ABSTRACT

TITLE STUDIES ON THE MORPHOLOGY AND DEVELOPMENT OF SOME MEMBERS OF THE FAMILY PARAMPHISTOMIDAE. FISCHOEDER 1901.

In introduction there is a brief review of the literature and an outline of the work embodied in the thesis. The thesis is divided into four main parts.

Part 1. Gametogenesis and early development in Gigantocotyle bathycotyle (Fischoeder 1901) Nasmark 1937.

This includes a description of the genitalia, with particular reference to the female organs and their associated ducts, an account of gametogenesis, egg shell formation and the early cleavage divisions.

Part 2. The species of the genus Paramphistomum Fischoeder 1901, which occur in the British Isles, with notes on some material from the Netherlands and France.

Two new species of Paramphistomum are described and they are compared with Paramphistomum cervi (Zeder 1790) Fischoeder 1901 hitherto believed to be the only mammalian paramphistome occurring in this country. Gametogenesis and early development are described briefly and compared with the processes in Gigantocotyle bathycotyle. The species obtained from the Netherlands and France are noted.

Part 3. The development of the miracidium of Paramphistomum hiberniae from the time of deposition of the egg, until hatching.

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Abstract (cont.)

Observations are based almost entirely on living material as only comparatively small numbers of eggs were available. The description of the miracidium includes notes on the staining reactions of various vital dyes and on the use of poly-vinyl alcohol as a means of keeping the miracidium still, without distortion. The process of hatching is described in some detail.

Part 4. The attempts made to infect snails experimentally with paramphistome miracidia.

An account is given of the collection and culturing of the eggs. Many hundreds of snails of various species were exposed to paramphistome miracidia. Results so far have been negative.

STUDIES ON THE MORPHOLOGY AND DEVELOPMENT OF SOME MEMBERS OF THE FAMILY PARAMPHISTOMIDAE.

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INTRODUCTION

The earliest reference to the occurrence of an amphistome according to Nasmark was in 1754 when Daubenton recorded the presence of one, without however naming it. It was not until 1790 that Zeder recorded and published a description of a parasite which he called Festucaria cervi, now Paramphistomum cervi (Zeder 1790) Fischoeder 1901. Zeder's description was closely followed by a publication by Schrank, in which he also described this parasite. Some doubt has been thrown on which was the original publication, Stiles and Goldberger, and some later authors ascribing it to Schrank. Actually it seems certain that it was that by Zeder.

Since then several hundred species have been described and eight main systems of classification proposed, with the result that the whole "Amphistome problem" has become greatly confused. The chief difficulty has been caused by a failure to agree on which characters constitute genuine specific and generic differences and which are variations in form caused by differing methods of fixation, the physiological state of the parasite immediately prior to fixation and its degree of maturity. All trematodes are subject to distortion during killing and fixing and the paramphistomes particularly so, being thick and fleshy and often with a comparatively thick cuticle.

The first serious attempt to classify the known species of Paramphistomes was made in 1901 and the following two years by Fischoeder. Unfortunately his observations were limited to mammalian parasites which made them necessarily incomplete. He set up the family Paramphistomidae and divided it into two sub-families, the Paramphistominae and the Cladorchinae. The genera and species were distinguished by such characters as the presence of a ventral pouch (Paramphistomum and Gastrothylax) the relative positions of the excretory pore and the opening of Laurer's canal, the proportion of the diameter of the acetabulum and the pharynx to the length of the body, the position of the genital pore, the length of the oesophagus, the form and position of the testes, etc.

In 1910 Stiles and Goldberger described a number of new genera and species and proposed the second system of classification. While using similar characters for differentiation as had Fischoeder they expanded the system to include the parasites of the amphibia. A Superfamily, the Paramphistomoidea, which was almost equal to the family Paramphistomidae Fischoeder was erected and this was divided into three families, the Gastrothylacidae, the Paramphistomidae and the Gastrodiscidae. Of these only the Paramphistomidae was further divided into four sub-families, the Paramphistominae, the Stephanopharynginae, the Cladorchinae and the Diplodiscinae.

Maplestone, in 1923, kept to the broad outlines of the system proposed by Stiles and Goldberger, but drastically reduced the number of genera and species by extensive synonomisation, which in many cases appears to be quite unjustifiable. As Stunkard has observed, Maplestone seemed unable to distinguish between actual differences and those caused by methods of fixation etc. As a result of this confusion many species have been recorded as Paramphistomum cervi and P. explanatum which in all probability are quite different. Maplestone only considered the parasites of mammals.

Two years later Stunkard published a review of the "Amphistome Problem" and proposed a system of classification based on his own observations and a critical study of the literature. In this he divided the family Paramphistomidae into nine subfamilies, as follows:-

Diplodiscinae	Cohn 1904
Schizamphistominae	Looss 1912
Pararphistominae	Fischoeder 1901
Cladorchinae	Fischoeder 1901
Gastrodiscinae	Monticelli 1892
Gastrothylacinae	Stiles and Goldberger 1910
Zygocotylinae	Stunkard 1916
Balanorchinae	Stunkard 1917
Brumptinae	Stunkard 1925

In 1929 Fukui, working on Japanese paramphistomes, introduced the study of the musculature of the pharynx and the acetabulum into the classification, and also followed up the

suggestion made by Looss that the excretory system was important. According to Fukui, the Gastrothylacinae are reduced from subfamily to generic rank and are included in the tribe Paramphistomatinea of the subfamily Paramphistominae. He retained the other eight subfamilies proposed by Stunkard, and erected two new subfamilies, the Pfenderinae and the Dadayinae. He accepted many of Maplestone's proposed synonyms.

Travassos in 1934 outlined a system of classification in which the superfamily Paramphistomoidea Stiles and Goldberger was re-erected. He divided it into six families, the Paramphistomidae, the Gastrodiscidae, the Opistholebetidae, the Gyliauchenidae, the Cephaloporidae and the Microscaphidiidae. The relationships of the last four are obscure and beyond the scope of the present work. The Paramphistomidae were divided into nine subfamilies, seven of which were the same as those proposed by Stunkard, but the Gastrodiscinae became a family, the Schizamphistominae became a genus of the subfamily Cladorchinae. Stephanopharynx became a subfamily and a new subfamily, the Kalitrematinae was erected for the single genus Kalitrema.

After working on a collection of paramphistomes from Malaya in 1936 Dawes revised the genera Paramphistomum Fischoeder

1901 and Gastrothylax Poirier 1883. In his opinion much of the synonomisation proposed by Maplestone was correct.

In 1937 Nasmark published "A Revision of the Trematode family Paramphistomidae." The first part of this work is an account of the comparative anatomy of the acetabulum, the pharynx and the genital atrium of the many paramphistomes which he examined. His observations were very extensive, some two thousand slides being examined. The second part of the work is a system of classification based on his anatomical observations. The characters on which he divides this subfamily up are mainly the musculature of the acetabulum, the pharynx and the genital atrium, which have the advantage that they do not vary with fixation or the age of the worm. The fifteen subfamilies which Nasmark proposes are:-

Pseudocladorchinae	Nasmark 1937
Schizamphistominae	Looss 1912
Stichorchinae	Nasmark 1937
Cladorchinae	Fischoeder 1901(reduced)
Pfenderinae	Fukui 1929
Diplodiscinae	Cohn 1904
Zygocotylinae	Stunkard 1916
Balanorchinae	Stunkard 1917
Paramphistominae	Fischoeder 1901
Gastrothylacinae	Stiles & Goldberger 1910
Brumptiinae	Stunkard 1925
Watsoniinae	Nasmark 1937
Gastrodiscinae	Monticelli 1892
Pseudodiscinae	Nasmark 1937
Stephanopharynginae	Stiles & Goldberger 1910

Nasmark confined himself to preliminary notes on all the subfamilies but the Paramphistominae which is dealt with in great detail. Within the Paramphistominae Nasmark proposed there

should be nine genera, namely:

Paramphistomum	Fischoeder 1901
Gigantocotyle	Nasmark 1937
Calicophoron	Nasmark 1937
Cotylophoron	Stiles & Goldberger 1910
Ugandocotyle	Nasmark 1937
Ceylonocotyle	Nasmark 1937
Nilocotyle	Nasmark 1937
Buxifrons	Nasmark 1937
Macropharynx	Nasmark 1937

While it might appear that Nasmark created a large number of new genera on comparatively slender grounds, his arguments are very convincing. The various acetabulum, pharynx and genital atrium types are, from his descriptions, quite distinct and where there is a fairly constant combination of two or more of these characters it is reasonable to assume that they constitute a genus. Nasmark also takes into consideration the geographical distribution, the location in the body of the host and the other anatomical features such as the arrangement and form of the testes, etc.

Before embarking on the experimental work described in this thesis a considerable time was spent in examining the slides of paramphistomes in the collection of the London School of Hygiene and Tropical Medicine. It was found that only by using Nasmark's scheme of identification could many of the specimens be identified. Only one aspect of his work seems to be of doubtful value; he asserts that the number of units in the bands of circular muscles in the acetabulum are more or less constant

within a given species. This may be true of an exactly median Sagittal section but it seems that as the dorsal and ventral circular muscles as seen in sections differ in number but are in fact continuous around the acetabulum some splitting of bundles must occur. Consequently successive sections may, and in fact do, show slightly different numbers. It is often impossible to be certain which of a series of sections is median, so that counts made of muscle bundles may be misleading. Since Nasmark actually makes little use of these numbers for specific diagnosis this does not detract from the value of his work. In support of his separation of a large number of genera and species it is interesting to note the extreme specificity of paramphistome miracidia for particular snail hosts which various workers in this field have reported.

The aim of the work described in the four main parts of this thesis was two fold. Firstly it was hoped that a more detailed study of the morphology, and particularly of the cytology would shed more light on the taxonomy of the group; secondly that the life cycle of P. cervi in this country could be worked out, together with the details of the germ-cell cycle. As far as the first object was concerned the chief difficulty encountered was that of obtaining material suitably fixed for cytological study. For the second paramphistomes are comparatively rare in this country and considerable time and

energy was expended in obtaining material and in trying to trace its exact source in order that a search could be made for the intermediate snail host. A number of other workers have very kindly given some of their material for this investigation and it was while comparing the various collections that it became apparent that not one but three species were present. No infected snails were obtained, but as many eggs as possible were collected and cultured and several hundred snails of various species were exposed to the miracidia thus obtained.

During these investigations some fifteen thousand serial sections were cut, stained and mounted and examined under the microscope and several thousand eggs were collected by sedimentation from faeces and from adult worms obtained on postmortem examination. The results are described in Parts 1-4 of this thesis.

GAMETOGENESIS, and EARLY DEVELOPMENT IN GIGANTOCOTYLE BATHYCOTYLE (Fischoeder,1901) Nasmark,1937.

INTRODUCTION

The cytology and germ cell cycles of digenetic trematodes are of particular interest in view of their complicated life histories, their hermaphroditism and the difficulties in taxonomy of these parasites.

There are numerous different opinions on synonomy in the family Paramphistomidae as expressed by Fischoeder (1901 etc.) Maplestone (1923), Stunkard (1925), Fukui(1929), Travassos (1934), Dawes (1936), and Nasmark (1937). Most of these consider different characters to be of generic and specific importance.

It is hoped that further light will be shed on the question by means of detailed studies on the chromosomes and germ cell cycles in a number of closely related members of the family. This paper deals with the processes in Gigantocotyle bathycotyle (Fischoeder, 1901) Nasmark, 1937.

Some difficulty was experienced in identifying the material. After study of a large number of slides in the collection of the London School of Hygiene and Tropical Medicine, it appears that the classification proposed by Nasmark in 1937 is reliable and the most easily workable. For the purposes of the present paper it is assumed that Gigantocotyle bathycotyle is not synonymous with Gigantocotyle (Paramphistomum) explanatum.

As many of the earlier papers which give an account of gametogenesis appear to be based on different interpretations of nuclear division, it is proposed to give a definition of terms used in this paper. These are based on those given by White (1946).

Leptotene corresponds to the earliest part of mitotic prophase. The chromosomes are very long slender threads with numerous chromomeres distributed along their length. According to White, Darlington believes that the chromosomes are unsplit at this time, and that this constitutes a distinction of primary importance between leptotene and the corresponding stage of somatic mitosis.

Zygotene; the homologous chromosomes come together, side by side, throughout their entire length.

Pachytene; the pairing process is complete. The appearance of the chomosome threads resembles that of mid-prophase chromosomes at mitosis.

Diplotene; the attraction between the homologous chromosomes seems to end and the pairs separate, remaining held together by chiasmata.

Diakinesis; corresponds to late prophase of somatic mitosis.

Bivalent; two homologous chromosomes which have completed the process of pairing and appear as one body.

MATERIALS AND METHODS

The material was collected from the rumen of Bos indicus at the Colombo Municipal Slaughter House, Ceylon. It was fixed in Carnoy (6:3:1) and preserved in 90% alcohol.

Material for sectioning was embedded in paraffin wax with ceresin, congealing point 52°C or 54°C, as it was found that after embedding in a wax with a higher congealing point, the material became very brittle.

Sections for the purpose of identification and study of general anatomy were cut at 20 μ and stained in Borax-carmine or Ehrlich's Haematoxylin, with Eosin as counter-stain.

For demonstration of spermatogenesis, sections were cut 4-6 μ in thickness and stained in Weigert's Iron Haematoxylin and Heidenhain's Iron Haematoxylin without counter-stain. Most satisfactory results were obtained with Heidenhain's Iron Haematoxylin, using the following method:

Mordant sections for two hours in 5% iron alum solution in 50% alcohol. Stain for 12-15 hours in 1% haematoxylin, differentiate with a saturated solution of picric acid in 70% alcohol. This differentiation is very rapid and should be watched under a binocular microscope.

For demonstration of oogenesis and early development sections were cut 4-12 μ in thickness and stained with Weigert's Iron Haematoxylin, Heidenhain's Iron Haematoxylin, without counter-

The Feulgen staining reaction was tried with little success. According to Britt (1947) it is more satisfactory if material is fixed for five minutes in Carnoy, followed by two hours in San Felice. When more fresh material is available, it is hoped to try this technique.

Except where otherwise stated, all drawings were made with the aid of a camera lucida.

ANATOMY OF THE GENITALIA

Male

There are two testes lying one behind the other, the posterior tending to be wedge-shaped. Both are very slightly lobed and smooth in outline. A vas deferens runs anteriorly from each testis joining to form a long, thin-walled vesicula seminalis. In mature worms this is packed with spermatozoa and much coiled. The vesicula seminalis leads into a pars musculosa which is not very strongly developed and this in turn passes into a pars prostatica. The cells surrounding this part of the duct agree very closely histologically with those of Mehlis gland. From the pars prostatica the ductus ejaculatoris opens into the genital atrium.

The following are measurements taken from thick hand sections. All the worms are mature and there is very little variation in size:

	Anterior testis.	Posterior testis.
Length (anterior-posterior)	1.5 mm.	1 mm.
Depth (dorsal-ventral)	2.5 mm.	2.5 mm.
Breadth (side-side)	2.5 mm.	3.5 mm.

Female (Figs. 1,2,3)

The single ovary lies posterior to the testes and dorsal to the acetabulum. It is pear-shaped and measures about 0.4 mm x 1 mm x 1 mm. Oogonia form a cap (See Fig. 3) opposite to the opening of the oviduct and occupy about one third of the ovary.

The oviduct is a slightly coiled narrow tube running posteriorly, dorsal to Mehlis gland. It is joined by Laurer's canal outside Mehlis gland, thus differing from Fasciola hepatica, in which the two ducts join within the gland (Stephenson, 1947). Laurer's canal opens to the exterior on the dorsal surface, in the mid line, about the level of the ovary. (See Fig.2) No spermatozoa nor surplus vitelline material have been found in Laurer's canal. The oviduct runs into Mehlis gland, where it is joined by the vitelline duct and forms the central chamber of the gland. The diameter of this is very variable according to the number of vitelline cells which it contains. No valve such as that described by Stephenson in F. hepatica has been observed.

The uterus leads from the central chamber and passes anteriorly. In the most proximal part of the uterus the eggs lie singly, but become closely packed in the more distal portions.

There is neither a receptaculum seminis nor a receptaculum uterinum, but in all the specimens sectioned there appears to be one region of the uterus into which spermatozoa are concentrated. This loop lies between the posterior border of the testis,

KEY TO LETTERING

a - acetabulum
al - anterior limit of acetabulum
c - gut caecum
cc - central chamber of Mehlis gland
e - excretory bladder
f - fortilisation membrane
j - junction of oviduct with Laurers canal
L - Laurers canal
Lo - opening of Laurers canal to exterior
M - Mehlis gland
o - ovary
oc - oocyte
od - oviduct
og - oogonia
os - fibrous layer surrounding ovary
ov - eggs in uterus
p - pronucleus
pbl - first polar body
pr - propagatory cell
s - spermatozoa in uterus
sc - spermatozoon in cytoplasm of oocyte
sh - shell
t - testis
ts - fibrous layer surrounding testis
u - wall of uterus
v - vitelline glands
vc - vitelline cells
vr - vitelline reservoir
w - wall of central chamber of Mehlis gland

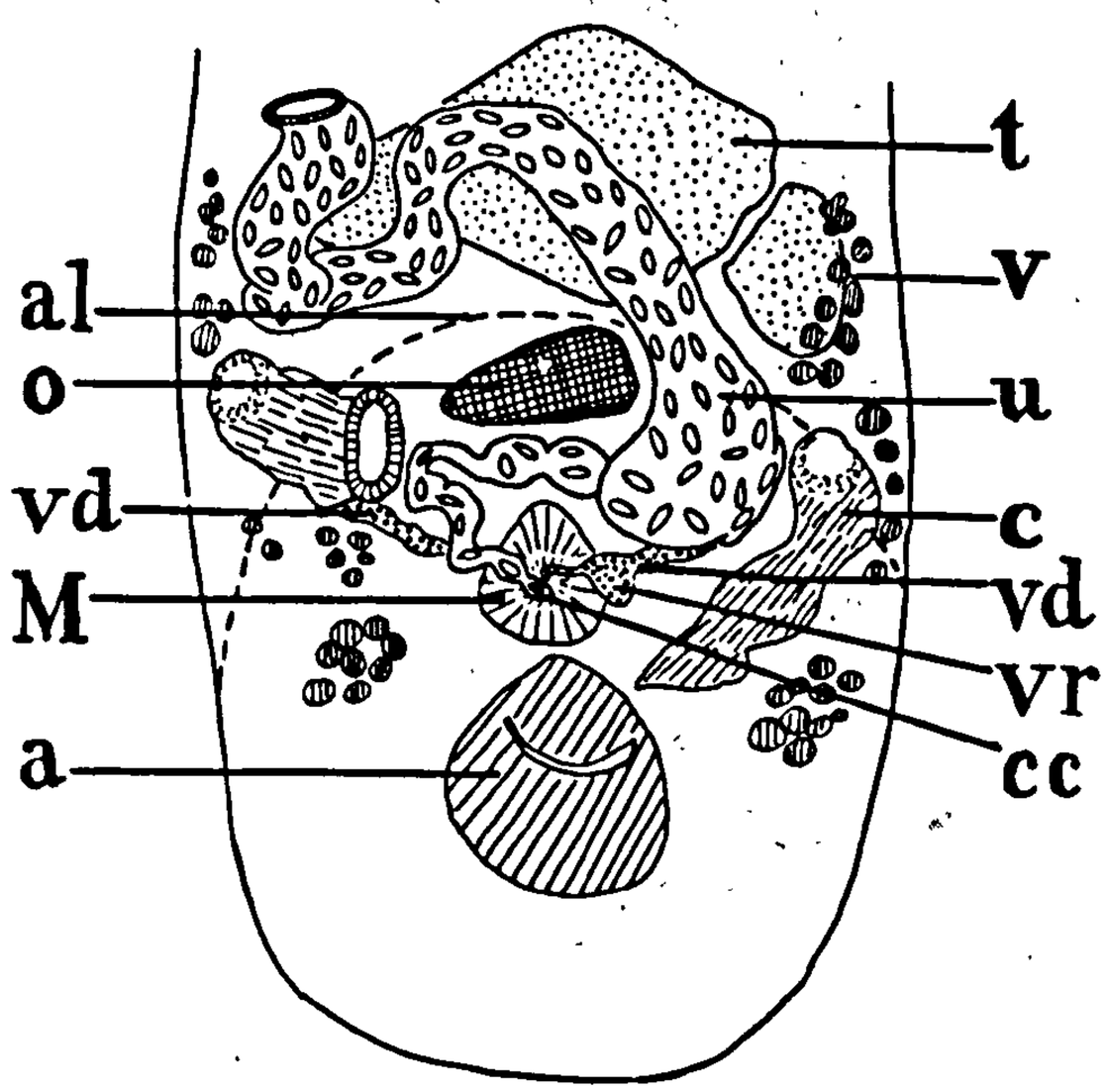


Fig. 1

Fig. 1. Diagrammatic representation of thick (hand) horizontal section. Oviduct and Laurer's canal omitted. Not to scale.

