887

LIGULA INTESTINALIS (L. 1758) - IN VITRO ~~VIVO~~ AND IN VIVO ~~VITRO~~ STUDIES

By

HILARY A. FLOCKHART, B.Sc. Hons., M.Sc.

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Department of Zoology and Applied Entomology,
Imperial College of Science and Technology
South Kensington, London, S.W.7.

This thesis is dedicated to the memory
of my mother, Rosa Avesia Flockhart.

ABSTRACT

Studies were made on the pseudophyllidean cestode, *Ligula intestinalis* (L. 1758), a parasite of the roach *Rutilus rutilus* in the Serpentine Lake, London, in order to learn more about the ecology, development and pathology of this parasite.

A survey of the roach population was carried out over a period of one year. Some aspects of the general ecology of the roach were examined, in particular the problem of correctly ageing the roach. Data were collected of the *Ligula* infections in these fish and when considering the data, particular attention was paid to the seasonal variation in intensity and incidence of infection and to the variation of intensity and incidence of infection in fish of different ages.

A comparative study of the development of adult *Ligula* was made between those cultured *in vitro* and those *in vivo*, in ducks and mice. The fertility of the eggs and the time taken to produce eggs were two of the criteria compared. Different *in vitro* culture methods were tried to improve the fertility and numbers of eggs produced. *In vivo* and *in vitro* culture of the coracidia were attempted.

The pathology of *Ligula* infections was reviewed and the effect of the plerocercoid on the host's metabolism was investigated further, using qualitative and quantitative methods. Changes in serum and liver protein, liver

glycogen and dry weight levels were determined. Actual liver damage was detected by assaying the levels of certain enzymes in the plasma.

The presence of an immune response, in the form of circulating antibodies, in roach serum to Ligula plerocercoids was investigated.

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CONTENTS

	PAGE
Abstract	3
Acknowledgements	5
Contents	6
List of Figures	11
List of Plates	13
List of Tables	15
Key to Abbreviations in the figures	17
General Introduction	18
Section I. Some aspects of the ecology of *Ligula intestinalis* infections in roach from the Serpentine	21
Chapter 1. An introduction to the parasite, its habitat and its fish host	22
The Parasite - *Ligula intestinalis* (L. 1758)	22
The life cycle of *L. intestinalis*	26
The Habitat - History and general features of the Serpentine	29
Physical and chemical features	30
The flora and fauna	31
Metereological Data	34
Transmission of *L. intestinalis* in the Serpentine	36
The Second intermediate host - *Rutilus rutilus*	37
Chapter 2. Collection and examination of roach from the Serpentine	39
Methods of sampling and examining the fish	39

PAGE

Age determination 41

The validity of age determination 42

Results and Observations 44

Age determination 44

The number of roach examined 49

Examination of roach for other parasites 49

Seasonal variations in the Ligula infections of roach 54

Variations in the Ligula infections of roach of different ages 65

Discussion 67

Summary of Section I 72

Section II. In vivo and in vitro culture 74

Chapter 3. In vivo culture 75

Introduction 75

The definitive host. 77

Maintenance and infection of ducks 77

Methods 77

Results 79

Histological examination 79

Implantation of Ligula plerocercoid fragments in the mouse peritoneal cavity 85

Method 85

Results 86

Discussion 90

PAGE

The first intermediate host. Laboratory maintenance of *Cyclops* spp. 92

Methods and Results 92

Infection of copepods 93

Chapter 4. *In vitro* culture 95

Introduction 95

Materials and Methods 97

Culture of plerocercoids to adults 97

Culturing coracidia 105

Results 107

Plerocercoids to adults 108

Coracidia 115

Types of eggs produced *in vitro* 115

Histological examination 117

Discussion 117

Summary of Section II 121

Section III. Aspects of the pathology and immunology of *Ligula* infections 123

Chapter 5. Pathology of *Ligula intestinalis* infections in the roach, *Rutilus rutilus* 124

Literature Review 124

Chapter 6. Electrophoresis of roach serum. A comparison between healthy individuals and those infected with *Ligula* 129

Introduction 129

PAGE

Materials and Methods 130

Disc electrophoresis 130

Gradient gel plate electrophoresis 132

Results 133

Disc electrophoresis 133

Gradient gel plate electrophoresis 133

Discussion 138

Chapter 7. The effect of the plerocercoids of Ligula intestinalis on the metabolism of Rutilus rutilus 145

Introduction 145

Materials and Methods 146

Total protein determination of serum 146

Total protein determination of liver 147

Glycogen determination of liver 147

Dry weight determination of liver 149

Periodic Acid Schiff staining of liver 149

Enzyme determinations on serum and plasma, 149

Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT)

Results 150

Discussion 156

Tissue damage 156

Disturbances in the host's metabolism 157

Chapter 8. Immunological studies on fish serum 160

Introduction 160

PAGE

Materials and Methods 160

Preparation of Ligula antigen 160

Extraction of excretory and secretory products 161

Collection of serum from roach 161

Preparation of agar plates 161

Disc immunodiffusion 162

Attempted immunization of uninfected fish 163

Results 163

Discussion 164

Summary of Section III 166

General Discussion 168

References 171

LIST OF FIGURES

Figure		PAGE
1.	The Life Cycle of Ligula intestinalis (L. 1758).	25
2.	Length frequency histograms for 0+, 1+, 2+ and 3+ age groups of Serpentine roach November 1975 to October 1976, plotted for each month.	45
3.	Length frequency histograms for 0+, 1+, 2+ and 3+ age groups of Serpentine roach November 1975 to October 1976, plotted for the whole twelve months.	46
4.	Length frequency distribution of all roach caught in the Serpentine, November 1975 to October 1976.	52 & 53
5.	Seasonal variations in the incidence and intensity of the Ligula infection in Serpentine roach, November 1975 to October 1976.	56
6a).	Frequency distribution of Ligula plerocercoids in roach from the Serpentine, November 1975 to October 1976.	59
6b).	Seasonal variation in the P.I. of Ligula infections in roach from the Serpentine, November 1975 to October 1976.	59
6c).	Seasonal variation in the weights of Ligula plerocercoids from Serpentine roach, November 1975 to October 1976.	59
7.	Seasonal variations in the frequencies of Ligula plerocercoids of different weights from Serpentine roach, November 1975 to October 1976.	62

Figure		PAGE
8a).	Variation in the intensity of infection with Ligula in Serpentine roach of different ages, November 1975 to October 1976.	66
8b).	Variation in the P.I. and the mean plerocercoid weight of Ligula in Serpentine roach of different ages, November 1975 to October 1976.	66
9.	Diagram of the genital anlagen of the plerocercoid of L. intestinalis.	81
10.	Diagram of the genitalia of adult L. intestinalis.	81
11.	Deep culture vessel used for the in vitro culture of L. intestinalis.	100
12.	Circulatory apparatus used for the in vitro culture of L. intestinalis.	102
13.	Adapted circulatory apparatus used for the in vitro culture of L. intestinalis.	103
14.	Culture chamber used for the in vitro culture of coracidia of L. intestinalis.	106
15.	Types of eggs produced in in vitro culture of L. intestinalis.	116
16.	Disc electrophoresis pattern of roach serum proteins. Serum from uninfected fish.	134
17.	Disc electrophoresis pattern of roach serum proteins. Serum from fish infected with L. intestinalis.	135
18.	Results of gradient gel plate electrophoresis of roach serum.	139
19.	Diagram of immunodiffusion plates.	162

LIST OF PLATES

Plate		Page
1.	A roach taken from the Serpentine, showing the typical ventral pouching caused by *L. intestinalis* plerocercoids.	27
2.	The same specimen with the body wall removed to show the plerocercoids of *L. intestinalis*.	27
3.	Transverse section of the plerocercoid of *L. intestinalis in situ* in the small intestine of a duckling, 4 hours post-infection.	83
4.	Development of *L. intestinalis* in the duckling. 24 hours post-infection. T.S.	83
5.	Development of *L. intestinalis* in the duckling. 48 hours post-infection. T.S.	84
6.	Development of *L. intestinalis* in the duckling. 48 hours post-infection, showing the receptaculum seminis full of spermatozoa and egg laying taking place.	84
7.	Development of *L. intestinalis* in the mouse peritoneum. 6 days post-infection. T.S.	88
8.	Development of *L. intestinalis* in the mouse peritoneum. 8 days post-infection. T.S.	88
9.	Development of *L. intestinalis* in the mouse peritoneum. 6 days post-infection. Plate showing receptaculum seminis.	89
10.	Deep culture vessel used for the *in vitro* culture of *L. intestinalis*.	99

Plate		Page
11.	*In vitro* culture of *L. intestinalis*. Section after 72 hours culture in medium 199 and 20% calf serum.	118
12.	*In vitro* culture of *L. intestinalis*. Receptaculum seminis, seen in Plate 11, full of spermatozoa.	118
13.	Disc electrophoresis of serum from uninfected Serpentine roach.	136
14.	Disc electrophoresis of serum from Serpentine roach infected with *L. intestinalis*.	136
15.	Gradient gel plate electrophoresis of roach serum. Protein stain.	140
16.	Gradient gel plate electrophoresis of roach serum. Glycoprotein stain.	140

LIST OF TABLES

Table		Page
1.	Meteorological Data November 1975 to December 1976.	35
2.	A comparison of the length ranges of the age groups of roach in cm.	47
3.	The number of roach in each age-group examined in each month from November 1975 to October 1976, from the Serpentine.	51
4.	Ligula intestinalis, percentage incidence of infection in Serpentine roach, November 1975 to October 1976.	55
5.	Ligula intestinalis, mean intensity of infection in Serpentine roach, November 1975 to October 1976, measured as the number of worms per fish.	58
6.	Ligula intestinalis, mean intensity of infection in Serpentine roach, November 1975 to October 1976, measured as the weight of plerocercoids per fish.	61
7.	Seasonal variation in the incidence and intensity of Ligula infections in Serpentine roach, November 1975 to October 1976. Summary of the data.	64
8.	Results of in vivo culture of Ligula intestinalis. Duck experiments.	80
9.	Results of in vivo culture of L. intestinalis. Mouse implantations.	87
10.	The species of copepods recorded as hosts for the procercoid of L. intestinalis.	94

Table		Page
11.	Results of *in vitro* culture of *L. intestinalis*, cultured with dialysis tubing intact and in the light.	110
12.	Results of *in vitro* culture of *L. intestinalis*, cultured in punctured dialysis tubing and in the light.	111
13.	Results of *in vitro* culture of *L. intestinalis*, cultured in punctured dialysis tubing and in the dark.	112
14.	Results of *in vitro* culture of *L. intestinalis*, cultured in 250 ml^3 Falcon flasks.	113
15.	Results of *in vitro* culture of *L. intestinalis*, fragments in 25 m^3 Falcon flasks.	114
16.	Percentage composition of the major fractions of roach serum.	137
17.	Results of total protein determinations of parasitised roach liver and serum.	151
18.	Results of total protein determinations of non-parasitised roach liver and serum.	152
19.	Results of glycogen and dry weight determinations on liver from parasitised roach.	153
20.	Results of glycogen and dry weight determinations on liver from nonparasitised roach.	154
21.	Results of enzyme determinations on plasma from parasitised and nonparasitised roach.	155

KEY TO ABBREVIATIONS IN THE FIGURES

c....cirrus

cp...cirrus pouch

e....egg

m....muscle layers

mg...Mehlis' gland

o....ovary

pc...cirrus pore

rs...receptaculum seminis

s....spermatozoa

sv...seminal vesicle

t....testis

u....uterus

up...uterine pore

v....vitellaria

vd...vas deferens

vp...vaginal pore

yd...yolk duct

yr...yolk reservoir

GENERAL INTRODUCTION

The aim of this project has been to study various aspects of the biology and ecology of a particular host-parasite relationship, *in vivo* and *in vitro*. The pseudophyllidean cestode *Ligula intestinalis* (L.1758) was chosen for this work as there was a source of material close to the laboratory in the roach, *Rutilus rutilus*, population of the Serpentine Lake, London. Also, the plerocercoids of *L. intestinalis* can be cultured in the laboratory *in vitro* (Smyth, 1976) and *in vivo* (Orr and Hopkins, 1969) to the adult stage and so provide plenty of material for experimental work.

The material presented in this thesis has been divided into 3 sections and detailed introductions have been written at the beginning of each section or chapter, as appropriate. However, a brief introduction to the work will be given here.

Section I examines some of the ecological aspects of the roach-*Ligula* relationship in the Serpentine. When the work for this thesis started, fish were collected in a random way to provide material for laboratory studies, but it was decided that it would be useful to combine the collection of material with an ecological study. So regular samples were collected once a month for 12 months. The conclusions drawn from a survey of this type are necessarily somewhat speculative, as any detailed ecological

study should be conducted over a period of years and the conclusions based on a more mathematical analysis, which is beyond the scope of this work. For this reason only certain aspects of the ecology of Ligula infections were looked at, in particular the seasonal variation in incidence and intensity of infection which had been found in rudd infections in England by Orr (1967) and also the variation in intensity and incidence of infection in fish of different ages which has been reported by a number of authors, e.g. Arme and Owen (1968) and Orr (1967).

Section II deals with the problems of in vitro culture of Ligula plerocercoids. The progenetic plerocercoids of Ligula are relatively easy to culture (Smyth, 1969, 1976), but the eggs produced are not always fertile. So different methods of culturing were investigated and the in vitro development of the plerocercoid compared to that in vivo.

Most of the pathological studies on Ligula-infected fish have concentrated on the dramatic reduction in gonad size and the inhibition of normal courting behaviour & reproduction. These effects have been reported extensively in the literature,but more specifically in roach in Great Britain by Arme (1968 and 1975), and Arme and Owen (1965 and 1968). In Section III some other aspects of the pathology of Ligula infections of roach have been studied, in particular liver damage and the inhibition of normal liver function were investigated.

The presence of Ligula plerocercoids elicits a tissue response within the body cavity of infected fish (Arme and Owen, 1970) and some preliminary experiments were carried out to try and detect any circulating antibodies in the serum of roach infected with Ligula. Molnar and Berczi (1965) had demonstrated the presence of precipitating antibodies in the serum of infected bream and it was hoped to be able to repeat this work with infected roach serum.

SECTION I

SOME ASPECTS OF THE ECOLOGY OF *LIGULA INTESTINALIS* INFECTIONS IN ROACH FROM THE SERPENTINE

CHAPTER 1

AN INTRODUCTION TO THE PARASITE, ITS HABITAT AND ITS FISH HOST

The Parasite - Ligula intestinalis (L.1758)

Early works, such as Cooper (1918), recognised only one species of the genus Ligula, L. intestinalis, and listed 63 fish host species from many families. Orr (1967a and b) described the geographical distribution of the plerocercoids of L. intestinalis and found them to be widespread, occurring in Europe, Russia, South Africa, India, Australia and North America. The most commonly infected family of fish is the Cyprinidae in both the Old and New World.

Dubinina (1950 and 1959) described the two forms of Ligula found in the Cobitidae and Gobiidae families as different species L. colymbi and L. pavlovskii. In 1964, Dubinina revised the taxonomy of the whole family Diphyllobothriidae and suggested that the two subfamilies should be recognised as independent families. Representatives of the family Ligulidae at the plerocercoid stage develop only in fresh water and in plankton-eating fish belonging to three orders and five families, Cyprinidae, Cobitidae, Gobiidae, Cottidae and Gasterosteiidae. The adults are found in fish-eating birds; they are not found in mammals since in general mammals masticate their food and destroy the plerocercoids. There have been reports of adult

Ligula being found in otter scats (Greer, 1955) but these could have resulted from an otter eating a piscivorous bird which was infected.

Dubinina (1964), on the basis of morphological differences, i.e. segmentation or no segmentation in the plerocercoid, has split the family Ligulidae into two subfamilies, Ligulinae and Schistocephalinae. The subfamily Ligulinae consists of two genera, Ligula, with a single arrangement of the genitalia and Digramma, in which the genitalia are double. The genus Ligula consists of three species, L. intestinalis the plerocercoids of which occur in the Cyprinidae, L. colymbi which occur in the Cobitidae and more rarely in the Cyprinidae and L. pavlovskii which occur in the Gobiidae.

The host specificity of the plerocercoid has been explained (Dubinina, 1964) as being due to the fact that in the genus Ligula the process of growth and the development of the genitalia take place at this stage and the development of the plerocercoid takes over 14 months; therefore, specific conditions are required which may only be found in certain groups of fish. However, the adult stage, where development is limited to maturation and egg formation, is not so host specific. Because of the host specificity of the plerocercoid stage it is possible that different 'strains' of L. intestinalis may develop in the various different fish hosts.

Taxonomy of the Family Ligulidae (Dubinina, 1964)

Class: Cestoda

Subclass: Eucestoda

Order: Pseudophyllidea

Family: Ligulidae

Subfamily: Ligulinae

Genus: Ligula

L. intestinalis (L. 1758)

L. colymbi (Zeder 1803)

L. pavlovskii Dubinina 1957

Genus: Digramma

D. interrupta (Rud, 1810)

D. nemachili Dubinina, 1957

Subfamily: Schistocephalinae

Genus: Schistocephalus

S. solidus (Müll, 1776)

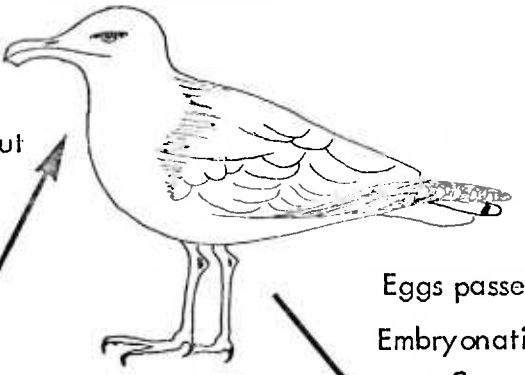
S. pungitii Dubinina, 1959

S. nemachili Dubinina, 1959

In Great Britain the plerocercoids of L. intestinalis have been reported from roach (Rutilus rutilus) (Baylis, 1928, 1939; Smyth, 1947; Kerr, 1948), gudgeon (Gobio gobio) (Baylis, 1939), brown trout (Salmo trutta) (Baylis, 1939), minnow (Phoxinus phoxinus) (Arme and Owen, 1968), rudd (Scardinius erythrophthalmus) (Owen and Arme, 1965; Orr, 1967), bleak (Alburnus alburnus) (Harris and Wheeler, 1974) and dace (Leuciscus leuciscus) (Chappell and Owen, 1969).

Figure 1. The Life Cycle of *Ligula intestinalis* (L. 1758)

Definitive Host



Plerocercoid develops in the gut to the adult worm in 48 hours

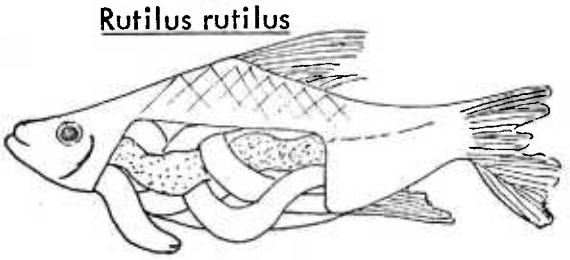
Eggs passed out in faeces
Embryonation takes 10 days at 25°C in water

Infected fish eaten by piscivorous bird



Embryonated egg

Rutilus rutilus

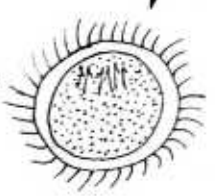


Egg hatches and free-living coracidium escapes

Plerocercoid penetrates gut wall & develops into plerocercoid in the body cavity taking about 14 months

Coracidium lives for 2 to 4 days

Eaten by fish



Eaten by Cyclops

Coracidium



Cyclops spp.

Coracidium sheds cilia & penetrates gut wall . Develops to procercoid in 10 to 15 days

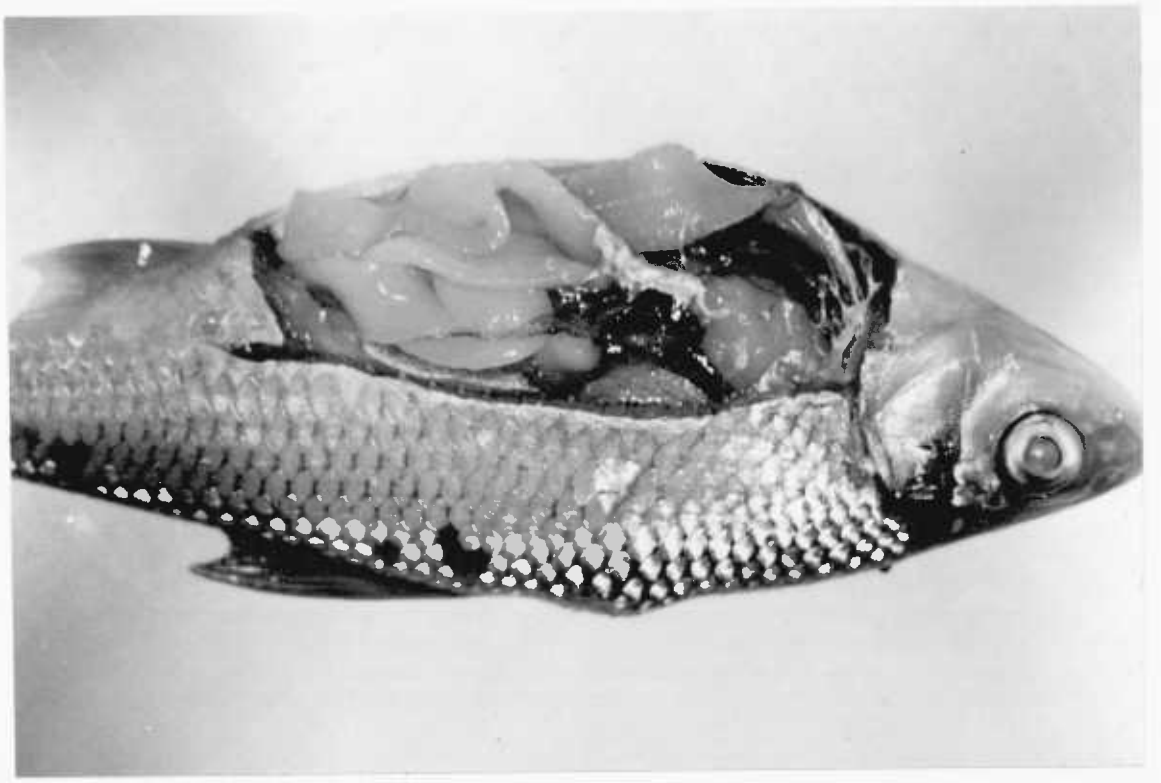
The Life Cycle of L. intestinalis

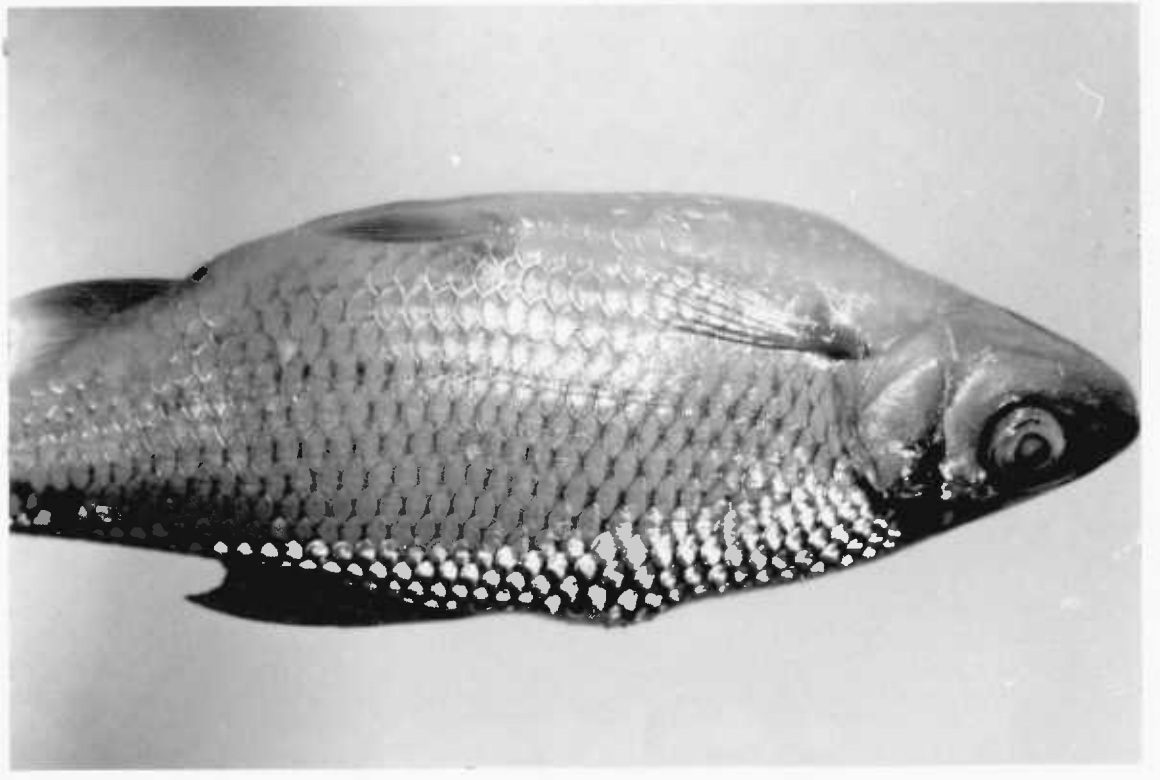
There are five stages in the life cycle of L. intestinalis (see Fig. 1). The operculate egg is passed out in the faeces of the definitive host and can only embryonate in water (Schaunisland, 1885). Development of the hexacanth embryo surrounded by a ciliated embyrophore takes 5-8 days depending on the temperature (Dubinina, 1953), after which time the egg hatches and releases a free-swimming coracidium. This stage is short-lived, and will only survive for 1-2 days unless ingested by a copepod, the first intermediate host. The coracidium sheds its ciliated embryophore in the gut of the copepod and migrates into the haemocoel where it develops to the procercoid in 10-15 days.

The second intermediate host is a species of cyprinid fish which ingests the infected copepod. The procercoid penetrates the gut wall and develops in the body cavity of the fish into the plerocercoid (see Plates 1 & 2). The development of the plerocercoid is the longest stage in the life cycle taking more than 14 months to develop (Dubinina, 1953). The plerocercoid is progenetic and the anlagen of all the genitalia are present. The life cycle is completed when the parasitised fish is ingested by any one of a number of piscivorous birds. The plerocercoid is released from the body cavity of the fish by the digestive processes of the bird and matures into the adult Ligula in the small intestine. Maturation takes 2 days

Plate 1. A roach taken from the Serpentine, showing the typical ventral pouching caused by L. intestinalis plerocercoids.

Plate 2. The same specimen with the body wall removed to show the plerocercoids of L. intestinalis.





(see Chapter 3) and the adult can remain in the intestine for up to 7 days, after egg-laying starts, although egg production tends to reach a peak on the fourth day and then fall off. The worms die due to senescence and are passed out with the faeces.

The Habitat

History and General Features of the Serpentine

(details from Lee, 1973)

The Serpentine is an artificial lake within the borders of two Royal Parks, Hyde Park and Kensington Gardens, in Central London. The parks and the lake are used extensively for recreational purposes and are managed by the Department of the Environment. The lake covers 164,000 square metres, approximately 40.5 acres; of this area 31 acres form the Serpentine itself and 9.5 acres, the Longwater, are an extension north of the road bridge. There is one small island covering ¼ acre in the lower basin.

The original Serpentine, in the form of a canal, was constructed in 1730. The Westbourne stream, sometimes referred to as the Kilburn or the Bays Water, was dammed at the lower end of an area of ponds and marshes (Foord, 1910). In 1820 it was extended by Nash giving it the appearance it has now. By 1849 it had become so badly polluted with raw sewage that it was drained and the bottom sealed with clay and concrete (London Illustrated News, 1857). The area was supplied originally with water from two spring-fed Hampstead streams - the Westbourne and the smaller Tyburn. The courses of these streams have now been lost. Other supplies of water to the Serpentine

are surface run-off from the parks and water from a well on Duck Island, St. James's Park, which is sometimes pumped up to the Serpentine (Pentelow, 1965). The outflow from the Serpentine is from the south-east corner. The exact course of the outflow water is uncertain. The course of the old Westbourne, now a flood sewer, was under Knightsbridge and through Sloane Square to the Thames. It may still be seen where it passes through Sloane Square tube station in a metal conduit (Barton, 1962). However some Serpentine water is believed to pass under Hyde Park Corner, into the pond in Buckingham Palace grounds and then into St. James's Park. If this is the course of the water then there is continual circulation of water since water is pumped back up to the Serpentine from St. James's Park.

Physical and Chemical Features

The Serpentine lies in the middle of the London Basin which is a downfold of chalk from the North Downs to the Chilterns. The basin is floored with impervious London clay with occasional sand and gravel deposits. The Serpentine itself has a partly artificial clay and concrete bottom. The lake is 1.6 km long. It gradually widens from 46 m at its northern end to 183 m at the south-eastern end. The depth of water varies from under half a metre near the fountains, to eight metres in the lower basin. There is a bathing area along the southern shore covering (335 x 37) m; the water in this area is lightly

chlorinated during the summer months. From October 1974 to December 1976 the height of the water only fell significantly during the hot summer of 1976, when it fell by approximately 50 - 75 centimetres. At no time during this period was the surface of the lake frozen. The monthly water temperatures for the period November 1975 to October 1976 are given in Table 1.

The Serpentine is a eutrophic lake. The well-oxygenated water from the fountain helps to sustain aerobic conditions throughout the year. The bottom consists of gyttya - a fine brownish sediment, characteristic of permanently oxygenated conditions (Ruttner, 1963). Despite being relatively sheltered from the wind the water is very turbid with inorganic and organic matter in suspension, resulting from the clay and sedimentary bottom and the abundance of plankton.

