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STUDIES ON SANGUINICOLA INERMIS PLEHN, 1905
FROM CULTURED CARP (CYPRINUS CARPIO L.) IN BRITAIN

A thesis presented for the degree of
Doctor of Philosophy to the University of Stirling

by

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1/85

To

My

Departed

Mother

D E C L A R A T I O N

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted, nor is being submitted for any other degrees. All the sources of information have been duly acknowledged.

.....N.A.M. Iqbal.....
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A b s t r a c t

Key words: Sanguinicola inermis, Digenea: Sanguinicolidae, freshwater blood fluke, fish parasite, morphology, developmental biology, effects on growth, blood parameters, histopathology.

Sanguinicola inermis Plehn, 1905, a recently introduced fish pathogen in Britain, has caused considerable damage to the carp industry. Two severely affected fish farms in England were included in the study. The incidence of fish infection for both farms was high, being 74-84% in 0+ and 1+ carp.

Studies were made on the infection process and details of route of entry, migration and maturation of the worm are presented. An apharyngeate, furcocercous, lophocercous cercaria which develops in the snail Lymnaea (Radix) peregra (Müll) was shown experimentally to develop into an adult Sanguinicola inermis. Maximum penetration of cercaria was achieved within 30 minutes and fins appeared to be the preferred site. Large numbers of worms were found to remain in the skin after penetration where they continued to develop to maturity, a previously unreported feature of S. inermis infection. The greatest migration to preferred loci occurred at 60 days post infection immediately prior to egg production. The migratory route used was found to be the loose connective tissue as well as the circulatory system. The distribution of mature worms in major blood vessels changed with season.

Light microscopical studies were made on the morphology of specimens collected from the two farms and these were identified as S. inermis. Scanning electron microscopy of the surface topography and transmission electron microscopy revealed details of the tegument and provided evidence for the absence of spines.

The major developmental features of the worm are described. A marked increase in size occurred up to 36 hours post penetration. Subsequent growth was slower. Egg production by mature worms began 10 weeks post infection at 15°C. Distribution and development of eggs in tissues is described. Egg production continued throughout the year with peaks during the summer months despite the constant environmental conditions.

The growth rate of 0+ fry was studied over a period of 16 weeks and heavily infected fish showed stunted growth and poor Specific Growth Rates, Protein Efficiency Ratios and Food Conversion Ratios. Haematological studies showed that infected fish suffered from hypochromic macrocytic anaemia with leucocytosis and an increased Erythrocyte Sedimentation Rate.

Three different phases of infection were recognized. During phase I, the invasive stage, mortality may occur if infection levels are extremely high, but lightly infected fish present as clinically normal. Phase II was found to be the most critical phase since the majority of fish (over 90%) died at this stage. Histopathology revealed the progress of the infection from cercarial invasion to worm migration, maturation, egg production and miracidial hatching. Pathological changes were observed in the skin in phase I. In phase II, the heart, gills and kidneys were severely affected by both mature worms and developmental stages. The infection reached a chronic stage during phase III evidenced by a granulomatous tissue reaction largely in response to residual eggs in tissues.

An attempt was made to integrate the phases of infection, development of the worm and pathogenesis in order to elucidate the host-parasite relationships.

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CHAPTER 1

GENERAL
INTRODUCTION

1. General Introduction

1.1 Present status and distribution

Since the first report on a blood-living digenetic trematode (Schistosoma haematobium, Bilharz, 1852) in man a great variety of blood flukes have been reported from mammals (family, Schistosomatidae), birds (family, Ornithobilharziidae), turtles (family, Spiror^cphiidae) and from freshwater and marine fish (family, Sanguinicolidae). Many species belonging to the four above mentioned families have been found to be pathogenic to their hosts, thus being of medical, veterinary and/or economical importance. This has resulted in an enormous number of published studies, particularly of schistosome species. Relatively very little attention has been paid to the blood flukes of cold-blooded vertebrates since their first report over a century ago.

The sanguinicolid blood fluke (Sanguinicolidae) was originally reported in fish by Odhner (1900). Since then very many species have been described from many different parts of the world in both wild and cultured fish. Fifty-one species of the family Sanguinicolidae, representing 12 genera have been recorded from a total of 91 fish species. Forty of these host species are from freshwater and 51 from the marine environment throughout the world. The literature on all the blood-flukes of cold-blooded vertebrates available up to December, 1971, was reviewed by Smith (1972). Only one more species has been described since then (Schell, 1974).

As far as freshwater fish species are concerned, blood-flukes of the genus Sanguinicola are known to occur in 12 families. In these 12 families, several species of Sanguinicola have been reported and

are listed in Table 1.1 with their host species. Four of these species are restricted to European countries including the USSR. These are Sanguinicola inermis, Plehn, 1905; S. armata, Plehn, 1905; S. intermedia, Ejsmont, 1926 and S. volgensis, Rasin, 1929. The infection of Sanguinicola species in salmonids is so far only reported in the USA. These are S. davisi, Wales, 1958; S. klamathensis, Wales 1958; S. alseae, Meade and Pratt, 1965, and S. idahoensis, Schell, 1974.

Sanguinicolid flukes of fish are of special interest amongst the digenea because they parasitize the circulatory system of fish. Some species of the genus Sanguinicola are regarded as dangerous parasites since they cause the disease 'Sanguinicoliasis' which is of great importance in fish culture. They are known to be associated with clinical problems in farmed cyprinids in Europe and the USSR and in farmed salmonids in North America. With the increasing practice of aquaculture in protein production, and the transportation of fish from one area to another, Sanguinicola infections are spreading at an alarming rate.

Of the four known European species, it is S. inermis which has received special attention, mostly from Russian scientists, because of its epizootological importance in carp, Cyprinus carpio L., where mortalities are frequently attributed to this parasite. Bauer, Grapmane, Uspenskaya and Chechina (1956), Chechina (1959) and Naumova (1961a) have all reported mass mortalities in rearing ponds in the Soviet Union due to acute infections of S. inermis. Losses due to Sanguinicola infection have also been reported from other European countries. Lucky (1964) described S. inermis from Czechoslovakia where most of the carp propagating ponds were found to be affected.

Table 1.1 Distribution of freshwater sanguinicolid blood-flukes

<u>Sanguinicola</u> species	Country	Host-fish	Reference(s)
<u>S. inermis</u> Plehn, 1905	Germany	<u>Cyprinus carpio</u>	Plehn, 1905 & 1908; Scheuring, 1920, 1921 & 1922; Ejsmont, 1926; Léger, 1930; Schäperclaus, 1954; Körting, 1982 (pers. comm.)
	USSR	<u>C. carpio</u>	Bauer, Grapmane, Uspenskaya & Chechina, 1956; Chechina, 1959; Naumova, 1960 & 1961a, b, & c; Bykhhovskaya-Pavlovskaya, Gusev, Dubinina, Izyumoud, Smirnova, Sokolovskaya, Shtein, Shul'man, and Epshtein, 1964.
		<u>Esox lucius</u>	"
		<u>Tinca tinca</u>	"
	Czechoslovakia	<u>C. carpio</u>	Lucky, 1964.
	China	<u>C. carpio</u>	Cheng-yen Hu, So Long & Wei-chu Lee, 1965.
	Britain	<u>C. carpio</u>	Sweeting, 1979.
<u>S. armata</u> Plehn, 1905	Germany	<u>C. carpio</u>	Plehn, 1905, 1908.
		<u>Tinca vulgaris</u>	
		<u>Scardinius erythrophthalmus</u>	
		<u>Tinca tinca</u>	Bykhhovskaya-Pavlovskaya <u>et al.</u> , 1964.

contd. ...

Table 1.1 Distribution of freshwater sanguinicolid blood-flukes (continued)

<u>Sanguinicola</u> species	Country	Host-fish	Reference(s)
<u>S. armata</u> (cont.) Plehn, 1905	USSR	<u>T. tinca</u>	Plehn, 1905, 1908; Ejsmont, 1926; Bykhovskaya-Pavlovskaya et al., 1964.
	China	<u>Mylopharyngodon piceus</u>	Cheng-yen Hu, So Long & Wei-chu Lee, 1965.
		<u>Hypophthalmichthys molitrix</u>	" "
		<u>Aristichthys nobilis</u>	" "
		<u>C. carpio</u>	" "
<u>S. intermedia</u> Ejsmont, 1926	USSR	<u>Carassius carassius</u>	Bykhovskaya-Pavlovskaya, et al., 1964. Markevich, 1963.
		<u>Tinca tinca</u>	
	Poland	Cyprinids	Ejsmont, 1926.
<u>S. volgensis</u> (Rasin, 1929) McIntosh, 1934 (Syn. <u>Janickia</u> <u>volgensis</u>)	Czechoslovakia	<u>Pelecus cultralis</u>	Rasin, 1929;
		<u>Alburnus alburnus</u>	Bykhovskaya-Pavlovskaya, et al., 1964. "
	Britain	<u>E. lucius</u>	Chappell, 1967; Chappell & Owen, 1969;
		<u>Rutilus rutilus</u>	Shillcock, 1972.
		<u>Leuciscus leuciscus</u>	
<u>S. magnus</u> Cheng-yen Hu, So Long & Wei-Chu Lee, 1965	China	<u>Ctenopharyngodon idellus</u>	Cheng-yen, So Long & Wei-chu Lee, 1965.

contd. ...

Table 1.1 Distribution of freshwater sanguinicolid blood-flukes (continued)

<u>Sanguinicola</u> species	Country	Host-fish	Reference(s)
<u>S. chalmersi</u> Odhner, 1924	Sudan	<u>Auchenoglanis occidentalis</u>	Woodland, 1923; Odhner, 1924; Khalil, 1969.
		<u>Synodontis schall</u>	Woodland, 1923; Odhner, 1924.
<u>Sanguinicola</u> sp.	Israel	<u>Garra rufus</u>	Paperna, 1964.
		<u>Tylognathus steinitziorum</u>	
<u>S. davisi</u> Wales, 1958	USA	<u>Salmo clarki</u>	Davis, 1953; Davis, Hoffman and Surber, 1961.
		<u>S. gairdneri</u>	Davis, 1953; Wales, 1958; Davis et al., 1961.
<u>S. Klamathensis</u> Wales, 1958	USA	<u>Salmo clarki</u>	Meade, 1967; Evans & Heckmann, 1973.
		<u>S. clarki henshawi</u>	Wales, 1958.
<u>S. alseae</u> (Meade & Pratt 1965) Holmes, 1971 (Syn. <u>Cardicola</u> <u>alseae</u>)	USA	<u>S. clarki</u>	Meade & Pratt, 1965.
		<u>S. gairdneri</u>	"
<u>S. lophophora</u> Erickson & Wallace, 1959	USA	<u>Notropis hudsonius</u>	Erickson & Wallace, 1959.

contd. . . .

Table 1.1 Distribution of freshwater sanguinicolid blood-flukes (continued)

<u>Sanguinicola</u> species	Country	Host-fish	Reference(s)
<u>S. occidentalis</u> Van Cleave & Mueller, 1932	USA	<u>Perca flavescens</u> <u>Stizostedion vitreum</u>	Fischthal, 1947. Van Cleave & Mueller, 1932.
<u>S. huronis</u> Fischthal, 1949	USA	<u>Huro salmonids</u> <u>Micropterus dolomieu</u> <u>dolomieu</u>	Fischthal, 1947 & 1949. "
<u>S. idahoensis</u> Schell, 1974	USA	<u>Salmo gairdneri</u>	Schell, 1974.
<u>S. argentinensis</u> Szidat, 1951	Argentina	<u>Prochilodus platensis</u>	Szidat, 1951.

There have been few reports in the scientific literature on S. inermis in Germany since the classical work of Marianne Plehn in 1905. However, damage done by the parasite to the carp industry is known to be considerable (Körting, pers. comm. 1980) not only because of losses in production but also because of the efforts and expenditure put into treatment and control of the parasite.

Of the four known salmonid sanguinicolid, two are reported to be of epizootological importance in the United States. A severe epizootic was described by Wales (1958) which occurred at California's Darrah Spring State hatchery, where several thousand rainbow trout, Salmo gairdneri and steelhead trout, S. gairdneri gairdneri died due to Sanguinicola davisi infection. In another instance mortalities occurred among Lohontan cutthroat trout fingerlings, Salmo clarki henshawi due to the infection of a second Sanguinicola species which Wales (1958) subsequently described as S. klamathensis. It was fifteen years after the original description of this species that Evans (1974b) carried out experimental infection studies on salmonids, and showed a dramatic decline in the growth of cutthroat trout, Salmo clarki infected with S. klamathensis.

1.2 Sanguinicola sp. infection in Britain

The first report of Sanguinicola infection in Britain was by Chappell (1967), who identified Sanguinicola sp. in Rutilus rutilus, Leuciscus leuciscus and Esox lucis from Lincolnshire drains and the River Bain. Apart from some minor differences, the species he described closely resembles S. volgensis. Since then there have been several more reports of the occurrence of Sanguinicola sp. Shillcock (1972) described S. volgensis in R. rutilus and L. leuciscus from Essex. There have been no reports on the epizootiological importance

and/or pathological effects of S. volgensis. It appears that S. volgensis occurs only in rivers or in flowing waters, and this may be a possible reason why it has been so rarely detected. However, very recently Sweeting (pers. comm. 1984) found S. volgensis causing problems in chub, Leuciscus cephalus in the River Loddon (a tributary of the River Thames). With increased popularity of fish culture, it seems likely that the sanguinicolids will continue to cause problems in this country in the future - a prediction made by Wynne Owen over sixteen years ago in 1967.

The first report of S. inermis infection in Britain was in 1978 by Sweeting who reported it from Cotswold carp farm, Gloucestershire. Subsequently it was reported from a carp farm in Nottingham by Smith in 1978 (Christina Sommerville, pers. comm, 1980); in Kent by Wilson (Sweeting, 1978) and in farms in the Lea Division of the Thames Water Authority by Dearsley in 1978 (Christina Sommerville, pers. comm. 1980). Apart from the original outbreaks in Gloucestershire and Nottinghamshire, other farms in Thames Water area are also affected. More recently (1982), the parasite was detected from an intensive carp farm in Suffolk and from a newly established farm in Devon. This type of scattered occurrence, as illustrated in Figure 2.1, would suggest that the parasite is spreading throughout the country. However, many of these outbreaks can be traced to the original Gloucestershire source as infected fish are still detected in fish marketed from there. In at least one case a repeat outbreak occurred within four years on the same farm, despite extensive efforts to eradicate it from the site.

To date there have been no records of infection from the major carp breeders in the North of England. Natural breeding of carp is restricted to Central and Southern England due to seasonal temperatures.

9

Sweeting (1979) investigated an outbreak of S. inermis from the Cotswold carp farm, Gloucestershire. Due to this outbreak over 90% of the year's fry were killed. Heavy losses were reported by the farmer as a result of mortality of the fry, treatment of the ponds and the cost incurred by the necessity to change husbandry methods.

S. inermis is an exotic species which has found its way into Britain possibly from South East Europe or Central Asia with either ornamental carps or with aquatic weeds containing infected snails in the late 60's and early 70's (Sweeting, 1979). S. inermis, however, may have spread through other host species as it was reported from wild carp as well as non-cyprinid fish (Bykhovskaya-Pavlovskaya et al., 1964).

1.3 Aims of the study

Though numerous outbreaks of S. inermis infection have been reported from the cyprinids, relatively little is known of the infection process, development and its pathogenicity. To obtain an understanding of the various developmental stages of infection and their effects on the host, it is very important that experimental studies are carried out in a controlled environment. The aim of the present study was, therefore, to carry out such a study on S. inermis, in its invertebrate host, Lymnaea (Radix) peregra (Müll) and its vertebrate host Cyprinus carpio L. with special reference to the host-parasite relationships.

CHAPTER 2

GENERAL
MATERIALS
AND
METHODS

2. General materials and methods

Some of the methods were common to all experiments and these are described below. Specific methods, where employed, have been described under the relevant chapters. Fish and snails were obtained from a variety of sources and are categorised below.

2.1 Source of fish and their groups

Group 'A'

Fish (Cyprinus carpio) in this group were collected from the Cotswold Carp Farm (farm 'A'), Bourton-on-Water, Gloucestershire. These were fry from a single spawn in June 1979. Fish of this group had <20% infection with a mean weight of 1.83 grams. Since it is impossible to detect the infection in living fish, this figure was estimated from a sample of 25 fish and determined accurately at post mortem.

Group 'B'

Fish (Cyprinus carpio) of this group were also collected from the Cotswold Carp Farm at the same time but from a different tank. These were from the same age group as group 'A' but the level of infection in this group was 100%.

Group 'C'

Ideally uninfected fish from the same farms would have been used as controls, but no uninfected fish were available from the infected farms. Fish, C. carpio, which were known to be uninfected were therefore brought from Newhay fish farm which is in the same vicinity. These were from approximately the same age and same size of group as infected fish. This group was treated as the control for group 'D'. Fish from this group were also used to study the cercarial infection.

Group 'D'

Fish (C. carpio) in this group were obtained from the Calverton Fish Farm, (Farm 'B'), Nottingham, and had 100% infection. In addition, fish in this group also had severe clinical signs of Sanguinicola infection.

Group 'E'

Fish in this group were obtained from the tropical hatchery of the Institute of Aquaculture at the University of Stirling. These fish were of the fish species Oreochromis niloticus (L.) (mean weight 6.5 g) and were used for the study of host specificity of the parasite.

Group 'F'

Rainbow trout, Salmo gairdneri R. with a mean weight of 7 g were obtained from Cloan Hatcheries, Vale of Glendevon, Perthshire and were used for host-specificity trials.

2.2 Source of snails and their groups

Group 'a' Cotswold Lymnaea (= Radix) peregra (Müll).

Some 4,000 snails were collected with a hand net from the inlets and outlets of infected ponds, from circular tanks and from ditches around the Cotswold carp farm.

Group 'b' laboratory-bred specimens of L. peregra, (parent stock from Cotswold carp farm).

Snails were hatched under laboratory conditions from eggs collected from group 'a' above and reared in the laboratory.

Group 'c'

L. stagnalis (L) were obtained using hand nets from a local canal in Broxburn, Stirlingshire.

Group 'd'

Laboratory-bred specimens from group 'c' hatches^d and reared under laboratory conditions.

Group 'e'

Laboratory bred specimens of L. auricularia (L.), L. peregra, L. palustris (Müll), Bithynia tentaculata (L.) and Physa fontinalis (L.) were obtained from cultures maintained in the Department of Biological Sciences, Portsmouth Polytechnic.

Group 'f'

L. peregra were collected by hand net from the River Devon, Stirlingshire.

Group 'g'

L. peregra were collected from the infected ponds of the Calverton fish farm. The snails were collected by digging out the soil of the drained ponds.

2.3 Transportation, quarantine and maintenance

Fish

Prior to transportation, the fish were packed in small numbers in polythene bags which were then filled with oxygen and were transported to Stirling by train or by car. On arrival at Stirling the fish were transferred to isolated aerated, stock tanks for two weeks and maintained at 15°C. Throughout this quarantine period fish were regularly screened for pathogens and parasites. During quarantine a few external parasites, mainly protozoa (Trichodina sp., Chilodonella sp. and Ichthyophthirius sp.), were encountered. They were treated with formalin and malachite green (Hoffman and Meyer, 1974). Fish used for experiments were thereafter free from any other known

parasites or pathogens with the exception of S. inermis unless otherwise stated. The fish were fed once a day with commercially available trout pellet No.2.

Snails

Transportation of the snails was carried out using the same method as described for the fish. On arrival at Stirling the snails were transferred to small plastic tanks (14" x 8" x 7"). Good aeration was ensured by placing an air-stone in the tank. Snails were fed with fresh lettuce, porridge sandwich (cooked porridge spread thinly between two filter papers and left to dry, and then cut into pieces and placed in the tank) or Tetraphyll*. Tanks were cleaned every second day and 50% of tank water was replaced with fresh water.

2.4 Experimental facilities

Most of the experiments were performed by using three different experimental facilities. These are described below.

System A

This is a modified recirculated water system, consisting of a series of fibreglass tanks (36" x 24" x 15") connected by a biological filter/storage tank. Each individual glass tank was divided into two halves by the use of netted frames. The purpose of this division was to ensure the same environmental conditions for the control and experimental groups. To prepare the biological filter, different sizes of gravel were used.

Throughout the experiment a continuous flow-through of copper free water into the storage tank was maintained. This ensured a good water quality and also compensated for water losses through

* Product of Tetra, West Germany.

evaporation etc. The copper-free tap water was drawn directly from the mains supply passing through plastic pipes. The required water temperature was maintained by using several immersion heaters in the storage tank. Each tank was individually well aerated. The water was kept circulating by use of a water pump. The gravel in the filter tank was removed at intervals of four weeks and thoroughly washed to remove deposited detritus. The flow rate and other water quality criteria were measured and analysed several times throughout the experiment using standard methods.

System B

Fibreglass tanks (36" x 24" x 15") were equipped with an inlet and outlet. The water from the mains was directed through the inlet at a controlled rate and an outflow pipe drained the excess water off. This continuous flowthrough of water was necessary to maintain a good water quality and to reduce the amount of work involved in cleaning. In order to maintain the required water temperature several immersion heaters were used.

System C

This is the aquarium at the Institute of Aquaculture where facilities were available to maintain a constant temperature. For part of the study snail/fish/miracidia etc. were kept in this aquarium. Tanks used varied in size and shape and are described where relevant in each chapter. The water was well aerated, and held at a constant temperature of 15°C.

2.5 Examination of fish organs for *S. inermis*

Prior to dissection, sample fish were anaesthetized with Benzocaine solution (80 mg/l). After thorough external examination with a stereo dissecting microscope ($\times 6.3$, $\times 16$, $\times 25$, $\times 40$), different organs were examined at high power in the following order -

- (1) Skin,
- (2) Gills,
- (3) Heart
- (4) Fins,
- (5) Kidney and ureters,
- (6) Liver, spleen,
- (7) Gall-bladder,
- (8) Swim-bladder,
- (9) Brain and
- (10) Eyes.

(1) Skin: The skin scrapings were taken from selected areas of the body and examined under high power.

(2) Gills: After removal of operculum the gills were cut out individually and immersed in 0.9% saline solution. Gill arches were then teased apart with fine needles. Blood vessels of the primary lamellae were also teased apart. The number and position of worms was noted. Primary lamellae were also squashed and examined under high power ($\times 10$ and $\times 40$) for the presence of eggs and miracidia.

(3) Heart: An incision was made along the ventral surface of the fish from the pectoral girdle to the cloaca. The heart, bulbus arteriosus, and afferent branchial blood vessels were carefully cut out and placed in small petridishes containing 0.9% saline solution.

These organs were then carefully opened up and examined.

(4) Fins: Fins were removed and examined individually. Usually worms, if present, were most visible using a $\times 10$ lens of a compound microscope. Worms were dissected out by teasing apart the connective tissues of the fin.

(5) Kidney and ureters: Kidneys and ureters were dissected out from the fish and were kept in saline. To detect the encapsulated eggs, miracidia and sometimes worms, a portion of the kidney was placed on a slide and a squash was made. Other portions were macerated in saline and examined for worms.

(6 - 9) Liver, spleen, gall-bladder, swim bladder and brain: These organs were dissected out individually from the fish and examined following the procedure described in 5 above.

(10) Eye: The eyes were carefully removed by making incisions through the connective tissue around the orbits and by severing the optic nerve and muscles. The eye was then opened up and examined. A squash was made from the lens and then examined.

2.6 Examination of snails

There are no external physical features to indicate the infection in snails, therefore two different methods were followed to isolate the infected snails. 1-5 snails were placed in vials (45 ml.) containing fresh water. Vials were then kept floating in a water bath to maintain the required temperature (20°C). A 60 W lamp was used to give constant illumination. Shed cercaria were then collected and counted using a stereo dissecting microscope.

The second way of examining snails was by direct examination of the internal organs. This was achieved by severing the head and foot. The shell was then crushed and the internal organs, including the digestive gland, removed. These dissected organs were then examined under both low and high powers of the microscope.

2.7 Descriptions of the farms

Farm 'A'

This is an extensive carp farm situated in Gloucestershire, England (Fig. 2.1). The ponds were excavated in gravel beds alongside the River Dickler. They vary in size from 1/20 to 12 acres and total water area is 17.2 acres. The ponds are normally non-drainable and many are interconnected. Depth of the ponds varies from 1½ to 16 feet. The farm also has five, 123 feet diameter circular tanks with individual pond water supply. The water supply to the farm is mainly from the water table and is hard and alkaline. The 'richness' of the ponds varies due to intermittent additions of manure. The farm provides small 0+ and 1+ fish for stocking and large fish for the table and has an average annual turnover of 20 tons. Snail species found on the farm are Lymnaea peregra, Bythynia tentaculata, Valvata cristata, Hydrobia ulvae, H. ventrosa and Planorbis sp.

Farm 'B'

This is a fish farm under the control of Severn-Trent Water Authority situated in Nottinghamshire, England (Fig. 2.1). The farm is divided into two units; a coarse fish experimental unit and a trout fish unit. It is the coarse fish experimental unit where the Sanguinicola infection has broken out.

The total area of the coarse fish unit is approximately four

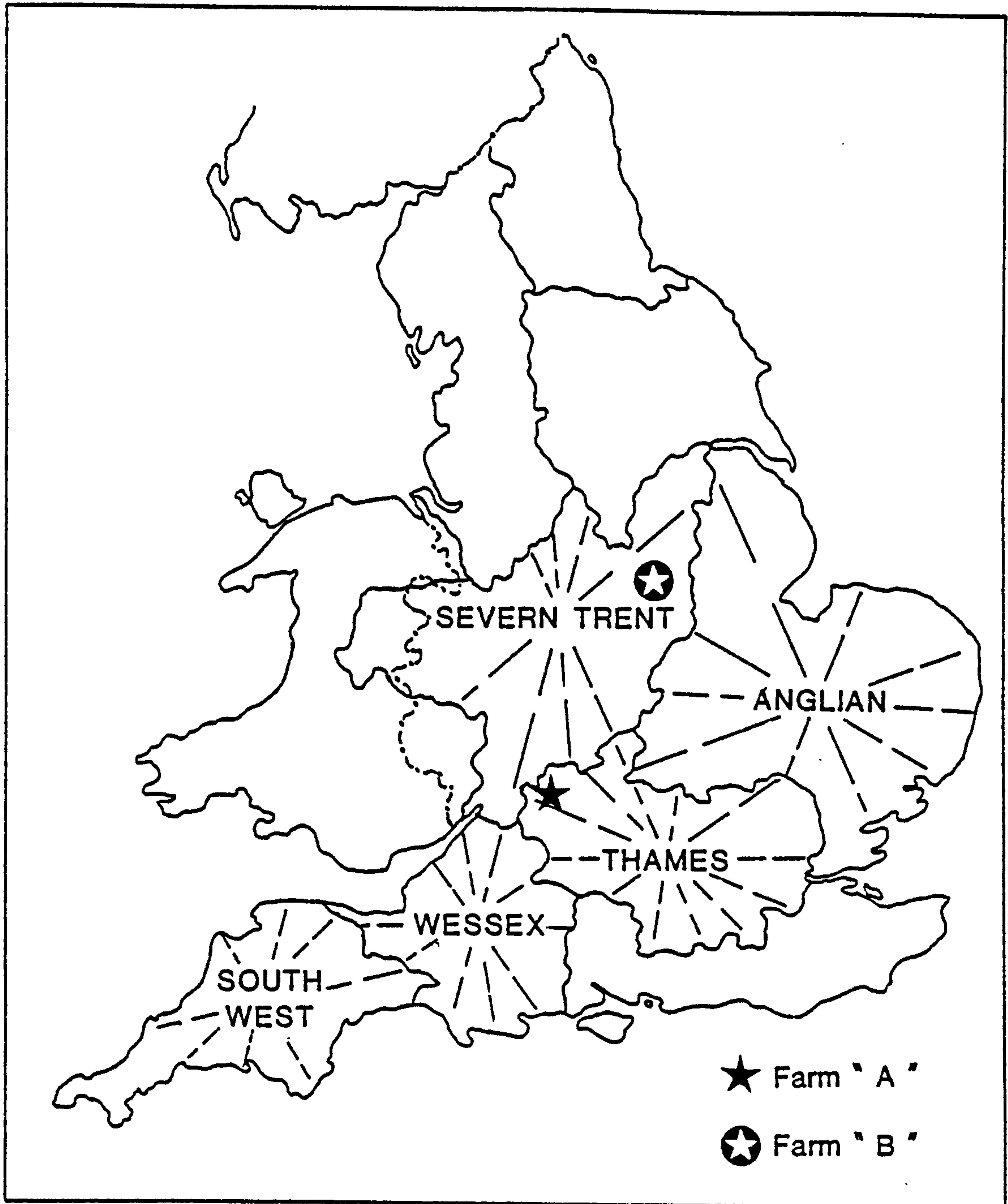


Fig. 2.1 Map of England and Wales showing the Sanguinicola affected areas under several Water Authorities with the location of the two severely affected farms of this study.

acres. The size of pond varies from 100 to 1200 metre square. The depth of the ponds also varies from 0.3 to 1.5 metres. Water supply is from a 20 m borehole which supplies both the trout and coarse fish units. The water is hard and alkaline. All ponds are separately drainable and also interconnected. The snail species infected by S. inermis was found to be L. peregra. The turnover of this experimental coarse fish unit is approximately 2 tonnes per annum.

CHAPTER 3

MORPHOLOGY

OF

SANGUINICOLA INERMIS

3. Morphology of *Sanguinicola inermis*

3.1 Introduction

Odhner (1900) was the first to report a sanguinicolid blood-fluke when he described *Aporocotyle simplex* from the gill of the dab, *Limanda limanda*. At that time it was thought to be an ectoparasite, but of uncertain zoological affinity. The first report of a freshwater sanguinicolid was given by Plehn in 1905 when she described *Sanguinicola inermis* and *S. armata* from cyprinid fish. Like Odhner she also incorrectly classified them, in this case as turbellarian endoparasites because of their ciliated coverings. Three years later Plehn (1908) transferred *S. inermis* and *S. armata* to the monozoic cestodes, even though this classification did not correlate with the internal anatomy of the worms. It was not until 1911 that Odhner clarified some of the misinterpretations and correctly classified *Sanguinicola* and *Aporocotyle* by placing them under Order Digenea.

Subsequently many more species of blood-flukes have been described from both marine and freshwater environments.

Since Plehn's (1905, 1908) original description on the genus *Sanguinicola*, various authors, namely Yamaguti (1953, 1958), Erickson and Wallace (1959), Bykhovskaya-Pavlovskaya, Gusev, Dubinina, Izyumova, Smirnova, Sokolovskaya, Shtein, Shul'man and Epshtein (1964), Hoffman (1967) and Radulescu and Ilie (1969) have prepared keys for the generic and specific identification of sanguinicolids. The most widely used key for the generic classification of sanguinicolids was prepared by Markevich (1951) and Bykhovskaya-Pavlovskaya et al. (1964).

There are, however, still conflicting views on the classification of blood-flukes into families and sub-families. The conflict lies in the justification for the division of the group into two families, one for freshwater and one for marine forms, i.e. Sanguinicolidae and Aporocotylidae respectively. After reviewing these conflicting views on the classification, Smith (1972) concluded that further life-cycle studies, particularly of the marine forms, may lead to the freshwater and marine forms being put under one family. This view of having one family was supported by Thulin (1980a), who, after elucidating part of the life-cycle for one marine form, Aporocotyle simplex Odhner, 1900, concluded that the pattern of life-cycle of A. simplex agreed extremely well with that of the few known life-cycles of freshwater sanguinicolids. This view was confirmed by Kjøie (1982) in her recent study of the morphology, development and ultrastructure of A. simplex.

According to the scheme proposed by Azimov (1970) and adopted by Smith (1972) the freshwater fish blood-fluke of the present study may be classified as follows.

Order Schistosomatida (Skryabin and Shults, 1937)

Suborder Sanguinicolata (Skryabin and Shults, 1937)

Family Sanguinicolida von Gaff, 1907

Genus Sanguinicola Plehn, 1905.

Sweeting (1978) identified the species causing mortalities in a carp farm in Gloucestershire, England, as S. inermis, since it lacked marginal spines. He also observed that the surface of the specimens was covered with diagonally arranged fine setae. Some doubts have arisen as to the species of Sanguinicola causing serious disease in carp farms in Germany (Körting, pers. comm.) since the problem of the presence or absence of spines has not yet been resolved. In view of

the various outbreaks of the disease in different parts of England it is necessary to study the surface structure of the worms in an attempt to elucidate the problems of identity. It was also hoped that this would provide additional information which would produce a firmer base for comparative morphological studies within the genus Sanguinicola and related genera. A greater knowledge of the morphological and biological features of Sanguinicola sp. would also assist in the understanding of the pathological processes involved. Morphological studies using light microscopy were therefore supplemented by the more recent techniques of stereoscan and transmission electron microscopy.

For the purpose of the present study the descriptions used for the genus Sanguinicola and the species S. inermis were adopted and modified from the above-noted texts. Details of this modified description of the genus Sanguinicola and the species S. inermis are presented in appendix 1.

3.2 Materials and Methods

Source of material

The materials studied were collected from two separate, naturally infected fish populations and from experimental laboratory infections. These fish belonged to the fish groups A, B, and D as detailed in Chapter 2. Fish were dissected according to the method described in Chapter 2.

Preparation of worms for light microscopic observation

The specimens were best studied alive and before fixation. Fresh specimens were transferred on a slide with the help of a finely drawn glass pipette. They were observed with or without slight coverglass pressure using bright-field and interference-contrast microscopy.

Live worms were found to be fragile and died within a few minutes, therefore, worms required for longer periods of observation were fixed in 3% buffered formal-saline. Specimens for stained whole mounts were fixed in 5% buffered formal-saline, Bouin's fluid or 70% alcohol. They were stained with Aceto-alum-carmin, Semichon's carmin and Ehrlich's iron haematoxylin (Methods in Appendix 2). The specimens were then cleared in Oil of Cloves, and mounted in Canada Balsam. Prior to staining, attachment of the worm on a coverslip was achieved by following the method described by Kennedy (1979). Measurements for taxonomic characters were made from the average measurements of fixed materials. Drawings were made from a large number of specimens with the help of a drawing apparatus.

Taxonomic indices

For the description of Sanguinicola sp. the following taxonomic indices were calculated from the measurements obtained from group 'A' and group 'B' worms.

Gut ratio

The gut ratio is defined as the ratio of the distance from the mouth to the posterior end of the gut to the length of the body.

Oötype ratio

The ratio of the distance from the oötype to the posterior end of the body to the length of the body.

Shape index

The shape index was calculated by dividing the total length of the body by the maximum width of the parasite and expressed as unit.

Preparation of worms for scanning electron microscopy (SEM)

To obtain a clear picture of the surface topography, it was found necessary to clean the adhesive substances (e.g. host mucus, serum etc.) from the surface of the worm as they formed an obscuring barrier to the SEM beam. Several washings with occasional flushing were therefore found to be absolutely essential. Problems arose with this technique as the worms were very delicate in nature and died within a few minutes of dissection. Washing was therefore performed as quickly and carefully as possible. They were first washed with saline solutions (0.9%) but it was found that this did not remove all the debris from the surface. It was found beneficial to follow the saline washes by a rapid rinse with a very weak solution (1%) of a surfactant ('Tween'-80*). However, the problem arising from the persistence of adhesive substances on the surface of the worm were only partly resolved by the use of 'Tween'-80. Worms were then fixed for three hours in 2.5% glutaraldehyde buffered with cacodylate to pH 7.4. They were then washed in buffer and postfixed for two hours in cacodylate buffered osmium tetroxide (1 hr. in a refrigerator at 5°C and 1 hr. at room temperature). Dehydration of the worms was achieved by transferring them through graded alcohols (30%, 50%, 70%, 90%) and to two changes of absolute alcohol. The specimens were then passed through Benzene-absolute alcohol 1:2 and 2:1 for five minutes each and through three changes of pure Benzene. To ensure rapid freezing, specimens together with a drop of benzene, were then dropped on aluminium foil lying on liquid nitrogen. The foil with the specimens was then placed in a small plastic petridish and left on a plate of ice in a freeze dryer** for 20 minutes. The dried

* Atlas Chemical Industries Inc.

** Pirani 11, Model EF03, Edwards High Vacuum

specimens were mounted on an SEM stub and coated with Gold using sputter coater.* The specimens were then examined in a I.S.I.60 scanning electron microscope operated at 8 kV and with a spot size of 8 nm. (This method for the preparation of worms was modified from the methods described by Norrevang & Winstrand, 1970; Thulin, 1980b and Kjøie, 1982).

Preparation of worms for transmission electron microscopy (TEM)

The methods followed for the preparation of worms for transmission electron microscopy (TEM) were the same as those described for SEM to the absolute alcohol dehydration stage. Absolute alcohol dehydrated specimens were then passed through two changes of 100% propylene oxide and then kept in a propylene-resin solution (50:50) for two hours. The worms were next transferred into the block cavity with 100% resin and left in an incubator at 37°C for three hours. The temperature of the incubator was then increased up to 70°C and left for 12 hours. Thin sections were cut with glass knives on an L.K.B.III ultramicrotome and mounted on bare grids. The sections were then stained with lead citrate and uranyl acetate for 15 minutes each. The material was examined on an JEOL.JEM.100C. electron microscope and micrographs were taken at magnification of 5,000 to 70,000.

* Edwards

3.3 Results

A. Adult worm

a. Light microscopical (LM) observations

The material studied was collected from two separate, naturally infected fish populations. These are namely specimens 'A' and specimens 'B' collected from the farm 'A' and farm 'B' located in England (see map, page 18). The descriptions of the farms can be found in Chapter 2. The specimens were recovered from the heart and from the major blood vessels of the gills of infected carp. The following descriptions of the species are based on thirty mature specimens collected from the two farms. The mean and range of the six different parameters measured are shown in the Table 3.1 together with various indices as defined in the materials and methods.

External features and the body wall

These are very small digeneans with a highly contractile, thin, semi-transparent body. Living juveniles of the species are greyish-white, while mature specimens are brownish-white in colour. Measurements of thirty specimens gave a wide range of dimension, varying from 455.9 microns to 1235.8 microns in length and 70.9 microns to 201.8 microns in width. These measurements along with other taxonomic characters are shown in Table 3.1.

The anterior end of all the specimens appeared to be elongated into a 'proboscis-like' (Fig. 3.1) projection which was seen to be more prominent and active during the early stage of development (see Chapter 4). As the development progressed the 'proboscis-like' projection tended to become blunt and less prominent. However, as the living specimen had the ability to vary in shape, this feature cannot be seen easily. To study the shape of this 'proboscis-like'

Table 3.1

Descriptions of the Sanguinicola sp. from the farm 'A' and farm 'B'
(measurements are in microns)

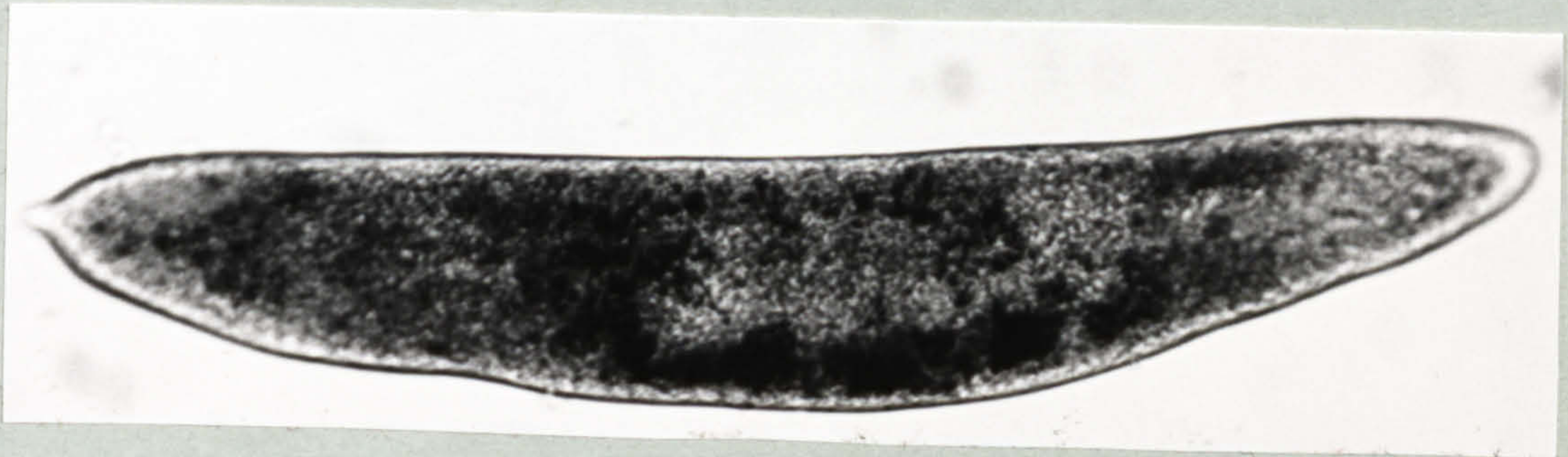
Characters	Mean	Range
Body length	899.6	455.9 - 1235.8
Body width	153.2	70.9 - 201.8
Length of oesophagus	295.2	152.0 - 378.3
Length of testes zone	263.7	68.3 - 378.3
Oötype distance	119.6	60.9 - 176.5
Size of mature egg	60.2 × 34.4	(43.0-75.0) × (17.7-41.0)
Gut ratio	1 : 3.1	
Oötype ratio	1 : 7.8	
No. of caecal lobes	4	
No. of pairs of testes	13	10 - 16
No. of ova in uterus at a time	1	
Shape index (Length/width)	6	5 - 8
Spination	Extremely minute setae or bristles, no spines.	

Fig. 3.1 Showing wet mounts of adults of S. inermis from the circulatory system of C. carpio.
 Diagram illustrates the major morphological features as observed microscopically.

A, specimen 'A' from farm 'A'. B, specimen 'B' from farm 'B'.

cp	:	cirrus pouch	ov	:	ovary
ev	:	excretory vesicle	pp	:	proboscis-like projection
ic	:	intestinal caecum	t	:	testes
led	:	lateral excretory duct	tcv	:	transverse connective vessel
m	:	mouth	ut	:	uterus
od	:	oviduct	vd	:	vas deferens
oe	:	oesophagus	vdt	:	viteline duct
ot	:	oötype	vit	:	vitellaria

A



B

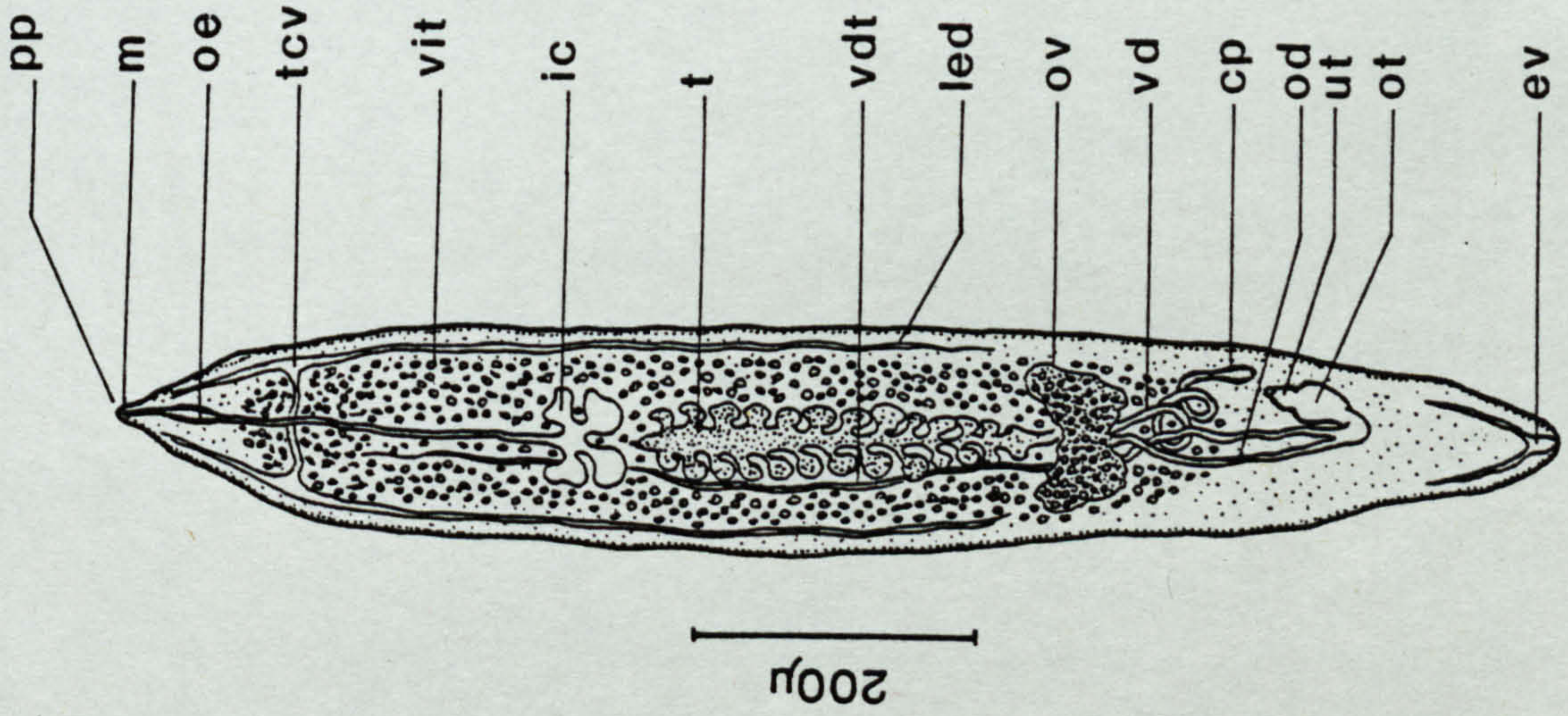
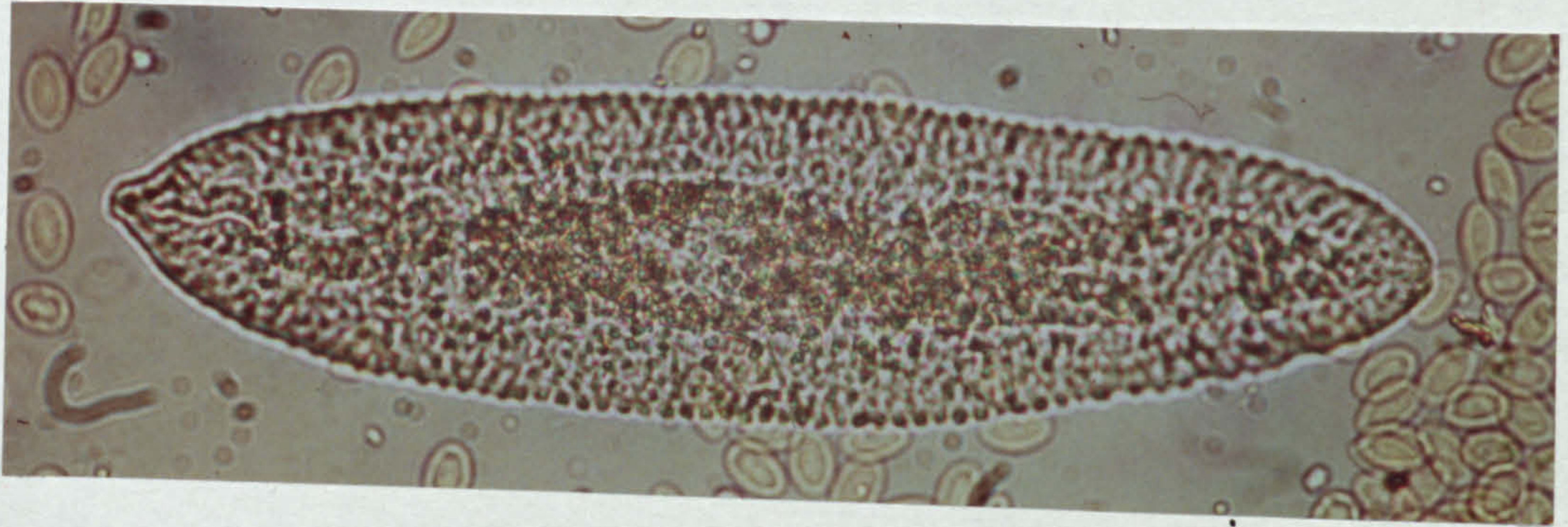


Fig. 3.1

projection, moribund or dead worms gave the best result. No constriction was seen between the projection and the body of the worm in any of the specimens studied. The posterior end of all worms appeared rounded.

The shape of the body of living specimens differed slightly from that of fixed specimens, since living specimens were constantly varying in shape but lanceolate at rest. Fixed specimens appeared spindle-shaped but sometimes their posterior end tended to be curled ventrally. This curling tendency appeared to be caused by contractions of the heavy musculature in the genital region. The shape index (length/width) varied with the size and age of the fixed specimens. In general, in the early stages of development, the worms were wider, but as they matured they became narrower (details of the development can be found in Chapter 4).

The outside margin of the body of the 'B' specimens was distinctly crenulated (Fig.3.1). However, no crenulations were seen at the outside margin of 'A' specimens. Minute 'setae-like' projections were also clearly visible on the crenulated margins of the 'B' specimens. These were arranged regularly along the body (Fig. 3.2) but were absent at the extreme anterior and posterior ends of the worms. Approximately three fine 'setae' appeared on each crenulation. The middle 'seta' was always longer than the adjacent two. These minute 'setae' were impossible to see in light microscopic observations if the specimens were first fixed in formalin. As with the marginal crenulations there were no 'setae-like' projections observed in the 'A' specimens. However, most of the 'A' specimens were fixed in formalin prior to examination. No marginal spines were observed on any of the specimens examined.

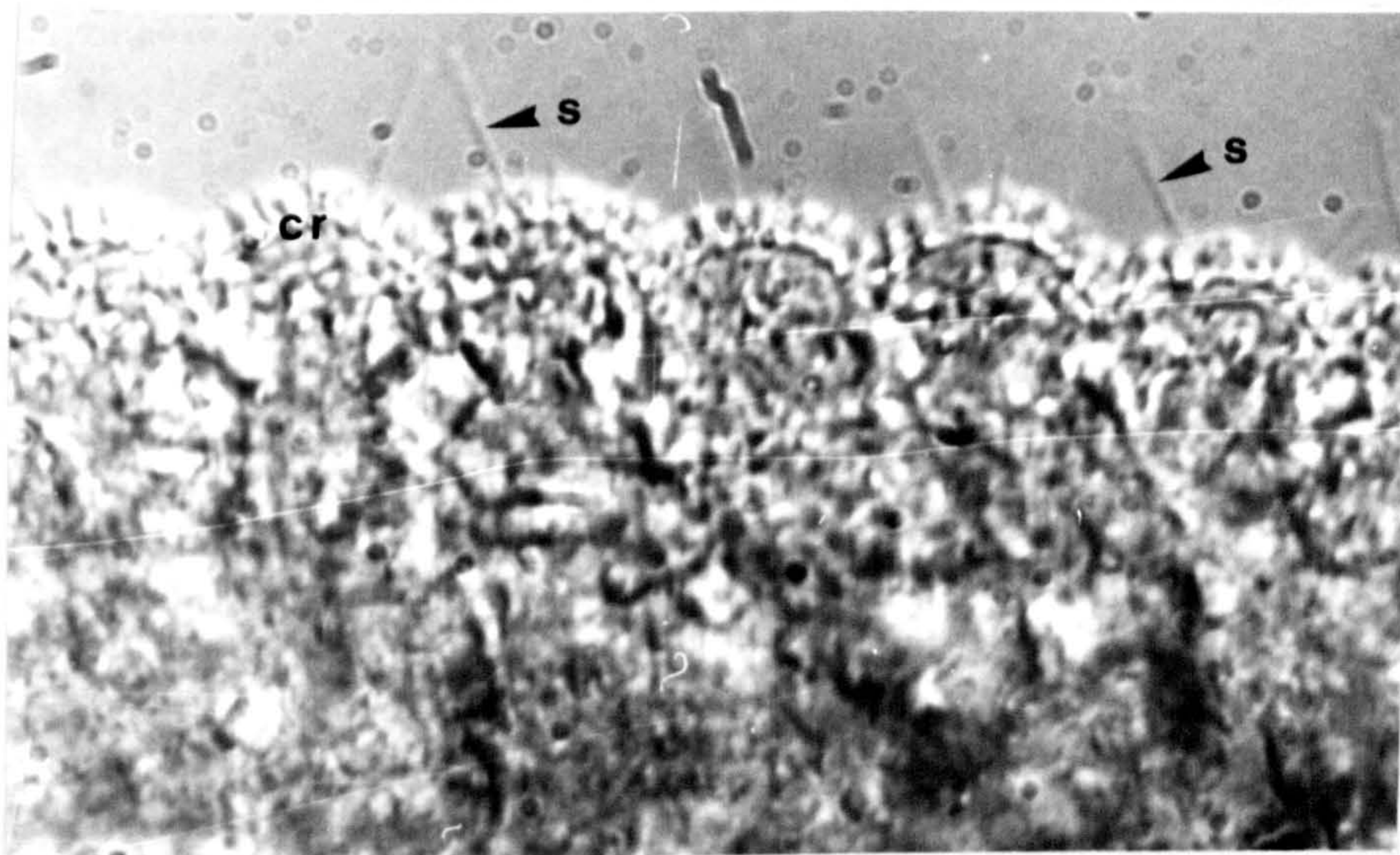


Fig. 3.2

Crenulated outside margin of the mid body region of S. inermis showing 'setae-like' projections ($\times 400$).

s: setae-like projections

cr: crenulated margin

Digestive system

The mouth opening was situated ventrally and close to the anterior tip of the body (Fig. 3.1). In the absence of a pharynx, the sub-terminal mouth led directly into a very long oesophagus of 152.0-378.3 microns (Table 3.1) which ended in a four lobed intestine. Prior to the opening into the intestine, the oesophagus formed a flask-like expansion which probably acts as a secretory reservoir. The length of the oesophagus in adult specimens was approximately one-third of the total body length. The ratio obtained for the length of the oesophagus to the length of the body is shown in Table 3.1. This ratio was found to be a consistent taxonomic character for both specimens 'A' and 'B'.

Reproductive system

The male reproductive system consisted of paired testes, situated immediately behind the intestinal caecum (Fig. 3.1). The testes consisted of 10-16 pairs of lateral vesicles of irregular shape and size which extended on both sides of a mid-line from the intestinal caecum posteriorly to ovary. The organ measured 68.3-378.3 microns in length and is shown in Table 3.1. They occupied centrally almost one-third of the whole body. The testes led into a centrally pointed vas deferens which travelled posteriorly and terminated in a copulatory organ near the posterior extremity of the worm. The vas deferens was convoluted into 2-3 loops just before passing into the cirrus pouch. The cirrus was small and papilla-like.

The female reproductive system as shown in Figure 3.1 consisted of a butterfly-shaped double ovary situated directly posterior to the testes. The oviduct, after emerging from the ovary, ran posteriorly and united with the vitelline duct just prior to forming the ootype.

The oötype was delimited by a constriction from the uterus. The metraterm contained only one immature egg at a time measuring 3.50 by 15.0 microns. The small vitellaria were numerous and were found to occupy the anterior region lateral to the gut and testes and extended as far back as the convoluted vas deferens.

Excretory system

In most of the specimens, short lateral excretory vessels were visible, extending from the anterior extremity towards the testicular region with a transverse connecting vessel partway along the oesophagus. A small excretory vesicle opened to the exterior through the sub-terminal excretory pore. Flame cells were not observed using the techniques described above.

b. Electron microscopical observation

From the light microscopical observation it was not possible to clarify some of the questions regarding the surface morphology of the worm which are such important taxonomic features. For example, the surface topography of the worm could not properly be seen due to the minute size of the worm. The nature of the 'setae-like' structures was difficult to determine using a light microscope as was their origin and arrangement. As details of the surface morphology are important in providing a morphological basis for the physiological activities at the host-parasite interface (Erasmus, 1970) it was decided to study these features further with the help of scanning electron microscope and transmission electron microscope.

Scanning electron microscopy (SEM)

The surface of the worms appeared to be covered by numerous transverse ridges as shown in Fig. 3.3. At higher magnification

these ridges were seen to be composed of irregularly branched microvillous-like projections which covered the entire body surface (Fig. 3.4). These projections were similar on both the ventral and dorsal surfaces and were variable in shape and size. The distal ends of the branched microvilli were tapered to an elongated point. Their size varied from 0.2 microns to 0.6 microns. Along the margins of the body the microvilli formed clusters (Fig. 3.5).

The surface topography of the anterior and posterior regions was similar to that found in other areas of the body but the covering of microvilli was less dense. No specialised structure could be seen in these regions.

Observations on several specimens revealed that there were no tegumental spines present. However, most of the specimens possessed the long setae-like structure arising from the surface of the body (Fig. 3.6). None of the specimens bore the surface pits, tubercles and spine bosses as described for other blood flukes of mammal and fish.

Transmission electron microscopy (TEM)

Sections of a small number of worms were examined using electron microscopy to study the origin and nature of the 'setae-like' structure and the microvilli of the tegument.

At higher magnification of 80,000 the tegument was seen to consist of two distinct layers (Fig. 3.7). The outer layer was composed of anucleated syncytium which was separated from the inner layer by a basement membrane. The inner layer was nucleated. The syncytial region was bounded externally by a trilaminate outer limiting membrane. The trilaminate membrane was present over the

Fig. 3.3

Scanning electron micrograph of the mid body region of adult S. inermis showing transverse ridges on the surface of the specimen (ventral view) ($\times 5,000$).

Fig. 3.4

Scanning electron micrograph of S. inermis to show the details of the branched microvillous-like^(mv) projection which covers the entire body surface ($\times 20,000$). Inset, higher magnification of the microvillous ($\times 30,000$).

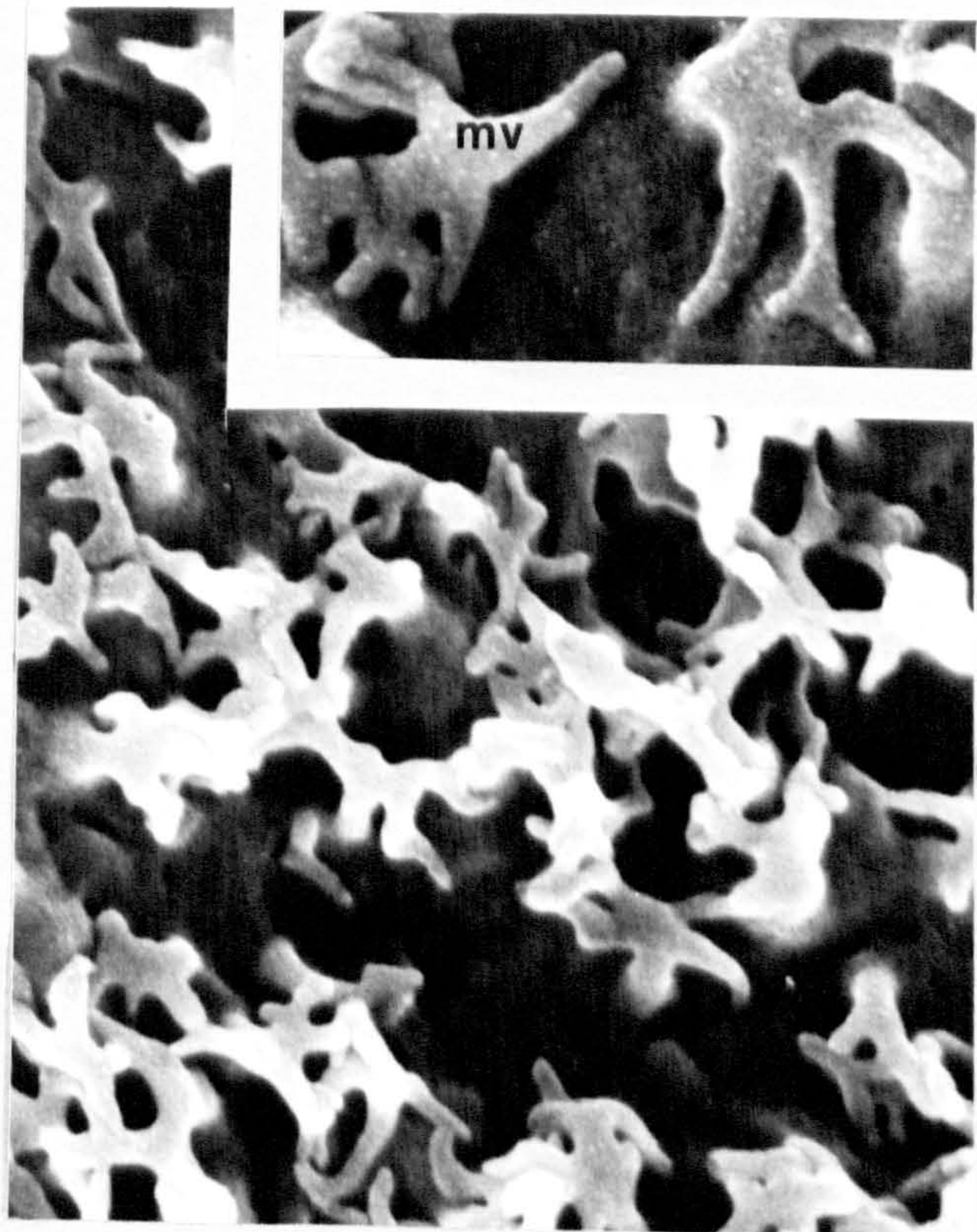
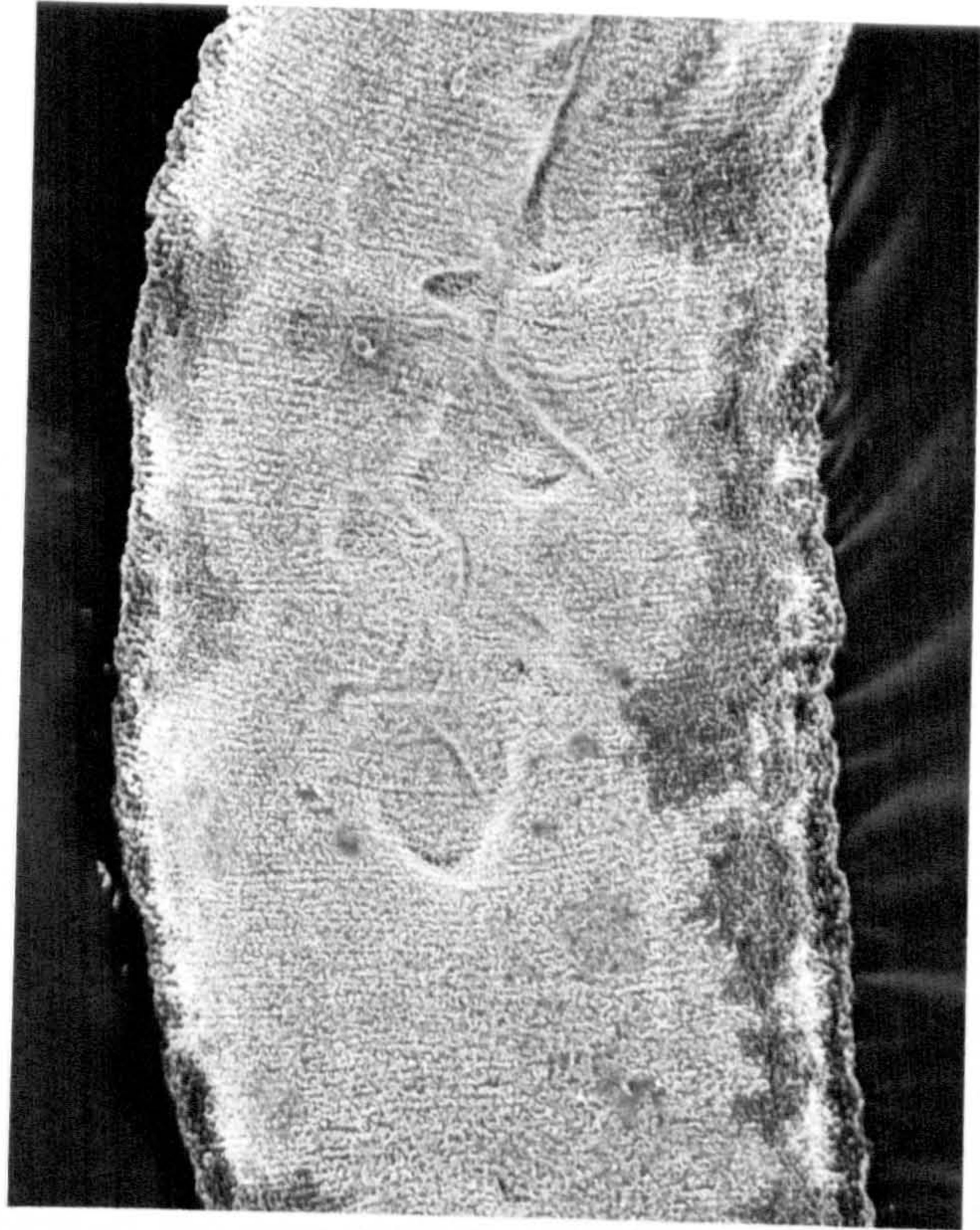


Fig. 3.5

Scanning electron micrograph showing the clusters of microvilli (cm) along the margins of the body ($\times 8,000$).