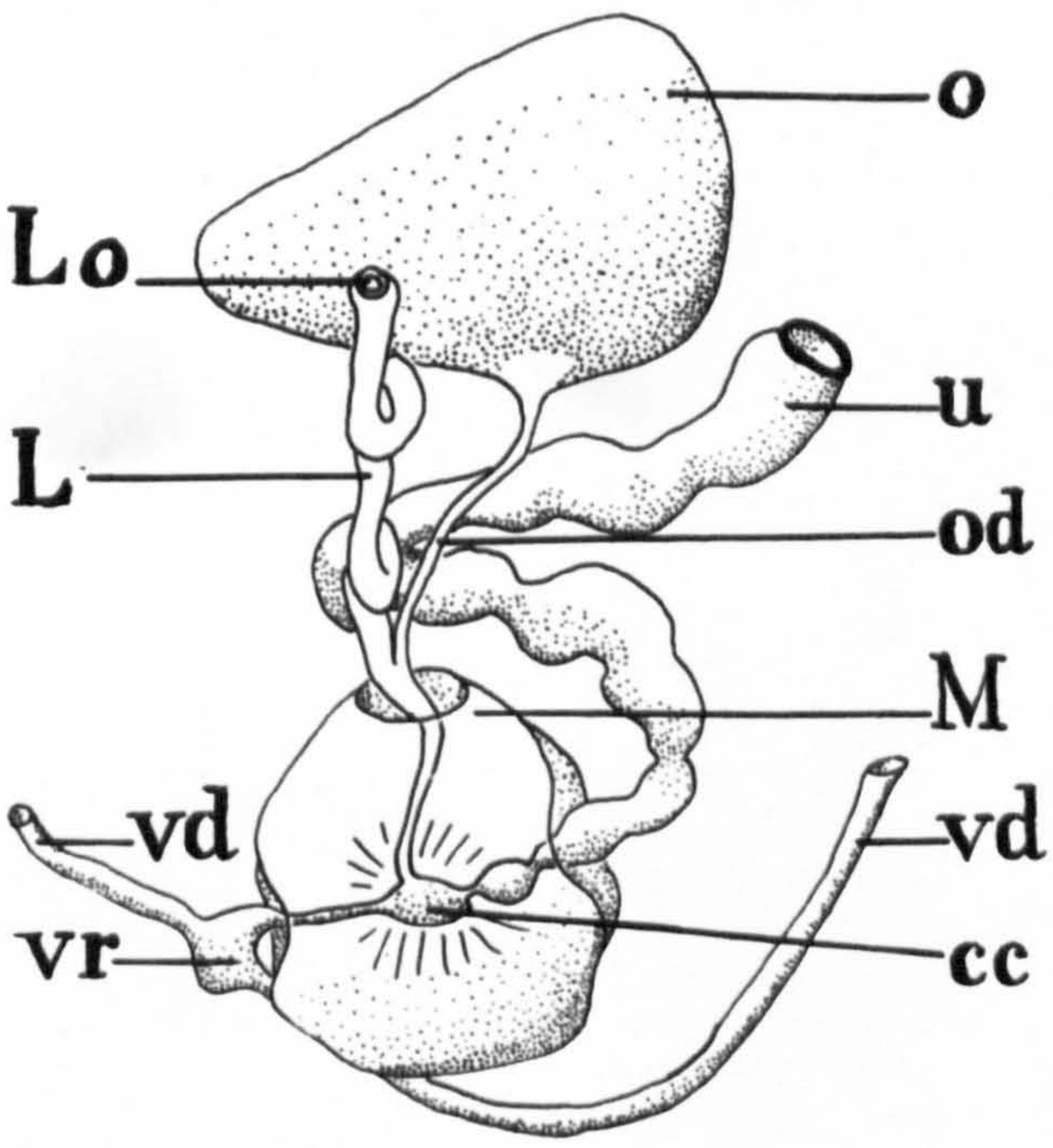


Fig. 2

Fig. 2. Ovary, Mehlis gland and associated ducts reconstructed from serial sections. Dorsal view. Scale only approximate.

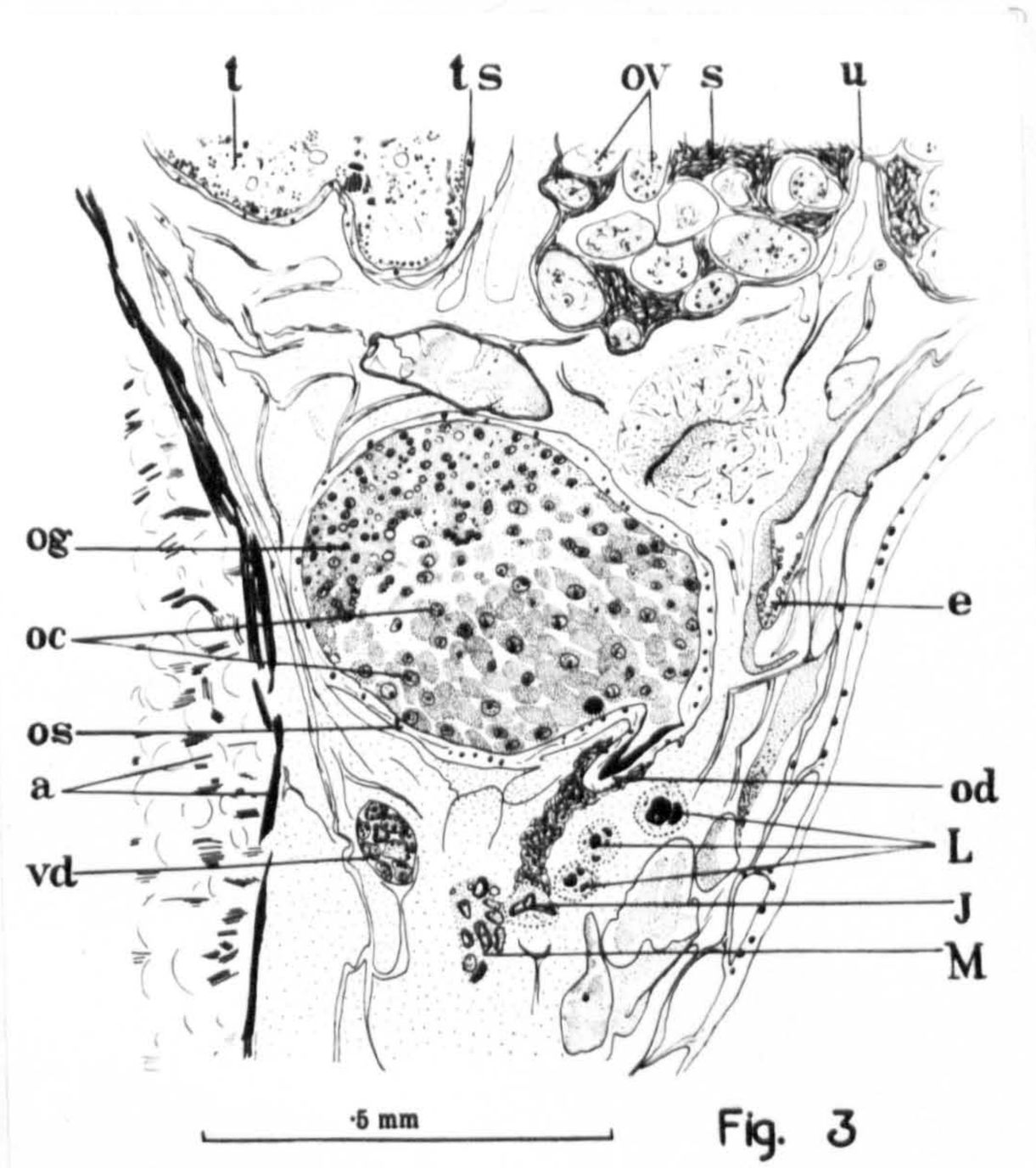


Fig. 3

Fig. 3. Sagittal section through ovary to show the junction of the oviduct and Laurers canal.

and the ovary. It was at first thought to be a receptaculum uterinum but further observations showed it to be continuous with the uterus and, in some specimens, to contain eggs. When packed with eggs the uterus becomes much convoluted and extends over almost the whole dorsal surface of the worm.

The metraterm, or vagina, is not clearly differentiated from the rest of the uterus and does not appear to be very muscular. It opens into the genital atrium just below the male opening.

Mehlis gland is compact and more or less spherical. The region of intracellular ducts is not as extensive as in F. hepatica (Stephenson, 1947).

The vitellaria are follicular and extend from the level of the pharynx to the middle of the acetabulum. A vitelline duct runs in dorsally to the acetabulum from each side and these join to form a vitelline reservoir adjacent to Mehlis gland. From this a single duct leads into the central chamber of the gland.

GAMETOGENESIS

Spermatogenesis. (Figs. 4-26).

The testes are bordered by a layer of fibrous tissue which appears to be derived from the cells of the parenchyma. Within this is a layer, from one to six cells in thickness, of primordial spermatogonia; in mature worms the usual thickness is two or three cells.

The nuclei of these cells are usually in the resting stage and there seems to be no distinct karyosome, (Fig. 4a). Division takes place rapidly and is a process of normal mitosis. As the cells are very closely packed and the nuclei vary considerably in size, measurement is difficult. During prophase nucleination of the chromosomes begins. (Fig, 4b). They become visible as double threads and in some cases it is possible to observe the pairs of chromomeres along the whole length of the chromosome. (Fig. 4c). It is unknown at what stage the longitudinal splitting of each chromosome into two chromatids takes place but as they appear as double threads, it is before prophase. It may be during the resting stage after the previous division (Darlington,1935) or earlier, according to some authors, as given by White (1948).

As nucleination and spiralisation continue the chromomeres become invisible and by the time metaphase is reached the chromosomes appear as compact densely staining bodies. The nuclear membrane disappears and a spindle is formed; neither astral rays nor centrosomes have been observed with certainty but this may be due to the small size of the cells. In one case a small darkly stained body was seen which was thought to be a centrosome. At metaphase the chromatids separate (Fig, 4d and e). No centromeres are visible in the chromosomes. During anaphase (Fig, 4f), the chromatids move apart to opposite poles of the spindle where they

enter into telophase and two daughter nuclei are reorganised. These pass into a resting stage and grow to the size of the parent cell. (Fig, 4g)

After an unknown number of mitotic divisions the cells move from the layer of primordial spermatogonia into the testis proper where the three spermatogonial divisions take place. There does not seem to be any zoning of the various stages in spermatogenesis as described by Pin Dji Chen (1937) in Paragonimus kellicotti All stages from primary spermatogonia to free spermatozoa were found throughout the testis and in many cases could be seen in the same section. It is possible that this is due to the degree of maturity of the worms. Cable (1931) working on Cryptocotyle lingua found that in mature worms the primary spermatogonia were located near the edge of the gonad.

The three spermatogonial divisions take place in the same plane and the daughter cells remain together. This results in a plate of eight cells which are the primary spermatocytes. It is at this stage that both nuclei and cells increase considerably in size. Although measurement of cells, other than primary spermatocytes was difficult, and not very accurate, the following table gives an idea of the amount of growth which takes place.

(See over,

Cells in peripheral layer of the testis.

	Primordial and Primary Spermatogonia,		Primary Spermatocytes	
	Diameter of Cell	Diameter of Nucleus	Diameter of Cell	Diameter of Nucleus
Minimum	6.4 μ	4.8 μ	8.0 μ	6.4 μ
Maximum	9.6 μ	7.2 μ	11.2 μ	8.8 μ
Average	7.7 μ	5.7 μ	9.6 μ	7.3 μ

No reduction in the number of chromosomes has so far taken place.

Sufficient stages of the reduction division have been observed to indicate that it is a normal meiosis. During leptotene the chromosomes become visible as long thin threads. (Fig, 5). They do not appear to be double but as the chromomeres are closer together and not as distinct as in a corresponding stage of mitosis, it is impossible to be certain. Zygotene has not been observed very frequently so that it seems likely that the actual pairing of homologous chromosomes takes place extremely rapidly. (FigC)

At pachytene, (Fig. 7) which seems to last a relatively long time, the bivalents become arranged as loops with all their free ends together at one side of the nucleus. Accordingto White (1948) this is a genuine phenomenon and not a fixation artefact.

Spermatogenesis in G. bathycotyle (contd.)

Fig. 21. Spermatod nucleus becoming ovoid and pushing out cell wall

Fig. 22. Spermatid nucleus still more elongated and becoming more densely staining. The nuclei appear to protrude through cell walls.

Fig. 23. Tail of spermatozoon being formed from spermatid nucleus. Chromosomes are still visible as twisted threads.

Fig. 24 Spermatozoa beginning to coil within the cytoplasmic mass.

Fig. 25 Spermatozoa tightly coiled, still lying on cytoplasmic ma

Fig. 26 Bunch of free spermatozoa lying in the testis.

Spermatogenesis in *S. bathycotyle*

Fig. 4. Stages of mitosis in cells of the peripheral layer from various sources.
a. Resting nuclei
b. Early prophase
c. Late prophase
d. Metaphase - polar view
e. Metaphase - side view.
f. Anaphase
g. Two daughter nuclei in resting stage.

Fig. 5. Leptotene

Fig. 6. Zygotene

Fig. 7. Pachytene

Fig. 8. Diplotene

Fig. 9. Diakinesis

Figs. 10 &11 First metaphase. Two sections through the same group of primary spermatocytes. x, x1 the same cell.

Fig. 12. Polar view of four primary spermatocytes in first metaphase.

Fig.13. Early first anaphase. Two bivalents still remain connected and are stretched on the spindle.

Fig. 14. Late first anaphase. Seven out of sixteen secondary spermatocytes are shown.

Fig. 15 Interphase.

Fig. 16. Polar view of second metaphase. Seven out of sixteen cells shown.

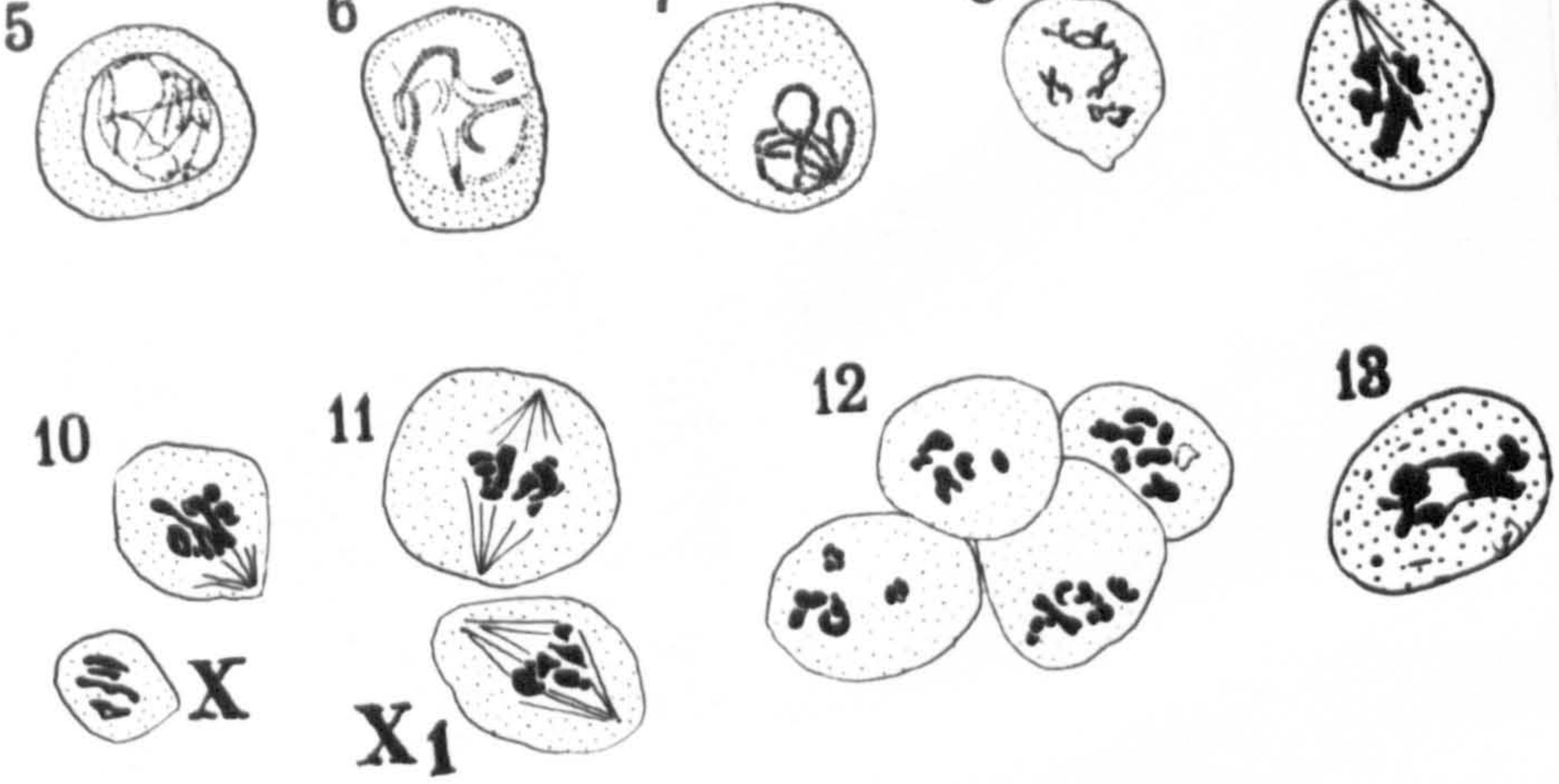
Fig. 17. Second anaphase.

Fig. 18. End of second maturation division. Chromomes are still distinct.

Fig. 19. Chromosomes from the cell in Fig. 9.

Fig. 20. Twelve out of thirty-two spermatid nuclei in the resting stage after the second maturation division.


A
A
B
4
C
G
F
E
D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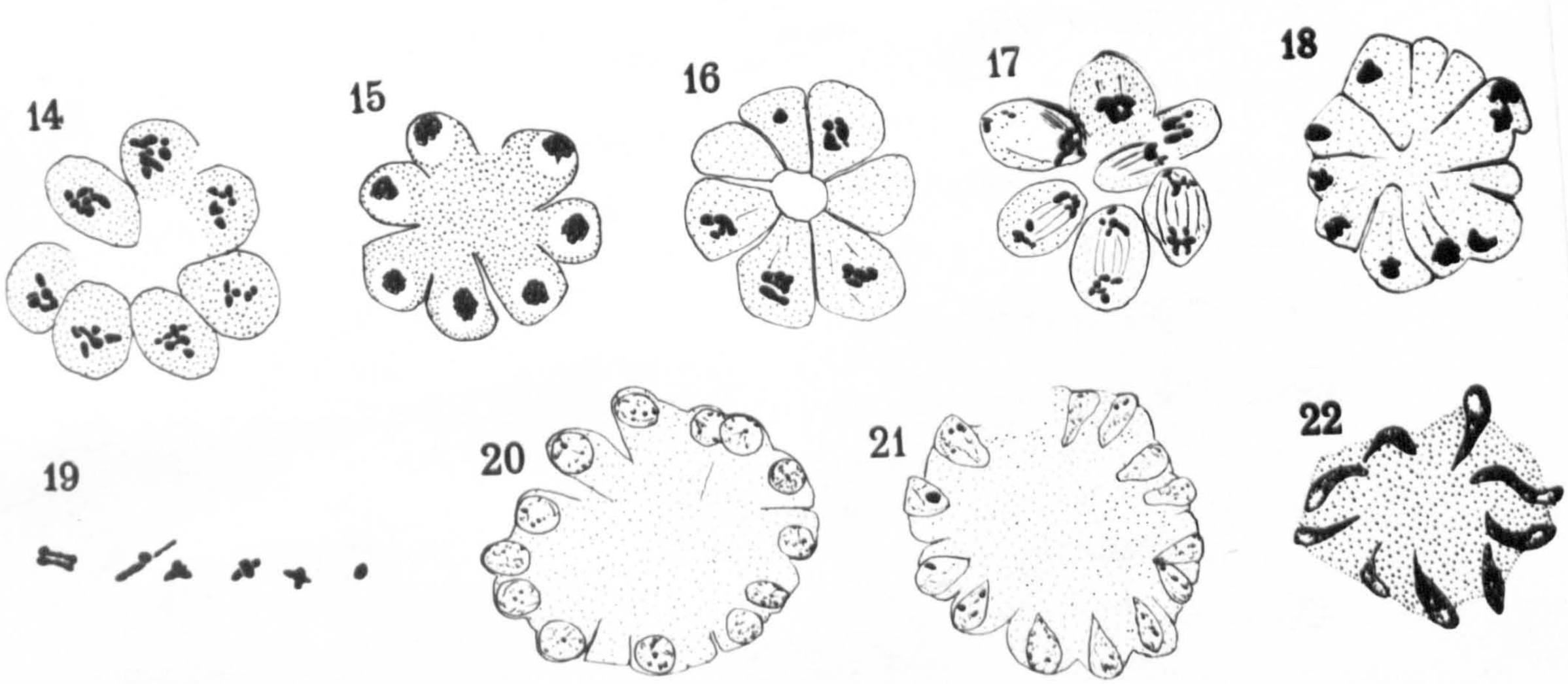
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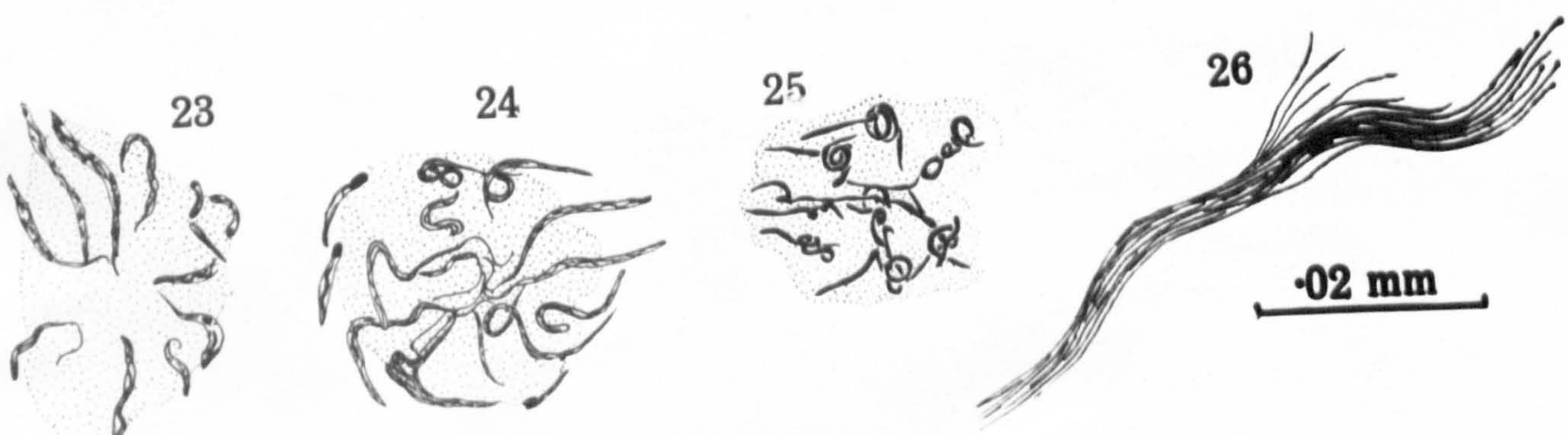
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Figs. 4-26

The chromosomes still retain a slightly woolly appearance which is not lost completely until the end of diakinesis. Condensation continues - during diplotene the pairs open out slightly, remaining held together only by chiasmata and lose their looped appearance. (Fig. 8)

The pairs spread out through the nucleus at diakinesis. (Fig, 9) The nuclear membrane disappears and a spindle is formed. In a few nuclei centrosomes are distinguishable, but they are extremely small and it is impossible to see if the centrioles have divided. No astral rays have been observed.