The Flora and Fauna

There are no clearly defined littoral or benthic zones in the Serpentine. The lake supports a balanced aquatic community and has been self-maintaining and self-regulating for many years. The only direct interference by man on the balance of the ecosystem is the occasional culling of the waterfowl, fishing, light chlorination of the water in the swimming area and the dredging of the bottom, which prevents the lake silting up. An extensive survey of the flora and fauna of the lake is

beyond the scope of this study but the following observations were made during the course of this work.

In the years 1975 and 1976 the zooplankton was abundant throughout the year, but appeared to show slight population peaks in the spring and autumn, which is quite normal. The major groups found were Copepoda, Cyclops vernalis americanus was the only species positively identified and this was borne out by Henderson (personal communication), although Lee (1973) reported finding Diaptomus spp. as well; Cladocera, in particular Daphnia spp., Isopoda, Asellus spp., and Ostracoda were also found. A wide variety of insect larvae, pupae, nymphs and adults were found at various times during the year. Molluscs were not abundant but Anodonta cygnaea (the swan mussel) were occasionally dragged up in the seine net. Lee (1973) also reported finding Planorbis spp., Lymnaea spp., and Sphaerium corneum. Tubifex sp., a Chaetopod Oligochaet was present in small numbers in the sediment.

The flora of the lake was not particularly abundant, apart from the phytoplankton. This was possibly due to the grazing activities of the numerous ducks. The rooted aquatic plants were mostly Elodea spp., and Potamegeton sp. was found around the shoreline.

The Serpentine supports a large population of water birds, migratory and resident species. The species seen were:

Canada geese	Branta canadensis (L)
Coots	Fulica atra (L)
Ducks - Mallard	Anas platyrhynchos
Mandarin	Aix galericulata
Pochard	Aythya ferima
Tufted	Aythya fuligula (L)
Great Crested Grebe	Podiceps cristatus
Gulls	Larus spp.
Moorhens	Gallinula chloropos (L)
Swans - Mute	Cygnus olor (L)

The fish fauna is diverse and abundant. It has not been interfered with since 1938 when some roach and tench were added. The following species were caught during 1975 and 1976:

Anguillidae	
Eel	Anguilla anguilla (L)
Cyprinidae	
Carp	Cyprinius carpio (L)
Gudgeon	Gobio gobio (L)
Roach	Rutilus rutilus (L)
Rudd	Scardinius erythrophthalmus (L)
Tench	Tinca tinca (L)
Percidae	
Perch	Perca fluviatilis (L)
Ruffe	Gymnocephalus cernua (L)

Roach and perch are the commonest species and Lee (1973) has suggested that there are 50,000 and 30,000

fish of each species respectively in the Serpentine, and that there are probably over 100,000 fish of all species present.

Meteorological Data

This was kindly made available by the Director-General of the Meteorological Office, London Weather Centre, Holborn, London. The records obtained were:

Total monthly rainfall (ins)

Air temperature (^{o}C), monthly mean.

The water temperature of the Serpentine was recorded at weekly intervals and a monthly mean calculated (^{o}C).

The figures are shown in Table 1.

Table 1. Meteorological Data November 1975 - October 1976

	Air temperature °C monthly mean	Total Rainfall in.	Water temperature °C
November	8.1	2.05	9.7
December	6.5	1.05	7.3
January	7.2	0.65	6.7
February	6.1	0.79	5.1
March	6.6	0.41	6.3
April	9.8	0.51	9.2
May	14.6	0.94	17.1
June	20.1	0.39	23.8
July	20.9	0.59	25.4
August	19.7	0.12	24.6
September	15.7	2.98	19.2
October	12.7	3.65	16.5

Transmission of L. intestinalis in the Serpentine

The first intermediate host of L. intestinalis are various species of Cyclops; quite a large number of species are susceptible (see page 94) but none of these were found in the Serpentine during the period of this survey, 1974 - 1976. It is likely that susceptible species were present and that the sampling methods used failed to collect any mature females, from which an identification could be made.

The second intermediate host is a cyprinid fish. In the Serpentine the most important fish host was the roach, the only other fish found to be infected was the gudgeon. The Serpentine supports quite a large bird population, but the most likely definitive host are the gulls. Ducks and coots were seen to take fish on occasions, so they probably play a part in the transmission of L. intestinalis.

The Second Intermediate Host

Class Osteichthyes

Superorder Teleostei

Order: Ostariophysi

Family: Cyprinidae

Genus: Rutilus

Species: Rutilus rutilus

This is one of the commonest freshwater fish in England. It is a widely spread species, except in Devon, Cornwall and West Wales, where it is rare and local. In Scotland its northern limits are Loch Lomond and the Taith and in the Lake District it is uncommon. It does not occur in Ireland (Hartley, 1947).

Rutilus rutilus is a variable species, and although the body is normally moderately deep and compressed at times it has a body as deep as its near relative the Rudd, or it may be slender like the Dace (Jenkins, 1936). The roach may be distinguished from the rudd firstly by the fact that the pharyngeal teeth are in one row (there are two rows in the rudd) and secondly that the dorsal fin originates only slightly behind the base of the pelvic fin (in rudd the dorsal fin originates a long way behind the pelvic fin).

The roach can be found in both still and running water; in general it is a fish of eutrophic alkaline waters. It is a fish of medium size, ranging

from 15 - 30 cm in length and sometimes up to 40 cm and attaining a weight of 1.4 kilos plus. It usually occurs in shoals and to some extent, avoids the light, being a bottom feeder, but in warm weather it does take flies from the surface. In the winter the roach retreat into deeper water. The fish spawn in April and May in shallow water; the adhesive, yellow eggs are deposited on water weeds and submerged plants. After 10 - 14 days incubation the young larvae hatch and then rest on the bottom for 8 - 10 days absorbing the yolk , then they swim around in dense shoals.

The literature on the biology and the parasitic fauna of the roach is quite extensive and a detailed survey of the literature is beyond the scope of this thesis. However some reference will be made to those studies directly concerned with the R. rutilus-L. intestinalis host-parasite relationship in Great Britain. Chubb (1962, 1963, 1964 and 1970) made an extensive survey of the parasite fauna of fish, including roach, in various habitats. Wilson (1971) and Wootten (1973) studied specific populations of roach infected with L. intestinalis and a survey of the occurrence and pathology of L. intestinalis in British fish was carried out by Arme and Owen (1968).

CHAPTER 2

COLLECTION AND EXAMINATION OF ROACH FROM THE SERPENTINE

Methods of Sampling and Examining the Fish

Samples of roach were removed from the Serpentine every month between November 1975 and October 1976. Roach were caught using a seine net 100 yards long, 8 feet deep with a mesh size of half-an-inch, knot to knot, and which was fitted with a bag. The fish were transported back to the laboratory alive, in plastic dustbins; over such a short distance oxygenation of the water was only necessary during the hot summer months of 1976. The fish were either killed by pithing and deep frozen to -20°C or kept alive in freshwater tanks, supplied with oxygenated water at 10-14°C from a rising main. No fish were kept alive for more than 72 hours before examination. Lee (1973) found that examination of frozen or freshly killed specimens made no significant difference to the weight and length characteristics of the fish or to the parasites it contains.

A record sheet was designed on which data obtained from each fish were recorded; each fish was regarded as one sampling unit. These data were later transferred to computer punch cards. The following data were obtained from each fish:

Standard length (cm)

Weight (gm)

Age (year group)

Number of parasites (plerocercoids)

Weight of individual plerocercoids (gm).

The standard length was measured from the tip of the nose to the end of the body, beginning of the caudal fin. For age determinations, scales were removed from an area just behind the dorsal fin and above the lateral line (Tesch, 1968). They were cleaned by wiping on tissue and stored in paper envelopes for examination at a later date. For examination the scales were mounted in glycerin or Canada balsam and observed under a dissecting microscope.

Sex determination, by examination of the gonads, was without problems in healthy, non-parasitized individuals. With parasitized fish sexing was extremely difficult as the gonads were usually completely reduced and histological examination was necessary. In the time available for this study it was decided that sex determination in all parasitized fish would be too time consuming, therefore in considering the data males and females are not separated.

Age Determination

The most frequent method of age determination in fish studies is the interpretation and counting of growth checks (annuli or rings) which occur in the hard parts of the fish. These are formed during alternate periods of faster and slower growth (or no growth) and reflect the various environmental and physiological influences, although the reason for annulus formation is not fully understood. The hard parts most often used are the epidermal scales, sagittal otoliths, opercular bones, fin rays and vertebrae. The use of annuli for ageing has been described extensively in the literature and has been reviewed by Graham (1929), Bryuzgin (1963) and Tesch (1968) among others.

Masterman (1922), Segesterale (1932), Hartley (1947) and Jones (1953) have described the roach scale in great detail. The scales of roach are relatively easy to read, as the growth checks are usually clearly marked. Age determinations, using scales, on roach populations have been carried out by a number of workers including Wilson (1971) and Evans (1978).

It is important to know the time of year when the growth checks occur. They should occur at approximately the same time every year, so that the check can be accepted for an annulus. Hartley (loc. cit.) showed that the growth check could occur at the edge of the scale

at any month of the year, except September and October, but that in most fish it was formed in May or June and coincided with the spawning season. Jones (loc. cit.) also concluded that the period of annulus formation was comparatively short and occurred during May, June and July. He found that the time of year of annulus formation varied from year to year, but that it was formed at the time of year when the roach spawned, although it was not possible to correlate its advent with the maturation and spending of the gonads.

For the purpose of this study, when fish were aged they were put into age groups, i.e. fish which were in their second year of life were put in the 1+ age group, those in their third year in the 2+ age group and so on.

The Validity of Age Determination

It is necessary to validate the method of age determination and one way of doing this is by analysing the size-frequency distributions. One of the methods used is that devised by Petersen (1892), which utilises length frequency distributions and is easy to employ if there is no large overlap in the size of individuals in adjacent age groups. Even if there is an overlap in the older age groups, the method can usually still be applied to the younger age groups. The length composition of a population will often exhibit modes among the smaller fish which correspond to the youngest age-groups. Since

the modes in a length frequency diagram suggest the mean fish lengths at successive ages, they may be compared with the lengths derived from age determinations made on scales or bones. When the modes correspond, at least approximately, it may be concluded that the marks used for age determination have been validated for their intended purpose. However this method does assume that growth is homogeneous throughout the population and this may not always be so, as the growth rate may vary with age, sex and maturity.

Results and Observations

Age Determination

The data can be plotted by months in which case different ages should appear as separate modes or if one age group is plotted throughout the year, then the mode should pass in a wave-like progression through the year. In Figure 2 the data are plotted throughout the year for the 0+, 1+, 2+ and 3+ age-groups, and show a confused pattern. Although modes are present in each month, there is overlapping in age-groups and no obvious growth progression through the year. In Figure 3 the length frequencies of these groups are plotted for the whole twelve months and it can be seen that although the 0+ and 3+ age-groups are obvious, the 1+ and 2+ age-groups do overlap considerably.

Hartley (1947a and b) states that the growth rate in roach is extremely variable and that examination of length frequency distributions gives a "minimum of information about growth and age". In Table 2 figures are shown of the length ranges of age-groups determined by various authors and this serves to illustrate the variation in length of the age groups. These figures are also compared with those obtained in this study.

Figure 2. Length frequency histograms for 0+, 1+, 2+ and 3+ age groups of Serpentine roach, November 1975 to October 1976, plotted for each month.

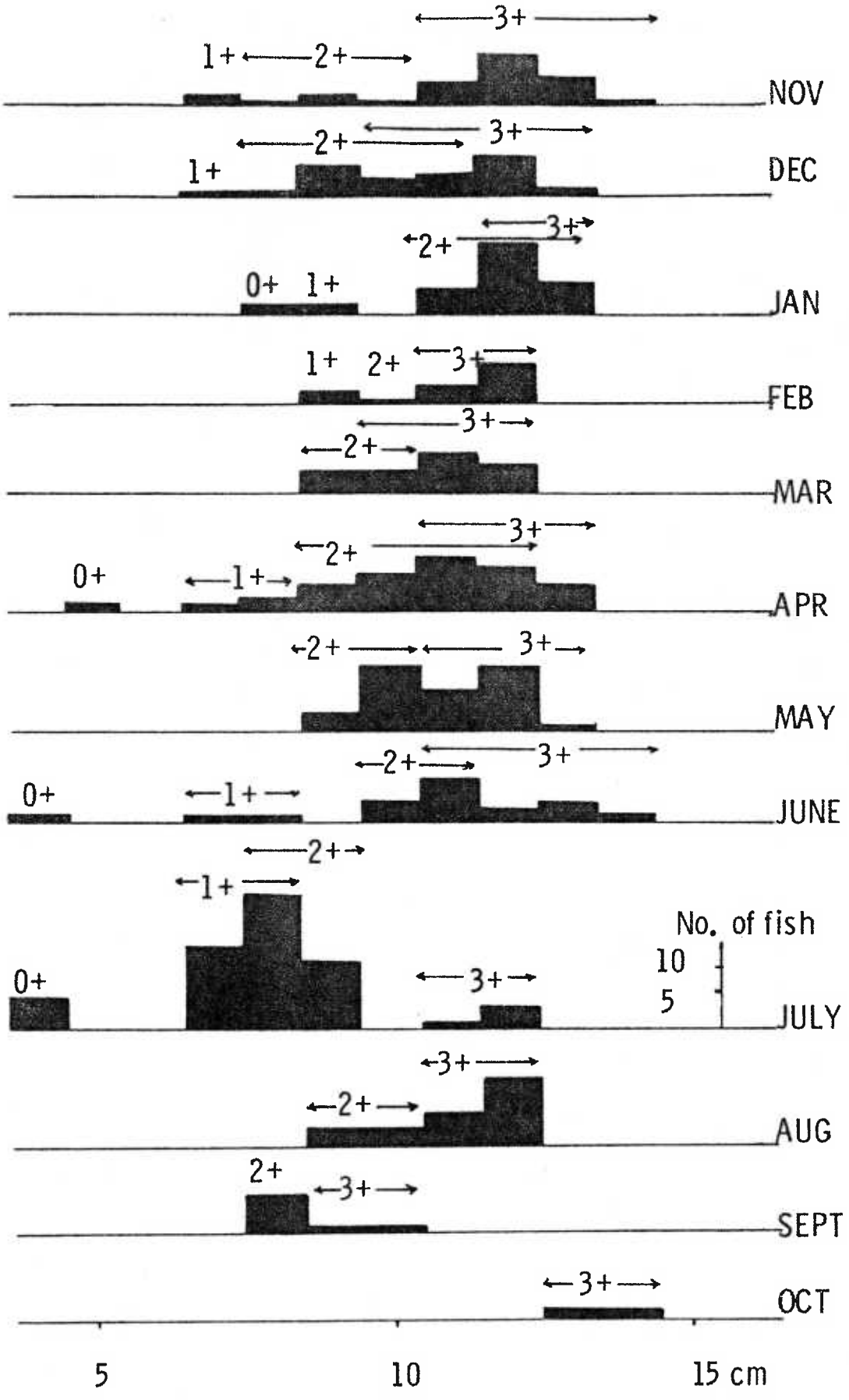
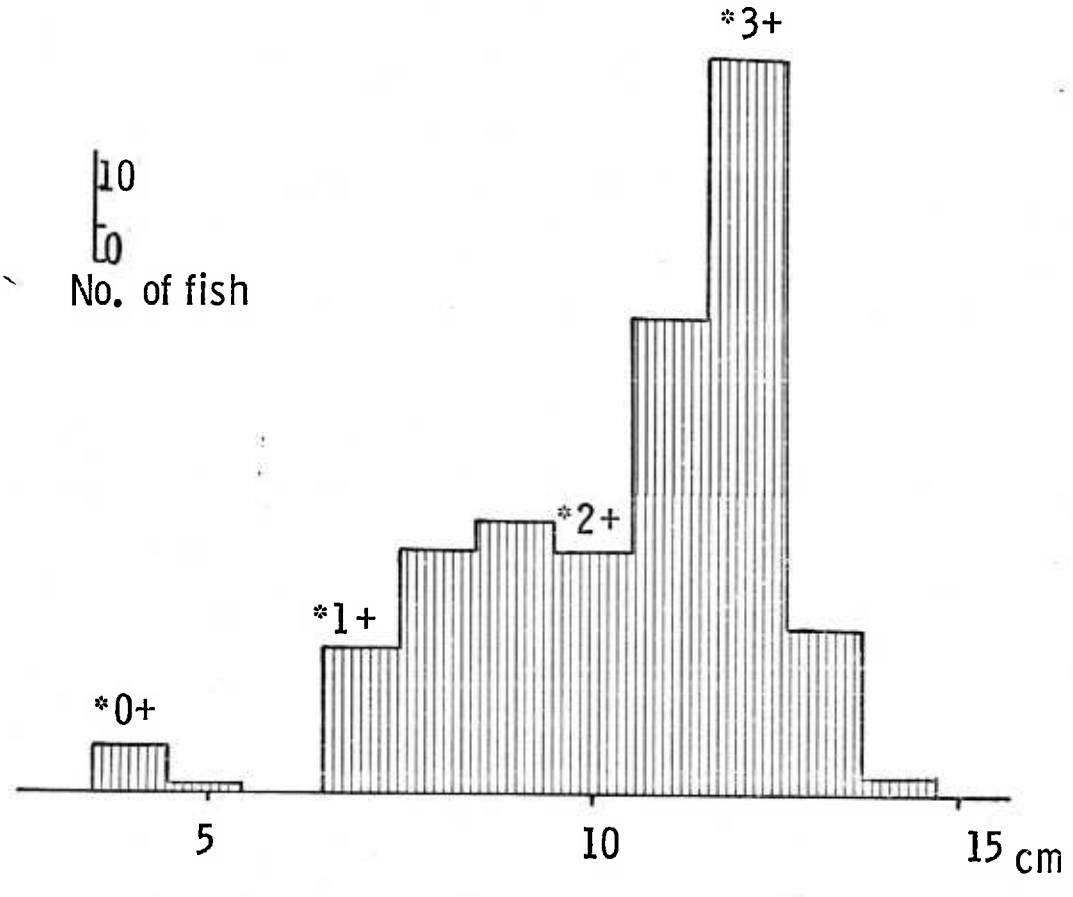

3+
1+
2+
NOV
2+
3+
1+
DEC
3+
2+
0+
1+
JAN
1+
2+
3+
FEB
3+
2+
MAR
3+
2+
0+
1+
APR
2+
3+
MAY
2+
3+
0+
1+
JUNE
2+
1+
No. of fish
10
5
0+
3+
JULY
3+
2+
AUG
2+
3+
SEPT
3+
OCT
5
10
15 cm

Figure 3. Length frequency histograms for o+, 1+, 2+ and 3+ age groups of Serpentine roach, November 1975 to October 1976, plotted for the whole twelve months.



* indicates the mode
the mean values are: 0+ - 5.1 cm
1+ - 8.1
2+ - 10.1
3+ - 12.2

Table 2. A Comparison of the Length Ranges of the Age-groups of Roach in cm.

Author	Jones (1953)	Jones (1953)	Wilson (1971)	Hartley (1947a & b)
Location	Llyn Tegid	Birket	Chew Valley	
Age 0+	-	-	0 - 7	
1+	6-8	6 - 8	7 - 14	
2+	7 - 14	5 - 10	14 - 21	Average 9.2
3+	11 - 18	6 - 12	21 - 25	11.5
4+	13 - 18	7 - 14	25 - 28	12.5
5+	-	7 - 15	28+	13.8
6+	-	10 - 17		15.0
7+				17.5

Table 2. (contd.) A Comparison of the Length Ranges of the Age-groups of Roach in cm. Serpentine November 1975 - October 1976.

	Mean	Minimum	Maximum	Range	Sample Size
0+	5.1	4.3	8.5	4.2	8
1+	8.1	7.0	11.3	4.3	38
2+	10.1	8.1	13.2	5.1	96
3+	12.2	9.6	15.0	5.4	139
4+	13.9	11.8	17.3	5.5	185
5+	15.6	12.4	17.8	5.4	113
6+	17.7	14.6	20.3	5.7	61
7+	19.2	17.5	21.3	3.8	41
8+	20.6	18.3	24.1	5.8	25
9+	22.5	19.7	25.8	6.1	15
10+	22.4	20.7	24.0	3.3	5

The Number of Roach Examined

The number of roach caught in the Serpentine by seine net between November 1975 and October 1976 was 728. The total number of roach caught and examined in each month and in each age group is shown in Table 3.

The greatest number caught were in the 6-20.9 cm length range. Fish smaller than this were hard to catch because of the mesh size of the net and fish larger than this were rare, as they were probably very old and therefore not present in large numbers due to natural mortality. As some fish grow faster than others in any population, the range would be expected to increase with the increasing age of the fish. This is true to some extent in the roach population in the Serpentine, but due to the great variation in size of all age-groups, the difference is not as marked as may be expected (see Table 2).

The length-frequency histograms of fish of all ages are plotted in Figure 4.

Examination of Roach for Other Parasites

Periodically roach were examined for the presence of any other parasites and the following parasites were found:

Monogenea	- *Dactylogyrus crucifer*	gills
Digenea	- *Diplostomum spathaceum*	lens of eye

Nematoda - *Eustrongylides* spp.	body cavity
Mollusca - *Glochidia* larvae	gills.

Table 3. The Number of Roach in Each Age-group Examined in Each Month from November 1975 - October 1976, from the Serpentine

Age Group	0+	1+	2+	3+	4+	5+	6+	7+	8+	9+	10++	All Ages
November 1975		2	4	19	2	2	6	2				37
December		1	11	11	8							31
January 1976	1	2	11	8	4	2						28
February		2	1	10	10							23
March			5	15	25	5	1	2		1		54
April	1	3	16	13	6	4	1	3	2	2		51
May			10	23	23	11	6	10	9	1		93
June	1	1	7	11	40	16	16	13	8	5	2	120
July	5	27	21	5	13	3	1		3			78
August			4	17	36	33	13	4	2		4	113
September			6	3	9	10	4	3	4		1	40
October				4	9	27	13	4	2	1		60
All months	8	38	76	139	185	113	61	41	30	10	7	728

10++ age group includes: 1 11+ June

1 12+ June.

Figure 4. Length frequency distribution of all roach caught in the Serpentine, November 1975 to October 1976. The data for all ages combined are shown as a continuous line. Data for each age group are shown as solid histograms with a scale on the right-hand side. Age groups 0+ to 4+.

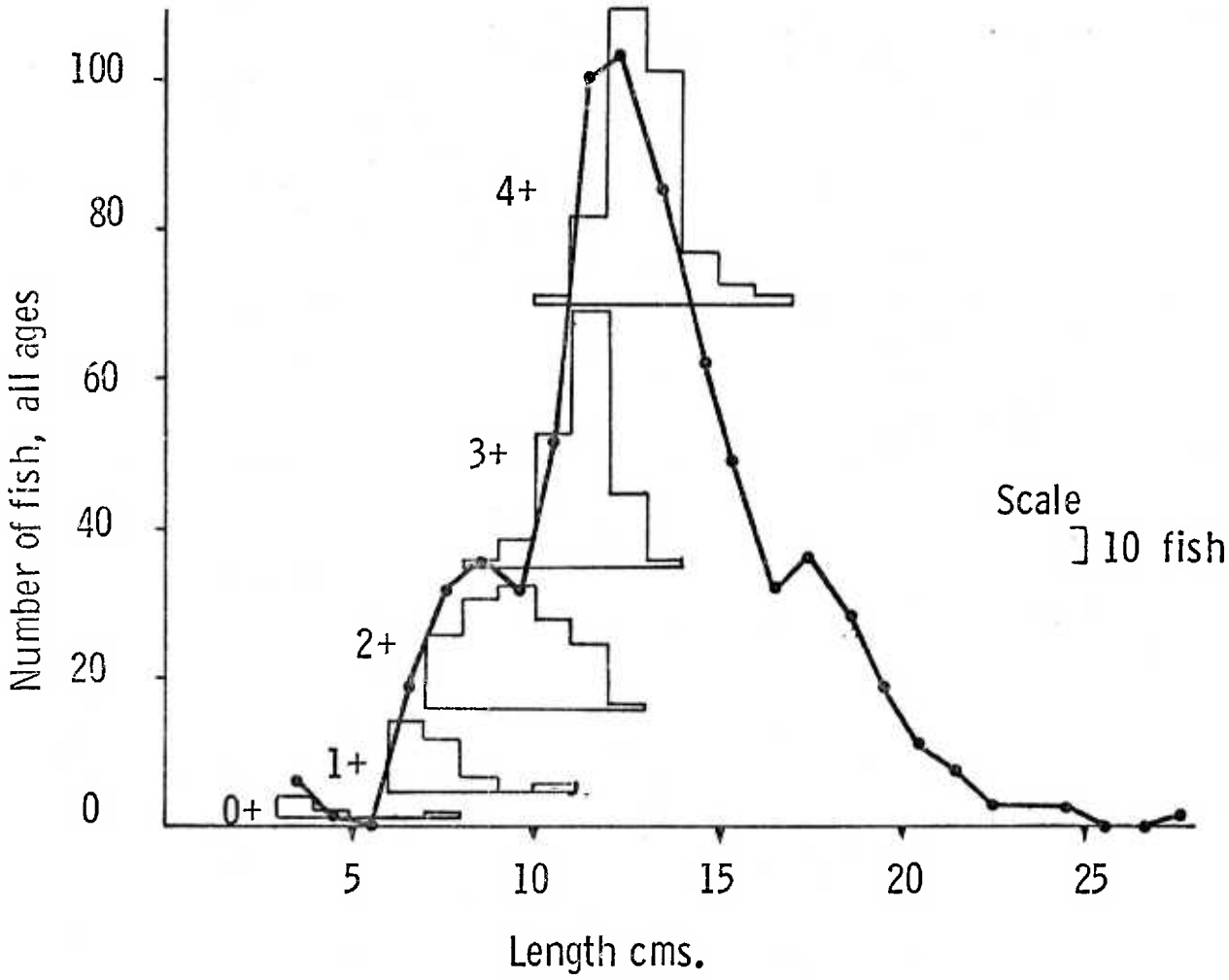
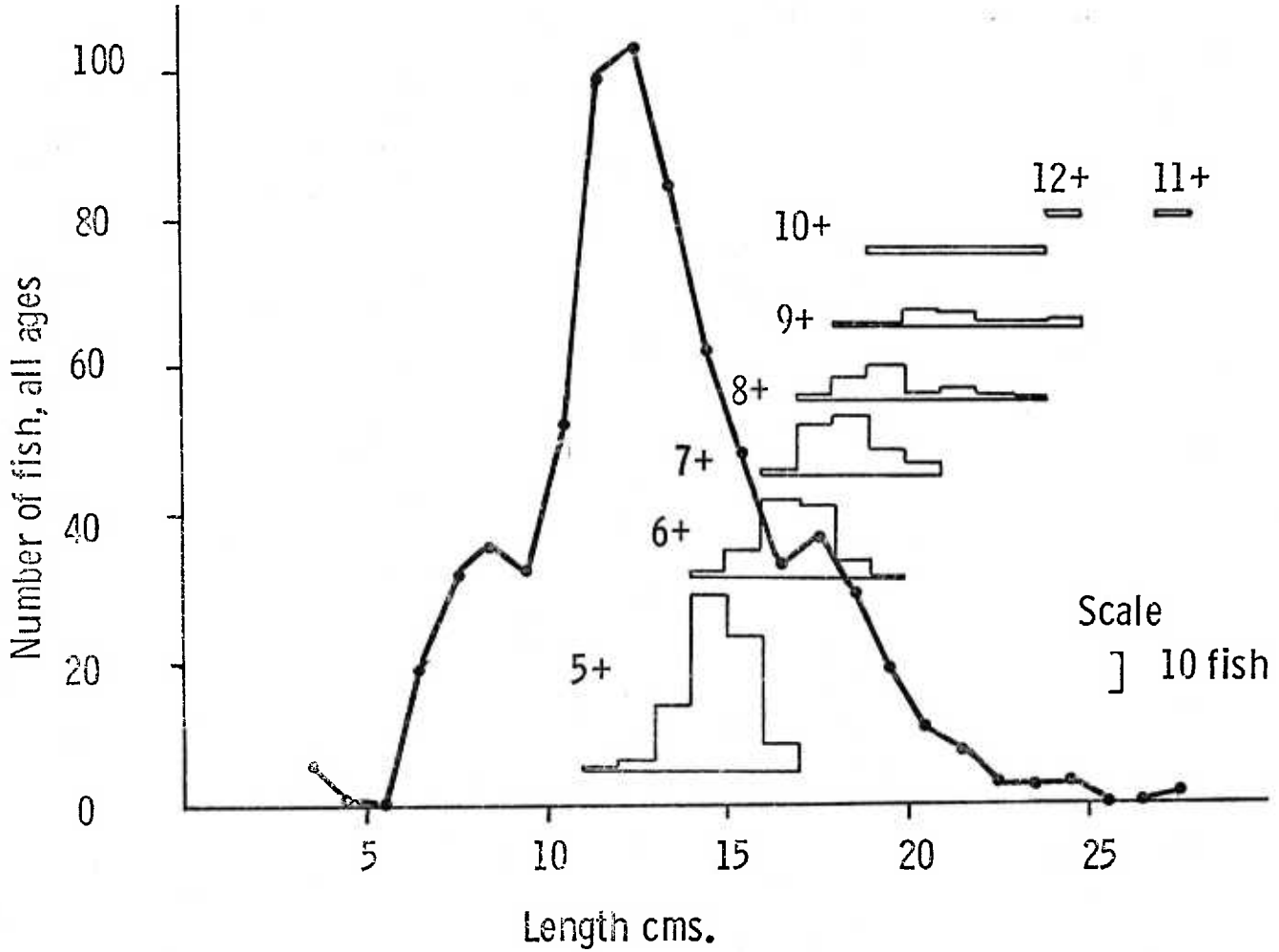

100
80
60
40
20
0
Number of fish, all ages
4+
3+
2+
1+
0+
Scale
] 10 fish
5
10
15
20
25
Length cms.

Figure 4. Length frequency distribution of all roach (contd.) caught in the Serpentine, November 1975 to October 1976. The data for all ages combined are shown as a continuous line. Data for each age group are shown as solid histograms with a scale on the right-hand side.