Fig. 3.6

Scanning electron micrograph of the posterior end of S. inermis showing 'setae-like' structures which extend from the surface of the body ($\times 6,000$).

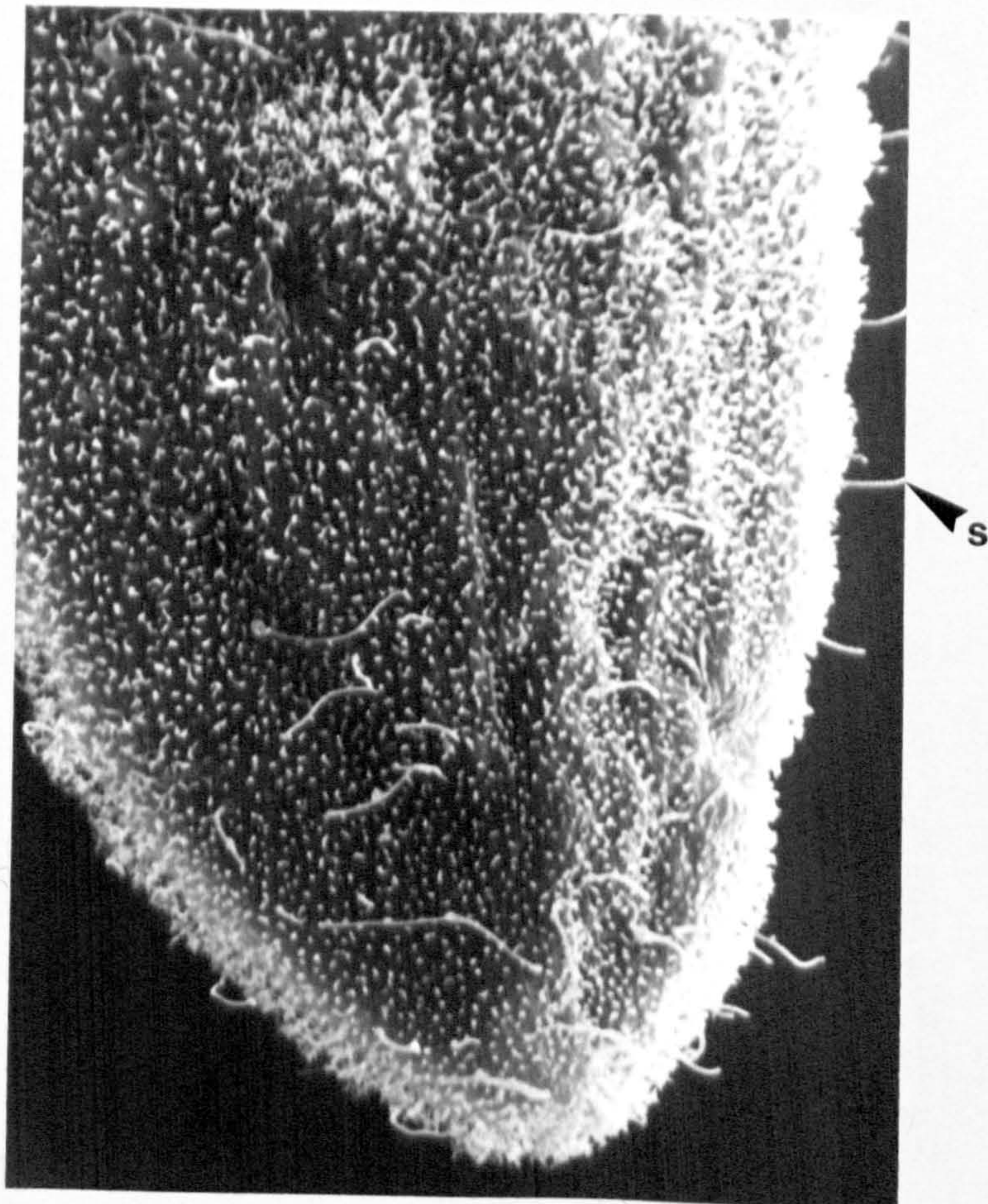
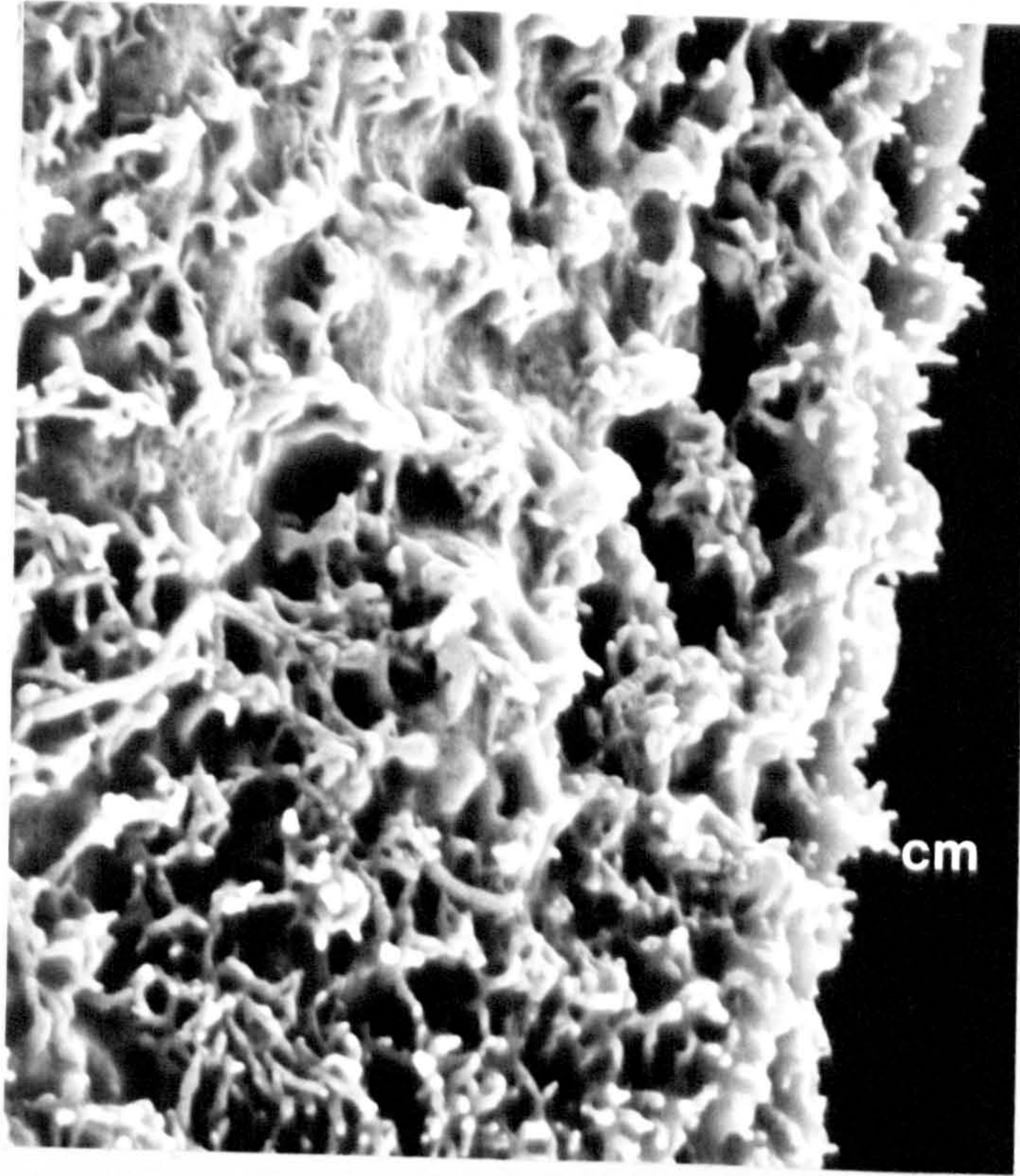


Fig. 3.5

Scanning electron micrograph showing the clusters of microvilli (cm) along the margins of the body ($\times 8,000$).

Fig. 3.6

Scanning electron micrograph of the posterior end of S. inermis showing 'setae-like' structures which extend from the surface of the body ($\times 6,000$).

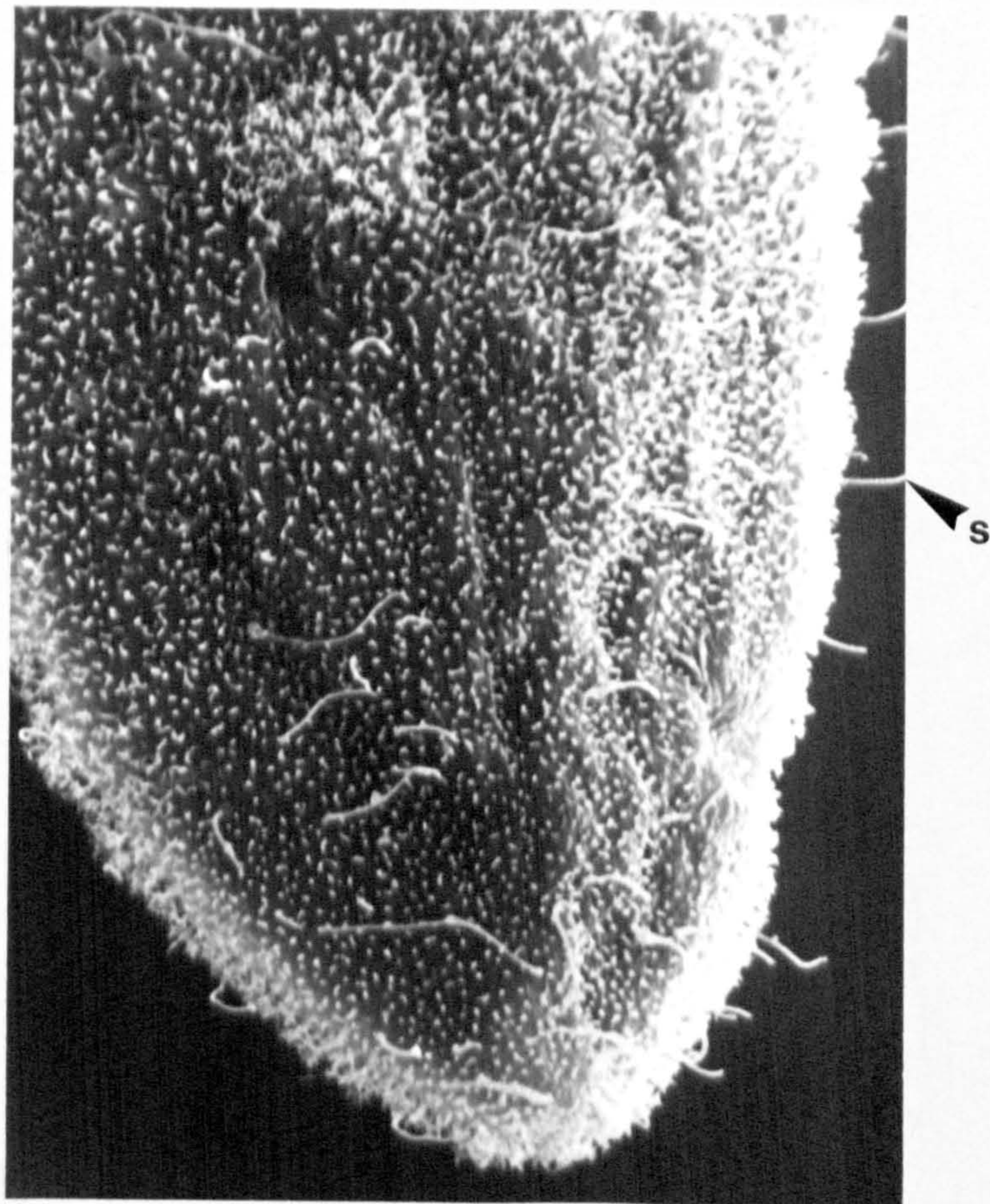
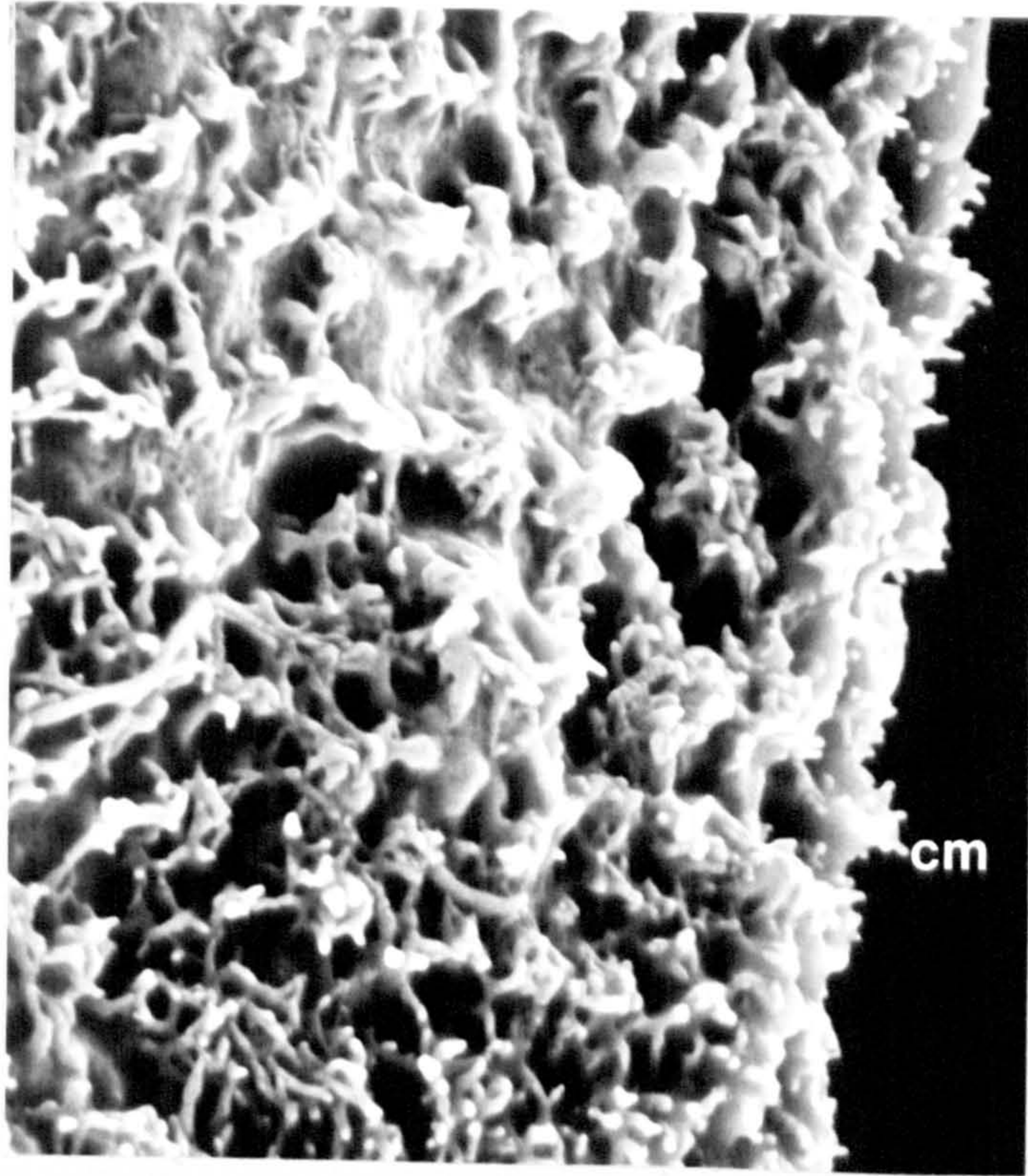


Fig. 3.7

Transmission electron micrograph of part of the body tegument.

A, Details of structure showing microvillous and trilaminate outer membrane ($\times 80,000$).

B, A microvillus to show clusters of electron dense granules at its base ($\times 210,000$).

bm : basal plasma membrane

eg : electron dense granules

mv : microvillus

np : nerve process from sense organ

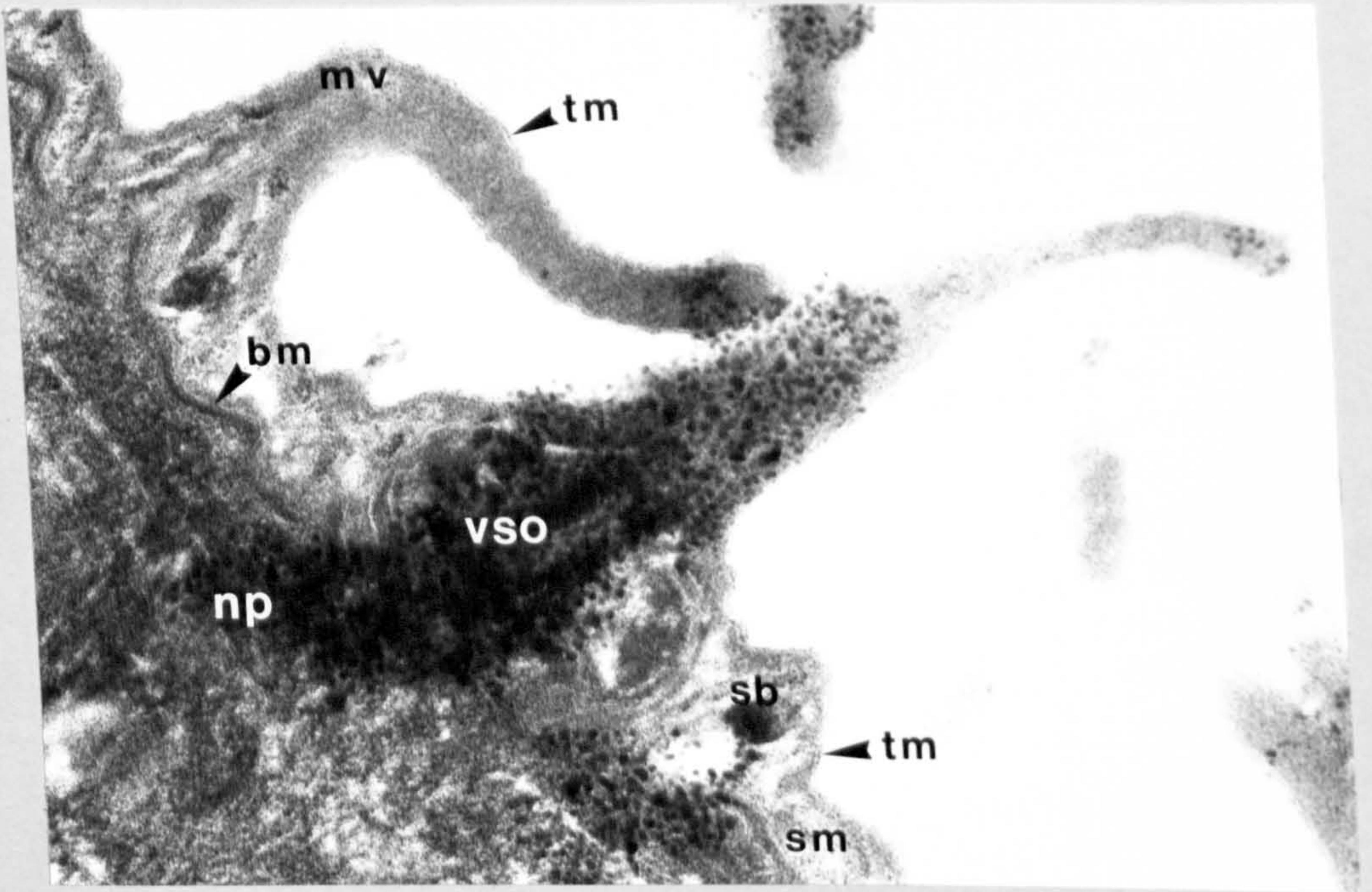
tm : trilaminate outer membrane

sb : secretory body

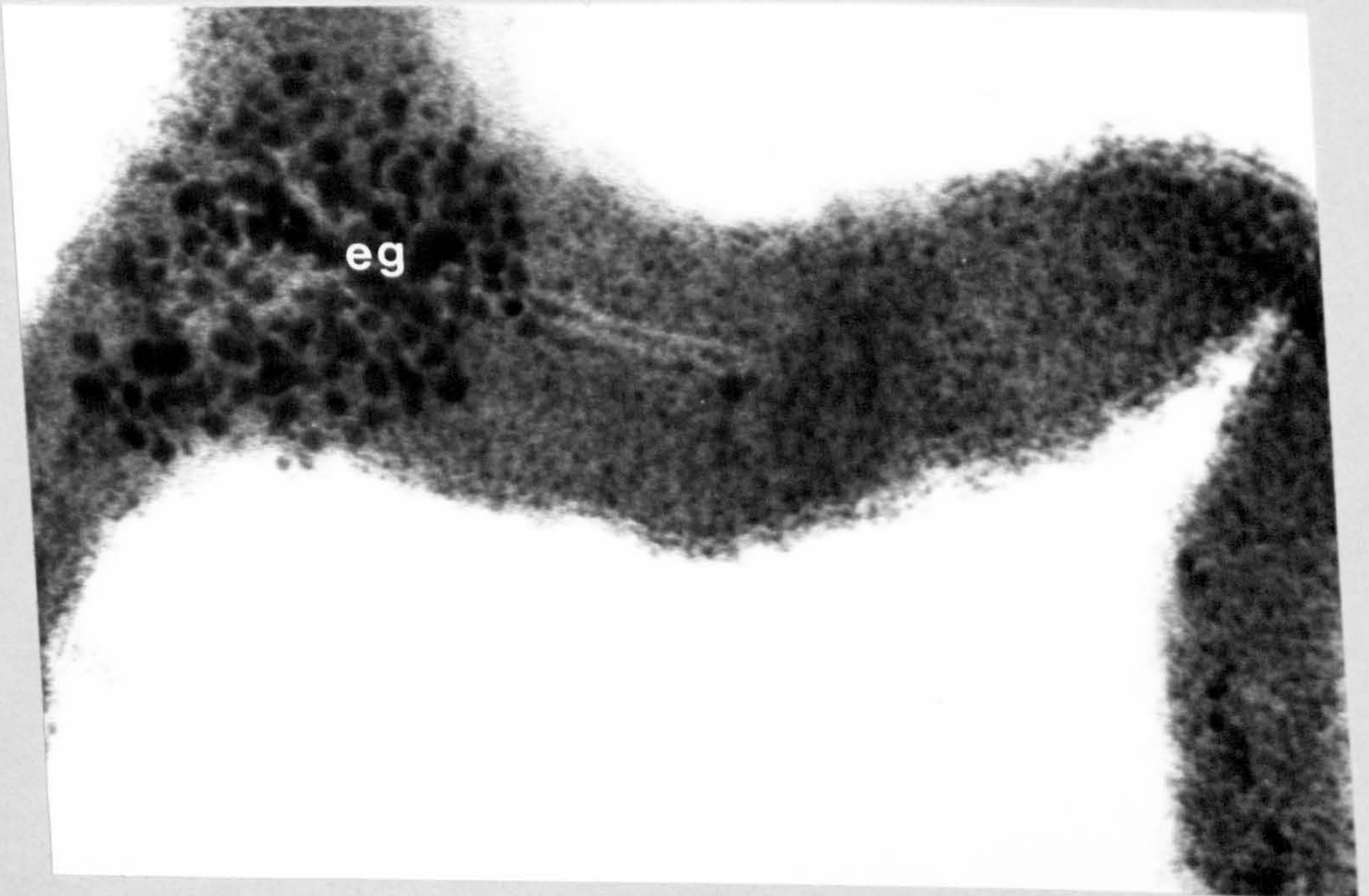
sm : syncytium

vso : vesicle of sense organ

A



B



surface of the tegument as well as over the entire surface of the microvilli (Fig. 3.7). Secretory bodies were seen as electric dense ovoid to spherical structures situated within the anucleated syncytium of the outer tegument (Fig. 3.7). Sensory structures were occasionally seen on the syncytium. These consisted of a long papilla which projected to the exterior of the tegument arising from a basal vesicle which traversed the basal membrane and continued into the inner nucleated layer by a nerve process (Fig. 3.7). Clusters of electron-dense granules were seen to accumulate on the top of the papilla and the tip of each microvilli. Similar clusters were also seen at the bases of the microvilli. The largest accumulation occurred on the vesicle of the sensory structure. No other surface structures, such as spines, were evident in the small number of sections examined.

Comparison of specimen 'A' and 'B'

Tables 3.2 and 3.3 present the details of taxonomic characters for the specimens 'A' and 'B' respectively. From the table it can be seen that there were some morphological variations between the specimens collected from the two localities. Table 3.4 clearly shows that the specimens from the farm 'A' had a greater mean length than the specimens of farm 'B'. The average length recorded for the specimens from farm 'A' was 1008.2 microns, whereas it was only 791.0 microns in the case of farm 'B'. However, the difference was found to be statistically insignificant ($p > 0.05$), considering the width of specimens the difference was reversed. Here the specimens of farm 'B' were shorter and wider (139.9 microns) compared to specimen 'A' (116.4 microns). This can be seen clearly from the 'shape-index' presented in Tables 3.2 and 3.3 for the specimens 'A' and 'B'

Table 3.2

Details of taxonomic characters of specimen 'A'
(measurements are in microns)

Characters	Mean	Range
Body length	1008.2	706.2 - 1235.8
Body width	116.4	100.8 - 201.8
Length of oesophagus zone	326.2	201.8 - 378.3
Length of testes zone	294.2	252.2 - 378.3
Oötype distance	143.4	100.8 - 176.5
Gut ratio	1 : 3.1	1 : 2.7 - 3.5
Oötype ratio	1 : 7.0	1 : 6.5 - 7.5
No. of caecal lobes	4	-
No. of pair of testes	14	13 - 16
Shape index	6.1	5.1 - 8.2

Table 3.3

Details of taxonomic characters of specimen 'B'
(measurements are in microns)

Characters	Mean	Range
Body length	791.0	455.9 - 962.4
Body width	139.9	70.9 - 176.5
Length of oesophagus	264.2	152.0 - 334.3
Length of testes zone	233.1	68.3 - 303.9
Oötype distance	95.8	60.8 - 126.1
Gut ratio	1 : 3.04	1 : 2.6 - 3.5
Oötype ratio	1 : 8.5	1 : 6.2 - 12.3
No. of caecal lobes	4	-
No. of pair of testes	13	10 - 15
Shape index	5.8	4.5 - 7.5

Table 3.4

Comparisons of taxonomic characters of the two specimens collected from farm 'A' and 'B'

Specimen	Length (micron)	Width (micron)	Shape index	Gut ratio	No. of caecal lobes	No. of pair of testes	Oötype ratio	No. of ova in uterus	Shape of egg
'A'	1008.2 + 136.8	116.4 + 28.9	6.1 + 0.9	1:3.1 + 0.2	4	14 (13-16)	1:7 + 0.4	0 - 1	Triangle
'B'	791.0 + 130.7	139.9 + 32.3	5.7 + 0.8	1:3.0 + 0.2	4	13 (10-15)	1:8.5 + 2.0	0 - 1	Triangle

respectively. However, the difference was found to be statistically insignificant ($p > 0.05$).

The crenulation on the outside margins of the body was seen only in the specimens 'B'. No crenulation was observed in the specimens 'A'. No 'setae-like' structures were observed on the 'A' specimens though it was not clear if they were present, or lost during processing.

The morphological details of the specimens studied were compared with those of other species of Sanguinicola, the major features of which are described in Table 3.5. The worms in the present study can be seen to agree most closely with the description of S. inermis as given by Plehn (1905) and adopted and modified by several authors.

B Morphology of some larval stages

a. Egg

The eggs were dissected out from the naturally and experimentally infected fish and were non-operculate. They appeared triangular in the longitudinal section and elliptical in transverse section. The size and shape were found to vary according to the age of the eggs. The new egg (EI, Fig. 3.8) was small, measuring 43.6×28.2 microns (Table 3.6a) with a long dorsal process on the convex edge. At an early stage, two large vitelline cells became associated with the primary oocyte. As development progressed, the nucleus of the oocyte became larger as the vitelline cells gradually disappeared. At this stage the zygote was seen to have several cleavages.

The embryonated eggs (EII, Fig. 3.9) were bigger, measuring $60.2 \times 34.4 \mu$ (Table 3.6b) and the dorsal process on the convex edge

Table 3.5 Comparison of the taxonomic characters of various species of Sanguinicola and the present species

Species	Maximum body length (mm)	Spination	Gut ratio	No. of caecal lobes	No. of pair of testes	Oötype ratio	Shape of egg	No. of ova in uterus at a time	Reference(s)
<u>S. inermis</u> Plehn, 1905	1.0	- With setae	1:3	3-4	15	1:7	Triangular (with short dorsal process)	1	Plehn, 1905 & 1908; Scheuring, 1920 & 1922; Ejmsont, 1926; Bykhovskaya-Pavlovskaya et al., 1964
<u>S. armata</u> Plehn, 1905	1.5	+ Regular with powerful marginal spine	1:3	5	10-15	1:7	Triangular	?	Plehn, 1905 & 1908; Ejmsont, 1926; Bykhovskaya-Pavlovskaya et al., 1964
<u>S. intermedia</u> Ejmsont, 1926	1.0	+ Regular with seta	1:3	4-5	10	1:7	Triangular (with long dorsal process)	?	Ejmsont, 1926; Markevich, 1963; Bykhovskaya-Pavlovskaya et al., 1964
<u>S. chalmersi</u> Odhner, 1924	1.228	+ Regular	1:3	Not lobed	6-7	1:14	?	?	Woodland, 1923; Odhner, 1924
<u>S. volgensis</u> Rasin, 1929	2.0	+ Regular	1:4	4	20+	1:13	Oval	1-9	Rasin, 1929; Bykhovskaya-Pavlovskaya et al., 1964; Shillcock, 1972
<u>S. davisi</u> Wales, 1958	0.58	+ Regular	1:3	4	Massive and irregular	1:11	Oval	?	Wales, 1958

contd. . . .

Table 3.5 (continued)

Species	Maximum body length (mm)	Spination	Gut ratio	No. of caecal lobes	No. of pair of testes	Oötype ratio	Shape of egg	No. of ova in uterus at a time	Reference(s)
<u>S. klamathensis</u> Wales, 1958	3.15	+ Regular with small spines	1:4	Not lobed	?	1:8	Spherical	Several	Wales, 1958; Meade, 1967; Evans & Heckmen, 1973
<u>S. lophophora</u> Erickson and Wallace, 1959	0.525	+ Regular	1:3	4	17-18	1:10	?	?	Erickson & Wallace, 1959
<u>S. alseae</u> (Meade & Pratt, 1965) Holmes, 1971 (Syn. Cardicola alseae)	1.414	+ Regular	1:4	4 (with long bran- ches)	6-7	1:12	Ovoid	Several	Meade & Pratt, 1965
<u>S. magnus</u> Cheng-yen Hu, So Long and Wei-chu Lee, 1965	2.93	+ Regular	1:4	4	27-29	1:13	Oval	Several	Cheng-yen et al., 1965

contd. ...

Table 3.5 (continued)

Species	Maximum body length (mm)	Spination	Gut ratio	No. of caecal lobes	No. of pair of testes	Oötype ratio	Shape of egg	No. of ova in uterus at a time	Reference(s)
<u>S. sp.</u> Chappell, 1967	1.24	+ Regular	1:3	4	12-19	1:7- 1:9	?	?	Chappell, 1967
<u>S. idahoensis</u> Schell, 1974	2.3	+ Regular	1:4	4-5	14-18	1:8	Oval	1-5	Schell, 1974
<u>S. inermis</u> Present study	1.235	- With extremely minute setae	1:3	4	10-16	1:7.8	Triangular (with short dorsal process)	1	Pers. Obs.

Fig. 3.8

Photomicrograph of eggs of S. inermis in early development described as EI (Interference Phase).

vc : vitelline cell

oo : oöcyte

Fig. 3.9

Photomicrograph of embryonated egg of S. inermis described as EII.

pe : pigmented 'eye spot'

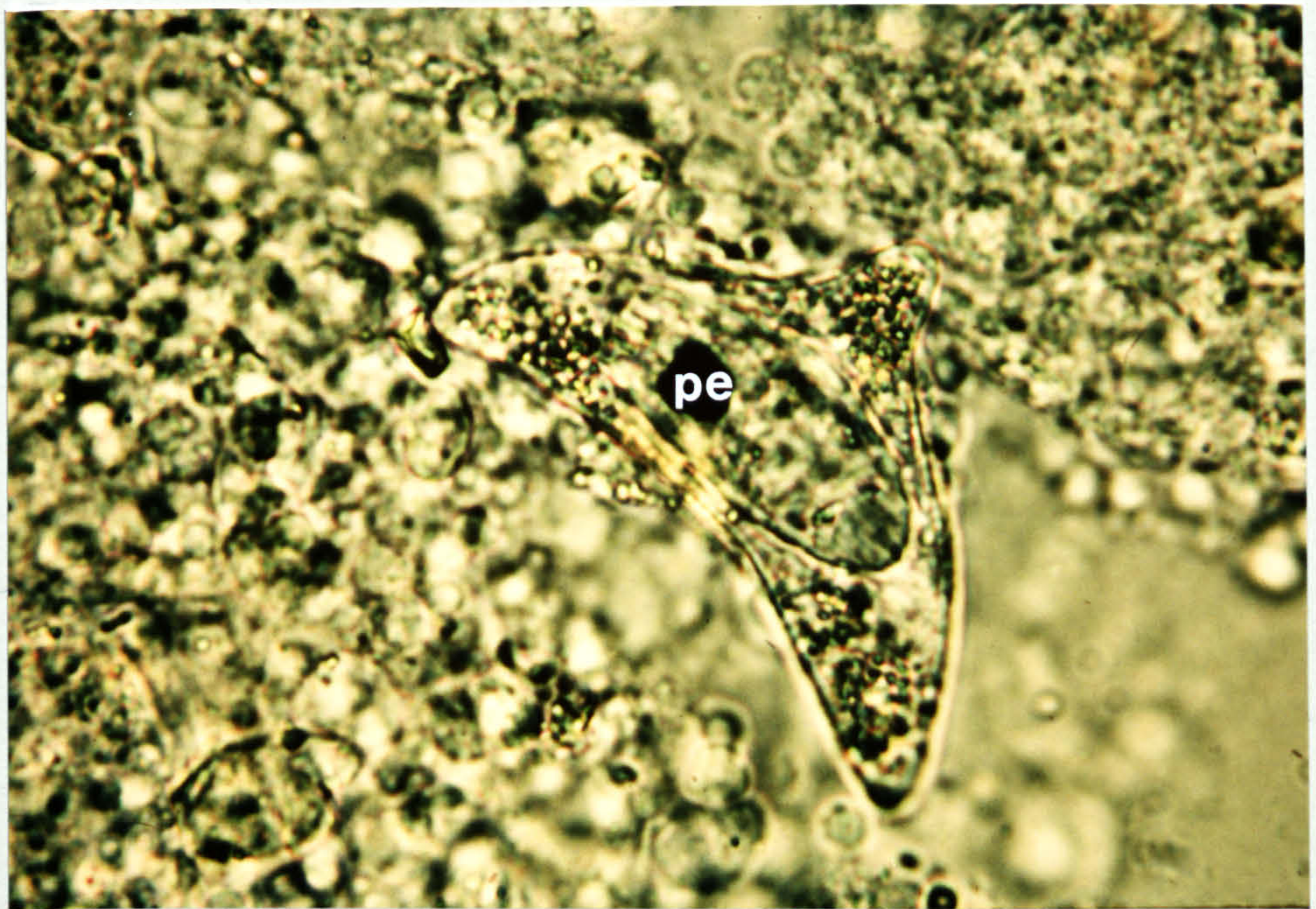
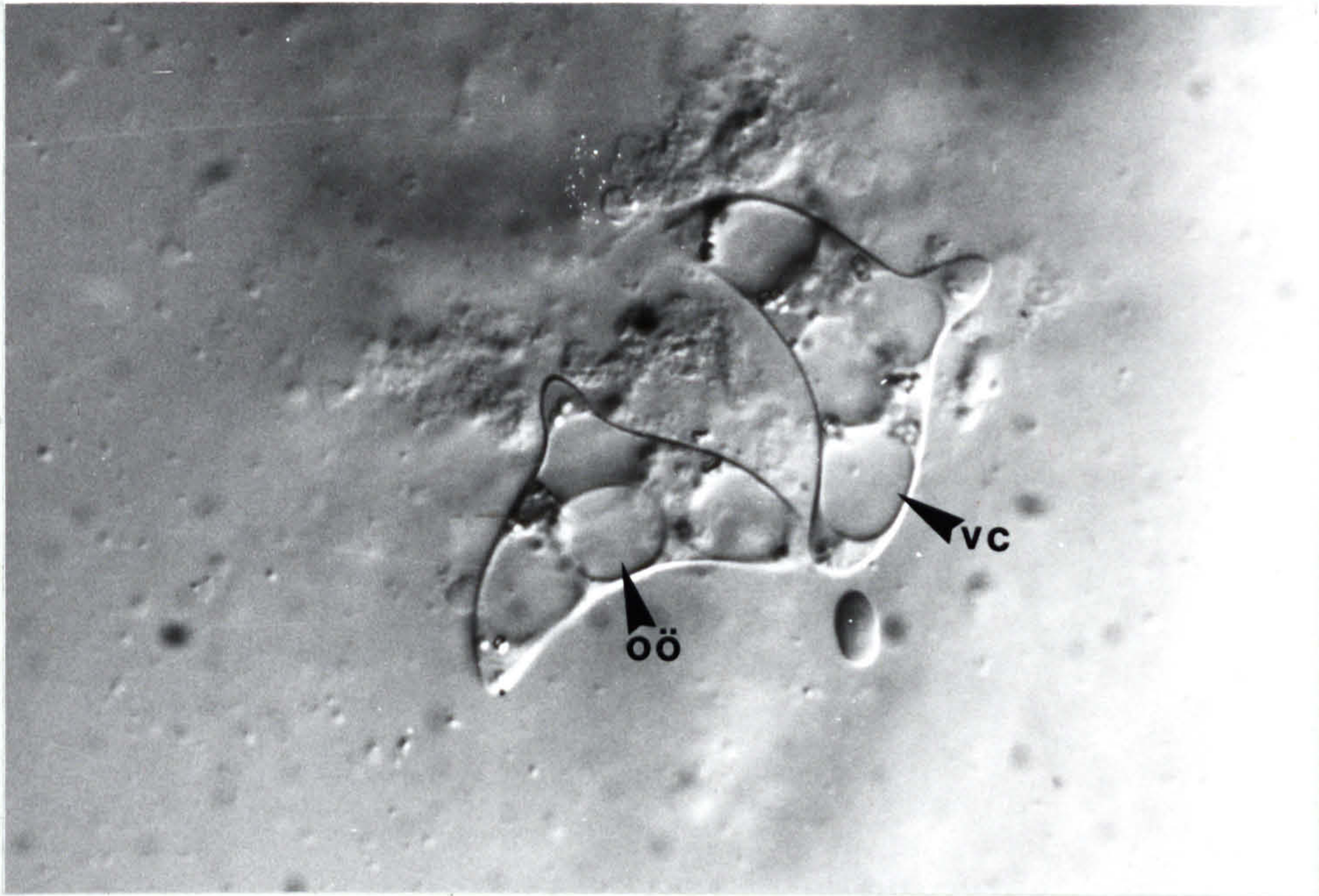


Table 3.6

Measurements of S. inermis eggs and miracidia (in microns)

a. Immature egg (EI)

	Length	Width (at highest point)
Mean (20)	43.6	28.2
Range	38.0 - 53.1	17.7 - 30.4

b. Mature egg (EII)

	Length	Width (at highest point)
Mean (20)	60.2	34.4
Range	50.7 - 70.9	27.8 - 40.5

c. Miracidia (MII)

	Length	Width	Diameter of eye-spot	Length of principal stylet
Mean (20)	41.8	19.9	9.8	20.1
Range	32.9 - 48.1	17.7 - 25.3	7.6 - 10.1	19 - 21

was proportionately reduced. These eggs which now contained a well-developed miracidium, had a thick conspicuous shell. EII was characterised by an accumulation of large pigmented ocelli, seen as a large black spot situated anterior to the nucleated mass. The diameter of these ocelli varied from 7-10 microns. A stylet was present at the anterior end of the miracidium together with a cluster of delicate light retracting rodlets. The size of the stylet was approximately 21 microns and the rodlets each measured approximately 5 microns.

b. Miracidia

Hatched miracidia (MII, Fig.3.10) were ciliated, free swimming larvae, measurements of which are given in Table 3.6c. The miracidium had an elongated ovoid form with the ability to change its shape from ovoid to elongate when swimming. When swimming, the anterior end was more pointed. The hatched miracidium was morphologically the same as described in the mature egg (EII).

c. Cercaria

The cercariae were recovered from the Lymnaea (Radix) peregra (Müll) of farm 'A' and 'B' and observed to develop into mature S. inermis. They were essentially of the apharyngeal, furcocercous, lophocercous type (Fig.3.11). The body of the cercaria was very small, measuring approximately 96 microns by 29 microns. The length of the body was almost half the length of the tail stem. The details of the measurements of fixed specimens are shown in Table 3.7.

These cercaria did not possess a sucker, instead they possessed a 'proboscis' like extension at the anterior tip, measuring approximately 15 microns long. This glandular extension of the penetration organ (a 'protrusible telescopic snout' as named by some authors) was

Fig. 3.10

Photomicrograph of a hatched, free-swimming miracidium of S. inermis described as MII.

a : anterior end
c : cilia
pe : pigmented 'eye spot'

Fig. 3.11

Photomicrograph of furcocercous cercaria of S. inermis recovered from the snail L. peregra (lateral view).

ao : anterior organ
b : body
f : fin-fold
fu : furcae
tf : terminal fin
ts : tail-stem

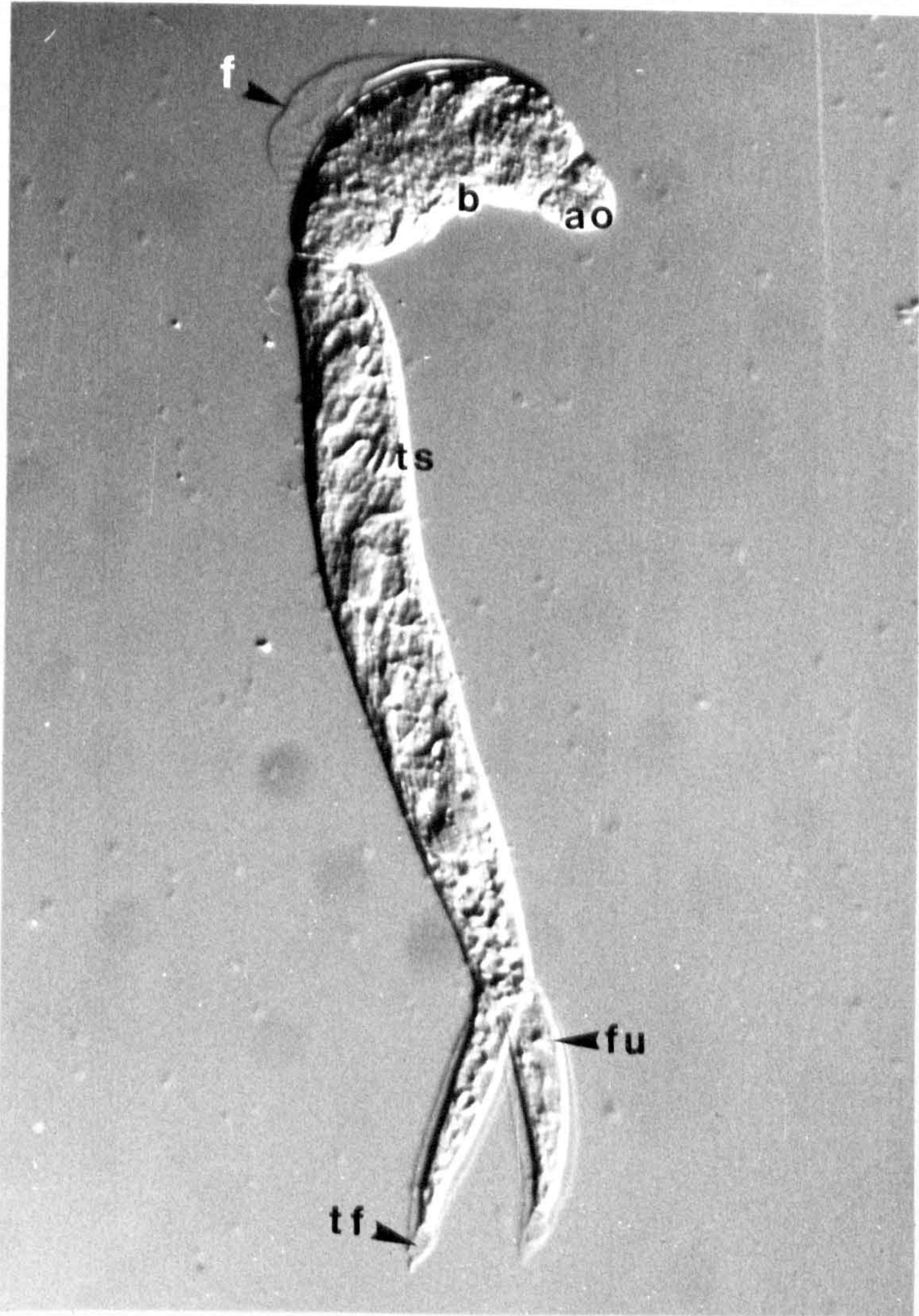
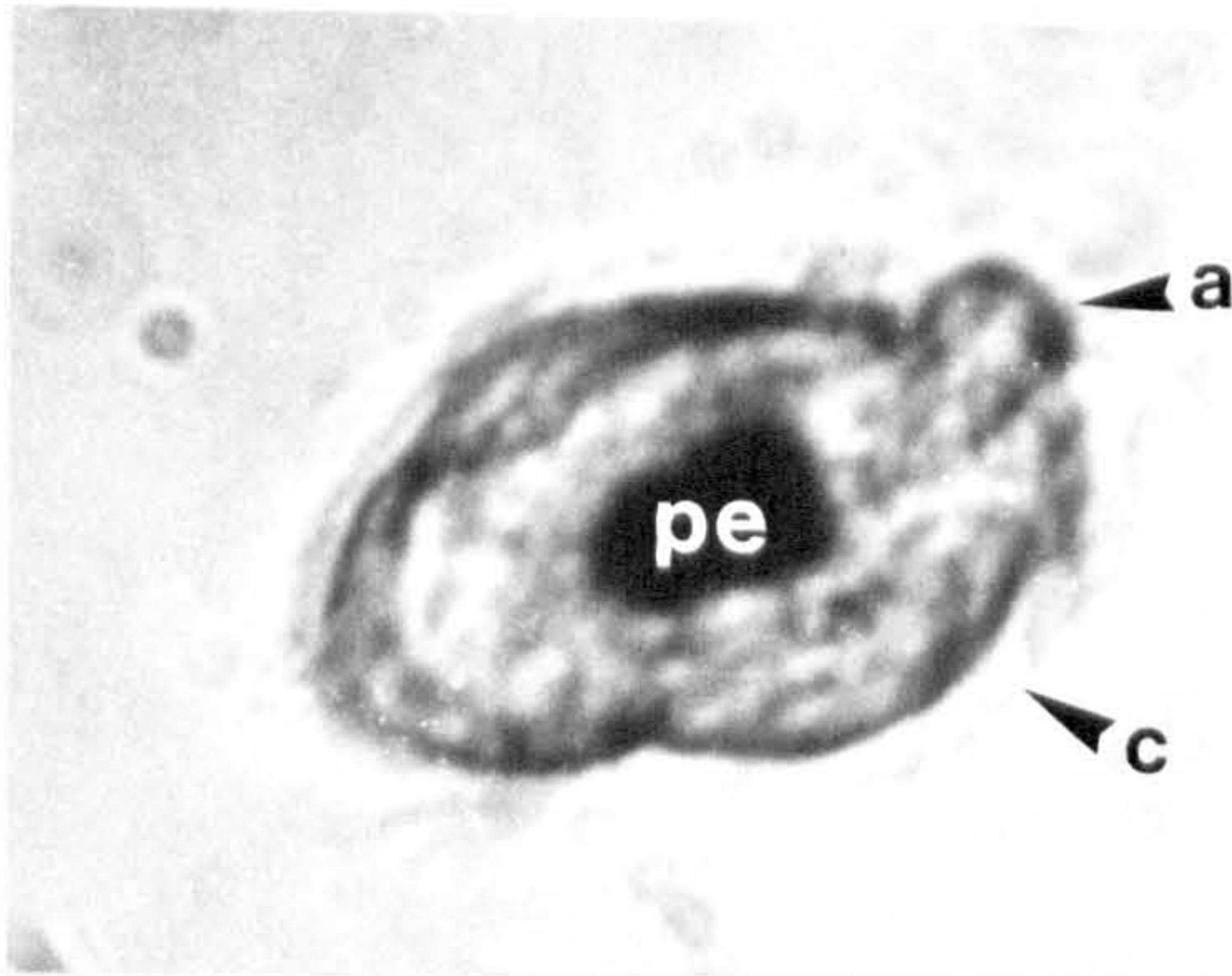


Table 3.7

Measurements of the cercariae of S. inermis (in microns) from farm 'A'

	Body		Fin-fold		Tail-stem		Length of furcae with terminal fin
	Length	Width at middle	Length	Highest width	Length	Width at middle	
Mean (20)	96.2	28.7	60.9	16.0	199.4	20.3	74.4
Range	81.0 - 111.4	25.3 - 32.5	50.7 - 70.9	15.0 - 20.3	192.5 - 208.5	20.3 - 21.0	65.8 - 82.0

markedly separated from the rest of the body by a constriction. There were no protusible terminal papilla as described for cercaria of Aporocotyle simplex by Køie (1982) or penetration spines observed.

The body of the cercaria curved ventrally (Fig.3.11) and there was a fin-fold or crest on the mid-dorsal line. The finfold was found to be reduced at either end of the body, starting from a point at a distance from the 'proboscis' and finishing a small distance in front of the junction of the body with the tail-stem. On contraction of the living cercarial body, the shape of the fin fold changed and became folded so that it appeared to be transversed by a number of ray-like lines. However, during the movement of the cercaria most of these fin-folds disappear on extension of the body. The ventral bending of the body and the presence of the median dorsal fin-fold caused the ceracriae to lie on one side when mounted on a slide.

In living and stained specimens, no mouth or gut was apparent. Several cells in the anterior body contained granular cytoplasm and prominent nuclei were probably gland cells. There was no eye spot. The excretory vesicle was not visible but two excretory ducts were seen to run alongside the tail stem. Each of these caudal excretory ducts passed into one of the furcae and opened into a cuplike organ at the tip.

The tail stem was more than twice the length of the body and there are several rounded caudal bodies extending throughout its length.

The furcae were short, laterally compressed, blade-like structures. They bore fin-folds on their dorsal and ventral sides, extending over the entire length and continuous over the terminal fins.

The descriptions of the cercariae of different species given by several authors are briefly compared with the species in the present study in Table 3.8. With minor differences, it appears that the cercaria are of S. inermis. However, the specific identification was confirmed through the cercarial infection of fish where they were found to develop to the adult stage of S. inermis (see Chapter 4).

3.4 Discussion

Adult Worm

Erickson and Wallace (1959) have listed eight features of importance in distinguishing between the adult worms of different species of the genus Sanguinicola. These features, with some additions, are shown in Table 3.5. From the table it can be seen that the specimens of the present study differ markedly from all species other than S. inermis. Worms of the present study were very similar in dimensions and general morphology to those described by Plehn, 1905 and 1908, Odhner, 1911 and Ejsmont 1926 as S. inermis. However, the maximum length of the specimens studied was greater, i.e. 1,235 microns compared to 1,000 microns (Bykhovskaya-Pavlovskaya et al., 1964).

An elongated anterior end has been described by many authors for Sanguinicola sp. Plehn (1905, 1908) described an extendable 'finger-like' proboscis in S. inermis. Wales (1958) also described a moderately well-developed proboscis for S. klamathensis. Thulin (1980a) and Kjøie (1982) described a 'snout-like' projection in the anterior end of Aporocotyle simplex. Worms collected from the two farms possess a poorly developed 'proboscis' like projection at the anterior end of the body which was more prominent during the early stage of the development. As the development progressed it became more blunt.

Table 3.8 Comparison of the morphology of the cercariae of Sanguinicola species given by several authors and the description of the cercariae from the present study (measurements in microns)

Species	Body		Fin-fold		Tail-stem		Length of furcae with terminal fin		Overall total length	Reference
	Length	Width	Length	Width	Length	Width	Length	Width		
<u>Cercariae cristata</u> (= <u>S. inermis</u>)	87-120	15-25	-	-	209-285	-	45-98	-	340-400	Scheuring, 1920, 1922
"	-	-	-	-	-	-	-	-	300-400	Ejmsont, 1926
<u>Cercariae cristata</u>	130-174	54.56	-	-	174	-	36-38	-	340-386	Chechina, 1959
<u>S. inermis</u>	94.0 (73-126)	25.0 (14-38)	-	-	196.0 (108-231)	17.0 (14-24)	61.0 (38-77)	-	351 (220-434)	Naumova, 1961b
<u>S. inermis</u> (Present study)	96.2 (81-111)	28.7 (25-30)	60.9 (50-71)	16.0 (15-20)	199.4 (193-209)	20.3 (20-21)	74.4 (66-82)	-	370 (349-385)	Pers. Obsn.
<u>S. klamathensis</u>	-	-	More than half of the body length	-	-	-	-	-	980	Wales, 1958
<u>S. klamathensis</u>	305	87	"	-	381	45	109	-	795	Mead, 1967
<u>S. klamathensis</u>	300	35	-	-	385	-	105	-	795	Evans & Heckmann, 1973
<u>S. davisi</u>	230	56	Absent	Absent	340	35	-	-	570	Wales, 1958

contd. . . .

Table 3.8 (continued)

Species	Body		Fin-fold		Tail-stem		Length of furcae with terminal fin length	Overall total length	Reference
	Length	Width	Length	Width	Length	Width			
<u>S. lophophora</u>	129	30	More than half of the body length	-	259	22	100	488	Erickson & Wallace, 1959
<u>S. alseae</u>	150	70	3/4 length of the dorsal body surface	-	250	30	50	450	Meade & Pratt, 1965
<u>S. idahoensis</u>	107-124	36-42	2/3 - 3/4 length of the dorsal body surface	-	271-327	28-30	64-78	442-529	Schell, 1974
<u>Cercaria kentensis</u>	93 (83-100)	20 (16-23)	Approx. 2/3 length of the dorsal body surface	19 (18-20)	182 (166-186)	18 (12-20)	63 (60-66)	338 (309-352)	Khan, 1961

Body spination appears to be a common feature for most of the sanguinicolids with the exception of S. inermis and S. argentinensis (Erickson and Wallace, 1959). S. armata, S. intermedia and S. volgensis all possess a marginal border of conspicuous spines arranged in an evenly spaced single row (Ejmont, 1926). In addition to spines S. intermedia also possessed 'setae'-like structures. Schell (1974) described numerous spines along the lateral body margins and a few scattered spines on the ventral surface of S. idahoensis from steelhead trout. S. klamathensis possessed two longitudinal columns of heavy spines extending nearly the length of the body, whereas S. davisii possessed columns of spines in three or four rows along each edge extending almost to the anterior and posterior ends of the body (Wales, 1958).

The presence of 'seta-like' structure on S. inermis have been reported by several authors (Plehn, 1905; Scheuring, 1920; Ejmont, 1926; Naumova, 1961b; and Sweeting, 1978). The Sanguinicola sp. of this study, though devoid of spines, were seen to possess minute 'setae-like' structure which extended from the crenulated margins of the body. These structures could only be seen in live specimens and those fixed in glutaraldehyde. When the specimens were fixed in formalin these structures were impossible to see. They were best seen in live specimens.

The only other species which possesses 'setae-like' structure is S. argentinensis. These were described by Szidat (1958) as 'bristles' on the body margins.

Most studies of the digenean tegument have concentrated on mammalian parasites of medical and economic importance, namely

schistosomes, liver-flukes etc. (e.g. Hockley, 1968, 1972; Hockley and MacLaren, 1973, 1977; McLarean and Hockley, 1976, 1977; Bennett, 1975; Voge, Price and Bruckner, 1978a, 1978b. Voge, Price and Jansma, 1978). Very few of such studies have been carried out on blood flukes of fish with which the present observation can be compared. Thulin (1980b) described the surface structure of mature A. simplex from the gill arteries of Hippoglossoides platessoides. Kjøie (1982) similarly described the surface structure of developing A. simplex from the lymphatic and blood system of Limanda limanda, Pleuronectes platessa and H. platessoides. The 'microvillous-like' projections observed in the present study were very similar to those observed by Thulin (1980b) and Kjøie (1982) in their specimens. However, these structures found on the surface of S. inermis were branched and long compared to the short structure described by Thulin (1980b) and lace-like structures described by Kjøie (1982) of A. simplex.

The surface topography of S. inermis appeared to be similar in a number of ways to that of male Schistosoma japonicum as described by Voge et al. (1978). S. japonicum is also devoid of spines and the pattern of the 'microvillus-like' projections is remarkably similar to those of S. inermis. Also in common with S. japonicum, S. inermis do not possess any tubercles or surface pits.

It appears that these surface structures may not be static, as some authors have described changes which take place at different stages in the life history. Kjøie (1982) found that the cephalic spines of A. simplex remained throughout its life, but the spines of the body decreased as the development progressed. Small tubercles or bosses appeared on the lateral margins. Initially these tubercles were without spines, but, as development proceeded, the number of

spines on the tubercles increased. Changes on the tegumental surface during the development of schistosomes were also described by many authors (Hockley and McLaren, 1973; Voge, Price and Bruckner, 1978; and Jean and Wilson, 1980 for S. mansoni). Lo, Hall, Allender and Klainer (1975) found that most of the sensory structures were lost during the development of an opecoelid cercariae to a metacercaria. Light microscopical observations were made of all stages of development in the present study as well as ultrastructural observations of pre and post egg stages and at no time were any spines evident.

A variety of sensory structures can be found in the syncytial region of the digenean tegument. These structures have been studied extensively for the tegument of juvenile-adult schistosomes and liver-flukes. Bennett (1975) showed the presence of three types of sensory receptors in the juveniles of Fasciola hepatica. Fujino, Ishii and Choi (1979) described four types of sensory structures on the tegument of Clonorchis sinensis. Page, Nadakavukaren and Huizinga (1980) described five different types of sensory structures that he found on the tegument of the cercaria of Ribeiroia manini.

The subdivision of different types of sensory structure was based on whether or not they cause protrusions in the tegument surface, the presence or absence of raised tegumental rings around the base of the cilium and the number and length of the cilia. Based on these criteria S. inermis possessed 'Type-1' (Page et al., 1980) sensory structure which was characterised by a bulb-like base with a single long cilium.

The functions of the various sensory structures depends on the type of stimuli in the surrounding environments. The ciliated

sensory structure observed in the specimens of the present study by TEM may be tangoreceptors and therefore involved in producing locomotor movements. It might also serve to detect direction of flow of a fluid medium (Erasmus, 1972).

The tegument of the adult specimen in the present study was seen to be bounded by a trilaminate membrane similar to that described by Clegg (1972) and McLaren and Hockley (1976) in the developing schistosomulum, whereas adult schistosomes have a double outer membrane consisting of two lipid bilayers (McLaren and Hockley, 1977), i.e. forming an heptalaminate tegument. Adult A. simplex also possess an heptalaminate tegument (McLaren and Hockley 1977) despite their apparent close relationship with S. inermis.

Hockley and McLaren (1973) observed that the outer membrane of adult schistosome though largely heptalaminate had small areas which retained the trilaminate structure of schistosomulum. In their study they also remarked on the rapid turnover rate of the tegument during the transformation of schistosomes.

Why the S. inermis possess a trilaminate rather than the apparently more usual heptalaminate outer membrane is not clear. One possibility is that it is an adaptation to contact digestion. The blood flukes so far described as possessing an heptalaminate outer membrane are Schistosoma spp., Spirorchis sp., Aporocotyle simplex and A. nosicanalis all of which possess a well developed gut with large caeca and contact digestion at the surface of the tegument may be less likely to occur in these species. On the other hand, the gut of S. inermis is small, thus, contact digestion is more likely. However, this interesting feature deserves a more careful and intensive investigation.

Biochemical studies of the known blood flukes, mostly the schistosomes, have indicated that the tegument has a protective, absorptive and secretory function (McLaren, 1980). This would also be true for the tegument of S. inermis and there are a number of similarities amongst them. The numerous 'microvillous-like' projections almost certainly serve to enormously increase the surface area for absorptive and secretory functions. They might also facilitate respiratory exchange. Lyons (1970) has suggested that the function of microvilli on the epidermis of the monogenean Entobdella soleae and Acanthocotyle elegans may be to support a layer of mucus or, alternatively, to increase the surface area and thus facilitate respiratory exchanges.

Sanguinicolid worms have chosen an environment (circulatory system of fish) which is very nutritive as well as hostile in nature. These worms lack any vestiges of oral and ventral suckers. To maintain their position in this hostile environment most of them, therefore, developed variously shaped and arranged spines, 'setae' or cilia. Some of them also adopt different behavioural patterns by which they managed to retain themselves in a particular area. Holmes (1971b) described the attachment of A. macfarlani in the lumen of blood vessels in a Sebastes species. A. macfarlani is a relatively short, fairly stout-bodied worm armed with patches of tegumental spines along the lateral margins of the body. He observed these worms pointed their anterior end into the blood current of the host, drift backwards until they reach a blood vessel small enough for the tegumental spines to wedge in to the walls of the vessel.

The present study has shown that the syncytical region of the tegument of S. inermis is devoid of any spines. The microvillous-

like structures of S. inermis could also possibly be an adaptation to increase the efficiency of attachment to the inner walls of the host's blood vessels. By the contractions of the tegument these microvilli might help anchorage in the lining of the blood vessels against circulating current. However, to what extent the microvilli aid in attachment is unknown. Chappell (1980) explained the possible functions of the microtriches which he found on the tegument of cestodes. In addition to increasing the surface area he considered that these microtriches might aid in attachment and maintenance of position in the host gut. This interesting feature also needs further investigation.

An examination of the internal morphological characters showed some minor differences between present specimens of S. inermis and those described by other authors. For example, the maximum number of pairs of testes in the specimens of this study were 16 compared to 15 for S. inermis (Bykhovskaya-Pavlovskaya et al., 1964). The gut ratio and the oötype ratio of this study was slightly higher compared to the ratios shown by Erickson and Wallace (1959) for S. inermis. The posterior part of the vas deferens of the specimens of this study occurred in 2-3 loops, compared to 2-4 loops for S. inermis (Bykhovskaya-Pavlovskaya et al., 1964). With respect to width, the present specimens were narrower.

The discrepancies among body dimensions and proportions may not necessarily be taxonomically critical, since they could be influenced by the size and age of the worm, as shown in the developmental study of the worm (Chapter 4). Morphological variation with age should be taken into account in taxonomic studies in the light of work carried out by Stunkard (1923) who found that in mature specimens of

Spirorchis haematobium some of the testes degenerate and disappear soon after sexual activity has declined. The method of fixation, age of the parasite, effects of the host and variation in localities could possibly account for some discrepancies (Markevich, 1951; Chappell, 1967 and 1980, Erasmus, 1972).