The chromosomes are now at their maximum density and lie on the equatorial plate of the spindle in a typical metaphase arrangement. (Figs. 10,11,12) At this stage the bivalents are widely separated from each other and it is comparatively easy to count them. There is no evidence of any chromosome remaining unpaired and forming a univalent. The homologous pairs separate and one chromosome from every pair passes to each pole. (Figs.13,14). The interphase between the first and second division is extremely short. (Fig, 15) As soon as anaphase is completed denucleination of the chromosomes proceeds until they resemble those in early prophase of a somatic mitosis. These then undergo a normal mitotic division which results in the formation of thirty-two nuclei, with the haploid number of chromosomes. (Figs. 16,17,18). Nuclear membranes are reformed but the cytoplasm

remains incompletely divided so that the spermatids do not separate but remain in a rosette. (Fig. 20)

Spermatozoa are formed from the spermatids without further division. After a short resting stage, during which the chromosomes do not become completely invisible, the spermatid nucleus begins to elongate. (Fig. 21) The side towards the outside of the cytoplasmic mass becomes pointed and pushes up against the cell wall. It then appears to protrude through the wall, but whether the wall is actually ruptured, or stretches to form a thin membrane surrounding the 'head' of the future spermatozoon, it is impossible to say. (Figs. 22&23).

The nucleus continues to elongate and to take stain more densely, and the individual chromosomes become indistinguishable. As the nucleus becomes longer and thinner, it coils within the cytoplasm. (Figs. 24&25). Finally the spermatozoa uncoil, free themselves from the cytoplasm and pass into the testis where they lie in bundles, gradually separating and entering the vas deferens. (Fig. 26).

The spermatozoa are long and threadlike, with a small indistinct 'head'. This is more apparent in the spermatozoa to be found in the uterus than in those lying in the testis and vesicula seminalis.

It seems that the whole spermatozoon is derived from nuclear material but it is not impossible that a small amount of

cytoplasm is involved. This seems unlikely, however, in view of the fact that the whole spermatozoon penetrates the oocyte prior to fertilisation. A similar state of affairs is described by Cable (1931) in Cryptocotyle lingua, Anderson (1937) in Proterometra macrostoma, Rees (1939) in Parorchis acanthus, and Markell (1943) in Probilotrema californiense. Pin Dji Chen is uncertain if this is the case in Paragonimus kellicotti and Woodhead (1931) working on the Bucephalidae states that the cytoplasm forms the tail of the spermatozoon.

Oogenesis. (Figs. 27-34)

Oogonia and primary oocytes only are found in the ovary, (Figs. 27 & 28). The nuclei of the oocytes differ from those of the spermatocytes in that they contain a distinct karyosome. This is usually spherical but does not appear to be homogeneous. The peripheral part stains very deeply and within this there appear to be two or three bodies which do not stain so intensely. The oogonia and oocytes are larger than the corresponding stages in spermatogenesis; oogonial divisions are normal mitoses.

The primary oocytes pass singly into the oviduct where it is assumed that they are penetrated by a spermatozoon although this has not been observed and may not be the case. The oocytes travel down the oviduct to the central chamber of Mehlis gland where they become surrounded by vitelline cells. The vitelline cells then give up the drops of shell-forming substances from their

Oogenesis in G. bathycotyle.

Fig. 27. Oogonia in ovary.

Fig. 28. Primary oocytes in ovary.

Fig. 29. Shell formation. Oocyte and vitelline cells in the central chamber of Mehlis gland.

Fig. 30. Primary oocyte - vitelline cells and shell omitted - with spermatozoon in cytoplasm. A spermatozoon which has failed to penetrate can be seen lying on the surface of the oocyte. The karyosome of the oocyte nucleus appears denser than in unpenetrated cells and the cytoplasm becomes more finely granular in appearance. The fertilisation membrane has not yet been formed.

Fig. 31. Oocyte with fertilisation membrane.

Fig. 32. First metaphase of the primary oocyte with three bivalents on the spindle and the spermatozoon becoming rounded.

Fig. 33. Second maturation division, with the nucleus of the first polar body in anaphase.

Fig. 34. Organisation of the male and female pronuclei.

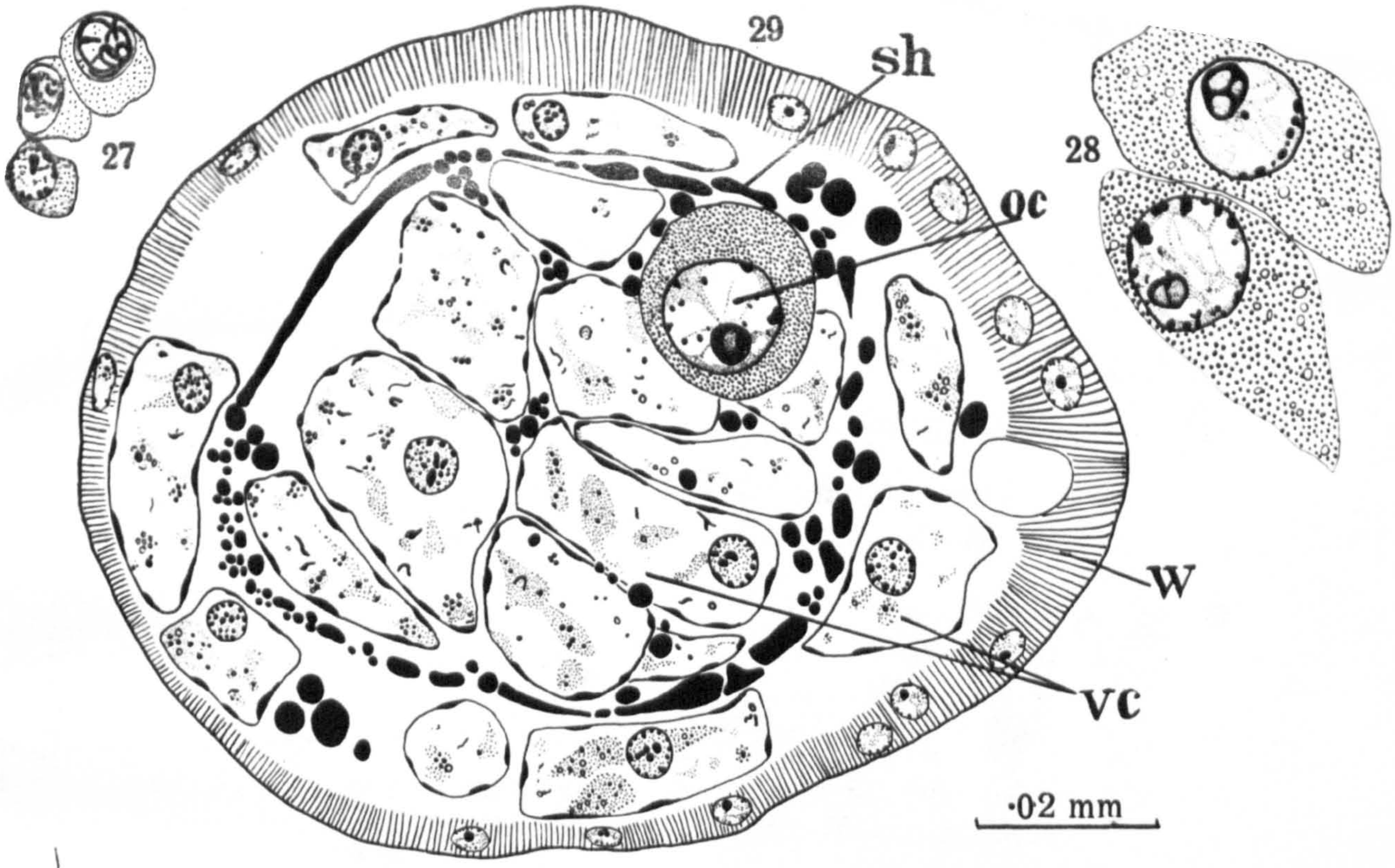
Cleavage in G. bathycotyle.

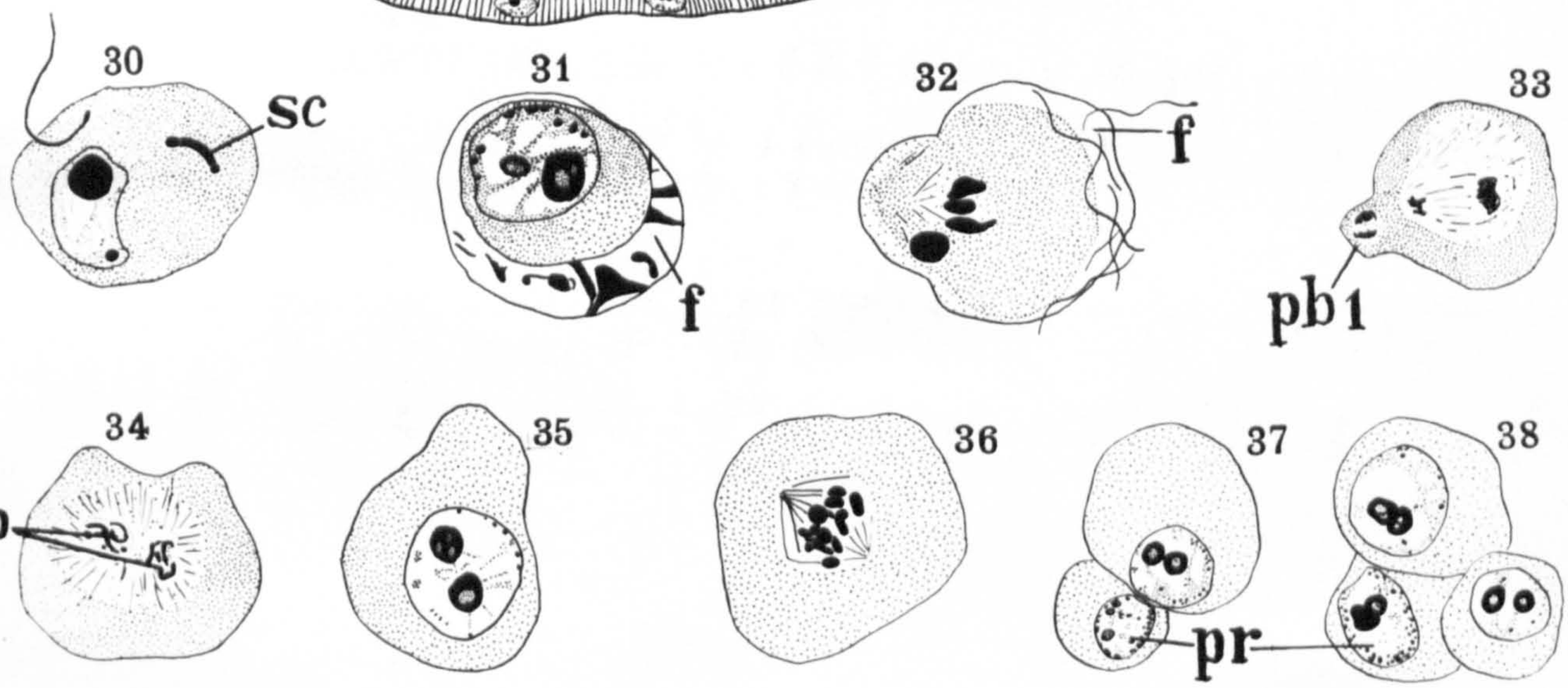
Fig. 35. Fertilised ovum with fusion nucleus.

Fig. 36. First cleavage division. Metaphase.

Fig. 37. Two celled stage.

Fig. 38. Three celled stage.



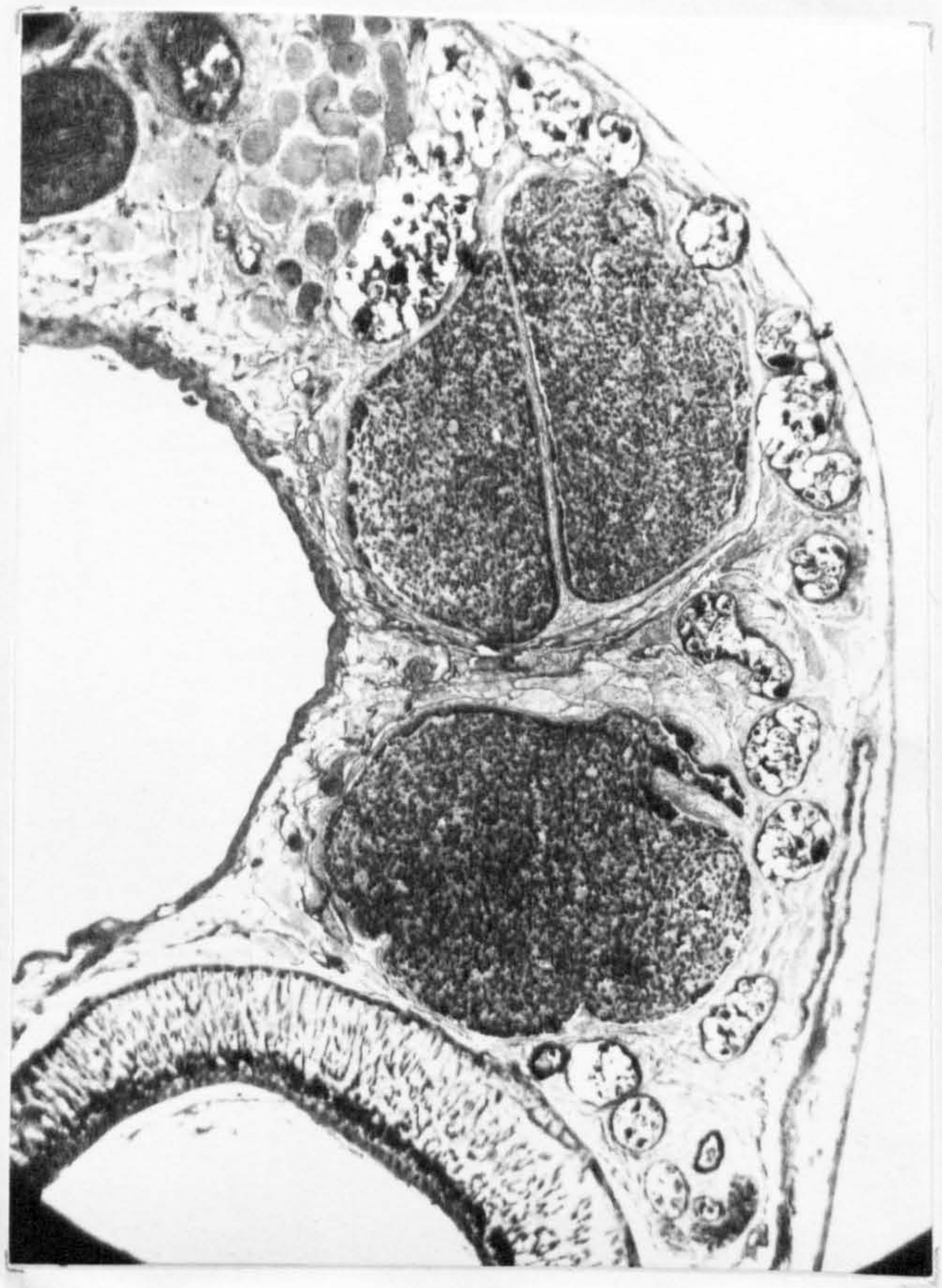


Figs. 27 – 38

cytoplasm and these drops pass to the outside of the group of cells where they coalesce to form the shell. (Fig. 29). At this stage the shell is very plastic. A few vitelline cells may remain outside the egg. Within the egg the cytoplasm of the vitelline cells breaks down but the nuclei persist for a considerable time.

In the most proximal part of the uterus a fertilisation membrane appears around the oocyte and the long threadlike spermatozoon can be seen within the cytoplasm. (Figs. 30 & 31). It seems probably that if the oocyte is penetrated by a spermatozoon in the oviduct the fertilisation membrane would be formed there. As, however, it is not apparent until the egg is in the uterus it is possible that spermatozoa are enclosed within the shell and that penetration does not take place until later. This membrane only persists for a short time and disappears by the time the spindle of the first maturation division is formed. (Fig. 32).

The primary oocyte nucleus remains unchanged until the egg has passed into the uterus. The spermatozoon within the cytoplasm becomes shorter and broader but remains a densely staining compact body for some time. When the spermatozoon has reached this stage the first division of the oocyte nucleus takes place very rapidly. None of the early stages of prophase have been observed, although a few nuclei were found in metaphase. A spindle is formed and six bivalents, resembling those of primary spermatocytes, appear on the equatorial plate. Anaphase follows and the first polar body



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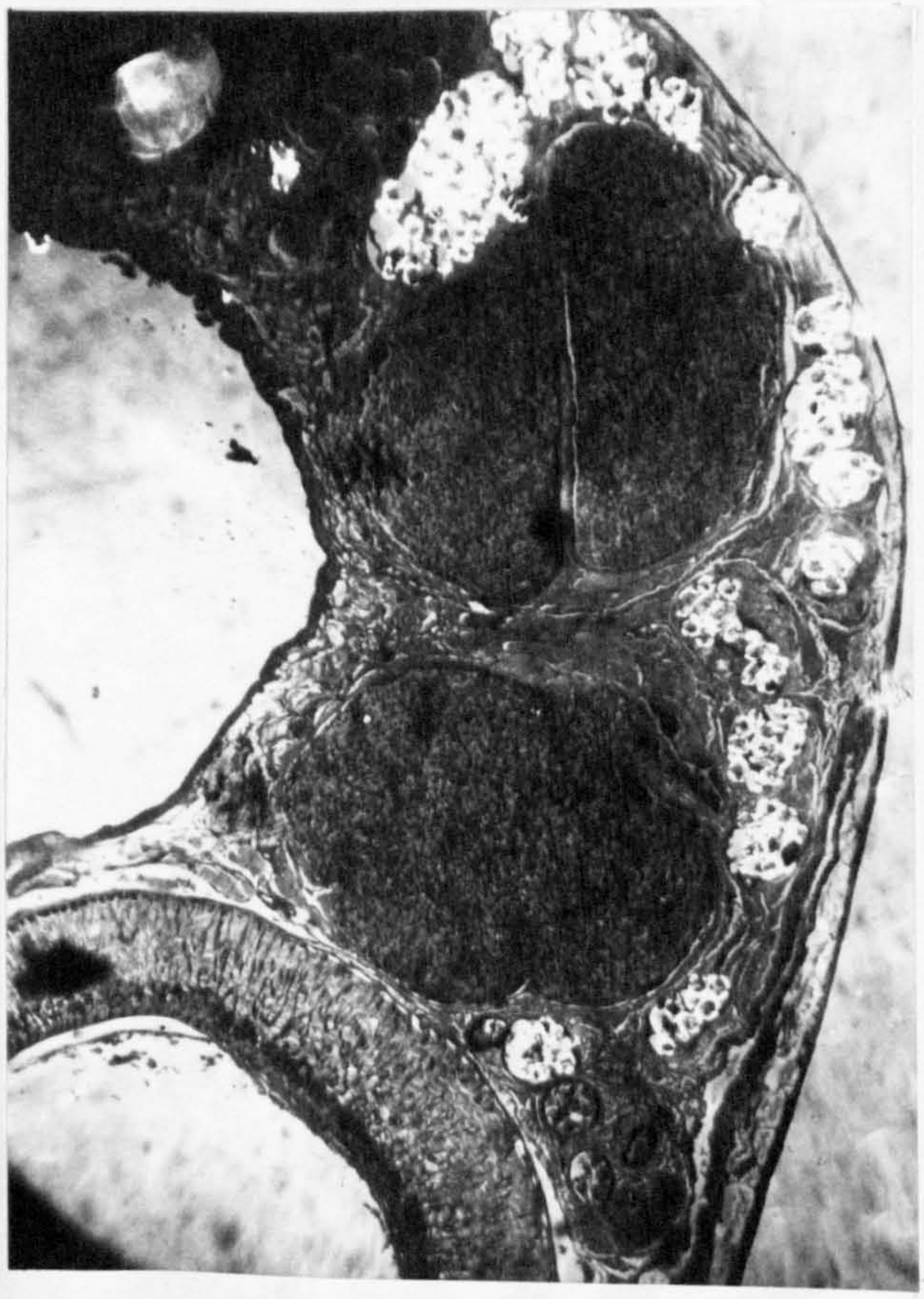

2

Photograph 1. Section of *A. bathycotyle* with ordinary light.