Age groups 5+ to 12+.


100
80
60
40
20
0
Number of fish, all ages
5
10
15
20
25
Length cms.
12+
11+
10+
9+
8+
7+
6+
5+
Scale
] 10 fish

Seasonal Variations in the Ligula Infection of Roach

The percentage of roach infected with Ligula (the incidence) was calculated for each sample and is shown in Table 4 and plotted in Figure 5a. It can be seen that there appears to be a very marked seasonal variation, the incidence being 100% in January and February and then declining over the Spring and Summer months. 32% of all fish were infected. The intensity of the infection can be measured as the number of worms per fish (see Table 5, Figure 5b). The maximum number of plerocercoids found in one fish was 18, caught in August 1976. The mean number of plerocercoids per fish was 1.3 and the mean number per infected fish was 3.1. As the size of the plerocercoids was very variable it was thought that the weight of the plerocercoids would give a better indication of the intensity of infection rather than the number, see Table 6 and Figure 5c. The mean weight per fish was 2.8 g whereas the mean weight per infected fish was 7.0 g. The largest plerocercoid weighed 6.7 g and was caught in August 1976 in a 5+ roach. The mean plerocercoid weight was calculated and plotted against the date of sample in Figure 6c. The frequency distribution of the number of plerocercoids per fish is shown in Figure 6a and it can be seen that of those fish infected, the greatest number had between 1 - 4 plerocercoids per fish. The distribution of the parasite

Table 4. Ligula intestinalis, Percentage Incidence of Infection in Serpentine Roach, November 1975 - October 1976. (The numbers of fish in each age/month category are given in Table 3.)

	0+	1+	2+	3+	4+	5+	6+	7+	8++	All Ages
Nov.		0.0	75.0	63.2	0.0	0.0	16.7	0.0		43.24
Dec.		100.0	100.0	90.9	100.0					96.80
Jan.	100.0	100.0	100.0	100.0	100.0	100.0				100.00
Feb.		100.0	100.0	100.0	100.0					100.00
Mar.			80.0	86.7	92.0	60.0	100.0	0.0	0.0	81.50
Apr.	100.0	66.7	56.3	15.4	33.3	50.0	0.0	33.3	0.0	37.25
May			20.0	21.7	17.4	27.3	0.0	0.0	0.0	13.98
June	0.0	100.0	28.6	0.0	35.0	18.7	0.0	0.0	0.0	17.50
July	0.0	14.8	23.8	40.0	23.1	0.0	0.0		0.0	18.00
Aug.			25.0	11.8	16.7	6.1	0.0	0.0	0.0	9.70
Sept.			0.0	33.3	22.2	0.0	0.0	0.0	0.0	7.50
Oct.				100.0	22.2	18.5	0.0	0.0	0.0	18.30
All months	25.0	31.6	64.5	49.6	42.2	17.7	3.3	2.4	0.0	

8++ includes: 9+ 10 individuals; 10+ 5 individuals; 11+ 1 individual; 12+ 1 individual.

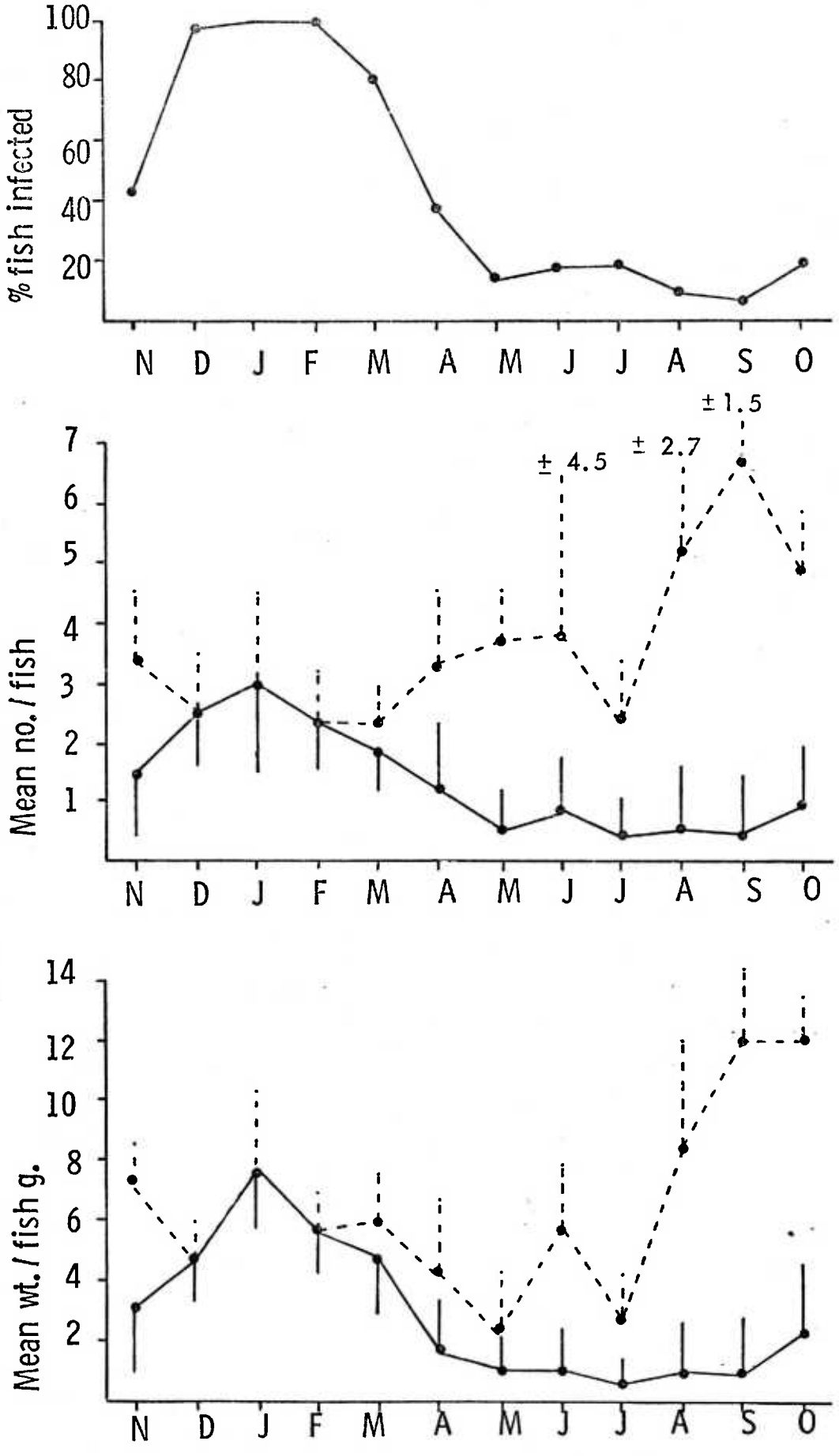
Figure 5. Seasonal variations in the incidence and intensity of the Ligula infection in Serpentine roach, November 1975 - October 1976.

a) Percentage of roach infected with Ligula.

b) Mean number of plerocercoids per fish.

c) Mean weight of plerocercoids per fish.

Confidence limits in Fig. 5b) and 5c) expressed as ½ the standard deviation.


% fish infected
100
80
60
40
20
N D J F M A M J J A S O
Mean no./fish
7
6
5
4
3
2
1
± 4.5
± 2.7
± 1.5
N D J F M A M J J A S O
Mean wt./fish g.
14
12
10
8
6
4
2
N D J F M A M J J A S O
------ no./wt. per infected fish
—— no./wt. per fish(whole sample)

within the host population was "skewed" and showed a marked overdispersion with the variance to mean ratio being significantly greater than unity (the expected value for a random distribution). Overdispersion of fish parasites has been described by several authors (e.g. Pennycuick, 1971; Anderson, 1974) and the factors which may cause such a situation have been reviewed by Crofton (1971). The weight-frequency distributions of the plerocercoids were also determined, the frequencies being expressed as number per fish (see Figure 7). The parasitization index (P.I.) as described by Arme and Owen (1968) was calculated for each fish and the mean determined for each sample, see Figure 6b. The P.I. is calculated thus:

$$\text{P.I.} = \frac{\text{weight of } \underline{\text{Ligula}}}{\text{weight of host + weight of } \underline{\text{Ligula}}} \times 100 .$$

Therefore if the weight of *Ligula* is equal to the weight of the host then the P.I. = 50.

The overall mean P.I. was 16.8, meaning that, on average, the weight of *Ligula* was between one fifth and one sixth of the weight of its host. The maximum P.I. was 48.9 which occurred in April 1976.

When sampling started in November 1975 the intensity of infection was quite high, as was the number and weight of worms. In December there was a dramatic rise in the incidence to 97% which then further increased

Table 5. Ligula intestinalis, Mean Intensity of Infection in Serp entine Roach November 1975 - October 1976.

$\bar{x}$ = mean number of worms per fish; St.E. = Standard Error of the Mean

	0+		1+		2+		3+		4+		5+		6+		7+		8++		All Ages	
	$\bar{x}$	St.E.	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E
Nov.			0.0		2.0	1.08	2.26	.59	0.0		0.0		.67	.67	0.0				1.49	.37
Dec.			2.0*		2.27	.45	2.5	.69	2.62	.57									2.45	.32
Jan.	2.0*		1.5	.50	2.18	.35	2.37	.37	2.5	.65	13.0	0.0							3.00	.57
Feb.			1.0	0.0	4.0*		2.9	.60	2.1	.43									2.43	.34
Mar.					1.2	.37	2.27	.41	1.88	.25	1.8	.92	5.0*		0.0		0.0*		1.87	.20
Apr.	1.0*		1.0	.58	1.56	.41	.15	.10	1.83	1.64	3.0	1.78	0.0		2.67	2.67	0.0		1.22	.31
May					.50	.40	.74	.35	.48	.26	1.27	.69	0.0		0.0		0.0		.50	.14
June	0.0*		1.0*		1.57	.81	0.0		1.33	.38	1.69	1.02	0.0		0.0		0.0		.77	.20
July	0.0		.15	.70	.29	.12	2.40	1.50	.77	.47	0.0		0.0*				0.0		.41	.14
Aug.					.25	.25	.18	.13	.94	.41	.57	.55	0.0		0.0		0.0		.50	.21
Sept.					0.0		1.33	1.33	1.78	1.22	0.0		0.0		0.0		0.0		.50	.30
Oct.							4.50	.87	.78	.52	1.04	.46	0.0		0.0		0.0		.88	.26
All months	0.38	.26	.39	.10	1.20	.15	1.50	.17	1.30	.16	1.20	.30	.15	.10	.19	.19	0.0			

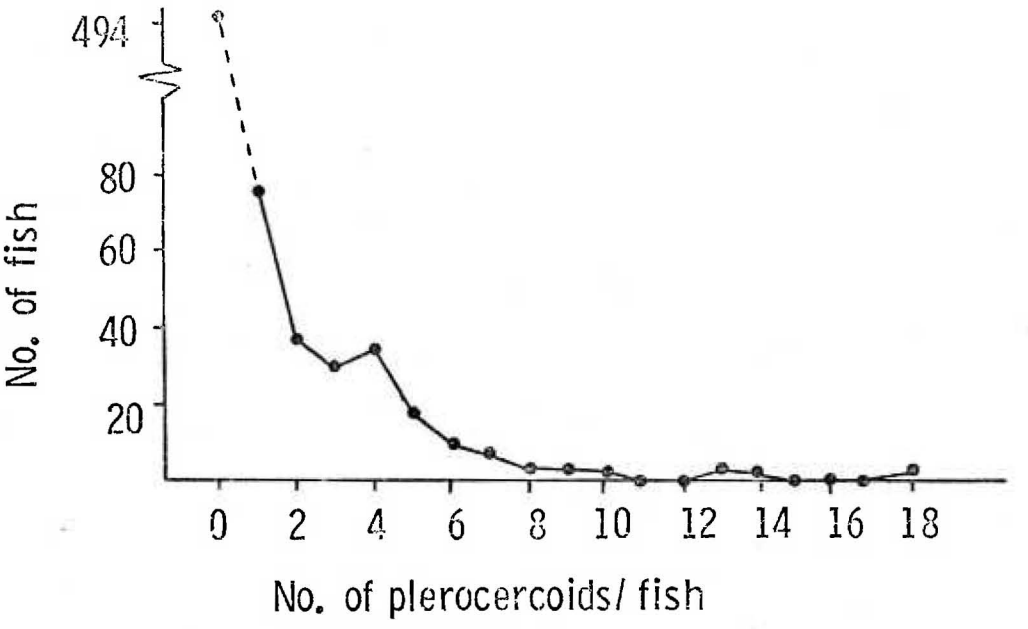
* Only one observation.

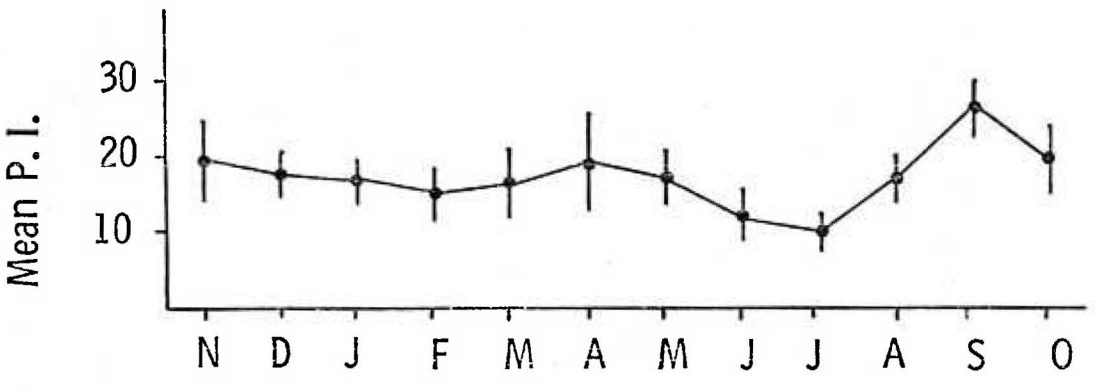
Figure 6a). Frequency distribution of Ligula plerocercoids in roach from the Serpentine, November 1975 to October 1976.

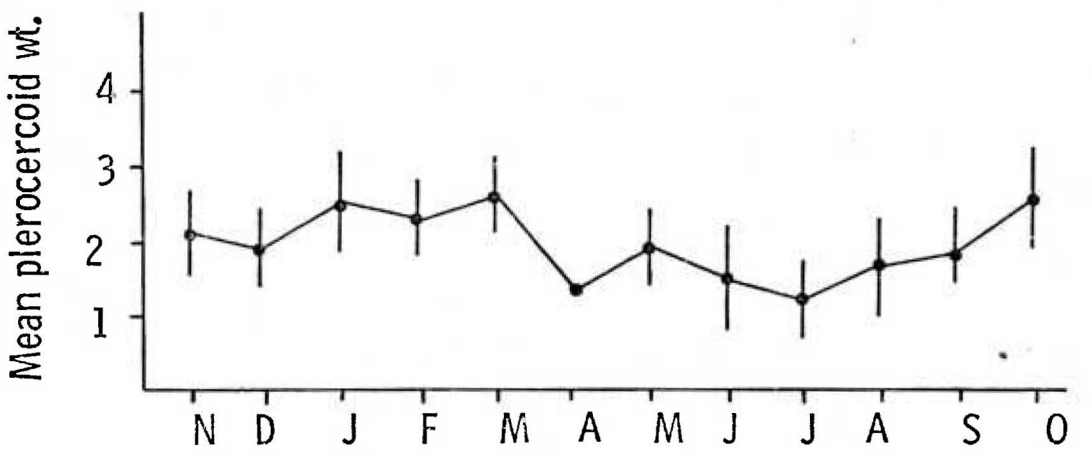
6b). Seasonal variation in the P.I. of Ligula infections in roach from the Serpentine, November 1975 to October 1976.

6c). Seasonal variation in the weights of Ligula plerocercoids from Serpentine roach, November 1975 to October 1976.

Confidence limits in Fig.6b) and 6c) expressed as ½ the standard deviation.


494
80
60
40
20
No. of fish
0 2 4 6 8 10 12 14 16 18
No. of plerocercoids/ fish


30
20
10
Mean P. I.
N D J F M A M J J A S O


4
3
2
1
Mean plerocercoid wt.
N D J F M A M J J A S O

to 100% during January and February. In December there was a drop in intensity, both in numbers and weight of plerocercoids. However the mean plerocercoid weight was 1.9-2.00 g and the weight-frequency showed that there was a decrease in the number of smaller worms at this time of year. It is possible that an infection which consists of a large number of small worms may be more deleterious to the host than an infection with one large worm and with the onset of winter, colder temperatures, shortage of food, the more heavily infected fish may well die or be predated upon.

In January there was a slight increase in the number and more particularly in the weight of plerocercoids. This could have been due to the growth of plerocercoids, but the mean weight of the plerocercoids dropped in January and there was an increase in the number of smaller worms. There was some natural mortality of older fish with large plerocercoids which would account for the decrease in the mean plerocercoid weight. From March to May there was a decrease in the incidence, but there was a steady increase in the numbers of plerocercoids and a drop in the weight. This was perhaps indicative of a new infection occurring in April and May; certainly in these months very small plerocercoids were seen in fish. The mean plerocercoid weight dropped in April, also indicating a new infection. In June the weight of plerocercoids suddenly rose and the number of small worms increased

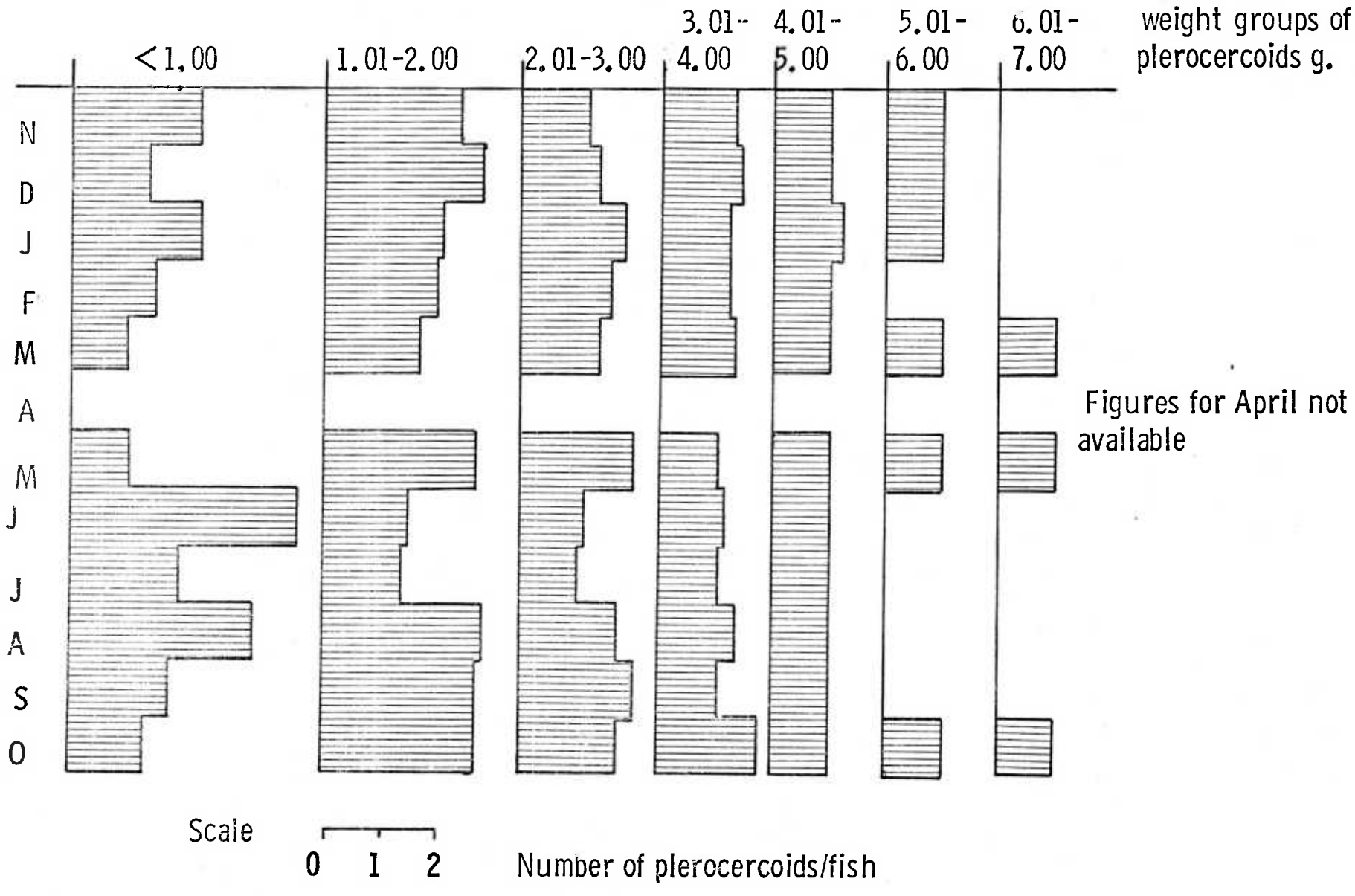
Table 6. Ligula intestinalis, Mean Intensity of Infection in Serptentine Roach November 1975 - October 1976.

$\bar{x}$ = mean weight of plerocercoids per fish; St.E = Standard Error of the mean.

	0+		1+		2+		3+		4+		5+		6+		7+		8++		All Ages	
	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E	$\bar{x}$	St.E
Nov.			0.0		3.83	1.61	4.39	.89	0.0		0.0		2.50	2.50	0.0				3.07	.67
Dec.			1.93*		3.23	.38	4.72	.88	6.58	.93									4.60	.47
Jan.	3.01*		3.62	1.80	6.91	1.01	6.58	.91	6.33	1.29	24.54	1.54							7.62	1.05
Feb.			1.93	.27	6.89*		6.07	.75	5.95	.81									5.70	.53
Mar.					2.29	.60	5.11	.88	5.10	.54	5.75	2.76	17.19*		0.0		0.0*		4.84	.51
Apr.	0.85*		.21	.14	1.63	.52	.05	.03	1.83	1.17	5.47	3.18	0.0*		6.34	6.34	0.0		1.56	.50
May	0.0*		0.08*		.78	.59	1.49	.67	.87	.52	2.79	1.70	0.0		0.0		0.0		1.00	.30
June					1.74	1.23	0.0		2.17	.60	1.44	.93	0.0		0.0		0.0		1.02	.26
July	0.0		.12	.06	.32	.15	2.66	1.89	1.17	.75	0.0		0.0*				0.0		.49	.19
Aug.					.58	.58	.46	.32	1.76	.75	.92	.74	0.0		0.0		0.0		.92	.33
Sept.					0.0		2.44	2.44	3.23	2.14	0.0		0.0		0.0		0.0		.91	.53
Oct.							10.27	1.50	2.52	1.67	2.56	1.07	0.0		0.0		0.0		2.21	.63
All months	0.42	.37	.45	1.7	2.09	.29	3.10	.32	2.77	.29	2.24	.52	.53	.37	.46	.46	0.0			

* Only one observation

Figure 7. Seasonal variations in the frequencies of *Ligula* plerocercoids of different weights from Serpentine roach, November 1975 to October 1976.


< 1.00
1.01-2.00
2.01-3.00
3.01-4.00
4.01-5.00
5.01-6.00
6.01-7.00
weight groups of plerocercoids g.
N
D
J
F
M
A
M
J
J
A
S
O
Figures for April not available
Scale
0 1 2
Number of plerocercoids/fish

dramatically (see weight-frequency Figure 7). The parasitization index was low at this time of year and so it can be concluded that quite a heavy infection was probably occurring.

In July the picture suddenly changed as there was a large decrease in intensity, the mean plerocercoid weight and P.I. also reached their lowest points. The summer of 1976 was extremely warm (see meteorological data, Table 1) and it is a possibility that heavily parasitised fish were even more open to predation than normal. It was also noticeable that in July very few large fish were caught, most were in the 1+ and 2+ group and the incidence of infection and intensity was low in these groups in July.

In August and September the incidence remained low, but started to increase in October. Both the weight and the number of plerocercoids increased dramatically to reach a peak of intensity in September. In August there was an increase in the number of small worms occurring showing that recruitment was taking place. However the mean plerocercoid weight started to rise in August, as did the P.I. which showed that plerocercoid infections already established were growing rapidly at this time of year. A summary of the data for infected fish is given in Table 7.

Table 7. Seasonal Variation in the Incidence and Intensity of *Ligula* Infections in Serpentine Roach, November 1975 - October 1976.
Summary of the Data

	No. fish	No. inf. fish	Total No. plero-cercoids	Total wt. pleroc. g	Mean wt. pleroc./ inf. fish g	Mean pleroc. wt. g	Mean No. pleroc./ inf. fish	Mean P.I.
Nov. 1975	37	16	55	113.76	7.11	2.07	3.43	20.02
Dec.	31	30	76	145.0	4.74	1.9	2.53	18.19
Jan. 1976	28	28	84	213.33	7.62	2.54	3.00	17.49
Feb.	23	23	56	130.99	5.69	2.34	2.43	15.21
Mar.	54	44	101	259.12	5.89	2.57	2.30	16.40
Apr.	51	19	61	79.99	4.21	1.31	3.21	19.71
May	93	13	47	92.17	7.09	1.96	3.61	17.61
June	120	21	81	122.16	5.82	1.51	3.86	12.82
July	78	14	32	38.44	2.75	1.20	2.30	10.43
Aug.	113	11	57	104.1	9.54	1.82	5.20	17.23
Sept.	40	3	20	36.42	12.14	1.82	6.67	26.20
Oct.	60	11	53	132.89	12.1	2.51	4.82	19.70

Variations in the Ligula Infections of Roach of Different Ages

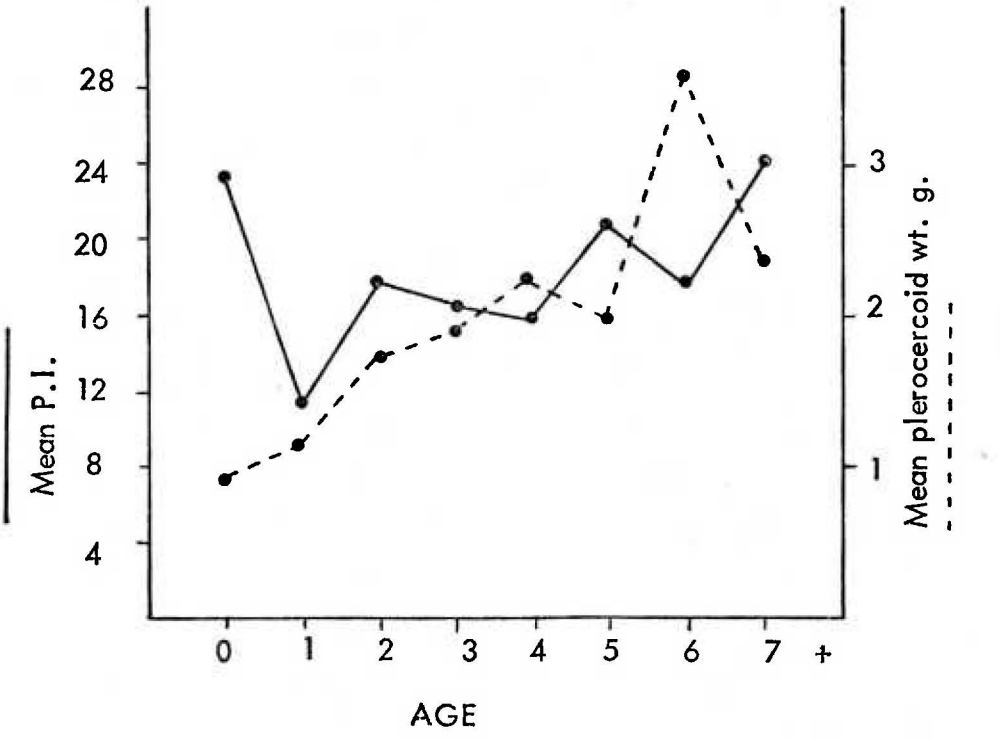
Table 4 shows the percentage incidence of infection in each age-group. This reached a peak in 2+ fish of 64.5%, remained just below 50% in 3+ and 4+ fish and then declined. No fish older than 7+ was found to be infected.

The intensity of the infection for the whole population of fish sampled is shown in Tables 5 and 6 and for infected fish only in Figure 8a. Figure 8a shows that as fish get older, intensity increases, both the weight and the number of plerocercoids increasing. It is to be expected that the plerocercoids would continue to grow, but it is surprising that the actual number of plerocercoids increases with age, as the dietary requirements of older roach normally breaks the cycle of transmission (see Discussion). The P.I. was very high, 23.54, in 0+ fish, but then decreased to 11.4 in the next group. This was probably due to high mortality in the heavily infected 0+ group. The P.I. then increased, erratically to 20.9 in the 5+ group, then decreased to 17.6 in 6+. The figures for the 7+ group cannot be considered to be reliable as only one specimen was caught. It is to be expected that the P.I. should start to decrease at some point in the older age groups, as the weight of the plerocercoids would become a smaller proportion of the weight of the fish. The mean plerocercoid weight increased throughout the age groups of the roach (Figure 8b).

Figure 8a). Variation in the intensity of infection with Ligula in Serpentine roach of different ages, November 1975 to October 1976. For numbers of fish in each age group see Tables 3 and 4.

8b). Variation in the P.I. and the mean plerocercoid weight of Ligula in Serpentine roach of different ages, November 1975 to October 1976.


No. plerocercoids/ infected fish
Mean wt. plerocercoids/infected fish g.
8
7
6
5
4
3
2
1
20
16
12
8
4
0 1 2 3 4 5 6 7 +
AGE


Mean P.I.
Mean plerocercoid wt. g.
28
24
20
16
12
8
4
3
2
1
0 1 2 3 4 5 6 7 +
AGE

Discussion

The apparent seasonal variation in the incidence of *L. intestinalis* in Serpentine roach, may be the result of biassed sampling. Arme and Owen (1968) and Wilson (1971) point out that it is extremely difficult to get an accurate idea of the incidence of infection, as netting is a very difficult method to standardise. It is well known that infection with *Ligula* plerocercoids causes changes in the hosts' behaviour. Dogiel *et al.* (1961) and Nikolskii (1963) state that the survival of fish heavily infected with *Ligula* is reduced as their ability to swim is impaired and they become easy prey for predators. This was borne out by Van Dobben (1952) who found that up to 30% of roach eaten by cormorants were infected with *Ligula*, whereas only 6.5% of those taken by fishermen in the same area contained this parasite. So there is strong evidence of the parasite increasing host susceptibility to predation.