The significance of minor morphological differences between specimens 'A' and 'B' is unknown. The absence of crenulation and 'setae-like' structure in the 'A' specimens is puzzling since they appear to be of the same species in all other respects. The difference may have been due to age, since the 'A' specimens were at least eight months old at earliest examination, compared to 4-5 months of 'B' specimens. However the 'B' specimens were observed to bear both 'setae' and crenulations even up to nine months of age. It is important to point out here that the two specimens studied were not only from two different localities but were also from two quite different outbreaks, one in 1979, another in 1981, in different parts of England.

Egg - miracidia

Odhner published five drawings (from the hand of Looss) representing different stages of development of eggs of Sanguinicola sp. in 1911. In a second paper, in 1924, he again published the same figures with some more explanation of the eggs regarding the existence of shell and the development of the germ-cells vesicles and granules. At that time the systematic position of the worm was in question, but he established that these eggs must be of vitellarian origin and that there must be an existence of ovary and vitellaria.

The descriptions of the developmental stages of eggs given by

Odhner (1911, 1924) for Sanguinicola sp. and the description of the species in the present study appear to be essentially the same. Ejsmont (1926) gave detailed morphological descriptions of the eggs of S. armata, S. inermis and S. intermedia. The comparison of the sizes of eggs of these species are shown in Table 3.9. Comparing the eggs of S. armata, S. inermis and S. intermedia, Ejsmont (1926) showed that eggs of S. intermedia were the smallest in size.

On the basis of shape, sanguinicolid eggs can be divided into two types. One type is rounded, ovoid to spherical in shape and the other type is distinctly triangular. The eggs produced by S. davisii (Davis et al., 1961), S. alseae (Meade and Pratt, 1965), S. klamathensis (Evans and Heckmann, 1973), S. idahoensis (Schell, 1974) and S. volgensis Rasin (1929) are of the first type. Eggs of S. inermis (Odhner, 1911, 1924; Scheuring, 1920; Ejsmont, 1926; Layman, 1957; Naumova, 1961b), S. intermedia (Ejsmont, 1926), S. armata (Ejsmont, 1926) and S. lophophora (Erickson and Wallace, 1959) are triangular and very similar in appearance.

The triangular-shaped egg of the four species described above could not be taxonomically critical as the present study shows that early eggs are very angular but, as they mature, the angles become blunt and the dorsal process is reduced. It seems likely that similar developmental changes occur in other species, but more study is required. The triangular eggs of S. lophophora described by Erickson and Wallace (1959) differ slightly from the present species in that the triangular form is suppressed somewhat by a greater rounding of apices. Of the few descriptions available, there appears to be little morphological difference in the early embryonic development of the triangular-shaped eggs.

Table 3.9

Comparison of size and shape of some sanguinicolid eggs and the eggs of present species

	Size (in microns)		Shape	Reference
	Length (range)	Width (range)		
<u>S. intermedia</u>	51.6 (52-59)	33.0 (28-37)	Triangular	Ejsmont, 1926
<u>S. armata</u>	67.7 (59-74)	40.2 (34-44)	Triangular	Ejsmont, 1926
<u>S. inermis</u>	65.5 (61-70)	30.7 (29-35)	Triangular	Ejsmont, 1926
<u>S. volgensis</u>	-	-	Oval	Rasin, 1929
<u>S. davisii</u>	(30-56)	-	Oval	Wales, 1958
<u>S. alseae</u>	24	20	Round to ovoid	Mead & Pratt, 1965
<u>S. klamathensis</u>	(17-27)	-	Spherical	Evans & Heckmann, 1973
<u>S. idahoensis</u>	(50-60)	(32-35)	Oval	Schell, 1974
Present species	60.2 (50.7-70.9)	34.4 (27.8-40.5)	Triangular	Pers. Obsn.

During the development of miracidium, the dimension of the eggs increases greatly and, in some cases, the size is doubled. This is presumably due to the absorption of nutrients from the surrounding host tissues. A substantial increase in size was also described by Meade and Pratt (1965) for the eggs of S. alseae.

Scheuring (1922) illustrated and described a cluster of little rods in the miracidium of S. inermis. The present species has the same cluster of rodlets but with a comparatively larger stylet. These rodlets in the developed embryos of S. inermis are comparable to the parallel fibres described by Schell (1974) for the miracidium of S. idahoensis. It appears from the descriptions of Schell (1974) that the miracidium of S. idahoensis does not possess any stylet. The only other sanguinicolid miracidium that possesses a stylet is S. davisii (Davis et al., 1961). The descriptions available for other sanguinicolid eggs are poor and the presence and arrangement of rodlets, fibres and stylets remain in doubt.

Ej-smont (1926), Szidat (1951) found Sanguinicola eggs in the blood vessels of fish without being able to find any flukes. It is not uncommon for eggs to be found in the fish when the adult worm is not present (C. Sommerville, pers. comm.). Also the adult worm is often difficult to locate because of its minute size, variable shape and transparency. The egg on the other hand is frequently and easily observed in gills and other tissues. Specific identification of the egg would thus be of great diagnostic value. However, from the taxonomic point of view, the usefulness of shape and size of eggs is doubtful as a number of them may look alike. Thus, prior to any specific identification, it is essential to illustrate the morphological characters during the entire development.

Cercaria

So far, lophocercous cercaria are known to occur in three different groups (Erickson and Wallace, 1959), the Clinostomatidae, the Spirorchidae, and the Aporocotylidae (= Sanguinicolidae). Since the new classification scheme proposed by Azimov (1970) Sanguinicolidae has now the priority over Aporocotylidae.

Cercaria cristata La valette, 1855, is thought to develop into S. inermis (Smith, 1972). This species was reported by many authors as a sanguinicolid cercaria from a variety of molluscs in various habitats and localities. It appears from the literature that the morphological descriptions of C. cristata vary markedly, which indicates that all of them may not necessarily develop into the one species of adult Sanguinicola. Early observations by Scheuring (1922) indicated that his C. sanguinicola inermis from the intermediate host Lymnaea auricularia and L. stagnalis was definitely distinct from C. cristata.

The cercaria of sanguinicolidae are known to be of the lophocercous type, possessing a typical fin-fold and developing in a sporocyst. However, this generalised statement is not true in all cases. For example, the sanguinicolid cercaria described by Wales (1958) as S. davisii are completely lacking the dorsal body fin-fold and furcal fin-fold. The cercaria of S. klamathensis (Evans and Heckmann, 1973) are also lacking furcal fin-folds and furcae are tipped with claw-like projections.

It appears from the work of Wales (1958) that redial stages as well as sporocyst stages of S. davisii occurred in the snail hosts Oxytrema circumlineata and O. silicula. So far this is the only report of redial stages in Sanguinicolidae.

During a study of the freshwater snails in Kent, Essex and Middlesex, Khan (1961) describes C. kentensis in the snail host L. peregra. This C. kentensis is very similar to the present specimen except for the body fin-fold which, in the present cercaria, extends almost the full length of the body behind the anterior organ, whereas it was reduced at the anterior as well as the posterior end of the body in C. kentensis. He also noted the presence of a rhabdocoele gut.

Scheuring (1922), Ejsmont (1926) gave elaborate descriptions of the cercaria of S. inermis. These descriptions agree with the descriptions of present cercaria. The more limited description, given by Naumova (1961b), also agrees with the present species. Chechina (1959) described a cercaria C. cristata which subsequently developed into a mature S. inermis. This C. cristata agrees with the S. inermis cercaria of present study in total length, but the body of his C. cristata is much longer and wider.

It seems from Table 3.8 that, among the known sanguinicolid cercaria^e, Cercaria S. inermis is the smallest in size in all respects. It is also very interesting that all European sanguinicolids which parasitise cyprinids are smaller compared to North American sanguinicolids which parasitise salmonids.

British sanguinicolids

The first description of a Sanguinicola sp. from Britain was given by Chappell (1967) who found specimens of this genus in the central aorta and afferent branchial vessels of Rutilus rutilus, Leuciscus leuciscus, and Esox lucius from Hobhole drain and River Bain in South Lincolnshire. He could not give any positive specific

identification because of some morphological variations with known species and a lack of sufficient specimens to allow detailed morphological study. However, he thought that his specimens, apart from some small differences, closely resembled S. volgensis. The slight differences in gut ratio, oötype ratio, number of testes and maximum length could, however, have been due to the age of the specimens and small number of worms studied. Furthermore, he did not find any eggs in oötype, thus adding to the difficulties of specific identification.

The presence of S. volgensis in Britain was subsequently confirmed by Shillcock (1972), who described the species from five sexually mature worms. There are, however, some obvious differences between S. volgensis described by Bykhovskaya-Pavlovskaya et al. (1964) and S. volgensis described by Shillcock (1972). For example, the specimens described by Shillcock (1972) were smaller than those given by Bykhovskaya-Pavlovskaya et al. (1964).

The only other description of Sanguinicola sp. found in Britain was by Sweeting (1979) from the specimens he found in cultured carp from Gloucestershire. His description corresponded with the description of S. inermis given by Plehn (1905) in Bykovskaya-Pavlovskaya et al. (1964), but differs from the species described by Chappell (1967) and Shillcock (1972) in that the latter had a spiny cuticle.

The descriptions of British sanguinicolids, i.e. S. volgensis (Chappell, 1967; Shillcock, 1972) and S. inermis (Sweeting, 1978) are compared with the species of Sanguinicola of the present study in Table 3.10. The present specimen differs from the specimens

Table 3.10 Comparison of British sanguinicolids
(measurements are in microns)

Species	Length (maximum)	Width (maximum)	Spination	Gut ratio	No. of caecal lobes	Oötype ratio	No. of pair of testes	No. of ova in uterus	Host-fish
<u>Sanguinicola</u> sp. (Chappell, 1967)	1240.0	545.0	+ Regular	1:3	4	1:7 - 1:9	12-19	None seen	<u>Rutilus rutilus</u> <u>Leuciscus leuciscus</u> <u>ESOX lucius</u>
<u>S. volgensis</u> (Shillcock, 1972)	1595.1	360.6	+ Regular with large spine	1:3	4	?	14-16	Ovate 3 at a time	<u>R. rutilus</u> <u>L. leuciscus</u> <u>L. cephalus</u>
<u>S. inermis</u> (Sweeting, 1978)	665.0	191.0	- Instead fine setae arranged diagonally	1:3	4	?	Approx. 15	Triangular, 1 at a time	<u>C. carpio</u>
<u>S. inermis</u> (Present study)	1235.8	201.8	- Extremely minute 'setae like' structure	1:3	4	1:7.7	10-16	"	<u>C. carpio</u>

described by Chappell (1967) and Shillcock (1972) in the absence of a spiny cuticle externally and s-shaped vas-deferens internally.

CHAPTER 4

BIOLOGY

OF

SANGUINICOLA INERMIS

4.1 Introduction

Life history

The earliest full report of a sanguinicolid life-cycle was published by Scheuring (1922) and was for S. inermis. Since then, sanguinicolid life-cycles have been described by Odhner (1924), Hirschmann-David (1953), Naumova (1961b) and Sweeting (1979), also for S. inermis; Wales (1958), Meade and Pratt (1965), Meade (1967) and Evans and Heckmann (1973) for S. klamathensis; Wales (1958), Davis (1961) and Rawstron (1971) for S. davisii; Meade (1965), Meade and Pratt (1965) for S. alseae; Erickson and Wallace (1959), Larson (1961) for S. lophophora and Schell (1974) for S. idahoensis.

The pattern of life-cycles of different sanguinicolid species is more or less the same and is summarised in diagram (Fig. 4.1). Non-operculate eggs are laid by the mature flukes which hatch in the blood vessels in the gills of the fish. Ciliated miracidia then break through the soft gill tissues of the fish and are free-living for a short period of time. The miracidia then penetrate into the intermediate host, a gastropod mollusc. In the snail host, miracidia develop into sporocysts and ultimately cercariae. The cercariae then emerge from the snail and, after a brief free-living existence, they penetrate into the final host - a fish, where they develop into adult worms. The details of different stages of the life-cycle are dealt with separately.

Eggs

Sanguinicolid eggs are non-operculate and their shells are generally thin, soft, pliable and contain elastin (Madhavi and Rao, 1971). Smith (1972) in his excellent review paper, suggested that the elastin type egg-shell may be characteristic of members of the family Sanguinicolidae.

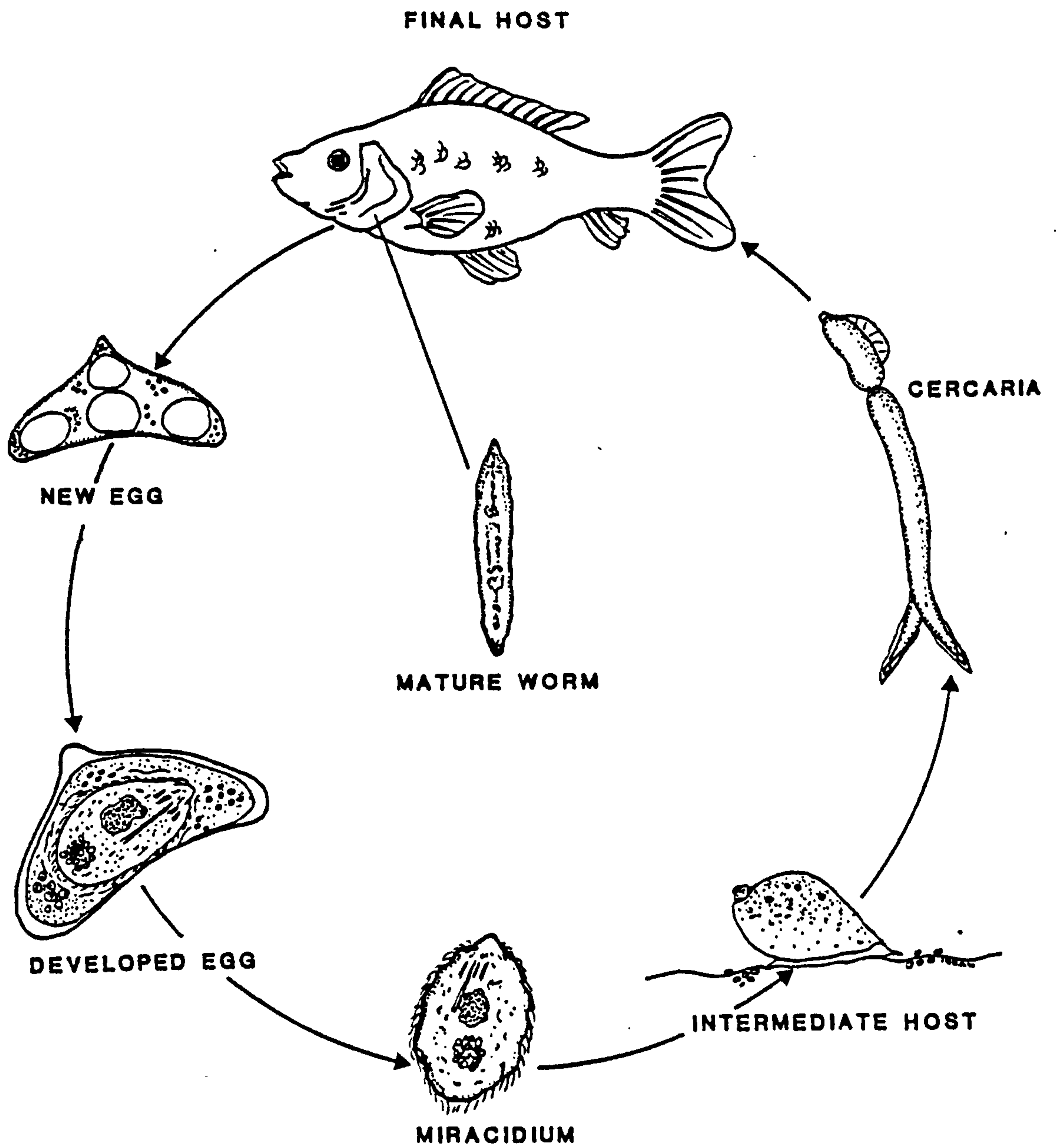


Fig. 4.1

Life cycle pattern of *Sanguinicola inermis* as observed in the British carp farms studied.

The shape and size of eggs of the S. inermis, S. intermedia, and S. armata, were redescribed by Ejsmont, 1925 and appear to be similar, being triangular in longitudinal sections and elliptical in transverse sections with a dorsal process on the convex edge. On the other hand, eggs of S. alseae are round and ovoid (Meade and Pratt, 1965). Davis, Hoffman and Surber (1961) mentioned that the early stage of S. davisii can be easily confused with the trophozoite stage of Myxosporidia because of their similar size and shape.

Though it was found by several authors that mature eggs of Sanguinicola occur most commonly in the secondary lamellae of the gills, they are also found in a variety of host-tissues, where they are ultimately caught and encapsulated by the host response. Various authors have described eggs of S. inermis in the liver, kidney, heart, muscle and spleen of fish (Léger, 1930; Schäperclaus, 1954; Martin 1960; Evans and Heckmann, 1973).

Miracidia

Sanguinicolid miracidia are complex ciliated larvae which are easily distinguishable by their darkly pigmented eye-spot. It is generally believed that the entire development of the miracidia takes place in the gills. The best description of a miracidium and its development to date was given by Davis et al. (1961) for S. davisii. According to this account, the most striking change in the miracidium, as development progresses, was the disappearance of certain nuclei which were present in the younger embryos. Scheuring (1922) working on S. inermis and Meade and Pratt (1965) for S. alseae and Schell (1974) working on S. idahoensis observed that as development progressed there was a substantial increase in size of the miracidium of those species.

Very little is known of the mechanism of emergence of the miracidium from the egg-shell. Plehn (1924) believed that the stylet of S. inermis was used for piercing the egg-shell. Contrary to this, Davis et al. (1961) described the function of the stylet as being primarily for penetration into the tissue of the snail^hhost.

This is obviously a very interesting area of research as one can envisage the effects of large numbers of miracidia emerging through host-tissue. Intense study is, therefore, essential, not only because of its fundamental interest, but also because of its practical value in fish-farming.

Sporocysts/cercariae

Development of sanguinicolid miracidia into cercariae in the gastropod molluscan host usually takes place through one or two generations of sporocysts. According to Cable (1965) distinction between rediae and sporocysts is not always clear. Most of the description of sanguinicolid sporocysts are of thin-walled sacs which are non-motile and ovoid in shape (Meade and Pratt, 1965). Schell (1974), however, described fully developed daughter sporocysts of S. idahoensis as spherical with a thin limiting membrane.

Various developmental stages of cercaria^e of S. davis were found scattered throughout the sporocysts lumen by Meade (1965), who reported that between 15 and 20 cercariae were usually present in various stages of development with only two or three mature cercariae in each sporocyst. In all cases development of sporocysts occurred either in the digestive gland and/or visceral mass of the infected snail.

Sanguinicolid cercariae are found to be of the furcocercous, brevifurcate, apharyngeate type.

At present little is known about the development of S. inermis in its intermediate host, and this is still to be determined.

Cercarial emergence and behaviour

Due to the little information available, it is very difficult to describe the overall pattern of cercarial emergence and behaviour. There are few reports on cercarial emergence and these vary from author to author (Meade, 1967; Erickson and Wallace, 1959; Meade and Pratt, 1965 and Naumova, 1961b). It would appear that variations occur mainly in the trigger environment for the cercarial release.

This environmental trigger or stimulus for S. inermis cercaria to emerge is not fully understood. For a better understanding of the host-parasite system and possible means of control, it is, therefore, important to find out the exact conditions for these trigger mechanisms to operate.

Unfortunately there ^(is) no data available on the host-finding behaviour of S. inermis cercariae and this also is another field which demands investigation.

Penetration in the definitive host

Penetration of the definitive host by cercariae was first observed by Scheuring (1922) for the cercariae of S. inermis when he found them attached to carp-gills. These cercariae then penetrated to the blood vessels.

Few reports are available on experimental infections. Schell (1974) described the experimental infection of S. idahoensis in

steelhead trout, Salmo gairdneri, where he found that the cercariae were unable to penetrate the skin of fish which exceeded 2.5 inch in length. Other experimental evidence shows that most of the sanguinicolid cercariae prefer soft areas of the fish as a point of penetration (Wales, 1958; Naumova, 1961b and Meade and Pratt, 1965).

Development and habitat in the definitive host

There are few studies of the development of *Sanguinicola* following penetration of the definitive host, and most observations are of mature infections.

Sexually mature sanguinicolids live mainly in the blood vessels of freshwater fish, particularly in the bulbus arteriosus, abdominal aorta and afferent gill arteries (Markevičh, 1951). Bauer et al. (1973) reported that S. inermis prefers to inhabit the chambers of the heart, bulbus arteriosus and ventral aorta, as well as the larger vessels of the gills.

This generalized statement rather than statement on the exact habitat of the worms seems to vary with seasonal differences and with the stage of maturity of the parasite. Thus Naumova (1960) stated that sexually mature S. inermis in carp appears to be localized in the large blood vessels of the gills. But in the winter, she found them in the bulbus arteriosus. Similar observations were also made by Evans and Heckmann (1973).

Most of the reports on the sanguinicolid habitat were determined from natural infections, so that there has been no investigation into the migration of the worm and its subsequent effects as it migrates.

A systematic account of the route of migration of the worms from a known infection is necessary for the evaluation of host-parasite interactions. One of the objectives of the present work is thus to carry out such experimental infections and to ascertain the subsequent pattern of migration of the parasite.

Seasonal dynamics of infection

The seasonal dynamics of infection have not been extensively studied. In the USSR, the infection is reported mostly in the warmer months of the year. Chechina (1959) observed outbreaks of Sanguinicola infection in carp during the summer in all rearing ponds of Byelorussian SSR and the highest mortality occurred during that time. Similar reports were given by Lucký (1964) where all carp propagating fish ponds in South and Central Moravia in Czechoslovakia were found to be infected only during the summer months.

Bobiatyn'ska (1964) reported that infection of carp took place several times during the year. In the farms of the Bialystok area of Poland, the first infection took place at the end of May and in June and the second during August, September and beginning of October. The multiple peaks of infection are apparently due to successive waves of cercarial invasion and overwintering of larvae producing peaks of mature worms in the following summer.

Migala (1969) pointed out that Sanguinicola infection was dependent on the presence of the intermediate hosts and their survival. He found that stocking density and the feeding regime of the farmed young carp indirectly influenced the course of Sanguinicola infection in farm ponds. Highly stocked ponds with no supplementary feed forced the fish to depend more on natural food including snails. He believed that this natural way of controlling snail populations

reduced the infection level. However, this could not be true of young fry as they are small in size compared to snails and as they are partially or fully dependent on supplementary feeding, particularly in an extensive farming situation.

4.2 Experimental infection of intermediate host by the miracidia of S. inermis

4.2.1 Materials and methods

Experimental infection of molluscs proved to be exceedingly difficult. A number of variables were tried over several months of the study to initiate molluscan infections. Several species of snails from group 'a' - 'f' (details in Chapter 2) were used since some of them are known to be the potential host for S. inermis and found in infected farms. To obtain a successful infection four different types of water were used: copper-free tap water, distilled water, artificial spring water and the water of the infected pond. Four different pH levels and three different temperatures have also been used as variables. These are summarised in the Table 4.1. All combinations of the variables were used.

Approximately 50 miracidia were introduced into small petridishes or beakers (25 ml - 100 ml) containing 2 - 10 snails. The infection was then allowed to proceed for at least five hours before transferring the snails to a 3ℓ circular tank. Batches of infected fish were also kept with uninfected snails for a longer period (over two months).

Examination of snails

Snails were examined regularly at intervals after an initial exposure to the miracidia. The methods of examination are described in Chapter 2.

4.2.2 Results

Miracidial Behaviour (attack)

The behaviour of the miracidium of S. inermis was observed with and without the presence of the snails. Most of the observations

Table 4.1

Showing the variables used for S. inermis infection.
All combinations of variables were used.

Snail species	Temperature °C	pH	Water	Methods
<u>Lymnaea peregra</u>				
<u>L. stagnalis</u>	Three different temperatures, 10; 15 and 20	Four different pH, 6.5; 7.0; 7.5 and 8.0	Copper-free tap water, Distilled water Artificial spring water and Pond water	Developedegg, Free miracidia, Infected gills and Infected kidney
<u>L. auricularia</u>				
<u>L. palustris</u>				
<u>Physa fontinalis</u>				
<u>Bithynia tentaculata</u>				

with snails were carried out using juvenile snails measuring 2×2.5 mm. Occasionally observations were also carried out using small pieces of tissue from matured snails.

Under laboratory conditions (room temp. $18^{\circ}\text{C} \pm 2^{\circ}\text{C}$), the miracidia of S. inermis showed a complex behavioural pattern. Generally, young, newly hatched miracidia were found to be negatively geotactic with positive phototactic responses. As time progressed, they tended to move to the bottom of the experimental vessel. Approximately five hours after hatching most of the miracidia (70%) were found to have moved to the bottom of the experimental dish. This was thought to be due to exhaustion owing to their continual movement.

Most of the observations were carried out under a dissecting microscope using incident light and the miracidia were found to move near the surface of the water under these conditions. Any discontinuation of light resulted in the movement of the miracidia towards the bottom of the dish. It is thus concluded that miracidia of S. inermis are photopositive. However, this photopositive behaviour was found to reduce gradually as time progressed.

Though miracidia always swim actively in the water, swimming behaviour was more vigorous in the absence of the snail. Immediately after the emergence from the egg, swimming movement was swift and restless with a straight darting motion. Frequent changes of direction at an angle of 180° in the horizontal plane were observed at this time (Fig. 4.2a). As it progressed the miracidium rotated continuously about its longitudinal axis. The direction of rotation being either clockwise or counter-clockwise (Fig. 4.2b). A rapid circular spinning motion was also frequently observed. During this motion

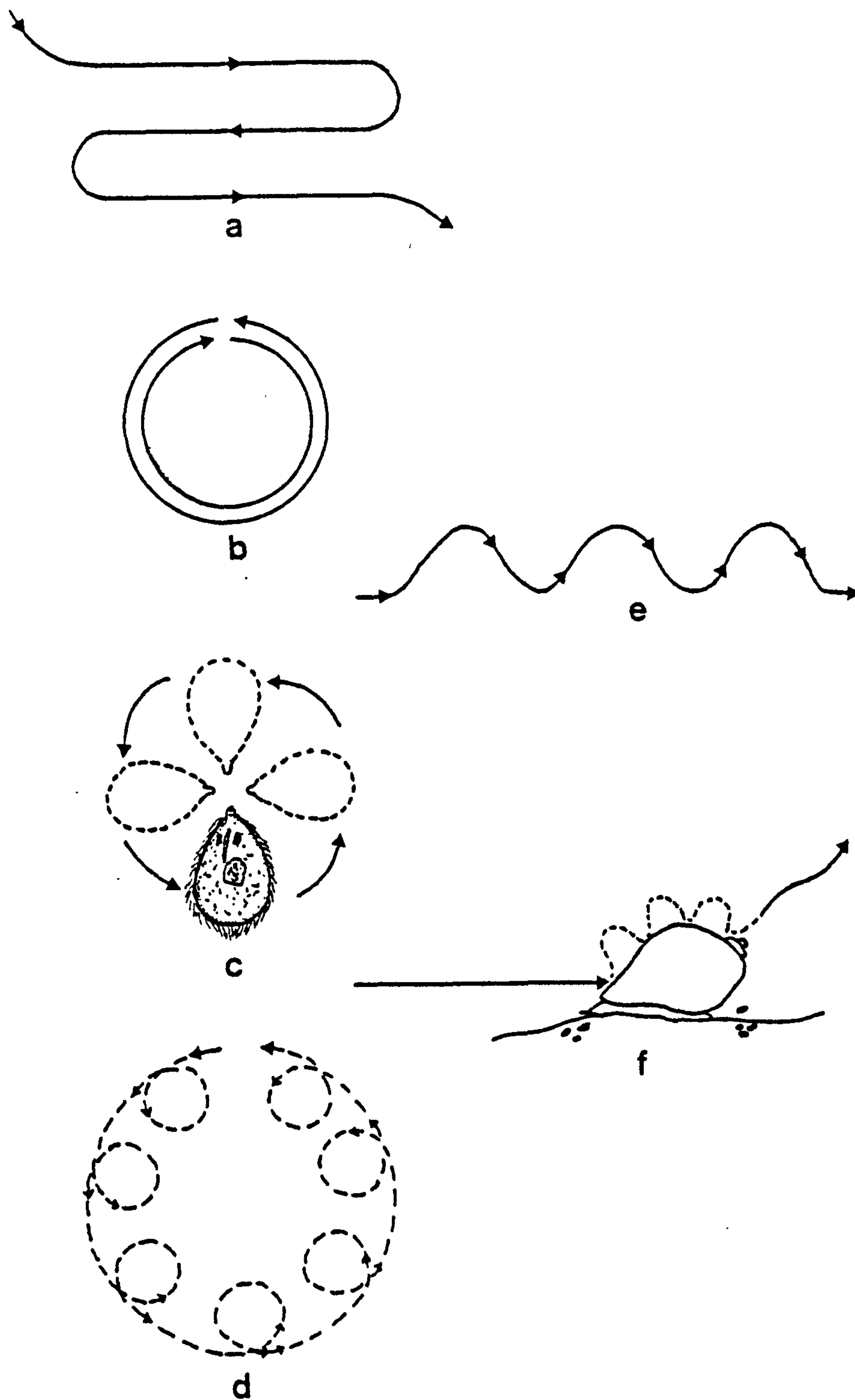


Fig. 4.2

Host finding behaviour of the miracidium of S. inermis.

- a. Quick "zigzag" with an angle of 180°
- b. Clockwise or counter clockwise rotation
- c. "Spinning" movement
- d. Circus movement with loops
- e. "Wigwag" movement
- f. Repeated dip investigation

the miracidium spins about an axis centralized at the anterior tip (Fig. 4.2c). Most of the time this type of motion was accomplished by a small circle with several loops (Fig. 4.2d). The spinning motion sometimes was found to stop as abruptly as it began, and the miracidium then swam a short distance away and repeated the same.

In presence of snails and/or snail tissue the pattern of swimming behaviour was found to be somewhat different. Miracidia coming into the vicinity of a snail (within the range of 2 - 5 mm) showed decreased speed with increased random turning. Within the vicinity of the snail they were swimming abruptly back and forth in a "wigwag" manner (Fig. 4.2e). When moving miracidia came into contact with the tissues of the snail, they stopped briefly before moving away from the snail. At this stage the miracidia made repeated contacts with the snail in a series of dips (Fig. 4.2f). This type of behaviour was found to continue until final penetration occurred. Most of the miracidia once in contact with the snail swam in and around the snail, and finally attached themselves to the snail. However, after their first contact a small number of miracidia swam away from the snail, started spinning without progressing, and ultimately died.

Penetration

Histological examination of serial sections of fixed snail tissue used in these experiments failed to show any evidence of miracidia or other forms of parasite, thus making it difficult to follow further penetration and development.

Experimental infection

Approximately 1,700 snails were examined using all combinations of the variables (Table 4.1) for experimental infections. Despite all attempts to experimentally infect snails no further development of the miracidium was found in the snails.

Some of the snails from group 'a', 'c', 'f' and 'g' were found to be infected with larval strigeids and polychaetes. These were groups of snails collected from natural populations. Snail groups 'b', 'd', and 'e' were laboratory bred and thus free from any other infection.

4.2.3 Discussion

The present study has shown that phototactic and geotactic responses occur in the miracidia of S. inermis, as well as other host-finding behaviour. As there is no published report on the host-finding behaviour of S. inermis miracidia, it is thus possible only to compare these findings with those of the few species from others so far studied.

Wright (1959, 1971) described three main ways by which miracidia are brought into contact with their molluscan hosts. In one of them the miracidia actively seek out their molluscan host. Miracidia of S. inermis fall into this group, as their movements after emergence from eggs were found to be very active. Following emergence from the egg the miracidia then responded to stimuli in the environment. The subsequent responses are primarily adapted to finding a host. This host finding behaviour supports the general statement made by Wright (1971) that miracidia respond to some physical stimulus such as light or gravity, and this response takes the miracidia to the general environment of the snail. The responses in the case of

S. inermis miracidia were positively phototactic and negatively geotactic in the presence of light.

Takahashi, Mori and Shigeta (1961) reported that miracidia of Schistosoma japonicum also exhibited phototactic and geotactic responses. Their finding showed that at 15°C the miracidia exhibited a positive response to any given light intensity but as the temperature rose, the intensity of the light had to be reduced to get a similar response. With the presence of light the miracidia of S. inermis showed a negative response to gravity. Takahashi et al. (1961) found the negative geotaxis of S. japonicum was disturbed by light intensities above 5,000 lux. As with S. inermis, Takahashi et al. (1961) also found that S. japonicum miracidial responses lapsed with time.

In the light of the responses of snail and miracidia of S. inermis it appears that similar interaction to those described by Takahashi et al. (1961) exists, i.e. under normal conditions (at a temperature of 20°C or above) both the snail and miracidia were found to move to the water surface.

Contrary to Takahashi's findings several authors believe that contact between snails and miracidia is a matter of chance (Stunkard, 1943; Chernin and Dunavan, 1962) and that trial and error are much more important than chemotactic response in bringing the miracidia to the proper snail (La Rue, 1951). Chernin and Dunavan (1962) concluded that no chemical attraction was involved in the selection of the snail host, Australorbis glabratus by the miracidia of Schistosoma mansoni. Etges and Decker (1963) also considered that both light and gravity are far more powerful stimuli in determining the orientation of S. mansoni miracidia than the chemical ones produced by their molluscan host.

However, MacInnis (1965) classified the behaviour of the miracidia of S. mansoni with and without the presence of chemical attractants. Without the presence of chemical attractants, what he called "normal" swimming behaviour was found. This swimming behaviour was similar to that of S. inermis miracidia when observed without the presence of snail^s. Most of the responses shown by the miracidia of S. inermis in the presence of the snail host were similar to that shown by S. mansoni when observed with test chemicals, with the exception of the speed of swimming. Observations on the speed of swimming of S. inermis during the behavioural study showed a decreased speed in the presence of the snail rather than the increased speed described by MacInnis (1965) for S. mansoni in the presence of test chemicals. This reduced speed of miracidia in the presence of the snail is considered to be advantageous. The reduced swimming speed of the miracidia occurred during the second phase of their host-finding behaviour pattern (Wright, 1971) which is characterized by its random movement with occasional turns. The final phase in the host finding behaviour of free-swimming miracidia, as several authors (Wright, 1966; MacInnis, 1965) believe, is governed by responses to chemical stimuli emanating from the snails themselves.

It is clear that L. peregra is able to act as vector for S. inermis since infected L. peregra were found in the infected farms, and it is therefore puzzling as to why no experimental infection took place. Although miracidia were observed to come into contact with L. peregra in the experiments, there was no evidence of penetration. There must, therefore, be further stimuli from the snail or from the surrounding environment which cause the miracidia to attack and penetrate the tissue. Wajdi (1966) describes four factors which he thinks play an

important part in the process of penetration. These are (i) ciliary action of the miracidia which beat strongly and press the miracidia against the snail; (ii) adhesion of the miracidium to the snail tissue by their mucoid contents of the adhesive glands; (iii) elongation of the anterior papilla which worked its way through the snail's epithelial cells; and (iv) contents of the penetration gland which apparently digest the tissue of the snail and thus help the miracidium to enter. It is not known if penetration by the miracidia took place or whether the penetrating miracidia failed to survive and this needs further study.

Lie, Heyneman and Kostanian (1975) experimentally showed that snails already harbouring Echinostoma lindoense were not capable of reinfection by the miracidia of the same species. They also confirmed that failure to reinfect snails by the miracidia was due to cannibalism exerted by rediae originating from the first infection. Thus pathogen-free laboratory bred snails were used in this study. Wilson and Taylor (1978), and Anderson (1978) suggested that the reduction of snail or parasite densities to low levels will make the chance of infection remote. However, in this study the parasite was always in close proximity to the potential host under the experimental condition. Failure to experimentally infect snails with S. inermis was also noted by Körting (pers. comm.) despite exhaustive experimental trials.

There is not enough evidence to come to any conclusions concerning the failure of the miracidia of S. inermis to infect L. peregra and five other species. However it is important to mention here that the level of infections amongst the naturally infected snails was very low. A total of 3,260 snails were examined from farm 'A' and 'B' but only seven of them (0.21%) were infected with S. inermis cercariae. A low

rate of infection of snails by a sanguinicolid cercaria was also reported by Radlett (1978) from Cave Castle Lake, England. Only 0.55% of L. peregra and 3.1% of L. auricularia were found infected with the Cercaria kentensis. Chappell (1967) also described a similar situation where he examined 1,000 freshwater gastropod molluscs (L. peregra, L. stagnalis, Bithynia tentaculata, Physa fontinalis and Planorbis corneus) from the River Bain, but none were found infested. Shillcock (1972) examined a large number of molluscs from the Sanguinicola infected River Roding, but none were found to be infected despite the presence of S. volgensis in fish from the river.

Whether the low infection rate of snails on the infected farms is a true reflection of the situation is not known for certain, because the infection was detected only when the mortality rate in fry started to rise and, by that time, the initial infection was estimated to be five months old. By the time the snails were examined the incidence in the molluscan host might possibly have declined. If this is the case, it suggests that the parasite is very pathogenic to the snail host, but requires further work.

4.3 Experimental infection of the definitive host with S. inermis cercariae and its development to maturity

4.3.1 Materials and methods

Source of fish: A total of 180 fish from three different families viz Cyprinus carpio, Oreochromis niloticus and Salmo gairdneri were used. These are the fish groups 'C', 'E' and 'F' (details of fish groups are shown in Chapter 2). The fish used in the experiment were small and fell within the range of 3.5 - 6.0 cm long.

Source of cercariae: Naturally infected Lymnaea peregra were collected from the rearing ponds of the Calverton Fish Farm (Farm 'B') in Nottingham. They were kept in a constant room temperature (System C, Chapter 2). Snails were fed with fresh lettuce, porridge sandwich or Tetraphyll. Tanks were cleaned every second day and half of the tank water was replaced with fresh water. Cercariae were collected by placing the individual infected snails in glass vials (45 ml). The vials were kept floating in a water bath to maintain the temperature at approximately 20°C. A 60w lamp was used for constant illumination. Shed cercariae were then collected and counted. Numbers of cercariae in the total volume were estimated using three aliquots of 1 ml per sample.

Experimental infections

Infections were carried out in the laboratory using small plastic tanks (8l) equipped with aerator. Fish were removed from the experimental tank after one hour exposure to the cercariae. They were transferred to either system C (Chapter 2) with a constant

temperature of 15°C or system B (Chapter 2) with a variable temperature of 15-18°C.

Trout and tilapia were exposed to 1,000 cercariae per fish. For carp three different levels of infection were used as follows -

- (a) 100 cercariae per fish,
- (b) 300 cercariae per fish and
- (c) 1,800 cercariae per fish.

For each level, three replicates were used.

Sampling schedule

To study behaviour, attachment and penetration of cercariae, two fish were sampled at intervals of 10, 20, 30, 50 and 60 minutes. Subsequent samples were taken at intervals 3, 6, 12 and 24 hours. Thereafter fish were sampled daily until 90 days post infection. Fish were either dissected or fixed for histology in 10% neutral buffered formalin. To study the pattern of migration, samples of five fish were dissected at monthly intervals and the position of the worms in the fish were noted. For the purpose of this study, gill and heart worms were added together. Experiments were carried out over a period of eight months.

4.3.2 Results

Of the three host species used, experimental infections of cercariae were successful only in carp. Infections with O. niloticus and S. gairdneri were negative. Thus, all subsequent results were obtained only from the infected carp. The data below is mainly from the fish kept in system 'B' unless otherwise indicated.

Cercarial emergence and behaviour

Cercariae emerged from infected snails during the day, but the majority of them emerged in late afternoon. They were emitted in large numbers and the number was enhanced by the change of water. The maximum number of cercariae emerged at water temperatures between 15 to 20°C.

After emergence the cercariae remained well distributed throughout the water column of the container (45 ml). Usually they hung motionless in water for long periods. When disturbed, however, with a pipette or when a shadow was thrown across the water, the cercariae became very active for a few seconds, swimming vigorously upwards tail first. When the disturbance ceased the cercariae reverted to their normal position. The tail-stem of some of the resting cercaria was curved to such an extent that the proximal half of the tail stem and the body came to lie against the distal half of the tail shaft as shown in Fig. 4.3.

The emergent cercariae survived longer at lower temperatures. They survived up to 24 hours at 12°C and 18 hours at 15°C, but at 20°C they survived only up to 11 hours.

Attachment, penetration and distribution

Cercarial attachment and penetration was observed either with living fish or with an isolated fish fin. After coming into contact with the host surface, the cercaria attached in the region of the fins body and gills possibly by muscular adhesion of the anterior region. After attachment, the activity of the cercaria was confined mainly to the body region. The penetration process was active, by means of vigorous extension and contraction of the entire body of the

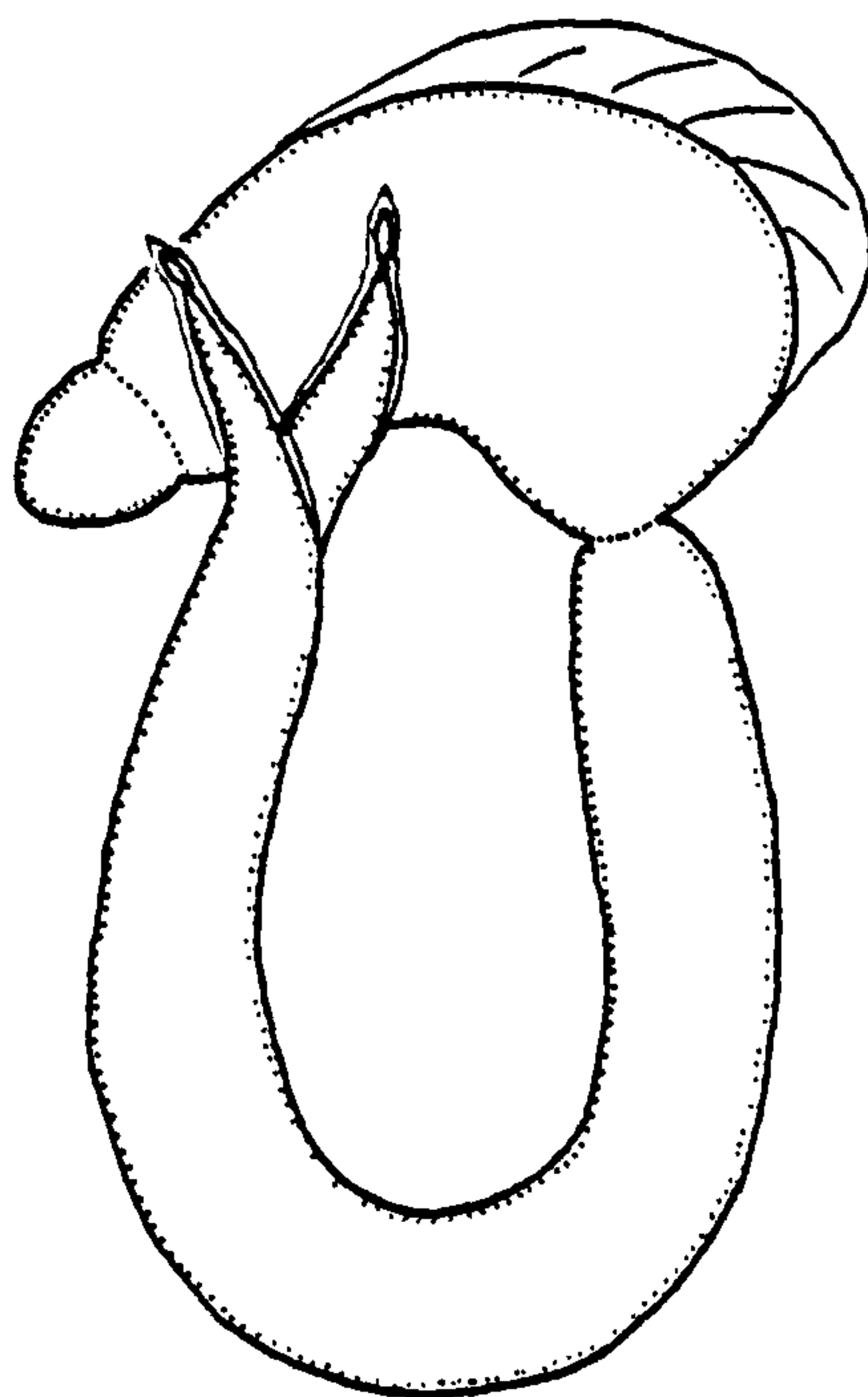


Fig. 4.3

Characteristic 'U' shaped resting position of
the cercariae of S. inermis

cercaria, until entry was achieved. The cercarial tail did not seem to play a great role in the penetration process and was shed soon after attachment or discarded on penetration.

The maximum number of cercariae had entered the fish within 30 minutes 25% of which were able to penetrate within 10 minutes and by 20 minutes as many as 95% of the total worms recorded had completed penetration.

To assess the most important site for penetration, the fish was divided into three main regions, namely Fins, Gills and Body surface. Table 4.2 shows the distribution of the successful cercariae in these

Table 4.2 Distribution of established worms (%)

Time	Cercarial load per fish	Fish Body		
		Fins	Body	Gills
60 minutes post infection	100	70	19	11
5 days post infection	365	73	16	11
	500	67	26	7
	1800	63	29	8
Mean percent		68.25	22.50	9.25

sites (calculated as the percentage of the total number of cercariae established). Cercariae were able to penetrate over the entire body surface, but within 60 minutes exposure 70% of the cercaria were found to have penetrated the fins whereas only 11% were in the gills and 19% over the remaining body surface of the fish. Among the fins, the caudal was found to be the preferred area as shown in Figure 4.4. Over 40 percent of the cercariae were penetrated through the caudal fin. The anal fin appeared to be the least preferred site. Of

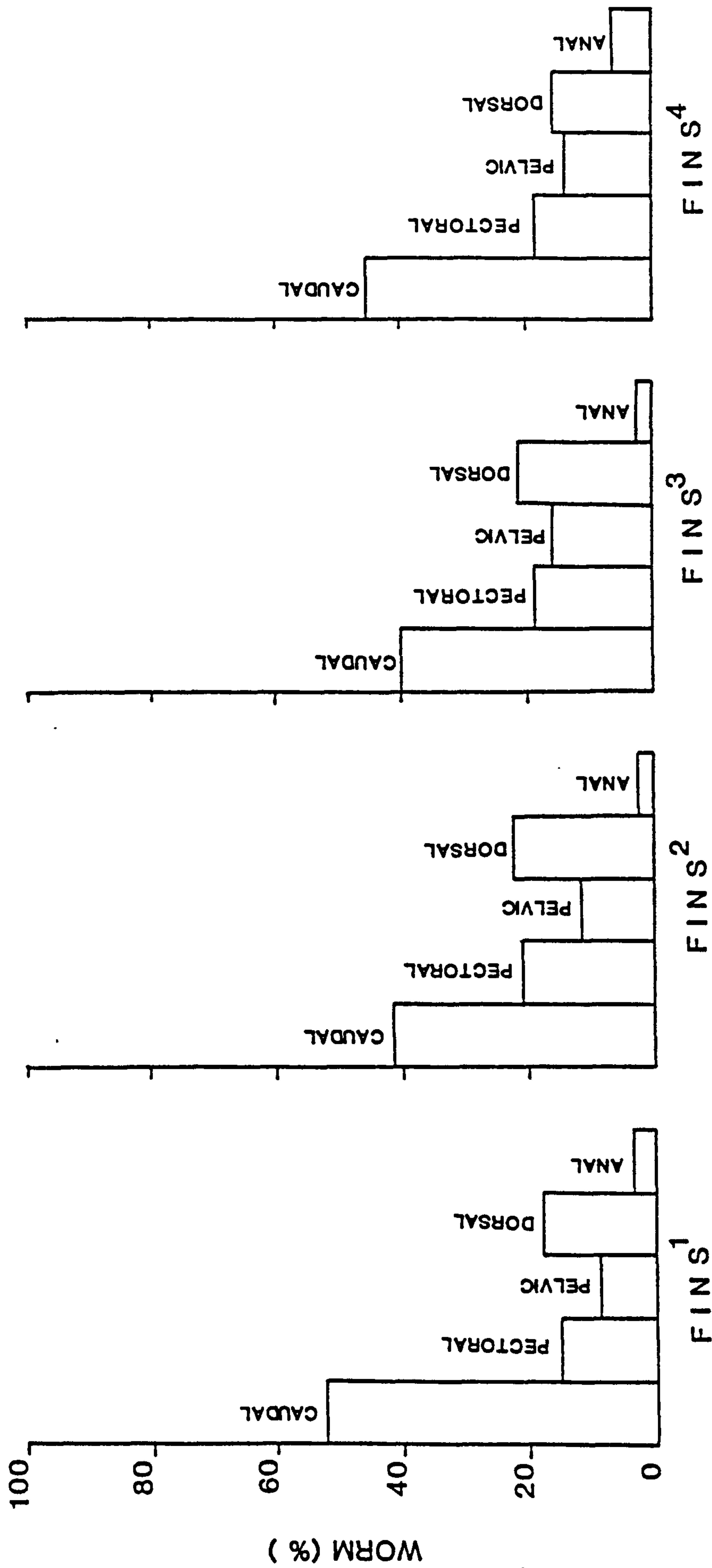


Fig. 4.4 Distribution pattern of *S. inermis* in the fins of the *C. carpio* at 60 minutes post infection.

- 1 100 cercariae/fish
- 2 365 cercariae/fish
- 3 500 cercariae/fish
- 4 1000 cercariae/fish

those which penetrated the body surface, other than the fins and gills, there seemed to be a preference for the scaleless areas of the body, e.g. ventral surface near the pelvic fins. The distribution pattern at 60 minutes and 5 days post infection was similar and the ratio of established worms in the three sites was not greatly affected by the initial cercarial load (Table 4.2). During this early period of establishment, fewer cercariae entered through the gills than any other site.

Success rate

The cercaria were considered to have succeeded in establishing themselves if they were present five days after the initial infection. It was found that the success of establishment varied with the size of the initial infection rate. Table 4.3 shows the percentage of successful worms with their means found in the skin and gill and/or heart region. With an exposure of 365 cercariae/fish the success rate was over 3% whereas this came down to 1.12% when 1800 cercariae per fish were used.

Sixteen percent of the worms were found to have successfully penetrated the fish 60 minutes after exposure. However five days post infection only 2.5% (1.12-3.18) worms had successfully established. The fate of the large populations of worms lost is unknown. No evidence was found of dead or moribund worms.

Migration

Figure 4.5 indicates the migration pattern of S. inermis over a period of eight months. Invading parasites did not settle at the site of entry of the host but were found to migrate.

The number of worms occurring in the skin shows a sharp decline

Table 4.3 Establishment of S. inermis cercariae in fry of C. carpio

Time	No. of cercariae exposed per fish	Mean no. of worms established in skin (Range)	Success (%)	Mean no. of worms established in gills and heart (Range)	Success (%)	Overall Success (%)
60 minutes post infection	100	14.00 (3-20)	14.00	1.80 (1-5)	1.80	15.8
5 days post infection	365	10.30 (1-15)	2.82	1.30 (0-3)	0.36	3.18
5 days post infection	500	14.20 (0-13)	2.84	1.10 (0-3)	0.22	3.06
5 days post infection	1800	18.6 (2-18)	1.03	1.60 (0-3)	0.09	1.12

starting at 60 days post infection. In the 90 day sample only 43% of the established worms were located in the skin. This declining trend was found to continue and, at the end of 150 days, no worms were located in the skin.

However, over the same period there was an increasing number of worms found in the gills and heart. Seven percent of the worms established in the gills at five days but this increased up to 57% at 90 days. It is interesting to note here that this was the period following the onset of egg production (section 4.4). The number of worms found in the gill and heart increased up to 120 days and thereafter they declined. This decline continued steadily until, at the end of 210 days, no further worms were located in gills and heart. The histogram (Fig. 4.5a) shows the total number of worms established in all sites which was found to decline over the experimental period, being steepest between 60 and 90 days. 93% of the worms established at five days survived until the age of two months, but thereafter they gradually died. Fig. 4.5b shows the spatial distribution of the invading worms. During the first two months of infection the majority of the worms occurred in the skin. However, at the 90 day sample and after, the majority of the worms were recovered from the gill and heart.

Growth and development of worms in the final host

Figure 4.6 shows the growth (increase in size) of skin and gill/heart worms up to 36 hours. During this early stage of development, growth was very rapid. The worms appeared to more than double their length within this short period of time. This growth pattern was found to be similar for both skin and gill/heart worms. The skin worms however, showed a greater increase in size than those in the

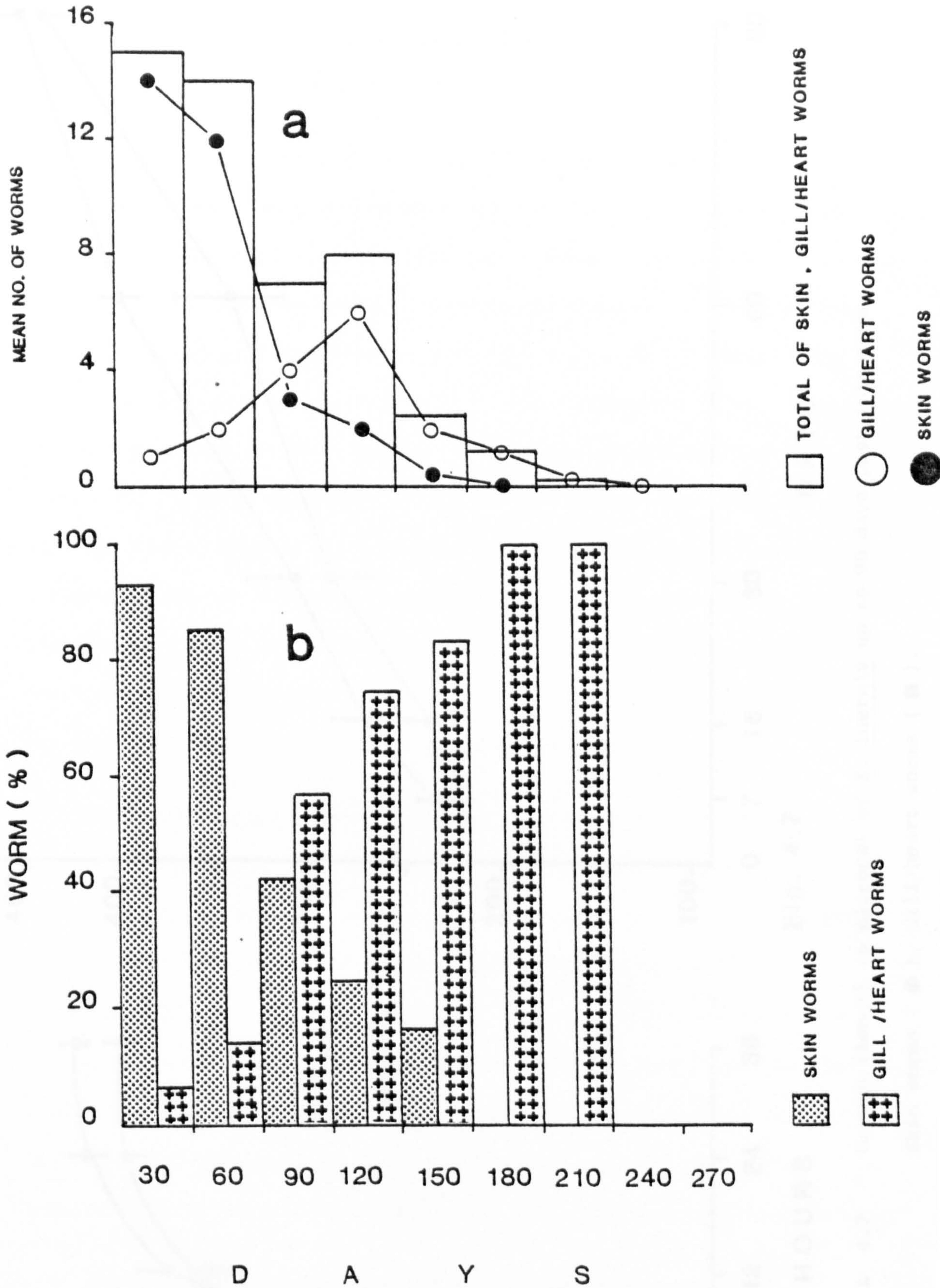


Fig. 4.5 Distribution pattern of skin and gill/heart worms in experimentally infected *C. carpio* up to 270 days post infection.

a, overall distribution;
b, spatial distribution.

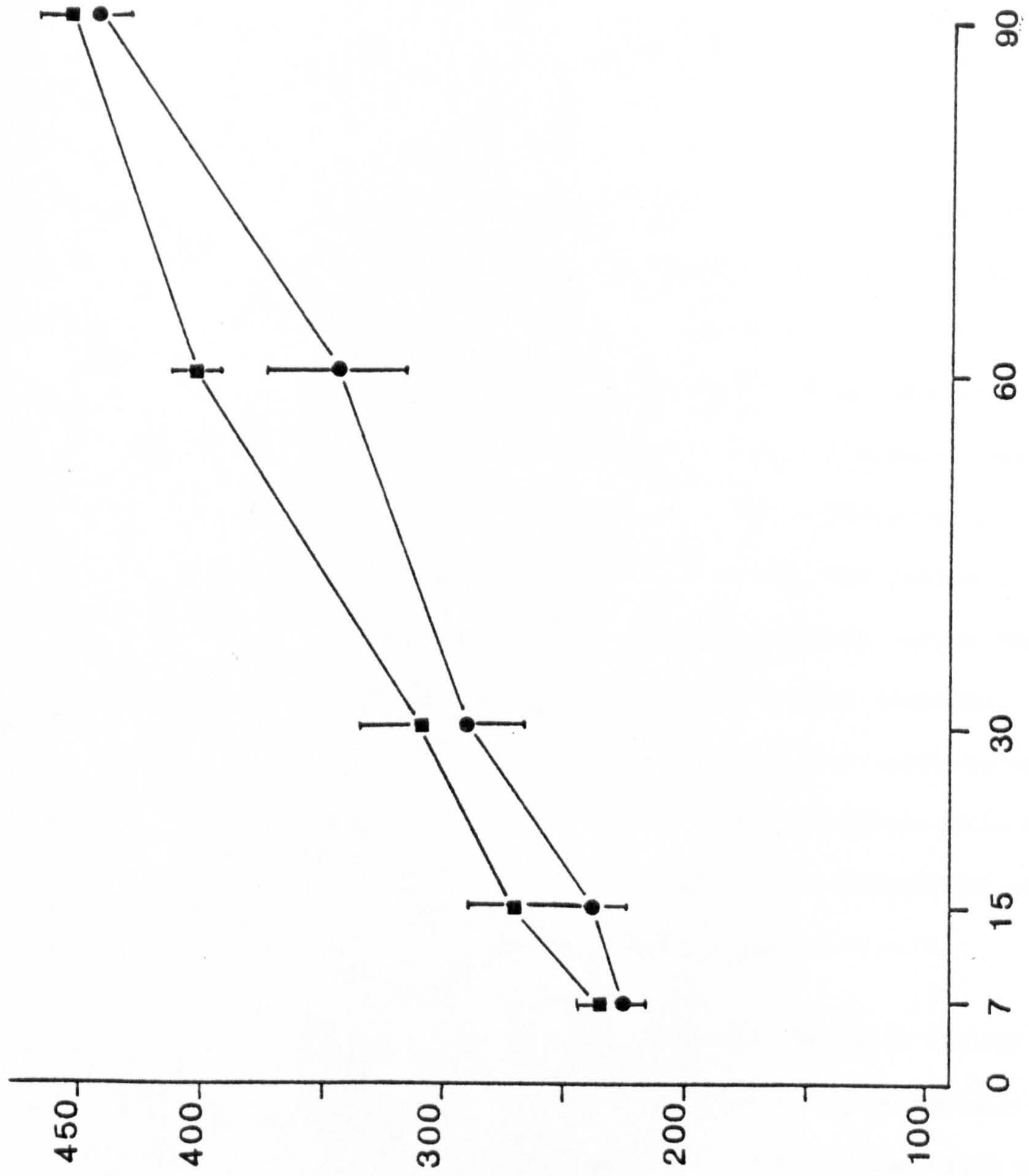


Fig. 4.7

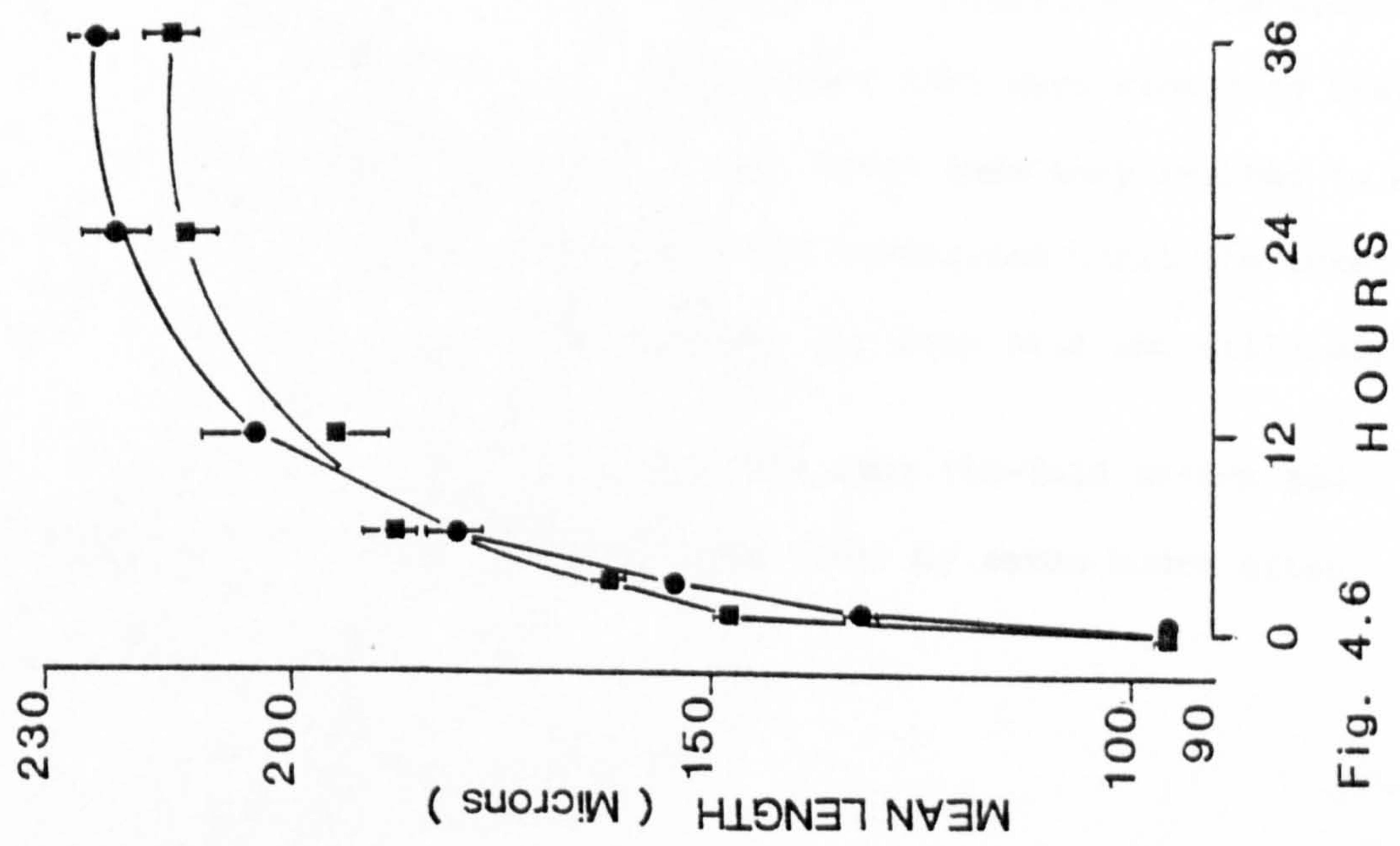


Fig. 4.6

Fig. 4.6 & 4.7 Growth (length in microns) of *S. inermis* up to 90 days post infection.

Skin worms (●), Gill/heart worms (■).

gill/heart region and this difference in size at 36 hours was statistically significant ($p < 0.05$).