Photograph 2. Section of *A. bathycotyle* with polarised light.

is extruded; this may, or may not, divide again, but in one case was observed in anaphase. There is no interphase, the second division following immediately and a second polar body is given off. (Fig. 33).

While these divisions are taking place, the spermatozoon rounds up to form the male pronucleus and chromosomal threads become distinguishable. The chromosomes of the secondary oocyte pass into a resting stage and a nuclear membrane is formed. This is the female pronucleus. Both pronuclei possess a single karyosome and are indistinguishable from one another. (Fig. 34).

Fusion of the pronuclei has not been observed but a number of cells show a single large nucleus in a resting condition, which contains two karyosomes, and it is assumed that this is a fusion nucleus. (Fig. 35).

Tanning of the egg shell takes place gradually along the whole length of the uterus. When first formed, the shell is not birefringent but becomes increasingly so as it travels up the uterus. (Photographs 1 & 2).

CHROMOSOMES (Fig. 19).

It is not possible to make accurate counts or descriptions of the chromosomes at any stage before diakinesis and metaphase of meiosis. At this stage six bivalents are distinguishable; it is therefore concluded that the diploid number of chromosomes for this

species is twelve, the complement being made up of eight short and four long chromosomes. It is difficult to distinguish individual chromosomes.

The only other member of the family Paramphistomidae which has been studied cytologically is Diplodiscus temporatus. In this form the chromosome number is given as sixteen (Cary, 1909).

CLEAVAGE (Figs. 36-38)

The first cleavage division is a normal mitosis and gives rise to two cells of unequal size, (Fig. 36). These probably correspond to the 'ectodermal' and 'propagatory' cells described by Ishii (1934) in the development of Fasciolopsis buski. Similar cells have been reported in Paragonimus kellicotti by Pin Dji Chen (1937) and in Parorchis acanthus by Rees (1939). The larger of the two cells then divides again, the 'propagatory' cell remaining unchanged, (Figs. 37 & 38).

Owing to the difficulty in getting fixatives to penetrate the egg shell and the extreme brittleness of the shell which results in tearing and distortion in the sections, it has not yet been possible to follow cleavage any further.

DISCUSSION

The number of papers on gametogenesis in trematodes is not large. The earlier work has been admirably reviewed by Brooks (1930). Since then descriptions have been published by Cable (1931)

in Cryptocotyle lingua, Woodhead (1931) in the family Fucophalidae, Pennypacker (1938 and 1940) in Pneumonoeces medioplexus and P. similiplexus, Anderson (1937) in Proterometra macrostoma, Chen (1937) in Paragonimus kellicotti, Rees (1939) in Parorchis acanthus, and Markell (1943) in Probilotrema californiense.

There are a number of differences in these accounts which seem to have arisen from three main causes, namely, a lack of definition of terms, variations in appearances caused by the use of different fixatives and different interpretations based on early literature on cytology. The only account which seems to contain really fundamental differences is that given by Woodhead (1931) in the BUCEPHALIDAE.

It seems unlikely in the light of recent cytological studies that the chromosomes are, at any stage, in the form of a continuous spireme as described by Woodhead, Chen and Anderson. They certainly do not appear to be in Gigantocotyle bathycotyle. Cable states that the filament may be continuous, but that such continuity has not been traced. He also notes the double appearance of the threads before loop formation (pachytene) showing that the homologous chromosomes have undergone pairing.

The account of the process in the Bucephalidae given by Woodhead is not very clear but the following differences from other accounts are apparent:

(a) Groups of spermatogonia fuse before their nuclei undergo reduction division.

(b) The nuclei of the spermatocytes become smaller before division takes place.

(c) The cytoplasm forms the tail of the spermatozoon.

Woodhead does not say if the whole of the spermatozoon penetrates the oocyte.

SUMMARY

1. The anatomy of the genitalia of Gigantocotyle bathycotyle is described.

2. An account is given of gametogenesis, egg-shell formation, and the first two cleavage divisions.

3. The chromosome number is given as n=6, 2n=12.

ACKNOWLEDGMENTS

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THE SPECIES OF THE GENUS PARAMPHISTOMUM FISCHOEDER 1901 WHICH OCCUR IN THE BRITISH ISLES, WITH NOTES ON SOME MATERIAL FROM THE NETHERLANDS AND FRANCE.

INTRODUCTION.

It has hitherto been assumed that the only paramphistome parasitising ruminants in the British Isles is Paramphistomum cervi (Zeder 1790) Fischoeder 1901. The incidence is slight and only three records of its occurrence have been found in the literature. It was recorded by Pillers (1922) from a cow in Cheshire, by Craig and Davies (1937) from sheep in Cheshire and by Kelly (1948) from cattle in several districts in Eire. In spite of this there have been a number of unconfirmed reports of its being found in various districts. Two veterinary surgeons state that they have found paramphistomes in the rumen of sheep in Herefordshire. The author has also been told of theirbeing found in the rumen of a cow in this country and it was suggested that the parasites had been introduced into Herefordshire by Canadian store cattle. There does not appear to be any definite evidence for this and in view of the specificity of the miracidia for particular snail hosts which several workers have reported it seems improbable that the trematode would have been able to establish itself. During the autumn of 1948 the abattoirs at Hereford and Leominster were visited but although

many hundreds of sheep and cattle from the surrounding districts were examined no paramphistomes were found.

The first specimens of paramphistomes from this country which were sent to the author were collected from a cow in the Isle of Mull. During a visit to the island only two beasts out of the two herds whose faeces were examined proved to be infected. The faeces of twenty sheep which had been grazed over the same area were also examined but all were free of paramphistome eggs. Unfortunately the infected beasts had not been bred on the island but bought on the mainland three or four years previously. A very large number of snails of various species were collected but none were infected. It therefore seems probable that the right species of snail to act as intermediate host was not present and that the parasite had been unable to establish itself.

Two visits were paid to the Municipal Abattoir in Glasgow. On the first occasion three rumens from Irish cattle were found to be infected and on the second out of some five hundred rumens of Scottish cattle examined only one was found to be infected. All the infections were heavy. A third collection, believed to be from Scottish cattle, and three collections from Eire were sent to the author. It has also been possible to examine two collections from the Netherlands and two from France.

While comparing sections of specimens from the various sources it became obvious that there were three different species present. One of these can be identified according to Nasmark's

system of classification as P. cervi but the other two both show a number of characters which are different from those of any hitherto described species. They are therefore regarded as new, and named and described below.

A very short account of gametogenesis and the early development is also given.

MATERIAL AND METHODS

The material collected from the abattoir, Glasgow, was fixed in 10% formalin, Bouin's fluid or Carnoy (6:3:1). The specimens from the Isle of Mull had been fixed in 10% formalin, those from Eire in formol saline, 5% formalin or 70% alcohol, those from the Netherlands in 70% alcohol or 10% formalin and those from France in 10% formalin.

Specimens for sectioning were cleared in cedarwood oil and embedded in paraffin wax with ceresin, congealing point about 54°C.

Transverse, horizontal and sagittal sections were cut, the thickness varying from 4 μ -10 μ. Those 6 μ -8 μ in thickness proved most satisfactory. Thick hand sections were also cut and a number of worms dissected under the binocular microscope in order to show the appearance of the testes.

Serial sections were stained with Ehlich's haematoxylin and eosin, Weigerts iron haematoxylin without counterstain or counterstained with van Gieson's picrosaurofuchsin and

KEY LETTERING.

a - acetabulum
bcm - basal circular muscle
c - gut caecum
cc - central chamber of Mehlis gland
cm - circular muscle
cu - cuticle
de 1 - dorsal external circular muscle, first series
de 2 - dorsal external circular muscle, second series
di - dorsal internal circular muscle
e - excretory bladder
ecm - external circular muscle
ed - excretory duct
elm - external longitudinal muscle
ep - excretory pore
ep' - epithelial cell of testis wall
f - fertilisation membrane
fi - fibrous tissue of testis wall
g - gland cell
ga - genital atrium
i - intracellular ducts
icm - internal circular muscle
ilm - internal longitudinal muscle
k - karyosome
L - Laurer's canal
Lo - opening of Laurer's canal to exterior
lm - longitudinal muscle
M - Mehlis gland
mcm - middle circular muscle
n - nerve
nf - nonfibrous layer of testis wall
o - ovary
oe - oesophagus
on - oocyte nucleus
pa - papilla
pa' - parenchyma
ph - pharynx
pm - para musculosa
pp - para prostatica
rm - radial muscle
sp - spermatozoon
st - spermatogonial tissue
t - testes
r - reservoir.

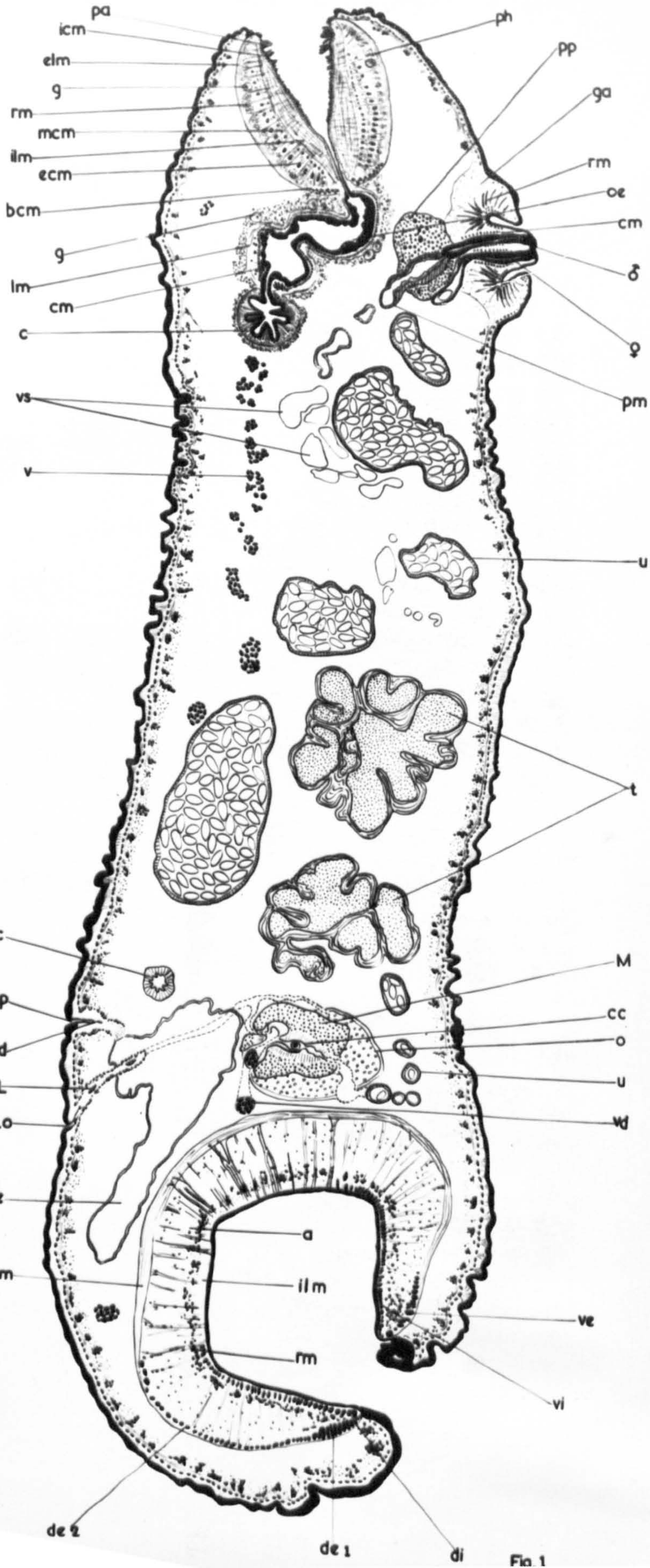
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Fig. 1

Heidenhain's iron haematoxylin without counterstain. Hand sections were stained with Borax-carmine.

Drawings were made with the aid of a camera lucida. The sagittal sections of the two new species are composite drawings. Measurements were taken on whole worms, and on sections.

Type material of the two new species is deposited in the collection of the Department of Parasitology of the London School of Hygiene and Tropical Medicine.

Paramphistomum hibernine n.sp.

Geographical distribution Ireland, Scotland, The Netherlands.

Host Bos taurus.

Habitat Rumen

Specific diagnosis

Length 4.9 mm, breadth 1.9 mm, dorsal-ventral 1.9 mm.

Dorsal line, very slightly curved.

Acetabulum, internal diameter, 0.95 mm.

Proportion to body length, 1:5.7. Type, Paramphistomum

Pharynx, length 0.71 mm. Proportion to body length, 1:7.7, Type, modified Liorchis.

Oesophagus length 0.49 mm.

Genital atrium. Type, Ichikawai, on a level with the oesophagus.

Testes one behind the other, small, almost spherical, extremely highly lobed with a muscular sheath.

Ovary ovoid to spherical, posterior to testes.

Excretory duct short.

Description

Habit Before fixation the worms were pinkish to red. They were found in large numbers at the bottom of the oesophageal groove and between the villi of the rumen. The body is straight or with an even slight curve.

Size Measurements taken after fixation.

Length:	4mm - 7mm	Average 4.9mm
Breadth:	1.5mm - 2.2mm	Average 1.9mm
Dorsal-Ventral	1.6mm - 2.2mm	Average 1.9mm

Acetabulum Paramphistomum type.

Measurements were taken on sagittal sections. The external diameter is taken from the membrane which delimits the tissue of the acetabulum from the body parenchyma; the internal diameter is the diameter of the cavity of the acetabulum.

External diameter	1.5mm - 1.7mm	Average 1.65mm
Internal diameter	0.9mm - 1.0mm	Average 0.95mm
Internal diam./body length	1/5.0 - 1/6.2	Average 1/5.7
Diameter of opening	0.2mm - 0.5mm	Average 0.38mm

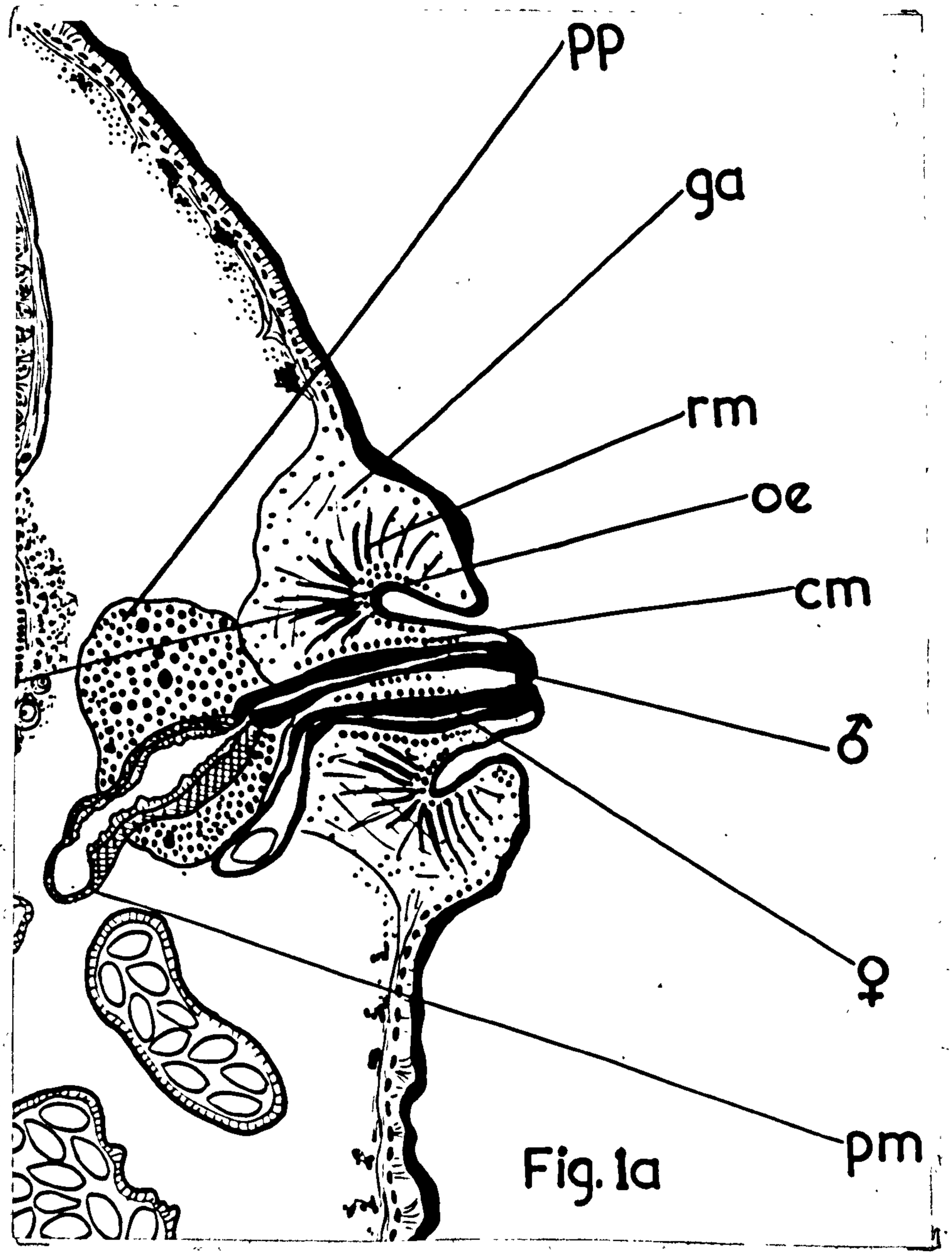

Fig. 1a

Fig. 1a. P. hiberniae, genital atrium.

Circular muscles	No. of units	Average
dorsal external 1	15-23	19
dorsal external 2	26-39	30
dorsal internal	42-48	46
ventral internal	47-53	50
ventral external	17-22	19

Pharynx (Fig. 1b)

Modified licrchis type.

The middle and external circular muscle layers are better developed in the posterior two-thirds of the pharynx. At the anterior end they are quite indistinct. The papillae are fairly long round the opening of the pharynx to the exterior but become progressively smaller towards the oesophageal end, where they are inconspicuous or lacking. Under an oil immersion objective it is possible to distinguish strands running into the papillae from amongst the band of longitudinal and radial muscles. These are believed to be nerves and the papillae to have a sensory function (Fig. 4) There are also a number of large uninucleate cells with clear cytoplasm which are believed to have a glandular function. Similar cells can be seen in the epithelium surrounding the oesophagus.

Length 0.64mm - 0.8mm Average 0.71mm

Length/Body length 1/5.4 -1/8.8 Average 1/7.7

Oesophagus

This is fairly straight, short.

Length 0.45mm -0.54mm Average 0.49mm

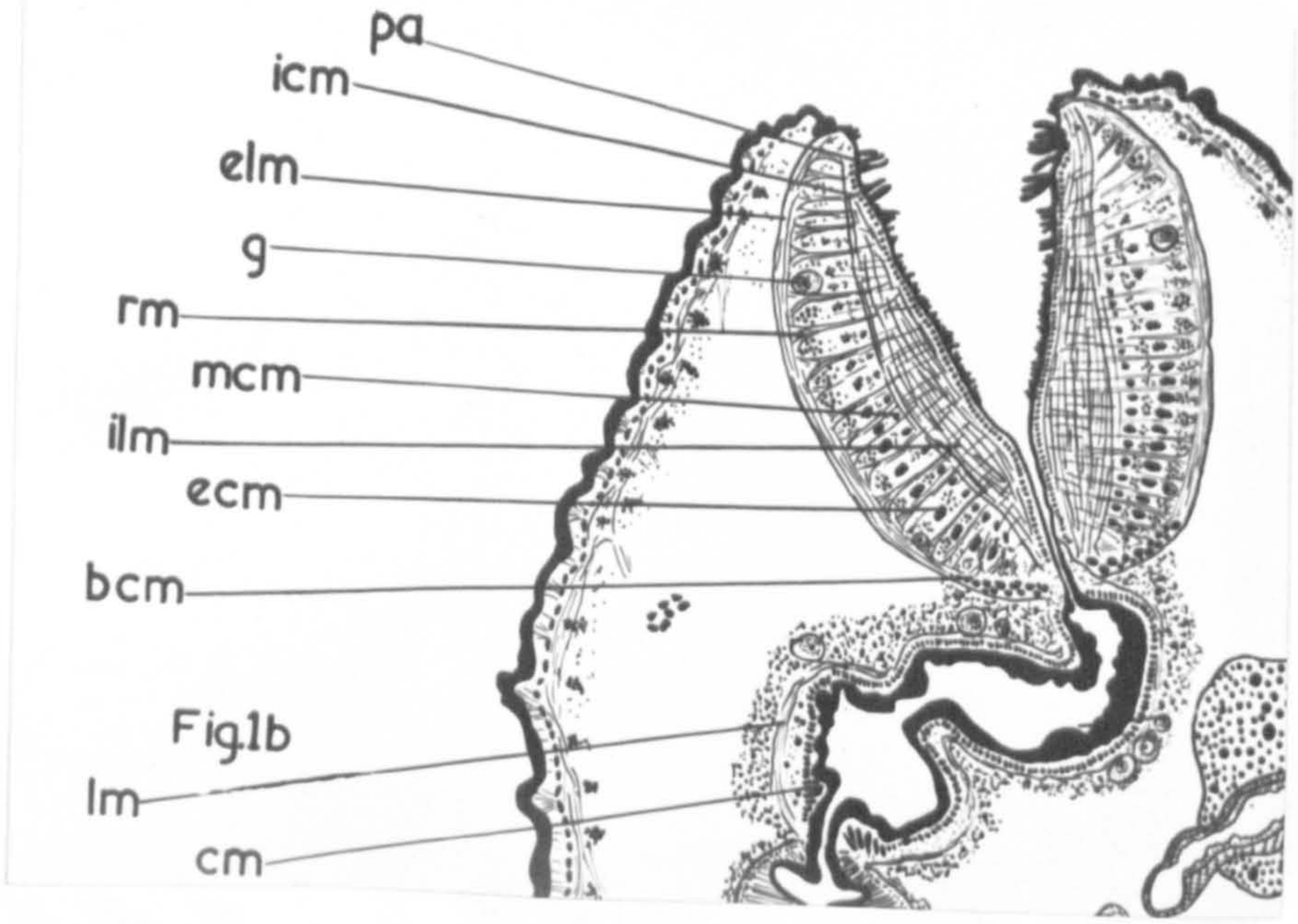

Fig. 1b. P. hiberniae, pharynx.