Infected fish tend to stay in shallow water and do not migrate in winter into the deeper water. Therefore, in the winter months only infected fish, on the whole, will be caught by seine netting from the shore. In the Spring and Summer when the water temperature rises, the healthy individuals migrate back into the shallower water to breed, so at this time of year a more mixed catch of healthy and infected individuals occurs, with a subsequent apparent drop in the incidence. Immature uninfected fish tend to

stay in the shallows during the winter, but none of these were caught in December, January or February. It is possible that these fish were shoaling in a different place to the point at which fishing was taking place, as it is well known that roach stay in age-group shoals (Wilson, 1971).

During the period of sampling, 32% of roach caught were infected. This compares with a mean incidence of infection of 48% in the jollytail in Australia (Pollard, 1974), 75% in rudd in Great Britain (Orr, 1966), 67% in bream and 40% in bleak in Russia (Kosheva, 1957), 35% in catastomids in Canada (Roussow, 1954) and 6.5% in roach in Holland (Van Dobben, 1952). In the Serpentine the mean number of plerocercoids per infected fish was 1.3, with a maximum of 18 occurring in one fish. This compares with the figures obtained by Pollard (1974) in the jollytail; this species had a mean number of plerocercoids per infected fish of 1.5 but the maximum number was 5 in one fish. Arme and Owen (1968) and Wilson (1971) found the maximum number of plerocercoids in any one infected roach was 53 and 63 respectively. Roussow (1954) found 1 - 2 worms per fish in catastomids and a maximum number of 20. Dence (1958) found that Notropis cornutus usually had only one plerocercoid and a maximum of 8.

The mean P.I. was 16.8, which means that on average in the Serpentine the parasite weighs between one-fifth and one-sixth of the weight of the host. In young

fish of the 0+ age-group the P.I. = 23. This was exceptionally high when compared with the figures of Arme and Owen (1968) where the P.I. for 0+ fish did not usually rise above 1. Only eight 0+ fish were collected and six of these were caught in June and July. It is thought that these were probably "late developers" and were in fact 1+ fish which had not yet formed their first annulus. In 1+ fish the mean P.I. was 11.4 which agrees quite closely with the results of Arme and Owen (loc.cit.). The P.I. continued to increase in the older age groups up to age group 5+ where it reached a peak of 20.9. In age group 6+ the P.I. decreased to 17.6 and it is to be expected that if the plerocercoid has reached its maximum size and the fish continues growing then the weight of the plerocercoid will become a smaller proportion of the weight of the fish.

The high incidence of infection found in young roach is due to their behaviour and their diet. Young roach feed mainly on plankton and algae, therefore their diet consists of a large proportion of copepods, which are the first intermediate host of Ligula. The high population of copepods in the summer coincides with the time when the fry start to feed. The exceptional summer of 1976 probably favoured the quick growth of copepods and shortened the development time of procercoids in the copepods and the incubation time of Ligula eggs, so that the summer infection in 1976 in roach fry was probably very heavy. At the age of 2+ or 3+ the diet of roach gradually

changes to a more omnivorous type, including plants, molluscs and insect larvae, so that the older fish which escaped infection when young are less likely to become infected during their lifetime. However, older fish are not completely cut off from the source of infection, as they do come into shallow water to breed in the spring and they often surface feed in the warmer weather.

It appears that new infections take place mainly throughout the summer months, this is shown by the increased levels of intensity (both in weight and numbers of plerocercoids), and also by the presence of numbers of small plerocercoids. Kennedy (1970) states that infection carries on throughout the year and it is possible that this is so in the Serpentine, although no very small worms were seen at any other time of year. This would suggest that the density dependent effects characteristic of cestode infections are occurring, i.e. the relatively small numbers of plerocercoids (compared with other studies, e.g. Arme and Owen, 1968; Wilson, 1971) are all able to develop to some extent in the fish host. However, the definitive hosts, gulls and ducks, are present all the year round as are the copepods, although these are reduced in number in the winter. It is also true that maturation of the procercoid and embryonation of the eggs of Ligula will be much slower at the lower winter temperatures, so the whole transmission cycle will take much longer.

In this study, fry were not specifically caught

and studied, as the main point of catching the fish was to collect parasite material for other experiments. However, it has been reported by Arme and Owen (1968) that infections become established in the first three months of life and by Wilson (1971) that roach fry usually contain 1 - 2 plerocercoids by the autumn. In fish aged 3+ and over it was unusual to find very small worms on their own, small worms (i.e. new infections) were only found in older fish if they had an already well established infection. Facilitated penetration of the procercoid larva has been suggested by a number of authors; Kosheva (1956) described the intestinal walls of fish infected with Ligula as being transparent and Shpolyanskaya (1953a) confirmed this change. Both Orr (1967) in rudd and Arme and Owen (1968) in roach observed that the presence of an established infection rendered the host more susceptible to further infection than a similar uninfected fish.

In roach, once the plerocercoid is established it appears to be able to survive as long as the host although the parasite does influence the host mortality rate. No evidence was found that the plerocercoids die in the host. There was no evidence that calcification occurred around the plerocercoids, as described by Owen and Arme (1965) and Orr (1967). However, the older plerocercoids were normally ensheathed in a thin layer of connective tissue. Wilson (1971) described finding roach with Ligula protruding from lesions in the body wall, but this was not seen in roach from the Serpentine.

SUMMARY OF SECTION I

1. In the Serpentine ,the plerocercoids of L.intestinalis were found in the body cavity of the roach(Rutilus rutilus) and the gudgeon (Gobio gobio).The first intermediate host, a cyclopoid copepod,was not found,but a variety of possible definitive hosts were seen ,including species of gulls,ducks and coots.

2. Samples of roach were removed by monthly seine-netting from November 1975 to October 1976 and data regarding the fish and the Ligula infection were collected.

3. Roach were aged by counting the annuli formed on the scales.Validation of the age determination,by examining the length-frequency distributions,proved to be difficult,because the growth rates of roach are extremely variable.

4. There was marked seasonal variation in the incidence and intensity of infection.The incidence was 100% in January and February and then declined over the Spring and Summer, before starting to increase in the late Autumn.The intensity was measured as the weight and as the number of plerocercoids per fish.When the data from the whole sample,including zero-infections,was considered,the variation in intensity was similar to that of the incidence i.e.high in the Winter and low in the Summer.Figures from infected fish only showed that the number of plerocercoids increased in June and again in September, reflecting the increase in the numbers of copepods at these times and hence the increased transmission of Ligula procercoids.The weight of the plerocercoids increased over

the Summer months and declined during the Winter,as heavily infected fish were predated upon or died.

5. Some seasonal variation was found in the P.I..Increased predation,climatic conditions and establishment of new infections probably accounted for the decrease over the Summer. The growth of the plerocercoids over the Summer was shown by a corresponding rise in the P.I..

6. An increase in the mean plerocercoid weight and in the numbers of small plerocercoids,as shown by the weight-frequency distributions,in the early Summer,were further indications that new infections were occuring at this time.

7. Variation in the intensity and incidence of infection with the age of the roach was found.With increasing age,the weight and the number of plerocercoids increased in infected fish.In the sample as a whole(including zero-counts)the intensity reached a peak in the 3+ age group.The highest incidence of 64.5% was found in 2+ fish and no fish older than 7+ was found to be infected.

8. From November to March the majority of fish caught were in the 1+ to 4+ age groups.Older fish were caught during the Summer when they moved back into shallow water.

9. The P.I. and the mean plerocercoid weight increased in infected fish with increasing age.

SECTION II

IN VIVO AND *IN VITRO* CULTURE

CHAPTER 3

IN VIVO CULTURE

Introduction

The definitive hosts of Ligula intestinalis are a variety of piscivorous birds; Cooper (1918) lists as many as 55 species of birds from which this cestode has been reported. As early as 1782 Bloch attempted to infect ducks with plerocercoids of Ligula but was unsuccessful, as was van Beneden (1858). Duchamp (1876) repeated these experiments and showed that the larvae became sexually mature in the duck intestine and that development was extremely rapid, within 48 hours egg production had started. These observations were confirmed by Donnadieu (1877), although his results were questioned by Smyth (1949) who suggested that Donnadieu was in fact examining Schistocephalus solidus and not Ligula; the development of S. solidus is more rapid than that of Ligula, occurring in 36-40 hours. All other evidence from feeding experiments with birds shows that eggs normally occur in the faeces after 3 days (Joyeux and Baer, 1942; Rosen, 1920).

Duchamp (1878) and Joyeux and Baer (1942) showed that a bird host was not essential for development and that the plerocercoid could reach maturity in the dog or cat, but the latter authors failed to infect the rabbit,

macaque monkey or man. Orr and Hopkins (1969) have been successful in maintaining the whole of the Ligula life cycle in vivo in the laboratory, using two species of copepod, Diaptomus fragilis and Mesocyclops leuckarti as the first intermediate host; a variety of tropical fish including Barbus sachsi (Schuberti barbs), Danio malabaricus (Giant Danios) and Tanichthys albonubes (White Cloud Mountain minnows) as the second intermediate hosts and ducks as the definitive host.

In the present study a series of in vivo experiments were carried out on domestic ducks, to provide information on the 'normal' development of the plerocercoid to adult in the definitive host, so that results obtained from subsequent in vivo and in vitro experiments could be compared and assessed. Implantations of plerocercoid fragments were carried out, into the coelomic cavity of an alien host, the mouse, as Clarke (1952) had found that S. solidus matured normally in this site. It was thought to be of some interest to see if Ligula could be maintained in this laboratory animal and whether or not it matured normally. Some attempt was made to infect Cyclops spp. in the laboratory with the aim of producing procercoids which could then be used in subsequent in vitro work.

The Definitive Host

Maintenance and Infection of Ducks

Methods

Day-old male, Khaki Campbell ducklings were supplied by a commercial duck farm. They were kept in wire-bottomed cages, with removable trays underneath, so that the faeces could be easily collected. The ducklings were fed on a diet of commercially prepared 'chick-crumbs' and were given a plentiful supply of water. Ducklings were normally infected at either 2 days or 7 days old, but occasionally a 2 day old duckling died after infection, as the plerocercoid ruptured the intestine, so after initial experiments, all infections were carried out on 7 day old ducklings.

Prior to infection the ducklings were starved overnight. A number of fish were dissected on the morning of the infections and the plerocercoids removed. Only those which were 6-8 cm long and weighing 1-2 gm were used. Ducklings were infected by holding the beak open and inserting the plerocercoid, scolex first, and pushing it down the oesophagus with a pair of blunt forceps. When all the pleroceroid had been swallowed the duckling's beak was held together to make sure that the plerocercoid was not regurgitated immediately. After infection the individual ducklings were put in separate cages for several minutes and watched to ensure that the plerocercoid had definitely been swallowed.

Faeces were collected from 72 hours post infection - the time that egg laying is reported to start in the definitive host. Faeces were mixed with a large volume of tap water, using a bench homogenizer (Thyristor Regler). Small quantities of this mixture were washed through a series

of sieves, the mesh sizes of which were 300 μ, 106 μ and 45 μ. The eggs were retained on the last sieve. Each sample was washed with about 3 litres of water and the sieves were continuously shaken to prevent blocking of the pores. The eggs were washed off the sieve into urine glasses and allowed to settle for 30 minutes, the liquid was then decanted. The eggs were washed and sedimented twice more with tap water and then washed in sterile water twice using the 'watch-glass' technique. This entailed putting a quantity of eggs into a watch-glass and rocking the watch glass backwards and forwards gently. The eggs rolled to the bottom and collected in a sheet in the centre of the watch glass and left any debris floating on the surface, this was then pipetted off. When the eggs were free of debris and had been washed at least twice, the watch glasses were placed inside petri dishes on top of damp filter paper. The eggs were incubated at 25°C for 10 days in the dark and hatched by exposure to bright light. The eggs were allowed to hatch for one hour and then aliquots were removed and the number of hatched and fertile eggs was counted so that the fertility could be calculated. A large amount of uric acid crystals were present in the duck faeces which made this an unsuitable method for quantifying the number of eggs produced from a known weight of faeces.

Ducks were sacrificed at 4, 24, 48 and 72 hours post-infection. The intestines were removed and the worms located. Pieces of small intestine with the worm *in situ* were removed and fixed for histological examination, as were pieces of worm which had been removed from the small

intestine. The fixatives used were Bouin and Carnoy. Sections were cut at 8 µm and stained in Ehrlich's haematoxylin and orange G or eosin.

Results

The most striking result was the high percentage fertility of the eggs produced in the duck, 87, 90 and 84% (see Table 8). Abnormal eggs like those seen in *in vitro* cultures (see Chapter 4) were never seen, although this is not to say that they did not occur under natural conditions. On examination of the digestive tract of the ducks the worms were nearly always found in the anterior third of the small intestine and orientated with the scolex towards the stomach.

Infections in ducks older than 7 days were never achieved and a reinfection of previously infected ducks could not be established. Whether this was due to the immune response of the animal expelling the worms or because of the difficulties associated with establishing an infection in older birds (see discussion) is not known.

Histological Examination

The detailed histology of the plerocercoid has been described by Smyth (1947) and is only summarized here:

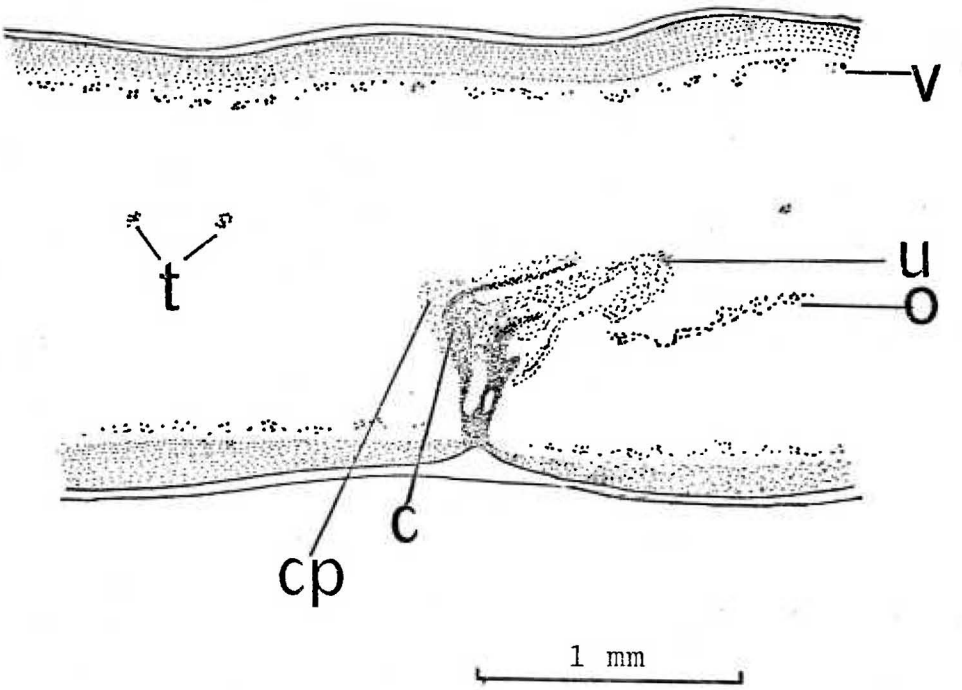
The anlagen of the genitalia are present, but are poorly developed and consist of undifferentiated cells which stain heavily with nuclear stains and show little

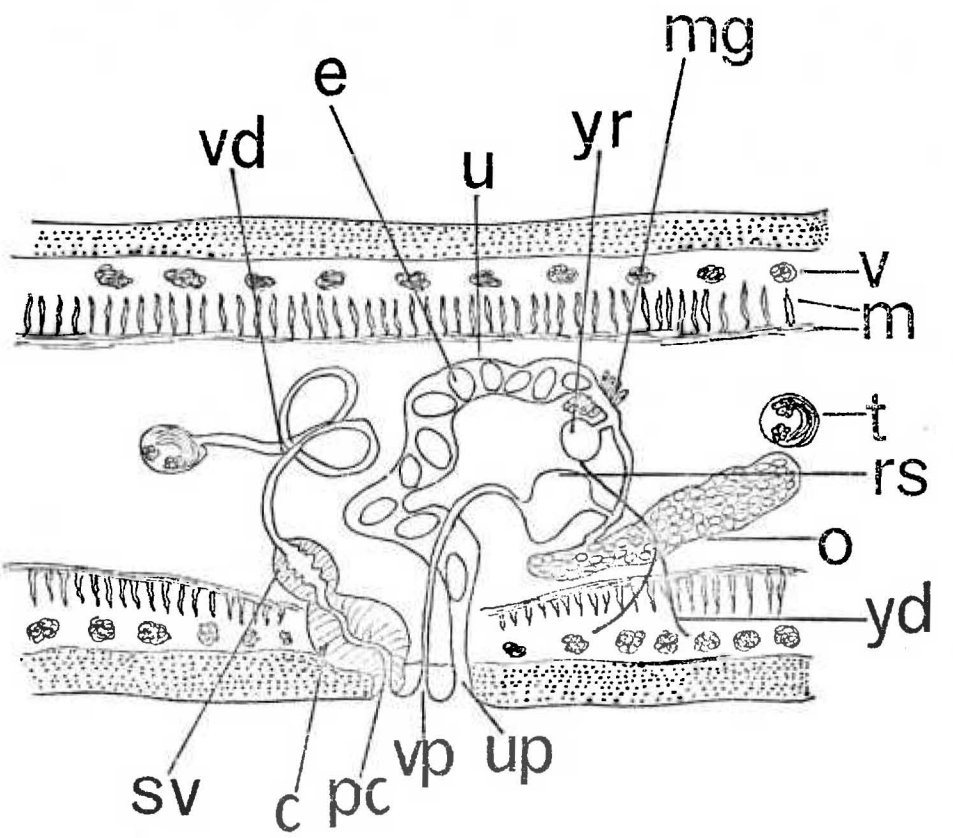
Table 8. *In Vivo* Culture of *Ligula intestinalis*. Duck experiments: Khaki Campbells

Experiment Number	Number of Plerocercoids / Duck	Age of Ducks	Number of Ducks	Duration of Infection	Comments	Fertility Rate of Eggs
1	1	1 month	2	Negative		
2	2	5 weeks	2	Negative	Reinfection of Experiment 1	
3	2	8 days	2	Negative		
4	1	2 days	6	Positive 7 days	All ducks infected	87%
5	1	7 days	5	Positive 7 days	All ducks infected	90%
6	1	11 days	2	Negative	Reinfection of Experiment 4	
7	1	2 days	11	Negative	All died due to ruptured intestine	
8	1	7 days	11	Positive 7 days	All ducks infected	84%
9	1	17 days	2	Negative	Reinfection of Experiment 8	
10	1	22 days	7	Negative	Reinfection of Experiment 8	
11	1	38 days	3	Negative	Reinfection of Experiment 8	

Figure 9. Diagram of the genital anlagen of the plerocercoid of L.intestinalis.
(After Smyth, 1947).

Figure 10. Diagram of the genitalia of adult L.intestinalis.
(After Smyth, 1947).


v
t
u
o
c
cp
1 mm


mg
e
vd
yr
u
v
m
t
rs
o
yd
sv
c
pc
vp
up

differentiation between nucleus and cytoplasm. Tubular organs, such as the cirrus, vas deferens, vagina and uterus, exist only as clumps of cells and show no sign of lumen formation. Genital pores are not present, the positions of the adult uterine, vaginal and cirrus openings being marked by thin, solid cords of cells (see Fig. 9).

Development at 4 hours: The plerocercoid was in position in the anterior third of the small intestine, but on examination little development had occurred from the plerocercoid condition (see Fig. 9 , Plate 3).

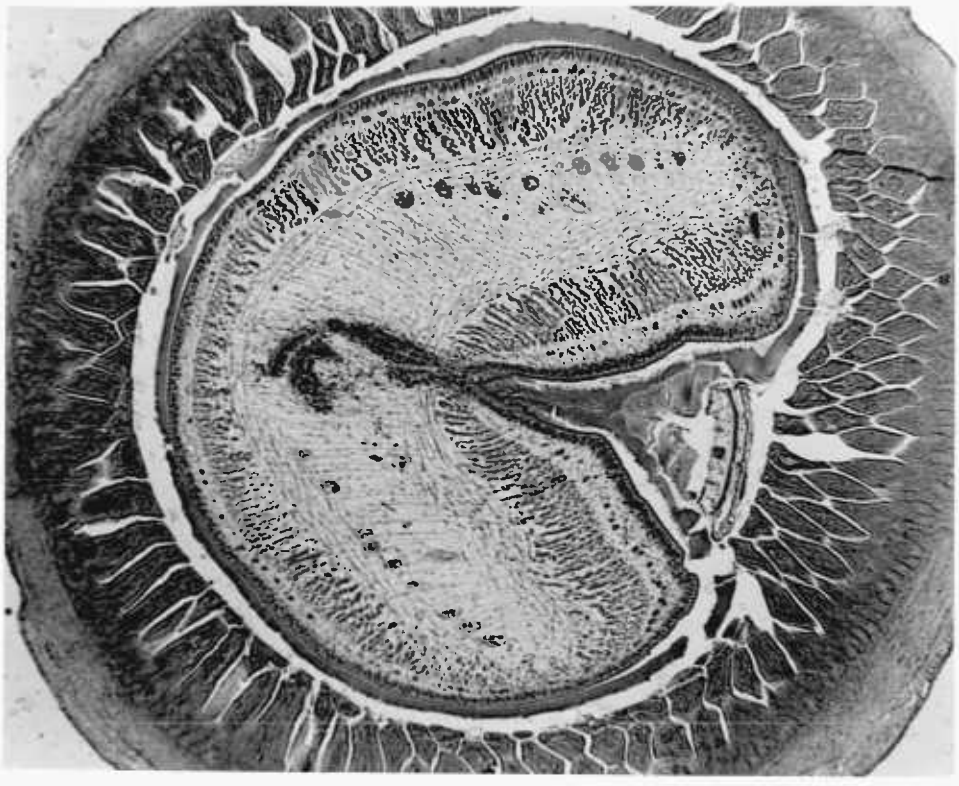
Development at 24 hours: a) Female genitalia. The female genitalia showed a considerable degree of development in 24 hours. The ovary, which in the plerocercoid consisted of a loose cluster of small cells, had enlarged and the nuclei of the developing ova stained deeply. The receptaculum seminis was formed and the vagina could be clearly seen (Plate 4). The uterus had started development into a tubular, lobed organ and the vitellaria had enlarged.

b) Male genitalia. The cirrus and the seminal vesicle were differentiating although the walls were not as clearly defined as the female tubular organs. The vas deferens was not seen. The testes were enlarged and clearly defined and spermatids were developed.

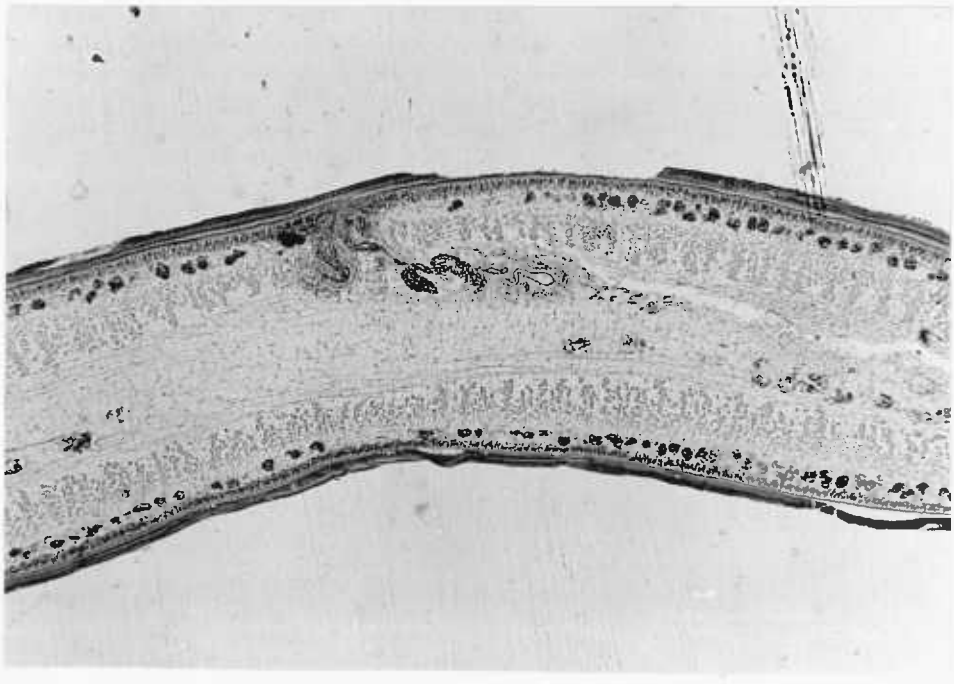
Development at 48 hours: Development was found to be complete at 48 hours and egg laying had commenced (Plate 5).

Plate 3. Transverse section of the plerocercoid of L. intestinalis in situ in the small intestine of a duckling, 4 hours post-infection. Haematoxylin and eosin.

Plate 4. Development of L. intestinalis in the duckling. 24 hours post-infection. T.S. Haematoxylin and eosin.



1 mm



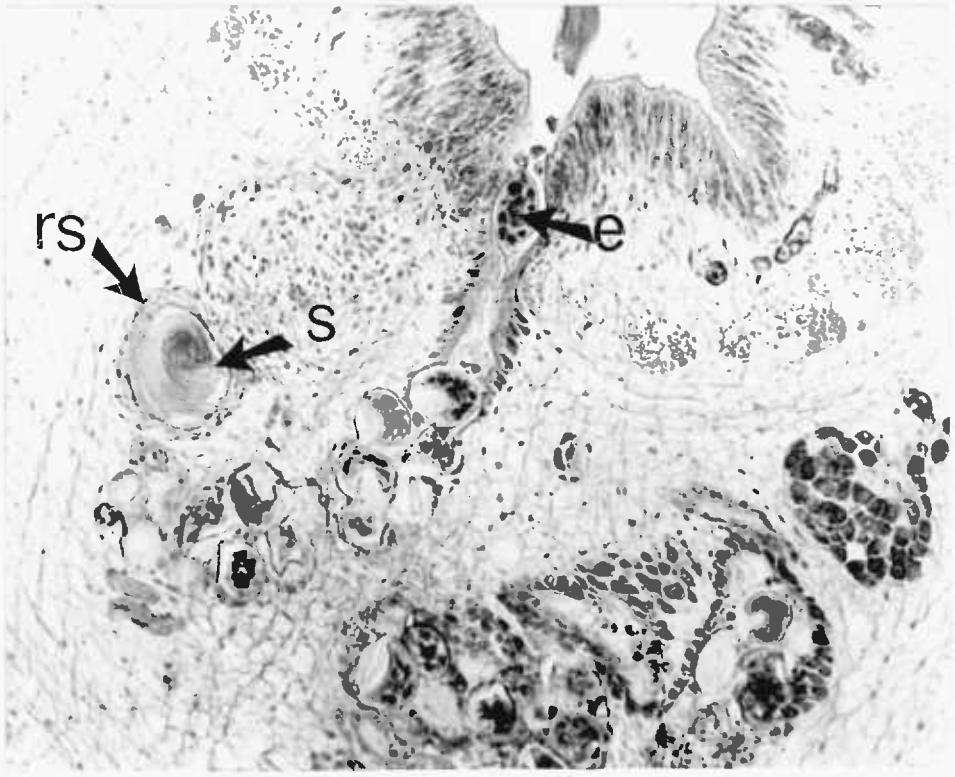
1 mm

Plate 5. Development of L. intestinalis in the duckling. 48 hours post-infection. T.S. Haematoxylin and eosin.

Plate 6. Development of L. intestinalis in the duckling. 48 hours post-infection, showing the receptaculum seminis full of spermatozoa and egg laying taking place. Haematoxylin and eosin.



1 mm



100 µm

The receptaculum seminis (Plate 6) was full of sperm, showing that insemination had occurred. The uterus was filled with eggs and vitelline cells. Histological examination showed the typical anatomical arrangement as found in adult Ligula and illustrated in Fig. 10.

Implantation of Ligula plerocercoid fragments in the mouse peritoneal cavity

Method

Plerocercoids were removed from roach under sterile conditions (see Chapter 4) and placed in medium 199 until required. Adult male mice were anaesthetized with penthrane carried on air. When unconscious the mouse was stretched out on a small animal operating board, on cotton wool and aluminium foil to maintain the body temperature. The front legs were held firmly in clips and the back legs were taped to the board. The abdomen was pushed up with a small bolster of cotton wool, which made it easier to work on. A penthrane mask was taped over the face of the mouse. The abdomen was swabbed with absolute ethanol and the fur shaved off. Using sterile instruments an incision was made 0.5 cm long through the skin and abdominal wall and a piece of plerocercoid 2.0 cm long was inserted into the abdominal cavity. The wound was stitched with fine catgut and the area sprayed with plastic skin and the mouse returned to a cage to recover.

Mice were dissected on days 4, 6, 7, 8, 9 and 13 post-implantation and the worms recovered. The abdominal cavity was examined for the presence of eggs and the intestines were removed and soaked in saline to extract any eggs. The rest of the carcass was soaked, with the abdominal cavity face downwards in a dish of saline for 2 hours. In cases where the eggs had provoked a cellular reaction and become encapsulated, the mass of tissue was placed in a solution of medium 199 + 0.25% trypsin pH 7.3-7.6 and incubated at 37°C. After 10-15 minutes most of the cellular material had been digested, and the eggs were spun down by centrifugation, washed twice in sterile distilled water and incubated as described previously.

