DEVELOPMENT AND IN VITRO CULTURE OF SOME DIGENEA.

VOLUME 1

by

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May 1976.

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ABSTRACT

Attempts were made to culture the metacercariae of Fasciola hepatica, Meiogymnophallus minutus and Microphallus similis to maturation in vitro in a wide range of media and under a variety of physical conditions.

In each case the normal development of the metacercaria was studied in a suitable laboratory host at both the light microscope and ultrastructural levels Various suitable criteria for assessing normal growth and development were drawn up.

The development of the tegument, digestive caeca, excretory system, parenchyma and surface features of the cultured metacercaria of all species were studied at an ultrastructural level and compared to the development in vivo. In the case of M. minutus and M. similis, the ultrastructure of the testes, ovary and vitellaria was also studied in detail.

The newly excysted juveniles of F. hepatica underwent some somatic growth in vitro but there was no significant development of the genital rudiment. Developmental stages were also removed from mice and transferred to culture.

The metacercariae of M. minutus and M. similis both possess partially developed genitalia. In vitro, the metacercariae of M. minutus produced sperm after 2 days and the vitellaria became positive for egg-shell precursors - a rate of development similar to that in the duckling. No normal eggs were produced but

clumps of tanned vitelline material appeared in the uterus after 3 days.

In vitro, the metacercariae of M. similis produced sperm after 18 hours and the vitellaria became positive. Up to seventy normal eggs were produced from the second day onwards. However, normal egg production ceased after the first few days of egg production and only abnormal eggs with no ovum were seen. Oviposition occurred in vitro.

The importance of in vitro cultivation as a biological tool and the problems involved are discussed.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor, Professor J.D. Smyth, for his continual encouragement and inspiration during this study and for reading and commenting on the manuscript.

The advice of Dr. R.B. Sinden, of this department, on the technical aspects of electron microscopy and on the interpretation of ultrastructural detail has been invaluable.

I am very grateful to Dr. C.P. Ollerenshaw of the Central Veterinary Laboratories, Weybridge, for providing me with a regular supply of Fasciola hepatica metacercariae throughout this study and also to Ms. Anna Sörmann of the Biology Department, University of York.

I am also grateful to Dr. B.L. James of the University College, Swansea for supplying me with infected cockles and oystercatchers, for the use of his laboratory on several occasions and for his discussion and advice.

I would like to thank Dr. C.E. Bennett for permission to quote from his thesis and for allowing me to reproduce some of his Textfigures.

Thanks are due to all my colleagues in this department who gave technical assistance, especially Mrs. I. Gadd, Mrs. J. Shepherd and Mr. I. French.

I am indebted to the Medical Research Council for the provision of a postgraduate research studentship 1972-1975 during the tenure of which this work was carried out, and to the Agricultural Research Council for providing funds for the research on Fasciola during the last two years of this project.

CONTENTS

VOLUME 1

Abstract 2

Acknowledgements 4

Contents 6

Chapter 1 : Introduction 8

Chapter 2 : General Methods 19

Chapter 3 : *Fasciola hepatica*

Part I - Growth and development *in vivo* 46

Part II - Growth and development *in vitro* 65

Part III - Ultrastructure of development *in vivo* 105

Part IV - Ultrastructure of development *in vitro* 140

Chapter 4 : *Meiogymnophallus minutus*

Part I - Development *in vivo* 182

Part II - Development *in vitro* 205

Part III - Ultrastructure of development *in vivo* 231

Part IV - Ultrastructure of development *in vitro* 265

Chapter 5 : *Microphallus similis*

Part I - Development *in vivo* 284

Part II - Development *in vitro* 299

Part III - Ultrastructure of development *in vivo* 310

Part IV - Ultrastructure of development *in vitro* 366

Chapter 6 : General Discussion 394

References 402

VOLUME 2

Fasciola hepatica : Plates 1-79 425

Meiogymnophallus minutus : Plates 80-125 . . 496

Microphallus similis : Plates 126 - 197 . . . 537

CHAPTER 1 : INTRODUCTION

The importance of the *in vitro* cultivation of helminths as an experimental tool is well recognised. It is of particular value in -

a) The study of the physiology and biochemistry of parasites in isolation from their host;

b) Research into their reactions to the immune response of their host;

c) Tests to determine the effects and usefulness of antihelminthic drugs.

The cultivation of helminths presents several basic problems, these have been discussed by Smyth (1966, 1969), and they can be summarised as follows -

a) An inadequate knowledge of the precise physico-chemical characteristics of the habitats of the larvae, developing and adult forms and the difficulty of reproducing them *invitro*.

b) The replacement of the complex nutritional materials available to the parasite *in vivo* by a defined media.

c) The sterilization of larvae obtained from a non-sterile site.

d) The efficient removal of toxic waste products.

e) The complex nature of the helminth life cycle and the incomplete knowledge of the conditions which stimulate their development from one stage to another.

The Digenea involved in this study are the metacercaria of Fasciola hepatica (Linnaeus, 1758), Meiogymnophallus minutus (Cobbold, 1859) comb. nov. (Gymnophallidae) and Microphallus similis (Jägerskiöld, 1900: Baer, 1943) (Microphallidae: Microphallinae).

The metacercariae of F. hepatica are minute (approximately 0.2 x 0.1mm.) and they have only a few cells in their simple genital rudiment. The egg-bearing adults, however, are comparatively large (up to 15.3mm. in length (Dawes, 1962)) and they possess a complex reproductive system. In contrast, the maturation of the metacercariae of M. minutus and M. similis mainly involves the final development of their already preformed genitalia; there is very little somatic growth. The adults of M. minutus begin to produce eggs after 3-4 days in their final host and those of M. similis after 2-3 days.

The cultivation of the metacercariae of F. hepatica to the adult stage therefore presents a much greater problem than the cultivation of M. minutus or M. similis.

Previous work on the cultivation of digenetic trematodes has been reviewed by Silverman (1965), Smyth (1966), Clegg & Smyth (1966, 1968), Taylor & Baker (1968) and Silverman & Hansen (1971). It has been emphasized by Kannangara & Smyth (1974) that only those metacercariae which possess well developed

genital primordia have been successfully cultured to egg-bearing adults (e.g. Basch et al, 1973). However, these authors have successfully cultured the metacercariae of Diplostomum spathaceum to egg production - these metacercariae are not progenetic*. (See footnote).

There have been many attempts to culture Fasciola hepatica since it is an important and harmful parasite of livestock. Early attempts concentrated on the maintenance of the adults (Stephenson, 1947; Dawes, 1954; Clegg, 1957; Rohrbacher, 1957). It is important to distinguish between maintenance cultures and those which allow growth, development and maturation. In the maintenance cultures the conditions are such that the cells are kept alive but the flukes do not grow or develop to maturity (in the case of the metacercariae), or continue to produce normal eggs and sperm (in the case of the adults). However, good maintenance cultures of F. hepatica have many uses (e.g. drug testing, physiological and biochemical studies) and work still continues in this field (Benex, 1966; Martinetto & Capucinelli, 1968; Ratcliffe et al, 1969; Foster, 1970; Locatelli & Paoletti, 1970).

*Footnote:

The meaning of the term "progenetic" is somewhat confused. Smyth (1965) states that "the term 'progenesis' has been used to describe advanced development in a larval form and a metacercaria with genital anlagen would be termed 'progenetic'". It is in this sense that the term "progenetic" is used here. Other workers only apply the term "progenetic" to larvae which have reached full sexual maturity e.g. the metacercaria of Proctoeces subtenuis in Scrobicularia plana.

The early maintenance media usually consisted of a balanced salt solution with a carbohydrate source. Stephenson (1947) maintained the adults of F. hepatica for 60 hours in such a medium and Dawes (1954), using aseptic technique, reported a maximum survival of 12 days during which time the worms were "more or less normal in appearance and in a state of active movement". Clegg (1957), using cellulose dialysis tubing, was able to increase the survival time to 17 days. However, this worker assessed the normality of the maintained adults in a more critical way than previous workers and he found that spermatogenesis became abnormal after only a few hours in culture and that egg production ceased. Rohrbacher (1957) supplemented the basic media with liver concentrate and extract which he found prolonged survival for up to 30 days. The continuous culture technique of Martinetto & Capucinelli (1968) used the dialysis tubing described by Clegg and a culture medium based on that of Rohrbacher.

Recent attempts at maintaining adult F. hepatica have used more complex media based on a carbohydrate supplemented saline with serum and blood cells (Bénex, 1966; Ratcliffe et al, 1969). In such a medium Bénex records a survival of 40 days. The condition of the adults was assessed using mobility, muscular movement and firmness of attachment to the culture vessel. Ratcliffe and his co-workers also used various other defined media (e.g. Medium 199) and tested the effects of various additives (e.g. bile, supplementary vitamins and amino acids) and solid bases. These they compared to the basic medium of glucose saline, serum and blood. The various media were compared by counting the number of viable eggs

produced by the adults during cultivation. This was then compared to the normal rate of egg production *in vivo*.

There is very little published work on attempts to culture the metacercariae of *F. hepatica*. Hughes & Christofinis in unpublished data cited by Dawes & Hughes (1964) have cultured the metacercariae for up to 108 days. The metacercariae grew to a size of 0.8 x 0.4mm. after a few weeks on culture. They compared this degree of growth to that attained by eight-day-old flukes from mice. No further growth occured and they observed that the flukes seemed to be in a state of "suspended animation". The cultivation conditions used in this experiment are described by Hughes & Harness (1972). The medium consisted of Medium 199 with 10% foetal calf and antibiotics (penicillin, streptomycin and mycostatin).

Wikerhauser & Cvetnić (1967) and Wikerhauser *et al* (1968) have studied the development of metacercariae in various defined media with or without monolayer cell cultures. The culture media that they used consisted of various combinations of the following:

Hanks salt solution or Eagle's medium;

lactalbumine hydrolyzate;

horse or calf serum (inactivated or not).

The monolayer cell cultures were:

calf testical cells;

bovine embryo kidney or liver cells;

pig kidney or embryo kidney cells;

monkey heart cells;

human amnion cells.

They found that metacercariae in cultures that contained cells survived for up to 14 days compared to a maximum of 5 days in those cultures without cells. If the media and/or cell cultures were replaced, the survival was prolonged for up to 29 days. They found evidence that the young flukes had been feeding but stated that there had been no growth or development.

Carillo de Albornoz & Guevara Pozo (1974) record the maintenance of F. hepatica metacercariae for up to 54 days in a culture medium of inactivated horse serum and sheep red blood cells at 38°C. The gas phase was 10% CO_2 in nitrogen and the media was changed every four days. The average length at 52 days was 0.52mm. and the maximum length achieved was 0.70mm. After 10 days in culture the digestive caeca possessed lateral outgrowths and after 52 days these had divided to produce secondary branches. After 34 days the two testes rudiments were separate and the outline of the cirrus sac was apparent. They also observed the formation of cellular aggregates along the sides of the cultured flukes and they suggested that this may represent the developing vitellaria.

In contrast to this, considerable success has been achieved with the cultivation of various progenetic metacercariae. An example of this is the work of Basch et al (1973) who cultured the tetracotyles of Cotylurus lutzi to the egg-bearing adults. The life cycle was completed experimentally using miracidia hatched from these eggs. Other recent successes have included the cultivation of Metagonimus yokagawi (Yasuaroka & Kojima, 1970), Sphaeridiotrema globulus (Berntzen & Macy, 1969) and Leucochloridiomorpha constantiae (Fried & Contos, 1973) to the ovigerous adults.

There have also been several reports of the successful cultivation of Gymnophallid and Microphallid metacercariae.

Bartoli (1963) cultured the metacercariae of Parvetrema timondavidi (Gymnophallidae) in Ringer's saline at 40°C. He recorded spermatogenesis, vitellogenesis and a limited amount of egg production. Yasuraoka et al (1974) cultured the same metacercariae in a complex medium of NCTC 109 supplemented with 20% inactivated bovine or chicken serum with a gas phase of 8% CO_2 in air at 41°C. using a roller tube technique. Sperm production began after 30 hours and egg production after 36 hours. This is similar to the rate of development observed in mice. No cultured flukes contained more than 51 eggs (a more usual figure being about 20), whereas flukes recovered from mice usually contained between 30 and 70 eggs. After 96 hours some cultured flukes contained a few embryonated eggs with motile miracidia. This was also observed in vivo at about this time. The flukes survived in culture for up to

11 days. If the bovine serum was not inactivated there was a marked inhibitory effect and the number of eggs produced was considerably reduced. It is interesting to note that these workers also attempted to culture P. timondavidi in Ringer's saline and, contrary to the results of Bartoli (1963), they recorded a maximum survival of 72 hours with no gametogenesis.

Bartoli (1965) attempted to culture the metacercariae of Gymnophallus fossarum in sea water and also in Ringer's saline at 41^{0}C. He found that there was some sperm production but that after 2-3 days in culture the worms died. This is particularly interesting since the life cycle and morphology of Meiogymnophallus minutus is almost identical to G. fossarum (see Chapter 4 part I). In view of these results Bowers & James (1967) attempted to culture the metacercariae of M. minutus in sea water at various temperatures. When the sea water was diluted by four parts to one and the cultures were kept at 31^{o}C. the metacercariae survived for 7-9 days. At a higher temperature the metacercariae died. There was no development.

Several Microphallids have also been cultured to egg production. Smyth (1966) records that Microphallus papillorbustus (a progenetic metacercaria containing sperm and mature vitellaria) will produce eggs after 4 hours in sea water. Hunter & Chait (1952) cultured the metacercariae of Gynaecotyle adunca (Microphallidae : Gynocotylae) in 1% sea water which was renewed daily. Sperm was observed in the cirrus 1-3 hours after excystment and normal eggs were shed 80 hours later. The maxiumum survival in culture was 8 days. James (1971) cultured the metacercariae of Microphallus pygmaeus in Medium 199

plus 10% bovine serum at 37°C. with a gas phase of 5% CO_2 in nitrogen and a pH of between 6.8 and 7.2. He records that one in ten metacercariae survived and that after 5 days they began to produce the first eggs with a maximum of 20 eggs after 8 days.

The criteria that are used to assess cultivation techniques are very important. Smyth (1969) states that

> "The completion of normal embryonic development and maturation are clearly the only satisfactory criteria for development."

A knowledge of the normal pattern of growth and development *in vivo* is therefore essential. With this knowledge the developmental process can be divided up into stages which can then be used as criteria for assessing development *in vitro*. Bell & Smyth (1958) suggest that these stages should be:

a) precisely definable (eg. the appearance of active spermatozoa);
b) readily recognisable - either by direct observation or by means of simple techniques;
c) cover the whole range of the maturation process.

They selected the following stages to describe the maturation of the streigid trematode *Diplostomum phoxini*.

Stage 1. Cell Multiplication. Characterised by an outbreak of intense mitotic activity which can be detected using the colchicine/aceto-orcein method.

Stage 2. Body Shaping.

Stage 3. Organogeny. Appearance of the genital anlagen.

Stage 4. Early gametogeny. Appearance of the early stages of spermatogenesis, oogenesis and vitellogenesis.

Stage 5. Late gametogeny. Maturation of sperm and ova.

Stage 6. Egg shell formation and vitellogenesis. This can be detected using the Fast Red Salt B or Catechol techniques.

Stage 7. Oviposition.

Criteria which have been used by other workers to assess cultured flukes include mobility, reaction to stimuli, size increase and various physiological and biochemical activities such as utilization of substrates or level of energy reserves. Most of these methods of assessment are unsatisfactory if used in isolation but they can be used to provide useful additional information. Although the stages of development described for streigids can be applied to other trematodes, it is probably better to compile more detailed and precise criteria for the cultivation of each particular metacercaria studied.

Although these types of criteria are very useful for general and day to day assessment of cultures, they do not usually provide information on why certain culture systems fail. Within the framework of these criteria more complex techniques can be used. Kannangara (1973) used histochemical, ultrastructural and ultrahistochemical techniques to examine his cultured trematodes. Ultrastructural examination of the tegument and the gut - which are in intimate contact with the culture media - enabled him to draw various conclusions about the nutritional processes of the cultured metacercariae.

Shaw (1973) studied the ultrastructure of the tegument, gut and reproductive system of adult Schistosoma mansoni maintained in Earles BSS and human serum. Robinson (1971) looked at the fine-structure of the gut epithelial cells of F. hepatica maintained in Hédon-Fleig saline with or without glucose. Hanna (1973a,b) studied the tegument and various other organ systems in slices of adult F. hepatica maintained in Hédon-Fleig saline. General features of the ultrastructure of cells and tissues in culture are discussed by Toro & Rappay (1972).

In this study the normal pattern of development of each of the three metacercariae (F. hepatica, M. minutus and M.similis) was determined and used as a basis for assessing growth and development in vitro. In addition a comparative ultrastructural study of cultured flukes, metacercariae and in vivo grown flukes was undertaken. The combined results were used to assess the cultivation techniques.

CHAPTER 2 : GENERAL METHODS

A. CULTURE METHODS

1. General sterile precautions

All the routine procedures of media preparation, setting up cultures and periodic changing of the culture media were carried out under sterile conditions. Only sterilized apparatus (bottles, pipettes, etc.) was used throughout and all the procedures were carried out inside a lamina flow cabinet which was regularly cleaned and swabbed with ethanol before use. Great care was taken not to touch the parts of the sterile apparatus which would come into contact with the media, cells or worms. The ends of pipettes and any unstoppered exits on a piece of apparatus such as the "Lift" or the Cellulose Tubing Culture Apparatus, were plugged with non-absorbent cotton wool before sterilization. The various items were thoroughly cleaned and dried and put in appropriate sized sterilization bags before autoclaving at 2kg./cm.^{2} pressure at 135^{o}C for 7 mintues under vacuum for pipettes, and at 121^{o}C for 20 minutes without vacuum for other glassware.

2. Physical Conditions of Culture

a) Culture Vessels

1. Leighton tubes (Bellco)

These are screw-capped glass tubes with one flat side near the lower end. Two sizes were used, 15ml. and 20ml. capacity.

2. Falcon Flasks (Flow)

These are sterile, rectangular, screw-capped plastic flasks which come in two sizes; the smaller 30ml. size was used.

3. Round Falcon Flasks (Flow)

These are sterile, round, screw-capped plastic flasks with two flat sides, containing about 60ml. of liquid; they will stand upright.

4. Universal containers (Sterelin)

These are sterile, screw-capped plastic vials containing about 25ml. of liquid. They have a pointed end similar to a centrifuge tube but they will stand upright.

5. Disposable plastic tubes (Sterelin)

These are sterile, screw-capped plastic tubes, 10cm. long,

1.5cm. in diameter, containing about 12ml. of liquid.

6. The "Lift"

This is a circulatory apparatus used by Smyth and Miller (1968) for the cultivation of Echinococcus granulosus. The upper part is partly based on Berntzen's recirculatory apparatus. The metacercariae were placed in three different types of conditions within the system.

1. In porous thimbles in the reservoir of the slowly circulating liquid phase.
2. In the cellulose tubing.
3. In the undisturbed liquid phase.

The system is described by Smyth (1969).

7. The Cellulose Tubing Apparatus

This apparatus is described by Smyth (1959, 1969), it was used for the cultivation of Schistocephalus solidus. Metacercariae were placed in the cellulose tubing with some media and allowed to sink to the bottom. The glass tube was filled with culture media.

b) Gas Phase

The following gas phases were used:

1. Air.
2. 5% CO_2, 15% O_2, and 80% N_2 (Aerobic)
3. 10% CO_2, 90% N_2 (Anærobic)

Sterile gas was bubbled through the stock bottles of the various media before use. Any large air-space in the bottles

was gased.

c) Agitation

Three different types of agitation were used.

1. Shaking water bath (Gallenkamp)

The culture vessels were gently and continuously agitated at about 80 shakes per minute.

2. Roller tube apparatus (TAAB)

The culture vessels were rotated horizontally at about two revolutions per minute in an incubator.

3. Rotator apparatus (Eschman)

The culture vessels were rotated vertically at about 25 revolutions per minute in an incubator.

d) Temperature

The routine temperatures used were 39-41°C for M. minutus and M. similis, and 37-38°C for F. hepatica.

e) pH.

The pH was maintained at approximately 7.4 using the colour change of the phenol red in NCTC 135 and Hanks saline as an indicator. Alkalinity was easily corrected by gasing the media.

f) Routine replacement of media

Depending on the particular protocol involved, the culture media was changed once or twice a week. This involved withdrawing the top three quarters of the old media and replacing it with fresh medium. In those cultures which contained red blood cells, these were replenished when the media was changed or as necessary.

3. Culture Media

a) Commercially obtained media

1. Hanks saline (DIFCO)

9.9g. of the commercially available powder and 0.35g. of sodium bicarbonate (Analar, BDH) were dissolved in a litre of triple distilled water (DIFCO). The solution was sterilized by vacuum filtration through a 0.2um. millipore filter (Gelman) into a sterile flask with various precautions to prevent contamination. The sterile media was decanted into sterile bottles, gased and stored at 4°C. The compsition of Hank's saline is given by Paul (1970)...

2. Medium NCTC 135 (GIBCO)

9.4g. of dried commercially available powder and 2.2g. of sodium bicarbonate were dissolved in one litre of triple distilled water and sterilized and stored as described for Hanks saline. In some cases glucose powder (Analar, BDH) was added to the powdered constituents. The composition of NCTC 135 is given by Paul (1970).

3. Medium 858

9.95g. of the powder and 2.2g. of sodium bicarbonate were reconstituted, sterilized and stored as described for Hanks saline. The composition of 858 is given by Paul (1970).

Other defined media (Flow):

Obtained in bottles as a sterile liquid and stored at 4oC.

4. Hams F12.

5. DMEM: Minimum Essential Medium (Dulbecco's Modification).

6. HBME: Basal Medium Eagles with Hanks salts.

7. MEM Vitamins (10x concentrate).

Antibiotics

8. Penicillin and Streptomycin (BDH)

These are supplied as a sterile powder and are reconstituted by adding 4.4ml. of sterile water (Evans Medical Ltd.). This solution is used at a concentration of 100 units each per ml.

of media. It is stored at 4°C. These antibiotics were routinely added to NCTC 135, 858 and Hanks saline before they were sterilized. They will henceforth be referred to as "antibiotics".

9. Amphotericin B. / Fungizone (Flow).

This is supplied in liquid form at a concentration of 250ug./ml. and is used at a concentration of 2.5ug./ml. of culture media. It is stored at -20°C.

10. Tetracycline.

This is supplied in a non-sterile powdered form.

Sera(Flow, Bio-Cult):

11. Foetal calf serum.
12. Calf serum.
13. Bovine serum.
14. Horse serum.
15. Lamb serum.
16. Swine serum.
17. Rabbit serum.
18. Chick serum.

The serum is supplied sterile and is stored at -20°C. After thawing, it may be kept for a short period at 4°C. Serum is inactivated, if necessary, by heating at 56°C for 30 minutes.

Red blood cells (Tissue Culture Services):

19. Sterile defibrinated horse blood.

20. Sterile defibrinated sheep blood.

21. Sterile chick cells (in Alsevers).

These cells are supplied in Alsevers and must be washed several times in Hanks saline before use. They are stored at 4°C.

Various additives:

22. Yeast extract (Oxoid).

A 5% solution (w/v) of yeast extract powder was made up in NCTC 135. It was filtered through Millipore filters of 5um, 1.2um and 0.45um pore size and then through a 0.2um pore size filter to sterilize it. It was stored in sterile bottles at 4°C.

23. Powdered liver concentrate (Sigma).

24. Rice starch (BDH).

25. Chick embryo extract CEE_{50} (Flow).

26. Bovine embryo extract BEE_{50} (Flow).

27. Vitamin C powder.

28. Glass beads 0.7mm. in diameter.

29. "Artificial gut" - nylon fur.

30. Insulin (Sigma).

31. Estrone-3- sulphate (Sigma).

32. L-thyroxine (Sigma).

33. Sodium taurocholate (prepared by Professor Haslewood).

Monolayer cell cultures (Flow):

34. Chang liver cells.

35. Mouse embryo cells.

36. Chick embryo cells.

37. Baby hamster kidney cells - BHK.

b) Prepared Media

1. Egg Macerate

This was prepared according to the method of Kannangara & Smyth (1974). Whole egg macerate was used. The surface of two hens' eggs were painted with ethanol and allowed to dry. The contents were then emptied into a sterile beaker. The yolk and albumen were mixed and coagulated by heating to 80°C for 1½ hours. The coagulated egg was transfered to a sterilized glass homogeniser with an equal volume of NCTC 135 with antibiotics. The mixture was homogenised for 15 seconds at a slow speed and the homogenate was stored in sterile bottles at 4°C.

2. Yolk Medium

This was prepared according to the method of Berntzen & Macy (1969). It is a suspension of the soluble parts of yolk and those parts which will go into a fine emulsion. In this case it was either made up in NCTC 135, in which case it was denoted as NCTC-Y, or with NCTC 135 plus 50% Foetal calf serum when it was denoted as (NCTC &FCS)-Y.

3. Chick Embryo Extract (CEE_{50})

This was prepared by the method described by Paul (1970) (page 79) according to the technique of Coon, Cahn & Cahn. The crude supernatant of the homogenate was separated into two fractions on a Sephadex column. Apart from the crude fraction a low molecular weight (L) and a high molecular weight (H) fraction were collected, transferred to sterile bottles and stored at -20°C.

4. Mouse Embryo Extract (MEE_{50})

This was prepared according to the general method described by Paul (1970) (page 81) for bovine embryo extract. The extract was stored in vials at -20°C. It was centrifuged before use and any solid matter was discarded.

5. Bile

a) Ox

b) Sheep

Sheep and ox bile were obtained from the gall bladders attached to livers brought into the laboratory for other purposes. It was collected under non-sterile conditions, centrifuged to remove any solid material and stored in vials at -20°C.

c) Duck

d) Mouse

This was collected from the gall bladders of livers removed from mice and ducks during various experiments.

It was stored at -20°C.

6. Sheep Liver Extract

The entire procedure was carried out at 4°C.

25 - 30g. of sheep liver was homogenised with 25 - 30ml. of distilled water in a blender. The resulting homogenate was diluted 1:4 with distilled water and centrifuged for one hour at 6,000 r.p.m. The supernatant was decanted and strained through three thicknesses of damp muslin. It was filtered through filters of 1.2um, 0.8um and 0.45um pore size and then through one of 0.2um to sterilize it. The extract was stored in vials at 20°C.

7. Mouse Liver Macerate

This was prepared by placing a mouse liver in the barrel of a syringe and expressing out the contents into a vial by inserting the plunger. The resulting macerate was washed several times in Hanks saline to remove blood cells. It was used immediately.

8. Duck Serum and Cells

Blood was collected from heavily anæthetized animals which were then killed. It was centrifuged at 5.000 r.p.m. for ten minutes. The serum was decanted into vials and stored at -20°C. The cells were washed twice in heperinized buffer (Tris/Ringers, pH 7.2) and stored in vials in fresh buffer at 4°C.

9. Duck Gut Extract

The upper small intestine of a Khaki Campbell duckling was washed several times with sterile Hanks saline containing antibiotics. The gut was homogenized in a Waring Blender with NCTC 135 under sterile conditions and the supernatant collected and stored at -20°C.

10. Solid bases

a) Serum bases

These are made from chick, calf, bovine or horse serum. A few mls. of the chosen serum was pipetted into the bottom of the culture vessel and heated at 75°C for about 1½ hours or until the base was firm. Different degrees of solidity in the base could be obtained by heating for different times. Some serum, e.g. chick serum, required longer for complete coagulation. In some treatments the surface of the base was pitted using a sterile Pasteur pipette, in others it was roughly chopped. Bases prepared in this way may be stored at 4°C.

Serum coagulated in this way can be used to make a macerate. The coagulated serum was transferred to a Waring Blender with an equal volume of Hanks saline. The speed and duration of the homogenisation determines the particle size of the macerate. A macerate was prepared by homogenising coagulated bovine serum with saline at a slow speed for 30 seconds. The homogenate was stored in sterile bottles at 4°C.

b) Coagulated serum and blood

The serum was mixed with homologous erithrocytes in the proportion of 3:1 and coagulated in the usual way in the bottom of the culture vessel.

c) Blood/Agar base

The preparation was adapted from the method of Novy, McNeal & Nicolle described by Smyth (1962).

1.4g. agar was dissolved in 90ml. distilled water. The solution was sterilized and cooled to 45 - 50°C. A third of its volume of sterile defibrinated blood was added and the blood/agar mixture was transferred in small amounts to culture vessels and allowed to set. The bases could be stored at 4°C.

4. Sources and Preparation of Metacercariae

a) Fasciola hepatica

Encysted metacercariae of F. hepatica were supplied by Dr. C.P. Ollerenshaw of the Central Veterinary Laboratories, Weybridge, Surrey and by Anna Sörmann of the Department of Biology, University of York. In both cases, the cercariae were allowed to encyst on cellophane and were stored in a screw-topped jar either in water, or with a piece of wet cotton wool to provide a humid atmosphere, at 4°C. The metacercariae were usually 2-4 weeks old when used but in some cases they were up to 6 months old.

Two different excystation procedures were used. The technique of Wikerhauser (1960) relies on the use of digestive enzymes whilst that of Sewell & Purvis (1969) uses the criteria for excystment laid down by Dixon (1965). These criteria are quoted by Smyth (1966) as being:

1. A temperature of about 39°C.
2. A low oxidation/reduction potential.
3. A CO_2 system.
4. The presence of bile.

The Method of Wikerhauser:

The cysts were treated for 2-3 hours in a freshly prepared solution of:

0.8% sodium chloride

0.5% pepsin (BDH, activity 1:2500)

in $N/_{20}$ hydrochloric acid (Analar, BDH)

at 38°C (pH approximately 2.0).

This solution was removed and the cysts were incubated for a further 2-3 hours in a freshly prepared solution of:

0.8% sodium chloride

0.4% trypsin (Hopkins & Williams)

1.0% sodium bicarbonate (Analar, BDH)

in distilled water

with 20% ox bile

at 38°C (pH approximately 7.4).

The Method of Sewell and Purvis :

To the cysts was added:

a) a volume of $^{N}/_{20}$ hydrochloric acid at 38°C.

b) an equal volume of a solution containing

20% ox bile

1.0% sodium bicarbonate

0.8% sodium chloride

at 38°C. The tube was capped immediately to avoid loss of carbon dioxide gas and the cysts were incubated for 4-5 hours at 38°C.

The cysts were either sterilized before excystation in which case the excystation was carried out under sterile conditions, or, more frequently, the excysted metacercariae were sterilized after a non-sterile excystment. In both cases, the sterilization procedure was similar. The cysts or metacercariae were washed under sterile conditions on a rotator in 10mls. of Hanks plus antibiotics in a Universal Container either at room temperature (cysts) or at 38°C (excysted metacercariae). Between each wash the worms were allowed to sediment, the old media was removed and fresh washing media was added. Six washes of ten minutes each were usually employed and in some cases about 3mg. of powdered tetracycline was added to the first two or three washes.

The sterile excysted metacercariae were then transferred to the culture media with Pasteur pipettes.

b) Meiogymnophallus minutus

Infected cockles (Cardium edule) were collected from the shore at Shoeburyness, Southend or from various areas of the Lhanrhiddian Sands near Swansea. The best source for M. minutus

metacercariae was from the cockles collected at Salthouse Point on the Lhanrhiddian Sands.

The cockles were examined as soon as possible after collection. If necessary, they were kept in tanks of aerated sea water.

The cockles were prised open to expose the unencysted metacercariae enclosed in the pallial line tissue near the hinge of the cockle. The pallial line tissue was removed and the metacercariae were teased out into Hanks saline or sea water under a dissecting microscope. The metacercariae were passed through a fine sieve to remove any cockle tissue or pieces of sand. They were then transferred to a sterile Universal Container and washed at room temperature according to the method described for F. hepatica. The sterile metacercariae were then transferred to the culture media.

c) Microphallus similis

Infected crabs (Carcinus maenas) were supplied by the University of London Biological Station, Millport, Scotland. The crabs were examined immediately or kept surrounded by moist seaweed for short periods. They were then pithed and the carapace removed. The digestive gland tissue was removed and the cysts of M. similis were teased out into Hanks saline or sea water under a dissecting microscope. The cysts were washed several times to remove any host tissue.

The encystment of the metacercariae was carried out as follows:-

They were treated for 30-60 minutes at 38°C in a freshly prepared solution of

0.5% pepsin in Hanks

brought to pH 2.0 with 1N hydrochloric acid. This was removed and they were treated for 10-15 minutes (or until excystment was complete) at 38°C in

0.4% trypsin (Sigma) in Hanks

brought to pH 7.6 by 1N sodium hydroxide.

The excysted metacercariae were transferred to a sterile Universal Container and washed at 38°C according to the method described for F. hepatica. The sterile metacercariae were then transferred to the culture media.

5. Treatment of Cultured Cells

a) Commercially obtained cell lines (Flow)

The cell lines used were:

Baby hamster kidney - BHK,

Chang liver,

Chick embryo,

Mouse embryo.

1. Maintenance

The cells were received as a confluent monolayer culture in round, flat-sided plastic flasks. They were incubated at 37°C. The maintenance medium was DMEM or HBME plus 10% calf serum for Chang liver or BHK cells, and DMEM or HBME plus 10% foetal calf serum for the embryo cells. 5mls. of the medium was used and the media was completely changed every 3 or 4 days. When the monolayer cultures were used in the cultivation of metacercariae the maintenance medium was removed and the appropriate experimental medium added.

2. Subculture

When the monolayer became confluent it was necessary to subculture the cells. The method is described in detail by Paul (1970), and involves two procedures:

a) suspending the cells from a monolayer culture

and b) inoculation of new vessels.

The culture medium was removed and 2mls of Earles balanced salt solution without calcium or magnesium (Flow) containing 0.25% trypsin (Flow) was added. After one minute, this solution was removed and the culture vessel was incubated at 37°C for 15 minutes or until the cells became detached from the surface of the flask. The cells were re-suspended in 5mls. of fresh growth medium and the number of cells per ml. was counted using a haemocytometer. Subcultures were prepared so that a final concentration of $1 - 5 \times 10^6$ cells per ml. of growth medium was obtained. The cultures were then incubated at 37°C and after a short time the cells formed a monolayer.

If the monolayer cultures contained trematodes, the trematodes were transferred to another flask containing the same cell line during the subculture of the original flask.

b) Prepared Cell and Tissue Cultures

1. Cell Cultures

Several attempts were made to culture disaggregated mouse hepatocytes using various different techniques, but these proved unsuccessful. Hepatocytes are very delicate cells and need special handling; they cannot be subcultured. In a mixed liver culture, the fibrocytes rapidly outgrow the hepatocytes and the culture contains only fibrocytes.

2. Tissue Cultures

Various techniques wereused in an attempt to grow fragments of mouse liver. The most successful method used the livers of 3-day-old mice. These were excised under sterile conditions and minced into small fragments of about 1 - 2 mm^3. The fragments were washed several times to remove blood cells and they were inoculated into culture vessels. The best culture system included a solid base of chick or calf serum with an overlying medium of NCTC 135 plus antibiotics with 20% foetal calf or chick serum in a round, flat-sided flask. The flask was incubated at 37°C. By day 3 of culture columns of hepatocytes had begun to migrate outwards. After 5 - 7 days there was considerably growth of cells from the explant and these cultures were now used for the cultivation of F. hepatica metacercariae (Plates 3 & 4).

B. ULTRASTRUCTURAL METHODS

1. Materials

Metacercariae, in vivo grown developmental stages, in vivo grown adults and cultured metacercariae of F. hepatica, M. minutus and M. similis were used.

2. Preparation for Transmission Electron Microscopy

(Modified from Lumsden, 1970)

The worms were fixed in 3% glutaraldehyde (TAAB) in Sorenson's 0.1M. phosphate buffer (pH 7.3) containing 3% sucrose for at least 2 - 3 hours at 4°C. They were washed in three changes of cold buffer containing 5% sucrose for at least 20 minutes each and then post-fixed in 1% osmium tetroxide (TAAB, EMscope) in Sorenson's buffer containing 1% sucrose for one hour at 4°C. The fixed worms were then quickly washed three times in cold distilled water. They were dehydrated with cold ethanol in stages from 50% to 95% and left to reach room temperature in 95% ethanol. This was then replaced with 100% ethanol using three changes of 10 minutes each. The ethanol was replaced with a 1:1 mixture of 100% ethanol and propylene oxide for 5 minutes. The worms were then taken through two 10 minute changes of pure propylene oxide and transferred to a 1:1 mixture of propylene oxide and epoxy resin (TAAB). They were left uncovered on a revolving stage overnight to allow gradual evaporation of the propylene oxide. In the morning they were transferred to fresh resin and allowed to infiltrate for a further 2 - 5 hours. The worms were then placed in fresh resin in Beams capsules (EMscope) or in a rubber block with rectangular cavities in which they could be orientated to give the correct plane of sectioning. The resin was polymerised at 60°C for 48 hours. The blocks were trimmed and thin sections (gold and silver) were cut using glass knives on a Huxley Mark 2 ultramicrotome. The sections were mounted on uncoated copper grids or grids coated with a film of Formvar. The grids were stained with saturated uranyl acetate in 50% ethanol for 20 minutes followed by 0.1% lead

citrate at a high pH for 1 - 3 minutes. (When staining with lead citrate, great care was taken to exclude carbon dioxide as this causes a precipitate of lead carbonate on the grids.) The sections were examined using a Phillips EM 300 electron microscope at 60 - 80 KV. Photographs were taken on Ilford N4E.50 8.2 x 10.8 cm photographic plates.

Thick sections of 0.2 - 0.3um (green and red) were also cut, mounted on glass slides, stained in 1% toluidine blue and examined on a light microscope. A drop of immersion oil was placed over the sections, a coverslip placed on top and the edges varnished. In this way virtually permanent preparations could be made.

Special techniques for M. minutus metacercariae

These metacercariae contained large numbers of calcareous granules in their excretory system. If they were processed in the normal way it was impossible to obtain thin sections that were not scored or torn - so the granules were decalcified by a method adapted from von Brand & Nylen (1970).

The worms were fixed in gluteraldehyde and osmium in the usual way and washed in cold distilled water. The metacercariae were dialyzed against cold EDTA (ethylene diamine tetracetic acid) for 72 hours and then dialyzed against cold water overnight. They were then dehydrated, infiltrated and embedded in the usual way.

3. Preparation for Scanning Electron Microscopy

The worms were fixed in glutaraldehyde and washed in buffer as previously described. They were then dehydrated in ethanol

at room temperature for ten minutes in each stage. (The stages used were 30%, 50%, 70%, 80%, 90%, and 95% ethanol). They were transferred to three ten minute changes of 100% ethanol and then to amyl acetate for two changes of 15 minutes each. The worms were then dried in a Critical Point Drying Apparatus (Polaron), mounted on metal stubs with double-sided Sellotape and coated with gold in a Polaron SE Coating Unit. The stubs were examined on a Cambridge Stereoscan Scanning Electron Microscope at 10 KV and photographs were taken using a 35mm. camera.

C. IN VIVO METHODS

1. Infection of Animals

a) F. hepatica

In order to obtain the developmental stages of F. hepatica, metacercarial cysts were fed by stomach tube to TF1 mice. The number of cysts administered depended on how many days after infection the mice were killed.

Number of cysts	Days after infection mouse killed
200	1
100	3-9
50	11-15

For some experiments, excysted metacercariae or cultured worms were injected into the peritoneal cavity. The number of worms injected again depended on the number of days the infection

was to last. If the worms were allowed to develop to egg-producing adults only 2 - 5 worms were injected.

Before intraperitoneal or oral infection, the mice were lightly anaesthetized with ether.

b) M. minutus

3 - 5 day old chicks and Khaki Campbell ducklings were fed metacercariae of M. minutus enclosed in cockle tissue. If the pieces of tissue were rinsed several times in Hanks saline to remove a lot of the salt, and if the chicks or ducklings had been starved for 12 hours prior to infection they ate the piece of tissue quite readily. Usually tissue from five cockles was fed to each animal, and they were thus exposed to about 5,000 - 10,000 metacercariae. Different feeding regimes were adopted since Bowers & James (1967) only managed to obtain a mature infection if the ducklings were fed on boiled minced cockles before and after infection. The feeding regimes used for the ducklings were:

1. Boiled minced cockles throughout;
2. Boiled minced cockles up to day 1 of infection and thereafter a normal diet of baby chick crumbs;
3. Baby chick crumbs throughout.

The young rats and mice were infected by stomach tube.

c) M. similis .

TF1 mice were infected by stomach tube with 100 metacercarial cysts each of M. similis. Several mice were

injected intraperitoneally with about 50 excysted metacercariae. Before these infections, the mice were lightly anaesthetized with ether.

2. Recovery of Worms

a) F. hepatica

The mice were killed by cervical dislocation after a light ether anesthetic.

1. Recovery from the peritoneum

The mouse was skinned and a large slit made in its abdomen. It was dipped repeatedly into a beaker containing warmed Hanks saline for about three minutes. The worms were allowed to sediment, the excess Hanks was removed and the worms were recovered under a dissecting microscope.

2. Recovery from the liver

The liver was removed and briefly rinsed to remove blood cells. It was chopped into small pieces, the size of which depended on the size of the worms to be recovered. The pieces of liver were incubated in Hanks saline at 37°C for 20 - 40 minutes. The worms were separated from the larger pieces of liver by sieving and recovered using a dissecting microscope.

3. Recovery of worms for culture

In this case every attempt was made to perform the above procedures quickly and under sterile conditions. The worms were washed six times in Hanks plus antibiotics at 37°C and transferred to the culture medium.

4. Recovery for electron microscopy

The time between death and recovery was kept as short as possible. The worms from the liver were not sieved in order to avoid causing any damage to their surface, they were picked out from the pieces of liver tissue after a short incubation of 5 - 10 minutes.

b) M. minutus

The animals were quickly killed by cervical dislocation. Their gut was removed, slit, divided into sections and incubated in Hanks for 20 - 30 minutes. The pieces of gut were removed and the sediment examined for worms. It was easier to recover the worms if the animals had been starved overnight before they were killed.

Adults were also recovered from oystercatchers (Haematopus ostralegus) shot on the Llanrhiddan Sands near Swansea. A winged animal was taken back to the laboratory of Dr. B.L. James at University College, Swansea, and killed by cervical dislocation. Its gut was removed, slit and incubated in Hanks saline at 37°C for 30 minutes. The adults were collected from the sediment under a dissecting microscope.

c) M. similis

The guts of mice killed by cervical dislocation were examined in the same way as that described for M. minutus. The peritoneum was examined following intraperitoneal injection of excysted metacercariae in the same way as that described for F. hepatica.

D. HISTOLOGICAL METHODS

1. Examination of Fresh Material

In many cases it was useful to examine slightly flattened, fresh specimens. The specimens were transferred to a slide in some saline and covered with a coverslip. Some of the saline was withdrawn with a piece of filter paper until the specimen was sufficiently flattened. Photographs of flattened specimens and of live worms in culture were taken on a Nikon Inverted Photomicroscope with a warmed stage chamber.

Fresh specimens were also stained in aceto-orcein at $37^{o}C$ for 10 - 30 minutes, depending on the age and batch of the stain. The worms were then washed in water, mounted on a slide and examined either whole or as a squash. Semi-permanent preparations could be made by painting the edge of the coverslip with varnish.

2. Fixed Preparations

a) Whole worms

The worms were fixed in buffered 10% formalin, 70% ethanol or with shaking in hot AFA (for the formula see Appendix). In some cases the live worms were flattened slightly and fixed in that position. For the morphological studies on F. hepatica development the worms were not flattened, they were all fixed in hot AFA. After fixation they were stained in Gower's Carmine for routine examination. A drop of glacial acetic acid added to the stain generally shortens the staining time. The development of the vitellaria was studied using the Fast Red Salt B

or Catechol techniques (Smyth, 1954b; Johri & Smyth, 1956).

b) Sectioned Tissues

The tissues were fixed in buffered 10% formalin or Bouin. When a piece of gut was fixed, it was ligatured at both ends and some fixative was injected into the lumen before immersion in the fixative. This helped to maintain the shape and allowed more rapid penetration of the fixative. The tissues were dehydrated, cleared and embedded in wax in the usual way. Sections were cut and stained in Haematoxylin and Eosin or according to the Periodic Acid Schiff technique. Sections of epon embedded worms were stained with 1% toluidine blue during transmission electron microscope processing (as previously described). Photographs of sectioned worms were taken on a photomicroscope.

CHAPTER 3: FASCIOLA HEPATICA

PART I: GROWTH AND DEVELOPMENT IN VIVO

Introduction and Methods

The life cycle of Fasciola hepatica is well known. In Britain the adults are mainly found in the bile ducts of cows and sheep although they may infect a wide variety of animals including man. Experimental infections can be produced in mice, rats and rabbits. The sporocyst and redial generations occur in the freshwater snail Lynnea truncatula and the cercariae, which emerge from these snails, encyst on the herbage.

The rate of development of the metacercariae varies according to the host. In the mouse, the flukes reach maturity after 5-6 weeks, whereas in the sheep maturation requires 10-14 weeks (Pantelouris, 1965b).

The mouse is a very convenient laboratory host and Dawes (1962) has studied the development of F. hepatica in this host from the metacercarial stage to the adult. However, this author does not state whether his observations relating to size were made on living or fixed flukes and this clearly makes comparisons difficult. It is well known that fixation produces shrinkage and that different fixatives produce different degrees of shrinkage. Also, it is not clear whether all the mice that were used by this author were of the same strain and sex, and of a

similar age or size. This may have a significant effect on the rate and pattern of development of the metacercariae. It was therefore decided to re-examine the development of Fasciola metacercariae in the mouse.

In this study, the fixative used throughout was hot AFA which is thought to fix the flukes in a relaxed position. The mice that were used were male, white TF1's weighing about 20g. at the time of infection. The dose, administration and recovery techniques are fully described in the General Methods. Twenty-one mice were infected and three mice were killed on each of Days 1,3,5,7,9,11 and 14 post-infection. The developing flukes were recovered from the abdominal cavity and the liver. Some worms were examined in a living condition, others were fixed and stained in Gower's carmine. Various measurements and observations were made on the fixed preparations.

Measurement of growth

The following measurements were taken and the standard errors of the means were determined:

a) Mean length.

b) Mean breadth at the widest point.

c) Mean oral sucker diameter.

d) Mean ventral sucker diameter.

e) Mean length between the middle of the ventral sucker and the anterior end.

From these measurements, the following parameters were determined:-

f) Ratio of oral sucker to ventral sucker.

g) Mean length between the middle of the ventral sucker and the anterior end as a percentage of the total length.

h) Diameter of the oral sucker as a percentage of the total length.

i) Diameter of the ventral sucker as a percentage of the total length.

Observations on development

Observations were made on the following:

a) The development of the digestive caeca.

b) The development of the genital rudiment.

c) The appearance of the anterior cone.

All these measurements were also made by Dawes (1962).

The study of the development in mice up to 14 days post-infection was a preliminary one and designed to produce criteria for assessing early growth in culture. It proved unnecessary, however, to extend this study any further since the metacercariae did not develop beyond this stage *in vitro*.

The present work was not designed to study the method or route of migration through the intestinal wall or the migrations within the liver. This has been studied in detail by Dawes (1963a,b).

Results

1. Recovery

The recovery, expressed as a percentage of dose, varied from 16-23% (Table 3I:1). By Day 3 about two-thirds of the young flukes were recovered from the liver, none were found in this site before Day 3. By Day 5 the majority of flukes had reached the liver, although a few could still be recovered from the abdominal cavity. Flukes were not found in the abdomen after Day 6.

2. Measurement of Growth (Table 3I:2, Text figure 1)

Up to Day 3 post-infection, or until the young flukes began to enter the liver, there was very little growth. Thereafter, up to about Day 9, there seemed to be a period of general elongation and during this time the rate of increase in length was faster than the rate of increase in breadth. This is reflected in the drawings of the various developmental stages (Text figure 2). The one day old fluke was plump and oval but there was a progressive elongation as the fluke developed up to about Day 9.

Between Day 9 and 14 the rate of increase in breadth was much faster than previously. The anterior cone began to appear at about this time and with the corresponding increase

in breadth the fluke began to assume the typical adult body shape. Although some of the flukes that were recovered at this time had a well developed cone and a broad body, others had only a poorly developed cone and retained a more elongate body. This produced considerable variation in breadth and the standard error of the mean was correspondingly large. This was also true, but to a lesser extent, of the standard error of the mean length. During the first 9 days of infection most of the flukes on any given day were of a very similar size, this resulted in very small standard errors of the mean for all measurements.

The diameters of the suckers as a percentage of the length decreased with development although the percentage remained fairly stable between Days 9 and 14. The diameter of the ventral sucker (expressed as a percentage of length) was consistently 1-3% larger than that of the oral sucker. The diameter of the suckers, however, depends to some extent on whether they were contracted or expanded at the moment of fixation.

There was also a gradual decrease in the distance between the centre of the ventral sucker and the anterior tip of the fluke (expressed as a percentage of length) during development. This figure reflected the size of the developing anterior cone region as a percentage of length. Initially the figure was 56%; it did not change radically for the first three days of infection but thereafter it gradually decreased until on Day 9 it was about 42%. It kept to this level until Day 14.

3. Observations on development

a) The development of the digestive caeca (Text figure 2)

In the newly excysted juvenile, the pharynx was very close to the oral sucker, there was no clearly defined oesophagus and the intestine was bilobed. The two caeca curved round either side of the ventral sucker and they appeared to have sacculations on their lateral surface. While the metacercaria was actually enclosed in the cyst the caeca were filled with a dark material; in the newly excysted juvenile the caeca were virtually empty. While the flukes were in the abdominal cavity there was very little development of the caeca. They showed some elongation and the sacculations became more pronounced. Some injested material was seen in the lumen.

Young flukes were first recovered from the liver on Day 3 post-infection. The caeca of these flukes were longer and showed much deeper sacculations than the caeca of flukes recovered from the abdomen at this time. If any flukes remained in the abdominal cavity after Day 3 there was no appreciable further growth or development of their caeca. By Day 5 the sacculations in the caeca of flukes recovered from the liver had developed to produce 11-13 definite lateral outgrowths. By Day 7 post-infection, these lateral outgrowths had become longer and in some cases the tips of the outgrowths had bifurcated slightly. On Day 9, these bifurcations were more prominent and several other secondary branches could be seen along the length of the lateral outgrowths. Although the first four lateral outgrowths had bulbous or slightly bifurcated tips,

they did not develop any proper secondary branches. By Day 11, the secondary branching had increased although, as yet, there was no tertiary branching. The anterior four lateral outgrowths still had no secondary branches and the outgrowths near the termination of the caeca were also less well developed. On Day 14, the branching of the lateral caeca had increased considerably and there was now some tertiary branching. The first four lateral outgrowths were still undivided. The greatest degree of tertiary branching occurred between the eighth and the twelfth lateral outgrowth.

b) <u>The development of the genital rudiment</u> (Text figure 2)

In the newly excysted juvenile the genital rudiment was in two parts. The anterior rudiment was situated dorsal to the anterior margin of the ventral sucker and the posterior rudiment was situated just posterior to the ventral sucker. The two were connected by a thick strand of cells.

During the first 24 hours of infection, the genital rudiment did not develop noticeably. In flukes recovered from the abdomen after 3 days, the connecting strand was slightly longer and thinner, in flukes recovered from the liver at this time it was longer still. As yet the two rudiments were still almost identical to those of the newly excysted juvenile.

In flukes recovered from the liver after 5 days the posterior rudiment had elongated and was constricted into three lobes. This elongation and constriction increased and by Day 7 post-infection the posterior rudiment was virtually divided into three. The three parts were usually still connected at this stage by a thin strand of cells but in some cases they had become quite separate. Although the connecting strand between the anterior of the posterior rudiments and the anterior rudiment was very difficult to see at this stage, it was still present.

The different parts of the genital rudiment develop as follows:

Anterior rudiment		- cirrus and cirrus sac
Connecting strand		- uterus
Posterior rudiment:	anterior	- shell gland complex (sgc) and ovary
	middle	- testes I
	posterior	- tests II.

By Day 9, the three posterior rudiments were quite separate. The most anterior one of them was usually bilobed, the lobe being slightly lateral and smaller than the rest of the rudiment. The testes were becoming rectangular in shape. The anterior rudiment appeared bilobed. By Day 11, it was clear that the anterior rudiment was taking on the shape of the cirrus and cirrus sac. The lateral lobe of the anteriormost posterior rudiment was growing out from the main body of the rudiment. This lateral lobe eventually becomes the ovary and the main body of the rudiment forms the shell gland complex. At this stage the two testes rudiments were large

and slightly lobed.

By Day 14, there was a considerable variation in the size of the flukes that were recovered. The smaller flukes were generally less well developed. In the larger flukes, the anterior rudiment was clearly developed into a cirrus and cirrus sac. The strand which originally connected the two rudiments in the newly excysted juvenile was thicker and it was usually slightly coiled. The ovary rudiment had grown out further from the sgc. rudiment and now had 2-3 branches. The two testes rudiments now had 4-7 lobes.

c) Appearance of the anterior cone

The anterior cone is a muscular and extensible region anterior to the ventral sucker. It is particularly important in locomotion and exploratory feeding movements.

It was not present at all in young flukes as a clearly definable region but, by Day 11, a constriction in the lateral surfaces near the ventral sucker began to be apparent. By Day 14 a definite shoulder had developed in this region and the posterior part of the fluke was much broader than before. At this stage the differentiation of the anterior cone was more marked in some flukes than in others which were smaller and generally less well developed.

Another very prominent feature of the very early development of F. hepatica metacercariae was the loss of the excretory

concretions - this occurred during the first day of infection. These concretions packed the two main arms of the excretory system of the encysted metacercariae. They are discussed in more detail in Part III of this chapter.

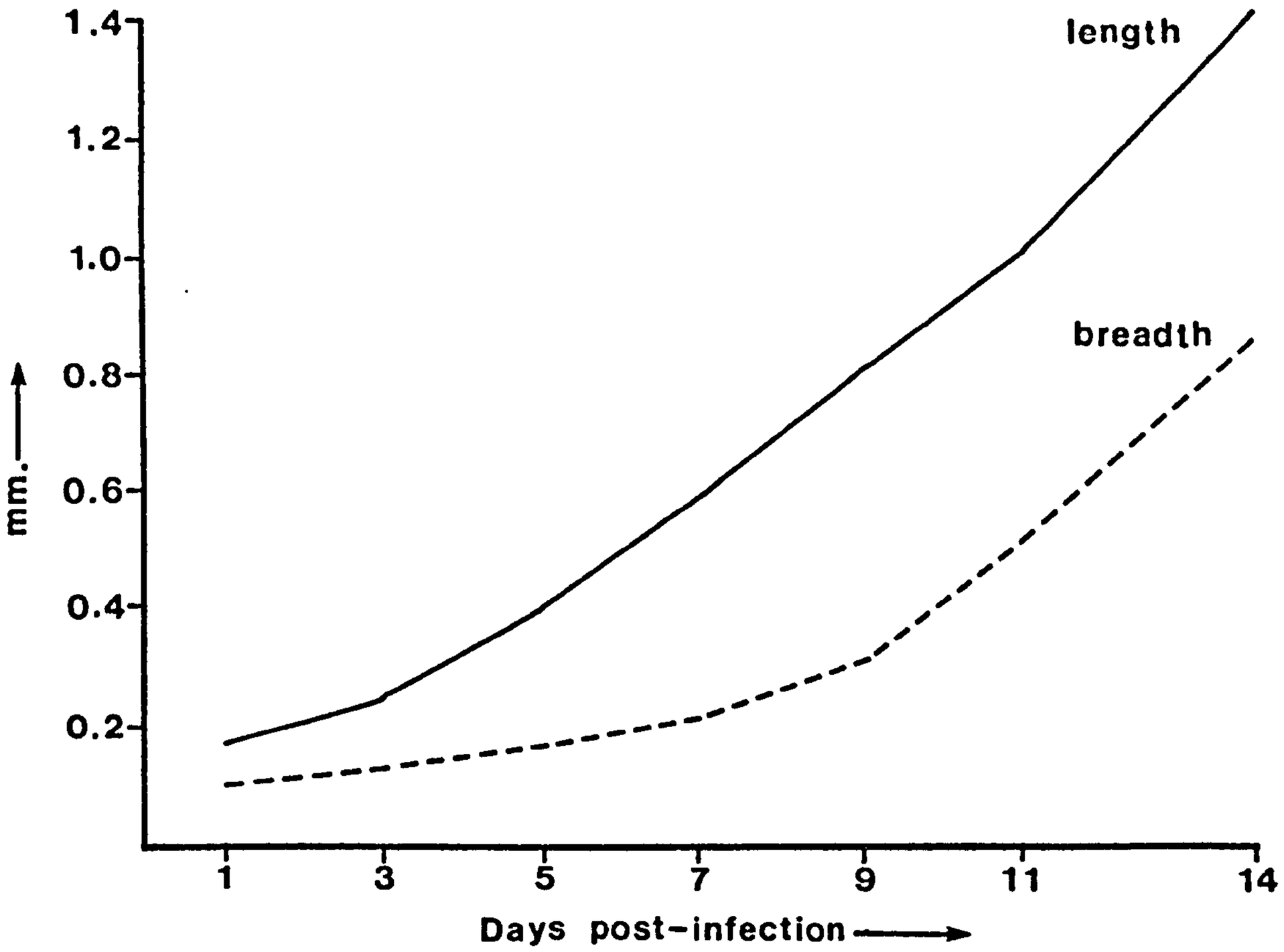
Table 3I:1 Fasciola hepatica. Recovery expressed as a percentage of cysts administered.