Genital atrium (Fig. 1a)

Ichikawai type, with very strongly marked radial muscles. The atrium does not lie very far anteriorly and is about on the level of the middle of the oesophagus.

Testes (Figs. 1,2,3)

These are small and spherical and lie one behind the other. Both are about the same size and they are usually some distance apart. The average measurements are:- the anterior testis, 0.62mm X 0.59mm X 0.60mm and the posterior 0.60 mm X 0.57mm X 0.62mm. They are extremely deeply lobed, both in longitudinal and transverse section and have a very fibrous wall in which both circular and longitudinal muscle elements are present, (Figs. 2 & 3).

The spermatogonial cells are small and only comparatively few spermatozoa are produced at a time. This is shown by the very few spermatozoa which are present in the vesicula seminalis although they are often numerous in the uterus. The peculiar development of the testis cannot be due to immaturity as in some specimens the uteri are packed with eggs.

Ovary (Fig. 1)

The ovary lies between the posterior testis and the acetabulum. It is roughly spherical and contains oogonia and primary oocytes. The oviduct leaves the ovary on the anterior border and runs dorsally and slightly posteriorly round Mehlis

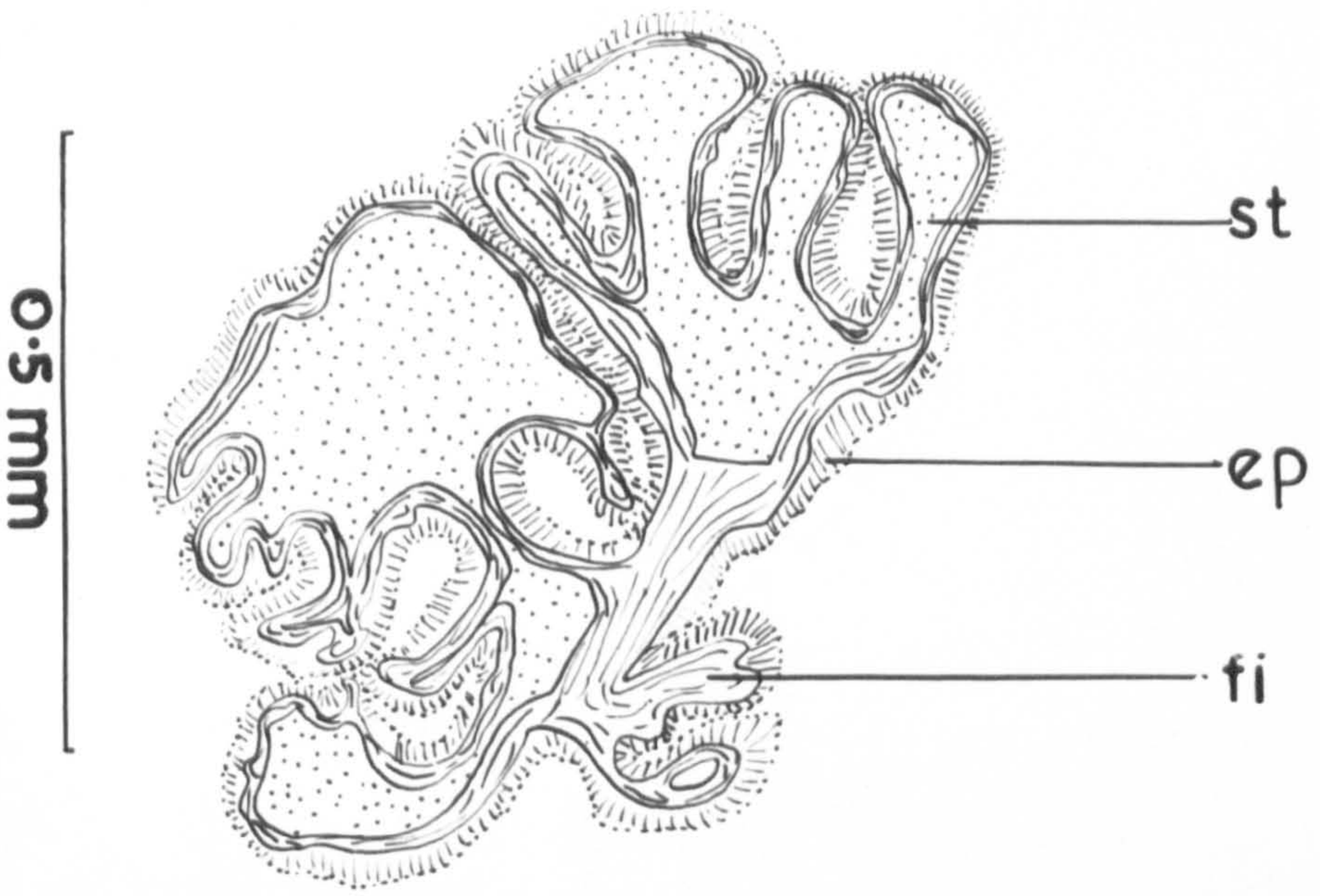

Fig. 2

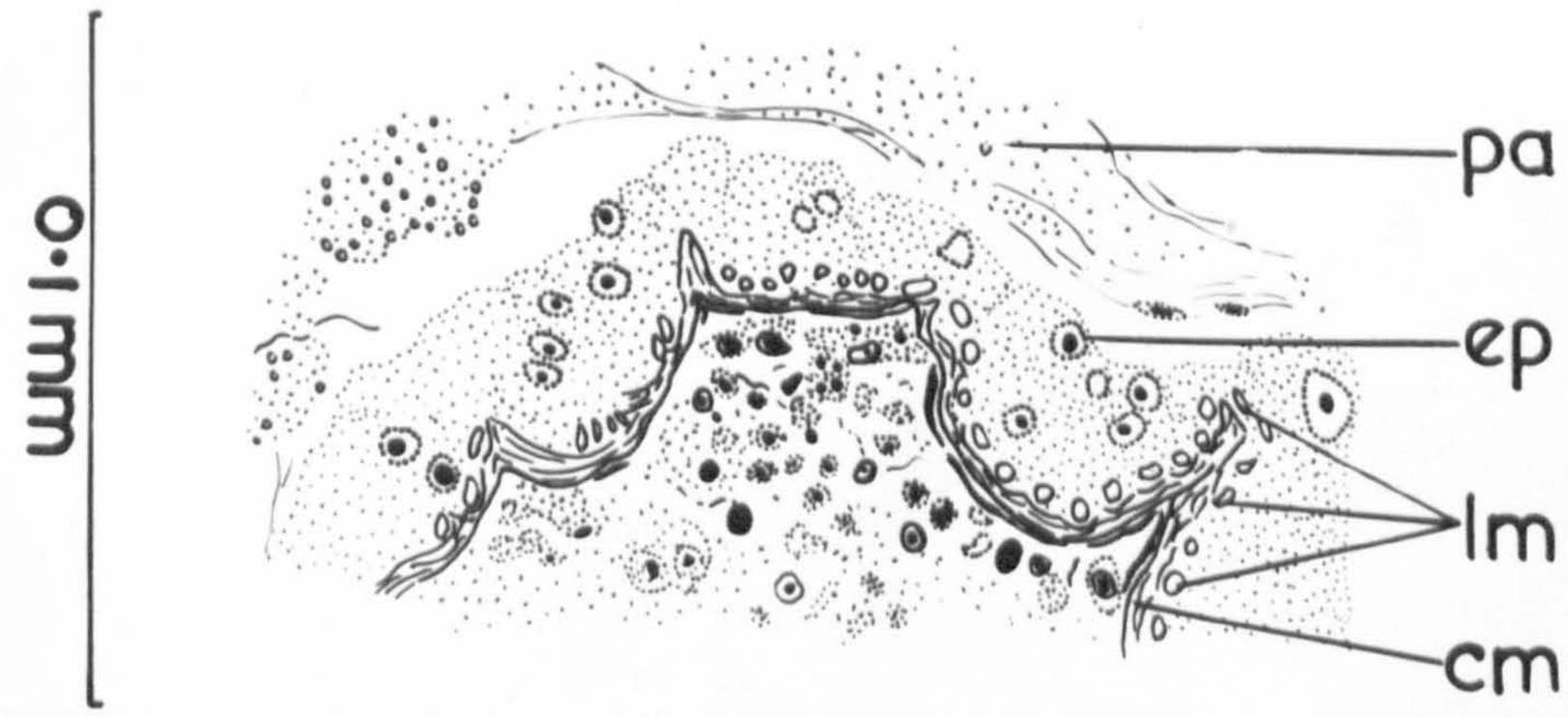

Fig. 3

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Fig. 2. P. hiberniae transverse section of testis.

Fig. 3. P. hiberniae higher magnification of testis wall.

gland until it joins Laurer's canal. It then runs into Mehlis gland and is joined by the vitelline duct, forming the central chamber.

Mehlis gland (Fig. 1)

This lies very close to, and almost on the same level as the ovary. There is a distinct region of intracellular ducts, very similar in extent to that described for Gigantocotyle bathycotyle. Laurer's canal runs from it and is joined by the oviduct just outside Mehlis gland. It then turns posteriorly and dorsally and opens to the exterior a short distance behind the opening of the excretory pore. Although neither vitelline material nor cells were found in Laurer's canal, they were visible in one series in that part of the duct between the central chamber and the junction of the oviduct with Laurer's canal. This indicates that Laurer's canal may serve as a way through which surplus vitelline material may be passed to the exterior as has been suggested.

Uterus In specimens with only a few eggs the uterus is narrow and only very slightly folded. As it becomes packed with eggs it spreads to occupy almost the whole body botween the oesophagus and the testes.

Vitellaria These are follicular and extend from the level of the pharynx almost to the posterior end of the worm. There are a pair of vitelline ducts which join between Mehlis gland and the acetabulum to form a small vitelline reservoir. From this a short

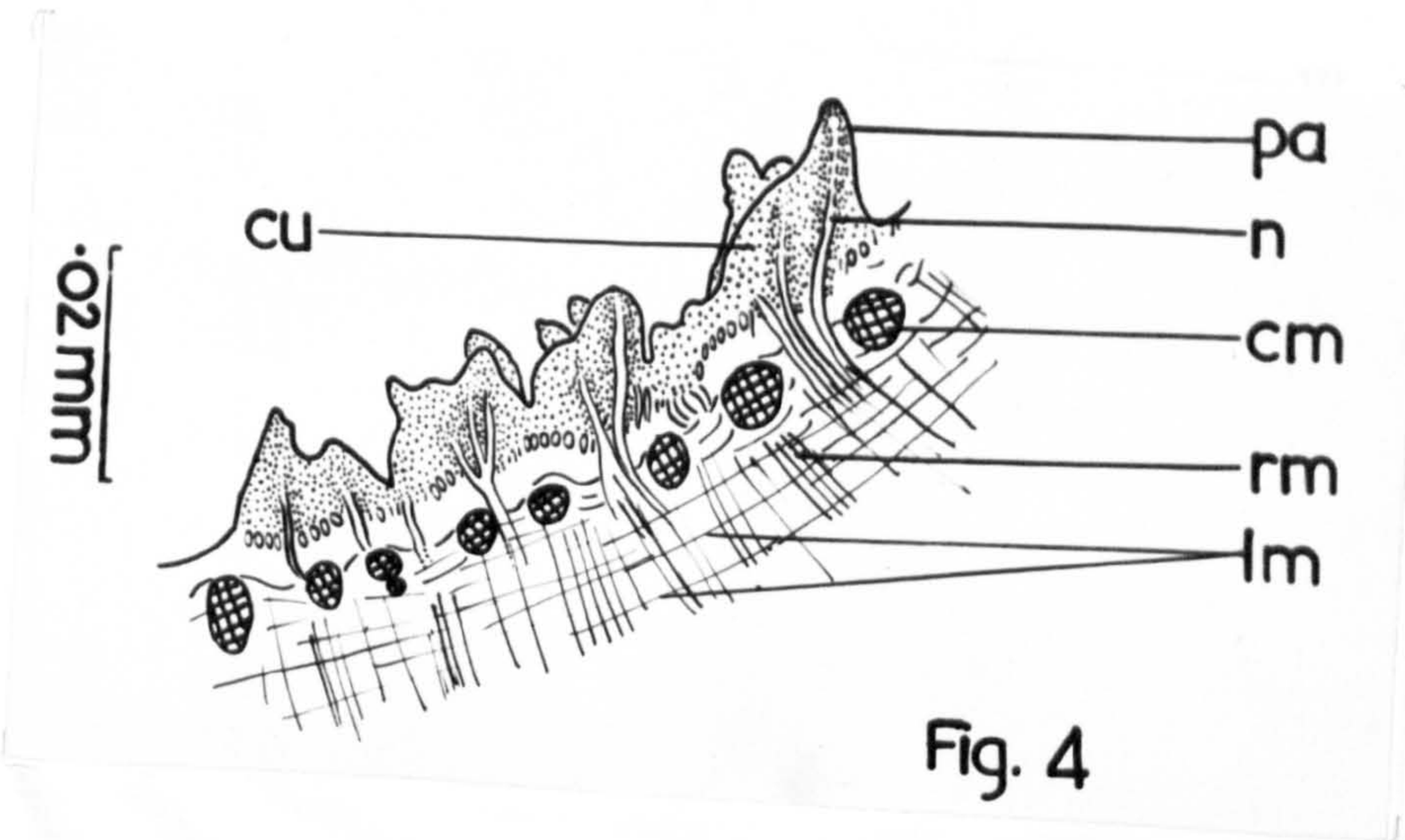

Fig. 4

Fig. 4. P. hiberniae pharyngeal papillae.

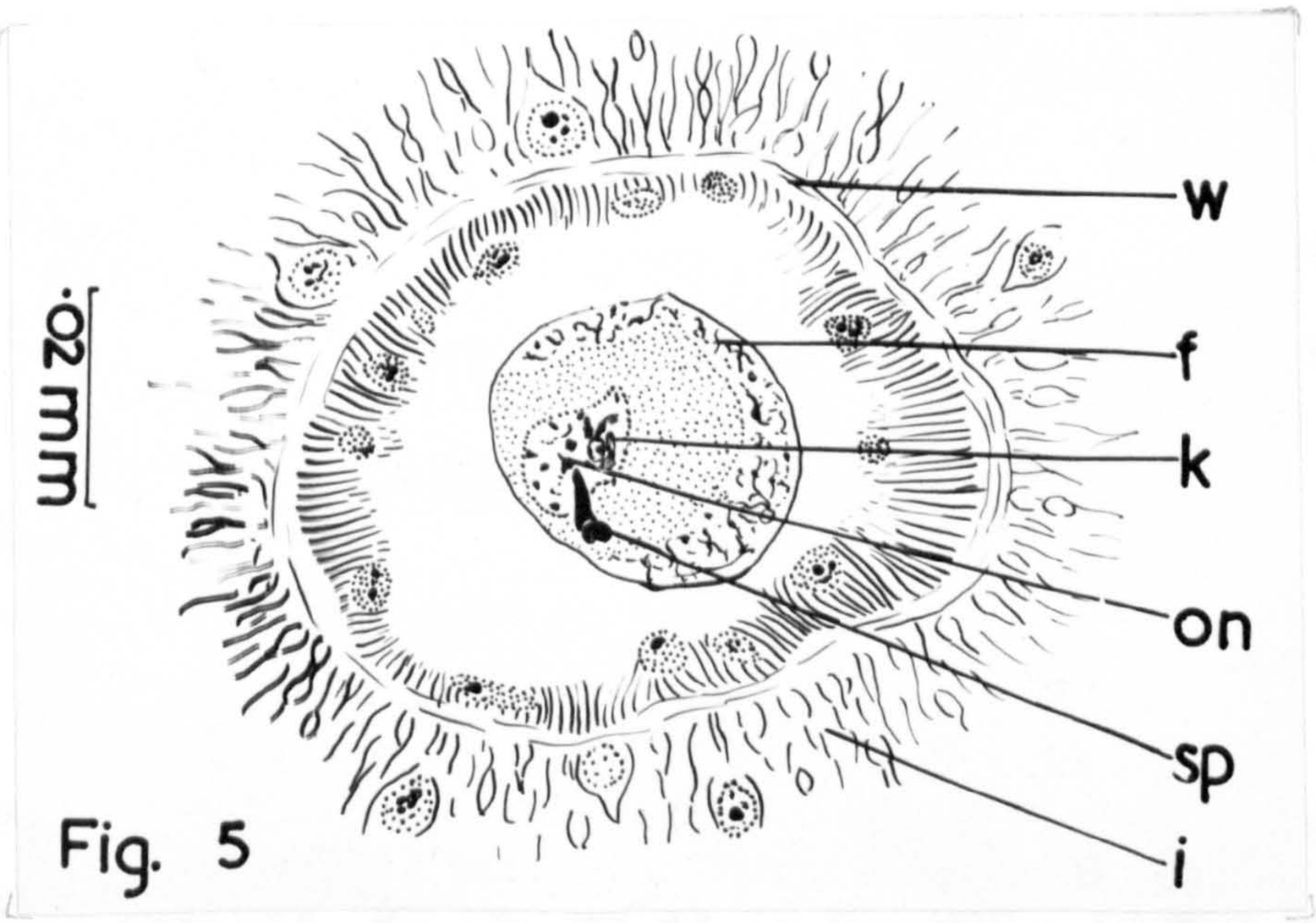

Fig. 5

Fig. 5. P. hiberniae primary oocyte in central chamber of Mehlis gland.

duct runs into the central chamber of Mehlis gland.

Excretory bladder (Fig. 1)

This is quite large and lies dorsally to the acetabulum but does not extend far anteriorly. It opens to the exterior by a very short duct, the pore being about the same level as the ovary.

Gametogenesis

Spermatogenesis.

Owing to the small size of the spermatogonial cells and the slowness with which divisions take place spermatogenesis is difficult to follow. As far as it is possible to say at present it proceeds in an exactly similar manner to that described for Gigantocotyle bathycotyle. A rosette is formed in which there are thirty-two spermatid nuclei, indicating that there are the usual three spermatogonial divisions followed by two reduction divisions. Very few nuclei could be picked out in stages of mitosis or meiosis and it is impossible to state with certainty how many chromosomes there are. In those cells where nuclear divisions could be seen normal spindles are formed and the chromosomes become orientated upon them at metaphase. (Figs. 5a &b) No centrosomes have been seen.

The haploid number of chromosomes is not less than six and not more than eight, eight occurring most frequently.

Oogenesis.

Only oogonial divisions take place in the ovary. The

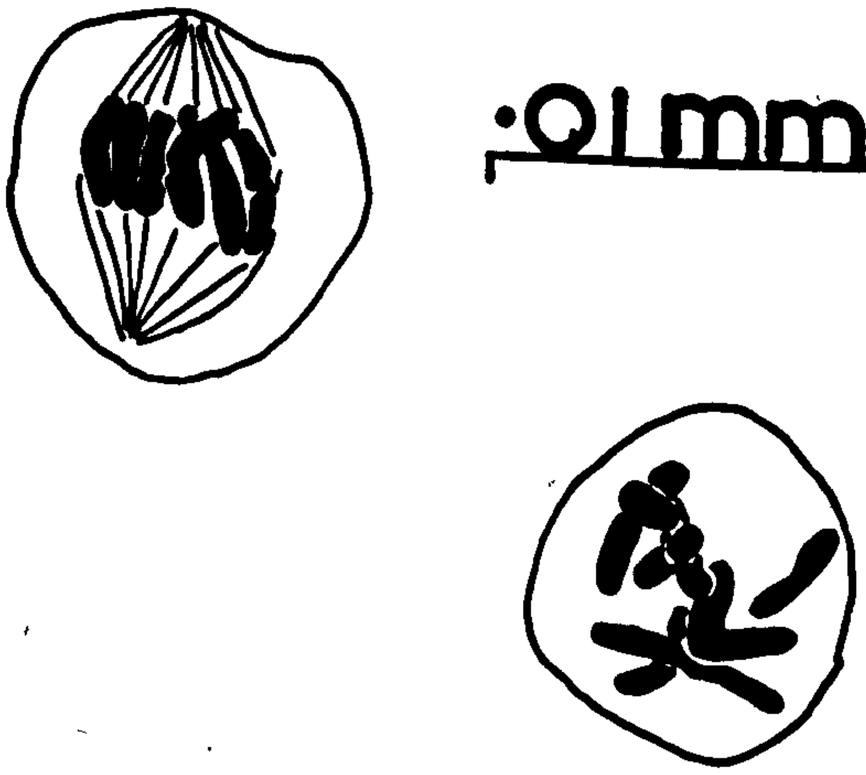

Fig.5a

Fig. 5a. P. hiberniae First metaphase chromosomes in primary spermatocytes.

oogonia form a cap on the outside of the ovary and the primary oocytes are in the centre and towards the opening of the oviduct. The primary oocytes enter the oviduct and are penetrated by a spermatozoon. The fertilisation membrane has formed by the time the oocyte reaches the central chamber of the Mehlis gland. (Fig. 5) The reduction divisions and the formation of the male and female pronuclei have not been observed.

