STUDIES ON THE TAXONOMY AND THE BIOLOGY OF DIPLOSTOMUM SPECIES (DIGENEA).

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by

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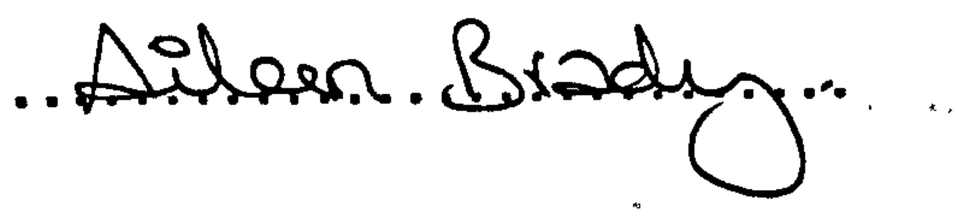
To My Loving Husband,

Joseph

DECLARATION

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



..Aileen..Brady...

ABSTRACT

For many decades the taxonomy of the genus Diplostomum von Nordmann, 1832 has remained in a state of confusion in the literature. The present study aimed at determining where the problems lie and what can be done to resolve them.

A brief survey of the farmed and wild populations of fish in Scotland indicated that there were four highly prevalent forms of Diplostomum metacercariae in the eyes of the fish. Type 1 were located in the lens of rainbow trout (Salmo gairdneri Richardson), Type 2 in the humour of rainbow trout, Type 3 in the retina of rainbow trout and Type 4 in the retina of perch (Perca fluviatilis Mitchell).

The four types were examined morphmetrically using Principal Components Analysis (PCA) and this revealed that the four types grouped separately. Type 1 metacercariae could be distinguished by the closeness of their lappets, Type 3 by their small size, Type 4 by their large size and high breadth to length ratio and Type 2 lacked any of these distinguishing features.

The culture of the metacercariae to adults revealed biological differences between the four types. Type 1 grew in both domestic chickens and herring gull chicks (Larus argentus Pontoppidan), but best in the latter. Type 2 also grew in both of these hosts, but best growth was achieved in the chicken. Chickens were refractive to Types 3 and 4, but both established and developed in herring gulls.

The adults obtained from the bird host were also examined morphometrically using PCA, and again the four forms grouped separately, indicating that they were morphologically distinct. In experimental infections it was found that the hind-body did not fully develop until day 16-18 p.i. and, therefore, morphological analysis was only carried out on worms which had been cultured in the bird host for more than 16 days. This was particularly significant in Type 1 adults where the relative position of the ovary was affected. Type 1 adults were distinguished from the others by the posterior position of the ovary in the hind-body, Type 2 by

the small dimensions of the ovary and its position at the intersegmental region, Type 4 by the anterior extent of the vitellarium and Type 3 adults lacked any of the distinguishing features.

Completion of the life-cycle of the diplostomes also revealed differences in the cercarial stage both morphologically and in the sensory papillar patterns determined by the use of chaetotaxy. The life-cycle was completed for all the diplostomes apart from Type 2. It was found that Type 2 miracidia would not establish in Lymnaea pereger, although the other three types established.

Infection of fingerling rainbow trout with Types 1, 3 and 4 cercariae revealed that the trout were refractive to Type 4 cercariae, but Types 1 and 3 established in the lens and retina, respectively. When perch were exposed to Type 4 cercariae it was found that metacercariae established in the retina. This indicated that the metacercariae are very site specific and also may show some host specificity.

Biochemical analysis of the metacercariae of Types 1-4 by analysing isoenzyme profiles with the use of Isoelectric Focusing (IEF) also revealed that there may also be some differences biochemically between the four types.

Identification of the four diplostomes was attempted using the keys (Dubois, 1970; Shigin, 1986) and information available in the literature. However, a confident identification could only be made for Type 1 which keyed down to D. spathaceum sensu Niewiadomska. The other three types could not, however, be identified with such confidence, but tentative identifications were made. The fact that the metacercarial, cercarial and adult stages were obtained and described for three out of the four diplostomes and still a positive identification could not be made perhaps reflects the inadequecies of the keys available in the literature.

The present study has successfully determined the problematic areas surrounding the taxonomy of the genus and has, therefore, cleared the way for future study.

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER 1:

GENERAL INTRODUCTION

INTRODUCTION

The cultivation of salmonids in temperate waters of the northern hemisphere has become a rapidly expanding industry in terms of both direct consumption, as fish are a rich source of protein, and in supplementing sport fisheries (Roberts, 1978). This ever increasing demand for fish has led to intensive culture farming techniques in which fish are cultured at very high stocking. Such high population densities provide the ideal environment for infectious diseases to present a constant and expensive threat to the success of fish culture (Ellis, 1988).

There are no reliable reports in the literature that parasitic diseases have been the cause of great economic losses in wild fish stocks (Egidius, 1983; Sinderman, 1987); but, within the confines of a fish farm, overcrowding increases the chances of contact with the parasite, thereby ensuring rapid spread throughout a population. There is also a potential for parasite numbers to build up to considerable levels as a direct result of environmental stress on the fish (Roberts & Shepherd, 1986). Parasites which appear benign in the natural environment may become highly pathogenic under these conditions (Sinderman, 1987).

Parasites belonging to the genus Diplostomum Nordmann, 1832 infecting the eyes of freshwater fish have been reported to cause disease in wild populations of fish (Ashton, Brown & Easty, 1969; Bottomley & Woodiwiss, 1969; Brown & Easty, 1969; Dogiel, Petrushevski & Polyanski, 1970), although there may be no great economic losses. However, in the farm situation severe infestations have been reported for many years (Ferguson & Hayford, 1941; Bauer, 1961). Nowadays, there are many reports from fish

farmers, especially in the hatchery of high infestations which can, and often do, result in economic losses in terms of productivity. Members of the Salmonidae appear to be particularly susceptible to these parasites, although this varies from species to species. In Scotland the native brown trout (Salmo trutta L.) is less susceptible to infection by D. spathaceum (Rudolphi, 1819) than the introduced rainbow trout (S. gairdneri Rich.) (Betterton, 1973). On the other hand, Speed & Pauley (1984) reported that coho salmon (Onchorhynchus kisutch Walb.) and cutthroat salmon (Salmo clarki Rich.) are more susceptible than rainbow trout.

Diplostomum infection in the fish host can cause serious damage or even death. Death may result from large numbers of cercariae penetrating the fish body, effectively breaking down the osmoregulatory function of the skin and small fry are especially vulnerable. This rarely occurs, however, and so the majority of fish invaded by Diplostomum cercariae survive the attack. The cercariae then go on to develop as metacercariae in the eyes or brain of the fish. In this study the Diplostomum species of interest are those infecting the eyes. Diplostomum species which infect the lens of the eye, induce cataractous changes in the lens which can result in partial or complete blindness, lens rupture and exopthalmia (Chappell, 1967; Dukes, 1975; Shariff, Richards & Sommerville, 1980). Other more general effects include stunted growth and abnormal feeding behaviour such as decreased visual acuity and lack of response to visual stimuli (Heckman & Palmieri, 1978; Hendrickson, 1978). This can have harmful effects in a farming situation where fish which do not achieve an optimal growth rate have little economic value.

Control of this parasite can be satisfactorily achieved by blocking out

various stages of the life-cycle. The life-cycle involves three hosts: a gastropod mollusc acts as a first intermediate host, a freshwater fish acts as a second intermediate host and a piscivorous bird acts as a final host (Fig. 5.1). There are of course some variations, but in general this is the case.

Control is normally best achieved by controlling the first intermediate host, the gastropod mollusc. Periodic draining and clearing of raceways and ponds to remove vegetation can keep the numbers of snails in a farm down to acceptable levels. Some farmers opt for the use of molluscicides on their farm. This method of control can be effective, but the chemicals used can also be very harmful to the fish and the environment, disrupting the delicate biological balance. A novel method of biological control of the larval stage of the parasite in the snail host has been effected through hyperparasitism by the protozoon Nosema strigeoidea. Hussey (1971), Palmieri, Heckman & Cali (1976) and Palmieri, Heckman & Evans (1976) found that Nosema was a specific hyperparasite of sporocysts of D. spathaceum, and thereby interfered with normal cercarial development.

Whatever the method of attack on the snail intermediate host, destruction of a large proportion of a population can prove detrimental to the water-body. Snails play an important role in the interplay between organisms in a water-body, are involved in determining the amount of calcium in the water and serve to re-cycle plant material, both living and dead (Wesenberg-Lund, 1934). Therefore, if a snail population is completely eradicated, there may be a complete biological change in the water-body that is detrimental to the fish.

The final host of Diplostomum is more difficult to control. If the source of snail infections is from the farm itself, then a good control method is to prevent the birds access to the farm and predating the stock. This can be achieved to some extent by either placing netting over the ponds or raceways or by placing a low fence around the perimeter of the ponds, thereby deterring birds from feeding at the edges of the ponds.

In conclusion, therefore, it appears that the most satisfactory method of control is to attack the infected snail populations, to prevent the fish coming into contact with the infectious cercariae and to control access of birds to the snail populations. However, this involves a knowledge of the life-cycle and especially the hosts involved. In general, if a parasite locates in its natural host to which it has become adapted, the level of pathogenicity is controlled by the defence mechanisms of the host. If, however, a parasite locates in an abnormal host where no such 'natural adaptation' has taken place the damage to the host can be severe. It is, therefore, essential to know the normal and abnormal hosts for each parasite species. in order to assess the potential pathogenicity. Without this knowledge great time and resources may be spent needlessly in controlling non-pathogenic species of diplostomes. Since 'natural adaptation' generally leads to a parasite becoming less pathogenic to a host many problems are caused when new host are introduced to the environment in a farming situation. Perhaps the best example of this in Britain is that of the introduced rainbow trout which, as indicated earlier, is more susceptible to infection and damage by diplostomes than the native brown trout.

The economic significance of Diplostomum has prompted many studies in

Britain. By far the most documented species is D. spathaceum, which infects the lens of many freshwater fish. This species perhaps attracts the most attention because of its harmful effects on the fish which are well documented (Chappell, 1967; Dukes, 1975; Shariff et al., 1980). It is assumed that other species of Diplostomum infecting the eyes of freshwater fish do have a deleterious effect on their host, although only Pennycuick (1971a) has shown harmful effects of a humoural/retinal form known as D. gasterostei Williams, 1966.

In most of the studies carried out on Diplostomum species in the UK, authors have made the assumption that lens parasites are D. spathaceum and that humoural and retinal parasites are D. gasterostei. In fact, in many reports this assumption has formed the sole basis for identification. A similar, although not identical, situation was at one time evident in the USSR. However, a more careful examination of the genus has revealed at least 13 species according to Shigin (1976).

Many studies have been carried out on Diplostomum in Britain investigating the epidemiology (Sweeting, 1974; Kennedy & Burrough, 1977; Kennedy, 1981; Stables, 1984) and immunology (Betterton, 1973; Stables, 1984; Whyte, 1989). Very few, however, have dealt with the taxonomy of the genus. In many cases this has been neglected and it is unclear what species of Diplostomum is actually being investigated or whether more than one species is involved. It is essential, therefore, as a fundamental need, that the true taxonomic situation is clarified. Although many species of Diplostomum have been documented worldwide, very few have been recorded in the UK. The nominal adult species documented in the UK are: D. spathaceum (Rudolphi, 1819), D. gasterostei Williams, 1966 (only from

an experimental infection), D. mahonae Dubois, 1953, D. phoxini (Faust, 1918) and D. parviventosum Dubois, 1932. The metacercarial stage of these species have been described in the UK with the exceptions of D. parviventosum and D. mahonae. Some species of metacercariae have also been documented to be present in the UK, although there are no records of the adults, e.g. D. truttae Lal, 1953, D. volvens (Nordmann, 1832) (see Irwin, Saville & Chubb, 1989), D. chromatophorum (Brown, 1931) and D. coregonus (see Faulkner, 1989).

The taxonomy of the genus Diplostomum is in a state of confusion in the literature. Perhaps a good example of the confusion surrounds D. spathaceum. Shigin (1977) claimed that the D. spathaceum described by Dubois (1970) as D. spathaceum spathaceum was in fact comprised of two species differing morphologically and biologically. Shigin named one species D. helveticum Dubois, 1929 and retained the name D. spathaceum (Rudolphi, 1819) for the other. Niewiadomska (1984), however, reported that the descriptions for D. helveticum corresponded to earlier descriptions for D. spathaceum (see Olsson, 1876; Krause, 1914). Therefore, she designated D. helveticum a synonym of D. spathaceum and named the other species, which Shigin had described as D. spathaceum, D. pseudospathaceum. Shigin (1986) questioned the validity of the name D. pseudospathaceum since the cercariae of this species were documented as Cercaria chromatophora Brown, 1931. Shigin (1986, 1987) proposed the name D. chromatophorum (Brown, 1931) Shigin, 1986 and designated D. pseudospathaceum a synonym of it. Niewiadomska (1989) pointed out that the cercariae described by Brown (1931) differed from the cercariae of D. pseudospathaceum, as described by herself (1986), in body size and proportions, body armature, penetration glands extent and flame cell

pattern. Since these differences were so marked, Niewiadomska declared that Cercaria chromatophora and cercaria D. pseudospathaceum must be recognised as separate species. What constitutes D. spathaceum (sensu stricto) and how British forms relate to it would appear, therefore, to be problematical.

The problems in classifying the genus Diplostomum really arise due to the fact that the metacercarial stage is the most common stage investigated because of its economic significance, because fish are readily available and because fish are more often studied than birds. However, the metacercarial stage has no obvious, good characters on which to distinguish different species. That is to say, morphologically they are very similar and, therefore, there are very few characters on which to base an identification key. Adult worms have more features than the metacercariae, since they have gonads and terminal genitalia. Again, however, even with the adults there appears to be problems in producing good keys, perhaps because natural, intraspecific variation has not been taken into account, since most descriptions are based on only one or two specimens. In the case of metacercariae, where there is plenty of material available, one of the methods of overcoming these problems would be to combine morphological features with statistical analyses in order to produce a more composite picture. There are also more sophisticated methods of comparing parasitic worms, such as the use of scanning electron microscopy, the study of larval chaetotaxy or the profiles produced by histochemical staining for enzymes.

The aim of this study was to attempt to clarify the taxonomy of the metacercarial stage of Diplostomum, since it is here that the biggest

problem exists and the most confusion lies. An initial survey was carried out to determine what metacercariae were abundant in the environment. From an economical point of view the most important metacercariae are those infecting farmed salmonids. Therefore, various farms in Scotland were sampled in order to determine the location of the metacercariae, their prevalence and intensity of infections. In the majority of farms, the fish had a mean of less than one metacercaria per eye. However, on surveying various farms it was found that farmed rainbow trout harboured lens, humoural and retinal metacercariae, but no one farm had all three. It was found that a fish farm in Perthshire had rainbow trout with a mean number of 4.2 lens metacercariae and 1.0 humoural metacercariae per eye. On the other hand, a farm on Loch Awe, near Oban had a mean number of 2.5 lens metacercariae and 3.4 retinal metacercariae per eye. Another farm in Dumfries and Galloway had a heavy infection of 25.6 humoural metacercariae per eye with no evidence of any lens or retinal metacercariae. It appeared, therefore, that at each farm the metacercariae were very site specific, suggesting that there was some inherent difference between them.

Wild fish were also examined to assess the parasite populations present in the natural environment. Loch Awe and Loch Tulla provided facilities for gill netting fish and it was interesting to compare both wild and farmed fish in Loch Awe. From both lochs brown trout (Salmo trutta L.), char (Salvelinus alpinus L.), pike (Esox lucius L.) and perch (Perca fluviatilis L.) were sampled. Only the perch harboured high numbers of Diplostomum metacercariae: these were located in the retina. Retinal metacercariae were also present in char and pike but only in a very small number of fish. Brown trout were infected with both humoural and lens metacercariae, but again very few fish were infected. On the otherhand all

the perch examined harboured retinal metacercariae with a mean intensity of 32.5 metacercariae per eye.

The study then centred around the three types of metacercariae in rainbow trout and the retinal metacercariae in perch, since these were by far the most abundant in the environment and a ready supply could be obtained. Having selected the metacercariae to examine, the problem of distinguishing them both morphologically and biologically was confronted. As noted above, most keys do not take into account natural variation, as many authors base their descriptions on only one or two specimens. Therefore, for statistical analyses carried out on morphometric data, the sample size must be relatively large to include such variation. There is also a problem with the choice of fixatives. Comparison of specimens fixed in different ways is very difficult and cannot be accurate. For the purpose of this study, therefore, it was decided that all material should be fixed in Berland's fluid, after consultation with the British Museum (Natural History) in London, since it was necessary to get the most accurate morphometric data for analysis. Berland's fluid was shown to be the best fixative for digeneans (Gibson, 1979).

For a good statistical package to compare morphological measurements staff of the Biometrics Section of the British Museum (Natural History) were consulted. Principal components analysis (PCA) is often used by taxonomists and provides the facility to look at organisms in the form of a multidimensional image, thereby enabling parasitic worms to be examined using many measurements at the same time. The package works blindly since measurements from all the different worm types are entered without notification of any grouping. Thus, any separation obtained from the

analysis is one based solely on the characters provided. From the results of the analysis it is relatively easy to decide, therefore, taking natural variation into account, whether Diplostomum metacercariae are indeed different from one another.

It is very apparent, however, that it is important to examine, not just the metacercarial stages of Diplostomum species, but also other stages in the life-cycle. Since a comprehensive key to adult diplostomes has been published by Dubois (1970) with reference to their hosts and locality, it would be sensible to look at the adult stage to determine similarities, if any, with the species described by Dubois. Also, since most taxonomic work and understanding of the genus is based upon the adult it is reasonable to assume that the greatest chance of solving the problem lies with this stage. Nevertheless, it is also important to study the cercarial stage because, as a free-living larva, it possesses more features likely to be useful as taxonomic criteria than the uniform metacercaria. Two keys have been produced for British freshwater cercariae (Blair, 1977; Nasir, 1984) and, therefore, this may help with the identification of this stage. The completion of the life-cycle in the laboratory is likely, therefore, to be a most important contribtion to the successful conclusion of this work.

Since many of the existing techniques have been so inadequate, it would be interesting to attempt to apply more sophosticated methods to this problem. Chaetotaxy has been used on the cercarial stage of a few species of Diplostomum by various authors (Richard, 1971; Shigin, 1973a, 1973b; Niewiadomska & Moczon, 1982; Eklu-Natey, Gauthey, Al-Khudri, Wuest, Vaucher & Huggel, 1985; Niewiadomska, 1986). Some comparisons could,

therefore, be made using this method. Specimens can also be compared with the use of scanning electron microscopy which allows the examinations of finite details, such as the sensory papillae and the surface tegument, of all stages in the lifecycle.

A completely new approach to Diplostomum would be to look at the isoenzyme profiles in order to determine whether or not different forms differed histochemically. This is a technique which has been very successful with the schistosomes.

In summary, it is considered that the best method to approach the problem of discerning different forms of Diplostomum metacercariae would be to combine morphometric data with statistical analyses so that natural variation is taken into account, to look at the morphology of the other important stages in the life-cycle, namely the adults and cercariae, to look at the biological differences that may be evident when completing the life-cycles and to attempt the novel approach of isoenzyme analysis. It is hoped that the combined results of all these investigations on the forms selected may help to clarify the confused situation which exists with regard to the taxonomy of Diplostomum metacercariae.

CHAPTER 2:

THE TAXONOMY OF DIPLOSTOMUM METACERCARIAE.

INTRODUCTION

The taxonomy of the genus Diplostomum von Nordmann, 1832 is somewhat confused in the literature. Almost 160 species names have been documented in as many years (Appendix 1). Sixty of these species have since been moved to other genera, however, and many of the remaining species are synonymous with one another. The actual number of valid species is, therefore, likely to be considerably less than the literature would at first indicate.

The metacercarial stage of this parasite has been found to be very difficult to identify. This is largely due to the remarkable uniformity of the morphology of this stage in the life-cycle and lack of useable characters. It is important, however, that the species of Diplostomum should be classified properly, since discrepancies may arise in the analysis of ecological data and also because several species may cause severe pathological effects among farmed fish (Ferguson & Hayford, 1941; Bauer, 1961; Shigin, 1965). It is important, therefore, to know what species are present so that, if there is an outbreak of disease, control of the correct species of molluscan or bird host can be attempted.

The adults of Diplostomum were first mentioned by Rudolphi (1819), who named them Distoma spathaceum. The metacercariae, however, were later described by von Nordmann in 1832, who took them to be adult trematodes and named two of them as Diplostomum volvens from the lens of the eye and D. clavatum from the vitreous humour. The latter species has since been recognised as the metacercaria of the genus Tylodelphys, i.e. T. clavata (von Nordmann, 1832). Streenstrup (1842) considered that the majority of

the life-cycle occurred within the eye itself and that D. volvens represented the adult form of which D. clavata and Holostomum cuticola (von Nordmann, 1832) were the 'larva' and 'pupa', respectively. Dujardin (1845) and others considered that all of these forms in the eye represented larval trematodes, which we now know to be the case. The true taxonomic position of Diplostomum was established six decades later by the Erkhardt brothers (Sudarikov, 1964), who obtained sexually mature adults in the common gull (Larus canus L.).

Keys to the metacercarial stage of Diplostomum have been produced by Sudarikov (1964; amended 1971 & 1975) (named species listed in Appendix 2) and Shigin (1976; 1986) (named species listed in Appendix 3). Until comparatively recently all Diplostomum metacercariae that infect fish in the USSR were considered to be D. spathaceum. However, a few Soviet authors (Iksanov, 1968; Razmashkin, 1969; Shigin, 1969) demonstrated that this was not the case. Shigin's key (1976) showed that a mixture of species were present with 'biological and morphological peculiarities'. In Shigin's later key (1986) 13 species of Diplostomum infecting the eyes of freshwater fishes and three infecting the brain were described.

A further three species of metacercariae infecting the eyes of freshwater fish have been described by other authors which are not mentioned by Shigin (1976; 1986) or Sudarikov (1964; 1971; 1975). These species are, D. adamsi Lester & Huizinga, 1977, D. pseudospathaceum Niewiadomska, 1984 and D. numericum Niewiadomska, 1988. D. adamsi is a North American species of Diplostomum restricted to the retina of perch (Perca flavescens). Lester & Huizinga (1977) described all stages in the life-cycle.

The nomenclatorial origin of D. pseudospathaceum is somewhat different from the others. Shigin (1977) claimed that the D. spathaceum described by Dubois (1970) as D. spathaceum spathaceum was in fact comprised of two species differing both morphologically and biologically. Shigin named one species D. helveticum Dubois, 1929 and retained the name D. spathaceum Rudolphi, 1819 for the other. Investigations carried out by Niewiadomska (1984) revealed that the descriptions for D. helveticum corresponded to earlier descriptions for D. spathaceum (see Olsson, 1876; Krause, 1914). Therefore, she designated D. helveticum a synonym of D. spathaceum and named the other species, which Shigin had described as D. spathaceum, D. pseudospathaceum.

D. numericum occurs in the vitreous humour of the eyes of Scardinius erythrophthalmus and Gymnocephalus cernuus in Poland. It is similar to D. baeri Dubois, 1937, but differs in some morphological dimensions. So far only the metacercarial stage has been described.

From this brief review it can be ascertained that the taxonomy of the metacercariae of Diplostomum spp. is somewhat confused, resulting from the fact that the categories used to separate the nominal species are inadequate. The aim of this study was to analyse the morphology of the metacercariae present in farmed rainbow trout (Salmo gairdneri) and wild perch (Perca fluviatilis), and as far as possible classify them according to previous descriptions. It was also an aim of this study to shed some light upon the characters important in distinguishing these metacercariae and to ascertain the value of different techniques.

MATERIALS AND METHODS

1. Source of Metacercariae.

Metacercariae were collected from five different sources:

1. Type 1 metacercariae were collected from the lens of farmed rainbow trout (Salmo gairdneri) from the River Earn in Perthshire.

2. Type 2 metacercariae were collected from the humour of farmed rainbow trout from Moffat Water, Dumfries.

3. Type 3 metacercariae were collected from the retina of farmed rainbow trout from Loch Awe, Argyll.

4. Type 4a metacercariae were collected from the retina of wild perch (Perca fluviatilis) netted in Loch Tulla, Glen Orchy.

5. Type 4b metacercariae were collected from the retina of wild perch netted in Loch Awe, Argyll.

Rainbow trout obtained from fish farms were hand-netted out of the ponds and cages. Perch obtained from Loch Tulla and Loch Awe were collected using a gill net.

2. Collection of Metacercariae.

After the fish were killed both eyes were removed and placed in 0.85% physiological saline. The eyes were stored at 4°C until it was time to remove the metacercariae. It was found that metacercariae would survive up to five days under these conditions but normally they were removed the same day the fish were sacrificed.

Using fine scissors the face of the eye was removed by cutting round the perimeter of the eye. Then, using fine forceps, the lens and humour were removed without disturbing the retina. The lens could then be easily separated from the humour. Each part of the eye was now ready for examination.

Metacercariae were dissected out of each part of the eye using fine dissecting needles and a fine pipette.

3. Treatment of Worms.

Light Microscope Analysis

Once the metacercariae were removed from the eye they were placed in physiological saline and washed two or three times to remove tissue debris. Worms were then fixed in Berland's fluid (19 parts glacial acetic acid. 1 part formalin), then put through three or four changes of 80% alcohol, placed in 10% glycerine in 90% alcohol and maintained on a hot plate to allow the alcohol to evaporate. Once this was completed the specimens were mounted unstained in glycerine jelly. Measurements were taken on an Olympus BHL microscope using interference phase contrast.

Scanning Electron Microscope Analysis

Once the metacercariae were removed from the eye they were placed in cacodylate buffer and washed four or five times by flushing with a pipette. They were then fixed in cacodylate buffered glutaraldehyde for 1 hour at 4°C, post-fixed in osmium tetroxide for 1 hour at 4°C, dehydrated through an acetone series, critical point dried, mounted on stubs and coated with gold. This method is used routinely at the British Museum

(Natural History) for schistosomes. Specimens were examined using an Hitachi Field Emission 800 scanning electron microscope.

4. Removal of Size Effect From Data.

Before PCA could be carried out it was necessary to remove the geometric size effect. To do this the data were logged, the mean log taken for each row and then the mean subtracted from all characters. This method of standardisation removes the effect of geometric growth without affecting allometric growth.

5. Principal Components Analysis (PCA).

PCA was carried out on data using the covariance matrix after the size effect had been removed by the method described above. Only the first three dimensions were plotted against one another, as these three axes account for the most variation in the PCA plot. Characters used in the analysis were:

1. Length (L).
2. Breadth at Brande's organ (B).
3. Length of oral sucker (LOS).
4. Breadth of oral sucker (BOS).
5. Length of ventral sucker (LVS).
6. Breadth of ventral sucker (BVS).
7. Distance from middle of ventral sucker to anterior (VS-ANT).
8. Distance between lappets (LAPP).

Additional measurements taken

1. Length of pharynx (LPH).

2. Breadth of pharynx (BPH).

3. Breadth/Length (B/L).

4. Ventral sucker to anterior/Length (VS-ANT/L).

5. Oral sucker (L x B)/Ventral sucker (L x B) (OS/VS).

6. Oral sucker (L x B)/Pharynx (L x B) (OS/PH).

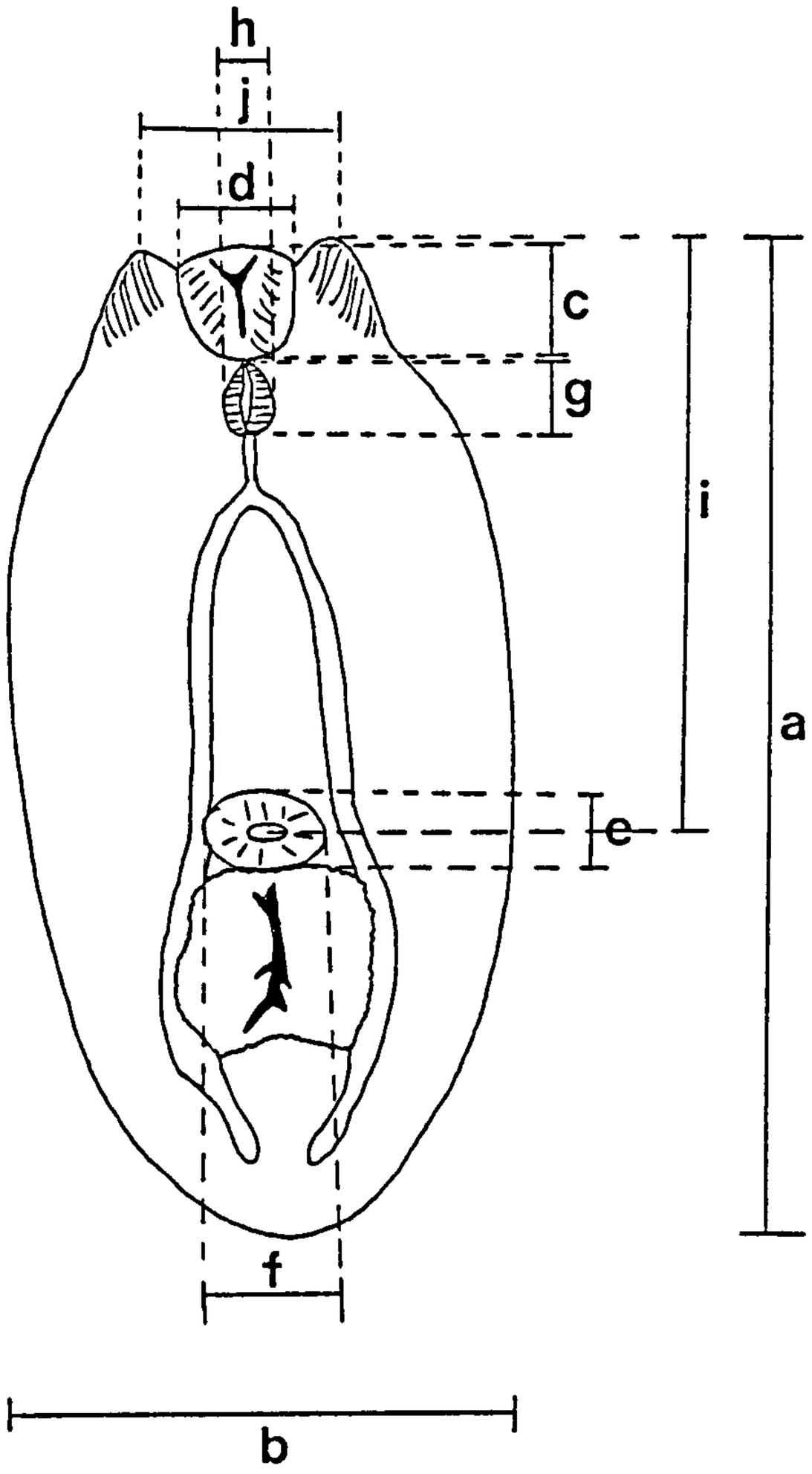
7. Square root of Breadth x Length (/B x L).

These measurements are illustrated in Fig. 2.1.

6. Histological Examination.

For histological examination whole eyes were fixed in 10% buffered formalin for at least 24 hours. After treatment in a tissue processor specimens were placed in wax mounts and 7 µm sections cut and stained in Mayers haematoxylin and eosin (H&E).

Fig. 2.1. A diagram of a Diplostomum metacercaria indicating the measurements taken.



a. L, b. B, c. LOS, d. BOS, e. LVS, f. BVS,

g. LPH, h. BPH, i. VS-ANT and j. LAPP.

Note: all the abbreviations are explained in Section 5.

RESULTS

1. Description of the metacercariae.

Metacercariae from five different sources were analysed using the light microscope. Morphological measurements for each type are given in Tables 2.1-2.4.

Correlation and regression analyses were carried out on all characters used in the principal components analysis (PCA) in order to determine which measurements significantly correlated with length. The results are displayed in Table 2.6. From the results it can be concluded that all characters used in the PCA positively correlate with length. Therefore, it was necessary to remove the effects of geometric size from all the data in order to obtain true separations.

Analysis of variance (ANOVA) was also carried out on specimens from the five sources in order to determine what characters were significantly different from one another. ANOVA was carried out on data with and without size effect removed for those characters used in the PCA. The results are displayed in Table 2.5. The results from 'un-standardised' data reveal geometric differences between the types, whereas results from the 'standardised' data reveal allometric differences. It is these allometric differences that are evident in the PCA.

Type 1 metacercariae

Measurements for lens of rainbow trout metacercariae are given in Table 2.1. They are medium length metacercariae but are fairly narrow, thus

having a low B/L ratio (0.513). The oral sucker measures 0.044 x 0.033 mm and the ventral sucker 0.035 x 0.043 mm. On average the two suckers are of comparable size (average ratio OS/VS is 1.007). The ventral sucker is located significantly more anteriorly than that of all the other metacercariae (VS-ANT/L ratio = 0.594). A significant feature of this type of metacercaria is the closeness of the lappets. The lappets are on average only 0.058 mm apart.

Type 2 metacercariae

Measurements for humour of rainbow trout metacercariae are given in Table 2.2. These worms are longer than those of Type 1 but are also broader. The B/L ratio is 0.549 which is significantly higher than that of Type 1. The suckers are also larger than those of Type 1 worms, the oral sucker being 0.048 x 0.039 mm and the ventral sucker 0.040 x 0.046 mm. However, the OS/VS ratio is comparable to that of Type 1 worms, i.e. the two suckers are equal in size. The ventral sucker in Type 2 worms is located more posteriorly than that of Type 1, the VS-ANT/L ratio being 0.566. The lappets of these worms are on average 0.085 mm apart.

Type 3 metacercariae

Measurements for retinal metacercariae of rainbow trout are given in Table 2.3. These worms are small in length, mean 0.312 mm, and hava a similar B/L ratio to those of Type 2 (0.545). The suckers of these worms are significantly smaller than those of all the other types. However, when standardised this feature is no longer evident. The oral sucker tends to be larger than the ventral sucker (OS/VS = 1.119). The position of the ventral sucker is comparable to that of Type 2 worms (VS-ANT/L = 0.567). The distance between the lappets of these worms is on average 0.072 mm,

which positions this metacercaria in the middle of the range between Types 1 and 2.

Type 4 metacercariae

Measurements for retinal metacercariae from wild perch are given in Tables 2.4a and 2.4b. From the 'non-standardised' measurements of these two populations of metacercariae it can be seen that they appear to be different from one another. Type 4a are the largest of all the metacercariae, whereas Type 4b are smaller than Type 1 metacercariae. Once size is accounted for by 'standardisation' of the data the two populations the difference is not so evident. This will be discussed in more detail later in this chapter. For the present Type 4a will be used to describe Type 4 metacercariae.

These metacercariae are large (mean length 0.430 mm) with a very high B/L ratio (0.610). Both suckers are large in size, although this is not so obvious when the measurements are 'standardised'. Both suckers are approximately equal in size, with a comparable OS/VS ratio to that of Types 1 and 2. The ventral sucker of these worms is positioned more posteriorly than that of all the others (VS-ANT/L = 0.533). The lappets of these worms are spread significantly further apart than those of all the others, with a mean distance of 0.095 mm between them.

TABLE 2.1. Morphological measurements (mm) of metacercariae obtained from the lens of farmed rainbow trout from the River Earn (Type 1).

Character	No.	Mean ± SD	Range
L	49	0.414±0.049	0.291-0.560
B	49	0.200±0.033	0.104-0.294
LOS	49	0.044±0.005	0.032-0.054
BOS	49	0.033±0.004	0.024-0.043
LVS	49	0.035±0.005	0.029-0.050
BVS	49	0.043±0.006	0.032-0.058
VS-ANT	49	0.216±0.025	0.180-0.284
LAPP	49	0.058±0.009	0.048-0.086
LPH	49	0.032±0.004	0.022-0.042
BPH	49	0.019±0.003	0.014-0.029
B/L	49	0.513±0.079	0.330-0.687
VS-ANT/L	49	0.594±0.024	0.542-0.641
OS/VS	49	1.007±0.233	0.453-1.544
OS/PH	49	2.552±0.593	1.463-4.184
/BxL	49	0.270±0.037	0.181-0.355

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

TABLE 2.2. Morphological measurements (mm) of metacercariae obtained from the humour of farmed rainbow trout from Moffat Water (Type 2).

Character	No.	Mean±SD	Range
L	67	0.366±0.055	0.302-0.397
B	67	0.210±0.025	0.155-0.262
LOS	67	0.048±0.004	0.030-0.056
BOS	67	0.039±0.004	0.029-0.048
LVS	67	0.040±0.004	0.029-0.049
BVS	67	0.046±0.005	0.032-0.064
VS-ANT	67	0.235±0.036	0.158-0.340
LAPP	67	0.085±0.011	0.054-0.112
LPH	67	0.035±0.005	0.027-0.053
BPH	67	0.020±0.000	0.016-0.029
B/L	67	0.549±0.079	0.347-0.704
VS-ANT/L	67	0.566±0.028	0.508-0.624
OS/VS	67	1.024±0.147	0.653-1.453
OS/PH	67	2.769±0.515	1.570-4.397
/BxL	67	0.294±0.029	0.225-0.354

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

TABLE 2.3. Morphological measurements (mm) of metacercariae obtained from the retina of farmed rainbow trout from Loch Awe (Type 3).

Character	No.	Mean±SD	Range
L	50	0.312±0.038	0.259-0.443
B	50	0.170±0.032	0.122-0.250
LOS	50	0.041±0.004	0.032-0.048
BOS	50	0.035±0.003	0.029-0.044
LVS	50	0.034±0.003	0.029-0.040
BVS	50	0.039±0.003	0.032-0.048
VS-ANT	50	0.177±0.022	0.144-0.281
LAPP	50	0.072±0.006	0.060-0.087
LPH	50	0.030±0.003	0.024-0.037
BPH	50	0.019±0.002	0.014-0.024
B/L	50	0.545±0.067	0.438-0.693
VS-ANT/L	50	0.567±0.020	0.495-0.634
OS/VS	50	1.119±0.134	0.865-1.480
OS/PH	50	2.656±0.425	2.014-3.569
/BxL	50	0.230±0.033	0.183-0.300

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

TABLE 2.4a. Morphological measurements (mm) of metacercariae obtained from the retina of perch netted in Loch Tulla (Type 4a).

Character	No.	Mean±D	Range
L	50	0.429±0.040	0.366-0.510
B	50	0.261±0.261	0.200-0.331
LOS	50	0.052±0.005	0.037-0.062
BOS	50	0.044±0.005	0.033-0.069
LVS	50	0.045±0.004	0.037-0.057
BVS	50	0.053±0.004	0.043-0.062
VS-ANT	50	0.229±0.023	0.191-0.285
LAPP	50	0.095±0.007	0.077-0.107
LPH	50	0.040±0.005	0.027-0.049
BPH	50	0.030±0.006	0.022-0.056
B/L	50	0.610±0.067	0.466-0.760
VS-ANT/L	50	0.532±0.026	0.476-0.664
OS/VS	50	0.969±0.028	0.870-1.351
OS/PH	50	1.999±0.393	1.079-3.147
/BxL	50	0.334±0.030	0.282-0.404

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

TABLE 2.4b. Morphological measurements (mm) of metacercariae obtained from the retina of perch netted in Loch Awe (Type 4b).