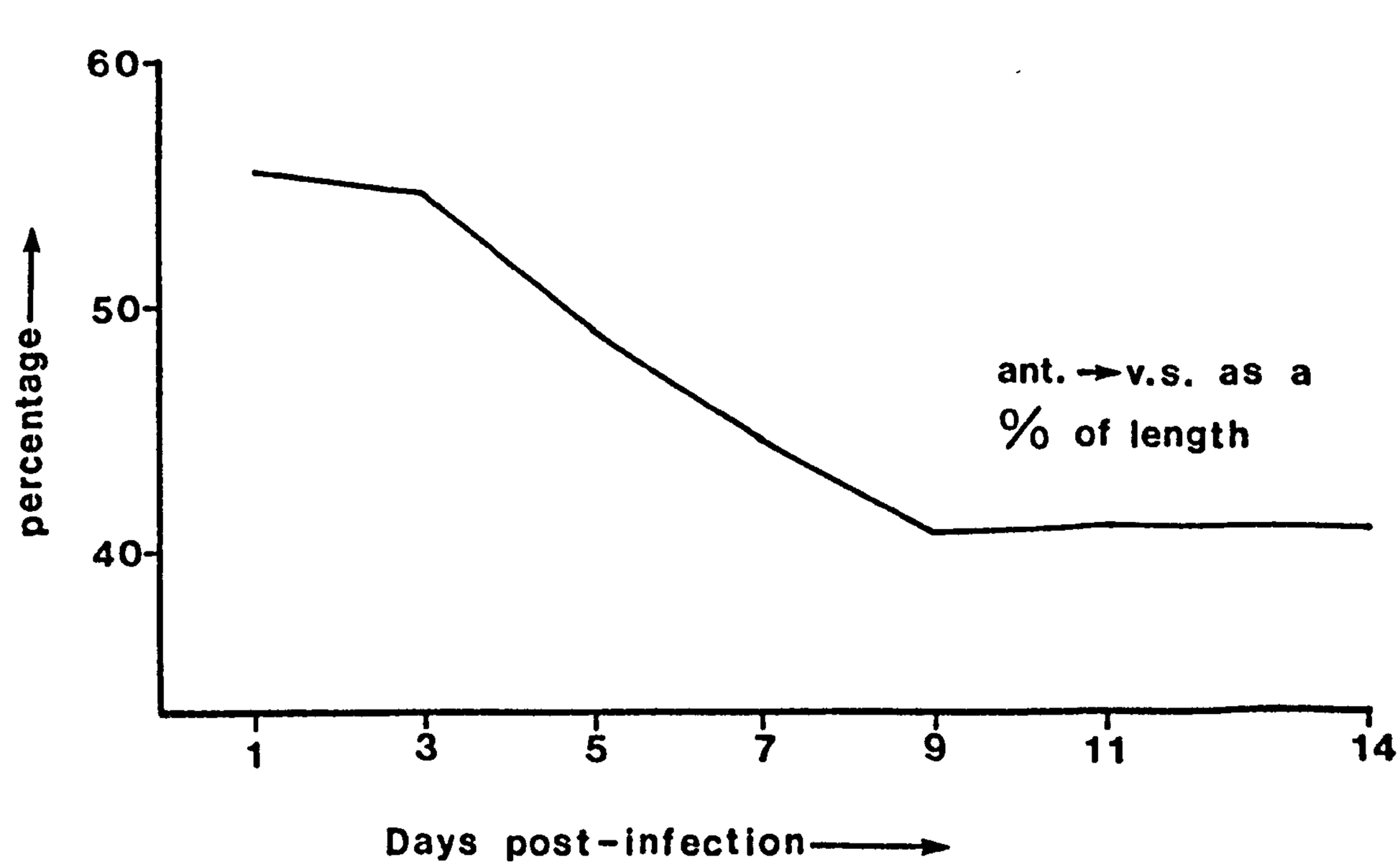
Day Post-Infection	Dose	% Recovery Abdomen	% Recovery Liver	Total % Recovery
1	200	21%	-	21%
3	100	13%	5%	18%
5	100	1%	22%	23%
7	100	-	22%	22%
9	100	-	16%	16%
11	50	-	17%	17%
14	50	-	19%	19%

DAYS POST INFECTION	N= No.of FLUKES EXAMINED	MEAN LENGTH (mm)	MEAN BREADTH (mm)	MEAN O.S. DIAMETER (mm)	MEAN V.S. DIAMETER (mm)	MEAN ANT.→ V.S.LENGTH (mm)	RATIO OS:VS	ANT.→ VS. AS A % LENGTH	DIAM. OS. AS A % LENGTH	DIAM. VS. AS A % LENGTH
1	37	0.178±0.004*	0.101±0.002	0.031±0.001	0.034±0.002	0.100±0.003	1.097	56.2	17.4	19.1
3 a)abdomen	33	0.221±0.006	0.121±0.002	0.037±0.001	0.043±0.001	0.122±0.004	1.162	55.2	16.7	19.5
3 b)liver	28	0.262±0.007	0.120±0.003	0.039±0.001	0.046±0.001	0.145±0.005	1.179	55.3	14.9	17.6
5	29	0.381±0.011	0.146±0.005	0.054±0.001	0.058±0.002	0.184±0.005	1.074	48.3	14.2	15.2
7	36	0.574±0.017	0.208±0.027	0.075±0.002	0.089±0.002	0.255±0.006	1.187	44.4	13.1	15.5
9	25	0.800±0.020	0.294±0.016	0.092±0.002	0.106±0.003	0.333±0.013	1.152	41.6	11.5	13.3
11	14	1.014±0.092	0.470±0.024	0.114±0.006	0.143±0.007	0.432±0.026	1.254	42.6	11.2	14.1
14	23	1.388±0.082	0.846±0.239	0.189±0.002	0.208±0.001	0.583±0.026	1.098	42.0	13.6	15.0

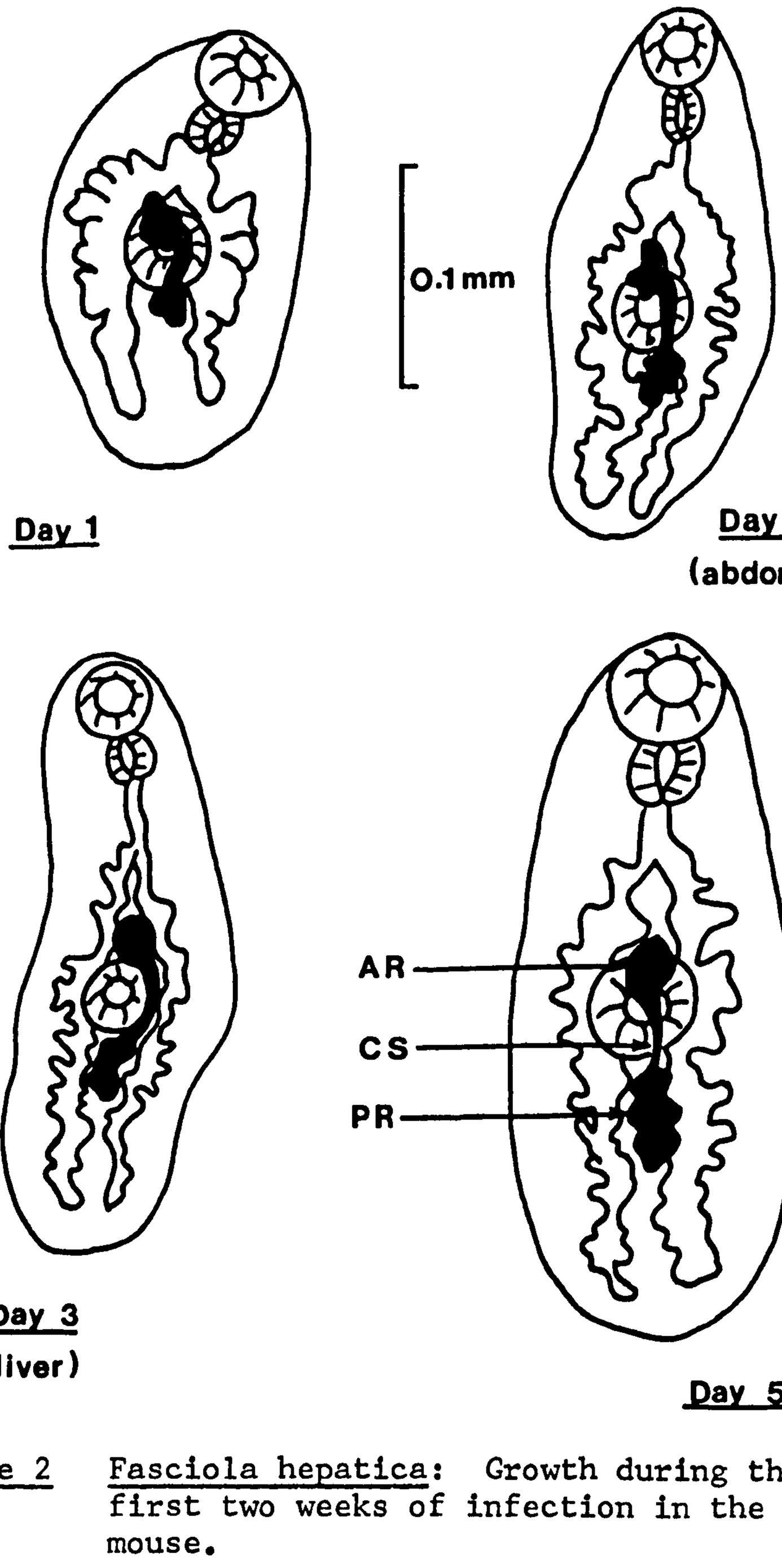
*with attached standard errors (3.d.p.)

TABLE 3I:2 F. hepatica Various measurements and parameters.





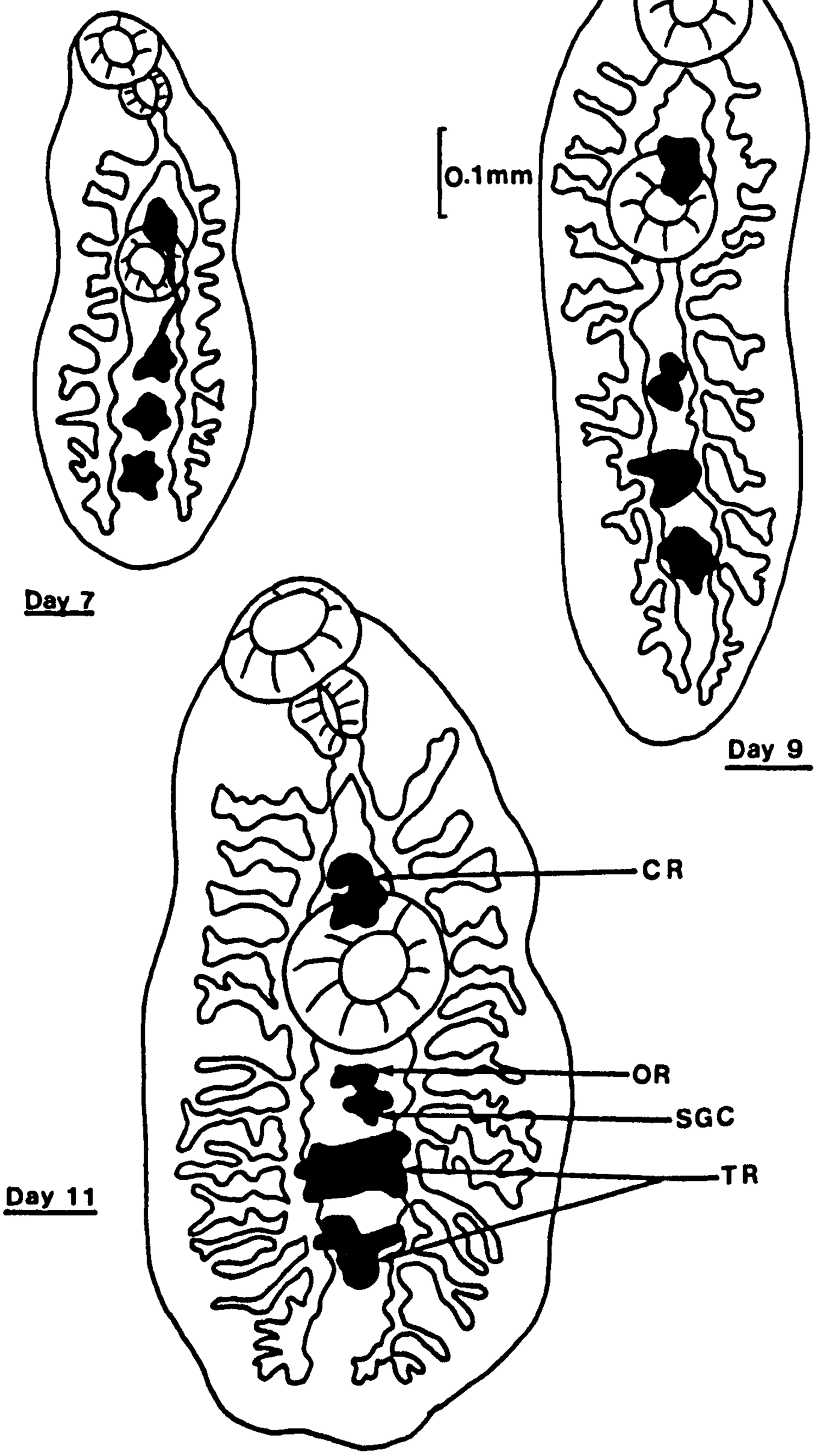
Text Figure 1 Fasciola hepatica: Development during the first two weeks of infection in the mouse.



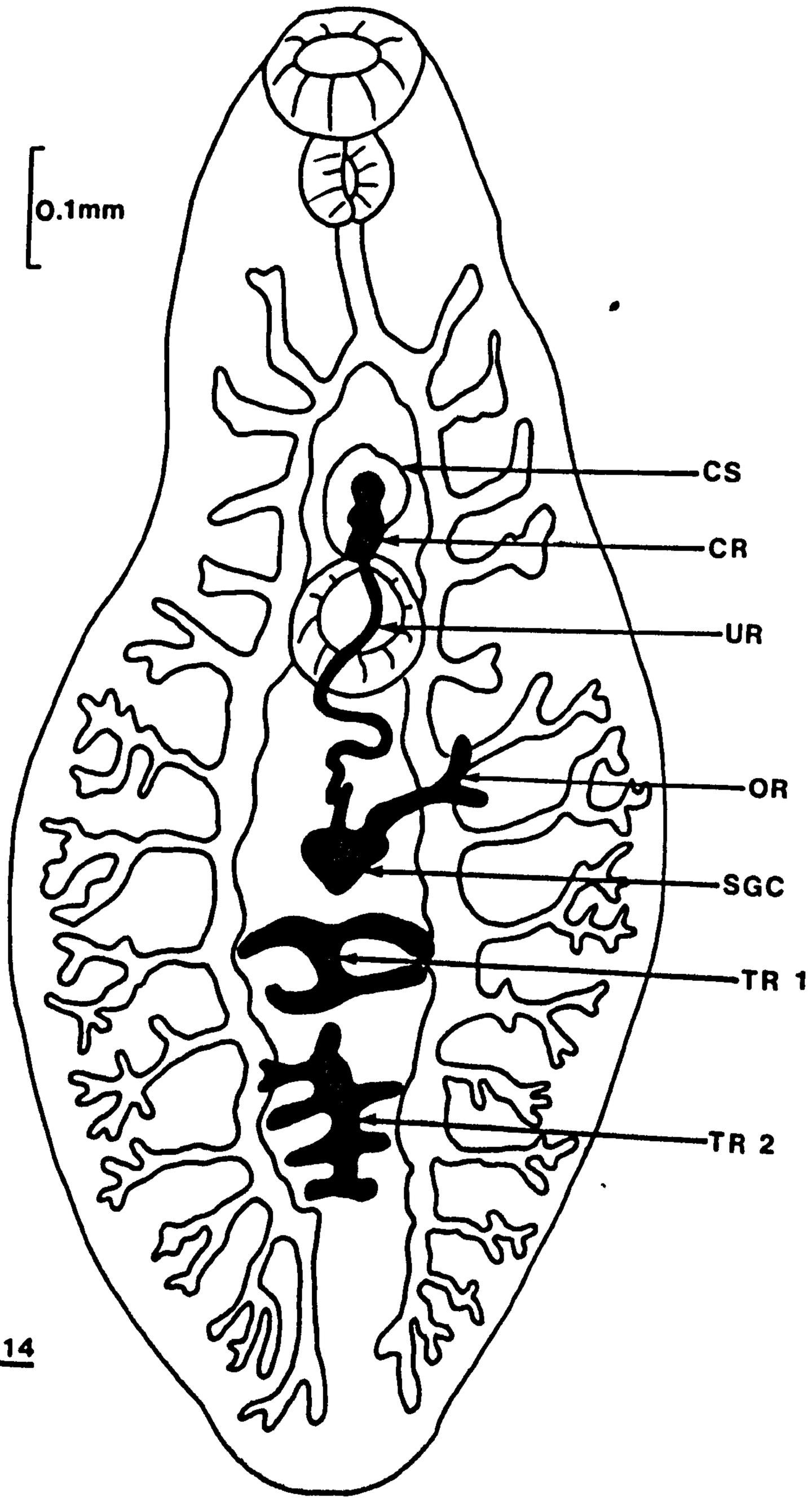
Text Figure 2 Fasciola hepatica: Growth during the first two weeks of infection in the mouse.

Key.

AR - anterior rudiment
CR - cirrus rudiment
CS - cirrus sac
OR - ovary rudiment
PR - posterior rudiment
S - connecting strand
SGC - shell gland complex
TR - testes rudiment
UR - uterus rudiment.



Text Figure 2. (continued)



Text Figure 2. (continued)

Discussion

The results presented here are, in general, similar to those obtained by Dawes (1962). Although the developmental pattern was identical the rate of development observed by Dawes was slightly faster (e.g. he records tertiary gut branching on Day 11). The measurements presented here are slightly, but noticeably, different to those recorded by Dawes although the trends are similar.

These slight differences could be accounted for by any one of the following:

a) Variations in experimental technique.

b) Variations in age and batch of cysts.

c) Possible non-standardisation of strain, age, sex or diet of the mice.

d) Different worm burdens.

e) Different fixation methods.

Dawes observed, however, that the diameter of the suckers and the distance between the ventral sucker and the anterior tip (both expressed as a percentage of total length) gradually decreased during development. The plateau in both these percentages between Days 9 and 14 which was observed in the present study (see Table 3I:2) was not recorded by Dawes. Its existence could be explained by the fact that there was a change in overall body shape during this period, the most obvious feature being a marked increase in breadth. However, this does not explain why it was observed in the present study and not by Dawes.

Bennett(1975c) has studied the numbers of young flukes recovered from the abdominal cavity of mice at various times between 2 hours and 4 days post-infection. He does not express his results simply as a percentage of dose but incorporates a measure of the viability of the cysts. Viability was assessed by the percentage of metacercariae which excysted in vitro which he recorded as 70%. He first recovered juveniles from the abdomen two hours post-infection. He found that the maximum number of flukes (65-68%) were recovered from this site after 6-7 hours and that this figure declined rapidly between 7-12 hours and more gradually between 1-4 days. After 4 days only 3% of the infection was recovered from the abdomen. If the different methods of assessing percentage recovery are taken into account, the recoveries obtained by this author on Days 1 and 3 are virtually the same as in the present study. (Bennett records a recovery of 29.5% on Day 1, and 10.5% on Day 3).

The fact that Bennett first recovered flukes after 2 hours indicates that excystment and penetration of the host's intestine can occur quite rapidly. A maximum recovery of 68.5% (even when the viability of the metacercariae are taken into account) could be due to inadequacies in the experimental technique or because a proportion of juveniles may have got "lost" during their journey across the host's intestine.

Conclusions

From these results various suitable criteria for assessing the early growth of F. hepatica were developed. Four separate criteria were used.

1. Increase in length with time.
2. Development of the digestive caeca:
   - Day 5 - production of lateral outgrowths;
   - Day 9 - secondary branching;
   - Day 11 - tertiary branching.
3. Development of the genital rudiment:
   - Day 3 - elongation of the connecting strand;
   - Day 5 - elongation of the posterior rudiment and its constriction into three parts;
   - Day 7-9 - division of the posterior rudiment into three separate parts;
   - Day 11-14 - Development of the cirrus sac, coiling of the connecting strand, outgrowth and branching of the ovary rudiment, lobing of the testes.
4. Development of the anterior cone and corresponding alteration of body shape (Day 11-14).

One very important point to emerge from these results is that by all these criteria young flukes which were recovered from the abdominal cavity had hardly developed at all. The normal pattern of development towards maturity only really began when the juveniles reached the liver. Those flukes which remained in the abdominal cavity after Day 3 did not grow or develop any further.

PART II: GROWTH AND DEVELOPMENT IN VITRO

Introduction and Methods

The techniques used for the excystment and cultivation of the metacercariae of F. hepatica have been described in the General Methods. Because of the fluctuating availability of metacercariae and the difficulty of counting out newly excysted juveniles while maintaining sterility, the number of flukes per culture varied. For this reason it was not possible to carry out a statistical analysis of the results.

The cultures were examined regularly (2 or 3 times weekly) under an inverted microscope in a warmed chamber. A record was kept of the flukes' mobility, gut contents and development, general shape, colour and length. Flukes were occasionally removed from culture during the course of the experiment to examine the development of the genital rudiment by staining in aceto-orcein.

Developing flukes were recovered from mice after various periods and transferred to an in vitro system in an attempt to answer the following developmental problems.

a) Is the penetration of the host's intestinal wall necessary in order to trigger further development in the way that penetration of the host's skin is necessary for the subsequent development of the cercariae of Schistosoma sp.?

b) Since very little development occurs unless the fluke reaches the liver (see Part I), is the subsequent development within the liver initiated by an initial "all or none" stimulus?

c) Once the fluke has reached a certain developmental stage within the mouse will it continue to develop further in culture or does it merely maintain that level of development or does it actually regress?

In order to test the suggestion of Hughes & Christofinis (quoted in Dawes & Hughes, 1964) that the cultured flukes are in a state of "suspended animation", metacercariae grown in a medium of NCTC, 20% foetal calf serum and horse red blood cells were injected intraperitoneally into mice after 4,8 and 12 days in culture. Five flukes were injected into each of three mice on each occasion. The liver and bile ducts of these mice were examined after a further 40 days.

Results

The results have been tabulated (Tables 3II:1-7) into groups of related experiments; they are not necessarily presented in the order in which they were performed. Where repeat experiments were carried out, only the results from the best experiment are included. If the cultures became contaminated before Day 14 the results are not included in the tables.

Except where otherwise stated, the flukes were cultured in 10mls. of media in a Leighton tube which was kept at a temperature of 37-38°C. in a shaking water bath; the media was gassed with the aerobic gas (N_2,O_2,CO_2); it was changed once a week. In those cultures which also contained monolayer cell cultures or explants the flukes were cultured in 5-7mls. of media in round Falcon flasks which were stored on their side at 37-38°C. in an incubator. The monolayer cells were subcultured as necessary.

The duration of the experiment is recorded in days and the reasons why a culture was terminated are given as follows:

C - the culture became contaminated (after Day 14).

D - all the flukes were dead.

T - the experiment was terminated. This was for a variety of reasons - e.g. the flukes had not grown or developed for some time and were becoming abnormal; no more media was available; the temperature of the water bath rose or fell drastically and the flukes were killed.

The measurements of the flukes' length refers to the optimum not the average length; they were measured alive and in a relaxed position using a measuring eyepiece.

The development of the genital rudiment was compared to the development in the mouse and the stage was described as the number of days post-infection when the same stage was observed *in vivo*. The development of the digestive caeca was described according to the various stages observed *in vivo* (see Part I of this chapter and Dawes, 1962). They were:

a) development of lateral branches;

b) bifurcation at the tips of the lateral branches;

c) development of secondary (2^{o}) branches;

d) development of tertiary (3^{o}) branches;

e) further branching ($4^{o}+$).

Table 3II: 1 Defined Media

Exp. No.	Media	Other Conditions	Duration	Results			Comments
				Length	Gut	Genitalia	
10c	Hanks saline w/o glucose	No serum	3(D)	No growth	No growth	0	Active for one day only
10b	Hanks saline	No serum	3(D)	No growth	No growth	0	"
10a	NCTC	No serum	5(D)	No growth	No growth	0	Active for two days only
	NCTC	Various sera					See Table 3II: 2
41a	120% NCTC	+20% FCS	15(D)	0.35mm	Lateral branchs	0-3	
41b	Hams F_{12}	+20% FCS	15(D)	0.35mm	Lateral branchs	0-3	

Table 3II: 2 NCTC and various sera

Exp. No.	Serum	Other conditions	Duration	Results			Comments
				Length Gut		Genitalia	
36e, 39c	20% Foetal Calf Serum (FCS)	W/o red blood cells (r.b.c.)	33(D)	0.35mm	Bifur-cation	0	
3a,b,13a 19,25a, 39d	20% FCS	+ horse r.b.c.	43(D)	0.55mm	2° branchs	0-3	Injesting blood (see Plates 1&2)
43a	20% chick serum (ChS)- Batch A	+ horse r.b.c.	18(C)	0.7mm	2° branchs	3-5	All very active & healthy, no black solids in gut. See Text Figures 3&4.
43b	50% " "	"		1.2mm	2° branchs	3-5	
48	100% " "	"	61(T)	0.55mm	Lateral branchs	0-3	Very dark, lots of lipid in excretory ducts
51a	20% ChS - Batch B	"	51(D)	0.44mm	Lateral branchs	0-3	Only moderately active black solids accumulated in gut
51b	50% " "	"	51(D)	0.44mm	Lateral branchs	0-3	

Table 3II:2 (continued)

Exp. No.	Serum	Other Conditions	Duration	Results			Comments
				Length Gut		Genitalia	
25 b	20% rabbit serum	+ horse r.b.c.	19(C)	0.55mm	Bifurcation	0-3	No difference
c	" (inactivated)	"	19(C)	0.55mm	Bifurcation	0-3	
20	20% lamb serum	+ horse r.b.c.	16(D)	0.33mm	No growth	0	Inactivated serum is better
21	" (inactivated)	"	24(T)	0.60mm	Bifurcation	0-3	
23 a	20% calf serum	+ horse r.b.c.	12(D)	0.30mm	No growth	0	Inactivated serum slightly better
b	" (inactivated)	"	24(T)	0.45mm	Lateral branchs	0	
24 a	20% horse serum	"	4(D)	No growth	No growth	0	No difference - both very toxic
b	" (inactivated)	"	6(D)	No growth	No growth	0	

Table 3II: 3 Diphasic Media

Exp. No.	Base	Media	Duration	Results			Comments
				Length Gut		Genitalia	
2 c	Coagulated calf serum (with & w/o holes)	NCTC +20%FCS	20(D)	0.30mm	No growth	0	Burrowed into base, got trapped. Not feeding.
36 a	Pieces of coag. calf serum	"	28(C)	0.33mm	Lateral branches	0	Feeding, burrowing.
36 b	Macerated coag. calf serum	"	28(C)	0.40mm	Lateral branches	0-3	Feeding
36 c	Semi-coagulated FCS	"	28(C)	0.40mm	Lateral branches	0-3	Feeding
L1	Coag. (calf serum & horse r.b.c.)	"	8(T)				See Table 3II:5
2 b	Nutrient agar/horse r.b.c.	"	10(D)	No growth	No growth	0	No burrowing, injest base, do not digest it.

Table 3II: 3 (continued)

Exp. No.	Base	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
2 a	Egg Macerate (5:1, yolk: albumen) 1 drop	NCTC + 20% FCS	28(T)	0.33mm	Lateral branches	0	Feeding
1 c	20%	"	5(T)	No growth	No growth	0	Dead when examined
36 d	Liver macerate	"	28(C)	0.40mm	Lateral branches	0	Feeding - macerate degenerates
50	Glass beads (0.7mm diam.)	NCTC + 50% Ch.S - Batch B + horse r.b.c.	69(D)	0.40mm	Bifur-cation	0-3	

Table 3II:4 Various additives

Exp. No.	Additive	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
	Horse or sheep r.b.c.						See Table 3II:2
	EMBRYO EXTRACTS (EE_{50})						
9 b	10% Chick EE_{50} - Batch A (commercial)	NCTC+20%FCS+ horse r.b.c.	70(C)	2.40mm	3° branches	?	Great variety of development, never able to repeat
42 a	10% Chick EE_{50} - Batch B (commercial)	NCTC+20%Ch.S (Batch A) + horse r.b.c.	15(D)	No growth	No growth	0	Sluggish throughout
19	" "	NCTC+20%FCS + sheep r.b.c.	28(C)	0.60mm	bifurcation	0-3	Moderately active
20	" "	NCTC+20%inac. lamb serum + sheep r.b.c.	16(D)	0.55mm	bifurcation	0-3	Sluggish
21	" "	NCTC+20% lamb serum + sheep r.b.c.	10(D)	No growth	No growth	0	Sluggish

Table 3II: 4 (continued)

Exp. No.	Additive	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
26 a	5% High Mol.Wt.CEE_{50} (prepared)	NCTC+20%FCS + horse r.b.c.	14(D)	0.30mm	Lateral branches	0	Inactive
b	5% Low Mol.Wt. CEE_{50} (prepared)	"	29(C)	0.55mm	2^{o} branches	0-3	Active
c	5% Crude CEE_{50} (prepared)	"	29(C)	0.50mm	bifur-cations	0-3	Moderately active
52 a	2% Mouse EE_{50} (prepared)	NCTC+20%Ch.S. (Batch B) + horse r.b.c.	63(T)	0.77mm	bifur-cations	0-3	Active throughout
54 b	"	NCTC+20%FCS + horse r.b.c.	55(T)	0.66mm	bifur-cations	0-3	
42 b	10% Bovine EE_{50} (commercial)	NCTC+20%FCS + horse r.b.c.	6(D)	No growth	No growth	0	Inactive

Table 3II: 4 (continued)

Exp. No.	Additive	Media	Duration	Results			Comment
				Length	Gut	Genitalia	
	LIVER EXTRACTS etc.						
4a	0.25% liver extract (commercial)	NCTC+20% FCS	12(D)	No growth	No growth	0	Inactive
4b	"	" +horse r.b.c.	31(D)	0.60mm	2^{o} branches	0-3	Feeding, mod. active
32a	10% prepared sheep liver extract	NCTC+20% rabbit serum	16(D)	No growth	No growth	0	Inactive, dark
32b	"	" sheep r.b.c.	20(D)	0.30mm	Lateral branches	0	Sluggish, dark
Control L1	"	NCTC+20%FCS + sheep r.b.c.	37(C)	0.60mm	2^{o} branches	0-3	Active
36a	Liver macerate						see Table 3II: 3

Table 3II: 4 (Continued)

Exp. No.	Additives	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
12	BILE 0.05% sheep bile	NCTC+20%FCS + horse r.b.c.	44(C)	1.35mm	2° branches	3-5	Dark. Great variation in growth & development, many stunted
14	0.1% sheep bile	"	8(D)	No growth	No growth	0	Dark & sluggish
39	0.05% mouse bile	"	23(D)	0.30mm	No growth	0	"
56b	0.04% taurocholate	"	53(T)	0.33mm	Lateral branches	0	
52a	VITAMINS 10% yeast extract	NCTC+20% ChS (Batch B) + horse r.b.c.	14(D)	0.33mm	Lateral branches	0	
39	0.1% vitamin C	NCTC+20%FCS + horse r.b.c.	27(C)	0.33mm	No growth	0	

Table 3II: 4 (Continued)

Exp. No.	Additive	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
	HORMONES						
53a	Insulin (10^{-3}mg/ml.)	NCTC+20% + horse r.b.c.	30(C)	0.45mm.	Lateral branches	0-3	
53b	Oestrogen (10^{-3}mg/ml.)	"	55(T)	0.66mm	2° branches	0-3	
56a	Thyroxine (10^{-3}mg/ml.)	"	53(T)	0.33mm	Lateral branches	0	
	CARBOHYDRATES						
9a	Rice starch	"	17(D)	0.35mm	Lateral branches	0	Injests starch granules
5a	Extra 1g/litre glucose	"	30(D)	0.33mm	Bifur-cation	0	N.B. No shaking ∴ compare to experiment 1 (Table 3II: 5)
5b	Extra 4g/litre glucose	"	30(D)	0.33mm	Bifur-cation	0	

Table 3II: 4 (continued)

Exp. No.	Additive	Media	Duration	Results			Comment
				Length	Gut	Genitalia	
	EGG PRODUCTS						
44a	NCTC- Y	No serum, no r.b.c.	12(D)	No growth	No growth	0	Sluggish & dark.
44b	"	20%ChS (Batch A) no r.b.c.	12(D)	No growth	No growth	0	
1c,2a	Egg macerate						see Table 3II: 3

Table 3II: 5 Physical Conditions

Exp. No.	Conditions	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
3a	Small Falcon flasks - 10 mls.	NCTC+20%FCS + horse r.b.c.					No difference - See Table 3II:2 for results
3b	Leighton tubes - 10 mls.	"					
1a	Stationary in water bath	"	30(D)	0.35mm	Bifur-cation	0	Shaking slightly advantageous
39d	Shaking in water bath	"	43(D)	0.55mm	2° branches	0-3	
57b	Rolling horizon-tally in incubator	"	13(D)	0.3mm	No growth	0	Sluggish, early build up of black solids in gut
57c	Rolling verti-cally in incubator	"	-				Always contaminated

Table 3II: 5 (continued)

Exp. No.	Conditions	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
13a	Changing media once a week	NCTC+20%FCS + horse r.b.c.	43(D)	0.55mm	2^{o} branches	0-3	See Table 3II: 2
13b	Not changing media	"	24(D)	0.33mm	Lateral branches	0	Become sluggish, black solids accumulate in gut
13c	Aerobic gas (N_2,O_2,CO_2)	"	22(C)	0.55mm	Bifur-cations	0	No difference, active, feeding
13d	Anaerobic gas (N_2,CO_2)	"	22(C)	0.55mm	"	0	
13e	Air	"	22(C)	0.55mm	"	0	
L1	"Lift": Static Diffusion system	Medium: NCTC+20%FCS	8(T)	No growth	No growth	0	Dead when examined
	Rapid Diffusion system	+horse r.b.c. Base: œag. serum/r.b.c.	8(T)	?	?	?	Burrowed into base and died there

Table 3II: 6 Cell Cultures

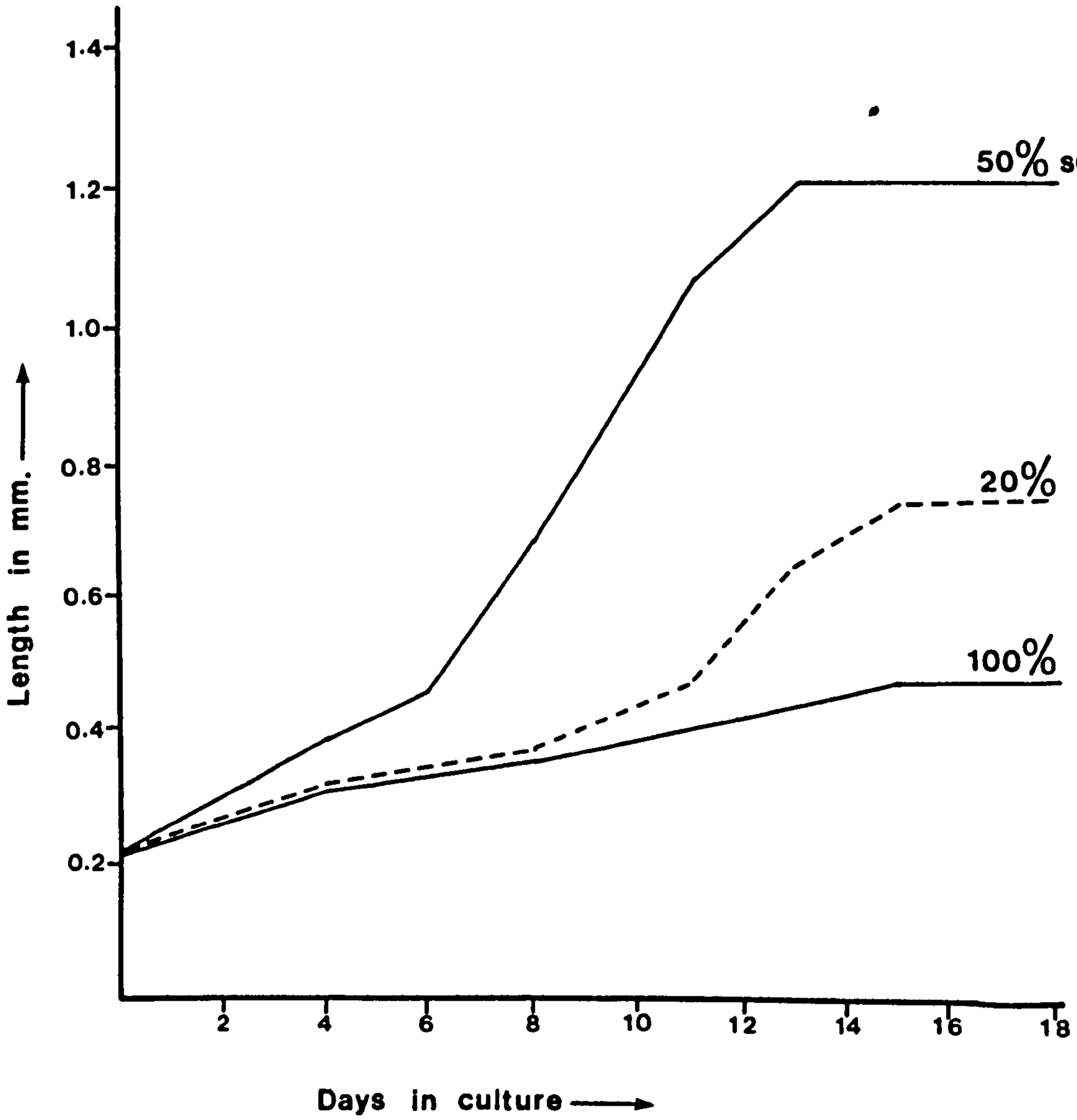
Exp. No.	Cell Type	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
39e	BHK cells (monolayer)	NCTC+20% FCS	17(T)	0.40mm	Lateral branches	0-3	Active, feeding
46a	Chang liver cells (monolayer)	"	28(D)	0.30mm	No growth	0	"
46b	Chick embryo cells (monolayer)	"	51(T)	0.40mm	Lateral branches	0-3	"
46c	Mouse embryo cells (monolayer)	"	51(D)	0.55mm	Lateral branches	0-3	"
45	Newborn mouse liver explant	Media:NCTC+20% Ch.S. Base:Coag.Ch.S. Explant: 7 days old (Plates 3 & 4).	20(D)	0.50mm	Lateral branches	0-3	For first 2 days burrowed in base, then fed on cells.

Table 3II: 7 Flukes from mice

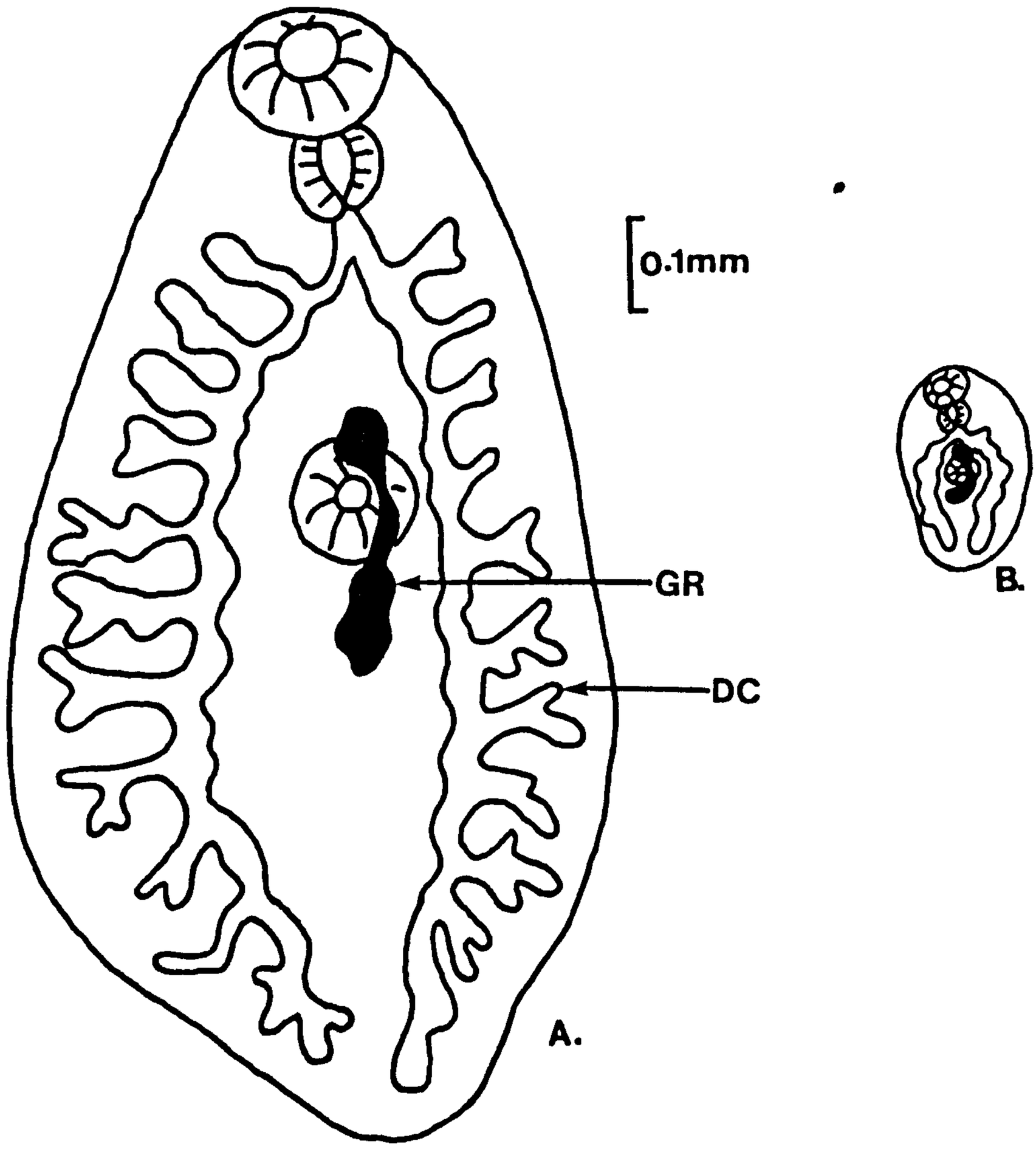
Exp. No.	Days in mice	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
8a	2	NCTC + 20%FCS	21(D)	0.35mm	Bifur-cations	3	
b	"	" + horse r.b.c.	27(D)	0.35mm	"	5	
c	"	" + 0.25% liver extract	8(D)	0.35mm	"	2 (no development)	
d	"	" + 0.25% liver extract + horse r.b.c.	11(D)	0.35mm	"	2 (no development)	
6a	10	NCTC + 20% FCS	62(T)	2.5mm	3^{o} branches	No development, some degeneration of testes	Injest blood throughout, clear.
b	"	" + horse r.b.c.	78(C)	4.0mm	$4^{o}+$ branches		
7a	10	" + 0.25% liver extract	49(C)	1.6mm	3^{o} branches	ditto	ditto
b	"	" " + horse r.b.c.	49(C)	2.3mm	$4^{o}+$ branches		

Table 3II: 7 (continued)

Exp. No.	Days in mice	Media	Duration	Results			Comments
				Length	Gut	Genitalia	
37a	7	NCTC+20%FCS	32(T)	1.6mm	Mainly 2^{o}, some 3^{o}branches	no development,testes degenerated	Active, rather dark
b	"	" +horse r.b.c.	32(T)	1.6mm	"	"	Active, feeding
c	"	" +macerated coag.calf serum	32(T)	1.6mm	"	"	" " , rather dark
38a	14	NCTC+20%FCS	31(T)	3.2mm	4^{o}+ branches	"	Moderately active, slightly dark
b	"	"+horse r.b.c.	31(T)	3.2mm	"	"	Active, feeding
c	"	" +macerated coag.calf serum	31(T)	3.2mm	"	"	" " , slightly dark



Text Figure 3. Fasciola hepatica: Growth in vitro in Chick Serum (Batch A).



Text Figure 4. Fasciola hepatica: Development in vitro -

A. A fluke from Culture 45 after 14 days

B. A newly excysted juvenile.

Key.

DC - digestive caeca
GR - genital rudiment

Discussion

A. General Points

1. Excystment of metacercariae

The methods of Sewell & Purvis and of Wikerhauser (see General Methods) for excysting the metacercariae of F. hepatica were both found to be equally satisfactory although the former was slightly quicker. The percentage excystment was usually between 60% and 80% depending on the batch of metacercariae and their treatment during storage. If the excystation procedure was carried out under sterile conditions - using either technique - then the time that was required for excystment was increased. This may be because the bacteria normally present in the excysting fluid (especially in the bile) provide reducing conditions. The presence of reducing conditions is considered to increase the rate of activation of the metacercaria within the cyst (Dixon, 1965).

If unexcysted but activated metacercariae were transferred to the culture medium, they did not usually continue to excyst. However, they remained active inside the cyst for up to two weeks. This may be because the "emergence" phase of excystment had not been triggered before the cysts were transferred to the culture media. Theoretically, unless the subsequent culture media contained bile this phase would never be initiated.

These observations conflict with the reports of Wikerhauser, et al (1973) who were able to recover developing flukes from the livers of mice which had been injected intraperitoneally with intact metacercarial cysts. These cysts could not have been stimulated to excyst by bile in the normal way.

2. Behaviour of flukes in culture

During the first few days in culture the flukes adhered firmly to the walls of the culture vessel. They also adhered to each other by means of their ventral suckers, usually in pairs but often as many as five were clumped together. Thereafter the flukes only adhered to the vessel after a media change. This behaviour may have occurred because the new media provided an energy source which had been depleted in the old medium, or it may be that the whole process of media change was a traumatic event and that this attachment to the vessel was the flukes' response.

During the first day in culture, the flukes did not feed. This is consistent with the observation made by Bennett (1973) that during the first 12-24 hours after excystment the gut functions as a penetration gland and that injestion does not occur. By the second day the flukes began to injest solid food material if it was available - it could be seen moving inside the caeca. In most of the cultures containing red blood cells, black solids were seen moving in the gut after only a few days. They could be expelled via the oral sucker but they often built up

until they packed the lumen of the caeca and distended it. The length of time which this took to occur varied with the media but once the caeca had become swollen in this way there was rarely any further growth or development. In some of the more successful cultures the build up of black solids in the gut was not so damaging or dramatic. (In culture number 43 (see Table 3II:2) there was no accumulation of black solids in the digestive caeca). This black material is probably indigestible or degenerating red blood cells.

One of the most prominent events of the first day of culture was the loss of concretions from the excretory ducts. This began as soon as the flukes had excysted and continued throughout the first day in the culture medium. The stimulus to void these concretions may have simply been the elevation of temperature, but there is no direct evidence to support this view.

During the first few days of culture the flukes were often rather sluggish. This is in contrast to their behaviour <u>in vivo</u> at this time when they are very active. In culture 45, which contained liver explants grown on a thin base of coagulated chick serum, the flukes burrowed through the solid base for the first few days and thereafter they fed on the liver cells and did not re-enter the base. This type of behaviour pattern corresponds to the situation <u>in vivo</u> during the first few days.

When solid bases were used in the culture system the flukes began to burrow into them during the first day. The burrows were extremely narrow and the flukes often appeared to get trapped in them. This was presumably because - unlike the hosts' intestinal wall - the solid base represented a dead-end situation. Some flukes made only very shallow burrows and looped back into the media. Whilst burrowing no injested pieces of the solid base were observed in the flukes' caeca, this suggests that they were not eating their way through the base.

3. Variations in batches of media

The different growth promoting properties of different batches of serum is well known; it has been discussed by Smyth & Davies (1974). It was strikingly demonstrated in the present study by the two different batches of chick serum that were used. They were both obtained from the same suppliers (Flow) so presumably the techniques involved in their preparation were the same. In Batch A somatic growth was significantly increased and the digestive caeca seemed to function well since no indigestible black solids accumulated. In Batch B, however, the results were similar to those routinely obtained in foetal calf serum; there was some somatic growth but the digestive caeca probably did not continue to function normally.

4. Inter- and Intra-culture variations

Within an individual culture, there was often a great deal of variation in growth and development. This was particularly noticeable in Culture 12 containing 0.05% sheep bile (Table 3II: 4) where the maximum length attained was 1.35 mm. In this culture there were many intermediate sizes and developmental stages, some had not grown or developed significantly from the newly excysted juvenile. This intra-culture variation was true of most of the cultures to some extent. In culture 43 (Table 3II:2), however, most of the flukes were of a similar size and there were very few stunted worms.

In each experiment two or three duplicates were set up using identical culture conditions. There was often a marked difference between the activity, growth or development of the flukes in these duplicates. This can only be explained by saying that our knowledge of the micro-environment which the fluke inhabits within the culture system is inadequate. Variations in this micro-environment could help to explain both inter- and intra-culture variations.

5. Development of cultured flukes in mice

Flukes removed from culture after 4, 8 and 12 days and injected into mice were recovered from the bile ducts of the liver 40 days later. In all but one of the mice one to three adult flukes were recovered. Most of these flukes contained normal eggs *in utero*. This supports the view that while the flukes are in culture they are held in a state of "suspended

animation" for several weeks until they eventually begin to degenerate and die.

B. Discussion of experiments

Table 1: Various defined media

Media which did not contain serum, either based on a saline or on a complex defined medium, did not maintain the metacercariae for more than a few days.

Hams F_{12} is a defined medium which has been found particularly useful for culturing hepatocytes, however it did not prove beneficial in the cultivation of the metacercariae of F. hepatica.

120% NCTC was tested because the addition of serum to normal strength NCTC has a diluting effect which may affect the osmotic pressure adversely. However, this culture media had no beneficial effects.

Table 2: NCTC plus various sera

The presence of serum prolonged survival considerably. Chick serum - Batch A was clearly the best serum; however, only a limited amount was available and the next batch (Batch B) of chick serum did not possess these growth-promoting properties. Foetal calf serum did not vary so severely from

batch to batch and was routinely used when assessing the effects of various additives.

Generally, inactivated serum was more beneficial than unheated serum which was rather toxic. However, although inactivation destroys complement it may also destroy other beneficial heat labile proteins in the serum.

It was very clear that serum from different species possessed different growth-promoting properties. It may be however, that the most beneficial serum possessed fewer inhibitors rather than more stimulatory factors. In contrast to the results obtained by Carillo de Albornoz & Guevara Pozo (1974) horse serum proved to be the most toxic. However, these authors have access to sera which has been specifically screened for growth-promoting properties *in vitro* (personal communication, Carillo de Albornoz).

Table 3: Diphasic media

Diphasic media has proved very successful in the cultivation of cestodes especially *Echinococcus granulosus* (Smyth, 1967). Kannangara (1973) and Kannangara & Smyth (1974) have attempted to culture *Diplostomum phoxini* in various diphasic media; these all proved unsuccessful. However, Kannangara (1973) records that -

> ".....*D. phoxini* actively penetrated a serum-agar base, but gradually became immobile during the course of 2-3 days and finally died as if they were trapped inside."

Similar behaviour was observed in the present study when solid serum bases were used in the cultivation of the newly excysted juveniles of F. hepatica. Ratcliffe et al (1969) observed that when adult F. hepatica were maintained in a diphasic media containing a base of coagulated serum they were comparatively inactive and that they buried their oral suckers in the base. However, none of the diphasic media used in this study had any favourable effect.

The egg macerate which proved so successful in the cultivation of Diplostomum sp. (Kannagara & Smyth, 1974) did not prove beneficial when used in small quantities; when used in larger quantities, the flukes died and disintegrated. This result was also obtained by Kannagara (1974) when he tried to culture the metacercariae of Paragonimus westermani in a similar media.

Table 4: Various additives

Red blood cells of sheep or horse origin increased somatic growth and prolonged normal activity and survival. They were routinely used in many cultures as a food source except in those where other types of food material were being assessed.

Although embryo extracts are considered to promote the growth of cells in culture, Clegg (1965a) found that chick

embryo extract inhibited the later stages of development of Schistosoma mansoni. Voge & Jeong (1971) found that it completely inhibited any growth or development of Cotylurus lutzi in vitro. In this study, this was true of Batch B of the chick embryo extract; Batch A, however, possessed considerable growth-promoting properties in vitro although the results were extremely variable. The commercial bovine embryo extract was definitely very detrimental. The prepared mouse embryo extract appeared to possess very slight growth-promoting properties.

Paul (1970) records that if chick embryo extract is fractionated into high and low molecular weight fractions, the former is inhibitory and promotes de-differentiation in cell cultures whereas the latter allows differentiation to be maintained. In this study the high molecular weight chick embryo extract was inhibitory, whereas the low molecular weight and crude extracts did not inhibit growth but neither did they appear to enhance it.

The various liver extracts that were tested did not promote development, the prepared sheep liver extract may even have been inhibitory. Clegg (1965a) observed that rabbit liver extract had an inhibitory effect on the growth of S. mansoni.

The effect of the addition of bile to the culture medium was rather difficult to assess. Sheep bile seemed to promote growth and development in small concentrations although the appearance and activity of flukes cultured in it was poor and there was a large population of stunted flukes present. Higher concentrations of sheep bile killed the flukes. Mouse bile was inhibitory and sodium taurocholate did not enhance growth. This is rather surprising since in the liver and, later on, in the bile ducts, the flukes are in an environment rich in bile.

Yeast extract, which is a rich source of amino acids and vitamins, especially certain B vitamins, had an inhibitory effect on growth. Wyllie et al (1960) found that the addition of yeast extract was necessary for egg production in Diplostomum phoxini whereas Kennangara (1973) was able to obtain egg production without the use of yeast extract. Smyth (1967) and Smyth & Davies (1974) have found yeast extract to be particularly useful in the cultivation of the sheep strain of Echinococcus granulosus.

Pantelouris & Hale (1962) have demonstrated the presence of Vitamin C in the excretory ducts of adult F. hepatica and suggest that its function is connected with the excretion of iron. Although NCTC already contains 49.9mg/litre of Vitamin C addition of extra Vitamin C to the culture media had a deleterious effect. This may be because the excess Vitamin C crystalised out and the micro-environment of the flukes therefore contained an extremely high concentration of this vitamin.

The effect of hormones on helminth growth and development has been reviewed by Solomon (1969) and on F. hepatica in particular by Pantelouris (1967). Pantelouris (1964) has demonstrated that insulin can be taken up through the tegument and that it exerts an effect on glycogen metabolism depleting the glycogen stores of the tegument and peripheral tissues. However, Hines (1969) and Sekardi (1966) have produced results which conflict with those of Pantelouris. Hines maintains that the addition of insulin to the incubation media has no effect on glycogen stores whereas Sekardi (using bissected flukes) found that it increased the rate of glucose uptake and glycogen synthesis. In mammals, insulin regulates the level of blood glucose and stored liver glycogen. Addition of insulin to the culture media in this case had no effect on the subsequent growth or development of the flukes.

The addition of thyroxine, a growth hormone produced by the thyroid gland, had little effect on flukes in culture - if anything it inhibited their growth and development slightly. Hutton et al (1972) incubated adult F. hepatica in media containing various host hormones in the presence of radio-actively labelled substrates; no significant effects were observed with thyroxine.

The effects of gonadectomy and the correlation of the reproductive cycle of the host with the development of their parasites has been noted by several workers. The monogenean Polystoma integerimum only produces eggs during the reproductive cycle of its host, the frog (Miretski, 1951). However,

addition of oestrogen to the cultivation medium of F. hepatica had no stimulatory effect on growth or development. Kannangara (1973) has studied the effects of insulin, thyroxine, somatotrophin, testosterone and oestrogen when added to the culture medium of D. phoxini. No significant differences, either stimulatory or inhibitory, were observed.

The addition of extra glucose or rice starch to the media does not enhance growth or development. It may be noted that NCTC contains 1g./litre of glucose.

The yolk medium of Berntzen & Macy (1969) is an emulsion of various components of yolk and is particularly rich in cholesterol (Bernard Fried, personal communication). Ether extracts of liver fluke tissue contains 19% cholesterol (von Brand, 1928), this is not accounted for by the lipid droplets in the excretory ducts which are cholesterol free (Stephenson, 1947). Hence, F. hepatica contains a relatively large amount of this sterol. Cholesterol is found in mammalian liver and in bile (Smyth & Haslewood, 1963) and may therefore play an important role in the development of juvenile F. hepatica. However, since cholesterol is a very insoluble substance, it was virtually impossible to test its effect except as a constituent of Berntzen & Macy's yolk medium. When this medium was used, with or without serum, there was no growth or development at all and the flukes became very dark due to a build up of lipid droplets in their tissues.

Im Se Wook (1969) found that F. hepatica adults 'survived' for longer in vitro when cholesterol was added to the incubation medium. Ratcliffe et al (1969) reported, however, that lg./litre of cholesterol had unfavourable effects on egg production.