Cleavage

The first cleavage division results in the formation of two unequal cells as has been described for a number of trematodes by various authors. These continue to divide until a vitelline membrane and an embryo of about eight to ten cells has developed. By this time the eggs are in the most anterior part of the uterus and are shortly laid. The vitelline membrane is probably derived from the ectodermal or larger cell of the first division, but again the brittleness of the egg shell causes a great deal of tearing in the sections and it is very difficult to follow the details of the divisions.

Paramphistomum scotiae. n.sp.

Geographical distribution Scotland, Eire.

Host Bos taurus

Habitat Rumen.

Specific diagnosis

Length 5.1mm, breadth 2.6mm, dorsal ventral 2.0mm.

Dorsal line strongly curved.

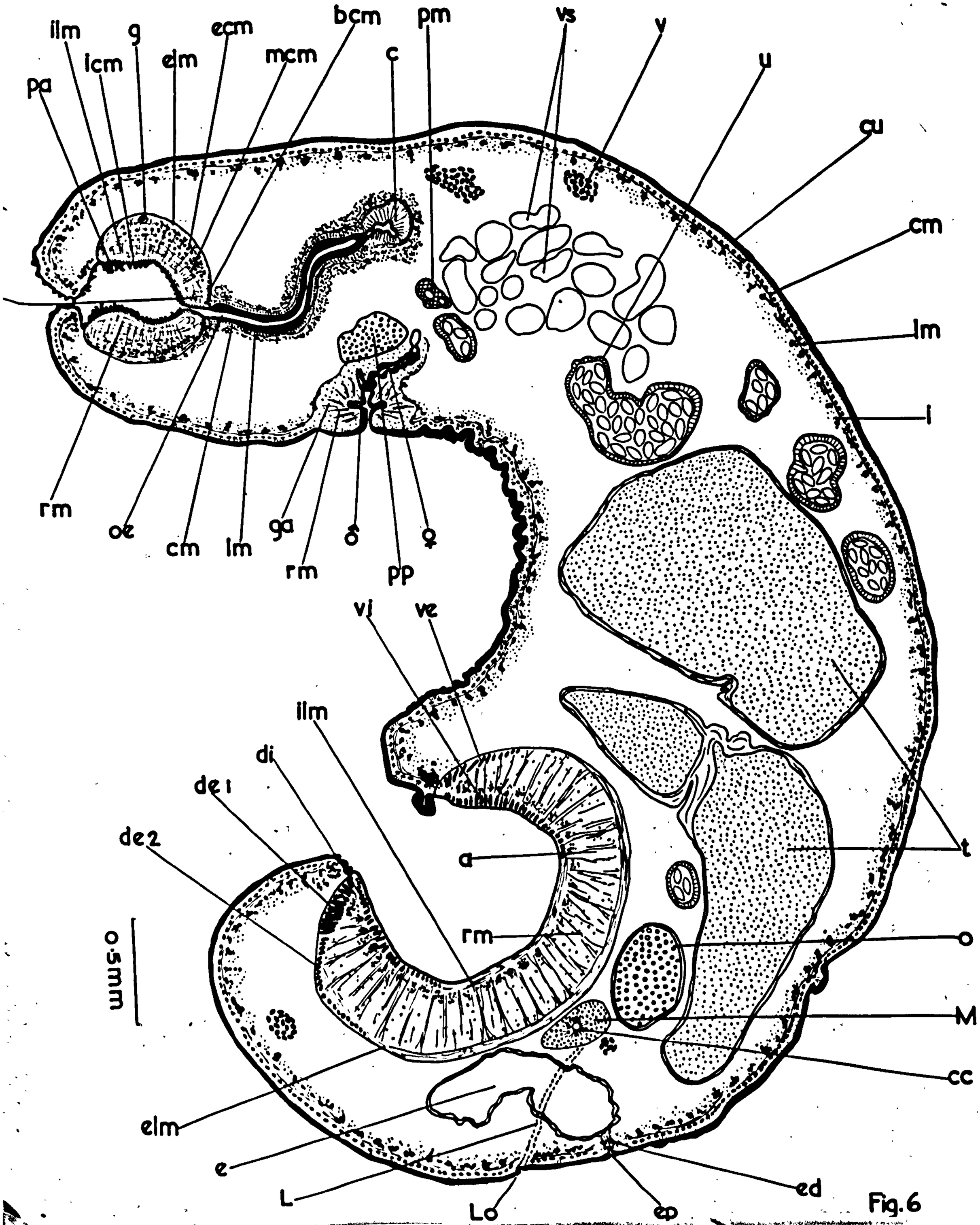

Fig.6

Fig. 6. Paramphistomum scotiae sagittal section.

Acetabulum, internal diameter 0.8mm, proportion to body length, 1:6.4. Type. Paramphistomum

Pharynx, length 0.62mm, proportion to body length 1:8.2. Type modified liorchis with well developed papillae.

Oesophagus, length 0.6mm.

Genital atrium. Type Epiclitum on a level with the posterior part of the oesophagus.

Testes, one behind the other, large with few lobes, the posterior often sickle-shaped in sagittal section, without a muscular sheath.

Ovary, ovoid to spherical, posterior to testis.

Excretory duct short.

Description

Habit These paramphistomes were not seen before fixation but they were reported to have been whitish. The body is strongly curved.

Size	Length	5.8mm - 6.0mm	Average 5.1mm
	Breadth	2.0mm - 3.0mm	Average 2.6mm
Dorsal-Ventral		0.9mm - 2.4mm	Average 1.0mm

Acetabulum Paramphistomum type.

Measurements were taken on sagittal sections:

External diameter	1.0mm - 2.0mm	Average 1.6mm
Internal diameter	0.4mm - 1.0mm	Average 0.8mm
Internal ~~drain~~ iam./body length	1/12.7 - 1/5.1	Average 1/8.5
Diameter of opening	0.2mm - 0.7mm	Average 0.4mm

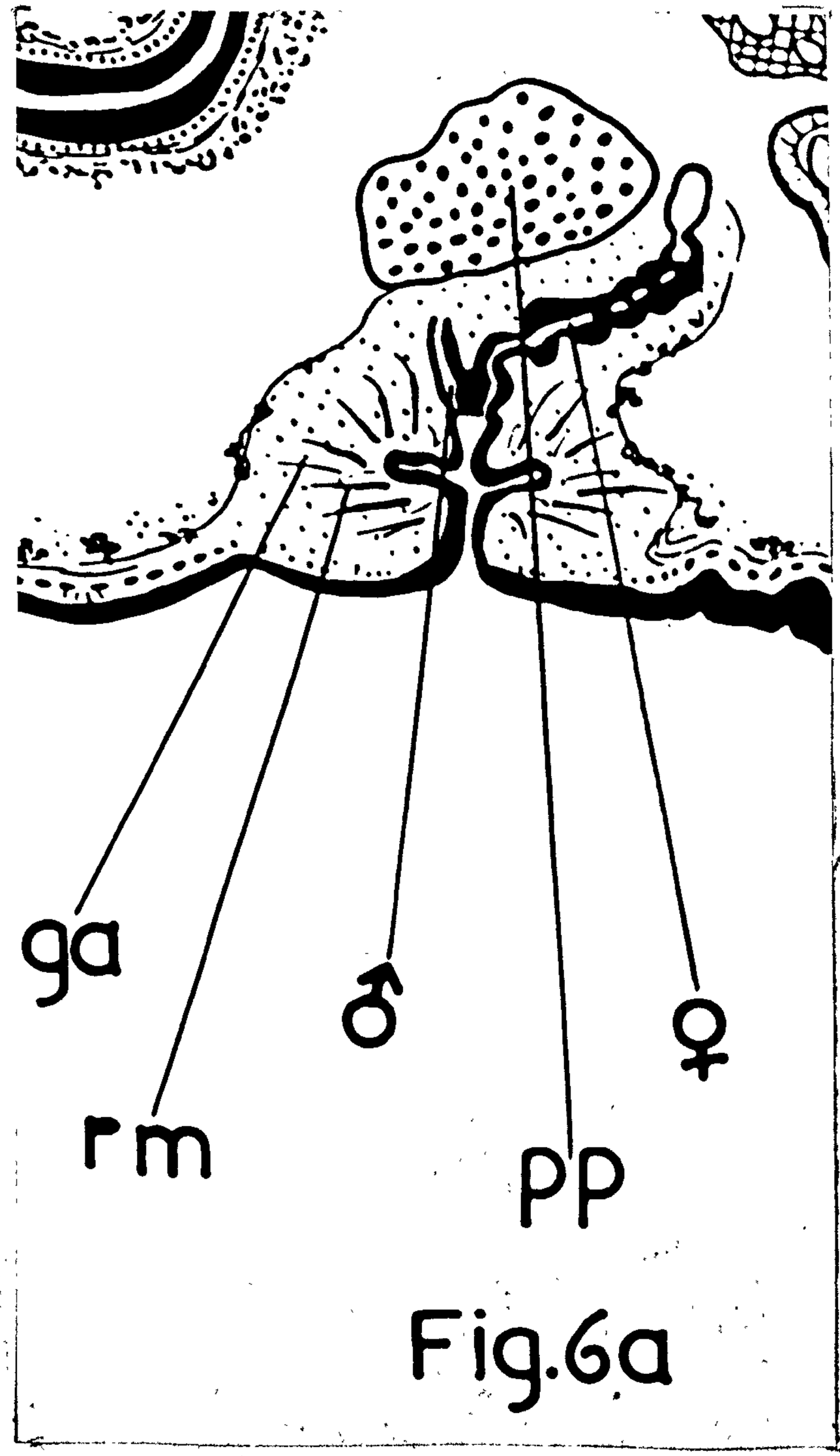

Fig.6a

Fig. 6a. P. scotiae genital atrium.

Circular muscles	No. of units	Average
dorsal external 1	13 - 21	18
dorsal external 2	18 - 30	27
dorsal internal	34 - 45	38
ventral internal	36 - 43	33
ventral external	13 - 16	15

Pharynx (Fig. 6b)

Modified liorchis type.

The pharynx is considerably retracted in all the specimens sectioned forming a funnel above the actual opening. The development of the muscular layers is very similar to that in P. hiberniae although the pharynx is shorter and more rounded than in this species. The papillae are more extensive than in P. hiberniae and seem to branch in many cases. (Fig 6c) Gland cells are present.

Length 0.5mm - 0.72mm Average 0.62mm

Length/body length 1/10 - 1/7 Average 1/8.2

Oesophagus (Fig. 6)

This is slightly longer than in the previously described species and not so straight.

Length 0.5mm - 0.9mm Average 0.56mm

Genital atrium (Fig. 6a)

Epiclitum type with fairly strongly developed radial muscles. It does not lie very far forward, being on a level with the posterior part of the oesophagus and the junction of the gut caeca.

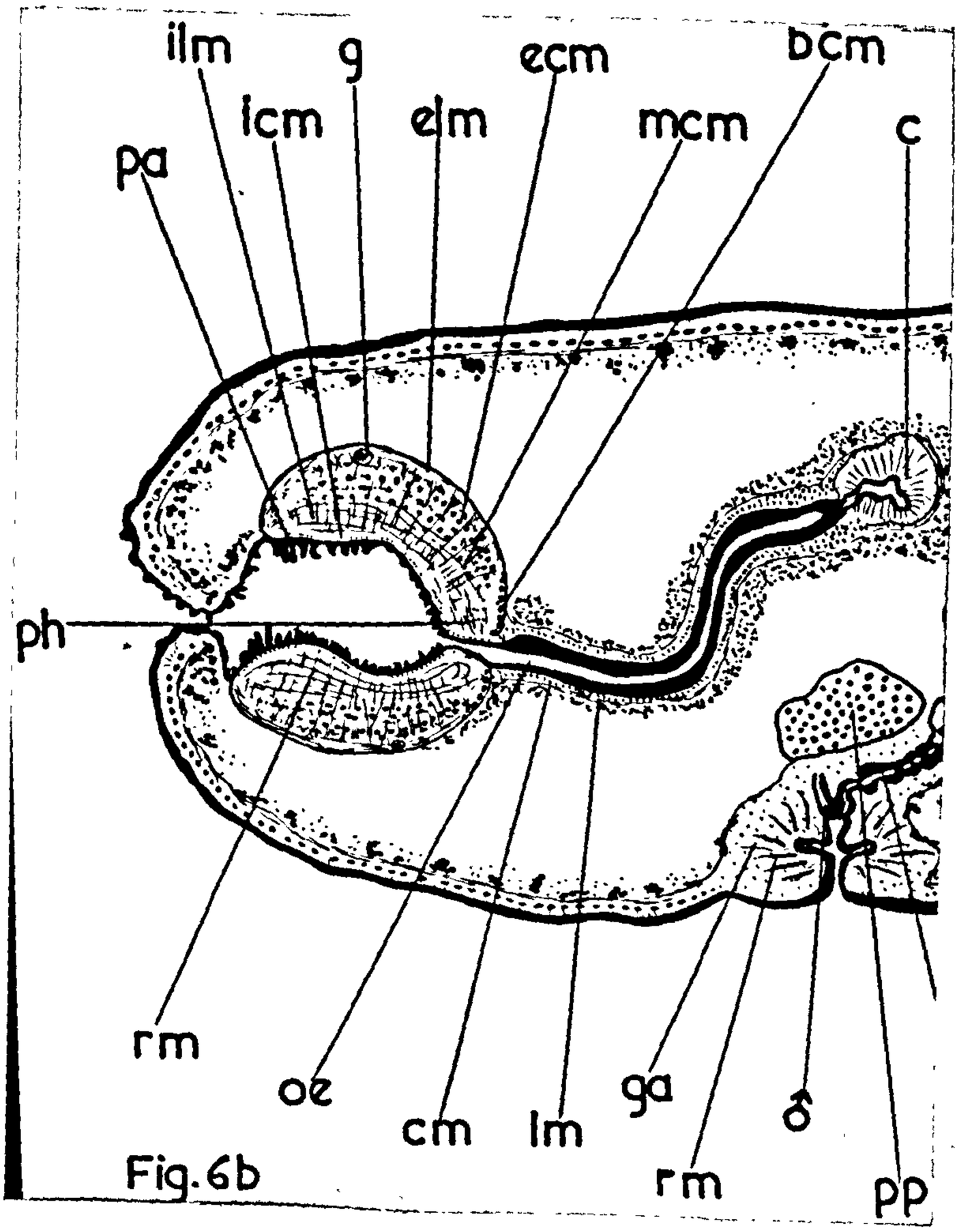

Fig. 8b. P. scotiae, pharynx.

Testes (Figs. 5 & 8)

These lie one behind the other and are comparatively large with a few lobes. The anterior one is rounded and the posterior often sickle-shaped in section. They are close together and the average measurements are:- anterior testis 1.1mm x 1.5mm x 1.2mm, posterior 1.2mm x 1.5mm x 1.6mm. The sheath which surrounds the testis contains no muscular elements, (Fig. 9).

The spermatogonial cells are much the same in size and appearance as in Gigantocotyle bathycotyle and nuclear divisions and spermatozoon formation goes on more rapidly than in P. hibernica

Ovary (Fig. 6)

The ovary is roughly spherical and lies posterior to the testes and dorsal to the acetabulum. There is a distinct region of cogonia and of primary oocytes. The oviduct leads from the latero-posterior border of the ovary to the outside of Mehlis gland, where it is joined by Laurer's canal. A joint duct leads into Mehlis gland.

Mehlis gland (Figs. 6 &78)

This lies behind and slightly to one side of the ovary. It is pearshaped or oval. The region of intracellular ducts is narrow and within it are a number of small round cavities which appear to be lined with some cuticular substance. There is no indication that they are large cells as the contents are quite

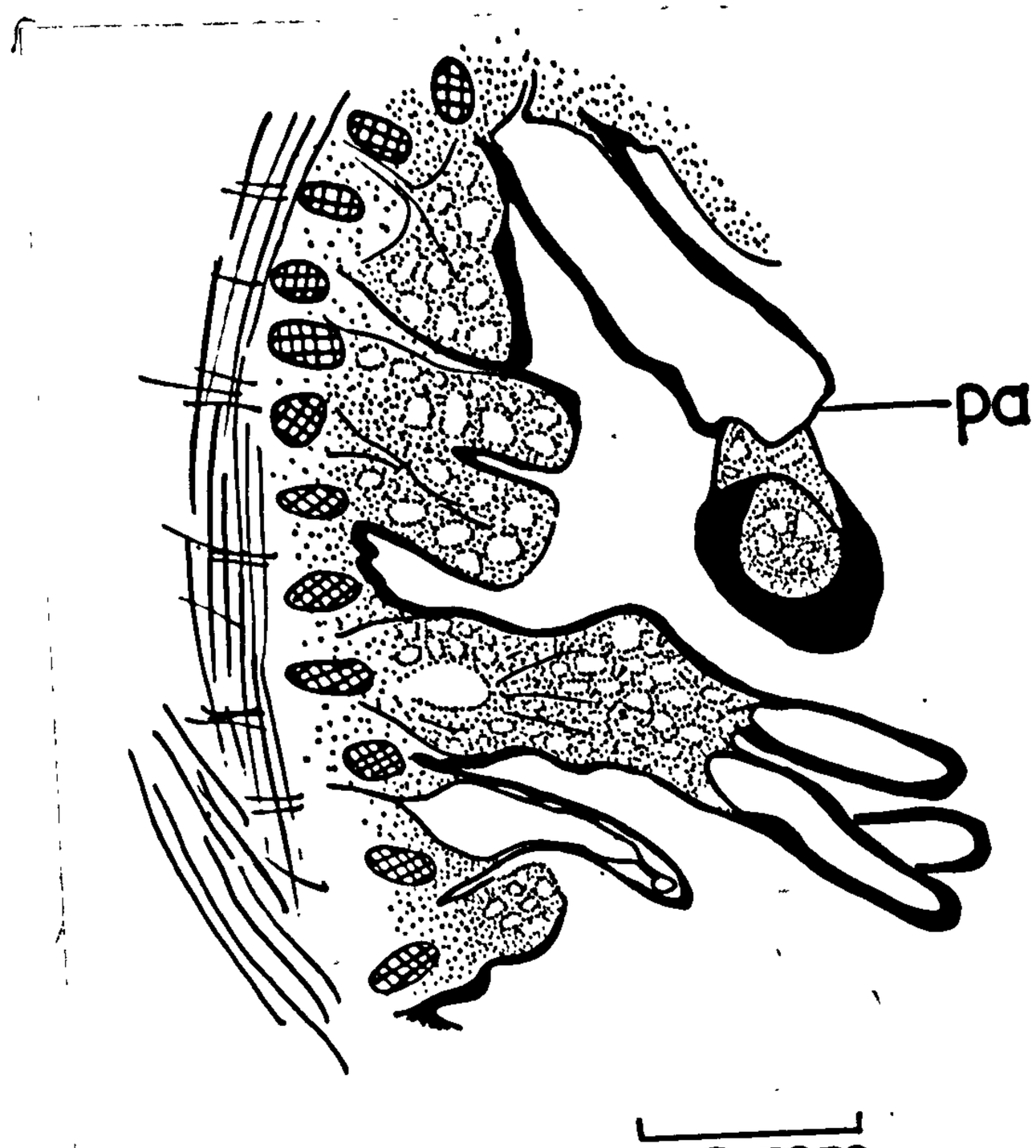

Fig. 6c

Fig. 6c. P. scotiae pharyngeal papillae.

clear and they are devoid of nuclei. It is thought that they may act as reservoirs for the secretion from the cells of Mehlis gland. They are particularly clear in that part surrounding the part of the uterus which lies within the gland.

Uterus

The uterus in all the specimens examined was packed with eggs and almost filled the dorsal surface and the space between the testes and the genital atrium.

Vitellaria

These are follicular and extend almost the whole length of the worm. A pair of vitelline ducts runs in and joins forming the vitelline reservoir, which lies just behind Mehlis gland, and from this a duct runs into the central chamber of the gland.

Excretory bladder

This is large and lies dorsal to the acetabulum. It opens to the exterior by a short duct which runs directly dorsally from the bladder.

Gametogenesis.