Character	No.	Mean±SD	Range
L	50	0.362±0.056	0.264-0.492
B	50	0.200±0.045	0.142-0.359
LOS	50	0.045±0.005	0.032-0.054
BOS	50	0.036±0.004	0.028-0.048
LVS	50	0.037±0.004	0.031-0.049
BVS	50	0.044±0.006	0.034-0.061
VS-ANT	50	0.199±0.029	0.151-0.281
LAPP	50	0.080±0.007	0.062-0.096
LPH	50	0.033±0.004	0.026-0.041
BPH	50	0.021±0.003	0.016-0.031
B/L	50	0.555±0.107	0.341-0.853
VS-ANT/L	50	0.554±0.059	0.397-0.889
OS/VS	50	1.020±0.199	0.567-1.554
OS/PH	50	1.870±0.199	0.990-2.569
/BxL	50	0.268±0.043	0.200-0.389

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

TABLE 2.5. Results of analysis of variance on metacercariael data.

Character	Type 1	Type 2	Type 3	Type 4a	Type 4b
L	0.414 b	0.366 c	0.312 a	0.429 d	0.362 b
L(st)	1.494 c	1.482 c	1.395 a	1.425 b	1.435 b
B	0.200 b	0.210 b	0.170 a	0.261 b	0.200 a
B(st)	0.807 ab	0.885 c	0.781 a	0.922 d	0.829 b
LOS	0.044 b	0.048 c	0.041 a	0.052 d	0.045 b
LOS(st)	-0.613 c	-0.659 b	-0.640 b	-0.695 a	-0.657 b
BOS	0.033 a	0.039 c	0.035 b	0.044 d	0.036 b
BOS(st)	-0.897 a	-0.885 ab	-0.776 d	-0.849 bc	-0.858 b
LVS	0.035 b	0.040 d	0.034 a	0.045 e	0.037 c
LVS(st)	-0.851 ab	-0.856 a	-0.828 bc	-0.843 ab	-0.834 b
BVS	0.043 b	0.046 c	0.039 a	0.053 d	0.044 b
BVS(st)	-0.638 c	-0.702 a	-0.694 ab	-0.673 b	-0.682 ab
VS-ANT	0.216 c	0.235 d	0.177 a	0.229 d	0.199 b
VS-ANT(st)	0.972 d	0.912 c	0.827 b	0.795 a	0.840 b
LAPP	0.058 a	0.085 d	0.072 b	0.095 e	0.080 c
LAPP(st)	-0.353 a	-0.099 b	-0.065 c	-0.081 bc	-0.072 c
LPH	0.032 b	0.035 d	0.030 a	0.040 e	0.033 c
BPH	0.019 ab	0.020 bc	0.019 a	0.030 d	0.021 c
B/L	0.513 a	0.549 b	0.545 b	0.610 c	0.555 d
VS-ANT/L	0.594 d	0.566 c	0.556 b	0.532 a	0.554 b
OS/VS	1.007 a	1.024 a	1.119 b	0.969 a	1.020 a
OS/PH	2.552 c	2.769 d	2.656 cd	1.999 b	1.870 a
/BxL	0.270 b	0.294 c	0.230 a	0.334 d	0.268 b

Note: st = standardised data. All other abbreviations are explained in Section 5 of the 'Materials and Methods'. All measurements are mean measurements. Means with the same postscript are not significantly different from one another; means with different postscripts are, $P < 0.05$. Postscripts are given in order of size.

TABLE 2.6. Results of regression and correlation analyses on the metacercarial data.

Character	Regression Equation	R-sq%	Correlation	Sig.
B	y = 0.037 + 0.452x	45.7%	0.676	P<0.01
LOS	y = 0.023 + 0.061x	42.6%	0.653	P<0.01
BOS	y = 0.017 + 0.054x	35.1%	0.592	P<0.01
LVS	y = 0.013 + 0.067x	55.2%	0.743	P<0.01
BVS	y = 0.016 + 0.077x	46.0%	0.678	P<0.01
VS-ANT	y = 0.018 + 0.517x	87.6%	0.936	P<0.01
LAPP	y = 0.018 + 0.157x	39.0%	0.625	P<0.01
LPH	y = 0.014 + 0.053x	36.2%	0.602	P<0.01
BPH	y = 0.005 + 0.042x	22.6%	0.476	NS

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

2. Principal Components Analysis (PCA).

Results of all PCA's are illustrated in Figs 2.7-2.13 and their corresponding Tables. For each PCA three graphs were plotted, although, only the graph which showed the best separation of the forms being examined is illustrated. The mixture of positive and negative loadings on all eigenvector values indicate that these are shape components (Blackith & Reyment, 1971) and, therefore, ensures that the effects of geometric size have been removed. Eigenvector values indicate how much each character contributes to each axis. Eigenvalues indicate how much each axis contributes to the overall variation. The most important differentiating characters are listed in Tables 2.7-2.13 These are represented by the highest values (ignoring sign) of eigenvectors in the most important axis.

PCA 1.

From Fig. 2.7 it can be clearly seen that Type 1 worms are separate from Type 2. The most important distinguishing characters, listed in Table 2.7, are also clearly defined. The distance between the lappets accounts for most of axis 1, since Type 1 worms have lappets which are very close together (0.058 mm) and Type 2 worms have ones which are spread fairly well apart (0.085 mm). The breadth of the worms is also a significant character. Again there is quite a marked difference between the two types. Type 1 worms have a low B/L ratio as they are long and slender, whereas Type 2 worms are shorter and stouter resulting in a high B/L ratio.

PCA 2.

In Fig. 2.8 it can be seen that Type 2 worms separate from Type 3 worms.

In Table 2.8 the most important separating character is the breadth of the worms. Type 3 worms have a B/L ratio of 0.545 which is in between those of Types 1 and 2 (Table 2.5). Therefore, Type 2 worms are again distinguished by their stoutness. Another important character is the position of the ventral sucker. From the 'standardised' figures for VS-ANT (Table 2.5), it can be seen that the ventral sucker of Type 2 worms is positioned more posteriorly compared to that of Type 3 worms. Furthermore, the breadth of the ventral sucker in Type 2 worms is significantly greater than that of Type 3.

PCA 3.

In Fig. 2.9 it can be seen that analysis of worms of Types 1, 2 and 3 shows how metacercariae from three different regions in the same host species separate from one another. Again separation occurs along axis 1 and from Table 2.9 this axis mainly reflects the distance between the lappets. This character alone could account for the order of separation of the three types. Type 1 worms have lappets which are very close together, Type 3 worms have lappets which are very far apart and Type 2 worms lie somewhere in the middle. The second most important character is the position of the ventral sucker. Again the order of separation is explained, since Type 1 worms have the most posteriorly positioned ventral sucker and the retinal forms have the most anteriorly positioned one. Similarly, the order holds true for the third most important character which is the breadth of the oral sucker. Type 1 worms have the smallest and Type 3 have the largest.

PCA 4.

Fig. 2.10 illustrates the result when two types of metacercariae from the

same location within the eye but from two different host species are compared to each other. Although there is some overlap, there still appears to be two different groupings. Analysis of axis 1 (Table 2.10) reveals that by far the most important separating character is the breadth of the worms. From Table 2.5 it can be seen that Type 4 worms are significantly broader than Type 3 worms. In fact Type 4 worms are the broadest of all the metacercariae. Another important separating character is the dimension of the oral sucker. From the 'standardised' data (Table 2.5) it can be seen that Type 3 worms have a significantly larger oral sucker than Type 4 worms.

PCA 5.

Fig. 2.11 shows the result when Type 2 worms are compared with Type 4 worms. Again there is some degree of overlap but there still appears to be two separate groupings. Analysis of axis 1 (Table 2.11) reveals that the most important separating character is the position of the ventral sucker. In Table 2.5 it can be seen that the ventral sucker of Type 4 worms is positioned significantly more anteriorly compared to that of Type 2 worms. The breadth and length of the worms are also very important separating characters. The significantly higher B/L ratio of Type 4 worms most likely accounts for this.

PCA 6.

Fig. 2.12 reveals the result of metacercariae from the same location within the eye and from the same host species but from two different water bodies being run together. There is a great deal of overlap of the two types (Type 4a and Type 4b). The only form of separation which can be seen is that Type 4b worms appear to have a higher variance than Type 4a.

However, there is also some separation of the two types along axis 1. In Table 2.12 the main characters of axis 1 are the breadth of the worms and the position of the ventral sucker. Analysis of Table 2.5 reveals that these two characters are significantly different in Types 4a and 4b when the data are 'standardised'. Type 4a worms are much larger in size than Type 4b. Therefore, it may be that the overall shape of the worms and the position of the ventral sucker are allometric and change with age. If this is the case then these characters may not be so useful in distinguishing the other types of metacercariae. The other possibility is that the two types of worms are indeed different from one another. This issue will be further discussed later.

PCA 7.

In Fig. 2.13 it can be seen that when the five types of worms are run together the retinal forms appear to overlap with one another. Certainly Types 1 and 2 separate from the others along axis 1. In Table 2.13 axis 1 is comprised mainly of the distance between the lappets and the position of the ventral sucker. The position of Type 1 worms can probably be accounted for by the fact that these worms have lappets which are very close together and they have the highest VS-ANT/L ratio (Table 2.5). The humoural forms also have a high VS-ANT/L ratio and this will account for their position in the separation. Type 3 and Type 4a worms appear to separate along axis 2. This axis is composed mainly of the breadth of the worms and the position of the ventral sucker. Differences in the breadth of these two types are discussed above (PCA 4). The ventral sucker of Type 4a worms is positioned more anteriorly than that of Type 3 worms. Type 4b worms overlap with both Type 3 and Type 4a worms.

TABLE 2.7. PCA 1. Metacercariae from the lens of rainbow trout vs the humour of rainbow trout.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.033	0.391	0.391
PC 2	0.020	0.239	0.630
PC 3	0.010	0.121	0.751

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	0.000	-0.467	-0.194
B	-0.356	0.440	-0.368
LOS	-0.196	0.125	0.768
BOS	0.148	0.074	0.355
LVS	-0.058	-0.063	-0.032
BVS	-0.307	0.291	-0.278
VS-ANT	-0.074	-0.647	-0.100
LAPP	0.843	0.246	-0.151

Analysis of eigenvector values for vector one reveal that the most important separating characters are;

1. Distance between lappets
2. Breadth
3. Breadth of ventral sucker

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.7. PCA 1. Metacercariae from the lens of rainbow trout vs the humour of rainbow trout.

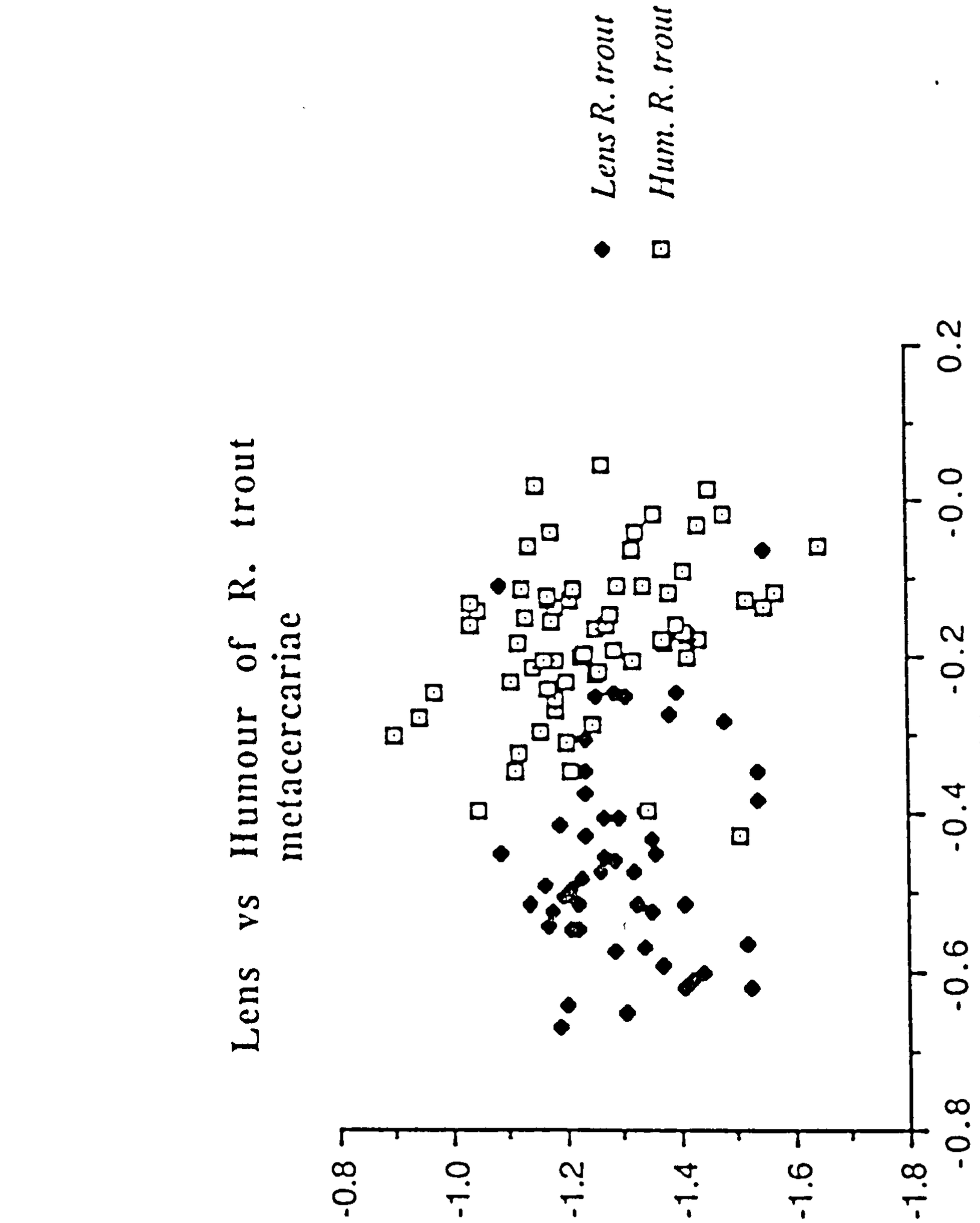


TABLE 2.8. PCA 2. Metacercariae from the humour of rainbow trout vs the retina of rainbow trout.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.021	0.370	0.370
PC 2	0.012	0.223	0.593
PC 3	0.010	0.183	0.776

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	-0.525	-0.149	-0.072
B	0.328	-0.794	-0.129
LOS	0.149	0.191	0.664
BOS	0.283	0.393	-0.448
LVS	0.039	0.280	0.269
BVS	0.259	-0.120	0.218
VS-ANT	-0.656	-0.049	-0.036
LAPP	0.124	0.247	-0.465

Analysis of eigenvector values for vector 1 reveals that the most important separating characters are;

1. Breadth
2. Breadth of oral sucker
3. Length of ventral sucker

Some separation also occurs along axis 2. The separating characters here are;

1. Distance from the ventral sucker to anterior
2. Length
3. Breadth

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.8. PCA 2. Metacercariae from the humour of rainbow trout vs the retina of rainbow trout.

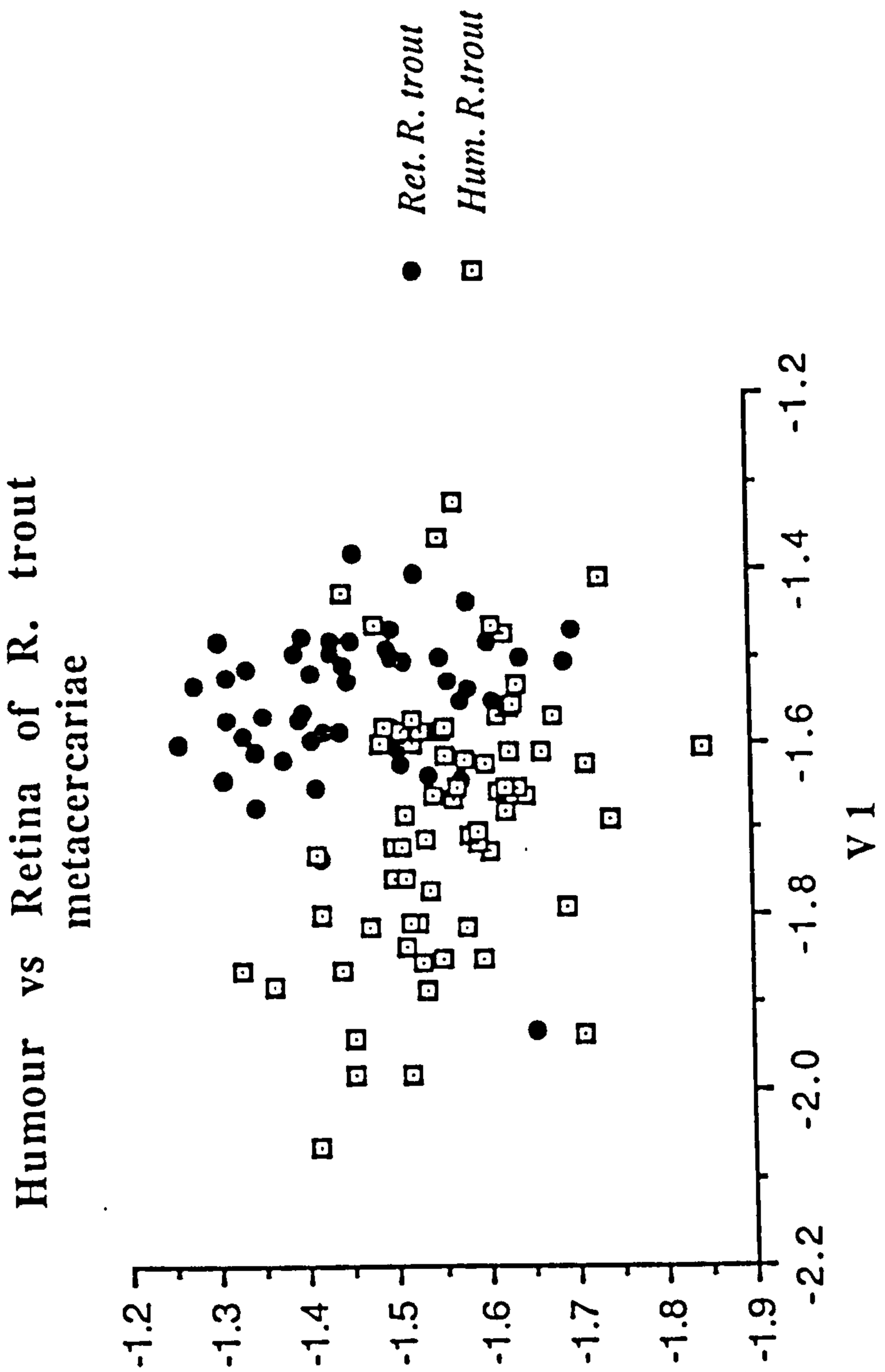


TABLE 2.9. PCA 3. Metacercariae from the lens vs the humour vs the retina of rainbow trout.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.033	0.410	0.410
PC 2	0.018	0.222	0.631
PC 3	0.010	0.128	0.760

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	-0.234	0.458	0.193
B	-0.292	-0.526	0.602
LOS	-0.078	-0.178	-0.618
BOS	0.309	-0.092	-0.204
LVS	0.019	-0.007	-0.264
BVS	-0.180	-0.354	-0.077
VS-ANT	-0.331	0.577	0.056
LAPP	0.785	0.122	0.312

Analysis of eigenvector values for vector 1 reveals that the most important separating characters are;

1. Distance between lappets
2. Distance from ventral sucker to anterior
3. Breadth of oral sucker
4. Breadth

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.9. PCA 3. Metacercariae from the lens vs the humour vs the retina of rainbow trout.

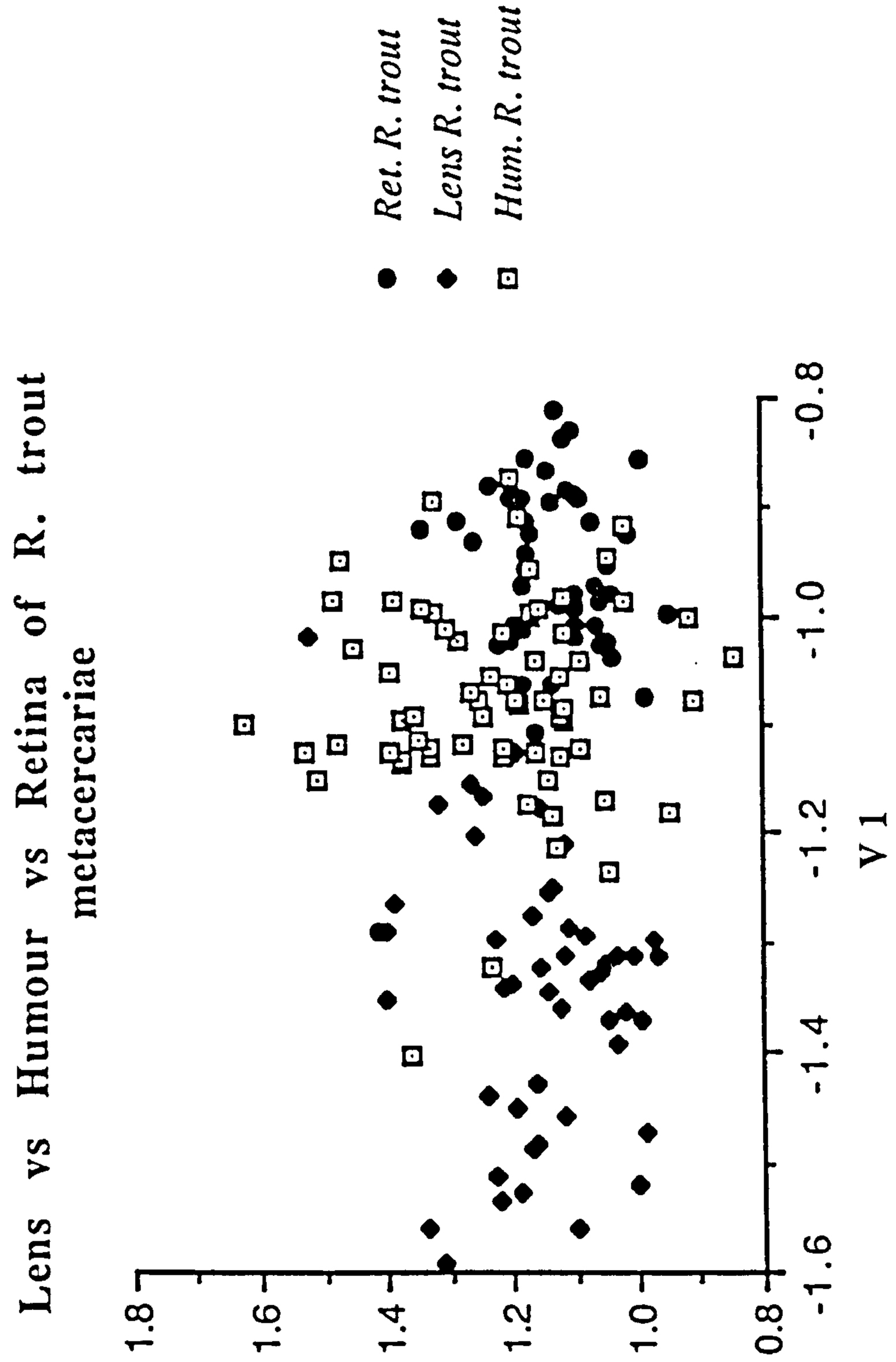


TABLE 2.10. PCA 4. Metacercariae from the retina of rainbow trout vs the retina of perch.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.018	0.384	0.384
PC 2	0.009	0.179	0.562
PC 3	0.007	0.137	0.699

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	-0.075	0.564	0.027
B	-0.874	-0.124	0.104
LOS	0.253	-0.233	0.835
BOS	0.335	-0.328	-0.277
LVS	0.114	-0.028	-0.228
BVS	-0.028	-0.328	-0.399
VS-ANT	0.178	0.615	-0.059
LAPP	0.095	-0.137	-0.003

Analysis of eigenvector values for vector 1 reveals that the most important separating characters are;

1. Breadth
2. Breadth of oral sucker
3. Length of oral sucker

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.10. PCA 4. Metacercariae from the retina of rainbow trout vs the retina of perch.

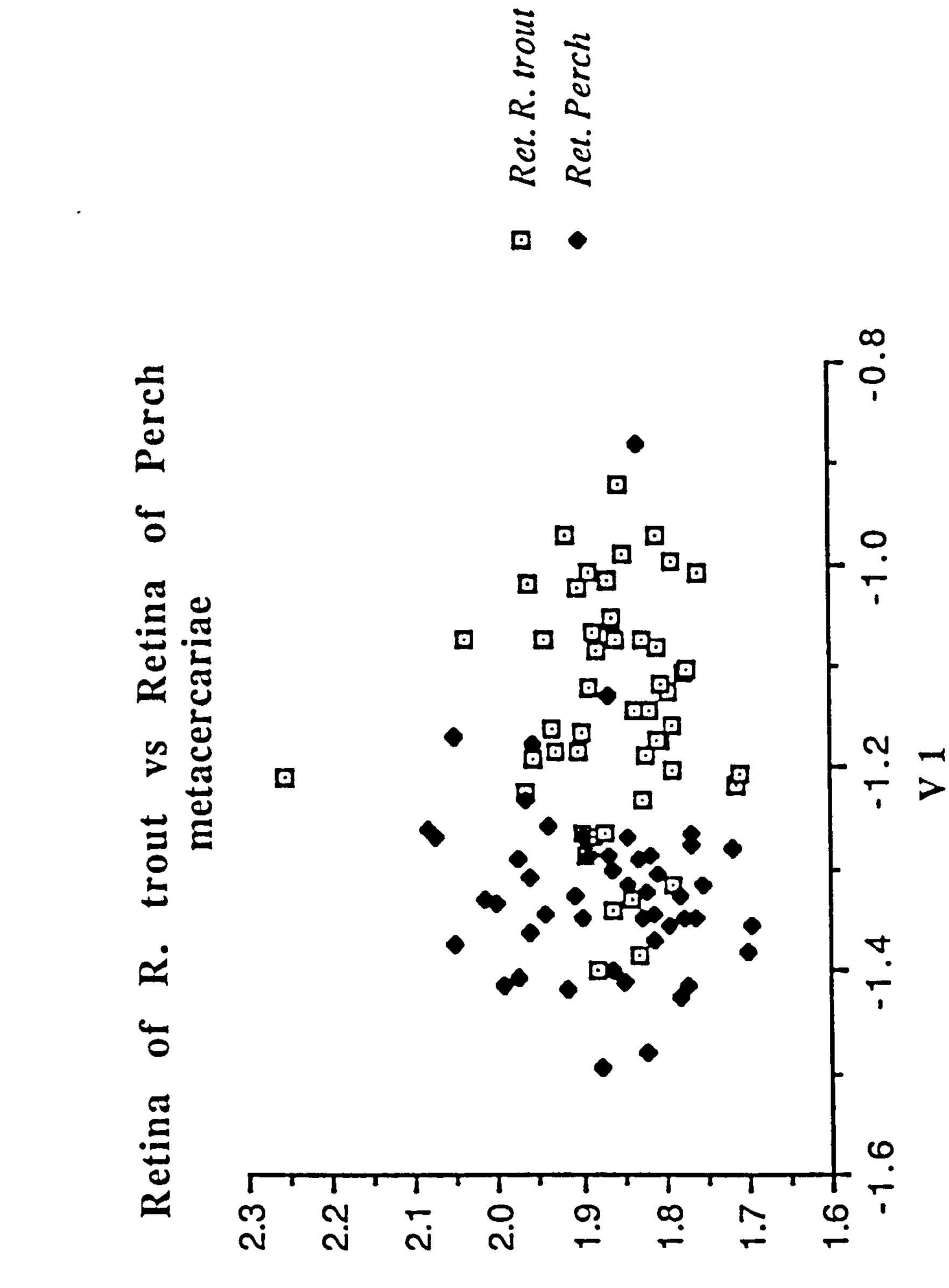


TABLE 2.11. PCA 5. Metacercariae from the humour of rainbow trout vs the retina of perch.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.026	0.440	0.440
PC 2	0.011	0.180	0.620
PC 3	0.008	0.135	0.756

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	0.400	0.015	0.264
B	-0.577	-0.106	0.498
LOS	-0.034	-0.741	-0.495
BOS	-0.121	0.480	-0.615
LVS	0.018	-0.142	0.197
BVS	-0.302	0.070	-0.001
VS-ANT	0.632	-0.005	0.138
LAPP	-0.017	0.428	0.014

Analysis of eigenvector values for vector 1 reveals that the most important separating characters are;

1. Distance from ventral sucker to anterior
2. Breadth
3. Length
4. Breadth of ventral sucker

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.11. PCA 5. Metacercariae from the humour of rainbow trout vs the retina of perch.

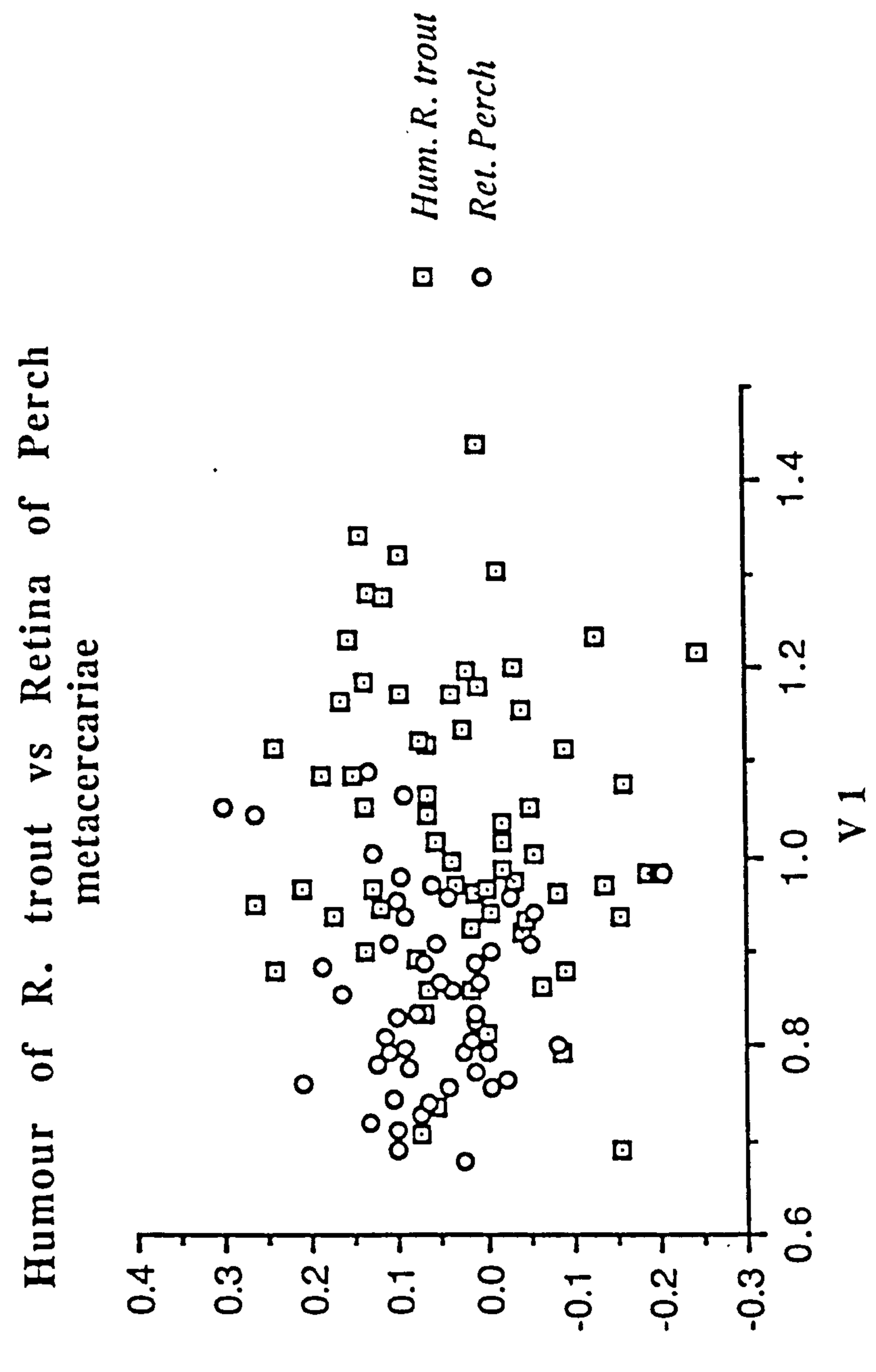


TABLE 2.12. PCA 6. Metacercariae from the retina of perch from Loch Tulla vs the retina of perch from Loch Awe.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.022	0.371	0.371
PC 2	0.011	0.191	0.561
PC 3	0.009	0.147	0.708

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	-0.190	-0.467	0.038
B	0.828	-0.220	-0.178
LOS	-0.093	-0.084	-0.624
BOS	-0.144	0.727	-0.268
LVS	-0.065	-0.004	0.508
BVS	0.208	0.270	0.497
VS-ANT	-0.436	-0.335	0.031
LAPP	-0.108	0.114	-0.004

Analysis of eigenvector values for vector 1 reveals that the most important separating characters are;

1. Breadth
2. Distance from ventral sucker to anterior
3. Breadth of ventral sucker

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.12. PCA 6. Metacercariae from the retina of perch from Loch Tulla vs the retina of perch from Loch Awe.

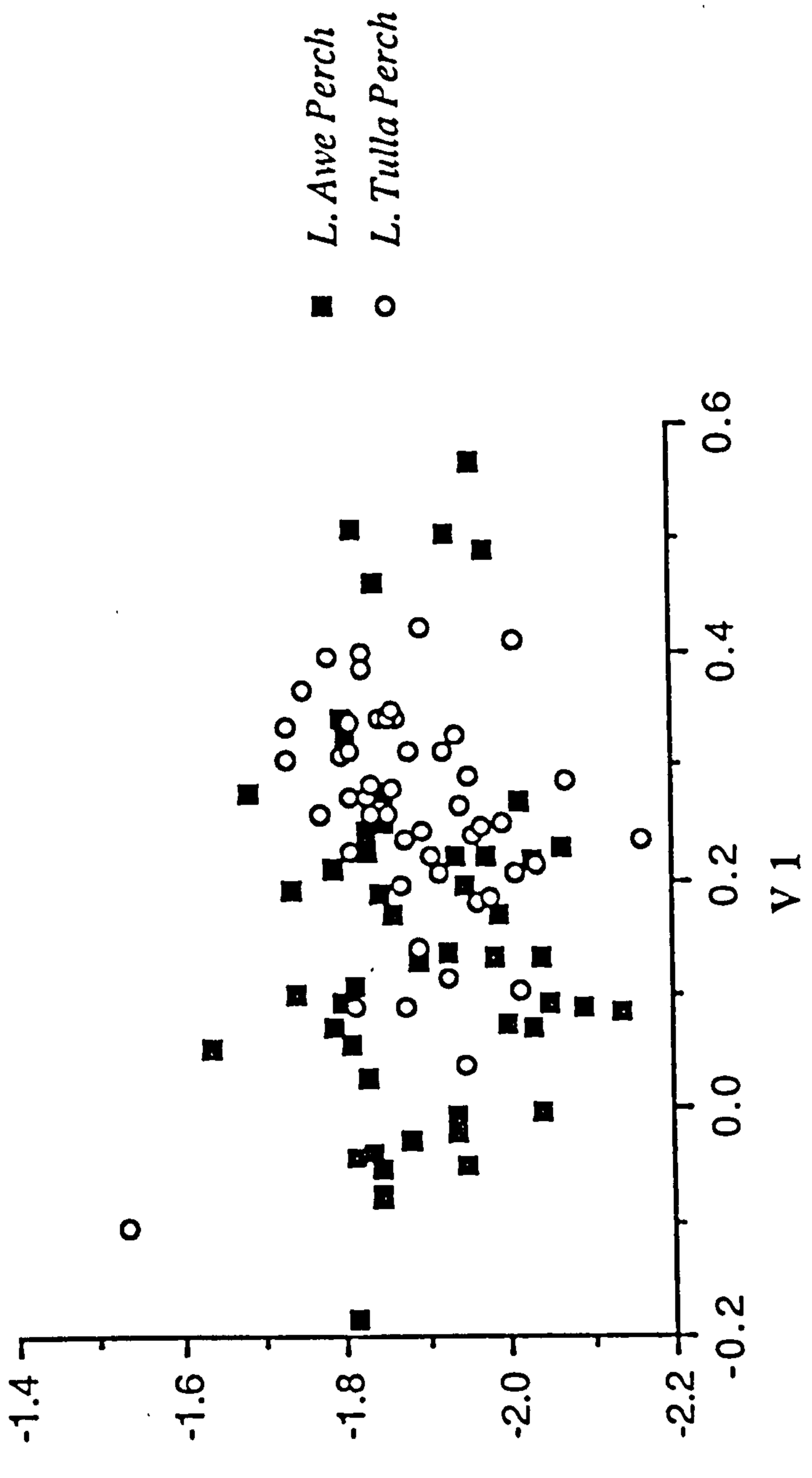


TABLE 2.13. PCA 7. Metacercariae from the lens of rainbow trout vs the humour of rainbow trout vs the retina of rainbow trout vs the retina of perch from Loch Tulla and Loch Awe.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.026	0.333	0.333
PC 2	0.020	0.262	0.595
PC 3	0.010	0.135	0.731

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	-0.299	-0.273	-0.352
B	-0.148	0.756	-0.375
LOS	-0.104	-0.017	0.574
BOS	0.320	-0.095	0.367
LVS	0.024	-0.038	0.133
BVS	-0.102	0.303	0.249
VS-ANT	-0.445	-0.475	-0.210
LAPP	0.753	-0.161	-0.385

Analysis of eigenvector values for vector 1 reveals that the most impotant separating characters are;

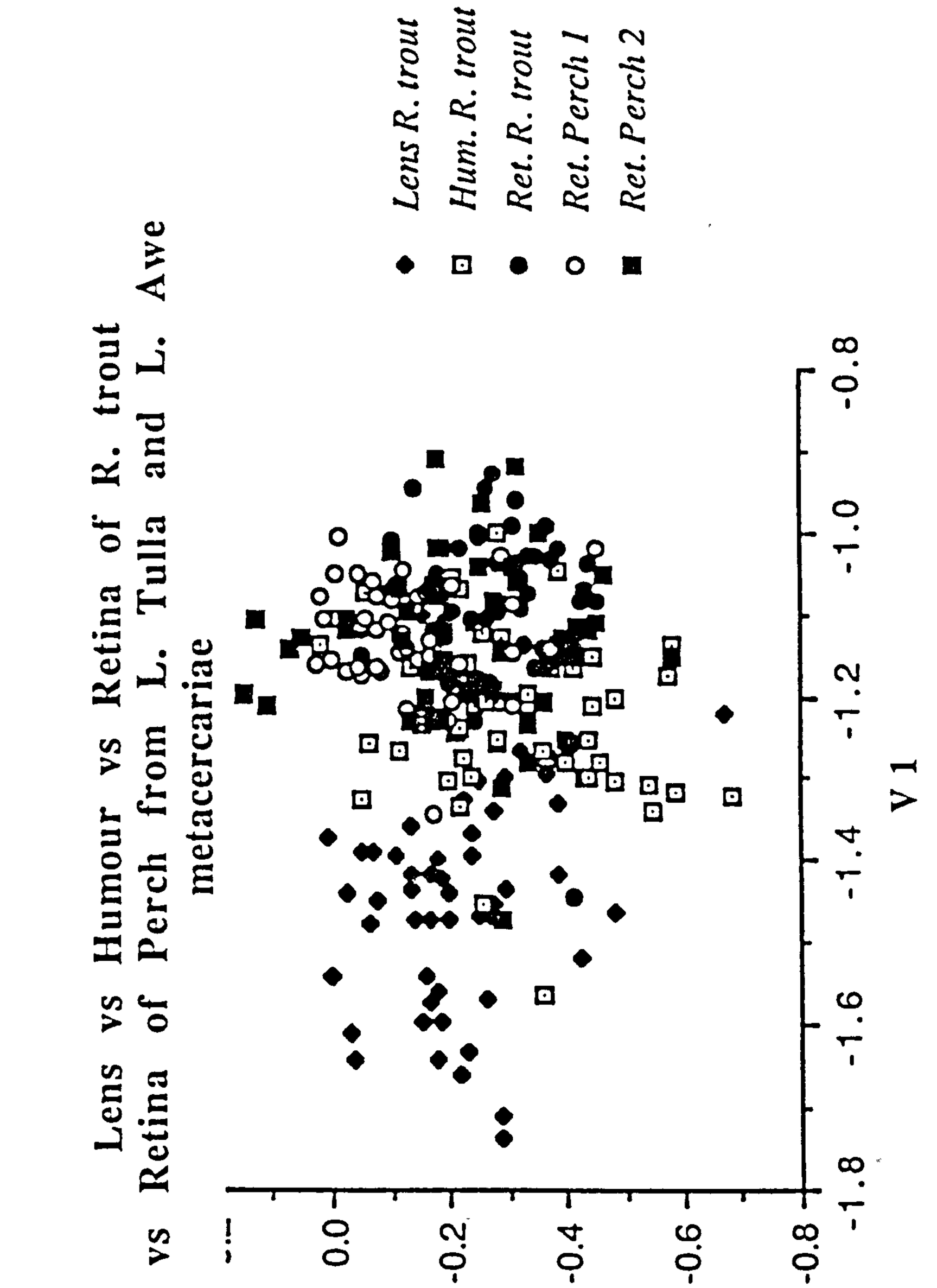
1. Distance between lappets
2. Distance from ventral sucker to anterior
3. Breadth of oral sucker
4. Length

Some separation also occurs along vector 2. Eigenvector analysis of this vector reveals that important separating characters are;

1. Breadth
2. Distance from ventral sucker to anterior
3. Breadth of ventral sucker
4. Length

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.13. PCA 7. Metacercariae from the lens of rainbow trout vs the humour of rainbow trout vs the retina of rainbow trout vs the retina of perch from Loch Tulla and Loch Awe.