Table 5: Physical Conditions

The use of Leighton tubes or small Falcon flasks had no significant effect on growth or development. The circulatory system or "Lift" (Smyth, 1969 - see General Methods) was, however, completely unsatisfactory. The metacercariae in the Static Diffusion system burrowed into the base and died there. The flukes in the thimbles placed in the Rapid Diffusion system were also dead when they were examined on Day 8. There was no evidence that they had been feeding or that there had been any growth or development.

If the media was not changed the flukes became sluggish and died after about three weeks, they didnot grow or develop as well as in those cultures where the medium was replaced weekly. This was presumably caused by an accumulation of excreted metabolites and a depletion of nutrients from the medium.

The cultures that were incubated in a gently shaking water bath were generally better than the stationary ones. Rolling cultures were adequate but they increased the opportunities for contamination. Shaking or rolling cultures have also been found to be beneficial in the cultivation of various other helminths, e.g. Sphaeridiotrema globulus (Berntzen & Macy, 1969),

Diplostomum sp. (Kannangara & Smyth, 1974). However, it has been known for some time that agitation inhibits the development of Schistosoma mansoni in vitro (Cheever & Weller, 1958; Clegg, 1965a).

The use of different gas phases, including an aerobic and an anaerobic mixture, had no effect on subsequent growth or development. Erasmus (1972) concludes that -

> "The information available suggests that adult parasite helminths are facultative anaerobes and, although they show a marked tolerance to lack of oxygen, are able to utilize it when available."

Rohrbacher (1957) has studied the effects of aerobic (Air + CO_2) and anaerobic (N_2) gasses in the maintenance of adult F. hepatica. In the best media, both survived for up to 20 days. The respiration of the digenea has been reviewed by Vernberg (1963) and von Brand (1966).

Table 6: Cell Cultures

The previous use of monolayer cell cultures in the cultivation of the newly excysted juvenile (Wikerhauser & Cvetnić, 1967; Wikerhauser et al, 1968) has been discussed in the Introduction. In the preset study, the young flukes fed on the cells and remained active and apparently healthy but they failed to develop further than those cultures which used red blood cells as a food source.

The cell line known as Chang liver cells is an established cell line. It consists mainly of liver fibrocytes. It is extremely difficult to prepare a monolayer culture of hepatocytes. Treatment of liver with the usual dissagregating agents (Trypsin or Versene) is unsuitable since they destroy the hepatocytes which are very delicate. The usual method that is employed is to perfuse the whole liver with collagenase. A culture of cells obtained by this technique contains hepatocytes and fibrocytes; however, under most conditions, the latter will rapidly outgrow the former. It is not possible to subculture hepatocytes since this involves the use of trypsin.
Hillis & Bang (1959) cultured fragments of embryonic and adult liver on a reconstituted collagen substrate (prepared according to the technique of Ehrmann & Gey, 1956) and obtained good outgrowths of cells which included hepatocytes. In the present study, fragments of newborn mouse liver were grown on a base of coagulated chick serum and a good growth of cells was obtained (Plates 3 & 4). The behaviour of newly excysted metacercariae in this system has been described previously. However, although they injested the liver cells, there was no beneficial effect on their growth or development.

Table 7: Flukes recovered from mice

Flukes recovered from the abdomen of mice after 2 days and cultured under various conditions did not survive as long as newly excysted juveniles under similar conditions. Although there was very little growth even in the best medium, there was some development of the digestive caeca and genital rudiment. Liver extract seemed to have a very inhibitory

effect on "2 day" flukes. The decreased viability of these young flukes may have been because they had used a lot of their energy reserves during their migrations and had not had time to build up new reserves before they were transferred to the culture medium. The result of this experiment suggests that migration across the hosts' intestine does not provide a "trigger" or stimulus which is essential for subsequent development.

When 7, 10 and 14 day old flukes were recovered from the liver, they survived for some time in culture. Again, liver extract was not beneficial although some growth occurred. In all cases, the best medium was NCTC, 20% foetal calf serum and horse red blood cells. In it, the growth was as follows:

	Original length (live)	Max. length in culture
"7 day" flukes	0.6mm.	1.6mm.
"10 day" flukes	1.3mm.	4.0mm.
"14 day" flukes	2.1mm.	3.2mm.

In all cases, the caeca continued to develop.

	Original development	Development in culture
"7 day" flukes	lateral branches only	2^{o} & 3^{o} branches
"10 day" flukes	2^{o} branches	4^{o}+ branches
"14 day" flukes	3^{o} branches	4^{o}+ branches.

In the "10 day" and "14 day" flukes, the anterior four lateral outgrowths had secondary branches; this development occurs on the 15th day in mice (Dawes, 1962).

The genital rudiment, however, did not show any development in culture. On the contrary, the testes showed some degeneration and this was complete in the case of the "7 day" flukes. This suggests that somatic and germinal development can become unsynchronised in culture and may proceed at different rates. This has been demonstrated quite dramatically by the development of monozoic forms of the cestode Echinococcus granulosus in culture (Smyth, 1971). These forms were sexually mature (although they did not contain shelled eggs) but there was little somatic growth and no segmentation. In the present situation the reverse condition occurs: there is some somatic growth but no germinal development.

The results of these experiments also suggest that normal development, especially of the genitalia, does not rely on an "all or none" stimulus when the fluke reaches the liver but needs the continued presence of various factors present in the hepatica parenchyma.

Conclusions

1. Cultured metacercariae were similar in many respects to the young flukes recovered from the abdomen of mice. As suggested by Dawes & Hughes (1964), they appeared to be in a state of "suspended animation". When injected intraperitoneally into mice, they were capable of normal development to egg-bearing adults.

2. Normal development *in vivo* will only proceed once the fluke has reached the liver (see part I of this chapter). Various liver extracts and products failed to stimulate development *in vitro*. The stimulation which the fluke receives in the liver was probably not provided by an initial "all or none" stimulus but by a complex, continuing interaction of various physico-chemical factors.

3. Significant growth in culture only occurred if the newly excysted juveniles were provided with a solid food source; red blood cells, embryonic cells or hepatic cells were equally satisfactory. None of these cells, however, provided the stimulus for continued growth.

4. Different sera and batches of the same type of serum possessed widely differing growth-promoting properties.

5. With either newly excysted juveniles or flukes removed from mice, the normal pattern of somatic and germinal development became unsynchronised in culture. Although some somatic growth was achieved, there was virtually no development of the germinative cells.

PART III: ULTRASTRUCTURE OF DEVELOPMENT IN VIVO

General Introduction

Bennett (1973, 1975a,b,c) and Bennett & Threadgold (1973, 1975) have produced a very full and detailed description of the ultrastructure of the metacercaria of Fasciola hepatica and its development to adulthood in the mouse, using both transmission and scanning electron microscopy. From thin sections, they have studied the development of four organ systems:

1. The tegument,
2. The digestive caeca,
3. The excretory system,
4. The parenchyma.

In scanning electron microscope studies, Bennett (1975b,c) concentrated mainly on:

1. The surface of the syncytium,
2. The development and distribution of the spines,
3. The sensory papillae.

The observations made by these authors on the ultrastructure of the metacercaria and its development up to two weeks post-infection in the mouse are reviewed here. Some of this work has been repeated by the present author; the resulting micrographs allow comparisons with those of Bennett but they also provide a visual comparison with the micrographs of cultured flukes. The developmental stages examined by the present author were:

1. The excysted metacercaria,
2. Young flukes recovered from the abdomen on Days 1-6,
3. Young flukes recovered from the liver on Days 3-14.

The work of Bennett and Bennett & Threadgold (loc. cit.) is reviewed and discussed here in two separate sections.

Section 1: Transmission Electron Microscopy (TEM).

Section 2: Scanning Electron Microscopy (SEM).

From the observations made by these authors, it is possible to draw up a series of ultrastructural criteria for assessing the normal growth and development of F. hepatica metacercariae in vivo. These are discussed in the Conclusions (page 134).

Section 1: Transmission Electron Microscopy.

Although Bennett (1973, 1975a) and Bennett & Threadgold (1973, 1975) have studied the development of the metacercariae up to the adult stage, only their results for the first two weeks of development will be reviewed here. The observations made by these authors on the development of the four organ systems are presented separately.

A. The Tegument

Introduction

The basic structure of the tegument of F. hepatica is common to the Digenea. It has been described by Threadgold (1963a,b;1967) and by Björkman & Thorsell (1964b). It consists of a surface syncytium connected by thin protoplasmic strands to the cell bodies which lie below the peripheral musculature. The syncytium contains various granules, mitochondria and spines. Its outer membrane is thrown up into folds. The projecting spines are enclosed within the surface membrane. Their bases lie in an evagination of the basal plasma membrane to which they are attached by a dense fibrous junction. The surface syncytium is separated from the underlying peripheral longitudinal and circular muscles by a layer of fibrous interstitial material. Various sensory receptors are found embedded in the surface synctium. The tegumental cell bodies (also called "perinuclear regions") contain mitochondria, ribosomes, granular endoplasmic reticulum (GER), Golgi complexes,

granules and a nucleus. These cells synthesise and package the various granules which fill the surface syncytium.

Bennett (1973) recognised three types of tegumental cells and granules in the early developing forms of F. hepatica, which he based on the classification of Threadgold (1963a,b;1967).

Type-0 cells

These cells are only found in the metacercaria and early developing stages. Between weeks one and two they gradually change to the adult type-1 cell. They are characterised according to the type of secretory bodies that they produce - these are termed T-0 granules. These granules are dense and biconcave to spherical in shape and are membrane bound. Type-0 cells have large masses of cytoplasm with pseudopodia-like ramifications.

Type-1 cells

These cells develop from type-0 cells and, in the adult, they are typically flask-shaped. They contain small spherical granules with dense material and elctron lucent areas interposed in such a way as to give a cartwheel-like appearance. They are termed T-1 granules.

Type-2 cells

These cells arise in the sub-muscular parenchymal regions very early on in development and by Day 4 they have fused with

the syncytium. They contain small, bi-concave, disc-like granules which are often stacked in parallel in small groups. These granules are membrane-bound and contain a dense granular material with an electron lucent medullary region. They are termed T-2 granules. In the adult, each type-2 cell is associated with two or three type-1 cells.

Bennett's observations on the ultrastructure of the tegument during the first two weeks of development are reviewed here. A summary of his results is quoted verbatim.

Review of Results (from Bennett, 1973)

(Refer to Plates 5-14 incl., produced by the present author)

"

(1) On moving out of the metacercarial cyst into the gut lumen and through the gut wall. (0 days to 12 hours)

(Refer to Plates 5-7)

a) The single type of tegumental cell termed 'type 0' appears to 'collapse' and its contained T-0 granules move up into the syncytium.

b) The number of T-0 granules in the syncytium increases and they often appear less dense than those remaining in the tegumental cells.

c) The ground cytoplasm of the syncytium becomes more electron dense.

d) The T-0 granules are secreted at the apical membrane of the syncytium by an eccrine mechanism, and a glycocalyx is formed.

(2) <u>In the abdominal cavity</u> (12 hours to 3 days)

(Refer to Plates 8-12)

a) The cytoplasm of the syncytium continues to become more elctron dense.

b) Macrophages are frequently seen attached to the surface glycocalyx and to each other.

c) Adult-like 'type 2' tegumental cells containing T-2 granules are seen for the first time at this stage. These cells lie below the peripheral musculature usually close to 'type 0' tegumental cells but as yet are not connected to the syncytium by protoplasmic tubes.

(3) <u>In the hepatic parenchyma</u> (3 days to 3 weeks)

(Refer to Plates 13 & 14)

a) The 'type 2' cells unite with the syncytium and T-2 granules appear in the latter.

b) The syncytial cytoplasm decreases in density but increases in depth from lum to 4.8um.

c) Mitochondria increase in numbers in the type 0 cells and the basal region of the syncytium.

d) Basal invaginations increase in numbers and size.

e) The synthesis of T-0 granules in the 'type 0' cells declines in this period.

f) The 'type 0' cells synthesise a new secretory granule which appears identical to the adult T-1 granule, and these T-1 granules ultimately dominate over the original T-0 granule. These cells may therefore be renamed the 'type 1' cells of the more mature juvenile and adult.

Discussion

Bennett (1973) drew the following conclusions about the three types of tegumental cells and their secretory bodies.

" 1. The T-0 granules are involved in a protective function, forming an initial glycocalyx.

2. The type-2 cells arise from embryonic cells.

3. The T-2 granules form a new glycocalyx and they probably contribute to the increase of plasma membrane necessary to produce the branching invaginations (of the adult).

4. The type-0 cells are transformed into adult type-1 cells containing adult T-1 granules. "

He also made the following points:

1. Since the young flukes in the abdomen are surrounded by macrophages, they are clearly recognised as "non-self". However, the macrophages do not seem to damage the flukes in any way. Bennett suggests that the inhibition of the macrophages' activity may be due to the physical or chemical properties of the glycocalyx of the fluke.

Lumsden et al (1970) suggest that the glycocalyx of trematodes may have adsorptive, transport, enzymic and/or immunological functions. Bennett suggests an additional function, that of protection - against the hydrolases of the host's gut lumen and cells, against the host's immune response and against attack by macrophages whilst in the abdomen.

2. Once in the liver the type-2 tegumental cells unite with the syncytium and release T-2 granules into it. At this time, production of T-0 granules by the type-0 cells is decreasing with a corresponding increase in the production of T-1 granules. Thus, in the twodifferent environments of abdomen and liver, there are two different populations of granules in the syncytium. Bennett suggests that there must be a need for these different types of granules (T-1 and T-2) in the new environment of the liver.

3. Dawes & Hughes (1964) observed that:

"Those (young flukes) remaining in the abdomen became stunted and although maintenance needs may have been satisfied by food available in the peritoneum, total needs were not satisfied and growth retardation was the result."

On the subject of growth, Bennett records that:

"If the fluke remains in the abdominal cavity on the third day, the growth of the syncytium is retarded in comparison with flukes of the same age which have penetrated the liver."

He suggests that there may be a component in the abdomen which inhibits growth. He suggests that such factors as hormones, pH, osmolarity, immunity or some other physical or chemical characteristic of the environment may be involved. An alternative explanation is that the energy derived from the nutritive supply available in the abdomen is used mainly for migration and protection against host defences and that little is left for growth.

B. The Digestive Caeca

Introduction

The ultrastructure of the digestive caeca of adult F. hepatica has been described by Robinson (1971). He states that there is one cell type only and that these gastrodermal cells can be divided into three groups.

Cells of the lateral outgrowths:

Group I - secretory phase cells

Group II - absorptive phase cells

Cells of the main caeca: Group III.

a) Lateral outgrowths

According to Robinson (1971), there is a gradual transition between secretory and absorptive phase morphology in the gastrodermal cells of the lateral outgrowths. The secretory and absorptive phase cells can be subdivided into early, transitional and late stages.

The general features of these cells are as follows:

1. They have a cuboid shape and are one cell thick.
2. The apical plasmalemma is thrown up into a series of lamellae, these have an electron dense core and a surface glycocalyx.
3. The basal membrane has many invaginations.
4. The nucleus is basal.
5. The GER and mitochondria are usually found in the basal 2/3 of the cell and have a base-apex orientation.

6. There are numerous Golgi complexes.
7. Secretory cells contain an apical concentration of oval, dense granules 0.5um in diameter.
8. Adjacent cells are connected at their apical surfaces by a terminal bar.
9. Junctional complexes are formed between the basal plasmalemma and projections of parenchymal cells.

b) Main Caeca

The gastrodermis of the main caeca is one cell thick. The cells are rather flattened, they have few lemellae and their GER and mitochondria are not orientated. They produce small amounts of secretions and these are often found concentrated in apical projections of the cytoplasm. Robinson (1971) considers that the function of the main caeca is mainly in the transport of injested food material and in the mixing of food with digestive secretions produced by the cells of the lateral branches.

Bennett and Bennett & Threadgold (loc.cit.) have studied the development of the digestive caeca of F. hepatica in the mouse. Their observations on the ultrastructure of the caeca of the newly excysted juvenile and its development during the first two weeks of infection are summarised here.

Review of Results (from Bennett, 1973, 1975a;
Bennett & Threadgold, 1973)

(Refer to Plates 15-22 incl., produced by the present author).

Day 0 (newly excysted juvenile)

The shape of the caecal lumen varies from virtually

occluded to greatly expanded; it is filled with a dense material. The lumen is surrounded by 2 or 3 voluminous cells which have sparse, short microvilli which are sometimes forked. These cells are filled with very large, electron dense, oval secretory granules about 1.3um. in diameter (in the present study they will be termed "juvenile" granules). They are often seen in the process of secretion into the lumen (refer to Plates 15 & 17). The gastrodermal cells also contain some small dense mitochondria, small groups of α and β glycogen, extensive GER with moderately electron dense contents, many ribosomes and polyribosomes but no Golgi complexes. There are no junctional complexes with the parenchymal cells at this stage.

12 hours (location: abdomen)

The cells are smaller than before because most of the granules have been secreted into the lumen which is distended. There is no injested material in the lumen. The cytoplasm is dominated by GER often with distended cisternae with a moderately dense content. Some small Golgi complexes are present. There are some basal invaginations and parenchymal projections, but no junctional complexes.

Day 3 (location: abdomen) (Text figure 5)

The cells are similar to those seen on Day 1 except that there is an increase in their volume with a corresponding decrease in the size of the lumen. Junctional complexes with parenchymal processes are observed for the first time (Plates 15 & 18).

Day 3 (location: liver) (Text figure 5)

In contrast to the gastrodermal cells of flukes recovered from the abdomen at this time, the majority of cells now have only a few "juvenile" granules. There are many Golgi complexes producing a new type of granule which is typical of the adult cell. These granules are electron dense and spherical measuring 0.5um. in diameter (in the present study they will be referred to as "adult" granules). They are secreted at the apical plasmalemma. The GER has a slight base-apex orientation. There are many junctional complexes with the parenchymal processes. The apical lamellae are tall and regular.

Day 5 (location: liver)

The ultrastructure of the cells in the main caeca and the lateral outgrowths is now different.

a) The ultrastructure of the main caeca at this stage is similar to that of the adult. The cells are flat, they contain non-orientated GER and mitochondria and some granules. The lamellae are infrequent and small.

b) At this stage, the lateral outgrowths are undergoing considerable growth and undifferentiated cells were frequently seen in the epithelium (Plates 19 & 21). The differentiated cells lining these outgrowths are similar to the corresponding cells of adult flukes except that the GER and mitochondria are not orientated and they still contain areas of α glycogen (Plate 22).

Text Figure 5. Fasciola hepatica.

A diagrammatic comparison of caecal cells of three day old flukes -

A. when located in the abdominal viscera

B. when located in the liver capsule.

(Reproduced from Bennett, 1973, 1975a)

Key.

BI - basal invagination

GC - Golgi complex

GER - granular endoplasmic reticulum

LA - lamellae

M - mitochondria

MU - muscle

MV - microvilli

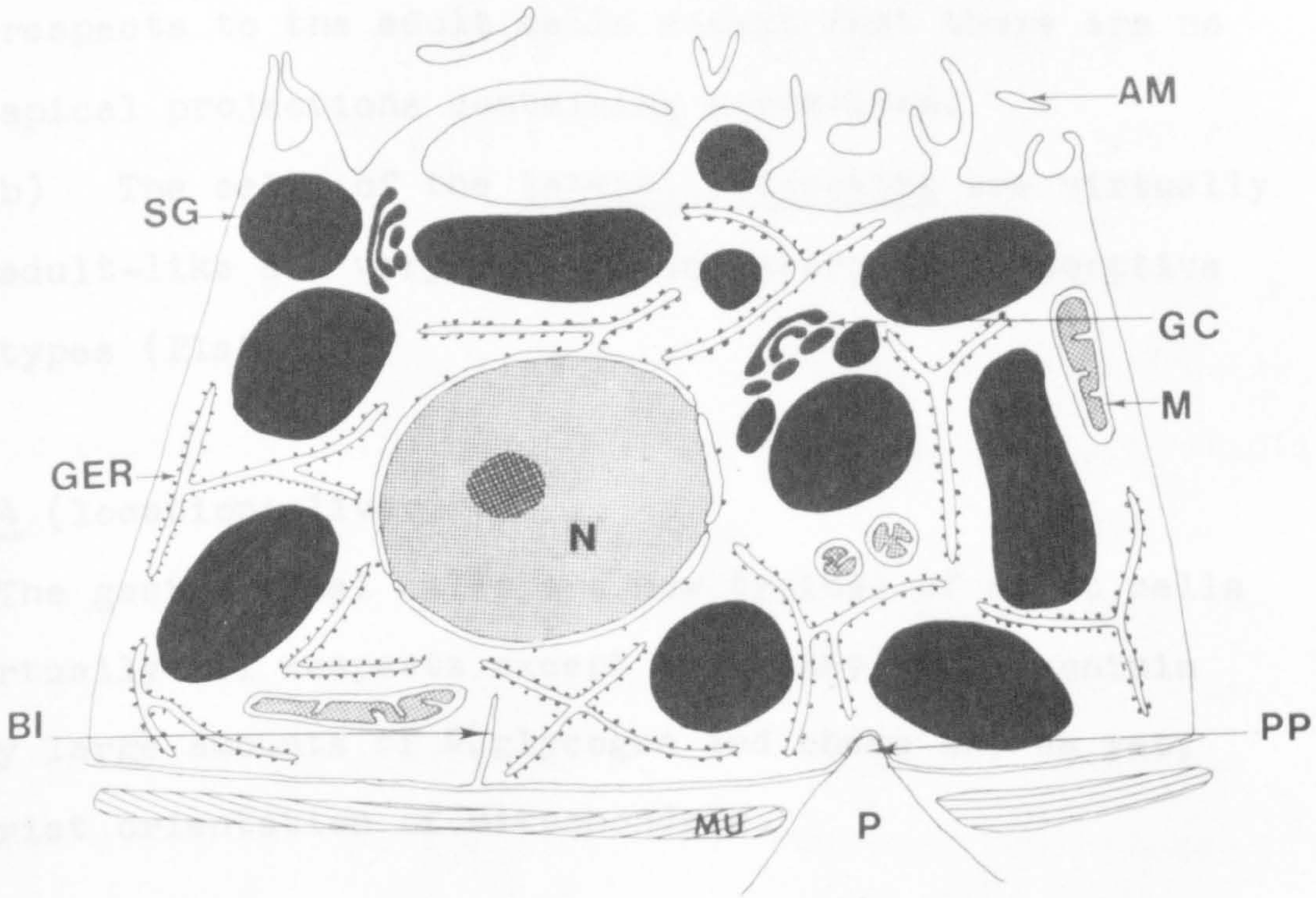
N - nucleus

P - parenchyma cell

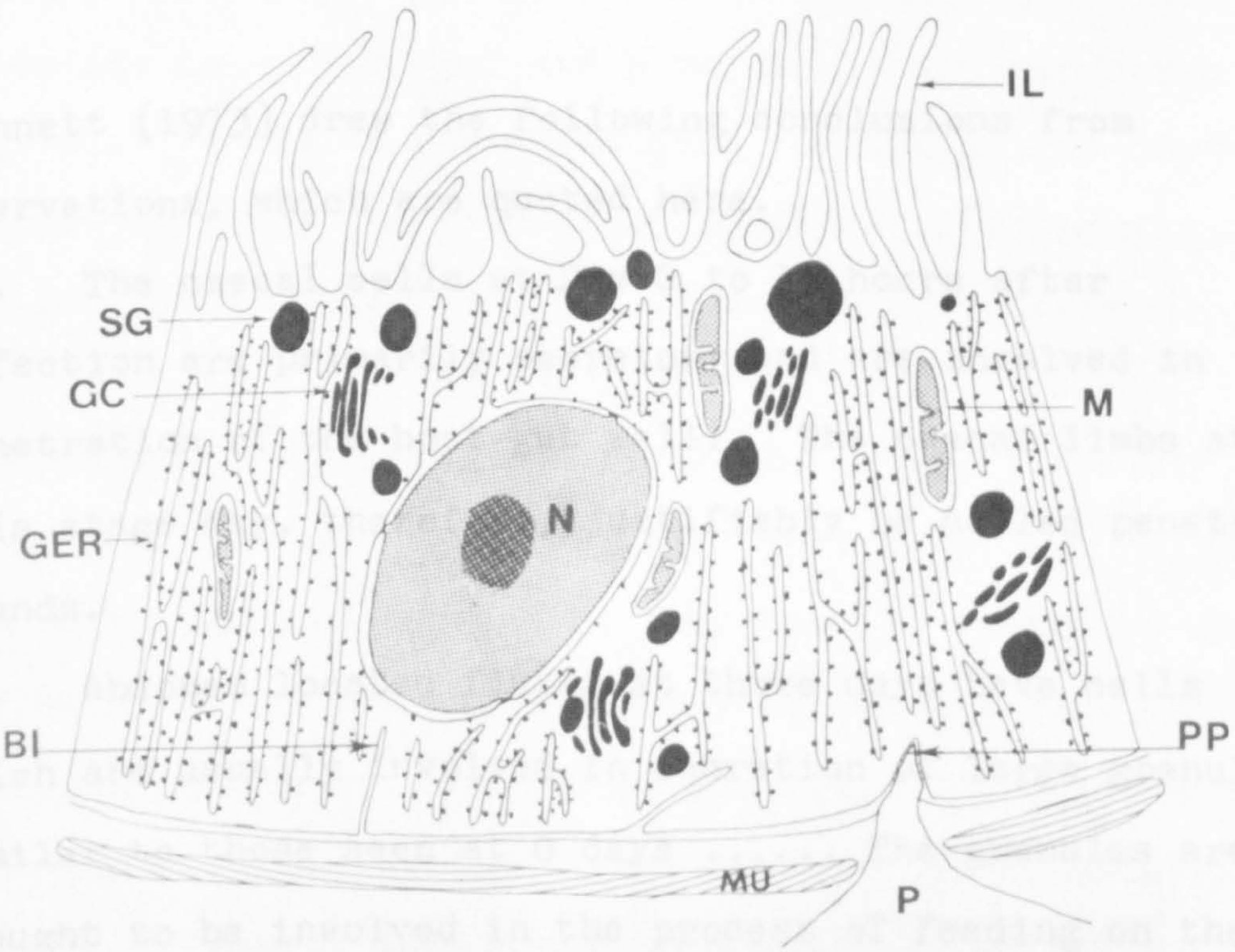
PP - parenchymal process

SG - secretory granule.

A



B



Day 7 (location: liver)

a) The cells in the main caeca are similar in all respects to the adult cells except that there are no apical projections containing secretions.

b) The cells of the lateral outgrowths are virtually adult-like and vary between secretory and absorptive types (Plate 20).

Day 14 (location: liver)

The gastrodermal cells are now typical of adult cells in virtually all respects except that they still contain fairly large amounts of ∝ glycogen and there is, as yet, no strict orientation of mitochondria.

Discussion

Bennett (1973) drew the following conclusions from his observations, which are quoted here.

"1. The caecal cells at Day 0 to 12 hours after infection are primarily secretory and are involved in penetration of the host gut wall. The caecal limbs at this stage may, therefore, justifiably be called penetration glands.

2. Abdomen located flukes at three days have cells which are usually involved in secretion of large granules similar to those seen at 0 days ..... The granules are thought to be involved in the process of feeding on the abdominal viscera and penetration of the liver capsule.

3. The dramatic changeover from the morphology of the penetration gland to that of the adult intestine is the

result of the fluke penetrating the liver.

4. The caecal cells of two and three week old juveniles are adult-like in morphology differing only in that the juveniles' cells contain glycogen stores."

He also suggests that growth of the caecal limbs may occur by de-differentiation of mature gastrodermal cells, cell division and subsequent re-differentiation of one of the daughter cells. In discussion he raises the following points:

- The absence of digested material, extensive lamellae or junctional complexes with theparenchyma cells in the caeca of flukes recovered during the first 12 hours of infection provides evidence that the gut is not adapated for uptake during this period. In flukes recovered from the abdomen, however, some injested material is found in the caecal lumen, small lamellae have developed and some junctional complexes are observed. There is obviously some adaptation for uptake at this time although the secretions characteristic of the "penetration gland" stage continue to be produced in large amounts.

- As soon as the fluke penetrates the liver, the cells become very well adapted for feeding. They produce a massive amount of a new type of secretion, they have an extensive system of lamellae and they have large numbers of basal invaginations and junctional complexes with the parenchyma. The gastrodermal cells aso begin to undergo the digestive/absorptive cycle typical of the adult. Bennett (1973) suggests that the stimulus which initiates this "dramatic changeover" could be:

a) A specific chemical trigger present in the liver parenchyma.

or b) A higher concentration of available nutrients in the liver.

An important point - which is relevant to the present study - is the very different morphology of the gastrodermal cells of young flukes which remain in the abdomen after Day 3 compared with those which penetrate the liver at this time. These differences are summarised in a diagram by Bennett (1973, 1975) which is reproduced here (Text figure 5). The distinction between the two types of morphology is very important when considering the ultrastructure of the gastrodermal cells of cultured flukes (see part IV of this Chapter).

C. The Excretory System

Introduction

The development and morphology of the excretory system of F. hepatica has been studied at the light microscope level by Kawana (1940). The ultrastructural morphology of the adult has been described by Dermott (1967) and Gallagher & Threadgold (1967). Bennett (1973) has studied the development of the excretory system at an ultrastructural level and his observations are reviewed here.

Review of Results (from Bennett, 1973)

(Refer to Plates 23-32 incl., produced by the present author)

He describes the ultrastructure of the flame cell in the newly excysted juvenile in great detail. It is basically similar to that described for the miracidium (Kümmel, 1960; Wilson, 1969) and the adult (Dermott, 1967). Briefly, the structure of the flame cell in the metacercaria, according to Bennett, is as follows:

The flame cell (refer to Plates 31 & 32) has an oval nucleus, mitochondria and GER. From one pole, a group of tightly packed cilia arise, each with the usual 9+2 arrangement of microtubules. The "flame" projects into the lumen of the conjoined excretory tubule. The junction between the flame cell and the cell enclosing the lumen of the excretory tubule is complex. It is composed of a series of interdigitating microvilli; adjacent microvilli are from different cells. These microvilli are in close association although they do not actually make contact with each other. Leptotriches arising from the flame cell project into the lumen surrounding the flame, whilst those from the duct cell extend into the thick layer of interstitial material.

The lumen of the conjoined tubule is extracellular and is enclosed by one or two cells. The morphology of the excretory tubules (ET) and the primary descending ducts (PDD) are similar. The cells are nucleated and the luminal membrane has occasional short microvilli (refer to Plate 29). Surrounding the ducts is a layer of interstitial material which is interrupted by junctional complexes between the duct cells and the parenchyma. The larger ascending ducts (CAD) have a similar morphology but their lumenal surface bears a number of short lamellae and

groups of three or more cilia which project into the lumen (refer to Plate 30). The two major descending ducts (MDD) and the excretory bladder (EB) are lined by a narrow, continuous syncytium. The apical surface of the MDDs and the EB has a moderate number of narrow lamellae. The cytoplasm contains α and β glycogen and lipid droplets but very little GER, Golgi complexes or mitochondria; at intervals large nuclei are observed projecting into the lumen. The basal plasmalemma has invaginations and forms junctional complexes with the parenchyma. The ducts are surrounded by interstitial material and bands of muscle. The lumen of the MDD contains large spherical concretions built up of concentric rings of granular or fibrous material of varying density (Plate 23) or of small aggregations of needle-like crystals (Plate 24 & 25).

During the development of the excretory system, the most prominent changes occur in the syncytium of the MDDs and EB and in the appearance of the CADs.

After 12 hours, the lumen of the MDDs and the EB has decreased in size and most of the concretions have been lost. Thereafter, there is a general trend towards an increase in the thickness of the syncytium; an increase in the amount of lipid drops in the syncytium and in the lumen; and a decrease in the amount of glycogen present (Plates 26 & 28 incl.). The CADs become virtually vestigial by about Day 16. On Day 7, they are no longer observed in the posterior part of the fluke, and when they are observed in the anterior, their lumen is often occluded. After 3 weeks, they are no longer observed.

Discussion

Bennett (1973) notes that:

"Of all the juvenile systems, the excretory most resembles that of the adult."

He draws various conclusions from his observations which are quoted here:

"1. In the early stages of growth up to 1 day the excretory ducts retain glycogen stored there in the cercaria. This glycogen is probably utilised for general cell functions and particularly in the excretion of lipid.

2. An adult-like method of lipid excretion exists in the newly excysted fluke.

3. Concretions are no longer produced nor remain in the main descending ducts of the reserve bladder after infection, at 12 hours.

4. The increase in numbers and size of lamellae in the descending ducts is thought to imply an increasing resorptive function.

5. The ciliated ascending ducts do not grow but recede.

6. The cilia of the ascending ducts may either be involved in movement of fluid due to the narrowness of the tubules or because of the pressure which the system must work against when in the encysted state."

The excretory concretions which pack the lumen of the MDD in the encysted metacercaria and the newly excysted juvenile of F. hepatica are probably calcareous. Erasmus (1967a) has demonstrated the presence of calcium in the concretions of

Cyathocotyle bushiensis. It has been suggested (Cable, 1965) that the excretory concretions of encysted metacercariae have a protective function and that they immobilise metabolic waste products which may otherwise prove toxic in the encysted state. In cestodes, the role of these granules is uncertain, but they have been implicated in CO_2 fixation (Martin & Bils, 1964) and in the buffering of acids (von Brand et al, 1960) - the subject is discussed by Smyth (1969).

D. The Parenchyma

Introduction

The parenchymal system of adult F. hepatica has been studied by Threadgold & Gallagher (1966), Dermott (1967) and Threadgold (1968). The ultrastructure of the parenchymal system during its development from the metacercaria has been studied by Bennett (1973) and his observations on the development of this system, during the first two weeks, are reviewed here.

Review of Results (from Bennett, 1973)

(Refer to Plates 33-35 incl., produced by the present author).

Day 0 (newly excysted juvenile) (Plate 33)

In the newly excysted juvenile, the parenchymal cells are polymorphic and have pseudopodia-like outgrowths. Between them is a layer of non-fibrous interstitial material. The parenchyma is associated with other cells by junctional complexes.

These are frequent with other parenchymal cells, muscle and tegumental cells but they do not occur with embryonic cells, the tegumental syncytium or the caecal cells at this stage. The position and shape of the nucleus varies. The cytoplasm contains mitochondria with a dense matrix and few cristae, but only small amounts of GER and ribosomes, and few Golgi complexes. Also present are frequent lipid droplets, large amounts of α and β glycogen and some primary lysosomes (δ bodies).

12 hours (location: abdomen)

The main difference in the parenchymal cells at this stage is an increase in their volume; an increase in the amount of free ribosomes and GER; and a drastic reduction in the glycogen content so that only dispersed β glycogen is observed.

Day 1 (location: abdomen)

Junctional complexes are first observed with the caecal cells on Day 1 although they still do not occur between parenchyma and embryonic cells or with the tegumental syncytium. The regions of GER and free ribosomes are now more frequent and are usually found in dense areas of cytoplasm near the nucleus, although some groups occur separately. The GER is now associated with some Golgi complexes (refer to Plate 34). The cell cytoplasm contains frequent lipid droplets and also large amounts of β glycogen and some α glycogen (refer to Plate 35). At this stage, autophagosomes (γ bodies) are also seen.

Day 3 (location: liver)

At this stage, there is a general increase in glycogen content, lysosomes and autophagosomes are now frequently seen and junctional complexes with the caeca are more common.

Subsequent development up to 2 weeks (location: liver)

After 2 weeks, the surrounding layer of interstitial material is slightly thicker. There are still no junctional complexes with the tegumental syncytium or the protoplasmic tubules leading to it (these do not occur until 4 weeks after infection when the flukes are in the host's bile ducts). Autophagolysosomes (mature lysosomes) are now observed in addition to the δ and γ bodies (refer to Plate 62, showing part of a parenchyma cell of a cultured fluke).

From Day 3 onwards, some of the parenchyma cells possess pseudopodia-like extensions which contain large, dense, spherical granules - these ramify into the space usually occupied by the interstitial material.

Discussion

Bennett (1973) draws the following conclusions based on his observations.

"(1) The parenchymal cells serve primarily as α and β glycogen stores in newly excysted juveniles.

(2) The cells take up fluid and therefore increase in volume during or immediately after crossing the host gut wall either by general uptake over the body surface or via the excretory bladder.

(3) On arrival in the abdominal cavity at 12 hours, all glycogen stores are very much depleted, due mainly to the locomotory movements of penetrating the host gut wall.

(4) By 24 hours glycogen stores are recovering and contain mainly the β form of glycogen. In all subsequent stages, both α and β glycogen is present.

(5) Parenchymal processes and junctional complexes with caecal cells occur for the first time at 24 hours after infection, allowing uptake from the caeca into the parenchymal cells.

(6) At 24 hours after infection, an adult-like system of glycogenolysis begins to operate and this is at least partly by autophagocytosis.

(7) The interstitial material which proliferates most when the fluke is in the bile ducts is not secreted by the parenchymal cells but by specialised cells producing large dense granules."

In discussion, the following points may be raised.

1. Dermott (1967) and Threadgold & Gallagher (1966) have suggested that junctional complexes may be sites where intercellular exchange occurs. Threadgold (1968) showed that acid and alkaline phosphatases are specifically located in the junctional complexes between the parenchyma cells and the cells of other organ system in adult F. hepatica. Bennett (1973) suggests that the absence of these complexes between parenchyma and caecal cells during the first 24 hours of infection indicates that during this time the caeca are functionally isolated and do not participate in the circulatory potential offered by the parenchymal cells. This is in accordance with the hypothesis that the caeca function as a penetration gland at this time.

2. Bennett (1973) regards the uptake of fluid during, or immediately after, crossing the host gut wall as an adaptation to become iso-osmotic with the environment. He also remarks that:

> "It would be of interest to discover if this influx of fluid into the parenchymal cells occurs in vitro during the cultivation of newly excysted flukes."

3. Threadgold & Gallagher (1966) and Dermott (1967) suggest that the general parenchyma produces the interstitial material. Bennett (1973) suggests that the ramifications of certain specialised parenchymal cells, which contain granules, are responsible for its production. However, this author never actually observed these granules in the process of secretion into the interstitial material.

Section 2: Scanning Electron Microscopy

Introduction

Bennett (1975b,c) has studied the surface features of the newly excysted juvenile, developing forms and adult *F. hepatica* under the scanning electron microscope. His observations on the structure of the newly excysted juvenile and the developmental stages up to two weeks post-infection are reviewed here.

Review of Results (from Bennett, 1975b,c)

a) Newly excysted juveniles

The shape of the newly excysted juvenile varied according to the phase of locomotion it was in but in all cases there was some degree of dorso-ventral flattening. The oral sucker measured 5 um. in diameter when opened and was directed antero-ventrally. The excretory pore was at the posterior tip of the fluke.

The surface of the juvenile was thrown up into many small irregular microvilli. When the body was constricted circumferentially, they showed anterior-posterior corrugations and when it was constricted along its length the corrugations ran in a transverse direction.

The spines were covered by the syncytium plasmalemma, they were single-pointed, backwardly directing and measured 0.5um. in length. Between the oral sucker and the posterior limit of the ventral sucker, the spines were arranged in

approximately 60 rings with 60-70 spines per ring. The spines in these rings formed a diamond pattern. Posterior to this region the spines were evenly scattered. No spines were found overlying the suckers or round the excretory pore.

Bennett described three types of (presumably) sensory papillae:

1. Pit type

These were composed of a spiral of tegument (1⅓ turns) apparently shielding a pit, there was no projecting cilium. A group of three was found on each side of the oral sucker.

2. Ciliated type

These were seen as elevated areas of tegument which surrounded a pit from which arose a single cilium 0.5um. long. Several of these were found scattered on the anterior ventro-lateral surface and a pair on the mid-anterior dorsal surface.

3. Domed type

No cilia or pits were observed in these papillae, only a smooth, domed area. A pair was observed on the dorsal lip of the oral sucker and a ring of six were regularly spaced around the ventral sucker.

TEM observations made by this author revealed the ultrastructure of the last two types of sensory papillae. The ciliated type consisted of a neurone which penetrated

the basal lamina of the syncytium to form a winged bulb within the syncytium. This was connected to the syncytium by a circular desmosome. A single cilium with a typical basal body arose from the bulb and protruded above the level of the tegument. The domed type differed from the ciliated type in that no cilium arose from the basal body to penetrate the bulb. The apex of the bulb was completely covered by the desmosome instead of being circled by it.

b) 1 week (location: liver)

This stage was very similar to the newly excysted juvenile. The anterior arrangement of spines was identical. They had not increased in number and so, because of overall body growth, they were less dense although they had grown in length to 1.5um. On the posterior surface, the overall number of spines had doubled but, compared to the newly excysted juvenile, the density was less. This trend continued throughout development. The spines in this region now measured 1.5um. in length.

In the anterior part of the fluke the syncytial plasmalemma was thrown up into circumferential corrugations which ran in an undulating manner around each ring of spines. On the posterior, the surface formed anterior-posterior corrugations.

The domed receptors around the ventral sucker often appeared as twin domes and sometimes there were two distinct sets of receptors.

c) 2 weeks (location: liver)

The anterior cone was now apparent. There was no increase in the number of spines in this region, they were still arranged in the same pattern and now measured 1.7um. in length. Those in the posterior were 1.4um. in length and were still distributed randomly. The corrugations of the surface were similar to those observed after one week.

At this stage, there was some sign of bifurcation at the tip of some of the spines. This was the earliest sign of the development of the spines; by 3 weeks post-infection, the older spines had up to 8 points. In the mature adult, the spines of the anterior cone had up to 15 points whilst those in the posterior part of the body may have had up to 30 points. There is also a general trend for the surface in the anterior cone region to become smooth whilst the surface of the rest of the body is thrown up into rectangular plateaux with surrounding valleys.

Discussion

In discussing the role of the spines, Bennett (1975b) points out that in the newly excysted juvenile movement is accomplished by waves of peristalsis with alternate attachment and release of the suckers. He suggests that the spines are probably important in maintaining position during locomotion and that the anterior ring of spines have an anchorage function if the grip of the suckers is lost. Since this regular array of spines on the anterior cone is maintained throughout development it probably has a permanent importance in locomotion. The development of the spines to the multi-tipped form and their

possible function will be discussed in Chapter 4 Part III in connection with the structure of the spines of Meiogymnophallus minutus.

Bennett also suggests possible functions for the three types of sensory receptors that he describes, although he provides no direct evidence to support these suggestions.

1. Pit type

He suggests that these may be chemical receptors used to determine the chemical nature of the environment during feeding movements.

2. Ciliated type

He suggests that these are tango receptors and that they may record the direction and degree of pressure acting on the spines.

3. Domed type

He suggests that these are pressure and/or stretch receptors and that they communicate the contact of the suckers with the substrate.

4. Conclusions

From these results it is possible to compile a list of very precise "markers" which chart the normal development of the metacercaria of F. hepatica up to two weeks post-infection in the mouse. These can be used as criteria to assess normal growth and development, against which in vitro cultured forms can be compared. It is probably most convenient to consider the four organ systems and the surface features separately because, in cultured flukes, it is quite possible for the rate and normality of development to vary between different systems. Only those developments which can be very precisely defined are used here as criteria.

Ultrastructural criteria for assessing the normal growth and development of F. hepatica metacercaria up to two weeks in mice. (Based on the results of Bennett and Bennett & Threadgold (loc. cit.)

1. Development of the Tegument

a) 12 hours-2 days (location: abdomen)

- type-0 cells are producing T-0 granules.
- surface glycocalyx formed.
- embryonic cells observed close to type-0 cells.

b) Day 3 (location: abdomen or liver).

- embryonic cells have differentiated to type-2 cells, producing T-2 granules. They are not joined to the syncytium.

c) Day 4-5 (location: liver)

- type-2 cells fuse with the synctium and T-2 granules begin to appear in it.

d) 1 week (location: liver)

- type-0 cells contain T-0 granules and a small amount of T-1 granules.

e) 2 weeks (location: liver)

- type-2 cells are adult-like.
- type-0 cells contain equal numbers of T-0 and T-1 granules.

2. Development of the Digestive Caeca.

a) 12 hours-Day 3 (location: abdomen)

- cells only contain "juvenile" granules.
- injested food material first observed in lumen on Day 1.
- junctional complexes with parencnyma first observed on Day 3, not very numerous.
- surface lamellae still relatively undeveloped.

b) Day 3 (location: liver)

- cells contain mainly "adult" granules.
- the volume of the cells is increasing.
- surface lamellae are longer and more numerous.
- frequent junctional complexes with parenchyma.

c) Day 5 (location: liver)

- morphology of main caeca and lateral outgrowths is now different.
- main caeca are virtually adult-like.
- lateral caeca are nearly adult-like. The GER has a base-apex orientation. The mitochondria are not orientated. There are many "adult" granules.

d) 1-2 weeks (location: liver)

- cells adult-like except there is no strict orientation of mitochondria and there is some ∝ glycogen present.

3. Development of the Parenchyma

The development of the parenchyma presents few useful criteria for assessing normal development. The most obvious changes occur whilst the young fluke is in the abdomen. Subsequently there is a gradual development to the adult state.

a) 12 hours (location: abdomen)
   - marked increase in the volume of the cells.
   - marked depletion of stored ∝ & β glycogen.

b) Day 1 (location: abdomen)
   - large amounts of stored ∝ & β glycogen.
   - Golgi Complexes first observed.
   - autophagosomes (γ bodies) first observed.
   - junctional complexes with caecal cells first observed.

c) 2 weeks (location: liver)
   - mature lysosomes (autophagolysosomes) now present.

4. Development of the Excretory System

As with the parenchyma, there are few obvious criteria to use for assessment during the development of the excretory system. In the newly excysted juvenile, this system is very similar to that of the adult and the process of maturation is a gradual one. However, the following criteria can be applied.

a) 12 hours (location: abdomen)
   - excretory concretions are lost.

b) 1 week (location: liver)
   - CAD are no longer seen in the posterior, in the anterior the lumen of the ducts are often occluded.

c) 2 weeks (location: liver)

- CAD are virtually vestigial.

5. Development of the Surface Features.

As with the two previous systems, the development of the surface features is a gradual process. However, there are two definite changes which occur during the first two weeks.

a) 1 week (location: liver)

- domed receptors around the ventral sucker divide.

b) 2 weeks (location: liver)

- spines bifurcate at the tips.

The most important and useful criteria for assessing normal development are found in the ultrastructural development of the digestive caeca and the tegument. This is particularly relevant since it is these systems which are in most intimate contact with the culture media during in vitro cultivation. If these systems are abnormal in cultured flukes, the normal processes of uptake of nutrients will be affected and this will in turn affect overall growth and development.

These criteria can be summarised as follows.

The Tegument

1. The development of type-2 cells.

2. The conversion of type-0 cells to type-1 cells.

Digestive Caeca

1. The production of "adult" secretions after a few days.

2. The development of a base-apex orientation of GER.

3. Adaptations for uptake:

- the development of surface lamellae;

- the development of junctional complexes with the parenchyma.

PART IV: ULTRASTRUCTURE OF DEVELOPMENT IN VITRO

Introduction and Methods

Because of the time-consuming nature of the procedures involved in the preparation and examination of material for electron microscopy, only flakes from selected cultures were studied. For transmission electron microscopy the following cultures were examined.

a) Culture 25b

Starting material:	excysted metacercaria.
Culture media:	NCTC plus antibiotics plus an extra lg/litre of glucose, with 20% foetal calf serum and defibrinated horse blood.
Other conditions:	10mls. of media in small Leighton tubes, incubated at 37-38°C in a shaking water bath. Media changed twice a week.
Fixation:	fixed and prepared for examination after 12 hours and 1,2,3,5,7 and 14 days.

b) Culture 43b

Starting material:	excysted metacercaria.
Culture media:	NCTC plus antibiotics, with 50% chick serum (Batch A) and defibrinated horse blood.
Other conditions:	as above.
Fixation:	fixed and prepared for examination after 12 days.

c) Cultures 37b and 38b

Starting material:	flukes removed from mice after 7 and 14 days respectively.
Culture media:	NCTC plus antibiotics and Amphotericin B, with 20% foetal calf serum and defibrinated horse blood.
Other conditions:	as above.
Fixation:	fixed and prepared for examination after 7 days.

The development of flukes from culture 25b was studied in some detail. Apart from the beneficial effect on somatic growth observed in culture 43b when chick serum was used, the culture conditions used in 25a were never bettered.

Flukes were removed from mice one and two weeks post infection and grown in culture for subsequent ultrastructural examination in order to determine whether their already partially developed organ systems degenerated, remained as they were or developed further.

In all cases only the largest, healthiest and most active flukes were fixed.

For scanning electron microscopy, flukes were fixed from cultures with the same media and conditions as those described for Culture 25b. Worms were fixed and prepared on Days 7 and 28.

The Results and Discussion are presented in two sections:

Section 1: Transmission Electron Microscopy (TEM).

Section 2: Scanning Electron Microscopy (SEM).

Section 1: Transmission Electron Microscopy (TEM)

Results

The most striking feature was the enormous amount of variation in morphology between one fluke and another grown in the same culture media. There was often a noticeable variation between the ultrastructure at different planes of section in the same fluke, this was particularly true of the digestive caeca.

The results from each of the cultures examined will be presented in turn and the four organ systems (tegument, digestive caeca, excretory system and parenchyma) will be dealt with separately.

Culture 25b

A. The Tegument

12 hours - 1 day (Plates 36, 37)

The outer membrane of the syncytium was covered with a fuzzy glycocalyx, the cytoplasm was electron dense and contained many T-O granules (see Page 108). Some biconcave T-O granules were aligned in groups at right angles to the outer membrane. The body of the tegumental cells also contained many T-O granules. The contents of the granules in the cells and the syncytium varied. Some were normal i.e. they were homogenous and moderately dense, others had granular contents of varying density. In some cases the contents had condensed

to give a cartwheel-like appearance. These granules were similar in appearance to the T-1 granules (see page 108) but remained the same size as the other T-0 granules (the diameter of normal T-1 granules is less than half that of T-0 granules). Golgi complexes were frequently seen and they were unusually prominent.

Some tegumental cells contained large areas of degenerating material with prominant lysosomal bodies.

Days 2-3 (Plates 38, 39)

The ultrastructure at this stage is essentially the same as that observed after 12 hours-1 day in culture. The contents of the T-0 granules in both the tegumental cells and the syncytium were much less dense than previously and many more of them appeared granular. In some cases the T-0 granules seemed to be virtually empty. Embryonic cells (see Discussion) were often seen at this time particularly in association with Type-0 cells.

Day 7 (Plates 40, 41, 42)

The width of the syncytium did not increase noticeably after one week in culture and was about the same as that observed by Bennett (1973) on Day 3 *in vivo* (see part III of this chapter). The ground substance was still very dense and there was an accumulation of dense mitochondria in the basal part. The granules in the syncytium were all of the T-0 type, they appeared to be virtually empty or to have a heterogeneous content. There was some very slight amount of vacuolation within the syncytium especially near the basal lamina. The underlying interstitial material was fibrous and about 0.2um. wide.

A new type of sensory ending was seen in a fluke removed from culture after one week (Plate 41). It probably corresponded to the "pit-type" receptors observed by Bennett (1975c) in the metacercaria (see part III of this chapter). It consisted of a small bulb which was connected to the basal part of the syncytium by a desmosome and which bore a short (1um) club-shaped cilium which appeared to lie within a large, domed, membrane-bound space within the syncytium. Fingers of syncytium projected towards it and the vacuole was completely enclosed by a thin (0.2um) strand of cytoplasm. The rootlet of the cilium and the nervous connection with the bulb were not observed in this case. This sensory receptor was seen on the lateral surface at the level of the ventral sucker; this is surprising since Bennett (1975c) only found the "pit-type" receptors grouped near the oral sucker.

The tegumental cells of one-week-old cultured flukes contained T-O granules only. The content of these granules was the same as those in the syncytium. The cells also contained an unusually large number of moderately electron dense mitochondria with 2 or 3 cristae. This concentration of mitochondria may have been illusory since the cells were generally less voluminous than previously. Few Golgi complexes were observed and the ground cytoplasm of the tegumental cells contained many free ribosomes (monosomes).

Day 14 (Plates 43-47)

The syncytium was still electron dense at this stage; it was of a similar thickness as before although in some areas it was quite thin and appeared "stretched". The T-O granules still appeared virtually empty or slightly granular although occasionally a denser type of granule was seen which was usually the same size as the T-O granules but which resembled a T-1 granule.

In some flukes, particularly in the region of the suckers, but also in isolated regions elsewhere, there was a considerable amount of vacuolation of the basal area of the syncytium (Plate 45). These vacuoles were not membrane bound, they were 1-4 um. in width and appeared to be filled with an amorphous material. In these regions the syncytium was highly abnormal; it contained few or no secretory granules and there were often deposits of α glycogen. Around the ventral sucker of such flukes, some of the sensory receptors were seen in the process of degeneration; these receptors were probably of the "domed-type" (Plates 46, 47) (Bennett, 1975c).

Occasionally, spines were seen which appeared to have sunk below the level of the syncytium. Their bases were usually surrounded by interstitial material.

The tegumental cell bodies (Plates 43, 44) were very similar to those observed in flukes removed from culture after one week. The secretory granules which they contained were similar to those found in the syncytium. The Golgi complexes appeared prominent probably because of the relative emptiness of the cells, they usually consisted of 8-10 sacs. No

mature granule-producing type-2 cells (see page 108) were ever seen but undifferentiated, embryonic cells were still observed in association with the type-0 cells.

Occasionally the tegumental cells were seen in various stages of degeneration with mature lysosomes and autophagic vacuoles. Disorganised keratin scrolls were also occasionally seen.

B. The Digestive Caeca

The following description of the development of the digestive caeca *in vitro* represents the best developed and most normal flukes. In some cases the gastrodermal cells had degenerated until there were very few recognisable organelles - apart from nuclei - and few or no secretory bodies.

12 hours - 3 days (Plates 48, 49, 50)

During the first three days in culture there were only a small number of "juvenile" granules (see page 115) in the cytoplasm - which was reduced in volume compared to the newly excysted juvenile (Plate 48).
This resulted in the terminal bars of adjacent cells projecting into the lumen. (This is identical to the situation observed *in vivo* at this time). There seemed to be areas of Golgi activity (Plate 49) although no definite Golgi complexes were seen. These areas were characterised by clumps of small irregular secretions of a similar density to the juvenile granules. The GER was short and had slightly dilated cisternae with moderately electron dense contents; it was not orientated. The mitochondria were moderately electron dense with 2-4 cristae. Concentrations of

α glycogen were occasionally seen (Plate 50). There were a few invaginations of the basal plasmalemma but no junctional complexes with the parenchyma. The surface microvilli were sparse and short as in the newly excysted juvenile. There was a tendency for the number of "juvenile" granules to increase during this period after the initial depletion.

Days 7-14 (Plates 51-56)

During this time the most prominent granules were of the "adult" type although some "juvenile" ones were occasionally seen (Plate 53). In extensive areas of caecal epithelium, however, there were no granules at all. In these areas the epithelium was very thin, contained only small amounts of fragmented GER and possessed very infrequent microvilli.

Where "adult" granules (see page 116) were observed, they were either scattered in the cytoplasm - especially near the lumen surface - or, more frequently, in projecting pyramids of cytoplasm often near the nuclei (Plate 52). This type of morphology is typical of the main caeca in vivo at this time (see part III of this chapter). Areas of possible Golgi activity were observed (Plate 54) and they were similar to those found during the first three days. However, no definite Golgi complexes were seen. In some cases, the "adult" secretions were not so dense as usual, they had a moderately electron dense centre but the periphery appeared granular (Plate 51).

The epithelium was not very thick (about 4um.) but this feature was very variable; the thicker areas of epithelium usually occurred in the region of a nucleus. The nuclei were large and oval with a smooth or gently undulating membrane (Plate 52). The nucleoplasm was less dense than the cytoplasm, it was homogenous and finely granular and usually lacked areas of heterochromatin. Where observed, the nucleolus was prominent and moderately dense.

The GER was similar to that observed during the first three days. In some areas, however, usually where there were "adult" granules, the GER was longer, did not have dilated cisternae and occasionally had a base-apex orientation.

The mitochondria were often fairly large and long with a dumbell shape and many cristae. Their matrix was slightly denser than the surrounding cytoplasm. They were not orientated.

The microvilli on the apical surface appeared to be more numerous than previously, especially in areas where there were "adult" secretions, although they were not so well developed as flukes recovered from the livers of mice (see part III of this chapter). They were occasionally seen to branch and anastomose. They were frequently cut in cross section and appeared round - indicating that they were probably microvilli rather than lamellae. They were often coated with a fuzzy glycocalyx.

The lumen was always filled with the same type of material (Plate 53). It was typically fibrous and probably represented the black solids which were seen to accumulate in the caeca of cultured flukes (see Part II of this chapter).

There were many basal invaginations (Plate 56) and during this period junctional complexes with the parenchyma developed and increased in number (Plate 55). One of the most prominent structural abnormalities in the digestive caeca of cultured flukes was the absence of a thick muscular coat around the limbs of the caeca. Short pieces of fairly thick muscle were observed but these often began and ended abruptly. The parenchymal cells were often directly adjacent to the caecal cells.

No difference in morphology was noted between the main caeca and the lateral outgrowths. No de- or re-differentiating cells were ever observed.