Results

(See Table 9). The fertility of the eggs produced ranged from 11.4 - 21.6% and it was found that egg production started at least two days later than in the normal bird host. Up until day 6-7 the recovered worms appeared healthy and motile, but after this time worms became encapsulated and by day 13 (Table 9) worms were beginning to deteriorate and die.

Histological examination of the worms at 6 and 8 days (Plates 7 and 8) revealed that the plerocercoid had undergone complete development and maturation. The receptaculum seminis was seen to contain some sperm,

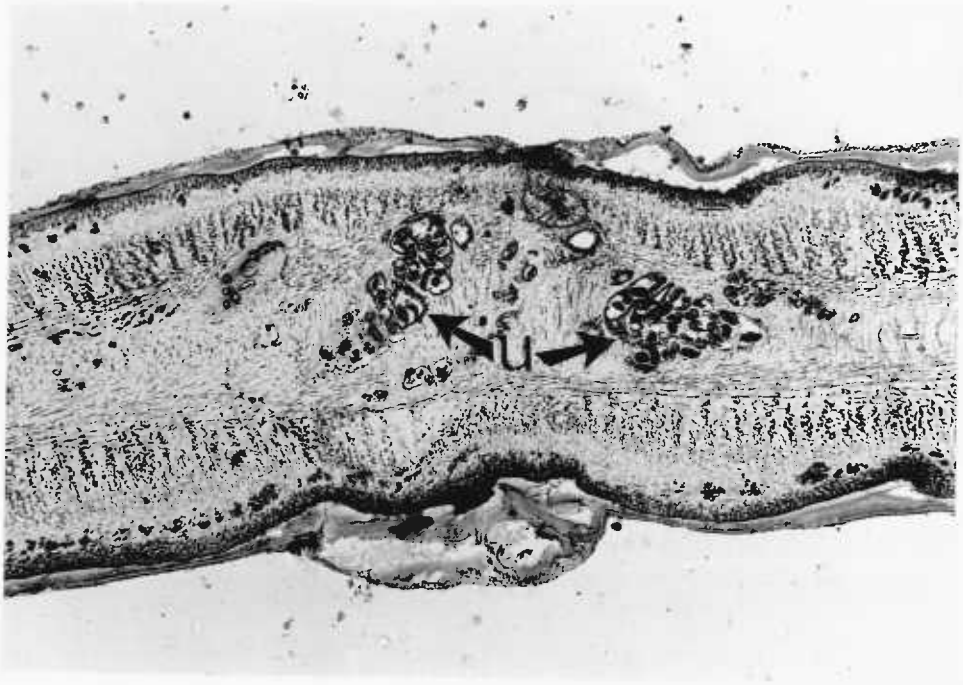
Table 9. In Vivo Culture of Ligula intestinalis implanted into the mouse peritoneum

Experiment Number	Number of days cultured	Egg Production	State of worm	Pathology in Mouse	Fertility rate of eggs
M1	4	2 eggs recovered	Healthy	Increase in peritoneal exudate. Spleen appeared enlarged and in several mice nectrotic lesions were present along the lower edge of the liver lobes. In M3 and M4 eggs only were encapsulated. M5 - M13 eggs and worm encapsulated. Increased vascularization of the abdominal wall.	-
M2	6	+ve	Healthy		* 11.4%
M3	7	+ve	Healthy		* 5.0 (eggs infected)
M4	8	+ve	Healthy		* 18.0
M5	9	+ve	Encapsulated - but alive		21.6
M6	13	+ve	Encapsulated - half of worm dead		14.0
M7	13	+ve	Encapsulated - alive		17.1
M8 - M13	7	+ve	Encapsulated - alive		20.0

* Ligula plerocercoid fragments cultured on in vitro. See Table 15.

Plate 7. Development of L. intestinalis in the mouse peritoneum. 6 days post-infection. The uterus can be seen to be full of eggs. T.S. Haematoxylin and eosin.

Plate 8. Development of L. intestinalis in the mouse peritoneum. 8 days post-infection. The uterus is nearly empty, indicating that egg production has stopped. T.S. Haematoxylin and eosin.



1mm

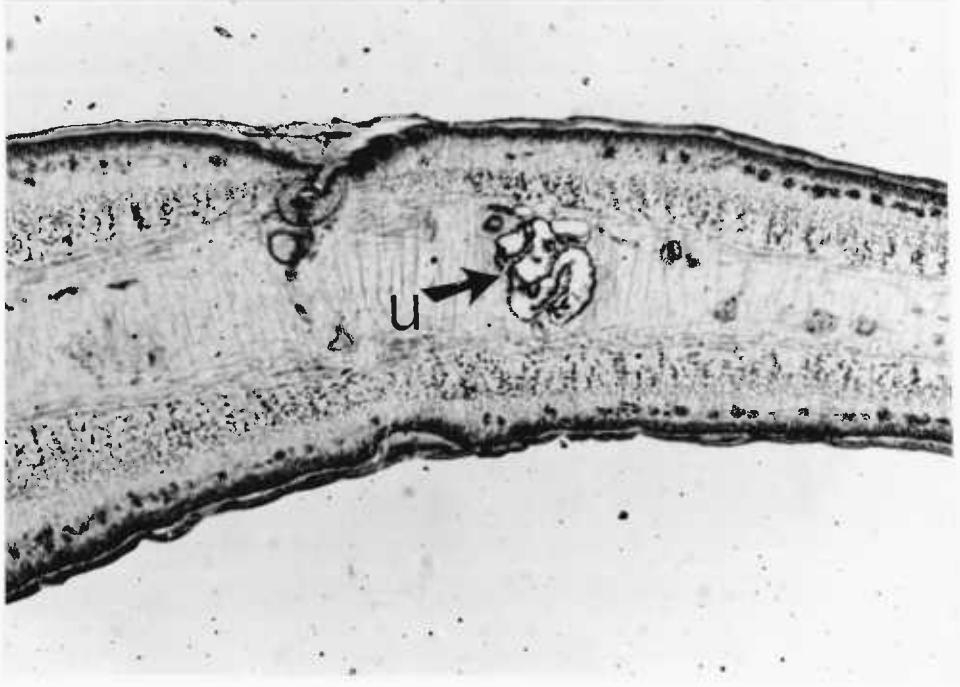


Plate 9. Development of L. intestinalis in the mouse peritoneum. 6 days post-infection. Plate showing receptaculum seminis filled with sperm.
Haematoxylin and eosin.



100 µm

showing that insemination had occurred to some extent, although the number of sperm in the receptaculum appeared to be much less than in the duck host (Plate 9).

Discussion

The plerocercoid of Ligula is progenetic and matures very rapidly when ingested by the definitive host. The time at which maturation is complete and egg laying commences has been the point of some debate in the literature. Duchamp (1876) and Donnadieu (1877) infected ducks and egg laying occurred at 48 hours. Dubinina (1953) infected the domestic duck, goose, lapwing, hen and rook and got development and the appearance of eggs in 48-60 hours in all these hosts. However Joyeux and Baer (1942) and Rosen (1920) claimed that development in the duck took 72 hours. In these experiments Khaki Campbell ducks were used, and egg laying had commenced by 48 hours and may have started earlier.

Infections were not achieved in ducks older than 7 days, but only a small number of experimental animals were used so the results are not conclusive. Orr (1967a) attempted to infect ducks aged 6 weeks and 3 months, but got a very low number of 'takes' and got no infections in ducks aged 6 months. Orr postulated that in older ducks the unprotected plerocercoid was probably killed by the digestive processes of the host. (In the natural state the plerocercoids are swallowed whilst still in the

body cavity of the fish host.) Ducks retain food in the stomach for approximately two hours at pH 2.33 and older birds have accumulated grit in the gizzard, unless fed on a grit-free diet.

The duration of infection in the duck was found to be around 10 days. This agrees with Orr's (1967a) findings but not with Dubinina (1964) who describes the infection of L. intestinalis as lasting only 2-5 days in the definitive host. The adult worm was always found in a similar position in the duck gut in the anterior part of the small intestine and was in position 4 hours after infection. McCaig and Hopkins (1963) found that Schistocephalus solidus occupied a specific site in the intestine for different hosts and was in position 6 hours after infection. In hosts where S. solidus occupied an anterior position, the worm was found to be swept further down the intestine and then to migrate back, whether this happened in Ligula infections was not observed.

In the experiments where reinfections were attempted no positive results were found. Joyeux and Baer (1936) claimed that there was an immune reaction in ducks which had been infected with Ligula, and that after an initial infection ducks could not be reinfected for 20 days after which time reinfection was possible. Attempts were made to repeat these experiments but no reinfections were found, but only 3 animals were used.

Clarke (1952) found that S. solidus matured in the mouse coelomic cavity in 48 hours and that the sex, age and maturity of the mouse had no effect on the maturation of the plerocercoid. Ligula was too large to implant

whole into a mouse, so fragments 2 cm long were implanted and maturation was found to take 4-5 days which was at least twice as long as the time taken in ducks. Dubinina (1953) found that development in the cat and dog took longer and thought that this was due to the lower body temperature of 37°C, 3°C lower than the bird. Mouse body temperature is 37°C (Clarke, 1952) and it is possible that this slowed down the development of the fragments in the mouse.

The First Intermediate Host: Laboratory Maintenance of Cyclops spp.

Methods and Results

Cyclops spp. were caught by plankton net in the Serpentine Lake, Hyde Park, London. Two methods of netting were used, a) hand-netting from around the boat-house pier, or b) by trailing the plankton net behind a boat. The Cyclops were kept in glass fish aquaria in London tapwater, which had been standing for at least a week before use. Cyclops seem to survive remarkably well in London tap water and there is no need to transport large volumes of pond water back to the laboratory. Large numbers of Daphnia were often present in the cultures and these were removed by bubbling CO_2 through, which has no effect on the Cyclops, but kills the Daphnia.

The Cyclops were fed on ciliate cultures produced from hay and lettuce infusions. Hay infusions were prepared by soaking sterilised hay in a plastic bucket and

seeding the infusion with pond water. Lettuce infusions were made by boiling up lettuce leaves and leaving them to soak for a few days and seeding with pond water. The copepods were fed once a week as described by Orr and Hopkins (1969).

Infection of copepods

Copepods were concentrated by pouring the cultures through a 105 μ sieve and washing the Cyclops, which were retained on the mesh, into a 500 ml beaker. Eggs which had already been exposed to the light for one hour and had started to hatch were added to the Cyclops and were left in bright light for about 8 hours and then transferred back to a larger tank.

The only species of Cyclops found in the Serpentine during 1974, 1975 and 1976 when sampling was taking place, was Cyclops vernalis americanus which is resistant to infection and this was borne out by P. Henderson (personal communication). Other species of Cyclops were collected from Osterley Lake and included MESO Cyclops leuckarti, C. bicuspidatus, C. languidus, C. viridis and C. vernalis americanus. These species were identified using the key of Harding and Smith (1960). However, none of these species were infected in the laboratory, although laboratory infections have been successfully reared by a number of authors (See Table 10). The cultures themselves survived very well and were maintained for up to 2-3 months.

Table 10

The species of copepods recorded as hosts for the procercoid of Ligula intestinalis

Species of copepod	Reference
Cyclops strenuus	Rosen, 1920 and Dubinina, 1953.
Diaptomus gracilis	Rosen, 1920 and Dubinina, 1953
Cyclops bicuspidatus	Joyeux and Baer, 1942
Acanthocyclops viridis	Dubinina, 1953 (experimentally not found naturally)
Mesocyclops leuckarti	Orr and Hopkins, 1969
Diaptomus fragilis	Orr and Hopkins, 1969

CHAPTER 4

IN VITRO CULTURE

Introduction

The study of any parasite demands that a constant source of material be made available, but this is not always possible, for a variety of reasons, with the parasite of choice, so the use of laboratory models, particularly for biochemical and physiological studies is very important. *In vitro* culture methods offer a means of maintaining parasites isolated from the laboratory hosts in a controlled environment. There are many uses to which *in vitro* techniques can be put e.g. for testing drug action - it is particularly useful as a primary screen for new compounds (Rogers and Denham, 1976); for studying metabolism, growth and development at various stages of the life cycle (Hopkins, 1952; Read and Rothman, 1958; Sinha and Hopkins, 1967); in the study of immunology, especially in the development of vaccines, since metabolic products are known to act as antigens in some cases (Denham, 1968, 1969a and b; Wilson, 1967).

In the case of cestodes, and in particular the *Pseudophyllidea* and *Cyclophyllidea*, a number of species have been successfully cultured to the adult stage and egg production has occurred, although fertile eggs have not always been produced. The majority of *in vitro* studies

have started with the larval stages of cestodes as these normally occur in a sterile position in the intermediate host. The plerocercoids of Ligula intestinalis are progenetic i.e. the primordia of the reproductive system are developed in the fish host and only need to mature in the definitive host, and they contain a sufficient amount of carbohydrate and protein reserves to meet the requirements of maturation without additional nutrient being given (Smyth, 1969, 1976).

Joyeux and Baer (1938b) cultured the plerocercoid of Ligula in a variety of media, including horse and dog serum, at 38-42^{o}C, but although some eggs were produced the development was abnormal as the testes failed to undergo spermatogenesis. Smyth (1946) studied the development of Schistocephalus solidus in peptone broth at 40^{o}C, but although development appeared to be normal the eggs produced failed to hatch as fertilisation had not taken place. Smyth (1947) applied his techniques used with S. solidus to Ligula and obtained a similar result in that egg production occurred but the eggs were infertile. Some fertile eggs were produced when Ligula was cultured in horse serum; Smyth (1948 and 1949) reported a fertility rate of 6%.

Clearly a more elaborate procedure was required to reproduce more closely the physico-chemical conditions found in the intestine of the definitive host. Smyth (1954, 1959) was successful in culturing the adult of S. solidus and producing fertile eggs. The new technique

included the use of a buffered medium to minimise the effects of the metabolic products, the use of deep tube culture bringing about semi-anaerobic or anaerobic conditions to prevent premature oxidation of the egg-shell precursors in the vitellaria, and the compression of the worm within dialysis tubing to encourage insemination and fertilisation. Orr (1967) attempted to use Smyth's (1954) technique with the plerocercoids of Ligula, but was not successful because the plerocercoids used were too large. However, these large plerocercoids did produce fertile eggs when cultured in horse serum in conical flasks at 40°C.

Smyth (1969, 1976) and Arme (1966) have used the deep tube culture technique to mature Ligula plerocercoids, but the fertility rate of the eggs has not been very high. The present work was undertaken to try and produce large numbers of fertile eggs by in vitro culture methods, so that the life cycle could then be continued in vivo by infecting copepods.

Materials and Methods

Culture of plerocercoids to adults

Plerocercoids for these experiments were removed aseptically from the body cavity of infected roach after the method described by Smyth (1946). A roach was pithed, thoroughly dried and firmly held by two pairs of large forceps, secured in clamp stands

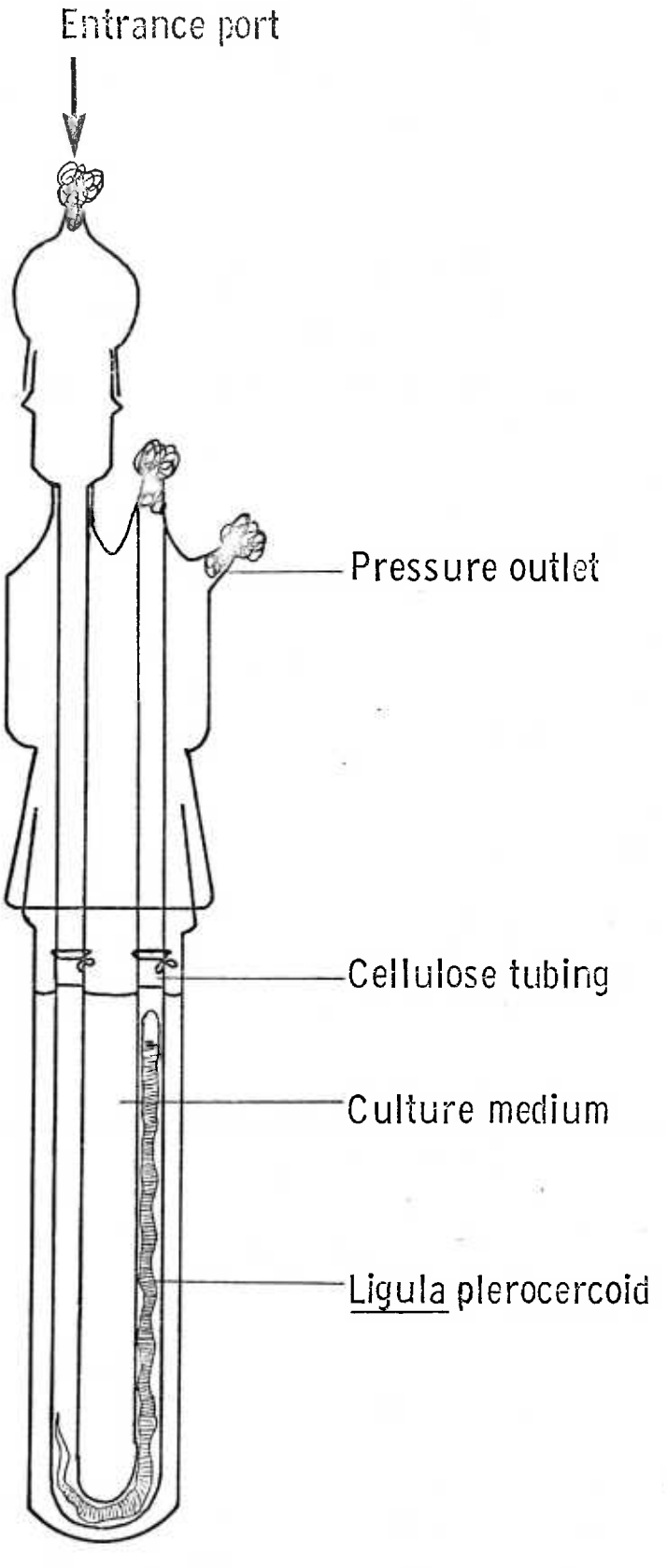
placed in a sterile cabinet. The ventral side was orientated towards the operator and painted with a 1% iodine in absolute alcohol solution. A second coat of iodine solution was applied and allowed to dry. Using sterile instruments the abdomen was opened along the ventral midline from the jaw to the anus. The plerocercoids were pulled out carefully one by one, care being taken not to rupture the intestine of the fish. The plerocercoids were placed in pre-weighed sterile petri dishes and the individuals were weighed. The plerocercoids were washed in two changes of sterile medium before being placed in the culture vessels.

Preliminary experiments were carried out in the deep culture vessel developed by Smyth (1954) for S. solidus. It consisted of a glass tube 8" long, which held the culture medium, suspended in which was a length of dialysis tubing (see Plate 10 and Fig. 11). Approximately 80 ml of culture medium were pipetted into the culture tube and the plerocercoid pushed into the dialysis tubing via the entrance port along with 2-3 ml of medium. The culture tube was placed in a basket in a shaking waterbath at 40°C, the temperature of the definitive host. A shaking waterbath was used to help with the dispersion of metabolic products throughout the culture medium (Smyth, 1954). The rocking movement was held at approximately 10-15 swings per minute, as rocking at any faster speed was found to cause fragmentation of the plerocercoids (Orr, 1967). The culture medium was changed every 24 hours as Smyth (1949) had shown that the removal of acidic metabolic

Plate 10. Deep culture vessel used for the in vitro culture of L. intestinalis.



Figure 11. Deep culture vessel used for the *in vitro* culture of *L. intestinalis*. (Smyth,1954).


Entrance port
Pressure outlet
Cellulose tubing
Culture medium
Ligula plerocercoid

products was necessary for normal development to occur.

Various slight modifications were made to the internal conditions in the culture tubes. Cultures 1-14 were carried out in the light whereas cultures 15-22 were wrapped in aluminium foil to simulate darkness. The first set of experiments, cultures 1-6, were carried out in intact dialysis tubing and it was felt that this did not allow sufficient circulation and diffusion of the metabolic products. Therefore holes were punched in the dialysis tubing with a 19 gauge needle, approximately 5 holes per cm and cultures 7-22 were carried out in this tubing.

Whole plerocercoids were also cultured in:

a) 250 ml Falcon tissue culture flasks in a 40°C incubator in the dark.

b) circulatory apparatus designed by Smyth (1972) for the cultivation of Echinoccoccus granulosus (Fig. 12). It was thought that as the plerocercoids of Ligula metabolise very rapidly in the first 24 hours of culture, a circulating system might be very suitable. The plerocercoids were put in the lower chamber and in the upper chamber.

c) modified circulatory apparatus (Fig. 13). The lower chamber of the original apparatus was removed and replaced by a 45 cm length of polythene tubing, 0.5 cm in diameter. Two or three large plerocercoids were put in the upper reservoir and they migrated down into the polythene tubing.

Both the circulatory systems were kept in a 40°C incubator in the dark and the gas used to circulate the

Figure 12. Circulatory apparatus used for the *in vitro* culture of *L. intestinalis* (Smyth, 1972).

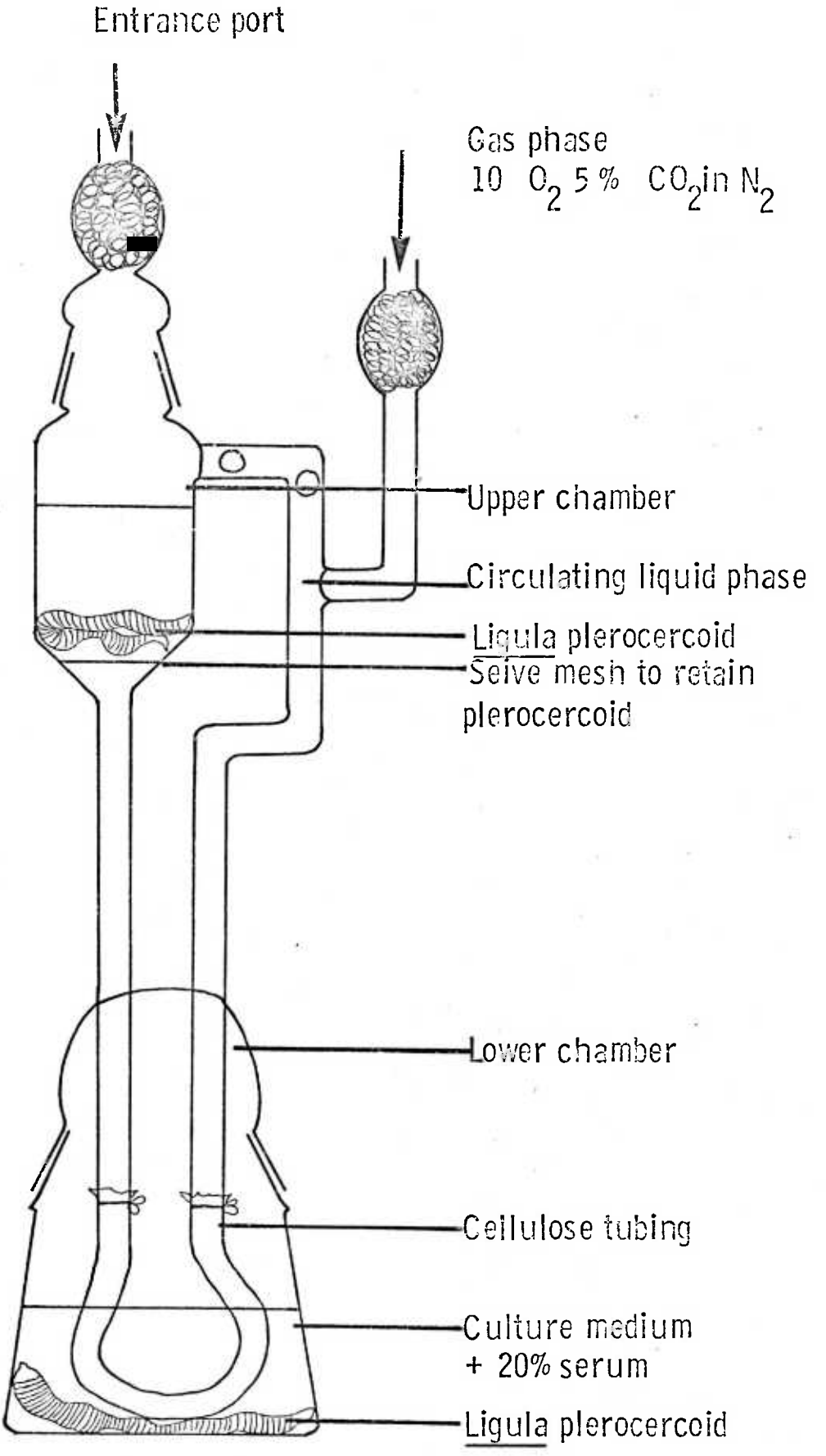
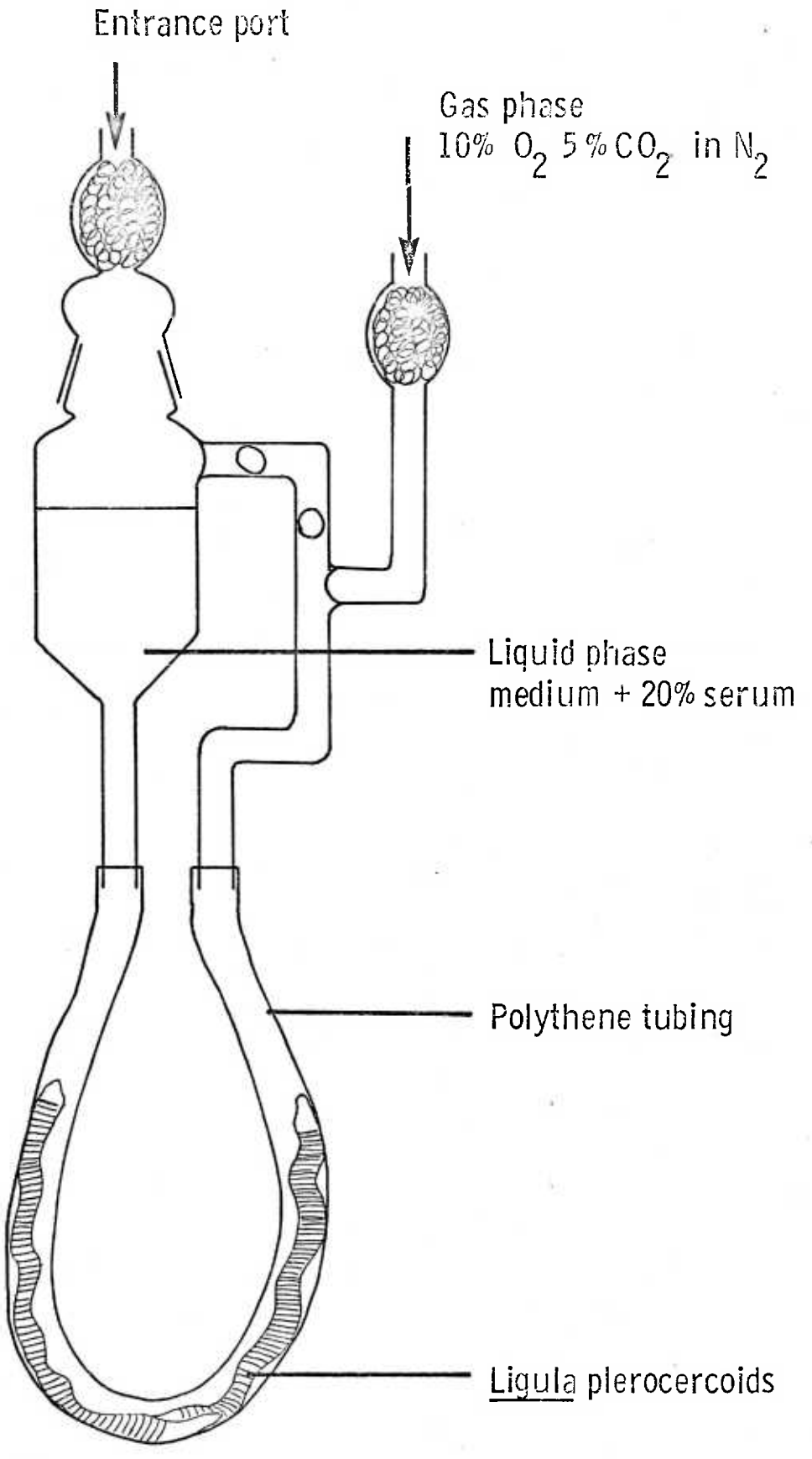

Entrance port
Gas phase
10 O_2 5 % CO_2 in N_2
Upper chamber
Circulating liquid phase
Ligula plerocercoid
Seive mesh to retain plerocercoid
Lower chamber
Cellulose tubing
Culture medium + 20% serum
Ligula plerocercoid

Figure 13. Adapted circulatory apparatus used for the *in vitro* culture of *L. intestinalis*.


Entrance port
Gas phase
10% O_2 5% CO_2 in N_2
Liquid phase
medium + 20% serum
Polythene tubing
Ligula plerocercoids

medium was 10% O_2, 5% CO_2 in N_2.

Fragments of plerocercoid, 2.5 cm long were cultured in 25 cm^3 Falcon tissue culture flasks at 40°C in the dark.

A variety of media and sera were used in these experiments including medium 199, NCTC 135, Hanks saline, calf and newborn calf serum (Flow Laboratories and Gibco Biocult) and foetal bovine serum (Flow Laboratories). All media contained antibiotics at the concentration of 100 µg/ml streptomycin and 100 IU/ml penicillin. Duck bile was used in some experiments at a concentration of 0.1 ml bile/10 ml medium, to see whether it had any effect on the development of the adult.