3. Comparison of Metacercariae Collected in this Study with Specimens from Shigin and Niewiadomska.

In an attempt to identify the four types of metacercariae some specimens were obtained from Dr A.A. Shigin. Samples of the following species were examined:

Species	No. of specimens
1. D. parviventosum	10
2. D. chromatophorum	10
3. D. nordmanni	10
4. D. paraspathaceum	10
5. D. rutili	10
6. D. pusillum	10
7. D. pseudobaeri	10
8. D. helveticum	5
9. D. mergi	2
10. D. volvens	2
11. D. pungiti	2
12. D. spathaceum	3

Measurements were made on these specimens and subjected to PCA. Mean measurements are given in Table 2.19

The measurements for 42 specimens of D. baeri were also kindly supplied by Dr K. Niewiadomska. Mean measurements for these specimens are given in Table 2.20.

PCA's were carried out to ascertain if any of the four types of metacercariae in this study resembled these species morphologically. D. spathaceum had to be deleted from the graphs as this species separated a clear distance from all other metacercariae in all analyses.

PCA 8.

Table 2.14 shows the result of a PCA carried out on Shigin's and Niewiadomska's specimens. In Fig. 2.14 it is evident that all the species separated into small groups. This illustrates well the usefulness of the analysis. From this graph it can be seen that D. baeri specimens group very close to D. pseudobaeri, highlighting how close these species are morphologically.

PCA 9.

Table 2.15 shows the result of a PCA carried out comparing Shigin's and Niewiadomska's specimens with 25 lens of rainbow trout metacercariae collected in this study (Type 1). In Fig. 2.15 it appears that Type 1 metacercariae group on their own. They do however, overlap with D. baeri and D. paraspathaceum. D. paraspathaceum is a lens form of Diplostomum which infects many species of freshwater fish in the USSR (Shigin, 1968).

PCA 10.

Table 2.16 shows the result of a PCA carried out on Shigin's and Niewiadomska's specimens compared with 25 humour of rainbow trout metacercariae collected in this study (Type 2). From Fig. 2.16 it appears that Type 2 metacercariae again group on their own. As in the case of Type 1 metacercariae, they group closest to D. baeri and D. paraspathaceum.

PCA 11.

Table 2.17 shows the result of a PCA carried out on Shigin's and Niewiadomska's specimens compared with 25 retinal metacercariae from rainbow trout collected in this study (Type 3). Again, in Fig. 2.17, Type 3 metacercariae appear to group on their own. However, there is some overlap with D. volvens, D. rutili and D. pseudospathaceum. D. volvens is the only one of these metacercariae to be located outside the lens of the eye and, therefore, it is more likely that Type 3 metacercariae would be related to this species.

PCA 12.

Table 2.18 shows the result of a PCA carried out on Shigin's and Niewiadomska's specimens compared to 25 retinal metacercariae from perch (Type 4a). In Fig. 2.18 it appears that Type 4 metacercariae do not group with any of the named species. There is some overlap, however, with D. baeri. This is a species of Diplostomum which resides outside the lens of the eye and has been reported to infect perch (Niewiadomska, 1988).

TABLE 2.14. PCA 8. Shigin's vs Niewiadomska's specimens.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.043	0.331	0.331
PC 2	0.027	0.211	0.542
PC 3	0.023	0.179	0.721

Eigenvectors

Variable	PC 1	PC 2	PC 3
L	0.364	-0.040	-0.035
B	-0.198	0.869	-0.252
LOS	-0.116	0.051	0.344
BOS	-0.076	0.080	0.058
LVS	-0.009	-0.066	0.132
BVS	-0.083	-0.039	0.261
LPH	-0.137	-0.228	0.101
BPH	-0.091	-0.326	-0.834
VS-ANT	0.811	0.062	0.024

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.14. Shigin's vs Niewiadomska's specimens.

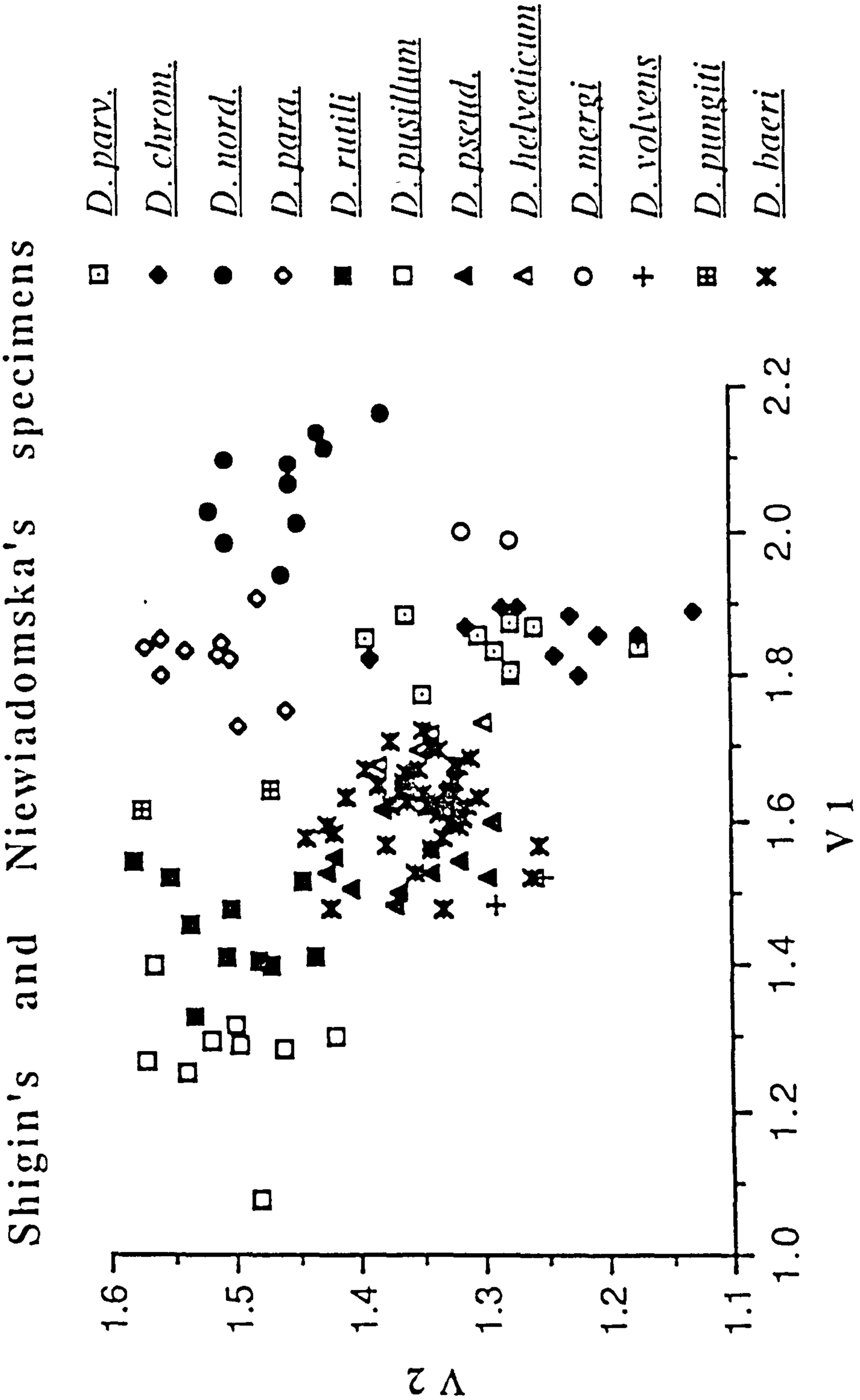


TABLE 2.15. PCA 9. Shigin's vs Niewiadomska's specimens vs metacercariae from the lens of rainbow trout.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.034	0.442	0.442
PC 2	0.020	0.255	0.697
PC 3	0.011	0.143	0.841

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	0.333	0.096	0.034
B	-0.558	0.723	0.099
LOS	-0.225	-0.217	-0.570
BOS	-0.115	-0.325	0.716
LVS	-0.014	-0.334	0.142
BVS	-0.127	-0.289	-0.357
VS-ANT	0.706	0.346	-0.064

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.15. PCA 9. Shigin's vs Niewiadomska's specimens vs metacercariae from the lens of rainbow trout.

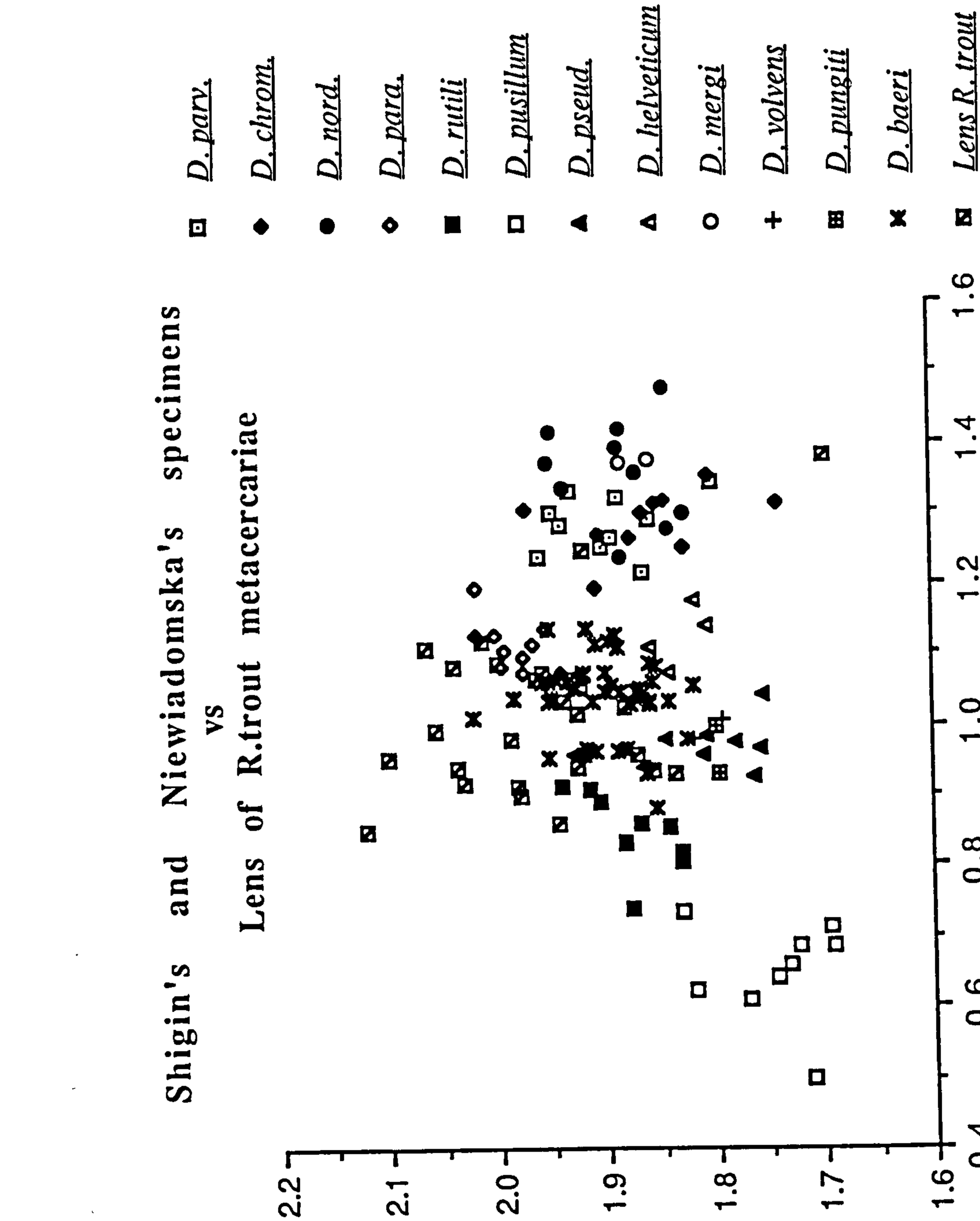


TABLE 2.16. PCA 10. Shigin's vs Niewiadomska's specimens vs metacercariae from the humour of rainbow trout.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.035	0.488	0.488
PC 2	0.019	0.271	0.759
PC 3	0.007	0.102	0.861

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	0.366	0.092	-0.130
B	-0.499	0.772	0.017
LOS	-0.210	-0.252	-0.493
BOS	-0.147	-0.247	0.793
LVS	-0.052	-0.324	0.103
BVS	-0.165	-0.308	-0.315
VS-ANT	0.737	0.267	0.025

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.16. PCA 10. Shigin's vs Niewiadomska's specimens vs metacercariae from the humour of rainbow trout.

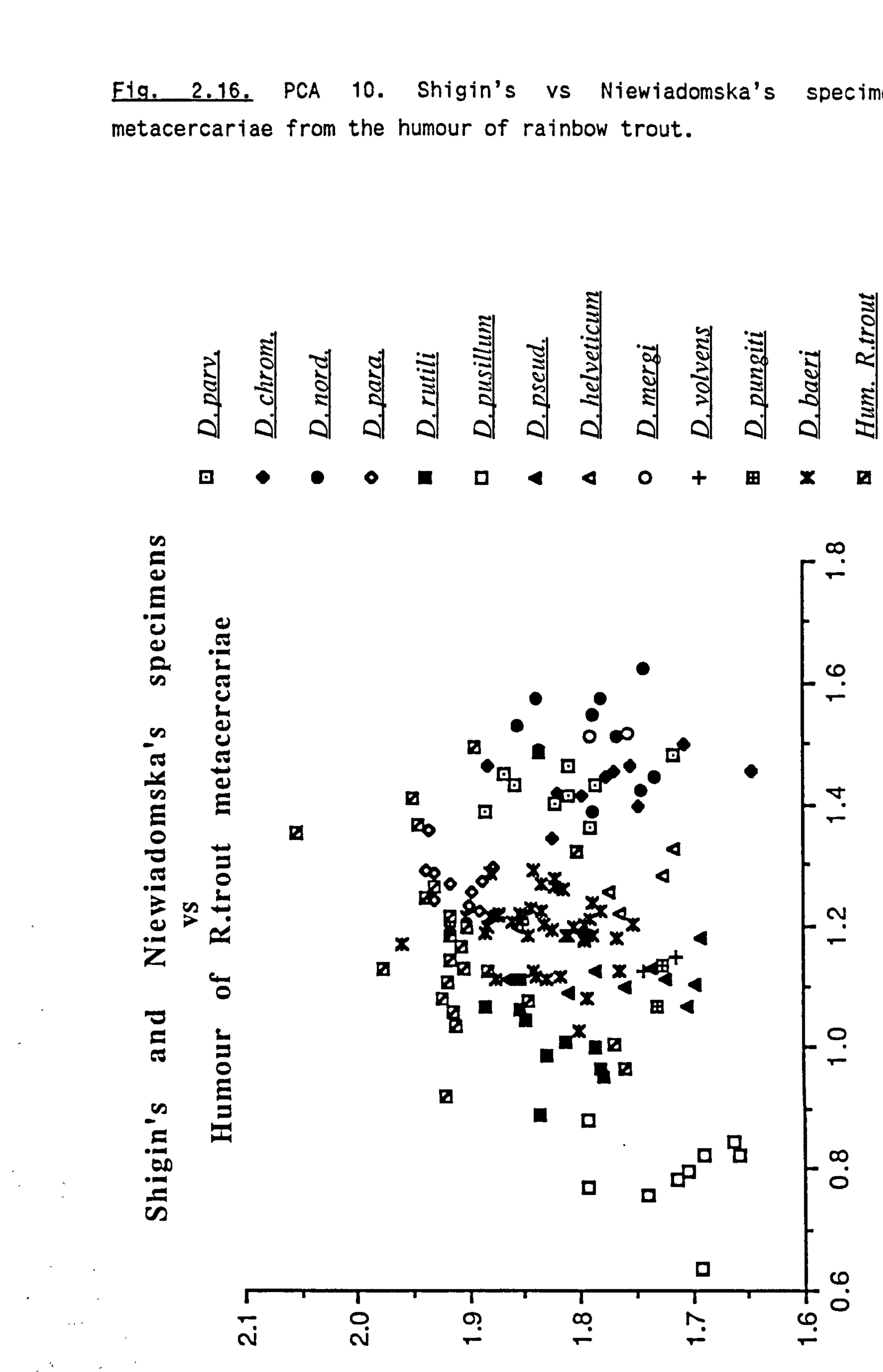


TABLE 2.17. PCA 11. Shigin's vs Niewiadomska's specimens vs metacercariae from the retina of rainbow trout.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.034	0.492	0.492
PC 2	0.020	0.292	0.784
PC 3	0.005	0.076	0.859

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	0.358	0.069	-0.032
B	-0.424	0.814	-0.062
LOS	-0.263	-0.329	-0.649
BOS	-0.174	-0.161	0.714
LVS	-0.082	-0.286	0.216
BVS	-0.163	-0.289	-0.105
VS-ANT	0.748	0.181	-0.081

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.17. PCA 11. Shigin's vs Niewiadomska's specimens vs metacercariae from the retina of rainbow trout.

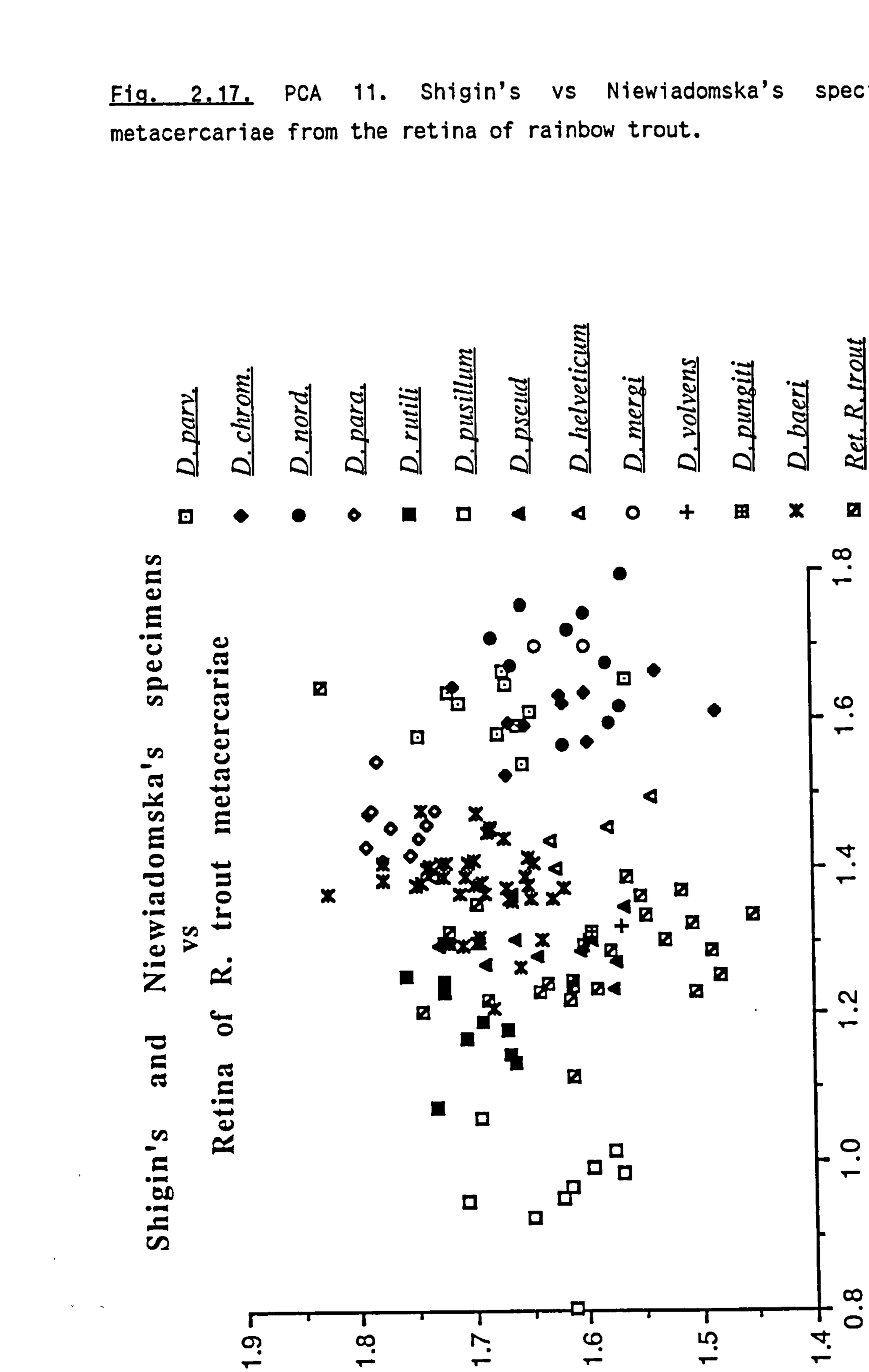


TABLE 2.18. PCA 12. Shigin's vs Niewiadomska's specimens vs metacercariae from the retina of perch.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	0.038	0.522	0.522
PC 2	0.019	0.265	0.786
PC 3	0.006	0.080	0.867

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
L	0.314	0.125	-0.149
B	-0.596	0.704	0.012
LOS	-0.185	-0.321	-0.483
BOS	-0.082	-0.258	0.836
LVS	-0.015	-0.303	-0.009
BVS	-0.134	-0.310	-0.213
VS-ANT	0.698	0.364	0.006

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

Fig. 2.18. PCA 12. Shigin's vs Niewiadomska's specimens vs metacercariae from the retina of perch.

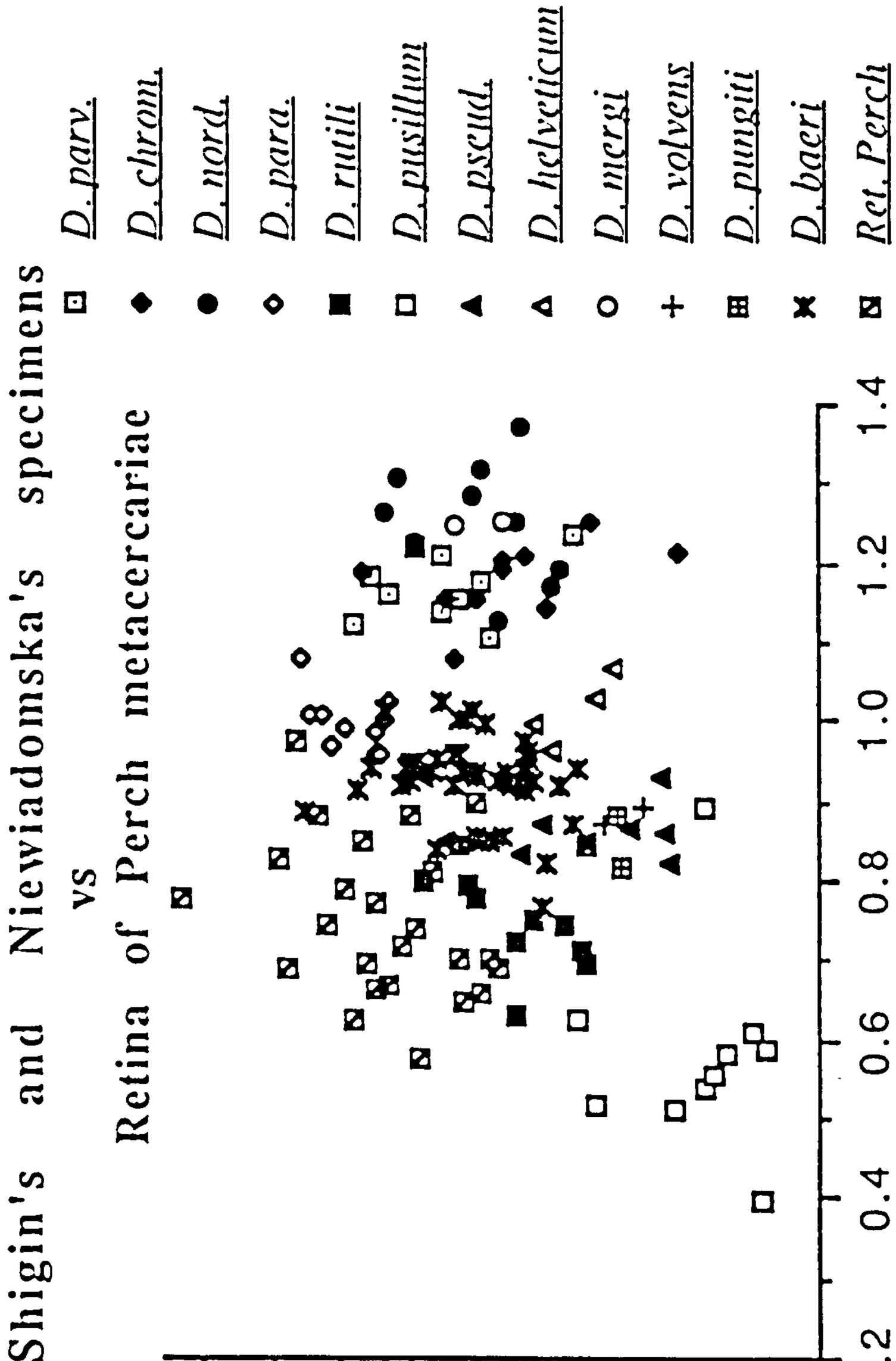


TABLE 2.19. Mean measurements (mm) ± SD for specimens obtained from Dr. A.A. Shigin.

Character	D. parviventosum (n=10)	D. chromatophorum (n=10)	D. nordmanni (n=10)
L	0.349±0.022	0.330±0.012	0.404±0.020
B	0.144±0.009	0.134±0.009	0.155±0.010
LOS	0.032±0.002	0.035±0.002	0.043±0.002
BOS	0.035±0.002	0.034±0.002	0.038±0.002
LVS	0.033±0.001	0.032±0.001	0.037±0.027
BVS	0.036±0.001	0.035±0.001	0.041±0.003
LPH	0.022±0.001	0.023±0.002	0.025±0.002
BPH	0.019±0.002	0.020±0.002	0.015±0.001
VS-ANT	0.217±0.007	0.215±0.008	0.274±0.014
LAPP	0.074±0.007	0.067±0.004	0.064±0.003

Character	D. paraspathaceum (n=10)	D. rutili (n=10)	D. pusillum (n=10)
L	0.395±0.047	0.376±0.018	0.353±0.016
B	0.209±0.028	0.222±0.028	0.224±0.038
LOS	0.045±0.005	0.048±0.002	0.053±0.003
BOS	0.044±0.008	0.044±0.002	0.051±0.002
LVS	0.040±0.005	0.041±0.002	0.046±0.003
BVS	0.044±0.004	0.048±0.002	0.052±0.003
LPH	0.027±0.002	0.029±0.002	0.032±0.001
BPH	0.023±0.003	0.019±0.002	0.024±0.001
VS-ANT	0.259±0.029	0.203±0.014	0.173±0.013
LAPP	0.075±0.011	0.116±0.010	0.120±0.007

Character	D. pseudobaeri (n=5)	D. helveticum (n=2)	D. mergi (n=2)
L	0.323±0.015	0.359±0.015	0.510±0.007
B	0.164±0.009	0.171±0.012	0.184±0.000
LOS	0.039±0.002	0.041±0.004	0.041±0.002
BOS	0.037±0.001	0.036±0.003	0.040±0.001
LVS	0.035±0.002	0.038±0.003	0.051±0.002
BVS	0.038±0.001	0.047±0.003	0.056±0.002
LPH	0.025±0.002	0.029±0.001	0.027±0.000
BPH	0.019±0.002	0.019±0.002	0.025±0.000
VS-ANT	0.176±0.008	0.221±0.009	0.303±0.005
LAPP	0.082±0.003	0.065±0.006	0.075±0.006

Character	D. volvens (n=2)	D. pungiti (n=2)	D. spathaceum (n=3)
L	0.389±0.016	0.457±0.010	0.393±0.003
B	0.189±0.000	0.252±0.007	0.393±0.003
LOS	0.048±0.001	0.057±0.000	0.039±0.002
BOS	0.037±0.000	0.051±0.001	0.040±0.002
LVS	0.043±0.001	0.059±0.000	0.035±0.002
BVS	0.051±0.002	0.066±0.000	0.037±0.001
LPH	0.032±0.000	0.035±0.004	0.022±0.000
BPH	0.024±0.002	0.022±0.001	0.020±0.000
VS-ANT	0.208±0.005	0.273±0.008	0.257±0.000
LAPP	0.085±0.001	0.100±0.002	0.067±0.005

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

TABLE 2.20. Mean measurements (mm) ± SD for 42 D. baeri specimens supplied by Dr K. Niewiadomska.

Character	D. baeri
L	0.476±0.027
B	0.229±0.014
LOS	0.052±0.004
BOS	0.047±0.003
LVS	0.047±0.004
BVS	0.051±0.003
LPH	0.036±0.004
BPH	0.028±0.003
VS-ANT	0.252±0.015

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'.

4. Identification Using Shigin's Key (1986).

Identification of the four types of metacercariae obtained in this study was not possible using specimens obtained from Dr A.A. Shigin and Dr K. Niewiadomska and subjecting them to PCA (Section 3). An attempt was made, therefore, to identify them using Shigin's key (1986).

Type 1 metacercariae

A Type 1 metacercaria is illustrated in Fig. 2.19 and Plate 2.1a. From these illustrations it is evident that the lappets are highly protruding, their extremities reaching beyond the anterior margin of the oral sucker. According to Shigin a lens metacercaria with this characteristic belongs

to the species D. rutili. However, Shigin also states that the VS-ANT/L ratio is smaller than 58%, whereas in Type 1 metacercariae this ratio has a range from 54% to 64% (Table 2.1). Furthermore, examination of collections of D. commutatum from Finland in the British Museum (Natural History), a synonym of D. rutili, revealed that the lappets protrude to a much greater extent than those of Type 1 specimens. Having decided that it is not D. rutili and continuing with the key, since the metacercariae have an ovoid body with the maximum width between the first and second third of the body and the L x B of VS = 1505, then Type 1 metacercariae key down to D. spathaceum.

Type 2 metacercariae

A Type 2 metacercaria is illustrated in Fig. 2.19 and Plate 2.1b. The average L x B of these metacercariae is 76,860 (Table 2.2). This classes them in Shigin's key as intermediate to large size. However, the average L x B of the suckers is <2000 which would class them as small metacercariae. Since the L x B is probably more important in classifying size, these metacercariae are classed as intermediate to large. In Table 2.2 it is evident that the B/L ratio ranges from 34.7% to 70.4% and the OS/VS ratio ranges from 1.57 to 4.40. These figures then place Type 2 metacercariae somewhere in between D. volvens and D. pusillum, as both ratios overlap with figures given for both of these species.

Type 3 metacercariae

A Type 3 metacercaria is illustrated in Fig. 2.21 and Plate 2.1c. In Table 2.3 it is evident that these are small metacercariae with L x B = 53,040. Furthermore, the L x B of the oral and ventral suckers is <2,000, and therefore, according to Shigin's key, these metacercariae belong to

the species D. pseudobaeri.

Type 4 metacercariae

A Type 4 metacercaria is illustrated in Fig. 2.22 and Plate 2.1d. In Table 2.4a it is evident that these metacercariae are of intermediate to large size (L x B = 111,969). Since the ranges for the ratios B/L and OS/PH overlap with D. volvens and D. pusillum in Shigin's key Type 4 metacercariae lie somewhere in between these two species. This is similar to the findings for Type 2 metacercariae.

5. Scanning Electron Microscopy (SEM).

Morphological analysis of the metacercariae was also carried out using the scanning electron microscope (SEM). Analysis revealed that a difference between the four types of metacercariae was evident in the shape of the lappets. Plate 2.2a shows an SEM of a lens metacercaria. It can be seen that the lappets are long and slender in shape. This clearly distinguishes Type 1 metacercariae from the others, for, as can be seen in Plate 2.2b-d, the other three types of metacercariae have round lappets. Plate 2.2d reveals just how much further apart the lappets of Type 4 metacercariae are as are those of Type 2 (Plate 2.2b). Type 3 metacercariae have lappets intermediary in position to those of Type 1 and Type 2.

6. Histological Analysis.

Histological analysis of the eyes of infected fish was carried out in order to determine the exact location of the metacercariae within the eye. Plate 2.3a-d shows lens, humoural and retinal metacercariae of rainbow trout and retinal metacercariae of perch. Lens metacercariae were often found to be clustered in one or two regions within the lens rather than dispersed throughout (Plate 2.3a). Humoural metacercariae, on the otherhand were found to be dispersed around one side of the eye, normally behind the lens, and not in such distinct clusters (Plate 2.3b). Both retinal metacercariae were found to be clustered in the one region. Retinal metacercariae from perch were found to be located in the pigment epithelial layer of the retina with the pigment much disturbed (Plate 2.3d). Many metacercariae collected from the retina of perch had a black pigment in their gut indicating that they were feeding on the cells containing pigment. Retinal metacercariae from rainbow trout, however, appeared to be located between the pigment epithelium and the outer limiting membrane with no disturbance evident in the pigment layer (Plate 2.3c). Few metacercariae collected from the retina of rainbow trout contained black pigment in their gut.

7. Comparison of Fixation Techniques.

A test was carried out to determine how different fixation techniques affect the morphology of metacercariae and thus contribute to the large amount of morphological variation. The fixative used in this study was Berland's fluid. No pre-treatment was used and afterwards the specimens were stored in 80% alcohol. Shigin and Niewiadomska, the leading workers

in the taxonomy of Diplostomum metacercariae, both use different techniques.

Shigin first places the metacercariae into tap-water for three hours prior to fixation. He believes that this is the only method by which immature specimens can be separated from mature specimens. Therefore, only those left alive after three hours are used. Shigin then fixes the specimens in acetic carmine, differentiates the stain with 1% HCl in 70% methanol, dehydrates through alcohols, clears in dimethylphthalate and mounts in canada balsam. Niewiadomska, on the other hand fixes her specimens in hot water.

In order to compare the different techniques, an infected rainbow trout with >100 metacercariae in the humour of one eye was sacrificed and 20 metacercariae from the humour were fixed by each method. The three batches of metacercariae were therefore, obtained from the same eye. Results are given in Table 2.21.

It is evident in Table 2.21 that metacercariae fixed by different methods do show a large amount of morphological variation. The length and breadth was not affected, but the position of the ventral sucker and the distance between the lappets were significantly different in all three fixatives. Niewiadomska's fixation method produced specimens which were larger than those fixed in Berland's fluid. This may be due to the fact that the fixation technique does not control swelling of the worms. Berland's fluid, on the other hand, incorporates formalin designed to stop swelling. Shigin's fixation method resulted in specimens being smaller than those fixed in Berland's fluid. This may be due to a more dilute solution of

acetic acid being used which may not work as quickly as Berland's fluid, hence resulting in contraction of the worms.

TABLE 2.21. Comparison of different fixation techniques using ANOVA.

Character	Shigin's technique (n=20)	Niewiadomska's technique (n=20)	Berland's fluid (n=20)
L	0.581±0.044 a	0.609±0.046 a	0.596±0.043 a
B	0.302±0.033 a	0.315±0.038 a	0.311±0.033 a
VS-ANT	0.327±0.029 a	0.371±0.028 c	0.357±0.031 b
LAPP	0.123±0.011 a	0.143±0.010 c	0.135±0.013 b

Note: The abbreviations are explained in Section 5 of the 'Materials and Methods'. Mean measurements (mm) given within rows. Means with the same postscript are not significantly different from one another ($P>0.05$). Means with different postscripts are significantly different ($P<0.05$).