C. The Excretory System (Plates 57, 58)

The excretory system remained the most normal of the four systems examined. The flame cells, collecting ducts and ciliated ascending ducts all appeared normal although there was no apparent degeneration of the latter as there was *in vivo*. The ciliated ascending ducts and major descending ducts had junctional complexes with the parenchyma (Plate 57).

The excretory concretions (Plate 57) were lost during the first day of cultivation. More lipid was produced in the major descending ducts *in vitro* than *in vivo* and the droplets were surrounded by α glycogen throughout (Plate 58). The cytoplasm of the major descending ducts remained quite thin and the nuclei projected into the lumen. The microvilli increased in length slightly but they were not so numerous or so long as *in vivo*.

D. The Parenchyma

12 hours - 2 days (Plate 59)

During the first two days of cultivation, there was no obvious increase in the volume of the parenchyma cells nor was there a depletion of stored glycogen as there was *in vivo* at this time. In most cells, there were large quantities of α and β glycogen and moderate amounts of lipid droplets which were sometimes quite large.

There were junctional complexes between the parenchyma cells and with the ciliated ascending ducts and major descending ducts of the excretory system (Plate 57). Groups of primary lysosomes (δ bodies) were often observed. The mitochondria were small and moderately electron dense with 2-4 cristae which were often short. They were found scattered throughout the cytoplasm or grouped together. There were also areas of free ribosomes, smooth membranes, GER and possible Golgi complexes.

Day 3 (Plate 60)

The ground cytoplasm began to assume a leached-out appearance and there was less stored α glycogen. Autophagosomes (γ bodies) were first seen at this time. Adjacent parenchyma cells often had different types of ground substance. In some cells, it was homogenous and finely granular whilst in others the granules had apparently clumped together to give a patchy appearance.

Day 7

The parenchymal cells were similar to those described on Day 3 except that they appeared to be more leached-out. The mitochondria had become more electron dense and around the nuclei the GER often had dilated cisternae with moderately dense, granular contents.

Day 12 (Plates 61, 62)

At this stage, the parenchyma cells were often seen to have numerous convoluted processes (Plate 61). The parenchyma-produced granules which Bennett (1973) associated with the formation of the interstitial material, were frequently observed. Primary lysosomes (δ bodies), autophagosomes (γ bodies) and autophagolysosomes were all seen at this stage (Plate 62).

Culture 43b

A. The Tegument (Plates 63, 64)

The syncytium (Plate 63) was electron dense with very dense mitochondria in the basal region. It contained spherical and biconcave T-0 granules which were usually virtually empty or had slightly granular contents. In some cases, the contents were condensed to give a cartwheel-like appearance; some of these granules were the same size as the T-0 granules but others were smaller and resembled normal T-1 granules.

(Plate 64)
The tegumental cells were similar to those observed in flukes after 7-12 days in Culture 25b. They contained areas of free ribosomes (monosomes) and many dense mitochondria with 2-4 cristae. There were very few secretory granules but those that were observed were similar in structure to those in the syncytium. Some dark, patchy areas were sometimes seen in the tegumental cells, these were probably autophagic bodies. No Golgi complexes were observed. Generally, the tegumental cells were much less voluminous than in vivo. No Type-2 tegumental cells with T-2 secretions were ever seen.

B. The Digestive Caeca (Plate 65)

The caecal epithelium of flukes from Culture 43b was more normal than that of flukes from Culture 25b. The cytoplasm was relatively thick (8-10um.) with areas of "adult" secretions usually near the lumen and often projecting into it in pyramids of cytoplasm. The secretory bodies were particularly abundant near the nuclei.

The GER lacked orientation, it was usually fragmented but was not swollen. However, in some areas, especially near the nuclei, there was some parallel GER. Some areas of possible Golgi activity, similar to those observed in culture 25b, were occasionally seen but no definite Golgi complexes were ever observed. The mitochondria were small and oval or longer and dumbell shaped, they had a moderately dense ground substance and were not orientated. The lamellae/microvilli projecting from the luminal surface were sparse but quite long, they occasionally had bulbous swellings at their tips. Some undifferentiated cells, with no GER or secretions, were occasionally seen in the basal part of the caecal epithelium. There was no difference in morphology between the main caeca and the lateral branches.

C. The Excretory System

The excretory system was similar to that observed in flukes from Culture 25.

D. The Parenchyma (Plate 72)

The parenchymal cells did not appear Eached out. They contained stores of α and β glycogen; lipid droplets, many dense mitochondria; δ and γ bodies and autophagolysosomes; dense areas of free ribosomes and GER often with distended cisternae with a moderately electron dense content.

An unusual crystalline body was seen in one of the parenchymal cells (Plate 72). Bennett (1973) has seen these bodies in flukes recovered from the abdomen of mice, he describes them as follows:

"The bodies are long and thin, tapering at both ends. They exhibit a regular pattern of longitudinal and transverse striations of densely granular material."

Cultures 37b and 38b

a) Culture 38b "14 day" flukes from mice

A. The Tegument (Plates 68, 69)

The most obvious abnormality in these flukes was the nigh level of vacuolation of the syncytium near the basement membrane. This was most frequently seen near the lateral edges and around the suckers. It was similar to the vacuolation described in flukes removed from Culture 25b after 14 days. However, in this case, whole sections of the syncytium had become detached from the underlying layers; this gave the impression that the syncytium was being "sloughed off". The vacuoles were not membrane-bound (Plate 69), they were filled with an amorphous material. In these regions, the syncytium was connected to the basement membrane by thin bridges of cytoplasm between the vacuoles. Spines were rarely observed projecting from the surface of the syncytium and in many cases the spines appeared to have sunk below the level of the syncytium and were embedded in the underlying interstitial material (Plate 68). The layer of interstitial material was fibrous and formed a wide band about 0.25um. thick. Unlike flukes recovered from Culture 25b after 12 days, the syncytium above these areas of vacuolation was not grossly abnormal. It contained some spherical T-O granules with granular contents, some T-1 granules and many moderately electron dense biconcave granules which may have been T-O or T-2 granules (it was not possible to say with any real certainty which they were). The T-1 granules may actually be T-O granules

whose contents have contracted, however, they were smaller than the spherical T-0 granules and were of a similar size to the normal T-1 granules. The syncytium was quite dense and contained apparently normal mitochondria.

Very few tegumental cell bodies were observed; this may have been because they had degenerated or because they had decreased in volume. No cytoplasmic bridges connecting the cells with the syncytium were seen. Occasionally, small areas of tegumental cell were seen but these contained only a few granules of the T-0 and T-1 type. No Golgi complexes were seen, there was very little GER but often relatively large amounts of free ribosomes and mitochondria. The nudei of embryonic cells were often observed in this area but mature type-2 cells were never seen.

B. The Digestive Caeca (Plates 70, 71)

The morphology of the main caeca appeared almost normal (Plate 70). The epithelium was about 10 um. thick and a moderate number of "adult" secretions were scattered throughout although they tended to be grouped together near the apical surface. The GER was not orientated; it was fragmented and had slightly swollen cisternae with moderately dense contents. The mitochondria appeared normal although they were not orientated. There were many basal invaginations and junctional complexes with the parenchyma. The boundaries and terminal bars between adjacent cells were particularly prominent. The muscle layer around the limbs of the main caeca was virtually continuous but quite narrow.

In the lateral branches there were few "adult" granules although pockets of them were occasionally seen. There was no orientation of the GER which was similar to that observed in the main caeca. The surface lamellae were long but rather sparse, they often had slightly bulbous ends. Some sunken, undifferentiated cells were seen in the gastrodermis (Plate 71) but this does not necessarily indicate that the process of de- and re-differentiation was active. Few basal invaginations and junctional complexes were seen. The layer of muscle around the lateral branches was thin and was not continuous.

C. The Excretory System

The cytoplasm of the major descending ducts was thin and the microvilli were sparse, only small amounts of lipid were being excreted. Otherwise, the excretory system appeared normal. The ciliated ascending ducts were not observed because the plane of section examined was posterior to the ventral sucker.

D. The Parenchyma (Plate 73)

The parenchyma was highly convoluted and the cells were separated by a thick layer of interstitial material (O.lum.). There were many junctional complexes between the parenchyma cells and with the digestive caeca and excretory ducts. Electron dense patches were seen in the cytoplasm and they contained free ribosomes, dense mitochondria and primary lysosomes (δ bodies). The

ground cytoplasm was granular and contained small amounts of lipid and moderate amounts of α glycogen especially in those parenchyma cells near the digestive caeca.

b) Culture 37b: "7 day" flukes from mice

A. The Tegument (Plate 66)

The ultrastructure of the syncytium was essentially the same as that described for the flukes from Culture 38b except that the degree of vacuolation was considerably less. Many vacuoles were present in the basal area but they were small. The tegumental cells were rarely observed and where they were seen, they were similar to those of flukes from Culture 38b. Cells containing T-2 granules were not seen.

B. The Digestive Caeca (Plate 67)

The main caeca and lateral branches were both similar in ultrastructure to the lateral branches of flukes from Culture 38b. There were few "adult" granules but they were occasionally seen concentrated in pyramids of cytoplasm projecting into the lumen. Basal invaginations and junctional complexes were observed but were infrequent. The GER was not orientated, it was fragmented and often branched with slightly dilated cisternae and moderately electron dense contents. The mitochondria were small, quite electron dense with 2-3 cristae and they were found mainly in the basal part of the cell. The cells were somewhat snrunken in volume and the terminal bars of adjacent cells were frequently seen projecting into the lumen.

C. The Excretory System

D. The Parenchyma

The morphology of both these systems was similar to that previously described for 14 day flukes from culture 45b.

Discussion

a) Cultivation of newly excysted juveniles

A. The Tegument

The *in vitro* production of secretory granules by the tegumental cells seemed to slow down and probably ceased altogether. However, it is very difficult to obtain a true picture of a dynamic process from thin sections which essentially provide a "morphological snap-shot".

In cultured metacercariæ, the granules in the Type-O tegumental cells presented a range of morphology; some were apparently normal, others contained varying amounts of heterogeneous granular material whilst others appeared to be virtually empty. In young flukes recovered from mice, the T-O granules in the tegumental cells were all filled with a moderately dense, homogenous material which was only lost when the granules reached the syncytium. It is quite apparent, however, that after a week of culture, the tegumental cells contained very few secretions and that the majority of these secretions appeared to be abnormal. The secretions may have become abnormal in the tegumental cells or they may have had an abnormal structure when they were produced. However, it is likely that a certain number of normal granules continued to be produced during *in vitro* cultivation. Although the Golgi complexes were quite prominent in the tegumental cells, they were not surrounded by the usual fringe of vesicles suggesting that they were not very active.

During cultivation, the ground substance of the syncytium remained very electron dense throughout. *In vivo* the syncytium increased in density whilst the fluke was in the abdominal cavity and decreased once it had reached the liver environment. Bennett (1973) suggests that, amongst other possibilities, this increase in density was due to some of the contents of the T-0 granules being released into the syncytium by a modified type of eccrine secretion (Kurosomi, 1961). If this is true, and if the granules in the tegumental cells lose their contents during storage in the cells, one would also expect the tegumental cells of cultured flukes to be noticeably electron dense. However, this was not the case. The morphology of the tegumental granules and their activity in culture presents a very complex and confused picture.

In vivo the Type-0 tegumental cell begins to convert to a Type-1 cell once the fluke has reached the liver. This did not seem to occur *in vitro* although some of the granules that were produced by the Type-0 cells resembled T-1 granules. Typically the T-1 granule contains dense material which is condensed to give a cartwheel-like appearance. Many of the T-0 granules, both in the syncytium and in the tegumental cells, had this appearance as early as 12 hours in culture. *In vivo*, the T-1 granules were not seen in the tegumental cells or the syncytium until 7 days post-infection and they were not present in large numbers until after two weeks (Bennett, 1973).

The cartwheel-like granules observed *in vitro* were probably modified T-0 granules rather than true T-1 granules. They were the same size as the T-0 granules,

T-1 granules being much smaller. This indicates that the T-1 granule may not necessarily be a different type of granule to the T-0 granule but rather a modification of it. The conversion from a T-0 to a T-1 type of morphology may depend on various environmental influences. However, no definite explanation can be offered for the early appearance of "T-1 like" T-0 granules *in vitro*.

A very prominent feature in flukes that had been cultured for several weeks was the decrease in volume of the tegumental cells. This was accompanied by an increase in free ribosomes and mitochondria. *In vivo*, the numbers of mitochondria increased when the fluke reached the liver so this increase in mitochondria *in vitro* may only be an apparent increase due to the decreased cell volume. However, it is possible that they were involved in providing energy for some energy-requiring process in the syncytium such as ion-pumping across the plasmalemma. Such a situation could have been caused by an incorrect balance of ions in the media. At this time, there were also increased numbers of mitochondria in the basal area of the syncytium. It is known that the close association of basal invaginations and mitochondria in other organs (e.g. the convoluted tubule of the mammalian kidney) is associated with ion-pumping and water exchange with the environment.

The increase in the number of free ribosomes probably represented the breakdown of the GER; further evidence for this suggestion is that the ribosomes were present as monosomes and not as polysomes.

The decrease in volume of the tegumental cells may be explained by saying that since they are no longer required as storage resevoirs for large numbers of granules they become redundant and eventually vestigial.

Although there were some embryonic cells* in close proximity to the Type-0 tegumental cells, they never differentiated into Type-2 granules as occurs *in vivo*. This may be related to the lack of development of the genital rudiment *in vitro*. It appears from these results that, in culture, the conditions are not favourable for the development and differentiation of embryonic cells.

The vacuolation of the syncytium in 12 day flukes from culture 23b will be discussed later.

*Footnote: The embryonic cells in the sub-tegumental area were similar in morphology to those of the genital rudiment. They possessed conspicuous nuclei with an electron dense matrix and dense patches of heterochromatin. The cytoplasm was usually relatively sparse and was undifferentiated i.e. it contained no specialised secretory granules. Bennett (1973) described how these sub-tegumental embryonic cells differentiate into granule-producing tegumental cells during the development of *F. hepatica in vivo*.

B. The Digestive Caeca

Initially the caecal epithelium of cultured flukes was similar to that of in vivo forms recovered from the abdomen of mice, except that the cells contained fewer "juvenile" secretions. Subsequently, however, there was a change-over to a more adult type of morphology. Although this changeover was by no means complete, "adult" secretions were produced in small numbers. In some areas, the GER was parallel and had a base-apex orientation. The surface microvilli became longer and more numerous and junctional complexes with the parenchyma developed. It seems that, if the fluke is in a sufficiently nutritionally rich environment, this changeover will occur. However, some factor or factors were obviously missing from the media since it was not possible to sustain this normal adult type of morphology. In many areas of the caecal epithelium, the morphology remained typical of flukes recovered from the abdomen but without the "juvenile" secretions. It was noticeable that the more normal adult type of morphology was often localised in the region of a nucleus. The messenger RNA, which is necessary for the synthesis of the secretions, is produced by the nucleus and it is possible that there was insufficient energy to transport this mRNA to more distant parts of the cell.

In Culture 43b, there were significantly more "adult" secretions in the caecal epithelium than in Culture 25b and the general morphology appeared more normal. This can be related to the build up of black solids in the lumen of the latter but not the former,

suggesting that this accumulation of material is due to an inadequate production of digestive secretions.

Also, in Culture 43b, unlike Culture 25b, there was appreciable somatic growth anddevelopment of the caeca. It was only in Culture 43b that undifferentiated cells were observed in the caecal epithelium. Although this does not prove that growth by de-differentiation, division and re-differentiation (as suggested by Bennett, 1973) was occuring, it was indicative of this process.

In the metacercaria, the projections from the apical membrane of the gastrodermal cells were in the form of short microvilli but during development *in vivo* lamellae were produced. *In vitro*, this production of lamellae - rather than microvilli - did not seem to occur since, in cross-section, the projections appeared to be round or oval.

One very consistent feature of the gastrodermal cells in culture was the fragmentation of the GER, often with a corresponding dilation of the cisternae and an accumulation of a moderately dense material within it. Robinson (1971) also observed this when he maintained adult *F. hepatica* in Hédon-Flieg saline prior to force feeding. He also recorded that these swollen GER fragments coalesced to produce a large apical concentration of secretory material which was eventually released by bursting. He regarded these secretions as "stressed secretions" and suggested that they by-passed the normal Golgi packaging process.

C. The Excretory System

The morphology of the excretory system of cultured metacercariae corresponded to the morphology *in vivo* during the first few days. The syncytium lining the major descending ducts was narrow and the nuclei projected into the lumen, the lipid secretions, which were numerous, were surrounded by ∝ glycogen. There were, in fact, very few obvious abnormalities. The ciliated ascending ducts remained similar to those of the newly excysted juvenile; they did not appear to become occluded during cultivation as they did *in vivo*.

D. The Parenchyma

The increase in the volume of the parenchyma cells during the first day post-infection in the mouse, which was recorded by Bennett (1973), was not observed *in vitro*, indicating that it was probably a response to environmental changes rather than an inbuilt reaction. There was no initial depletion and subsequent replacement of the glycogen stores (as observed by Bennett (1973) *in vivo*) they remained fairly constant throughout although there was some leaching out of the ground susbstance. The development of the bodies associated with glycogenolysis - lysosomes, autophagosomes and autophagolysosomes - also occurred *in vitro*. The production of the secretions associated with the synthesis of interstitial material did not seem to be imparied. The thickness of the layer of interstitial material between cells and around organs was generally greater in cultured flukes than in flukes of a corresponding size *in vivo*.

An interesting feature was the crystalline body that was seen on one occasion in a parenchyma cell of a fluke from Culture 43b. Because these bodies were only observed by Bennett (1973) in flukes removed from the abdomen and never in those recovered from the liver or bile ducts, he suggested that they may be a crystallisation of a substance taken up from the abdominal viscera or peritoneal fluid. He also points out, however, that they are reminiscent of viral material. Although the serum present in the media may contain similar components to the peritoneal fluid, it is also quite possible that this body represents viral material such as coat protein.

b) Cultivation of flukes removed from mice after 7 and 14 days

A. The Tegument

There were many similarities between these flukes and cultured metacercariae: The syncytium was dense; the tegumental cells decreased in volume during cultivation; the mitochondria and free ribosomes in the tegumental cells apparently increased in numbers; the synthesis of T-0 and T-1 granules by the tegumental cells decreased and may have ceased; the T-0 granules had a variety of contents; the GER became less abundant.

The Type-2 cells which had already differentiated from embryonic cells and which were producing T-2 granules when the flukes were removed from mice, were not observed *in vitro* when the flukes were examined after 7 days. It is difficult to say whether there were any T-2 granules in the syncytium since they are very similar to the biconcave T-0 granules. It is probable, however, that they were not present. The results seem to indicate that the Type-2 cells degenerated or de-differentiated during the period of *in vitro* cultivation. It is possible that, not only will the cultivation conditions not allow the differentiation of embryonic cells, they will also not maintain the morphology of any embryonic cells which have already differentiated. This is substantiated to a certain extent by the degeneration or de-differentiation of the rudimentary testes of these flukes which is described in part II of this chapter.

The most conspicuous feature of the syncytial tegument of these flukes was the formation of vacuoles (which were not membrane-bound) near the basal lamina. This was also observed to a lesser extent in the ventral sucker region of flukes after 12 days in culture 25b.

This vacuolation has also been observed by Hanna (1973a) who incubated slices of adult F. hepatica in various defined media without serum. In Hédon-Fleig saline, vacuolation was observed after only a few hours and after 20 hours he recorded that large areas of the tegument had broken up and floated away from the slices leaving a bald basal lamina.

Wilson & Barnes (1974) found that, if adult Schistosoma mansoni were incubated in Hanks saline for only 30 minutes, they developed vauolations of the syncytium and the tegumental cells. After 4 hours, the syncytium became grossly abnormal. Schistosomes that were incubated in Eagles medium or NCTC for 5 hours did not show these abnormalities. However, they noted that a rapid wash in Eagle s medium produced a swelling of the basal invaginations to form large extracellular vacuoles. This abnormality apparently disappeared on subsequent incubation in Eagles medium. They suggest that since the basal invagination are probably sites of Na/K transport the vacuoles could have resulted from "localised sodium transfer from the tegument cytoplasm to interstitial spaces coincident with an osmotic flow of water."

In the present case, the vacuolation of the syncytium was similar to that observed by Hanna (loc.cit.) and by Wilson & Barnes (loc. cit.) in Hanks saline; it did not appear to involve the basal invaginations. The cause of this vacuolation is not clear; it may have resulted from an osmotic imbalance between the media and the tissues of the fluke. However, it must be borne in mind that it is the tegument of the fluke which is in continual and intimate contact with the culture medium and that it may be exposed to a variety of toxic or unfavourable conditions which could affect its morphology. There is no apparent reason, however, why the vacuolation of the syncytium is most severe around the suckers.

B. Digestive Caeca

The morphology of the lateral and main branches of the digestive caeca showed various differences after only 5 days in vivo (see part III of this chapter), but this difference was not maintained in the "7 day" flukes which were transferred to culture. The ultrastructure of the caeca, especially the lateral branches, seemed to regress. In many ways it was similar to that of the best developed flukes from cultures which started with the newly excysted juvenile. It was also similar to that of the main caeca of flukes recovered from the hepatic parenchyma of mice - there were few secretions, there was no orientation of the GER, the epithelium was thinner, and the lamellae were less abundant than in the lateral branches. Robinson (1971) suggests that the main caeca have a

mainly transporting function and, although they may allow the injested food to be mixed with the digestive secretions produced by the epithelia of the lateral branches, they are not directly involved in digestion to any great extent. All this tends to indicate that the caeca of the cultured "7 day" flukes were not particularly active.

In the "14 day" flukes, the main caeca maintained its normal morphology but the structure of the lateral branches degenerated and they were obviously not fulfilling their digestive functions. Apart from the loss of secretions and the degeneration of the GER, the frequency of junctional complexes and basal invaginations decreased. This would seem to imply that, when they were no longer important as sits of interchange of digested materials, they degenerated or regressed.

It is apparent from these results that the production of "adult" granules is not initiated by a "once and for all" trigger. Although the culture media was nutritionally very rich, it obviously lacked certain factors which were necessary for the continuing, normal production of digestive secretions. This may have been because certain precursors were missing or because insufficient energy was provided for such massive protein synthesis, or it may have been because the general metabolism of the cell was not functioning normally.

C. The Excretory System

Although the excretory system remained essentially normal, there was some regression; the epithelium of the major descending ducts became thinner and the lamellae were less frequent. The general morphology was reminiscent of the early stages *in vivo*.

D. The Parenchyma

The only prominent abnormal features of the parenchyma cells was their high degree of convolution and the relative thickness of the interstitial material. The reason for these differences is not immediately apparent. However, a possible explanation may be that the various components of the normal somatic growth process had become unco-ordinated during *in vitro* cultivation.

Section 2: Scanning electron microscopy (SEM)

Results: (Plates 74-79)

The arrangement, distribution and size of the spines (Plates 78, 79) as well as the overall body shape (Plate 74) of cultured flukes was almost identical to that of the newly excysted juvenile (Bennett, 1975c - see part III of this chapter). No gross abnormalities were seen on the surface of the cultured flukes; the tegument plasmalemma was thrown up into many small irregular microvilli. There seemed to be no degeneration of surface morphology after 28 days in culture, and in most respects it was the same as that of flukes removed from culture after 7 days.

Numerous sensory receptors were seen around the suckers and scattered over the anterior ventro-lateral surface. The tegument overlying the oral sucker was relatively smooth and many "domed" receptors were seen in this region (Plate 75). However unlike the pair on the lateral lip described by Bennett (loc. cit.), these were not very prominent. After 7 days in culture, the "domed" receptors on the ventral sucker had begun to divide (Plate 76) and after 28 days this process was nearly complete (Plate 77). However, the "daughter" receptors remained very close together and did not form a separate ring.

In some cases, the cilia of the "ciliated-type" receptors projected out from the bulb for some distance (Plate 78). This was only observed after 28 days in culture and in most cases the sensory receptors were quite normal and had a similar morphology to those of the newly excysted juvenile.

Discussion

Although the development of the surface features is a very gradual process *in vivo* there are certain differences between the newly excysted juvenile and the 7 day fluke from the mouse (see part III of this chapter). When these are taken into consideration, the cultured flukes most resembled the newly excysted juvenile. In the 7 day fluke from the mouse, the spines on the anterior region are more widely spaced and they are slightly larger than those in the posterior region; they are about three times the length of the spines on the newly excysted juvenile. The surface of the 7 day fluke from the mouse is different to that of the newly excysted juvenile, and the cultured flukes in that it is thrown up into more regular corrugations which, in the anterior region, run in an undulating manner around the spines.

The main differences between the cultured flukes and the newly excysted juvenile are with respect to the sensory receptors. In the newly excysted juvenile (Bennett, 1975c) and probably in the 7 day fluke from the mouse (Bennett, 1975b), there is only one pair of "domed" receptors on the oral sucker and these are on the dorsal lip. In cultured flukes, there are, in addition to this pair of receptors, numerous other "domed" receptors around the oral sucker. They are much less prominent than the pair on the dorsal lip. It is likely that they are also present in the newly excysted juvenile and in the young fluke, but, because the overlying syncytium is so much more folded under normal conditions, they are not visible. In cultured flukes, the tegument overlying the oral sucker is relatively smooth and so the

ring of "domed" receptors can be seen. It is quite likely that they are a different population of receptors to those on the dorsal lip and, if the suggestion of Bennett (1975c) that they are pressure receptors is correct, they may respond to different thresholds of stimulation.

In vivo the "domed" receptors on the ventral sucker begin to divide by 7 days post infection and eventually the daughter receptors form a separate ring. Although these receptors begin to divide during cultivation, they always stay very close together and never form a separate outer ring.

In several cases, the cilia of the ciliated-type of receptors project out for an abnormally long distance. This will be discussed in the corresponding section on cultured Microphallus similis (Chapter 5: part IV), however, it may be the result of sensory deprivation in the culture media.

Considering the degree of abnormality in the ultrastructure of the syncytium and the tegumental cells seen in thin sections, it is surprising that the surface features remain virtually unchanged from the newly excysted juvenile even after up to 4 weeks in culture.

Conclusions

A. The Tegument

(1) The ground substance of the syncytium of cultured metacercariae remained electron dense throughout and the syncytium did not increase in depth. *In vivo*, the ground substance became progressively less electron dense once the fluke had reached the liver and the syncytium gradually increased in depth during development.

(2) The production of secretory granules by the tegumental cells slowed down during cultivation and probably ceased altogether.

(3) The embryonic cells near the Type-0 cells did not differentiate into Type-2 cells. *In vivo*, these cells differentiated and began to produce T-2 granules after 3-5 days (see part III of this chapter). The flukes removed from mice after 7 and 14 days already possessed functioning Type-2 cells but they seemed to degenerate or de-differentiate during cultivation.

(4) The Type-0 cells did not convert to Type-1 cells *in vitro* although granules similar to the normal T-1 granules were produced after only 12 hours in culture. *In vivo* the Type-0 cells began to convert to Type-1 cells after 7 days and by three

weeks the transformation was complete.

(5) The T-O granules in the tegumental cells and in the syncytium were abnormal in that they were virtually empty. In vivo the granules in the tegumental cells were homogenous and finely granular, they only began to lose some of their contents once they reached the syncytium.

(6) The tegumental cells appeared to decrease in volume during cultivation.

(7) The presence of vacuoles (which were not membrane bound) in the basal part of the syncytium was a common abnormality.

B. The Digestive Caeca

1. During the first three days of cultivation, the caeca were similar to those of flukes recovered from the abdomen of mice.

2. Subsequently, "adult" granules were produced in small numbers although the caeca did not develop the full adult-type of morphology typical of flukes from the liver.

3. In cultures originating from newly excysted juveniles, there were no significant differences

between the ultrastructure of the main caeca and that of the lateral branches (where they were present). In vivo there were noticeable differences after 5 days post infection.

4. In cultures originating from flukes removed from mice, the lateral branches degenerated or de-differentiated whereas the main branches remained more normal.

5. Although the culture media was probably able to initiate or support the synthesis of small amounts of "adult" granules, it was unable to maintain the level of production found in vivo.

6. Junctional complexes with the parenchyma developed in cultures of newly excysted juveniles. In vivo, they began to develop by the third day post-infection.

C. The Excretory System

This system remained the most normal although there was very little development. It was similar to that of flukes recovered from the abdomen of mice.

D. The Parenchyma

1. Parenchymal stores of glycogen and lipids were maintained during cultivation.

2. Junctional complexes with other parenchyma cells and organ systems developed and were maintained.

3. The interstitial material continued to be produced.

4. The components of glycogenolysis (lysosomes, autophagosomes and autophagolysosomes) apparently developed normally.

5. There was some leaching out of the ground substance of the parenchymal cells during cultivation.

The outer surface

1. The outer surface of the cultured flukes was similar to that of the newly excysted juvenile although there was some development of the "domed" sensory receptors.

2. The cilia of the "ciliated-type" receptors were unusually long.

Although only the most active and healthy cultured flukes were fixed for electron microscopy, a study of their ultrastructure revealed that they ranged from almost normal to grossly abnormal. This was not only true of different flukes from the same culture but also of the morphology of the same organ systems in different planes of sections in the same fluke. From these results, it is

apparent that cultured flukes - which seem to be thriving by all outward signs - possessed fundamental abnormalities at an ultrastructural level.

Dawes & Hughes (1964) have suggested that newly excysted juveniles, cultured under similar conditions, were in a state of "suspended animation". From the present study, it appears that, at an ultrastructural level, cultured flukes are more similar to flukes recovered from the abdominal cavity of mice, than those removed from the liver. It is interesting to note that Dawes (1962) observed that, although young flukes are able to survive for some time in the abdominal cavity, they do not undergo any further growth or development unless they reach the liver, i.e. they, too, are in a state of "suspended animation".

CHAPTER 4 : MEIOGYMNOPHALLUS MINUTUS

PART I: DEVELOPMENT IN VIVO

Introduction

1. Life Cycle

The morphology, ecology and life cycle of Meiogymnophallus minutus have been studied by Bowers & James (1967). The life cycle is summarized in Text figure 6.

The adults parasitize the small intestine of the oystercatcher (Haematopus ostralegus occidentalis) and the common scoter (Melanitta nigra L.) - one bird may be infected with 12,000-60,000 adults of M. minutus. Until recently, the first intermediate host was unknown but Bowers & James (personal communication) have now recovered the sporocysts and cercariae of this species from the marine lamellibranch Scrobicularia plana. However, the percentage infection appears to be very low. The metacercariae are found in the second inter mediate host, the cockle Cardium edule L. (=Cerastoderma edule). Presumably, the furcocercous cercariae leave the first intermediate host and enter the cockle via the opening between the two valves of the shell. Young metacercariae similar to tailless cercariae have been found free in the space between the mantle and the shell by Loos-Frank(1971). The mature metacercariae are unencysted but are enclosed in a proliferation of the pallial line epithelium which lies in the wedge-shaped cavity of the shell below the hinge of the cockle (Plate 80). From 1 to 700 metacercariae may be found per cockle and where

the infection is present virtually all the cockles in the area are infected. The infestation increases with the age of the cockle and varies with the seasonal migration of the final host (Bowers & James, 1967). The final host becomes infected by eating infected cockles. One oystercatcher may eat up to 300 cockles a day (Drinnan, 1957) and if the average number of metacercariae per cockle is 90, the oystercatcher could become infected with up to 27,000 metacercariae each day. Indeed, the infection rate may be much higher since cockles recovered from Salthouse Point on the Llanrhidden Sands may contain up to 1,500 metacercariae each (personal communication, Dr. B.L. James).

There has been some controversy about the classification of this trematode and the validity of the genus Meiogymnophallus; this question will be discussed later.

2. Morphology (according to Bowers & James, 1967)

a) Metacercaria

The morphology of the mature metacercaria is shown in Text figure 9 and its various dimensions are recorded in Table 4I: 1. Developing testes and ovary are already present at this stage but the most conspicuous feature is the voluminous, Y-shaped excretory vesicle. The fully formed vesicle is packed with excretory granules 0.003-0.004 mm. in diameter and it almost fills the body, obscuring most of the morphology. During the development of the metacercaria, this vesicle becomes progessively larger and more densely packed with granules. Loos-Frank (1971) has studied this development in more detail.

Bowers & James (1967) consider that these granules accumulate because their excretion is hindered due to the metacercaria being completely enclosed by host tissue. It is also possible that they may have a function similar to that suggested for the encysted metacercaria of F. hepatica - namely that of immobilizing toxic waste products whilst in an enclosed environment.

Bowers & James (loc. cit.) record that some of the metacercariae were hyperparsitized by microsporidian protozoa (Nosema sp.). They observed that:-

> "Whenever infestation occurred, most of the metacercariae within the cockle were affected. The spores of the hyperparasite cause the body to enlarge, sometimes to almost twice the normal size, and to become opaque and greyish white. It appears that the metacercaria is killed by this microsporidian".

This hyperparasitization has been studied by Canning & Nicholas (1974); they have described the microsporidian and have transferred it to the genus Unikaryon re-naming it Unikaryon legeri. It was previously recorded by Giard (1897), Dollfus (1912, 1946), Guyénot et al (1925) and more recently by Boyden (1970).

b) Adult

The morphology of the adult is shown in Text figure 7 and its various dimensions are recorded in Table 4I: 1.

The morphology (according to Bowers & James, loc. cit.) is very similar to that of the metacercaria, except that:-

a) The excretory granules have been lost and the excretory vesicle is less voluminous,

b) The genitalia and associated structures have developed to maturity.

Bowers & James (loc. cit.) describe the genitalia as follows:-

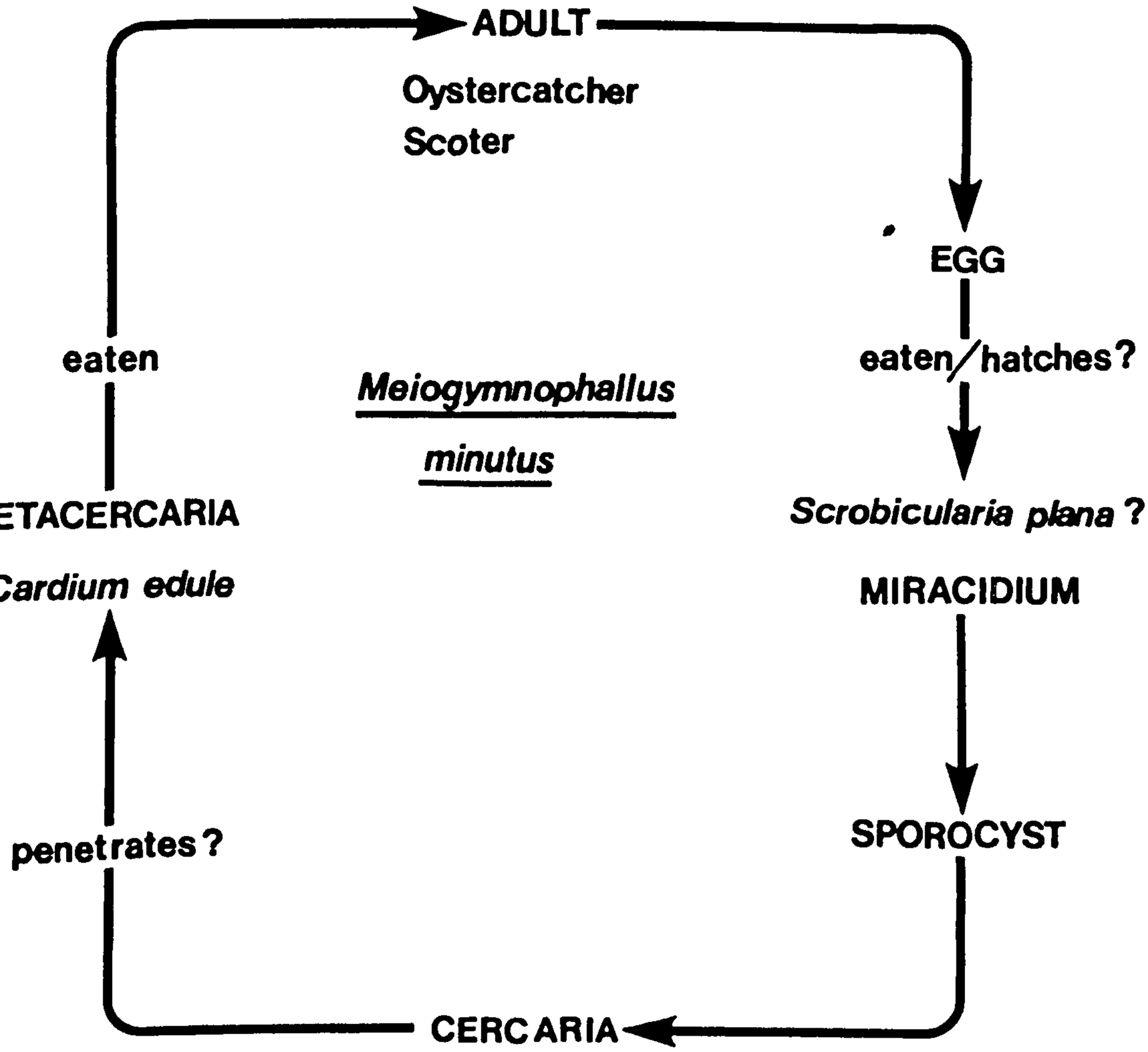
"The testes are oval and lie, one on each side, near the lateral body wall, level with the posterior border of the ventral sucker. The cirrus and cirrus pouch are absent. The vesicula seminalis is large, somewhat rectangular and undivided; it lies on the left side of the body just off the mid-line, between the ventral sucker and the gut caeca. The pars prostatica opens directly into the genital atrium. The genital pore lies on the anterior lip of the ventral sucker. The genital atrium is tubular. The ovary lies just anterior to the right testes and level with the anterior border of the ventral sucker. Laurer's canal, the central chamber of Mehlis's gland and an ovijector are present. Mehlis's gland lies just off the mid-line below the ventral sucker. The receptaculum seminis lies immediately behind Mehlis's gland, near the lateral body wall and close to the posterior end of the body. The vitelline glands are paired, compact, almost spherical and situated one on each side of the ventral sucker.

The uterus is extensive but confined to the anterior two-thirds of the body in front of the ventral sucker. The gravid uterus contains up to 97 eggs. They are oval, thin shelled, light yellow, operculated and embryonated".

Loos-Frank(1971) disagrees with some of these observations. She considers the vesicula seminalis to be bi-lobed, but points out that since the two parts usually overlie each other, the vesicula generally appears undivided. This author also failed to observe the receptaculum; she suggests that Bowers & James (loc. cit.) may have mistaken it for the beginning of the uterus which could have been full of sperm.

3. Experimental Hosts

In an attempt to find a suitable laboratory host for the adult of M. minutus, Bowers & James (loc. cit.) used day-old Khaki Campbell ducklings. These only became infected if they had been fed on boiled minced cockles prior to and during infection and if they were force-fed with metacercariae enclosed in host tissue rather than with free metacercariae. If the ducklings were fed on baby chick crumbs, they failed to become infected. They suggested that a marine diet may be important in determining the susceptibility of the host to this parasite. However, they also recorded that ducklings fed on boiled minced cockles were very stunted in growth and were probably suffering from malnutrition. In view of this, they point out that malnutrition, rather than the direct effect of a marine diet, may cause the ducklings to be susceptible to infection.



Text Figure 6. Meiogymnophallus minutus: Life cycle.

Text Figure 7. Meiogymnophallus minutus.

Morphology of -

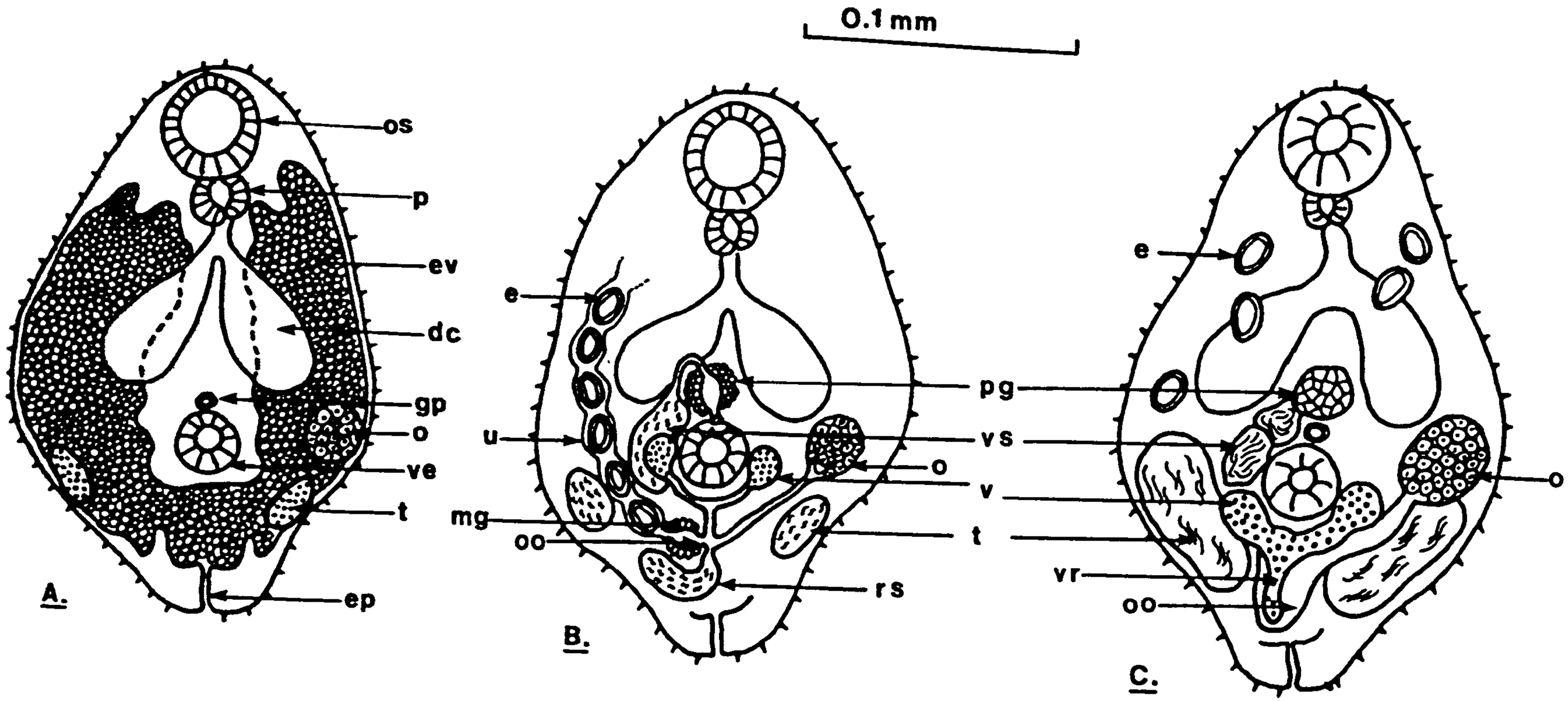
A. The metacercaria (redrawn from Bowers & James, 1967).

B. The adult (redrawn from Bowers & James, 1967).

C. An adult recovered from a duckling three days post-infection (from camera lucida drawings of live flukes).

Key.

dc - digestive caeca
e - eggs
ep -excretory pore
ev - excretory vesicle
gp - genital pore
mg - Mehlis' gland
o - ovary
oo - ootype
os - oral sucker
p - pharynx
pg - prostate gland
rs - receptaculum seminis
t - testes
u - uterus
v - vitellaria
ve - ventral sucker
vr - vitelline resevoir
vs - vesicula seminalis



Text Figure 7.

Experiments

The following experiments were designed to study

a) the normal pattern and rate of development *in vivo*;

b) the suitability of various laboratory animals as hosts for the adults of *M. minutus*;

c) the effect of diet on susceptibility to infection.

Experiment 1: Boiled minced cockles throughout

Methods

Ten one-day-old Khaki Campbell ducklings were fed on a diet of boiled minced cockles for three days prior to infection and throughout the subsequent experiment. When they were four days old, they were force fed with metacercariae (enclosed in host tissue) from eight cockles which had been collected at Salthouse Point on the Gower Peninsula, South Wales. Since there were an average of 976 metacercariae per cockle (Dr. B.L. James, personal communication), the ducklings were probably exposed to approximately 8,000 metacercariae.

Two ducklings were killed on each of Days 1,2,3,4 and 6 post-infection. Their guts were divided into five sections:-

1. Duodenum
2. }
3. } Ileum - divided into three equal sections
4. }
5. Colon and caeca.

The pieces of gut were slit and incubated in saline at 40°C for 20-30 minutes when the sediment was examined for worms. The number of flukes recovered was not counted

but a rough estimate was made of the percentage infection.

In order to study their development, observations were made on:-

a) live flukes;

b) flukes stained in aceto-orcein;

c) flukes fixed in ethanol and treated with Fast Red Salt B to determine the presence of egg-shell precursors (Bell+Smyth, 1958);

d) flukes fixed in buffered formalin and stained in Gower's carmine for permanent preparations;

e) flukes fixed and prepared for transmission electron microscopy (see part IV of this chapter for results).

Results

On Days 1 and 2 about 100-400 flukes per duckling were recovered (1-5% infection) from the ileum, mainly from the third section. On Day 3, there were only about 20 flukes per duckling, whilst on Days 4 and 6 no flukes whatsoever were recovered.

Development

Day 1

The excretory vesicle no longer contained the granules that were so abundant in the metacercariae. The prostate gland - which was not evident in the metacercariae - was packed with irregular secretions. The testes contained spermatids but no mature spermatozoa. The vesicula seminalis and receptaculum were empty. In some cases, the vitellaria were positive for egg-shell precursors.

Day 2

In all cases, the vitellaria were positive for egg-shell precursors and they assumed a typical Y-shape. The testes contained bundles of spermatozoa and in most cases the vesicula seminalis was full of sperm. When the fluke was flattened slightly, the vesicula seminalis was clearly seen to be bipartite. It is likely that the two lobes overlie one another and that they can only be distinguished when the fluke is flattened. The receptaculum was not observed. Very occasionally, one or two shelled eggs were seen in the uterus.

Day 3

The vesicula seminalis was always packed with sperm and sperm continued to be seen in the testes, however, the receptaculum was never seen. Up to 6 shelled eggs were seen in the uterus (Text figure 7C). There was no significant somatic growth or body-shaping during development to the adult. The length, breadth and diameter of the suckers was similar to that of the metacercariae.

It became apparent during this study that, not only were the vesicula seminalis and vitellaria different from the description of Bowers & James (loc. cit.), the testes and ovary were larger. Measurements were made on the testes and ovaries of 10 live flukes recovered on Day 3 post-infection. In Table 4I: 2, these measurements are compared to those made by Bowers & James (loc. cit.) and also to those on Gymnophallus fossarum made by Bartoli (1972).

Experiment 2: Boiled minced cockles prior to infection

Methods

The ducklings were fed on boiled minced cockles for the three days prior to infection and thereafter on baby chick crumbs (the normal laboratory diet for ducklings). Otherwise, the experimental details were the same as for Experiment 1.

Results

The results obtained in this experiment were virtually identical to those recorded for Experiment 1. On Days 1, 2, and 3, the recovery was 1-5%, on Days 4 and 6, no flukes were recovered. The developmental pattern was as described in Experiment 1.

Experiment 3: Normal diet throughout

Method

The ducklings were fed on baby chick crumbs throughout, both before and after infection. Otherwise, the experimental details were as before.

Results

On Days 1, 2 and 3, small numbers of flukes (0.1-0.5% infection) were recovered from the ileum, especially the posterior third. On all these days, and especially on Day 1, a large number of dead flukes were seen - many of these still possessed granules in their excretory bladder. On Day 3, a small number of flukes contained up to 5 eggs.

Some flukes recovered on Days 2 and 3 still possessed large quantities of granules in their excretory system; these flukes were alive and active but contained no eggs and there had been no development of their vitellaria or testes. On Days 4 and 6, no flukes were recovered.

Experiment 4

Methods

Attempts were made to infect several other laboratory hosts.

a) Suckling TF1 mice

b) Young TF1 mice (one month old)

c) Suckling Wistar rats.

These aniamls (a-c) were fed by stomach tube with about 600 free metacercariae each. Their guts were examined for worms after three days.

d) Adult TF1 mice

Four adult mice were each force-fed with approximately 1,000 metacercariae enclosed in host tissue. Their guts were examined on Days 2 and 4 post-infection.

e) 5-day-old chicks

In this experiment half of the chicks were fed on baby chick crumbs and the other half on boiled minced cockles. They were each force-fed with about 1,000 metacercariae enclosed in host tissue. One chick from each group was killed on Days 1, 2,3,4 and 6 post-infection and their guts were examined in five portions as in Experiment 1.

Results

None of the animals used in this experiment became infected with M. minutus.

Experiment 5

Methods

In order to examine the adults of M. minutus in their natural definitive host, two oystercatchers were shot on the Llanrhidian Sands in South Wales. They were winged and taken back to the laboratory of Dr. B.L. James at University College, Swansea, where they were killed and their guts examined. Several pieces of ileum were fixed in buffered formalin, processed for wax sectioning and stained in Haematoxylin and Eosin in order to examine the location of the adult within the host's gut.

Results

Many thousands of adult M. minutus were recovered from the ileum of each oystercatcher. Various cestodes and large numbers of the Gymnophallid trematode Parvetrema affine were also present.

The flukes were embedded between the villi but they did not penetrate deeply into the crypts of Lieberkühn. They were often located in a small vacuole and the surrounding host cells were frequently seen to be degenerating. The fluke was attached by means of its ventral sucker, the oral sucker usually contained a plug of macerated host tissue (Plates 81 & 82).

Table 4I:1 M. minutus and G. fossarum (dimensions in mm.)

	M. minutus metacercaria (Bowers & James, 1967)	M. minutus adult (Bowers & James, 1967)	G. fossarum adult (Bartoli, 1972)
Length	0.12-0.30	0.13-0.31	0.182-0.268
Breadth	0.052-0.14	0.06-0.17	0.118-0.147
Diameter of Oral Sucker	0.032-0.042	0.032-0.048	0.032-0.043
Diameter of Ventral Sucker	0.019-0.028	0.021-0.032	0.024-0.030
Diameter of Pharynx	0.016-0.018	0.016-0.018	0.015-0.024
Length : OS	6 : 1	6 : 1	6 : 1
OS : VS	1.5 : 1	1.3-1.5:1	1.08-1.52:1
Testes - length	0.026-0.037	0.026-0.035	0.039-0.058
Testes - breadth	0.010-0.014	0.010-0.013	0.017-0.029
Ovary - length	0.019	0.026-0.034	0.037-0.056
Ovary - breadth	0.010	0.016-0.022	0.030-0.043
Vesicula seminalis	-	"large"	0.029-0.054x 0.015-0.032
Receptaculum	-	0.041-0.046x 0.016-0.017	"small"
Pars prostatica	-	0.015x0.019	0.033-0.055x 0.019-0.035
Vitellaria	-	0.022-0.028x 0.010-0.017	"voluminous"
Eggs	-	0.016-0.024x 0.011-0.016	0.017-0.022x 0.011-0.014

Table 4I:2 M. minutus - genitalia in the adult.

(dimensions in mm.)

	M. minutus (present study)	M. minutus (Bowers & James,1967)	G. fossarum (Bartoli, 1972)
Testes:length	0.057-0.067	0.026-0.035	0.037-0.062
breadth	0.023-0.033	0.010-0.013	0.016-0.031
Ovary:length	0.037-0.043	0.026-0.034	0.037-0.056
breadth	0.027-0.030	0.016-0.022	0.030-0.043

Discussion

a) Diet

In Experiment 1, the ducklings were very weak and stunted and were probably suffering from malnutrition, they ate very little of the food that was offered to them. In Experiment 2, the ducklings were in a similar condition prior to and during infection but as soon as they were returned to a normal diet they began to grow and became healthier. Since the worm burden and general pattern of infection in both these sets of ducklings was virtually the same, it is likely that starvation rather than the direct effect of a marine diet made them more susceptible to infection than normal healthy ducklings.

The type, quality and quantity of diet is known to affect the avian intestine in various ways.

a) The nature of the foodstuff may cause the gastric pH to vary (Farner, 1943), this could affect the efficiency of peptic activity.

b) In the Domestic Fowl, the rate of gastric secretion is affected by the quality of the food (Farner, 1960).

c) In herbivorous birds, the thickness of the musculature of the gizzard isfairly readily modifiable as a direct function of the hardness of the food (Farner, 1960).

d) In the Domestic Fowl, starvation reduces the rate of bile secretion (Schmidt & Ivy, 1937).

Any of these factors could account for the variation in susceptibility to infection between cockle-fed ducklings and those fed on a normal diet. The weakened condition of the birds in Experiments 1 and 2 may also have made them generally less resistant to infection.

In healthy ducklings, the function of the gizzard is two-fold.

1. It is a very powerful organ which aids in the mechanical digestion of food by grinding.

2. It is a site of preliminary acid proteolytic digestion.

The metacercariae of M. minutus are relatively unprotected and the gizzard is a potentially hostile environment. This could result in a very high mortality rate and account for the low infection in healthy animals. If starvation or variation in diet affects either or both of these functions adversely, the mortality rate in the gizzard of such ducklings may be less.

The presence of undeveloped metacercariae in the small intestines of healthy ducklings (Experiment 3) on the second or third day of infection is rather difficult to explain. Normally, metacercariae begin to lose their excretory granules as soon as they are freed from host tissue and transferred to a warm environment (e.g. saline at 38oC). This implies that the metacercariae that were recovered from the intestines of these ducklings had only just been released from the surrounding cockle tissue. It is unlikely, however, that this process would take 2 or 3 days. Such metacercariae were never recovered from the intestines of ducklings in the other experiments.

b) Host specificity

It seems that the duck is rather a poor host for M. minutus. Although no figures are available for the percentage of dose which becomes established in the ileum of the oystercatcher, it is unlikely to be so low.

The basic morphology of the avian intestine is modified and adapted according to feeding habits and diet. There are various general differences in the intestines of carnivorous and herbivorous birds. Since the oystercatcher is exclusively carnivorous and the duck is generally herbivorous, these differences could account for the low infection in the duck. According to Farner (1960), some of the major differences are as follows:-

1. In herbivores, the gizzard has a thick muscular wall and a well-developed, grinding koilin layer. In carnivores the gizzard is thin-walled and bag-like and, in those birds which feed on soft foods, the koilin layer is thin.

2. The morphology of the villi of the ileum varies - in herbivores they are leaf-like, in carnivores they are finger-like.

3. In carnivores the ileum is shorter and the food is retained for a shorter period compared to herbivores.

Smyth & Haslewood (1963) point out that the chemical composition of bile varies greatly between different animal species and that it may represent one of the most variable factors in host intestinal physiology. They record that there is a tendency for the bile salts of herbivorous animals to be conjugated with glycine and those of carnivorous animals to be conjugated with taurine.

There are probably a wide variety of physical, physiological and biochemical differences between the guts of Khaki-Campbell ducks and oystercatchers which makes the latter a good host for M. minutus and the former a poor one.

c) Elimination of worm burden

In the preliminary experiments and in those reported here, the worm burden was shed between days 3 and 4 post-infection irrespective of diet. Bowers & James (1967) failed to notice this since they examined all their experimental ducklings after 3 days. It is possible that (for the various reasons outlined above) the flukes never really became established in the small intestine of the ducklings. However, their elimination could also be due to some cell-mediated, non-specific host response. Further research on this subject was beyond the scope of this project.

d) Development of the metacercaria

There was no growth or body-shaping during the development of the metacercaria to the adult. Apart from the loss of excretory granules during the first day of infection, the main changes affected the reproductive system. The associated glands e.g. the vitellaria and the prostate gland began to produce secretions, and the testes and ovary presumably completed the final stages of spermatogenesis and oogenesis prior to sperm and egg production.

It was apparent that there were various discrepancies in the description of the morphology of the adult between the present study and that of Bowers & James (1967). These will be discussed in the next section.

e) Comparison of M. minutus and Gymnophallus fossarum

The classification of the Gymnophallidae has been reviewed by Stunkard & Uzmann (1958), James (1964) and Loos-Frank (1971), however, there is some dispute as to the validity of the genus Meiogymnophallus (Loos-Frank, 1971).

Gymnophallus fossarum is similar in many respects to M. minutus; Bartoli (1972, 1973, 1974) has described the life-cycle and development of this closely related species. The metacercaria are indistinguishable but the adults show various morphological differences.(see Table 4I: 1). The life-cycle of the two species are virtually identical. According to Bartoli (1972, 1973) the most important differences between them are as follows:-

1. The vesicula seminalis - this is undivided in M. minutus but bipartite in G. fossarum.

2. The shape and size of the prostate gland - that of M. minutus is tubular and smaller than the ventral sucker, that of G. fossarum is oval and larger.

3. The vitellaria - these are compact and spherical in M. minutus whereas they are voluminous and almost touching one another in G. fossarum.

4. The genitalia have different dimensions - those of M. minutus are smaller.

5. The second intermediate host of M. minutus is Cardium edule, not Cardium glaucum. In Provence in France, the metacercariae of G. fossarum are found in C. glaucum - C. edule does not occur in this area.

6. The metacercariae of M. minutus are only found under the hinge of the cockle and are always enclosed in a proliferation of the pallial line tissue. The metacercariae of G. fossarum

are mainly found along the edge of the valves between the shell and the mantle and also to a lesser extent under the hinge, they are not enclosed in the pallial line tissue.