The growth of worms, from 36 hours, was much slower and during this period there was a marked reversal in the relative sizes of skin and gill/heart worms, i.e. the gill/heart worms achieved a greater mean size than the skin worms. Between 36 hours and seven days skin worms increased 1.8% compared to 8.9% of the gill/heart worms, thus overtaking the skin worms (Fig. 4.7). The difference in size (length) of the two groups of worms at day 7, however, was insignificant ($p > 0.05$). At 60 days post infection gill/heart worms had increased 323% in length, whereas skin worms showed a 258% increase. The size difference between the two groups at 60 days was statistically significant ($p < 0.05$). The size difference between skin and gill/heart worms was maintained till the end of the experiment at 90 days but the difference was no longer significant ($p > 0.05$).

In overall shape of the worms was also found to change as the development progressed. To give some quantitative measure of the change in shape, a shape index was calculated as the ratio of length/width. The size and shape index for each sample up to 90 days is shown in Table 4.4. During the first 36 hours, when the growth in length was very rapid, the worms became much more elongated thereby losing the cercarial shape. After seven days they started to become wider and flatter and this shape was maintained until the worms matured. This pattern was similar for both skin and gill/heart worms.

During the first few hours, the body fin-fold shrank and deteriorated and was absorbed completely by seven hours after penetration.

Table 4.4 Mean size and shape index of skin and gill and heart worms of S. inermis up to 90 days post infection

Age of worms	Size				Shape			
	Skin worms		Gill and Heart worms		Skin worms		Gill and heart worms	
	Length (μ)	Width (μ)	Length (μ)	Width (μ)	Length/width	Length/width	Length/width	
0 hour	96 + \pm 10.45	25 + \pm 3.83	96 + \pm 10.0	25 + \pm 3.86	4	4	4	
1 "	133 + \pm 6.32	14 + \pm 1.25	148 + \pm 3.54	15 + \pm 1.60	10	10	10	
3 "	155 + \pm 3.81	15 + \pm 1.61	163 + \pm 2.97	15 + \pm 1.61	10	10	11	
6 "	181 + \pm 8.44	17 + \pm 1.02	188 + \pm 8.47	16 + \pm 1.90	11	11	12	
12 "	205 + \pm 13.91	19 + \pm 2.04	195 + \pm 16.01	18 + \pm 1.02	11	11	11	
24 "	221 + \pm 10.45	19 + \pm 2.51	213 + \pm 11.95	20 + \pm 3.24	12	12	11	
36 "	223 + \pm 7.46	18 + \pm 2.49	214 + \pm 5.90	21 + \pm 1.90	12	12	10	
7 days	227 + \pm 9.88	20 + \pm 2.79	233 + \pm 9.48	23 + \pm 1.90	11	11	10	
15 "	240 + \pm 15.34	30 + \pm 6.79	271 + \pm 23.90	41 + \pm 9.16	8	8	7	
30 "	291 + \pm 24.77	43 + \pm 7.67	310 + \pm 26.25	48 + \pm 4.08	7	7	6	
60 "	344 + \pm 32.15	45 + \pm 5.02	406 + \pm 10.45	49 + \pm 4.10	8	8	8	
90 "	443 + \pm 11.95	47 + \pm 8.20	455 + \pm 37.01	58 + \pm 6.14	9	9	8	

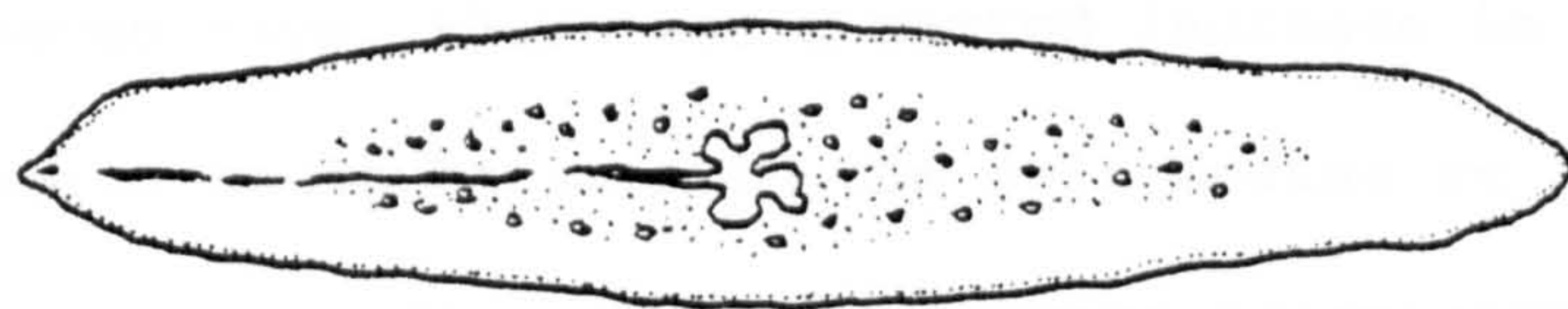
Morphological development of worms in the fish host

Worms were examined for morphological changes with respect to major organs developed and this development is shown diagrammatically in Figure 4.8.

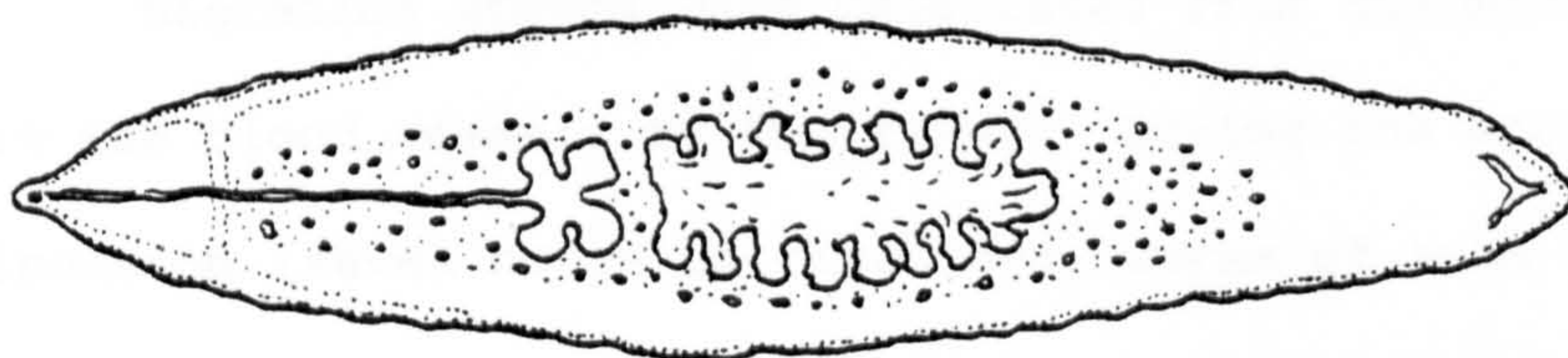
The gut caeca were first observed at seven hours and were situated in the mid body region. As the development progressed the gut caeca^c gradually came to occupy the anterior third of the worm as gonadal development took place in the posterior part of the body. In the 30-day-old worm the length of the oesophagus was about half the length of the body, whereas in most 60-day-old worms and in all 90-day-old worms, this length was about one-third of the body length.

No reproductive organs were observed in the 30-day-old sample. Development of the testes was first observed at 60-day sample. However, there was no trace of ovary. Development of the ovary occurred sometime after 60 days. Thus, the 90-day-old worms were found to have complete gonadal development taking the form of mature worm as shown in Figure 4.8. Though the reproductive system of 90-day-old worms was fully developed, no uterine eggs were seen. Uterine eggs were seen occasionally in the 120-day-old worms. However, it is probable that some of the worms may become sexually mature as early as 79 days as eggs were found lying freely in the skin and gill squash. Therefore maturity was achieved between 60 and 90 days.

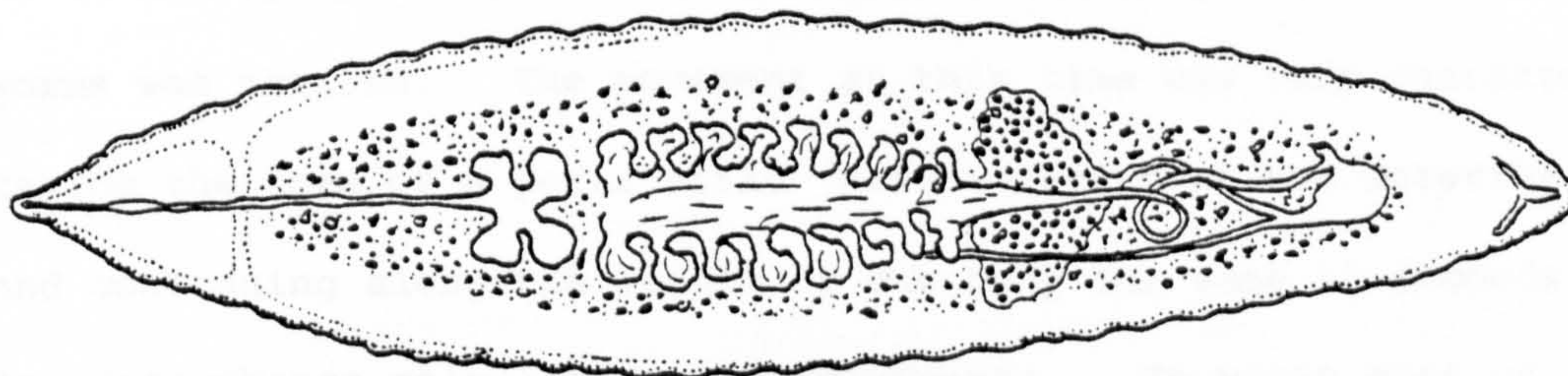
The crenulated margin and the 'setae-like' structures were first observed at 60-day-sample. These features were retained throughout the experimental period, i.e. up to 210 days.



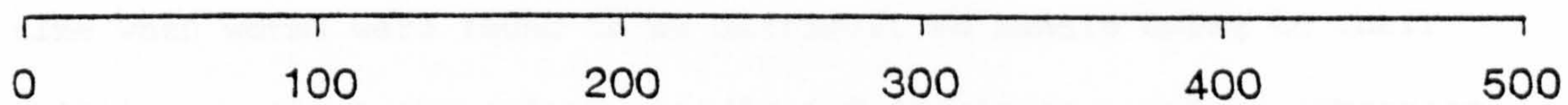
30 DAYS



60 DAYS



90 DAYS



LENGTH (Microns)

Fig. 4.8 S. inermis in experimentally infected C. carpio.

Behaviour and activity of worms

During the first 36 hours worms were found to be sluggish. Their movements were mainly confined to the proboscis region but, after seven days, there was a marked increase in activity. At this time worms could be seen in the skin moving in all directions, but when quiescent, fin worms were always orientated towards the anterior of the fish. The movement was characterized by the expansion of the anterior half and then withdrawal of the rest of the body, thus body advanced forward.

Migration speeds were calculated from the movements of the worms in the blood vessels of the fins. During the early stage of invasion (seven hours to five days) worms of such location were found to move with a speed of 0.15 mm per hour, i.e. they travel more than half of their body length/hour.

Thirty days onward worms showed greater elasticity and were capable of considerable changes of shape when observed in vitro. At 70 to 90 days post infection, a vigorous swimming activity of the worms was noticed. The movement at this time was very characteristic, taking the form of a peristaltic wave starting at the anterior tip and continuing along the length of the body for some 15 seconds. Any disturbance stimulated these movements. In vitro most of the worms were found to float dorsally. Occasionally worms crawled on the smooth surface by creating peristaltic movements. This was the time when worms were found to be difficult to handle owing to their ability to attach themselves rapidly and firmly to a smooth substrate, e.g. the bottom of a petridish so that they were difficult to detach. At 90 days and after, the worms showed a decrease in activity in vitro and old senile worms showed little or no activity.

Worms collected from the skin, generally were more active than gill/heart worms. Skin worms also showed a greater ability to survive in vitro. Worms of 90-day-old and after appeared to lose their ability to survive in vitro.

4.3.3 Discussion

It appears from the observation of cercarial emergence that the majority of them emerged during late afternoon. Other sanguinicolid cercariae which have been shown to emerge during late afternoon or at night are Cercaria kentensis (Khan, 1961), cercariae of S. alseae (Meade and Pratt, 1965) and cercariae of S. idahoensis (Schell, 1974). Some cercariae prefer to emerge in the early morning, e.g. S. klamathensis (Meade, 1967). Yet other cercariae emerge at various times of the day, e.g. cercariae of S. lophophora (Erickson and Wallace, 1959). However, the variation in the time of cercarial emergence from the snail host could be related to various biotic and abiotic factors of a particular water body which enhance the emergence. Water temperature, presence or absence of the potential host and their densities must have an influence on the pattern of cercarial emergence. Very few cercariae appear to emerge from molluscs in water below 12-15°C (Erasmus, 1972) although Meade and Pratt (1965) reported that the emergence of cercariae of S. alseae occurred in cold temperature at 12.8°C. In the present study, cercarial output decreased in temperatures below 15°C and above 20°C. Naumova (1961b) observed S. inermis cercarial release at 12°C or above but did not specify the temperature for optimal cercarial emergence.

Cercariae of Sanguinicola seem to be stimulated into swimming activity by any disturbance in the water as found in this study (Erickson and Wallace, 1959; Meade, 1967; and Schell, 1974). In

common with S. inermis Erickson and Wallace (1959) also found that the release of cercariae was enhanced simply by a change of water.

The resting position, with the furcae uppermost and the body and tail-stem bent into a 'U' shape seems to be a characteristic of the sanguinicolid cercariae since they were noted for S. lophophora (Erickson and Wallace, 1959), Cercaria kentensis (Khan, 1961), S. klamathensis (Meade, 1967) and S. idahoensis (Schell, 1974).

The longevity of cercariae is limited to a maximum of 24 hours at 12°C. However Naumova (1961b) found that the cercariae of the same species survived for 48 hours at 13°C and 22-29 hours at 20°C.

The experimental study has shown that penetration by the cercariae of S. inermis occurred through the body skin, gill and fins of the fish but marked local concentration occurred, namely in the region of the fins. This localization of penetration was evident at a very early stage of infection. Site preference for penetration has been shown to occur in other cercarial species invading the fish. For example, Erasmus (1959) studied the distribution and penetration of the Cercariae X Baylis and showed a marked concentration of the cercariae in the head region of the three-spined-stickleback (Gasterosteus aculeatus). A similar pattern was described by Betterton (1974) for the cercariae of Diplostomum spathaceum in brown and rainbow trout. Sommerville (1982) also showed a similar concentration on the fins of rainbow trout after an experimental infection of the cercariae of Haplorchis pumilio. The reason why the majority of cercariae preferred the fins, particularly the caudal fin, as a route of entry is not clear. This preference could possibly be related to the relative ease of penetration of the soft tissues between the fin rays but more likely because of the proximity of the

blood vessels to the surface in these areas.

From the few observations on the route of entry made by other authors it appears that other sanguinicolids prefer the soft areas of the body, particularly the fins. Wales (1958) found that the cercariae of S. davisii entered through the tissues of the fins of rainbow trout fingerlings but did not clarify which fins were preferred. Evans and Heckmann (1973) assumed from the histological findings of their experimental infection that the cercariae of S. klamathensis penetrated the pectoral and pelvic fins of cutthroat trout (Salmo clarki). S. lophophora also penetrated through the fins of Notropis hudsonius (Erickson and Wallace, 1959). None of these authors (Wales, 1958; Erickson and Wallace, 1959; Evans & Heckmann, 1973) however, indicated the cercarial selection for fin.

As with the fins, the body skin was also found to be an important site for cercarial invasion as reported by Meade and Pratt, 1965; Meade, 1967; Schell, 1974 and K oie, 1982. Meade and Pratt (1965) found that cercariae of S. alseae were only successful in penetrating the ventral surfaces of the trout fingerlings and were unsuccessful elsewhere.

As a site of penetration gills appeared to be the least preferred area for the cercariae of this study. Contrary to this, Scheuring (1922) in his study of S. inermis noted that cercariae attached themselves to carp gills and penetrated to the blood vessels. Looss's experiments with S. inermis (reported by Odhner, 1911, 1924) noted the presence of young worms on the mucous membrane of the buccal cavity and on the gills of carp and goldfish kept with infected Lymnaea auricularia. Layman (1957) and Naumova (1961b) claimed that

the gills of carp were a preferred site for invasion by S. inermis cercariae. However, none of these studies were quantitative and the time factor was not known.

Size of the fish may influence the site selection of the penetrating cercariae. In smaller fish the area covered by the fins is much greater than the total surface area of the whole fish. It is generally accepted that cercariae contact the host by chance (Erasmus, 1972; Betterton, 1974), therefore the chance of the fins of a smaller fish being a site of entry is much greater than the bigger fish. The size of fish can also influence the success rate of penetrating cercariae. For example, Schell (1974) observed that the cercariae of S. idahoensis were only able to penetrate the steelhead trout fingerlings of 1½ to 1¾ inches long but were unable to penetrate the fish size of 2½ to 3 inches. The fish used in the present experiment were small (3.5 - 6.0 cm in length) and therefore the small variation in size did not affect the success of penetration.

Little is known about the method of penetration of sanguinicolid cercariae. Host locating mechanisms employed by cercariae are surrounded by controversy but it is generally accepted that cercariae contact the host by chance. The present study has shown that only after coming into contact with the host surface does the cercaria attach itself and this is achieved by the muscular adhesion of the anterior region. No evidence was observed of directed attack but attachment was initiated as soon as contact was made. Contrary to this observation, Layman (1957) noted that cercariae of S. inermis swam toward the carp and, with a stylet on the front end of the body, they pierced the epithelium of the gills.

Immediately after attachment, the cercaria showed vigorous activity by the extension and contraction of the whole body. This type of vigorous activity obviously indicated the active penetration of the fish tissues. It is interesting to note here that, similar to schistosomes (Stirewalt and Kruidenier, 1961; Matthews, 1977) and some strigeids (Erasmus, 1972) the cercarial tail did not play any role in penetration and was found to be cast off before penetration. Active penetration was also reported by Meade (1967) when S. klamathensis cercariae were found to push their apical papillae against the host tissue. However, in this case, the tail seemed to play an important role. The discrepancies that exist with regard to the role of the cercarial tail during the penetration process could possibly be due to the differences in species, since behaviour of cercaria of Schistosoma mansoni was found to vary with different mammalian skin (Stirewalt and Hackey, 1957).

The penetration of S. inermis cercariae into the fish host appeared to be very rapid; only 30 minutes on average for the majority to enter a potential host. This seems to be similar to those findings for other sanguinicolids. For example, Wales (1958) reported that the cercariae of S. davisii entered the fish-host Salmo clarki within a few minutes of their attachment. Meade and Pratt (1965) described the cercarial penetration of the skin of S. clarki fingerlings by S. alseae which took 15-20 minutes. Similarly Meade (1967) found the cercariae of S. klamathensis under the skin of Salmo clarki fingerlings within 15 minutes of their attachment. Schell's (1974) experimentation on the penetration process of the cercariae of S. idahoensis revealed that the cercariae required 10-20 minutes to enter Salmo gairdneri fingerlings. Penetration amongst the

Sanguinicola species is therefore vary rapid but is by no means as rapid as some other cercarial species as some reports indicate. For example, the Cercaria flexicauda accomplished penetration in 7-8 minutes (Ferguson, 1943) whilst a remarkably rapid penetration was found for the cercariae of Exorchis oviformis where entry into the fish took about 3-5 minutes (Erasmus, 1972).

The rapid penetration of the cercariae is, certainly, an adaptation to their parasitic existence. The ability of free-swimming cercariae to attach to a host and penetrate is very susceptible to the influence of the external environment such as water current and movement of fish. The energy that is stored by the non-feeding cercariae is very limited, thus, rapid location and penetration of the host is essential for their survival. In the natural situation, where host-parasite interactions are more complex, one could assume that a rapid penetration rate would result in a higher success rate. If the infection rate in the mollusc was as low as is indicated by the few observations made here, i.e. seven out of 3,260, then there must exist a very efficient infection mechanism to account for the high incidence and intensity of the parasite in the fish population in the affected pond.

Migration of cercariae in fish has been described by several authors, but only a few studies were based upon experimentation. Results obtained from dissection of fixed specimens and, in some cases, histological findings, confirmed that S. inermis cercariae used loose connective tissue as a migratory route as well as the circulatory system. Sommerville (1981 and 1982) has shown that the cercariae of Haplorchis pumilio and Stephanochasmus baccatus use the loose connective tissue as a route of migration to their fish host. However, some

controversy exists on the route used by digenean cercariae to reach the preferred locus. In studies on strigeid cercariae, Ferguson (1943) and Johnson (1971) considered the blood stream to be the prime migratory route whereas Erasmus (1959) claimed connective tissue as a prime one. Early observations by Blochmann (1910), Szidat (1924), Dubois (1929), Wesenburg-Lund (1934) as cited by Erasmus (1972) suggested that the blood stream forms the main route for cercarial migration. Weather and Wilson (1979) have shown that schistosomula leave the skin mainly via the blood vessels. As migration into the dermis took place, increasing numbers of schistosomula were observed closely applied to venules. In the case of S. inermis which live primarily in the major blood-vessels of gills and heart, as do schistosomes, a circulatory route would seem the most direct. Several arguments can be put forward in favour of the blood stream route, e.g. the non-random nature of migration (Ferguson, 1943; Erasmus, 1959; Johnson, 1971). The involvement of the circulatory system would provide passage to the anterior region where the heart and other large vessels are situated. The circulatory system as a migratory route has also been reported for schistosomes (Stirewalt, 1959) and other tissue penetrating helminths, e.g. hookworms (Erasmus, 1972). The route of migration of other sanguinicolid cercariae has not been adequately studied but, as most of the cercariae were reported to penetrate through the fins, a circulatory route would probably serve as the most direct route of migration.

The studies of Ferguson (1943), Erasmus (1959), Johnson (1971), Weather and Wilson (1979) and Sommerville (1981 and 1982) all have shown that cercariae, after penetrating the host and migrating towards their preferred locus remain in this site undergoing further develop-

ment. The cercariae of S. inermis as shown in the present study were able to migrate up to 210 days post penetration. However, some of the worms were able to remain in the skin and to continue to develop there. This ability to continue to develop in the skin without further migration possibly indicates the presence of specific stimuli in the skin which allow development. However, why some of the worms migrate and some do not is not known but may be due to the presence/absence of correct stimuli or immunological response (Lewert, 1958; Betterton, 1974).

There is a suggestion of a relationship between exposure density and penetration of cercariae in to the host. Up to a certain exposure density, the relationship appeared to be linear, but a departure from linearity occurred at higher exposure densities (365/fish). Brassard and Curtis (1982) have shown similar relationships for the cercariae of Diplostomum spathaceum where linearity deviated with an exposure density of 250 cercariae per fish. The significant decrease in the proportion of penetrating cercariae which established themselves in the skin and gill/heart at a high exposure density, suggests that many cercariae never reach their destination. The mortality may result from an immunological response by the host resulting in cercaricidal agents or as a result of other inflammatory process (Lewert, 1958).

It is possible that cercariae did not penetrate the other experimental host Salmo gairdneri and Oreochromis niloticus due to lack of the necessary stimuli in the mucus or skin. There are also other factors responsible for the success of penetration of cercariae as well as the absence of appropriate stimuli such as the thickness and viscosity of the mucus layer, the arrangement of scales and the

toughness of the fibrous layer beneath the scales. If penetration was achieved in an unsusceptible host, the cercariae might subsequently be killed by an inflammatory response.

S. inermis worms do not possess any recognisable metacercarial stage in their life-cycle. Penetrated cercariae were observed to develop directly into adult worms.

The present investigation revealed a very interesting feature of Sanguinicola infections. It has been generally assumed by previous workers that S. inermis cercariae on penetrating the fish undergo migration to the major blood-vessels of the heart and gills since these were the sites where they were always found. With the exception of S. idahoensis (Schell, 1974) no sanguinicolids have been described from the skin. It was also believed that worms developed to maturity in the major blood-vessels of the heart and gills. This study has shown that the worm may remain in the skin and continue to develop to maturity, even producing eggs in the skin, a previously unreported feature of Sanguinicola infection. Kjøie (1982) found that the cercariae of Aporocotyle simplex penetrated the skin of fish and developed into mature worms in the lymphatic system as well as the circulatory system of Limanda limanda, Pleuronectes platessa and Platichthys flesus.

It is not understood why the worms in this case remained in the skin. Study of the similar outbreaks in the Gloucestershire farm (Chapter 4.4) showed no skin worms. It may be that the fish from the Calverton farm (Chapter 4.4) were observed at an early stage before the migration took place. Whereas those at the Gloucestershire farm were at a later stage of the infection. However, the initial burden of the cercariae may also have some influence on migration.

During the early stage of development the growth of the worms was very rapid. For example, worms doubled their length within the first 36 hours after penetration. This rapid increase in length was possibly due to the sudden absorption of nutrients or changes in the tegument resulting in a change in osmotic balance. The purpose of the rapid increase in size may also be to assist in anchorage of the worms in blood vessels, as S. inermis unlike some other sanguinicolids has no spines.

Soon after penetration, the body fin-fold of the cercaria of S. inermis was observed to shrink and disappeared within seven hours. A similar rapid disappearance of the body fin-fold was noted by Schell (1974) for the cercariae of S. idahoensis where it was absorbed completely within 4-5 hours of penetration. The fin-fold assists the cercaria to float in water until it comes into contact with a potential host. But, after penetration the rapid disappearance of the fin-fold is necessary to facilitate the migration of the cercaria to the preferred locus.

The maturation of the worms inhabiting the two localities occurred at the same rate, i.e. the onset of egg production occurred simultaneously in both skin and gill/heart worms at 79 days. The developmental features of worms inhabiting two locations was also observed by Kjøie (1982) for Aporocotyle simplex who found that both the worms, collected from lymphatic and blood system, at 180 day post infection had uterine eggs.

Very little is known about the maturation rate of sanguinicolid worms in relation to temperature.

In general, the various developmental stages of S. inermis have been reported as being active in the warmer months of the year. There seems to be a general agreement that the development is facilitated by warm water temperature (Scheuring, 1923; Chechina, 1959; Lucký, 1964 and Naumova, 1961b). All the outbreaks investigated during this study also occurred during the summer months and it is thus likely that warm temperatures are required for development of certain stages. However, incidental observations of worms in fish kept at either 15°C or 18°C showed that maturity, i.e. egg production was achieved at the same time, i.e. 2½ months indicating that temperature might not be so important to development once infection of fish has been achieved. Certainly egg production rate is independent of ambient temperature. Egg production of the worm continued throughout the year but a peak appeared in the months of July-September suggesting a cycle of events of development and maturation which is independent of ambient temperature. This is further supported by the evidence reported in section 4.4. However, this very interesting aspect of biology of S. inermis is requiring further study.

4.4 Dynamics of fish infections

4.4.1 Materials and methods

Farm 'A': Batches of C. carpio were collected from farm 'A' (details in Chapter 2) for parasitological examinations. They were kept in system 'B' (details in Chapter 2). A constant temperature ($18^{\circ}\text{C} \pm 2^{\circ}\text{C}$) was maintained throughout the experimental period. Fish were fed with commercial trout pellet No.2 once a day. Sampled fish were from a single spawn and original infection occurred in the month of July, 1979. The infections were recognised by the presence of developmental stages as well as by the presence of adult worms in major blood vessels of gills and heart.

Farm 'B': A total of 50 carp (mean length 5.98 cm) were dissected for parasitological examinations over a period of 12 months starting from October 1981 up to September, 1982. Sampled fish were from a single spawn and original infection was estimated to occur during the month of August 1982 when the fry were released to the earthed pond of the farm containing infected snails. These are the fish of the group 'c' (Chapter 2) which were kept in the system 'B' (Chapter 2) at a temperature $18^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The infections were recognised by the same procedure as described for farm 'A' i.e. by the presence of any developmental stages as well as by the presence of adult worms in the dissected fish.

4.4.2 Results

Farm 'A': A total of 222 fish were dissected over a period of 24 months from December 1979 to December, 1981. The prevalence and intensity of infection of S. inermis in each monthly sample of fish is shown in Table 4.5. The overall prevalence of infection from December 1979 to December 1981 was found to be high. During this period a mean of 74% of the fish as indicated in Figure 4.9 were infected. Despite the constant conditions in the aquarium the incidence of infection varied and was found to decrease with age of infection. Figure 4.9 shows that this decline was not apparent until September, 1980. The level of infection then decreased gradually until June 1981 when the decline was more rapid. This was approximately two years after the onset of infection. The overall drop in prevalence was 60% by the end of October 1981 and 83% by the end of 1981 as no further infection took place after the original exposure in July, 1978.

Seasonal variation in the intensity of infection was found to occur throughout the experimental period. Worms were not always recovered from infected fish. Figure 4.9 shows the mean number of worms recovered per fish during the 24 months of observation. Only mature worms were found in the heart, gills and major blood vessels. The maximum intensity of infection occurred at the end of 1980 when the age of infection was approximately 16 months. The mean worm burden had increased slowly to November 1980, probably as a result of migration of maturing worms to these sites. The maximum number of worms per fish was 53. However, this number was elevated owing to one very heavily infected fish in the November sample when a total of 193 worms were recovered from the major blood vessels of the gills

Table 4.5 The prevalence, intensity of infestation and parasite index of *S. inermis* infected *C. carpio* from farm 'A'

Month	Size of fish (mean length cm)	Number examined	Number infested	% Prevalence	Total no. of worms	Mean intensity ¹	Mean parasite index ²
1979 December	2.59	15	12	80	0	0	0
1980 January	2.78	5	3	60	0	0	0
February	3.10	5	3	60	4	1.33	0.8
March	2.42	5	4	80	0	0	0
April	2.69	10	8	80	3	0.38	0.3
May	2.72	10	10	100	4	0.40	0.4
June	3.21	10	8	80	12	1.50	1.2
July	3.62	10	8	80	20	2.5	2.0
August	3.35	40	36	90	127	3.53	3.18
September	3.05	10	10	100	40	4.0	4.0
October	3.83	6	5	83.3	17	3.4	2.83
November	4.02	5	4	80	213	53.25	42.6
December	4.05	10	9	90	2	0.22	0.2
1981 January	4.00	5	4	80	0	0	0
February	3.80	5	3	60	0	0	0
March	4.62	5	4	80	0	0	0
April	5.32	5	3	60	3	1	0.6
May	5.12	20	12	60	15	1.25	0.75
June	5.20	8	6	75	0	0	0
July	5.47	6	4	66.7	0	0	0
August	6.38	6	3	50	0	0	0
September	6.50	5	2	40	0	0	0
October	7.02	5	1	20	0	0	0
November	7.66	5	1	20	0	0	0
December	7.78	6	1	16.7	0	0	0

¹ Mean intensity = $\frac{\text{Total no. of parasites found}}{\text{Total no. of fish infected}}$

² Mean parasite index = $\frac{\text{Total no. of parasites found}}{\text{Total no. of fish examined}}$

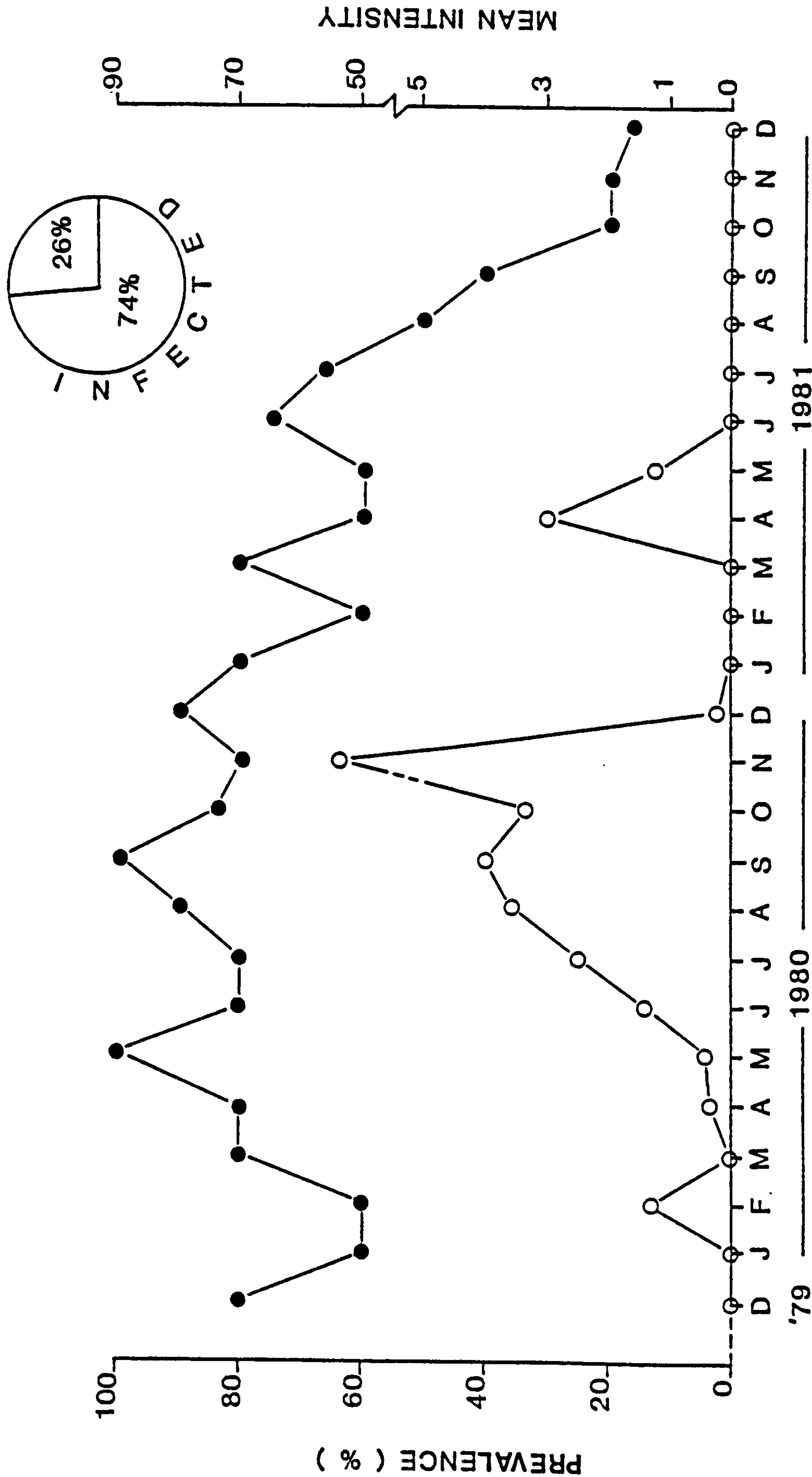


Fig. 4.9 Seasonal variation in prevalence and intensity of *S. inermis* infection found in *C. carpio* from farm 'A' during the period of 1979-1981.

Percent prevalence (●); Mean intensity (○).

N.B. Worms estimated to be 5 months old at start of study.

and from the heart. If this extreme case is excluded from the mean, the intensity at this time was seven worms per fish.

Spatial distribution

The main habitat of the matured worm was found to be the bulbus arteriosus. The majority of the worms (over 50%) were recovered from this site and are shown in Figure 4.10. As an habitat, gills played a secondary role where 33% of the totals were found to occur. The remaining 16% of the worms were recovered from the heart.

Seasonal distribution of worms

Figure 4.10 shows the seasonal distribution of worms over the period of January 1980 to May 1981. In general during the first half of the year (February - July) large numbers of worms were recovered from the bulbus arteriosus but in the second half fewer were located in this site. The gill worms showed a reverse of this pattern during this time. A seasonal variation of the distribution of the worms is clearly illustrated in Figure 4.11. In the winter and spring the majority of the worms stayed in the bulbus arteriosus and in the heart. During summer and autumn the majority of the worms were located in the major blood vessels of the gills. The minimum number (7-10%) of worms occur in the heart in summer months. This apparently seasonal distribution occurred despite the constant experimental conditions of light, temperature etc.

Farm 'B': The prevalence and intensity of infection of S. inermis for each monthly sample of fish is shown in Table 4.6. The overall prevalence of infection from October 1981 up to September 1982 was found to be high. During this period of time a mean of 84% of the fish had infections of both active worm as well as other developmental stages of the parasite. Figure 4.12 shows the prevalence of the

Table 4.6 The prevalence, intensity of infestation and parasite index of S. inermis infected C. carpio from farm 'B'

Month	Size of fish (mean length cm)	Number examined	Number infested	% Prevalence	Total no. of worms	Mean intensity	Mean parasite index
1981 October	5.43	5	5	100	1134	226.8	226.8
November	5.42	5	5	100	473	94.6	94.6
December	5.54	5	5	100	301	60.2	60.2
1982 January	5.74	5	5	100	158	31.6	31.6
February	5.88	5	5	100	92	18.4	18.4
March	5.80	5	5	100	47	9.4	9.4
April	5.98	5	4	80	13	3.3	2.6
May	6.52	5	3	60	5	1.7	1.0
June	6.52	5	3	60	3	1.0	0.6
July	-*	-	-	-	-	-	-
August	-	-	-	-	-	-	-
September	6.98	5	2	40	0	0.0	0.0

* Negative sign indicates no data available.

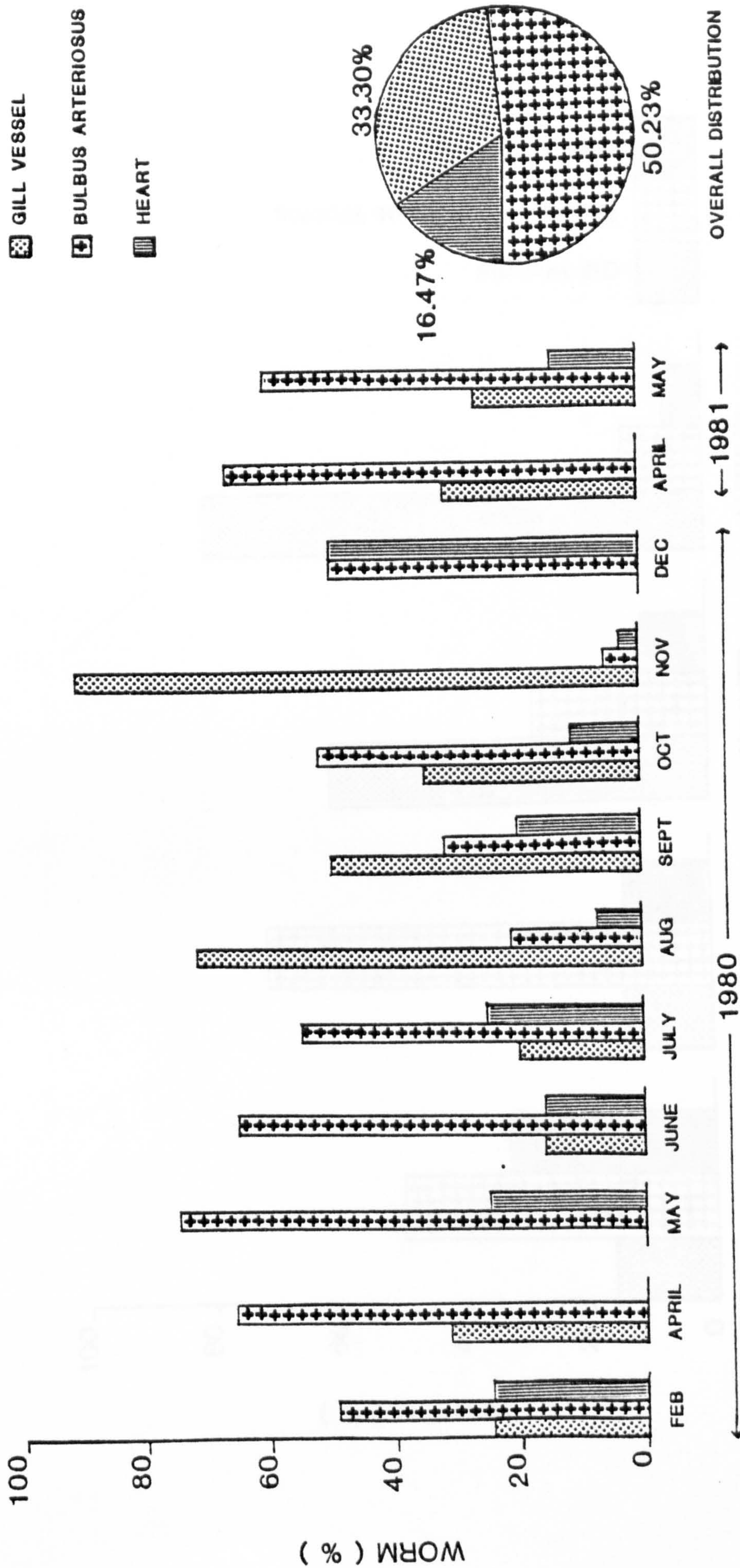


Fig. 4.10 Spatial distribution of *S. inermis* in *C. carpio* from farm 'A' during the period of 1980-1981.

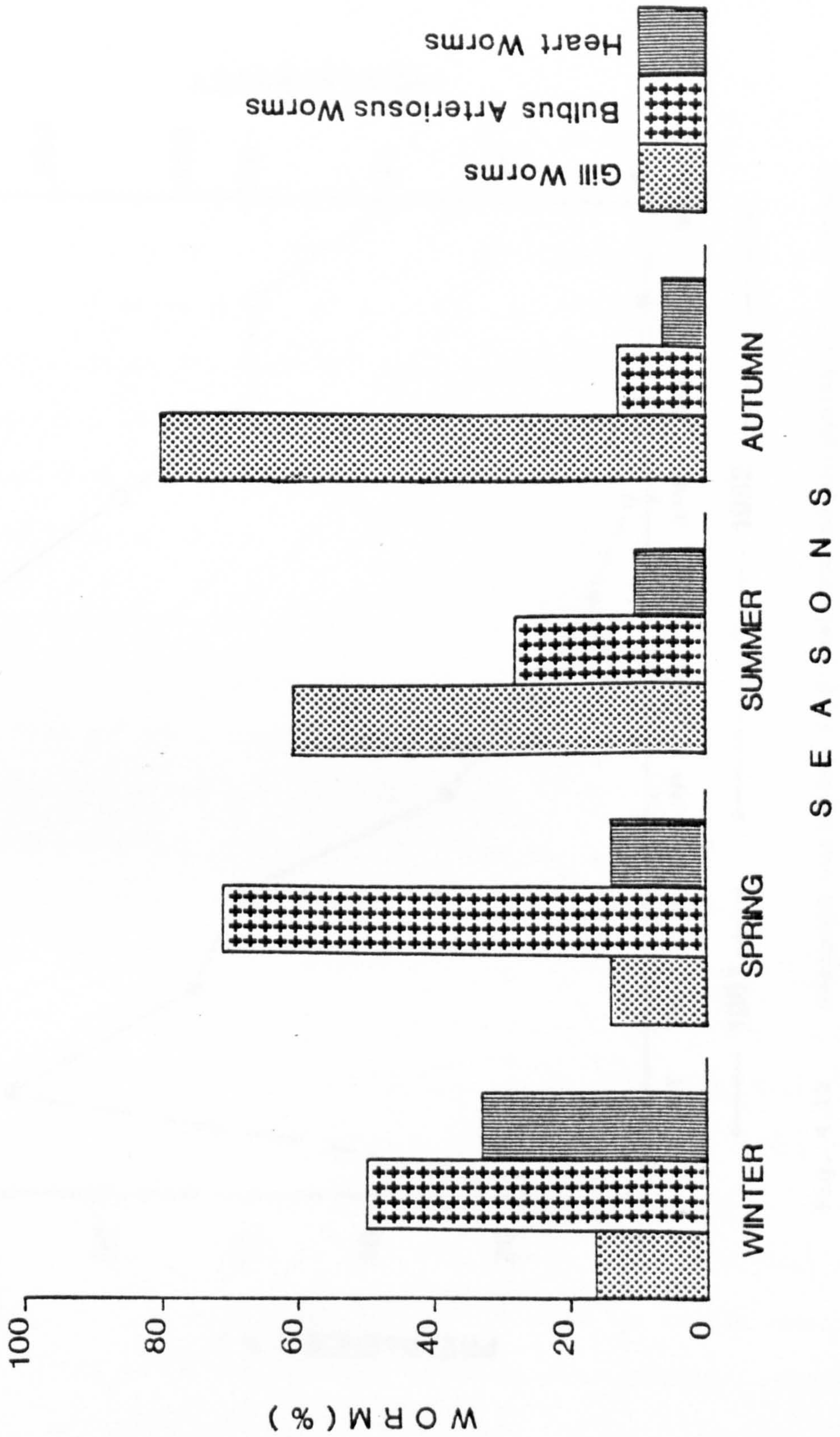


Fig. 4.11 Seasonal variation in the distribution of *S. inermis* in *C. carpio* from farm 'A' during the year 1980-1981.

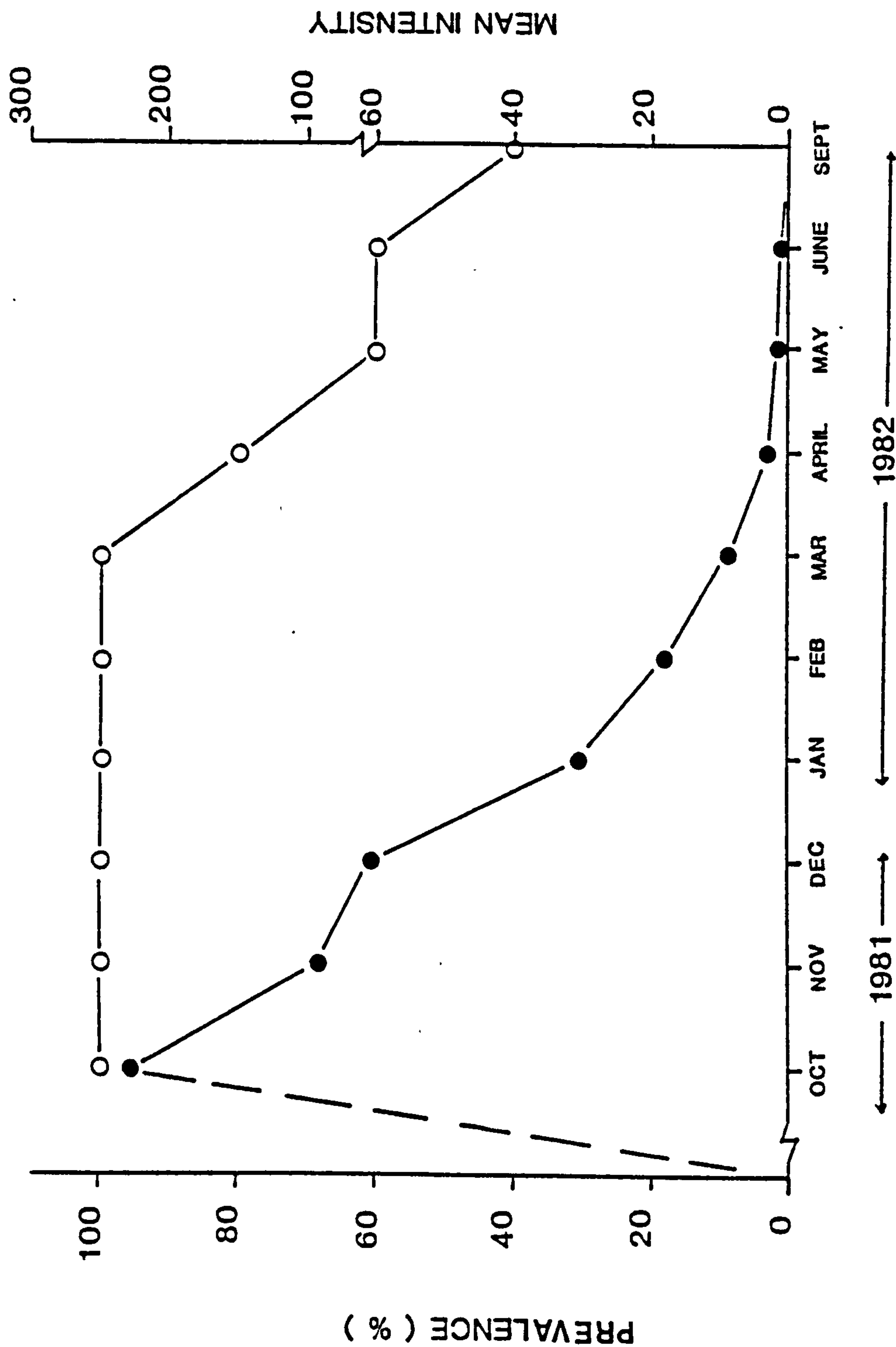


Fig. 4.12 Seasonal variation in prevalence and intensity of *S. inermis* infection in the *C. carpio* from farm 'B' during the period of 1981-1982. Percent prevalence (●); Mean intensity (○).

N.B. Worms estimated to be 2 months old at start of study.

infection being high (100%) up to March 1982, even though initial infection occurred seven months earlier. After March there was a decline in the percentage of infection which continued down to 40% at the 12 months sampling.

Figure 4.12 also indicates the high intensity of infection (over 200 worms/fish) which occurred at the time of the first sample, i.e. approximately 60 days after the original exposure to infection. However, monthly sampling showed a sharp decline in the intensity of infection during the first few months, followed by a gradual decline to the final sample in September 1982. At 12 months after the original exposure to infection no worms were present either in the skin or internal organs.

In order to examine the distribution of worms during this period, the location of mature and immature worms was recorded. Figure 4.13 shows the mean number of worms found in the skin and gill/heart locations during the experimental period. Initially the number of skin worms was very high, about six times the number in the gill/heart combined. However, the number of worms occupying the skin showed a sharp decline which began approximately 60 days after the initial infection. This decline coincided with an increase in the number of worms in the gill/heart and was assumed to be a result of migration of worm from the peripheral sites to the gill/heart. At this time there was also an overall reduction in the number of worms. The decline continued for both skin and gill/heart worms throughout the sampling period. After approximately 240 days the sample revealed no skin worms. Gill/heart worms appeared up to 300 days: thereafter no worms were recovered.

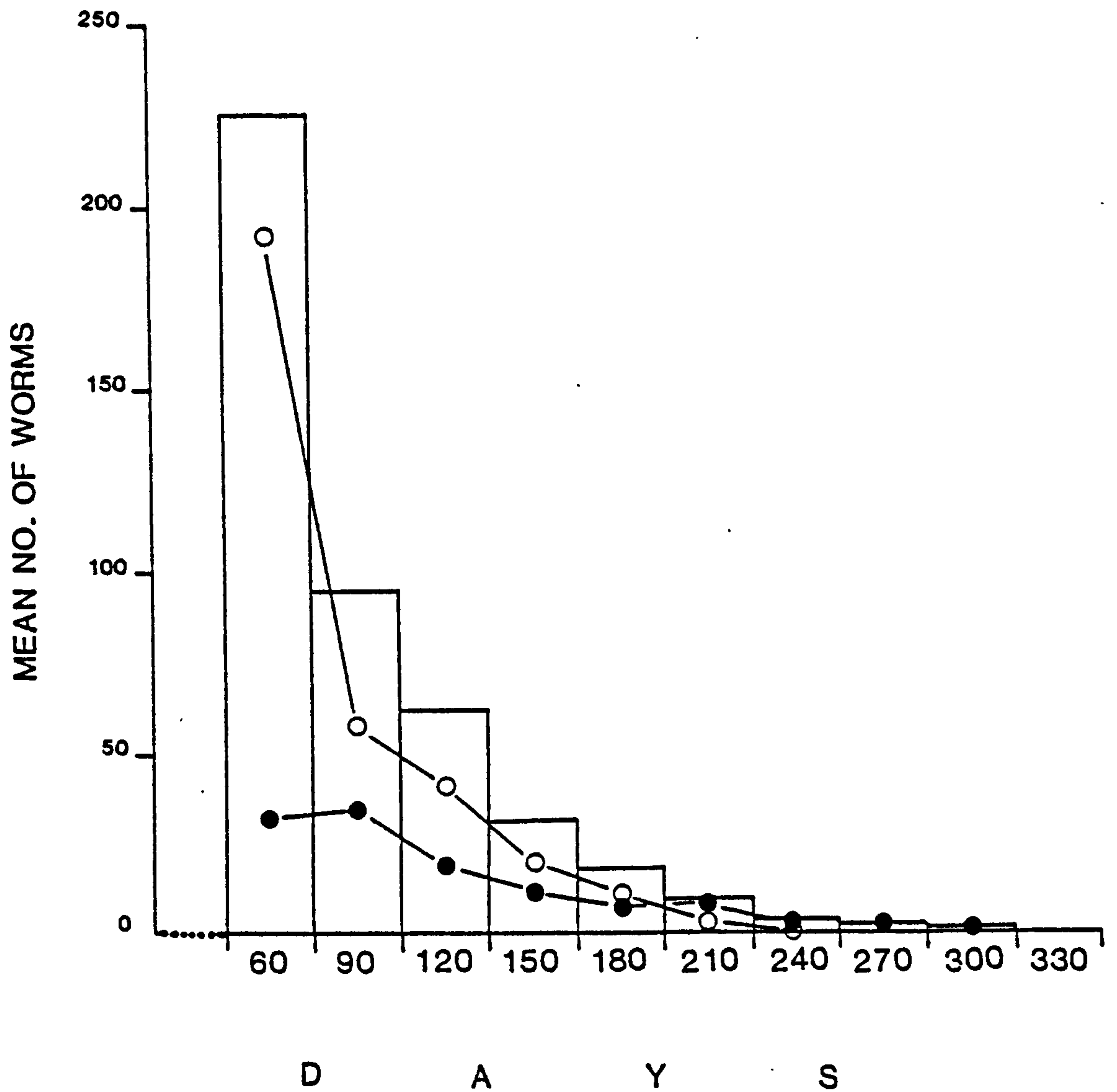


Fig. 4.13

Distribution pattern of skin and gill/heart *S. inermis* in *C. carpio* from farm 'B' up to an estimated 330 days post infection.

Skin worms (○); Gill/heart worms (●);
Total of skin and gill/heart worms (□).

Figure 4.14 illustrates details of the distribution of worms in the skin and gill/heart sites for each monthly sample. It is evident from the figure that during the first six months of infection, over 68% (62-85%) of the worms occupied the skin. There was always a small percent of worms located in the gill/heart region which remained relatively constant until the sixth month, thereafter the figure changes, and there is a greater occurrence of worms in the gill/heart region.

Egg production

Materials and methods

A minimum of five fish was sampled each month from all infected groups of fish and were dissected as described in Chapter 2. Egg production was calculated from squash preparations and by counting the eggs over a surface area of 22 × 22 mm under the low power of a light microscope. Organs included are gills, heart, kidneys, gall-bladder, liver, brain and the skin. In all cases random samples were taken.

Results

The production of eggs by the mature worms was found to be continuous throughout the year. Figure 4.15 shows the average egg production throughout 1980 to 1981. These are the means of the total collection of eggs from the skin, gill, heart, kidney, gall-bladder, liver and brain. At the beginning of 1980, i.e. approximately five months after the onset of infection, egg production was found to be small. The production increased from late spring and reached a peak during the months July - August. The pattern of egg production in the year 1980 was the same as 1981, despite the constant conditions of the aquaria and the absence of any new infections. The only

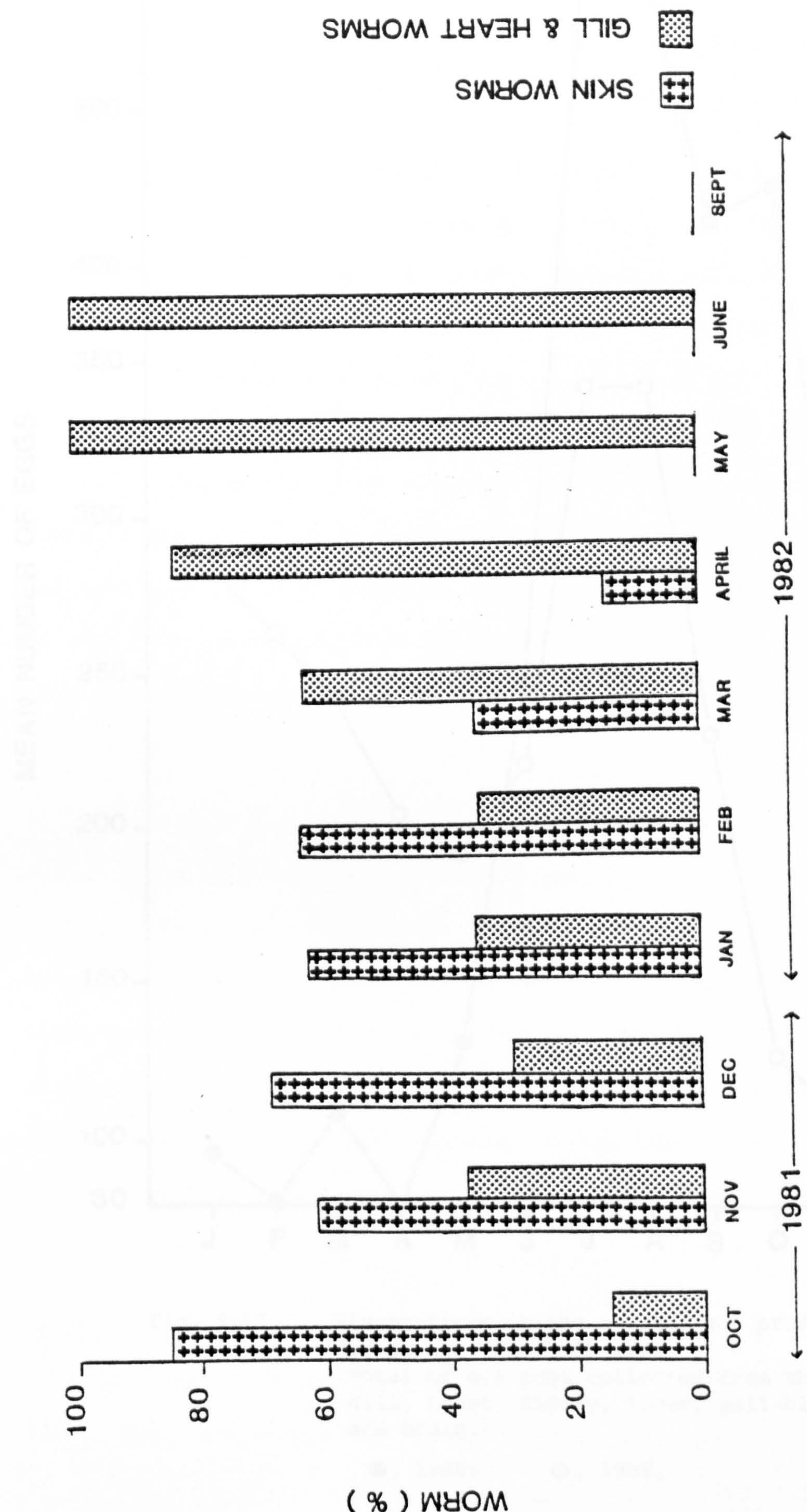


Fig. 4.14 Spatial distribution of *S. inermis* in *C. carpio* from farm 'B' during the period of 1981-1982.

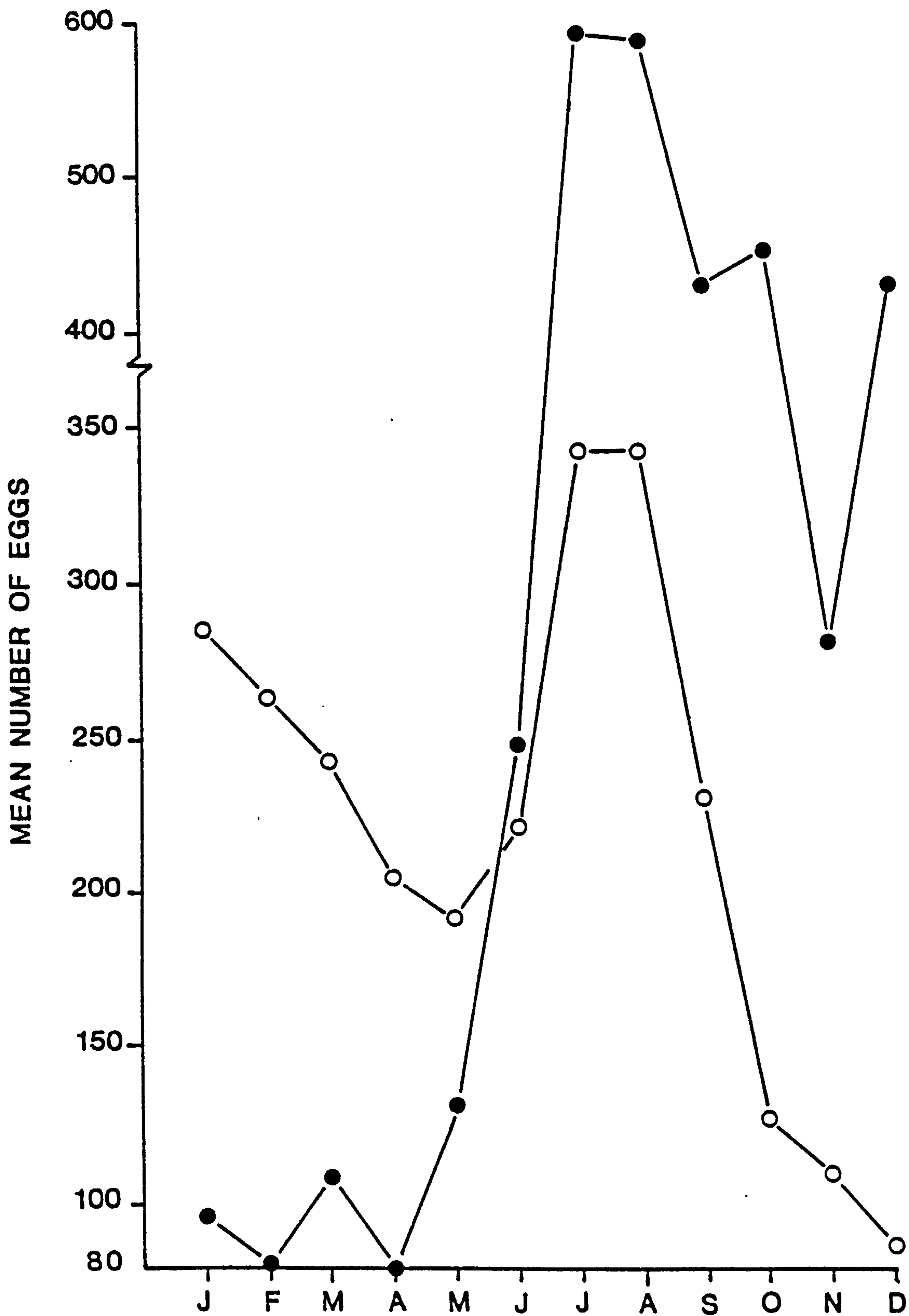


Fig. 4.15

Fluctuations in the overall egg production during the year 1980-1981. (Total of all eggs collected from the skin, gill, heart, kidney, liver, gall-bladder and brain.)

●, 1980; ○, 1981.

difference between the 1980 and 1981 observations was the actual level of egg production, i.e. the overall production during 1981 showed a decreased output compared to the production of 1980.

For a better understanding of the production of eggs and their subsequent stages, eggs were categorised into New Egg (EI), Developed Egg (EII), Pre-hatched Egg (MI), Hatched Miracidia (MII) and the Encapsulated Egg (EE). A quantitative study was performed by means of differential egg/larva counts over the period of 1980 - 1981. For this purpose the two major affected organs, gill and kidney were studied. Figure 4.16 shows the distribution of different stages found in the gill. It is interesting that the maximum number of all developmental stages appeared in July - September months. It is even more interesting that the pattern is similar for both years. Degenerated encapsulated eggs occurred in highest numbers than any other stage.

Figure 4.17 indicates the distribution pattern of different egg stages found in the kidneys. Here, a very similar pattern of distribution to that observed in the gills emerged. The maximum number of all actively developing stages, coupled with degenerate eggs, appeared in July - September months. Occurrence of all these stages appeared to be least in winter months, i.e. a period during January - March and October - December of the year. As in the gills, degenerated encapsulated eggs appeared in higher numbers than other stages.