Incubation of eggs

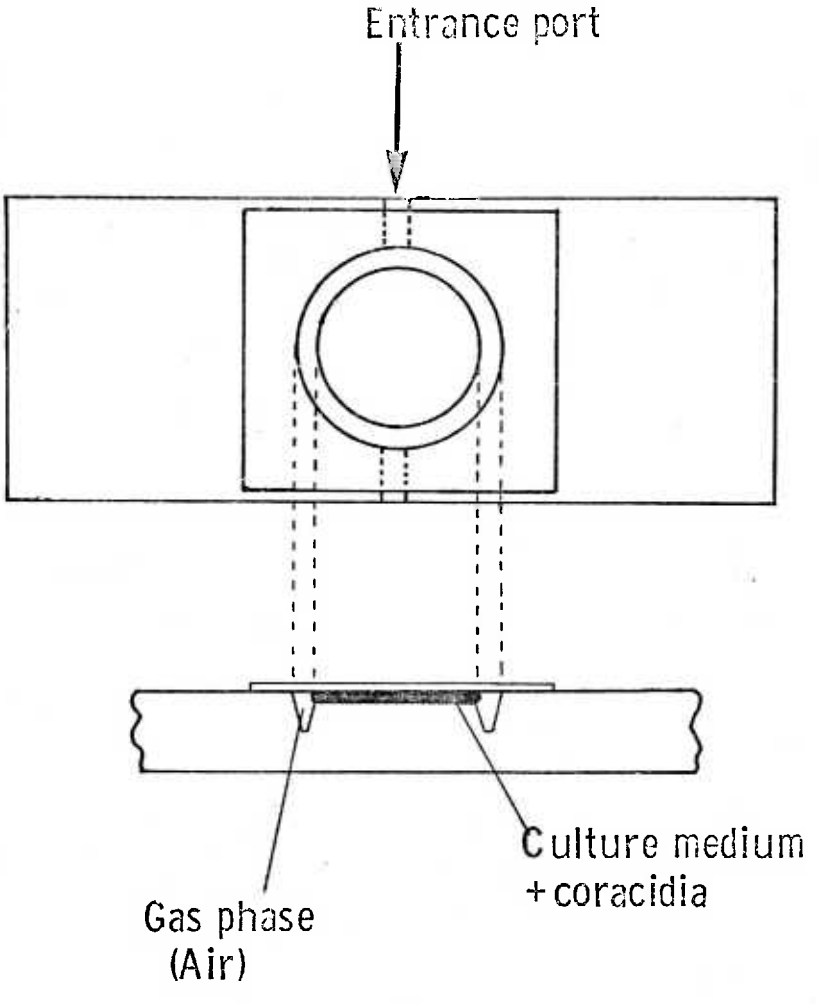
Where the plerocercoids had been cultured in dialysis tubing the eggs were collected by flushing the tubing through with distilled water. In the cultures where the tubing had been punctured, the majority of eggs were still retained in the tubing although any which had passed through the holes could be seen as a brown film on the base of the culture tube and could be collected. In the Falcon tissue culture flasks the culture medium was carefully pipetted off and the eggs washed out with sterile distilled water. Eggs were collected sterilely and washed using the 'watch-glass' technique described previously, incubated and the fertility calculated as before.

Culturing coracidia

As a ready supply of coracidia was available from hatched eggs an attempt was made to culture this stage as it had not been achieved for any member of the Pseudophyllidea. The chamber used was that devised by Cruickshank, Cooper and Conran (1959) for cell cultures. The main feature of the chamber (Fig. 14) is the retention of a drop of culture medium against the under-surface of a glass coverslip by a transparent central pillar, so that cultures can be easily observed. A peripheral moat surrounds the upper surface of the pillar so that the medium is retained in position by surface tension only and its peripheral surface is exposed to the gas phase in the moat. Access to the medium and the gas phase is provided by two opposed ducts of 3 mm diameter. The chamber is completed by cementing a 1½" coverslip to the top surface of the block with beeswax.

On the day of the experiment the eggs were brought out into the light to hatch and the excess water poured off, so that the coracidia would be more concentrated. While the eggs were hatching the chamber was sealed with the coverslip and beeswax, using sterile technique and working in a sterile cabinet. Working with a microscope as many coracidia as possible were picked out of the watch glass with a 1 ml syringe fitted with a 25 gauge needle. The coracidia were washed out of the syringe into approximately 0.3 ml of culture medium held in a small glass

Figure 14. Culture chamber used for the *in vitro* culture of coracidia of *L. intestinalis* (Cruickshank *et al.*, 1959).


Entrance port
Culture medium
+coracidia
Gas phase
(Air)

tube. The culture medium and coracidia were transferred by syringe into the culture chamber and then the chamber was sealed with silicon plugs and beeswax, placed in a petri dish and incubated at 25°C.

Coracidia were cultured in Grace's medium for insect tissues (Gibco Biocult) and lobster haemolymph (Grand Island Biological Company) at a ratio of 2:1, and also in medium 199 + 20% calf serum.

Pieces of worm for histological examination were fixed in Bouin or Carnoy. Sections were cut at 8 µm and stained with Erlich's haematoxylin and orange G or eosin.

Results

In these experiments the success of a particular *in vitro* culture technique was assessed by considering a number of criteria. Firstly, whether the plerocercoid actually survived or not, and whether it was active and metabolising. As described in the method, the culture medium was changed every 24 hours and this medium contained phenol red which acts as an indicator by changing colour from red to yellow when acid metabolites are produced. Secondly, whether sexual maturity was attained and the timing of development of the reproductive system as compared with the *in vivo* development (see Chapter 3) and the time at which egg production occurred. Thirdly, if egg production occurred it was important to determine

the fertility of the eggs and to assess the relative number of eggs produced by each culture, although no direct quantitative method was attempted. As the fertility of the eggs was compared to that obtained *in vivo* it was necessary to incubate the eggs with great care, as they are easily infected with bacteria and fungi. This problem is not so severe with eggs produced *in vitro* as these are already sterile, but eggs produced *in vivo* have to be washed and sieved out of faeces.

Plerocercoids to adults

Cultures 1-6 were conducted in intact dialysis tubing whereas number 7-14 were in punctured tubing (Tables 11 and 12). A difference was seen in the activity of the worms, those in the punctured tubing being much more active, contracting and expanding and moving up and down the dialysis tube, so presumably the holes allowed better diffusion of metabolic products. The fertility of the eggs did not seem to be greatly affected by the presence or absence of holes in the dialysis tubing; the fertility of eggs from cultures 1-6 ranging from 0-8.69% (Table 11) and that of eggs from cultures 7-14 ranging from 0-12.2% (Table 12).

Cultures 1-14 were conducted in the light and although egg production started on day 3 and continued in most cases up until day 7 the fertility of the eggs was low (Tables 11 and 12). The best results were obtained with cultures which were kept in the dark and where the

worm was held in punctured dialysis tubing (Table 13). The most satisfactory medium / serum mixture was medium 199 + 20% calf serum, Cultures 15 and 18, which gave the highest fertility of eggs of 27.1 and 31.0% (Table 13).

Where whole worms had been cultured in 250 ml^3 Falcon flasks (Table 14) they matured and produced eggs but the number of eggs produced was low as was the fertility ranging from 5.8 to 7.3%. All the adult worms were dead or dying by day 6 whereas in the deep culture vessel they were still alive on day 7. Egg production tended to start later on day 4 rather than day 3.

The presence of bile in cultures 16 and 17 (Table 13) reduced the total number of eggs produced and the fertility was lower being 11.2 and 13.4% respectively compared with 27.1 and 31% for the cultures without bile. The circulatory apparatus (Table 14) was not satisfactory as diffusion was too slow and the worms died without producing any eggs. However the modified apparatus was more successful and eggs were produced on day 3 and had a fertility of 20%.

The cultures of plerocercoid fragments in 25 ml^3 Falcon flasks (Table 15) were quite successful. Egg laying was again delayed until day 4 and the fertility ranged from 11.4 to 19.3%. In some cases the fragments remained alive until day 9 when the culture was terminated. The presence of bile in culture 33(Table 15) inhibited egg production and the fertility of the eggs that were produced was low, 6.2%.

Table 11 In vitro culture of Ligula intestinalis.

Conditions: Deep culture tube, 40°C, dialysis tubing intact, light.

Medium 199 + 20% foetal bovine serum	Days 1	2	3	4	5	6	7	Egg Fertility %	Comments
Culture 1	+	+	+	+	D			0	Culture infected
2	+	+	EP	+	+	+	CT	8.69	Worm sluggish
3	+	+	EP	+	+	+	CT	6.2	Worm sluggish
4	+	+	EP	F				6.5	Worm sluggish
Medium 199 + 20% calf serum									
Culture 5	+	+	EP	+	+	+	D	7.0	Worm sluggish
6	+	+	EP	+	+	+	D	7.4	Worm sluggish

Key + Alive D Dead F Fixed for histology

EP Egg production commenced CT Culture Terminated

Table 12 In vitro culture of Ligula intestinalis

Conditions: Deep culture tube, 40°C, dialysis tubing punctured, light.

Medium 199 + 20% calf serum	Days 1	2	3	4	5	6	7	Egg Fertility %	Comment
Culture 7	+	+	EP	+	+	+	CT	9.3	
8	+	+	EP	+	+	+	CT	0	Eggs infertile
9	+	+	EP	+	+	+	CT	8.6	Very few eggs produced
10	+	+	EP	+	+	+	CT	7.4	Worm very inactive
Medium NCTC 135 + 20% calf serum									
Culture 11	+	+	EP	+	+	+	D	5.7	Good egg production
12	+	+	EP	+	+	D		0	No eggs produced
Medium 199 + 20% newborn calf serum									
Culture 13	+	+	EP	+	+	+	CT	10.0	Worm very active
14	+	+	EP	+	+	+	CT	12.2	Worm very active

Table 13 In Vitro culture of Ligula intestinalis

Conditions: Deep culture tube, 40°C, dialysis tubing punctured, dark.

Medium 199 + 20% calf serum	Days 1	2	3	4	5	6	7	Egg Fertility %	Comments
Culture 15	+	+	EP	+	F			31	
16	+	+	EP	+	F			11.2	+ Bile 0.1 ml/10 ml
17	+	+	EP	+	+	D		13.4	+ Bile 0.1 ml/10 ml
18	+	+	EP	+	+	+	CT	27.1	
Hanks + 20% newborn calf serum									
Culture 19	+	+	EP	+	+	CT		11.3	
20	+	+	EP	+	+	CT		10.4	
NCTC 135 + 20% newborn calf serum									
Culture 21	+	+	EP	+	+	CT		10.6	
22	+	+	EP	+	+	CT		9.5	

Table 14 In vitro culture of Ligula intestinalis

Conditions: 250 ml^3 Falcon Flask, 40°C, dark.

Medium 199 + 20% calf serum	Days 1	2	3	4	5	6	7	Egg Fertility %	Comments
Culture 23	+	+	EP	+	+	D		6.1	Low egg production from all of these cultures. Worms very inactive by day 5.
24	+	+	+	EP	+	D		5.8	
25	+	+	+	EP	+	D		7.3	
26	+	+	+	EP	+	D		6.6	
Conditions: circulatory apparatus, 40°C.									
27	+	+	D					0	Culture infected
28	+	+	+	+	+	+	D	0	Worm died. No egg production.
Conditions: modified circulatory apparatus, 40°C.									
29	+	+	D						Culture infected
30	+	+	EP	+	+	+	CT	20.0	

Table 15 In vitro culture of Ligula intestinalis fragments

Conditions: 25 ml^3 Falcon Flask, 40°C, dark.

Medium 199 + 20% calf serum	Days 1	2	3	4	5	6	7	Egg Fertility %	Comments
Culture 31 (x 4)	+	+	+	EP	+	+	CT	11.4	Out of the 4 cultures, one piece fixed at 24, 48 and 72 hours.
32 (x 2)	+	+	+	EP	+	+	CT (day 9)	12.7	Control for culture 33
33 (x 2)	+	+	+	EP	+	+	CT (day 9)	6.2	+ Bile 0.1 ml/10 ml. Very few eggs produced.
34	EP	+	+	+	+	CT		21.0	Mature fragments transplanted from mice.
35	EP	+	+	+	+	CT		5.0	Culture 35 eggs heavily contaminated with bacteria.
36	EP	+	+	+	+	CT		18.0	
37 (x 5)	+	+	+	EP	+	+	CT	15.0	Eggs pooled for examination of fertility
NCTC 135 + 20% calf serum									
Culture 38 (x 2)	+	+	+	EP	+	+	CT	19.3	Eggs pooled for examination of fertility.

Coracidia

The cultures were examined daily under a microscope. The coracidia remained alive until day 2 in both types of culture medium, but did not shed their cilia or undergo any development.

Types of eggs produced in vitro

After incubation it was observed that several types of embryonated eggs were produced from *in vitro* cultures (see Fig. 15):

a) normal embryonated eggs, which contained normal coracidia and appeared to be similar to eggs produced *in vivo* in ducks. These eggs hatched normally on exposure to light, and produced normal coracidia (Fig. 15a and b).

b) Abnormally embryonated eggs containing aberrent coracidia, most commonly these were seen as "small" coracidia with the coracidium lying outside the embryophore (see Fig. 15 c).

c) Embryonated eggs containing "small" coracidia, about half the size of a normal coracidia (see Fig.15 d), but otherwise appearing normal.

Neither eggs of type c) or d) were ever seen to hatch, although they were operculate. The abnormal eggs were the same size as the normal eggs and the egg shell had a normal appearance.

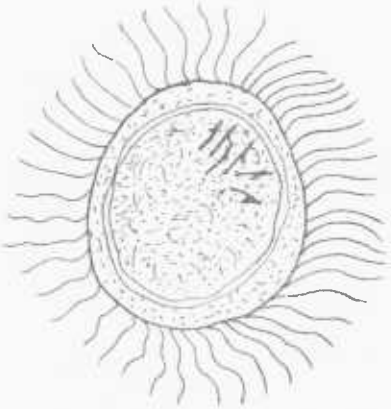
Figure 15. Types of eggs produced in *in vitro* culture of *L. intestinalis*.

a)



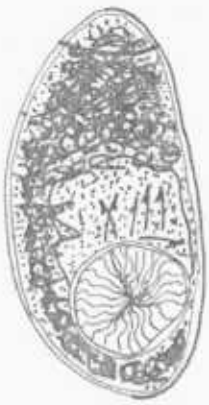
Normal embryonated egg

b)



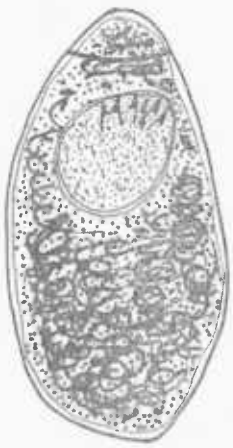
Normal coracidium

c)



Small embryo egg

d)



Small embryo egg

Histological examination

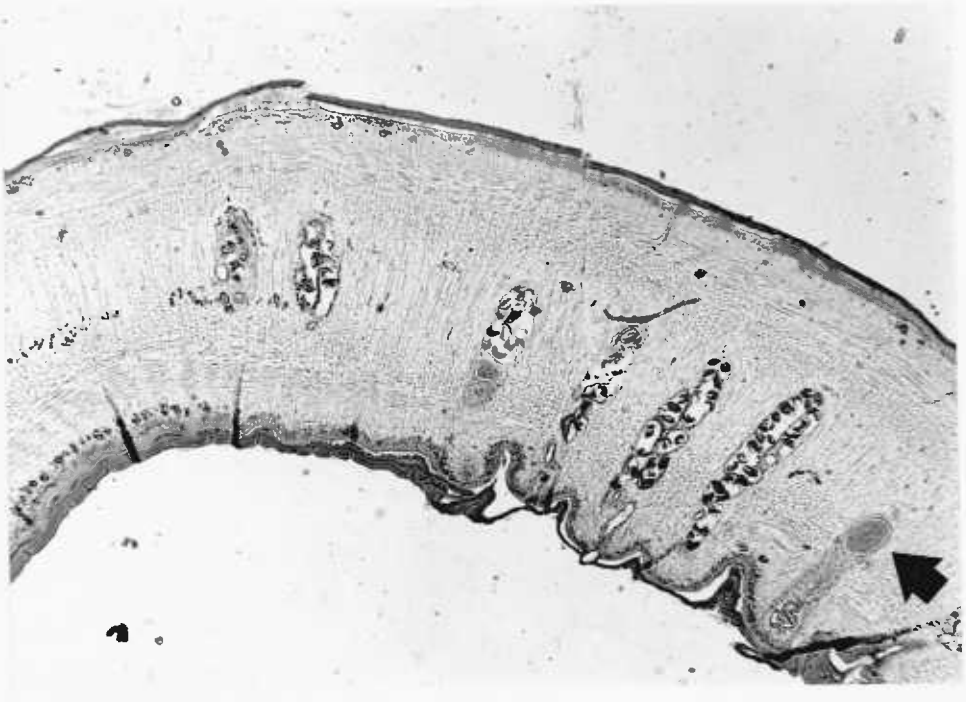
Histological examination of the plerocercoids of L. intestinalis cultured in medium 199 and 20% calf serum showed that maturation was not completed until 65 - 72 hours. The delay of 24 hours between in vivo and in vitro development appeared to occur in the first 24 hours of culture. In vivo this is when development of the tubular organs occurs, but in in vitro cultures this development was not seen until 48 hours. Development at 72 hours appeared to be normal and comparable with that obtained at 48 hours in vivo. The receptaculum seminis was not always seen to contain sperm, so obviously insemination was not always achieved whereas in in vivo all the sections examined had full receptacula (see Plates 11 and 12).

Discussion

The ability to culture the life cycle of a pseudophyllidean cestode, such as L. intestinalis, completely in vitro, would be of considerable use to parasitologists, as it has a number of stages in its life cycle occurring in different hosts; it would be an advantage to be able to study the biochemistry and physiology etc. of all stages. The life cycle of a cyclophyllidean cestode Hymenolepis nana has been cultured in vitro by Berntzen (1962 and 1970) and Graham and Berntzen (1970). The plerocercoids of Spirometra mansonoides (Pseudophyl-

Plate 11. *In vitro* culture of *L. intestinalis*.
Section after 72 hours culture in medium 199 and 20% calf serum.
Haematoxylin and eosin.

Plate 12. *In vitro* culture of *L. intestinalis*.
Receptaculum seminis, seen in Plate 11, full of spermatozoa.
Haematoxylin and eosin.



1 mm



100 µm

lidea) have been cultured to egg-producing adults *in vitro* by Berntzen and Mueller (1964 and 1972).

The most successful culture method in these experiments was found to be incubation in a deep culture tube, under anaerobic conditions and compressed in dialysis tubing (see Table 13). Even so, the percentage of fertile eggs produced did not exceed 30%, which is considerably lower than the 90% fertility obtained *in vivo*. Histological examination of the adults from these *in vitro* experiments showed that fertilisation did not always occur and this probably accounts for the low fertility of the eggs. Smyth (1954) used this technique with *S. solidus* and achieved a percentage fertility of 77%. It was obvious that the compression in the dialysis tubing did have some effect on insemination rates, as those adults cultured in Falcon Flasks only produced eggs with a fertility of 7.3%. It is possible that there are some other factors affecting fertility rate possibly the spermatozoa produced are not normal or that cirrus development is abnormal.

Development *in vitro* was not completed until 65-72 hours and this is slower than *in vivo*. The reasons for this are not known at the present time, but there are many lines of investigation which could be pursued. It is possible that the presence of antibiotics in the medium has a toxic effect on the plercercoid, which inhibits or slows down development. Berntzen (1962) found that antibiotics were toxic to *H. nana* and *H. diminuta*. Other possible lines for future research are the importance

of various ion concentrations such as Na^+, K^+ or Cl^-, and the importance of the gas phase.

The production of abnormal embryonation has been reported before by Smyth (1950) when culturing S. solidus in vitro. He concluded that as no fertilisation had occurred these eggs were haploid and had undergone parthenogenetic development. The stimulus which causes parthenogenetic development is not known, but it could occur during the actual culturing or when the eggs are removed from the culture and incubated. Abnormal embryonations in L. intestinalis cultures were very similar to those produced in S. solidus cultures and so these embryos were probably haploid too.

Smyth (1949) cultured fragments of L. intestinalis which produced eggs but these were not fertile. In the present experiments, fragments were cultured in media and sera (see Table 15) and fertile eggs were produced with a fertility of 12.7%. However, egg laying was delayed until day 4, which means development takes twice as long as in in vivo. Preliminary attempts to culture the coracidia of L. intestinalis were not successful. It is felt that, using this technique, some success may be gained if the ciliated embryophore is removed first, this is normally shed during digestion in the copepod gut.

SUMMARY OF SECTION II

1. *Ligula* infections were established in Khaki Campbell ducklings by force feeding them with plerocercoids dissected from roach.

2. Successful establishment of infection was determined by the presence of eggs in the faeces. 100% infections were obtained in ducklings aged 2 and 7 days, but were not obtained in ducklings any older than this or in ducklings which had been previously infected.

3. Egg production commenced 48 hours post-infection and continued for up to 10 days post-infection. Eggs were recovered from the faeces by washing and sieving. The eggs were embryonated and hatched, and the fertility of these was between 84-90%.

4. Histological examination of worms removed from the duck intestine at 4, 24, 48 and 72 hours was carried out, to trace the development of the adult stage from the plerocercoid.

5. Plerocercoid fragments were implanted into the peritoneal cavity of mice.

6. Eggs were produced from day 4 post-infection and the worms remained alive up to day 13 post-infection, but were becoming encapsulated and starting to deteriorate by this time.

7. Eggs were removed from the mouse peritoneal cavity, embryonated and hatched. The fertility of these eggs was 11.4-21.6%.

8. Histological examination of the adult worms showed that the development appeared to be normal when compared to

that in the duck and that insemination had taken place.

9. Cyclops spp. were maintained in the laboratory.Attempts to infect Cyclops from the Serpentine with Ligula were unsuccessful,as only one species was caught there and this was refractory to infection with Ligula.Suitable susceptible species were collected from Osterley Lake,but infections could not be established in these species either.

10. The in vitro culture of plerocercoids was undertaken, using a variety of techniques and conditions.These included a deep tube culture vessel (Smyth,1954),a circulatory system (Smyth,1972) and Falcon Flasks.The following media and sera were used:Medium 199,NCTC 135,Hanks saline,calf,newborn calf and foetal bovine serum.

11. The fertility of eggs produced from the various cultures was determined and the best result(31%) was obtained from cultures carried out in a deep tube culture vessel in medium 199 and 20% calf serum,incubated at 40°C in the dark.

12. The in vitro culture of plerocercoid fragments was achieved using Falcon Flasks and eggs were produced with a fertility of 12.7%

13. Comparison of development of adults produced in vitro with those in vivo ,showed that the former took 24 hours longer to mature and start egg-laying and only survived up to 7 days.Fragments of plerocercoid started egg production on day 4 of culture and also survived for 7 days.

14. The in vitro culture of coracidia was attempted using Grace's insect tissue medium and lobster haemolymph.

SECTION III

ASPECTS OF THE PATHOLOGY AND IMMUNOLOGY OF *LIGULA* INFECTIONS IN ROACH

CHAPTER 5

PATHOLOGY OF LIGULA INTESTINALIS INFECTIONS IN THE ROACH, RUTILUS RUTILUS

Literature Review

The literature on the pathology of *Ligula intestinalis* infections is extensive. The majority of studies have been conducted in Russia and Eastern Europe where fish farming is an important industry. Figures concerning actual losses caused by ligulosis are difficult to find but Reshetnikova (1967) estimated a loss of 4,600 - 8,900 tons per annum of bream from the Tsimlyansk reservoir alone, due to infection with *Digramma interrupta*. Brylinski (1970) found that in the Wdsydze lake complex in Poland, 72.3% of 3,173 bream were infected, with an annual mortality of 50%. Mortality was highest among young fish and it was calculated that 17% of the whole bream population died as a result of the infection.

Probably the most important and interesting effect of ligulosis is the inhibition of gonadal development in infected fish of both sexes. This has been observed by a number of authors including Fuhrmann (1934), Kerr (1948), Voore (1950), Kirschenblat (1951), Jara (1953), Dence (1958), Arme (1968 and 1975), and Arme and Owen (1965 and 1968). These studies, particularly those of the latter authors, Arme, Arme and Owen, on roach, *Rutilus rutilus*, have revealed

the almost complete absence of gonads prior to the breeding season. On closer examination the gonads could be seen as small mesenteric-like pieces of tissue and the sex of the specimen could only be determined by histological examination. The size of the gonads in infected fish appeared to remain almost unchanged irrespective of the size of the fish. Histological examination of the gonads of infected roach showed that in the ovary of an infected female only oogonia and primary oocytes could be seen and in infected males spermatogenesis was arrested at the stage of spermatogony. This condition was found irrespective of the season and apparently persisted for the life of the fish.

In parasitised roach Kerr (1948) found that the size and granulations of the basophil cells in the meso-adenohypophysis of the pituitary to be markedly reduced, while other cells were not affected. Kirschenblat (1951) confirmed Kerr's suggestion that these roach showed a considerable reduction in the secretion of gonadotrophins. Arme (1968) described the cytological changes in the basophils of the mesoadenohypophysis. He found that experimentally infected fish showed signs of the inhibition of gonad development similar to the testosterone treatment of other uninfected individuals. It was suggested that the effect could be caused by a specific steroid produced by the pleroceroid which acts as an anti-gonadotrophin. However Arme (1978) reported that no steroid activity could be detected in Ligula extracts.

It is also possible that the compound 'produced' by

the plerocercoid may be a non-steroid with anti-gonadotrophic activity. Non-steroids can block the action of pituitary gonadotrophins on gametogenic and endocrine tissue. Hoar, Wiebe and Wai (1967) injected α-methyl-allylthiocarbamyl-2-methylthiocarbamylhydrazine into three species of fish, Carassius auratus, Gasterosteus aculeatus and Cymatogaster aggregatus and the processes of maturation were suspended in the gonads of both sexes of all three species.

Ligula plerocercoids have been shown to inhibit gonad development in other species of fish including bream, Abramis brama (Willer, 1912; Voore, 1950; Brylinski, 1972), gudgeon, Gobio gobio (Fuhrmann, 1934), common shiner, Notropis cornutus frontalis (Dence, 1958) and Galaxias maculatus (Pollard, 1974).

As well as causing parasitic castration, Orr (1966) suggested that parasitised rudd failed to participate in normal breeding behaviour. Of 200 rudd removed from a shoal exhibiting courting behaviour only one was parasitised by Ligula, whereas of 75 rudd netted at random 56 were infected. From this work he suggested that parasitised rudd failed to produce the necessary sterohormones which are normally released into the environment to stimulate spawning. Brylinski (1972) conducted tagging experiments on bream in the Kaszubian Lake District and also concluded that infected fish did not take part in spawning. Pollard (1974) also noticed the inhibition of Galaxias maculatus movement to the spawning grounds.

A number of biochemical investigations have been carried out on the hosts of Ligula plerocercoids to see if this cestode has other pathological effects on the fish host. Jarzynowa (1971) found that the presence of plerocercoids had no effect on the protein or fat content of the muscle or viscera of the bream host. Safonov (1972) found a highly significant difference between infected and healthy bream in the Tsymlyansk reservoir in the mean values of size, weight (without viscera) and the fatness coefficient (according to Clark). The differences in the mean values of weight with viscera and of the fatness coefficient (according to Fulton) were not significant.

Changes in the blood picture of infected gudgeon have been described by Sadkovskaya (1953) who found that the average monocycte and polymorphonuclear leucocyte counts increased in infected fish. Shpolyanskaya (1953b) obtained similar results with Carassius carassius. The latter author found that the haemoglobin levels decreased and Kosheva (1956) confirmed this in bream as well as finding a decrease in the fat content of the host. The packed-cell volume and haemoglobin content showed a significant inverse relationship with the parasitic index of roach (Arme and Owen, 1968) and that the mean liver weight was significantly lower in infected individuals. Kosareva (1961) found that the glycogen content in the liver of three species of Cyprinid fish infected with Ligula was significantly lower than that of healthy fish of the same age and catch.

It is fairly obvious from this literature review that the great majority of work concerned with the pathology of *Ligula* infections has been devoted to the inhibition of host reproduction and it was for this reason that it was decided to investigate some other aspects of fish pathology and this work will be described in the following chapters.

CHAPTER 6

ELECTROPHORESIS OF ROACH SERUM: A COMPARISON BETWEEN HEALTHY INDIVIDUALS AND THOSE INFECTED WITH LIGULA INTESTINALIS

Introduction

Electrophoretic investigations of intraspecific variations in fish serum proteins have shown that differences may be effected by the genetic constitution of the individual, by physiological factors such as age, sex and breeding condition and by environmental factors such as temperature, nutrients and amount of light. There is a great wealth of literature on this subject which has been reviewed by Booke (1964) and Harris (1971) and a bibliography has been written by Hawkins and Mawdesley-Thomas (1972). However little is known regarding changes in fish serum protein fractions as a result of parasitic infection and only Guttowa and Honowska (1973) have reported changes in serum protein patterns of bream infected with *L. intestinalis*.

In the present work the serum of healthy roach was compared with that from roach naturally infected with *L. intestinalis* after electrophoresis on polyacrylamide gels. Polyacrylamide gel electrophoresis was the method selected for these studies as it has several advantages over the other methods of starch gel and paper electrophoresis. The polyacrylamide support medium forms a network of polymer chains, the pore size of which can be varied to accommodate the dimensions of the molecules to

be separated. Thus separation by polyacrylamide gel utilises both protein mobility and molecular size and this vastly improves the separation.

Materials and Methods

Disc Electrophoresis

The fish used in these studies were captured by seine netting in the Serpentine Lake, London, at monthly intervals between August 1975 and February 1976. The size and age of the fish used were noted.

The fish were bled either one or two days after capture. They were firstly anaesthetised in a solution of 0.01% tricaine methane sulphonate (MS 222) and then bled by cardiac puncture. The blood was left to stand in glass clotting tubes for approximately one hour, at room temperature, and then left at 4°C overnight. Serum was separated from the clot by centrifugation and electrophoresis was carried out on the fresh serum samples.

Polyacrylamide gel electrophoresis was carried out on apparatus supplied by the Shandon Scientific Company Ltd, Model no. SAE-2734. A 7.5% acrylamide gel was used, the rods being prepared the day before and stored overnight at 4°C. 10 µl of sample was applied to each rod, the sample consisting of serum diluted 1:3 with 20% sucrose solution. Bromophenol blue dye (0.05%) was added to the buffer in the upper reservoir to act as a marker.

