separate white plastic trays. In the above manner all the snails were examined. The process was repeated every week, as some infested snails delayed shedding cercariae for as long as 114 days.

*Collection of metacercariae* (see Fig. 1)

The principles used for the collection of metacercariae were based on Durie's (1955) technique with a few modifications:—

- (1) Infested snails were placed in aquaria painted black on the inner surface to prevent encystation of cercariae on the sides.
- (2) A 40 watt, yellow electric bulb (A) was suspended so that its lower surface was 1 to 1½ inches above the surface of the water in the centre of the aquarium. An ordinary metal electric light shade (B) was attached above the globe. When switched on, the yellow light was the only light source, other light being excluded by the small blinds previously mentioned.
- (3) It was necessary to provide a base on which the cercariae could encyst. To do this 5½ inch long strips of cellulose sausage casing (1⅞ inches inflated diameter) (C) were wrapped around a cylindrical flat-bottomed, specimen bottle (D), 2 inches high with a diameter of 1½ inches. The cellulose which had a ½ inch overlap was fixed with a small strip of "sellotape", the latter was not allowed to make contact with the water.
- (4) This vessel with its surrounding cellulose wrapping was placed in the centre of the aquarium immediately beneath the light bulb. It was essential that the cellulose strip formed a continuous surface from the bottom of the aquarium to above the water level.
- (5) Since snails will ingest metacercariae, a circular fibre glass "mosquito" gauze "cage" (E) was placed around the outside of the glass vessel. This allowed free passage of cercariae but prevented snails from passing through.
- (6) The light was switched on for a period of five hours. Since the yellow light was concentrated on the cellulose wrapped cylinder on which cercariae encyst the collection of metacercariae was relatively easy.

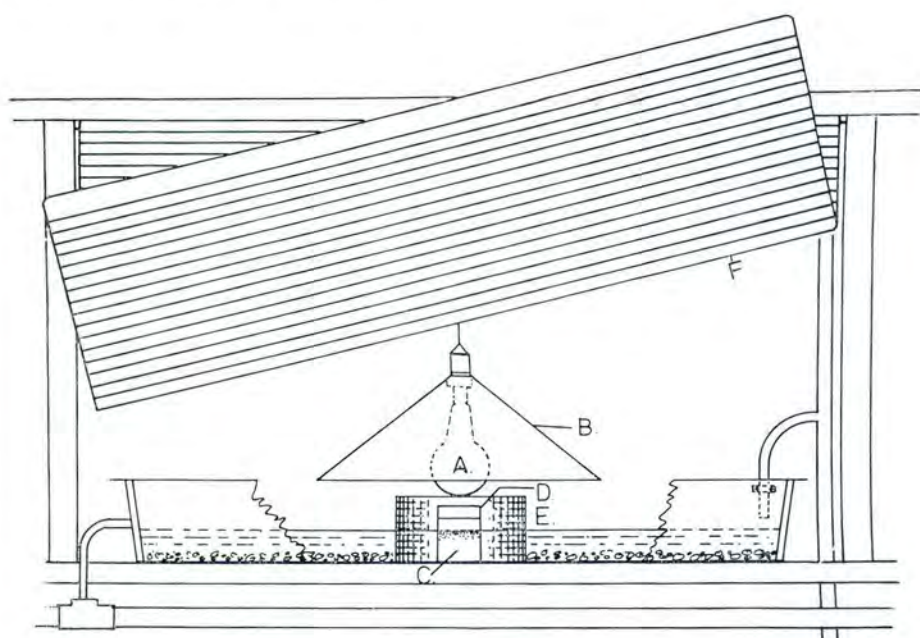


FIG. 1.—Collection of metacercariae

A. 40 watt yellow electric light bulb; B. metal shade; C. strip of cellulose sausage casing ( $1\frac{7}{8}$  inch diameter inflated); D. cylindrical flat-bottomed specimen bottle, 2 inches high,  $1\frac{1}{2}$  inch diameter; E. glassfibre gauze cage; F. blind.

*Periodicity of shedding cercariae*

According to Lengy (1960) "under strong illumination, the largest number of cercariae emerge during the second or third hour after exposure of the snails to light". It is not clear what intensity of light Lengy implies but elsewhere in the text he mentions a 75 watt light bulb at 30 cm distance.

In extensive experiments with a 40 watt yellow light bulb,  $1\frac{1}{2}$  inches above the surface of the water, and collecting metacercariae by the method described, the periodicity of cercarial shedding was tested.

Every hour after exposure of infested snails to the yellow light the cellulose strips on which the cercariae encysted were removed and replaced with fresh strips. The metacercariae on these strips were counted. From the counts the following pattern emerged, expressed as a percentage of the total.

1 Hour	2 Hours	3 Hours	4 Hours	5 Hours	6-8 Hours
%	%	%	%	%	%
5	27	35	27	4.6	1.4

The emergence of cercariae was not very marked for the first hour but rapidly increased to reach a peak two to three hours after exposure to the light source. Five hours after exposure to the light source very few cercariae were still being shed and only 1.4 per cent thereafter.

Although Lengy's (1960) observations are confirmed, that the largest numbers of cercariae are released two to three hours after exposure to the light in these experiments 98.6 per cent of the cercariae were released within five hours of exposure to yellow light. In Lengy's trials the cercariae were released up to nine hours after exposure.

On the days of collection the snails were exposed to yellow light from 8 a.m. to 1 p.m. only.