Spatial distribution of eggs

Eggs were found to occur in gills, heart kidneys, gall-bladder, liver, brain and skin. The spatial distribution pattern of eggs

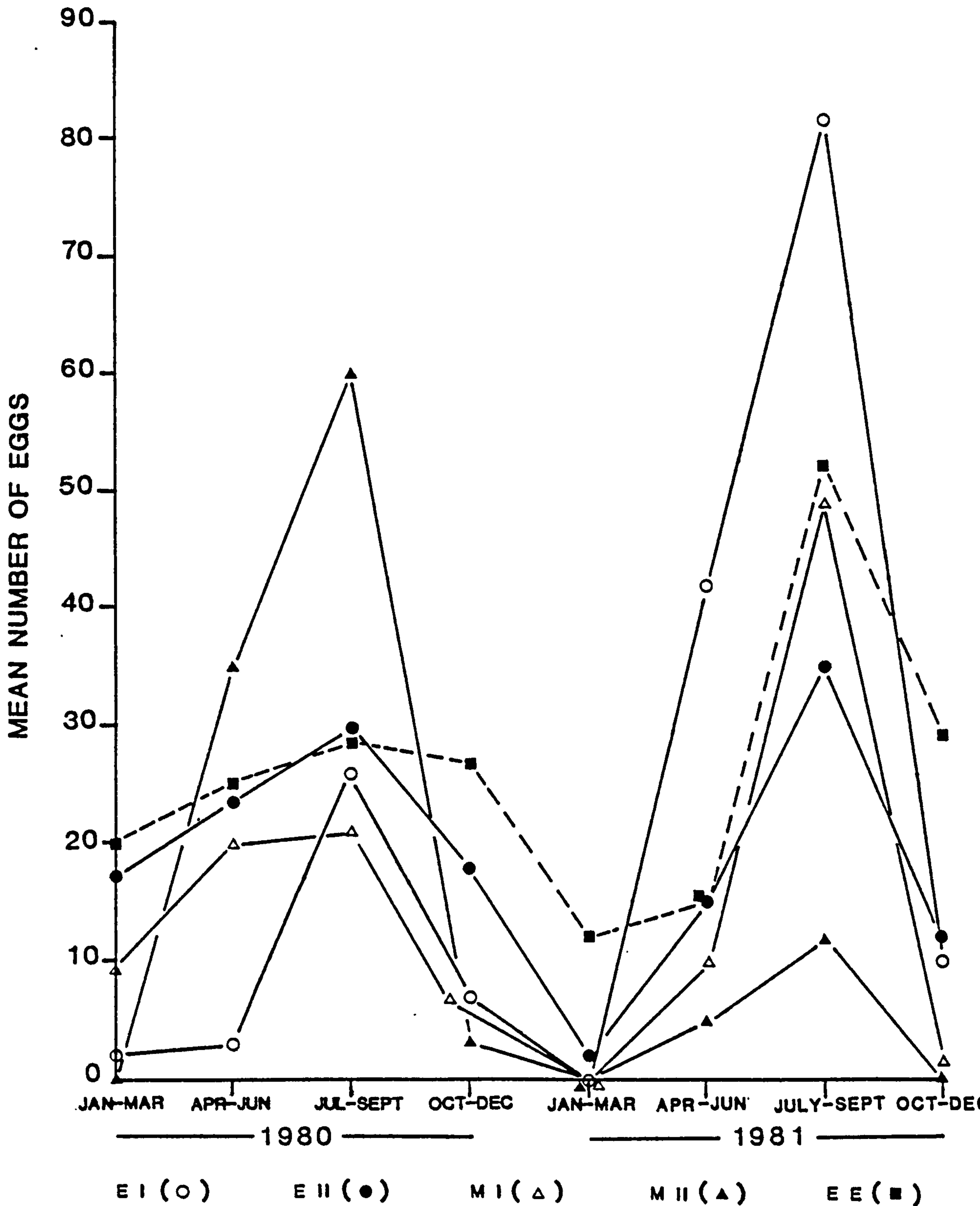


Fig. 4.16

Distribution of different developmental stages of eggs and miracidia (EI - EE) of *S. inermis* in the gills of infected *C. carpio* during the year 1980 and 1981.

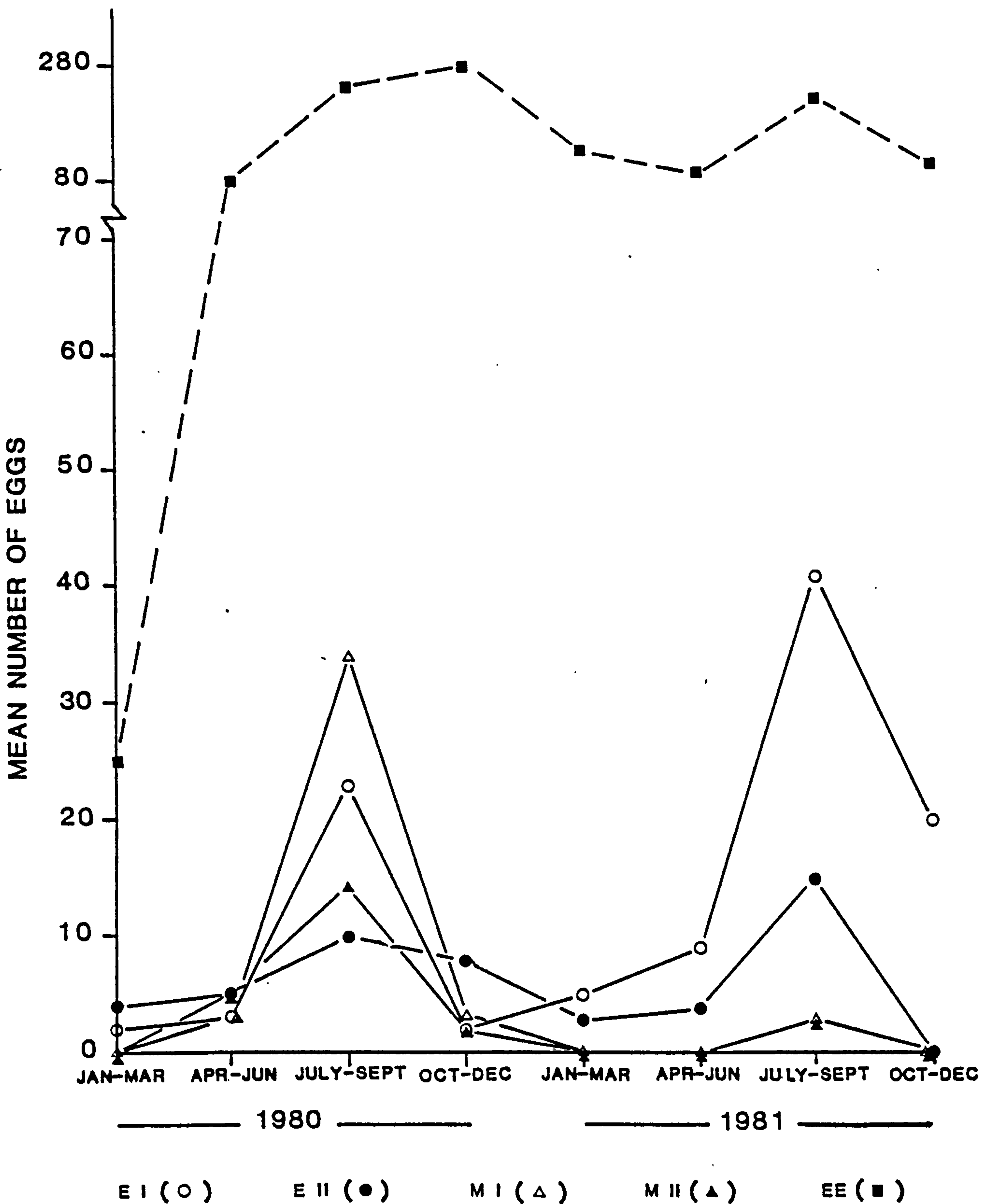


Fig. 4.17 Distribution of different developmental stages of eggs and miracidia (EI - EE) of *S. inermis* in the kidneys of infected *C. carpio* during the year 1980 and 1981.

recovered from these seven different organs of fish for the year 1980 are shown in Figure 4.18. It appears from this figure that the highest percentage of all stages throughout the year was found in the kidney. The eggs passed into the kidney throughout the year; old eggs degenerated eventually becoming encysted and at the same time new eggs were deposited. The highest number in gills were found in the summer months declining to a minimum of 17% during winter. Few eggs were ever encountered in the skin. A similar pattern of spatial distribution was also observed during the year 1981. Figure 4.19 clearly illustrates that the highest number of eggs occur in the kidneys with a single exception when the majority of eggs occurred in gills during the July of the year. No skin worms were located during this period.

Longevity of worms

In general, the life-span of the worms in the fish was found to be much greater than expected. Three different patterns of longevity were evident from farm 'A', 'B' and from the experimental infections. Worms in fish collected from farm 'A' and kept in the aquarium at a constant temperature of $18^{\circ} \pm 2^{\circ}\text{C}$ and a light regime with a 12 hours periodicity, were found to survive up to 24 months after the estimated time of the initial infection. No further infection could have taken place as care was taken to ensure no intermediate hosts were present. The survival of worms collected from fish in farm 'B' and kept in the same conditions was much lower. In this case, worms survived only up to 10 months after the estimated time of infection. However, the longevity of the worms was lowest in the experimentally infected fish where worms survived only up to seven months after exposure to infection despite the same environ-

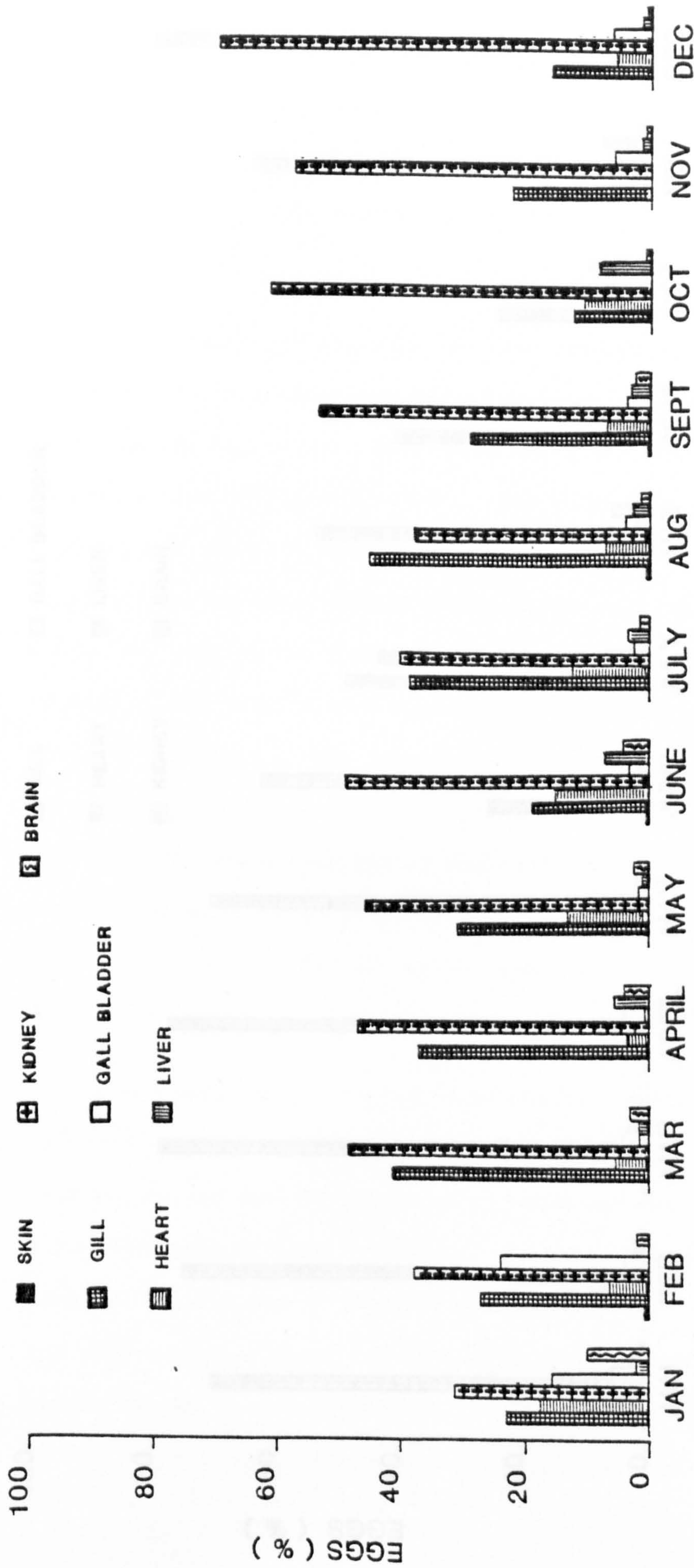


Fig. 4.18 Spatial distribution of eggs of *S. inermis* in 7 different locations in *C. carpio* from farm 'A' during the year 1980.

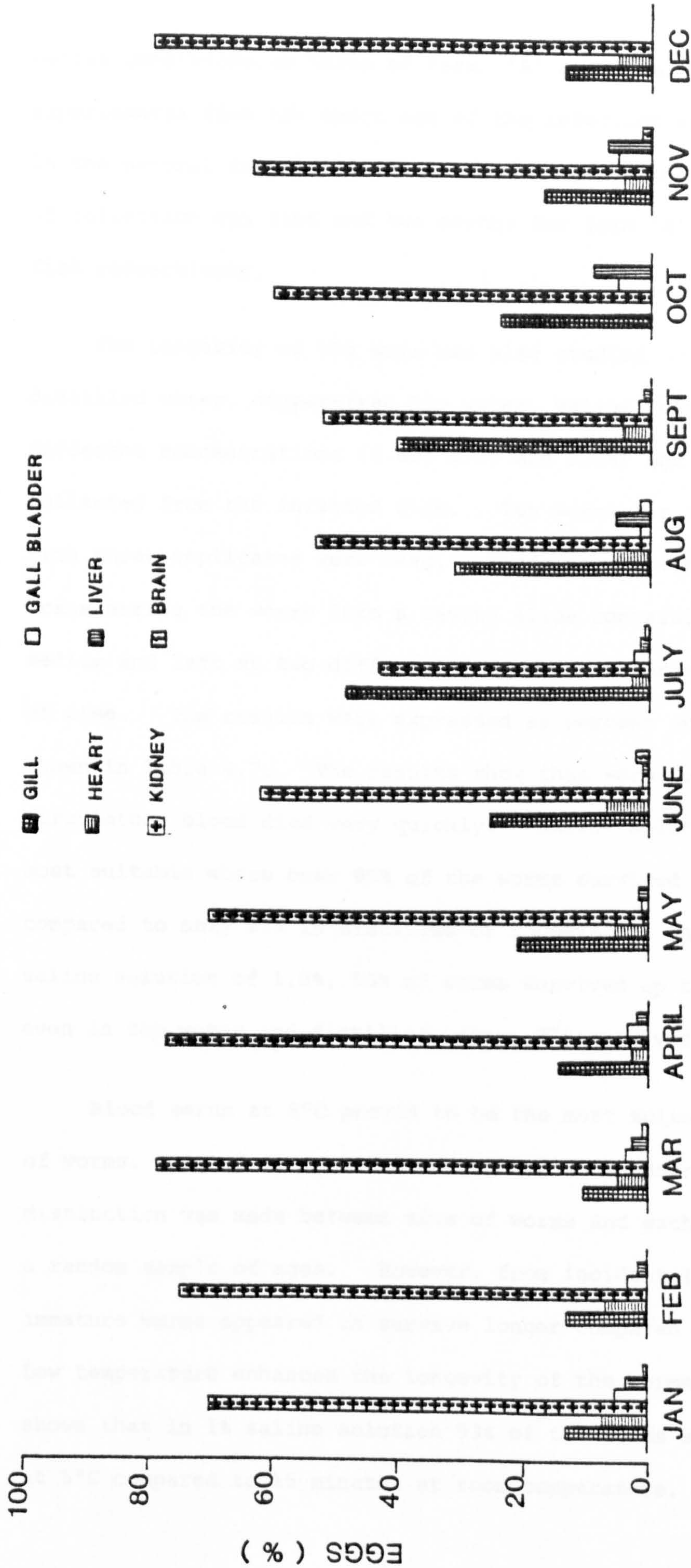


Fig. 4.19 Spatial distribution of eggs of *S. inermis* in 7 different locations in *C. carpio* from farm 'A' during the year 1981.

mental conditions as those of farm 'A' and farm 'B' fish. In the experimental fish the exact age of the infection was known, whereas in the natural infections the estimated age of infection at the time of collection was five and two months for farm 'A' and farm 'B' fish respectively.

The longevity of the worm was also studied .. in vitro using distilled water, copper-free tap water, saline solutions of three different concentrations (0.80, 0.90 and 1.0%) and blood serum collected from the infected fish. Ten worms for each time interval with three replicates were used. Experiments were carried out by transferring the worms into a cavity slide containing the test medium and left at two different temperatures for various periods of time. The results were expressed as percent survival and are shown in Table 4.7. The results show that worms outside the circulatory blood died very quickly. Saline solution of 1% was most suitable where over 90% of the worms survived up to 30 minutes, compared to only 27% in distilled or copper-free tap water. . In saline solution of 1.0%, 50% of worms survived up to 60 minutes, but even in tap water and distilled water, 27% survived 30 minutes.

Blood serum at 5°C proved to be the most suitable for the survival of worms. 3% of worms survived as long as 36 hours. No distinction was made between ages of worms and each test contained a random sample of ages. However, from incidental observations, immature worms appeared to survive longer compared to matured worms. Low temperature enhanced the longevity of the worms. Table 4.7 shows that in 1% saline solution 93% of the worms survived 60 minutes at 5°C compared to 15 minutes at room temperature. Similarly in

Table 4.7 Longevity of S. inermis worms (% survival) in different media

Time	15 - 18°C						5°C		
	Copper free tap water	Distilled water	0.85	Saline solutions (%) 0.90	Serum 1.0	Serum	Serum	Serum	Saline 1.0
2 minutes	100	93	100	100	100	100	100	100	100
5 "	83	73	100	100	100	100	100	100	100
10 "	60	57	80	93	97	100	100	100	100
15 "	57	47	80	93	93	100	100	100	100
30 "	27	27	73	73	93	45	100	95	95
60 "	—*	—	7	30	50	30	90	93	93
2 hours	—	—	—	—	7	11	87	80	80
3 "	—	—	—	—	3	—	83	73	73
4 "	—	—	—	—	—	—	80	65	65
5 "	—	—	—	—	—	—	63	47	47
6 "	—	—	—	—	—	—	60	33	33
12 "	—	—	—	—	—	—	33	—	—
24 "	—	—	—	—	—	—	10	—	—
36 "	—	—	—	—	—	—	3	—	—
48 "	—	—	—	—	—	—	—	—	—

* Negative sign indicates zero survival

100% of the worms survived up to 30 minutes at 5°C compared to five minutes at room temperature.

4.4.3 Discussion

Long term observations were carried out on both groups of fish collected from the two localities situated in England. The overall annual pattern of incidence and intensity is a dynamic process (Chubb, 1979) which involves at least four recognisable components. The first component is the gain of worms from the intermediate host. Cercarial invasion in fish of farm 'A' and 'B' was thought to occur during the month of July 1979 and August 1981 respectively, i.e. when fish fry from the hatchery were released into the earth nursery ponds of the farm containing infected snails. Thus by the time the fish were first examined in the laboratory the age of infection in farm 'A' was estimated to be five months, and farm 'B' two months old. This estimate is based on the age of the fry when they were released into the ponds, i.e. at 1-2 months old and assuming that the infection was already present in the snails.

It appears that invasion of fishes by sanguinicolid cercariae usually occurs at an early age. Scheuring (1922) found most S. inermis in young rather than old carp. Naumova (1961c) found that carp may become initially infected with S. inermis when only a month old. Sapozhnikov (1976) also observed that the young carp ponds were most affected. Invasion can even be achieved as early as 10 days old as Poljansky and Koulemina (1965) observed juvenile Sanguinicola in 2% of 10-day-old roach, Rutilus rutilus. There are at least three explanations for the invasion of early/young fish. First, an abundance of infected snails which coincides with the spawning period of the fish. Secondly, cercarial output also

coincides with the spawning period of fish, thus facilitating the infection. Thirdly, the softer skin tissue of young fish certainly facilitates penetration. On the other hand Naumova (1961c) reported one and two-year-old fish to be infected with S. inermis but it is possible that, in the light of the great longevity of some worms, that these were infected as fry.

The second component of a dynamic process (Chubb, 1979) is maintenance of a population of worms in the definitive host. The present study has shown that the overall prevalence is high for both farms, i.e. 74% and 84% in farm 'A' and 'B' respectively. However, in both groups the prevalence was found to decrease with the increase in the age of the infection. Layman's (1946) observation on incidence and intensity of infection by the same species revealed that there was a decrease in the incidence and intensity of infection with the increase of the host age, though he did not provide any detailed data. Any apparent increase in incidence in the laboratory-based fish occurring up to 100% in May and in September 1980, must be due to sampling error, since there was no new infection throughout the experimental period. However, the gradual decline from March 1981 is almost certainly due to worm mortality because, after this time, no apparent increase in incidence occurred.

The prevalence, intensity and parasite index in this study were very high compared to known records of other sanguinicolid infection in Britain, as shown in Table 4.8. It is noteworthy that most of these reports were of infection in wild populations of the fish, thus the exact time of the invasion was not known.

The information available on seasonal dynamics of Sanguinicola

Table 4.8 The occurrence, prevalence and intensities of infestation of Sanguinicola sp. in Britain

Species identified	Locality	Host fish	No. of fish examined	No. of fish infected	Prevalence %	No. of worms	Mean intensity	Mean parasite index	Reference
<u>Sanguinicola</u> sp.	South Lincolnshire (Hobhole Drain and River Bain)	<u>Rutilus rutilus</u> <u>Leuciscus leuciscus</u> <u>Esox lucius</u>	283 30 27	4 3 1	1.40) 10.00) 3.70)	9	1.13	0.03	Chappell, 1967
"	Essex (River Roding)	<u>L. leuciscus</u> <u>R. rutilus</u> <u>L. cephalus</u>	42 62 12	7 3 3	16.70 4.80 25.00	14 3 6	2.0 1.0 2.0	0.30 0.05 0.50	Shillcock, 1972
"	Kent	<u>C. carpio</u>			data not found				Sweeting, 1978
<u>S. volgensis</u>	Essex (River Roding)	<u>R. rutilus</u>	91	5	5.50	7	1.4	0.10	Shillcock, 1972
<u>S. inermis</u>	Gloucestershire	<u>C. carpio</u>	46	19	41.00	-	1-200/ fish	-	Sweeting, 1978
"	Lea Division of Thames Water	<u>C. carpio</u>			data not found				Sweeting, 1978
"	Gloucestershire	<u>C. carpio</u>	222	164	73.87	460	2.80	2.07	Present study
"	Nottinghamshire	<u>C. carpio</u>	50	42	84.00	2237	53.26	44.74	"

infection is contradictory, though little experimental work has been done on this aspect. In general, the dynamics of infections appear to be temperature dependent as infections are reported mostly in the warmer months of the year, even though Martin (1952) reported that the development of the larval sanguinicoid, Cercariae hartmanae, can proceed in Antarctic waters where temperatures never exceed 1.6°C. Layman (1951) stated that the highest incidence of infection of carp with S. inermis was in summer. Similar infections by this worm in warmer months were reported by Chechina, 1959; Lucký, 1964 and Naumova, 1961c. Bobiatyn'ska (1964) reported that infection of carp took place several times during the year. The first infection took place at the end of May and in June, and the second during August, September and beginning of October in the farms of the Bialystok area of Poland. Lucký (1964) reported that the heaviest intensity of infection was reached at the end of July and in August in the carp propagating fish ponds in South and Central Moravia of Czechoslovakia. Contrary to all reports, high incidences in winter were found by Ivasik (1953, 1957) and Ivasik and Svirepo (1971). The latter authors observed that 14% of young carp on a fish farm in the Lvov area, USSR, died in wintering ponds owing to S. inermis infections.

Thus worms were present throughout the year. The actual level of infection at any particular time might not reflect the time of invasion. In the present study maturity was shown to occur at 2½ months after the original invasion. Further, worms might develop at different rates, depending on environmental conditions, e.g. late summer/autumn infections might overwinter without much development taking place. Maturity might not then be achieved until the following spring when eggs are first produced. When this is the case,

the maximum life-span is six months. The results obtained from the farm 'A' fish showed that worms survived up to 24 months, thus surviving two successive winters in the constant ecological conditions.

The habitat of the worms of the present study was found to vary throughout the year, even though the fish were kept in constant conditions. The variation coincided with season and/or age of maturity of the worm. For example, the results from farm 'A' showed that in Winter and Spring, the majority of the worms occurred in the bulbus arteriosus of the fish, but in summer they moved to the major blood vessels of the gills (Fig. 4.11). This behaviour in constant aquarium conditions reflects that occurring under natural conditions in the farms of the USSR, Germany, Czechoslovakia and USA, shown in Table 4.9. Naumova (1960) noted that, during summer, sexually mature S. inermis were localised in the large blood vessels of the gills. However, in winter and in spring they were concentrated in the bulbus arteriosus of the fish. Lucky (1964) has also shown that in spring and in early summer 80% of the worms occurred in the heart and ascending aorta of carp, but in July and August 95% of worms moved to the gill arteries. Studies have shown that parasites have distinct microhabitat, within their vertebrate host (Holmes, 1971b; Chappell, 1969), although temporarily they may migrate away from these microhabitats. There may be some other factors which determine the habitat of the worms. For example, the geometry of the ventral aorta of the fish and its branches and the relative flow of blood through them appeared to determine the habitat of Aporocotyle macfarlani, a sanguinicolid blood fluke (Holmes, 1971b). The results of this study suggest that the mature worm possessed innate

Table 4.9 Habitats of some sanguinicolid blood flukes in definitive host

Species	Habitat(s)	Host(s)	Source	Reference(s)
<u>Sanguinicola inermis</u>	Branchial blood vessels (mature flukes in summer); Bulbus arteriosus (mature flukes in winter and spring); Vessels of other internal organs	<u>Cyprinus carpio</u>	Natural	Naumova, 1960
"	Heart, conus arteriosus, aorta ascendus up to the branches of the gill arteries (flukes in pre spawning time) 95% of the worm in the gill arteries (during spawning time)	"	"	Lucky, 1964
"	Aorta, branchial arteries	"	"	Layman, 1959
"	Anterior chambers of the heart, major afferent vessels leading to the gills and gill capillaries	"	"	Sweeting, 1979
<u>S. volgensis</u>	Heart, ventral aorta, branchial vessels	<u>Rutilus rutilus</u> <u>Leuciscus leuciscus</u>	"	Chappell, 1967
"	Bulbus arteriosus of the heart	<u>Rutilus rutilus</u>	"	Shillcock, 1972

continued/

Table 4.9 (continued)

Species	Habitat(s)	Host(s)	Source	Reference(s)
<u>S. idahoensis</u>	Adult flukes - ventral aorta, branchial arteries, brain surface, connective tissues around the eyeballs, choroid coat, iris stroma. Vitreous humor Young flukes - muscles, connective tissues in all parts of the body	<u>Salmo gairdneri</u>	Experimental	Schell, 1974
<u>S. klamathensis</u>	Gill vessels, kidney, heart	<u>Salmo clarki</u>	Experimental	Evans & Heckmann, 1973
"	Adult fluke - efferent renal vein Immature fluke - skin, all parts of the blood circulatory system	<u>Salmo clarki</u>	Experimental and Natural	Meade, 1967
<u>S. davisi</u>	Arteries which extend from the heart to the gill arches	<u>Salmo gairdneri</u> <u>S. clarki</u>	Natural	Davis et al., 1961
<u>S. alseae</u>	Blood vessels of the gills, liver, mesenteries and kidneys	<u>Salmo gairdneri</u> <u>S. clarki</u>	Experimental	Meade & Pratt, 1965
<u>S. lophophora</u>	Blood vessels throughout body; Immature flukes observed actively moving in the fins and muscles	<u>Notropis hudsonius</u>	Experimental	Erickson & Wallace, 1959
<u>S. inermis</u>	Immature flukes - blood vessels throughout body including skin, connective tissue Mature flukes - heart, bulbus arteriosus, aorta - winter and spring. Major blood vessels of the gills - summer, autumn	<u>Cyprinus carpio</u>	Experimental and Natural	Present study

'seasonal rhythms' which govern their life-cycle pattern which are independent of seasonal variations as described by Worms (1972).

The habitat of the worms of farm 'B' fish showed a different pattern compared to farm 'A' in that no skin worms were observed in farm 'A' fish. A very similar distribution of worms as in farm 'B' was obtained from the experimental infection of S. inermis cercariae (which are described in the previous section 4.3) where at a particular age or stage of maturity the worms migrate towards their preferred locus. A similar situation has been described by Evans and Heckmann (1973) who recovered immature S. klamathensis from gill vessels, kidney and heart of Salmo clarki. They believed that there was a migration of worms to "larger areas of the circulatory system, such as the dorsal aorta, efferent renal vein or heart chambers" as the worm became mature. Meade and Pratt (1965) recovered young Cardicola (= Sanguinicola) alseae from the gills, liver, mesentric vessels and kidneys of the infected Salmo gairdneri and found a migration as the worms mature. Meade (1967) also reported immature Cardicola (= Sanguinicola) klamathensis in all parts of the circulatory system of Salmo clarki, but the mature adult flukes occurred only in the efferent renal vein. Thus the reason for the absence of skin worms in fish of farm 'A' is possibly the age of the infection. The first observations of these fish were carried out when the infection was at least five months old and when worms had already migrated to the internal organs.

The third and very essential component of dynamic process is the production and accumulation of eggs in the host tissues (Chubb, 1979). It seems that, once the worms attain maturity, egg production begins and continues throughout the year until death of

the worms. However, the rate of egg production decreased in autumn and winter and increased in summer, indicating a seasonal periodicity in production, even though the fish were being kept in constant conditions. High egg production coincided with the warmer months, perhaps to facilitate the infection of the intermediate host which has a seasonal prevalence, i.e. very few Lymnaea overwinter and egg production by the worms during winter would be very uneconomic. However, the increase in egg production during July and August occurred in two successive years in fish from farm A. The stimuli for the increased egg output are not at all evident but may be associated with the physiological condition of the fish, e.g. variation of the blood constituents of the fish which may stimulate the worm to produce eggs. Unfortunately very little information is available on the long term observation of blood of carp kept in constant conditions. Long term observations on the blood parameters of adult carp held in a closed water system (22° - 27°C) showed no remarkable seasonal fluctuations (Hilge, 1980) whereas Hilmy Badawi and El-Domiaty (1978) recorded distinct seasonal variations of total serum protein of carp from a natural environment. However, the variations in the blood components, particularly variation in urea, uric acid and ammonia may act as stimuli for the production of eggs. It has been known that the host provides a physiological stimulus to initiate the developments of Ascaris lumbricoides (Fairbairn, 1961). A similar situation possibly exists in the case of S. inermis but this interesting aspect of the study deserves further work.

Eggs, deposited by mature worms, were carried by the circulating blood and were found throughout the fish body/tissues, including

gills, heart, brain, liver, gall bladder, kidney skin etc.. It was not clearly understood why the maximum number of all developing stages appeared in the gills. It may be because of the close proximity between the worms and the tissues of the gills since the majority of matured worms were recovered either from the bulbus arteriosus or from the major blood vessels leading to the gills, i.e. afferent vessels and gill capillaries of the infected fish. It may also be associated with the triangular shape of the eggs for which they were caught in the capillaries of the lamellae. However, a large number of eggs were also carried to the kidneys by the circulating blood. Since the eggs are filtered out ultimately by the kidney this represents a "dead end". Eggs can develop further to advanced miracidial stages but eventually became encapsulated. The continuous deposition and retention of the eggs in kidneys was thought to be the reason of maximum numbers in these areas.

The fourth and final component of a parasite dynamic process is the loss of worm (Chubb, 1979). Once invasion of the host is achieved, it was followed by growth, development and the production and liberation of eggs, after which the parasites die. Surprisingly, worms of the farm 'A' survived up to 24 months compared to 10 months of farm 'B' and in the case of experimental worms, seven months only. The differences found in the life-span of these worms are puzzling. However, this could be due to biotic and abiotic factors (Chubb, 1979) which determine the longevity of the worms. The strain of the fish may also play an important part in determining the longevity of the worms (Smithers & Worms, 1976). Slowed development overwinter may increase longevity but this does not explain the 17 months difference found in the longevity of the worms from different sources in this study. However these deserve further investigation.

CHAPTER 5

EFFECTS

ON

THE FISH HOST

5.1 Effects of infection on growth performance and mortality of fish

5.1.1 Introduction

There have been reports of outbreaks of Sanguinicola infection from fish propagating ponds in several parts of the world and adverse effects on growth and survival have been reported. For example, in Germany and the USSR infection resulting in impaired growth and mortalities of fish were described by Schäperclaus, 1954; Bauer et al., 1956; Bauer, 1964; Layman, 1957; Chechina, 1959; Naumova, 1961a, etc.. Layman (1957) reported that S. inermis has been a problem in the fish-farms of the USSR since 1932. Bauer (1964) also reported that S. inermis was a serious problem, particularly in the central and southern zones of the USSR. Chechina (1959) claimed it to be widespread (over 51%) throughout the pond farms of the Byelorussian SSR and Naumova (1961a) assessed the economic significance of the disease to the Russian carp industry in terms of mass mortalities and reduced growth.

The devastating effects of S. inermis infection in the Czechoslovakian carp-propagating farms have been reported by Lucký (1964). Similar outbreaks of Sanguinicola infection have also been found to occur in other countries of central and southern Europe including Britain. Mass mortality of the year's fry were reported from at least two farms in England (Sommerville, Pers. Comm., Sweeting, 1979).

Apart from European species, two American species of Sanguinicola have also been reported to cause mortalities. These are S. davisii and S. klamathensis (Wales, 1958; Evans and Heckmann, 1973). Evans (1974b) showed also a dramatic decline in the growth rate of

cutthroat trout infected with S. klamathensis.

There is no laboratory based information concerning the growth of carp infected with S. inermis. The reports noted above are entirely on the field basis and these have not, in general, been conducted under defined conditions of temperature, water quality, feeding levels etc., but largely in terms of reduced production.

The present sixteen-week growth study was undertaken to determine the effects of S. inermis on the growth and mortality of carp and in order to provide useful data on the growth performance, food conversion and mortality of infected fish.

5.1.2 Materials and methods

Source of fish

A total of 300 fish (mean weight 1.40 g) were divided into two groups of 150 each; 150 heavily infected fish (incidence 100% - Group 'B') and 150 with a low infection rate (incidence <20% - Group 'A'). Each group was divided into three replicates each containing 50 fish. During this experiment no batches of fish, completely free from infection, were available as controls and therefore the group 'A' of lightly infected fish was compared with group 'B' (full descriptions of the fish groups are shown in the general materials and methods, Chapter 2). All fish were from the same spawning and were 14 months at the start of the experiment.

Experimental system

The experimental facility used for this study was a modified recirculated water system consisting of a series of glass tanks (described as system A in Chapter 2). Each of the glass tanks was divided into equal sections by the use of netted frames. Each tank

contained one replicate each of group 'A' and group 'B'. The purpose of this division was to ensure the same environmental conditions for the high and low infected fish groups. At the end of the quarantine period the fish were transferred to these experimental tanks. The temperature of the water was gradually raised to 20°C and fish were acclimated at this temperature for seven days. All growth experiments were carried out at 20°C \pm 0.5. An artificial photoperiod was maintained with a total of 14 hours light and 10 hours of darkness throughout the experiment.

Experimental diet

The diet used in this study was a commercially available fingerling trout pellet no. 2 supplied by the Omega Quality Fish Foods (Ewos/Baker Ltd.). The proximate composition of the pellets is shown in the table 5.1.

Table 5.1 Proximate analysis of 'Trout Pellet'

Moisture	8%
Oil	8%
Protein	49%
Fibre	3%
Ash	11%
NFE*	21%

* Nitrogen Free Extractives

Feeding rate

All fish were fed 5% of total fish body weight per day. Carp are continuous feeders in nature and hence it was decided to divide the daily ration into four portions per day. Each feeding period was 10-15 minutes to ensure total consumption of the whole ration.

The quantity of food fed was adjusted weekly after weighing.

Sampling and weighing

Weighing of individual fish was performed fortnightly. Prior to weighing, fish were starved for 12 hours to ensure complete evacuation of intestinal content. Weighing was performed under anaesthetic (details in Chapter 2). Anesthetized fish were blotted with a paper towel, weighed and returned to fresh water. Intermediate weekly weighings were done in bulk for the purpose of adjusting the food ration. In this case anaesthetized fish were allowed to drain for some seconds in a net with gentle shaking. They were then transferred to a bucket of water on a balance. The weight of the fish were calculated by the difference.

Analysis of experimental data

The following indices were calculated from the data obtained.

Specific growth rate

The specific growth rate is defined by the percent weight gain per day and was calculated using the following equation.

$$\text{SGR (\%/day)} = \frac{\log_e W_2 - \log_e W_1}{T_2 - T_1} \times 100$$

where, W_2 is the weight in grams at time T_2

W_1 is the weight in grams at time T_1

$T_2 - T_1$ is the time interval between weighing W_2 and W_1 in days.

(Ref. Brown, 1957).

Food Conversion Ratio (FCR)

The food conversion ratio is defined as the amount of dry food fed per unit live weight gain of fish.

$$\text{FCR} = \frac{\text{Food fed, g dry food}}{\text{Live weight gain, g wet fish}}$$

Protein utilization

Protein utilization or the efficiency with which fish were able to utilize dietary protein was demonstrated by calculating Protein Efficiency Ratio (PER), defined as the gain in wet weight of fish per gram of crude protein consumed.

$$\text{PER} = \frac{\text{Weight gain, g wet fish}}{\text{g Crude Protein Fed}}$$

(Jauncey, 1979)

5.1.3 Results

Growth performance

The growth performance was studied over a period of sixteen weeks and is shown in table 5.2. No batches of fish, completely free from infection, were available as controls. Therefore the group 'A' of lightly infected fish was compared with group 'B'. From the table 5.2 it is apparent there was a significant difference ($p < 0.05$) between the final mean weights of infected fish group 'B' compared to group 'A'. Although initially, fish group 'B' had a slightly lower mean weight compared to the group 'A', i.e. 0.95g and 1.83g respectively, this was not shown to be significant ($p > 0.05$).

The marked differences in growth performance was also supported by specific growth rate and daily live weight gain parameters. SGR (%) was 1.62% for group 'A' compared to 1.41% for group 'B' and daily live weight gain (mg) being 83.75 and 32.92 for each group respectively. The percentage increase in body weight was also calculated in order to clearly show the differences in gain over the experimental period allowing for the variation in the initial mean weights of fish.

The increase in mean weight of fish throughout the experimental

Table 5.2 Growth performance, nutrient utilization and mortality rates for carp (Cyprinus carpio) infected with S. inermis at a mean water temperature of 20°C

Parameter	Group	
	'A' (<20% infected)	'B' (100% infected)
Mean initial weight (g)	1.83 ± 0.07	0.95 ± 0.82
Mean final weight (g)	11.17 ± 0.16	4.59 ± 0.75
Specific growth rate (%/day)	1.62	1.41
Percent increase in body weight (%)	510.38	383.16
Feeding level (%)	5.00	5.00
Daily live weight gain (mg)	83.75	32.92
Daily food intake (mg)	240.83	109.17
Food conversion ratio (FCR)	2.88	3.32
Daily protein intake (mg)	118.01	53.49
Protein efficiency ratio (PER)	0.71	0.62
Mortality (%)	2.60	30.60

± standard deviation

period is shown in figure 5.1. From the graphical representation of the growth of fish a consistent suppression was observed for the infected group 'B' fish compared to group 'A' fish. Fish within group 'A' grew logarithmically whilst those of the group 'B' showed a suppressed growth. The percentage increase in the mean final weight of group 'A' fish was found to be 510.38% compared to 383.16% of group 'B'.

Food conversion and nutrient utilization

The efficiencies of feed utilization of fish in the present experiment are shown in table 5.2. The food conversion ratios were in general, very poor for both groups. However, there was evidence that daily food intake was more greatly affected in carp with a high level of infection i.e. 109.17 mg/d in low level infection 240.83 mg/d. This difference closely reflects the growth performance. Figure 5.2 shows the mean FCRs over the 16 weeks experimental period. FCRs can be seen to fluctuate over this period being poorest between week 2 and 6 which coincided with the heavy parasite egg production (Chapter 4).

Like FCRs the protein efficiency ratios were also poor in both groups reflecting the poor FCRs. However, group 'A' showed slightly higher value than the heavily infected group but the changes were probably too small in order to account for the differences in growth performance.

Mortality

Mortality between the two groups of fish during the sixteen weeks experimentation is shown in fig. 5.3. During this period death rates in group 'A' were low, approximately 3%. However, over 30% of

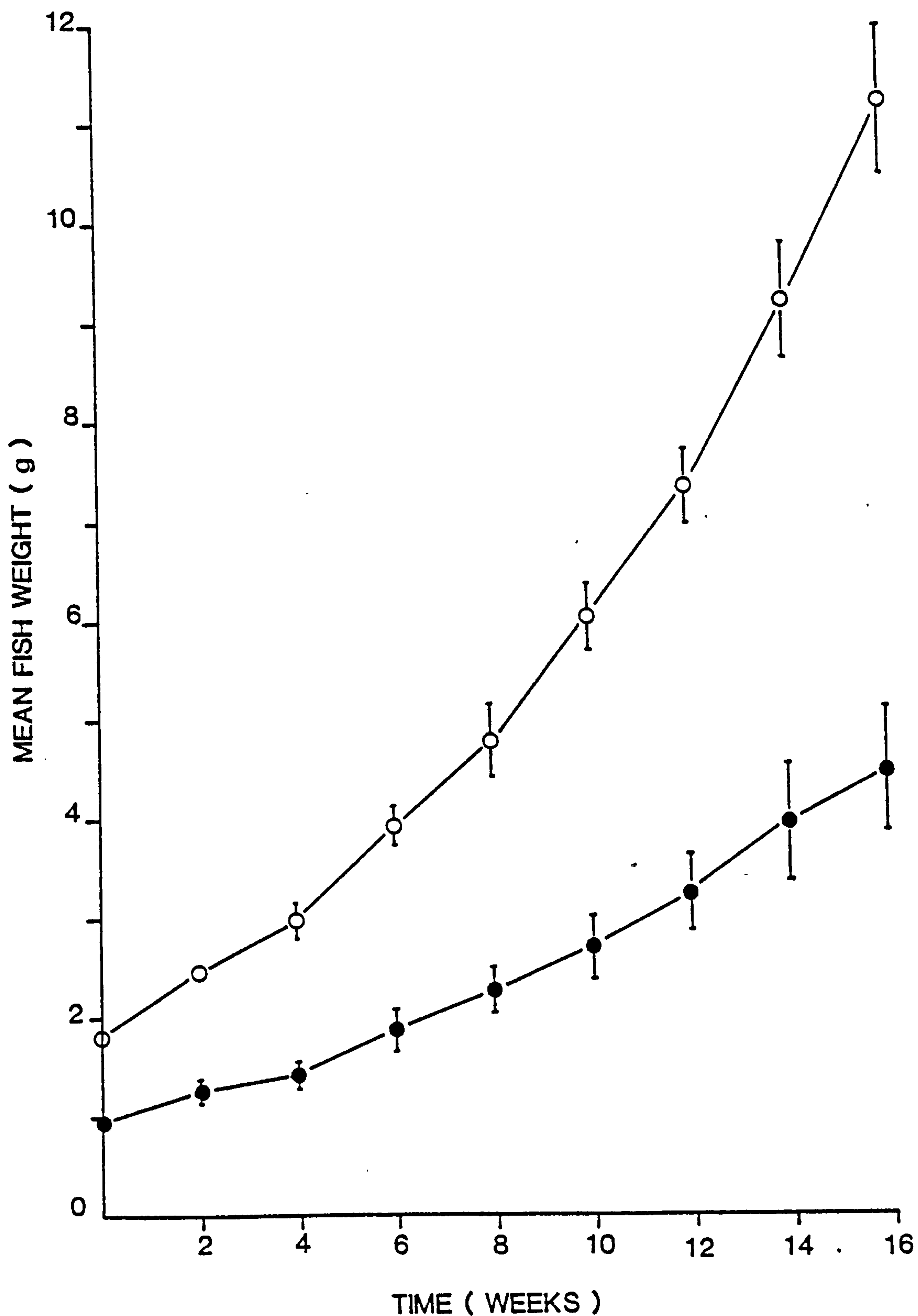


Fig. 5.1 Growth pattern of C. carpio infected with S. inermis.

○ , Group 'A' with light infection;
● , Group 'B' with heavy infection.

(Bars represent the standard deviation of the mean.)

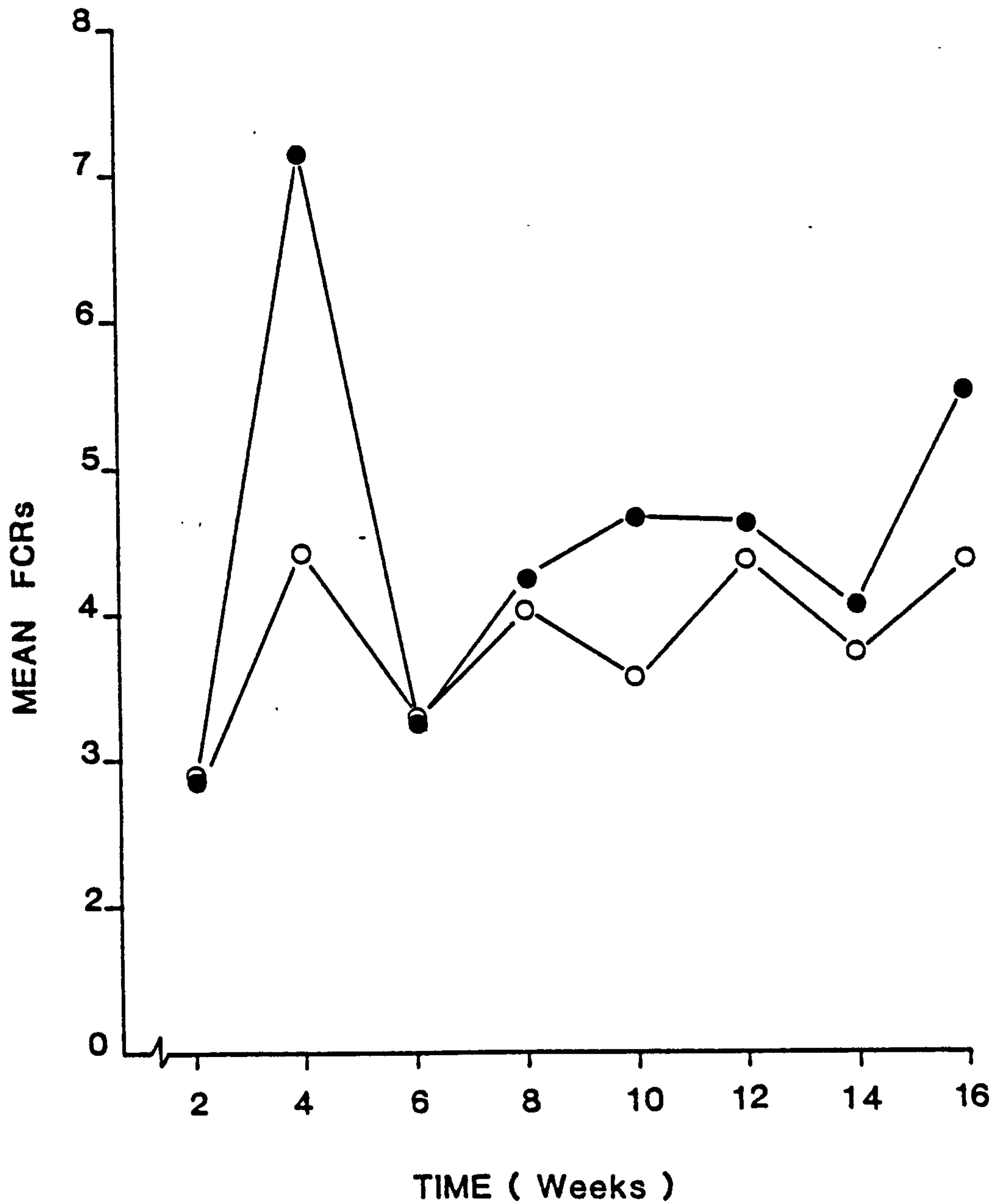


Fig. 5.2 Food conversion ratios (FCR) of C. carpio with S. inermis.

○, Group 'A' with light infection;
●, Group 'B' with heavy infection.

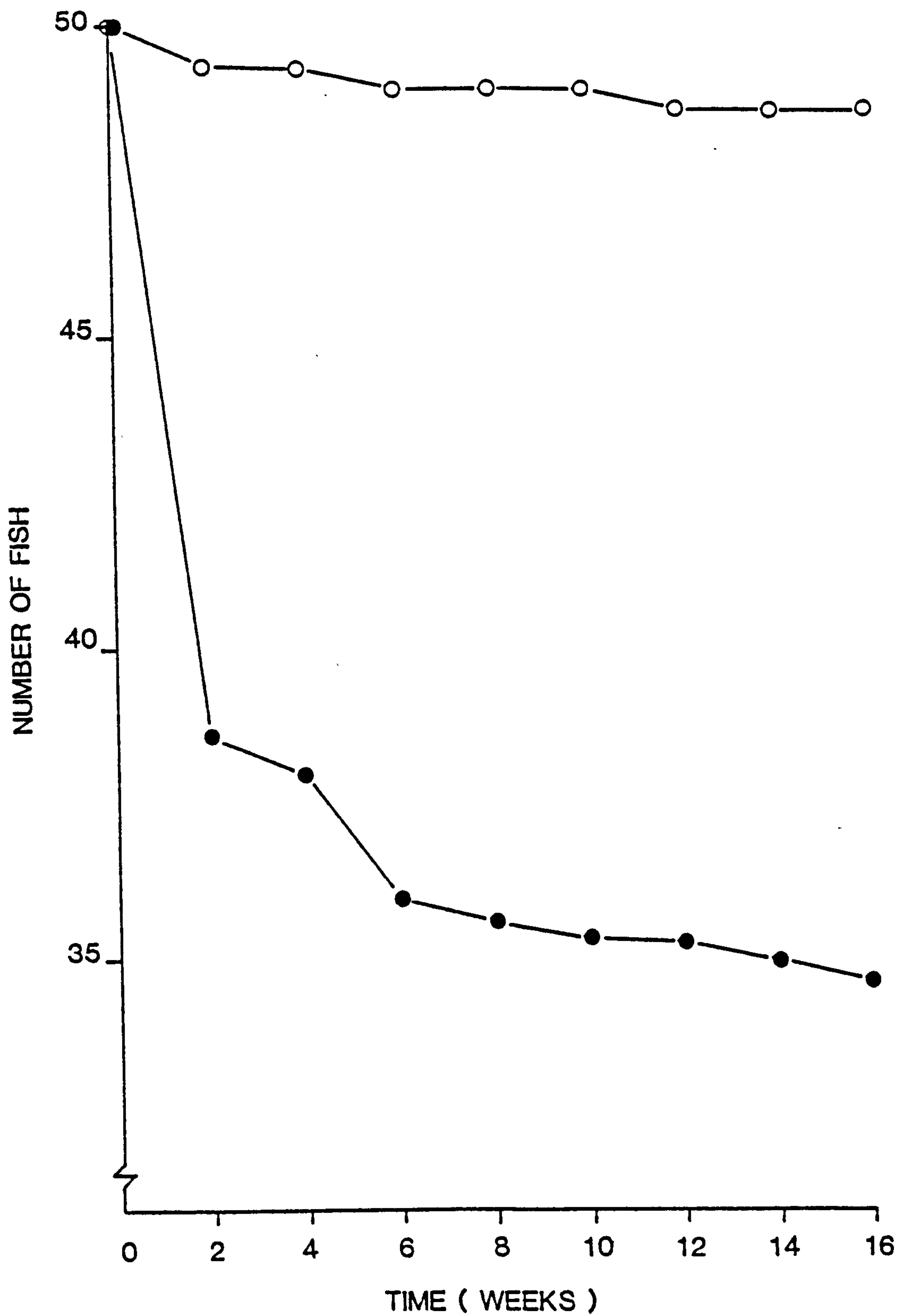


Fig. 5.3 Survival pattern of C. carpio infected with S. inermis.

○, Group 'A' with light infection;
●, Group 'B' with heavy infection.

the fish in group 'B' did not survive. The greatest mortality occurred during the initial stage of experiment, i.e. between week 1 and week 2. This is estimated to be approximately 12 months after the initial infection.

5.1.4 Discussion

A marked suppression in growth was evident in heavily infected fish during the study. Suppression also occurred in lightly infected fish, compared to normal carp (Jauncey, 1979) fed with a similar diet and ration but to a lesser extent than in heavily infected fish. The results obtained from the present investigation are in agreement with the findings of Evans (1974b) which is the only report regarding the growth of Sanguinicola infected fish. He reported a dramatic reduction in the growth of cutthroat trout after an experimental infection with S. klamathensis. Evans demonstrated a significant difference in length (F ratio = 11.5) and weights (F ratio = 11.1) between control and experimental group, three months after infection.

Specific growth rate, daily live weight gain, and daily food intake were all decreased in the heavily infected fish of the present study. Atack, Jauncey and Matty (1979) measured the specific growth rate of normal carp under different dietary treatments and found a variation between 1.13 and 2.78%/day at a temperature of 25°C. Jauncey (1979) found that at a temperature of 20°C and with 3% ration of trout pellet, carp fingerling displayed SGRs of 1.15%. With a feeding rate of 6% body weight and using the same diet this SGRs increased to a value of 1.65. The SGRs of the present investigation are low for both groups compared to those values noted above. This was certainly the result of infection.

The histopathological findings (Chapter 5.3) showed severe destruction of gills, kidneys, liver, brain and other major organs of infected fish by the different developmental stages of the parasite. Damage to the different organs is interconnected with all body functions. For example, gills are responsible for regulating the exchange of gas, water and ions and play a major role in the excretion of nitrogenous waste products. Therefore, slight structural damage can thus render a fish very vulnerable to osmoregulatory as well as respiratory difficulties (Ellis, Roberts and Tytler, 1978). Extensive skin damage by the cercarial invasion or gill damage by the miracidial emergence results in both inflow of water from the exterior and loss of tissue fluids which possibly in turn results in poor growth.

Feed utilization together with food conversion ratio and the protein efficiency ratio of both light and heavily infected groups were inferior to those reported for normal carp (Gribanov et al., 1966; Korneev, 1969). The food conversion ratios of normal carp varies from 1.14 to 2.54 (Jauncey, 1979; Atack et al., 1979) compared to 1.88 to 6.33 (fig 5.2) of the present study.

Poor SGRs and PERs and inferior FCRs are the result of reduced food intake and possibly poor assimilation of nutrients during digestion. Systemic disbalance due to the infection was thought to be an important factor in the reduced food intake of fish (Vahl, 1979). Reduced feeding response was noted to be a clinical feature characteristic of phase II of the infection (Chapter 5.3). Chechina (1959) and Naumova (1961a) also reported loss of appetite in S. inermis infected fish. In addition, Naumova (1961a) emphasized the economic aspect of the disease. In one example, she showed one and a half times loss in production in infected 'O' group fish compared to

uninfected controls. The average weight of infected fish was 1.8 grams, whereas controls were 6.7 grams with an individual maximum of 14 grams.

The poor growth of the fish may also result due to the sharing of the essential nutrients with the parasite. Results have shown that digeneans, i.e. Schistosoma, Fasciola can absorb nutrients, e.g. glucose, amino acid etc. through the gut and body surface (Pappas & Read, 1975). Though no work has been done on the ability of the absorption of nutrients by S. inermis, similar results from Schistosomes suggest that this also to be the case for S. inermis. In addition to the gut S. inermis possessed numerous microvillus like projections on the tegument (Chapter 3) which may also facilitate the absorption of essential nutrients (Smithers & Worms, 1976), and thus interfere with normal tissue repair and growth of fish.

Mortality

In the present study about 31% mortality was observed in the heavily infected group and about 3% in the lightly infected group during the 16 weeks experiment. An accurate figure for mortality due to infection by the parasite in this spawn was not available since most mortalities occurred before the fish were obtained. However, an estimate of 90% mortality on the farm within the first two weeks of infection was made by Sweeting (1979).

Chechina (1959) reported mass mortalities in 0₊ carp in the rearing ponds of the Soviet Union due to acute infection of S. inermis. The highest incidence of mortality took place in the warmer months, i.e. July-August period but no figures were reported. Those fish which survived the infection were said to be considerably weakened.

East European literature, similarly, lacks the actual figures of

the growth data and mortality of infected fish. Lucky (1964) reported frequent mortalities attributed to S. inermis in farmed carps of South and Central Moravia of Czechoslovakia. Damage done by this parasite to the carp industry in Germany has been found to be considerable (Schäperclaus, 1954). Though he did not give a figure he observed a great loss among 6 cm carp in the large ponds of the Peitz fishery. Similar observations' in Germany have also been made more recently by Körting (pers. comm.).

In some cases new Sanguinicola sp. have only come to light after they have caused considerable losses, e.g. the first salmonid sanguinicolid came to the knowledge of an American parasitologist only when approximately 300,000 rainbow trout and steelhead trout died in Darrah Spring State hatchery, California in 1958. A new species of Sanguinicola (S. davisii) came to light only after an epizootic and was described by Wales (1958). In another instance a batch of 5,000 Lohontan cutthroat trout were killed by another salmonid sanguinicolid which Wales (1958) subsequently described as S. klamathensis.

Following three months exposure to the S. klamathensis Evans (1974b) observed 80% mortality among experimental cutthroat trout.

In recent years there have been several outbreaks of S. inermis infection in Britain, causing considerable economic losses to the carp farms affected. Heavy losses have been reported from a farm in the Lea Division of Thames Water Authority (Dearsley, pers. comm.). Recurrent infection has appeared in the farms despite all efforts made to control the parasite since 1978 (Sommerville, pers. comm.). A second round of infection approximately four years following the first outbreak caused one farm to suffer more than 80% mortality of yearlings.

At the beginning of infection, the rate of mortality was approximately 2-5% per day which reached 10% per day at the peak of infection (Ian Pocock, pers. comm.).

More recently Sweeting (1979) reported the devastating effects of S. inermis infection in Gloucestershire where 90% of the year's fry were killed due to this infection. Mortalities began approximately three months post-hatching with several hundred carp dying per day. Though he did not record the weights of the fish, a reduced growth rate was noted.

Despite the records of the devastating effects of Sanguinicola infections Rawstron (1971) showed good annual harvests and survival rates and good growth of rainbow trout heavily infested with S. davisii. His estimation of a good annual harvest was based on a creel census and performed in an open water reservoir of 1,000-acres. Therefore, how such fish would respond when planted in other environments with artificial diet was not known.

From the basis of the present investigation it can be concluded that nutritional parameters, like food conversion ratio, protein efficiency ratio, daily protein intake etc. of carp, were directly or indirectly affected by the infection of S. inermis.

5.2 Blood Parameters

5.2.1 Introduction

Haematology is a useful tool for the ichthyologist and fishery biologist as an indication of the physiological condition of the experimental and hatchery fish stock (Hickey Jr. 1976). With more information, this might also prove to be a useful tool to assess the state of diseases due to parasites and other sources and stress due to change in environmental conditions.

The most frequently used parameters in fish haematology are haematocrit values, haemoglobin concentration and numbers of erythrocytes and leucocytes. Among haematological parameters the most widely and readily determined parameter under field or hatchery conditions is the haematocrit. Thus Houst^o and De Wilde (1972) have demonstrated significant correlations between haematocrit values, haemoglobin and erythrocyte numbers and suggested that haematocrit alone could be used as a general index of haematological status in the case of carp. For brook trout, Salvelinus fontinalis (Mitchill), however, they found no significant correlations amongst these values.

Many authors agree that haematological methods are valuable in disease diagnosis, provided blood samples are drawn with care. Inconsistency of results due to stress factors and handling techniques is one of the major difficulties with haematological studies of fish. Fisheries biologists have stressed the need for standardised techniques and the establishment of normal haematological values in fish with a view to the diagnosis of disease (Hesser, 1960; Snieszko, 1960; Larsen & Snieszko, 1961; Summerfelt, 1967). Thus Blaxhall (1972)

and Blaxhall and Daisley (1973) have suggested routine haematological methods for fish blood and attempted to establish a normal range of values for trout, Salmo trutta blood. There have, however, been no such attempts to establish routine haematological methods for carp. Many researchers have demonstrated that the values of blood parameters of carp vary with ecological conditions and geographical locations (Field, Elvehjem and Juday, 1943; Shiro Murachi, 1959; Chiba, 1965; Houst^o and De Wilde, 1968; Hines and Spira, 1973; Fourie and Hattingh 1976; Fourie and Vuren, 1976; Vuren and Hattingh, 1978; Mayo and Chien, 1980; and Hilge, 1980).

From the literature, therefore, it is very difficult, or rather impossible to establish a normal blood profile for carp, because blood parameters of carp vary with different environments, artificial diets, starvation and, above all, differences in methods used to measure the blood. One of the earliest reports on carp blood was given by Field et al (1943) who tried to define clinically normal fish under laboratory conditions. More recent studies provide information on the seasonal fluctuations in the blood of carp under field and laboratory conditions (Murachi, 1959; Fourie and Hattingh, 1976; Vuren and Hattingh, 1978).

Sexual differences have been observed in some blood values of C. carpio, Tilapia zilli and Barbus holubi (Ezzat, Shabana and Farghally, 1974; Van Vuren and Hattingh, 1976; and Fourie and Hattingh, 1976). Male carp showed higher red blood cell number, haemoglobin concentration and haematocrit values throughout the year (Fourie and Hattingh, 1976). Again Vuren and Hattingh (1978) found no difference in blood parameters of wild carp irrespective of sex difference.

Cyclic changes of blood parameters found in carp and other species are greatly affected by water temperatures, dissolved oxygen content, breeding phase of the fish, starvation, etc. Young carp which had starved for seven weeks showed higher ESR and lower haematocrit levels, haemoglobin concentration and mean cell haemoglobin concentration (Murachi, 1959; Chiba, 1965; and Guanghua, 1980).

Houston and De Wilde (1968b) investigated the acclimatization process of carps within a range of temperatures close to the upper and lower lethal limits. Their observations on fish maintained under summer, autumn and winter conditions showed that erythrocyte numbers, packed cell volumes and haemoglobin levels tend to vary directly with temperatures, though there were no significant differences between the summer and winter groups.

Considering the blood profile as an indicator of a particular disease state in fish, little progress has been made except with anaemias. Kawatsu (1969) described a macrocytic anaemia in brook trout, Salvelinus fontinalis with symptoms similar to human pernicious anaemia. Smith and Halver (1969) found megaloblastic macrocytic anaemia in Coho salmon, Oncorhynchus kisutch after using a diet deficient in folic acid. Starvation also caused a definite fall in various haematological values of the fish and resulted in anaemia (Kawatsu, 1966; Joshi, 1979).

The only report of changes in blood parameter due to a sanguinicolid infection was by Evans (1974b), who experimentally infected cutthroat trout with S. klamathensis and found a significant decrease in packed cell volumes and oxyhaemoglobin levels.

Until now there have been very few data available on the blood profile of fish infected with S. inermis. Naumova (1961a) first reported that the level of haemoglobin in the blood of S. inermis infected carp was noticeably lower than the controls and the number of erythrocytes was 25% lower in the infected fish. There were no further studies until Karrar in 1979 investigated the changes in the blood parameters of cultured carp chronically infected with S. inermis. In this case, the blood values of the infected group showed lower erythrocyte number, lower haematocrit value, as well as a lower level of haemoglobin. Karrar was unfortunately unable to obtain fish which were infected only with S. inermis. Her results were complicated by multiple infections with other parasitic agents. The present study was therefore performed with fish infected only with S. inermis.

Neither of these studies mentioned above examined the effect of S. inermis infection on the blood profile throughout the entire course of infection taking the developmental stages of the disease into account. It was therefore one of the aims of this study to evaluate the effects on blood parameters taking these factors into account.