Electrophoresis was started using a current of 1mA per tube for the first 15 minutes and increased to 4mA per tube for 45 minutes. After electrophoresis, the gels were removed from the rods by the irrigation needle method.

Several methods for staining for total protein were used:

(a) Weber and Osborn (1969) - 1.25 g Coomassie brilliant blue R 250 in 45 ml of 50% methanol and 46 ml of glacial acetic acid. This solution was filtered and stored at room temperature. Gels were put into this stain and left overnight. They were destained in a solution of 75 ml acetic acid, 50 ml methanol and 875 ml H_2O. Destaining took several changes of solution and a number of days.

(b) Gels were fixed and washed in a 12.5% solution of trichloroacetic acid (T.C.A.) for 10 minutes, then stained in a 0.25% aqueous solution of Coomassie brilliant blue G 250 (2.0 mls/40 mls T.C.A.) for three hours.

(c) Gels were fixed and stained in a 1% amido black in acetic acid solution. Staining took one hour. Destaining took 24-36 hours and was carried out using successive changes of 7% acetic acid solution.

Method (b) gave the most satisfactory results and was the method adopted for further experiments.

Gels were stored in universal containers in a 7% acetic acid solution. Photoelectric scans were obtained using a Gilford Spectrophotometer 240, at a wavelength of 550 nm.

Gradient Gel Plate Electrophoresis

Electrophoresis was carried out on Pharmacia Electrophoresis Apparatus GE-4, loaded with gradient gel plates PAA 4/30. 10 µl of serum in a sucrose solution containing between 10 - 20 mg/ml of protein were applied to each chamber of the marker column. Marker proteins were run at the same time.

The plates were prerun at 125 V for 15 minutes then subjected to pre-electrophoresis 20 minutes at 70 V and finally electrophoresis for 15 hours at 125 V. Tris-HCl buffer (pH 8.35) was employed.

After separation, the plates were removed from the cassettes and stained with Coomassie brilliant blue G 250 (as previously described). Glycoproteins were stained with periodic acid Schiff's (PAS) reagent according to Felgenhauer (1970). For lipoproteins the method devised by Perrier et al. (1974) was used. This staining solution contained 0.5 g Sudan black, 5 ml acetone, 15 ml acetic acid and 60 ml water. Staining took four hours and destaining was done in a solution of 50 ml 90% ethanol, 10 ml acetic acid and 40 ml water. The gels were stored in 5% acetic acid and retained their colour in this fixative.

Results

Disc Electrophoresis

In all a total of 26 serum samples were examined, 9 from healthy fish and 17 from roach infected with Ligula intestinalis plerocercoids. The protein patterns obtained were variable and in fact, no two samples were identical. However, it was possible to describe the general picture of roach serum proteins after electrophoresis and to show some of the differences between healthy and infected roach serum.

In the majority of samples 6-8 major protein fractions were evident (see Fig.16&17, Plates 13&14). These were labelled 1-7 in order of decreasing mobility and could be tentatively identified as pre-albumin band 1, albumin band 2, the α-, β- and γ-globulins bands 3, 4, 5, 6 and 7. 3 or 4 minor bands were occasionally resolved, nearly always in the β-region.

A quantitative comparison of the relative amounts of protein in the fractions was obtained using the densitometer traces of the stained gels (see Table 16).

Gradient gel plate electrophoresis

Protein stain

5-9 bands were evident and the separation was better than that achieved with disc electrophoresis. Bands 2 and 6 were seen to be very much reduced in infected serum samples (see Fig.18 , Plate 15).

Glycoprotein stain

5 glycoprotein bands were developed on the plate, numbers 1, 3, 5, 6 and 7 (see Fig. 18, Plate 16).

Figure 16. Disc electrophoresis pattern of roach serum proteins. Serum from uninfected fish.

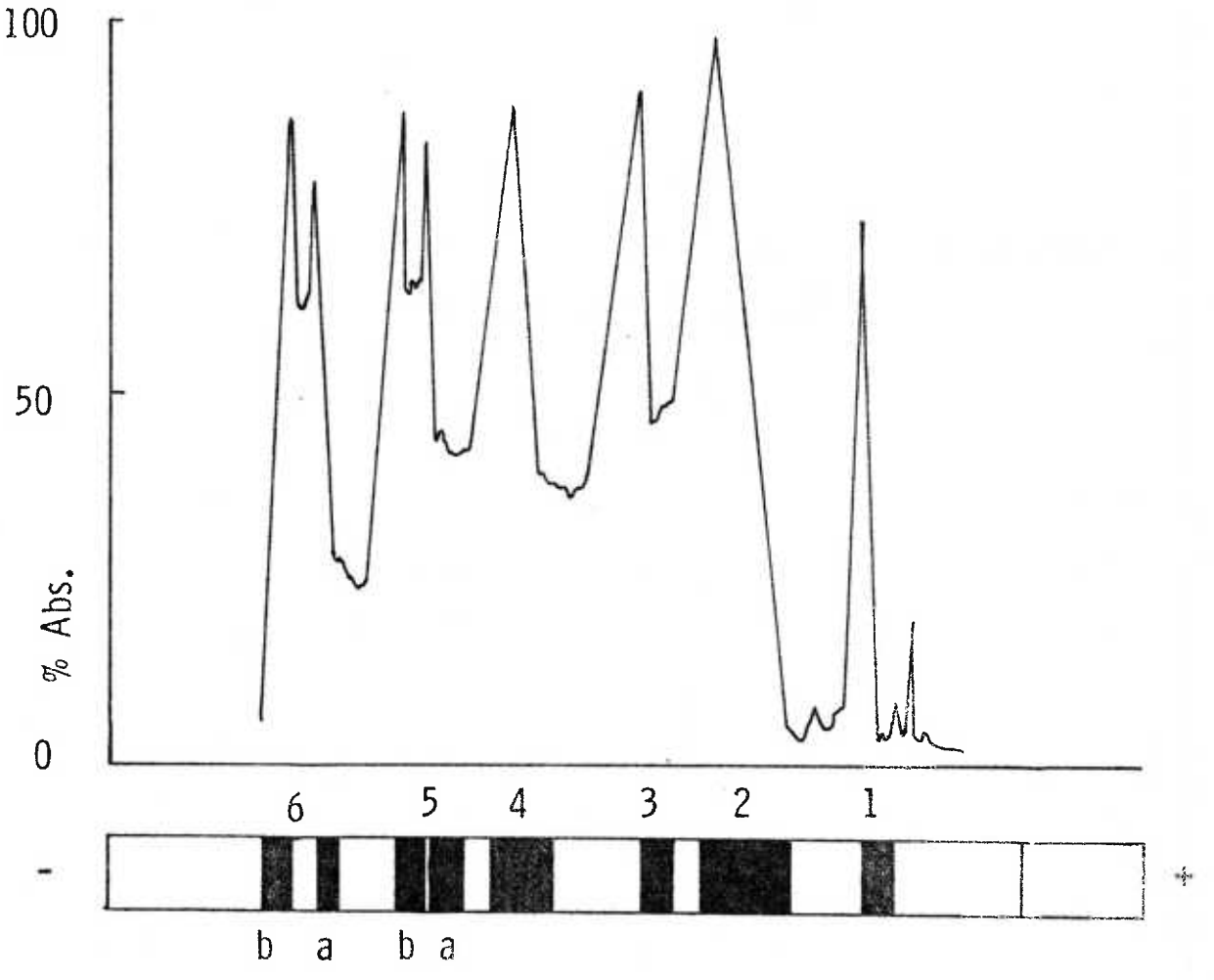

100
50
0
% Abs.
6
5
4
3
2
1
-
+
b
a
b
a

Figure 17. Disc electrophoresis pattern of roach serum proteins. Serum from fish infected with *L. intestinalis*.

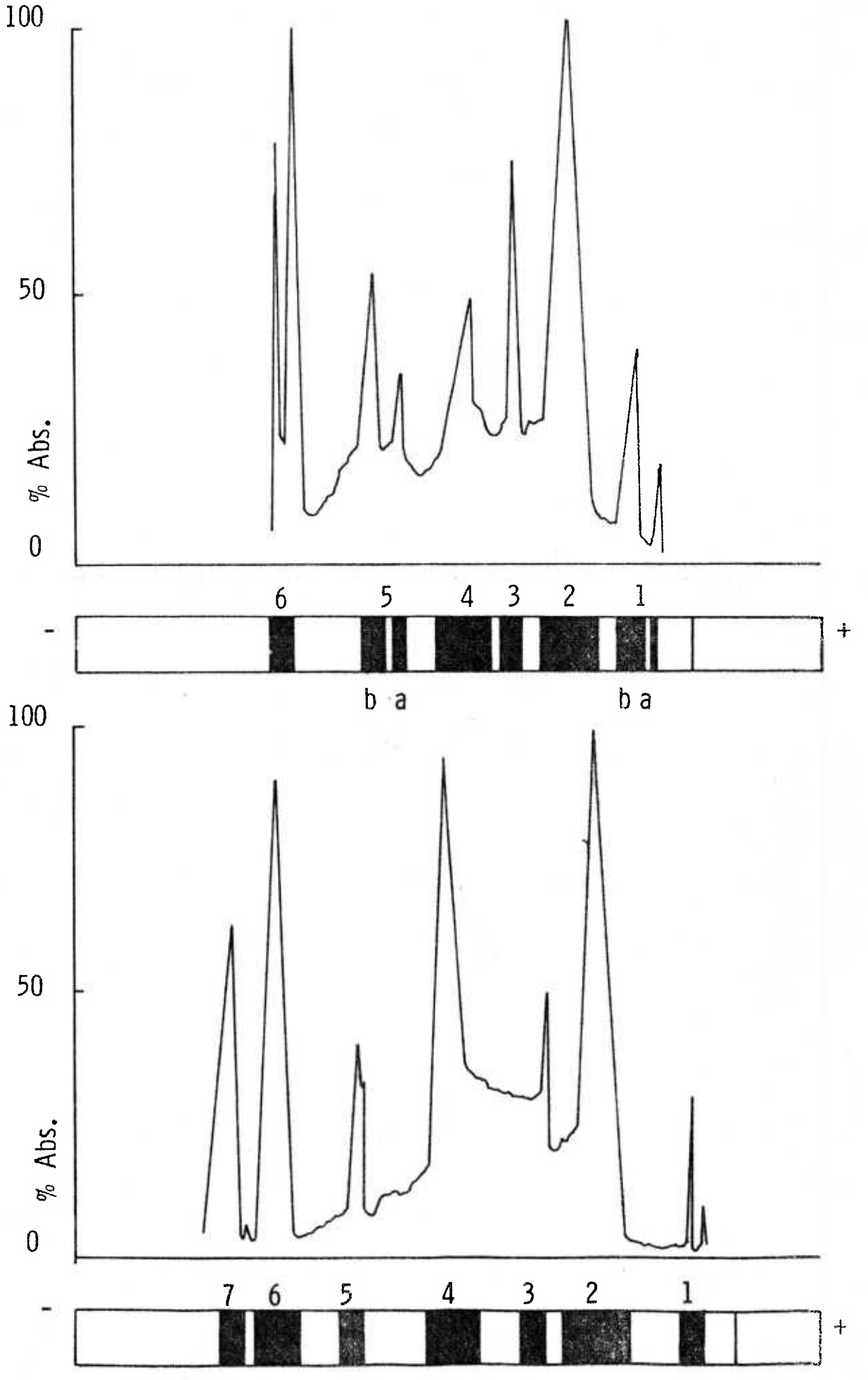
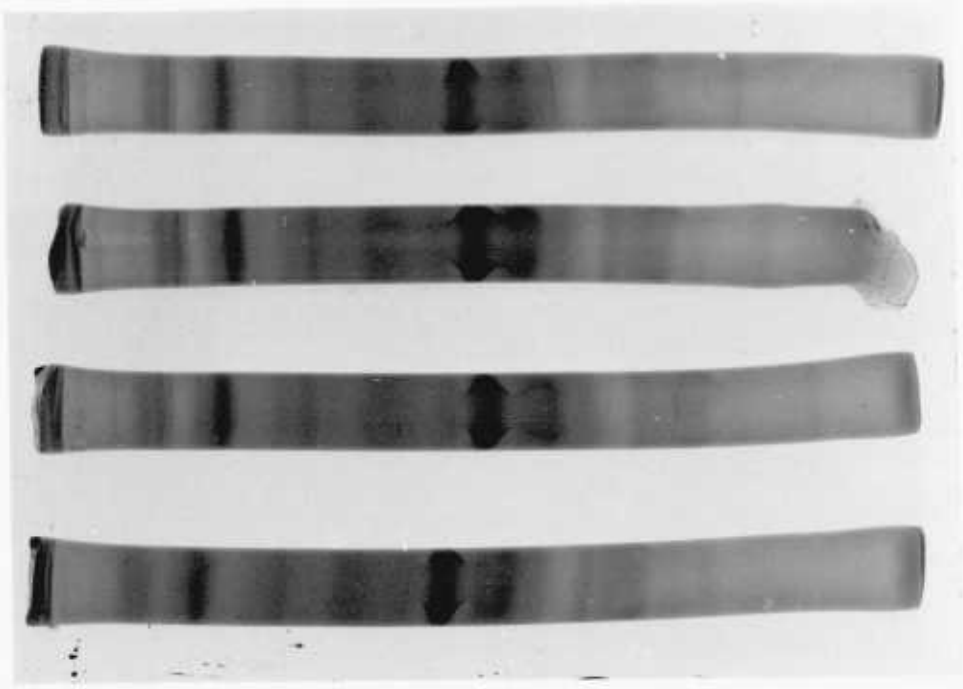

100
50
0
% Abs.
6
5
4
3
2
1
-
+
b a
b a
100
50
0
% Abs.
7
6
5
4
3
2
1
-
+

Plate 13. Disc electrophoresis of serum from uninfected Serpentine roach.

Plate 14. Disc electrophoresis of serum from Serpentine roach infected with *L. intestinalis*.



-ve

+ve

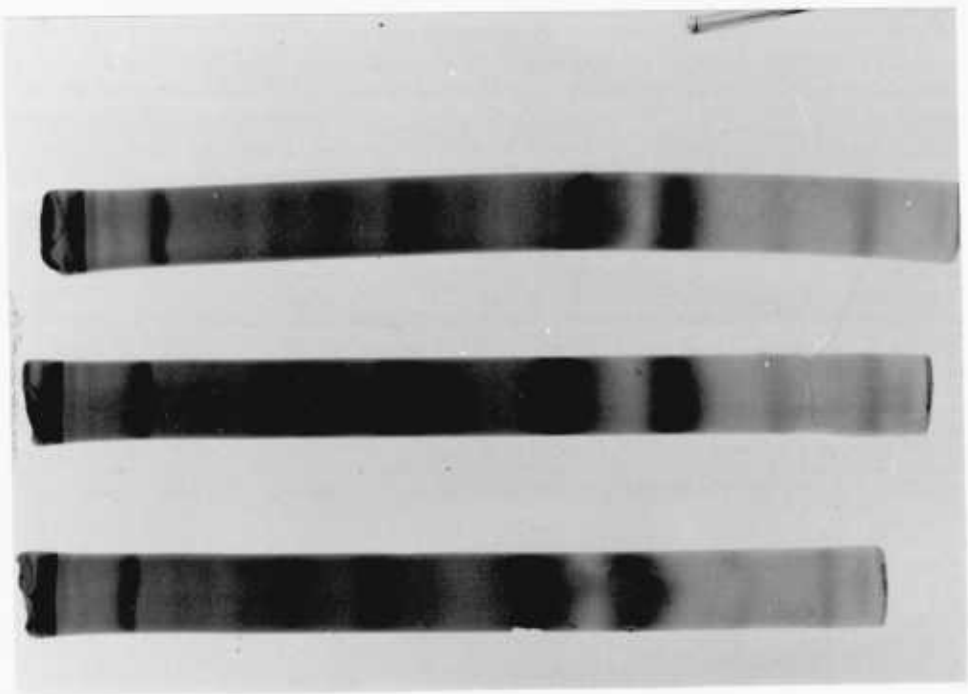


Table 16
Percentage composition of major fractions of Roach serum

January 1976

	Age	Fraction 1	2	3	4	5	6	7	A/G ratio
Healthy	4+	8.6	23.4	20.3	18.0	1.26	8.6	8.6	.47
	4+	8.1	22.0	18.04	25.5	14.6	11.7	-	.43
Infected	5+	9.5	35.2	26.65	14.3	12.7	1.6	-	.81
	4+	6.5	45.8	23.2	13.5	6.8	3.5	0.8	1.09

The albumin to globulin ratio was calculated as the sum of fractions 1 + 2 / the sum of fractions 3 + 4 + 5 + 6 + 7.

Lipoprotein stain

One band was shown up as a lipoprotein - band 3. This band also stained with the glycoprotein stain and is thus a glyco-lipoprotein complex.

Molecular weights

The molecular weights of the serum proteins separated by gradient gel electrophoresis were obtained by running a series of marker proteins of known molecular weight (see Fig. 18).

In general, the mobilities of the fish serum components appeared to be less than those of human serum.

Discussion

No two serum samples ever gave identical protein patterns after disc or plate electrophoresis, although repeat runs of the same serum samples under similar conditions gave reproducible results. It is very difficult to correlate these differences with any of the numerous parameters which must be considered such as age, sex, or health of the fish, although correlations with some of these parameters have been demonstrated and can be found in the reviews of Booke (1964) and Harris (1971). Much of the published work has been carried out on fish populations whose aetiology is known and whose environment can be controlled, e.g. trout raised in hatcheries. Interpre-

Figure 18. Results of gradient gel plate electrophoresis of roach serum.

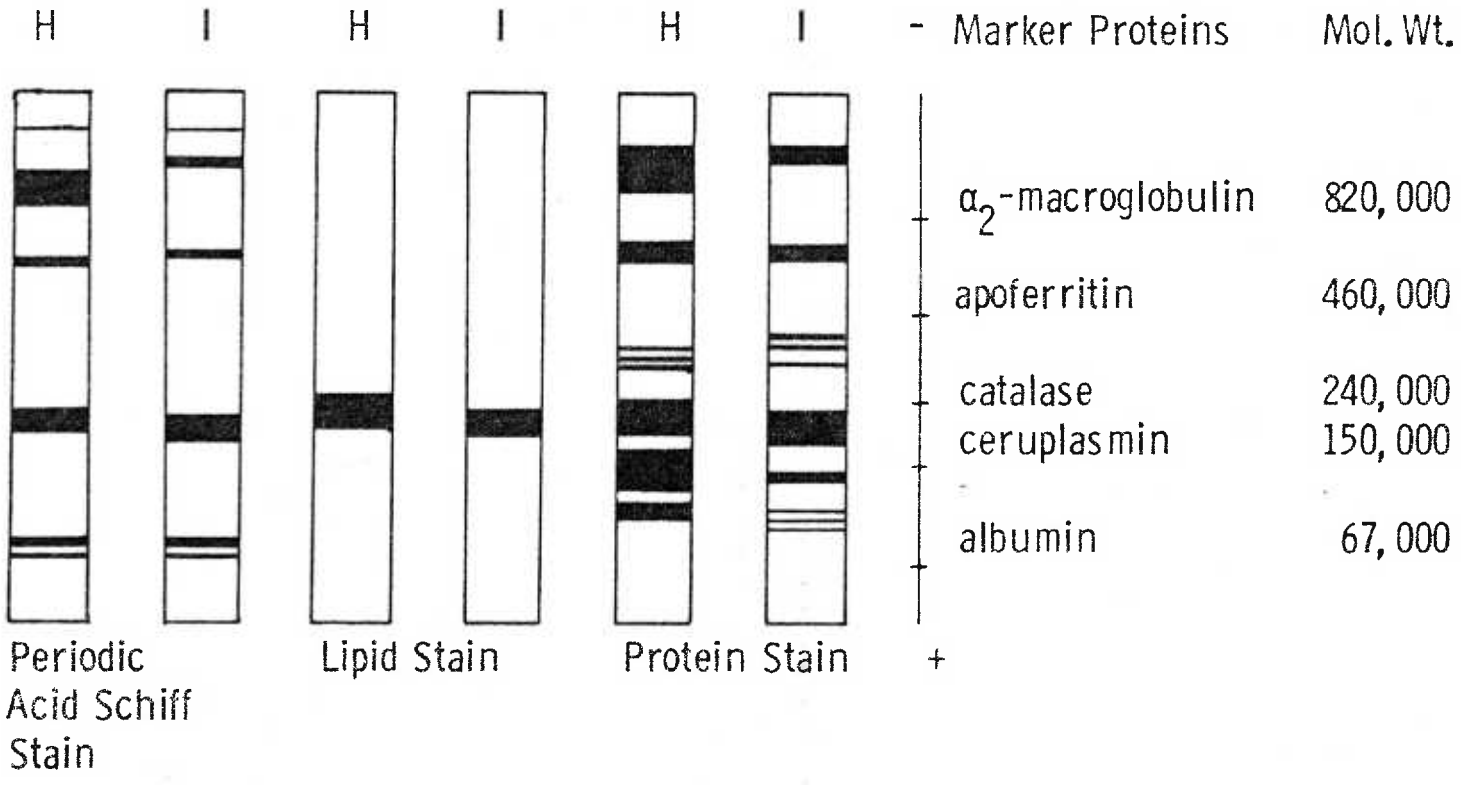
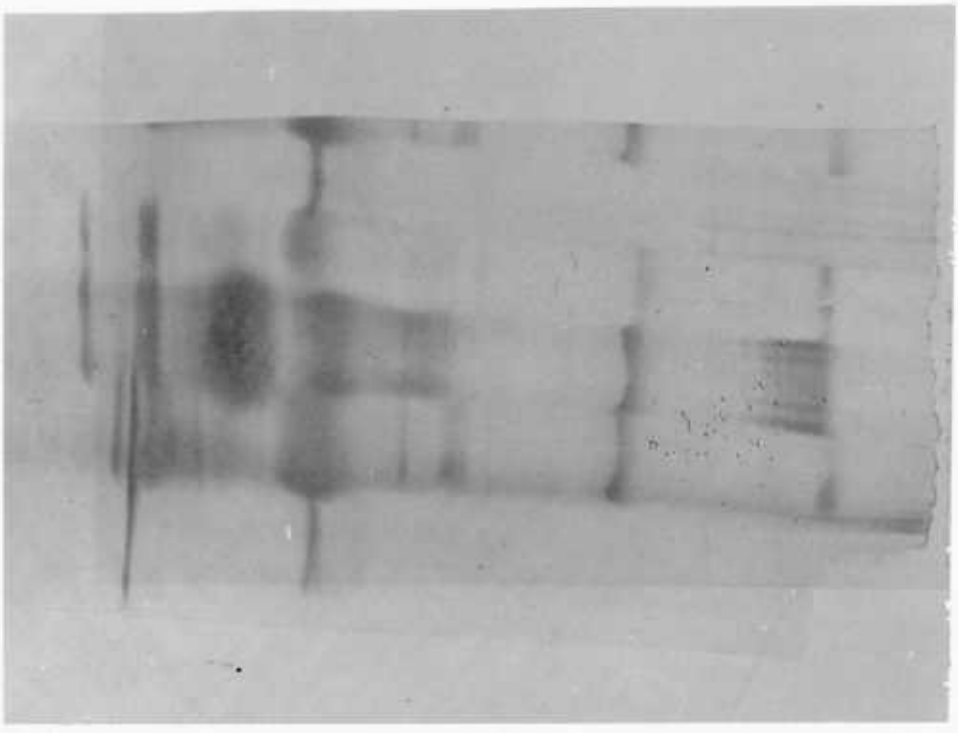

H
I
H
I
H
I
- Marker Proteins
Mol. Wt.
α2-macroglobulin
820, 000
apoferritin
460, 000
catalase
240, 000
ceruplasmin
150, 000
albumin
67, 000
Periodic Acid Schiff Stain
Lipid Stain
Protein Stain
+
H- Healthy Roach
I-Infected Roach

Plate 15. Gradient gel plate electrophoresis of roach serum. Protein stain.
H - uninfected roach serum
I - serum from roach infected with *L. intestinalis*.

Plate 16. Gradient gel plate electrophoresis of roach serum. Glycoprotein stain.
Key as above.

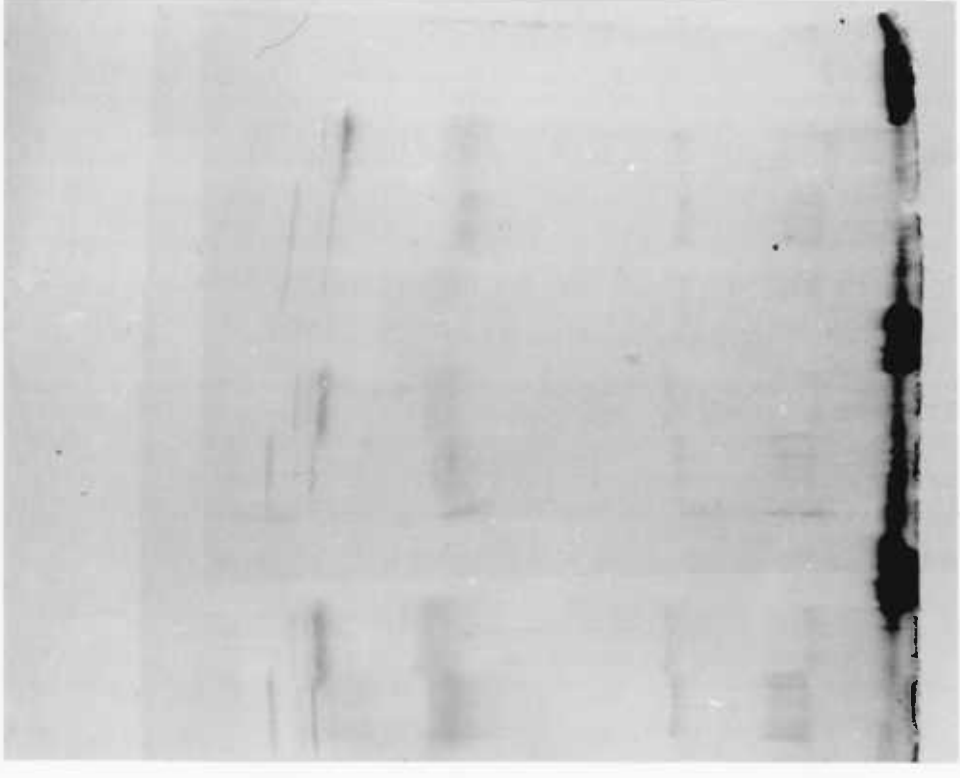


H

I

+ve

-ve



I

H

tation of results is much easier in these cases than if a wild population is being studied, as in the present investigation.

After electrophoresis of roach serum, 6 - 8 protein fractions were identified although Nyman (1965) using starch gel electrophoresis described 9 - 12 fractions and Child (1975) described 4 - 6 fractions.

The albumin to globulin ratio in samples separated by disc electrophoresis (Table 16) showed a marked difference between healthy and infected individuals, being 0.45 and 0.92 respectively. These figures are relatively low when compared to those of other cyprinid fish such as dace, 1.4 - 1.9 (Harris, 1974), chub, 1.18 - 1.31 (Planas and Gras, 1957) menhaden, 1.28 - 2.08 (Lepkovsky, 1929), although Guttowa and Honowska (1973) reported figures of 0.49 - 0.42 for healthy and parasitised bream respectively.

The plate electrophoresis separations showed a noticeable difference in the albumin fraction, which was significantly reduced in infected individuals. A similar decrease in albumin was reported by Guttowa and Honowska (1973) during the infection of bream with Ligula and by Einszporn-Orecka (1970) during ergasilosis infection in tench and by Sorvachev et al., (1962) in serum of carp with rubella infections. The decrease in albumin in fish with Ligula infections may be due to the disruption of liver function, where primary metabolism of serum proteins occurs. The majority of these electrophoresis experiments were carried out over the winter months and a decrease in albumin and/or the albumin to globulin ratio over the winter

months for freshwater fish generally has been reported by several authors (Saito, 1957; Khailov, 1963) and a decrease in the total serum protein occurs at this time (Saito, 1957; Sorvachev, 1957a and b). Changes in serum patterns brought about by starvation are comparable to the changes which occur over the winter (Sorvachev, 1957a and b; Love, 1970).

The globulin fraction of serum in human blood is normally split into three parts, α, β and γ-globulin and the same terminology has been applied to fish serum in much of the available literature. No significant changes were seen in the α and β fractions of roach serum but the γ-fraction was considerably smaller in infected individuals. In contrast Cuttowa and Honowska (1973) found a regrouping of globulins in bream during Ligula infections, resulting in a decrease of α-globulins, an increase of β-globulins and no change in the γ-globulins. Sorvachev et al. (1962) found a decrease in α- and β-globulins and an increase in the γ-globulins.

Specific humoral antibodies in rainbow trout (Salmo gairdneri) immune serum were found in the electrophoretically least mobile protein fraction (Post, 1966), and these proteins coincided most closely with the β-globulins and possibly to the least mobile α-globulin of higher animals. Molnar and Berczi (1965) demonstrated a precipitating antibody in the serum of bream infected with Ligula, so if the immune status of infected roach has been changed this may lead to changes in the globulin fractions of the serum. A precipitating antibody has yet to be

demonstrated in roach infected with Ligula (see Chapter 8).

Only one lipoprotein was observed and this was in the α-region. Harris (1974) reported two lipoproteins from dace, one in the α-region and one in the β-region, and that the β-lipoprotein was only found in females and spent fish and never in males. A lipoprotein has been shown to be involved with changes in maturity and the onset of egg production in females of cyprinid and salmonid fish (Vanstone and Ho, 1961; Krauel and Ridgway, 1963; Summerfelt, 1964). However, band 3 of the roach separation as well as staining for lipoprotein also stained with PAS, so is obviously a glycolipoprotein complex. A similar type of band has been reported from the plasma of eleven fish species (including roach) by Perrier et al. (1974).