Although the ecological differences between the two species are indisputable, the morphological differences are not so significant. Loos-Frank (1971) states that - contary to the observations of Bowers & James, (1967) - the vesicula seminalis of M. minutus is bi-partite; the same observations were also made in this study. Bowers & James (loc. cit.) describe the vitellaria of M. minutus as compact and spherical, I have found them to be much more similar to the voluminous, Y-shaped vietllaria of G. fossarum. In this study, the dimensions of the genitalia of M. minutus were larger than those recorded by Bowers & James (see Table 4I: 1) and they are more like those of G. fossarum. The prostate gland is not tubular in M. minutus (in the description of the adult by Bowers & James (loc. cit.) the term "pars prostatica" is confused with the term "prostate gland "). Taking all these considerations into account, there are now no important morphological differences between the adults of the two species.

Bartoli (personal communication) believes that M. minutus and G. fossarum are two species in the process of speciation and that they are already distinctly separate from each other. He also suggests that the purely morphological concept of species should be abandoned in favour of a biological concept.

Conclusions

1. Cockle-fed ducklings are better hosts for the adults of M. minutus than ducklings fed on a normal diet, although the maximum infection expressed as a percentage of dose was only 5%.

2. Chicks, suckling mice and rats, young and adult mice will not act as laboratory hosts for the adults of M. minutus.

3. In ducklings, the infection is eliminated after 3 days, irrespective of age.

4. During their development to adults, the metacercariae of M. minutus do not increase in size. Their already preformed genitalia complete their development - sperm is produced on Day 2 and eggs on Day 3 post-infection - and the associated glands (vitellaria, prostate) begin to produce their secretions.

5. Various differences in the shape or size of certain organs (vitellaria, vesicula seminalis, receptaculum, ovary, testes) were seen in the present study when compared with the observations of Bowers & James (1967).

6. Gymnophallus fossarum and M. minutus are very closely related species whose only real difference appears to be the distribution of the metacercariae within the cockle and the species of cockle which they parasitize.

PART II: DEVELOPMENT IN VITRO

Introduction and Methods

The techniques used for the recovery and cultivation of the metacercariae of M. minutus have been described in the General Methods.

The cultures were examined daily under an inverted microscope in a warm chamber (38°C). A record was kept of the flukes' mobility, development and general appearance. Flukes were occasionally removed from culture during the course of the experiment to examine the development of the genitalia; they were examined eitner alive and slightly flattened or stained in aceto-orcein. Permanent preparations were made by fixing in buffered formalin or 70% ethanol and staining in Gowers Carmine. The presence of egg-shell precursors in the vitellaria was examined by treating with Fast Red Salt B or with Catechol (Bell & Smyth, 1958).

Because the metacercariae came from a non-sterile environment, the problem of de-contamination prior to cultivation was particularly difficult. Great care was taken to exclude host tissue and to perform the preliminary washes thoroughly - tetracycline was always used in the initial washes. In spite of these precautions, many cultures became contaminated. However, it was discovered that metacercariae freed from cockle tissue could be stored for 2-3 days in Hanks saline at 4°C. If the test culture became contaminated the stored metacercariae could be washed again and the process repeated once more. This reduced the wastage of expensive media and culture constituents.

If a culture contained many hyperparasitized metacercariae, then, very shortly, many of the developing flukes appeared to harbour spores. This was probably not due to contamination of uninfected flukes with spores from the hyperparasitized flukes since the appearance of spores in previously uninfected metacercariae was so rapid. A more likely explanation is that, when a cockle contains hyperparasitized metacercariae, many of the apparently normal flukes in that cockle will contain the earlier developmental stages (schizonts, sporonts and sporoblasts) of the microsporidian. The higher temperature of the culture media may cause the pre-spore stages to complete their development more rapidly. For this reason, if a cockle contained obviously hyperparasitized metacercariae, none of the flukes from that cockle were used for <u>in vitro</u> culture.

Since the treatment of the metacercariae prior to cultivation was quite different to the conditions they would normally encounter in the definitive host's digestive tract, an experiment was designed to determine the effects of pre-treatment with enzymes. Pieces of pallial line tissue containing metacercariae were incubated in:

a) 0.05% pepsin in Hanks at pH2.0 for 1 hour at 40°C;

b) 0.05% trypsin in Hanks at pH7.0 for 1 hour at 40°C;

c) 0.05% pepsin for ½ hour, then 0.05% trypsin for ½ hour at 40°C;

d) Hanks saline at 40°C for 1 hour.

Any flukes which werefreed from the pallial line tissue during this treatment were removed and prepared for cultivation in the usual way.

Results

The results have been tabulated (Tables 4II: 1-5) into groups of related experiments, they are not necessarily presented in the order in which they were performed. If the culture became contaminated with bacteria or fungus, the results were not included in the tables.

Except where otherwise stated, the flukes were cultured in approximately 8 mls. of media in plastic tubes or Leighton tubes (capacity 15 mls.) which were kept at a temperature of 39-40°C. in a shaking water bath. The media was gassed with air or the aerobic gas mixture (N_2, O_2, CO_2). If the cultures were kept for longer than 7 or 8 days, the media was changed. In those experiments which also contained monolayer cells, the flukes were cultured in 5-7 mls. of media in round Falcon flasks which were stored on their side at 37-38°C. in an incubator.

The duration of the experiment is recorded in days and the symbols used to describe why a culture was terminated are the same as those employed for F. hepatica in Chapter 3, Part II.

Except where otherwise stated, the development of the metacercariae conformed to the optimum development described below.

Development of the metacercariae in vitro

Day 1

The digestive caeca contained large spherical globules. The vitellaria were not observed; they were negative for egg-shell precursors when treated with Fast Red Salt B or with Catechol. In most cases, the prostate gland contained irregular,

globular secretions and was easily recognisable. Spermatids but not sperm were seen in the testes. The vesicula seminalis and receptaculum were empty.

Day 2

Secretions were clearly seen in the vitellaria and the vitelline reservoir and they were weakly positive for egg-shell precursors. Sperm were seen in the testes and in some cases in the vesicula seminalis.

Day 3

Sperm were seen in the testes and vesicula seminalis but not in the receptaculum. The vitellaria and vitelline reservoir were straw coloured and a few granular clumps of orange material were seen in the uterus in the anterior part of the fluke. This material was strongly positive for phenols (staining red) and was termed "egg-shell material" (esm.).

Day 4

By Day 4, the colour of the secretions in the vitellaria and vitelline reservoir had become darker. The clumps of egg-shell material in the anterior part of the fluke had increased. In many cases, they were roughly oval and approximately 0.03mm. long (true eggs are 0.016-0.024mm. long, Bowers & James 1967). They often appeared in columns (Text figure 8A) but in many cases, the uterus was seen to be filled with an amorphous mass of material (Text figure 8B). Hyper-parasitized flukes filled with spores did not contain egg-shell material or sperm; they usually died after 3-4 days in culture. No free ova were ever seen moving in the uterus. The ovary was never clearly seen either in live flukes or in those stained in aceto-orcein. Sperm were seen in the vesicula seminalis

and occasionally in the testes; the receptaculum was never seen. Where present, the testes often seemed contracted and empty of sperm - in some cases, they were not observed at all.

The digestive caeca were packed with large spherical granules - many more than on Day 1.

Days 5-8

There was a small increase in the amount of egg-shell material in the uterus during this period but the vitellaria and vitelline reservoir tended to lose their contents and they became only weakly positive for egg-shell precursors. "Oviposition" of these clumps of egg-shell material probably did not occur in vitro. No normal eggs were ever observed, either shelled or unshelled. As cultivation progressed, no further sperm were seen in the testes although the amount of sperm in the vesicula seminalis did not appear to diminish.

Longevity

The flukes appeared clear and active up to Day 8 in culture. By Day 12 only 20% of the flukes were alive and by Day 16 most of them were dead.

Pretreatment with enzymes

a) Pepsin

In Pepsin, the cockle tissue began to be digested almost immediately. The metacercariae were very active within the host tissue and a large proportion freed themselves from it. However, the freed metacercariae were very sluggish and were obviously affected by the low pH of the media. When these metacercariae were transferred to culture, the majority died within the first day and they were all dead by Day 4 with no development.

b) Trypsin

Trypsin appeared to have some effect on the cockle tissue although digestion was not as rapid or extensive as in the Pepsin solution. The metacercariae were very active inside the host tissue and many freed themselves. At this pH (approximately pH 7.0) the freed metacercariae remained very active. In culture, they remained active and clear but did not exceed the optimum development described previously.

c) Pepsin then Trypsin

Some metacercariae were freed in the Pepsin solution and further metacercariae were freed when treated with Trypsin. In culture, some of the flukes became dark and sluggish and died after 4 days, others remained active and clear and achieved the optimum development.

d) Hanks saline

The metacercariae became active inside the cockle tissue and several freed themselves from it. In culture, these flukes remained clear and active and achieved the optimum development.

Table 4II:1 Defined media and sera

Exp. No.	Media	Duration	Results
7	Hanks saline a) without glucose b) with glucose	4(D) 4(D)	Became dark and sluggish. Some spermatogenesis.
1,2,4	858 + 20% foetal calf serum (FCS) a) basic medium b) with added glucose (1g./litre)	9(T) 9(T)	Optimum development.
10	NCTC + Chick serum (ChS) - Batch A a) 10% serum b) 20% c) 30% d) 40% e) 50%	10(D) 14(D) 14(D) 16(D) 16(D)	Optimum development. Most esm. in uterus observed in 40% Ch.S.

Table 4II: 1 (continued)

Exp. No.	Media	Duration	Results
40b	Chick serum alone-Batch A	8(D)	Became dark and sluggish but achieved optimum development.
40a.	NCTC + 40% Ch.S. - Batch B	8(T)	Optimum development. (As Batch A).
27	NCTC + 40% Ch.S. - Batch A		
	a) serum untreated	8(T)	) No difference.
	b) serum inactivated	8(T)	) Optimum development in both.
33	NCTC + 40% Duck serum (inactivated)	6(D)	Became dark and sluggish. Only small amounts of esm. in uterus.

Table 4II: 2 Diphasic Media

Exp. No.	Media	Solid phase	Duration	Results
29	NCTC + 40% Ch.S.	a)coag.calf serum + holes	3(D)	Dead worms in burrows. No development.
	"	b)coag.calf serum in pieces	4(D)	Became dark and sluggish. Small amounts of esm.
	"	c)coag.calf serum macerated	4(D)	Optimum development. Large amounts of esm.
12, 32a)	"	a)Coag.Ch.S., with + w/O holes (small Falcon flasks)	6(D)	) Worms burrow into) base and die there.) No development.
	"	b)Coag.Ch.S., with + w/O holes (Leighton tubes)	6(D)	) ")
38b)	"	Coagulated Chick serum	8(T)	Optimum development. Burrowing in larger pieces.

Table 4II: 2 (continued)

Exp. No.	Media	Solid Phase	Duration	Results
38	NCTC + 40% Ch.S.	Glass beads (0.7mm. diameter)	8(D)	Many dead by Day 2. Small amounts of esm.
24	"	Artificial gut (nylon fur)	7(D)	V. dark and sluggish throughout. Only small amounts of esm.

Table 4II: 3 Feeding Experiments

Exp. No.	Media	Food Material	Duration	Results
30b	NCTC + 40% ChS.	Chick red blood cells	7(T)	Optimum development. Probably feeding.
20a	"	Sheep red blood cells	6(D)	Not feeding. Dark, sluggish. Small amounts esm.
31	NCTC + 20% FCS	a) Epithelial cells (BHK)	7(T)	Very active) Optimum development.
		b) Embryo cells (Chick)	7(D)	Not very active) Probably feeding.
21	NCTC + 40% ChS.	a) 2 drops egg macerate	6(T)	Optimum development.
		b) egg macerate only	9(T)	No live flukes recovered.
29c	NCTC + 40% ChS.	Macerated coagulated calf serum.	8(D)	Optimum development. Large amounts esm.

Table 4II: 4 Various Additives

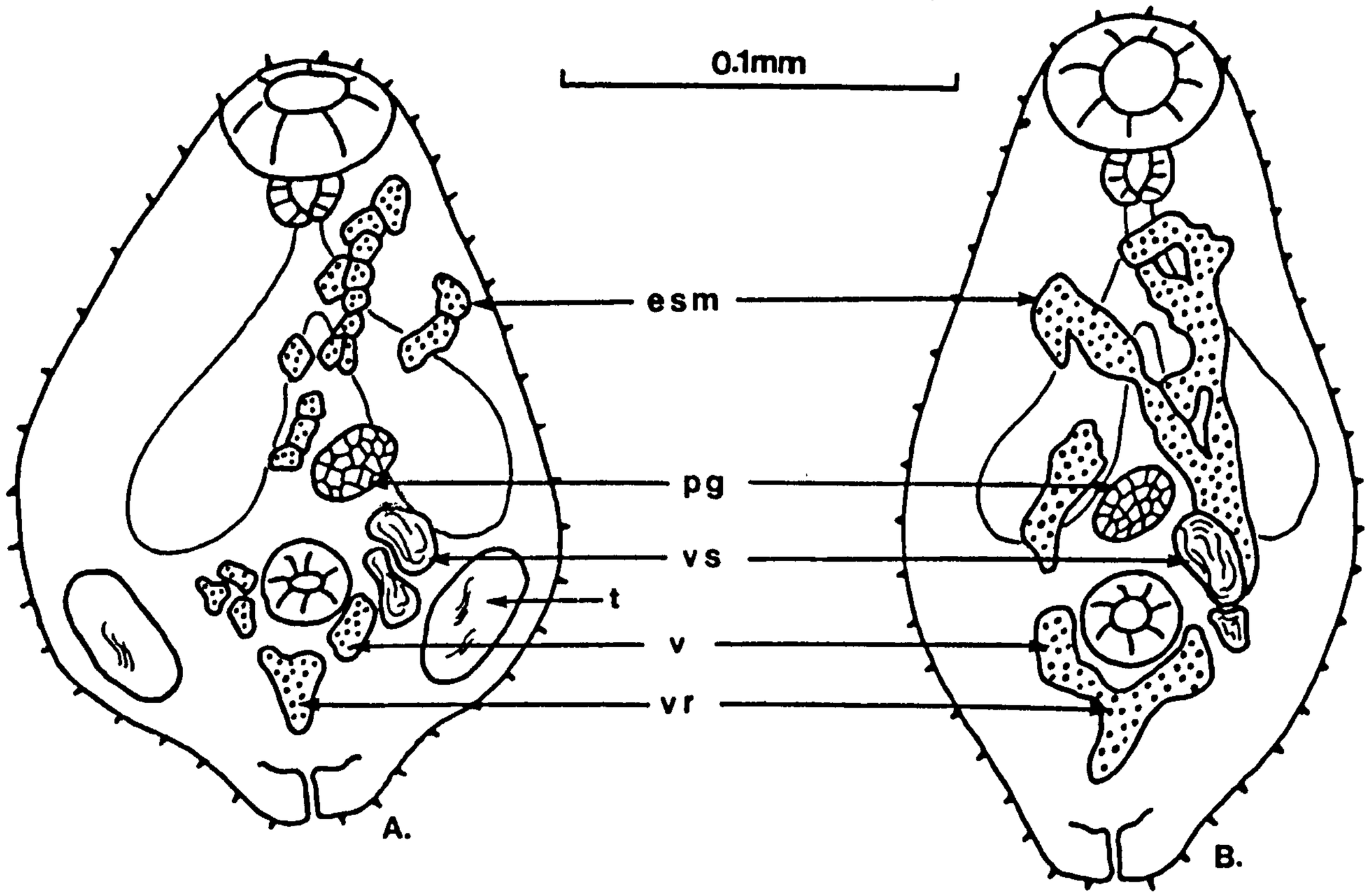
Exp. No.	Media	Additives	Duration	Results
13	NCTC +40% Ch.S	10% CEE_{50} -Batch A	8(T)	Optimum development. Large amounts esm.
23b	"	10% CEE_{50} -Batch B	6(D)	Dark and sluggish. Very little esm.
23a	"	10% crude, prepared CEE_{50}	6(T)	Became rather dark, but produced small amounts esm.
19	"	10% Sheep liver extract	7(D)	V. dark, not v. active but optimum development.
11	"	0.02% Duck bile	6(D)	Very dark and sluggish.. No development.
35a	None	NCTC - y	6(D)	} Not very active, deformed.
35b	None	NCTC - y + 40%ChS	6(D)	} Only very small amounts esm.

Table 4II: 4 (Continued)

Exp. No.	Media	Additive	Duration	Results
30a	NCTC + 40% ChS	0.5% Yeast extract	5(D)	Dark, sluggish. Small amounts esm.
14	"	a) 0.2% Vitamins	6(T)	} Optimum
	"	b) 0.2% Amino acids	6(T)	) development.
39	"	a) Insulin (10^{-4}mg./ml.)	8(D)	} Dark and sluggish.
	"	b) Seratonin (10^{-4}mg./ml.)	8(D)	) No development.
	"	c) Oestrogen (10^{-4}mg./ml.)	8(T)	Optimum development. Large amounts esm.

Table 4II: 5 Physical Conditions

Exp. No.	Media	Conditions	Duration	Results
24	NCTC + 40% ChS	a) Small Falcon flasks	7(D)	Became dark and sluggish.
	"	b) Sterelin vials	7(D)	Very little development.
	"	c) Leighton tubes	7(T)	Optimum development.
3	"	a) Shaking	8(T)	Optimum development.
	"	b) Stationary	8(T)	Culture (a) more active.
22	"	a) Aerobic gas (N_2,CO_2,O_2)	8(T)	No difference.
	"	b) Anaerobic gas (N_2,CO_2)	8(T)	Optimum development.
	"	c) Air	8(T)	
34, 36	"	Cellulose Tubing Apparatus	6(T)	All dead when examined.



Text Figure 8. Meiogymnophallus minutus: Cultured flukes showing the distribution of tanned egg-shell material (esm).

Key.

esm - eggshell material
pg - prostate gland
t - testes
v - vitellaria
vr - vitelline reservoir
vs - vesicula seminalis

Discussion

Development in culture

The early development of the metacercariae of *M. minutus* *in vitro* was similar to that observed *in vivo* in the duck. During the first few days in culture, the prostate gland and the vitellaria began to produce their secretions although the vitellaria became positive for egg-shell precursors slightly later than *in vivo*. On the first day *in vitro* and *in vivo* spermatids were seen in the testes and by the second day they had developed to spermatozoa which were occasionally seen stored in the vesicula seminalis. However, subsequent development *in vitro* became abnormal.

Ova were never seen in the oviduct or ootype of cultured flukes and there were no normal, shelled eggs in the uterus. It is likely that the ovary degenerated during cultivation since it was never seen in live, flattened specimens or in those stained in aceto-orcein.

On the third day in culture the material packing the vitellaria and vitelline reservoir appeared straw coloured and during the next few days it darkened until it was orange or brown. At this time similar dumps of material were also seen in the uterus. These dumps were often roughly oval in outline and were similar in size to normal eggs, they were probably moulded by the ootype. They were dense and granular, however, and there was no suggestion that they enclosed an ovum.

In most trematodes studied, some form of tanning process occurs and the egg-shell is composed of a sclerotin-type protein. In many species, it appears that quinone tanning occurs but in others where phenolase activity cannot be demonstrated, some other mechanism for protein hardening must occur. In M. minutus the precursors of the quinone tanning system can be demonstrated histochemically using Fast Red Salt B to test for the presence of phenols and Catechol to test for polyphenol oxidase (phenolase). The process of quinone tanning has been described in detail by Smyth (1966). Phenol is enzymically converted to quinone by polyphenol oxidase in the presence of oxygen. This quinone then cross-links with the free NH_2 groups of protein chains to produce a "tanned" protein or sclerotin. The process may, however, be more complex than it appears (Ramalingam, 1973).

In cultured flukes, it is likely that the vitelline secretions become tanned prematurely whilst still in the vitellaria and the vitelline reservoir. A possible regulator in the quinone tanning process is oxygen since it is only in the presence of molecular oxygen that phenolase will convert phenol to quinone. The precise availability of oxygen in vivo may ensure that tanning of the vitelline secretions occurs at the right time and in the right place. Crompton et al (1965) have measured the oxygen tension within the intestine of the duck. They have shown that it approaches zero in the lumen of the gut but that it is substantially higher in the reigon of the mucosa - the highest tension recorded was equivalent to 3.3% oxygen. If the culture media is gassed with air or with the aerobic mixture of

gasses (15% O_2) the amount of oxygen available may be excessive and may cause this premature tanning within the vitellaria. However, contrary to expectation, gassing with an anaerobic mixture (N_2,CO_2) did not prevent premature tanning. Clegg (1957) records that, when Fasciola hepatica adults were maintained in vitro under aerobic conditions, the shell globules tan and the vitellaria become dark brown.

It is unlikely that this premature tanning of the vitelline secretions was the main reason why normal, shelled eggs were not produced since the most vital constituent - the ova - appeared to be missing.

After the initial production of vitelline secretions no further secretions appeared to be produced and by Day 6 the vitellaria were usually empty. This was also true of sperm; after the initial production of sperm, which were stored in the vesicula seminalis, the testes shrank and probably degenerated. It is likely that the precursors of spermatogenesis and vitellogenesis were present in the metacercariae and that, once they had been used, no further precursors were produced. This could be because the culture conditions did not provide sufficient energy for their production or because certain vital components were missing from the media.

It is unlikely that cross- or self-insemination occurred in vitro, no sperm were ever seen in the receptaculum or anywhere in the uterus. In an attempt to obtain fertilization in vitro various conditions were devised in which the flukes were compressed - this has been shown to be necessary for insemination and fertilization

in the intestinal cestodes Schistocephalus solidus (Smyth, 1954a) and Ligula intestinalis (Smyth, 1947) in culture. The various techniques that were employed are discussed later but none were successful.

Longevity

The longevity of M. minutus in vivo is not known since, in the only suitable laboratory host, the duckling, the adults are eliminated after three days. It is unlikely that this also happens in the oystercatcher. Relatively little work has been done on the longevity of digenean adults although it is known that the adults of Schistosoma and Fasciola can survive for many months in vivo. James (1971) has demonstrated that Microphallus pygmaeus only survives for 9-13 days in the intestine of experimental mice. It is quite possible that progenetic intestinal digenea have only a short life span in their natural hosts. If this were so, a maximum survival of 16 days in vitro would not be abnormal.

Pretreatment of metacercariae with enzymes

Pretreatment with enzymes had no beneficial effect on subsequent development in vitro. On the contrary, exposure to pepsin at a low pH seemed to damage the flukes irreversibly. It is likely that the elevation of temperature to about 40°C was mainly responsible for activating the metacercariae within the cockle tissue and that, once they were activated, they could wriggle out.

Discussion of results

Table 4II:1 Defined media and sera

Bartoli (1965) reported that the metacercariae of Gymnophallus fossarum showed some spermatogenesis when they were cultured in sea-water or Ringers saline at 41°C but that they died after 2-3 days in culture. A similar situation was observed when the metacercariae of M. minutus were cultured in Hanks saline.

The best development in vitro was obtained in a complex media such as Medium 858 or NCTC 135 enriched with serum - either foetal calf or chick serum. In order to standardise the results a basic medium of NCTC plus 40% chick serum was generally employed. The two batches of chick serum which had such different growth-promoting properties on F. hepatica showed no difference when they were used in the cultivation of M. minutus. Inactivation of the serum (to inhibit complement activity) was not beneficial, neither was it inhibitory.

Duck serum appeared to be toxic. However, the duck from which this serum was collected, had previously been infected with Raillietina cesticillus, and may have possessed non-specific antibodies against helminths.

Table 4II:2 Diphasic Media

Although diphasic media has been very successful in the cultivation of the cestode Echinococcus granulosus (Smyth, 1967), it was not useful in the cultivation of the metacercariae of M. minutus. The behaviour of these flukes in a diphasic medium with a solid base was similar to that described for F. hepatica (see Chapter 3, part II),

Diplostomum phoxini (Kannagara & Smyth, 1974) and Paragonimus Westermani (Kannangara, 1974). The metacercariae burrowed into the base and apparently became trapped there and die.

Various other diphasic media (e.g. artificial gut, glass beads, pieces of coagulated serum) were used in an attempt to compress the fluke during development and so obtain insemination, but none of these were successful.

Table 4II: 3 Feeding Experiments

In vivo the developing adults of M. minutus presumably feed on the epithelial cells of the intestinal villi. Various solid and semi-solid food materials were examined in vitro (epithelial and embryonic cells in monolayer, red blood cells, egg macerate and macerated coagulated serum) and although the flukes often appeared to injest the food material it did not enhance their longevity or development in vitro.

Table 4II: 4 Various additives

Many of the additives used in the cultivation of M. minutus have also been tested on F. hepatica metacercariae and they are discussed in Chapter 3, part II.

The two different batches of chick embryo extract that were used to culture M. minutus produced different results: when Batch A was used optimum development was achieved and large quantities of egg-shell material were produced whereas when Batch B was used the flukes became dark and sluggish and their development was retarded.

This batch difference was also observed in the cultivation of F. hepatica. The crude, prepared chick embryo extract also inhibited development and the flukes became dark and sluggish.

Duck bile was found to retard development in vitro. Unfortunately oystercatcher bile was not available. Bile is known to act as a 'trigger' or stimulus in the excystment of some metacercariae (e.g. F. hepatica, Paragonimus westermani)and it may playan important role in the establishment and development of intestinal helminths.

When sheep liver extract was added to the basic culture media, the flukes became dark and were only moderately active although they achieved optimum development. A similar result was obtained when sheep liver extract was used to culture F. hepatica.

Oestrogen was added to the media in an attempt to enhance oogenesis. The effect of the host's reproductive cycle on the development of various helminth parasites has been discussed previously (Chapter 3, part II); it is probably correlated with the production of sex hormones by the host. Although oestrogen failed to stimulate oogenesis in vitro it apparently enhanced vitellogenesis.

The addition of extra vitamins or amino acids to the culture media did not affect development but the addition of yeast extract, Berntzen & Macy's (1969) yolk medium and the hormones seratonin and insulin appeared to be detrimental.

Table 4II: 5 Physical Conditions

The presence or absence of oxygen in the gas phase had no visible effect on the cultured flukes. The question of whether trematodes are aerobes or anaerobes has been discussed in Chapter 3, part II.

The use of small Falcon flasks or Sterelin vials rather than Leighton tubes was found to be disadvantageous; a similar observation was made in the cultivation of F. hepatica. When Falcon flasks were used, the flukes were scattered over the base of the vessel, in the Sterelin vials they were concentrated together in the conical base and in the Leighton tubes they were in close proximity to each other but were not packed together.

Although the media in these different vessels was the same the flukes' micro-environment obviously varied considerably and this clearly had a significant effect on their development.

The cellulose tubing apparatus that was so successful for the in vitro cultivation and insemination of S. solidus and L. intestinalis (Smyth, 1947, 1954a) was of no value in the cultivation of M. minutus. The metacercariae probably died quite early on in the experiment since when the cellulose tubing was examined after 6 days many of the dead flukes still possessed excretory granules.

The effects of shaking on the development of M. minutus in vitro was not marked although slightly more egg-shell material was produced. Shaking probably prevents the accumulation of excreted metabolites in the vicinity of the flukes.

General Comments

The adults of M. minutus are apparently very host specific and it seems unlikely that this is merely determined by feeding behaviour. However, the specificity may be indirectly linked with the food material since the guts of birds with a similar diet will have similar modification of the digestive tract. It is likely that the metacercariae of M. minutus require a very precise physical and chemical environment in order to develop to maturity. It is usually relatively simple to culture metacercariae with such well developed genital organs, but this is not the case with M. minutus. It would have been very interesting to study the effects of oyster-catcher bile in vitro, however, it was not available. James (1971) correlates the degree of host specificity with ease of cultivation. Speaking generally, he suggests that where the adult stage is relatively host specific it is more difficult to culture them in vitro.

Conclusions

1. During the first few days in culture, the vitellaria, prostate gland and testes of M. minutus all apparently developed normally. Thereafter, development became abnormal.

2. No normal eggs were produced in vitro; the ovary probably degenerated. The addition of oestrogen or egg derivatives to the culture media did not encourage oogenesis.

3. There was a premature tanning of the vitelline secretions in vitro and clumps of tanned material were seen in the uterus. It is unlikely that this was due to excessive oxygen in the media.

4. Normal production of sperm and vitelline secretions probably ceased by Day 6.

5. It is unlikely that copulation or insemination took place in vitro.

6. Pretreatment with digestive enzymes did not improve the subsequent development of the metacercariae in vitro.

7. A basic medium of NCTC 135 or Parker 858 plus serum at 38°C in plastic or Leighton tubes produced the best results. This development was not improved on by the use of diphasic media or various additives and foodstuffs.

8. Flukes survived for a maximum of 16 days in culture. It is likely that longevity *in vivo* may also be quite short.

9. The adult stage of *M. minutus* is very host specific and probably requires very precise conditions for completely normal development *in vitro*.

PART III: ULTRASTRUCTURE IN VIVO

Introduction and Methods

To the knowledge of the present author there are no published descriptions of the ultrastructure of Gymnophallid metacercariae or adults. In the present study, the development of the various organ systems of the metacercaria of Meiogymnophallus minutus has been studied using the transmission electron microscope. Particular attention was paid to the tegument, digestive caeca and reproductive system. The external morphology of adult M. minutus was studied using the scanning electron microscope. It was not possible to study the surface features of the metacercaria since it was covered by a thick surface glycocalyx.

The study of the normal development and ultrastructure of the metacercarial and adult stages of M. minutus was undertaken in order to provide a comparison with the ultrastructure of in vitro cultured flukes.

The general techniques involved in the preparation of flukes for scanning and transmission electron microscopy have been described in Chapter 2: General Methods. The special techniques required for the preparation of the metacercariae of M. minutus have also been described.

The developmental stages examined for transmission electron microscopy (Section 1) were:-

1. Metacercariae (in situ in cockle tissue).
2. Developing metacercariae recovered on Day 1 post-infection from ducklings.
3. Adults recovered from ducklings on Day 3 post-infection and from oystercatchers.

Adults recovered from oystercatchers were also examined under the scanning electron microscope (Section 2).

SECTION 1: TRANSMISSION ELECTRON MICROSCOPY (TEM)

Results

The Tegument

A. METACERCARIA (Plates 83, 84, 89)

The basic structure of the tegument of the metacercariae and adults of Meiogymnophallus minutus is similar to that described for the adults of F. hepatica by Threadgold (1963 a,b). It consists of a surface syncytium connected by protoplasmic strands to the cell bodies which lie below the peripheral musculature.

The surface syncytium of the metacercaria was covered by a thick, fibrous glycocalyx 2.5-3.0um. in depth. The underlying syncytium contained spines, granules and mitochondria. The outer surface of the syncytium was thrown up into irregular folds. The basal lamina was flat and often quite dense, there were relatively few invaginations. The underlying layer of interstital material was very thin and often appeared to be non-existent. The spines were present over the whole surface of the fluke and pointed posteriorly. The tips were rounded and, as in Microphallus similis (page 335), they had a fringed or "toothed" appearance (refer to Plate 95). The projecting part of the spine was enclosed by the surface membrane of the syncytium which ran closely around the "teeth". The spines possessed the typical crystalline sub-structure seen in other trematodes. The base of the spines was flat and usually lay in, and was attached to, a fold of the basement membrane (refer to Plate 94). In the metacercaria, the syncytium was approximately 1.8um. wide. Its ground substance was finely granular and moderately electron dense. Mitochondria were scattered throughout the syncytium and they possessed 2-4 cristae depending on their size.

There appeared to be three types of morphologically distinct granules in the syncytium of the metacercaria(see Plate 83)

Type O

These spherical granules were membrane-bound and measured approximately 0.16um. in diameter. They were scattered throughout the syncytium and contained a small amount of granular material. The appearance of these granules varied but they often appeared virtually empty and were less dense than the surrounding syncytium.

Type 2

These granules were small and spherical or oval measuring approximately 0.8um. in diameter. They were membrane-bound and their dense heterogeneous contents were often contracted together in the centre of the granule. They were found scattered throughout the syncytium although they had a tendency to be localised in the apical parts of the syncytium. They were less frequent than the Type O or Type 3 granules.

Type 3

These biconcave granules were membrane-bound with dense contents. They measured approximately 0.16um. in length. They were mainly localised in the apical parts of the cell, particularly near the spines, and they were often arranged at right angles to the apical plasma membrane.

The tegumental cell bodies of the metacercaria contained a nucleus, mitochondria and free ribosomes but no GER or Golgi complexes; secretory granules were abundant and were of Type O only. Protoplasmic strands connecting the cell bodies to the syncytium were rarely observed.

Unicellular gland cells (refer to Plate 94) were found scattered amonst the tegumental cell bodies over the whole surface of the fluke. These gland cells were packed with oval or spherical secretory bodies (0.12-0.18um. in diameter) which were membrane-bound and contained a homogeneous, dense material. The cells

contained a nucleus but, apart from the secretory granules, they appeared to be devoid of any other organelles. Ducts from these cells led up between the peripheral muscle blocks and penetrated the syncytium. The ducts were lined with a ring of peripheral microtubules and, where they passed through the syncytium, they were connected to it by a desmosome. Similar gland cells were also seen in the forebody of Microphallus similis (Chapter 5, part III)

B. ADULTS (Plates 94, 95, 96)

In adult M. minutus the thick, fuzzy glycocalyx which covered the surface of the metacercaria was no longer present although the surface membrane of the syncytium was coated with a thin glycocalyx.

In addition to the Type 2 and Type 3 granules, that were present in the syncytium of the metacercaria, there was now another type of granule. This granule (Type 1) apparently replaced the Type 0 granule of the metacercaria and was similar to it except that its contents were moderately dense and homogeneous rather than sparse and granular. In all other respects, the syncytium of the adult was similar to that of the metacercaria.

The tegumental cell bodies contained free ribosomes, mitochondria, Golgi complexes and areas of GER especially around the nuclei (Plate 96). The secretory granules were of Type 1 and tegumental cells containing Type 2 or Type 3 granules were never seen.

The unicellular gland cells associated with the tegumental cells of the metacercaria were also present in the adult and they appeared to be identical with those previously described (Plate 94).

The Parenchymal Cells

A. METACERCARIA (Plates 85 & 86)

Parenchyma cells were not abundant in M. minutus. Where they were observed, their ground substance was granular although they did not appear to contain large stores of α glycogen. Organelles, apart from mitochondria and nuclei, were not seen. No junctional complexes, either with the cells of the digestive caeca or with other parenchyma cells, were ever observed.

B. ADULT

The parenchyma cells of the adult were infrequent and similar to those described for the metacercaria.

The Digestive Caeca

A. METACERCARIA (Plates 85 - 88)

Thé oesophagus was lined with tegumental syncytium which was similar to that covering the surface of the fluke except that it did not contain spines and there were many invaginations of the basal lamina (Plate 85). The syncytium lining the oesophagus was joined to the gastrodermal cells of the digestive caeca by a desmosome which ran from the luminal surface to the basement membrane. The surface of the oesophageal syncytium was smooth or gently undulating whereas that of the caecal cells was thrown up into long but fairly sparse lamellae. Adjacent gastrodermal cells were connected by a desmosome (Plate 86). The nuclei of the caecal cells were rarely seen indicating that the cells are prdably quite voluminous. The apical membrane and the surface lamellae were coated with a thin, fuzzy glycocalyx (Plate 87 & 88). The lumen of the gut and the oesophagus contained some granular material and some large droplets

measuring up to 8.0um. in length which resembled the lipid droplets seen in the excretory system (Plate 85). The cytoplasm of the gastrodermal cells contained GER, mitochondria, Golgi complexes, secretory granules and large lipid-like droplets. Areas of parallel GER were seen and they were often orientated parallel to the surface membrane (Plate 88) or to the plasmalemmas of adjacent cells. Vesicles with moderately dense contents were often seen budding off from the GER (Plate 88).

The caecal cells contained many mitochondria which were often concentrated together in thicker areas of cytoplasm. Golgi complexes were frequently seen, they had a dense content similar to that of the secretory granules (Plate 87). The secretory granules were spherical (approximately 0.25um. in diameter), membrane-bound and with a homogeneously dense content. They were scattered throughout the epithelium. Large lipid-like droplets were frequently seen in the cytoplasm and they were often seen in the process of extrusion into the lumen (Plate 86).

There were few basal invaginations of the basal plasma membrane (Plate 88) and no junctional complexes were seen with the parenchyma cells. The layer of interstitial material surrounding the caeca was very thin. Generally, the epithelium lining the digestive caeca was thin - measuring only 1.5-2.0um. in depth.

B. ADULT

Owing to the small size of the adult flukes and the difficulty in accurately determining the plane of section, the digestive caeca of the adult were never seen.

The Excretory System

A. METACERCARIA (Plates 89 & 90)

The most conspicuous feature of the metacercarial stage was the voluminous excretory bladder, the two arms of which occupied a large portion of the body (refer to Plate 80). In the metacercaria, the excretory bladder was packed with excretory granules. The cytoplasm of the excretory bladder was extremely thin, its luminal surface was thrown up into long, tenuous lamellae and less regular cytoplasmic projections. In certain places, the lining of the bladder was similar to the tegumental syncytium; it contained Type 0 and Type 2 granules but no Type 3 granules or spines (Plate 89, arrowed). Nuclei similar to those of the major descending ducts (MDD) and excretory bladder of F. hepatica (see Plate 27) were not observed in the cytoplasm lining the excretory bladder of M. minutus. Lateral cell membranes were never seen and it is likely that the bladder lining is syncytial rather than epithelial.

The decalcified excretory granules or concretions measured 0.5-1.0um. in diameter and consisted of two or three concentric rings of fibrous material (Plate 90). The inner one or two rings were often roughly spherical whereas the outer ring was usually undulating. The rings of material were connected by radially arranged fibrous strands. The concretions were often closely associated with the surface lamellae. The cytoplasm of the excretory bladder also contained small lipid droplets, about 1.0um. in diameter. They were not very frequent however, and they were never seen free in the lumen.

The collecting ducts and flame cells of the metacercaria were similar to those of the adult.

B. ADULT (Plates 97, 98 & 107)

The excretory granules present in the metacercaria were not seen in the excretory bladder of the adult. The cytoplasm lining the excretory bladder was extremely thin; it contained ribosomes, glycogen and occasional mitochondria. Tegumental-type granules, nuclei or cell junctions were never seen. The apical membrane was thrown up into long lamellae which were often pressed against the surface of the excretory bladder. Large lipid drops were frequently seen in the process of extrusion into the lumen. The excretory bladder was considerably less voluminous than in the metacercaria.

Sections through smaller excretory ducts were occasionally seen (Plate 97). These were presumably the collecting ducts which carried the excretory products from the flame cell to the excretory bladder. The cells of these ducts contained mitochondria, lipid droplets and some microtubules. The luminal surface was thrown up into many long lamellae and, in addition, there were up to six cilia with a typical "9+2" configuration and typical basal bodies. The surface of these cilia tended to be rather irregular. At the base of the lamellae, occasional possible micropinocytotic vesicles were seen (Plate 97, arrowed). Invaginations of the basal plasmalemma were frequent and extensive. It was uncertain whether the cytoplasm lining the collecting ducts was cellular or whether it was a syncytium.

The flame cells of M. minutus (Plate 98) were similar in morphology to those described for F. hepatica (Dermott, 1967; Wilson, 1969; Bennett, 1973). The "flame" consisted of a bundle of tightly packed cilia with the typical 9+2 arrangement of filaments. The cilial basal bodies were embedded in the body of the flame cell. The junction between the flame cell and the

cell enclosing the collecting duct consisted of a series of interdigitating microvilli. These microvilli were in close association with each other and there was a dense plaque within the microvilli in the area of the association. They were separated from each other by a band of dense material. Internal leptotriches arising from the flame cell projected into the lumen surrounding the flame. There appeared to be no external leptotriches as in the flame cells of the miracidium and metacercaria of F. hepatica (Wilson, loc.cit.; Bennett, loc.cit.). The flame cell of M. minutus was surrounded by a fairly thick layer of interstitial material.

The Female Reproductive System

A. METACERCARIA

Occasionally, sections were obtained which showed the gonads of the metacercaria. However, because the exact plane of section was unknown, it was not possible to say with any real certainty whether the gonads were ovary or testes. The gonad shown in Plate 84 contained several large irregular cells. The ground substance of these cells was granular and moderately dense, the only recognisable organelles were small, dense mitochondria and some ribosomes. The nuclei were large and oval. The ground substance of the nucleus was less dense than the surrounding cytoplasm, nucleoli were occasionally present but patches of heterochromatin were usually lacking. Groups of cells were often seen around the periphery of the gonad which were smaller and had smaller nuclei and a denser cytoplasm than the cells in the central regions. Some semi-circular nuclei were seen around the periphery of the gonad - they were probably the nuclei of the nurse cells. The cytoplasm of the "nurse cells" was granular and contained

small mitochondria.

The cells of Mehlis' gland and those of the vitellaria were not seen in the metacercaria.

B. ADULT

Mehlis' gland

The cells of Mehlis' gland were not observed during the present study.

Vitellaria (Plates 106 & 107)

Developing vitelline cells contained a nucleus with a prominent nucleolus and patches of heterochromatin. These cells contained an extensive GER system, many free ribosomes, Golgi complexes, occasional lipid droplets, rod-like mitochondria and clusters of secretory droplets. The secretory droplets consisted of spherical aggregates (up to lum. in diameter) of irregular, dense bodies. In the mature vitelline cells, the only recognisable organelle was the nucleus; the rest of the cell body was filled with secretory granules and lipid droplets. In these cells, the secretory bodies were no longer associated in spherical granules.

Ovary

No publishable micrographs were obtained of the ovary of the adult of M. minutus. However, sections through the ovary were occasionally seen. Its ultrastructure appeared to be similar to that described for F. hepatica by Björkman & Thorsell (1964a) and Gresson (1964). On the periphery of the uterus, close to the thick basement membrane, were the semicircular nuclei of cells which were probably nurse cells.

In the centre of the ovary were several large cells with irregular outlines and large nuclei. The cytoplasm of these cells contained elongated mitochondria, GER, ribosomes, lipid-like droplets and small, spherical, dense granules. The granules weremainly found around the periphery of the cells but some were scattered in the cytoplasm. On one occasion, an oocyte was seen entering the oviduct. The cytoplasm of this cell was dense and contained GER, free ribosomes and some mitochondria. Several large lipid-like droplets were seen in the cytoplasm and the small dense granules were now all located around the periphery of the cell. On several occasions, unshelled ova were seen in the uterus surrounded by sperm. The cytoplasm of these ova contained a prominent network of GER, free ribosomes and some mitochondria. The nucleus appeared irregular and was often bilobed, it contained dense patches of heterochromatin and no nucleolus. The nuclear membrane was apparently intact. The dense cortical granules and the lipid-like droplets were no longer present at this stage. Fertilised, but unshelled, ova were probably not seen. Shelled ova were badly preserved due to the inadequate penetration of the fixative.

The Male Reproductive System

A. METACERCARIA

(See "The female reproductive system", page 239.)

The cells of the prostate gland were not seen in the metacercarial stage.

B. ADULT

The Prostate Gland (Plates 104 & 105)

In the adult, the cells of the prostate gland were completely filled with irregular, dense secretory bodies measuring up to 0.4um. in diameter. It is uncertain whether or not these granules were membrane-bound. The ground substance of the cells between the secretory bodies was granular. The prostate gland cells protruded into the lumen (pars prostatica) of the gland which was usually virtually occluded. Long lamellae projected from the apical surface of the prostate gland cells into the lumen. Adjacent cells were connected at their luminal surface by a short desmosome which was approximately 0.2um. long (Plate 105). Secretions were never observed free in the lumen of the prostate gland.

Testes (Plates 100-103)

Various stages of spermatogenesis were seen in the testes of adult M. minutus. The central part of the testes contained large cells with large, oval nuclei (probably spermatocytes) and developing spermatids. Mature sperm were mainly found around the periphery of the testes. In the developing spermatids (Plates 100-103) the condensed nucleus, "centriole-like body", peripheral microtubules and the "9+1" axial filaments with their basal bodies and tapering striated rootlets could all be seen. The cytoplasm of the spermatids contained mitochondria, free ribosomes, ∝ glycogen and Golgi complexes. However, since the development and morphology of the spermatids of M. minutus were very similar to those of Microphallus similis, this subject is described and discussed in more detail in Chapter 5: part IV.

The mature sperm seen in the testes and vesicula seminalis (Plate 99) were typical of those Digenean spermatozoa that have been previously studied (see page 359). Cross sections through sperm from the vesicula seminalis revealed three types of morphology:

1. Two "9+1" axial filaments, a small mitochondrion, a nucleus and varying numbers of peripheral microtubules.
2. Two axial filaments, a mitochondrion and peripheral microtubules.
3. Two axial filaments and peripheral microtubules.

These three different morphologies probably represented sections through the sperm at different levels.

Sections through the vesicula seminalis were occasionally obtained which indicated that it was bilobed, i.e. it consisted of two separate parts.

Microsporidian Spores

A. METACERCARIA (Plates 91-93)

Metacercariae were occasionally seen whose body was entirely filled with the spores of the microsporidian Unikaryon legeri (refer to Plate 80). Prespore stages (sporonts and sporoblasts) were often seen embedded in the epithelium of the excretory bladder of what, otherwise, seemed to be uninfected metacercariae (Plate 93). In heavy infections, the spores were scattered throughout the body although they were particularly abundant in the sub-tegumental area. The ultrastructure of the spores has been described by Canning & Nicholas (1974); the coiled filament, bands of helically arranged ribosomes and the posterior membraneous area were all clearly visible. However, certain bodies were seen in the cytoplasm of the

metacercaria in the vicinity of the spores which were not observed by these authors (Plates 91 & 92). The first (Plate 91, "A") was an irregular, membraneous, convoluted body. This was usually the same size as the spores (about 2.0um.) or larger, and occurred randomly amonst the spores. The second type of body (Plate 91,"B") was frequently seen around the spores and occasionally around developing sporoblasts. They did not appear to be physically connected to the spore wall although their association was quite close. In longitudinal section, these bodies, which measured about 0.05um. in width and up to 0.4um. in length, appeared ladder-like except that the "rungs" were oval. In cross section, these bodies appeared as one or two concentric rings (Plate 92). It was not possible to say with any certainty whether these bodies were membraneous or whether they had a subunit structure since they were never clearly resolved and always appeared rather fuzzy.

B. ADULT

Spores were never seen in adult M. minutus from oyster-catchers or from ducklings. However, they were observed on two occasions in flukes recovered from ducklings one day post-infection. These flukes possessed secretory granules in their prostate and vitelline glands and there were developing spermatids in their testes.

Discussion

Previous work on the ultrastructure of the various organ systems of trematodes is reviewed in Chapter 5 in connection with the ultrastructure of M. similis; it is not discussed here unless it is directly relevant.

The Tegument

The basic structure of the tegument of M. minutus is similar to that described for F. hepatica by Threadgold (1963a,b).

The tegument of the metacercaria of M. minutus is coated by a thick fibrous glycocalyx which disappears once the fluke begins its development to adulthood. Lumsden (1975) points out that:-

> "Some metacercariae do not form cysts, but may bear a thick coating of mucopolysaccharide which could subserve some of the functions of the cyst walls formed by other trematodes."

He defines the function of these "encapsulating structures" as being protective,

a) in protecting the parasite from direct contact with the intermediate host's tissues (or, in the case of free-living metacercariae, the external environment);

b) in shielding the parasite from unfavourable conditions (e.g. gastric fluids) encountered during entry of the definitive host.

Different populations of secretory granules are probably present in the tegument of the metacercaria and the adult. In both the adult and the metacercaria of M. minutus the biconcave (Type 3) and the oval (Type 2) secretory granules were seen in the syncytium. Bennett(1973) has suggested that the biconcave granules in the tegument of F. hepatica may be collapsed spherical

granules whose contents have been secreted (either into the syncytium or onto the surface of the fluke). Erasmus (1967b) believes that the biconcave and the spherical granules represent different sections through the same disc-like secretory body.

Oval secretory granules similar to those seen in the syncytium of M. minutus were also present in the tegumental cell bodies and the syncytium of F. hepatica (Bennett, 1973) and in adult Microphallus similis (page 316). In M. minutus the oval (Type 2) granules were never observed in the tegumental cell bodies. Since the tegumental syncytium of trematodes does not possess the organelles involved in the secretory process (e.g. GER, ribosomes, Golgi complexes), it is unlikely that the oval granules were actually produced in the syncytium. It is possible that the oval granules may be modifications of the spherical-type granules which are known to be present in the tegumental cell bodies or that the tegumental cells which produce the oval granules are relatively infrequent.

In both the adult and the metacercaria, spherical secretory bodies were present in the syncytium and the tegumental cell bodies. However, those of the metacercaria were electron lucent (Type 0) whereas those of the adult were electron dense (Type 1). It is unlikely that the electron lucent granules seen in the syncytium of the metacercaria represented granules whose contents had been secreted, as in juvenile F. hepatica (Bennett, 1973), since the granules in the cell bodies were also similarly "empty". On one occasion, sections were obtained through an immature fluke recovered from an oystercatcher. It had lost its thick surface glycocalyx and excretory granules but the spherical secretory granules in the tegument were typical of the metacercaria rather

than the adult i.e. they were electron lucent rather than electron dense. The production of the denser spherical granules in the adult may have been associated with the development of a GER system in the tegumental cell bodies. In the metacercaria, the ribosomes in the tegumental cell bodies are not associated with membranes. However, it must be born in mind that the metacercadiae were not processed in exactly the same manner as the adults and that the process of decalcification may have had some effect on the appearance of the secretory granules.

The surface of the fluke was covered with rows of distinctive "t o othed" spines - they are discussed in more detail in the next section on scanning electron microscopy.

The unicellular gland cells associated with the tegument of the adults and metacercaria of M. minutus appear virtually identical with those on the forebody of Microphallus similis. The similarity between these gland cells and those of the lappets of certain strigeoids (Erasmus, 1969a, 1970) is discussed in Chapter 5, page 343. However, it must be noted that, although these gland cells show a remarkably similar ultrastructure, they are not necessarily similar in chemical composition or function. This is substantiated when the ultrastructure of the unicellular gland cells of M.minutus and Microphallus similis, that have been grown in vitro under virtually identical conditions, are compared (page 380).

The distribution of the unicellulr gland cells and the toothed spines on the body surface of both M. minutus and Microphallus similis in relation to their relative positions in the intestine of their deinitive hosts is discussed in Chapter 5, page 345. The various functions which these gland cells may fulfil is also discussed.

Parenchymal Cells

The parenchymal cells of the metacercaria of M. minutus do not apparently function as a significant glycogen store, as they do in Microphallus similis (page 317) and F. hepatica (Bennett, 1973; Bennett & Threadgold, 1973). However, the metacercariae of M. similis and F. hepatica are both enclosed within a cyst and so are presumably unable to take up nutrients across the tegument or the gut epithelium. Although the metacercaria of M. minutus is covered with a thick surface coat, this does not necessarily preclude the uptake of nutrients. The cells of the digestive caeca of the metacercaria possess secretory granules which may have a digestive function (this is discussed later). It seems possible, then, that uptake of nutrients may occur during the metacercarial stage and so the accumulation of large amounts of glycogen to use as an energy reserve may be unnecessary.

Junctional complexes with other parenchymal cells and organ systems are apparently absent in both the metacercarial and adult stages of M. minutus. Threadgold & Gallagher (1966) have suggested that junctional complexes are sites of transport of metabolites. Junctional complexes develop during the maturation of F. hepatica (Bennett, 1973) and their presence may reflect the increasing size of the fluke, the decreasing surface area to volume ratio and the resulting problems in diffusion and transport of metabolites. M. minutus is a very small trematode with a large surface area to volume ratio and a relatively small number of cells; such specialised transport sites may be unnecessary under these conditions.

The Digestive Caeca

The digestive caeca of M. minutus are cellular rather than syncytial and adjacent cells were connected by desmosomes. The oesophagus was lined with tegumental-type syncytium and, as in Microphallus similis (page 370), it was joined to the cells of the digestive caeca by a desmosome. The nuclei of the epithelial cells of the digestive caeca were rarely seen indicating that the cells were voluminous. The caecal cells of the metacercaria contained a GER system, apparently active Golgi complexes, dense secretory granules and surface lamellae. This indicates that, even in the metacercaria, the gut is already modified for the production of secretory material and, possibly, for the uptake of nutrients. The metacercaria is not immobile within the cockle tissue although it is not very active. It is quite possible that the metacercaria browses on the cells of the pallial line tissue and on the debris in the space which it occupies. Some debris was seen in the gut lumen of the metacercaria and this may represent undigested food material. Although M. minutus does not increase in size significantly during the metacercarial stage, there is considerable development of the gonads and this, of course, would require energy.

Unfortunately, due to technical difficulties, the ultrastructure of the digestive caeca of the adult was not observed. It would be of interest to determine:-

a) whether the GER/Golgi produced granules continue to be synthesized, and whether they increase or decrease in number;

b) whether the lipid-like secretion continues to be produced and in what quantity;

c) whether there is a proliferation of the surface lamellae;

d) whether junctional complexes develop between the cells of the digestive caeca and adjacent parenchyma cells.

It is likely that the food injested by adult M. minutus is similar to that of Microphallus similis and that it consists of a mixture of host epithelium and partially digested food from the host's gut lumen. (The possible role of the unicellular tegumental glands in extracorporeal digestion is discussed in relation to Microphallus similis in Chapter 5, page 343. It is possible that the gland cells of M. minutus fulfil a similar function in predigesting host tissue.)

Excretory system

The excretory bladder of the metacercaria of M. minutus was filled with excretory granules which, considering their structure when decalcified, probably had an organic "skeleton". The possible function of excretory granules (or concretions) in the metacercarial stage has already been discussed (page 123) in connection with F. hepatica. Cable (1965) suggests that the excretory concretions may have a protective function and that they may serve to immobilise waste products which could prove toxic to the flukes under enclosed conditions. Bowers & James (1967) note that:-

> "the excretory products appear to accumulate during the life of the metacercaria, perhaps because excretion to the exterior is hindered by the metacercaria being completely enclosed by host tissue."

These authors suggest, then, that the excretory granules are themselves the excretory product.

The ultrastructure of the bladder wall of M. minutus, both in the adult and the metacercaria, is similar to that of other trematodes (Erasmus, 1972) in that it is a thin-walled syncytium bearing surface lamellae and producing lipid droplets which are extruded into the lumen. In M. minutus the production of lipid increases during the development of the metacercaria to the adult. This may suggest, either that the metacercaria is metabolising very slowly, or that the lipid is absorbed by the excretory granules as soon as it is produced, or that the excretory product is not lipoidal.

The collecting ducts of M. minutus are ciliated. The suggestion of Bennett (1973) that in F. hepatica the cilia are necessary to overcome the pressure which the excretory system must work against whilst in the encysted state cannot be used as a generalisation. Although ciliated ducts are present in the metacercaria of F. hepatica (encysted), they are not present in Microphallus similis (encysted), whereas they are present in M. minutus (not encysted). Bennett (loc. cit.) also suggests that they may assist the movement of fluids in very narrow tubules where capillary action may inhibit the flow due to fluid drag.

The possible micropinocytotic vesicles and the surface lamellae in the collecting ducts may be involved in the absorption of material from the excretory fluid in the lumen. Bennett (loc.cit.) discusses micropinocytotic uptake in the excretory system of trematodes with particular reference to the juvenile stages (up to 4 weeks) of F. hepatica. He concludes that, in juvenile F. hepatica, "pinocytosis is involved in improving the efficiency of food retention and utilization."

The flame cells of M. minutus are similar to those described by Wilson (1969) for the miracidium of F. hepatica and by various other workers (see page 121). However, as with Microphallus similis (page321), external leptotriches seem to be absent from the flame cells of M. minutus, Wilson (loc.cit.) suggests that external leptotriches may serve a support function by holding the other cells at a distance and creating and maintaining a fluid filled cavity around the flame cell. The flame cells of M. minutus are surrounded by a thick layer of interstitial material and are obviously able to function without the aid of external leptotriches.

Female Reproductive System

Vitellaria

The cells of the vitellaria were not distinguished in the metacercarial stage. This is in contrast to the metacercaria of Microphallus similis where the cells of the vitellaria contained abundant GER and were clearly distinguishable. It is likely that the vitellaria of Microphallus similis were better developed in the metacercarial stage than those of M. minutus and that, in M. minutus, the vitelline cells were indistinguishable from the parenchymal cells or other undifferentiated cells.

The vitellaria of the adult were very similar to those of Microphallus similis (page 327) and those of F. hepatica (Irwin & Threadgold, 1970); they contained abundant GER, lipid drops and typical secretory droplets. The mature cells were similar to those of M. similis and the cytoplasm of the cell was almost entirely replaced by secretory droplets which were no longer associated together in aggregates. The outline of the droplets

was roughly spherical, rather than irregular or faceted as in the developing cells, presumably because they were no longer being compressed by the surrounding cytoplasm.