Fig. 2.19. A metacercaria from the lens of rainbow trout (Type 1).

Scale-bar: 50 um.

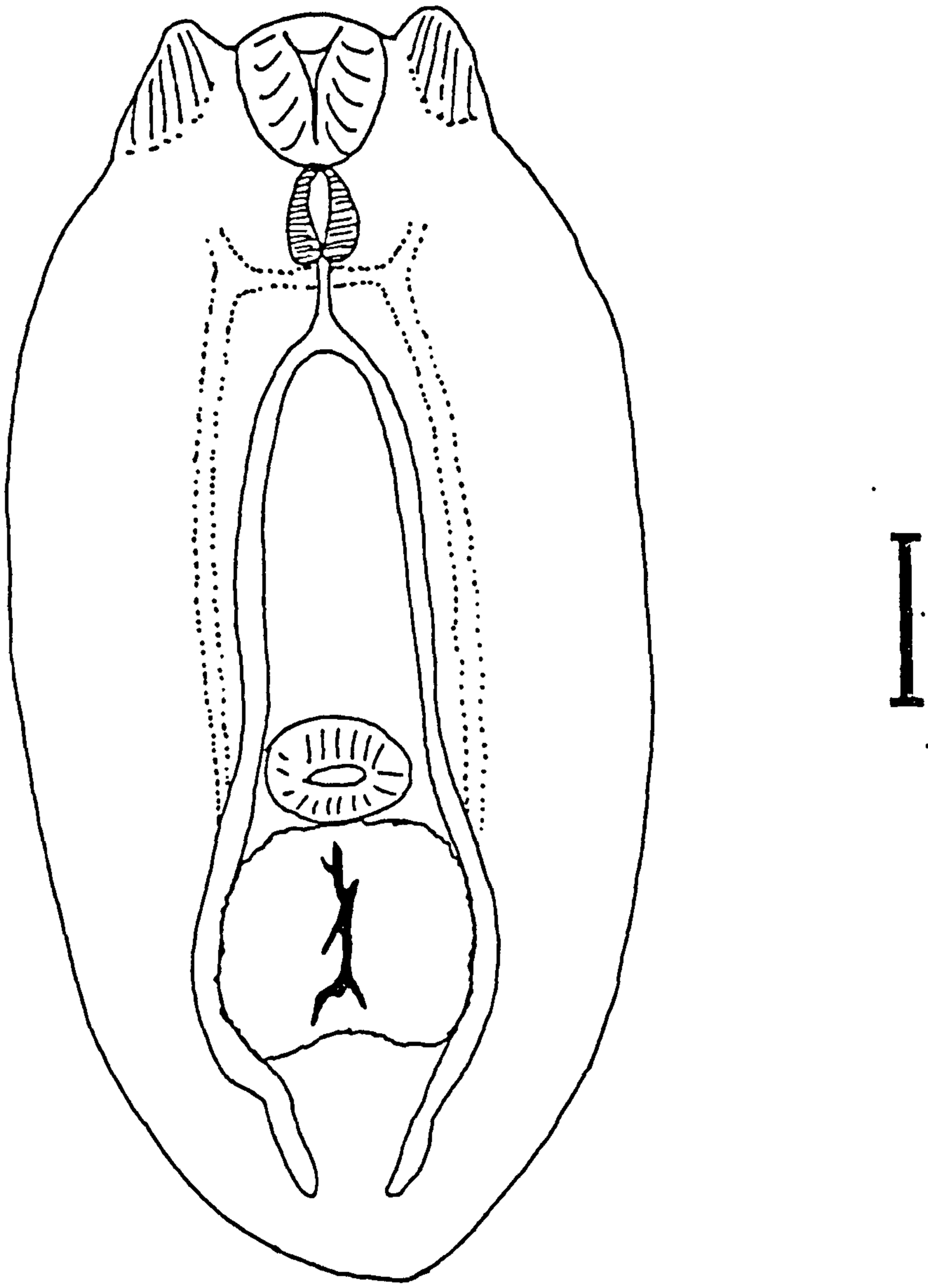


Fig. 2.20. A metacercaria from the humour of rainbow trout (Type 2).

Scale-bar: 50*um*.

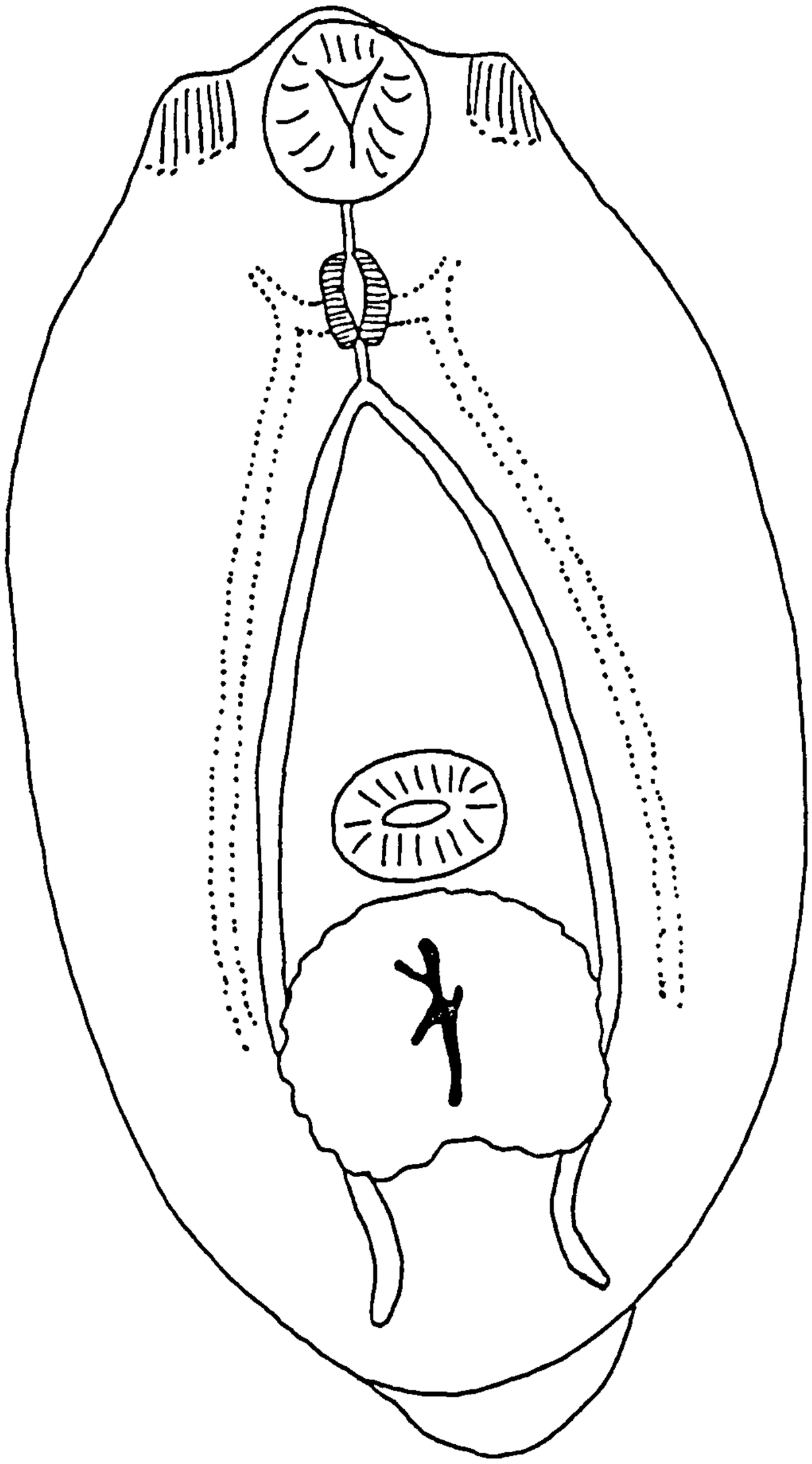


Fig. 2.21. A metacercaria from the retina of rainbow trout (Type 3). Scale-bar: 50 μm.

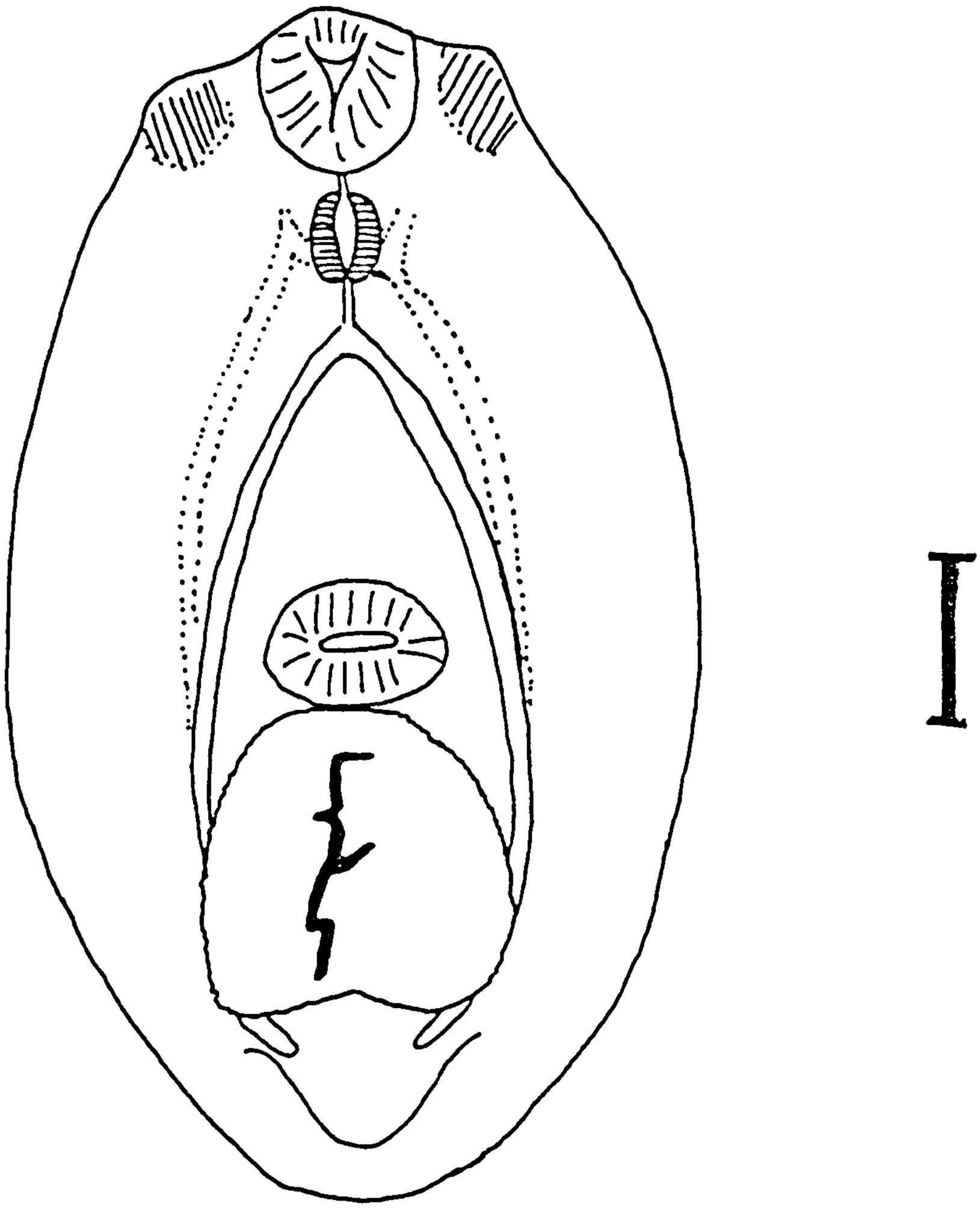


Fig. 2.22. A metacercaria from the retina of perch (Type 4).

Scale-bar: 50*um*.

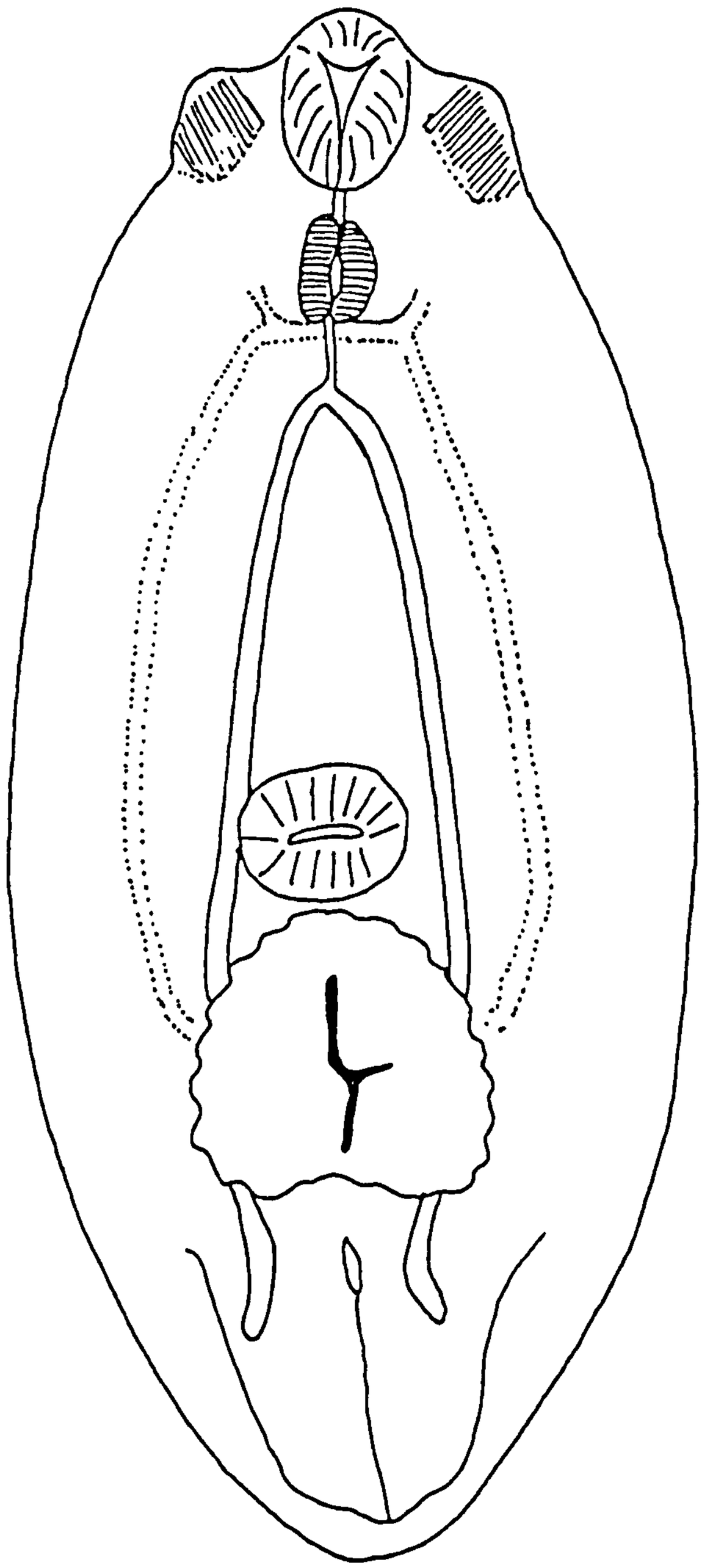
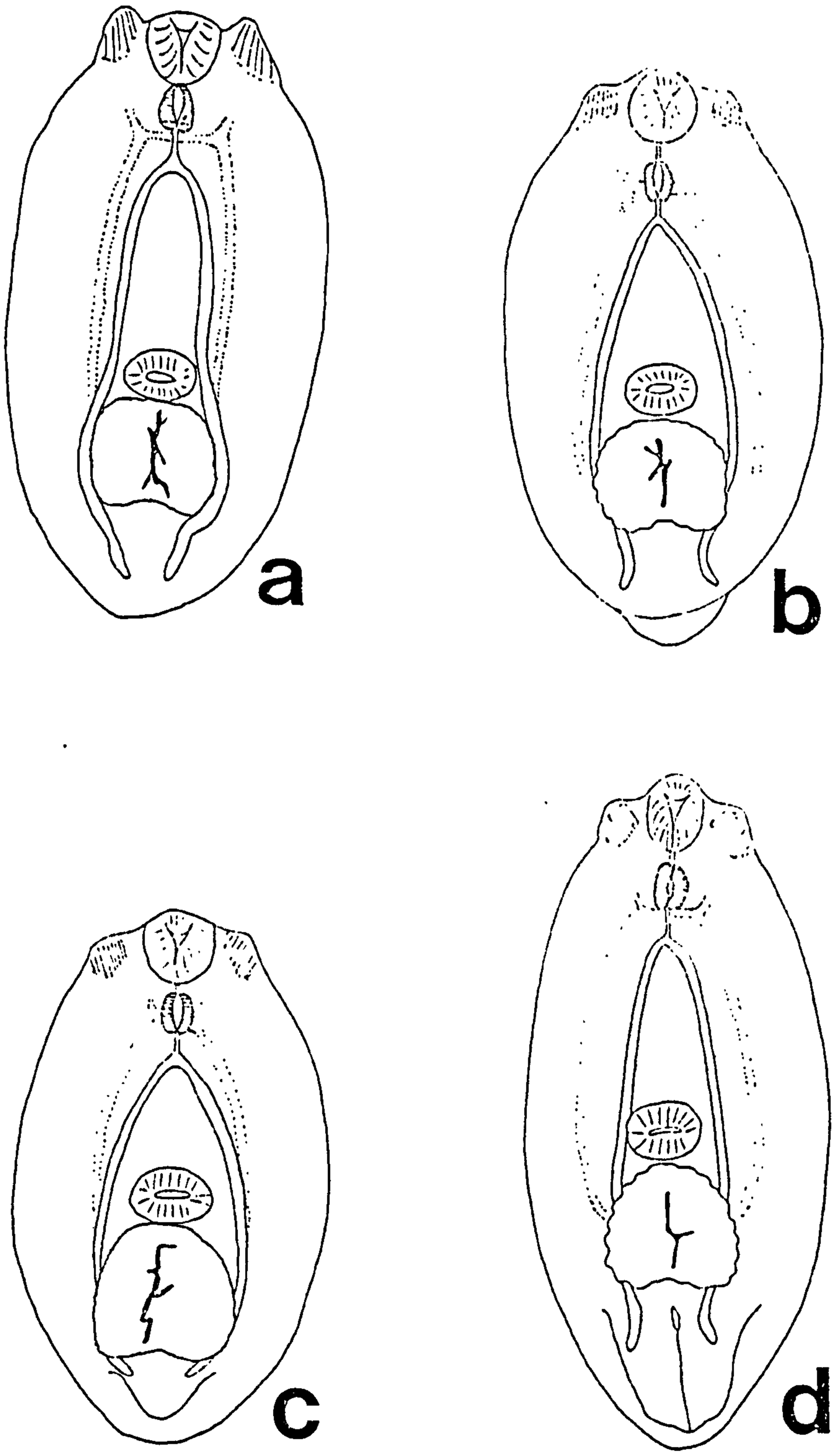


Fig. 2.23. A comparison of the four types of metacercariae.

Scale-bar: 50um.



a: Type 1 b: Type 2 c: Type 3 d: Type 4.

PLATE 2.1. Photomicrographs of the four types of metacercariae.

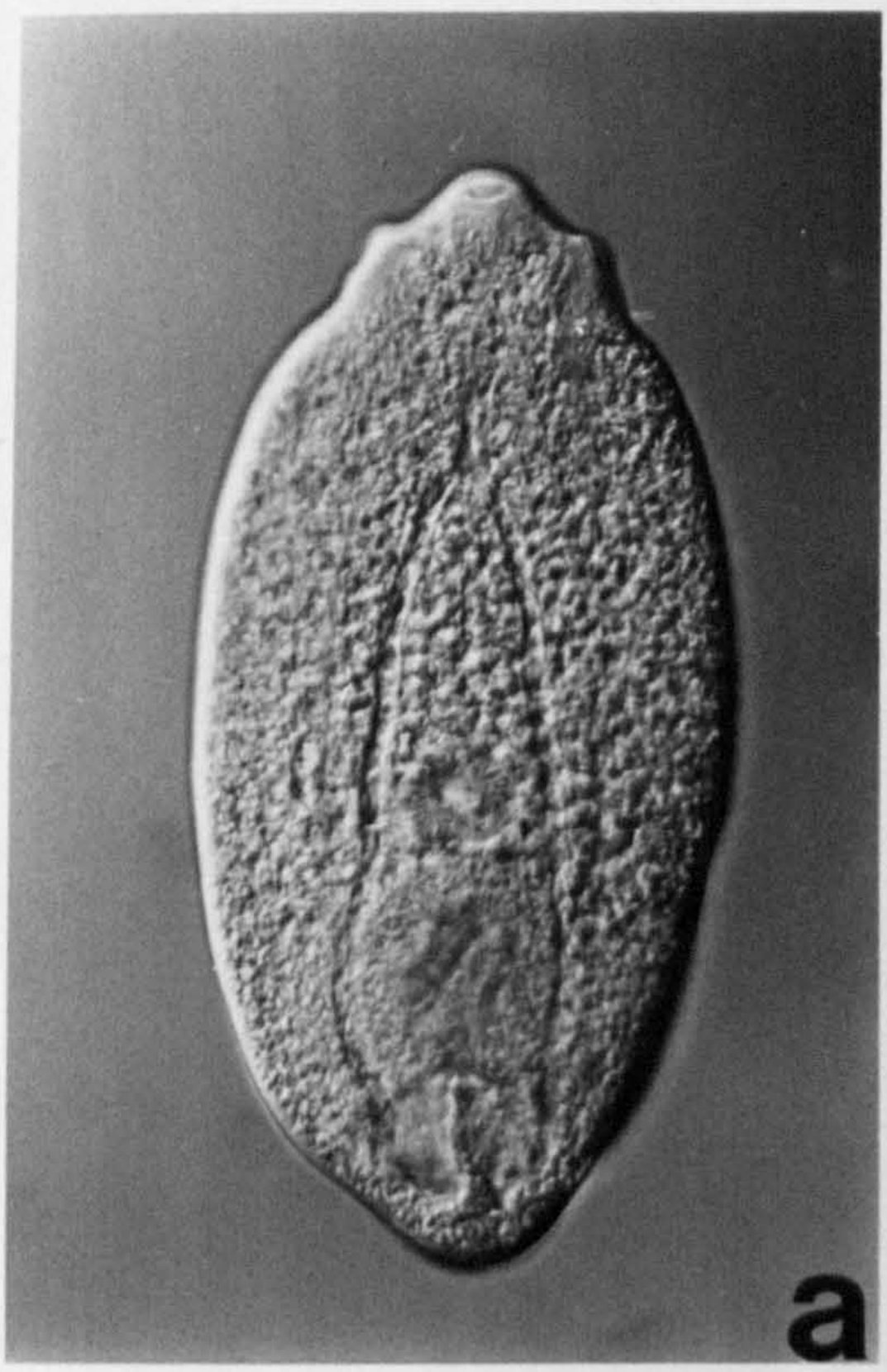
Scale-bar: 100μm.

a: Metacercaria from the lens of rainbow trout (Type 1)

b: Metacercaria from the humour of rainbow trout (Type 2)

c: Metacercariae from the retina of rainbow trout (Type 3)

d: Metacercaria from the retina of perch (Type 4)


a


b


c


d

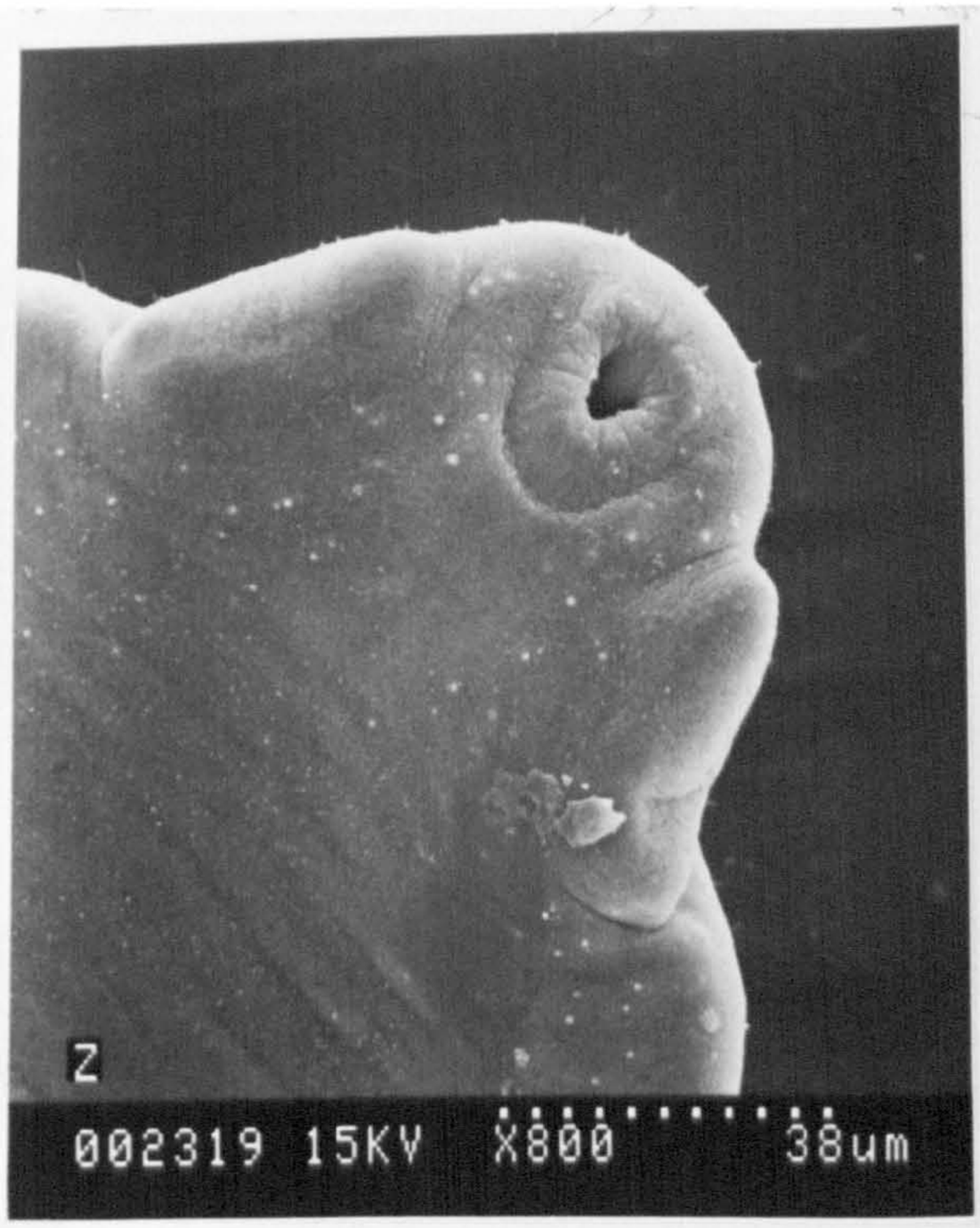
PLATE 2.2. Scanning electron micrographs of the four types of metacercariae.

a: Metacercaria from the lens of rainbow trout (Type 1)

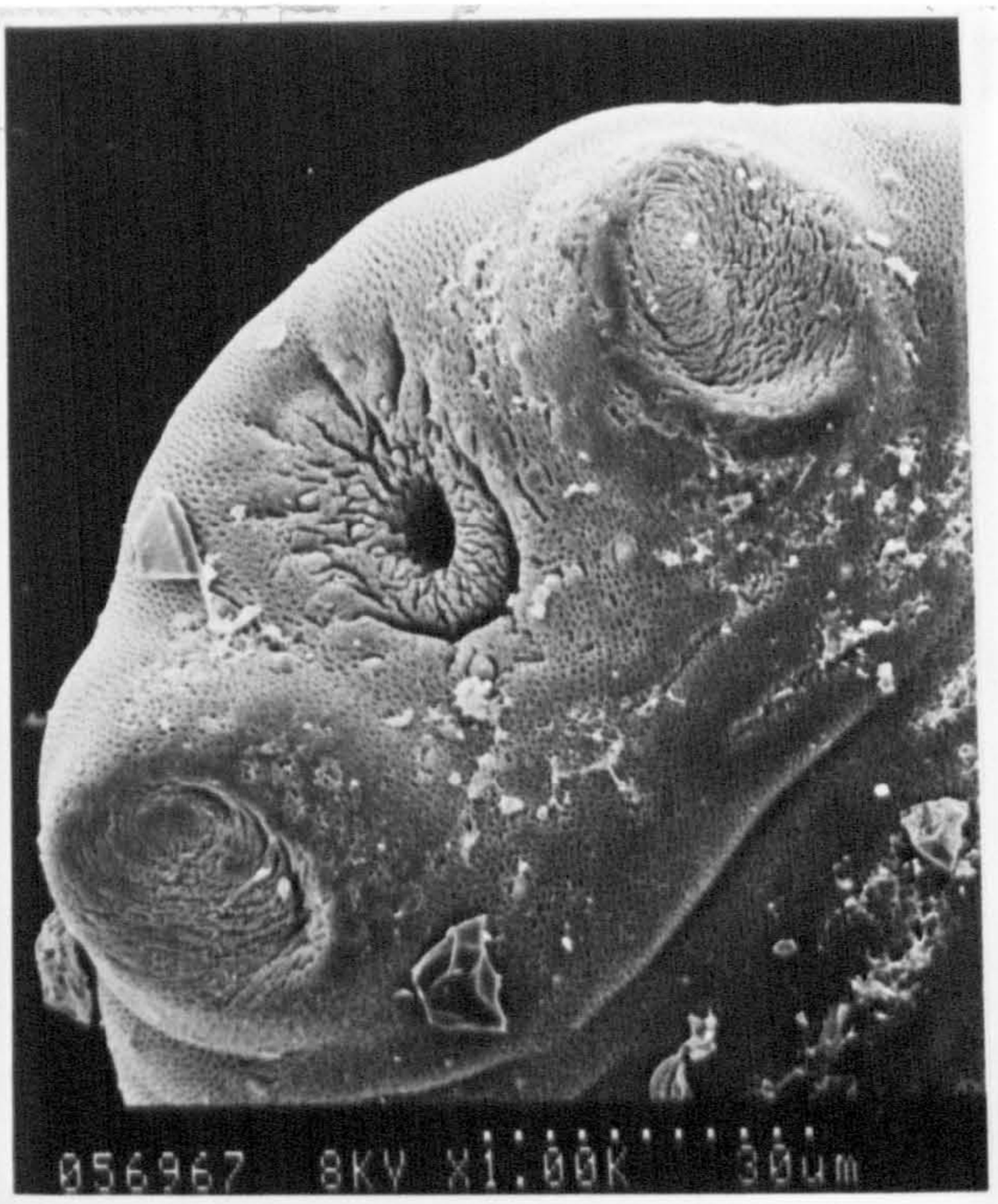
b: Metacercaria from the humour of rainbow trout (Type 2)

c: Metacercariae from the retina of rainbow trout (Type 3)

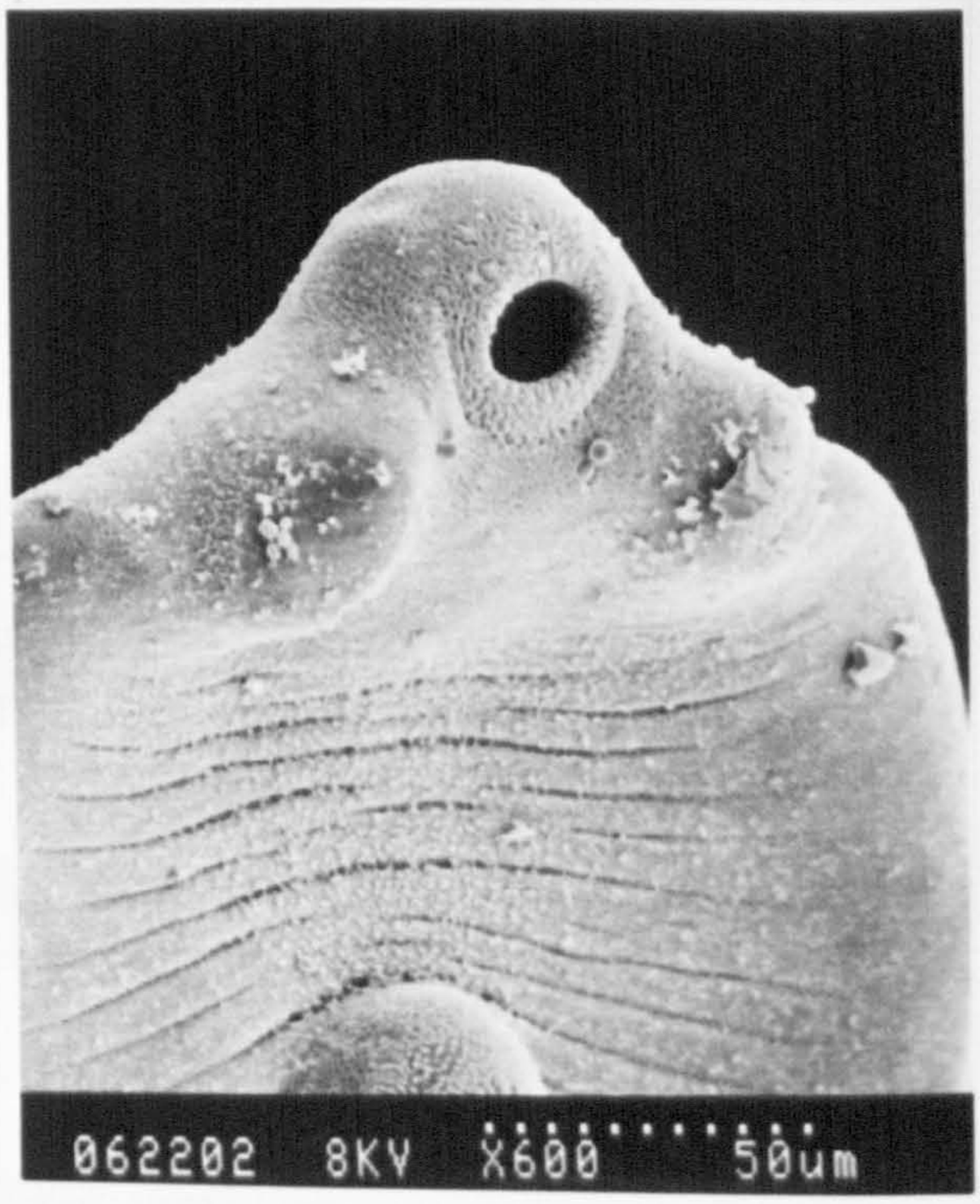
d: Metacercaria from the retina of perch (Type 4)


Z
002319 15KV X800 38um

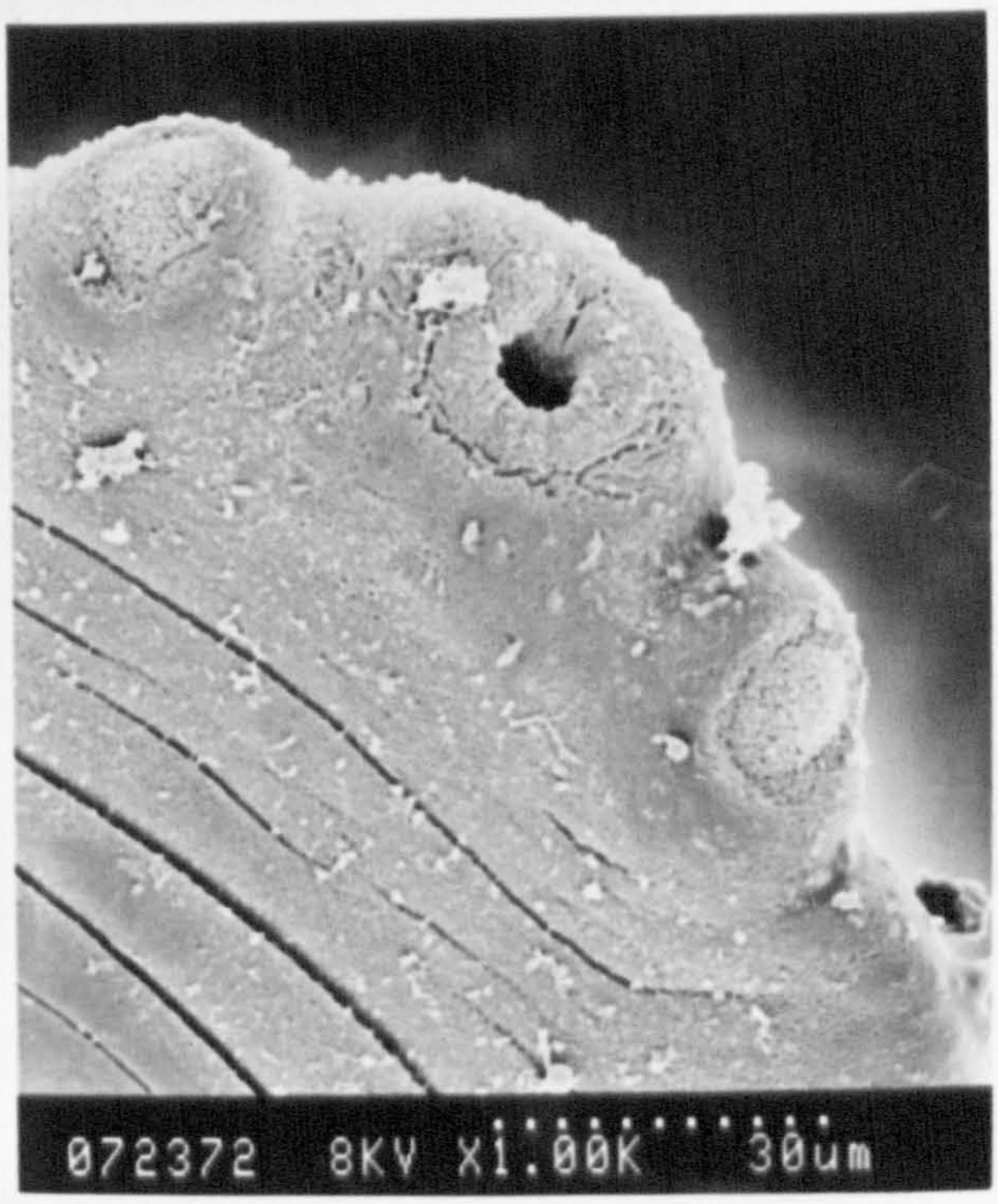
a


056967 8KV X1.00K 30um

b


062202 8KV X600 50um

c


072372 8KV X1.00K 30um

d

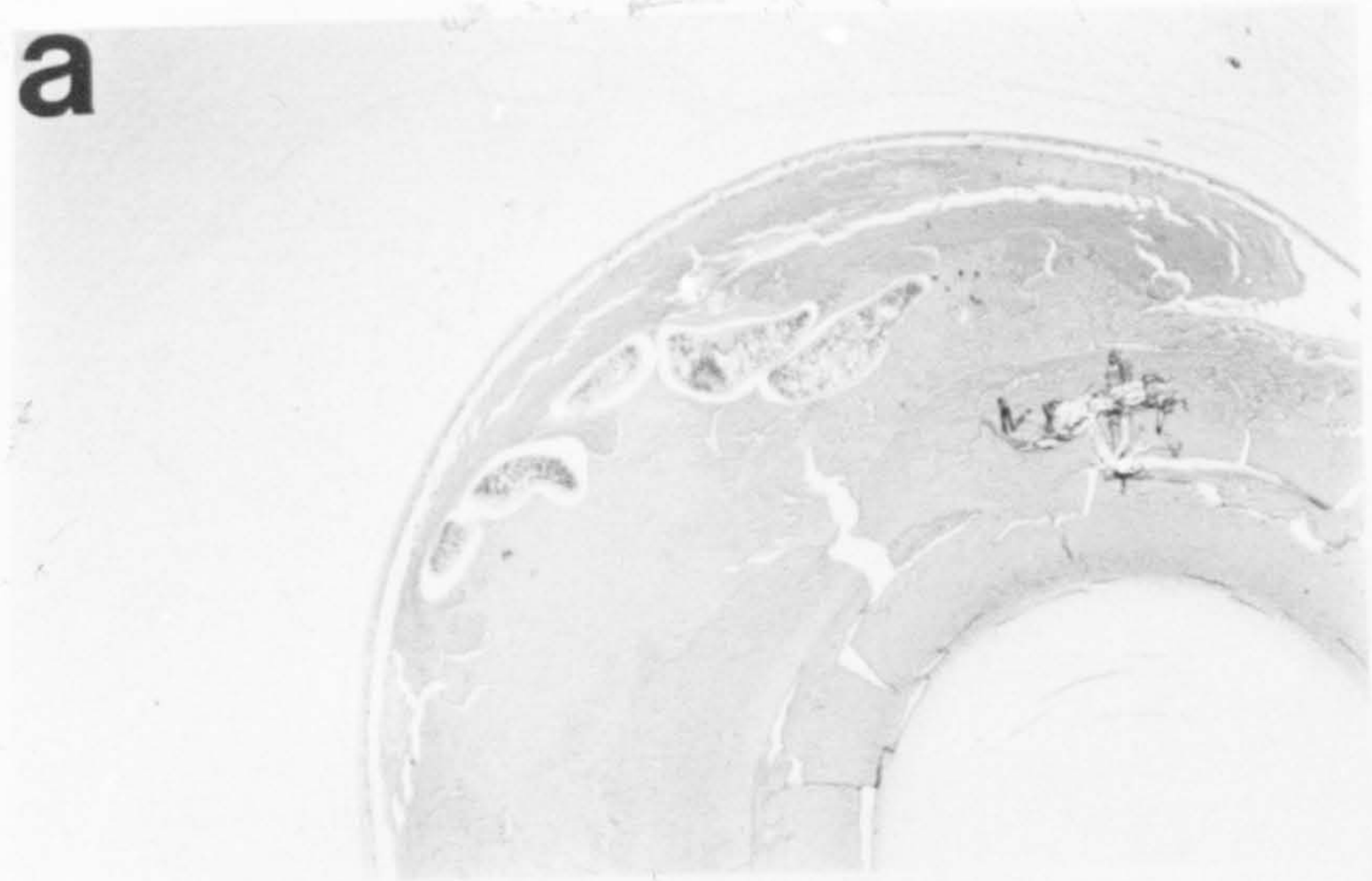
PLATE 2.3. Photomicrographs of histological sections through eyes infected with the four types of metacercariae.