5.2.2 Materials and methods

Source of fish

Haematological studies were carried out from the fish group 'C' and 'D' details of which are shown in Chapter 2. For better understanding of the effects on the blood, it was decided to divide the fish group 'D' into 5 subgroups on the basis of the developmental stages of the worms, so as to assess the effects on the blood as the disease progressed over the first 12 months of infection. These subgroups are:

Infected stage 1 : Group sampled at 8 weeks after initial infection i.e. pre-egg production stage of the worm. At this stage a large number of worms occurred in the skin.

Infected stage 2 : Group sampled at 12 weeks after infection. This was the stage when the majority of the worms migrate to internal loci. This was also the peak egg production period of the worms.

Infected stage 3 : Group sampled at 6 months after infection when the egg production of the worm was low.

Infected stage 4 : Group sampled at 9 months after infection. At this stage the majority of the worms had died.

Infected stage 5 : Group sampled at 12 months after infection. At this stage no worms were located but the major organs contained degenerating eggs.

Fish in group 'C' were used as a control for group 'D', which were also divided into five subgroups in order to match subgroups of 'D'. These were control stage 1 to control stage 5.

Experimental system

The experimental facility used in the present study was a 'flow through system', and described in Chapter 2 as system 'B'. Fish groups 'C' and 'D' were kept in this system for the whole of the experiment.

Diet

Fish groups 'C' and 'D' were fed four times a day, with commercially available grade 2 trout pellets. The ration given was 5% of the body weight per day.

Blood sampling technique

Careful collection of blood was the most important factor prior to any blood analysis as the stress reaction results in abnormal values. In order to minimise the variations in the blood values, the fish were caught gently using a small net. The fish to be sampled were then kept in a small bucket (8 l) for at least two hours prior to blood sampling. This procedure was followed to acclimate the fish into the laboratory and minimise the handling stress (Smit, Hattingh & Burger, 1979). Sampled fish were then anaesthetised by using neutralised benzocaine hydrochloride (NBH) which was found to be best for carp. The concentration of neutralised benzocaine hydrochloride was 80 mg/l aquarium water at 20°C.

The anticoagulant used for the present investigation was heparin. The concentration of the heparin used was 4 mg/l which provided the best results (Smit and Hattingh, 1980). Heparinization was carried out by drawing the heparin solution into the plastic syringe and drying in a hot oven at 20°C.

Blood collection

To obtain the blood sample, a fish was removed from the anaesthetic as soon as it lost equilibrium and became immobile. To avoid contamination with mucus and water the fish was wiped gently with a paper towel. Blood was then collected anaerobically directly from the caudal vein. A 1 ml disposable syringe with a 22 gauge needle (1½ inch) was inserted at a right angle to the surface of the skin in the caudal region, posterior to the anal fin and ventral to the lateral line. The caudal vein lies in the haemal arches ventral to the aorta. The needle was directed anteriorly towards the ventral column until the needle struck. The needle was then withdrawn slightly from the vertebral column. Blood was then drawn under gentle aspiration. This method allowed for the repeated sampling of blood from individual fish (Klontz and Smith, 1968) and reduced the dilution possibilities with tissue fluids which occurred if the caudal peduncle was severed. The use of plastic syringes helped in delaying the clotting time because of its smooth surface.

Blood cell counts

For the present investigation erythrocytes (RBC), leucocytes (WBC) and thrombocytes were counted. Since three types of cells were counted simultaneously, Rees-Ecker solution (Appendix 3) was used as it was found by Wedemeyer and Yasutake (1977) to be the most convenient for staining the three types of cells. Standard RBC diluting pipettes and standard haemocytometer (in this case, Improved Neubauer Counting Chamber) were used.

Blood samples were drawn directly into the red blood cell pipette just beyond the 1.0 mark on the stem of the pipette. The tip of the pipette was wiped with soft absorbant tissue to adjust the volume

exactly to the 1.0 mark. Then the pipette was immediately filled to the 101 mark with Rees-Ecker solution to make a 1:100 dilution. The use of lower dilution was to reduce the coefficient of variation and thus increase accuracy (Blaxhall and Daisley 1973; Wedemeyer and Yasutake 1977). Blood was then mixed for a few seconds by slight agitation. Immediately after mixing, the first few drops of the diluted blood was discarded and the tip of the bulb was wiped. The tip of the pipette was then touched at the edge between cover slip and the counting chamber and the fluid was allowed to flow in. The cells were allowed to settle for several minutes before the counting. Counting was performed manually. For the erythrocyte and thrombocyte counts $1/5 \text{ mm}^2$ were counted. For the leucocyte counts 5 mm^2 or if the numbers appeared low, then 9 mm^2 on each side of the counting chamber were counted. Calculations were made from the mean of the two counting areas on each chamber using the formula given by Baker and Silverton (1976).

Haematocrit values (packed cell volume; PCV)

To measure the volume of packed cells in plasma, blood was drawn into three-fourths of an heparinised microhaematocrit tube (75 mm × 1.1-1.2 mm). The distal end was then sealed by flame. The tube was centrifuged in a microhaematocrit centrifuge for five minutes at 10,500 rev./minute. The readings were made with the aid of an Hawksley microhaematocrit reader and the result was expressed as the volume of the erythrocytes per 100 cm^3 of blood. To avoid variability due to cellular swelling, clot formation etc., blood was centrifuged as quickly as possible after collection.

Haemoglobin (Hb) estimation

The method used for the haemoglobin estimation was the cyanmet-

haemoglobin method. In this method the haemoglobin is converted into methaemoglobin by ferricyanide. Methaemoglobin then reacts with cyanide to form cyanmethaemoglobin which is measured with a spectrophotometer.

Procedure

0.02 ml of blood was placed into a bijou bottle containing 5 ml of Drabkin's solution (Appendix 4). The solution was gently mixed by inversion and allowed to stand for at least fifteen minutes at room temperature. This ensured the complete conversion of haemoglobin into cyanmet-haemoglobin. The solution was then centrifuged if necessary to remove suspended cellular material as suggested by Stevenson McCarthy, and Roberts (1973). Transmittance of cyanmethaemoglobin was then read on a Cecil spectrophotometer at a wavelength of 540 nm. The transmittance of the commercially available standard haemoglobin solution* was recorded at the same time.

The haemoglobin in g per 100 ml of blood was calculated using the following formula:

$$\text{Haemoglobin (g/100 ml)} = \frac{\text{Tu(CS)}}{\text{Ts} \times 1000} \times \text{Dilution factor.}$$

where Tu = Transmittance of unknown

Ts = Transmittance of standard

Cs = Concentration of standard in mg/100 ml

Dilution factor = 251

(after Dacie and Lewis, 1968)

Erthyrocyte sedimentation rate (ESR)

ESR was determined by using heparinized microhaematocrit tube of the same size as described above. Blood was drawn up to three-fourths of the total length of the tube. The end which was filled with blood

* Product of B.D.H.

was sealed with 'cristaseal'. The tube was then allowed to stand vertically for exactly sixty minutes at room temperature. The erythrocyte sedimentation rate was calculated by measuring the distance, the erythrocyte had sedimented, and was expressed in terms of 'volume percent'.

Haematological indices

Haematological indices calculated for this study were Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH) and Mean Cell Haemoglobin Concentration (MCHC). These volumes were calculated mathematically from red cell count, haemoglobin content and packed cell volume. The formula used was the same as that described by Baker and Silverton (1976) and shown in Appendix 5.

5.2.3 Results

Table 5.3 to 5.9 show the blood values of control fish (uninfected) and Sanguinicola infected fish measured at five different stages over the period of one year. These stages refer to the different phases of infection. Though aquarium conditions were constant, stage 1 to 2 occurred during the autumn and stage 3 in the late winter. Fish were sampled at stage 4 and 5 in late spring and late summer respectively. Statistical comparisons between means of the five individual stages were made by multiple analysis of variance using Duncan's Multiple Range of F-test (Duncan, 1955) and the results are shown in Table 5.3 and 5.4 for the control and infected group respectively. Comparison of the means between control and infected group for a particular stage were made by two-way sample 't' test using Minitab programme (Ryan, Joiner and Ryan, 1976) and results are shown in Tables 5.5 to 5.9. The values obtained are described individually under the following headings -

Control group

During the one year study period the values of all the blood parameters for the control group (stage 1 - stage 5) fell within the range of those found by other workers for clinically normal carps though in most other studies, no parasitological examination was indicated. However, fluctuations in the normal values of the different parameters did occur through the study period despite the maintenance of a constant environment and these are evident from Table 5.3.

The numbers of red blood cells varied significantly at stage 3 and 5 when the number of RBCs were reduced by 7.86 and 16.43% respectively. These reductions in the numbers of red blood cells were significant ($p < 0.05$).

The white blood cell numbers were fairly consistent throughout the sampling period until the final sample, i.e. 12 months from the onset of the study (August, 1982) when the number of WBC was significantly lower than the earlier values ($p < 0.05$).

The values of the packed cell volume followed closely the pattern for the erythrocyte counts, being significantly lower ($p < 0.05$) during the stage 3 but in this case it remained at the lower level at stage 4 after which it was found to return to the levels expressed at stage 1 and 2.

Erythrocyte sedimentation rate showed only very minor fluctuations during the study. The rate of sedimentation was maximal at stage 3 and differed significantly from stage 2.

As with the other parameter, fluctuations in the thrombocyte counts were evident but were only significantly different at stage 3 when it was at its lowest.

Table 5.3 The values of blood parameters for control C. carpio during one year sampling period (Means \pm S.D.)

Parameters	Control stage 1 (N = 10)	Control stage 2 (N = 10)	Control stage 3 (N = 10)	Control stage 4 (N = 10)	Control stage 5 (N = 10)
Length (cm)	5.82 \pm 0.11	6.07 \pm 0.24	6.86 \pm 0.65	7.71 \pm 0.93	8.25 \pm 0.72
Weight (g)	7.65 \pm 1.32	7.96 \pm 0.84	11.33 \pm 2.19	15.86 \pm 5.04	20.39 \pm 6.70
Age (months)	6+	8+	9+	12+	15+
RBC (million/mm ³)	1.40 ^c \pm 0.04	1.38 ^{bc} \pm 0.05	1.29 ^b \pm 0.17	1.40 \pm 0.05	1.17 ^a \pm 0.12
WBC (thousand/mm ³)	7.50 ^b \pm 0.89	6.75 ^b \pm 1.27	7.30 ^b \pm 0.56	7.00 ^b \pm 1.24	5.50 ^a \pm 1.53
Hc (%)	35.40 ^b \pm 1.96	35.80 ^b \pm 2.23	32.00 ^a \pm 1.79	35.70 ^b \pm 2.53	34.20 ^{ab} \pm 2.99
Hb (g/100 ml of blood)	10.57 ^b \pm 0.37	10.57 ^b \pm 0.33	8.54 \pm 1.03	8.36 ^a \pm 0.70	10.05 ^b \pm 0.57
ESR (%)	1.67 ^{ab} \pm 0.82	1.15 ^a \pm 0.55	1.90 ^b \pm 0.48	1.28 ^{ab} \pm 0.64	-
MCV (μm^3)	251.83 ^a \pm 17.10	258.77 ^a \pm 20.15	251.19 ^a \pm 29.61	254.51 ^a \pm 15.01	293.40 ^b \pm 34.37
MCH (pg)	75.27 ^c \pm 3.14	76.33 ^c \pm 3.39	66.56 ^b \pm 6.85	59.67 ^a \pm 5.39	86.83 ^d \pm 7.00
MCHC (%)	29.64 ^c \pm 1.67	30.63 ^c \pm 2.53	26.63 ^b \pm 2.26	23.46 ^a \pm 1.81	29.59 ^c \pm 3.65
Thrombocyte (million/mm ³)	0.20 ^b \pm 0.02	0.20 ^b \pm 0.02	0.14 ^a \pm 0.03	0.18 ^b \pm 0.04	0.17 ^b \pm 0.01

Figures in each parameter with common superscripts are insignificantly different ($p < 0.05$)

As with the major parameters noted above, the values of the indices were also found to fluctuate throughout the study period. In the case of mean cell volume (MCV) the only obvious change occurred at the final stage and here MCV was found to be significantly higher ($p < 0.05$) than the rest of the values. Significant changes can also be found in the mean cell haemoglobin (MCH) and mean cell haemoglobin concentrations (MCHC) particularly at stage 3 and stage 4, when they appeared with minimum values.

Infected group

Table 5.4 shows the values for blood parameters in the infected group obtained during the one year sampling period. As with the control groups the values recorded here also fluctuated during the period and are described below.

In general, the values of red blood cells obtained from the infected groups were very low. The only significant fluctuations in the number of red blood cells occurred at stage 2 which was the pre-egg stage of the S. inermis infection when the red blood cell number was at its minimum. The values found in stage 1, 3, 4 and 5 did not differ from each other significantly ($p > 0.05$).

The white blood cell counts throughout the experiment were found to fluctuate but these minor fluctuations were insignificant with an exception of the final sample, when the numbers were significantly lower ($p < 0.05$) compared to the values of stage 1, 2 and 4. The maximum white cell count was evident at stage 2, i.e. post-egg stage of the infection when it was 52.64% higher than it was at stage 5.

The values for packed cell volume, in general, decreased over the sampling period. A significant drop in the values of packed cell

Table 5.4 The values of blood parameters for infected C. carpio during one year sampling period (Means \pm S.D.)

Parameters	Infected stage 1 (N = 10)	Infected stage 2 (N = 10)	Infected stage 3 (N = 10)	Infected stage 4 (N = 10)	Infected stage 5 (N = 10)
Length (cm)	5.51 \pm 0.30	5.81 \pm 0.25	6.68 \pm 0.19	6.71 \pm 0.54	7.14 \pm 0.86
Weight (g)	5.57 \pm 0.82	5.90 \pm 0.54	8.78 \pm 1.47	9.03 \pm 1.75	11.91 \pm 2.47
Age (months)	6+	8+	9+	12+	15+
RBC (million/mm ³)	0.90 ^b \pm 0.08	0.77 ^a \pm 0.13	0.89 ^b \pm 0.14	0.91 ^b \pm 0.13	0.88 ^b \pm 0.09
WBC (thousand/mm ³)	11.00 ^b \pm 2.11	12.44 ^b \pm 4.49	9.55 ^{ab} \pm 1.74	11.95 ^b \pm 2.69	8.15 ^a \pm 2.19
Hc (%)	31.70 ^c \pm 0.57	26.40 ^a \pm 1.85	29.10 ^b \pm 1.92	26.90 ^{ab} \pm 2.30	24.90 ^a \pm 2.12
Hb (g/100 ml of blood)	6.33 ^{ab} \pm 0.54	5.82 ^a \pm 0.36	6.40 ^b \pm 0.56	6.70 ^b \pm 0.61	6.33 ^{ab} \pm 0.65
ESR (%)	2.96 ^a \pm 1.13	3.12 ^a \pm 1.14	2.50 ^a \pm 1.08	2.39 ^a \pm 0.92	-
MCV (μm^3)	355.87 ^c \pm 35.73	333.82 ^{bc} \pm 26.27	336.49 ^c \pm 58.14	299.75 ^{ab} \pm 23.57	283.94 ^a \pm 26.51
MCH (pg)	70.71 ^a \pm 5.95	71.53 ^a \pm 6.18	74.00 ^a \pm 19.37	74.71 ^a \pm 6.87	72.34 ^a \pm 8.60
MCHC (%)	20.09 ^a \pm 1.76	22.16 ^{ab} \pm 2.35	22.91 ^{bc} \pm 3.48	24.95 ^{cd} \pm 1.66	25.62 ^d \pm 3.59
Thrombocyte (million/mm ³)	0.19 ^c \pm 0.03	0.14 ^b \pm 0.01	0.21 ^c \pm 0.07	0.14 ^b \pm 0.02	0.10 ^a \pm 0.03

Figures in each parameter with common superscripts are insignificantly different ($p < 0.05$)

volume occurred at the second stage and again at the final stage when they were 20.08% and 27.31% lower compared to stage 1.

The levels of haemoglobin followed closely the pattern found in erythrocyte counts. Here also the lowest levels were observed at stage 2 and were found to be significantly different ($p < 0.05$) from other stages.

Erythrocyte sedimentation rate, though fluctuating over the sampling period, was found to change insignificantly. The maximum values, however, were observed during post-egg stage of infection.

Marked fluctuations in the thrombocyte counts were also evident throughout the sampling period, being highest at stage 3 and lowest at stage 5.

MCV values were elevated significantly over the entire experimental period being highest at stage 1, i.e. initial stage of the infection. Along with the progression of the infection MCV values gradually decreased and they were at the lowest at stage 5, when they were significantly lower than stages 1, 2 and 3. Fluctuations among the values of MCH appeared to be insignificant ($p > 0.05$). Lowest values of MCHC occurred at the initial stage (stage 1) of the infection and were significantly lower than those of stages 3, 4 and 5.

Comparison of infected and control groups

Data obtained from the infected and control groups were compared and are presented graphically in Figures 5.4 to 5.6. From Figure 5.4 it can be seen that the RBC, Hc and Hb were also lower in the infected group irrespective of stage of infection. The value of Hb and Hc follows the pattern of RBC. A depression of these values was evident from the very earliest stage examined, i.e. at the pre-egg stage of

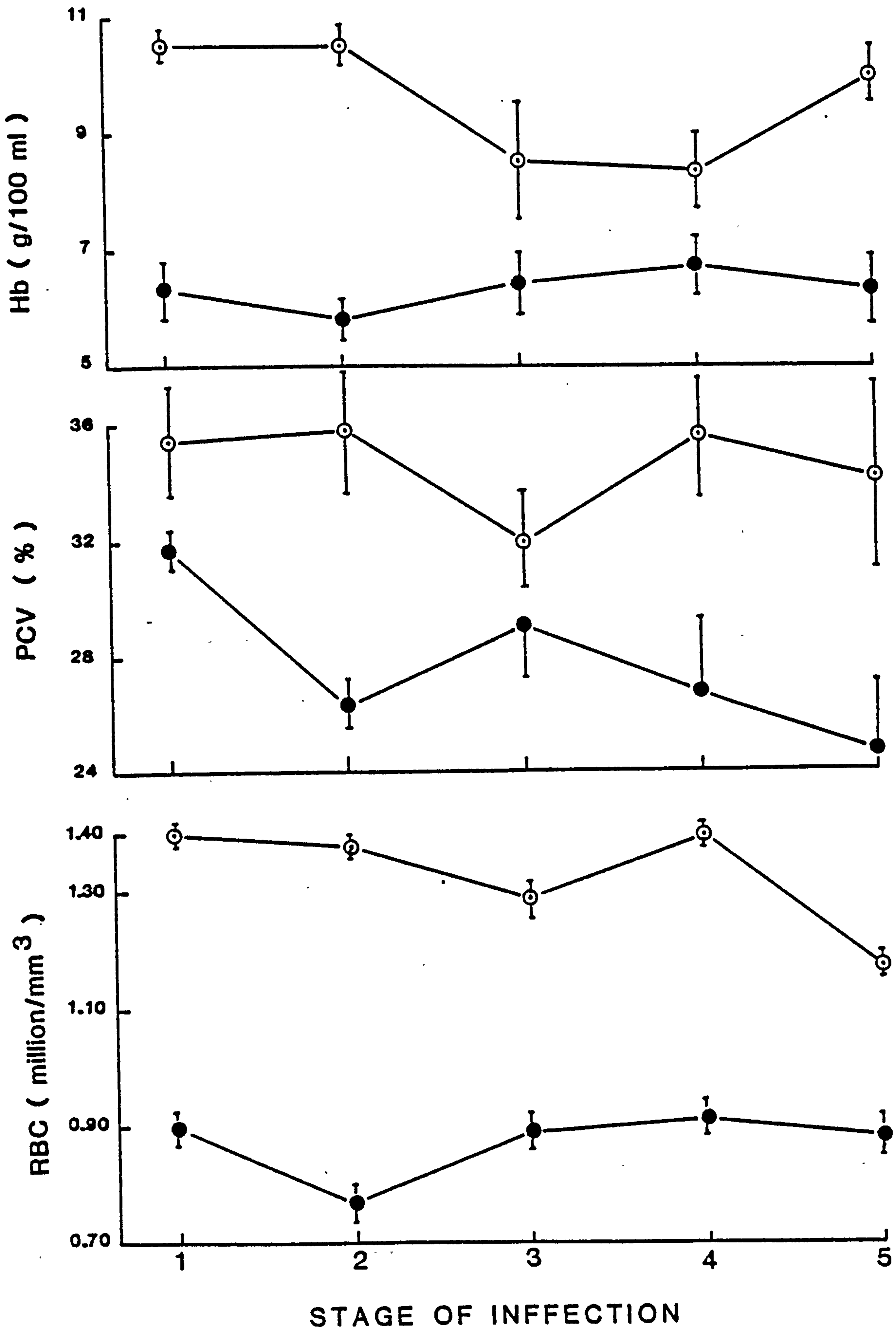


Fig. 5.4

Graphs showing RBC, PCV and Hb of control (O) and *S. inermis* infected (●) *C. carpio* during the different stages of infection. (Bars represent the standard deviation of the mean.)

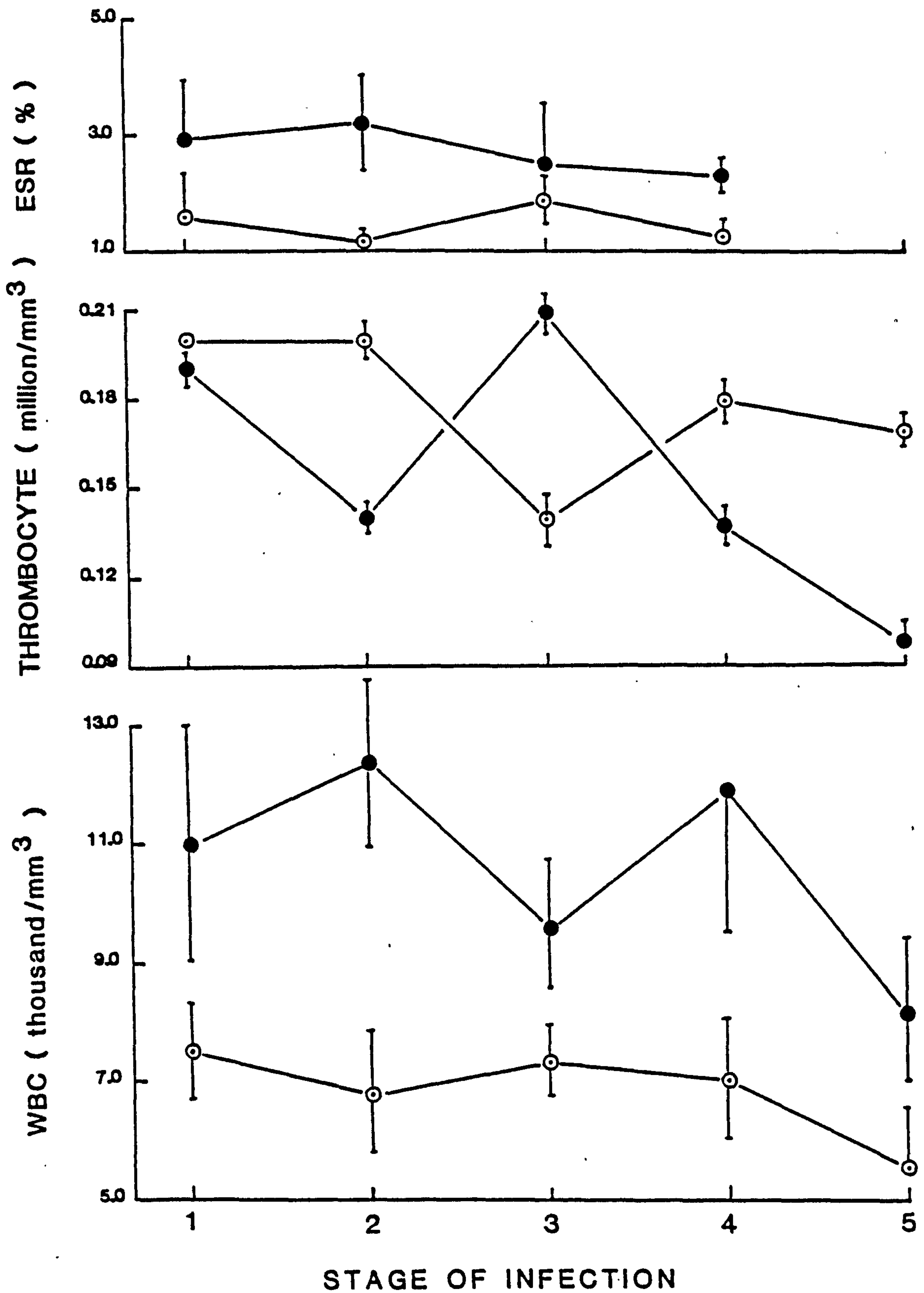


Fig. 5.5

Graphs showing WBC, Thrombocyte number and ESR of control (O) and *S. inermis* infected (●) *C. carpio* during the different stages of infection.

(Bars represent the standard deviation of the mean.)

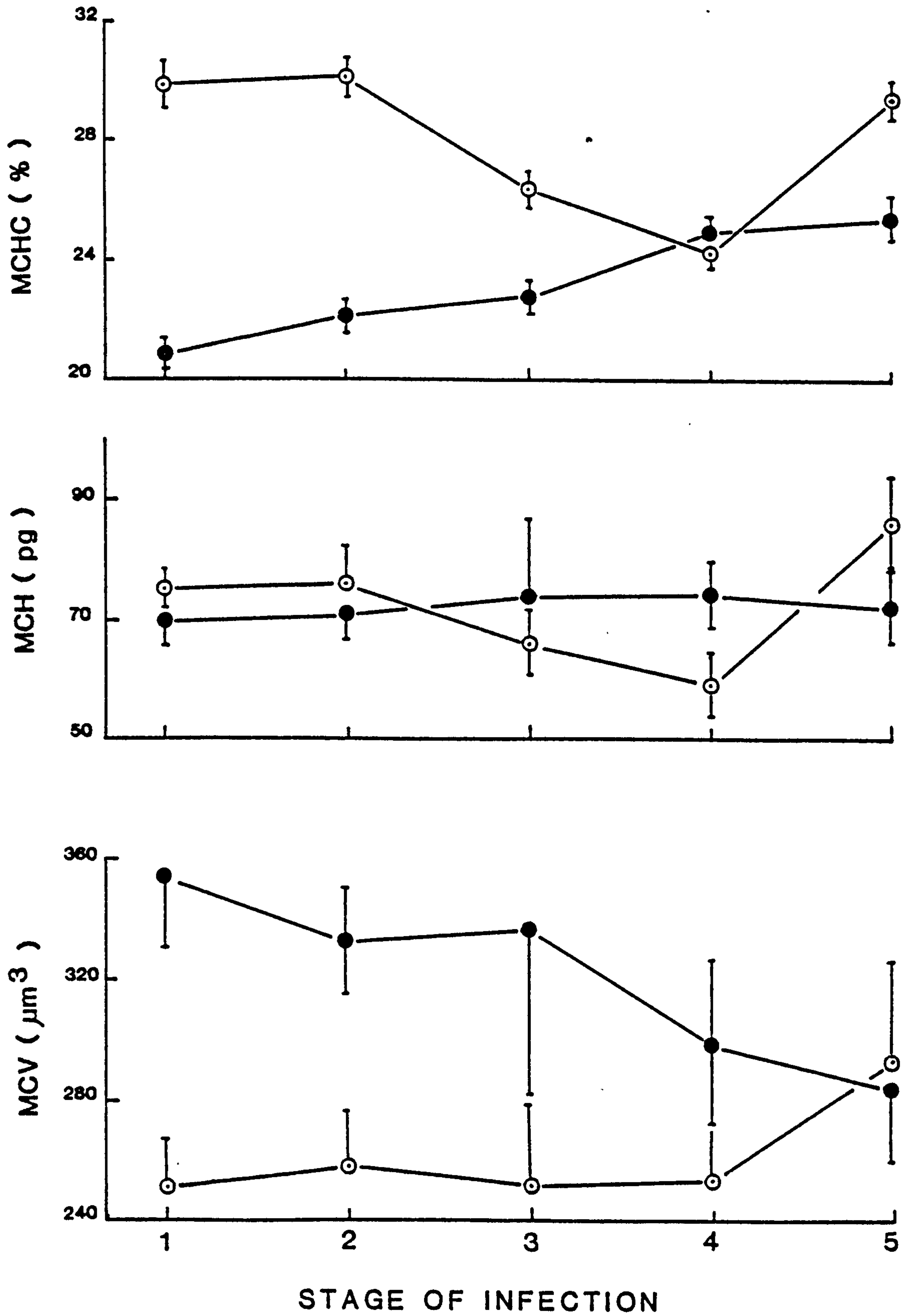


Fig. 5.6

Graphs showing MCV, MCH and MCHC of control (○) and *S. inermis* infected (●) *C. carpio* during the different stages of infection. (Bars represent the standard deviation of the mean.)

infection. The number of erythrocytes at stage 1 was more than 35% lower in the infected group compared to controls. This difference in the number of RBCs, between the two groups was continued throughout the sampling period, being at its highest at stage 2 when the infected group showed a 44% lower RBC number than the controls. The low number of erythrocytes indicates the loss of blood from the body by external or internal haemorrhage, the destruction of blood cells in the body or even a decrease in the production of blood cells. The greatest difference in the RBC counts between control and infected groups occurred at stage 2 and these were almost certainly due to the loss of blood through the damaged gill tissues following miracidial emergence.

Fig. 5.5 shows high WBCs and ESR and low thrombocyte numbers in the infected group when compared with controls. At the initial stage of infection the white blood counts of infected fish were about 47% higher than the control group. The maximum difference in white blood counts occurred in infected fish when the WBC increased 13% at stage 2. At this stage the difference between control and infected fish was nearly 85%. However, the high white blood cell counts were maintained till the end of the experiment, i.e. well after 12 months of infection, though in the later stages the difference was smaller.

ESR of the infected fish, again, showed high values compared to control fish. The high values observed throughout the experimental period, being highest at stage 2 when the difference between two groups was 171%.

MCV, MCH and MCHC of the infected and control group were compared and are presented in Figure 5.6. During the initial stage of infection MCH and MCHC showed low values, whereas MCV showed high values in the

diseased group compared to controls. The difference in the values of MCV and MCHC decrease with the increase of the age of infection.

In summary, it can be seen from the Tables 5.5 - 5.9 that RBC, Hb, Hc and MCHC are significantly low at all stages of infection. Similarly WBC of infected fish at all stages remained significantly high compared to controls. The MCH was also significantly higher in infected fish throughout the experiment with a single exception at stage 5, when it was lower than the controls. In the case of MCH the only significant difference appeared at stage 4 and 5. Compared to controls, a significantly high ESR was also observed in the infected group throughout the period of study.

The observed decreases in erythrocyte number, haemoglobin concentration, packed cell volume and mean corpuscular haemoglobin concentrations indicate that the fishes in the infected group were anaemic. The anaemia was characterised as hypochromic macrocytic anaemia.

5.2.4 Discussion

Relatively few haematological studies have been carried out on carp, particularly from closed water systems. Therefore, the data obtained from the fish in this study were compared with those obtained from fish of different areas as well as with the fish of similar or near similar origin.

Haematological parameters can only be useful if 'normal' values are known for a particular species (Reichenback-Klinke & Elkan, 1965). The published results on carp blood are found in the work of Field et al. (1943), Murachi (1959), Houston and De Wilde (1968b) Hines and Yashouv (1970), Fouri and Hattingh (1976), Vuren and Hattingh (1978),

Table 5.5 Comparison of haematological values for control and infected C. carpio at stage 1 (Means + S.D.)

Parameters	Infected stage 1 (N = 10)	Control stage 1 (N = 10)	"t" values	Level of Significance
RBC (million/mm ³)	0.90 ± 0.08	1.40 ± 0.04	- 16.928	0.0000
WBC (thousand/mm ³)	11.00 ± 2.11	7.50 ± 0.89	4.583	0.0000
Hc(%)	31.70 ± 0.54	35.40 ± 1.96	- 2.940	0.0107
Hb (g/100 ml of blood)	6.33 ± 0.54	10.57 ± 0.37	- 19.422	0.0000
ESR (%)	2.96 ± 1.13	1.67 ± 0.82	2.761	0.0139
MCV (µm ³)	355.87 ± 35.73	251.83 ± 17.10	7.880	0.0000
MCH (pg)	70.71 ± 5.95	75.27 ± 3.14	- 2.032	0.0631
MCHC (%)	20.09 ± 1.76	29.64 ± 1.67	- 11.770	0.0000
Thrombocyte (million/mm ³)	0.19 ± 0.03	0.20 ± 0.02	- 0.709	0.4895

Table 5.6 Comparison of haematological values for control and infected C. carpio at stage 2 (Means + S.D.)

Parameters	Infected stage 2 (N = 10)	Control stage 2 (N = 10)	"t" values	Level of Significance
RBC (million/mm ³)	0.77 ± 0.13	1.38 ± 0.05	- 2.490	0.0344
WBC (thousand/mm ³)	12.44 ± 4.49	6.75 ± 1.27	6.575	0.0000
Hc (%)	26.40 ± 1.85	35.8 ± 2.23	- 1.820	0.0960
Hb (g/100 ml of blood)	5.82 ± 0.36	10.57 ± 0.33	- 1.133	0.2740
ESR (%)	3.12 ± 1.14	1.15 ± 0.55	4.684	0.0000
MCV (µm ³)	333.82 ± 26.27	258.77 ± 20.15	6.801	0.0000
MCH (pg)	71.53 ± 6.18	76.33 ± 3.39	- 2.059	0.0603
MCHC (%)	22.16 ± 2.35	30.63 ± 2.53	- 7.350	0.0000
Thrombocyte (million/mm ³)	0.14 ± 0.01	0.20 ± 0.02	- 1.121	0.2913

Table 5.7 Comparison of haematological values for control and infected C. carpio at stage 3 (Mean \pm S.D.)

Parameters	Infected stage 3 (N = 10)	Control stage 3 (N = 10)	"t" values	Level of Significance
RBC (million/mm ³)	0.89 \pm 0.14	1.29 \pm 0.17	- 5.458	0.0000
WBC (thousand/mm ³)	9.55 \pm 1.74	7.30 \pm 0.56	3.658	0.0044
Hc (%)	29.10 \pm 1.92	32.00 \pm 1.79	- 3.314	0.0041
Hb (g/100 ml of blood)	6.40 \pm 0.56	8.54 \pm 1.03	- 5.499	0.0000
ESR (%)	2.50 \pm 1.08	1.90 \pm 0.48	1.512	0.1565
MCV (m ³)	336.49 \pm 58.14	251.19 \pm 29.61	3.922	0.0018
MCH (pg)	74.00 \pm 19.37	66.56 \pm 6.85	1.256	0.2351
MCHC (%)	22.91 \pm 3.48	26.63 \pm 2.26	- 2.663	0.0177
Thrombocyte (million/mm ³)	0.21 \pm 0.07	0.14 \pm 0.03	2.719	0.0175

Table 5.8 Comparison of haematological values for control and infected C. carpio at stage 4 (Mean \pm S.D.)

Parameters	Control stage 4 (N = 10)		"t" values		Level of Significance
	Infected stage 4 (N = 10)	Control stage 4 (N = 10)			
RBC (million/mm ³)	0.91 \pm 0.13	1.40 \pm 0.05	-	10.327	0.0000
WBC (thousand/mm ³)	11.95 \pm 2.69	7.00 \pm 1.24		5.014	0.0000
Hc (%)	26.90 \pm 2.30	35.70 \pm 2.53	-	7.718	0.0000
Hb (g/100 ml of blood)	6.70 \pm 0.61	8.36 \pm 0.70	-	5.354	0.0000
ESR (%)	2.39 \pm 0.92	1.28 \pm 0.64		2.957	0.0093
MCV (μm^3)	299.75 \pm 23.77	254.51 \pm 15.01		4.827	0.0000
MCH (Pg)	74.71 \pm 6.87	59.67 \pm 5.39		5.166	0.0000
MCHC (%)	24.95 \pm 1.66	23.46 \pm 1.81		1.826	0.0854
Thrombocyte (million/mm ³)	1.14 \pm 0.02	0.18 \pm 0.04	-	2.952	0.0121

Table 5.9 Comparison of haematological values for control and infected C. carpio at stage 5 (Mean \pm S.D.)

Parameters	Infected stage 5 (N = 10)	Control stage 5 (N = 10)	"t" values	Level of significance
RBC (million/mm ³)	0.88 \pm 0.09	1.17 \pm 0.12	- 5.806	0.0000
WBC (thousand/mm ³)	8.15 \pm 2.19	5.50 \pm 1.53	2.973	0.0090
Hc (%)	24.9 \pm 2.12	34.2 \pm 2.99	- 2.324	0.0425
Hb (g/100 ml of blood)	6.33 \pm 0.65	10.05 \pm 0.57	- 2.636	0.0271
ESR (%)	-	-	-	-
MCV (μm^3)	283.94 \pm 26.51	293.40 \pm 34.37	- 0.792	0.4399
MCH (pg)	72.34 \pm 8.60	86.83 \pm 7.00	- 3.921	0.0011
MCHC (%)	25.62 \pm 3.59	29.59 \pm 3.65	- 2.324	0.0328
Thrombocyte (million/mm ³)	0.10 \pm 0.03	0.17 \pm 0.01	- 1.502	0.1590

Hilge (1980) etc.. These authors reported RBC counts ranging from 0.84 to $2.96 \times 10^6 \text{ mm}^3$, haematocrit values from 24.5 to 37.5%, haemoglobin concentrations from 5.8 to 10.5g and white blood cell counts from 1.45 to $10.4 \times 10^3 \text{ mm}^3$ for clinically normal fish (Table 5.10.) It can be seen from this table that even within clinically normal fish the range of values varies. The values for the normal fish in the present study fall within the ranges found in the literature despite the large variation in size, age and techniques reported.

Seasonal changes could affect the blood parameters of fish since physiological condition of the fish depends mainly on the surrounding medium. Seasonal studies on blood of fish were reported by Ezzat et al. (1974), Denton and Yousef (1975), Hattingh (1976), Vuren and Hattingh (1978) etc.. In these reports little distinction has been made between results obtained from fish in the field or from laboratory aquaria, Fourie and Hattingh (1976) and Van Vuren and Hattingh (1976) though, studied the seasonal changes in laboratory acclimated carp and yellowfish, Barbus holubi, the sources of the fish were different and the temperatures were uncontrolled, thus they cannot be compared with the present study.

Murachi (1959) looked at seasonal variations and reported that the blood of carp possessed lower haematocrit and haemoglobin in winter but a reverse pattern of responses in the blood of wild carp was observed by Vuren and Hattingh (1978) where peak haemoglobin and haematocrit occurred in the colder season. The latter authors also observed that the number of leucocytes peaked during summer months, whilst the number of erythrocytes were more variable and showed a different seasonal peak. ESR and MCH were both higher in the winter.

Table 5.10 Haematological values (mean) of clinically normal Cyprinus carpio reported in the literature with the values of clinically normal carp of the present study.

Mean weight (g)	RBC (million/ mm^3)	WBC (thousand/ mm^3)	Thrombocyte (million/ mm^3)	Hc (%)	Hb (g/100 ml)	MCV (μm^3)	MCH (pg)	MCHC (%)	Reference(s)
26.82	0.842	3.67	-	31.3	10.5	311	72.0	-	Field et al., 1943
212-304	1.43-2.96	1.45-7.84	-	30.54-42.47	5.67-10.0	125.0-301.4	32.4-61.6	18.3-29.1	Murachi, 1959 Fourie and Hattingh, 1976
over 400.0	1.43 (17°C) + 0.13	-	-	24.5 (20°C) + 1.0	5.8 (20°C) + 0.3	183.0 (27°C) + 5.0	48.0 (20°C) + 1.0	-	Houston and De Wilde, 1968
400-600	1.012	37.25	-	34.1	-	-	-	-	Hines and Yashouv, 1970
751-1114	1.22-1.78	3.3-10.4	-	21.4-43.3	5.5-8.6	141-309	42.3-67.3	23.9-31.9	Vuren and Hattingh, 1978
7.65-20.39	1.17-1.40	5.50-7.50	0.14-0.20	32.0-35.8	8.36-10.57	251.19-293.40	59.67-86.83	23.46-30.63	Present study

The values of the blood parameters at different seasons described in the literature are so contradictory that no pattern could be defined. The results were complicated by variations in species, age, sex, diet and environmental temperature etc. of the fish studied as well as the differences in wild and cultured fish.

In the present investigation, no seasonal fluctuations were evident throughout the experiment. This was to be expected since all fish were kept in a constant environment. The constant environment eliminated any changes due to seasonal fluctuations, thus revealing differences most likely to be due to the progress of the disease itself. The results are in good agreement with data given by Hilge (1980) who found no seasonal fluctuations in the haematological values of mature carp held in a warm water system.

In some species age and particularly breeding activity seem to be determining factors affecting haematological parameters (Murachi, 1959). In the present study this was not a factor as all fish used were immature. To understand individual variations and different seasonal responses Van Vuren and Hattingh (1978) have stressed the importance of the water quality. In other words, to get a consistent result, a good water quality should be maintained. In the present study a modified recirculation system and a flow through system were used to maintain a good water quality. Periodic sampling of water from these systems showed that the values of O_2 , CO_2 , pH, NH_3 , temperature etc. were stable throughout the sampling period.

The values for blood parameters of all groups of infected fish were lower than those found for clinically normal fish in the present study. They were also lower than those described by Houston and De Wilde (1968b) and Fouri and Hattingh (1976) for normal fish. The

differences in levels obtained for fish infected with S. inermis resulting in hypochromic macrocytic anaemia were almost certainly due to the effects of infection. This type of anaemia is characterised by a decrease in erythrocyte number, haemoglobin and mean cell haemoglobin concentration with corresponding increase in mean cell volume (Kawatsu, 1969). Kawatsu in a series of papers (1966, 1968, 1969 and 1978) described anaemia of fish due to starvation, repeated bleeding, disease due to parasite and of other origins. Kawatsu, in these papers, has stressed the need to establish a classification of anaemia similar to that which exists for human disease. For example, Komiya (1960) classified the human anaemia into four types depending on the volume index. These are 'normocytic', 'microcytic', 'macrocytic' and 'megalocytic'. On the basis of colour index, human anaemia was again divided into 'hypochromic', 'normachromic' and 'hyperchromic' one.

The anaemic condition of the infected fish might have been brought about by a variety of pathological processes, for example, haemorrhaging both internally and externally were features of several stages in the course of the infection.

Invasion of fish by the cercariae of S. inermis resulted in external haemorrhaging. Internal haemorrhages were observed to occur during the migration of the cercaria and subsequent developmental stages through the tissues of the infected fish (Chapter 5.3). Noticeable difference in the severity of anamia occurred at stage 2, i.e. at the post-egg stage of infection. The significant decrease in MCH and MCHC was possibly due to constant bleeding through haemorrhages externally and internally caused by the activity of the different stages of the worm. The loss of blood disturbs the iron

balance of the body, thus resulting in non-availability of iron which eventually reduces the haemoglobin synthesis (Mahajan et al., 1979). Hoffman and Lommel (1984) described a mild anaemia in Salmo gairdneri and Leuciscus idus due to repeated blood sampling from these fish. The anaemia demonstrates a decrease of RBC counts, haemoglobin and PCV.

Probably the major contributory factor in the anaemia at different stages of infection was haematopoiesis. Haematopoiesis in the infected fish was probably disturbed by the direct physical damage to the kidney and spleen due to the migration of the worms through these tissues. At the most critical stage 2, large numbers of eggs occur in the kidney. Eggs blocking capillaries, glomeruli etc. and possibly miracidial hatching in kidney causes further damage. The occurrence of granulomas (Chapter 5.3) due to the presence of degenerating eggs in the spleen and kidney might also reduce the haemopoietic activity, thus resulting in the decrease of packed cell volume and oxyhaemoglobin concentration. The formation of granulomas in response to egg/miracidia in kidney at later stages, i.e. stages 3 and 4, prolonged the anaemia and possibly inhibited recovery. Decreased erythropoiesis caused by the destruction of haematopoietic tissue of the kidney by S. klamathensis was also reported by Evans (1974b).

A third contributory factor to the anaemic condition might be haemodilution resulting from osmoregulatory failure. Damage to the epithelium of skin and gill due to cercarial invasion, emergence of miracidia etc. would result in the imbalance of osmoregulatory process and in some acute cases be a direct cause of death.

The anaemia in fingerlings which had a long term S. inermis infection could also be attributed to other factors as well as those proposed above, such as starvation due to loss of appetite (Chapter 5.1). Blaxhall (1972) reported that carp starved for several weeks gave abnormal blood values. Kawatsu (1966) also described a microcytic anaemia of rainbow trout caused by starvation. Starvation caused a definite fall in various haematological values of Clarias batrachus, Heteropneustes fossilis and Channa punctatus (Joshi, 1979). Erythrocytes, thrombocytes and neutrophils were reported to be most sensitive to starvation. From five weeks onwards of starvation, a sharp decline in these cells in the blood of Channa punctatus were reported by Mahajan and Dheer (1983).

The white blood cell counts for all stages of the infected fish were elevated above those of control fish. They were also high compared to the number described by Hines and Yashouv (1970), Fouri and Hattingh (1976) and Vuren and Hattingh (1978) and two to three times higher than those claimed by Field et al. (1943) for normal carp. Leucocytosis was observed throughout the experiment indicating a response to the cellular injury resulting from the migration and subsequent activity of the worm in the tissues of the fish. Kawatsu (1968) produced evidence that the number of leucocytes increased immediately after experimental blood was removed from rainbow trout but decreased to the original level after the 6th sample. The present study has shown that the number of leucocytes was at its highest during the initial stage of infection (12 weeks post infection) and was $12.44 \times 10^3 \text{ mm}^3$ but reduced over the infection period, so that in the chronic phase, it was down to near normal values, $8.15 \times 10^3 \text{ mm}^3$.

Leucocytosis in the present study may well be in part a response

to the continued haemorrhaging resulting from the activity of the miracidium as well as the extensive tissue damage by the presence of the parasite. Naumova (1961a) also reported leucocytosis in carp fingerlings infected with S. inermis. In her study the leucocyte number was doubled. Kazadaev (1954) cited by Dogiel et al. (1961) reported that S. armata in tench caused a doubling of polymorphonuclear agranulocyte count and a slight rise in the monocyte and neutrocyte counts. In the present study no differential white cell counts were made but it is likely that there would be an increase in the cells of the monocyte series in response to the parasite products and tissue damage.

The thrombocytes have long been known to be responsible for the clotting process and this phenomenon has been well described by Wardle (1971). The reason for the steep increase in thrombocyte numbers at stage 3, i.e. six months after the initial infection, is not clear. However, this was the period when a lot of worms had died and the egg production was low. Since the function of thrombocyte mainly, is clot formation, one could suspect their presence in highest numbers at stage 2 when maximum haemorrhages occurred. It may be that the stimulation of thrombocyte numbers at stage 2 in response to the blood loss was not evident in the circulating blood owing to their accumulation at the clotting site. This may be followed by a lag phase in which the clotting requirement has been reduced, reflected by a corresponding decrease in thrombocyte numbers. Otherwise an increase in thrombocyte numbers may indicate some stress factor (Ellis 1977) or even indicate the phagocytic activity as reported by Yokoyama (1960), and Fange (1968) and cited by Ellis (1977).

Raised ESR was observed in infected groups of the present study.

It has been known that ESR is usually raised with increased tissue destruction both in acute and chronic infections, heavy metal poisoning, nephritis etc. (Blaxhall, 1972). Brook trout, Salvelinus fontinalis infected with furunculosis always showed raised rates of fall (Schumacher, Hamilton & Longtin, 1956). In human medicine the ESR is a non-specific reaction giving a measure of the presence and intensity of disease processes in the body (Baker & Silverton, 1976).

The plasma of the carp used in the present study was colourless. Contrary to this, Field et al. (1943) reported that carp had yellow plasma. It is unlikely that this has any disease significance but was possibly because of the food used, since Ezzat et al. (1974) claimed that the diet had a direct effect on the blood constituents. The carps of this study were fed on trout pellet, whereas Field et al. (1943) used yellow corn to feed their fish.

Changes in the blood parameters of S. inermis infected fish had already occurred at the initial stage of infection and were evident at the first sampling. These changes were sustained throughout the experiment. During the active stage of the infection, it appears that stage 2 is the most critical stage. At this stage, hypochromic macrocytic anaemia occurred with highest white cell number and highest ESR. During the stage 2 of infection, numerous miracidia which developed in the gill make their way out through the soft gill tissues.

In general, there is a paucity of information concerning the blood parameters of diseased fish. Saprolegnia diclina infected brown trout, Salmo trutta showed a significant decrease of major ions in the serum (Richards & Pickering, 1979). Karpenko and Vaslyushko (1961) found carp with infectious dropsy of viral etiology showed a

decrease in erythrocyte number and haemoglobin content with a doubling in leucocyte number. The result obtained from the infections of S. inermis are in good agreement with these findings. According to Watson, Guenther and Royce (1956) in a study of virus-diseased sockeye salmon, Onchoryhnchus nerka, haematocrit values fell to 16% by the 8th day after exposure to the virus compared to 47% in the controls. Anderson and Conroy (1970) pointed out that fish suffering from a Vibrio infection showed marked anaemia characterised by lowered red cell counts, PCV and haemoglobin concentrations. Thorpe and Roberts (1972) observed neutrophils in the inflammatory exudate caused by an aeromonad infection in brown trout.

The interaction of haemoparasites with fish blood is even less well studied. Khan (1977) has reported lower levels of haemoglobin and haematocrit, and higher ESR values with increased parasitemia in Atlantic cod, Gadus morhua, after an infection with Trypanosoma murmanensis. Similar results on blood parameters of carp have been reported by Lom and Suchaukova (1974) following an infection with T. danilewsky.

Hines and Spira (1973a) while observing leucocyte response of carp infected with the protozoan ectoparasite, Ichthyophthirius multifiliis, found changes in differential leucocyte counts though the overall leucocyte number in infected fish was normal.

Some of the external parasites appear to cause severe anaemia, even though the parasite burden is small. Kawatsu (1978) found hypochromic microcytic anaemia in crucian carp which resulted from the infection by the blood feeding digenean Diplozoon nipponicum. As the number of parasites increased there was a corresponding decrease in the haemoglobin level.

The effects of the digenean Isoparorchis hypselobagri on an air-breathing fish, Channapunctatus were investigated by Mahajan, Agrawal, John and Katta (1979), who found a significant decrease in haemoglobin and haematocrit values resulting in hypochromic macrocytic and normochromic-normocytic anaemia with an increase in total leucocyte number. These parasites do not inhabit the blood, though some may well feed on blood products.

In a similar study related to blood flukes Smitherman (1964) (cited by Evans, 1974b) reported decreased PCV in bluegill, Lepomis macrochirus infected with a trematode larvae Posthodiplostomum minimum.

Until now, very little data have been available on the blood profile of carp infected with S. inermis. Naumova (1961a) reported anaemia in young carp suffering from S. inermis infection. She reported that the level of haemoglobin and the number of erythrocytes in infected carp were noticeably lower than the controls. The number of leucocytes in the fish suffering from S. inermis infection was twice as much as in the controls. It appears that Naumova's (1961a) work on the blood parameters was carried out during a single stage of the infection. Karrar (1979) investigated the blood values of carp infected with S. inermis at two separate stages of infection, and described a normocytic anaemia (Table 5.11). Her results were however, complicated by infection with other haemoparasites as well as ectoparasites and the infection levels were considerably lower than those found in the fish during the present study.

The present study defines the blood parameters of at least four different well defined stages of S. inermis infection in fish free from other pathogens and shows that the values of different parameters change at the very earliest stage of infection. Signs of recovery were only apparent after one year of active infection.

Table 5.11 Comparison of haematological values (mean) between present study and a similar study by Karrar (1979) on S. inermis infected Cyprinus carpio

Parameters	Control group		Sanguinicola infected group	
	Karrar (1979) (N = 22)	Present study (N = 50)	Karrar (1979) (N = 15)	Present study (N = 50)
Length (cm)	7.9	6.94	7.0	6.37
Weight (g)	8.2	12.64	5.6	8.24
Age (months)	9	6-15	9	6-15
RBC (million/mm ³)	0.94	1.33	0.79	0.87
Hc (%)	35.50	34.62	31.30	27.80
Hb (g/100 ml of blood)	7.26	9.62	5.90	6.32
ESR (%)	-	1.50	-	2.74
MCV (µm ³)	375.00	261.96	403.00	321.97
MCH (pg)	76.00	72.93	80.00	72.66
MCHC (%)	18.00	27.99	20.00	23.15
Thrombocyte (million/mm ³)	0.71	0.18	0.34	0.36

Haematological techniques:

Fish held in a closed water system were exposed to conditions that differed from those of natural environments. Since blood parameters are directly related to the environmental conditions in which the animal lives, it is invalid to compare the blood parameters of these fish with those of fish from different areas of the world. In fish haematology, the most important aspect is the determination of normal values. The normal and non-pathological values must be known in order to recognise the pathological states involved (Hesser, 1960; Hickey, 1970). That is why consideration was given to the comparison of the values of the infected fish with those of the controls of the same study.

The various experimental errors which can occur with the use of haematological techniques in fish have been stressed by Bouck and Ball (1966), Love (1970), Blaxhall (1972), Blaxhall and Daisley (1973), Hattingh (1973 and 1975). The method of capture and time which elapsed before blood sampling is extremely important (Van-Vuren and Hettingh, 1978). Fish in this study were always acclimatised to the laboratory and this reduced the variation in results due to handling/transportation.

Techniques employed in the present study for the determination of haematological parameters were essentially the same as those described by Blaxhall (1972) and Blaxhall & Daisley (1973) with the exception of the use of MS 222 and EDTA as anaesthetic and anticoagulant respectively.

Use of MS 222 as an anaesthetic was discontinued in the present study since its action involves neuro-endocrine stimulation (Ferreira,

Smit and Schoonbee, 1981). It has also been shown by Wedemeyer (1970) that unbuffered MS 222 is a powerful stressing agent for fish.

Heparin as an anticoagulant was chosen because of its stability in haematological values for carp (Smit and Hattingh, 1980). Heparin is distinguished as a most suitable and well known antithrombin anticoagulant for the blood of freshwater fish when used for haematocrit, haemoglobin and sedimentation rate techniques (Hesser, 1960; Klontz and Smith, 1968; Hattingh, 1975 and Smit & Hattingh, 1980). Above all coating of syringes and capillary tubes with heparin was carried out easily since it is readily soluble in water.

To determine haemoglobin, the cyanmet-haemoglobin method was used. It was found to be an accurate method of determination of haemoglobin in carp as the absorption curves of cyanmethaematin derived from haemin and from haemoglobin agreed within the range of maximum absorption band 545 nm (Murachi, 1959). The superiority of this method was also described by Larsen and Snieszko (1961), Blaxhall (1972) and Blaxhall and Daisley (1973) because of the consistency of the results, the stability of the reagent, and the commercial availability of the standards.

5.3 Pathological changes

5.3.1 Introduction

Members of the genus Sanguinicola have been known to cause considerable pathological change in cultured fish (Bauer et al., 1973). Most of the earlier work on the pathology of this genus has been carried out on carp by German, Czech and Soviet workers Schäperclaus, 1954; Layman, 1957; Chechina, 1959; Naumova, 1961a; Lucky, 1964; Htond et al., 1977, and more recently by workers in the USA (Wales, 1958; Evans, 1974a and Schell, 1974) on salmonids.

From the literature, it appears that severe disease caused by Sanguinicola can occur in an acute and fatal form, or in a chronic form which is rarely fatal. As categorized by Layman (1957) for S. inermis in carp, the acute branchial form occurs mainly in fry and yearlings, whereas the chronic form appears mainly in the older fish. In addition to the acute and chronic form, Lucky (1964) described a condition of the disease in carp which considered it to be a pre-acute form whereby no pathological changes could be seen. However, he considered gross changes only and did not report findings in infected fish at the tissue level.

To date there has been little study of the effects of the initial infection, which gives rise to the pre-acute stage, particularly the invasion of Sanguinicola sp. cercariae either from natural or from experimental infections. Most of the pathological studies have been performed only when the mortality was at a peak, i.e. 2-3 months after the onset of infection, or when the infection became severe enough to draw the attention of researchers. To identify the specific effects

of S. inermis at various stages it would be necessary to study the pathogenesis in experimentally infected fish. This study was thus undertaken to examine the various effects of the disease on fish at known stages of infection.

5.3.2 Materials and methods

Source of fish

Natural and experimentally infected fish were taken into account for the study of histological changes due to infection with S. inermis. Fish were sampled on the basis of age of infection and stage of infection.

Fixation and histological techniques

Anaesthesia (Chapter 5.2) was employed prior to sampling. Most of the fish were fixed whole after being cut into small blocks of suitable sizes. From some fish, organs were dissected out and fixed separately. The organs removed always included gill, heart, brain, kidney, liver, spleen and gall-bladder.

All tissues were fixed in 10% neutral buffered formalin. Processing of the tissue blocks was achieved using an automatic tissue processor over a period of 20 hours. The programme is shown in Appendix 6. Prior to sectioning, waxed blocks were decalcified for approximately two hours. Blocks were cut at 5 μ on a rotary microtome. Sections were then stained routinely in Haematoxylin and Eosin (H & E).

5.3.3 Results

The early stages of the disease could only be observed during experimental infections as naturally infected fish were not available until approximately 60 days after the onset of infection. None of the experimental infections were as heavy as those found to occur naturally owing to the small number of infected molluscs available.

The clinical features and pathological changes observed in the infected fish fell into three distinct patterns characterising the different phases of the infection. These are designated phase I, phase II and phase III. Phase I began from the moment of cercarial invasion up to a period prior to egg production of the worm. Phase II emerged around 90 days post infection and continued approximately up to 150 days. This was the period of high egg production. The third phase emerged at a later stage when the age of infection increased from 4-5 months after initial infection and was still extant at the end of the study period, i.e. 24 months after the initial infection. The descriptions below are arranged according to the phases of infection for convenience. In general, the initial stage of phase I is comparable to the pre-acute phase, whereas phase II is referred to as acute and phase III as chronic stage.

Phase I

Gross changes

Infections during the early period of invasion with low levels of cercarial load showed no obvious clinical signs. However, in a few cases, 4-5 fish were exposed to infected snails continuously for 2-3 hours. These fish showed erratic movements, became restless and died within a few hours of infection. Wet preparations of the freshly dead fish contained numerous cercaria-form worms especially in the preparations from the dermis of fins and from the gill epithelium.

Histological changes

Observations made at the very early stage, i.e. the period of cercarial invasion showed no obvious pathological changes. It should be noted however that the earliest observations were made on laboratory infected fish and these resulted in much lower parasite burdens than natural infections. The worms were thus more difficult to find and only a few specimens were observed. Fish examined at twenty-four hours after infection showed cercari-form larvae lying between the fin rays, in loose connective tissue and usually in the vicinity of the blood vessels (Fig. 5.7). Again, no damage was evident in the locality of the worm. Slight ruptures were observed on the edge of the epidermis which were possibly the result of mechanical damage occurring during the process of invasion. The eosinophil granulocytes appeared to be more numerous at the site adjacent to fin parasite at tenth day of infection (Fig. 5.8).

Prominent signs of a reaction did not appear until 30 days after infection, when several eosinophil granule cells were seen in the vicinity of the worms as seen in Fig. 5.9. Slight separation of malpighian cells of the epidermis and some vaculation of dermis could be seen at this stage.

The epidermis of the infected fish appeared very thin as shown in Fig. 5.10. The reduced epidermal goblet cells and mucous cells were prominent during the pre-egg production stage of infection whilst many of the worms were found in the skin. In some cases marked epidermal erosion up to dermis was also evident.