Ovary

The gonad seen in the metacercaria may have been the ovary or the testes. It appeared to be in a state of quiescence; the cells, judging by the morphology of the nuclei, were in interphase and synaptonemal complexes[1.] were absent. Since the cercaria of M. minutus probably contains no recognisable gonads[2.], the anlagen of the ovary and the testes must develop during the metacercarial stage. Since eggs or sperm are never seen in the metacercaria, it is likely that spermatogenesis and oogenesis proceed up to a certain point in the metacercarial stage and then stop until the parasite is eaten by its definitive host. In order for this to happen, the process of gametogenesis must be very finely controlled in the metacercaria and some blocking mechanism must exist to prevent the later stages of gametogenesis occurring. Such a control is probably genetically determined but it may depend on the availability of nutrients or other factors such as temperature.

Footnotes.

1. Synaptonemal complexes/occur during both oogenesis and spermatogenesis. They are a universal feature of the primary spermatocyte nuclei of higher animals and they also occur in invertebrates where they may be particularly complex. They represent a bivalent pair of chromosomes during the zygotene/pachytene stage of meiosis (Ford, 1973).
2. The cercaria of Gymnophallus fossarum, which is very similar to M. minutus (see page 202), does not possess any recognisable gonads (Bartoli, 1972, 1974).

For technical reasons no publishable micrographs of the ovary of adult M. minutus were obtained; the sections were either badly scored, creased or contaminated by lead deposits. However, it was possible to make a description of the adult ovary. Nurse cells were probably present in the metacercarial and adult stages but the nuclei of such cells were only seen in the gonad of the metacercaria. Cortical granules, similar to those seen in Schistosoma mansoni (Erasmus, 1973) and F. hepatica (Björkmann & Thorsell, 1964a; Gresson, 1964), were present in M. minutus and they appeared to move towards the periphery of the cell during the development and maturation of the ovum. The "ribosomal bodies" seen in the ova of F. hepatica (Björkmann & Thorsell, loc. cit.) were apparently absent from the ova of M. minutus.

On those occasions when sections through the ovary were obtained, only mature or nearly mature cells were observed - developing stages were not seen. If the developmental stages were zoned within the ovary so that the least developed cells were furthest away from the oviduct and the best developed nearest the oviduct (rather than the ovary being lined with early developmental stages as in F. hepatica), this could be accounted for.

It appears that, in M. minutus, the cortical granules are lost during the passage of the ovum down the oviduct and into the uterus, since the micrographs of ova within the uterus show no sign of cortical granules. Erasmus (loc.cit.)has also observed this phenomenon in S. mansoni and he correlates the loss of the granules with the process of fertilization. In the present study, the ova in utero, which lacked the cortical granules, were surrounded by sperm although fertilized ova were not seen. (The ultrastructure of fertilization has been described by Burton (1967b) in H. medioplexus.)

Male Reproductive System

Prostate Gland

The cells of the prostate gland were not seen in the metacercaria. In the adult the cells were filled with secretory granules; the GER and the Golgi complexes, which must have been involved in their synthesis, were never seen. The ultrastructure of the prostate gland of F. hepatica has been described by Threadgold (1975). However, it appears to be very different to that of M. minutus. In F. hepatica the secretory product consists of "large electron-lucid carbohydrate-rich secretory vesicles" and "a very dense granular and fibrillar material." The secretions are produced in unicellular gland cells and they pass down ducts lined with microtubules which open into the ejaculatory duct to which they are joined by desmosomes. Threadgold (loc.cit.) points out that the morphology of the prostate gland of F. hepatica is basically similar to that of the Mehlis gland (Threadgold & Irwin, 1970), it also bears similarities to the unicellular gland cells associated with the tegument of M. minutus and Microphallus similis. Threadgold (loc.cit.) suggests various functions for the prostate gland secretions:

"a) the stimulation of the spermatozoa previously stored in a quiescent state in the seminal vesicle;

b) an additional energy source for the spermatozoa in addition to the intraspermatozoan glycogen;

c) to stimulate or in some way influence the female reproductive ducts."

In M. minutus the lumen of the prostate gland is presumably the ductus ejaculatoris and it is surprising that, in the many sections through the gland that were seen, sperm were never present in the lumen. Secretions were never seen free in the lumen neither were they seen in the process of secretion. It is possible that the secretions are not released in packets but that they are released by a form of eccrine secretion (Erasmus, 1972; Kurosomi, 1961 - Extrusion Type IV) whereby the membrane enclosing the secretory body fuses with the limiting membrane of the prostate gland cell releasing the contents into the lumen.

Testes

Previous work on the ultrastructure of the Digenean testes and the process of spermatogenesis is reviewed in Chapter 5, page 359 . Spermatogenesis and spermateleosis in M. minutus is very similar to that described for Microphallus similis (pages 330 & 387) and for H. medioplexus (Burton, 1972); it is discussed in more detail in Chapter 5. The ultrastructure of the spermatozoon of M. minutus is similar to the general pattern described for other Digenes (Morseth, 1969; Hershenov et al, 1966; Shapiro et al, 1961; Tulloch & Hershenov, 1967; Burton, 1972). It consists of two lateral axial "9+1" filaments, an elongated nucleus with condensed chromatin, mitochondria (or one long mitochondrion?), and peripheral microtubules. Unusual arrangements of components, such as those seen in the sperm of Microphallus similis, were never observed in M. minutus.

From the present study, there is evidence to support the suggestion made in part I of this chapter (page192) that the vesicular seminalis is bilobed rather than undivided as described by Bowers & James (1967).

Microsporidian Spores

The ultrastructure of the microsporidian spores and pre-spore stages which hyperparasitise the metacercariae of M. minutus have been described by Canning & Nicholas (1974). However, these authors made no mention of the membraneous and "ladder-like" structures associated with the spores. The membraneous bodies are probably produced by the damaged or degenerating host cells but the "ladder-like" structures appear to be much more intimately associated with the sporoblasts and the spores. If these "ladder-like" structures were produced by the microsporidian, they were probably formed during the sporoblast stage before the production of the thick spore wall; these structures were never seen in association with earlier stages. The function of these bodies remains a mystery.

Apparently normal spores were seen in the tissues of developing adults recovered from ducklings. They were never seen in egg-producing adults and, although this does not necessarily mean that mature adults cannot harbour visible spores, it may indicate that hyperparasitised metacercariae are unable to complete their development.

SECTION 2: SCANNING ELECTRON MICROSCOPY

Results

A. METACERCARIA

The surface features of the metacercariae of M. minutus were not examined since, at this stage, the flukes were covered by a thick glycocalyx.

B. ADULT (Plates 108 - 110)

The adults of M. minutus exhibited a wide variety of shapes after fixation but Plate 108 shows the typical shape assumed by the adults in a resting position. The body was roughly pear-shaped although the area around the excretory pore protruded slightly. There was some slight doso-ventral flattening of the body.

With the exception of the lips of the suckers and a fold of tegument posterior to the ventral sucker, the body of the adult was covered with spines. These spines were arranged in rings around the body and pointed posteriorly. They were flattened (rather than wedge-shaped as in F. hepatica) and their tips, which were rounded, were toothed or fringed with up to six "teeth" (Plate 109). In the region of the bases of the spines the surface of the fluke appeared indented.

The oral sucker was at the anterior tip of the fluke and the ventral sucker was about two-thirds of the way down the body. There were no spines on the lips of the suckers; the tegument in this region was relatively smooth or else it was thrown up into folds or small microvilli. The bulbs of sensory endings with their short protruding cilia were present on the lips of the suckers (Plates 109 & 110), in the general area of the oral sucker (Plate 109), and - to a lesser extent - scattered

over the body surface (Plate 108). In the region of the ventral sucker the spines were often smaller and appeared to be sunk into pits in the tegument which was thrown up into folds. The genital pore, observed at the light microscope level by Bowers & James (1967) just anterior to the ventral sucker of adult M. minutus, was not observed in the present study. However, the tegument was often folded in this region and the genital pore may have been obscured. Posterior to the ventral sucker was a conspicuous transverse ridge which was bare of spines (Plate 108). Just anterior to this ridge was a deep furrow and there were often several lesser furrows posterior to the ridge. The appearance of this ridge and associated furrows varied according to the position in which the fluke was fixed.

Discussion

The surface of adult M. minutus was covered from anterior to posterior (with the exception of the suckers) with rows of distinctive "toothed" spines which all pointed posteriorly. Similar spines have also been seen on the forebody of Microphallus similis (page 311) on adult F. hepatica (Bennett, 1975b) - especially on the anterior cone, and on the cercariae and adults of Neophasis lageniformis (Køie, 1973). The adults of M. minutus, M. similis and N. lageniformis are all parasites of the intestine. Køie (loc.cit.)suggests that the "toothed" spines of adult N. lageniformis assist the fluke in maintaining its position in the intestine of the final host. Bennett(loc.cit.) has suggested that the spines of F. hepatica may help to abrade the host's bile duct epithelium and render it easier to ingest. The function of the spines in M. minutus (and M. similis) may combine both these roles. It is interesting to note (see page 345) that, on both M. minutus and Microphallus similis, the spines only occur on that part of the fluke which is in most intimate contact with the host's tissues. The adults of M. minutus are completely embedded between the host's villi and are covered with spines, whereas adult M. similis only bear "toothed" spines on their anterior surface and it is only the forebody which is embedded between the villi - the posterior part of the fluke protrudes into the host's gut lumen.

The sensory papillae were mainly associated with the suckers and they were also found in the area around the oral sucker. It is significant that they were most frequent on the organs of attachment and on that part of the fluke which makes exploratory feeding movements. They are probably mainly

tangoreceptors and assist the fluke in site location, maintenance of position and feeding.

The apparent absence of the genital pore, as described by Bowers & James (1967), was interesting. However, if it was very small, it may have been obscured by the folding of the tegument in this region. The significance of the spine-free ridge posterior to the ventral sucker was puzzling. It appears to develop during the maturation of the adult, since, in cultured metacercariae (page 279), it was not present. In that case, it is likely that it is either connected with egg production in some way or that it is related to the new environment in which the fluke finds itself.

Conclusions

1. The body of adult Meiogymnophallus minutus was roughly pear-shaped and was covered (except for the suckers) with rows of backwardly-pointing, toothed spines. Ciliated sensory endings were scattered over the whole body but were mainly located on and around the suckers. The genital pore described by Bowers & James (1967) was not seen. A ridge of tegument, which did not bear spines, was present just posterior to the ventral sucker.

2. The tegument of the metacercaria and adult of M. minutus consisted of a surface syncytium connected by protoplasmic strands to the cell bodies which lay below the peripheral musculature.

3. GER was present in the tegumental cell bodies of the adult but not in those of the metacercaria.

4. The tegumental secretory bodies in the syncytium were of three basic types - spherical (Types 0 and 1), oval (Type 2), and biconcave (Type 3). In the metacercaria the spherical granules had an electron-lucent content, whereas in the adult the contents were moderately dense. The tegumental cell bodies only appeared to possess the spherical granules.

5. Unicellular gland cells containing dense secretory bodies opened via slender ducts, which were lined with microtubules, onto the surface of the fluke over the surface of the whole body. The ducts were connected to the syncytium by a desmosome.

6. The parenchymal cells were infrequent and did not act as significant stores of α glycogen. Junctional complexes were not present.

7. The oesophagus was lined with tegumental-type syncytium. The lining of the digestive caeca was cellular. In the metacercaria, the epithelial cells contained small, dense secretory granules, GER, Golgi complexes, lipid drops and surface lamellae. The gut of the metacercaria may be functional. The digestive caeca of the adult was not observed.

8. In the metacercaria the excretory bladder was filled with inorganic excretory granules (concretions) which had an organic matrix. These granules were lost during development.

9. The lining of the excretory bladder was probably syncytial and bore long, tenuous surface lamellae.

10. The amount of lipid in the lumen and the lining of the excretory bladder increased during development.

11. The collecting ducts of the excretory system were ciliated in both the metacercaria and the adult.

12. The basic pattern of the flame cell was similar to that described for other flukes (e.g. the miracidium of F. hepatica - Wilson, 1969) except that external leptotriches were absent.

13. Vitellogenesis was similar to that described for other trematodes (e.g. Microphallus similis, F. hepatica). The vitelline cells of the metacercaria were unrecognisable as such.

14. The gonad (testes/ovary?) of the metacercaria was apparently in a state of quiesence.

15. The ova in the ovary of the adult possessed cortical granules but no ribosomal bodies. The cortical granules were lost during the passage of the ovum down the uterus.

16. The cells of the prostate gland of the adult contained irregular, dense secretory bodies which were never seen free in the lumen or in the process of secretion. The ultrastructure of the prostate gland of M. minutus was quite different to that described for

F. hepatica (Threadgold, 1975).

17. The processes of spermatogenesis and spermateleosis in M. minutus were similar to those described for H. medioplexus (Burton, 1972).

18. The spermatozoa of M. minutus were similar to those described for other trematodes and consisted of an elongate nucleus, two "9+1" axial filaments, mitochondria (or one long mitochondrion?) and peripheral microtubules.

19. Sporoblasts, spores and the pre-spore stages of the microsporidian Unikaryon legeri were present in the metacercaria and in developing adults. Membraneous and "ladder-like" bodies were seen in association with the spores and sporoblasts.

PART IV: ULTRASTRUCTURE IN VITRO

Introduction and Methods

The ultrastructure of six day old cultured metacercariae from several different cultures was examined. The culture conditions were as follows:-

Medium: NCTC 135 plus antibiotics, with 20% serum (foetal calf, chick or rabbit).

Gas phase: Aerobic (10% O_2, 5% CO_2, 85% N_2) or Anaerobic (5% CO_2, 95% N_2).

Temperature: 39°-40°C.

Other Conditions: 8-10 mls. media in a Leighton tube with continuous shaking.

Cultured flukes were examined using the scanning and the transmission electron microscope; the techniques involved are described in Chapter 2, General Methods. Particular attention was paid to the surfaces which come into contact with the culture media, i.e. the tegument and the digestive caeca, and to the development of the reproductive system.

SECTION 1: TRANSMISSION ELECTRON MICROSCOPY

Results

The Tegument (Plates 111 & 112)

The syncytium of cultured flukes contained numerous secretory granules (Plate 112). The most common types were the spherical, electron lucent (Type 0) and the small, oval (Type 2) granules (see Page 233 for a description of these granules). The biconcave (Type 3) granules were rarely seen and the spherical, electron dense (Type 1) granules were absent. The Type 2 granules were probably more abundant in the syncytium of cultured flukes than in the metacercaria or the adult. The mitochondria in the syncytium of cultured flukes were prominent; they were large and slightly more dense than the surrounding cytoplasm with well developed cristae which were long, closely packed and often looped. The layer of interstitial material underlying the syncytium was thicker than that of the metacercaria or the adult. In some areas, especially on the inner surface of suckers and the pharynx, the syncytium was vacuolated producing a bubbling effect (Plate 111), these vacuoles were not membrane-bound. In addition, large lipid-like droplets (up to 1 um. in length) were also observed, they often occupied the entire width of the syncytium.

The tegumental cell bodies contained Type 0 and Type 2 secretory bodies, some small mitochondria, numerous free ribosomes, very sparse GER, Golgi complexes and some large oval bodies which were probably lipid droplets.

Gland cells associated with the tegument (Plates 113 & 114)

The ultrastructure of the unicellular gland cells associated with the tegument (Plate 113) of cultured flukes was similar to that of the adult and the metacercaria (page 233). In several instances, developing gland cells were seen which contained mitochondria, ribosomes and possible Golgi complexes; the nuclei of these cells contained dense patches of heterochromatin. The secretory bodies packing the gland cells were occasionally irregular in shape with abnormal electron lucent areas (Plate 114).

Digestive Caeca (Plate 115)

The caecal epithelium of cultured flukes appeared abnormal and disorganised. Adjacent cells were connected by a desmosome near their apical surfaces. The surface lamellae were long and tenuous and often branched. The epithelial cells were usually quite narrow (1 - 2 um.) and contained mitochondria, free ribosomes and small lipid-like droplets; the dense secretory bodies and GER were sparse. Some large lipid-like droplets (4 - 5 um. in width) were present in the lumen of the gut, these often had some cytoplasm adhering to them and were enclosed by surface lamellae. Occasionally, some membrane-bound bodies containing ribosomes were seen free in the lumen, these were presumably blebs or projections from the surface of the caecal cells.

Excretory System (Plates 121 & 122)

The excretory system of cultured flukes was similar to that already described for the metacercaria and the adult (pages 237 & 238). The excretory bladder contained many large lipid drops (1 - 2 um. in diameter) which were often enclosed

by long, thin lamellae (or microvilli) which projected from the apical surface. The lining of the bladder appeared to be syncytial and the cytoplasm contained free ribosomes, mitochondria, occasional very small vesicles, infrequent GER and some lipid droplets. Occasionally some excretory granules (concretions) were still present in the excretory bladder after several days of in vitro cultivation (Plate 122). These granules (which had not been decalcified prior to processing as in the metacercaria) had a thick, dense, cortical region and an electron-lucent medullary region in which there was often an additional ring of dense material. These granules appeared to be in close association with the surface lamellae. The collecting ducts and flame cells of cultured flukes were similar to those already described for the adult and the metacercaria.

Parenchymal cells

The parenchymal cells of cultured flukes were similar to those seen in the metacercaria and the adult (page 235). They contained some stored α glycogen but they often appeared rather leached out and the cytoplasmic granules appeared clumped.

Female Reproductive System

Mehlis' Gland

The cells of Mehlis' gland were never observed in cultured flukes.

Vitellaria (Plate 119)

The vitelline cells of cultured M. minutus were similar in many respects to those of adults recovered from oystercatchers.

They possessed fairly extensive GER, numerous free ribosomes, areas of Golgi activity, nuclei with nucleoli and patches of heterochromatin, and typical secretory droplets. As in the normal adults, the droplets, which were probably membrane-bound, consisted of aggregates of smaller, irregular, secretory bodies However, unlike that of the normal adults, the secretory material produced by cultured flukes was not uniformly dense and the secretions within the droplets, often had a "ragged" appearance. In addition, some lysosomes and membraneous bodies were present in the vitelline cells.

Unfortunately, sections through the clumps of prematurely tanned vitelline material seen in the uterus of cultured flukes (see page 208), were not obtained.

Ovary (Plate 117)

The ovary of cultured flukes appeared rather disorganised. Plate 117 probably shows an oblique section through the edge of the ovary since it is considerably smaller (0.009mm. or 9um. in length) compared to the dimensions described by Bowers & James (1967) (0.026-0.034 x 0.016 - 0.022mm.). Two cells appear to be present in this section and since they possess cortical granules, they are probably ova (or mature oocytes) rather than earlier developmental stages. In addition to the cortical granules, the cells possess a nucleus, mitochondria, oval electron lucent areas (lipid drops?), small electron lucent vesicles and whorls of lamellae. Apparently extracellular mitochondria were present and these were larger and denser than those of the ova. These mitochondria may belong to the ovarian nurse cells although the nuclei of such cells were not seen. The ovary was bounded by a thick basement membrane

and, although there were isolated muscle cells around the periphery, there was no continuous muscular coat.

At the light microscope level, the ovary of cultured flukes was very difficult to discern and it has been suggested (page 220), that it may degenerate during cultivation.

Male Reproductive System

Prostate Gland

The ultrastructure of the prostate gland of cultured *M. minutus* was similar to that of the normal adult. However, secretory droplets were occasionally observed which had electron-lucent areas similar to the abnormal secretory granules of the tegumental gland cells of cultured flukes.

Testes and Vesicula Seminalis (Plates 116 & 118)

After six days of *in vitro* cultivation, the testes usually appeared very disorganised with large areas of vacuolation and lysosomes (Plate 118). Some sperm were seen in the testes. In cross section many of these sperm were similar to those seen in the normal adult, i.e. they contained two "9+1" axial filaments, a condensed nucleus, a mitochondrion and several peripheral microtubules. However, quite a large proportion of the sperm present in the testes and vesicula seminalis were apparently abnormal in that they contained four filaments rather than the usual two (Plate 116). In these abnormal sperm, there was only one nucleus although there were one or two mitochondria. Much more rarely, sperm were seen with one filament, some peripheral microtubules and a nucleus. These abnormalities in sperm ultrastructure were never observed *in vivo*.

Microsporidian spores

Microsporidian spores were often seen in cultured flukes. They appeared similar to those described by Canning & Nicholas (1974). They were present in flukes which were producing sperm and whose vesicula seminalis was full. The associated bodies described on page 244 (Plate 91) were present in cultured flukes.

Discussion

The Tegument

The tegument of six day old cultured flukes was very similar to that of the metacercaria. The spherical secretory bodies were electron lucent (Type 0) rather than dense (Type 1) like those of the adult. Also, as in the metacercaria, the ribosomes in the tegumental cell bodies were free and were not associated with membranes to any significant extent. The oval (Type 2) and the biconcave (Type 3) secretory bodies were also present in the syncytium of cultured flukes. However, unlike both the metacercaria and the adult, the oval (Type 2) secretory bodies were also present in the tegumental cell bodies. This may indicate that, *in vitro*, the transport of the oval granules does not proceed as fast as it does *in vivo* and that the granules are able to accumulate, or, that the oval granules are, in fact, modified spherical granules (i.e. spherical granules which have lost some of their contents). The number of oval granules in the tegument as a whole appeared to be greater than in the metacercaria of the adult. Assuming that they are a different type of granule to the spherical granules, this may represent a response to the abnormal conditions which the fluke encounters in the culture medium.

Fairly large lipid drops were seen in the syncytium of cultured flukes. The accumulation of lipid in the syncytium is indicative of abnormality and it may be connected with the bubbling of the syncytium typical of cultured flukes. Wilson & Barnes (1974) have described a similar vacuolation of the syncytium in adult *Schistosoma mansoni* maintained in culture. However, in *S. mansoni* the vacuolation was, in fact, a swelling of the invaginations of the basal lamina, and the vacuoles were membrane-bound. In *M. minutus* the vacuoles were not membrane-bound;

they may have been the result of the accumulation and subsequent extrusion of the lipid droplets seen in the syncytium, this would presumably result in the production of a space or "hole" within the syncytium which was not membrane-bound.

The large mitochondria with the numerous, well-developed cristae seen in the syncytium of cultured flukes may reflect the increased availability of oxygen for aerobic respiration in the culture media compared to the normal environment between the host's villi. However, the mitochondria of flukes grown in culture media with an anærobic gas phase were similar suggesting that either this explanation is incorrect or else the gassing procedure did not produce truly anaerobic conditions.

The increase in the amount of interstitial material between the syncytium and the underlying peripheral musculature was also observed in cultured F. hepatica (page 144) and in cultured Microphallus similis (page 367).

Unlike those of Microphallus similis (page 369), the gland cells associated with the tegument of M. minutus appeared virtually normal in cultured flukes, In the gland cells of cultured M. similis, the secretory product coalesced to form large masses within the cell bodies of the glands; this did not occur in M. minutus even though the culture conditions were virtually identical. This re-enforces the danger of assuming that, because the morphology of a particular structure is similar to that of another, they must necessarily have a similar composition or similar functions.

The Digestive Caeca

Although the digestive caeca was probably rather abnormal, it is difficult to assess the effects of cultivation since the ultrastructure of the normal adult gut is unknown. The GER in the caeca of cultured flukes was sparse although there were large numbers of free ribosomes. It is probable that, as in cultured F. hepatica, the GER breaks down during cultivation. No recognisable Golgi complexes were seen in the gut epithelium cells of cultured M. minutus and so it is to be expected that the number of secretory granules was small. The apparent degeneration of the secretory apparatus and the decreased number of granules may be a response to abnormal cultivation conditions or it may reflect the normal adult condition - further work would be necessary in order to clarify this problem. Since there was no solid food in the culture medium, the cells of the caecal epithelium may have become abnormal as a result of dis-use.

The production of lipid by the cells of the digestive caeca increased during cultivation and this may reflect the normal developmental pattern. The lipid in the digestive caeca of Microphallus similis also increased during development in vivo and it has been suggested (page 320) that the lipid drops produced by the gut may function as a lubricant or that the digestive caeca may function as a subsidiary excretory organ; these functions may also apply to M. minutus.

Junctional complexes between the caecal cells and adjacent parenchyma cells failed to develop during cultivation but it is not known whether they are produced during normal development in vivo. Junctional complexes were not observed between parenchyma cells of normal adults and it has been suggested

(page 348) that the large surface area to volume ratio in M. minutus may render them unnecessary as specialised sites of transport.

Excretory System

The ultrastructure of the excretory system of cultured M. minutus was very similar to the adult except that less lipid was present in the excretory bladder. This suggests that cultured flukes are not metabolising at the same level as normal adu l ts. This is not surprising when the abnormalities in the ultrastructure of those surfaces involved in the uptake of nutrients - the tegument and the caecal epithelium - are considered.

The presence of excretory granules in the bladder of cultured flukes, even after several days cultivation, indicates that these granules are not voided merely as a response to the elevation of temperature. Such a conclusion was also suggested when apparently intact metacercariae were recovered from the intestines of ducklings which had been fed on baby chick crumbs (i.e. a normal diet) rather than boiled minced cockles (page 199).

Parenchyma Cells

Occasionally, the parenchyma cells of cultured M. minutus appeared rather "leached out". A similar situation was also observed in cultured F. hepatica (page 167) and it is possible that this may indicate an osmotic imbalance between the culture media and the cells of the fluke.

Female Reproductive System

The Vitellaria

Virtually normal vitelline secretions were produced *in vitro* and the ultrastructure of the vitelline cells was essentially similar to those of the normal adult. However, the secretions had a rather "ragged" appearance, as did those of cultured *Microphallus similis*, and they may correspond to the prematurely tanned vitelline droplets seen under the light microscope. Premature tanning at an ultrastructural level is discussed, in connection with *M. simillis*, on page 384 . Unfortunately, mature prematurely tanned vitelline cells were not seen in the uterus in the present study. However, they were probably very similar to those described for *M. similis* (page 371 , Plate 182).

Ovary

The ultrastructure of the ovary of cultured *M. minutus* appeared rather disorganised and abnormal, early stages of oogenesis were never seen. It has been suggested in part II of this Chapter (page 220) that the ovary degenerates during cultivation and it is often very difficult to distinguish the ovary of cultured flukes under the light microscope. It appears then that oogenesis becomes abnormal during cultivation and that, after six days, the ova - although they possess cortical granules - have considerable cytoplasmic abnormalities.

Unshelled ova were never seen free in the uterus of cultured flukes either under the light microscope or at the electron microscope level. The absence of normal eggs in cultured flukes may depend on -

a) cytoplasmic abnormalities in the ova;

b) non-release of ova from the ovary;

c) the premature tanning of the vitelline droplets in the vitellaria.

Also, sperm were never observed in the uterus of cultured flukes and it is possible that insemination and fertilisation fails to occur in culture. In addition, it cannot be assumed that the Mehlis' gland secretions are normal in cultured flukes and these secretions are known to play an important role in egg-shell formation (Clegg, 1965b; Erasmus, 1972).

The Male Reproductive System

Prostate Gland

The prostate gland produced large numbers of secretions most of which appeared normal. However, as in normal adults, these secretory bodies were not seen in the process of secretion nor were sperm seen in the ductus ejaculatoris which forms the lumen of the gland.

Testes

After six days of *in vitro* culture, the testes appeared grossly abnormal with large lysosomal bodies and vacuolations. It was virtually impossible to discern the various stages of spermatogenesis within the testes. As with the ovary, the testes of cultured flukes appeared to degenerate and was often difficult to see in fresh or stained preparations at the light microscope level (see page 209). Sperm were present in the testes and vesicula seminalis of cultured flukes. However, a large proportion of them were abnormal in that they contained four

rather than two axial filaments. It has been suggested (Davies, 1975) that this abnormality results from the doubling up of the filament-producing apparatus at the spermatid stage. It is unlikely that it results from the fusion of two sperm since, although the abnormal sperm often contained two mitochondria, they were never seen to possess two nuclei. This abnormality was never seen in normal adults from oystercatchers or from ducklings. The same abnormality in sperm ultrastructure was also seen in cultured Microphallus similis (page 389) although it was much rarer.

Microsporidian Spores

Spores and pre-spore stages of the microsporidian Unikaryon legeri were often seen in cultured flukes. They appeared normal (Canning & Nicholas, 1974) and they were associated with both the membraneous and the "ladder-like" bodies seen in the metacercariae and in immature adults (page 244). It appears that, as in developing flukes recovered from ducklings, the presence of the microsporidian does not impede spermatogenesis or the production of vitelline or prostate gland secretions.

SECTION II: SCANNING ELECTRON MICROSCOPY

Results

The surface features of cultured Meiogymnophallus minutus were similar in many respects to those of adults recovered from oystercatchers (page 258). The major differences were as follows:-

1. Compared to the normal adult, cultured flukes did not exhibit such a wide variety of shapes. The typical shape of in vitro grown flukes is shown in Plate 123. Its shape was oval to pear-shaped and there was a pronounced ventral curling of the lateral edges.

2. The appearance of the oral sucker varies according to whether it was "open" or "closed" at the moment of fixation. However, in cultured flukes the oral sucker was usually "wide open" (Plate 123 cf. Plate 108) and appeared very large. The tegument covering the lip was very smooth and bore no spines. Bulbs of sensory endings were seen in this region and the projecting cilia appeared unusually long (Plate 124 cf. Plate 109).

3. The ventral sucker (Plate 125) was not very prominent and the spines surrounding it often appeared completely sunk into pits below the surface of the fluke. In other respects, the ventral sucker of cultured flukes was similar to that of normal adults (cf. Plate 110). The ridges and furrows posterior to the ventral sucker were apparently not present.

4. The most conspicuous abnormality in cultured flukes were the "tegumental bubbles". Where these bodies were present they were scattered over the surface of the fluke although they were most frequently seen in the oral sucker region. They appeared to be dome-shaped extensions of the surface. They did not possess spines and their surface was irregular.

Discussion

Cultured M. minutus did not exhibit a very wide range of shapes compared to the adult and there was often a pronounced curling of the lateral edges. Cultured Microphallus similis (page 390) also showed a similar curling at its edges.

The cilia of the sensory papillae of cultured M. minutus often projected an unusual distance from the bulb; this abnormality was also seen in cultured Microphallus similis (page 390) and in cultured F. hepatica after prolonged cultivation (page 174). It is suggested (page 176) that this abnormality may be due to sensory deprivation. If the sensory endings in any way control or influence the activity of the muscles of the oral sucker, the lack of stimulation could account for the gaping of the oral sucker which was frequently seen in cultured flukes. However, the gaping of the sucker - and also the generally decreased muscular activity as evidenced by the uniformity of shape of cultured flukes - could also be explained by an inadequate energy supply. If the uptake of nutrients in culture was low, the energy produced may be insufficient to allow normal muscular activity.

The spine-free ridge just posterior to the ventral sucker which was so prominent in adult flukes was not present in cultured flukes. Unfortunately, because of the thick glycocalyx which covered the surface of the metacercaria, it was not possible to say whether the ridge was present at this stage. However, it does seem likely that it develops during the maturation of the fluke and it has been suggested (page 261) that it may in some way be connected with the maturation of the reproductive system or with the new environment between the host's intestinal villi.

The bubbles present on the surface of cultured M. minutus were also present on the surface of cultured Microphallus similis. It is suggested, in relation to M. similis (page 390), that these bubbles may be due to an osmotic imbalance or that they may be the result of a variety of toxic or unfavourable conditions in the culture media. However, smaller bubbles have been observed in thin sections and it is suggested (page 273) that they may result from the accumulation of lipid in the syncytium.

Conclusions

1. The tegument of cultured M. minutus was more like that of the metacercaria than that of the adult. The spherical secretory bodies were electron-lucent.

2. Unlike the tegument of the metacercaria or the adult, the oval secretory granules were seen in the tegumental cell bodies.

3. The accumulation of lipid droplets in the syncytium of cultured flukes may be connected with the "bubbling" of the tegument.

4. The mitochondria in the syncytium were unusually large with many cristae, irrespective of gas phase.

5. The gland cells associated with the tegument were similar to those of the metacercaria and the adult. Abnormalities similar to those seen in cultured Microphallus similis were not present.

6. The gut epithelial cells were probably abnormal. The GER had probably broken down, Golgi complexes were not seen and there were only very small numbers of secretory bodies.

7. The amount of lipid produced by the gut epithelial cells increased during cultivation.

8. Junctional complexes did not develop between the cells of the digestive caeca and adjacent parenchyma cells.

9. The excretory system was similar to that of the adult except that the amount of lipid that was produced was less and occasionally some concretions remained in the bladder.

10. The parenchyma cells sometimes appeared rather "leached out".

11. Vitellogenesis appeared almost normal in culture although the secretory droplets often appeared rather "ragged".

12. The ultrastructure of the ovary was probably rather abnormal although the ova possessed cortical granules.

13. Unshelled ova were not seen free in the uterus.

14. Sperm were not seen in the uterus of cultured flukes.

15. The ultrastructure of the prostate gland was similar to that of the adult.

16. The testes began to degenerate during cultivation although masses of sperm were produced.

17. A significant proportion of the sperm were abnormal in that they contained four rather than two axial filaments and sometimes two mitochondria.

18. The overall shape of cultured flukes, as revealed by scanning electron microscopy, was similar and there was often a ventral curling of the lateral edges.

19. The cilia of the sensory papillae often projected an unusual length from the bulb.

20. The oral sucker was frequently wide open and gaping.

21. The spine-free ridge just posterior to the ventral sucker which was seen in normal adult flukes was not present in cultured flukes.

22. Tegumental bubbles were often seen on the surface of cultured flukes.

CHAPTER 5: MICROPHALLUS SIMILIS

PART I: DEVELOPMENT IN VIVO

Introduction

1. Life cycle and Morphology of the metacercaria and adult

The life cycle of Microphallus similis has been clarified and described by Stunkard (1957); it is summarized here in Text Figure 9 . Its ecology, development and host specificity have been studied by James (1969, 1971).

Larval Stages

The mother and daughter sporocysts are found in most of the varieties and subspecies of the prosobranch molluscs Littorina saxatilis, L. littoralis and L. littorea. In Britain they are most frequently found in L. saxatilis rudis and to a lesser extent in L. saxatilis tenebrosa.

The egg is injested by the first intermediate host and it hatches in the host's intestine to release the miracidium. The miracidium penetrates the intestine wall and enters the visceral haemocoel of the digestive gland and gonad where it develops to the mother sporocyst. When the mother sporocyst is mature, it bursts to release the first generation of daughter sporocysts, these in turn produce several more generations of daughters which eventually pack the digestive gland and gonad. The daughter sporocysts produce the cercariae. These monostome xiphidiocercariae (stylet-bearing) have no gut but bear four pairs of unicellular penetration glands and numerous cystogenic glands of two basic types.

The cercariae are released from the snail and are carried by respiratory currents over the gills of the second intermediate host. In Britain this is usually the common shore crab Carcinus maenas and, to a much lesser extent, Cancer pagarus. Stunkard (1957) suggests that the cercariae "enter the body at the bases of the gills, pass by way of the vascular system to all parts of the body and localise principally in the connective tissue of the digestive gland." James (1971) records that crabs can become infected by eating infected L. saxatilis and suggests that the cercariae may also enter the crab by penetration of the gut wall.

Metacercaria (Plates 126 & 130)

The cercaria encysts in the digestive gland of the crab. The smallest metacercarial cysts are only about 0.05mm. in diameter and the cyst wall is very thin. As the metacercaria grows, it loses its stylet, the digestive tract and ventral sucker develop and the cyst wall increases in thickness. A fully grown cyst may measure up to 0.55mm. in diameter, the cavity containing the metacercaria being 0.35 - 0.4mm. in diameter (see Text Figure 10). According to James (1969) the cyst consists of two walls - an outer, radially striated wall (up to 0.05mm. thick) and an inner wall (up to 0.02mm. thick) which consists of two layers. Munroe (unpublished results, 1975) has shown that the outer wall consists of two layers - a thin outer layer and a thicker, striated inner layer. A four-layered cyst has also been described for Microphallus opacus by Strong & Cable (1972). They suggest that the thin outermost layer is produced by the host and the other layers are produced by the parasite. James (1967) suggests that the two different populations of cystogenous cells present in

the cercaria of Microphâlus similis produce the two different cyst walls. The large dorsal cells contain neutral mucopolysaccharides and probably produce the inner wall. The small cells, which mainly occur ventrally and laterally, contain acid and neutral mucopolysaccharides, mucoprotein and glycoprotein all of which have been demonstrated in the outer cyst wall.

Within the cyst, the metacercaria is folded ventrally. Released, flattened specimens are almost fully grown, measuring 0.25 - 0.8mm. in length and 0.16 - 0.45mm. in width. Their reproductive system is virtually fully-formed although the gonads do not contain mature ova or sperm. However, James (1969) reports that he found two precocious encysted metacercariae which contained a few eggs in the uterus.

Adult (Plates 127 & 132)

The adults of M. similis occur in the small intestine of Larus gulls and other sea birds. James (1969, 1971) has successfully infected a variety of laboratory hosts with the metacercariae of M. similis. He suggests that the adult is less host specific than the metacercaria, but that under natural conditions host specificity may be due primarily to the feeding behaviour of the vertebrate host. The morphology of the adults has been studied by Stunkard (1957). His measurements (Table 5I:1) and a summary of his descriptions are included here.

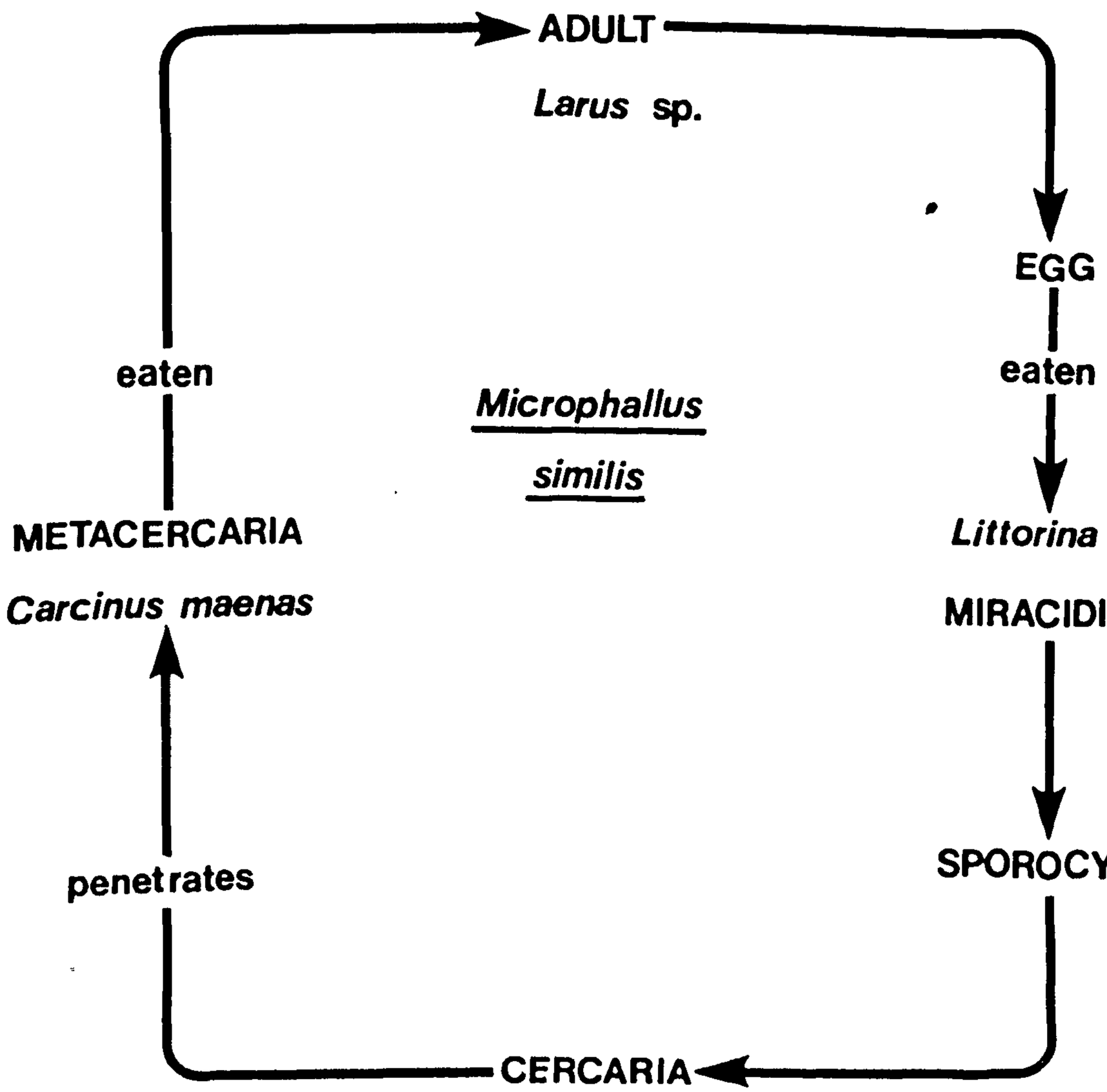
The body is oval to pyriform and there is often a constriction between the more mobile forebody and the inert hindbody which is filled with eggs (see Text Figure 10). The surface of the forebody is covered with conspicuous spines but those on the hindbody are smaller and sparser. The suckers are approximately equal in size; the oesophagus is long; the caeca are straight, they diverge at an obtuse angle and terminate level with the ventral sucker. There is a posterior bilobed excretory bladder which connects to the exterior by an excretory pore; the flame cell formula is 2 (2 + 2) + (2 + 2). The testes are dorsal, lateral and opposite; the vasa deferentia unite to form the oval vesicula seminalis which lies anterior and dorsal to the ventral sucker. A coiled ejaculatory duct, enclosed in glandular cells (prostate gland), leads to the muscular male papilla which almost fills the genital atrium, situated to the left of the ventral sucker. The ovary is oval to triangular and is located on the right side of the body at the level of the ventral sucker. The coiled oviduct expands to form a "fertilization space" (receptaculum seminis) from which Laurer's Canal winds to the dorsal surface of the body, opening just behind the ventral sucker. The oviduct continues and receives a short common vitelline duct; it enlarges to form the ootype which is lined with cilia and enclosed in the cells of Mehlis' gland. The vitellaria are large, lobed glands, situated below and behind the testes; ducts from the two sides unite to form the common vitelline duct which discharges into the ootype. The uterus passes backward from the ootype

then loops forward in coils on the ovarian side of the body as far as the end of the digestive caeca, then backward almost to the posterior end of the body where it crosses to the opposite side and forms a corresponding series of loops on the left side eventually emptying into the left side of the genital atrium. The eggs are operculate and are embryonated when laid. Initially the egg shell is thin, flexible and transparent (these eggs are located on the ovarian side) but they become thicker, harder and bright yellow (when they are located on the antovarian side).

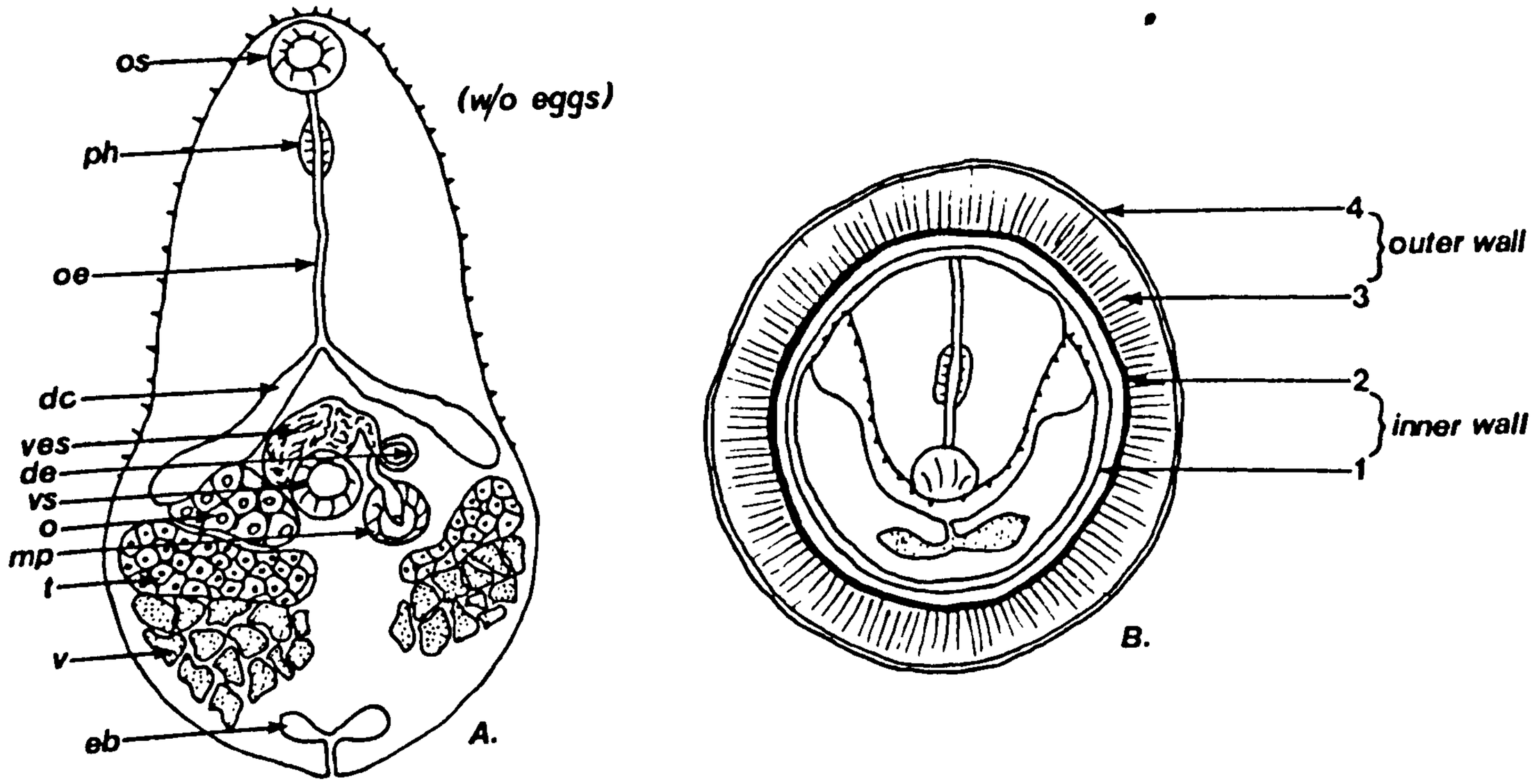
James (personal communication) has studied the mode of attachment of the adults in the intestine of gulls. The anterior end of the fluke is embedded between the villi and the posterior end protrudes into the lumen of the host's intestine. No information is available as to the longevity of the adult in its final host.

2. Experimental Definitive Hosts

James (1971) has force-fed various species of gulls (Larus argentatus, L. fuscus, L. marinus), ducklings, pigeons, guinea-pigs, rats and mice with encysted metacercariae of M. similis. On dissection all the animals were found to be infected with adult M. similis. The recovery (expressed as a percentage of dose) varied from 2.2% (L. argentatus) to 9.9% (mice). The experimental hosts and the natural hosts (gulls) appeared to be equally satisfactory.



Text Figure 9. Microphallus similis: Life cycle.



Text Figure 10. Microphallus similis: Morphology of -

A. The adult (the eggs are not shown).

B. The encysted metacercaria.

Key.

dc - digestive caeca
de - ductus ejaculatoris
eb - excretory bladder
mp - male papilla
o - ovary
oe - oesophagus
os - oral sucker
ph - pharynx
t - testes
v - vitellaria
ves - vesicula seminalis
vs - ventral sucker

Methods

In view of the relatively high recovery obtained by James (1971) from mice, the following experiment was designed to study the development of M. similis metacercariae in vivo.

Fifteen TF1 mice were infected orally using a stomach tube with 100 metacercariae each. Three mice were examined after 18 hours, 24 hours, 48 hours, 4 days and 7 days post infection. (The location of the parasites within the host's gut was not examined in the present study). The whole of the small intestine and duodenum was slit and incubated in Hanks saline at 40°C for 20 minutes. The sediment was examined for flukes under a binocular microscope. The adults were examined in various ways:-

a) alive and slightly flattened;

b) stained in aceto-orcein;

c) treated with Fast Red Salt B or Catechol for egg-shell precursors (Bell & Smyth, 1958);

d) fixed and stained in Gowers Carmine for permanent preparations;

e) prepared for scanning or transmission electron microscopy.

Fifteen excysted metacercariae were injected intraperitoneally into each of two mice to test whether they coulddevelop to adulthood in an abnormal site. The abdomens of the mice were examined on Day 4 post-infection according to the technique described in the General Methods (Chapter 2).

The *in vitro* excystation of the metacercariae of *M. similis* is described and discussed in part II of this chapter.

Results

Intra-peritoneal Infection

A total of 4 worms were recovered from the two mice. They were all dead and were encapsulated in a thick granular layer of host tissue which was up to 0.55mm. thick. No eggs were seen in the dead flukes.

Oral Infection

a) Recovery

Although an accurate record was not kept of the numbers of flukes recovered, it was estimated that it was approximately 10% (expressed as a percentage of dose).

b) Development

18 hours

Clusters of spermatids and some free sperm were seen in the testes; the vesicula seminalis was empty. The vitellaria had increased in size from those of the metacercaria (Plates 130 and 131) and were now positive for egg-shell precursors. No eggs were seen in the uterus.

24 hours

The testes were filled with sperm and in most cases the vesicula seminalis also contained moderate amounts of sperm. The vitellaria had increased further in volume and the vitelline secretions were seen in the vitelline ducts and vitelline reservoir. In some flukes 1 or 2 thin-shelled, pale eggs were seen in the uterus on the ovarian side.

48 hours

The testes and vesicula seminalis were packed with sperm - the latter appeared orange. Sperm were seen in the ductus ejaculatorius. The glandular cells (prostate gland) surrounding the ductus were not very prominent but they contained globular secretions. Up to 27 normal, pale, thin-shelled eggs were seen in the uterus on the ovarian side.

4 days (Plate 132)

The posterior part of the fluke was filled with numerous eggs which formed a cup-shaped mass around and posterior to the ventral sucker. The eggs were pale and thin walled on the ovarian side and thick walled and darker on the antovarian side. The testes and vesicula seminalis were still packed with sperm.

Day 7

There was an increase in the number of eggs and those on the antovarian side appeared orange. Otherwise there was no further development.

Table 5I:1 M. similis: Dimensions of the adult according to Stunkard (1957)

(Fixed preparations)

	size range in mm.
Length	0.36 - 0.7
Width	0.22 - 0.36
Ventral sucker, diameter	0.048 - 0.065
Oral sucker, diameter	0.05 - 0.065
Pharynx, diameter	0.02 - 0.03
Male papilla, diameter	0.038 - 0.058
Testes	0.10 x 0.06 - 0.16 x 0.09
Vesicula seminalis	0.06 x 0.04 - 0.09 x 0.064
Ovary	0.08 x 0.05 - 0.01 x 0.062
Eggs	0.022 x 0.011 - 0.027 x 0.012

Discussion

Intra-peritoneal Infection

When excysted metacercariae were injected into the peritoneal cavity of mice, they were obviously recognised as foreign by the defence system of the host. Encapsulation and death of the fluke probably occurred quite rapidly since no eggs were seen in the dead flukes. This situation is in contrast to the abdominal stages of F. hepatica where, although the flukes may become surrounded by macrophages, they are not destroyed or apparently damaged.

Oral Infection

a) Recovery

The recovery of adults is quite low when compared to the dose. This is not because the mouse is an unsuitable experimental host since the recovery is similarly low in the natural definitive host (James, 1971).

This low recovery could be explained if the cysts passed through the host's intestine too rapidly to allow for complete excystation. However, as will be shown in Part II of this chapter, excystation occurs quite rapidly and it is more likely that a significant proportion of metacercariae excyst in the acid stomach of the host and are destroyed there. The low recovery rate is not a reflection of the viability of the cysts which is usually about 90% in vitro (see part II of this chapter).

However, a low infection rate could be an important device to ensure that the worm burden does not become too great since every time a gull eats a crab it may be exposed to thousands of cysts.

b) Development

The development of the metacercaria to maturity is a very rapid process. Spermatogenesis and oogenesis reach an advanced stage of development in the metacercaria and the precursors of the secretions produced by the vitellaria and prostate glands must have been available. A great deal of somatic and germinal growth and development undoubtedly occurs within the metacercarial cyst. In effect, the metacercaria only needs to be "switched on" by the environment found in the intestine of the final host in order to become fully mature.

Summary

1. Microphallus similis has a progenetic (see Footnote to page 10) metacercaria which produces sperm within 24 hours and shelled eggs after 1-2 days post-infection in its final host. The vitellaria are positive for egg shell precursors after 18 hours and large numbers of eggs are present by Day 4 post infection.

2. Although its natural definitive host is the gull (Larus sp.), a similar percentage infection can be obtained in laboratory hosts such as mice (James, 1971). In the mouse the level of infection is probably about 10% (recovery expressed as a percentage of dose).

3. Excysted metacercariae are unable to survive within the abdominal cavity of mice and are probably destroyed quite rapidly.

PART II: DEVELOPMENT IN VITRO

Introduction and Methods

Excystment of the metacercaria

The technique used to excyst the metacercariae of *Microphallus similis* prior to *in vitro* cultivation has been described in the General Methods. In unpublished results, Munroe (1975) has studied the roles of the digestive enzymes pepsin and trypsin in the excystment process and has investigated the effects of various stimuli which are known to trigger excystation in other species. This author considers excystation to be a four stage process; the first two stages require the presence of pepsin at a low pH, the third requires the presence of trypsin and the final stage is achieved by the parasite itself.

Stage 1: Shedding and partial digestion of cyst wall layer 4.

Stage 2: The swelling and digestion of cyst wall layer 3.

Stage 3: The digestion of cyst wall layer 2.

Stage 4: The rupture of cyst wall layer 1 by active movements of the parasite using, in particular, the oral sucker. (An enzymic secretion produced by the parasite may also be involved).

Munroe found that various osmotic gradients, the presence of bile or bile salts and high CO_2 concentrations were all unable to trigger excystation.

Interpreting these results, the excystation of the metacercariae of *M. similis* appears to be a passive process - with the possible exception of the final stage - requiring the presence of host digestive enzymes.

Using the technique for excystation described in the General Methods about 90% of the metacercariae excysted.

Cultivation of the metacercariae

The techniques employed in the cultivation of M. similis metacercariae have been described in the General Methods. The cultures were examined daily and a record was kept of the flukes' development and activity. Flukes were removed from culture at various intervals to study the development of the genitalia and associated gland cells.

Results (Plates 128,129 & 133)

Except where otherwise stated, the metacercariae were cultured in 15mls. of media in Leighton tubes which were incubated at a temperature of 40-41°C in a stationary water bath. The media was gassed with the aerobic gas mixture and it was changed after a week.

Only a small number of experiments were performed and the results are tabulated in Table 5II:1. The number of days the flukes survived in vitro (Duration) is recorded. The development of the metacercariae in vitro is described according to various criteria:-

1. The day when the vitellaria become positive for egg-shell precursors (i.e. positive when treated with Fast Red Salt B or with Catechol.)
2. The day when sperm are first seen in the testes.
3. The day when sperm are first seen in the vesicula seminalis.

4. The day when the vitelline reservoir is formed.
5. The day when normal eggs are first produced.
6. The maximum number of normal eggs produced.
7. The maximum number of abnormal eggs produced.
8. The day oviposition first occurs.

For reference, the developmental pattern *in vivo* is included at the end of the table.

Table 5II: 1

Medium	Duration (days)	Criteria * 1	2	3	4	5	6	7	8	Comments
Hanks saline alone	4	1	1	2	3	3	6	8	-	Only one worm with any eggs. Flukes became dark, sluggish and curled up.
Hanks saline + 20% FCS (Flow)	14	1	1	2	2	2	70	70	5	Generally active and clear. Vitellaria and reservoir contained tanned material.
NCTC alone	4	1	1	2	3	3	5	8	-	More flukes have eggs than in Hanks alone, slightly more active.
NCTC + 20% FCS (Bio-Cult)	12	1	1	3	3	-	0	10	5	Only abnormal eggs produced. When some flukes were transferred to medium with Flow FCS on Day 3 they produced up to 60 normal and 63 abnormal eggs
NCTC + 20% (Flow) FCS (aerobic or anaerobic gas)	10	1	1	2	2	2	33	82	5	Generally active and clear

*see page 300 .

Table 5II: 1 (continued)

Medium	Duration (days)	Criteria 1	2	3	4	5	6	7	8	Comments
NCTC + 20% Chick serum (Flow, Batch B)	3	-	2	2	-	-	-	-	-	Sluggish, dark, curled. Very few produced sperm.
NCTC + 20% FCS (Flow) + coagulated Calf serum base. Small Falcon flasks	8	2	1	2	3	2	12	34	-	Do not burrow into base or become attached to it.
Development in vivo in the mouse	?	1	1	1	1	1	100s	-	?	Are not curled like cultured flukes, are more active

Discussion

General Appearance

Unlike flukes recovered from mice, the cultured flukes assumed a very characteristic abnormal shape. This was achieved by a ventral curling of the lateral and anterior edges of the body and a general contraction. This is clearly demonstrated by scanning electron microscopy (see part IV of this chapter, Plate 195). Because of this contraction, the cultured flukes often appeared rather dark. In some media, this darkening of the body was real and not just apparent, it was probably due to an accumulation of lipid droplets in the degenerating cells.

The "tegumental bubbles" seen in cultured F. hepatica and Meiogymnophallus minutus were frequently seen over the whole body surface of M. similis but particularly in the anterior part and around the oral sucker.

The flukes did not attach themselves to the culture vessel by means of their suckers and although they showed vigorous activity this was often only spasmodic.