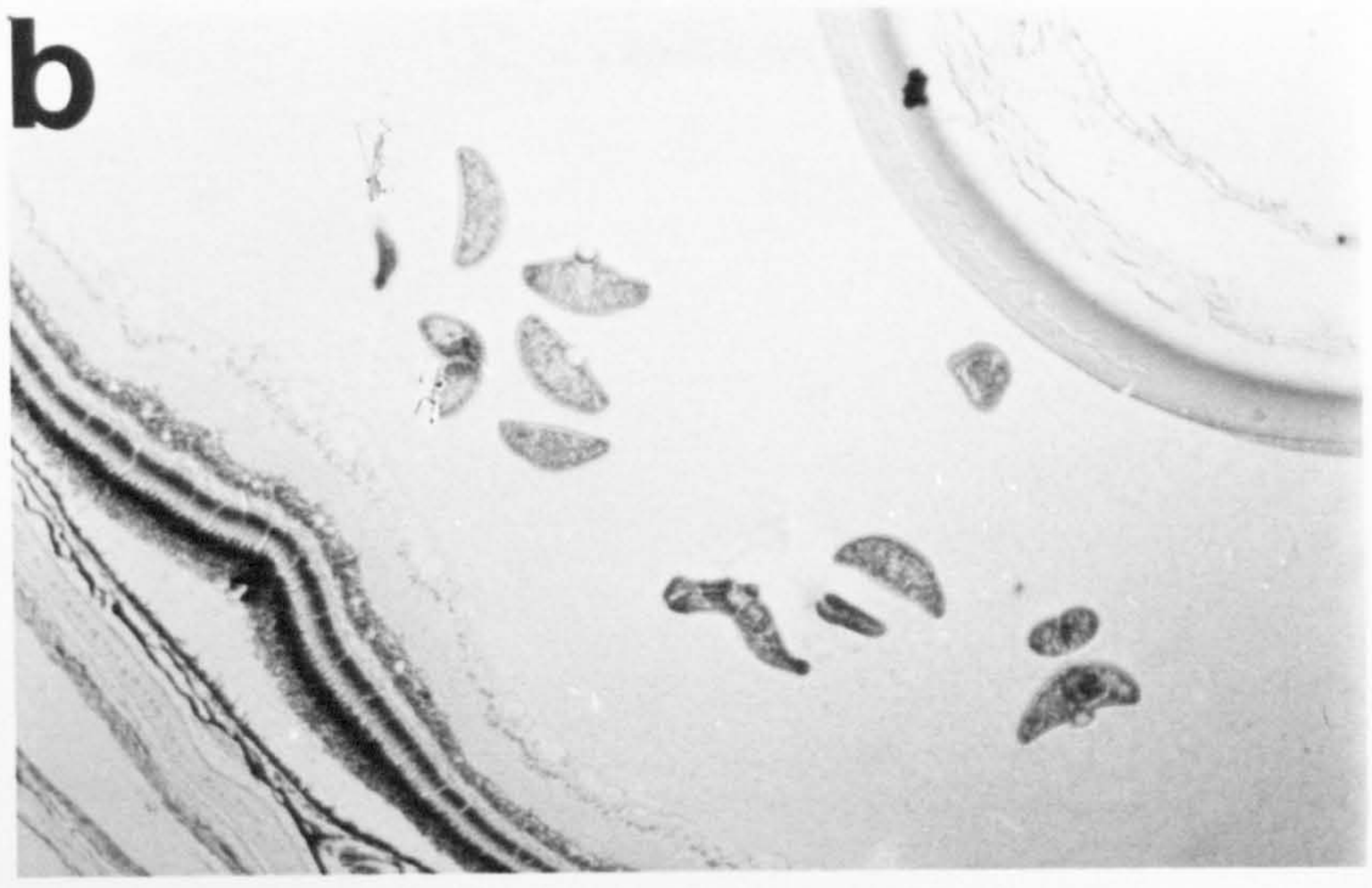
a: A section through the lens of rainbow trout showing Type 1 metacercariae.

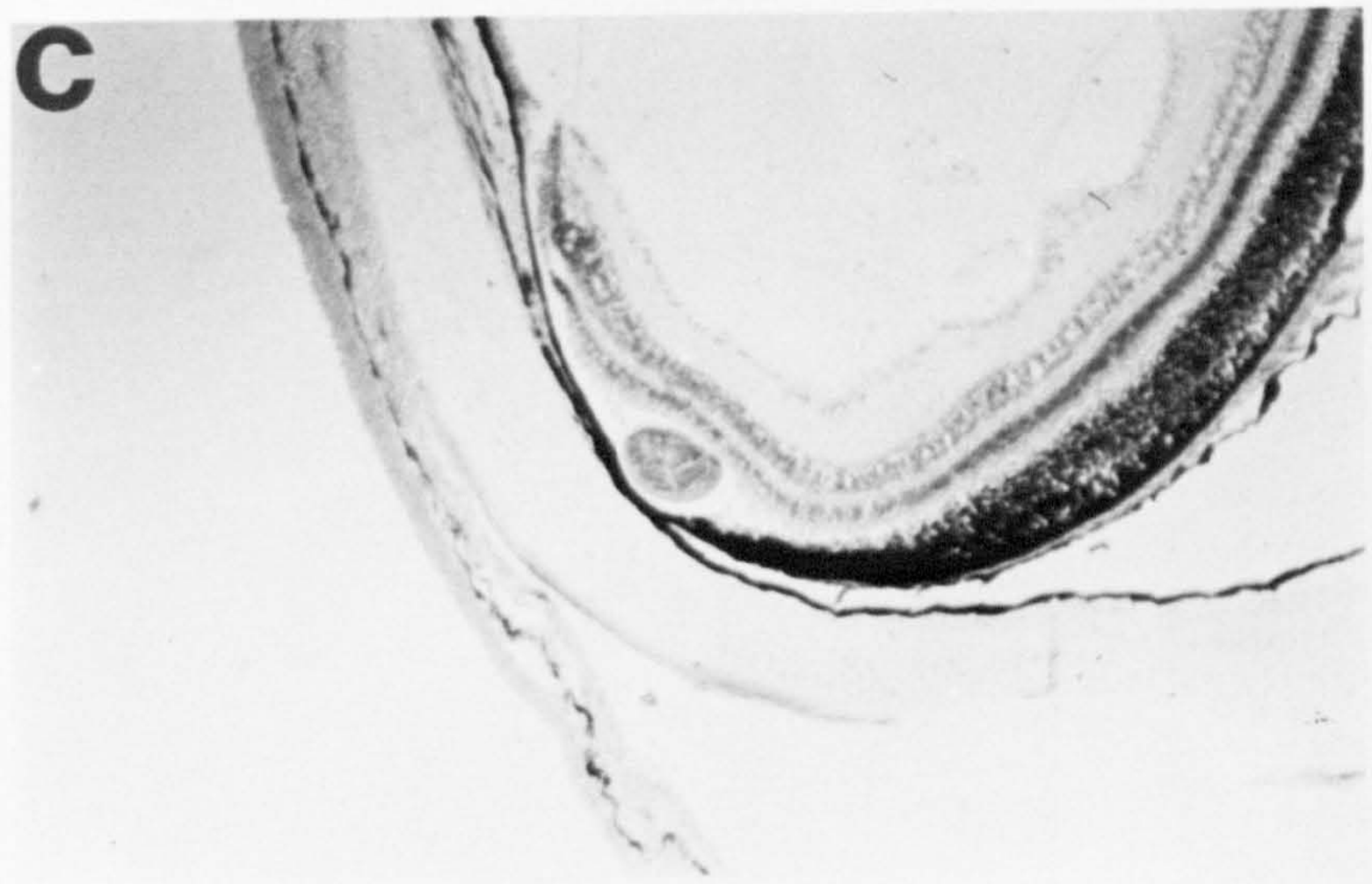
b: A section through the humour of rainbow trout showing Type 2 metacercariae.

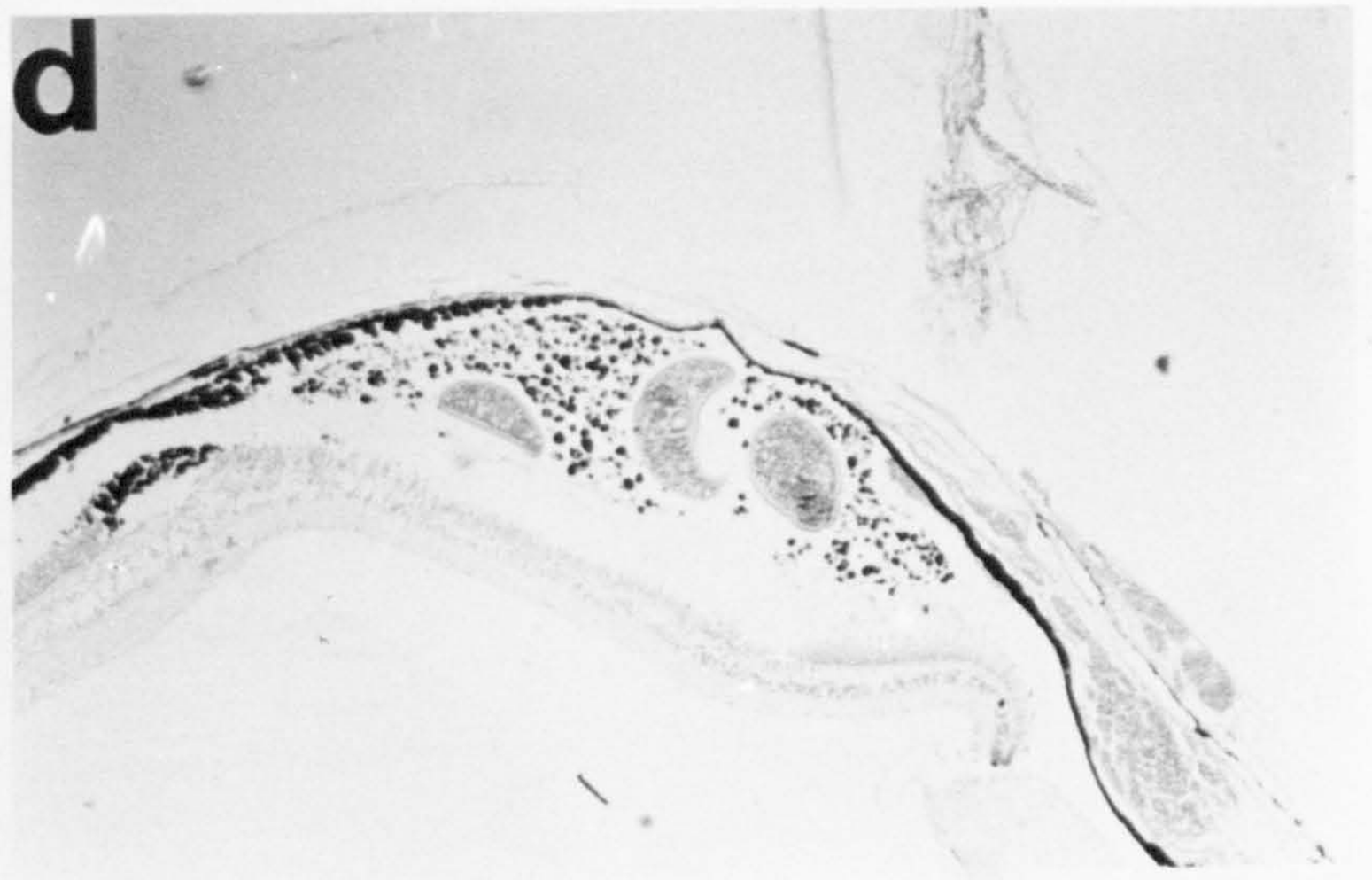
c: A section through the retina of rainbow trout showing a Type 3 metacercaria.

d: A section through the retina of perch showing Type 4 metacercariae.


a


b


c


d

DISCUSSION

Analysis of the metacercarial stage of Diplostomum present in rainbow trout and perch revealed that four different types were present. Types 1 to 3 were present in the lens, humour and retina of rainbow trout, respectively, and Type 4 was present in the retina of perch. Using Principal Components Analysis (PCA) the four types grouped separately, illustrating that they are different morphologically. PCA has been previously used in studying the taxonomy of Diplostomum species. Gibson et al. (1985) demonstrated that it was potentially useful for distinguishing different species of Diplostomum in a small study of mainly Finish specimens. Hoglund and Thulin (1988) also used PCA to separate three species of Diplostomum in Sweden. These metacercariae were from the lens and retina of perch and the lens of roach. Shigin (1986) and Niewiadomska (1988) used a similar type of cluster analysis which also demonstrated the potential use of biometrics in taxonomy of these digeneans. PCA has also been used by Canadian workers to demonstrate morphological variation within the adults of the species D. baeri bucculentum (Shostak, Tomkins & Dick, 1987). However, in this study they failed to separate lens and humoural forms and, therefore, separations obtained are likely to be due to separation of two species rather than separation of the same species due to the influence of the final host.

In this present study PCA demonstrated that almost all the characters used were very useful at separating the four types of metacercariae. Perhaps the most important characters were the distance between the lappets and the position of the ventral sucker. However, the dimensions of the suckers and the breadth of the metacercariae also contributed to

the separation. The length of the worms, however played an insignificant role in most of the analyses. This is most likely to be due to the fact that the measurements were standardised and, therefore, allometric growth effect are of prime importance.

Having demonstrated that four different types of metacercariae were present, the difficult task was to decide which species they represent. Specimens of named species of Diplostomum metacercariae were obtained from Shigin and Niewiadomska and PCA was carried out using measurements for these specimens and those for the metacercariae obtained in this study. This proved unsuccessful in identifying them as the metacercariae from the present study mostly grouped on their own and only a slight overlap was seen with some of the named species. This was thought likely to reflect the difference in the fixation techniques, since both Shigin and Niewiadomska's fixation techniques resulted in metacercariae which were different morphologically from those fixed in Berland's fluid used throughout the present study. Furthermore, the differences found to be due to the use of different fixation techniques, were in the distance between the lappets and the position of the ventral sucker, which were the two main characters which contributed to the separation of the four types in the present study. Shigin fixed his specimens in acetic carmine which one might have expected not to produce significantly different results to Berland's fluid, since the main component of both fixatives is acetic acid. However, before fixation was carried out by Shigin the metacercariae were placed in tap-water for three hours, as Shigin believes that this is the only method to distinguish immature specimens from mature ones. Only those which are left alive after three hours are considered by Shigin to be mature. This treatment before fixation may alter the morphological

dimensions, since tap-water is osmotically stressful to endoparasites such as diplostomes, therfore, this may cause damage of the tegument rendering the parasites more susceptible to contraction when placed in the acetic carmine. Furthermore, although the fixatives both contain acetic acid, acetic carmine contains a more dilute solution than Berland's fluid. The fixation technique used by Niewiadomska was somewhat different. The metacercariae were fixed in hot water which was found to cause swelling of the worms relative to Berland's fluid and acetic carmine. Therefore, worms fixed by these different methods were rendered somewhat incomparable using PCA.

The fact that different fixation method produce artifactual morphological differences renders the value of existing keys questionable unless the same fixation technique is used for all specimens to be identified. Shigin (1986) in fact stated that his key could only be used for specimens which had been treated in exactly the same manner as he had treated his. It was not unexpected, therefore, that when identification of specimens collected in this present study was attempted using Shigin's key (1986) the outcome was unclear. Only the lens metacercariae from rainbow trout gave a clear result: these Type 1 metacercariae keyed down to D. spathaceum. The other three types of metacercariae, however, could not be identified with any degree of confidence.

Problems with the identification of specimens fixed using different methods may be one of the major the causes of confusion in the taxonomy of Diplostomum metacercariae. Different fixation methods may well be the cause of many mis-identifications and false new taxa. For this reason, in this study great care was taken to ensure that all specimens were treated

in exactly the same manner in order that any differences which did occur were not fixation artifacts. However, this does not solve the problem of identifying the metacercariae obtained.

The name D. spathaceum has been used for many, indeed most, metacercariae in the UK. These metacercariae are reported to be site-specific for the lens in many species of freshwater fish (Hoffman, 1960; Sudarikov, 1971). The metacercariae collected from the lens of rainbow trout in this study do appear to conform to the description given by Niewiadomska (1986) for D. spathaceum and also appear to key down to this species using Shigin's key (1986). D. spathaceum has been reported from the lens of rainbow trout by many other authors (Betterton, 1973; Dick & Rosen, 1981; Stables, 1984; Bortz, Kenny, Pauley & Bunt-Milam, 1988). It does not seem surprising, therefore, that these metacercariae correspond to this species.

British parasitologists have tended to name all metacercariae found in the humour and retina of the eyes of freshwater fish D. gasterostei Williams, 1966 (see for example, Connelly & M^{c}Carthy, 1984). However, the ranges of measurements for Type 2 and Type 3 metacercariae did not fit well with the measurements published for D. gasterostei by Williams (1966). It is unlikely, therefore that they belong to this species.

Metacercariae from the humour and retina of rainbow trout in Canada have been identified as D. baeri bucculentum Dubois & Rausch, 1948 (Dick & Rosen, 1981; Ching, 1985). Shigin (1977) designated this species synonymous with D. baeri Dubois, 1937. When PCA was carried out with D. baeri specimens from Niewiadomska and the metacercariae collected in the present study it was found that Type 2 metacercariae grouped closely to D. baeri. However, when measurements of both types of metacercariae were

compared, it was found that Type 2 metacercariae were somewhat smaller than D. baeri. Nevertheless, this could be due to artifactual differences caused by the use of different fixation techniques as discussed earlier.

Type 3 metacercariae grouped closely to D. volvens when compared to Shigin's specimens on the PCA. Although this species is specific to the retina of the eye (Shigin, 1986), it has not been reported from salmonids. Using Shigin's key (1986) Type 3 metacercariae keyed closest to D. pseudobaeri which although it has been recorded from salmonids, it has not been recorded to occur in Europe and is located in the humour of the eye. However, the size ranges for these two metacercariae are similar.

Metacercariae from the retina of perch collected in this study posed more of a problem than the other types since two populations were examined and quite different measurements were found from each population. Metacercariae from perch netted in Loch Tulla were considerably larger than those collected from perch netted in Loch Awe. When size was accounted for by standardising the data the differences were not so great. However, the two populations were still significantly different in their breadth, the length of the oral sucker and the position of the ventral sucker. If these two populations are the same species, then this may lay some doubt on the value of these measurements in distinguishing between other types of metacercariae. The breadth of the worms may change allometrically in relation to length with age, as might the position of the ventral sucker. Since in this study the metacercariae were collected from natural infections, the age of the metacercariae could not be determined. It is possible, therefore, that in this instance we may only be seeing the effect of age. On the other hand PCA on Types 4a and 4b

revealed that the two populations did not separate very well into two groups. This is a promising result in that the analysis did not separate two different age groups, if that is what we are dealing with. The morphological differences between Types 4a and 4b may not, however, be due to differences in age. It may be that the two populations have different size ranges due to some other factor. For instance, the difference may lie in the host species. The population of perch in one loch may have a better diet than the other, or even be more immunologically competent. The retina of the eye has a good blood supply and, therefore, unlike the lens, it is not an immunologically priviledged site, thus the host may have some effect on the development of the metacercariae in the retina. If this is the case, then we may be seeing only a natural difference between two populations of the one species of parasite.

The answer to the problem of reducing variance within the same species of metacercariae may lie in controlled experimental infections in the laboratory so that effects of differences in the condition of the host and the age of the metacercariae may be ruled out. However, this does not reduce the problem that scientists in the field have of identifying metacercariae from natural infections since these variations cannot be controlled in nature. However, this may be worthwhile in order to produce voucher specimens with which natural infections could be compared.

Identification of Type 4 metacercariae was attempted using the key of Shigin (1986). Yet again this was found to be unsuccessful. The measurements for these metacercariae overlapped with those for D. volvens and D. pusillum. Adults of D. pusillum have been isolated from birds in Switzerland, France, Rumania, USSR, Asia and North America, but not in the

UK. It is possible, therefore, that this species does not occur in UK. D. volvens has been found outside the lens of the eyes of perch (Nordmann, 1832). Irwin et al. (1989) identified specimens collected from fish in North Wales as D. volvens, indicating that this species is apparently present in the UK. Furthermore, D. volvens is a retinal metacercariae unlike D. pussilum which Shigin describes as being ' free in the tissues of the eye or in a thin-walled cyst'. However, measurements for Type 4 metacercariae are outside the ranges of measurements given for D. volvens. These difference could, however, be due to differences in the methods of fixation.

This study has further outlined the difficulties present in identifying the metacercarial stages of the genus Diplostomum by conventional methods. It has illustrated the need for comprehensive keys, although if, as indicated in this study, the variance between populations of metacercariae is very high, such keys may still be of limited value. For the identification of the metacercariae obtained in this study it is necessary to find better features and to look at other stages in the life-cycle. There is a key to adult strigeoids by Dubois (1970) which is comprehensive and, therefore, may lead to the possibility of identifying these metacercariae from their adults. It may be that identification of metacercariae is not possible in natural infections without first looking at other stages in the life-cycle. Until the situation is further clarified, the problems of the identification of the metacercariae of Diplostomum must remain largely unresolved.

CHAPTER 3:

THE CULTURE OF METACERCARIAE TO ADULTS.

INTRODUCTION

Metacercariae of the genus Diplostomum develop naturally to the adult stage in piscivorous birds. In the laboratory, however, the natural host is not always readily available, even when its identity is known. Many workers have, therefore, attempted to culture the metacercariae to adults using other methods.

Some workers have tried to culture digenean metacercariae in vitro. The first attempt was made by Stunkard (1930) using Cryptocotyle lingua. With regard to Diplostomum, a technique for the in vitro culture of D. phoxini was described by Bell & Hopkins (1956) using a serum based medium containing egg-yolk. Survival was only achieved for a maximum of seven days, and in this short time, development of the genitalia was very poor. Bell & Smyth (1958), using a chicken egg-yolk and albumen medium, succeeded in obtaining almost complete maturation. The only abnormality observed was the development of the vitellarium which correlated with the failure to produce normal egg-shells. Wyllie, Williams & Hopkins (1960) and Williams, Hopkins & Wyllie (1961) failed to achieve better results using a yeast extract. However, Kannangara & Smyth (1974), using a new egg macerate medium, obtained improved vitelline development and growth response in D. phoxini.

Heatly (1958) and Wilson (1960) attempted to culture metacercariae of Diplostomum spathaceum in vitro. They used a variety of media, including some containing egg-yolk, and various animal sera, but failed to achieve any gonadal development. Kannangara & Smyth (1974) cultured D. spathaceum to the stage of egg production for the first time, but they did not test

to see if the eggs were viable.

For many years the value of _in vitro_ culture of parasitic helminths has been well recognised (Silverman, 1965; Silverman & Hansen; 1971). The main advantage in this technique is that it eliminates the need to use and maintain laboratory animals, which can be expensive, time consuming and ethically undesirable. On the other hand, there are major disadvantages, such as: the developmental rate of the parasite is often considerably slower; it can be difficult to maintain the parasites in a sterile environment; and also, from a taxonomic point of view, the parasite may develop in an abnormal manner, making morphological analyses somewhat difficult. For these reasons it was decided that for the purpose of the present study, it was best not to use _in vitro_ culture of the metacercariae.

It is well established that chicken embryos provide a highly convenient, inexpensive and, therefore, attractive method by which micro-organisms may be cultured. Levaditi (1906) was the first to use the chicken embryo for the propagation of an infectious agent when he cultured spirochaetes. The embryo is a naturally sterile environment, protected by the shell and its membrane, and this provides an ideal situation, therefore, where cross-infection may be ruled out. Although the embryo can produce antibodies, the young embryo is relatively immunologically incompetent and may, therefore, be very receptive to infection.

Many authors have attempted with much success to culture parasitic protozoa as well as helminths in this embryonic environment. As early as 1934, McCoy was able to obtain development of the nematode _Trichinella_

spiralis. The majority of early workers, however, cultured protozoan parasites, such as Trichomonas vaginalis (see McNutt & Trussell, 1941), Plasmodium gallinaeceum (see McGhee, 1949), Hexamita meleagridis (see Hughes & Zander, 1954) and Toxoplasma gondii (see Jacobs & Melton, 1954). Later work, has also focused on helminth cultures.

A number of digeneans have been cultured on the chick chorioallantois and the technique generally used for implanting worms has been described and modified several times (Woodruff & Goodpasture, 1931; Zwilling, 1959; Fried, 1962 ; Fried, 1973). Using this method Philophthalmus sp. (see Fried, 1962), Sphaeridiotrema globulus (see Fried & Huffman, 1982), Echinostoma revolutum (see Fried & Butler, 1978; Fried & Pentz; 1983; Fried & Fujino, 1984; Wisnewski, Fried & Halton, 1986) and Diplostomum spathaceum (see Leno & Holloway, 1986; Irwin et al., 1989) have been cultured with some degree of success.

Kannangara & Smyth (1974) observed development of D. spathaceum in vitro, but failed to achieve any gonadal development when they attempted chorioallantoic transplantation of the metacercariae. Leno & Holloway (1986), however, obtained ovigerous adults using a serial transfer of metacercariae through seven to nine day old eggs after three to eight days. These adults not only released eggs, but the eggs hatched to produce viable miracidia. This, therefore, is a method by which viable miracidia may be obtained all year round. The only apparent disadvantage seems to be that the adult worms developed slowly and no more quickly than those in vitro (Kannangara & Smyth, 1974). As the metacercariae grow on live tissue, there would appear to be a much better chance that the adults obtained would be more normal in morphology than those cultured in vitro.

For this reason this method of obtaining adults was attempted.

In taxonomic studies it is most important that the end result produces parasites which resemble morphologically their natural counterparts. For this reason it was decided that the metacercariae should also be cultured in the bird intestine. Many reports have shown that Diplostomum can be easily cultured in the domestic chicken (Berrie, 1960a; Khalil, 1963; Ching, 1984; Niewiadomska, 1986, 1987; Niewiadomska & Moczon, 1987), in ducklings (Bell & Hopkins, 1956; Berrie, 1960a; Berrie, 1960b; Williams, 1966; Sweeting, 1976; Shigin, Chupilko & Klochova, 1985) and even in pigeons (Williams, 1966). From these three species of birds the most promising and consistent results have been from domestic chickens.

In the natural environment D. spathaceum is a parasite of birds belonging to the Family Laridae. Many reports have revealed heavy infestations of D. spathaceum in Larus ridibundus (see Sulgostowska, 1958; Jennings & Soulsby, 1958; Pemberton, 1963), L. argentus and L. fuscus (see Pemberton, 1963; Fraser, 1973) and L. marinus (see Fraser, 1973). Not all species of Diplostomum are natural parasites of the gull family. For example, D. parviventosum is reported to be an obligate parasite of fish-eating ducks, for example goosanders (Mergus merganser) (Sudarikov, 1964; Dubois, 1938, 1970).

In the laboratory, gull chicks appear to be very good hosts of Diplostomum. Many metacercariae have been successfully raised to the adult stage in the small intestine of gull chicks, for example D. baeri bucculentum (see Dick & Rosen, 1981; Shostak et al., 1987), D. spathaceum indistinctum (see Dick & Rosen, 1981), D. pseudospathaceum (see

Niewiadomska, 1986) and D. spathaceum (see Stables, 1984; Niewiadomska, 1986; Whyte, 1989). The main disadvantages of maintaining the parasite in gull chicks are: the birds have to be removed by licence from the wild in the UK; they must be hand reared; and they are very expensive to maintain on a fish diet. However, they are reported to be very good hosts for Diplostomum and so this far outweighs the disadvantages.

The aim of this part of the study was to provide a means by which adult Diplostumum could be obtained in a manner which provided morphologically 'normal' adults. So-called 'normal' adults are those most closely resembling adults obtained from natural infestations. It was assumed that the best specimens would be obtained from herring gulls, which would be the most natural environment; but it was important to know if the other techniques could provide material in useful condition and quantities. This work was also intended to determine how long it is necessary to culture the metacercariae and what effect time has on egg production, worm size and recovery rate.

MATERIALS AND METHODS

1. Collection and Maintenance of Birds.

Chickens

Day-old chickens were obtained from three different sources:

a. Muirfield Hatchery at Kinross (Black Leghorn chicks)

b. Marshall's Chunky Chickens at Newbridge (Broilers)

c. Marshall's Chunky Chickens at Whitburn (Broilers)

The chicks were then maintained in small indoor pens. A commercial chick meal was fed ad libitum. The diet was also supplemented with cooked fish.

Ducklings

Six one-day-old Karkie Campbell ducklings were obtained from a small hatchery in Ayrshire. The ducklings were maintained in small indoor pens similar to the chickens and were fed ad libitum on a commercial chick meal which had been previously soaked in water to make the diet more moist.

Pigeons

One adult male, one adult female and one young pigeon bred in isolated cages in the Stirling University Animal House. The adult pigeons were maintained on a commercial pigeon grain and the young pigeon was still being fed naturally on 'pigeon milk' from it's parents (uninfected birds). All the pigeons were kept in an indoor aviary.

Gulls

In the summer of 1987 herring gull (Larus argentus L.) eggs were collected from the Bass Rock and herring gull chicks were collected from the Island

of Fidra, both situated off the coast of North Berwick. In the following season (1988) a mixture of herring gull and Lesser black-backed gull (Larus fuscus L.) eggs were collected from the Island of Inchmickery in the Firth of Forth. From the hatched-out chicks it was ascertained, however, that they were almost all herring gulls. All collections were carried out under a Nature Conservacy Council (NCC) Licence and the NCC also assisted with the collections.

The gull eggs were transported as quickly as possible to Stirling and placed in a purpose built incubator set at 39±1°C with a relative humidity of 70%. Eggs were rotated four or five times per day. After hatching, the chicks were maintained in the incubator until their feathers had dried. Young chicks were then placed in an artificial nest made from pressed paper in a cardboard box. Chicks which hatched out at the same time were kept together. Normally the maximum in one box was three. Chicks which hatched out at more than a day apart could not be maintained together as the competition from the older chick(s) was too fierce. Chicks were maintained on a diet of cooked whiting supplemented with some bran. All chicks were hand fed four times per day. Once per day the water given to the chicks was supplemented with multivitamins.

Chick Embryos

Fertile chick eggs were obtained from Muirfield Hatchery, Kinross. On arrival at Stirling, eggs were placed into an incubator set at 39°C and a humidity of 70%. They were rotated four times per day until 12 hours before the metacercariae were administered.

2. Preparation of Metacercariae.

The sources of metacercariae were the same as those detailed in Chapter 2, except that only perch retinal forms from Loch Tulla were used and lens metacercariae from experimental infections in the laboratory were used as well as those from the Perthshire fish farm.

Bird Infections

The eyes were removed from the fish and rinsed in sterile physiological saline. For lens metacercariae the lens was removed and placed in a chilled embryo dish. The humour was treated in the same manner except that excess humour material was removed so that only pieces which contained parasites were retained. This reduced the amount of material which had to be given to the bird, and was especially necessary when dealing with small chicks. For metacercariae from the retina of the eye, the lens and humour were carefully removed and the back of the eye, containing both the retinal material and the metacercariae, was administered whole. In all cases some sterile physiological saline was added to the chilled embryo dish to prevent desiccation. The number of parasites in the lens and humour tissue could be readily counted under a dissecting microscope. The retinal parasites could not, however, be easily counted and, therefore, only an approximation of the number of retinal parasites administered was possible. This was achieved by examination of a sample of 15-30 fish to obtain an estimate of the number of parasites per retina in the fish population.

Chorioallantoic Culture

The eyes were removed from the fish and washed in sterile physiological saline. The parasites were then carefully dissected out, as described in Chapter 2, Section 2 of the 'Materials and Methods', into either:

(a) Sterile Ringers Solution.

(b) Sterile Hank's Balanced Salt Solution (HBSS).

To both of these solutions, 500 units Penicillin/ml and 500 µg Streptomycin/ml were added. The metacercariae were rinsed twice in their respective solutions and then held in the solution at 35°C until required.

3. Administration of Metacercariae.

Birds

For all young birds the lens and humour parasites were administered by placing them into the oesophagus using a piece of plastic tubing mounted on a 5ml syringe. Older birds were fed the lens and humour tissue wrapped in a small piece of fish muscle. Retinal metacercariae were administered by placing the excised tissue to the back of the mouth using a pair of blunt forceps. The birds normally then swallowed the eyes voluntarily. Older birds could, however, simply be offered the eyes which they ate without assistance.

Birds less than 10 days old were killed by dislocation of the neck. Birds more than 10 days old were killed by exposing them to an increasing concentration of CO_2. These methods are listed under Schedule 1 of the Animals (Scientific Procedures) Act, 1986.

Chick embryos

Method 1. (Fried, 1962; 1973)

After five days of incubation the fertile eggs were candled and the position of the air-sac and embryo noted. After this time the eggs were no longer rotated. On day six, an area 1-1.5 cm from the air-sac was marked. It was necessary to ensure that the area was clear of large blood vessels and positioned over the chorioallantoic membrane (CAM). Using a small scalpel blade a triangle was cut out of the shell. Care was taken not to puncture the shell membrane. Once the triangular piece of shell had been removed, the air-sac, situated at the blunt end of the egg, was punctured by drilling a small hole with a sharp dissecting needle.

A small drop of sterile saline was pipetted onto the shell membrane over the area where the triangle was removed. Whilst candling the egg, the shell membrane was punctured using a flamed dissecting needle. For best results, the needle was inserted at an obtuse angle. It was at this point that the CAM could be seen to fall and, therefore, a false air-sac was produced, displacing the natural air-sac. The metacercariae could then be innoculated onto the CAM using a sterile syringe. Three different sizes of needles were used; 19, 21 and 23 gauge. The metacercariae were innoculated suspended in 0.1 ml of either Ringer's Solution or Hank's Balanced Salt Solution, both of which were sterile and supplemented with 500 units of Penicillin and 500 µg of Streptomycin per ml.

The aperture created in the egg-shell was then sealed using freshly cut cellotape. The eggs were replaced into the incubator, with the culture cavity uppermost, and were not rotated. The eggs were candled every day to ensure that the embryo was still alive.

Method 2. (Irwin & Saville, 1988; Irwin et al., 1989).

This method is similar to Method 1, except for the following changes:

1. Eggs were incubated for seven to eight days prior to infection.

2. Metacercariae were innoculated into the chick chorioallantoic space (CAS).

3. Each day after innoculation of the parasites, 0.2 ml of chicken serum was innoculated into the CAS.

4. After seven days of incubation the parasites were serially transferred into new seven to eight day old embryos.

All chick embryos were killed by chilling to 4°C. This method is listed under Schedule 1 of the Animals (Scientific Procedures) Act, 1986.

4. Removal of Worms from Chicks Prior to Infection or Re-infection.

The anthelminthic Droncit was administered at a dose of 15 mg/kg body weight of bird in order to kill the parasites in the intestine. All gull chicks brought in from the wild were given this dose to ensure any natural infections were removed. This dose was also given to birds in which the first infection did not take well, in order that they might be re-infected.