A number of authors have shown that the major portion of teleost serum is not comparable to human albumin but to α-globulin (Summerfelt, 1964; Snieszko et al., 1966). Perrier et al. (1974) found that the holoprotein fraction which predominated quantitatively as serum albumin in mammalian plasma does not occur in trout and Post (1966) found that the three major fractions of trout serum do not coincide with the electrophoretic mobility of the classic albumin, α, β and γ globulin separation of higher animals.

In conclusion, the interpretation of the results of electrophoresis of roach serum and a comparison between healthy and infected individuals is complicated by all the other parameters which must be considered when studying a

natural population. However, a generalised picture of roach serum patterns has been described and some possible reasons given for some of the observed differences between healthy fish and those infected with Ligula intestinalis.

CHAPTER 7

THE EFFECT OF THE PLEROCERCOID OF LIGULA INTESTINALIS ON THE METABOLISM OF RUTILUS RUTILUS

Introduction

The plerocercoid of L. intestinalis resides in the body cavity of cyprinid fish and is in close association with the intestines and liver of the host. A significant decrease in the size and weight of the liver of infected fish has been reported by Arme and Owen (1968). Organ damage can be traced by the assay of specific enzymes which have leaked from that tissue into the blood. Andrews et al. (1961) found that elevation of serum glutamate pyruvate transaminase (GPT) activity reflected the migration of larval Ascaris suum through the liver of pigs and that elevation of serum glutamate oxaloacetate transaminase (GOT) indicated the migration of larvae not only through the liver but also through the lungs. In albino rats infected with Fasciola hepatica, there was a close relationship between serum GOT activity and migration of flukes through the liver, the period of maximum liver damage and the extent of liver damage (Thorpe, 1965). Bundesen and Jannsens (1971) found that the migrating behaviour of F. hepatica in mice could be followed using serum assays of GPT.

It is characteristic of many parasitic infections to bring about changes in blood proteins. Some of the changes which occur in Ligula infections have already been

demonstrated qualitatively by electrophoresis (Chapter 6). Changes in protein metabolism may also be detected by measuring the total protein levels of serum and liver tissue (von Brand, 1973).

Disturbances in carbohydrate metabolism manifest themselves in changes in blood sugar and/or glycogen reserves. Ascaris larvae migrating through the liver were studied by Munnich (1958) and the glycogen contents of the parasite foci were reduced but that of the surrounding tissue was unaffected. Kawazoe (1961) found a decrease in the host's liver glycogen and blood sugar in Ascaris infections. Kosareva (1961) showed that the glycogen content in the liver of three species of cyprinid fish infected with Ligula was lower than in healthy specimens.

The present study was thus undertaken to see if any tissue damage had occurred and whether any changes in protein or carbohydrate metabolism could be found in roach infected with L. intestinalis.

Materials and Methods

Total protein determination of serum

Serum was obtained from fish by the method described previously (Chapter 6). Total protein was determined using the biuret method. Biochemica test kits were used and these were supplied by Boehringer Mannheim. The optical density was read at 546 nm in glass cuvettes on a Unicam SP 600 spectrophotometer.

Total protein determination of liver

The fish were pithed and an incision made along the mid-ventral line and one side of the body wall removed. This made the liver easily accessible in healthy and infected fish, although in some heavily infected individuals the liver was difficult to separate from the connective tissue and intestinal mesenteries. Between 150 - 200 mg of liver tissue were used for each determination and each sample was accurately weighed on a small piece of aluminium foil. The tissue was dissolved in 2 ml of 4N sodium hydroxide (NaOH) and then diluted to 80 ml with 0.5N NaOH. This particular dilution was determined by running a series of preliminary experiments using varying dilutions and the dilution of 200 mg tissue in 80 ml NaOH gave the range of optical density readings desired.

A set of standards was made using bovine serum albumin (BSA). It was found convenient to prepare a stock solution of 1 mg BSA/ml in 0.5N NaOH and appropriate aliquots were used to give 20, 50, 100 and 200 µg protein. The dilutions were carried out with 0.5N NaOH to give equivalence with the tissue samples.

The total protein determination was carried out using the Lowry (1951) method. The optical density was read at 750 nm in glass cuvettes in a Unicam SP 600 spectrophotometer.

Glycogen determination of liver

The liver was removed from fish as previously

described, weighed and its glycogen content determined using the anthrone method (Roe and Dailey, 1966). Approximately 100 mg fresh weight of liver was dissolved in 2 ml 30% potassium hydroxide (KOH) in a glass test tube. The solution was heated for 15 - 30 minutes until the tissue had dissolved. The tube was cooled and 3 ml 95% ethanol added and then replaced in the heating block and shaken until the mixture began to boil. The solution was cooled and centrifuged at 3000 r.p.m. for 15 minutes. The supernatant was decanted and the tube inverted and allowed to drain over filter paper.

The glycogen precipitate was dissolved in distilled water and washed into a 50 ml volumetric flask and made up to 50 ml. 1 ml of glycogen solution to be tested was put into a test tube and 5 ml 0.05% anthrone reagent added. It was stirred well and boiled at 100°C for 15 minutes, then cooled to room temperature and the optical density read at 600 nm.

Distilled water was used as a reagent blank and a glucose solution containing 0.1 mg/ml was used as a standard.

The amount of glycogen was calculated as follows:

$$\text{Amount of glycogen} = \frac{OD_1 \times 100}{OD_2 \times 1.11} \text{ µg in 1 ml sample} \times 50.$$

OD_1 = optical density of unknown solution

OD_2 = optical density of standard

1.11 = Factor for the conversion of glucose to glycogen.

Dry weight determination of liver

Liver tissue remaining from livers removed for glycogen determinations, was used for the dry weight determinations. The piece of liver tissue was placed on a piece of aluminium foil and its fresh weight accurately weighed. It was dried in an oven at 105°C for 24 hours, reweighed and the percentage dry weight calculated.

Periodic Acid Schiff Staining of Liver

8 µm sections of liver from healthy and infected fish were stained with PAS to see whether any difference in the glycogen content of the hepatic cells could be seen.

Enzyme determinations on serum and plasma Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT)

These enzyme determinations were carried out on fresh serum in the first instance. Biochemica test kits were used, supplied by Boehringer Mannheim. The initial results were unsatisfactory because some haemolysis had occurred which caused artificially high results.

The cause of the haemolysis of the serum at this time could not be traced although it is known that fish blood is very difficult to handle. It was thus decided to use plasma for the enzyme assays. The plasma was obtained using heparin as the anticoagulant. A stock solution containing 100 I.U./ml of buffer was prepared and 0.1 ml

of heparin solution was taken up into a 1 ml syringe with 0.9 ml of blood. The blood and heparin solution were mixed thoroughly and the cells spun down at 2500 r.p.m. for 20 minutes at room temperature. Enzyme assays were then carried out on the fresh plasma.

Results

There was a slight, but not significant decrease in total serum protein of roach infected with L. intestinalis, $P < 0.1$ (see Tables 17 and 18).

Liver protein, $P < 0.1$ (see Tables 17&18) the dry liver weight ratio of liver ($P < 0.01$, see Tables 19 &20) were reduced but glycogen content was increased ($P < 0.02$, Tables 19& 20) in the liver of infected fish.

Histochemical examination of the liver tissue by PAS staining did not provide any evidence that there was a real difference in the glycogen levels of the hepatic cells of parasitised roach liver.

Although time only allowed a preliminary set of experiments, the results obtained were encouraging, as a highly significant difference between enzyme levels in parasitised and non-parasitised fish was observed. There was a highly significant increase ($P < 0.001$) in the levels of GOT and GPT in the plasma of infected roach (see Table 21).

Table 17

Total protein determinations of parasitised and nonparasitised roach liver and serum

Parasitised

Date	Age	Sex	Length cm	Weight gm	T.P. Serum g/100ml	T.P. Liver g/100ml	P.I.
Nov. 1975	3+	*	12.0	25.3	4.3	9.9	28.37
	3+	F	12.2	26.5	4.7	10.24	22.36
	3+	*	13.0	35.7	4.3	6.5	19.81
	3+	*	12.5	30.16	4.2	4.5	23.63
	3+	*	12.5	32.9	6.0	5.1	11.93
	3+	*	14.0	32.9	3.9	-	22.04
Dec. 1975	3+	*	12.2	26.38	5.1	-	26.21
	2+	*	14.4	16.75	6.3	-	19.71
	2+	*	10.7	16.75	5.0	-	21.69
	2+	*	9.9	11.93	3.8	-	13.80
	2+	*	11.6	23.16	4.5	-	13.24
	2+	*	11.4	24.83	4.9	-	11.98
Jan. 1976	1+	*	11.3	22.41	4.1	-	19.48
	0+	*	8.5	8.34	3.7	-	26.52
	5+	*	15.0	57.34	4.8	-	31.26
	5+	*	16.7	75.97	4.7	-	23.24
	4+	M	13.9	44.46	5.8	-	7.70
	2+	M	12.8	35.47	4.8	-	13.91
	2+	M	12.7	38.46	6.4	-	10.70
Feb. 1976	4+	*	14.1	44.78	5.4	-	9.13
Oct. 1976	5+	*	15.8	57.05	-	7.6	23.2
				Mean =	4.84	=7.31	g/100 ml

*=sex not determined

Table 18

Total Protein determinations of parasitised and nonparasitised roach liver and serum

Nonparasitised

Date	Age	Sex	Length cm	Weight gm	T.P. Serum g/100ml	T.P. Liver g/100 ml
Nov. 1975	6+	F	19.0	128.9	6.2	-
	7+	F	20.3	155.3	6.7	-
	7+	F	19.2	130.52	5.5	-
	6+	F	18.8	119.7	7.56	5.13
Oct. 1976	7+	M	19.2	120.2	4.02	9.6
	9+	F	23.3	236.2	3.42	7.8
	8+	F	22.6	230.6	4.52	7.7
	6+	M	18.1	103.6	3.84	8.3
	7+	M	19.4	136.33	-	10.2
	6+	M	17.5	97.63	-	6.8
	6+	F	17.4	93.14	-	10.2
				Mean =	5.22	=8.22

Total protein of serum

The difference between the means of parasitised and nonparasitised individuals was not significant at the 5% level, $P < 0.1$.

Total protein of liver

The difference between the means of parasitised and nonparasitised individuals was not significant at the 5% level, $P < 0.1$.

Table 19

Glycogen determination on roach liver and dry weight estimation

Parasitised

Date	Age	Glycogen	Dry weight	P.I.
Aug. 1976	4+	5.97	14.94	26.26
	5+	3.48	19.91	8.2
	4+	7.46	19.87	30.77
	5+	1.65	21.6	28.59
Dec. 1975	4+	5.6	-	19.71
	4+	8.35	-	13.14
	4+	7.82	-	13.8
	4+	9.97	-	7.49
	3+	9.58	-	11.90
	4+	8.13	-	10.03
	3+	11.48	-	7.92
	2+	8.09	-	17.97
	2+	8.34	-	22.45
	2+	7.96	-	10.65
	2+	6.78	-	18.00
	3+	6.93	-	12.01
Mean =		7.35 mg/100 mg tissue		=19.08 g

Glycogen Determination

The difference between the means was statistically significant at the 2% level, $P < 0.02 > 0.01$.

Dry weight

The difference between the means was statistically significant at the 1% level, $P < 0.01$.

Table 20

Glycogen determination on roach liver and dry weight estimation

Nonparasitised

Date	Age	Glycogen	Dry weight
Aug. 1976	5+	6.09	21.85
	5+	11.1	24.83
	5+	8.6	21.75
	4+	5.02	19.71
	5+	4.19	22.7
	6+	2.81	18.66
	5+	6.08	19.76
	4+	4.45	24.92
	5+	10.05	24.1
	8+	6.11	24.91
	6+	5.36	24.22
	6+	5.4	27.1
	5+	5.29	25.2
	10+	1.93	22.67
	6+	2.21	22.6
	4+	1.49	24.8
	6+	5.00	27.2
	5+	1.55	25.4
	5+	6.5	19.7
	4+	5.1	26.8
Dec. 1975	3+	6.79	-

Mean = 5.3 mg/100 mg tissue = 23.44 g

Table 21

Enzyme determinations of roach plasma

Parasitised

GOT	GPT	P.I.	Age	Sex
-	38.22	21.22	2+	M
-	57.2	21.92	2+	*
-	37.33	7.86	3+	M
310	31.13	31.26	5+	*
210	19.73	23.24	5+	*
456	44.2	7.70	4+	M
390	19.3	13.91	2+	*
390	33.2	10.70	2+	*

Mean = 337.7 =33.1 u/l

*=sex not determined

Nonparasitised

GOT	GPT	P.I.	Age	Sex
68.1	18.0	0	5+	M
76.0	17.0	0	5+	M
38.0	12.8	0	6+	F
56.7	18.7	0	4+	F
33.8	8.64	0	5+	M
94.2	14.2	0	5+	M
39.3	11.2	0	5+	F
62.0	10.8	0	4+	F

Mean = 58.5 =13.9 u/l

The difference between the means was significant at the .1% level for both GOT and GPT, $P < 0.001$.

Discussion

Tissue Damage

The results of these preliminary experiments would seem to indicate that there is some effect on the liver of roach infected with *Ligula* plerocercoids. Raised levels of GOT and GPT activity in the plasma of infected individuals were observed and this elevation of enzyme activity has been shown by a number of authors to be indicative of liver damage (Andrews *et al.*, 1961; Thorpe, 1965; Bundesen and Jannsens, 1971).

However, the roach used in these studies were from a wild population and were naturally infected and it is felt that it would be useful to continue this work by using fish infected in the laboratory with a known number of plerocercoids. This would enable the pattern of enzyme release to be traced throughout an infection and to see whether it indicated the extent and duration of tissue damage, as found by Gallagher (1969), whether there was any relationship between the extent of the damage and the number and size of the plerocercoids and at what stage in the infection did liver damage occur. It would also be interesting to investigate whether there is a reciprocal decrease in enzyme activity in the liver as the serum enzyme activity increases. Schmidt *et al.* (1958) showed this occurs in humans with acute hepatitis and Asada (1958) demonstrated this with rats with experimental liver necrosis.

Disturbances in the host's metabolism

The results showed a slight increase in glycogen levels of parasitised fish livers and there are some instances where parasites have induced an increase in polysaccharide synthesis. During Clonorchis infections of rabbits, polysaccharide granules appeared in previously polysaccharide-free epithelial cells of proliferating bile ducts (Kuwamura, 1958) and the mucin production of bile ducts parasitised by Dicrocoelium dendriticum is greatly increased (Dhar and Singh, 1963). It is more common for a decrease in glycogen to occur e.g. during Ascaris infections (Munnich, 1958), Fasciola hepatica infections in rabbits and guinea pigs (Kublickiene and Goldbergiene, 1966) and with F. gigantica infections in buffaloes (Mostafa et al., 1964).

It is possible that a decrease in glycogen does occur in the early stages of the infection, before the tissue reaction envelopes the plerocercoid. The majority of fish used in these experiments had relatively large plerocercoids and the infections were obviously quite well established as tissue reactions were present in all specimens. Histochemical studies have shown that the hepatic cells in the vicinity of young cysticerci of Taenia taeniformis lack glycogen, but when they are encapsulated the glycogen content of adjacent cells reverts to normality (Lewert and Lee, 1955).

The increased glycogen level could also be

explained by the fact that the liver in some infected roach is so reduced, that there may be a concentrating effect of glycogen in the remaining hepatic cells.

One further point to consider is whether the damage to the liver is caused mechanically by the plerocercoid or whether there is another explanation such as digestion and utilisation by the plerocercoid. There is some evidence that cestodes could utilise large molecules such as proteins either by direct uptake or by membrane digestion (Smyth, 1976). Membrane bound enzymes are believed to be capable of digesting large molecules with which they may come into contact (Ugolev, 1965 and 1968). However, the occurrence of proteolytic enzymes in cestodes has not been reported widely but Smyth (1969) has speculated the existence of such enzymes in Taenia, Diphyllobothrium and Echinococcus. Davydov and Kosenko (1972) found a considerable amount of amylase in the surface layer of Ligula plerocercoids and it was proved experimentally that amylase, capable of splitting starch to glucose was secreted into media in which the live plerocercoids were placed. These findings and also the presence of microvilli on the body surface suggest strongly that L. intestinalis is capable of contact digestion and absorption of food from its host.

Charles and Orr (1968) have also investigated the structure of the tegument of Ligula plerocercoids and found that it suggests uptake of nutrients may occur by three methods, a) mass flow through the pores, b) diffusion, involving no specific structural relationship

between the membrane and the diffusing material and c) active transport. They found no evidence for pinocytosis which would be necessary for the uptake of large molecules such as proteins.

As the plerocercoid of Ligula is not an intestinal parasite, no pronounced interference with protein absorption from the gut would be expected. Any interference with protein metabolism may be due to the reduction of the liver function. However no significant changes were seen in the levels of total protein in the serum or in the liver tissue.

CHAPTER 8

IMMUNOLOGICAL STUDIES ON FISH SERUM

Introduction

The fundamental principles of fish immunology are well documented, reviews being presented by Clem and Leslie (1969), Cushing (1971) and Corbel (1975), but relatively little work has been involved with the role of immunity in parasitic disease. Nigrelli and Breder (1934) and Hines and Spira (1974) demonstrated the presence of acquired protective immunity in fish to parasitic agents. Harris (1972) and Cottrell (1977) characterised both the antigen and antibody involved in a fish host - helminth parasite system. Of particular interest is the report by Molnar and Berczi (1965) indicating the presence of precipitating antibodies in the serum of bream (Abramis brama) infected with Ligula plerocercoids.

A ready supply of roach naturally infected with Ligula was available and an attempt was made to demonstrate the presence of precipitating antibodies.

Materials and Methods

Preparation of Ligula antigen

The method used in these experiments was modified

from Harris (1972). Plerocercoids were removed from the body cavity of the roach and weighed. They were homogenized in phosphate buffered saline (PBS) pH 7.2 (1 volume of worms to 2 volumes of PBS) using a Thyristor Regler mixer for 15 minutes. The solution was kept on ice during the mixing. The worm debris was removed by centrifugation and the supernatant delipidated with anhydrous ether and centrifuged. The fatty layer was removed and the resulting solution was concentrated with Lyphogel to approximately half its original volume and stored at -20°C. The antigen extract was found to contain on average 2.1 g/100 ml of protein by the Biuret method.

Extraction of Excretory and Secretory Products

Method after Harris (1972). The plerocercoids were removed from the fish and weighed. They were placed in a tissue culture flask in 0.5% NaCl at 10°C for approximately 5-6 days. After incubation the plerocercoids were removed and the solution was concentrated with Lyphogel.

Collection of serum from roach

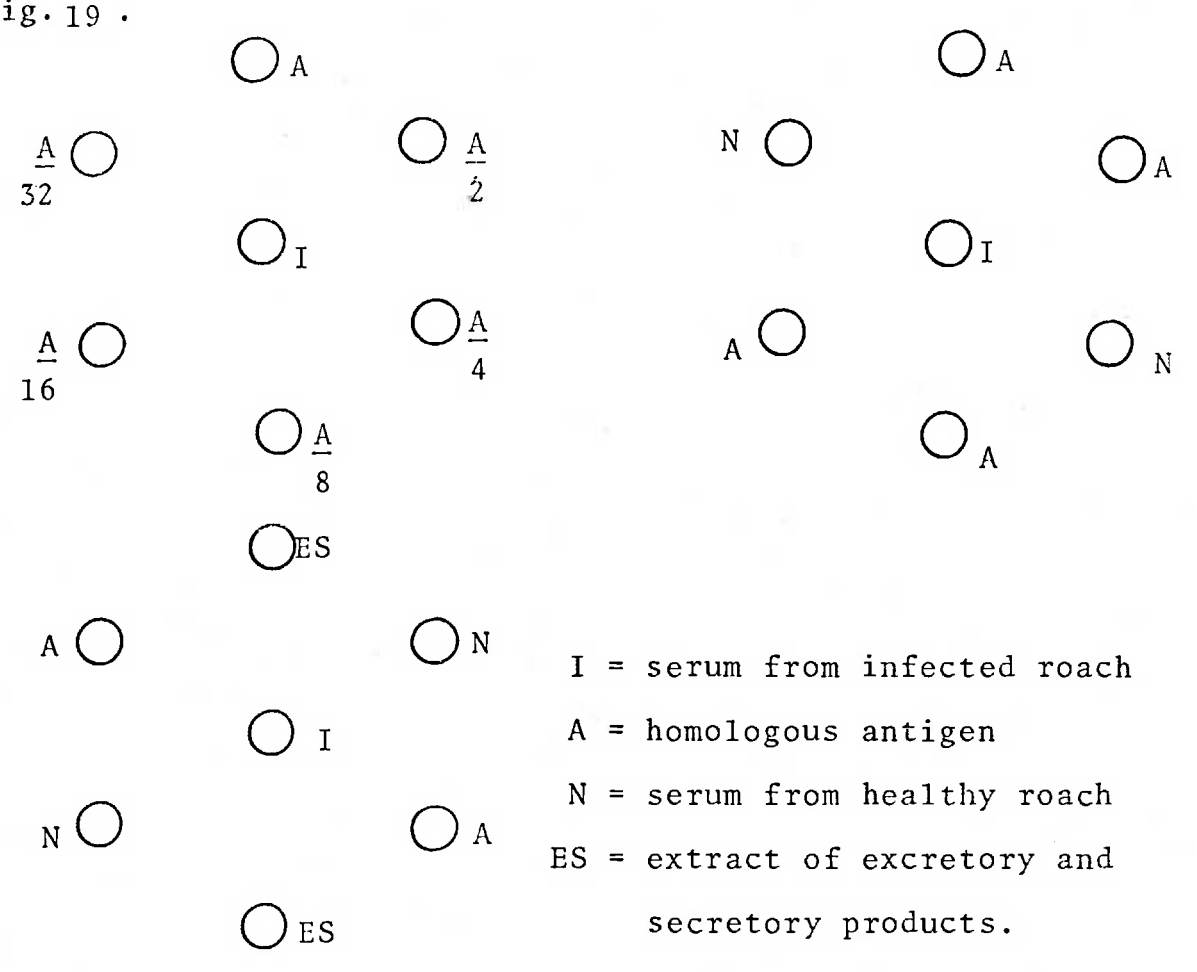
Method as described on page 130.

Preparation of agar plates

Immunodiffusion using Ouchterlony (1968) double diffusion method, was carried out in a 1% Ionagar No. 2

(Oxoid) made up in PBS pH 8.2 and containing 0.02% sodium azide as a fungicide. The serum and antigen were plated as shown in Fig. 19.

Fig. 19 .



Immunodiffusion was conducted at 25°C and after 48 hours the plates were washed in several changes of PBS. The plates were stained with Coomassie brilliant blue as described in Chapter 6.

Disc Immunodiffusion

Polyacrylamide gel electrophoresis (PAGE) of

the Ligula antigen was carried out by the method described in Chapter 6. PAGE gel rods were cut in half longitudinally and one half immersed in warm (50°C) 1% agar and the latter allowed to gel. Troughs (2 mm) were cut in the agar allowing a 4 mm diffusion distance between the trough and the embedded PAGE rod. One trough was filled with infected roach serum and the other with normal roach serum. The plates were allowed to diffuse at 4°C for two weeks, then the gel rods were removed and the agar plates washed and stained.

Attempted immunization of uninfected fish

Two previously uninfected roach were injected with 1 ml Ligula antigen extract intraperitoneally on days 1, 12 and 28 (after Harris, 1972). They were bled by cardiac puncture on day 60 and the serum plated against the Ligula antigen extract.

Results

The sera of twelve infected roach were tested and no precipitin bands were seen in the straight double-diffusion test. The sera from two roach were used in the disc-immunodiffusion test and no precipitin bands were observed. These results were not surprising as the level of precipitating antibodies in natural infections may be low .

The Ouchterlony test was repeated using pooled

sera which had been concentrated with Lyphogel, but again no precipitin bands were visualised.

The attempt to immunize uninfected roach with Ligula antigen extract was not successful as no precipitin bands were seen.

Discussion

The failure of these experiments to detect circulating antibodies cannot be taken as evidence that they are not produced at all or that an immune reaction is not involved in the roach-Ligula plerocercoid system.

Failure to detect circulating antibodies has been reported by Kennedy and Walker (1969) in the dace host-Caryophyllaeus laticeps (Cestode) system and by Harris (1972) in the dace, grayling, perch and roach host-Pomphorhynchus laevis (acanthocephalan) system.

When considering adult cestode infections, it is not known whether the host tissue needs to be damaged to allow antigen access or whether antigen can be transported through the intact epithelium (Smyth, 1969b). Rees (1967) stated that responses to adult cestodes can only develop if the mucosa of the hosts' intestine is invaded.

The plerocercoid of Ligula does not cause damage to the intestinal mucosa of its host and in fact never comes in contact with it. It is the procercoid, ingested inside a copepod, which penetrates the intestinal wall and develops into the plerocercoid in the body cavity, see page 26. So any antibody production would be directed

against the procercoid. The plerocercoid of Ligula elicits a tissue response from the roach and this is evident in roach fry as young as three months (Arme and Owen, 1970). So although Ligula infections may be long-lived the plerocercoid is only exposed for a relatively short time and is then encapsulated in connective tissue.

Antibody production by fish has been shown to be temperature dependent (Bisset, 1948; Ridgway et al., 1966; Cottrell, 1977). Kennedy and Walker (1969) suggested that if fish were held at a temperature below 15°C then the chances of antibody production were lowered. During parenteral immunization experiments Cottrell (1977) kept immunized plaice at 20°C whereas Harris (1972) kept immunized chub at 10°C and both got positive results with the Ouchterlony test. The fish used in the present work were kept at 11-12°C and it is possible that antibody production was inhibited at this temperature.

It is also possible that no precipitating antibody was demonstrated because the techniques used were not sensitive enough or were unsuitable. Only twelve samples of roach serum were examined and this was probably not a large enough sample. Harris (1972) found precipitating antibodies in 20 out of 38 chub whereas Cottrell (1977) found only 2 out of 23 plaice.

SUMMARY OF SECTION III

1. The literature on the pathology of Ligula intestinalis was reviewed.

2. Disc and plate PAGE were carried out on serum samples from non-parasitised and parasitised roach. Serum protein patterns were compared using protein, glycoprotein and lipoprotein stains. Between 5-9 protein fractions could be separated using these techniques and of these 5 were glycoproteins and 1 was a lipoprotein fraction.

3. Plate PAGE showed a decrease in the albumin and the γ-globulin fractions in infected roach serum.

4. Serum and liver total protein levels were determined in parasitised and non-parasitised roach by the biuret and Lowry methods. Both levels were LOWER in infected roach. Liver glycogen concentrations were estimated by the anthrone method and the dry/wet weight ratio of the liver calculated. The former was increased in infected fish, whilst the latter was decreased.

5. PAS staining of liver sections did not reveal any differences in the histology or glycogen content of the liver cells.

6. Assays of the enzymes GOT and GPT in parasitised and non-parasitised roach plasma, showed a significant increase in the levels of both enzymes in plasma from infected fish.

7. Attempts were made to demonstrate the presence of precipitating antibodies in serum from roach infected with Ligula. The Ouchterlony double-diffusion plate and disc-immunodiffusion

methods were used and antigen was prepared from the plerocercoids and from the excretory and secretory products.

GENERAL DISCUSSION

In conclusion, it is felt necessary to sum up the results of the experimental work covered in this thesis, to point out some of the limitations of the study and to suggest some of the ways in which the work could be continued in further studies.

Seasonal variation and variation with the age of the host,were found in the incidence and intensity of infection with Ligula plerocercoids in roach from the Serpentine. Some possible explanations were put forward but the limitations of conclusions drawn from such a survey were stressed. The survey was carried out over a period of 12 months only and the method of sampling (seine netting) was not ideal.

Further study would be facilitated by improving the sampling technique. Statistical analysis would be made easier by making the sample groups the same size and by having the same number of fish in each age group. It would also be necessary to catch fish by a variety of methods, such as rod and line and fishtraps, as well as netting.

In the present work the fish caught were not sexed, so it would be interesting to discover what the balance of the sexes is in the Serpentine and whether there is any correlation between sex and the Ligula infection. In the light of the results from the ageing and length frequency analysis of the roach in this study, further work,on the possible effect of Ligula on the growth of the fish,may explain why the lengths of the different age groups was so variable.

The importance of being able to maintain a para-

site in the laboratory, either *in vivo* or *in vitro* has been discussed in Section II. Successful *in vivo* culture of the plerocercoid to the adult stage was carried out in ducks and mice, and these were used in comparative studies with those cultured *in vitro*. Various methods of *in vitro* culture were used in an attempt to improve the fertility of the eggs, and this was achieved by using the techniques originally described for *S.solidus* and *E.granulosus*. However, the eggs produced were not as fertile as those produced *in vivo* and maturation took slightly longer,so further investigation is required.

Some of the ways in which *in vitro* culture may be developed can be suggested here. The importance of the gas phase, ion concentrations,use of antibiotics and use of other media are all possible lines of further study. Further histological studies on the development of the adult, possibly incorporating some electron microscope work, may reveal any abnormalities e.g. in the development of the sperm, which could account for the lower fertility of eggs produced *in vitro*.

Some aspects of the pathology and immunology of the *Ligula* infection in roach were studied, in particular possible damage to the liver and subsequent impairment of function were investigated. Qualitative differences in the serum proteins were detected by electrophoretic methods and some attempt made at interpreting the results obtained. However it was felt that more control over the large number of variables, such as physical conditions,age,sex and season, was required, before any conclusions could be reliably drawn. This work could be carried out on fish which had been raised in the laboratory and experimentally infected,and kept under controlled conditions.

The preliminary experiments to detect raised enzyme levels in the plasma, produced very interesting results and indeed suggested that there was some liver damage occurring during Ligula infections. It would be necessary to carry out more experiments of this kind to verify the results reported here, as only a limited number of experiments were performed. It is also suggested that further work could be done on the levels of protein in the serum and the liver as although the decrease in levels found in this study was not significant, they were lowered, and this could be indicative of some impairment of liver function.

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