A severe cellular infiltration by leucocytes, mainly of macrophages and cells closely resembling lymphocytes were seen in the

Fig. 5.7

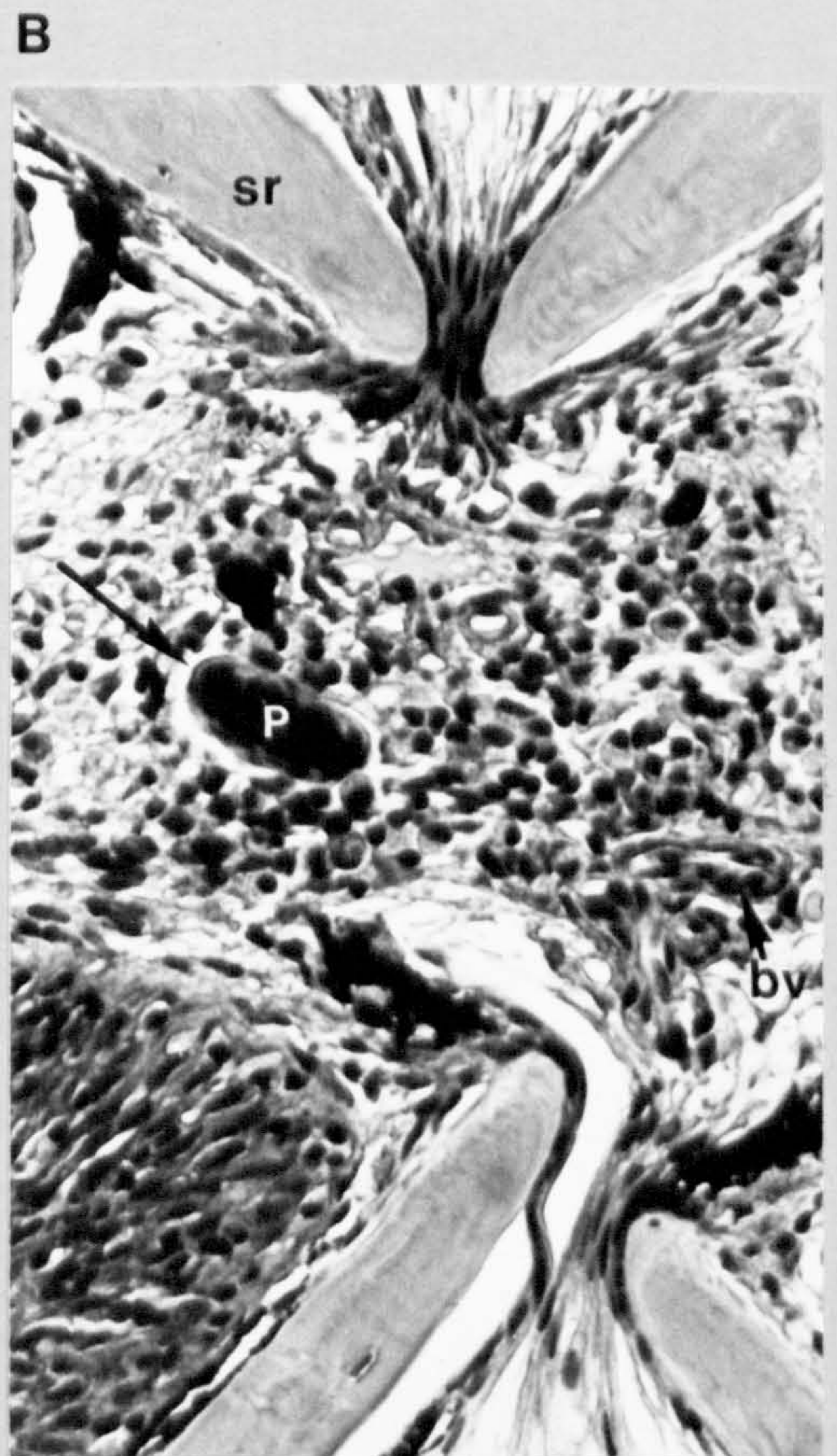
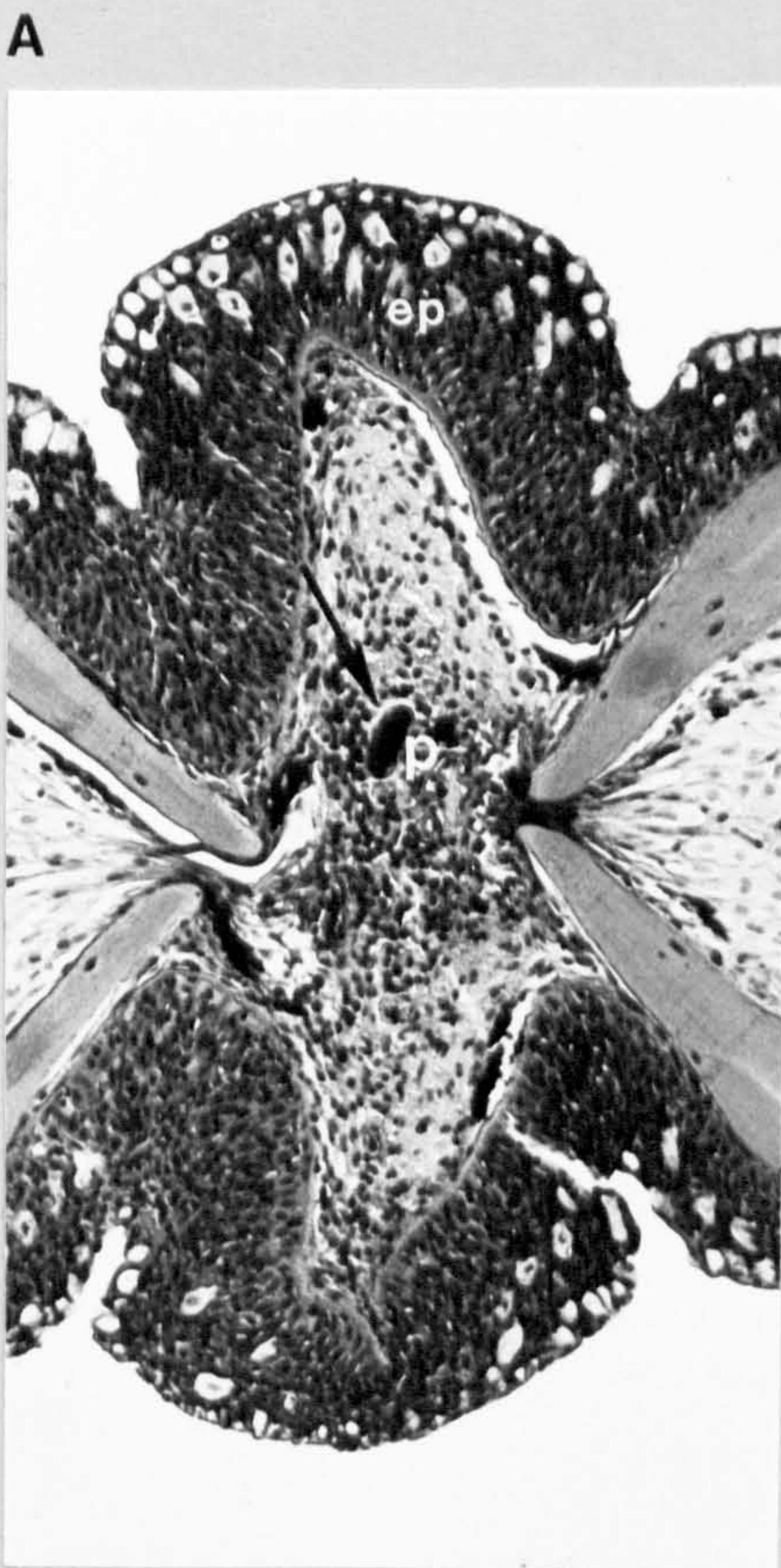
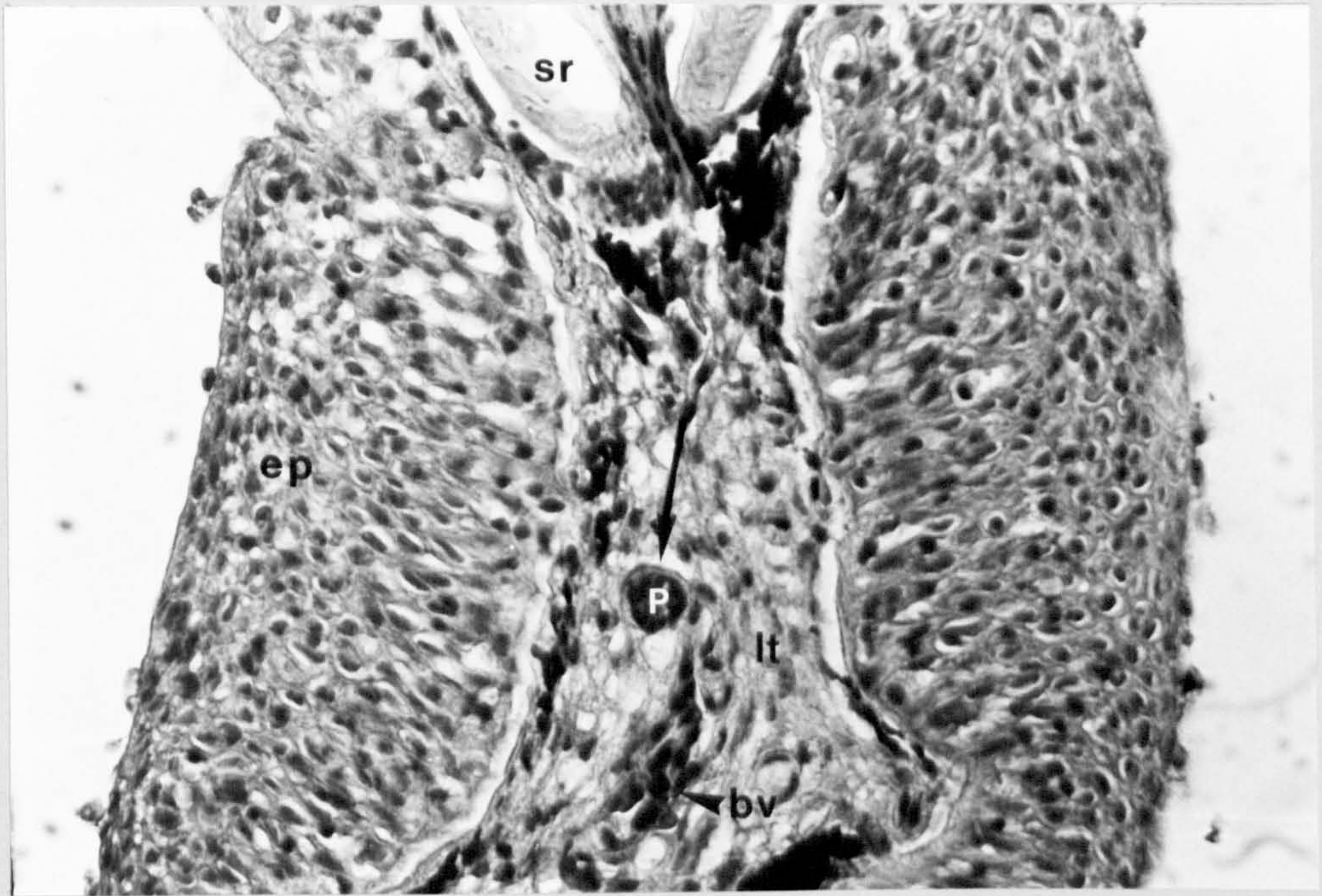
Section of caudal fin at 24 hrs post infection showing S. inermis cercaria migrating through connective tissue (arrowed) (H & E, × 400).

bv : blood vessel
ep : epidermis
lt : loose connective tissue
sr : soft rays

Fig. 5.8

Section of caudal fin at 10th day post infection showing a developing S. inermis with a more flattened body (arrowed). Note the eosinophil granulocytes. (H & E, A, × 100 B, × 250).

bv : blood vessel
ep : epidermis
sr : soft rays



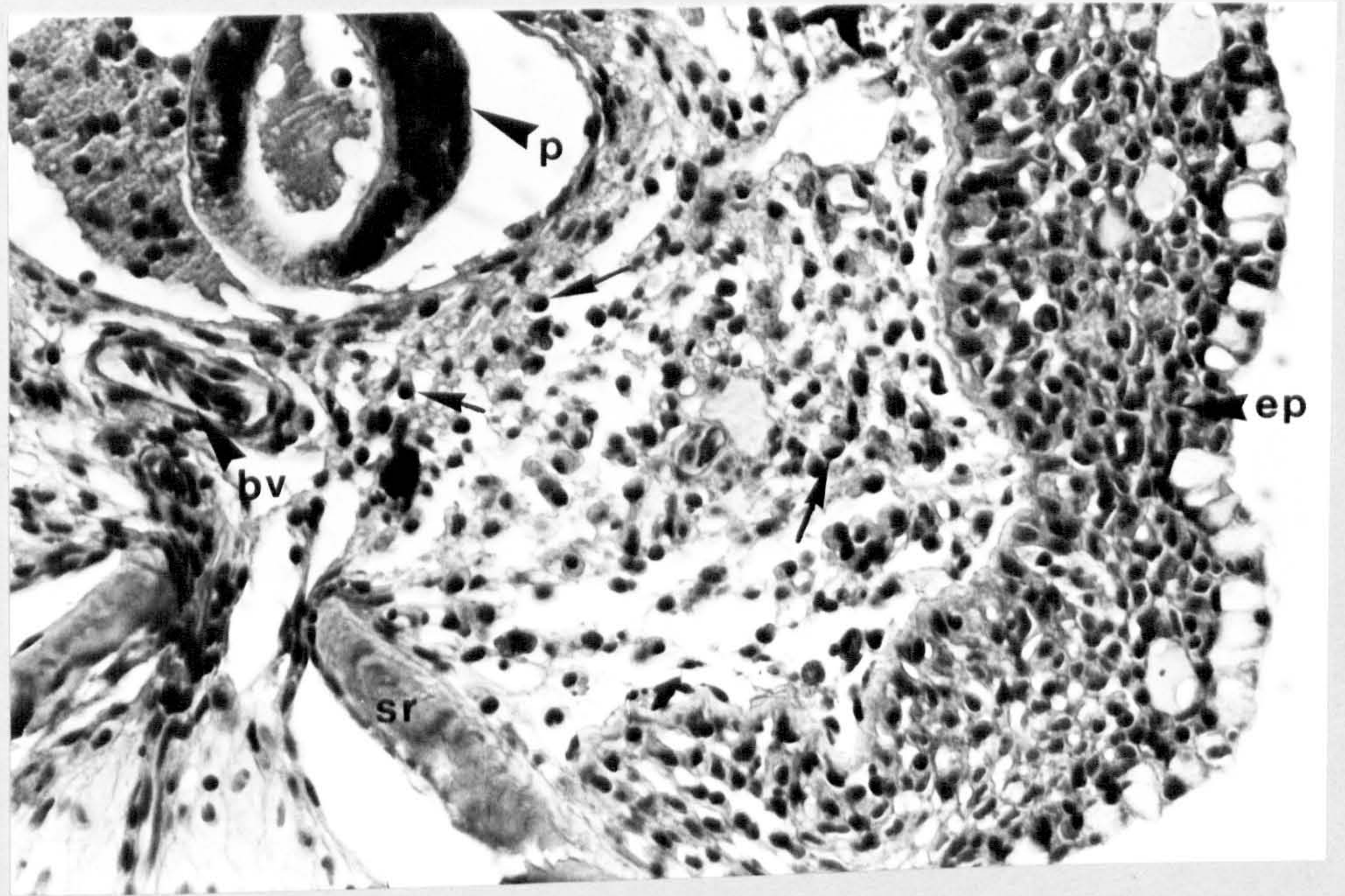


Fig. 5.9

Section of caudal fin at 30th day post infection showing vacuolation of the dermis adjacent to the worm. The degenerated tissue shows numerous eosinophil granulocytes (H & E, $\times 400$).

bv : blood vessel

ep : epidermis

p : parasite

sr : soft rays

Fig. 5.10

A. Section through skin and muscle of C. carpio at 60 days post infection showing reduction of epidermal cell layer (arrowed) and extensive cellular infiltration in the muscle tissues (H & E, × 100).

d : dermis

e : epidermis

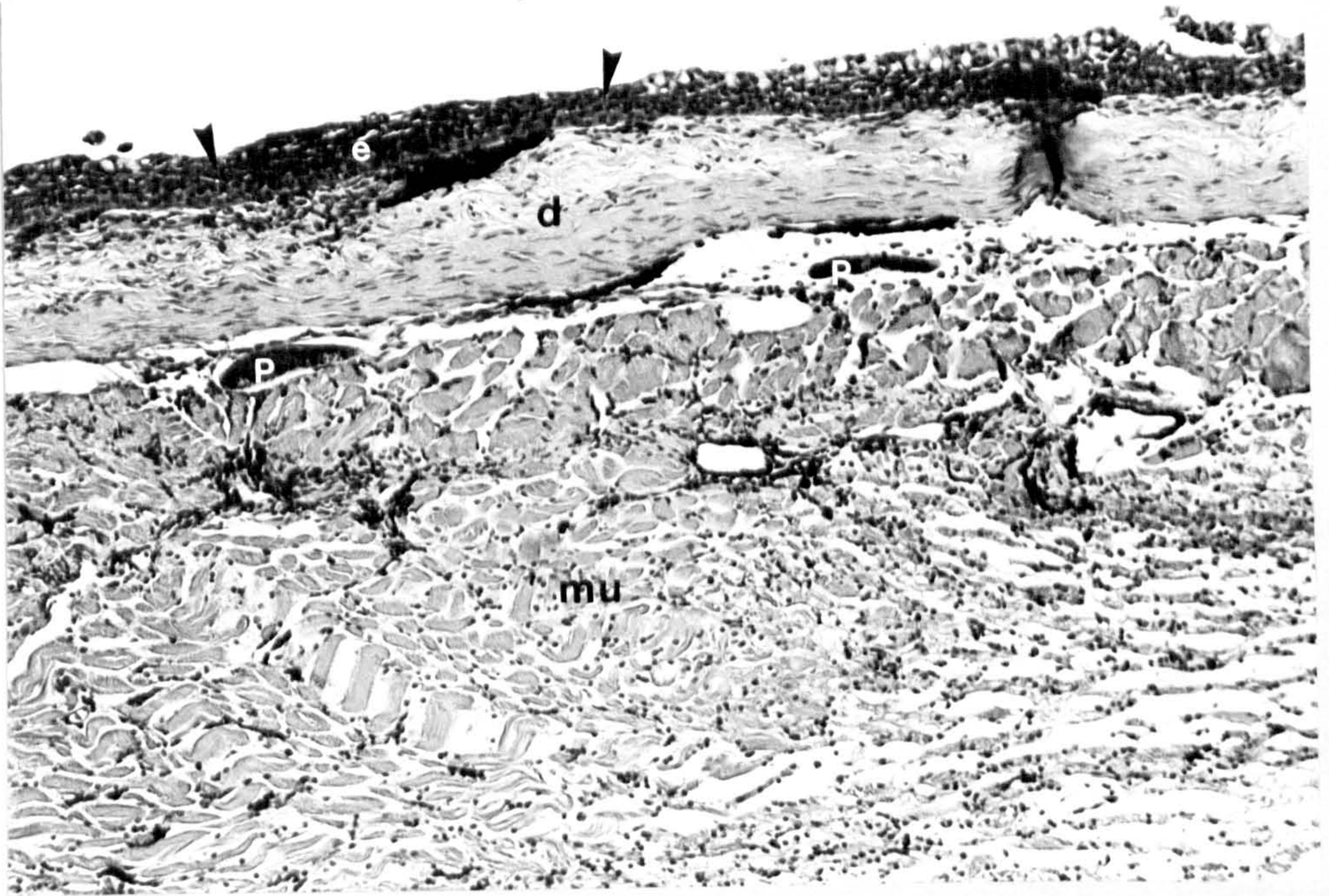
mu : muscle fibre

p : parasite

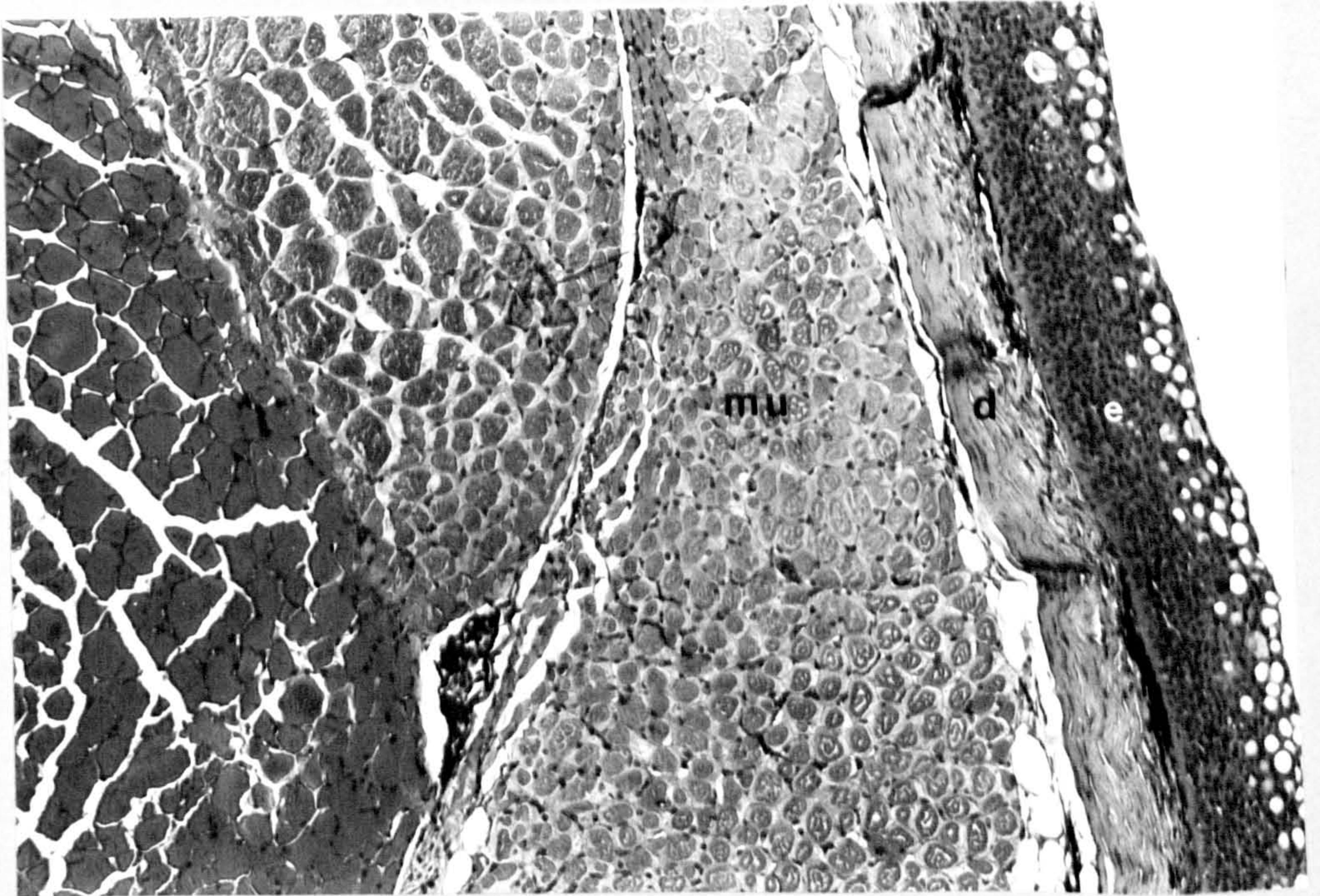
B. Normal tissue for comparison with 5.10A

(H & E, × 100)

A



B



dorsal and ventral muscle tissues as shown in Fig. 5.10. This was a period between 70 and 90 days post infection when most of the migration took place. Cloudy swelling of the muscle fibre in these areas with the disappearance of the striation was a common feature during this stage of infection. Since the majority of the worms migrate during this period, worms were found in larger blood vessels of viscera (Fig.5.11).

Prior to the maturation of the worm, i.e. the period prior to egg production, little pathological change was seen in the tissue of the gills. However, in some cases hyperplasia and haemorrhages were evident in inter lamellar spaces of the gill (Fig.5.12). During this pre-egg production period of the worm, the endothelium of the heart became hyperplastic. This hyperplasia was most marked in the auricle (Fig. 5.13). Leucocyte infiltration of the spaces amongst the muscle fibres of the auricle and ventricle was also evident during this period. Little or no damage was seen in kidneys and other organs.

Phase II

Gross changes

At phase II dermal haemorrhages became visible externally all over the body, particularly the areas near the head and eyes, external surface of the operculum, base of the fins and ventral surface beneath the pectoral fins (Fig. 5.14). Blisters of watery exudate appeared between the dermis and the epidermis in the region around the eyes, operculum and caudal peduncle. Microscopical examinations of these severely affected fish revealed numerous worms from skin preparations. Worms were observed to be most numerous in the areas immediately beneath the watery exudate in the epidermis of the skin. Subsequently,

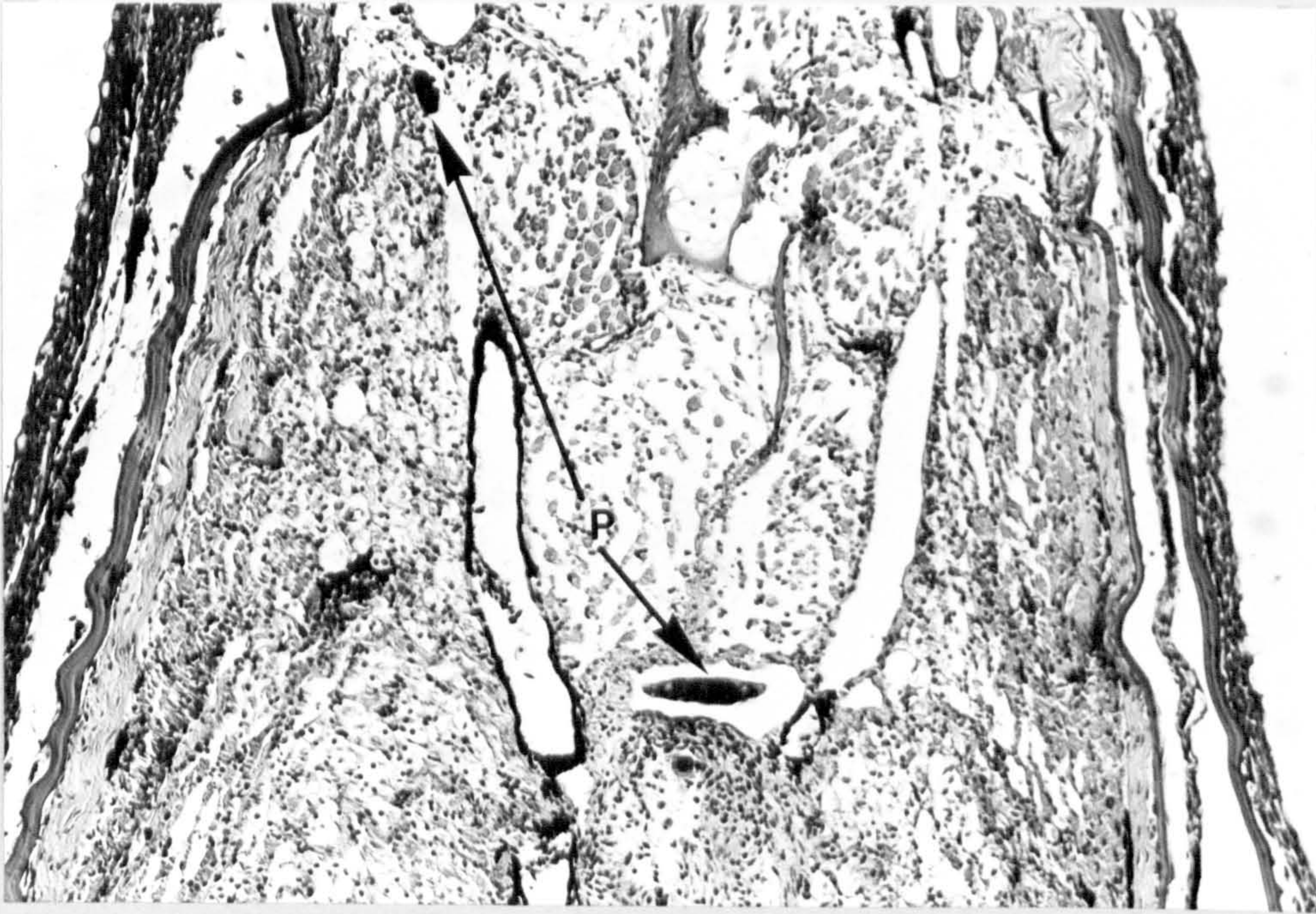


Fig. 5.11

Section through post abdominal region of C. carpio at 80 days post infection showing loss of structure of myotomes of the skeletal muscles and degeneration of muscle fibres

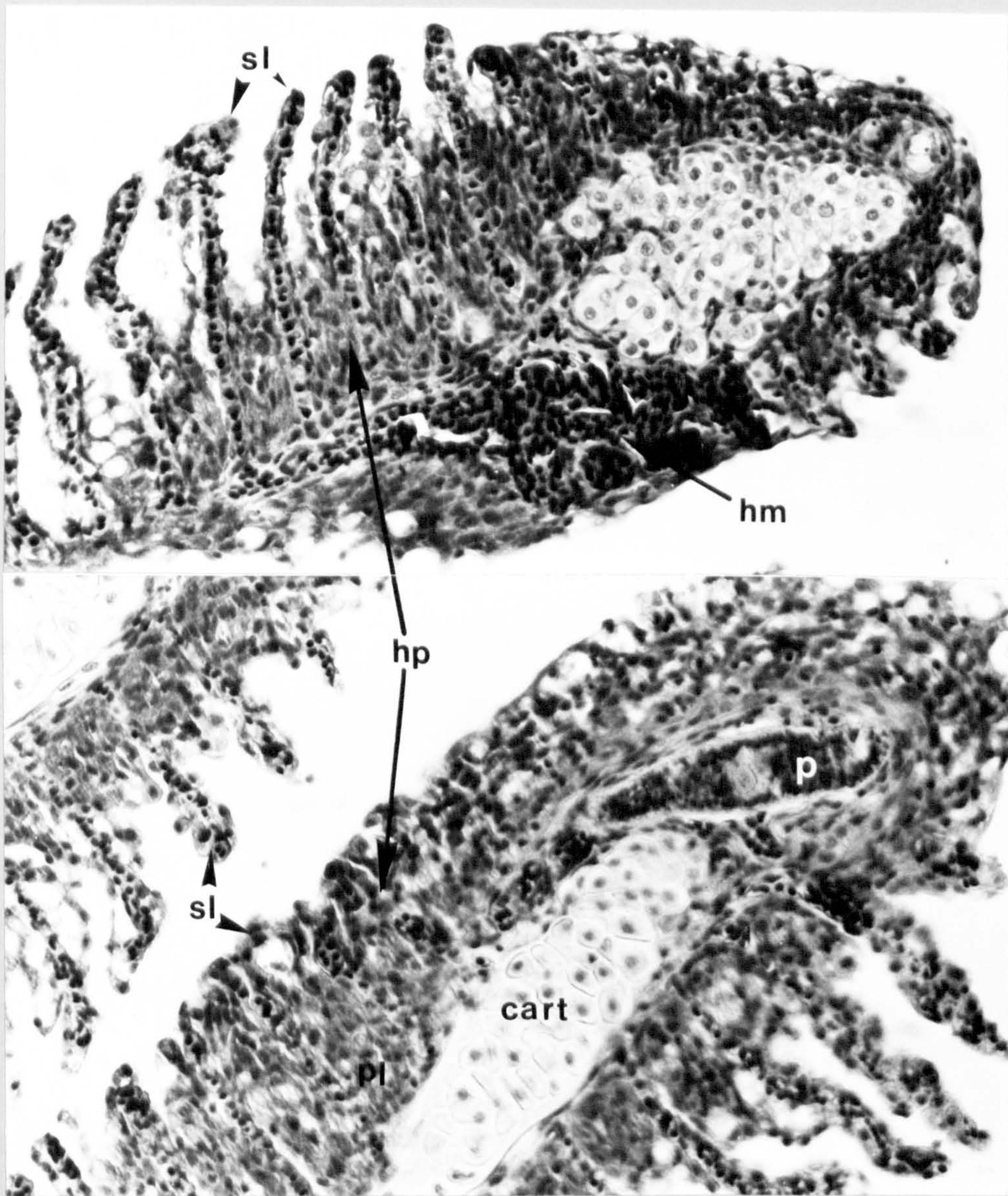
(H & E, $\times 100$).

p : parasite

Fig. 5.12

Section of primary lamellae of gill at 60 days post infection showing hyperplasia in inter lamellar spaces and haemorrhages (H & E, × 250).

cart	:	cartilage
hm	:	haemorrhage
hp	:	hyperplasia
pl	:	primary lamella
sl	:	secondary lamella



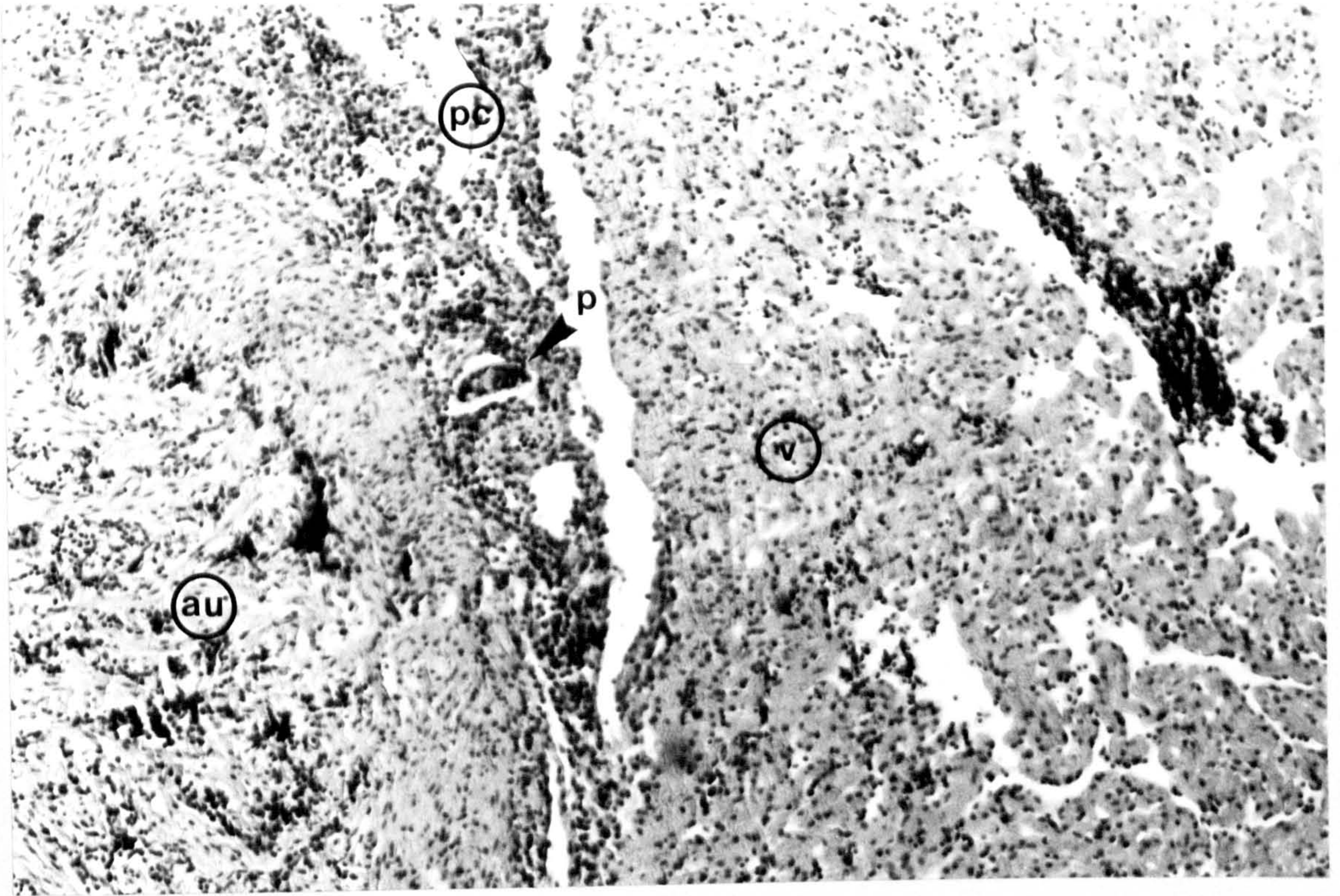


Fig. 5.13

Leucocyte infiltration in the muscle fibres of auricle and ventricle during pre-egg production period of the worm. Note thickened pericardium containing developed worm (H & E, $\times 100$).

au : auricle

p : parasite

pc : pericardium

v : ventricle

the operculum became distended as a result of the swelling of the gills (Fig.5.15). After examination, the gills revealed extensive haemorrhages due to the presence of worms in the major blood vessels and to the release of miracidia from the eggs embedded in the gill tissue. The most dramatic behavioural changes in infected fish were observed during this second phase. At this period, the affected fish showed a loss of equilibrium, swimming slowly, occasionally in a spiral fashion describing a short helical sequence. This time (5/1) ? respiratory distress resulted in frequent surfacing to gulp air. The most affected fish were lethargic and showed increased irritability. They reacted slowly to any external noise and/or disturbances and showed reduced feeding behaviour. During feeding they appeared at first to be attracted to the food item and thus / swam towards it / but soon lost interest and swam away without taking food. Listless fish were also seen to accumulate near the outflow or the inflow of the tank. These sick fish could easily be caught by hand. This was a very critical period for fish as mass mortality occurred during this stage.

Histological changes

Pathological changes during the period of phase II were severe. Haemorrhages and mononuclear infiltration of the dermis accompanied this phase of the infection (Fig. 5.16). This was the period when egg production was at its greatest. In the skin, necrosis of the dermal cells was evident, and in some cases dermal ulceration. Extensive oedema and swollen connective tissue were seen associated either with worms or deposited eggs in the tissues (Fig. 5.16). Fibroblasts aggregated to form a connective fibrous capsule around the deposited eggs in the dermis during this phase and these are shown in Fig. 5.16.

Fig. 5.14

C. carpio at phase II showing dermal and periorbital haemorrhages.

A, lateral view. B, ventral view.

(scale in mm.)

Fig. 5.15

C. carpio at phase II showing distended operculum and swelling of the gill.

(scale in mm.)

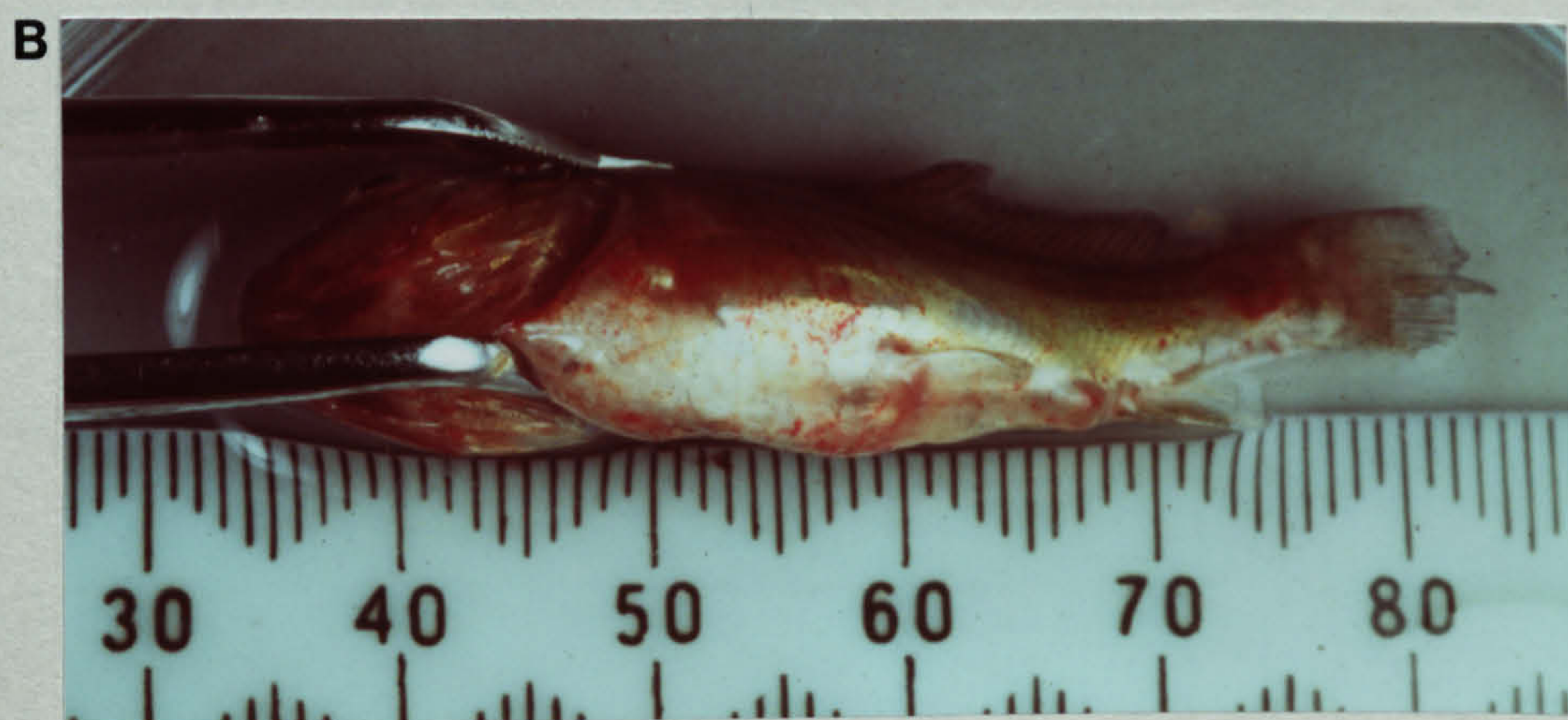


Fig. 5.16

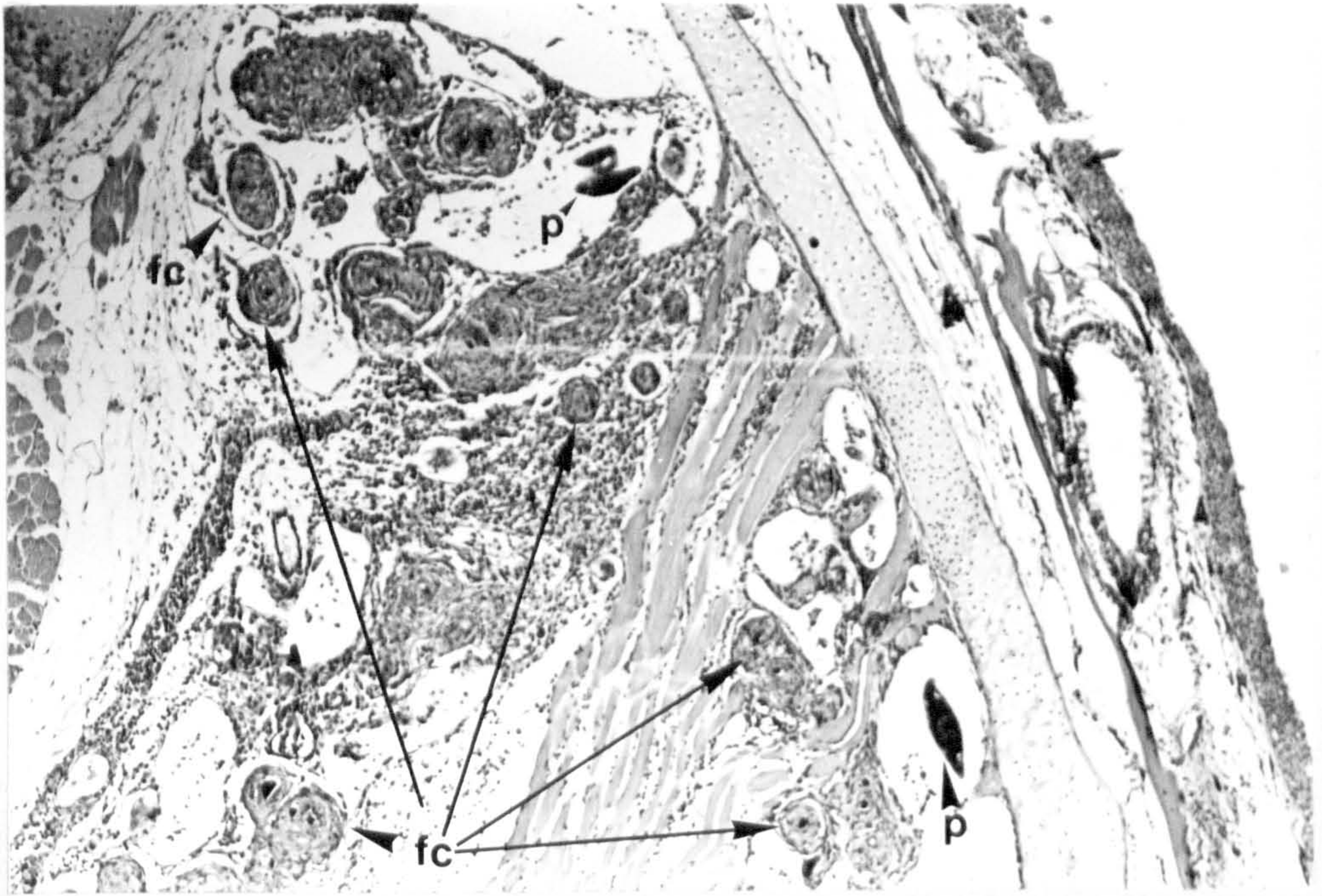
Sections of C. carpio at phase II (during heavy egg production) showing massive cellular infiltration and tissue necrosis in the vicinity of eggs and mature worms.

(H & E, A, × 63. B, × 100. C, × 250.)

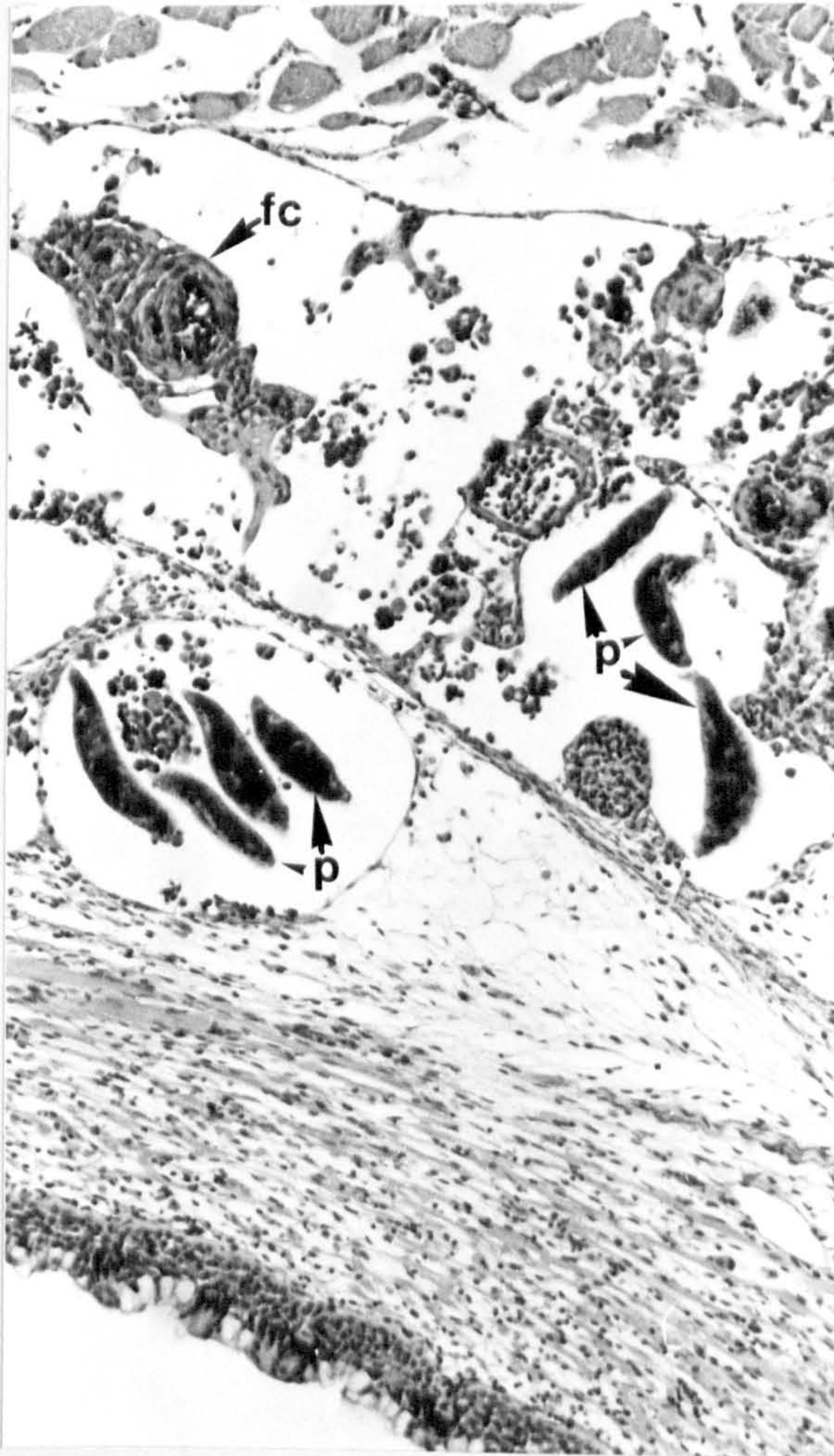
fc : fibrous capsule

p : parasite

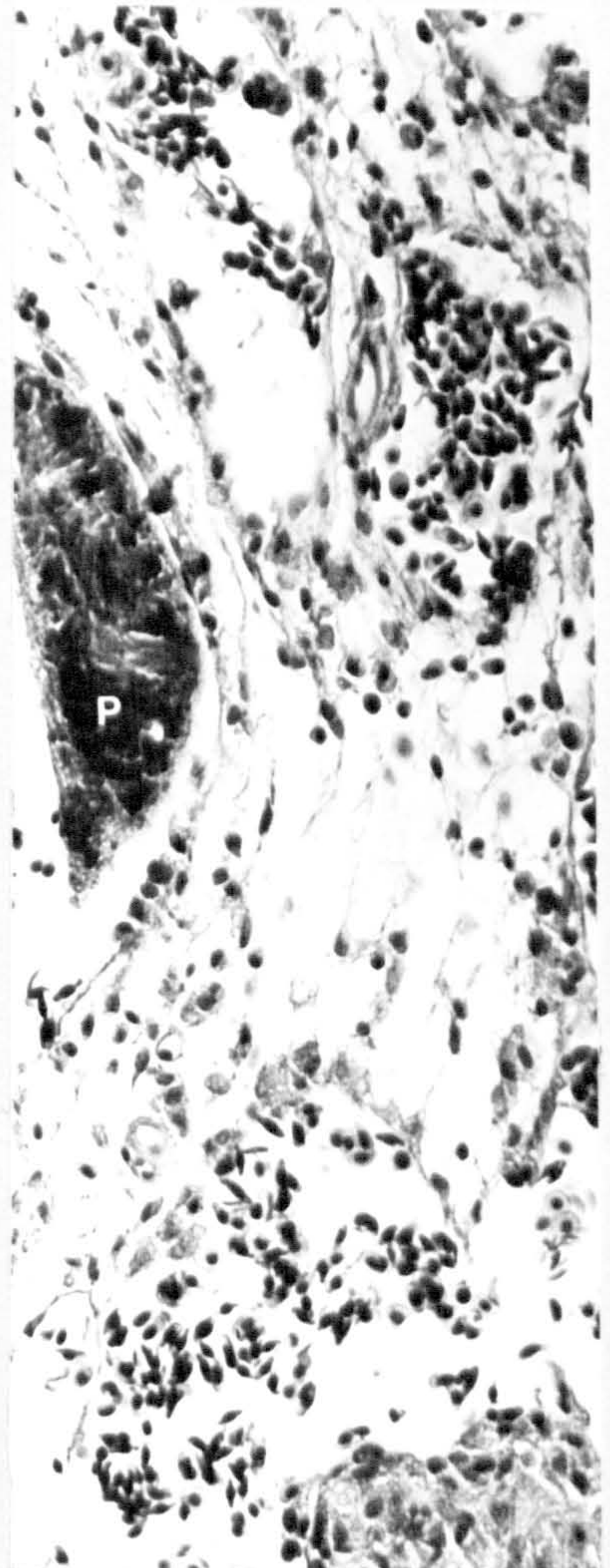
A



B



C



The first signs of effects on the gill appeared in the early stages of phase II and became progressively severe towards the peak of egg production around 120 days post infection. The gill tissues during this phase showed proliferation of epithelium and connective tissue elements causing fusion of secondary lamellae. This marked epithelial hyperplasia in the branchial tissues was due to the presence of the numerous eggs and miracidia (Fig. 5.17). Frequent thrombi could be seen in blood vessels of the lamellae causing secondary lamellae to dilate. During this period of enlarged congested arteries containing several mature worms (up to three worms) were a common feature in tissue sections (Fig. 5.18). In these vessels internal hyperplasia on the inner lining of the blood vessels caused raised areas of cells which project into the lumen thus adding to the reduction in the vessel diameter.

As the infection progressed, the granulomatous inflammatory reaction became more evident in the pericardium (Fig. 5.19) as a result of the deposition of eggs in these areas. As these eggs degenerated in the pericardial connective tissue numerous granulomas were formed as shown in Fig. 5.19. The kidney at phase II showed damage due to the presence of different developmental stages of the worm. Eggs deposited in the circulating blood probably drifted to the kidneys where they accumulated in the glomeruli and adjacent tissues and caused extensive alteration of the tissues (Fig. 5.20). Here the normal tissues of the kidney are displaced by the fibrous tissue as a result of stimulation by miracidia.

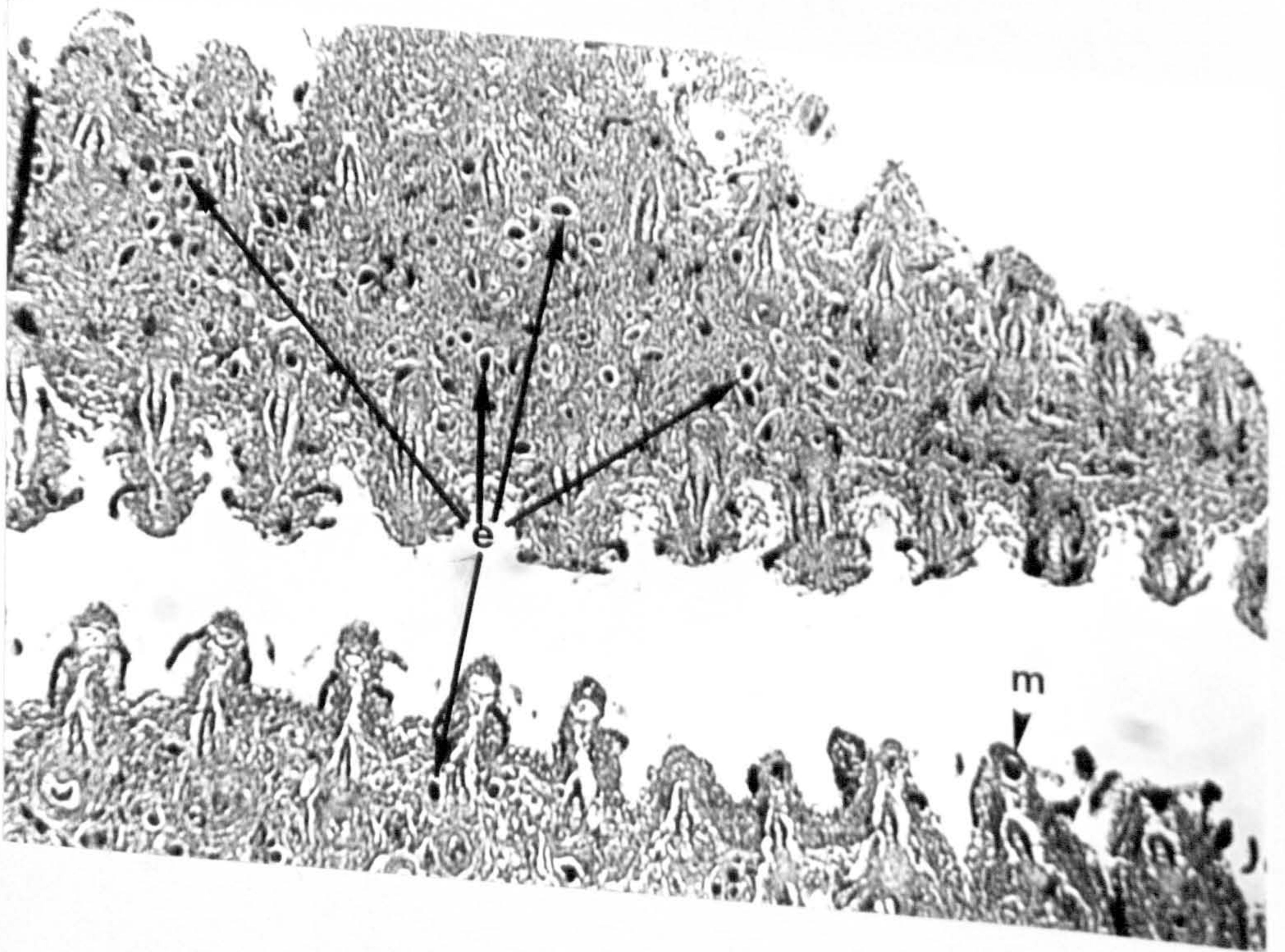
Fig. 5.17

Gill sections during peak egg production period showing breakdown in structure of the lamella. Numerous eggs and developing miracidia are embedded in the hyperplastic tissue.

(H & E, A, × 63. B, × 250.)

- e : egg
- m : miracidia
- pl : primary lamella
- sl : secondary lamella

A



B

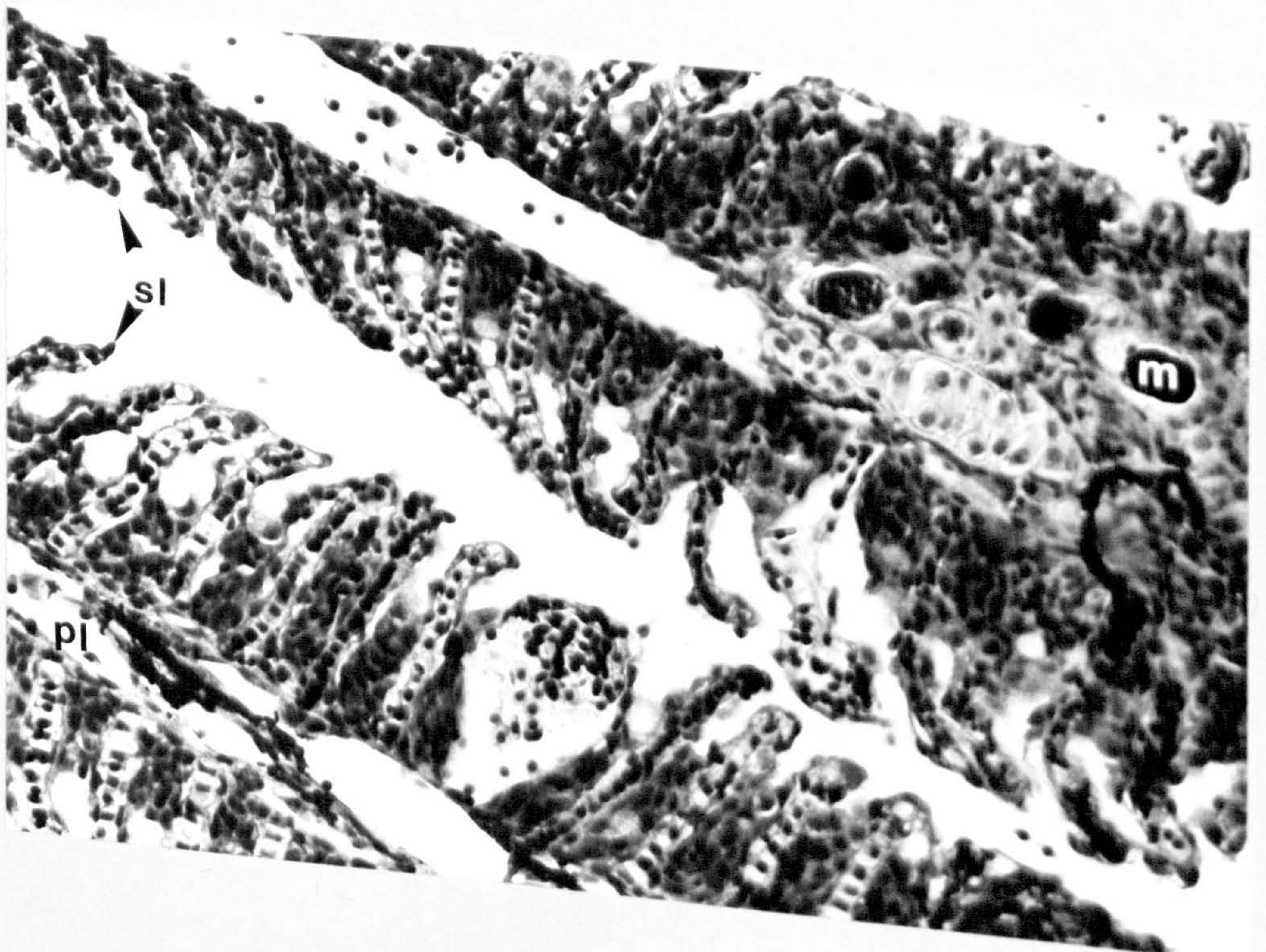


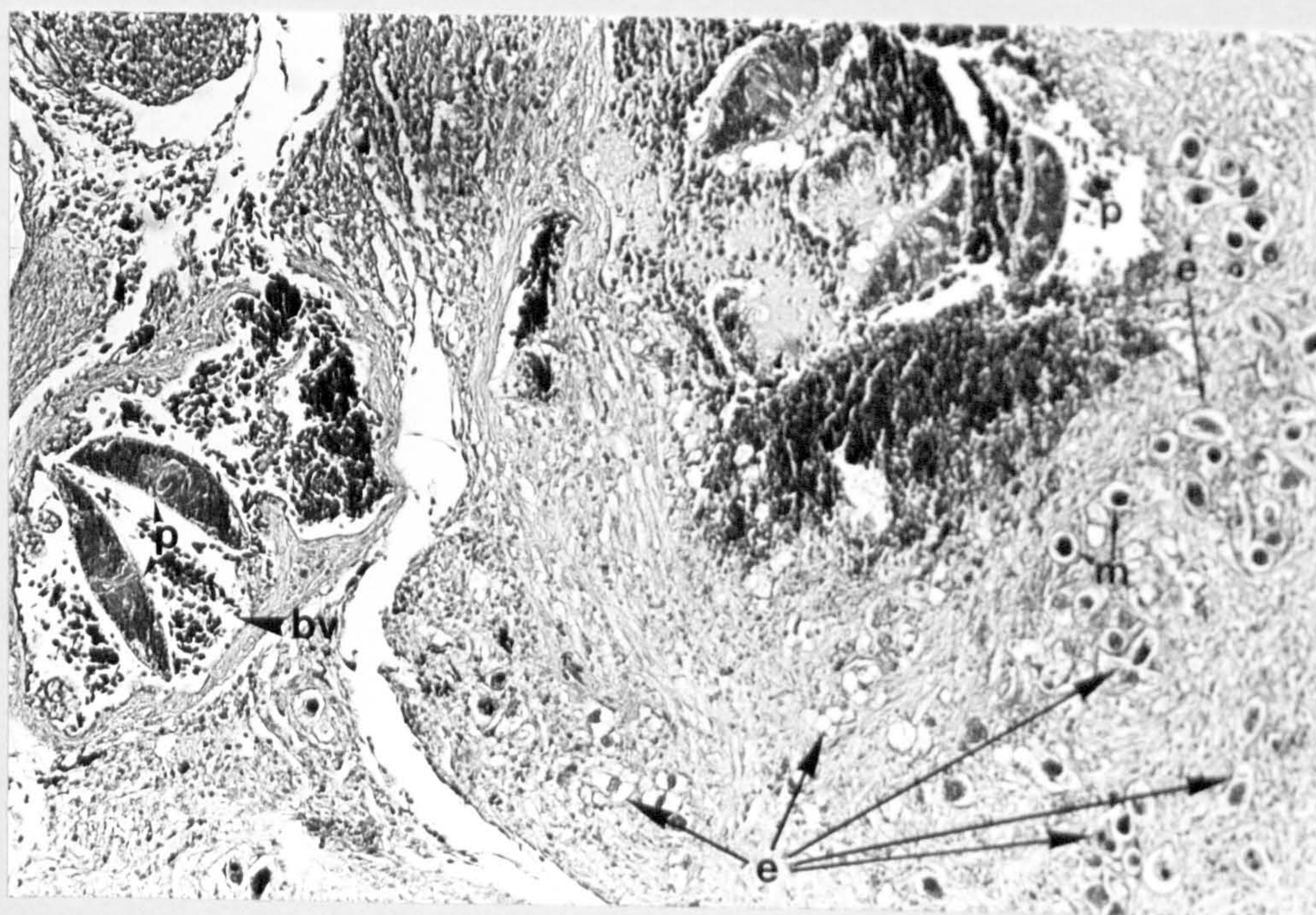
Fig. 5.18

Section showing matured S. inermis in the branchial blood vessel and numerous eggs and miracidia in the branchial tissues. Note marked epithelial hyperplasia in the branchial tissues (A) and hyperplasia on the inner lining of the blood vessel (B).

(H & E A, × 100 B, × 250.)

bv : blood vessel
e : egg
m : miracidium
p : parasite

A



B

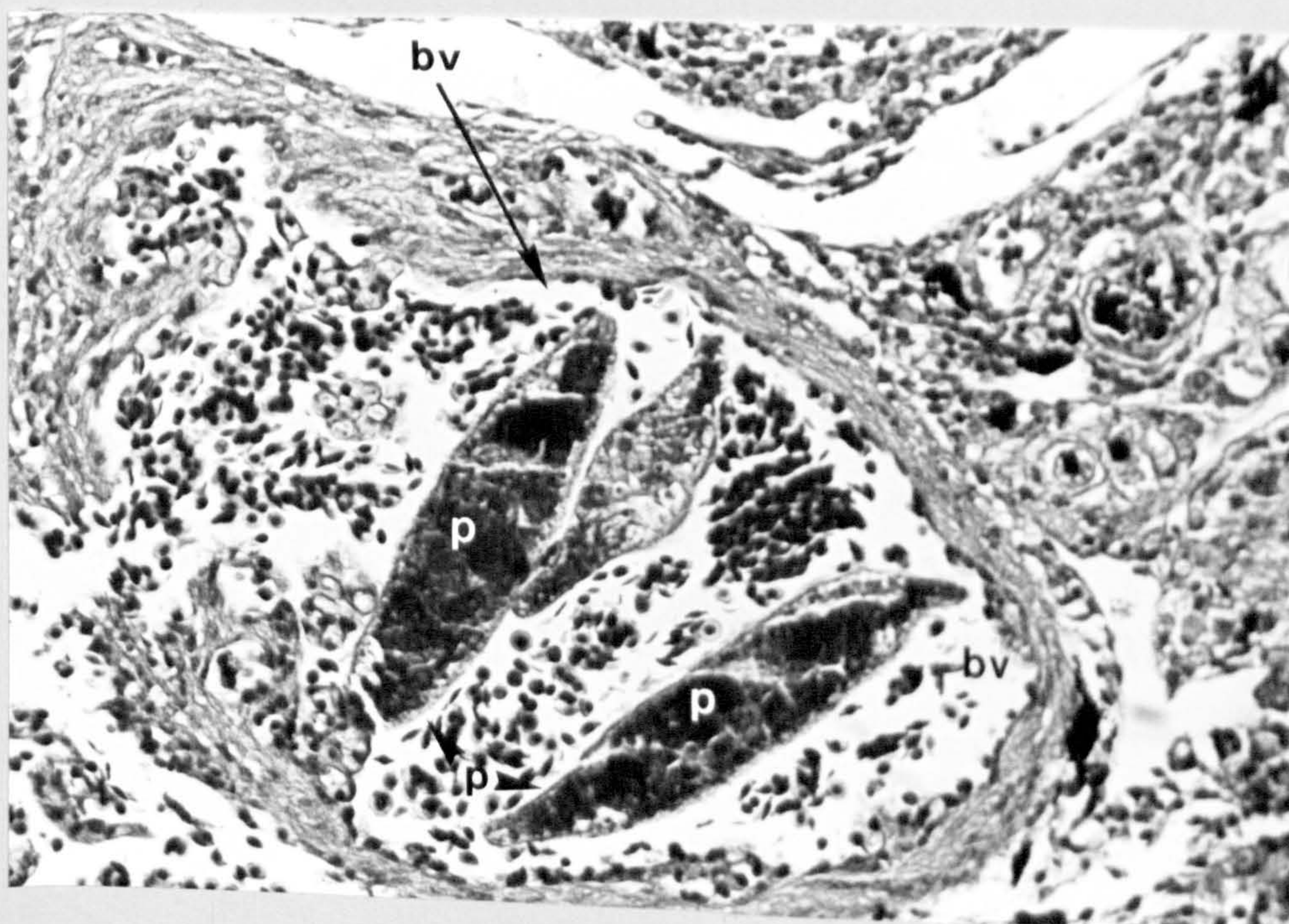


Fig. 5.19

Sections showing numerous granulomas containing degenerate eggs and hatched miracidia in the pericardium of heart.