In all cases Droncit was administered in a powder form placed into small pieces of food and fed to the birds.

5. Examination of Faeces for Eggs and Egg Counts.

Fresh faecal samples were collected from the bird's enclosure. Samples were suspended in 5-10 ml of distilled water. The volume of faeces collected was measured by allowing the faeces to settle in a graduated test-tube before the wet volume was read. The faeces were then gently homogenised for approximately 1 minute. They could then be sieved through a series of micro-pore meshes. Firstly, large faecal material was removed when the sample was washed through a 150 µm and a 100 µm mesh. The eggs in the remaining suspension were washed by pouring the suspension through fine meshes of 63 µm and 45 µm. In these meshes the eggs were retained and could, therefore, be flushed with distilled water in order to remove any remaining faecal matter. The eggs were then washed off the meshes into a large petri dish for counting. Total counts were, therefore, taken from known volumes of faeces (normally 2ml of faeces was analysed at one time).

6. Recovery of Adults.

From birds.

After killing the bird, the intestine was removed and placed in a large petri dish containing a small amount of physiological saline. 3 cm portions of the intestine were opened and examined in sequence under a dissecting microscope with the aid of dissecting needles. All adults were removed from the intestine by the gentle action of a small brush and placed into saline until fixation in either Berland's fluid or cacodylate buffered glutaraldehyde. The position in the intestine in which the adult worms were recovered was recorded.

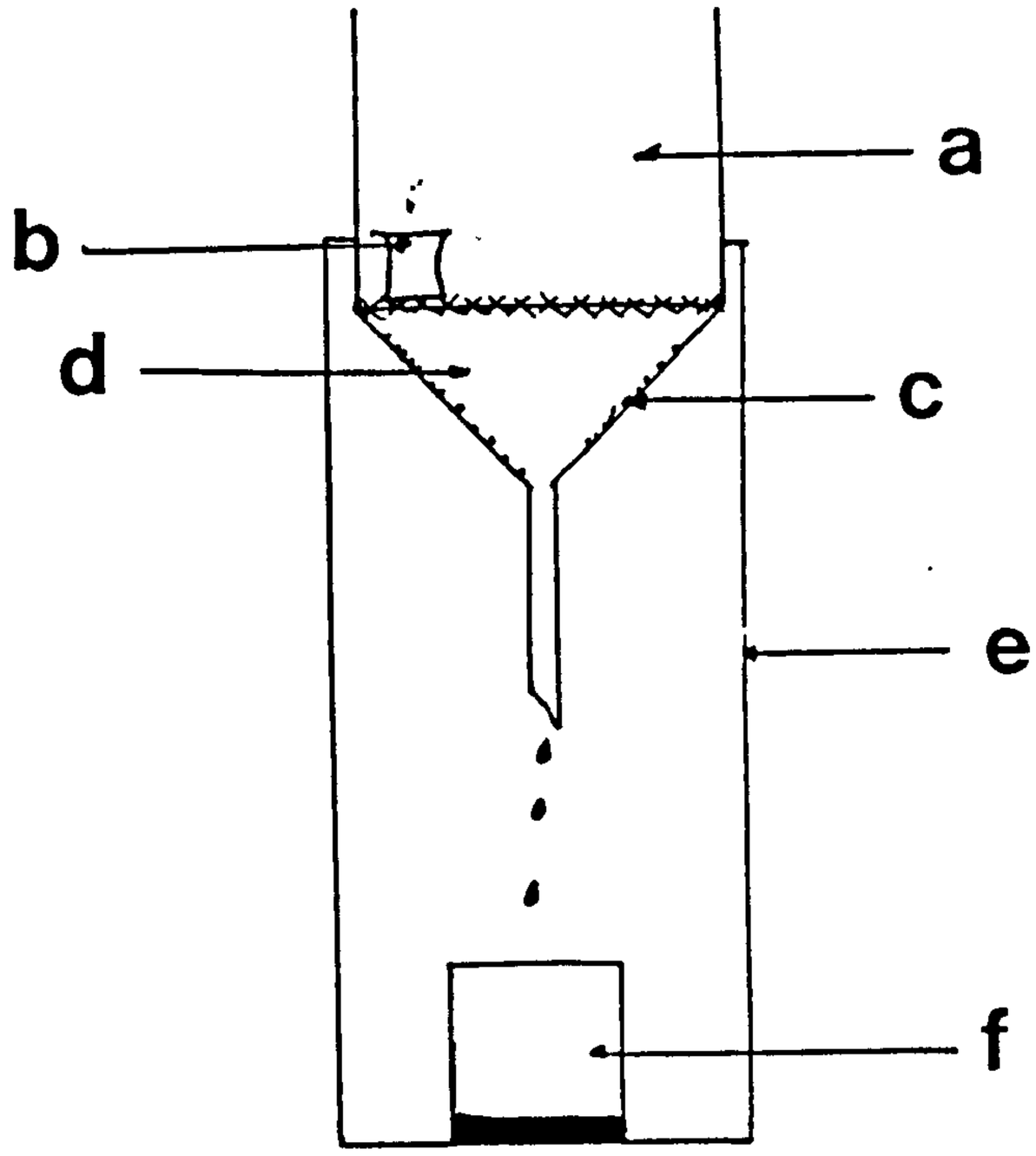
From chick embryos.

After chilling the embryo, the egg was cut along the long axis with a pair of scissors. The embryo and yolk-sac were turned out of the shell: the CAM could then be peeled off the egg-shell in one piece. In method 1 the parasites were found on the top surface of the CAM. In method 2 the parasites were found on the under-surface of the CAM or occasionally in the embryonic fluid.

7. Recovery of Adult Parasites from Birds after the Administration of Droncit.

In order to recover adults a dose of 10mg/kg Droncit was given which was thought to be sub-lethal to the parasite. The gull chick was then placed in an apparatus shown in Fig. 3.1. The funnel was kept constantly wet in order that faeces could flow down and collect into the beaker. Adult worms were retrieved by washing the faeces through a series of 150 µm, 100 µm and 63 µm meshes. Worms were collected into physiological saline and subsequently fixed in Berland's fluid.

Fig. 3.1. The apparatus used to collect faeces from birds containing adult Diplostomum after administration of anthelmintic.



a: The bird is placed here.

b: A beaker of water to prevent dehydration.

c: Droplets of water to maintain moisture for the faeces.

d: Funnel.

e: Stand.

f: Beaker to collect faeces.

RESULTS

1. Analysis of Faecal Egg Counts.

Faecal egg counts were monitored throughout the infection in herring gulls (Table 3.1). Unfortunately, it was extremely difficult to extract eggs from the faeces of chickens. This was mainly due to the fact that they were fed a high fibre diet and the eggs became enmeshed or attached to these fibres. It was fairly easy, however, to obtain clean batches of eggs from the faeces of gull chicks. Gull chicks are maintained on a diet of cooked fish and so had very little fibre in their faeces, thus facilitating the collection of eggs. The gulls were given bran after the faecal samples were collected each day, but the fibre had cleared from their faeces by the next collection.

Egg counts were used mainly as an assessment of establishment of infection in the birds. For this reason only irregular samples of faeces were analysed and so total egg counts were not determined. If few eggs were found in a number of samples from a single bird, then it was considered to be an unsatisfactory infection and was terminated using the method in detailed Section 4. The bird was then available for re-infection.

All lens and retinal infections established, but humoural infections did not take so well. Three birds previously infected with humoural forms were dosed with Droncit and re-infected 1 week later.

Egg counts were also used to determine the longevity and peak of

maturation in the herring gull host. Table 3.1 shows the mean number of eggs recovered per 1 ml sample of faeces over 61 days. From this table it can be determined that egg counts remained fairly constant after 10 days p.i., although a slight peak appeared at 21 days p.i. Egg counts also appeared to decline after 49 days p.i. Infections were only followed for a maximum of 61 days, and, therefore, the longevity of infection was considered to exceed this.

Table 3.1. Results of faecal egg counts from herring gull chicks infected with lens and retinal metacercariae. The figures given are mean values.

No. of chicks	Days post-infection	No. eggs/ml of faeces
19	7	25
19	10	48
17	14	50
16	18	40
10	21	65
4	28	50
3	35	47
3	42	45
2	49	35
2	56	32
2	61	27

2. Development of the Hind-body of the Worms.

Using adults obtained from lens of rainbow trout metacercariae as a guide for hind-body development, it was found that the hind-body did not fully develop until 16-18 days p.i. It was assumend that the hind-body was fully developed once it had reached a maximum size. In the case of the lens forms from rainbow trout, the hind-body accounted for approximately 60% of the total body length. Therefore, although adult worms mature and release eggs from day 4, maximum growth was not obtained until 12-14 days later. Worms obtained before day 16 p.i. were not used for morphological assessment.

3. Recovery of Adult Worms.

No adult worms were recovered from 6 one-day-old ducklings infected with a range of 35-78 metacercariae per bird and the birds were examined 2-6 days p.i. One adult pigeon had 45 lens metacercariae administered, and the other had 59 humoural metacercariae. They were both examined 4 days p.i. The young pigeon had 46 lens metacercariae administered and was examined 3 days p.i. No infection was established in either the adult or the young pigeons. From a total of 50 chicken embryos infected with lens, humoural and both types of retinal metacercariae no infection was found to establish. Since the same method is used successfully by Irwin et al. (1989) it is unclear why this method proved so unsuccessful.

Recovery from chickens

From Table 3.2a it can be seen that infection only established in 3 out of 26 (11.5%) Marshall's Broiler chickens infected with lens of rainbow trout metacercariae. These lens forms also established in 1 out of 4 (25%) Black Leghorn chickens (Table 3.3).

Establishment of infection with humoural metacercariae of rainbow trout was considerably higher. In Marshall's Broilers 9 out of 16 (56%) were successfully infected (Table 3.2b) and 3 out of 7 (43%) Black Leghorns were successfully infected (Table 3.3).

Success was not so evident with retinal metacercariae from rainbow trout or perch. No adult worms were recovered from 10 Marshall's Broilers infected with a mean of 48 retina of rainbow trout metacercariae dissected 10-14 days p.i. Similarly, no infection established in 6 Marshall's Broilers infected with a mean of 60 retina of perch metacercariae dissected and 10-16 days p.i. As indicated in Table 3.3 retinal metacercariae from rainbow trout did not establish in Black Leghorn chickens. The infection of retinal metacercariae from perch was not attempted in this strain of chicken.

From these results it appears that Diplostomum metacercariae will grow to adults in the domestic chicken, although it appears to be a satisfactory host only for humoural metacercariae from rainbow trout.

TABLE 3.2a. Recovery of adult worms from Marshall's Broiler chickens infected with lens of rainbow trout metacercariae.

Dose	Recovery (%)	Days p.i.
34	10 (29.4)	1
60	0	10
75	0	11
43	0	11
34	0	12
43	0	12
37	0	12
28	0	14
36	0	14
30	0	14
50	0	14
53	0	14
59	0	14
50	0	14
75	1 (1.3)	16
76	0	16
49	0	16
58	0	16
112	2 (1.8)	17
94	0	17
47	0	17
47	0	17
51	0	17
53	0	17
48	0	18
75	0	18

A total of 26 chickens were infected with lens metacercariae from rainbow trout.

TABLE 3.2b. Recovery of adult worms from Marshall's Broiler chickens infected with humour of rainbow trout metacercariae.

Dose	Recovery (%)	Days p.i.
114	24 (21.1)	10
80	0	12
50	0	13
64	2 (3.1)	14
28	1 (3.6)	14
68	8 (11.8)	16
92	9 (9.8)	16
54	18 (33.3)	17
85	4 (4.7)	17
64	0	17
27	1 (3.7)	18
76	5 (6.6)	18
45	0	18
25	0	18
57	0	18
55	0	18

A total of 16 chickens were infected with humoural metacercariae from rainbow trout.

TABLE 3.3. Recovery of adult worms from Black Leghorn chickens infected with lens, humour and retina of rainbow trout metacercariae.

Source of metacercariae	Dose	Recovery (%)	Days p.i.
Lens r. trout	38	2 (5.3)	12
"	47	0	12
"	65	0	14
"	54	0	18

A total of 4 chickens were infected with lens metacercariae from rainbow trout.

Humour r. trout	66	2 (3.0)	11
"	61	0	11
"	51	5 (9.8)	12
"	59	0	12
"	49	0	14
"	59	0	16
"	74	1 (1.4)	18

A total of 7 chickens were infected with humoural metacercariae from rainbow trout.

Retina r. trout	?	0	10
"	?	0	14

A total of 2 chickens were infected with retinal metacercariae from rainbow trout. Note: the dose of metacercariae was estimated to be in the range 40-60.

Recovery from gull chicks

Infection of the gull chicks was readily established with metacercariae from the lens of rainbow trout (91%), as can be seen from Table 3.4. However, only 50% of the chicks infected with humoural metacercariae harboured adult worms at post-mortem.

Gulls were also infected with retinal metacercariae from rainbow trout and perch (Table 3.5). In this instance the number of worms administered could not be counted as retinas were fed to the chicks intact. However, it was estimated that the numbers ranged between 45 and 61 metacercariae. Infection established in all gulls administered retinal metacercariae from perch and only 1 out of 5 infections (20%) did not take in those administered metacercariae from the retina of rainbow trout.

In Table 3.6 it is evident that the herring gull is a better host for lens and retinal metacercariae than the domestic chicken. However, when humoural metacercariae are examined it can be seen that the recovery rate of adult worms in the domestic chicken is double that from the herring gull. For this metacercaria, therefore, the chicken is considered a better host than the gull.

TABLE 3.4. Recovery of adult worms from herring gull chicks infected with lens and humoural metacercariae from rainbow trout.

Source of metacercariae	Dose	Recovery (%)	Days p.i.
Lens of r. trout	26	6 (23.1)	5
"	27	1 (3.7)	6
"	39	14 (35.9)	18
"	50	25 (50.0)	18
"	61	13 (21.3)	18
"	100	0	18
"	30	1 (3.3)	23
"	80	5 (6.3)	24
"	51	2 (3.9)	25
"	40	5 (12.5)	44
"	42	16 (38.1)	61
Humour of r. trout	36	4 (11.1)	6
"	40	1 (2.5)	6
"	49	0	9
"	57	3 (5.3)	10
"	59	0	11
"	45	0	12
"	64	1 (1.6)	18
"	40	0	21

TABLE 3.5. Recovery of adult worms from herring gull chicks infected with approximately 45-61 retinal metacercariae from rainbow trout and perch.

Source of metacercariae	Recovery	Days p.i.
Retina of r. trout	6	13
"	1	14
"	6	18
"	0	18
"	8	19
"	1	24
Retina of perch	34	11
"	3	18
"	1	24
"	14	24
"	11	28
"	2	61

TABLE 3.6. Comparison of establishment and recovery rates of adult worms from Marshall's Broiler chickens and herring gull chicks.

Source of	Herring Gulls		Chickens	
metacercariae	% Est.	% Recovery	% Est.	% Recovery
Lens r. trout	91	19.8	11.5	10.8
Humour r. trout	50	5.1	56.3	10.8
Retina r. trout	80	?	0	0
Retina perch	100	?	0	0

Analysis of number of metacercariae administered and duration of infection vs percentage recovery.

Fig. 3.2 shows the results of a regression analysis on the number of the four types of metacercariae administered compared to the percentage of adult worms recovered in both chicken and gull chick infections. From these graphs it can be seen that there is no linear relationship between these variables (P>0.05).

Fig. 3.3 shows the results of a regression analysis of the duration of infection compared to the percentage of adult worms recovered. Again there is no linear relationship between these variables (P>0.05).

4. Number of eggs vs size and days p.i. of adult worms.

Tables 3.7-3.9 show the number of eggs in the uterus of each adult worm measured. Fig. 3.4 illustrates plots made of the length of adult worms compared to the number of eggs present in the uterus. Regression analysis of this data for all types of adults shows that there is no linear relationship between these variables (P>0.05). Therefore, it would seem that the number of eggs produced by an adult worm is not determined by its length.

TABLE 3.7. Results of infections of herring gull chicks with lens metacercariae from rainbow trout. The number of eggs in the uterus in relation to days p.i. and the length of the adult worms.

Lens of r. trout (1)[a]			Lens of r. trout (2)[a]		
No. of Eggs	Days p.i.	Length of worm(mm)	No. of Eggs	Days p.i.	Length of worm(mm)
2	18	2.300	4	18	2.375
8	18	2.163	3	18	2.038
8	18	2.425	4	18	1.963
7	18	2.375	5	18	1.925
17	18	2.433	18	18	2.100
1	18	2.550	2	18	1.825
9	18	2.000	2	18	2.125
8	18	2.050	10	18	2.000
3	25	1.825	10	18	2.050
8	25	2.350	1	48	2.125
10	33	1.450	2	48	2.225
8	33	1.313	6	48	2.250
6	33	1.450	3	48	2.038
8	33	1.750	4	48	2.288
3	33	1.575	5	61	2.525
7	33	1.825	3	61	2.250
5	33	1.825	9	61	2.300
9	33	1.500	5	61	2.200
6	33	1.950	5	61	2.325
3	33	1.925	1	61	2.325

[a] Lens of r. trout (1) are adults obtained from lens metacercariae from farmed rainbow trout from Loch Awe. Lens of rainbow trout (2) are adults obtained from metacercariae from experimental infections of rainbow trout in the laboratory.

TABLE 3.8. Results of infections of herring gull chicks with retinal metacercariae from rainbow trout and perch. The number of eggs in the uterus in relation to days p.i. and the length of the adult worms.

Retina of r. trout			Retina of perch		
No. of Eggs	Days p.i.	Length of worm(mm)	No. of Eggs	Days p.i.	Length of worms(mm)
4	18	1.638	10	24	1.525
15	18	1.475	3	24	1.475
4	18	1.100	6	24	1.300
7	18	1.225	5	24	1.088
1	18	1.075	1	24	1.000
15	18	1.450	3	24	1.250
6	19	1.650	1	24	1.175
13	19	1.275	11	24	1.425
5	19	1.025	2	24	1.388
11	19	1.100	2	24	1.225
6	19	1.175	12	24	1.288
8	19	1.075	1	32	1.425
6	19	1.000	4	32	1.650
14	19	1.225	5	32	1.400
13	24	1.600	5	32	1.375
			3	32	1.300
			5	32	1.400
			1	61	1.563
			2	61	0.950
			1	69	0.975

TABLE 3.9. Results of infections of Marshall's Broiler chickens with humoural metacercariae from rainbow trout. The number of eggs in the uterus in relation to days p.i. and the length of the adult worms.

Humour of r. trout		
No. of Eggs	Days p.i.	Length of worm(mm)
8	17	0.698
7	17	0.859
2	17	0.827
4	17	1.049
12	17	0.978
3	17	1.067
9	17	0.954
4	17	1.002
3	17	0.763
2	17	0.779
6	17	0.906
6	19	1.049
5	19	0.763
7	19	0.970
9	19	1.193
3	19	0.994
7	19	1.145
7	19	1.065
7	19	0.763

5. Recovery of Adult Worms after Treatment with a Sub-lethal Dose of Droncit.

Having been infected with 61 metacercariae from the lens of rainbow trout, a gull chick was administered a sub-lethal dose of Droncit and its faeces examined for worms. 13 adult worms were recovered within 1 hour 30 minutes of dosing. The worms were dead, but morphological analysis revealed that there was little contraction caused by the drug.

6. Description of Adult Worms.

A full morphological analysis of the adult worms is given in Chapter 4. Table 3.10 gives the mean measurements for adult worms for each period post-infection. Table 3.11 shows the results of t-tests carried out to compare the lengths of adult worms at various times post-infection. None of the results were significantly different apart from the lens of rainbow trout adults when compared at 18 and 33 days p.i. and 18 and 61 days p.i. A significant decrease in worm size from 18 to 33 days p.i. was an unexpected result in that it would suggest that the worms decreased in size. However, it is more likely that this may be explained by intrinsic differences in the hosts which carried these worms. That is to say that the host which harboured the worms until 33 days p.i. may not have provided optimal conditions for worm growth and so the worms may have been small throughout the infection. On the other hand the differences might be due to the small number of worms involved.

TABLE 3.10. Mean lengths (mm) of adult worms at various days p.i.

Source[a]	Days p.i.	No.	Mean Length	Range	S.D.
1	18	8	2.287	2.000-2.550	0.197
1	25	2	2.088	1.825-2.350	0.371
1	33	10	1.656	1.313-1.950	0.226
2	18	9	2.044	1.825-2.375	0.154
2	48	5	2.185	2.038-2.288	0.102
2	61	6	2.321	2.200-2.525	0.111
3	18	6	1.327	1.075-1.650	0.228
3	19	8	1.191	1.000-1.650	0.209
3	24	1	1.600	-	-
4	24	11	1.285	1.000-1.525	0.162
4	32	6	1.425	1.300-1.650	0.118
4	61	2	1.257	0.950-1.563	0.433
4	69	1	0.975	-	-
5	17	11	0.898	0.698-1.067	0.123
5	19	8	0.993	0.763-1.193	0.159

[a] Sources are:

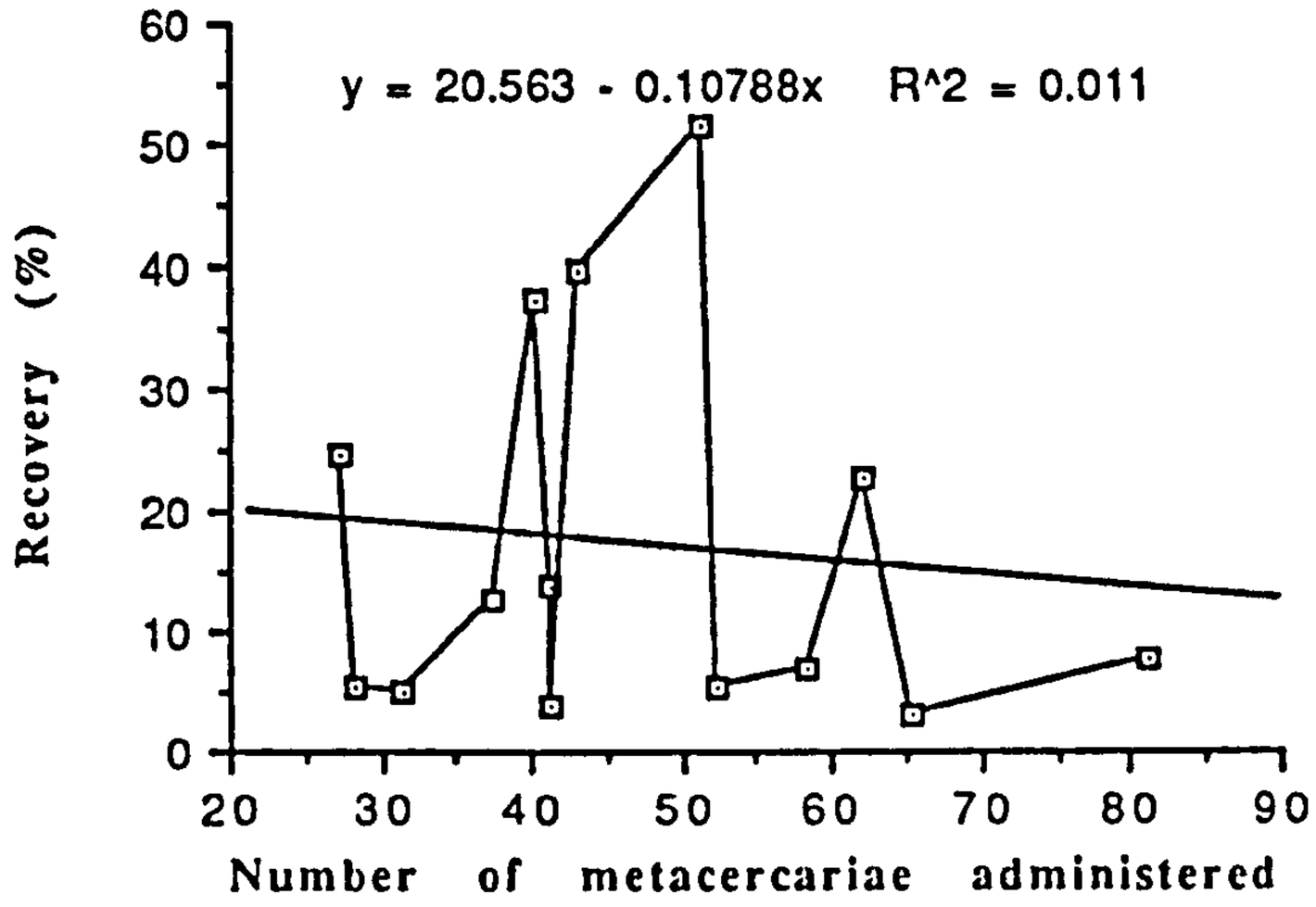
1. Lens of rainbow trout metacercariae from a farm on Loch Awe.
2. Lens of rainbow trout metacercariae from experimental infections in the laboratory.
3. Humour of rainbow trout metacercariae from a farm on Moffat Water.
4. Retina of rainbow trout metacercariae from a farm on Loch Awe.
5. Retina of perch metacercariae from Loch Tulla.

TABLE 3.11. t-statistics comparing the length of adult worms after 18 days p.i.

Source[a]	Days p.i.	T value	DF	Probability	Sig.
1	18/33	6.33	15	P< 0.001	S
2	18/48	2.05	11	P> 0.050	NS
2	18/61	4.03	12	P< 0.050	S
2	48/61	2.11	8	P> 0.050	NS
3	18/19	1.15	10	P> 0.050	NS
4	24/32	2.04	13	P> 0.050	NS

[a] Sources are explained in Table 3.10.

Fig. 3.2. Percentage recovery of adult worms from herring gull chick and Marshall's Broiler chick infections in relation to the number of metacercariae administered.



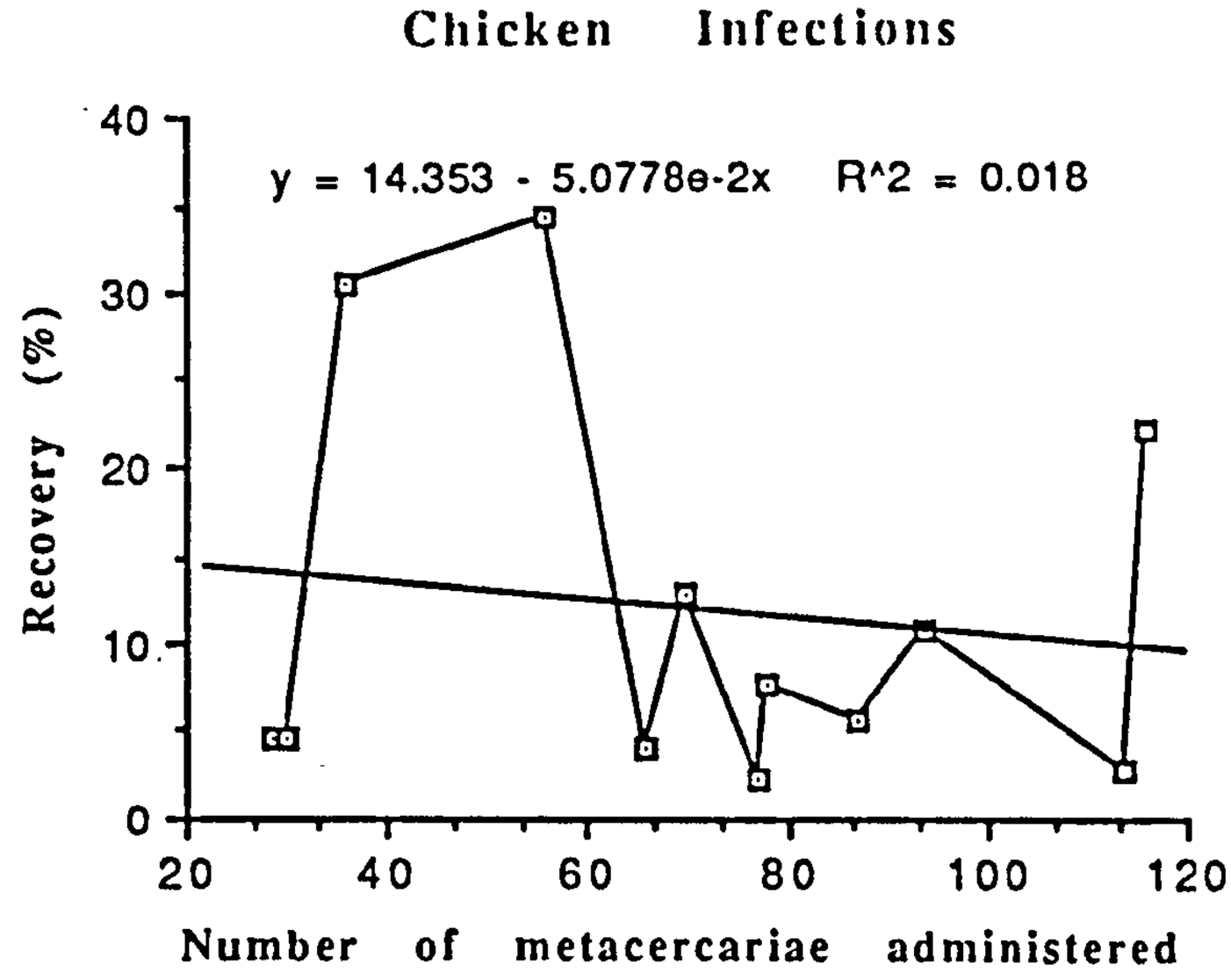


Fig. 3.3. Percentage recovery of adult worms from herring gull chick and Marshall's Broiler chick infections in relation to days p.i.

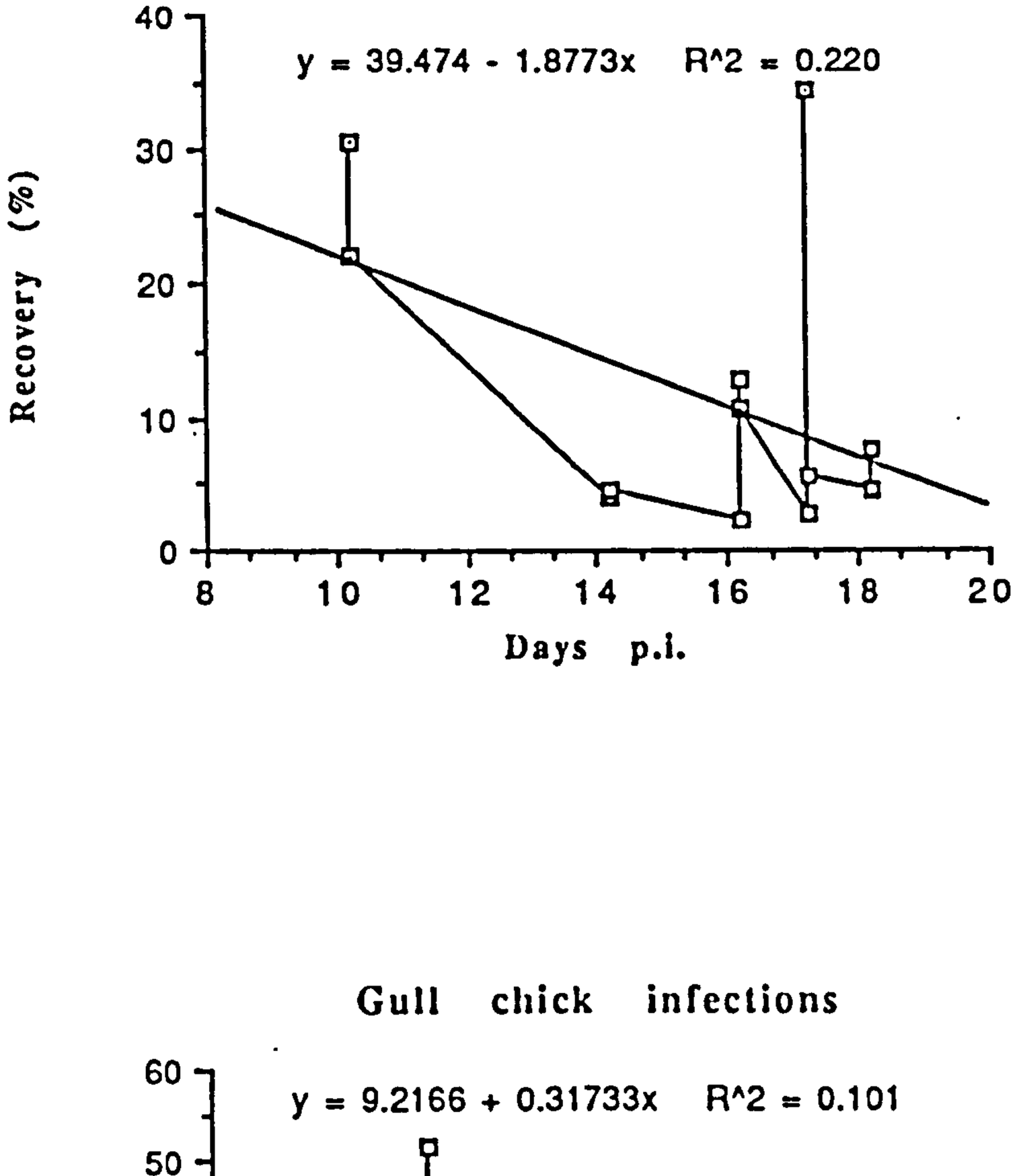
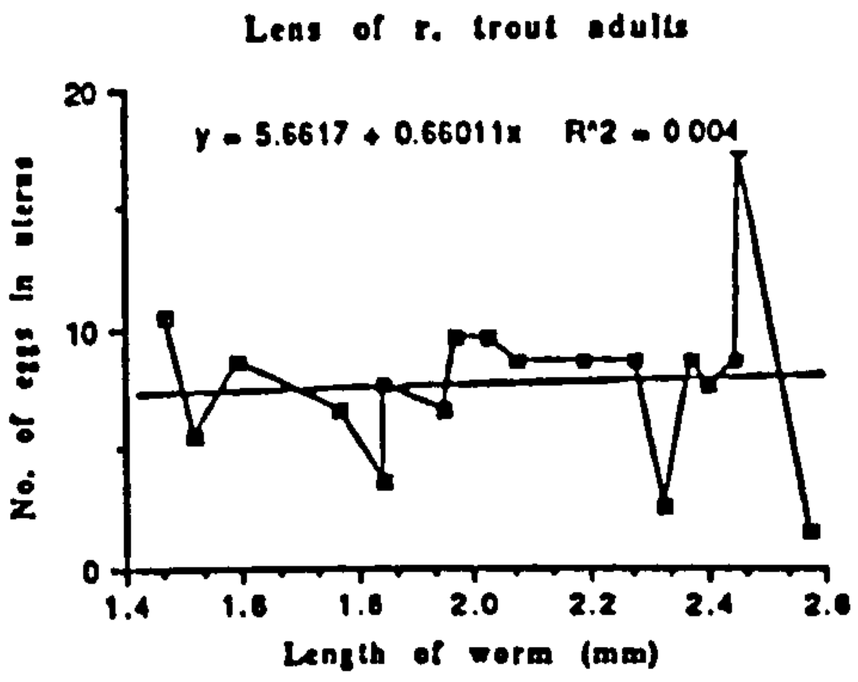
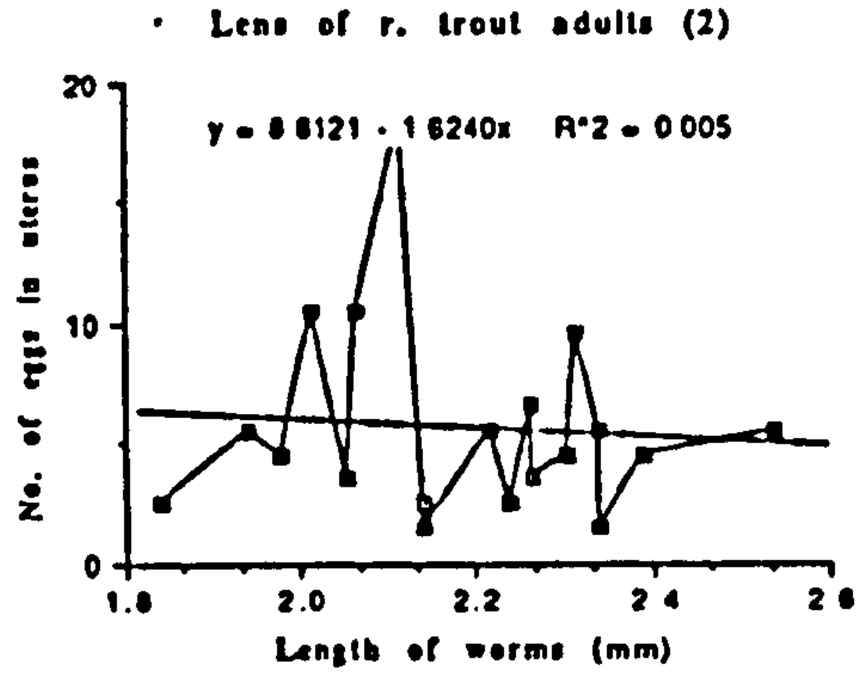
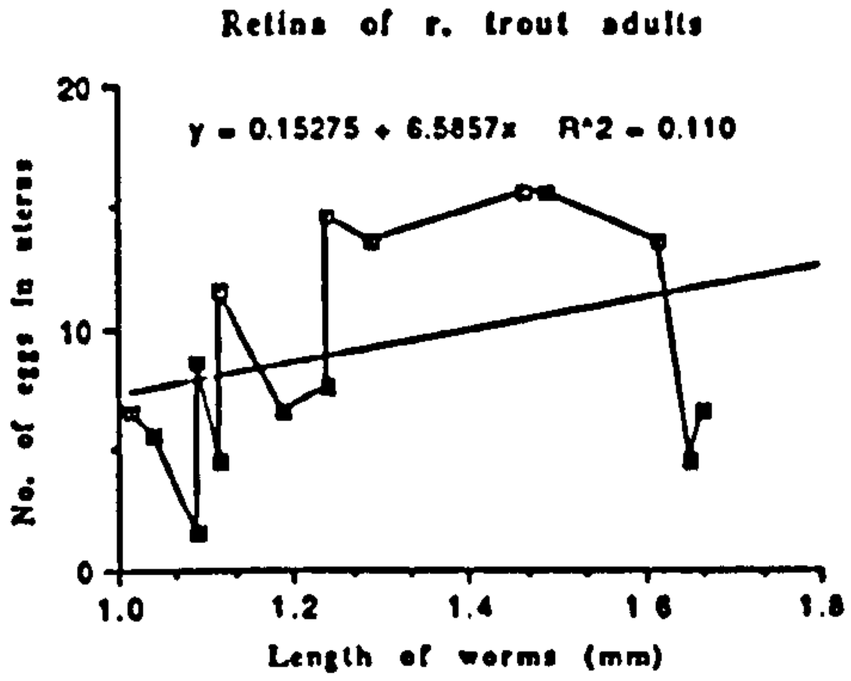
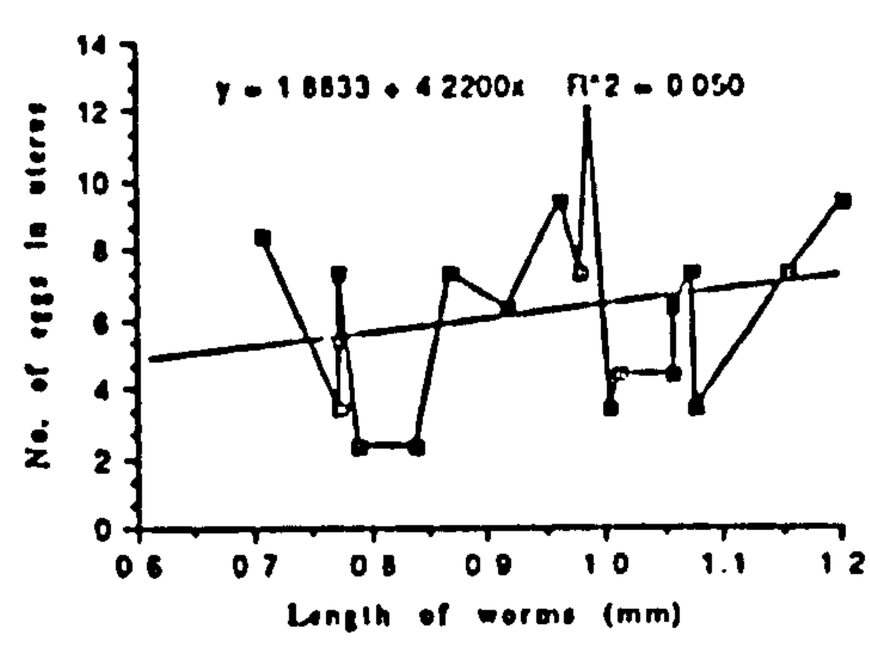


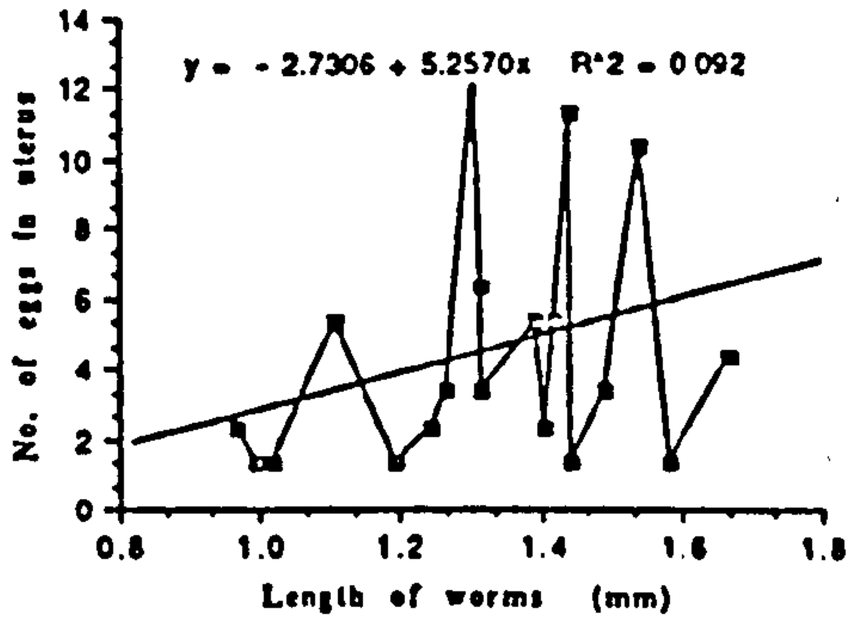
Fig. 3.4. The number of eggs in the uterus of adult worms in relation to the length of the worm.