Spermatogenesis. The germinal tissue is made up of larger cells than in P. hiberniae and it was therefore possible to see most of the stages. The nuclear divisions and spermatozoon formation appear to take place exactly as in Gigantocotyle bathycotyle. The chromosome number as seen in meiotic metaphases is haploid number, eight, diploid, sixteen. Spindles are formed but no centrosomes were seen.

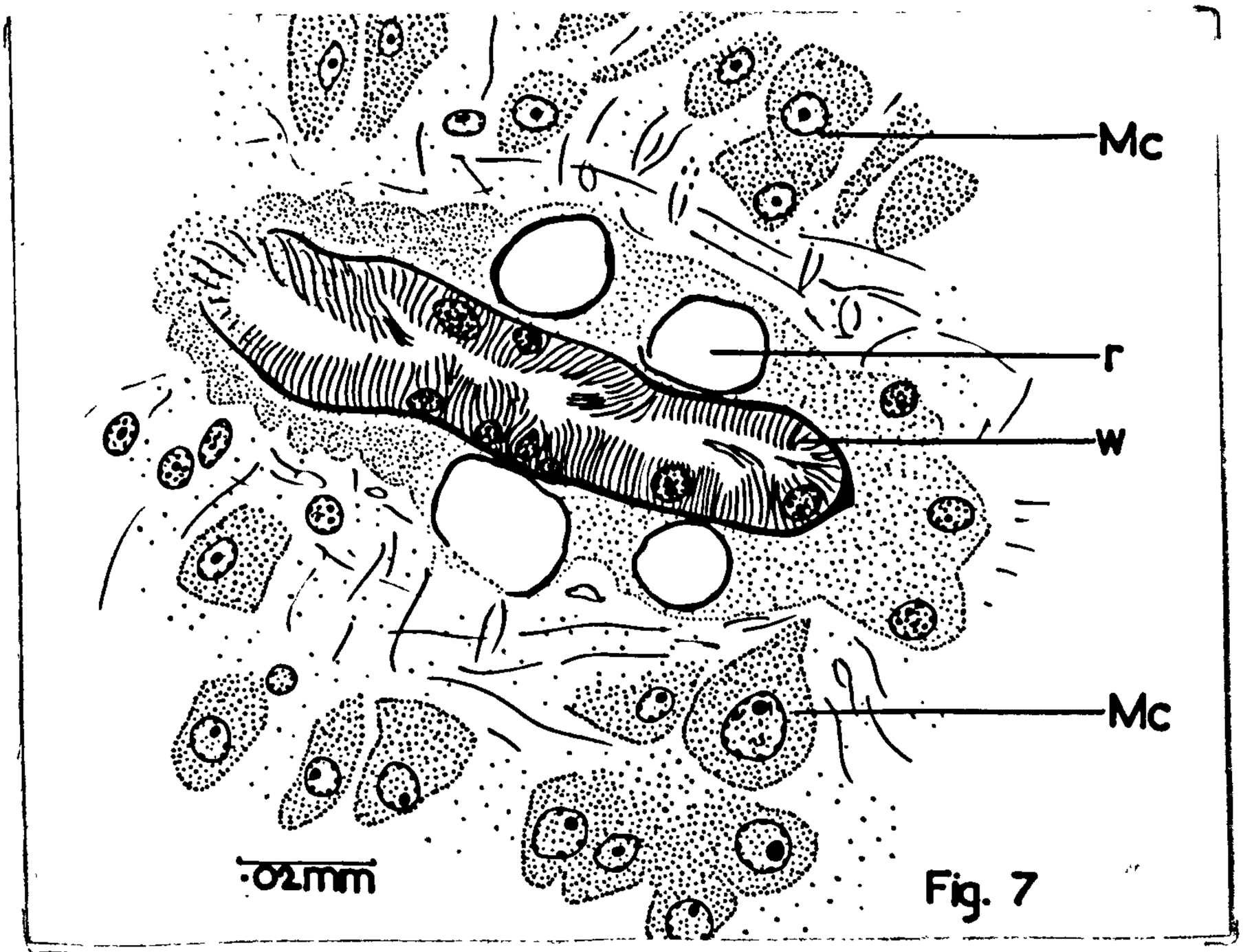

Fig. 7

Fig. 7. P. scotiae uterus in Mehlis gland.

Oogenesis. As before, only oogonia and primary oocytes are found in the ovary, reduction division only taking place after the oocyte has been penetrated by a spermatozoon. This was not observed in this species.

Cleavage

This appears to follow the same plan as that previously described in G. bathycotyle and P. hiberniae. A vitelline membrane is formed and the embryo is at about the eight to ten cell stage when the egg is laid. Even greater difficulty than before was experienced in getting good sections of the anterior end as the egg shells seemed particularly hard and tore the sections badly.

MATERIAL FROM THE NETHERLANDS AND FRANCE

Two collections from the Netherlands have been received; both were labelled Paramphistomum cervi and neither was in a very good state of preservation. One collection had been fixed in 70% alcohol and the other in 10% formalin but in neither could any details of histology or cytology be made out. The acetabulum in the Paramphistomum type, the pharynx, the diorchis type and the genital atrium the Ichikawai type. These characters, combined with highly lobed testes indicate that this material is identical with P. hiberniae.

The three specimens labelled P. cervi from France are better preserved. There has not, however, been time to make

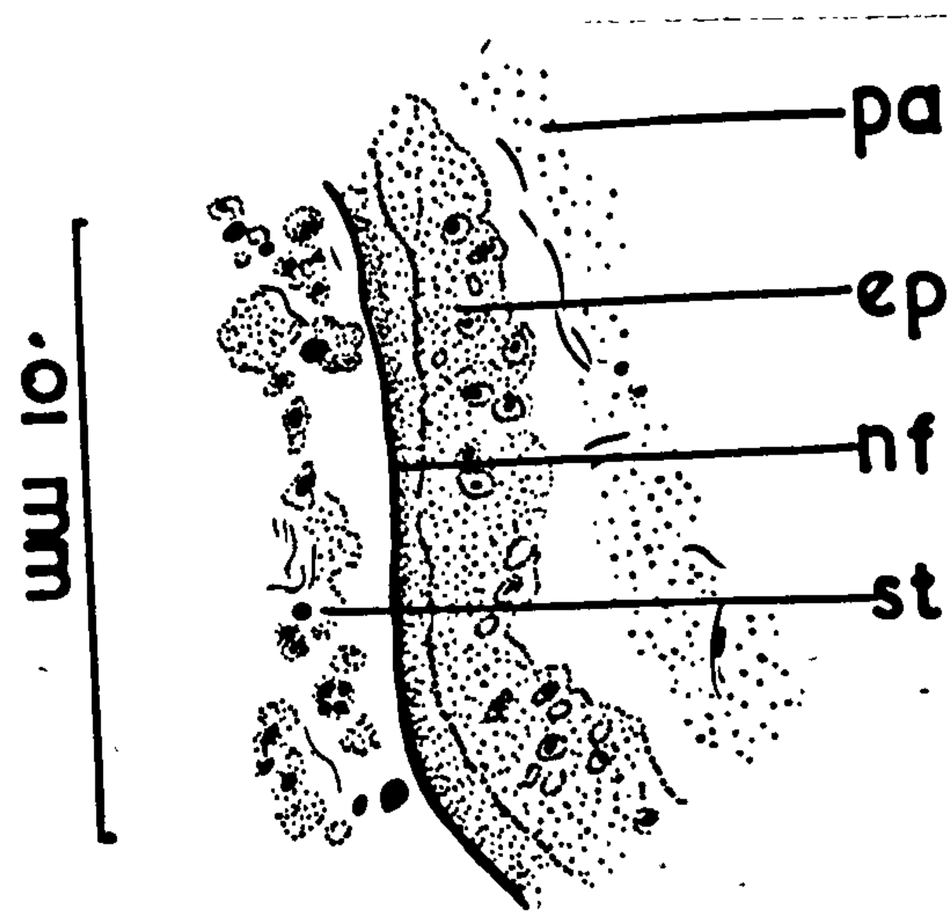

Fig. 8

Fig. 9

Fig. 8. P. scotiae testis wall.

Fig. 9. P. scotiae First metaphase chromosomes in primary spermatocytes.

a detailed study of them. From preliminary observations they are very much larger than any of the specimens of P. scotiae and P. hiberniae, being 8-9mm long, 2.5-4 mm in breadth and 2-2.5mm in dorsal-ventral measurement.

Three specimens identified as Cotylophoron cotylophorum have also been received from France. The pathology of this species, and of P. cervi has been studied by Guilhon and Priouzeau (1945) who state that C. cotylophorum has only once been found in France and that from the district of Mourthe-et-Moselle, in the South East. The papillae in the pharynx of thtse species are small and inconspicuous.

COMPARISON WITH P. CERVI

As has already been stated it was assumed at the beginning of this work that P. cervi was the only species present. There proved, however, to be so many points of difference which were constant between the specimens from the different collections that it could not be just a question of individual variation. Unfortunately the two collections which contained P. cervi were not fixed by the author and are in rather a poor state of preservation. The genital atrium of these specimens does, in some sections show occasional strands of muscle fibres, but these are so irregular and insignificant that the atrium may still be classified as the Gracile type. (fig 10)

If the specific diagnosis is based on the acetabulum,

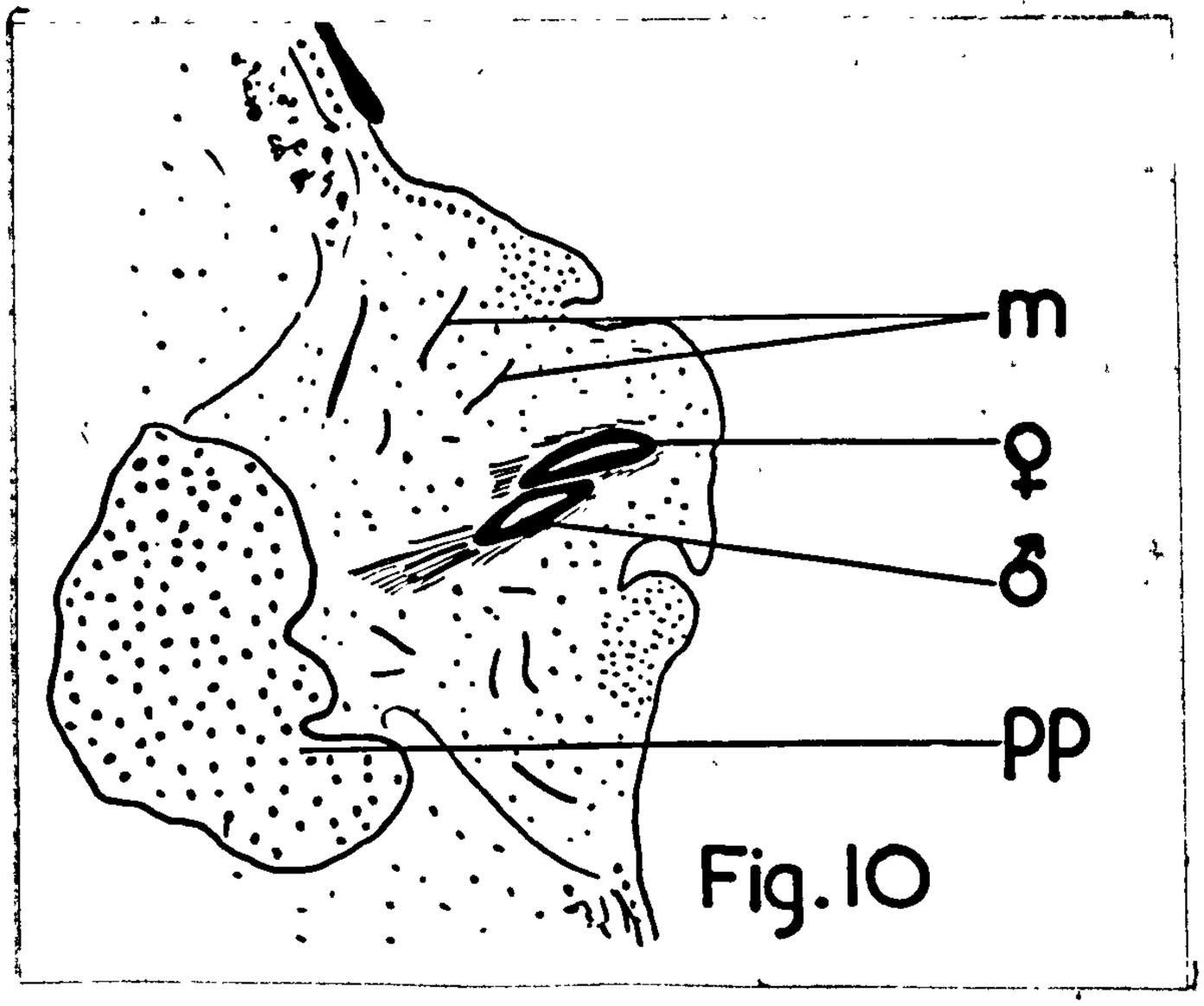

Fig.10

Fig. 10. P. cervi genital atrium.

pharynx and genital atrium type only, P. scotiae might be considered as a synonym of P. Leydeni Nasmark 1937 but there are a number of other morphological differences which seem to be sufficient to warrant its description as a new species.

A comparison of the four species P. cervi, P. Leydeni, P. scotiae and P. hiberniae is given in tabular form below. The figures for P. cervi and P. Leydeni are after Nasmark.

(For Table see over.

TABLE I.

	P. corvi	P. hiberniae	P. Leydeni	P. scotiae
1. Length	8.89mm	4.9mm	8.4mm	5.1mm
2. Acetabulum diameter/ Body Length	1/4.4	1/5.7	1/3.6	1/6.4
3. Pharyngeal papillae	Small, inconspicuous	Well developed anteriorly	Well developed	Very well developed
4. Genital atrium	Gracile type	Ichikawai type	Epiclitum type	Epiclitum type
5. Position of genital atrium	Level with posterior part of oesophagus	Level with oesophagus	Level with most anterior part of pharynx	Level with posterior part of oesophagus
6. Testes	Large, slightly lobed	Small, highly lobed	Often disc-shaped	Large, slightly lobed
7. Excretory bladder	Dorsal to acetabulum	Dorsal to acetabulum	Dorsal and anterior to ovary and acetabulum	Dorsal to acetabulum
8. Excretory duct	Fairly long	Very short	Very long	Very short
9. Excretory pore	Front of posterior testis	Middle of posterior testis	Anterior border of anterior testis	Posterior border of posterior testis.

Although not a great deal of importance is attached to the number of circular muscle units in the acetabulum it is interesting to compare the average numbers of P. hiberniae and P. scotiae with those given by Nasmark for P. cervi. He does not give figures for P. Loydeni.

TABLE II.

Circular muscle units.	P. cervi	P. hiberniae	P. scotiae
dorsal external 1	14	19	18
dorsal external 2	37	30	27
dorsal internal	41	46	38
ventral internal	40	50	38
ventral external	19	19	15

SUMMARY.

Two new species of Paramphistomum from cattle are described and named, P. hiberniae and P. scotiae, first collected from Irish and Scottish cattle respectively.

A brief account of gametogenesis and early division is given.

The chromosome number for P. scotiae is n=8, 2n=16, for P. hiberniae no definite number is given but it is believed to be n=8(6-8), 2n=16(12-16). Notes are made on some specimens received from the Netherlands and France.

P. hiberniae and P. scotiae are compared with P. cervi and P. Leydeni.

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THE DEVELOPMENT OF THE MIRACIDIUM OF PARAMPHISTOMUM HIBERNIAE FROM THE TIME OF DEPOSITION OF THE EGG UNTIL HATCHING.

INTRODUCTION

Since the description by Looss in 1892 of the life history of Diplodiscus subclavatus and in 1896 of the miracidia of Gastrothylax crogarius, G. aegyptiacus and Paramphistomum cervi there have been very few accounts of paramphistome life histories. Cary (1909) published an account of the life history of Diplodiscus temporatus which Cort considered to be incorrect, Krull and Price (1932) repeated the work on the same species; Beaver (1929) published an account of all the developmental stages of Allassostoma parvum except the sporocyst; Brumpt (1936) described the cercaria and experimental infection of the intermediate and final hosts of P. cervi in Corsica; The fullest account has been given by Bennett (1936) of a paramphistome which was identified as Cotylophoron cotylophorum but which Price and Mackintosh (1944) consider to be a new species of the genus Paramphistomum, namely P. microbothioides.

It was hoped that it would be possible to give a complete account of the life history of at least one of the specie of Paramphistomum which occur in this country but failure to

obtain an experimental infection of snails, or to collect naturally infected ones has made it impossible to continue beyond the stage of the miracidium.

MATERIAL AND METHODS

Eggs were collected from adult worms by placing them in tap water and leaving overnight. The water was then poured off, the eggs allowed to settle and then washed several times, finally being put into a small amount of water with some well washed rumen content. It was found that the eggs developed better when kept in a small quantity of filtered tap water, to which either sterile rabbit faeces or rumen content had been added. When kept in too much water, or water without vegetable matter in it they collapsed after a few days. No decaying animal tissue was left in the culture. The water was not changed at all, simply being added to if it showed signs of drying up. This is quite different from the technique employed by Bennett (1936), in which the water on the eggs was changed at least twice daily to ensure the highest percentage of hatching.

No attempt was made to fix and section eggs, all observations being carried out on living material.

Miracidia after hatching were treated with various vital dyes of which methylene blue was the most successful. An account of these is given in the description of the miracidium. For

observations on unstained miracidia an aqueous suspension of polyvinyl alcohol was found most useful for slowing down their movements with the minimum amount of distortion. A few drops of the suspension were put onto a slide and allowed to evaporate until they were of a treacly consistency. A few miracidia were then pipetted into the centre of the drop and the coverslip put on. Miracidia treated in this way live for up to an hour, and accurate observations can be made under the high power of the microscope. Chloral hydrate was also used for slowing down the miracidia but even when very dilute this caused them to contract violently and killed them in a very short time.

For demonstration of the epidermal cells the silver nitrate technique described by Lynch in 1933 was used with great success. An attempt was made to demonstrate nerve endings by fixing in gold chloride and then reducing this with formic acid but unfortunately the gold deposited indiscriminately over the whole body surface of the miracidium.

EGGS.

The eggs are oval with a thin transparent shell. The opercular end is usually the narrower and at the opposite end there may be a thickening of the shell, or sometimes a small projection similar to that described by Bennett in the eggs of P. microbothrioides (Cotylophoron cotylophorum)- There is

considerable variation in the size of the eggs but whether this can be correlated with the size of the parent as in P. microbothrioides is not known. They range from 132 μ x 83 μ to 183 μ x 100 μ and the average is 154.6 μ x 91.1 μ. This is larger than those of P. microbothrioides where the range is from 115 μ x 58 μ to 147 μ x 76 μ with an average of 129 μ x 68 μ.

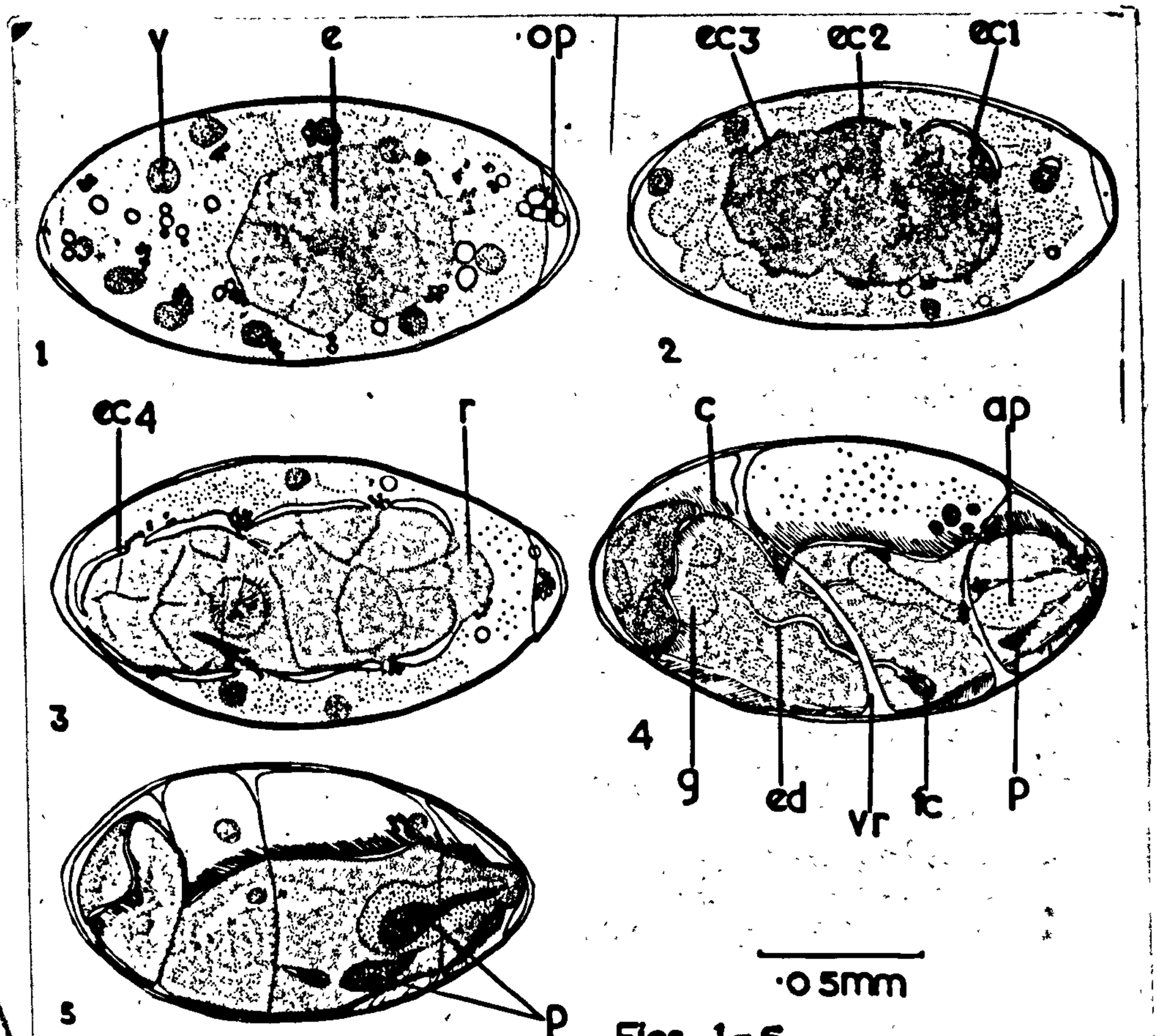
DEVELOPMENT

When the egg is laid the embryo appears as a small sphere of cells with very granular contents. Surrounding it are the cells of the developing vitelline membrane, which have large nuclei and comparatively clear cytoplasm. The origin of this membrane has not been traced but it seems probable that it is derived from the "ectodermal" cell as in Parorchis acanthus as described by Rees in 1940. Eggs collected from the faeces by sedimentation do not appear to be any more advanced in development than those collected directly from the adult, indicating that some external stimulus, such as drop in temperature is required before development will proceed.