Metacercariae were collected daily from Monday to Saturday. The numbers of metacercariae collected on Monday and Tuesday, were invariably larger than the collections on the other days of the week. This apparently was due to the rest period over Sunday.

Lengy (1960) observed that snails that had released cercariae under strong illumination, required a rest period of at least 24 hours before being capable of releasing cercariae again in any appreciable number.

Various patterns of collection followed by rest periods were carried out and the total number of metacercariae collected over a seven day period used as an index of the best method. The following pattern proved the most satisfactory: Collections on Monday, Tuesday, Thursday and Friday and resting the snails on Wednesday, Saturday and Sunday.

This did not entirely confirm Lengy's observations but it must be pointed out that even on consecutive collecting days snails were not exposed to any light for 19 hours. The longer rest periods varied from 43 to 67 hours.

At present there are 1,000 infested snails in aquaria from which metacercariae are being collected and counted (*vide infra*). The daily production from 1,000 snails is 41,000 metacercariae. This is only 41 metacercariae per snail per day. It must also be mentioned that a small proportion of the cercariae encysts on the surface of the water, is not collected and is therefore lost. In an attempt to obtain the actual number of metacercariae released per snail a single infested *B. tropicus* in a pyrex crucible was exposed to a 300 watt (quartz lamp) ultra-violet light at a distance of 20 cm for four hours daily except Sundays. No increased production was noted on Mondays. In a period of 134 days this snail shed 16,616 metacercariae, with an average of 124 and a range of three to 597 per day. Large numbers of cercariae were still recovered from the snail after it had died. It is interesting to note that the average daily production of metacercariae is 41 under the influence of a 40 watt yellow light, but that this can be increased to 124 when a 300 watt ultra-violet light is used as the stimulus.

It has not yet been determined whether the intensity of light or the ultra-violet rays is the stimulus for this increased production. Tests carried out by Dr. Koen of the Pretoria University have shown that the vessel used, when filled with water, permits the passage of ultra-violet light up to 2,800 Å. Experiments are under way to clarify this interesting observation.

## STUDIES ON PARAMPHISTOMIASIS II

### *The counting of metacercariae*

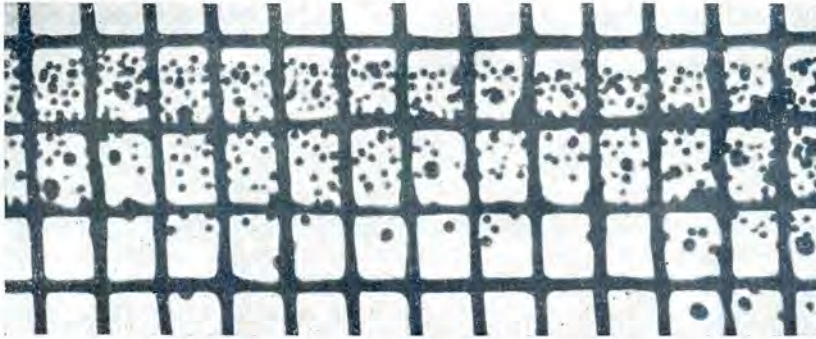


FIG. 2.—Grid system for counting metacercariae. Microphotograph of metacercariae on cellulose strips placed on glassfibre gauze for counting purposes.

When the trials started, each metacercaria was counted individually. The daily production has, however, occasionally exceeded 130,000 and it is impossible to count such numbers.

The cellulose strips were removed from the glass vessel and left overnight in a beaker of water. When the cellulose strip was removed from the beaker and laid flat, a black band of metacercariae was noted along the length of the strip. The top edge was straight and darker than the rest of the band. This had large numbers of metacercariae encysted on it while beneath the surface fewer encysted with an uneven distribution. The whole band was approximately 1 cm wide; these metacercariae were counted and used for infestation.

Metacercariae were counted on a grid system using the lowest magnification of a dissection microscope. Fibreglass gauze 3 by 2 inches and with 2 mm square apertures was placed flat in a petri-dish of 4 inch diameter. Each cellulose strip was cut into two equal lengths of approximately  $2\frac{1}{2}$  inches and one of them placed flat on top of the gauze. The band of metacercarial encystation was parallel to the length of gauze and the cellulose strip did not overlap it. The metacercariae were counted by moving the petri-dish across the field of the microscope.

Since the encystation of metacercariae on the cellulose strip was uneven, one average series of squares across the band of encystation out of every block of ten was counted. This process was repeated until the end of the strip was reached.

The total number counted multiplied by 10 = the total number of metacercariae on the strip.

The other half of the cellulose strip was similarly counted.

### *The longevity of infested snails*

One hundred newly infested snails were placed in an aquarium on 15 April, 1961 and a 40 watt yellow light used as a stimulus for cercarial shedding and collection of metacercariae. The snails died over a period of 10 months.

SUMMARY

(1) Methods of collecting paramphistome eggs, largely freed of faeces, and their hatching are described.

(2) Young *B. tropicus* were readily infested with paramphistomes: only 12·4 per cent of adult snails were infested.

(3) Two methods of infestation are described.

(4) The mass production, collection and counting of metacercariae are described.

(5) After exposure to a 40 watt yellow electric light bulb at 1½ inches above the water surface 98·6 per cent of cercariae were shed within five hours.

ACKNOWLEDGEMENTS

The Chief, Veterinary Research Institute is thanked for facilities and permission to carry out these investigations and publish the results. Drs. R. A. Alexander and I. G. Horak are thanked for their help with the manuscript.

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