DISCUSSION

Adult worms of the genus Diplostomum were readily obtained from both chickens and gull chicks. Although previous authors have obtained adults successfully from ducklings and pigeons (see 'Introduction'), no adults were obtained from either in this study. The reason for the unsuitability of these birds as hosts in this study is not clear, but perhaps susceptibility depends on the strain and immunological competence of the bird. There have been other reports of failures to obtain adults from ducklings and pigeons, e.g. Lester & Huizinga (1977) after infection with D. adamsi metacercariae.

No adults were recovered from chicken embryo infections. The reasons for this are not fully understood, as other authors have readily obtained adults using this technique (Leno & Holloway, 1986; Irwin et al., 1989). It is possible that technical problems were at fault, e.g. the metacercariae may have been damaged on innoculation with the metal syringe used. For future attempts rubber tubing as used by Irwin & Saville (1988) may be more suitable. Another possibility is that the strain of chicken used may not have been susceptible, for, although chick embryos are relatively immunologically incompetent, they are capable of producing antibodies as well as a cellular response (Long, 1978). The results of Irwin et al. (1989) yielded adults which were smaller than those from natural infections even though they were gravid. Therefore, when worms are required for morphological analysis, the chicken embryo may not be an ideal host, although they might still provide an environment for the propagation of eggs if tests revealed that the eggs obtained were viable.

From the present study it appears that chickens provide the best environment for the development of humoural metacercariae from rainbow trout. It also appears that the strain of chicken is important as the metacercariae established in Marshall's Broilers much better than they did in Black Leghorn chicks. Nevertheless, metacercariae from the other three sources, the lens and retina of rainbow trout and the retina of perch, exhibited a better establishment and development in gull chicks. This difference in suitability of hosts may indicate a biological difference between the humoural form and the other three. This aspect, however, will be discussed in Chapter 4.

The recovery rate in both bird hosts was low, ranging from 1.33-33.33% in chickens and 1.56-50.00% in gull chicks. These low recovery rates have also been found by other authors: Lester & Huizinga (1977) recorded a 20 % recovery rate from herring gulls infected with D. adamsi, and Shostak et al. (1987) recorded recovery rates of 1.0-15.2% with ring billed gulls (Larus delawarensis Ord.) and 17.2-48.2% with herring gulls infected with D. baeri bucculentum. Perhaps a low recovery may be attributed to one or more of the following: a high percentage of the metacercariae may not have reached the infective stage; a proportion of metacercariae may have been damaged during innoculation; or perhaps these bird species are not entirely suitable hosts. Certainly chickens are not natural hosts of these digeneans and, therefore, may not provide the best physiological requirements for the establishment and development of Diplostomum. Herring gulls are not the natural host for all Diplostomum spp., but some species do occur in this bird naturally and this would suggest that it is more likely to be a more suitable host. There is also the possibility that, even if the worms establish in the birds, they may later be expelled if

an immunological response is initiated. In fact, Sweeting (1976) found that D. petromyzi fluviatilis would only develop in ducklings if they were immuno-suppressed. Normal ducklings expelled the metacercariae within 12 hours. It is more likely, however, that the natural establishment rate of this parasite, like many other, is relatively low. This is readily compensated for by their high reproductive potential.

The recovery rate of adult Diplostomum does not appear to be influenced by the number of metacercariae initially fed to the bird. Similarly, the recovery rate is not influenced, at least not during the period covered by this study, by the number of days after infection the adults are retrieved. This would suggest that adult Diplostomum are not expelled after establishment in any significant numbers. The large variance in recovery rates may be attributed, therefore, to variation in the establishment rates from host to host. This is a common phenomenon in parasitic infections.

The recovery rate is also not apparently influenced by the method of retrieving adults since a herring gull given a sub-lethal dose of Droncit showed a recovery rate of 21%. Certainly this could not be confirmed until many more birds were tested, but it does approximate to the expected figure. It was unfortunate that all the worms recovered were dead: perhaps it might be possible to use a lower dose of Droncit and obtain live worms. This method of recovery could prove very useful in the field, since the analysis of worm burden could be carried out without killing wild birds, some of which are protected in Britain. The fact that the worms were recovered within two hours is also encouraging, as the wild birds would not need to be caged for long periods.

Using adults derived from the lens of rainbow trout metacercariae as a guide, it was found that the hind-body of the adult worms was not fully grown until 16-18 days p.i. This seems rather a long time, as other workers, e.g. Niewiadomska (1984, 1987), have obtained fully grown adults within five or six days. In the present study, however, it was found that, if the rainbow trout lens adults were recovered prior to about 16 days post-infection, the ovary in the hind-body was situated very close to the intersegment. After 16 days the hind-body was much larger and the ovary was situated half way down the hind-body. Such a dramatic change was not obvious in the other three forms; but, for the taxonomic descriptions in Chapter 4, only worms recovered after 16 days were used. The time recorded for the full development for adult worms remains a major discrepancy between infections with gulls and chickens obtained by Niewiadomska (1984, 1987) and the results of this study.

Often the number of eggs present in the uterus is used as a guide to the maturity of adult worms. However, in this study it was found that there was no significant correlation between the total length of the worm and the number of eggs in the uterus. This is most probably due to the sporadic release of eggs from the uterus over a period of time.

After 18 days p.i. the length of the adult worms varies considerably. For the majority of worms it appears that, at least for the period of this study, they continue to increase in length the longer they remain in the bird host. Whatever the reason, it appears that the lengths of adult worms recovered are highly variable. It may be expected, therefore, that in the wild similar results may be found. Hence, it is essential that a good

variety of lengths are represented in each of the four forms for taxonomic analysis in Chapter 4.

In summary, therefore, humoural metacercariae from rainbow trout were best cultured in one day-old chickens, whereas the other three forms developed better in herring gull chicks. The recovery rate did not appear to be influenced by dose or time and, therefore, the same percentage of worms are likely to be recovered whether 30 or 300 metacercariae are cultured for 6 or 61 days. Results after 61 days p.i. are not available and, therefore, expulsion of worms may occur after this time. The number of eggs present in the uterus did not correlate with worm length and, therefore, it is not a good character to assess maturity. If the length of hind-body is used as a measure of maturity, it would appear that, in the lens form at least, full maturity of the worms is not attained until after 16 days post-infection.

CHAPTER 4:

THE TAXONOMY OF DIPLOSTOMUM ADULTS.

INTRODUCTION

The adults of the genus Diplostomum Nordmann, 1832 have been found to be much more easily classified than the larval stages. Adults of Diplostomum were first mentioned by Rudolphi (1819) who named them Distoma spathaceum. The true taxonomic position of Diplostomum was not established until decades later when the Erkhardt brothers obtained sexually mature specimens, referred to as Hemistomum spathaceum, from the common gull (Larus canus L.) which had been fed metacercariae (Sudarikov, 1964).

In classifying the genus Diplostomum, Dubois (1970) divided the genus into six subgenera. Dubois amalgamated the six subgenera into the one genus on the grounds that the adult stages are very similar. These were: Diplostomum, Tylodelphys Diesing, 1850, Dolichorchis Dubois, 1961, Adenodiplostomum Szidat & Nani, 1951 and Glossodiplostomoides Dubois, 1970. In Dubois' key (1970) adults of the subgenus Diplostomum are differentiated from the other subgenera by virtue of the fact that they lack a genital cone. In this comprehensive key 22 species of Diplostomum are described, and six others mentioned (Appendix 4). Of these 28 species, however, the full life-cycle is described for only six, namely, D. spathaceum (Rudolphi, 1819), D. mergi mergi Dubois, 1932, D. phoxini (Faust, 1918), D. gasterostei Williams, 1966, D. scudderi (Olivier, 1941) and D. micradenum (Cort & Brack, 1938). Each of these species have metacercarial stages present in the eyes or brain of freshwater fish, with the exception of D. micradenum which parasitises the central nervous system of frogs.

Another earlier key to strigeoids was produced by Sudarikov (1964). The

conception of the subgenus Diplostomum recognised by Dubois was treated as a full genus in this key due to the fact that the larval stages are somewhat different, especially the cercariae. Although over 20 species are described in Sudarikov's key (Appendix 2), only three which have a metacercarial stage in freshwater fish have a full account of their life-cycle, namely, D. spathaceum (Rudolphi, 1819), D. flexicaudum (Cort & Brooks, 1928) and D. murrayense (Johnston & Cleland, 1938). Sudarikov (1971, 1975) later revised this key and added another seven species to the list (Appendix 2).

Leading authors in the field of Diplostomum taxonomy, Shigin and Niewiadomska, have also produced descriptions of adult stages. Shigin (1961, 1965, 1968, 1977) showed that D. spathaceum described by Dubois (1970) did in fact comprise of two species differing both morphologically and biologically. He named one D. helveticum and retained D. spathaceum for the other. Niewiadomska (1984) pointed out, however, that the original description given by Shigin (1977) for D. helveticum corresponded to earlier descriptions given for D. spathaceum (see Olsson, 1876; Krause, 1914). Therefore, the earlier name D. spathaceum should in fact be used for this form. What Shigin (1977) named D. spathaceum was then re-named by Niewiadomska (1984) as a new species, D. pseudospathaceum. This species was further described and the life-cycle verified by Niewiadomska (1986, 1987). Shigin (1986) then declared that D. pseudospathaceum was in fact a synonym of D. chromatophorum (Brown, 1931), but this was disputed by Niewiadomska (1989).

Although there is some confusion as to the names which should be assigned to the various adult species of Diplostomum, there are very good

descriptions available in Dubois' 'Synopsis' (1970) and in work by Shigin (1977), Shigin et al. (1985) and Niewiadomska (1984; 1986; 1987). It is the aim of this chapter to try and identify the adults obtained in Chapter 3, since the taxonomy of the metacercariae is so confused (Chapter 2). In addition to describing the adults, an attempt was made to look at the differentiating characters and to decide what features appear to be taxonomically important for these forms of Diplostomum.

MATERIALS AND METHODS

1. Collection of Worms.

Adult worms were obtained by culturing metacercariae in chickens and herring gull chicks as described in Chapter 3. Only worms >18 days p.i. were used.

Metacercariae were obtained from the following five sources.

1. The lens of farmed rainbow trout from Loch Awe, Argyll (Type 1a).
2. The lens of rainbow trout experimentally infected in the laboratory (Type 1b).
3. The humour of farmed rainbow trout from Moffat Water, Dumfries. (Type 2).
4. The retina of farmed rainbow trout from Loch Awe, Argyll. (Type 3).
5. The retina of perch from Loch Tulla, Glen Orchy. (Type 4).

Two sources of lens metacercariae from rainbow trout were used in order to determine if the population of metacercariae influence the morphology of the resulting adults.

Twenty specimens of Types 1a, 1b and 4 adults were obtained from herring gull chicks, 15 specimens of Type 3 adults were obtained from herring gull chicks and 15 specimens of Type 2 adults were obtained from Marshall's Broiler chickens.

2. Treatment of Worms.

Light Microscope Analysis.

Adult worms were collected and washed two or three times in physiological saline to remove any mucus from their surface. All worms were fixed in Berland's fluid, stained in Mayer's paracarmine, cleared in Beechwood creosote and mounted in Canada Balsam. Measurements were taken on an Olympus BH2 microscope using bright light field.

Scanning Electron Microscope Analysis.

Adult worms were collected and washed four or five times by flushing with in sodium cacodylate buffer using a pipette. All worms were fixed in cacodylate buffered glutaraldehyde for 1 hour at 4°C, post-fixed in osmium tetroxide for 1 hr. at 4°C, dehydrated through an acetone series, critical point dried, mounted on stubs and coated in gold. Specimens were examined using an Hitachi Field Emission 800 microscope.

3. Removal of Size Effect From Data.

Before PCA could be carried out on the data it was necessary to remove the size effect. To do this the data were logged, the mean log taken for each row and then the mean subtracted from all characters. This method of standardising data removes the effects of geometric growth without affecting allometric growth.

4. Principal Components Analysis (PCA).

PCA was carried out on data, which had the size effect removed, using the covariance matrix. Only the first three dimensions were plotted against one another as these three axes account for the most variation. Characters used in the analysis were;

1. Total body length (BL).
2. Length of anterior segment (AL).
3. Breadth of anterior segment (AB).
4. Length of posterior segment (PL).
5. Breadth of posterior segment (PB).
6. Length of ovary (LOV).
7. Breadth of ovary (BOV).
8. Distance from middle of ventral sucker to anterior (VS-ANT).
9. Distance from middle of ovary to intersegment (OV-INT).
10. Extent of vitellaria from intersegment (INT-VIT).
11. Distance between lappets (LAPP).

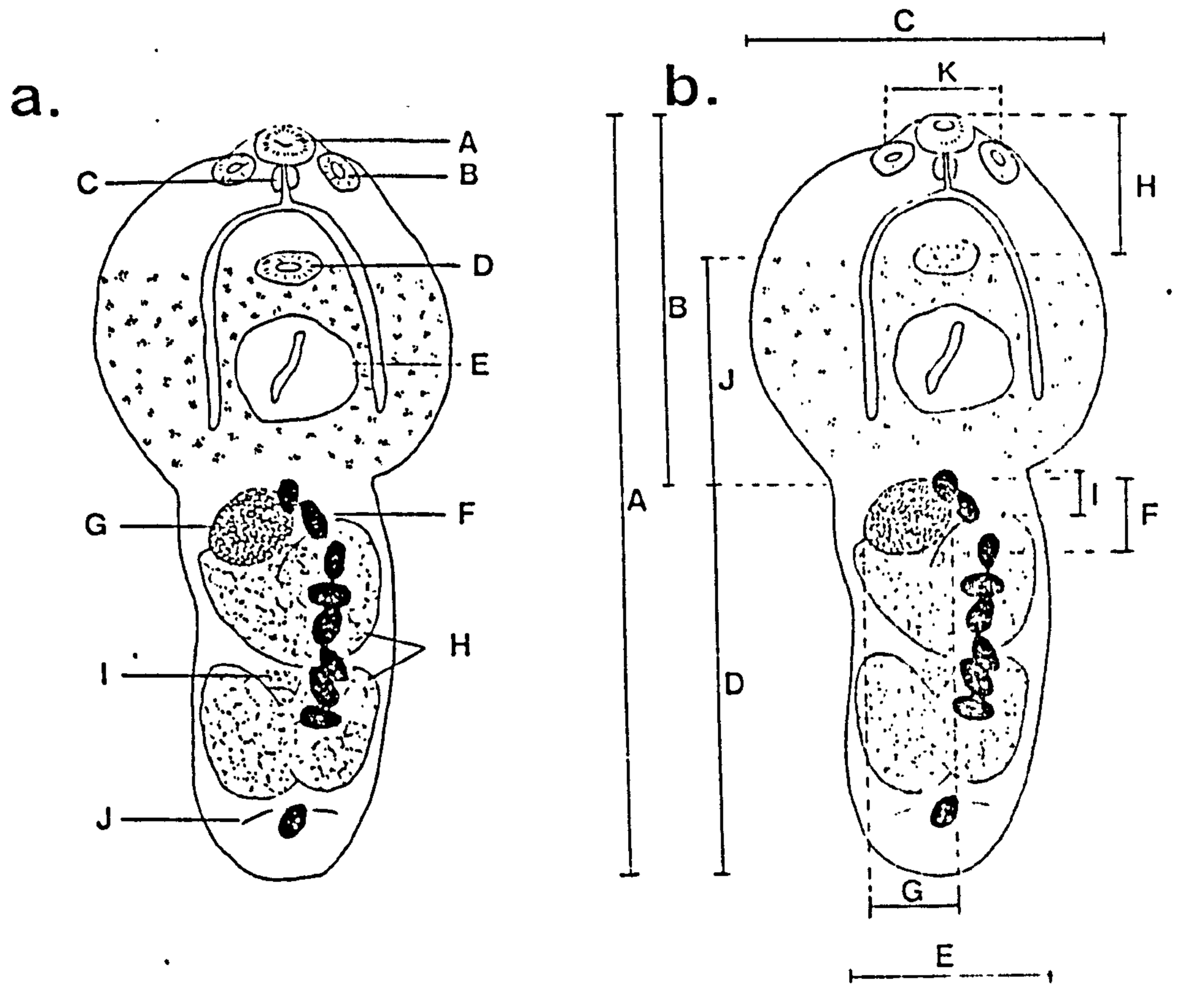
These measurements are illustrated in Fig. 4.1.

PCA and analyses of variance, correlation co-efficients and regression analyses were carried out using a Minitab package on a VAX mainframe computer.

Additional Measurements Taken.

1. Length of oral sucker (LOS).
2. Breadth of oral sucker (BOS).
3. Length of ventral sucker (LVS).
4. Breadth of ventral sucker (BVS).
5. Length of pharynx (LPH).
6. Breadth of pharynx (BPH).
7. Length of egg (L.EGG).

Fig. 4.1. Diagrams of Diplostomum adults indicating the internal organs and measurements taken.



a. A: oral sucker B: lappet C: pharynx D: ventral sucker E: Brande's organ F: egg G: ovary H: testes I: vitelline reservoir J: excretory pore.

b. A: BL B: AL C: AB D: PL E: PB F: LOV G: BOV H: VS-ANT I: OV-INT J: INT-VIT K: LAPP.

Note: all the abbreviations are explained in Section 4 of the 'Materials and Methods'.

RESULTS

1. Description of Adults.

Adult worms from the five different sources of metacercariae were analysed using the light microscope. Morphological measurements of each type are given in Tables 4.1-4.4. Measurements between the five types were analysed using analysis of variance (ANOVA). This was carried out on data with and without size effect removed for those characters used in the PCA. Results from the 'un-standardised' data reveal the real differences between the various types, whereas results from 'standardised' data reveal what differences would appear in the PCA. Results of the ANOVA are given in Table 4.7.

Correlation and regression analyses were carried out on all characters in order to determine which measurements significantly correlated with length. Results are illustrated in Table 4.8. It is concluded that out of the 11 characters used in PCA only the breadth of the ovary and the breadth of the posterior segment did not significantly correlate with length. This, therefore, justified the removal of the size effect before carrying out the PCA, otherwise false separations would be achieved merely between small and large worms. The size effect has not been removed, however, from the breadth of the ovary or the posterior segment as this would also produce false results.

Comparison of Types 1a and 1b (Table 4.7), both of which are adults obtained from lens of rainbow trout metacercariae, but from two different sources, reveal many differences in 'unstandardised' data (BL, AL, AB, PL,

VS-ANT, INT-VIT). However, on analysis of 'standardised' data, they only appear to differ in the distance between lappets. This again illustrates the usefulness of removal of the size effect in that without this step two similar worms would be falsely distinguished from one another. The fact that two populations differ in the distance between the lappets may cast some doubt on the use of this character. However, since the measurements for both of the populations were so significantly different from the others, this character is still very useful in separating Type 1 worms from the other three.

Type 1 adults (Fig. 4.16 & Plate 4.1a, 4.2a,b)

These adults obtained from lens metacercariae cultured in herring gull chicks are relatively large in size, ranging from 1.313 to 2.550 mm overall. The hind-body accounts for the majority of the length (approx. 62%). The intersegmental region is clearly defined.

In length the oral and ventral suckers are fairly similar (0.076mm and 0.077mm, respectively), however, in breadth the ventral sucker is by far the largest (0.093mm compared to 0.077mm). The ratio of the length multiplied by the width of the oral to ventral sucker is 0.785. The ventral sucker is situated approximately 55.3% along the anterior segment (from the anterior end), normally very close to Brandes' organ. The lappets are relatively close together, 0.100-0.150mm.

The vitelline follicles are well developed and extend approximately 44.7% along the anterior segment, normally anterior to the ventral sucker.

The ovary varies in shape from being ellipsoid in some specimens to

almost circular in others. It is situated approx. one third of the way down the posterior segment just anterior to the anterior testes. The testes are transversely elongate. The posterior testis is bilobed, measuring 0.220-455 x 240-385mm. The anterior testis is single lobed, measuring 0.210-340 x 195-390mm. The eggs measure 0.075-0.100 x 0.045-0.060mm.

Type 2 adults (Fig. 4.17 & Plate 4.1b,4.2c,d)

These adults were obtained from humoural metacercariae from rainbow trout. They did not grow very well in herring gull chicks, but developed better in domestic chickens. All the specimens described were cultured in domestic chickens.

These adults are relatively small in size, 0.698-1.193mm. This may be accounted for by the fact that they are grown in such an unnatural host. However, these are the best specimens available. The intersegmental region is not so clearly defined compared to the other worm types.

The oral sucker tends to be longer but less broad than the ventral sucker. Dimensions of oral and ventral sucker are 0.066-0.090 x 0.073-0.092 and 0.054-0.079 x 0.072-0.103mm, respectively. The ratio of oral to ventral sucker, calculated as above, is 1.10, making the oral sucker slighly the larger of the two. In some specimens, however, they are equal in size. The ventral sucker is situated 59% along the anterior segment (from the anterior end), just anterior to Brandes' organ. The lappets are spread well apart, 0.127-0.254mm.

The vitellarium is not so clearly defined and this may reflect again the unsuitability of the final host. However, it extends 51% along the anterior segment, to the level of, or just anterior to, the ventral sucker.

The ovary is very small in size, 0.056-0.081 x 0.054-0.078mm, and is almost circular in shape. The middle of the ovary is level with the anterior region of the anterior testis. Often the ovary is located in the intersegmental region, half into the posterior segment and half into the anterior segment. Testes are transversely elongate, the posterior one being bilobed. The anterior testis measures 0.100-0.190 x 0.110-0.200mm and the posterior testis measures 0.120-0.200 x 0.115-0.230mm. The eggs measure 0.081-0.101 x 0.046-0.055mm.

Type 3 adults (Fig. 4.18 & Plate 4.1c,4.3a,b)

These adults were obtained from retina of rainbow trout metacercariae cultured in herring gull chicks. They are avarage in size compared to the other types of adults, measuring 1.000-1.650mm. In the majority of the specimens the posterior segment is shorter than the anterior, but in a few the posterior segment is the larger. The intersegmental region is clearly defined.

The ventral sucker is slightly larger than the oral sucker. The ratio of oral to ventral sucker is 0.85. The ventral sucker is situated 52% along the anterior segment (from the anterior end), and on average the distance from the posterior margin of the ventral sucker to the anterior margin of Brandes' organ is 0.015-0.025mm. The lappets are relatively far apart,

0.130-0.210mm.

The vitellarium is clearly defined and extends 54% along the anterior segment to the anterior margin of the ventral sucker.

The ovary is ellipsoid in shape, measuring 0.070-0.125 x 0.095-0.150mm. It is situated just posterior to the intersegmental region anterior to the anterior testis. The testes are transversely elongate, the posterior one being bilobed. The anterior testis measures 0.115-0.250 x 0.115-0.405mm, and the posterior one measures 0.150-0.275 x 0.220-0.420mm. The eggs measure 0.080-0.115 x 0.040-0.070mm.

Type 4 adults (Fig. 4.19 & Plate 4.1d, 4.3c,d)

These adults were obtained from retina of perch metacercariae cultured in herring gull chicks. In length they are very similar to Type 3 adults ranging from 0.950-1.650mm. The posterior segment is normally shorter than the anterior segment, although in some worms they were of equal length. The intersegmental region is evident but not so clearly defined as Types 1 and 3.

The oral sucker is noticeably smaller than the ventral sucker in most specimens, the oral to ventral sucker ratio being 0.84. The ventral sucker is situated 55% along the anterior segment (from the anterior extremity) and the posterior margin of the ventral sucker is on average 0.020mm away from the anterior margin of Brandes' organ. The lappets are spread well apart at 0.125-0.188mm.

The vitellarium is clearly defined and extends 60% along the anterior segment, to the level of, or just anterior to, the anterior margin of the ventral sucker.

The ovary is ellipsoid in shape and is situated just posterior to the intersegmental region, but anterior to the anterior testis. The testes are tranversely elongate, the anterior measuring 0.105-0.225 x 0.125-0.275mm, the bilobed posterior one measuring 0.135-0.305mm. The eggs measure 0.090-0.105 x 0.035-0.070mm.

No difference was evident in the terminal genitalia of the four types of adult worms when histological sections were examined under the microscope. (Histological preparations were kindly made by Mr D.W. Cooper of the British Museum (Natural History), London).

TABLE 4.1a. Morphological measurements (mm) of gravid adults obtained from lens of rainbow trout metacercariae (Type 1a)

Character	No.	Mean SD	Range
BL	20	1.952±0.375	1.313-2.550
AL	20	0.718±0.156	0.440-1.000
AB	20	0.529±0.042	0.430-0.610
PL	20	1.222±0.253	0.775-1.550
PB	20	0.371±0.046	0.300-0.470
LOV	20	0.114±0.012	0.090-0.130
BOV	20	0.122±0.017	0.090-0.150
VS-ANT	20	0.393±0.091	0.200-0.570
OV-INT	20	0.411±0.130	0.200-0.610
INT-VIT	20	0.320±0.057	0.210-0.450
LAPP	20	0.127±0.015	0.100-0.155
LOS	20	0.075±0.008	0.060-0.088
BOS	20	0.076±0.009	0.063-0.090
LVS	20	0.074±0.009	0.060-0.085
BVS	20	0.090±0.009	0.073-0.100
LPH	20	0.064±0.006	0.053-0.073
BPH	20	0.046±0.004	0.040-0.053
L.EGG	20	0.090±0.006	0.080-0.100

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

TABLE 4.1b. Morphological measurements (mm) of gravid adults obtained from lens of rainbow trout metacercariae (Type 1b).

Character	No.	Mean SD	Range
BL	20	2.163±0.173	1.825-2.525
AL	20	0.825±0.108	0.620-0.980
AB	20	0.578±0.076	0.390-0.770
PL	20	1.342±0.104	1.125-1.525
PB	20	0.392±0.077	0.300-0.510
LOV	20	0.129±0.091	0.080-0.125
BOV	20	0.120±0.016	0.090-0.150
VS-ANT	20	0.461±0.075	0.330-0.590
OV-INT	20	0.418±0.062	0.275-0.540
INT-VIT	20	0.369±0.068	0.290-0.500
LAPP	20	0.124±0.015	0.095-0.150
LOS	20	0.077±0.008	0.065-0.095
BOS	20	0.071±0.007	0.060-0.085
LVS	20	0.080±0.011	0.060-0.100
BVS	20	0.096±0.012	0.070-0.115
LPH	20	0.063±0.007	0.050-0.078
BPH	20	0.042±0.005	0.035-0.053
LEGG	20	0.091±0.007	0.075-0.100

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

TABLE 4.2. Morphological measurements (mm) of gravid adults obtained from humour of rainbow trout metacercariae (Type 2).

Character	No.	Mean SD	Range
BL	19	0.938±0.143	0.698-1.193
AL	19	0.485±0.064	0.349-0.636
AB	19	0.492±0.070	0.318-0.581
PL	19	0.465±0.127	0.260-0.808
PB	19	0.374±0.052	0.254-0.457
LOV	19	0.066±0.020	0.056-0.081
BOV	19	0.093±0.018	0.054-0.078
VS-ANT	19	0.288±0.035	0.221-0.368
OV-INT	19	0.017±0.031	0.001-0.095
INT-VIT	19	0.249±0.042	0.190-0.349
LAPP	19	0.162±0.029	0.127-0.254
LOS	19	0.077±0.007	0.066-0.090
BOS	19	0.082±0.005	0.073-0.092
LVS	19	0.064±0.009	0.054-0.079
BVS	19	0.090±0.008	0.072-0.103
LPH	19	0.068±0.008	0.051-0.079
BPH	19	0.046±0.007	0.038-0.058
L.EGG	19	0.087±0.006	0.081-0.101

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

TABLE 4.3. Morphological measurements (mm) of gravid adults obtained from retina of rainbow trout metacercariae (Type 3).

Character	No.	Mean SD	Range
BL	15	1.274±0.229	1.000-1.650
AL	15	0.633±0.119	0.470-0.880
AB	15	0.515±0.095	0.265-0.640
PL	15	0.627±0.139	0.410-0.930
PB	15	0.367±0.061	0.210-0.470
LOV	15	0.100±0.014	0.070-0.125
BOV	15	0.127±0.018	0.095-0.150
VS-ANT	15	0.331±0.055	0.265-0.445
OV-INT	15	0.083±0.051	0.001-0.175
INT-VIT	15	0.339±0.090	0.200-0.500
LAPP	15	0.165±0.023	0.130-0.210
LOS	15	0.082±0.012	0.055-0.100
BOS	15	0.095±0.012	0.075-0.115
LVS	15	0.086±0.012	0.065-0.105
BVS	15	0.106±0.011	0.090-0.125
LPH	15	0.073±0.008	0.060-0.090
BPH	15	0.053±0.008	0.040-0.065
L.EGG	15	0.094±0.009	0.080-0.115

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

TABLE 4.4. Morphological measurements (mm) of gravid adults obtained from retina of perch metacercariae (Type 4).

Character	No.	Mean SD	Range
BL	20	1.309±0.195	0.950-1.650
AL	20	0.687±0.113	0.500-0.850
AB	20	0.523±0.058	0.450-0.700
PL	20	0.609±0.106	0.400-0.770
PB	20	0.320±0.034	0.240-0.380
LOV	20	0.088±0.011	0.070-0.105
BOV	20	0.106±0.013	0.090-0.130
VS-ANT	20	0.378±0.062	0.260-0.465
OV-INT	20	0.061±0.026	0.001-0.100
INT-VIT	20	0.411±0.075	0.275-0.550
LAPP	20	0.146±0.014	0.125-0.188
LOS	20	0.077±0.006	0.070-0.095
BOS	20	0.088±0.011	0.070-0.110
LVS	20	0.087±0.010	0.070-0.100
BVS	20	0.093±0.007	0.085-0.110
LPH	20	0.072±0.047	0.065-0.080
BPH	20	0.050±0.005	0.040-0.060
L.EGG	20	0.097±0.006	0.090-0.105

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

2. Identification.

Having described the four types of worms, an attempt to identify them using Dubois' 'Synopsis' was made. Type 1 adults keyed down to Diplostomum spathaceum. Measurements for Type 1 adults can be compared with those given for D. spathaceum sensu Niewiadomska (1984) in Table 4.5.

Identification of the other three types of adult worms was not readily achieved. All three appeared to be similar to D. mergi, D. parviventosum and D. mahonae. Measurements of these three species are given in Table 4.6.

TABLE 4.5. Mean measurements (mm) for D. spathaceum sensu Niewiadomska (1984) compared to Type 1 adults.

Character	Niewiadomska 1984	Type 1 (mean)
BL	up to 4mm	2.058
AL	1.11-1.48	0.772
AB	0.59-0.85	0.554
PL	1.56-2.92	1.282
PB	0.56-0.66	0.382
LOV	0.138-0.222	0.122
BOV	0.163-0.236	0.121
LVS	0.078-0.095	0.077
BVS	0.089-0.102	0.093
LPH	0.059-0.074	0.064
BPH	0.051-0.074	0.044

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

TABLE 4.6. Mean measurements (mm) for D. mergi, D. parviventosum and D. mahonae according to Dubois (1970).

Character	D. mergi	D. parviventosum	D. mahonae
BL	1.600	1.500	1.440
AL	0.52-1.08	0.66-0.81	0.54-0.74
AB	0.23-0.56	0.34-0.50	0.33-0.56
PL	0.25-0.70	0.54-0.81	0.48-0.70
PB	0.19-0.54	0.36-0.49	0.31-0.49
LOV	0.058-0.100	0.100-0.172	0.080-0.104
BOV	0.063-0.140	0.117-0.135	0.107-0.150
LOS	0.036-0.076	0.040-0.050	0.068-0.095
BOS	0.036-0.083	-	0.078-0.107
LVS	0.040-0.114	0.072-0.097	0.073-0.094
BVS	0.050-0.136	-	0.073-0.094
LPH	0.040-0.070	0.054-0.060	0.065-0.095
BPH	0.021-0.050	0.043-0.045	0.057-0.094
VIT limit	ant. margin of VS or further	ant. margin of VS or further	30-39% from anterior

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

TABLE 4.7. Results of analysis of variance on adult data.

Character	Type 1a	Type 1b	Type 2	Type 3	Type 4
BL	1.952 c	2.163 d	0.938 a	1.274 b	1.309 b
BL(st)	1.613 ab	1.651 b	2.337 c	1.491 a	1.529 ab
AL	0.718 c	0.825 d	0.485 a	0.633 b	0.687 bc
AL(st)	0.608 a	0.681 ab	1.681 d	0.791 bc	0.883 c
AB	0.529 a	0.578 b	0.492 a	0.515 a	0.523 a
AB(st)	0.322 a	0.325 a	1.693 c	0.581 b	0.618 b
PL	1.222 c	1.342 d	0.465 a	0.627 b	0.609 b
PL(st)	1.141 b	1.174 b	1.611 c	0.774 a	0.760 a
PB	0.371 b	0.392 b	0.374 b	0.367 b	0.320 a
LOV	0.114 bc	0.229 c	0.066 a	0.100 b	0.088 b
LOV(st)	-1.214 b	-1.261 b	-2.063 a	-1.046 b	- 1.169 b
BOV	0.122 c	0.120 c	0.090 a	0.127 c	0.106 b
VS-ANT	0.392 b	0.461 c	0.288 a	0.331 a	0.378 b
VS-ANT(st)	-0.002 b	0.095 b	1.160 c	0.144 bc	0.285 d
OV-INT	0.411 c	0.418 c	0.017 a	0.083 b	0.061 ab
OV-INT(st)	0.023 d	-0.001 cd	-3.413 a	-1.771 b	-1.757 bc
INT-VIT	0.320 b	0.369 cd	0.249 a	0.339 bc	0.411 d
INT-VIT(st)	-0.194 a	-0.130 a	1.010 d	0.149 b	0.365 c
LAPP	0.127 a	0.124 a	0.162 c	0.165 c	0.146 b
LAPP(st)	-1.107 b	-1.214 a	0.579 d	-0.545 c	-0.656 c
(Characters not used in PCA)					
LOS	0.075 a	0.077 ab	0.077 ab	0.082 b	0.077 a
BOS	0.076 a	0.072 a	0.082 b	0.095 c	0.088 b
LVS	0.074 b	0.080 c	0.065 a	0.086 c	0.087 c
BVS	0.090 a	0.096 a	0.090 a	0.106 b	0.093 a
LPH	0.064 ab	0.063 a	0.068 bc	0.073 d	0.072 cd
BPH	0.046 a	0.043 a	0.047 ab	0.053 c	0.050 bc

Note: st = standardised data. All other abbreviations are explained in Section 4 of the 'Materials and Methods'. The measurements given are means. Means with same postscript are not significantly different from one another; means with different postscripts are significantly different, $P < 0.05$. Postscripts are given in order of size.

TABLE 4.8. Results of regression and correlation analyses on adult data.