When a solid base was used, the flukes did not penetrate it, neither did they become attached to it or feed on it.

Egg production

Normal egg production only continued for one or two days. Normal eggs were oval; the ovum with a nucleolus occupied about two-thirds of the egg and at one pole there was a cluster of yolk granules. The whole was enclosed in a shell which was originally quite pale in colour but became progressively darker. However, subsequently, the eggs became abnormal (see Plate 133). There were three types of abnormal eggs:-

1. An ovum with an associated mass of tanned vitelline material but with no shell.
2. A shelled mass of tanned vitelline material with no associated ovum.
3. An unshelled mass of tanned vitelline material with no associated ovum.

This situation may have arisen because the medium was in some way deficient in precursors or other elements necessary for normal oogenesis and so only a small number of ripe ova were produced. It could also be explained if the vitelline secretions became tanned before they were associated with an ovum. The latter explanation is probably more likely since unshelled ova which were not associated with vitelline cells were often seen in the uterus.

Egg production is a very complex process requiring the precisely controlled interaction of a variety of factors, i.e. the release of an ovum, fertilization, the production and release of Mehlis' gland and vitelline secretions (Smyth & Clegg, 1959). If any part of the process is disturbed normal egg production will be affected.

The eggs that were produced during the first few days appeared normal in every way although they were not embryonated, this may indicate that they were not fertilized. Stunkard (1957) records that when the ova of M. similis are oviposited in vivo they are embryonated.

Spermatogenesis

Some degree of spermatogenesis occurred in all the cultures that were investigated, however deficient the media were in nutrients. In those cultures that continued to survive after 10 days the amount of sperm in the testes and vesicula seminalis probably decreased. The sperm in the vesicula seminalis and ductus ejaculatorius could be seen to be moving vigorously. It appears then, that, unlike oogenesis, the requirements for spermatogenesis are fulfilled in the culture media.

Vitellogenesis

During the early stages of in vitro culture, vitellogenesis was apparently normal. In most media the vitellaria became positive for egg-shell precursors on Day 1 as in vivo. However, as with Meiogymnophallus minutus, the vitelline secretions in the vitellaria and vitelline reservoir became prematurely tanned after about 5 days in vitro. Similarly, when the medium was gassed with a mixture of gasses which contained no oxygen, this did not prevent the premature tanning of the vitelline secretions or the production of abnormal eggs (see Chapter 4, part II for a discussion of premature tanning in vitro).

Comparison of media

Normal and continuing development of the metacercariae seemed to rely on some factor or factors present in the serum component of the culture media. Although a certain amount of development occurred in simple or complex media without serum, the flukes rapidly degenerated and died. It seemed, however, that once this serum requirement was fulfilled the synthetic medium component did not necessarily have to be a complex one. Indeed, in Hanks saline plus foetal calf serum the flukes survived for a longer time and produced a larger number of normal eggs than when the complex defined medium NCTC 135 was used.

As with F. hepatica, the developing metacercariae seemed to be very sensitive to different types and batches of serum. The chick serum which produced optimum development when used in cultures of Meiogymnophallus minutus rapidly killed the metacercariae of M. similis. The two different batches of foetal calf serum that were used in the cultivation of .. M. similis produced very different results. It seemed that the foetal calf serum supplied by Bio-Cult laboratories was in some way inhibitory to oogenesis since no normal eggs were produced. However, when flukes cultured in this serum were transferred to a medium containing foetal calf serum from Flow laboratories, they began to produce normal eggs.

Further experiments

The above experiments were of a preliminary nature and, using these results as a basis, further experiments could be designed.

For example:-

1. Various supplements such as embryo extract, egg products or hormones could be used in the culture media in an attempt to obtain continuing, normal oogenesis *in vitro*.

2. Feeding could be studied *in vitro* using a variety of foodstuffs e.g. macerated serum, blood cells, monolayer cells.

3. The effects of bile or bile salts *in vitro* could be studied.

4. Attempts could be made to mimic more closely the physical environment of the fluke's natural habitat.

5. It would be of great value to determine whether the apparently normal eggs produced *in vitro* are capable of embryonation and subsequent continuation of the life cycle.

6. Further attempts to solve the problem of premature tanning of the vitelline secretions *in vitro* may include the use of reducing agents or the use of a circulatory system (e.g. The "Lift") which is continuously gassed with an anaerobic gas.

Summary

1. In a serum containing medium, the metacercariae of *Microphallus similis* were capable of spermatogenesis, vitellogenesis and, probably, oogenesis.

2. The flukes survived up to two weeks *in vitro* and produced a maximum of 70 normal eggs.

3. Oviposition occurred *in vitro*.

4. The vitelline secretions in the vitellaria and vitelline reservoir became prematurely tanned after about 5 days *in vitro*.

5. Normal eggs were produced during the first one or two days of egg production only. Subsequent egg-production became abnormal and clumps of tanned vitelline material with or without an associated ovum were frequently seen.

6. Cultured flukes assumed a characteristic, abnormal shape.

7. Various further experiments based on these results are suggested.

PART III: ULTRASTRUCTURE IN VIVO

Introduction and Methods

The aim of the present study was to examine the normal development of the various organ systems of the metacercaria of Microphallus similis to the adult stage at an ultrastructural level. These observations can then be used as a means of assessing the development of the metacercaria in vitro (this will be dealt with in part IV of this Chapter).

The only published work dealing with the ultrastructure of Microphallids appears to be that of Stanier et al (1968) describing the tegument and metacercarial cyst formation in Spelotrema sp. encysted in Carcinus maenas.*

The organ systems examined in the present study were:-

1. The tegument.
2. The digestive caeca.
3. The parenchyma.
4. The excretory system.
5. The female reproductive system.
6. The male reproductive system.

In addition, the surface features of the excysted metacercaria were examined using the scanning electron microscope.

* Footnote.

Spelotrema is now believed to be synonymous with Microphallus and the species referred to by Stanier et al (loc.cit.) is probably M. primus.

The techniques used for processing flukes for scanning and transmission electron microscopy have been described in the General Methods (Chapter 2). Excysted metacercaria and adults removed from mice, four days post infection, were processed for transmission electron microscopy, and excysted metacercaria for scanning electron microscopy.

Results

TRANSMISSION ELECTRON MIGROSCOPY

The Tegument

A. METACERCARIA

a) The Syncytium

The surface of the metacercaria was covered by a moderately electron dense syncytium which was connected to the underlying tegumental cell bodies by fine protoplasmic strands which passed between the peripheral musculature. There appeared to be three morphologically distinct types of surface: the anterior surface, the posterior surface and the lateral surfaces posterior to the ventral sucker.

The anterior surface (Plate 134) was characterised by the regular array of large spines which were "toothed" at their tips. These spines had a typical crystalline structure, they pointed posteriorad and their bases were embedded in pockets of the basal lamina. At the base of the spines was a more electron dense layer. The tip of the spine had a fringed or toothed appearance with up to 13 "teeth". There were two types of granule in the outer syncytium; one was biconcave, the other was spherical.

The biconcave granules were membrane-bound, about 0.2um in length with dense contents. They were scattered throughout the whole syncytium but were especially frequent in the apical part where they were often aligned at right angles to the outer membrane. The spherical granules were membrane-bound and measured 0.1-0.2um in diameter. They possessed a variety of contents, ranging from virtually empty to quite electron dense. In many cases the contents of the granules were contracted into the centre producing an electron lucent peripheral region. These granules were distributed throughout the whole syncytium but were most common in the basal part.

Small spherical or rod-like mitochondria were scattered amonst the spherical granules in the basal part of the syncytium. Their ground substance was of a similar density to that of the syncytium. The surface of the syncytium was irregular, but in many areas it appeared quite flat. The basal lamina was undulating and quite thick and there were few or no basal invaginations. The thickness of the underlying, fibrous interstitial material varied - in places it was up to 0.7um.thick.

The syncytium covering the posterior part of the fluke was similar to that of the anterior except that the surface was more folded and the spines were smaller and less frequent without the toothed tips.

Just posterior to the ventral sucker, on each side of the fluke, were two morphologically distinct areas of tegument (Plate 135). In these regions, the surface was thrown up into folds which were themselves folded. No spines were

seen in this region and the basal lamina possessed many invaginations. In all other respects the syncytium in this region was similar to that which covered the anterior part of the fluke i.e. it possessed the usual secretory granules and mitochondria.

b) Tegumental cell bodies (Plates 134, 136, 137)

The tegumental cell bodies were situated below the peripheral musculature. They were irregular in shape and possessed nuclei with a moderately dense ground substance and irregular patches of heterochromatin which often adhered to the nuclear envelope. The cytoplasm of the cell bodies contained many free ribosomes and frequent Golgi complexes with dilated cisternae; GER was not present. The granules present in the cell bodies were similar to the spherical granules of the syncytium. They were membrane-bound, 0.1-0.2um. in diameter and they showed a variety of contents which were frequently condensed into the centre of the granule.

The protoplasmic strands connecting the cell bodies with the syncytium were long and slender; their connection with the syncytium was only infrequently seen.

c) Associated cells: "Mitochondria cells" (Plate 135)

Underlying the modified lateral areas of syncytium were groups of cells which contained only mitochondria and no other recognisable cellular organelles apart from nuclei. Processes from these cells ran upwards towards the syncytium but no unequivocal connections with the syncytium were seen. The mitochondria packing these cells had an electron lucent matrix and up to ten cristae depending on the size of the mitochondrion. They ranged from 1.5 to 3.0um. in length and were usually spherical or "sausage-shaped".

In some cases, the cytoplasmic processes from the cells appeared to unite and form a thin, dense layer underneath the basal lamina of the syncytium but above the layer of interstitial material.

d) Associated cells : Gland cells (Plate 134, 136)

Underlying the tegumental cell bodies in the anterior region of the fluke (anterior to the ventral sucker) were the cell bodies of numerous unicellular gland cells. These were virtually identical with those previously described for the metacercariae and adults of M. minutus (pages 233 and 234). The cell bodies were packed with electron-dense, membrane-bound, secretory bodies which varied in size but measured up to 0.4um. in diameter. In some instances, the secretory bodies were spherical but they often appeared to be rather irregular in shape, probably due to compression. Nuclei were occasionally seen in the gland cells but more frequently there were no other recognisable cellular

organelles and the cytoplasm had a granular appearance. Long projections or ducts arose from these cells, they passed between the tegumental cells and the peripheral musculature and penetrated the syncytium where they opened to the outside of the fluke. The ducts from the gland cells were lined with a ring of microtubules which were aligned parallel to the walls of the ducts. When the ducts were cut in cross section the peripheral ring of microtubules was clearly seen. The duct of the gland cell was connected to the syncytium by a desmosome.

No secretory material - either as secretory bodies or as a diffuse mass - was seen on the surface of the fluke. Developing gland cells were never seen, all the cells appeared to be mature.

B. ADULTS

a) <u>The Syncytium</u> (Plates 148, 149)

The syncytium of the adult was similar in most respects to that of the metacercaria. The three morphologically different surfaces were still present. The main difference was in the number and type of secretory granules. Very few spherical granules were seen and these were mainly found in the basal part of the syncytium. The bulk of granules in the syncytium were of the biconcave type. The mitochondria were scattered throughout the syncytium but were often found in groups, their ground substance was more electron dense than in the metacercaria and they were spherical or rod-shaped.

b) Tegumental Cell Bodies (Plate 150)

As in the metacercaria, onlyspherical granules were found in the tegumental cells. However, in the adult, these seemed to fall into two separate populations. The first type (Type A) was spherical, membrane-bound, about 0.2um. in diameter with homogenous, dense contents which were often concentrated into the centre of the granule to produce a lighter area around the periphery. The second type of granule (Type B) was smaller, more oval in shape and contained clumps of dense, granular material.

Many free ribosomes were present in the cytoplasm of the cell bodies but there was no GER. The sacs of the Golgi complexes were considerably dilated. Mitochondria were only infrequently seen. The nuclei were similar to those of the metacercaria except that the patches of heterochromatin were larger. Protoplasmic connections with the syncytium were infrequent.

c) Associated cells: "Mitochondria cells"

These cells were identical to those already described for the metacercaria except that the ground substance of the mitochondria was more electron dense enabling the cristae to be seen more clearly.

d) Associated cells: Gland cells

The tegumental gland cells were identical with those previously described for the metacercaria. Secretory products were never seen on the outside of the worm.

Parenchyma cells

A. METACERCARIA

Large numbers of parenchymal cells were present between the various organ systems particularly in the anterior part of the fluke. The parenchymal cells were quite large with an irregular outline and ramifying processes. Nuclei were only infrequently seen, indicating that the cells had a large volume. The nuclei were usually located in dense areas of cytoplasm, they had a moderately dense ground substance and some dense patches of heterochromatin.

The majority of parenchyma cells had a granular cytoplasm, many of them - especially those amongst the tegumental cell bodies and those around the limbs of the digestive caeca - contained large stores of ∝ glycogen. Occasional areas of free ribosomes were seen in the cytoplasm, these were often associated with membraneous whorls or With mitochondria. The mitochondria were often quite large (up to 2um. long) and were irregular in shape, although their cristae were usually short and infrequent.

A thin layer of interstitial material was present between adjacent parenchymal cells. Junctional complexes between parenchymal cells were very rare and were only seen on a few occasions; they were never seen between parenchymal cells and the cells of the other organ systems.

B. ADULTS

The parenchymal cells of the adult were very similar to those of the metacercaria. Stores of glycogen were still present although adjacent parenchyma cells often had very different types of ground substance (Plates 160, 161). There were occasional junctional complexes between the parenchymal cells and the cells of the digestive caeca.

Digestive Caeca

A. METACERCARIA (Plate 138)

In the metacercaria the lumen of the digestive caeca was packed with an irregular dense material and occasionally some small lipid droplets.

The caecal epithelium was one cell thick. Adjacent caecal cells had undulating boundaries, the cells were connected near the luminal surface by a prominent thick desmosome which often had lengths of GER in close proximity to it. The nuclei were basal, they were irregular in shape with a moderately electron dense ground substance and dense patches of heterochromatin which often adhered to the nuclear envelope. The cytoplasm of the caecal cells showed two different types of morphology. There were areas of cytoplasm which were relatively electron lucent and contained stores of ∝ glycogen and occasional mitochondria and lipid droplets. Interspersed with these areas were areas of denser cytoplasm. These denser areas were often associated with the nucleus; they contained free ribosomes, GER and small mitochondria. The surface of the caecal cells was thrown up into short lamellae, 1.0-1.5um. in length. Adjacent lamellae were of a similar length

and were closely pressed together. There was a fine layer of dense material between adjacent lamellae. No secretory bodies were seen in the cells of the caecal epithelium of the metacercaria although small lipid droplets (usually less than 1.0um. in diameter) were occasionally present. The basal membrane was much invaginated and there were many short stumpy parenchymal processes which projected into the caecal cells. These processes often contained stores of ∝ glycogen. There were, however, no junctional complexes between the parenchymal process and the caecal cells. The caecal limbs were surrounded by a layer of interstitial material. There were many muscle cells around the caeca and they appeared to run in a variety of different directions.

B. ADULTS (Plates 151, 152, 153)

The cells of the digestive caeca of the adult were similar to those of the metacercaria in many respects, but there were certain important differences:-

a) The patches of cytoplasm containing stored ∝ glycogen were no longer present.

b) Secretory granules were present in the caecal cells and they appeared to be of two types (Plate 152). Small numbers of spherical, membrane-bound, dense granules measuring about 0.15um. in diameter were seen scattered throughout the cytoplasm. In addition, there was a larger type of granule which was more common and had less dense contents, it measured about 0.3um. in diameter. The fine structure of the larger granules was very difficult to determine.since they were of a similar density to the ground substance of the caecal cells.

They may have been fragments of dilated GER.

c) Large lipid droplets, 1-2um. in diameter, were frequently seen in the cytoplasm (Plates 151, 153).

d) Processes arising from the parenchymal cells were occasionally connected to the caecal cells by junctional complexes (Plate 152).

e) The mitochondria in the epithelial cells were denser and more frequent than in the metacercaria.

f) The lumen was virtually occluded and was relatively empty, although some areas of granular material were seen (Plate 153).

g) The surface lamellae were longer (up to 2um. in length). The relationship between the adjacent lamellae could be seen more clearly. The lamellae were bounded by the usual trilaminate membrane, and, in places, the narrow space between the surface membranes of adjacent lamellae was filled with a dense material of similar density to the smaller secretory bodies (Plate 153). In areas where the lumen was narrow the lamellae from opposite sides of the caecal limbs appeared to interdigitate. (The arrangement of the lamellae may, however, be more complex than this).

h) No definite Golgi complexes were seen although the small dense secretory bodies were often clustered together.

Excretory System

A. METACERCARIA

The excretory system of the metacercaria consisted of flame cells which were connected, via collecting ducts, to the bilobed excretory bladder.

a) Flame Cells (Plate 139)

The flame cells, whilst conforming to the basic pattern described for other trematodes (Kummel, 1960; Wilson, 1969; Gallagher & Threadgold, 1967; Bennett, 1973), showed an unusual modification in their morphology.

The cell body of the flame cell appeared to consist of two separate parts which were connected by cytoplasmic strands. The main part of the cell contained the nucleus; the cytoplasm of this part contained free ribosomes, GER and mitochondria. It was connected by strands of cytoplasm to that part of the flame cell which contained the basal bodies of the cilia of the "flame". This part of the flame cell body was cup-shaped and although most of the cytoplasm was finely granular, the peripheral cytoplasm contained some ribosomes, mitochondria and microtubules. The typical ciliary basal bodies were embedded in the apical cytoplasm of the cup-shaped portion of the cell. They possessed slight dense swellings on their outer surface. The striated roots were branched and tapered off into the cytoplasm.

The cilia of the 'flame' were arranged in a hexagonal pattern. They had the typical 9+ 2 arrangement of micro-tubules with 9 outer doublets and a pair of central filaments. There were many internal leptotriches. External leptotriches arising from the conjoined tubule cell were not present although projections pointing towards the barrel of the flame cell did arise from the periphery of the cup-shaped portion of the flame cell body. The interdigitating microvilli at the junction of the flame cell and the conjoined tubule

were similar to those described for the metacercaria of F. hepatica by Bennett (1973) (see page 121). The cell of the conjoined tubule appeared to have an irregular surface near its junction with the flame cell. The cytoplasm of the cell of the conjoined tubule contained ribosomes, GER and mitochondria; the nucleated parts of the cell were not observed during this study. The whole flame cell was embedded in a thick layer of non-fibrous interstitial material.

It is probable that the nucleated part of the flame cell body is displaced to one side of the cup-shaped portion since it was only seen in oblique sections through the flame cell - in longitudinal sections it was never seen (see Plate 194).

b) Collecting ducts (Plate 141)

The collecting ducts were lined by one or, more probably, two cells joined by a desmosome. The cytoplasm was dense and contained many free ribosomes. The nuclei of these cells were rarely seen but on several occasions they were observed embedded in the adjacent parenchyma. The nucleus had a moderately dense nucleoplasm, dense patches of heterochromatin and a dense, oval nucleolus.

The apical surface of the collecting duct was thrown up into lamellae which were similar to those seen in the excretory bladder, but they were less frequent and shorter; they were often pressed against the side of the duct. Cilia or basal bodies were not seen in the collecting ducts.

Occasionally lipid droplets were seen in the lumen of the ducts. The ducts were surrounded by a layer of interstitial material. There were frequent invaginations of the basal membrane and these often contained interstitial material.

c) Excretory bladder (Plate 140)

The excretory bladder of the metacercaria was lined with cytoplasm of varying thicknesses. The cytoplasm was quite dense with large quantities of GER which was often arranged in parallel arrays or in whorls. The nuclei were prominent and often projected into the lumen of the bladder, they were similar in appearance to the nuclei of the collecting ducts. The basal plasmalemma had large numbers of invaginations. The apical surface was thrown up into long lamellae which were sometimes branched; they often filled the lumen. Occasionally, lipid drops were seen in the process of extrusion into the lumen. The surrounding interstitial material was apparently quite thin. Muscle cells were seen around the periphery of the excretory bladder but they did not form a continuous coat.

Although the lining of the excretory bladder was probably syncytial the possibility of it being epithelial in nature could not be ruled out.

B. ADULT (Plate 160)

The flame cells, collecting ducts and excretory bladder of the adult were similar to those described for the metacercaria, except that large quantities of lipid droplets were seen in the lumen and in the cytoplasm of the excretory bladder.

Female Reproductive System

A. METACERCARIA

Mehlis' Gland

The cells of the Mehlis' gland could not be distinguished in the metacercaria. Undifferentiated cells were seen in various parts of the body and some of these probably represented the undeveloped cells of the Mehlis' gland.

Vitellaria (Plate 144)

Immature vitelline cells were irregular in outline. They contained a prominent nucleus with a granular ground substance, a dense oval nucleolus and dark patches of heterochromatin which often adhered to the nuclear membrane. The cells were packed with free ribosomes, some GER (which was often arranged in parallel arrays or in whorls), frequent Golgi complexes and occasional electron lucent mitochondria. Very occasionally, clusters of vitelline droplets were seen but the most prominent cytoplasmic organelles were the free ribosomes.

Uterus (Plate 142)

The lining of the uterus was cellular and the nuclei projected into the lumen. The cytoplasm around the nudeus and near the luminal surface of the uterus was dense and it contained free ribosomes, lengths of GER and occasional mitochondria. The bulk of the cytoplasm of the cells was granular and contained stored ∝ glycogen and some mitochondria. The apical surface of the uterus was thrown up into irregular, finger-like processes which were often branched; they contained free ribosomes and appeared electron

dense. Adjacent cells were connected at their apical surfaces by a short desmosome. The uterus was surrounded by a layer of interstitial material which often extended into the frequent basal invaginations of the plasmalemma. There were no parenchymal processes or junctional complexes with the parenchyma cells.

The ootype had the same basic structure as the uterus except that the areas of dense cytoplasm with free ribosomes predominated. The desmosomes connecting adjacent cells were longer and thicker than in the uterus. The lumen of the ootype contained cilia with the typical "9 + 2" arrangement of filaments. The cilia were not arranged in any regular pattern (as in the flame cell). Basal bodies were occasionally seen embedded in the cytoplasm of the cells lining the ootype. The apical surface of the cells was irregular with finger-like projections and lamellae. In addition, there were thicker projections of the cytoplasm which were often cut in cross-section. The basal plasmalemma of the ootype had more invaginations than that of the uterus. The ootype was surrounded by a thick layer of interstitial material and in several places the muscular coat appeared to be continuous.

Ovary (Plate 145)

The cells of the ovary appeared to be of one basic type. They were spherical in outline with a spherical or oval nucleus which had a granular matrix, dense patches of heterochromatin, and, occasionally, a spherical nucleolus. The cytoplasm of the cells was filled with free ribosomes. The mitochondria, which were usually spherical in shape, were frequently clustered

together in small groups, they had a heterogeneous content and 1-4 cristae. Golgi complexes were present but GER was rarely observed. The developing germ cells were separated from each other by the cytoplasm of the nurse cells which contained some short lengths of GER, free ribosomes and small dense mitochondria. The nuclei of the nurse cells were rarely observed but, when they were present, they were located around the periphery of the ovary. The ovary was surrounded by a thick basement membrane and a layer of interstitial material. There did not appear to be a continuous muscular coat.

No dividing cells were seen in the ovary of the metacercaria and no synaptonemal complexes (see page 253) were seen in the nuclei of the cells. The presence of the nucleolus indicated that the cells were in interphase since the nucleolus disappears during cell division. It is not possible to determine, from this information, which stage of oogenesis the cells present in the ovary represent.

A. ADULT

Mehlis' Gland (Plate 161)

The cells of the Mehlis gland were seen rather infrequently. They were located in the region of the ootype although ducts leading from the cells into the ootype were not recorded. The cells were irregular in shape; they possessed a nucleus, GER, free ribosomes, small Golgi complexes and secretory bodies. The secretory bodies were in the form of irregularly shaped sacs measuring up to 0.3um. in length. The sacs often appeared empty although some dense material was often seen adhering to the internal surface of the

bounding membrane.

Vitellaria (Plate 162)

The vitellaria of the adult contained cells in various stages of development.

(i) Immature cells

These cells had an electron-lucent cytoplasm with occasional free ribosomes and mitochondria. No secretory bodies were present.

(ii) Developing cells

These cells were similar to the vitelline cells of the metacercaria although they contained varying amounts of vitelline droplets. The vitelline droplets were usually spherical and were approximately 1.0um. in diameter; they were composed of aggregates of dense secretory bodies. It is uncertain whether or not these secretory bodies were membrane-bound.

(iii) Mature cells

In these cells, large quantities of vitelline and lipid droplets had almost replaced the cell cytoplasm although some patches of GER, free ribosomes and mitochondria were occasionally seen.

Ovary (Plate 163)

The ultrastructure of the ovary of the adult was not studied in great detal.

Most of the cells of the ovary seemed to be similar in morphology. They were irregular in shape and possessed a prominent, roughly oval nucleus with a granular ground

substance, irregular patches of heterochromatin and a dense granular nucleolus. No dividing cells or synaptonemal complexes were seen. The density of the cytoplasm varied; it was finely granular, probably consisting of small clusters of ribosomes. There appeared to be some short lengths of GER but these were infrequent. Golgi complexes were not seen. Mitochondria were scattered throughout the cytoplasm, they were irregular in shape, their ground substance was heterogeneous and generally less dense than the surrounding cytoplasm; depending on their size, they possessed 1-3 cristae. The basement membrane and the surrounding interstitial material and musculature were all similar to those of the metacercaria.

The dense cortical granules seen in the oocytes of Meiogymnophallus minutus (page 241) and in F. hepatica by Gresson (1964) and by Björkman & Thorsell (1964a) were not seen in M. similis.

Male Reproductive System

A. METACERCARIA

Prostate Gland

The cells of the prostate gland were not observed in the metacercaria.

Testes (Plates 146 & 147)

The cells of the testes rested on a thick basement membrane which was surrounded by a thin layer of interstitial material and a thin, continuous muscular coat.

The germ cells were surrounded by the cytoplasm of the nurse cells which were finely granular with oocasional mitochondria. The nuclei of the presumed nurse cells were very rarely seen. On the periphery of the testes were groups of small cells with a relatively dense cytoplasm and nucleoplasm. These cells contained mitochondria and occasional Golgi complexes, their granular cytoplasm probably cons isted of small clusters of ribosomes. The nuclei of these cells contained frequent synaptonemal complexes (Plate 146).

The cells packing the rest of the testes were large and irregular. The nuclei of these cells were large, their nucleoplasm was less electron dense and they contained small, dense areas of heterochromatin and frequent synaptonemal complexes. The nuclei were cut obliquely on several occasions and the nuclear pores were clearly visible (Plate 147). The cytoplasm of these cells was also less dense; it contained mitochondria, small aggregates of ribosomes, Golgi complexes, membraneous whorls and, occasionally, centrioles.

B. ADULT

Prostate gland

The cells of the prostate gland were not observed in the adult.

Testes (Plates 154-157)

The periphery of the adult testes was similar to that of the metacercaria - the basal lamina was thick and it was surrounded by a layer of interstitial material and a thin muscular coat. Around the periphery, usually in groups, were the smaller cells

with denser cytoplasm and nucleoplasm which have been described in the metacercaria. These cells were probably spermatogonia or young spermatocytes.

The central regions of the testes contained developing spermatocytes and spermatids and bundles of spermatozoa. Some spermatocytes were similar to those seen in the metacercarial stage and they appeared to be non-dividing; others possessed centrioles in preparation for cell division. In addition to this, some spermatocytes were seen joined together in a "cytophore" (Plate 154). The individual cells were joined by a short, fat stalk to a central mass of residual cytoplasm. These cells were often seen in the process of cell division and the dense chromatin could be clearly seen. Centrioles with associated spindle fibres were frequently seen in these cells (Plate 155). The ground substance of the developing spermatoctyes was finely granular and included clusters of free ribosomes. The mitochondria were dense and elongate and there were many large Golgi complexes. The mitochondria and Golgi complexes were usually found in the area between the nudear material and the residual cytoplasm of the cytophore (Plate 154).

Spermatids in various stages of development were also present (Plates 154-157), they were often associated together in a cytophore to form the typical "morula" of light microscopy. The development of the spermatidto the spermatozoon (spermateleosis) is described in detail in Part IV of this Chapter in connection with the ultrastructure of cultured flukes (page 387).

Bundles of sperm were seen in the central regions of the testes, around the periphery and packing the vesicula seminalis. Cross sections through sperm from the vesicula seminalis showed eleven quite different types of morphology (Plates 158 & 159), these are drawn semi-diagrammatically in Text Figure 11 and can be described as follows:

A. A laterally situated filament and a filament consisting of 9 or fewer disorganised outer doublets with no central core. The mitochondrion and peripheral microtubules were apparently absent.

B. A laterally situated nucleus and a normal 9 + 1 filament. Peripheral microtubules were present in some cross-sections but not in others. Mitochondria were sometimes present (C).

D. A nucleus, a normal 9 + 1 filament and a second filament with 9 or fewer disorganised doublets and no central core. (This was only observed on two occasions and, in each case, the mitochondrion and peripheral microtubules were lacking.)

E. Two normal lateral 9 + 1 axial filaments, a central nucleus and mitchondrion and two groups of peripheral microtubules. The number of microtubules varied from 3 - 10 and they were sometimes present in 2 or 3 rows.

F. Two lateral 9 + 1 filaments and a central mitochondrion. The peripheral microtubules were as in E.

G. Two lateral filaments. The peripheral microtubules were either as in E. (Ga) or they were absent (Gb).

H. A 9 + 1 lateral filament and a second lateral filament which had a 9 + 1 configuration but was condensed (0.15um. cf. 0.20ùm. in diameter) and had a thick band of dense material between the central core and the peripheral doublets. The central core was often smaller than in the normal axial filaments. Mitochondria and peripheral microtubules were usually lacking.

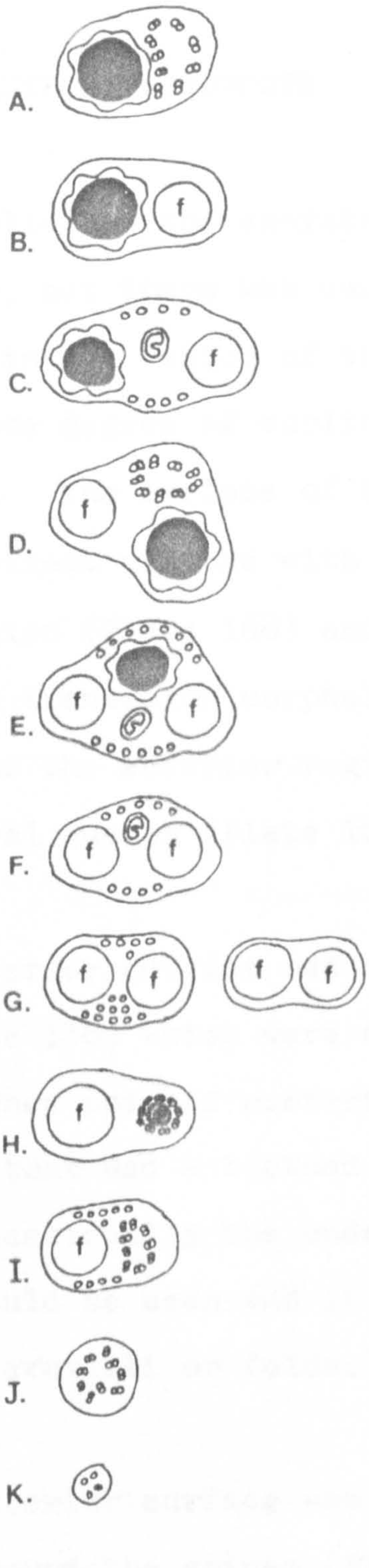
I. A 9 + 1 lateral filament and a second filament with 9 or fewer disorganised outer doublets and no central core. Mitochondria were absent but one or two rows of peripheral microtubules were usually seen.

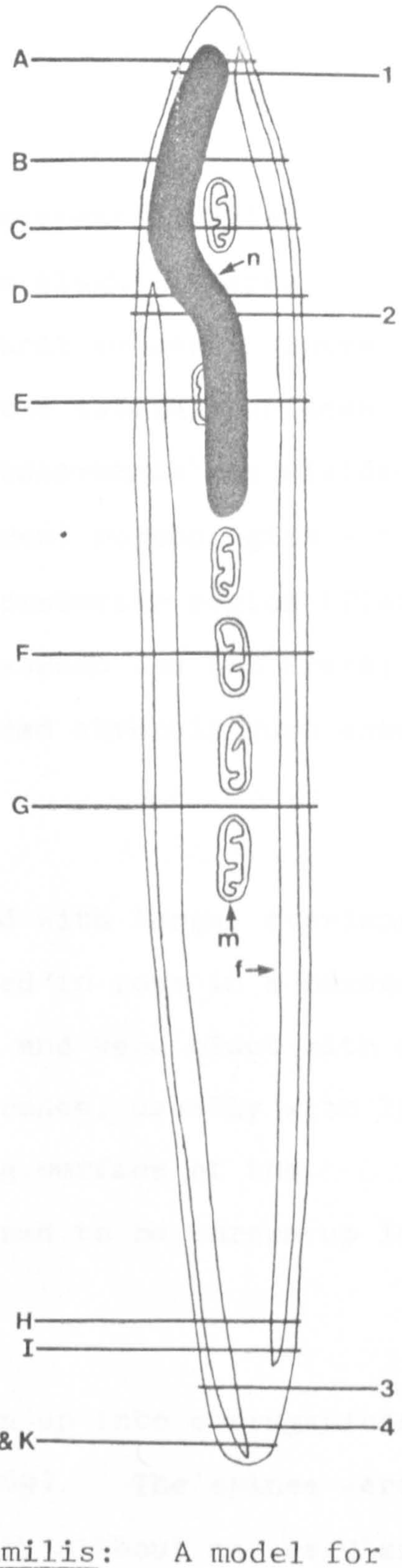
J. One filament with 9 or fewer disorganised doublets and no central core.

K. Various numbers of single microtubules.

The fine structure of the typical axial filament can be seen in the inset to Plate 189. It consisted of 9 outer doublet microtubules with two short arms projecting from one of the tubules. The arms all pointed in the same direction i.e. all clockwise or all anticlockwise. The central complex consisted of a dense central core surrounded by an electron lucent medullary region and a dense sheath. The central complex was connected to the 9 outer doublets by 9 radial "spokes" which were often rather indistinct. The sperm nucleus was usually

quite electron dense although occasionally the strands of dense chromatin were interspersed with lighter areas. The membranes of the nuclear envelope often appeared ruffled. The mitochondrion was usually quite small and spherical. The ground substance of the mitochondrion was moderately dense and it was often difficult to distinguish the cristae. It was not clear whether there were a series of mitochondria along the length of the sperm or whether there was one long, continuous mitochondrion. Dense plaques, usually a pair on each side, were often associated with the inner surface of the plasma membrane of the sperm. The peripheral microtubules varied considerably in number. In some sections, microtubules were consistently absent (Types A,J,K) and in others they were observed on some occasions but not others (Types B,G,H). It appears from these observations that the microtubules are not continuous from one end of the sperm to the other. In some regions several rows of microtubules were seen (Types E,F,G,I).





Text Figure 11. Microphallus similis: A model for the fine structure of the spermatozoon incorporating the various different types of morphology seen in cross section (A-K). Cross sections at 1-4 were not observed. (See Plates 158 & 159).

Key.

f - filament
m - mitochondrion
n - nucleus

SCANNING ELECTRON MICROSCOPY

The outline of the excysted metacercaria varied considerably, but there was usually a slight lateral constriction in the region of the ventral sucker. There was often some degree of curling of the lateral surfaces (Plate 164). The surface of the metacercaria was divided into two distinct regions with different morphologies - the anterior region (Plate 168) and the posterior region (Plate 169). There was no transition morphology between the two areas; the morphology of the anterior region ended abruptly just anterior to the ventral sucker (Plate 166).

The anterior surface was covered with large, overlapping spines (Plate 188) which were arranged in rows in a hexagonal pattern. They pointed posteriorly, and were blunt with a rounded tip that had a toothed appearance, usually with 13-14 "teeth". Occasionally the underlying surface of the syncytium could be seen and it appeared to be thrown up into small irregular microvilli or folds.

The posterior surface was thrown up into corrugations which ran around the spines (Plate 169). The spines were small and peg-like and were apparently without any regular arrangement. Around the excretory pore there were no spines. The pattern of the surface corrugations in the posterior region was quite complex. On the ventral surface, the corrugations were shallow and usually circumferential. Just posterior to the ventral sucker there was a series of deep anterior-posterior folds. The lateral surface, just posterior to the ventral sucker, was particularly complex. It was thrown up into about

six deep circumferential folds. Each fold was divided by five or six shallower folds running in an anterior-posterior direction and the normal surface corrugations ran anterior-posteriorly. There were no spines in this region. (This lateral region of the tegument probably overlay the clusters of "mitochondria cells" seen in thin sections, (see page 314, Plates 129 & 135)). In the most posterior part of the fluke, the corrugations were less regular, although they appeared to converge in the excretory pore region.

The lip of the oral sucker bore no spines and was thrown up into radial corrugations (Plate 167). There were many sensory papillae around the oral sucker - these appeared to be of two types. One type was similar to the "ciliated receptors" and the other to the "domed receptors" recorded by Bennett (1975c) on the metacercaria of F. hepatica. The distribution of the sensory papillae on and around the oral sucker appeared to exhibit a bilateral symmetry. Sensory papillae of the ciliated type were scattered over the fore-body, they were less frequent on the hind-body.

The lip of the ventral sucker was covered with toothed spines and the cavity of the sucker was lined with a spine-free syncytium. Several sensory papillae - mainly of the "ciliated" type - were scattered around the ventral sucker. Posterior to the ventral sucker was a deep fold which extended round the right hand side of the sucker where it ended in a definite orifice which probably corresponded to the opening of the genital pore.

Discussion

The Tegument

The ultrastructure of the tegument of trematodes has been reviewed by Lee (1966, 1972) and by Lumsden (1975). In those species that have been studied the ultrastructure is similar, in basic pattern, to that originally described for Fasciola hepatica by Threadgold (1963a,b). The ultrastructure of the tegument of several species of trematodes have been described e.g. F. hepatica (Theadgold 1963a,b, 1967; Björkman & Thorsell, 1964b; Bennett & Threadgold, 1973, 1975), Schistosoma sp. (Senft et al, 1961; Morris & Threadgold, 1968; Smith et al, 1969; Hockley, 1973), Haematoloechus medioplexus (Burton, 1964; Bogitsh, 1971), Gorgoderina sp. (Burton, 1966), Acanthoparyphium spinulosum (Bils & Martin, 1966), Cyathocotyle bushiensis (Erasmus, 1967a), Posthodiplostomum minimum (Bogitsh & Aldridge, 1967), Megalodiscus temperatus (Bogitsh, 1968), Diplostomum phoxini (Bibby & Rees, 1971).

The basic structure of the tegument of Microphallus similis conforms to that described for F. hepatica by Threadgold (1963a,b). It consists of a surface syncytium (containing spines, mitochondria and secretory bodies) which is connected to the sunken cell bodies by protoplasmic strands. However, as with certain strigeoids (Erasmus, 1969a,b,c, 1970), M. similis possesses certain specialised areas of tegument.

The most common type of secretory body present in the tegument of those trematodes studied is the spherical or discoid type. These granules are present in the cell bodies and syncytium of M. similis metacercaria and adults. The function of these granules has not been clearly demonstrated. Burton (1964, 1966) and Wilson & Barnes (1974) have suggested that they break down within the syncytium to form the general ground substance, whereas Oaks & Lumsden (1971) and Erasmus (1967) have suggested that they contribute to the surface glycocalyx.

In the tegument of M. similis, there are two types of granules in the syncytium - spherical and biconcave. It is likely that these granules are, in fact, one population. Bennett (1973) also found these two types of granules in the syncytium of the metacercaria of F. hepatica. He suggests that the biconcave granules represent collapsed spherical granules whose contents have been secreted, either into the surrounding syncytium by a modified form of eccrine secretion (Kurosomi, 1961), or onto the surface to form a glycocalyx. Erasmus (1967) suggests that in Cyathocotyle bushiensis these two types of granules represent different sections through the same body. However, this is difficult to envisage in the present case since the distribution of the two types of granules is always the same regardless of the plane of section. One of the functions of the biconcave granules may be to contribute membrane to the outer plasmalemma thus allowing growth which, in M. similis, is considerable during the encysted stage. In the adult, where there is little growth, they may allow a considerable turn-over of the surface membrane. A similar function has been suggested

for the multilaminate vesicles in the tegument of S. mansoni (Hockley & McLaren, 1973; Wilson & Barnes, 1974).

In the metacercarial stage, the type of material enclosed within the spherical granules in the syncytium and the cell bodies varied considerably. Although these granules were very similar in shape and size they may have been very different in composition. It is also possible that they represented different stages in the secretion of their contents into the surrounding cytoplasm.

In the adult, syncytium, the tegumental secretory bodies were almost exclusively of the biconcave type. If the biconcave granules are collapsed spherical granules, this would indicate that once in the syncytium the spherical granules rapidly lose their contents. It may be inferred from this that, whatever functions the spherical granules fulfil, they play a more important role in the adult than in the metacercaria since the biconcave granules are more frequent in the syncytium of the adult.

The oval, granular secretory bodies seen in the tegumental cells of the adult were never observed in the syncytium and the role of these granules is not clear.

An unusual feature of the tegumental cells of the adults and metacercaria of M. similis was the absence of GER. The presence of a substantial GER system is a typical feature of the tegumental cell bodies of trematodes. In the present case, the ribosomes were not associated with membranes but were free in the cell cytoplasm. The Golgi complexes were prominent

in these cells and the typically flattened cisternae were much swollen although they usually appeared electron lucent and apparently empty.

The lateral tegument just posterior to the ventral sucker was modified from the basic pattern and cells, which apparently contained only mitochondria, were found amonst the tegumental cells. The surface of the syncytium in this region was different to the anterior tegument and the rest of the posterior tegument in that it was much folded and bore no spines. This modification of the posterior tegument was found in both the adult and the metacercaria.

The "mitochondria cells" undoubtedly sent up protoplasmic processes towards the syncytium but no unequivocal connections were seen between the cells and the syncytium. The syncytium in this region does not contain an unusually large number of mitochondria and although the protoplasmic processes leading from the cells contain mitochondria, they differ noticeably in size from those in the syncytium. In the event that the cells and the syncytium are connected, it seems extremely unlikely that the mitochondria in the cells are being "secreted" into the syncytium.

The role of these cells is very difficult to imagine. They have been observed by Bowers & James (1975) who suggest that they may be involved in providing energy for the enormous amount of secretory activity connected with egg production which occurs in this region. (The "mitochondria cells" are in close proximity to the vitellaria.) If this is so, it is remarkable

that similar cells have not been seen in other trematodes which produce large quantities of eggs. Also, the secretory process in itself does not have a very high energy requirement.

One possibility is that these cells may be specialised respiratory cells. It is interesting to note that this region of "mitochondria cells" occurs just posterior to the ventral sucker. When the fluke is attached to the host's intestine in its usual position (see page 288) this region would lie near the surface of the intestinal mucosa. There is evidence to suggest (Rogers, 1949; Crompton et al, 1965) that oxygen tension approaches zero in the lumen of the gut but is substantially higher in the region of the mucosa. If this is true, the fluke may have become adapted to the particularly high level of oxygen available in this region. The increased surface area of the overlying syncytium may present a large area over which diffusion of gasses could occur. Erasmus (1972) suggests that the respiratory rate of adult trematodes may be directly proportional to the oxygen available in the environment.

However, the validity of the interpretation of data obtained by oxygen electron probes has been questioned by various workers (cited by Mettrick & Podesta, 1974) for a variety of reasons. Work on the intestines of dogs (Hamilton et al, 1968), rats (Maggi et al, 1970) and cats (McIver et al, 1926) indicates that oxygen diffuses freely between the aqueous phase of the luminal contents and the intestinal mucosa and that such a steep pO_2 gradient does not exist. It would be of interest to determine whether other gut-living trematodes which are partially embedded in the intestinal mucosa, e.g. strigeoids, possess similar cells.

Another possible explanation as to the nature of these cells is that they are the cell bodies of the longitudinal muscle fibres which, for some reason, are grouped together in one region. From the micrographs, it is not possible to determine, with complete certainty, whether the protoplasmic processes from the "mitochondria cells" lead to the syncytium or to the peripheral musculature. However, if these cells were the cell bodies of the longitudinal muscles one would expect to find them as a band around the periphery of the fluke rather than as two separate clumps of cells. Also, this explanation does not account for the modification of the overlying syncytium.

The tegument of the anterior part of the fluke is also modified. It contains numerous unicellular gland cells which were never seen in the posterior part of the body. These gland cells appear to be fully-formed in the metacercaria and developing cells were rarely seen. It is likely that they release their contents to the outside of the worm by a holocrine type of secretion (Kurosomi, 1961).** Erasmus (1972) discusses the various types of secretory processes that have been described for trematodes. He suggests that the penetration gland cells of miracidia and cercaria and the lappet gland cells of strigeoids show a holocrine type of secretion.

** Holocrine secretion is defined as "the complete conversion or replacement of cell cytoplasm with secretory products so that the gland cells exhibiting this type of secretion have a restricted survival time terminating with the accumulation of secretion." (Erasmus, 1972)

The gland cells of Meiogymnophallus minutus and M. similis are similar in ultrastructure to gland cells associated with the general tegument or specialised areas of the tegument of other helminths e.g. the lappets of Apetemon gracilis and Diplostomum phoxini (Erasmus, 1969a, 1969c); the forebody of Posthodiplostomum minimum (Bogitsh & Aldridge, 1967); the bothria of pseudophyllidean cestodes (Öhman-James, 1973); the oral sucker region of Haplometra cylindracea and Opisthioglyphae ranae (Halton & Dermott, 1967); the ventral haptor and the forebody of Aspidogaster conchicola (Bailey & Tompkins, 1971; Halton & Lyness, 1971). Although all these gland cells have a remarkably similar ultrastructure, it must be appreciated that ultrastructural similarities do not necessarily imply similarities in chemical composition or in function. Three possible functions for the secretions produced by these tegumental gland cells have been suggested:-

a) that of adhesion to the host,

b) that of extracorporeal digestion, and

c) that of protection against host digestive enzymes.

The forebody gland cells of Posthodiplostomum minimum (Bogitsh & Aldridge, loc. cit.) and the lappett gland cells of Diplosomum and Apetemon (Öhman, 1965, 1966) are positive for esterases and Bogitsh & Aldridge (loc. cit.) and Lee (1962) suggest that the secretions produced by these glands may be involved in extracorporeal digestion. No enzymic function was demonstrated for the secretions produced by the gland cells associated with the bothria of Diphyllobothrium ditremum and Öhman-James (loc. cit.) suggests that the secretions are proteinaceous and that they possibly help in attachment and have an adhesive function. Halton & Dermott (loc. cit.)

have shown that the secretions produced by the gland cells in the region of the oral sucker of Opisthoglyphae ranae are positive for mucopolysaccharides and they suggest that these secretions may function to protect the parasite against host enzymes.

If any of the functions which have been described for the tegumental gland cells of other helminths can be applied to those of M. similis, the presence of fully-formed glands in the encysted metacercaria is puzzling. However, mature lappett gland cells are also present in the metacercarial stages of strigeoids. Erasmus (1969c) suggests that since Diplostomum develops to adulthood very rapidly in its final host (egg-laying occurs 3 days post-infection) an efficient feeding mechanism would be required from the outset. He implies that this is provided for by the secretions from the mature lappett gland cells which have a digestive function. In the metacercaria of Posthodiplostomum minimum the secretions from the gland cells pass into the tegumental syncytium rather than to the outside as in the adult, Bogitsh & Aldridge (loc. cit.) These authors suggest that this is a homeostatic mechanism allowing for the disposal of the secretions, and they suggest that this mechanism continues to function in this manner until the fluke reaches its definitive host.

A histochemical study on the secretions produced by the tegumental gland cells of Meiogymnophallus minutus and M. similis would be valuable in helping to determine the function of these gland cells. If the secretions were enzymic in nature it may implicate them in a digestive role as in Diplostomum and Apetemon.

It is interesting to note that whereas the tegumental gland cells are only found on the forebody of M. similis, they are scattered over the whole surface of Meiogymnophallus minutus. It has been shown (page 195) that M. minutus is completely embedded between the villi whereas M. similis only has its forebody embedded (Dr. B.L. James, personal communication). It would seem then that these gland cells are only present in areas of intimate host-parasite contact.

The distribution of spines over the surface of the metacercaria, as revealed by scanning electron microscopy, was unusual. The large toothed spines were only found in the anterior region of the fluke and it is this part which is embedded between the host's villi (see page 288), the posterior region of the fluke protrudes into the lumen of the intestine. The spines on the forebody were backward pointing and could thus provide a very effective means of anchorage once the fluke has reached its site of attachment. Another function which these spines may perform is that of abrading the host's tissue. Bennett (1975b) has seen similarly shaped spines on adult F. hepatica and he suggests that they may abrade the host's bile duct epithelium. The tegumental gland cells discharge in the region of the toothed spines in M. similis. It is possible that, if the gland cell secretions are enzymic, the toothed spines physically disrupt the host's intestinal epithelium and the secretions may partially digest the resulting debris so that it may be more easily injested. In connection with this, Meiogymnophallus minutus bears toothed spines and gland cells over its whole surface and, in this case, the adult fluke is completely embedded between the host's villi.

The hind body of M. simlis, which projects into the host's gut lumen, bears only very small spines but has an increased surface area due to the folded nature of the tegument. It seems possible that the hind body, with its large surface area, may be specially adapted for the uptake of partially digested food material which is available in the host's intestinal lumen.

The much-folded lateral regions of the tegument in the hind-body probably correspond to the areas of "mitochondrial cells" seen in thin sections. The possible functions of this area have already been discussed.

The sensory endings are particularly abundant around the oral sucker and over the fore body, and, to a lesser extent, around the ventral sucker. It seems then that the sensory receptors are most common in the area of intimate host/parasite contact. As might be expected, the probing oral sucker region is particularly rich in sensory endings and it is in this region that the possible "domed" receptors are found which Bennett (1975) suggests may be pressure receptors.

Digestive Caeca

The first description of the ultrastructure of the digestive caeca of a trematode was made by Gresson & Threadgold (1959) on F. hepatica. Since then several other species have described e.g. Schistosoma mansoni (Senft et al, 1961; Morris, 1968), Haematoloechus medioplexus (Davis et al, 1968; Dike, 1967) and Cyathocotyle bushiensis (Erasmus, 1972). More

detailed studies have been made on F. hepatica by Thorsell & Björkman (1965a), Robinson (1971), Robinson & Threadgold (1975) and Bennett (1973, 1975a).

Certain characteristics are common to most of the species that have been studied:-

a) The presence of abundant GER.

b) The presence of membrane-bound bodies with dense contents.

c) A complex infolding of the basal plasmalemma.

d) An elaboration of the surface plasmalemma to form lamellae or microvilli.

The lining of the caeca of F. hepatica and H. medioplexus is epithelial whereas that of G. amplicava and S. mansoni (Morris, loc. cit.) is described as being a syncytium. The caecal lining of M. similis is cellular and adjacent cells are joined at their luminal surface by a desmosome. The absorptive/secretory cycle described for the gastrodermal cells of F. hepatica has also been observed in C. bushiensis, however, it appears to be absent in H. medioplexus (Davis et al, loc. cit.) and was not recorded in M. similis in the present study.

Junctional complexes with the parenchymal cells have been described for adult F. hepatica and S. mansoni (Morris, loc. cit.) Bennett (loc. cit.) has described how they are formed during the development of F. hepatica from the metacercaria to the adult. Similarly, in M. similis, junctional complexes with the parenchymal cells were absent in the metacercaria although they were present in the adult - they presumably developed during the fluke's maturation in the definitive host.

Bennett associated the development of junctional complexes with the parenchymal cells with an increase in the absorptive function of the caecal cells. This is probably also true of M. similis since the metacercarial stage is non-feeding and, during this stage, the gut presumably has a limited digestive function.

The two types of secretory granules seen in the present study have also been recorded in the caecal cells of H. medioplexus by Davis et al (loc. cit.). These authors suggest that the large secretory bodies with the less dense contents are probably fragments of swollen GER. This may also be true of the large secretory bodies seen in adult M. similis. As with H. medioplexus, no clearly defined Golgi complexes were seen in M. similis although clusters of small dense secretory bodies were occasionally observed. The absence of secretory bodies in the metacercarial stage is further evidence that the digestive function of the caeca is limited. The large areas of α glycogen in the gastrodermal cells of the metacercaria indicates that one of its main functions is that of an energy store. This store of glycogen is depleted during development to the adult.

The luminal surface of the caeca of all the trematodes studied so far appears to be thrown up into lamellae rather than microvilli with the exception of G. amplicava (Dike, loc.cit.) where spherical cross-sections through obvious microvilli were seen. The frequency of these lamellae varies considerably; in F. hepatica they are abundant, whereas in S. mansoni they are sparse. In the present case, the lamellae

were relatively short although they were very closely packed. The thin layer of material between the laemllae was similar in density to the contents of the dense secretory bodies. It may therefore represent secretions from these bodies (although none of them were ever seen in the process of secretion) or it may be a condensation of the surface coat. However, this dense layer was also seen between the caecal lamellae of the metacercaria although at this stage no secretory bodies were present in the gastrodermal cells.

Lipid-like droplets were also seen in the cytoplasm of the gastrodermal cells of both the metacercaria and the adult, and in the metacercaria they were occasionally seen in the lumen. Similar droplets have been recorded in the gastrodermal cells of S. mansoni by Morris (loc. cit.) who suggests that they represent injested haemaglobin which has been partly digested. It is unlikely that the lipid-like droplets seen in the digestive caeca of M. similis represent injested food material. It is more likely that they are secretory or excretory products. Although they were not seen in the process of extrusion into the lumen in the metacercariae this was observed in the adults where they appeared to be more abundant. It is possible that these droplets may have a lubrication function or else that the digestive caeca may have a subsidiary excretory function. Erasmus (1972) suggests that the alimentary tract may be a possible route for elimination of waste substances.

Compared to F. hepatica, only small quantities of secretory bodies appear to be produced by the gastrodermal cells of M. similis. This could reflect the differences in their food material : F. hepatica injests hepatic and bile duct epithelial cells which have not been pre-digested, whereas the food material of M. similis is already partially digested by the host's digestive enzymes.

Parenchymal cells

The parenchymal cell system of developing and adult F. hepatica has been studied in some detail by Bennett (1973), Threadgold & Gallagher (1966) and Gallagher & Threadgold (1967). The work of these authors and unpublished work on strigeoids referred to by Erasmus (1972) indicates that the parenchymal system of trematodes is cellular and that the concept of large fluid-filled spaces referred to by earlier workers is erroneous.

The parenchymal cells of M. similis are similar to those described for F. hepatica. However, whereas junctional complexes are comparatively frequent between the parenchymal cells of F. hepatica, they are rare in M. similis. As with F. hepatica, junctional complexes with the digestive caeca (Bennett, 1973, 1975a) develop during the maturation of the metacercaria. However, the junctional complexes reported with the parenchymal cells and those of the tegument and the excretory system in F. hepatica (Gallagher & Threadgold, loc. cit.) were not seen in the present study.

The parenchymal cells of the metacercariae and adults of M. similis appear to act as glycogen stores. Erasmus (1972)

suggests that this is typical of the parenchymal cells of those trematodes which have been studied. The metacercaria (Bennett, loc. cit.) and the adult (Threadgold & Gallagher, loc. cit.) of F. hepatica are rich in stored glycogen. Threadgold & Gallagher (loc. cit.) suggest that the functions of the parenchyma system (of F. hepatica) may include the storage of glycogen; the transport of metabolites (via junctional complexes); and the production of the fibrous interstitial material which packs the space between the tegumental cells and which may act as a type of 'skeleton'.

Excretory system

The ultrastructure of the excretory system of F. hepatica has been described by Pantelouris & Threadgold (1963) and Gallagher & Threadgold (1967). The development of the excretory system at an ultrastructural level has been described for F. hepatica by Bennett (1973) and for Cyathocotyle bushiensis by Erasmus (1967a). The fine structure of the flame cells of various helminths have been described in some detail by Kümmell (1960 - F. hepatica), Wilson (1969- F. hepatica), Senft et al (1961 - S. mansoni), and Howells (1969 - Monezia expansa).

The excretory bladders of F. hepatica and C. bushiensis are considered to be lined by a syncytium rather than a layer of epithelial cells. Cell boundaries were not observed in the excretory bladder of M. similis and it is likely that it too is lined by a syncytium. The GER of the bladder lining in F. hepatica and C. bushiensis is not very extensive,

it is usually present as short strands. In M. similis, however, the cytoplasm of the excretory bladder was dense with large quantities of GER. The presence of such extensive GER is more characteristic of cells involved in secretion.

Although there was some lipid present in the cytoplasm and lumen of the excretory bladder in the metacercarial stage, the number of droplets increased markedly during development to the adult. This suggests that an adult-like method of lipid excretion exists in the newly-excysted metacercaria. These observations are similar to those made by Bennett (1973) on F. hepatica but in contrast to those made on C. bushiensis by Erasmus (1967a) who found that lipid droplets only began to be excreted after about three days and that, until then, the typical metacercarial excretory corpuscles continued to be produced.