(H & E A, × 100 B, × 250.)

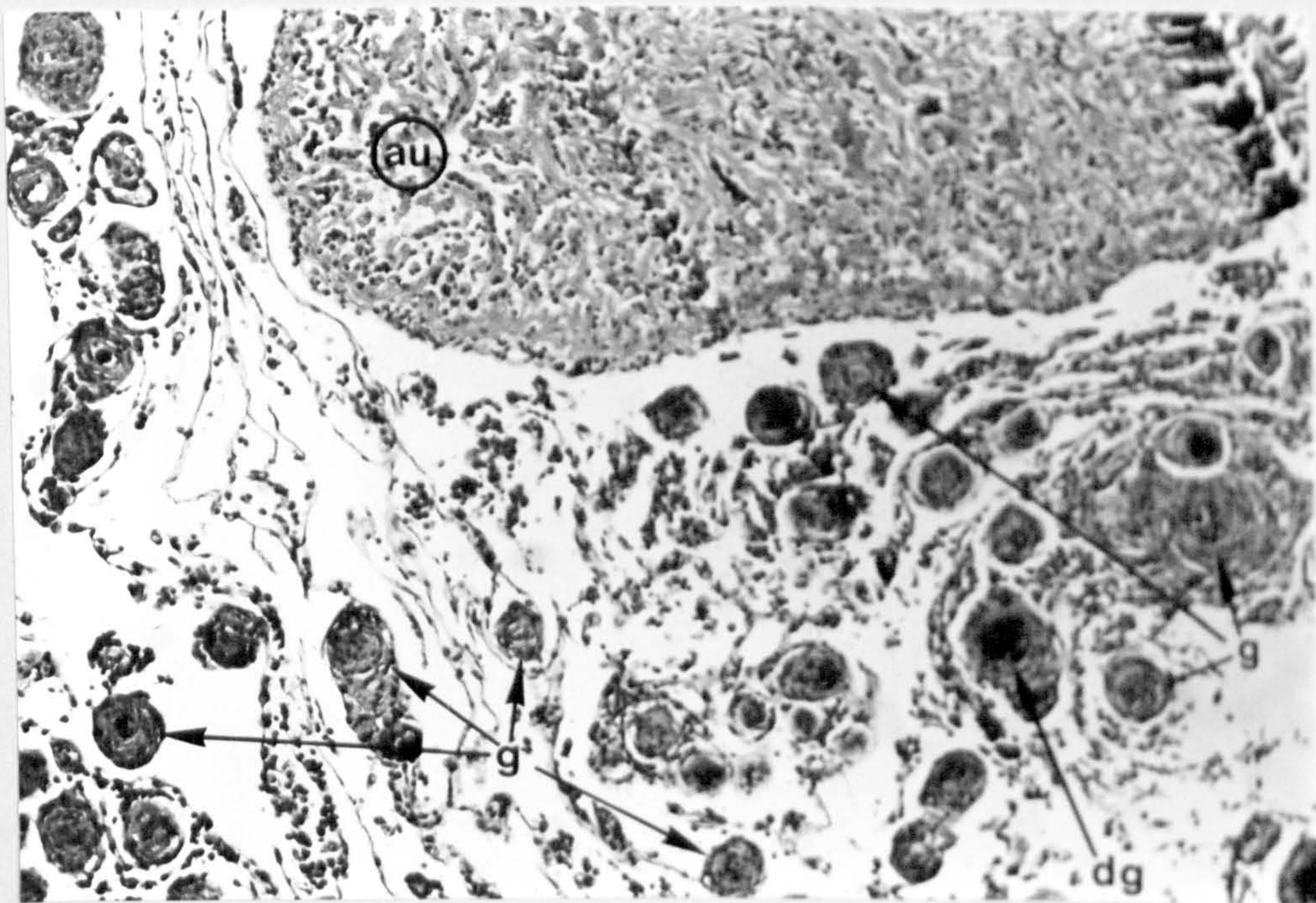
au : auricle

dg : degenerated egg

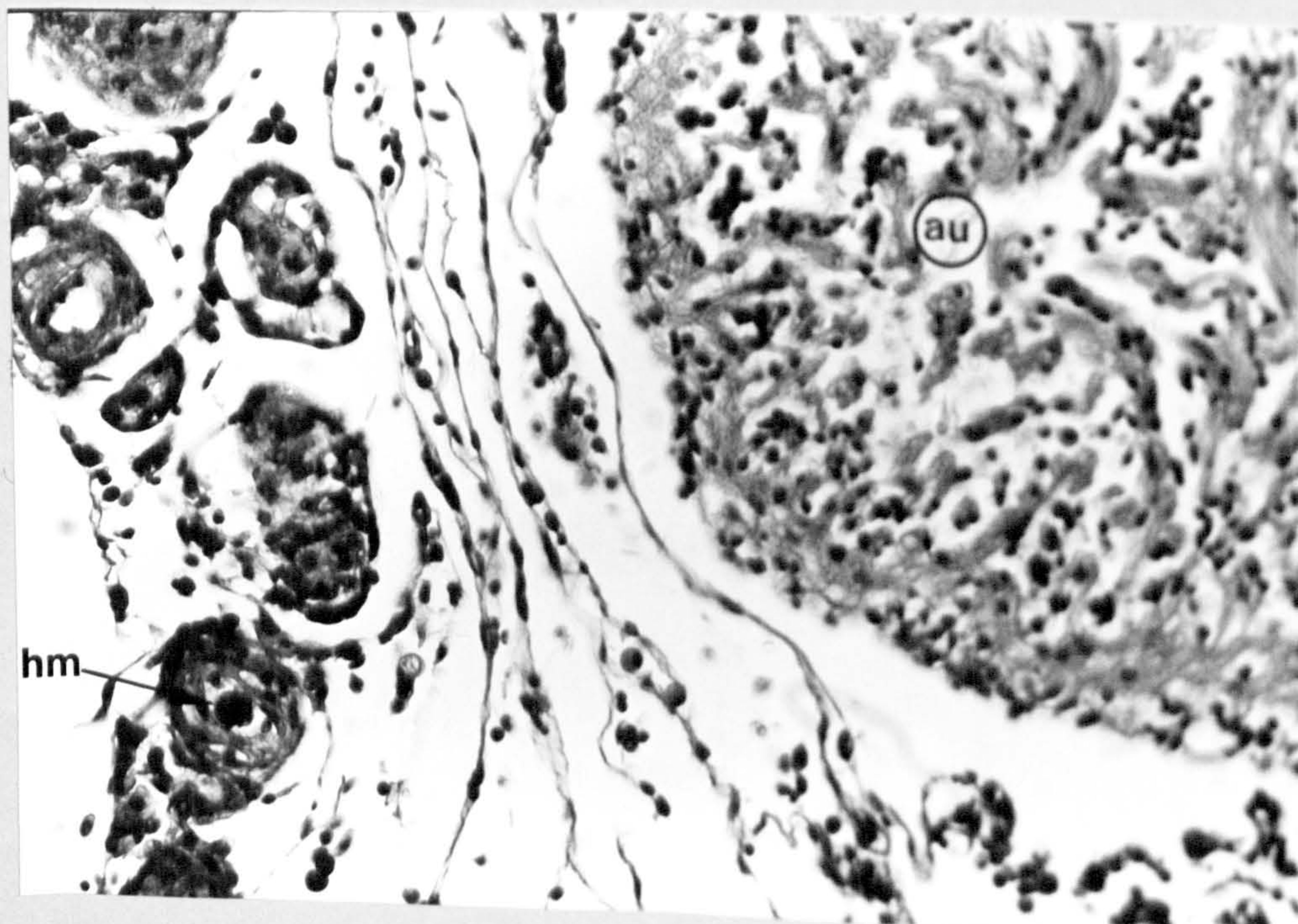
g : granuloma

hm ; hatched miracidium

A



B



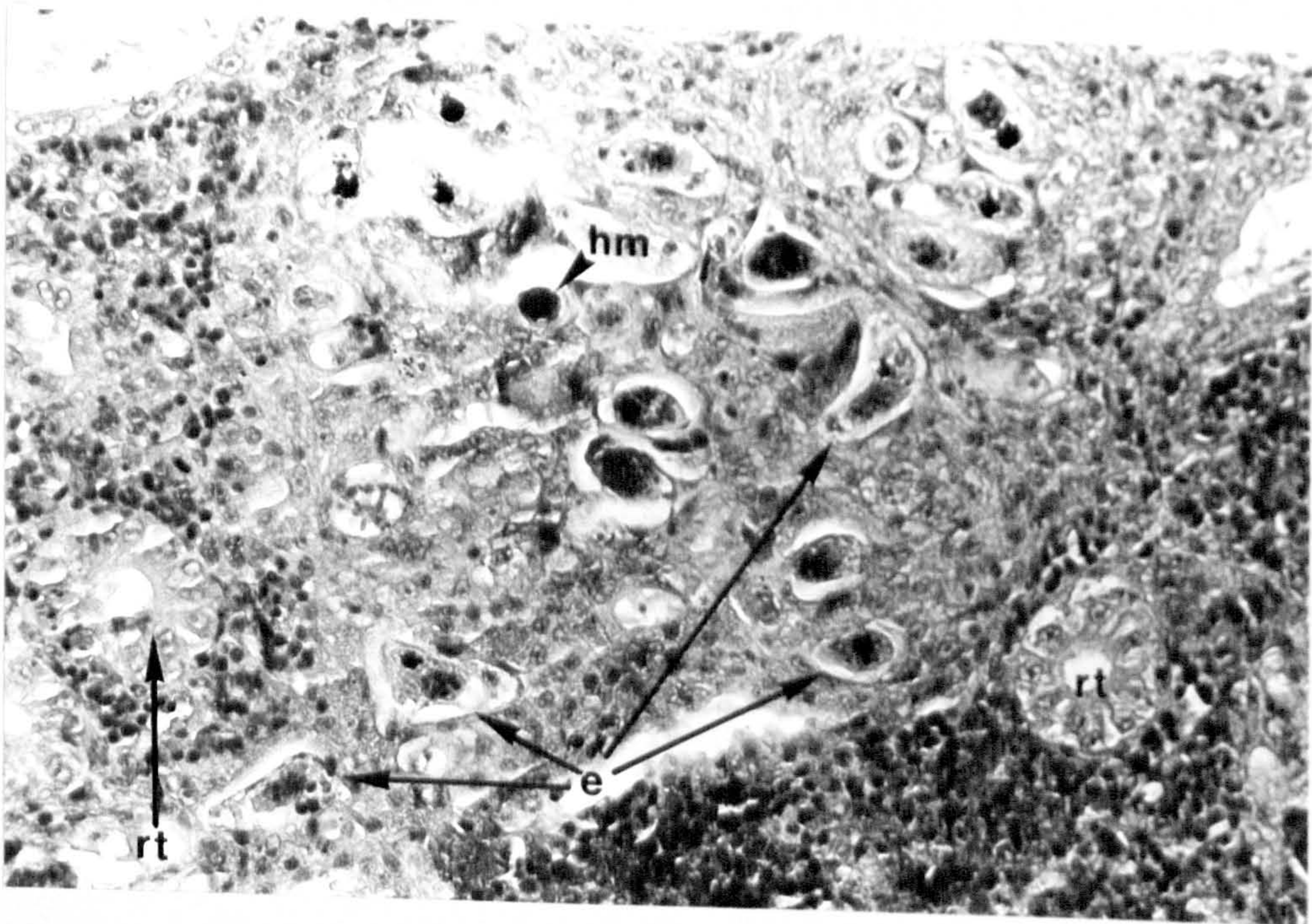


Fig. 5.20

Section of kidney showing extensive alteration and necrosis of kidney tissue due to the presence of *S. inermis* eggs and hatched miracidia (H & E, × 250).

e : egg

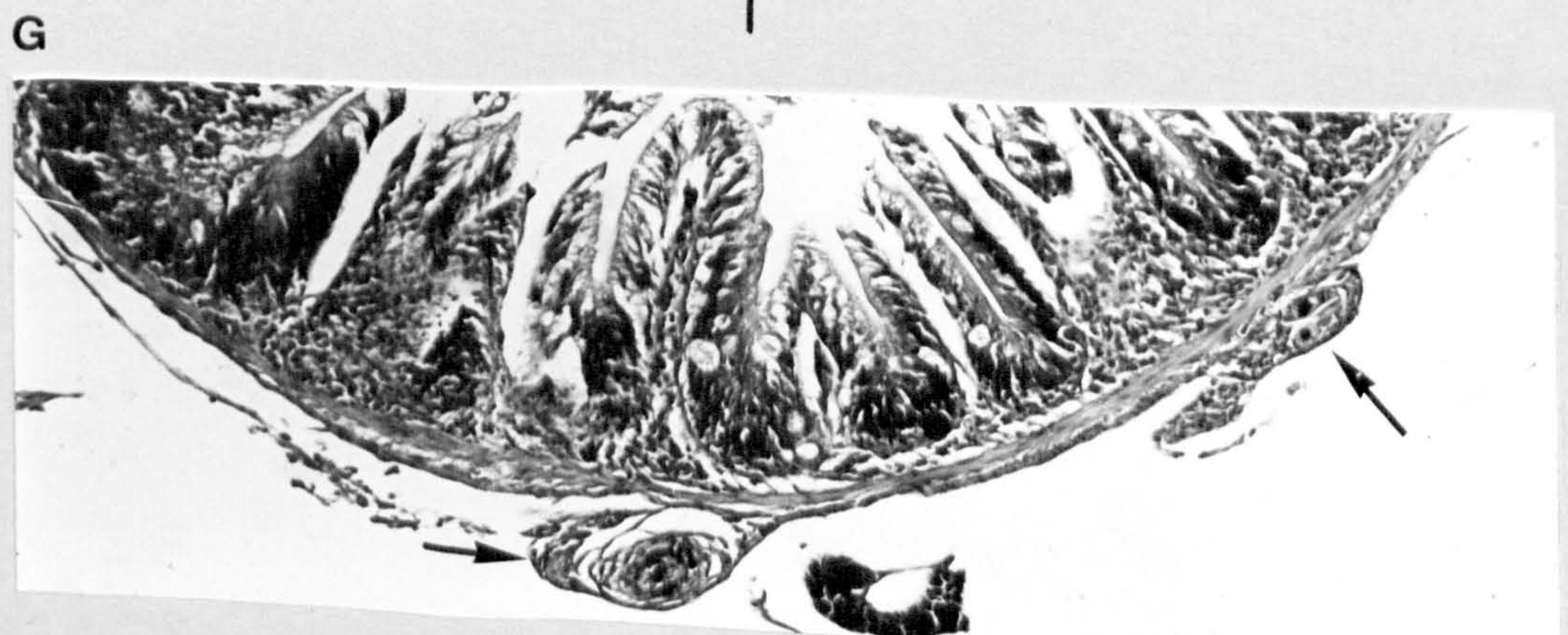
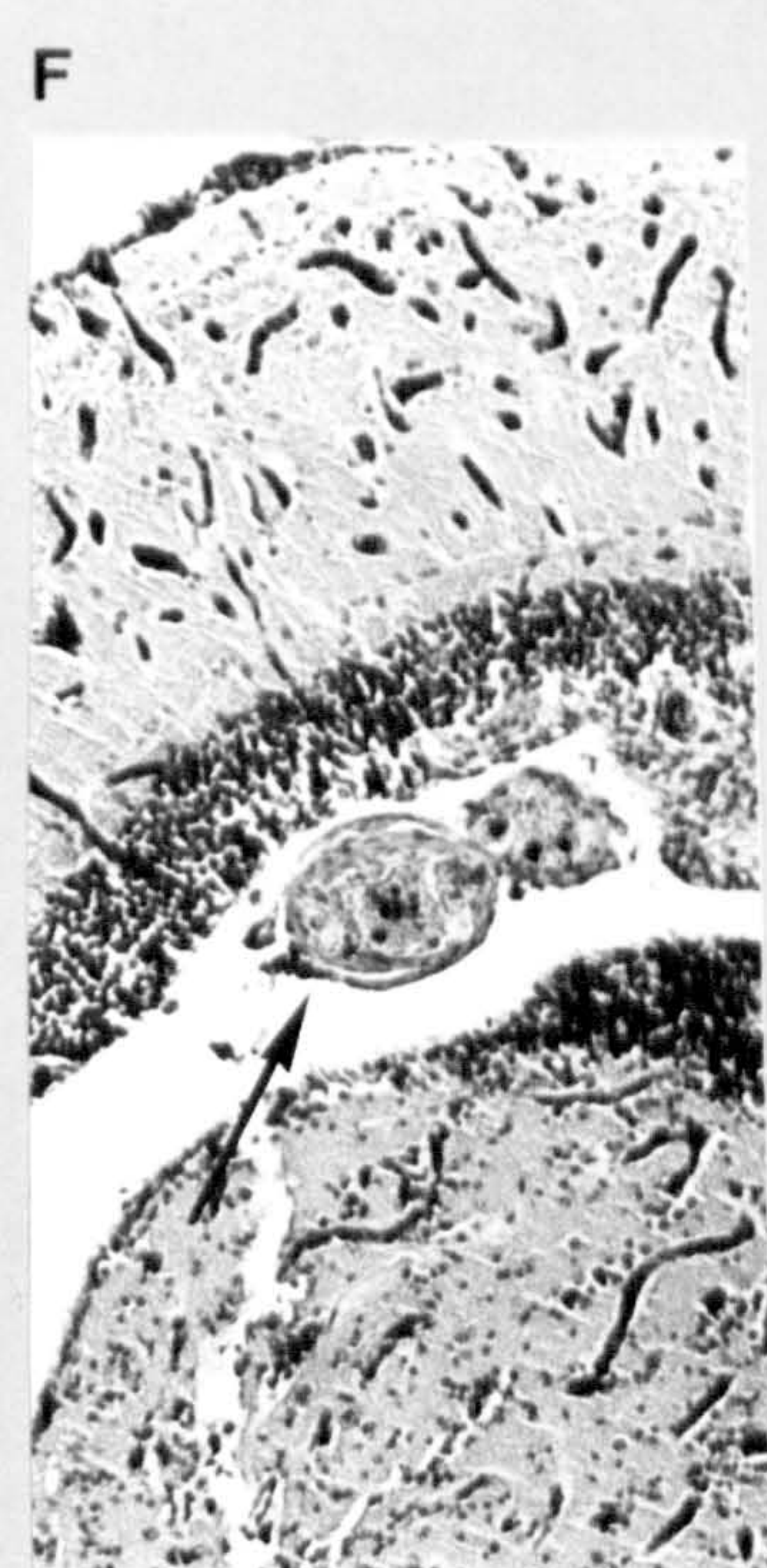
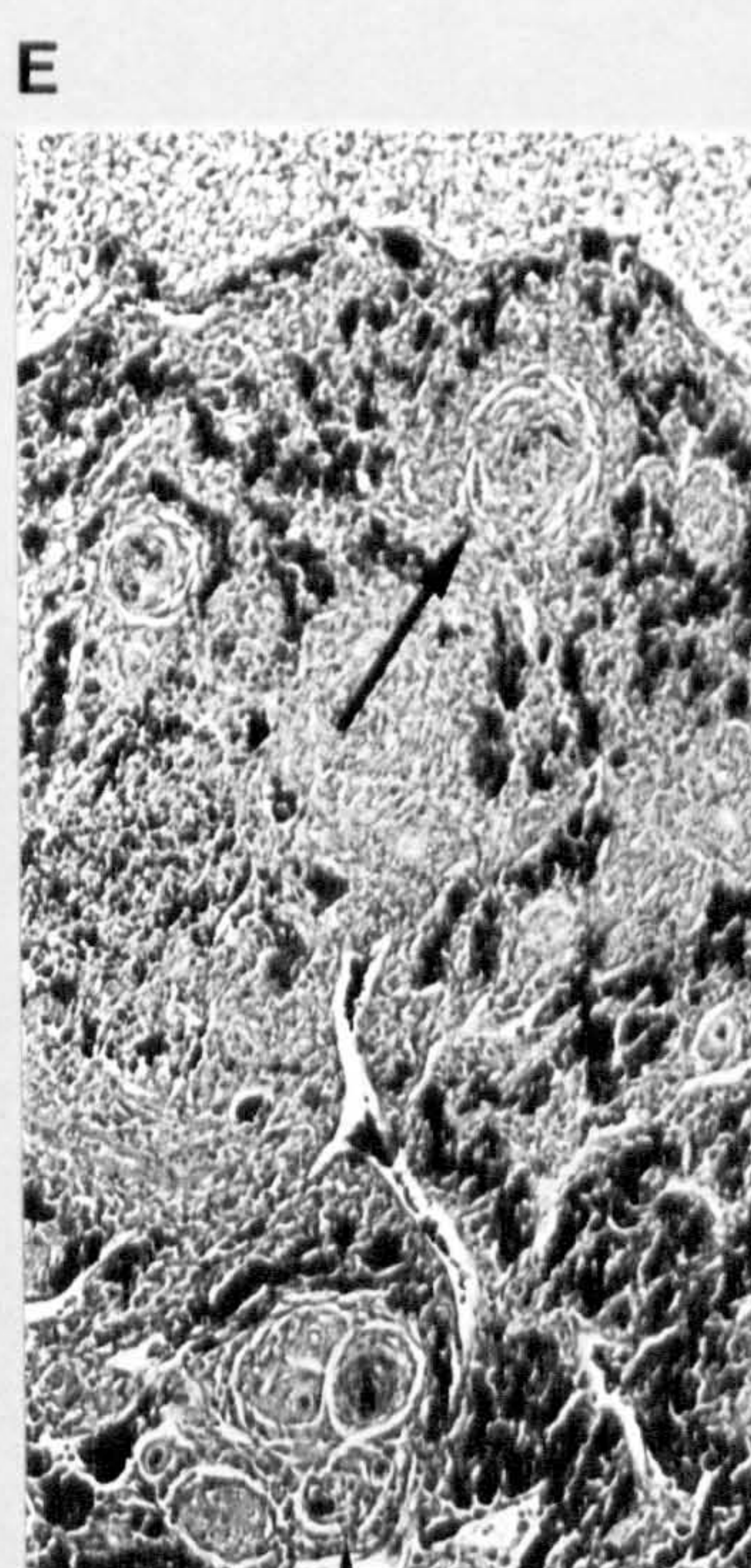
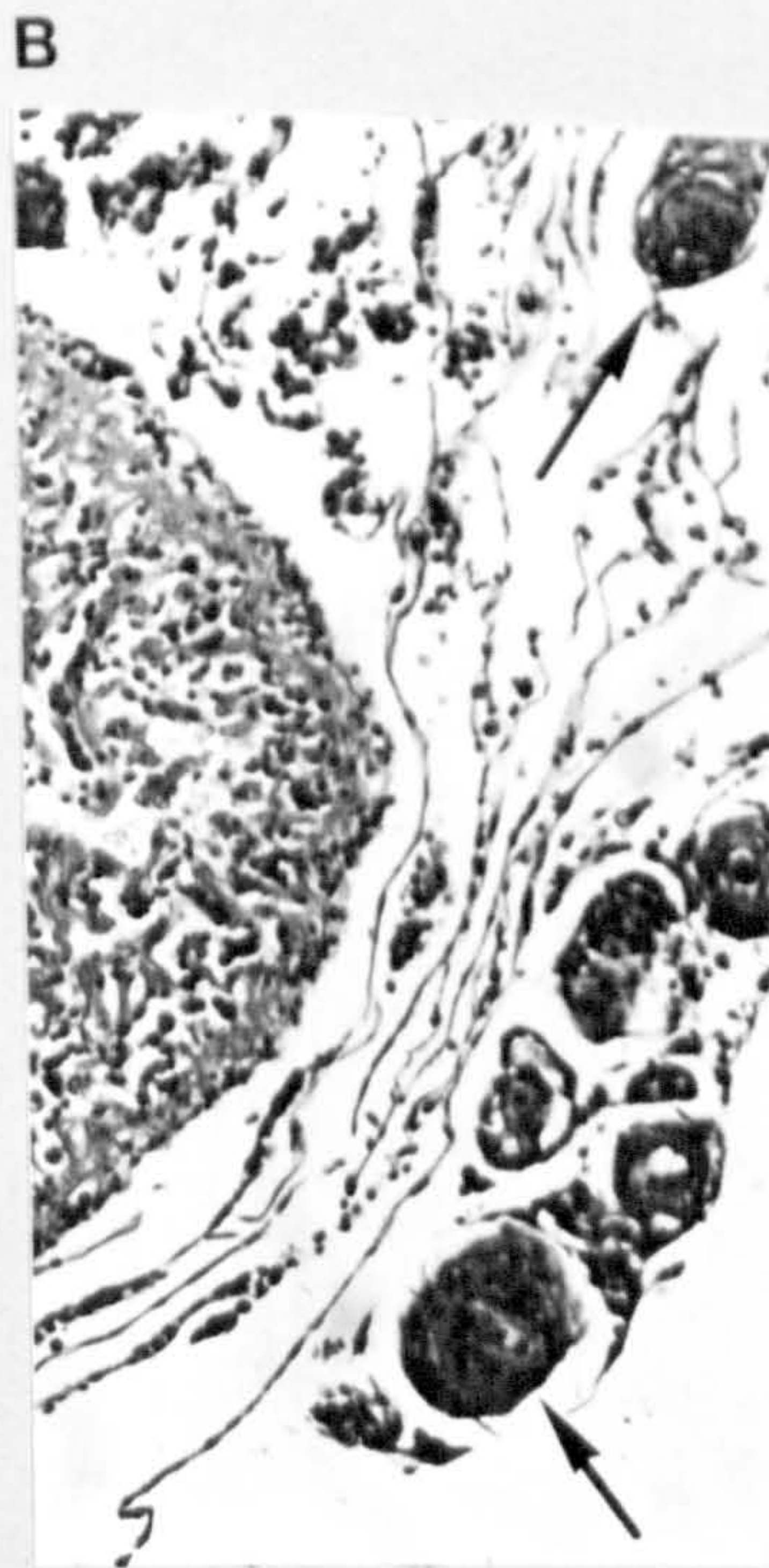
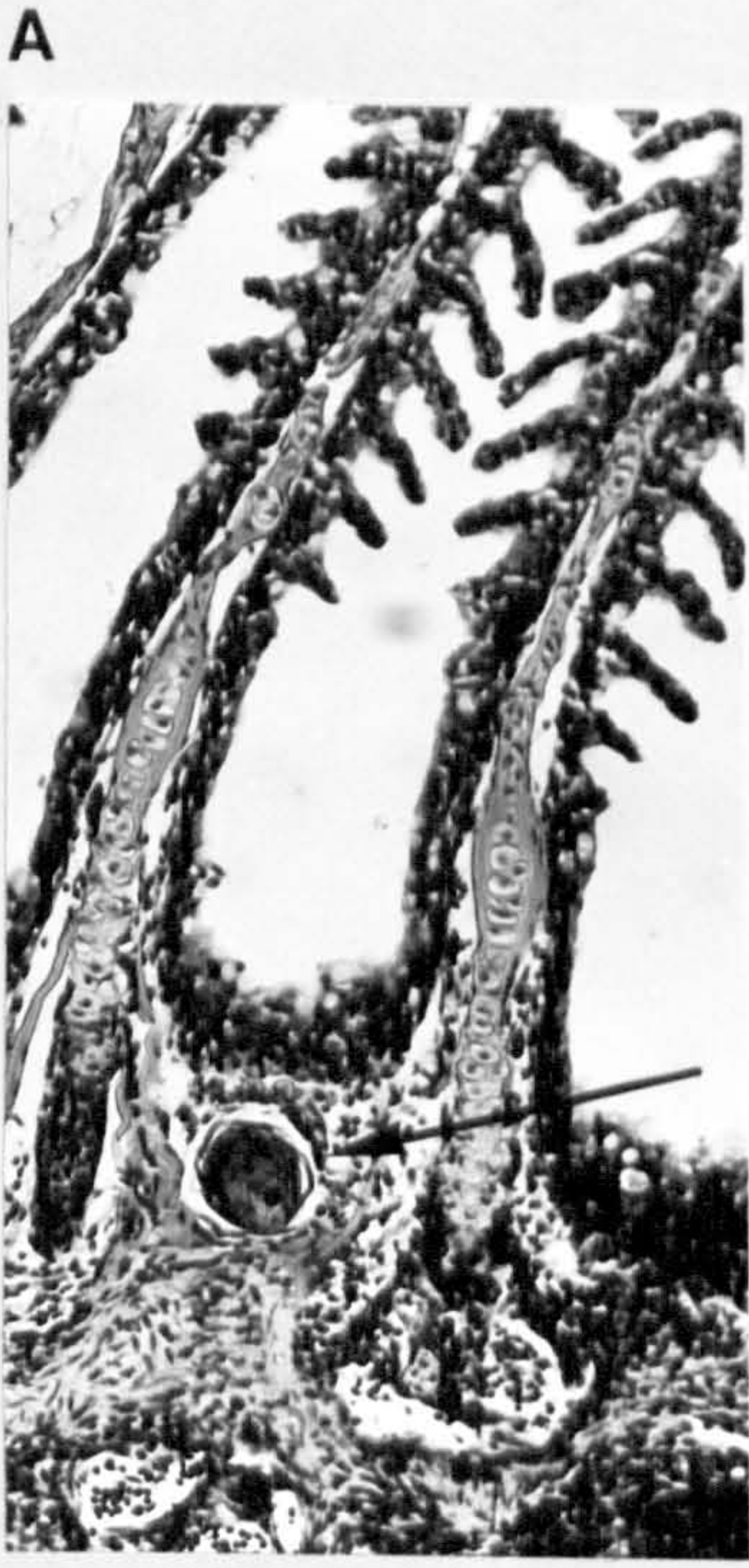
hm : hatched miracidium

rt : renal tubule

Fig. 5.21

Granulomatous tissue response (arrowed) to the presence of degenerated eggs and miracidia in tissues.

- A. section of gill (H & E, × 100)
- B. section of heart (H & E, × 100)
- C. section of kidney (H & E, × 100)
- D. section of liver (H & E, × 100)
- E. section of spleen (H & E, × 63)
- F. section of brain (H & E, × 100)
- G. section of gut (H & E, × 100)



Phase III

Gross changes

This phase emerged at a later stage, i.e. 4-5 months after the initial infection. Fish at this stage were very dark in colour, the head being more prominent (pinhead) as fish became emaciated. In some cases exophthalmia was evident. In fish which recovered from phase II, the clinical signs disappeared, but growth remained retarded (Chapter 5.1) and blood showed abnormal values (Chapter 5.2). No remarkable behaviour amongst the infected fish was seen during the third phase of infection with some exceptions where inappetance continued.

Histological changes

During the third phase of infection, the pathological change evident in the tissues of different organs was the continued development of a granulomatous response to the presence of eggs. Granulomata were seen in a variety of organs such as gills, heart, liver, kidney, pancreas, spleen, brain etc., wherever eggs were present and are shown in Fig. 5.21.

It was not always possible to differentiate phase III from phase II since production of eggs continued for a long period but at a reduced level (Chapter 4), thus some of acute responses could be seen even at phase III of the infection. The chronic phase continued after all the worms were dead in response to residual eggs in tissue.

5.3.4 Discussion

Studies of the pathological features of S. inermis infected carp revealed three different phases. At an early stage of phase I it was very difficult to recognize the diseased fish without careful dissection. Restlessness and erratic movements observed in a few

cases were the only signs indicating disturbance due to the invasion of cercariae through the epidermis of fins and body skin. The erratic swimming behaviour and ultimate loss of equilibrium prior to death seen in the present study was very similar to those described by Johnson (1971) and Sommerville (1982) for the penetrating cercariae of Cotylurus erraticus and Haplorchis pumilio respectively. Erasmus (1972) suggested that the swimming disturbances are associated with the cercarial migration. Wesenberg-Lund (1934) cited by Erickson and Wallace (1959) found that goldfish became sluggish in 24 hours and invariably died in 1-2 days when invaded by numerous cercariae of a blood fluke (possibly S. inermis). Death of fish was also reported by Meade and Pratt (1965) amongst Salmo clarki and S. gairdneri fingerlings on account of massive penetration by cercariae of S. alseae.

Though sanguinicolid cercariae have been known to enter through skin, there were no reports of any pathological changes from these areas.

Histopathological changes associated with the early hours of infection were not evident from the present study. It may be that small wounds made by the parasite heal very quickly as no change could be seen. Anderson and Roberts (1975) found the epithelial covering of small wounds in Atlantic salmon was achieved in two hours at 20-25°C. In addition, gland cells of cercaria may possess some lubricative content that produce little friction and/or damage during the process of invasion. Stirewalt and Kruidenier (1961) have suggested that one of the functions of the postacetabular gland secretions by the cercaria of Schistosoma mansoni is lubricative. In view of the little effects caused by the S. inermis cercaria it is more likely that penetration is achieved by separation of epidermal cell aided by lubricative secretion.

Sommerville (1977) also found a similar situation where flatfish showed little response at the early hours of Stephanochasmus baccatus infection. Lucky's (1964) description of the pre-acute form of S. inermis infection would possibly fall in this phase since no expressive pathological changes were found. Mortality can occur at this stage however, when infection rate is high and it is most likely that this is due to osmoregulatory stress as cercariae penetrate the skin in large numbers simultaneously.

Of the three phases described, phase II appeared to be the most critical. This is the period which was described by other authors as acute and it was at this stage that mortality amongst infected fish reached a peak. Thrombi in branchial capillaries and proliferation of the branchial epithelium and connective tissue elements resulting in fusion of lamellae appeared to be a major feature of the acute form of the disease. Thrombosis occurred as a result of blockage of capillaries by the numerous eggs followed by necrosis of the gill tissue distal to the thrombus.

Early works by Schäperclaus (1954) also found a very similar condition of carp which died due to the infection of S. inermis. However, Lucky (1964) believed that lack of oxygen from blockage of the gill circulation by S. inermis eggs or adults is more effective in killing carp than the later necrosis of gill filaments. In view of the observations of present study it is likely that the fusion of lamella reducing the functional surface of the fills and thus causing a lack of oxygen could cause the mortality of fish. However, it is more likely that the displacement and destruction of the resident tissue of the gills by the hatched miracidia would certainly result in osmoregulatory failure and thus the death of fish. Serious injury

to the gills caused by developing miracidia of S. inermis was also reported by Hirschmann-David (1953) and Naumova (1961a). Causes of heavy mortality amongst S. davisi-infected trout fingerlings were also reported to be the hatched miracidium in the gills (Davis et al., 1961).

During the acute form of disease a large number of eggs drifted to the different organs of the fish. Organs affected included heart, kidneys, liver, gall-bladder, spleen, gut wall, eyes, brain etc. Some of the deposited eggs developed in these organs and the miracidial activity caused considerable damage. Since all organs except gills are a blind alley as far as their future development is concerned, they became encapsulated by the host as the chronic stage of the disease developed.

The chronic granulomatous inflammatory reaction observed in tissues of various organs was induced by eggs and miracidia. Necrosis and hypertrophy of renal epithelial cells and granuloma formation possibly obstructing the vessels caused reduced erythropoiesis (Chapter 5.2). Obstruction of the renal vessels was also known to occur in cutthroat trout by the migrating S. klamathensis worms and eggs (Evans, 1974a).

The occurrence of the adult or juveniles of S. inermis was associated with obstruction of blood vessels and destruction of the surrounding tissues. With the absence of any specialized organs of attachment like tegumental spine S. inermis worms may have to struggle continually against the current of the blood. It is possibly the result of this continuous irritation which causes inflammation and hyperplasia of the adjacent tissues. The production of toxic metabolic

by-products might possibly change the blood enzymes and hormone activity of the fish host, as suggested by Smith (1972), and contribute to the pathological process.

The maximum mortality of infected fish occurred during the period of maturation of the worm.⁵ The 90% mortality observed in farms coincided with what was experimentally defined as the critical period. Schell (1974) also reported that heavily infected trout fingerlings tended to die about the time the parasite becomes sexually mature. The 80-90% mortality described by Hønd et al. (1977) was also during the peak egg production period of the worm, thus indicating a relation between maturation of the worm and the mortality of the fish.

As categorized by Layman (1957), the acute form of the infection occurs mainly in fry and fingerling and the chronic form appears mainly in the older fish. It appears from the present investigation that the separation of these phases is not always possible since they were found to overlap each other irrespective of age of infection. Bauer et al. (1956), for instance, reported fatal results in 24 carp (weighing 100-150g) infected with S. inermis. On the other hand, Chechina (1959) reported 0+ carp showing chronic symptoms of S. inermis infection.

It is apparent from the study that the blood fluke S. inermis is very devastating to its carp host. The entry of this fluke into fish, its development, egg production and their hatching and/or possible chemical secretion into an organ either from the metabolic by-product or from the dead parasite itself, caused destruction and/or displacement of resident tissue components thus manifesting a clinical feature which was known to be common for many helminth diseases.

CHAPTER 6

GENERAL DISCUSSION
AND CONCLUSIONS

6. General Discussion and Conclusions

Sanguinicola sp. is a recently introduced fish pathogen into Britain. Several outbreaks of this pathogen have already caused considerable losses to the carp industry. Little was known about the species which was causing problems and its biology. An attempt has therefore been made to clarify aspects of the biology, morphology and development of the species in order to understand the host-parasite relationships.

Initially, attempts were made to create the right conditions for the life-cycle to be maintained entirely under controlled conditions. But experimental infections of molluscs proved to be exceedingly difficult. Though a number of variables were tried over a period of several months to initiate molluscan infections, the results were negative. It was not clear why the fully developed, hatched miracidia of S. inermis failed to infect any of the five snail species presented, including the L. peregra from the affected farms. On the farms the prevalence of infections in fish was always very high but the infections in the snail collections from the ponds was very low. Low levels of snail infections have also been shown for Sanguinicola from the work of Chappell (1967), Shillcock (1972) and Radlett (1978). Whether the low infection rate of snail^s on the infected farm is a true reflection of the situation is not known for certain. It may be that the peak of molluscan infection was passed by the time the infection became evident in fish. This would also suggest that the pathogenicity of the larvae in molluscs was severe, causing possibly early mortality of snails. Future investigation of natural snail infections might shed further light on the problem.

Since infection by the miracidium of S. inermis was negative, subsequent experimental infections of definitive hosts were performed with cercaria which had emerged from naturally infected snails. Of the three host fish species used, experimental infection with cercaria was successful only in carp, indicating a strong host-specificity. Though cercaria penetrated throughout the body, they showed a marked local concentration in the region of fins. The reason why the majority of cercariae preferred the fins, particularly the caudal fin, as a route of entry, is not clear. This preference could possibly be related to the relative ease of penetration of the soft tissues between the fin rays or more likely because of the close proximity of the blood vessels to the surface in these areas.

The present study revealed a very interesting feature of Sanguinicola infections. Invading cercaria did not migrate immediately towards the heart and gills but settled at the site of entry. During the developmental period most of the immature worms migrated to the preferred loci. Those which remained in the skin continued to develop to maturity, even producing eggs in the skin, a previously unreported feature of Sanguinicola infection. However, the reason why some of them delayed their migration and matured in the skin is not understood. It has been known for some larval filarial worms to complete development to the adolescent stage, principally in the subcutaneous and muscle tissue before migrating to the heart (Orihel, 1961; cited by Smithers & Worms, 1976). One possibility is that there is a regulation mechanism controlling the actual number of worms entering the vascular system. Though the percentage of worms in the major blood vessels increased, the overall number of worms decreased continually, thus making it difficult to justify the parasite regulation mechanism.

It was also not clear whether the eggs produced by the worms located in the skin were able to continue their life-cycle. Fully developed miracidia were seen but it is not certain if they were able to exit through the skin. There is no apparent reason why the miracidia should not make their way out by this route since the majority of miracidia were seen to hatch in the gill and came out through the gill epithelium. However, this very interesting phenomenon deserves further work.

The absence of skin worms from one outbreak was puzzling. Since worms were only recovered from gill/heart region of Cotswold fish, the initial thought was that they might be altogether a different species compared to the species of the Calverton outbreak. However, the difference is now thought to be due to the stage and size of the infection. The first observations of fish of the Cotswold farm were carried out when the infection was at least five months old and when worms had already migrated to the intravascular areas. In the outbreak at Calverton farm the parasite burden was excessively high, and at an earlier stage (two months) thus giving the different picture compared to the Cotswold farm. Experimental infections with a low cercarial load showed a very similar pattern to that observed in the Cotswold farm.

Worms collected from the two farms when studied in detail appeared to be the same and were identified as Sanguinicola inermis. However, there were some morphological differences between worms of the two farms. First, the mean size of worms collected from the Calverton farm was smaller than that of the Cotswold farm. Secondly, the outside margins of the body of Calverton worms possessed crenulation, but no such crenulation was found in worms of the Cotswold farm.

Thirdly, in addition to the crenulations, Calverton worms bore minute 'setae-like' structures all over the body. These 'setae-like' structures were absent from the worms of the Cotswold farm.

The morphological variations described above are considered to be most likely due to age of the worm. In general, worms of the Cotswold farm at the time of the study of the morphology were much older, i.e. more than 10 months. Whereas the Calverton worms were studied as early as three months, i.e. the earliest stage at which sexually mature worms were available but all under 10 months since no worm was detected after that period. As with the increase of age of Calverton worms, the crenulated margins of the body and 'setae-like' structure were seen to reduce, indicating an age-dependent phenomenon.

The physical characteristics of the vascular environment obviously influence the morphology of the parasite. The habitat essentially is a tube, therefore it is not surprising to find a tendency of worms to become elongate in shape and achieve streamlining which serves to reduce friction and turbulence in a moving medium.

As a morphological adaptation to this environment, most Sanguinicola species possess spines of various sizes and shapes originating from the tegument. It has been suggested that these spines serve to maintain the position of the worms within the blood vessels against the circulating blood flow (Holmes, 1971b). It seems likely that the 'setae-like' structure described by others for S. inermis serve the same purpose. However, mature worms beyond the age of 10 months in this study lacked 'setae-like' structures.

SEM and TEM studies also showed that S. inermis of six months lacked organs of attachment or tegumental spines and it is not known

how they maintain themselves in situ within the vascular system. The SEM study revealed numerous branched microvillous-like projections all over the body surface. These projections were thought to be an adaptation to increase the efficiency of attachment to the inner walls of the host's blood vessels. By the contractions of the tegument these microvilli might help anchorage to the lining of the blood vessels, thus securing a stable position in the circulating blood. Worms in vitro attached firmly to the plastic substrate and in some histological sections, they appeared to be closely applied to the vessel walls. However, to what extent the microvilli aid in attachment and to what extent they increase surface area for absorption of nutrients is unknown. These would be very interesting features for further study.

TEM study of the worm revealed that the tegument of the adult specimens are bounded by a trilaminate outer membrane, covering both the surface of the body as well as the entire surface of the microvilli. Why S. inermis possesses a trilaminate rather than the apparently more usual heptalaminate outer membrane for blood flukes (McLaren and Hockley, 1977) is not clear. One possibility of this was thought to be an adaptation to contact digestion. However, this interesting feature deserves a more careful and intensive investigation.

The results of the present study suggest that the mature worms possess 'innate seasonal rhythms' which govern their life-cycle pattern independent of environmental changes. The migratory activity of the worms within the small and large vessels and the egg production showed a seasonal preference despite the constant environmental conditions which govern the host.

The great variation in the longevity of naturally occurring worms was surprising. Worms of the experimental infection survived only

up to seven months compared to 24 months of natural infections. Several factors, such as initial load of worms, the strain of fish host used, biotic and abiotic factors (Chubb, 1979) might all be involved. Slowed development over winter may also increase longevity but does not readily explain the 17 months difference in age found in some groups. These also deserve further investigation, but would obviously be aided by experimental infections.

Several kinds of effects were shown to occur due to the infection of S. inermis. Amongst them, the most dramatic one was the mortality which occurred in most of the infected fry. The parasite, therefore, must be regarded as one of the major 'killer' diseases since a high mortality has been reported from many carp propagating farms of central and southern Europe and the USSR. The few farms in Britain which were affected by this parasite also suffered heavy losses, not only due to mortality of the fish and subsequent poor performance of survivors, but also because of the cost incurred by the radical treatment, disinfection of the farm and the changes in the farms structural system and husbandry practice necessary to try to eliminate the disease. Despite these efforts the disease reappeared in some farms.

Fish which survived the infection showed significantly slowed growth, poor SGRs, PERs and FCRs resulting in unprofitable production.

Fish suffering from the infection were anaemic. The anaemia was of the hypochromic macrocytic type which was brought about by a variety of pathological processes during the course of infection. The pathological processes contributing to the anaemia include external and internal haemorrhages which were seen to occur during the early and acute phase of infection. During the acute phase, numerous eggs

hatched in the gills making their way out by destroying the gill epithelium. The destruction of the gill epithelium would certainly enhance the failure of osmoregulation and result in haemodilution. The possible reduction in haematopoiesis due to the destruction of kidney and spleen by the activity of the different developmental stages, i.e. deposition of the eggs and the movement of the hatched miracidia in these tissues was also thought to be another possible factor contributing to the anaemia. Starvation due to loss of appetite and unidentified reasons such as toxic by-products of parasite metabolism or toxins released on the death of parasites, may also add to the development of anaemia. Noticeable difference in the severity of anaemia occurred at stage 2, i.e. the peak egg production stage.

Leucocytosis was observed throughout the infection period, indicating a response to the cellular injury resulting from the migration and subsequent activity of the worm in the tissues of the fish.

Pathological changes due to infection, as seen histologically, appeared in three distinct phases. During the early stage of infection little change was seen, possibly because of low level of the experimental infection. However, in farms with very heavy infections mortality occurred at the invasive stage. The effects of infection at phase II were severe and acute in all fish. Cellular infiltration, haemorrhages and proliferation of connective tissue elements in the affected tissues were common features at this stage. The most badly affected areas at this stage were the skin and gills.

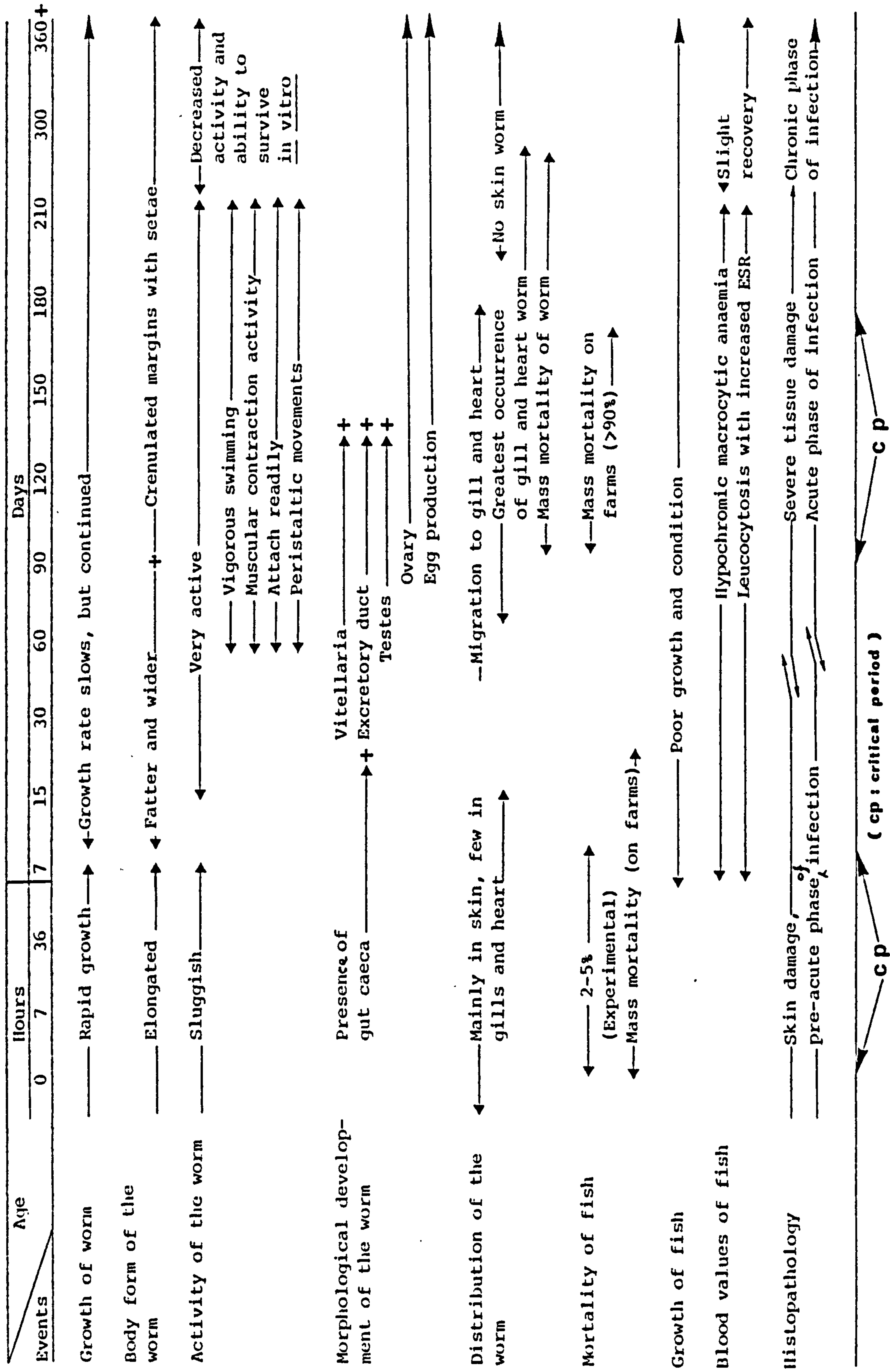
A chronic granulomatous tissue reaction was seen during the last phase of infection. This was the period when the degenerated eggs

and dead worms became encapsulated in different tissues, especially the kidneys and heart. Though the fish at this stage had recovered from the worst effects of the infection, they continued to show a reduced growth rate. During the chronic phase of infection, the values of different blood parameters showed a little recovery, but remained abnormal compared to controls.

The major feature of S. inermis infection and its development in the C. carpio along with the effects on the fish, as studied, are summarized in Fig. 6.1. These have all been transcribed onto the same time scale in order to integrate the features in an attempt to clarify some of the host-parasite interactions.

It can be seen that the most critical periods for the host fish occurred at two different stages of infection. The first was during the early hours of infection when mortality was attributed to the massive cercarial invasion. The second critical period did not appear until the maturation of the worm, when the majority of them either migrated from skin to internal loci or disappeared as a result of mortality amongst the developing worms. This was also a period of greatest egg production and when numerous miracidia hatch out and leave the fish by destroying the gills. A second major mortality amongst the infected fish was a feature of this period. Infected fish which survived these critical periods showed poor growth and condition which continued even two years after the original infection.

Fig. 6.1 Summary of major events in developments of S. inermis in carp (C. carpio) fry and their effects on fish



R E F E R E N C E S

R E F E R E N C E S

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APPENDICES

APPENDIX 1

Description of the genus *Sanguinicola*, Plehn, 1905

External morphology: Trematodes of small size, with highly contractile thin semi-transparent body, constantly varying shape, but lanceolate at rest. The body is dorsally convex and narrowed at both ends. Anterior end is elongated into a proboscis.

Digestive organ: Digestive organ consists of a long oesophagus which begins at the proboscis tip, with aperture opening. Oesophagus forms a flask like expansion, a secretory reservoir, on passing into intestine. Intestine consists of 4-6 short sacs of irregular lobes which reach about the second third of the body.

Nervous system: Nervous system is represented by a paired ganglion and one or more pairs of lateral, longitudinal nerve trunks.

Male reproductive system: Male reproductive system consists of testis, usually behind intestinal bifurcation, which has a large number of paired seminal vesicles in the middle of the body. Vas deferens somewhat curved at distal portion, turning into copulatory organ opening at dorsal surface of body at the beginning of last quarter. It is an exception amongst the digeneas in that sanguinicolids do not possess seminal vesicle, bursa of cirrus or prostatic part of semen ejaculating canal. All are hermaphrodite.

Female reproductive system: Directly behind the male sexual glands is a butterfly-shaped double ovary, from which emerges a long oviduct, reuniting with the vitelline duct. Vitellaria are well developed, medial and occupying anterior half of body. Female genital pore

lies next to the male, postovarially. Ootype delimited by a constriction from the short uterus which is represented by a metraterm only.

Excretory system: Excretory system consists of two longitudinal trunks forming a v-shaped excretory vesicle at the posterior end.

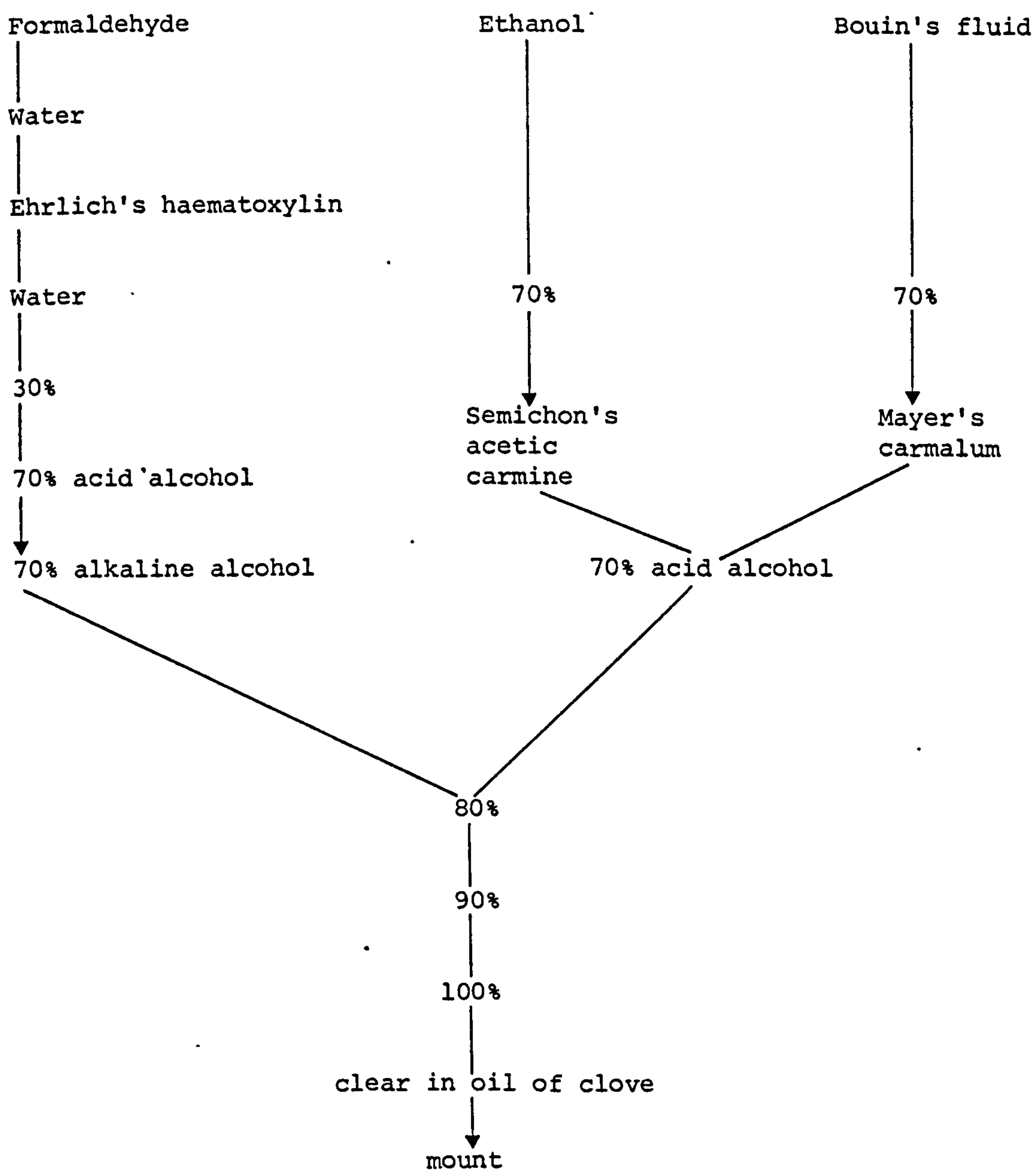
Adult worms are parasitic in the circulatory system of freshwater fish. Sexually mature Sanguinicola live mainly in bulbus arteriosus, ventral aorta and afferent branchial arteries.

Description of the species S. inermis, Plehn, 1905

Body highly contracted with no cuticular spines. Maximum body length is approximately 1 mm and width 0.3 mm. Surface of the body covered with extremely minute seta or hairs arranged diagonally. Mouth very small, situated at the anterior body tip, from which emerges a long oesophagus, approximately one third the total body length. Intestine represented by 4, more rarely 5 or 6 small lobate sacs. One unbranched nerve trunk on each side of body. Excretory canal is short and weakly developed. Fifteen pairs of testes are usually present. Posterior part of vas deference forms 2-4 loops. Cirrus sac very poorly developed in form of papilla. Uterus, a short metraterm, containing one egg which measures approximately 0.061-0.070 × 0.029-0.035 mm. Dorsal process of the egg is short. The gut ratio is approximately 1:3 and oötype ratio is approximately 1:7.

(Modified from texts)

APPENDIX 2

Summary of staining procedures

% refers to ethanol concentration

APPENDIX 3

Rees-Ecker Solution

1.	Sodium citrate	3.8 g
2.	Neutral 37% formalin	0.2 ml
3.	Brilliantcresyl blue	0.5 g
4.	Distilled water	100.0 ml

APPENDIX 4

Drabkin's Reagent

1.	Potassium ferricyanide	20 mg
2.	Potassium cyanide	50 mg
3.	Distilled water	1 l

Drabkin's reagent is commercially available in powder or tablet form from B.D.H.

APPENDIX 5

Haematological Indices

Mean Cell Haemoglobin (MCH)

This expresses the average haemoglobin content (in picograms) of a single red blood cell. The most convenient method for determining the MCH is to multiply the haemoglobin content in gram per 100 ml of blood by 10 and divide by the number of millions of red cells per mm^3 .

$$\text{MCH} = \frac{\text{Hb g per 100 ml of blood} \times 10}{N}$$

where N = number of millions of red blood cells per mm^3 .

Mean Cell Volume (MCV)

This is the average volume of a single red cell expressed in cubic microns (μm^3). MCV was calculated by dividing the packed cell volume (PCV) by number of millions of red cells per mm^3 and then multiplying the result by 10. In practice the formula is

$$\text{MCV} = \frac{\text{PCV} \times 10}{N}$$

where N = number of millions of red blood cells per mm^3 .

Mean Corpuscular Haemoglobin Concentration (MCHC)

This refers to the percentage of haemoglobin in 100 ml of red blood cells, as opposed to the percentage of haemoglobin in 100 ml of whole blood, giving the concentration of haemoglobin in the cells.

$$\text{MCHC} = \frac{\text{Hb g/100 ml of blood}}{\text{PCV}} \times 100$$

APPENDIX 6

Histological Methods

Processing schedule

50% Methylated Spirits	1 hour
80% " "	2 "
8% Phenol in methylated spirits	3 "
8% " " "	2 "
8% " " "	2 "
Absolute alcohol	2 "
" "	1 "
Chloroform	1 "
"	1 "
Wax	2 "
"	2 "
"	1 "

APPENDIX 6 (continued)

Haematoxylin and Eosin Stain (H & E)

1.	Xylene	5 minutes
2.	Absolute Alcohol	2 "
3.	Methylated Spirit	1½ "
	Wash	
4.	Mayer's Haematoxylin	10 "
	Wash	
5.	1% Acid Alcohol	1 - 3 dips
	Wash	
6.	Scott's Tap Water Substitute*	Till Blue
	Wash well	
7.	Eosin	5 minutes
	Wash	
8.	Methylated Spirit	30 seconds
9.	Absolute Alcohol I	2 minutes
10.	Absolute Alcohol II	1 minute
11.	Xylene	5 minutes

* After Scott's Tap Water Substitute, sections are examined microscopically and

if too dark, differentiated again in 5.

if too light, process is repeated from 4.

APPENDIX 7

List of species

Host species:

Alburnus alburnus (L.)Aristichthys nobilis (Richardson)Auchenoglanis occidentalis Cuvier & ValenciennesAustralorbis glabratus (Say)Barbus holubi SteindacherBithynia tentaculata (L.)Carassius auratus (L.)C. edrassius (L.)Channa punctatus (Bloch)Clarius batrachus (L.)Ctenopharyngodon idellus (Cuvier & Valenciennes)Cyprinus carpio L.Esox lucius L.Gadus morhua L.Garra rufa (Heckel)Heteropneustes fossilis (Bloch)Hippoglossoides platessoides (Fabricius)Huro salmonids (Lacepede)Hydrobia ulvae (Pennant)H. ventrosa (Mont)Hypophthalmichthys molitrix (Cuvier & Valenciennes)Lepomis macrochirus Rafinesque

Leuciscus cephalus (L.)

L. idus (L.)

L. leuciscus (L.)

Limanda limanda (L.)

Lymnaea auricularia (L.)

L. palustris (Mull)

L. (Radix) peregra (Müll)

L. stagnalis (L.)

Micropterus dolomieu dolomieu Lacépède

Mylopharyngodon piceus (Richardson)

Notropis hudsonius (Clinton)

Oncorhynchus Kisutch (Walbaum)

O. nerka (Walbaum)

Oreochromis niloticus (L.)

Oxytrema circumlineta

O. silicula (Gould)

Pelecus cultratus (L.)

Perca flavescens (Mitchill)

Physa fontinalis (L.)

Platichthys flesus (L.)

Pleuronectes platessa L.

Prochilodus platensis Holmberg

Rutilus rutilus (L.)

Salmo clarki Richardson

S. gairdneri Richardson

S. gairdneri gairdneri Richardson

S. salar (L.)

S. trutta L.

Salvelinus fontinalis Mitchill

Scardinius erythrophthalmus (L.)

Stizostedion vitreum (Mitchill)

Synodontis schall Bloch-Schneider

Tilapia zilli (Gervais)

Tinca tinca (L.)

Tinca vulgaris Cuv.

Valvata eristata Müll

Parasite species:

Acanthocotyle elegans Monticelli

Aporocotyle macfarlani Holmes

A. spinosicanalis Williams

A. simplex Odhner

Ascaris lumbricoides L.

Cercaria cristata La Val.

C. flexicauda

C. hartmanae Martin

C. kentensis Khan

Clonorchis sinensis (Cobbold)

Cotylurus erraticus (Rudolphi)

Diplostomum spathaceum (Rudolphi)

Diplozoon nipponicum Goto

Echinostoma lindoensis Sandground & Bonne

Entobdella soleae (Beneden & Hesse)

Fasciola hepatica L.

Haplorchis pumilio (Looss)

- Ichthyophthirius multifiliis (Fouquet)
Isoparorchis hypselobagri (Billet)
Posthodiplostomum minimum Hughes
Ribeiroia marini Faust & Hoffman
Sanguinicola alseae Meade & Pratt
S. argentinensis Szidat
S. armata Plehn
S. charlmersi Odhner
S. davisii Wales
S. huronis Fischthal
S. idahoensis Schell
S. inermis Plehn
S. intermedia Ejsmont
S. klamathensis Wales
S. lophophora Erickson & Wallace
S. magnus Cheng-yen Hu, So Long & Wei-chu Lee
S. occidentalis Van Cleave & Mueller
S. volgensis (Rasin)
Saprolegnia diclina Willoughby
Schistosoma japonicum Katsurada
S. mansoni Sambon
Spirorchis haematobium Stunkard
Stephanochasmus baccatus Nicoll
Trypanosoma danilewskyi Laveran & Mesnil
T. murnanensis Nikitin