Owing to the comparatively small number of eggs which were available at any one time it was not possible to make a detailed study of the organogeny, but eggs were examined daily under the high and low powers of the microscope and the general process of development and the order in which the various tissues were derived observed as closely as possible.

KEY TO LETTERING OF FIGURES

ap - apical gland
b - basal granules of cilia
c - cilia
e - embryo
ec, 1-4 - epithelial cells, tiers 1-4.
ed - excretory duct
ep - excretory pore
ev - excretory vesicle
fc - flame cell
g - germ cells
gm - germ cell matrix
n - central nerve mass
op - operculum
p - penetration gland
pp - opening of penetration gland
r - rostrum
se - subepithelial layer
v - vitelline membrane
vr - remains of vitelline membrane



Figs. 1-5.

Figs. 1-5. Development within the egg.

Fig. 1. Five days after deposition.

Fig. 2. Seven days " "

Fig. 3. Nine days " "

Fig. 4. Eleven days " "

Fig. 5. Immediately before hatching.

Development does not proceed at a uniform rate, some embryos developing much more slowly than others. The number of days given for each stage can therefore only be regarded as approximate.

Up to five days after deposition the embryo does not change greatly in shape or size, but the vitelline membrane grows rapidly and becomes applied to the inside of the shell, (Fig. 1) The embryo proper then becomes elongated and by the end of seven days the epidermal cells can be seen arranged in four tiers. The cilia are not visible at this stage, (Fig. 2.) After another two days the rostrum is distinct and there is an indication that the flame cells have started to function, (Fig. 3) At this stage the vitelline membrane starts to break down, some of its products presumably being resorbed by the embryo proper. The miracidium increases considerably in length and the penetration glands and the apical gland appear. Cilia can now be seen, but their basal granules are never visible while the miracidium is still within the egg. By the end of the twelfth day the flame cells are working actively, the germ cells at the posterior end of the miracidium are dividing and the vitelline membrane remains only as a viscid substance surrounding the embryo which lies coiled in the shell, (Fig. 4.) At this stage the miracidium appears almost ready to hatch. The penetration glands have attained their maximum size and the

miracidium makes occasional movements within its shell.

In none of the eggs observed could a distinct mucoid plug, such as described by Barlow (1925) in Fasciolopsis buski be detected but there did seem to be a slight thickening of the viscid remains of the vitelline membrane between the rostrum and the operculum.

The miracidium can remain at this stage for several weeks. At laboratory temperatures most of the eggs hatched between fourteen and twenty days after collection but some quite viable eggs were seen in a culture after six weeks. Hatching can be induced by warming the eggs up to 26°C or exposing them to a bright light.

HATCHING

The actual process of hatching may take anything from thirty minutes to several hours after stimulation of the eggs, but the usual time is about thirty to fortyfive minutes.

During the first few minutes after exposure to light the flame cells become extremely active, and the body of the miracidium becomes more granular in appearance. After about ten minutes the cilia of the first tier of epidermal cells begin to beat and the anterior end of the body begins to move. The rostrum moves rapidly backwards and forwards across the base of the operculum; presumably the combination of the ciliary action

and this movement serves to break down mechanically any accumulation of waste matter which may be there. This is followed by the cilia of the second, third and fourth tiers commencing to beat and the miracidium begins to move actively within the shell. The remains of the vitolline membrane can be seen as large bubbles which the miracidium pushes round and round inside the shell. This movement usually continues for about twenty minutes although it may do so for longer. In some instances the miracidium may rotate completely within the egg, or even go round and round on the equator but the rostrum is usually kept pointing towards the operculum. After this period of intense activity the miracidium withdraws slightly from the operculum and becomes quiescent. A few minutes later the operculum bursts open and the miracidium is once more stimulated to great activity and swims through the opercular opening. This may take several seconds as the posterior part of the miracidium is broader than the opening through which it has to pass. Quite a number of miracidia have been observed swimming actively with the shell still attached to the posterior end.

The actual bursting of the operculum does not appear to be caused by physical pressure exerted by the miracidium. From observations made on free miracidium by means of dark ground illumination it seems that the secretion from the penetration glands, and the apical gland is forced out by the contractions of the body as a whole. It seems reasonable to conclude that this

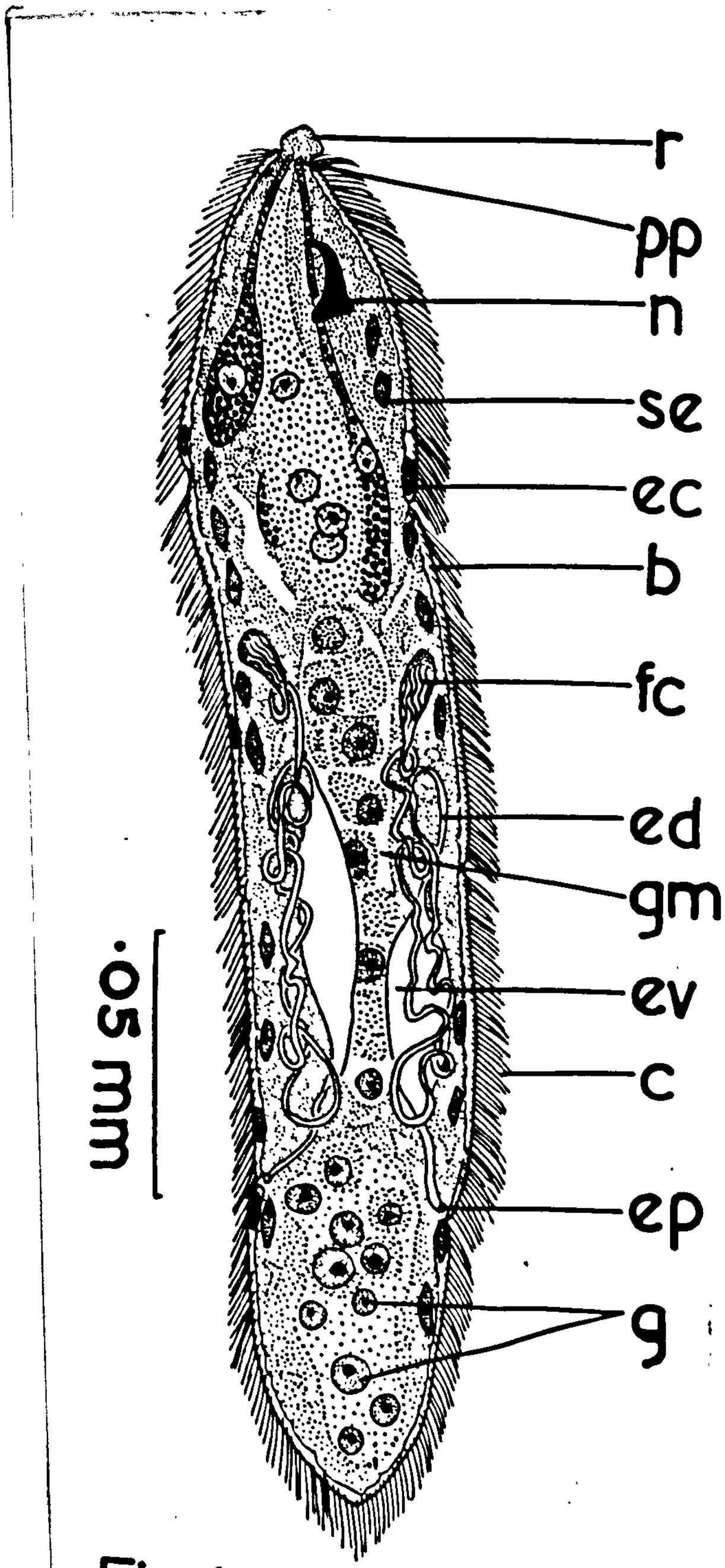


Fig. 6

Fig. 6. The free swimming miracidium.

is also the case when the miracidium is still within the egg and consequently that these secretions may play some part in dissolving the cementing substance which holds the operculum closed.

MIRACIDIUM Fig. 6

The miracidium, once free of the egg shell, is long and narrow with a permanent rostrum. It swims actively, rotating on its own long axis and is capable of considerable and rapid change in shape.

Measurements

Measurements on living miracidia were very difficult to make on account of their ceaseless movement and even treatment with polyvinyl alcohol produced a slight contraction. It was, however, found that if a number of miracidia were put into a very small drop of water on a slide they swam to the edge and there were held by surface tension for sufficiently long for fairly accurate measurements to be made.

They were found to be approximately 259 μ long by 35 μ broad when fully extended, the maximum breadth being at the anterior end of the second tier of cells. This is considerably longer than the miracidium of P. microbothioides the maximum size of which is given by Bennett (1930) as 210 μ the average size being 184 μ x 39 μ.

Epithelial cells Fig. 7

These were demonstrated by the silver nitrate technique.

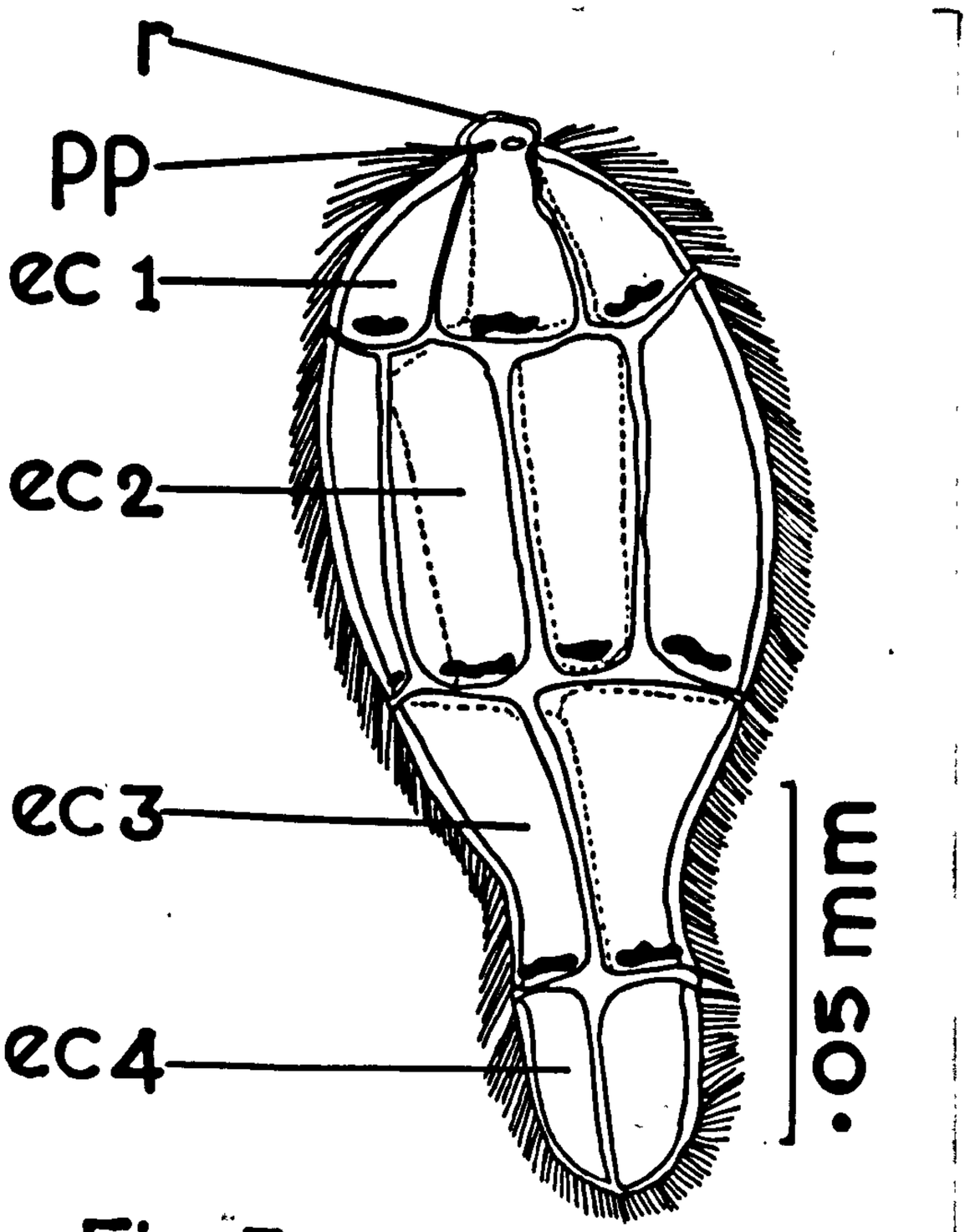


Fig. 7

Fig. 7. Miracidium treated with silver nitrate to show arrangement of epithelial cells.

Only a few miracidia (about 20) were used and the numbers in these were constant. It is possible, however, that had more been examined some variation would have been noted. They are arranged in four tiers, of six, eight, four and two cells respectively. The six cells of the most anterior tier are thicker than the others. In all the cells the basal granules of the cilia are visible and the nuclei are extremely irregular in shape. There is a distinct gap between each tier and the rostrum has no epidermal covering. The arrangement is shown in Figure II and can be expressed as a formula (Bennett 1936) 6:8:4:2.

Muscles

The presence of both longitudinal and circular muscles is demonstrated by the great amount of contraction of which the miracidium is capable. They are however extremely difficult to distinguish and only the circular muscles of the first tier have been seen as very faint striations. The longitudinal muscles have not been seen at all. The better development of the circular muscles in the anterior region of the body may be associated with the need for forcing out the secretions of the glands.

Glands

There are four penetration glands. These are uni-nucleate, flaskshaped cells with ducts opening to the exterior at the base of the rostrum. The contents are clear and do not

take up vital stains easily.

The apical gland or primitive gut is sac-like and larger than the penetration glands. There are usually four nuclei present. The duct from it appears to open at the tip of the rostrum, but is not always easy to distinguish.

Nervous system and Sense organs.

In living material the nervous system is difficult to see and all that can be made out is a fibrous mass which varies in shape from pyramidal to spherical. This lies by the side of the penetration glands, either at the level of the first tier of epidermal cells, or the anterior part of the second. No nerves could be seen leaving this central nerve mass.

There are no eye spots, nor other sense organs such as have been described by a number of workers. What were at first taken to be lateral sensory papillae such as described by Heisinger (1923) and Bennett (1936), Lynch (1933) and Rees (1940) or anterior or lateral ducts according to Cort (1919) and Faust and Meleney (1924) were found to be nothing but extrusions of cytoplasm between the first and second tiers of epidermal cells. This extrusion takes place when the miracidium is becoming moribund and is later followed by similar extrusions between the other tiers of cells. Care must be taken in distinguishing between these and sense organs from which a nerve can be traced to the central nervous mass.

An attempt was made to demonstrate superficial nerve

endings by means of gold chloride but this was unsuccessful.

Excretory system

This consists of two flame cells situated towards the anterior end of the second tier of cells. The flame cell nuclei were not seen and it is possible that as in Schistosoma haematobium as described by Meisinger (1923) the nuclei are lacking. The ducts are long and coiled and each opens into a large excretory vesicle. From this a short duct leads to the excretory pore which lies laterally, between the third and fourth tier of epidermal cells. No duct nuclei or accessory excretory cells such as described by Bennett were seen. The discharge of excreta to the exterior is not periodic and seems to depend on the contractions of the body as a whole.

Germinal tissue

The germ cells appear to be arranged in two groups in the same way as has been described by previous workers. Between the posterior end of the apical gland and the level of the excretory pore the cells seem to be embedded in a clear substance, called by Looss the germ cell matrix. At the posterior end of the body they are closer together and appear to lie freely.

Length of Life

At laboratory temperatures (20°C - 22°C) the miracidia swim actively for six to eight hours. After this they sink to the bottom of the container and continue to rotate slowly for about another hour during which time the anterior end of the miracidium

swells up until it becomes pearshaped. Occasionally they were seen to be quite active after ten hours, but all were always dead after fifteen hours.

Staining reactions

Neutral red. Kills instantly whether in saline or distilled water and however dilute. Stains cytoplasm diffusely, the nuclei of the germ cells, epidermis and subepidermal tissue standing out distinctly.

Methylene blue. In distilled water the germcells, gland nuclei and other nuclei purple, the cytoplasm blue and the excretory vesicles pale, slightly greenish blue. The anterior tier of cells and the rostrum stain very much more deeply than the rest of the body.

In saline, stains central nerve mass dark blue, the rest of the body not so deeply as when in distilled water. The anterior tier and the rostrum stain more deeply, as before.

Miracidia live in this for some time.

Brilliant crosyl blue
Toluidine blue } Stain very much as does methylene blue, with the anterior tier deeper than the rest, but both kill the miracidia far more quickly.

Dilute alizarin Stains diffuse yellow and kills rapidly. Red does not come up until after death of the miracidium.

Yworts Stains whole body pinkish, kills fairly rapidly but does stain what look like bubbles of a secretion from the anterior end,

DISCUSSION

The miracidium of P. hiberniae resembles that of P. microbothricides (C. cotylophorum) extremely closely. The chief differences are in size, both the egg and the free swimming miracidium being appreciatively larger in P. hiberniae, in the lack of sense organs and the presence of large excretory vesicles.

The number of epithelial cells is twenty which is in agreement with the two other members of the family in which these cells have been counted, namely Diplodiscus temperatus and P. microbothrioides.

SUMMARY

The eggs and the development and anatomy of the miracidium of P. hibernicae are described and compared with P. microbothrioides.

An account is given of the process of hatching and the length of life of the miracidium.

The staining reactions of various vital dyes are noted.

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A SERIES OF ATTEMPTS TO INFECT VARIOUS SPECIES OF SNAILS WITH SEVERAL SPECIES OF PARAMPHISTOME MIRACIDIA.

As has already been stated in an earlier part of this thesis the number of accounts of paramphistome life histories is very small.

From an experimental infection of Paramphistomum microbothium Fischoeder 1901 and of an unidentified species of paramphistome from N. Rhodesia, in sheep, it was hoped to be able to make an experimental study of the whole life cycle.

The eggs of these two species were collected from the faeces by sedimentation. As the infections were extremely light this was a very laborious process. They were incubated in small tanks containing filtered tap water, with a small amount of washed rumen content; the tanks were aerated and kept at a temperature of 25°-30°C. Hatching commenced after ten to fourteen days. When hatching started young snails of the following species were put into the tanks - Physopsis africana, Bulinus tropicus, Limnaea spp, Planorbis spp and B. truncatus. The cercariae of P. microbothium which had produced the experimental infection had been collected from B. truncatus. The snails were left in the tanks until hatching had ceased. They were then kept at 25°-30°C. Several died during the following fortnight

but careful examination showed no infection with sporocysts or rediae. No cercariae were secreted and snails killed and examined several months later showed no sign of any infection.

The eggs of P. hiberniae were collected by placing the adult worms in water and leaving overnight. The eggs were then washed and kept at room temperature (about 19°C) in a small quantity of filtered tap water containing a comparatively large amount of rumen content. It was found that with too much water the eggs collapsed. The few eggs of P. scotiae which were collected in a similar fashion were kept in water with sterile rabbit faeces added. Both these species took fourteen to twenty-one days before hatching commenced. The miracidia were then picked out and four or five put into small watch glasses. One snail was put in with each lot of miracidia and left overnight. Several hundred snails of the following species were exposed in this manner, Limnaea truncatula, L. peregor, L. glabra (only one or two), L. stagnalis, Planorbis, three species, an unidentified operculate snail. These snails were then kept at either room or outside temperature. Some died about seven days after exposure, but did not show any infection. The rest have been kept, but after five months have not liberated any cercariae, nor have any killed and examined showed any infection so that it appears unlikely that they will do so now.

It is interesting to note that in none of the four species whose eggs have been incubated does development proceed

properly in cultures without some vegetable matter, and that for P. hibernise and P. scotiae the amount of water required in the culture is very much less than for P. microbothium and Paramphistomum sp.

Durie (1949) has reported similar very great difficulty in getting laboratory infections of snails by P. cervi and P. cotylophoron miracidia in Australia. These miracidia exhibit great specificity for the snail host.

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