The extracellular nature of the collecting ducts, which consisted of two or more cells joined by desmosomes, which was observed in M. similis, was also recorded in F. hepatica and C. bushiensis. However, whereas the collecting ducts of M. similis, F. hepatica, S. mansoni and Diplostomum trituri (Lautenschlager, 1961) bore lamellae projecting into the lumen, those of C. bushiensis did not. In the metacercaria and juvenile forms of F. hepatica (Bennett, 1973) some of the collecting ducts also bore cilia; these were not observed in M. similis. Bennett (loc. cit.) regards the lamellae of the collecting ducts and excretory bladder as adaptations which allow uptake and possibly resorption.

Although the basic morphology of the flame cell corresponds to that described for F. hepatica by Kümmel (1960), Wilson (1969) and Bennett (1973), it differs in certain important respects. The body of the flame cell was divided into two portions which were connected by thin strands of cytoplasm. One portion, which was probably displaced to one side, contained the nucleus, most of the mitochondria, free ribosomes and some GER. The other portion of the cell contained the ciliary basal bodies and it was this portion which interdigitated with the cell of the conjoined tubule. In adult F. hepatica (Gallagher & Threadgold, 1967), in the miracidium of F. hepatica (Wilson, 1969) and in C. bushiensis, the flame cell body contained large vacuoles and the cytoplasm between these vacuoles was often rather attenuated. It may be that this type of structure gave rise to the peculiar morphology seen in the flame cell of M. similis. It is very difficult to imagine, however, what possible advantage could be obtained by the fluke from the unusual structure of its flame cells.

External leptotriches were apparently not present in the flame cells of M. similis. In other trematodes, these structures project out from the cells of the conjoined tubule in the region of the "flame". It has been suggested by Wilson (1969) that, if they are rigid, they may function by holding other cells at a distance thus creating and maintaining a fluid-filled space around the flame cell. In M. similis projections pointing along the barrel of the flame cell did arise from the cup-shaped portion of the flame cell body but these may have been oblique sections

through the strands connecting the two parts of the flame cell body. Although the wall of the collecting duct in the region of its interdigitation with the flame cell body is rather irregular and bears finger-like processes, these do not resemble the external leptotriches described by Wilson (loc. cit.). The absence of external leptotriches does not apparently prevent the flame cell from lying within a large extracellular space filled with interstitial material.

The Female Reproductive System

Mehlis' gland

The ultrastructure of the Mehlis gland of trematodes has been described by Thorsell & Björkman (1965b) and Threadgold & Irwin (1970) in F. hepatica; by Burton (1967a) in Haematoloechus medioplexus; by Erasmus (1973) in S. mansoni; and in the monogenea by Stranock & Halton (1975). In F. hepatica and H. medioplexus the secretory cells of Mehlis' gland are of two basic types. The most prevalent type in both cases is the cell which produces curved, membrane-bound, sac-like secretory bodies. The other cell type produces spherical, dense secretory bodies. Both of these types of secretory cell are, in fact, unicellular gland cells which open into the lumen of the ootype by slender ducts. In S. mansoni (Erasmus, loc. cit.) there is only one type of gland cell and this produces the spherical, dense type of secretory body. In M. similis only cells producing the sac-like bodies were seen. However, these cells were seen rather infrequently and the presence of the second type of secretory cell cannot be excluded. The apparent scarcity of

the Mehlis gland cells and the failure to observe ducts leading into the ootype indicates that, in M. similis, Mehlis gland is a rather small organ. This is rather surprising considering the large number of eggs that are produced by the adult. The role of Mehlis gland is uncertain; several suggestions have been made as to the functions of the secretions and these have been reviewed by Erasmus (1972). At present, the most widely accepted theory is that it produces a lipoprotein secretion which forms a membrane around the ovum and accompanying vitelline cells and which may serve as a template for the deposition of shell material (Clegg, 1965b).

The Vitellaria

The ultrastructure of the vitellaria of F. hepatica has been described by Björkman & Thorsell (1963) and Irwin & Threadgold (1970); of H. medioplexus by Tulloch & Shapiro (1957); and of S. mansoni by Erasmus (1973). It has been studied from a developmental point of view by Irwin & Threadgold (loc. cit.), Erasmus (loc. cit.) and by Halton et al (1974) in the Monogenea.

The development of the vitelline cells of M. similis was essentially similar to that described for F. hepatica except that the mature cells did not appear to act as a glycogen store. The developmental process involved the formation of large amounts of GER which, in association with the Golgi complexes, produced the secretory globules which formed into clusters and which eventually replaced most of the cell cytoplasm. Considering the small size of the

follicles of the vitellaria of M. similis compared to F. hepatica the developmental progression from immature cells on the periphery to mature cells in the central regions was not seen. The nurse cells which surrounded the vitelline cells of F. hepatica were not seen in the present study. Irwin & Threadgold (loc. cit.)suggest that these cells facilitate transport of nutrients between the parenchymal cells and the vitelline cells via junctional complexes. Because of the smaller size of M. similis compared to F. hepatica such a complex mechanism may not be necessary. No junctional complexes between the vitelline cells and the parenchymal cells were seen in M. similis.

Lipid droplets were very infrequent in F. hepatica although the "yolk granules" observed by Björkman & Thorsell (loc. cit.) are reminiscent of lipid droplets. Lipid drops were, however, seen in the vitelline cells in the present study and also in S. mansoni. In S. mansoni and F. hepatica whorls of ribosomes were seen in the vitelline cells - these were not present in M. similis. Erasmus (loc. cit.) suggests that, in S. mansoni, the secretory globules go to form the egg shell whereas the lipid droplets and ribosomal whorls are retained in the vitelline cell after the formation of the egg shell.

The Uterus and Ootype

Erasmus (1973) has described the ultrastructure of the uterus and ootype of S. mansoni. In this trematode, the uterus is apparently tegumental in nature. It is lined with an anucleate syncytium which is connected to the nucleated cell bodies by thin protoplasmic strands; typical tegumental secretory bodies can be seen in the cell bodies. The fine structure of the uterus of M. similis is in complete contrast to this. It is lined with a nucleated epithelium which acts as a glycogen store in the metacercaria. Whereas the surface of the uterus of S. mansoni is described as folded and irregular, that of M. similis metacercaria possesses definite finger-like projections.

The lining of the ootype of M. similis metacercariae was similar to that of the uterus except that it did not contain such extensive glycogen stores and the invaginations of its basal plasmalemma were more numerous. In M. similis the ootype was ciliated - this was easily seen in living specimens. In S. mansoni the ootype did not possess cilia although they were present in the common duct which receives the vitelline ducts and oviduct and which joins the posterior end of the ootype. The ootype of S. mansoni was not tegumental, it was lined with epithelial cells and was otherwise similar to the ootype of M. similis. However, in S. mansoni the ducts of Mehlis gland entered the ootype near its junction with the oviduct and vitelline ducts; no Mehlis gland ducts were seen entering the ootype of M. similis. As has been previously suggested, this may be because Mehlis gland is a rather small organ in M. similis.

Ovary

The ultrastructure of the trematode ovary has been described by Erasmus (1973) for S. mansoni and by Björkman & Thorsell (1964a) and Gresson (1964) for F. hepatica. These authors described the various developmental stages from the oogonia around the periphery of the ovary to the oocytes or ova in the central regions. In both these flukes, the mature oocytes or ova contained dense, spherical cortical granules. These granules were produced by the Golgi complexes in S. mansoni. In F. hepatica Golgi complexes were not seen although large clusters of ribosomes ("ribosomal bodies") were present and Björkman & Thorsell (loc. cit.) suggest that they may be invovled in the production of the dense, cortical bodies. The role of these cortical granules is not clear; Gresson (loc. cit.) suggests that they may have a nutritive function whereas Erasmus (loc.cit.) implicates them in the process of fertilization since they are lost in that part of the oviduct where fertilization occurs.

These cortical granules are apparently absent from M. similis, although they were present in Meiogymnophallus minutus (page 241) but further work would be necessary to substantiate this.

(It is possible that the sections obtained in the present study passed through immature regions of the ovary since the ultrastructure of the cells was similar to that of the immature oocytes of S. mansoni or the earlier developmental stages described for F. hepatica (Björkman & Thorsell, loc. cit.). In these two flukes cortical granules were only present in mature oocytes or ova and not in the earlier developmental stages.)

The Male Reproductive System

The Prostate Gland

The prostate gland was never observed in the adults of M. similis (although on one occasion what appeared to be secretions of the gland were seen in the lumen of the ductus ejaculatoris of a cultured fluke (see page 373)). This is in contrast to Meiogymnophallus minutus (page 242) where the prostate gland was frequently seen. In adult M. minutus, the prostate gland was clearly visible under the light microscope, whereas in M. similis it was not as obvious.

The Testes

The ultrastructure of the testes and the process of spermatogenesis has been described by Gresson & Perry (1961), Sato et al (1967), Hendelberg (1962) and Burton (1972) for trematodes; by Tuzet & Hédi Ktari (1971) for monogeneans; and by Rosario (1964) and Featherstone (1971) for cestodes. The fine structure of the spermatozoon has been studied by Morseth (1969, Echinococcus granulosus and Dicrocoelium dendriticum), Hershenov et al (1966), Shapiro et al (1961, Haematoloechus medioplexus), Świderski (1968, Monezia expansa), Tulloch & Hershenov (1967).

Spermatogenesis and spermateleosis in M. similis is similar to that described for Paragonimus miyazakii by Sato et al (loc.cit.) and for H. medioplexus by Burton (loc.cit.); it will be discussed in Part IV of this chapter since most of the observations were made on cultured flukes.

In most of the trematodes that have been studied, the fine structure of the sperm is remarkably similar. It consists of two lateral axial filaments with a 9+1 arrangement of microtubules, an elongated nucleus with condensed chromatin, mitochondria (or one long mitochondrion?) and two sets of peripheral microtubules. Generally the sperm is divided into two main regions - the "head" region (which contains the nucleus) and the "tail" region (Sato et al, 1967). In contrast to this, M. similis exhibits a wide variety of sperm structure in cross-section. It is possible that those sperm which do not conform to the basic pattern represent abnormal or malformed sperm but, because of the frequency with which these unusual structures were seen, it seems more likely that they represent sections at different levels through sperm whose structure is modified from the usual pattern. One possible model which incorporates all the different types of morphology seen in cross-section is described in Text Figure 11. However, it may be possible to construct other models from the same observations which are equally satisfactory. The model which is described here does not provide any information on the relative lengths of each of the sections. In order to do this - assuming the model to be true - it would be necessary to determine the relative frequency of each of the various different types of sperm cross-section.

This model presents certain problems. It is difficult to envisage the longitudinal displacement of one of the filaments with respect to the other since they are both formed from adjacent basal bodies in the spermatid. The fine structure of the axial filament tip cannot be unequivocally described from

this study. The condensed filament was only seen in Section H; in other parts of the sperm (according to the model) where one would expect to find a similar structure, it was not seen (e.g. Sections 1,2 and 3) and there appeared to be no transition between the normal filament structure and that of the core-less filament with nine or fewer disorganised doublets. In order to retain the rigidity and some of the functions of the normal filament, it could be argued that a more likely structure at the axial unit tip would be the gradual loss of the peripheral doublets so that only the central core remained. However, during spermatid development, the central core complex appears to arise a short distance away from the basal body. If the mature sperm become detached from the residual cytoplasm of the spermatid just below the basal body, there would be a region of axial filament which possessed no central core complex and it is quite likely that, without the influence of the basal body or a central core, it may become rather disorganised and appear collapsed.

In order to substantiate unequivocally such a model, further work would be necessary.

a) to study the relative frequencies of the various types of morphology;

b) to determine whether the "missing" sections (Sections 1,2,3 and 4) are actually present; and

c) to study longitudinal sections of sperm, especially in the "head" region and at the tip of the "tail".

In this study of the ultrastructure of the adult, it must be remembered that the mouse is not the natural definitive host of M. similis. This may lead to certain abnormalities in morphology and developmental pattern. Ideally, adults should be studied from the natural definitive host, the seagull.

Conclusions

1. The tegument of M. similis metacercariae and adults consisted of a surface syncytium connected to sunken cell bodies by protoplasmic strands. It contained typical secretory bodies although their distribution changed during development.
2. In contrast with other trematodes, GER was not present in the tegumental cell bodies of M. similis.
3. Specialised cells containing mainly mitochondria were found amongst the tegumental cell bodies in the posterior/ lateral regions.
4. Unicellular gland cells containing dense secretory bodies opened by slender ducts to the surface of the forebody of the metacercaria and the adult. The ducts were lined by microtubules and joined to the syncytium by a desmosome.
5. The forebody of the metacercaria was closely covered with rings of oval, backwardly-pointing spines with toothed tips. The hind-body bore irregularly-placed, peg-like spines and the surface of the syncytium was thrown up into folds which were often quite complex. Sensory endings were located around the suckers and over the forebody.
6. In the metacercaria, the digestive caeca acted partly as a glycogen store.
7. Junctional complexes between the cells of the digestive caeca and the surrounding parenchymal cells developed during maturation to the adult.

8. The apical surface of the caecal epithelium was thrown up into closely pressed lamellae; a thin layer of dense material was present between adjacent lamellae.
9. Secretory bodies were absent from the caecal epithelium of the metacercaria and in the adult two different types of bodies were present.
10. Lipid-like droplets occurred in the caecal epithelium of both metacercariae and adults.
11. The parenchymal cells contained stores of ∝ glycogen. Junctional complexes between adjacent parenchymal cells were present but were infrequent.
12. The amount of lipid droplets in the lumen and the lining of the excretory bladder increased during development.
13. The lining of the excretory bladder was probably syncytial but it contained an unusually extensive GER system.
14. The apical membrane of the lining of the excretory bladder and excretory ducts was thrown up into long, tenuous lamellae.
15. The basic pattern of the flame cell was similar to that described for other flukes except that the flame cell body was divided into two parts connected by cytoplasmic strands, and there were apparently no external leptotriches.
16. The Mehlis gland appeared to be small and it produced sac-like secretory bodies. No ducts were seen emptying into the ootype.

17. The lining of the uterus and the ootype were cellular and in the metacercaria the cells acted as a glycogen store. The ootype was ciliated.

18. Large quantities of vitelline droplets were produced during the development of the metacercaria. The vitelline cells were similar to those described for other trematodes. Cells exhibiting a range of development were seen in the vitelline follicles of the adult.

19. In the present study, the sections through the ovary of adult flukes probably only showed immature developmental stages. No "ribosomal bodies" or cortical granules were seen in these germ cells.

20. Spermatogenesis and spermateleosis were essentially similar to that described for H. medioplexus (Burton, 1972).

21. The sperm of M. similis consisted of an elongate nucleus, two axial filaments, mitochondria (or one mitochondrion) and peripheral microtubules. The arrangement of these components within the sperm may be different to that described for other trematodes and an alternative model is suggested.

PART IV: ULTRASTRUCTURE IN VITRO

Introduction and Methods

The ultrastructure of apparently healthy flukes from Culture 6 was examined on Day 4 of culture both under the electron microscope (Section 1) and the scanning electron microscope (Section 2). The culture conditions were as follows:-

Medium: NCTC 135 plus antibiotics, with 20% foetal calf serum (Flow)

Gas phase: Aerobic (10% O_2, 5% CO_2, 85%N_2).

Temperature: 40°C.

Other conditions: 15mls. media in a Leighton tube without shaking.

Particular attention was paid to the surfaces which came into contact with the culture medium i.e. the tegument and the lining of the digestive caeca. A preliminary study showed that spermatogenesis was apparently normal in vitro. In order to study the various stages of spermatogenesis and spermateleosis, flukes were removed from culture and fixed after 2,6,12 and 18 hours, and 1,2 and 4 days. The culture medium in this experiment was as previously described.

The techniques used for the preparation of material for electron microscopy have been described in the General Methods (Chapter 2).

Section 1: TRANSMISSION ELECTRON MICROSCOPY (TEM)

Results

1. The Tegument

a) The Syncytium. (Plates 170 & 171)

The tegumental syncytium of cultured flukes was separated into the three separate regions previously described for the metacercaria (page 311), and the distribution of spines and the general surface morphology was essentially similar. The ground substance of the syncytium was moderately dense. Small, spherical mitochondria with a dense matrix were scattered throughout, although they were more frequent in the basal half of the syncytium. The distribution and type of secretory bodies in the syncytium of cultured flukes was similar to that described for the metacercaria (page 311). Both biconcave and spherical granules were present (Plate 170). However, the syncytium immediately above the basement membrane appeared abnormal (Plates 170, 171); it was granular and contained degenerating secretory bodies and vesicles. The basal lamina possessed few invaginations but was much thicker (about 0.1 um.) than in the metacercaria or the adult. Underlying the basal lamina was a thick layer of fibrous interstitial material which extended around the peripheral muscles, it varied in thickness from 0.3-1.5um.

The posterior, lateral syncytium was similar to that described for the metacercaria and the adult (pages 314 & 316), although the basal regions exhibited the abnormalities described above. Protoplasmic strands connecting the syncytium with the "mitochondria cells" were never demonstrated unequivocally. The thin, dense layer of cytoplasm - probably derived from the "mitchondria cells" - that was seen under the basal lamina of the syncytium of the metacercaria, was never seen in cultured flukes.

b) Tegumental cell bodies (Plates 170, 172, 174 & 175)

The tegumental cell bodies contained two types of granules (Plate 175) - the spherical, "type A" granule previously seen in the tegumental cells of adult flukes and a dense, rod-like or biconcave granule similar to those seen in the syncytium (type C). The spherical granules were more typical of those seen in the metacercaria than those of the adult since they possessed a wide variety of contents ranging from uniformly dense to virtually empty. The "type B" granules present in the adult were not seen in the tegumental cells of cultured flukes.

The cytoplasm of the tegumental cell bodies contained free ribosomes, mitochondria and frequent Golgi complexes with much dilated cisternae. The Golgi complexes were often associated with small membrane bound sacs or vesicles which usually appeared empty. Also present were small amounts of lipid droplets and some dense bodies which were probably lysosomes. The nuclei of the tegumental cells had a granular nucleoplasm and dense patches of heterochromatin.

The tegumental cells of cultured flukes often contained large, usually oval, crystalline bodies which were not seen in the metacercaria or the adult (Plates 174 & 175). These bodies were not membrane-bound but often had biconcave, "type C" granules associated with them (Plate 175). The crystalline structure of these bodies was similar to that of the spines in the syncytium.

c) Associated cells: "Mitochondria cells". (Plate 171)

The "mitochondria cells" were similar to those previously described for the metacercaria (page 314) except that - as in the adult - the mitochondria had a denser ground substance.

d) Associated cells: Gland cells (Plates 172 & 173)

The unicellular gland cells associated with the tegument were similar to those described for the metacercaria (page 314). However, the gland cells of cultured flukes also contained large masses of finely granular, moderately dense material which was not membrane-bound. The secretory granules present in the gland cells were frequently seen in the process of disintegration and their contents appeared to contribute to these large, amorphous masses of material (Plate 173). Ribosomes and nuclei were the only other organelles seen in the gland cells.

2. Parenchyma cells (Plate. 175)

The parenchyma cells of cultured flukes were similar to those described for the metacercaria (page 317). Stores of ∝ glycogen were present.

3. The Digestive Caeca (Plates 176, 177 & 178)

The cells of the digestive caeca of cultured flukes were different in several ways to those of the metacercaria or the adult (Pages 318 & 319). The cells were much less voluminous and the lumen of the caecal limbs was thus considerably larger. The cytoplasm of the caecal cells contained free ribosomes, GER and possible Golgi areas. Secretory granules similar to the small dense granules recorded in the adult were present, especially near the lumen surface (Plate 178). The larger, less dense secretory bodies seen in the adults were not present. The surface lamellae had apparently not grown from those of the metacercaria, they were 1.0-1.5um. in length. The thin layer of dense material between the closely pressed lamellae was present

in cultured flukes although, in many areas, it appeared to have been extruded and it was often seen clumped together in large masses (Plates 177 & 178). These masses were very dense but their substructure could occasionally be seen - they consisted of stacks of long, thin, dense elements similar to those seen between the lamellae. This material usually remained associated with the lamellae although it was occasionally seen free in the lumen. In the region of the lamellae, the lumen often contained a granular substance (Plates 177 & 178).

Adjacent caecal cells were connected at their apical surface by a desmosome. Compared to the adult, the boundaries of adjacent cells were relatively straight. Invaginations of the basal lamina were occasionally present (Plate 176) but parenchymal processes were not seen. The layer of interstitial material around the limbs of the digestive caeca was quite thick (about 0.4um.) and fibrous (Plate 176).

The oesophagus was lined with tegumental syncytium containing mainly biconcave granules. Its surface was generally smooth but it was thrown up into occasional folds. The syncytium was joined to the caecal cells by a desmosome.

4. <u>The Excretory System</u>

The excretory system of cultured flukes was similar to that of the adult (page 323), except that fewer lipid droplets were seen in the lumen of the ducts or in the cytoplasm of the cells lining them. A long section through a flame cell was seen (Plate 194); the ultrastructure appeared normal and was consistent with that described for the metacercaria (page 321).

5. The Female Reproductive System

a) Mehlis' Gland (Plate 179)

The cells of the Mehlis' gland of cultured flukes seemed identical with those described for the adult (page 320, cf. Plate 161). They contained the typical sac-like secretory bodies, free ribosomes, strands of GER, mitochondria and a nucleus. No ducts emptying into the ootype were seen and the gland cells were relatively infrequent.

b) Vitellaria (Plates 181 & 182)

The morphology of the developing and mature vitelline cells was similar to that described for the adult (page 327). The developing cells contained a nucleus, GER, free ribosomes, Golgi complexes and typical vitelline droplets. In some cells, large clumps of vitelline secretory bodies were seen which had irregular, ragged outlines.

Clumps of dense vitelline droplets were frequently seen in the uterus, they were often associated with an ovum. In Plate 182, a vitelline cell with a nucleus, GER and some large, irregular, dense secretory bodies was seen associated with an ovum. Although there appears to be a thin fuzzy layer of material around the ovum, there was no true egg-shell.

c) Uterus (Plates 181 & 182)

The uterus was similar to that described for the metacercaria (page 324). The cells lining the uterus were thin and, compared to the metacercaria (Plate 142), they appeared "stretched". The cells contained ribosomes, short lengths of GER and nuclei. The surface was thrown up into lamellae and slightly thicker finger-like pro-

jections; adjacent cells were joined by a desmosome. Bands of muscles ran around the uterus.

d) Ovary (Plate 180)

Although the ultrastructure of the ovary of cultured flukes was not studied in very great detail, it was similar to that previously described for the adult (page 327). The cells were irregular in shape and contained a prominent, large nucleus which was roughly spherical or oval. The nucleus had a granular ground substance with occasional, small, dense patches of heterochromatin. Most of the nuclei contained a dense, oval nucleolus, about 1.8um. long, at one pole. The cytoplasm of the cells was granular and probably consisted of small clusters of ribosomes. Groups of moderately dense mitochondria of various shapes and sizes were frequently seen. Also present in the cytoplasm of these cells were large, roughly oval, dense, granular bodies. These were similar in size and appearance to the nucleoli although they were more coarsely granular. They did not appear to be membrane-bound. The small, dense bodies seen in the peripheral cytoplasm of the ooqytes of M. minutus (page 241) and in F. hepatica by Gresson (1964) and by Björkman & Thorsell (1964a) were not seen. No Golgi complexes were observed.

Some unshelled ova were seen free in the uterus (Plate 182). These cells were similar to those seen in the ovary although they were more regular in outline. The nuclei, however, were much less regular and often had a bilobed appearance. They contained prominent nucleoli, a granular ground substance and dense patches of heterochromatin. The cytoplasm of the cells in the uterus was similar to those in the ovary; it contained irregularly-shaped, dense mitochondria, some small lipid droplets and the large, dense,

granular bodies. Again, the small, dense granules seen in the ova of M. minutus and F. hepatica were not seen in cultured M. similis.

6. The Male Reproductive System

a) Prostate gland (Plate 193)

The prostate gland proper was not seen; however, on several occasions, the ductus ejaculatoris in the male papilla region was seen to contain some dense, secretory bodies in the lumen; These bodies were assmed to be secretions of the prostate gland because of their position and because of their similarity to the prostate gland secretions of M. minutus (page 242). They were usually clumped together and were either free in the lumen or they were associated with some cytoplasm. The wall of the ductus ejaculatoris in this region appeared to be lined with tegumental syncytium. Although it was rather abnormal it contained the usual biconcave secretory bodies found in the tegumental syncytium.

b) Testes (Plates 183-191)

The development of the testes in cultured flukes was studied in some detail. Flukes were removed from culture and examined after 2,6,12 and 18 hours, and 1,2 and 4 days.

2 hours

No dividing cells were seen and the ultrastructure of the testes was similar to that recorded for the metacercaria.

6 hours

Spermatocytes in various stages of development were seen. In many cases, they were similar to those of the metacercaria, i.e. with an intact nucleus, although centrioles were frequently

seen. In other parts of the testes, rosettes of dividing spermatocytes associated together in a cytophore could be seen (Plate 183). Centrioles and spindle fibres were often observed. In some cases, developing spermatids were seen, in no instances did they possess condensed chromatin in their nuclei.

12 hours

The dividing and non-dividing spermatocytes were still seen at this stage and continued to be present in all the stages examined up to Day 4. Many more developing spermatids were seen at this stage but no mature sperm were present. The detailed development of the spermatid (spermateleosis) is described later (page 375).

18 hours

Many free axial filaments and membrane-bound bodies containing peripheral microtubles were seen. These represented sections through the central and lateral processes of adjacent spermatids before they united to form the mature sperm.

24 hours

Sperm were seen for the first time at this stage. Bundles of sperm were especially abundant around the periphery of the testes. All the various stages of spermatogenesis, from dividing spermatocytes to free spermatozoa, could be seen.

2 days

Large numbers of mature sperm were present.

4 days

As 2 days.

Spermateleosis: development of the spermatid.

(Plates 154,157 and 184-189)

The following description of spermateleosis in M. similis is based mainly on observations made on the development of the testes of cultured flukes but also on observations on the ultrastructure of the testes of adults recovered from mice.

During spermatogenesis the spermatocytes divide to produce the spermatids, these remained attached to the residual cytoplasm of the cytophore by a slender stalk (Plate 184). The recently formed spermatids possessed a nucleus, several dense mitochondria and frequent large Golgi complexes. The mitochondria were large and irregular in shape although they often appeared bilobed or horse-shoe shaped. The mitochondria and Golgi complexes were distributed throughout the cytoplasm of the spermatid, they were not localised in the stalk region as in the spermatocytes.

During their development, the nuclei of the spermatids became elongated and their chromatin material began to condense into short dense threads. As the nucleus elongated it grew into a protrusion of the cytoplasm which arose from the outer surface of the spermatid (Plates 185 & 186). This protrusion of cytoplasm eventually grew to form the central process or filament of the developing spermatid. It was lined with a single layer of microtubules. As the central filament grew outward from the body of the spermatid the plasma membrane on either side of it folded inwards to form a furrow (Plate 155). In cross-section, this was seen as a groove which was frequently crescent-shaped but was often seen to completely encircle the central filament, separating it from the body of the spermatid (Plates 157 & 188).

During the early stages of spermatid development buds of cytoplasm developed on either side of the central process. These extended to produce the two lateral processes, each of which contained a single axial filament. The fine structure of the axial filament is shown in the Inset to Plate 189 and is described on page 332. At the base of the developing axial filament was a basal body which lay at right angles to the longitudinal axis of the spermatid. Attached to each basal body was a tapering, striated rootlet which pointed backward along the longitudinal axis of the spermatid, (Plates 184 & 186). Between the basal bodies was a "centriole-like" structure (Plates 185-187 & 157). This body measured 0.4um. in length and approximately 0.3um. in width. It was orientated parallel to the long axis of the spermatid and appeared to consist of nine parallel layers of dense material stacked in three groups of three. The inner core of three dense layers was usually quite distinct whereas the layers forming the outer sheath were usually rather fuzzy and indistinct (Plate 185). This "centriole-like" body was not seen in cross-section. It was closely associated with the basal bodies although the nature of the connection, if it existed, was not clear. The basal bodies were seen in cross-section (Plate 187), they had the typical basal body structure of nine sets of triplet tubules tilted slightly with respect to one another with no central filament. In the region of the basal body, the axial filament probably lacked the central core complex (Plate 186), which appeared to arise at a short distance distil to the basal body. During the development of the spermatid the basal bodies changed their orientation so that the long axis of the two lateral filaments came to lie parallel to the central filament.

As the development of the spermatid proceeded the nucelus elongated considerably and appeared as a twisted structure in the cytoplasm of the spermatid which was often cut many times in cross-section (Plate 188). At this stage, the chromatin was condensed into sheets of dense material which ran along the length of the nucleus. In the mature sperm, the nuclear material was condensed into a dense rod (Plate 190). During development, this elongated nucleus migrated down into the central filament and it was presumably accompanied by a number of mitochondria. It is not clear whether these mitochondria eventually fused or whether they remained separate.

Cross-sections through the lateral and central filaments were frequently seen (Plate 156). The central filament contained two rows of microtubules opposite to one another, there were usually 3-5 microtubules in each group. Between the two groups of microtubules were two areas of dense material associated with the bounding membrane of the filament. Each of the areas consisted of two dense, rectangular plaques which were often connected to each other by a bridge of dense material. Similar dense material was also seen in mature sperm (Plate 189) and occasionally on either side of the nucleus in the early stages of the development of the spermatid (Plate 157). As development proceeded, the lateral and central filaments fused and when the development of the sperm was complete it became detached from the body of the spermatid. The apparatus associated with the production of the axial filament i.e. the basal body "centriole-like" body and the rootlet, were never seen in the mature sperm and presumably remained in the residual cytoplasm.

The fine structure of the sperm of M. similis is described in Part III of this chapter (page 331). The more usual type of sperm

cross-section with two lateral filaments, a nucleus and a mitochondrion was observed in cultured flukes and so were sperm with one filament and a lateral nucleus (Plates 190-192).

Abnormal sperm produced in vitro - Plate 192

On a few occasions sperm which possessed four axial filaments were seen in the vesicula seminalis. These were similar to those seen in cultured Meiogymnophallus minutus (page 270). This abnormality was never seen in adult flukes recovered from mice.

Discussion

The Tegument

The distribution of secretory bodies in the syncytium of cultured Microphallus similis was similar to that of the metacercaria rather than the adult, both biconcave and spherical granules were present in roughly equal proportions. However, the basal regions of the tegument of cultured flukes were abnormal and appeared to be degenerating. A similar type of abnormality was seen in the tegument of cultured F. hepatica (see Chapter 3, part IV). The basal plasma membrane of the tegument is considered to be important in ionic exchanges and the translocation of dissolved substances (Erasmus, 1972). Abnormalities in the region of this metabolically active surface must have a considerable effect on the normal processes of uptake via the tegument in in vitro cultured flukes.

Unlike the tegumental cell bodies of cultured F. hepatica, those of M. similis apparently continued to produce secretory bodies although the presence of these bodies in the tegumental cells may indicate a defective transport process rather than a normal secretory process. The oval, granular secretory bodies seen in the tegumental cells of the adults, but not the metacercaria, were not seen in cultured flukes. It seems then that the tegument of cultured flukes does not develop significantly in the direction of the typically adult-type of morphology but retains the morphology of the metacercaria.

A prominent feature of the tegumental cell-bodies of cultured flukes was the presence of large, crystalline masses of material. The composition of these, usually oval, masses was similar to that of the spines of the syncytium. Rod-like or biconcave

granules were often seen associated with these masses. Normally, such granules were only seen in the syncytium and were not present in the cell bodies. A possible explanation for this abnormality is that some of the spherical secretory bodies (whose function may be to provide material for the production or repair of spines in the syncytium) are losing their contents in the tegumental cell bodies and that this accounts for the large masses of spine-like material seen there. In order for this to occur, the mechanism which controls the normal secretion of tegumental cell bodies must have been disturbed.

The unicellular gland cells associated with the tegument of adults and metacercariae were also seen in cultured flukes. However, the cell bodies of these glands often contained large amorphous masses of moderately dense granular material. The normal secretory bodies which are produced by these gland cells were often seen disintegrating into this mass of material. It appears then that the mechanism which controls the normal processes of packaging and transport of gland cell secretions is faulty in these cultured flukes. It is surprising that the tegumental gland cells of M. minutus do not appear to be affected in a similar way by in vitro culture. This may indicate that different control procedures are in operation and possibly that the secretory bodies have a different composition.

It is interesting that, in M. similis, in vitro culture has such similar effects on the secretory process in two different types of cells - the tegumental cells and the unicellular gland cells associated with the tegument.

The "mitochondria cells" in the posterior/lateral regions of cultured flukes were similar to those seen in the adults and did not appear to have degenerated in anyway. However, it is not

possible to say, from thin sections, whether the mitochondria were active or functioning normally.

Digestive Caeca

Instead of the gastrodermal cells increasing in bulk, which occurred during development *in vivo*, they appeared to decrease in volume in cultured flukes. A parallel situation was also recorded in the cultivation of *F. hepatica* metacercariae (see Chapter 3, part IV). The organelles present in the gastrodermal cells of adults were also seen in those of cultured flukes. The GER system was not as extensive as in the adult and it was usually present as short fragments. In cultured *F. hepatica* the GER fragments had distended cisternae which contained a granular material, this was not seen in the present case. The large secretory bodies seen in adults from mice (which may be composed of swollen GER - see page 319) were not present in cultured forms although the smaller denser bodies were seen. The junctional complexes with the parenchyma cells, which developed during normal *in vivo* maturation, were not seen in cultured flukes. This is in contrast to cultured *F. hepatica* where junctional complexes between parenchymal and caecal cells appeared to develop normally. The non-development of junctional complexes with the parenchymal cells may reduce the transport of digested food material from the caeca. Threadgold & Gallagher (1966) suggest that junctional complexes are sites of transportation and Threadgold (1968) has demonstrated that acid and alkaline phosphatases are specifically located in these regions. Bennett (1973, 1975a) regards the development of junctional

complexes in the digestive caeca of juvenile F. hepatica as evidence of the increasing absorptive function of the gastrodermal cells.

The dense material between the lamellae of adults and metacercariae was also present in cultured flukes. However, in many areas, it appeared to have been extruded from between the lamellae and to have clumped together. It has been suggested (page349) that this material represents secretory material similar to that seen in the dense bodies in the cytoplasm, or that it represents a condensation of a surface coat or glycocalyx. The extrusion of this material from between the lamellae may be a purely physical reaction resulting from the reduction in volume of the gastrodermal cells during cultivation. The surface coat of the apical plasmalemma of the digestive caeca of trematodes does not appear to have been studied although its presence has been recorded by several authors (Dike, 1967; Morris, 1968; Robinson & Threadgold, 1975). The nature and properties of the glycocalyx covering the tegument of trematodes has, however, been studied in some detail. In most of the species so far examined, it has been shown to contain mucopolysaccharides and the adsorptive properties of the glycocalyx are well documented (Lumsden, 1975). It is possible that the surface glycocalyx of the digestive caeca is similarly "sticky" and that - if extruded from between the lamellae - it may clump together.

Parenchymal Cells

As with the adults removed from mice, the parenchymal cells of cultured flukes contained stores of glycogen. This suggests that the flukes are able to utilize the glucose available in the culture medium.

Excretory system

The excretory system of cultured flukes was similar to that of the adult except that less lipid was apparently being excreted. This decrease in the amount of excretory products may be due to a poor diet, since the culture medium did not include any solid or semi-solid food material.

Female Reproductive System

Mehlis' gland, Uterus and Ootype

The Mehlis gland, uterus and ootype of cultured flukes were essentially similar to those of adults recovered from mice. *In vitro* cultivation does not appear to have any deleterious effect on the development of the Mehlis gland or the production of its secretory bodies.

The Vitellaria

In most respects, the vitelline cells of cultured flukes were similar to those of adults removed from mice. However, some of the vitelline droplets appeared slightly abnormal and they were rather ragged in outline. These may correspond to the prematurely tanned vitelline droplets seen under the light microscope (see part II of this Chapter). Since, in *F. hepatica* at least, both the substrate (phenolic protein) and the enzyme (phenolase) are located in the clusters

of vitelline droplets (Smyth & Clegg, 1959), it is surprising that tanning does not normally occur within the vitellaria. Irwin & Threadgold (1970) suggest that the enzyme may lie in the space between the individual membrane-bound droplets which make up the globule and that this physical separation may account for the inhibition of the tanning process in the vitellaria under normal conditions. The ragged appearance of the globule droplets in cultured forms may indicate that there is some defect in the packaging system (as in the tegumental cells and associated gland cells, q.v.) which allows the substrate and the enzyme to come into contact with each other prematurely. Smyth & Clegg (loc.cit.) suggest that the controlling factor in F. hepatica may be the presence or absence of oxygen, however, this can probably be ruled out in the present case since, in an anerobic medium, premature tanning still occurred (see part II of this Chapter).

Mature vitelline cells associated with an ovum were often seen in utero. In these cells, the vitelline droplets had formed into large, dense amorphous clumps of material and there was no proper egg shell. These masses of materialundoubtedly represented the prematurely tanned material seen in the uterus of cultured flukes under the light microscope. In Schistosoma mansoni (Erasmus, 1973) the vitelline cells enclosed in the egg capsule contain none of the vitelline globules seen in the cells of the vitellaria, these globules are considered by the author to have contributed to the formation of the shell. If this is so in M. similis, the large dense masses of secretory material in the vitelline cells associated with the ovum may account for the lack of normal egg-shell production in this case. Some apparently normal eggs were undoubtedly produced in vitro

and it is likely that premature tanning of the vitelline droplets and the concomitant failure of egg-shell production occurs as a result of continuing cultivation.

The Ovary

In contrast to the results from the brief observations made on the ovary of adult M. similis recovered from mice, cultured flukes possessed the cytoplasmic "ribosomal bodies" seen by Björkman & Thorsell (1964a) in the oocytes of F. hepatica. As has been suggested earlier (page 358), it is likely that the sections through the ovary of adult M. similis only showed immature oocytes or early developmental stages. The ova or oocytes of cultured M. similis (either in the ovary or the uterus) did not contain the cortical granules seen in Meiogymnophallus minutus adults (page 241), F. hepatica (Gresson, 1964; Björkman & Thorsell, loc.cit.) and in S. mansoni (Erasmus, 1973). Erasmus (loc.cit.) notes that these granules are absent from the ova of unisexual infections of S. mansoni. The production of these granules may thus appear to be sensitive to abnormal conditions. Erasmus implicates these granules in the process of fertilization and, if this is also the case in M. similis, this may help to explain the cessation of normal egg production after the first few days. Björkman & Thorsell (loc. cit.) suggest that the large "ribosomal bodies" seen in the oocytes of F. hepatica are involved in the production of cortical granules. The presence of one and not the other in cultured M. similis renders this rather unlikely in the present case.

A more detailed study of the ovary of cultured flukes and adults recovered from mice would be necessary in order to confirm these suggestions.

The Male Reproductive System

The Prostate Gland

The secretions of the prostate gland were observed on one occasion only in the lumen of the ductus ejaculatoris. The body of the gland was never seen, nor was it seen in adults recovered from mice. This suggests that, in M. similis, the prostate gland is probably quite small (cf. Meiogymnophallus minutus).

The Testes

The development of the male gametes of M. similis in vitro and in vivo is very similar to that described for the frog lung fluke Haematoloechus medioplexus by Burton (1972) and for Paragoniumus miyazakii by Sato et al (1967).

It is likely that the early stages of spermatogenesis (from the primary to the tertiary spermatogonia) occur during the development of the cercaria to the metacercaria within the cyst. During the early stages of development of the newly excysted metacercaria, the germ cells of the testes divide and then become associated together in a cytophore when they divide again. Sato et al (loc.cit.) suggest that the formation of the cytophore is associated with the meiotic division which occurs between the primary and secondary spermatocytes. For these reasons, the majority of the germ cells in the testes of the metacercaria are probably tertiary spermatogonia or primary spermatocytes although there are undoubtedly a population of earlier development stages around the periphery of the testes. In the present study, the association of the secondary spermatocytes and the spermatids as a cytophore could be seen very clearly.

Spermateleosis (development of the spermatid)

Burton (1972) has described spermateleosis in H. medioplexus in considerable detail. Spermateleosis in M. similis appears to agree with Burton's description in virtually all respects.

The term "centriole-like body" was used by Burton to describe the lamellate body located between the two basal bodies of the developing spermatid. The term is used here to avoid confusion. However, Burton points out that:

> "... the structure seen in the lung fluke spermatids is not a typical centriole."

He suggests that the function of the "centriole-like body" is to provide a pool of monomeric subunits from which the doublet tubules of the axial filaments can be polymerised and that the basal bodies serve as nucleation sites for the formation of the doublet tubules. He also suggests that since the central core arises some distance away from the basal body, its assembly may not be directly under the influence of the basal body.

The dense plaques seen in the present study in cross-sections of the central processes and in the mature sperm were also seen in H. medioplexus by Burton. He suggests that the fusion of the three filaments occurs rapidly and he implicates the dense plaques in this process. He also remarks that the migration of the nucleus down the central process (which was also recorded in Fasciola and Dicrocoelium dendriticum by Hendelberg, 1962) appears to be correlated with the lateral fusion of the three separate processes "as if the migrating nucleus induced fusion at the level of its translocation down the shaft". No evidence was obtained in the present study to determine whether the

nucleus migrated down the central process during the later stages of spermatogenesis. However, this would probably be revealed by light microscope observation. Hendelberg (loc.cit.) suggests that the nucleus migration observed in Dicrocoelium and Fasciola may be common to the Digenea.

Burton records that the structures associated with the production of the axial filaments ("centriole-like body", basal bodies, striated rootlets) were never seen in mature sperm. This observation was confirmed in the present study. Burton suggests that:

> "... although these structures play an important role during sperm differentiation, once the process is completed, they are left behind in the residual cytoplasmic mass to degenerate. Of course, if the centriole-like bodies do indeed represent a pool of microtubular subunits, little of their mass should remain upon completion of the assembly of axial unit doublet tubules."

Since the peripheral microtubules apparently vary in number in different sections through the sperm, it is likely that they are not continuous along the length of the spermatozoon. Similar observations were made by Sato et al (loc.cit.) who record that in the head region of P. miyazakii there were 10-15 peripheral microtubules and that in the main portion of the tail the number was 20-22, whereas at the tip of the tail, it was 30.

Sperm Fine Structure

The fine structure of the sperm of cultured flukes was, in the majority of cases, similar to that of normal adults. It is discussed on page 360 and a possible model for the arrangement of the components of the sperm is suggested.

Abnormal Sperm in Cultured Flukes

Occasionally abnormal sperm with four axial filaments were observed in cultured M. similis. These were similar to those seen, in larger numbers, in cultured Meiogymnophallus minutus. It is suggested in relation to M. minutus (page 278), that this abnormality arises from a doubling up of the "centriole-like body"/basal body complex in the spermatid. This particular type of abnormality was never observed in vivo. However, it is important to assess the type and frequency of abnormal sperm morphology in vivo before abnormalities in sperm structure can be used to assess cultivation techniques (see page 278 and Davies, 1975). Featherstone (1971) has noted a doubling of the usual number of axial filaments in Taenia Hydatigena. Morseth (1969) has observed that, in E. granulosus, the axial filament may lack the central core or that one of the pairs of peripheral doublets may be replaced by an adherent central core complex. The same author records that in abnormal sperm of D. dendriticum, the central core complexes of the two axial filaments may be very closely associated with the loss of some of the peripheral doublets.

SECTION 2: SCANNING ELECTRON MICROSCOPY (SEM)

Results

Cultured flukes assumed a very typical abnormal position (Plate 195). The lateral edges of the flukes were curled toward the median line of the ventral body surface. In this position the ventral sucker, and the ventral surface generally, was infrequently observed. The tegument covering the anterior and the posterior parts of the fluke was similar to that seen in the metacercaria (page 335). However, irregular bodies similar to those seen on the surface of cultured Meiogymnophallus minutus (page 279) were also fairly frequent on the surface of cultured M. similis (Plate 197). Another prominent abnormality which was also observed in cultured M. minutus was the unusually long cilia of the ciliated sensory endings around the oral sucker (Plate 196).

Discussion

The bubbles seen on the surface of cultured M. similis under the scanning electron microscope were also observed on the surface of Meiogymnophallus minutus (and on F. hepatica in thin sections). As has been suggested previously (page 281) this bubbling up of the syncytium may be due to an osmotic imbalance or it may be the result of a variety of unfavourable or toxic conditions in the culture media.

The unusually long cilia protruding from the sensory bulbs of cultured M. similis were also frequently seen in cultured M. minutus and they were also present in F. hepatica after prolonged in vitro cultivation. It is possible that, in the definitive host, these cilia may "test" the surrounding

surfaces. Under <u>in vitro</u> conditions, which do not include a solid substrate (apart from the glass wall of the vessel), these cilia may protrude from the bulb of the sensory ending for an unnatural length as a result of "sensory deprivation".

Conclusions

1. The distribution of the tegumental secretory bodies in the syncytium of cultured flukes was similar to that of the metacercaria rather than that of the adult.
2. Large masses of crystalline, spine-like material were found in the tegumental cell bodies of cultured flukes. Biconcave secretory bodies were often closely associated with these masses. This abnormality was not seen in adult flukes from mice.
3. The cell bodies of the glands associated with the forebody tegument contained large, amorphous masses of secretory material which was not membrane-bound - this was never seen in normal adult flukes.
4. The surface features of cultured flukes were similar to those of the metacercaria except that there were some tegumental bubbles and the cilia of the ciliated-type sensory endings often protruded for an unusual length.
5. The "mitochondria cells" associated with the tegument in the posterior/lateral regions were similar to those of the metacercaria and the adult.
6. The cells of the digestive caeca of cultured flukes were reduced in volume; the larger, less dense secretory bodies seen in the adults were not present; there were no junctional complexes with the parenchymal cells as in the adult; and the dense material between adjacent lamellae often appeared to have been extruded and was clumped together.
7. As in the metacercaria and the adult, the parenchymal cells contained stores of α glycogen.

8. The excretory system of cultured flukes was similar to that of the adult although less lipid was present.
9. The Mehlis gland of cultured flukes apparently developed normally.
10. Vitellogenesis occurred *in vitro*. The vitelline droplets in the vitellaria often had a ragged outline and they may correspond to the prematurely tanned secretions seen under the light microscope. Vitelline cells associated with a non-shelled ovum were often seen in the uterus - the secretions in these cells were present as large, dense masses of material.
11. The ova (or oocytes?) in the ovary of cultured flukes possessed "ribosomal bodies" but no cortical granules.
12. The prostate gland apparently produced secretions *in vitro* although the body of the gland was never seen.
13. Spermatogenesis and spermateleosis were apparently normal in cultured flukes and were essentially similar to that described for *H. medioplexus* (Burton, 1972). Large masses of mainly normal sperm were produced *in vitro*. Very occasionally sperm with four, rather than two, axial filaments were seen.

CHAPTER 6 : GENERAL DISCUSSION

The basic problems involved in the *in vitro* cultivation of helminths have been summarised in the Introduction. During the present study, one of the major problems has been the inconsistency of the results. The variations between different cultures where the same media was used (inter-culture variation) and between duplicate treatments within the same culture (intra-culture variation) were often very striking. The micro-environment which the fluke inhabits within the cultivation system is obviously of the utmost importance. It depends on a variety of factors including the shape of the culture vessel, whether the vessel is agitated, how frequently the media is changed, how many flukes there are per culture and their proximity to each other. The media in the micro-environment is a delicately balanced mixture of the flukes' secretory products and waste materials and the cultivation medium. This may affect a variety of physico-chemical conditions such as pO_2, pCO_2 and pH in the immediate vicinity of the flukes. During the *in vitro* cultivation of *Schistosoma mansoni* schistosomules Clegg (1965,a) found that although the majority of flukes developed up to Stage 4 (appearance of sperm in the male) or Stage 5 (appearance of egg-shell protein in the vitellaria) only one fluke reached full development (Stage 6) and produced a shelled egg. The author suggests that this may have been due to some unusual physical condition such as the fluke being held in a fold in the cellulose sac, i.e. the micro-environment contained freak perfect conditions which enabled the fluke to complete its development.

The presence of suitable physical conditions within the

cultivation system may be as important as the chemical nature of the cultivation medium. In the cultivation of Echinococcus granulosus protoscoleces (Smyth & Davies, 1974) strobilisation and maturation is only achieved in a diphasic medium with a solid base. However, fertilisation does not occur in vitro and shelled eggs are not produced; the authors suggest that the correct physical conditions may not be present in the cultivation medium and that compression of the strobila in some way may be necessary for insemination to occur. A similar problem was solved in the case of Schistocephalus solidus by compressing the worms in narrow cellulose dialysis tubing (Smyth, 1969); however, this has proved unsuccessful for E. granulosus (Smyth & Davies, loc. cit.).

Another factor which is very important when inter-culture variation is considered is the origin of the non-defined or natural components such as sera, embryo and liver extracts. It has become strikingly apparent during the present study that different batches of foetal calf and chick sera have very different growth-promoting properties and that some have a pronounced inhibitory effect. Also, whereas one particular batch of serum may be particularly suitable for the cultivation of one trematode, it may be unsuitable for the cultivation of another. It is necessary, therefore, to screen each batch of serum before it is routinely used in the cultivation medium. Smyth & Davies (1974) have discussed the problems involved in the cultivation of the cestode Echinococcus granulosus and they stress the inevitable variation caused by the use of non-defined components in the culture medium. Although many attempts have been made to replace serum with a more defined substitute (e.g. Lasfargues et al, 1973;

Hayashi & Sato, 1976), the results have often been equivocal and although the substitute may support the growth of one particular cell line, it may be unsuitable for another. The particular constituents of the serum which are responsible for promoting the growth of cells in culture are still unknown (Clarke & Stoker, 1971).

With the development of defined media such as Parker 858 and NCTC 135 which contain a complex mixture of amino acids, vitamins, co-factors and nucleotides based on a balanced salt solution, their use has become widespread in cell and tissue culture and in the cultivation of helminths. However, such complex media may not always be required especially in the case of well-developed metacercariae which mature quickly and contain food reserves. e.g. the "best" medium for the cultivation of Microphallus similis metacercariae was Hanks saline plus serum - this was slightly superior to the more complex NCTC 135 plus serum. The precise requirements necessary for the growth in vitro of helminths are unknown and the tendency is to base the cultivation medium on a complex media which contains as many nutrients and substrates as possible. However, it is possible that a high level of nutrients may prove to be inhibitory - for example, an incorrect balance of amino acids may prevent their uptake due to competitive inhibition at the active transport sites. It is important to investigate the lower limits of a cultivation system by using a variety of defined media of increasing complexity, starting with the simplest, and using various levels of serum and other non-defined supplements.

From the results presented here, it appears unlikely that a "universal" medium could be developed for the cultivation of digenea. Adult trematodes inhabit a wide variety of habitats, e.g.

<u>Schistosoma</u> sp. - blood system

<u>Fasciola</u> sp. & <u>Dicrocoelium dendriticum</u> - bile ducts

<u>Diplostomum</u> sp. - intestine

<u>Paragonimus</u> sp. - lungs,

and many, e.g. <u>Gorgodera</u> sp., <u>Haematoloechus</u> sp., <u>Haplometra cylindracea</u> and <u>Proctoeces subtenuis</u> have, as their final host, a cold-blooded animal such as a fish or an amphibian. The environments presented by these habitats will vary considerably and so, presumably, will the requirements of the parasites which are found in them. It even seems unlikely that a generalised cultivation medium can be designed for each particular habitat. Strigeoid trematodes, e.g. <u>Diplostomum phoxini</u>, and <u>Meiogymnophallus minutus</u> both inhabit the intestines of water birds and, whereas, <u>D. phoxini</u> thrives in an egg yolk medium (Kannangara, 1973; Kannangara & Smyth, 1974), no live flukes were recovered when <u>M. minutus</u> metacercariae were cultured in an identical medium.

The usefulness of ultrastructural techniques to assess cultivation methods has been established in the present study. Using these techniques, a much fuller picture can be obtained of the effects of <u>in vitro</u> culture at a cellular level. Although certain abnormalities which occur during <u>in vitro</u> cultivation can be studied at a light microscope level, e.g. the premature tanning of the vitelline secretory droplets in <u>Meiogymnophallus minutus</u> and <u>Microphallus similis</u>, more information can be obtained

when these abnormalities are studied under the electron microscope. Some processes which appear to occur normally *in vitro*, e.g. spermatogenesis in *M. minutus*, show conspicuous abnormalities when studied at an ultrastructural level. In addition, a detailed study of the tegument and caecal epithelium can only really be achieved at the electron microscope level.

The development of *M. similis* metacercariae during the first few days of *in vitro* cultivation appears quite normal when examined under the light microscope. However, at an ultrastructural level, it is apparent that conspicuous abnormalities develop in the tegumental cell bodies, the cell bodies of the unicellular gland cells and the cells of the caecal epithelium at a very early stage. It is interesting to note that the unicellular gland cells of *M. similis* were morphologically identical to those of *M. minutus* but that under similar cultivation conditions, the gland cells of *M. similis* became abnormal whereas those of *M. minutus* remained apparently normal. This underlines the danger of assuming that a similar morphology implies a similar function or chemical composition.

It is important that those surfaces of the fluke which form the host-parasite interface during *in vivo* development should develop normally during *in vitro* culture - it is these surfaces which come into intimate contact with the culture medium. It is particularly important that the adsorptive and digestive surfaces of the cultured flukes, i.e. the tegument and the caecal epithelium, should develop normally in culture. Even if the culture medium contains all the necessary nutrients, they will be of no value to the fluke if it is unable to take them up. Smyth (1966) states that:-

> "... unsuitable cultivation conditions could indirectly affect the availability of nutriment by failing to provide the conditions under which normal feeding can take place."

Abnormalities in the tegument and the caecal epithelium at an ultrastructural level were observed in all three flukes examined during the present study and this is a very important factor to consider when development in vitro fails to occur or becomes abnormal.

Further work, at an ultrastructural level, could include the use of ultra-histochemical and ultra-autoradiographical techniques which could be used to provide additional information on the distribution of enzymes, e.g. phosphatases, and the uptake of nutrients and substrates in cultured flukes. Kannangara (1973) has studied the distribution of acid phosphatases in the tegument of cultured Diplostomum phoxini and compared it to the distribution in the metacercariae. Hanna (1975), using autoradiographical techniques, has studied the incorporation of labelled amino acids into the secretory bodies in the caecal epithelium of F. hepatica slices maintained in culture.

Although it is obviously more difficult to culture a metacercaria such as Fasciola hepatica which must undergo a considerable amount of growth and differentiation before it reaches adulthood, than it is to culture a progenetic metacercaria such as those of Meiogymnophallus minutus and Microphallus similis which possess well-developed genital anlagen and develop to

adulthood within a few days, the development *in vitro* of such progenetic metacercariae may present a very useful biological model. In *M. minutus* and *M. similis* a definite sequence of developmental processes occurs in a relatively short period of time before the flukes become mature. The study of the development of these flukes could help in the understanding of certain basic problems - for example, why does gametogenesis proceed so far and no further in the metacercarial stage, how is this process controlled and what "triggers" continued development once the fluke reaches its final host?

The cultivation of progenetic metacercariae, although relatively easy, is not without its problems. The premature tanning of the vitelline secretions of *M. minutus* and *M. similis* which results in the production of abnormal eggs or no egg-production at all, also occurs when adult *Fasciola hepatica* are maintained *in vitro* (Clegg, 1957). Such a problem may prove to be fundamental in the cultivation of trematodes, and the study of vitellogenesis in such flukes as *M. minutus* and *M. similis* could prove particularly useful. If premature tanning was understood and could be induced *in vivo* it may be possible to make use of it as a control measure since normal egg-production would be prevented.

The co-ordinated differentiation of the germinal and somatic cells which produces the normal pattern of development seen *in vivo* can become unco-ordinated during *in vitro* culture. In the present study, there was considerable body or somatic growth during the cultivation of *F. hepatica* metacercariae although the cells of the genital rudiment showed no development or

growth whatsoever. In addition, there was some evidence that when partially developed flukes were transferred to culture media, the developing genitalia regressed (either by degeneration or by de-differentiation) although the somatic growth continued for some time. A completely opposite situation has been recorded during the cultivation of Echinococcus multilocularis (Smyth & Davies, 1975) and, to a lesser extent, with E. granulosus (Smyth, 1971). In E. multilocularis (Smyth & Davies, loc.cit.) there appears to be a supression of mitosis in those cells (somatic cells) which give rise to the strobila resulting in the production of sexually mature monozoic or unsegmented forms. These authors suggest that the induction of sexuality and the suppression of segmentation in cultured E. multilocularis could be linked to certain factors which inhibit cell division in the somatic growth region. This hypothesis is based on one which has been developed to account for the initiation of sexuality in Hydra sp. (Burnett & Diehl, 1964) where such factors as starvation, overfeeding, temperature and pCO_2 variations are known to induce the development of sexuality. Smyth & Davies (loc.cit.) suggest that factors present in the serum or complex media or such physical factors as pCO_2 could inhibit somatic growth and so produce monozoic development. Problems such as these are basic in developmental biology and it is becoming increasingly recognised that parasites, when cultured in vitro, may be used as biological models in the study of the differentiation and development of cell and organ systems.

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