Character	Regression Equation	R-sq%	Correlation	Sig.
AL	y = 0.266 + 0.264x	73.2%	0.855	P < 0.01
AB	y = 0.458 + 0.046x	10.4%	0.323	P < 0.01
PL	y = -0.270 + 0.736x	94.3%	0.971	P < 0.01
PB	y = 0.345 + 0.013x	1.2%	0.111	NS
LOV	y = 0.064 + 0.023x	44.0%	0.663	P < 0.01
BOV	y = 0.106 + 0.007x	3.6%	0.188	NS
VS-ANT	y = 0.163 + 0.136x	63.8%	0.798	P < 0.01
OV-INT	y = -0.324 + 0.343x	84.0%	0.916	P < 0.01
INT-VIT	y = 0.221 + 0.076x	21.2%	0.460	P < 0.01
LAPP	y = 0.182 - 0.025x	25.0%	-0.500	P < 0.01
LOS	y = 0.077 + 0.000x	0.1%	0.023	NS
BOS	y = 0.096 - 0.009x	16.1%	-0.402	P < 0.01
LVS	y = 0.066 + 0.008x	10.3%	0.322	P < 0.01
BVS	y = 0.090 + 0.003x	1.6%	0.127	NS
LPH	y = 0.074 - 0.004x	6.8%	-0.260	P < 0.01
BPH	y = 0.051 - 0.002x	3.1%	-0.177	NS

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

3. Principal Components Analysis (PCA).

Results of all the PCA's are illustrated in Figs 4.9-4.15 and their corresponding tables. For each PCA three graphs may be plotted, although only the graph which shows the best separation of the forms being examined is illustrated. The mixture of positive and negative loadings on all eigenvector values indicate that these are shape components (Blackith & Reyment, 1971) and, therefore, ensures that the effects of size have been removed. The most important differentiating characters are listed in Tables 4.9-4.15. These are represented by the highest values (ignoring sign) of eigenvectors in the most important axis.

PCA 1.

From Fig. 4.9 it can be clearly seen that Type 1 worms are different morphometrically from Type 2. The most important separating characters (Table 4.9) are also clearly defined. The position of the ovary in the posterior segment is the strongest character, which is not surprising since, in the Type 1 worms, it is situated one third of the way down the posterior segment, whereas, in Type 2 worms it is situated at the intersegment. Type 2 worms also have a very small ovary compared to the other types. Furthermore there is a distinct difference in the distance between the lappets, since in Type 1 worms they are very close together, whereas in Type 2 worms they are spread fairly well apart. This differentiating character was also apparent in the PCA of the metacercarial stage of these types in Chapter 2.

PCA 2.

From Fig. 4.10 it can be seen that Type 1 worms also differ morphometrically from Type 3 worms. In this instance there are only two characters which contribute to any great extent to the separation (Table 4.10). The very high value of 0.915 for the distance between the ovary and the intersegment reveals what a good distinguishing character this is. Type 1 adults seem to be distinguished from the other types in rainbow trout by virtue of the fact that the ovary is situated one third of the way down the posterior segment. They can also be distinguished by the fact that the lappets are positioned very close together, as this character is again important in the separation of these two forms.

PCA 3.

From Fig. 4.11 it can be seen that Type 2 adults also separate out morphometrically from Type 3 adults. However, there are two specimens of Type 2 adults which have separated alongside Type 3. This could be due to some Type 2 metacercariae being fed to the herring gull chicks when being infected. However, it was found that Type 2 metacercariae did not establish well in herring gulls. It is more likely, therefore, that for some reason these are Type 2 adults but have characters very similar to those of Type 3. This may be due to the high amount of variation found in the adult worms so that some appear very different compared to the others. The most important separating character here is the dimensions of the ovary (Table 4.11). As discussed earlier, the ovary of Type 2 worms is very small in size compared to the others. This appears, therefore, to be the most important distinguishing character which separates Type 2 adults from the others in rainbow trout. The distance from the intersegment to the ovary is also an important character. From Table 4.7 it can be seen

that in Type 2 adults the ovary is situated significantly closer to the intersegmental region than all the other types.

PCA 4.

This analysis was carried out to illustrate how the adult forms of the different rainbow trout metacercariae compare to each other. It can be seen from Fig. 4.12 that Type 1 and Type 2 worms are furthest apart with Type 3 worms in the middle. This can perhaps be explained by the fact that the distance from the ovary to the intersegment is an important character in the separation. In Type 1 worms the ovary is situated furthest along the posterior segment, Type 3 worms the second furthest, and Type 2 worms are the closest to the intersegmental region.

PCA 5.

Fig. 4.13 reveals that metacercariae from the same site in the eye, but from two different host species, produce morphologically different adults. Types 3 and 4 differ mainly in the extent of the vitellarium. In Table 4.7 it can be seen that the vitellarium extends further anteriorly in Type 4 adults (60%) than in Type 3 (54%). From the descriptions of the two adults it is evident that in Type 3 adults the vitellarium extends just to the anterior margin of the ventral sucker, whereas in Type 4 adults it often extends further forward than the ventral sucker. This is accentuated by the fact that the ventral sucker in Type 4 worms is also situated more anteriorly than that of Type 3. The distance from the ovary to the intersegment also makes up quite a large proportion of the separating axis. This character is not, however, significantly different in the two types of worms and this may be the cause of the overlap between the two forms.

PCA 6.

From Fig. 4.14 it can be seen that Types 2,3 and 4 separate fairly well from one another. Types 2 and 4 are furthest apart with Type 3 in the middle. As expected from the previous PCAs with Type 2 adults the most important differentiating characters are the position of the ovary and its dimensions (Table 4.7). From Table 4.14 a similar picture to that in Fig 4.14 is seen in that the ovary is most closely situated to the intersegment in the Type 2 worms, and the furthest away from the intersegment in Type 3 worms. This character may account for the order of separation of the three forms.

PCA 7.

As indicated in Table 4.15 separation in Fig. 4.15 in not very clear. However, along Vector 3 some separation of Type 1 and Type 4 worms occurs. This axis is made up mainly of the distance from the intersegment to the ovary and the length of the posterior segment. The extent of the vitellarium perhaps accounts for the position of the forms on the graph. The vitellarium extends the furthest in Type 4 worms (60%) and least in Type 1 worms (44.7%) with Type 3 worms in the middle (54%). The same is also true for the length of the posterior segment, in that it is longest in Type 1 adults and shortest in Type 4 adults. These features may well account for the large amount of overlap in Fig. 4.15.

TABLE 4.9. PCA 1. Lens of rainbow trout (Type 1) vs humour of rainbow trout (Type 2) adults.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	12.282	0.930	0.930
PC 2	0.729	0.055	0.985
PC 3	0.114	0.009	0.994

	Eigenvectors		
Variable	PC 1	PC 2	PC 3
BL	-0.127	-0.028	-0.366
AL	-0.173	-0.013	-0.206
AB	-0.215	-0.022	0.292
PL	-0.091	-0.064	-0.549
PB	-0.226	0.025	0.227
LOV	0.445	0.427	-0.074
BOV	0.457	0.475	0.151
VS-ANT	-0.183	-0.007	-0.102
OV-INT	0.556	-0.766	0.107
INT-VIT	-0.193	-0.003	-0.117
LAPP	-0.250	-0.025	0.438

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

Analysis of eigenvector values for vector 1 reveal that the most important separating characters are;

1. Distance from ovary to intersegment
2. Dimensions of the ovary
3. Distance between lappets

Fig. 4.9. PCA 1. Lens of rainbow trout (Type 1) vs humour of rainbow trout (Type 3) adults.

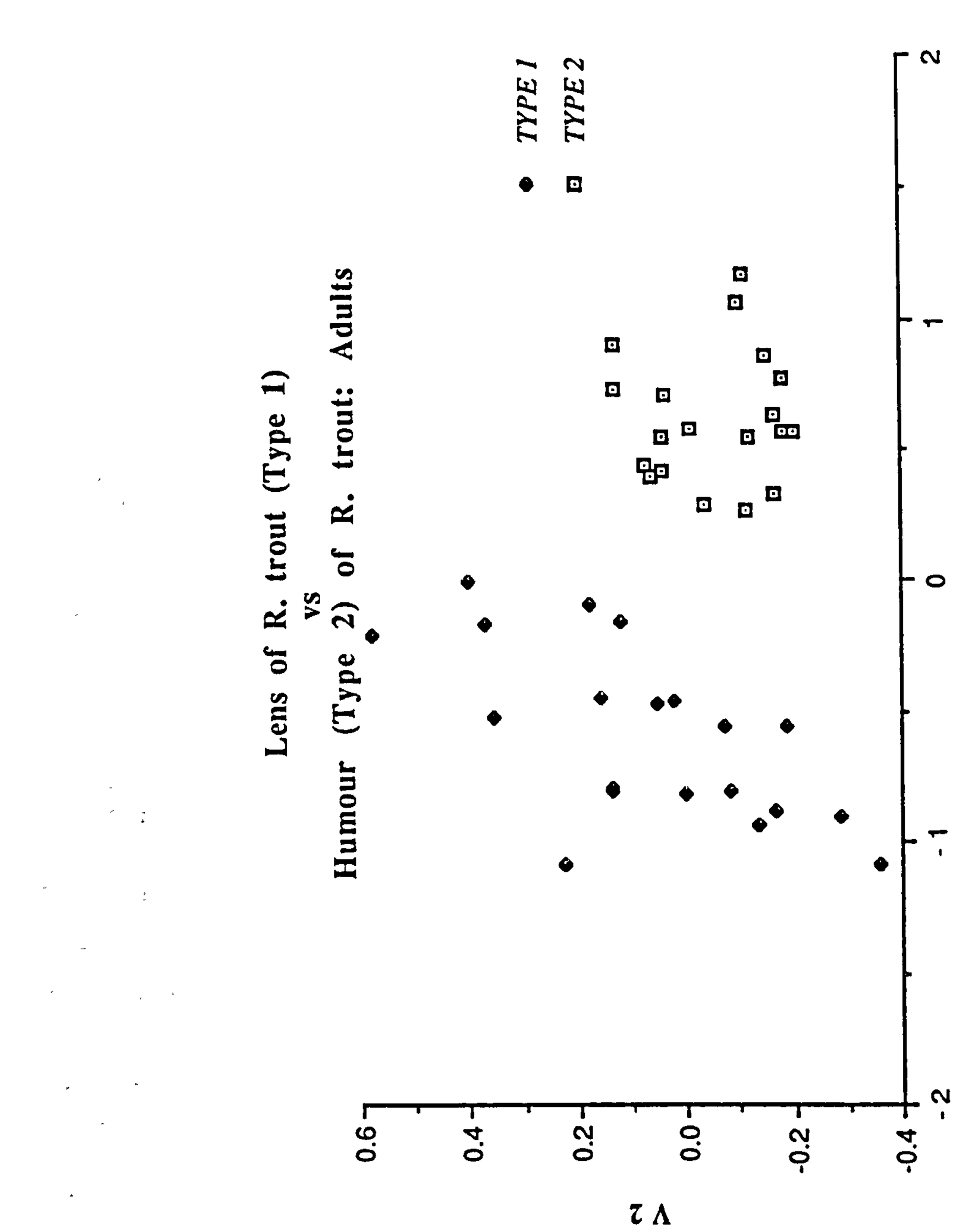


TABLE 4.10. PCA 2. Lens of rainbow trout (Type 1) vs retina of rainbow trout (Type 3) adults.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	1.446	0.854	0.854
PC 2	0.094	0.056	0.910
PC 3	0.054	0.032	0.942

	EIGENVECTORS		
Variable	PC 1	PC 2	PC 3
BL	0.018	0.318	0.105
AL	-0.077	0.371	-0.062
AB	-0.134	-0.309	-0.148
PL	0.106	0.324	0.197
PB	-0.159	-0.252	-0.281
LOV	-0.083	-0.281	0.862
BOV	-0.167	-0.255	-0.082
VS-ANT	-0.072	0.426	-0.087
OV-INT	0.912	-0.195	-0.120
INT-VIT	-0.103	0.166	-0.174
LAPP	-0.239	-0.214	-0.210

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

Analysis of eigenvector values for vector 1 reveals that the most important separating characters are;

1. Distance from ovary to intersegment
2. Distance between lappets

Fig. 4.10. PCA 2. Lens of rainbow trout (Type 1) vs retina of rainbow trout (Type 2) adults.

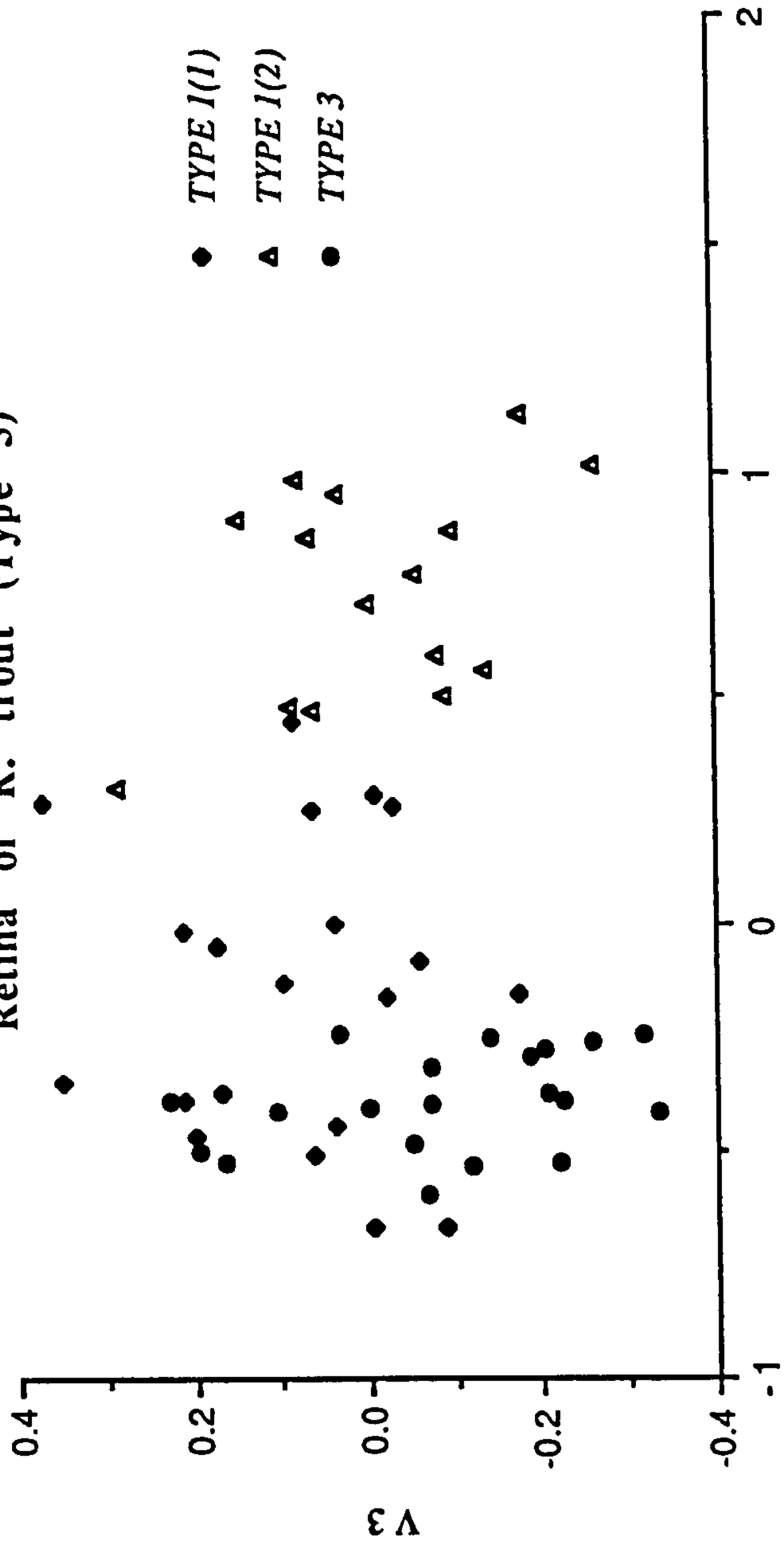


TABLE 4.11. PCA 3. Humour of rainbow trout (Type 2) vs retina of rainbow trout (Type 3) adults.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	10.067	0.837	0.837
PC 2	1.795	0.149	0.986
PC 3	0.091	0.008	0.993

	EIGENVECTORS		
Variable	PC 1	PC 2	PC 3
BL	-0.163	0.020	0.256
AL	-0.170	0.038	0.237
AB	-0.206	0.017	-0.456
PL	-0.159	-0.017	0.378
PB	-0.214	0.037	-0.257
LOV	0.516	0.327	0.038
BOV	0.556	0.368	-0.079
VS-ANT	-0.185	0.035	0.037
OV-INT	0.393	-0.867	-0.038
INT-VIT	-0.168	0.019	0.400
LAPP	-0.199	0.022	-0.216

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

Analysis of eigenvector values for vector 1 reveals that the most important separating characters are;

1. Dimensions of ovary
2. Distance from ovary to intersegment
3. Breadth of anterior segment

Fig. 4.11. PCA 3. Humour of rainbow trout (Type 2) vs retina of rainbow trout (Type 3) adults.

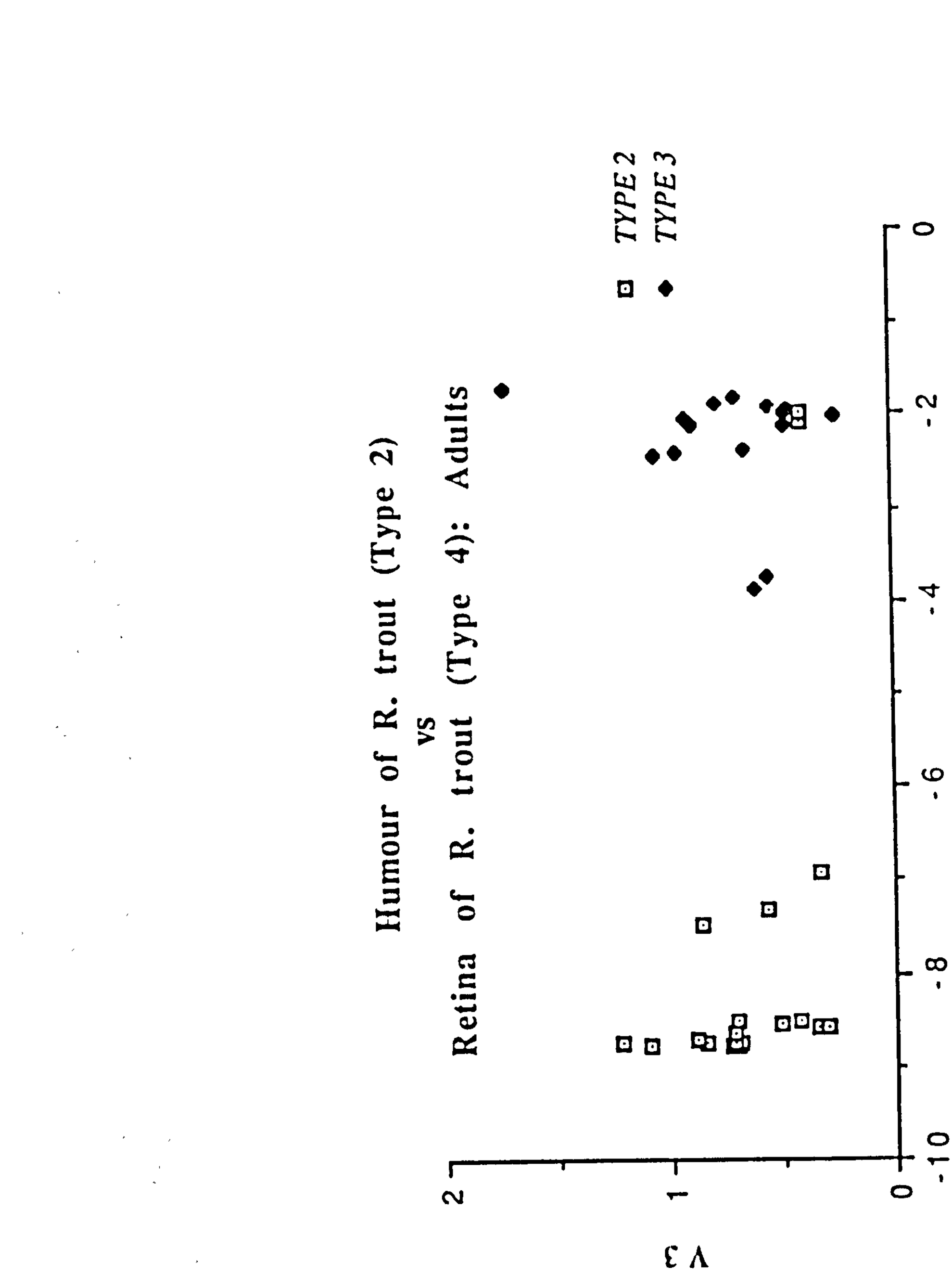


TABLE 4.12. PCA 4. Lens (Type 1) vs humour (Type 2) vs retina of rainbow trout (Type 3) adults.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	8.322	0.859	0.859
PC 2	1.141	0.118	0.977
PC 3	0.107	0.011	0.988

	EIGENVECTORS		
Variable	PC 1	PC 2	PC 3
BL	-0.127	-0.074	-0.362
AL	-0.169	-0.013	-0.207
AB	-0.216	0.001	0.288
PL	-0.094	-0.141	-0.517
PB	-0.230	0.026	0.184
LOV	0.450	0.413	-0.139
BOV	0.457	0.487	0.116
VS-ANT	-0.180	-0.022	-0.170
OV-INT	0.551	-0.749	0.198
INT-VIT	-0.185	0.003	-0.064
LAPP	-0.256	0.069	0.472

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

Analysis of eigenvector values for vector 1 reveals that the most important separating characters are;

1. Distance from ovary to intersegment
2. Dimensions of ovary
3. Distance between lappets

Fig. 4.12. PCA 4. Lens (Type 1) vs humour (Type 2) vs retina (Type 3) of rainbow trout adults.

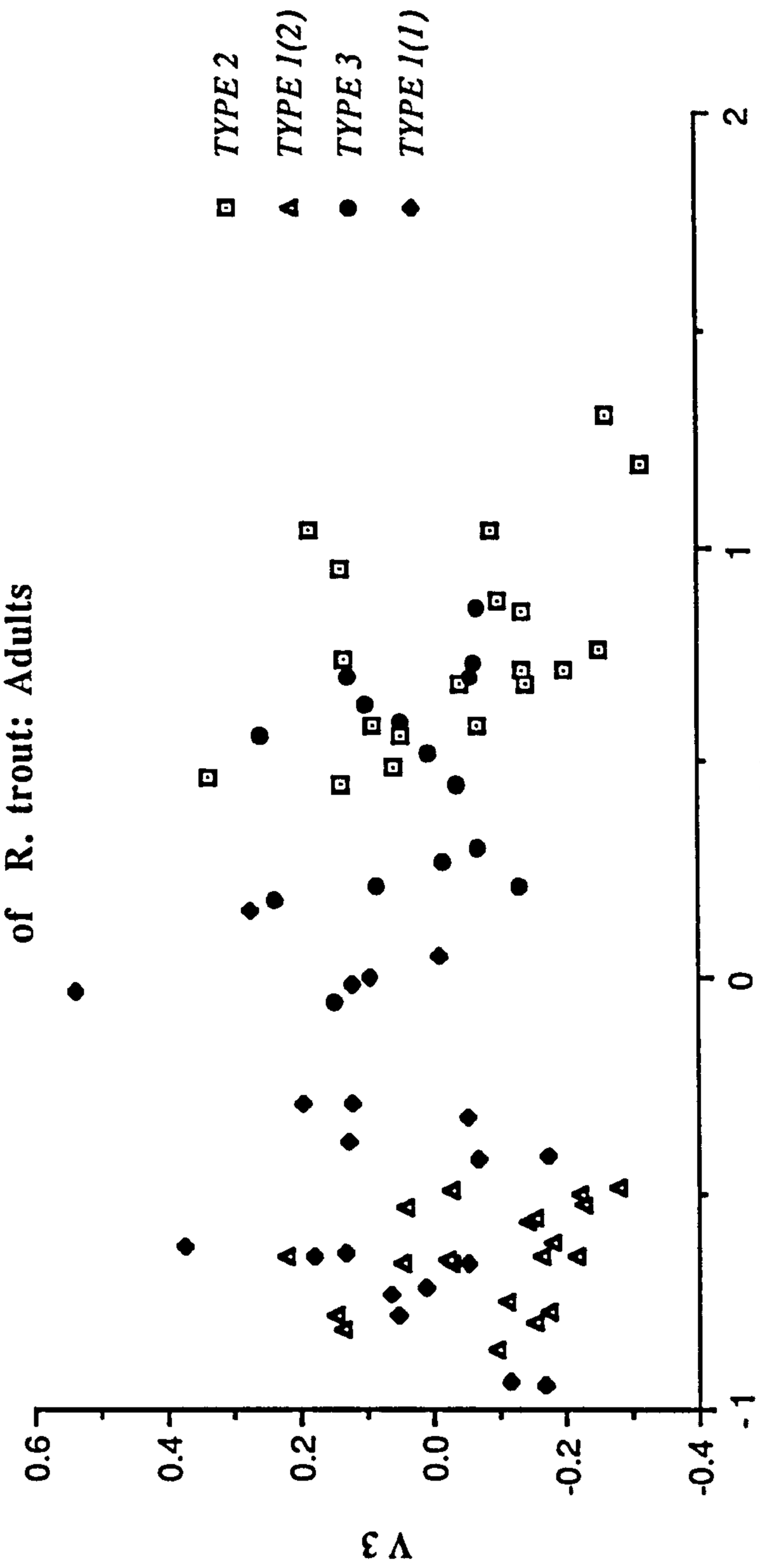


TABLE 4.13. PCA 5. Retina of rainbow trout (Type 3) vs retina of perch (Type 4) adults.

Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	1.465	0.876	0.876
PC 2	0.114	0.068	0.944
PC 3	0.037	0.022	0.966

	EIGENVECTORS		
Variable	PC 1	PC 2	PC 3
BL	-0.081	-0.226	-0.082
AL	-0.106	-0.322	-0.019
AB	-0.105	0.271	0.651
PL	-0.038	-0.142	-0.152
PB	-0.123	0.161	0.244
LOV	-0.077	0.188	-0.417
BOV	-0.111	0.201	-0.223
VS-ANT	-0.114	-0.315	0.169
OV-INT	0.950	0.033	0.023
INT-VIT	-0.077	-0.500	0.098
LAPP	-0.119	0.250	0.008

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

Analysis of eigenvector values for vector 2 reveals that the most important separating character are;

1. Extent of vitellaria from intersegment
2. Length of anterior segment
3. Distance from ventral sucker to anterior

However some separation also occurs along vector 3. Analysis of eigenvector values for this vector reveals that the next most important separating characters are;

1. Breadth of anterior segment
2. Length of ovary
3. Breadth of posterior segment

Fig. 4.13. PCA 5. Retina of rainbow trout (Type 3) vs retina of perch (Type 4) adults.

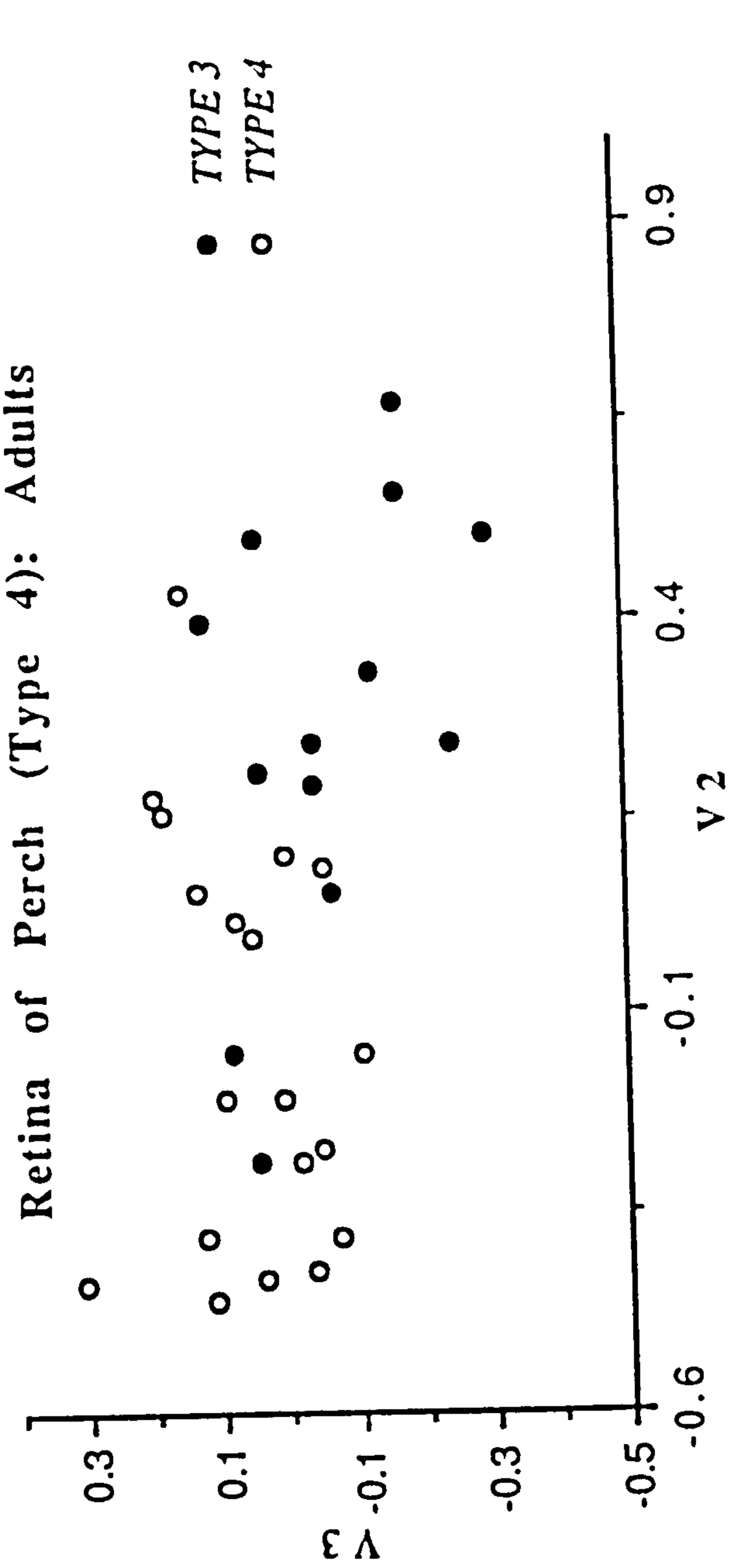


TABLE 4.15. PCA 7. Lens of rainbow trout (Type 1) vs retina of rainbow trout (Type 3) and perch (Type 4) adults.

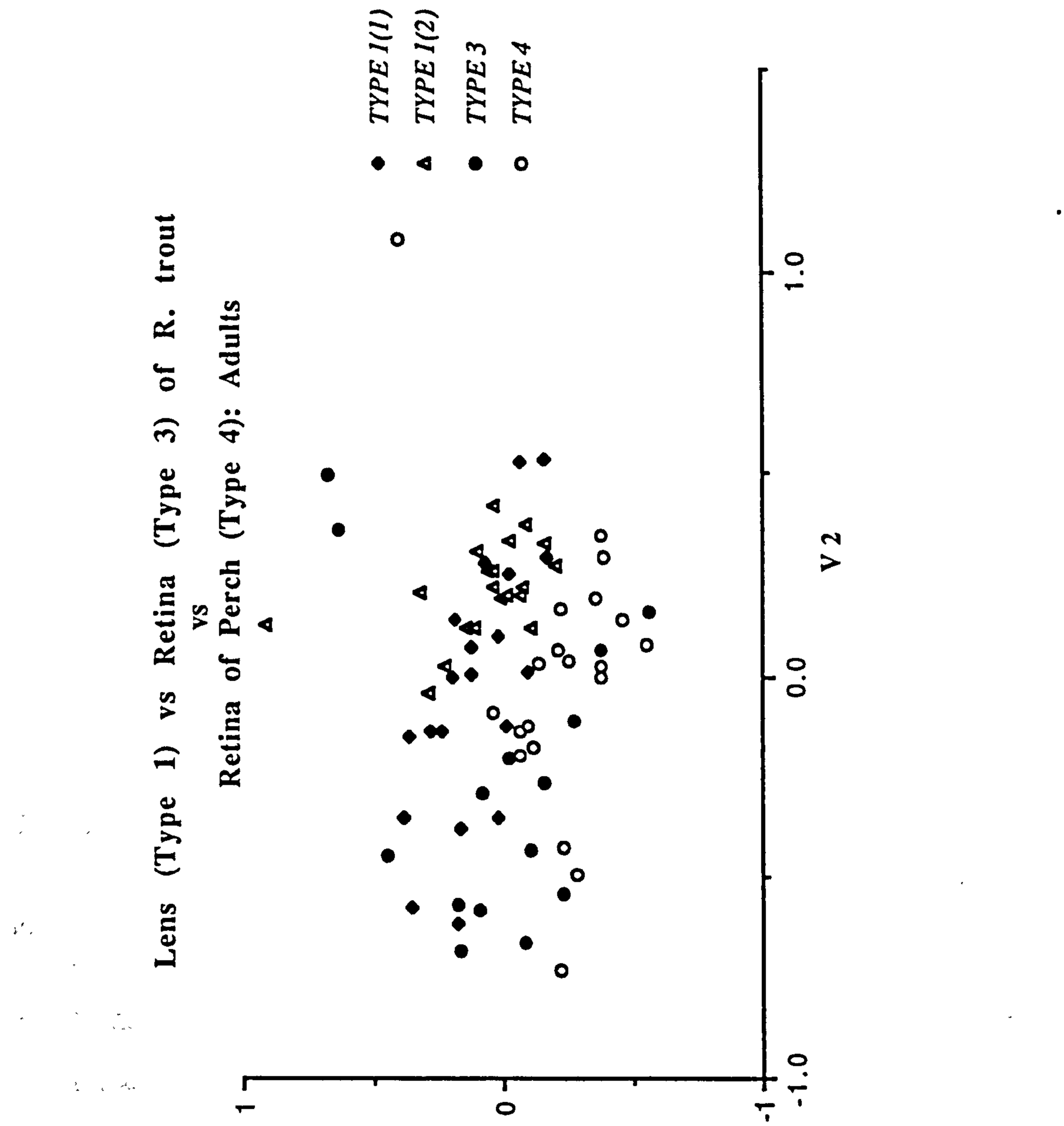
Component	Eigenvalue	Proportion	Cumulative Proportion
PC 1	1.689	0.869	0.869
PC 2	0.096	0.049	0.919
PC 3	0.062	0.032	0.951

	EIGENVECTORS		
Variable	PC 1	PC 2	PC 3
BL	0.009	0.311	-0.251
AL	-0.099	0.349	0.119
AB	-0.131	-0.285	0.029
PL	0.110	0.305	-0.524
PB	-0.128	-0.186	-0.090
LOV	-0.067	-0.231	-0.427
BOV	-0.135	-0.189	-0.052
VS-ANT	-0.098	0.388	0.113
OV-INT	0.916	-0.138	0.216
INT-VIT	-0.160	0.262	0.531
LAPP	-0.218	-0.288	0.337

Note: The abbreviations are explained in Section 4 of the 'Materials and Methods'.

Unfortunately separation is not clear here at all and it is very difficult to ascertain what feature separate them. Therefore, it is best to analyse results from previous PCA's. A similar result was obtained when a PCA was carried out on all four types, although the results for this are not illustrated.

Fig. 4.15. PCA 7. Lens of rainbow trout (Type 1) vs retina of rainbow trout (Type 3) and perch (Type 4) adults.



4. Light Microscopy.

Drawings and micrographs were recorded from the light microscope in order that direct comparisons of the four types of adult worms may be made. Figs 4.16-4.19 show drawings of Types 1-4, respectively. Plate 4.1 shows photomicrographs of Types 1-4.

5. Scanning Electron Microscopy (SEM).

Each of the four types of adult worms were studied under the scanning electron microscope. Plates 4.2-4.3 show the body shape of each type, which all correspond to those revealed under the light microscope. Apart from differences in shape, the SEM revealed some differences in size of sensory papillae between Type 4 and the other adults (Table 4.16). Sensory papillae around the oral sucker region of Type 3 adults are the smallest (Plate 4.3a) and those around the oral sucker of Type 4 adults are the largest (Plate 4.3b).

Sensory papillae were found to be present around the oral and ventral suckers, and also round the periphery of the anterior segment. Two types of papillae were present and similar to those recorded on Megalodiscus temperatus by Nollen & Nadakavukaren (1974) and on Leucochloridiomorpha constantiae by Font & Wittrock (1980). Type A papillae have a smooth surface with a single central cilium projecting from a pit (Plate 4.3d), whereas Type B are dome shaped papillae which consist of a basal bulb with a short apical knob (Plate 4.3c).

TABLE 4.16. Measurements of ciliated sensory papillae around the oral sucker of Types 1-4 adults.

Type 1	Type 2	Type 3	Type 4
2.450a	2.501a	2.465a	3.217b

Note: Mean values (*u*m). Means with different postscripts are significantly different from one another.

Fig. 4.16. A Type 1 adult.

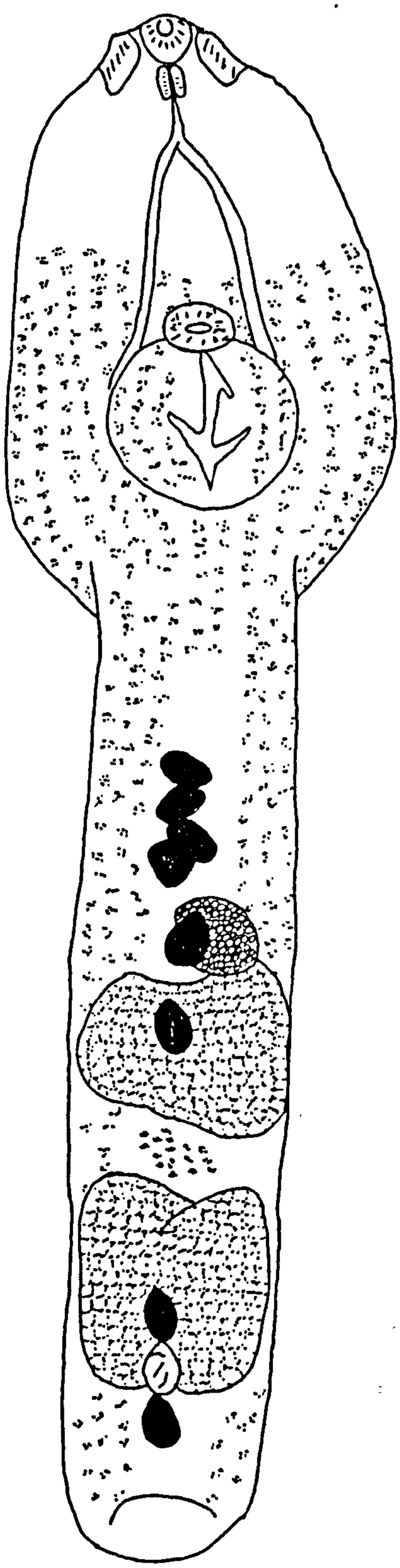


Fig. 4.17. A Type 2 adult.

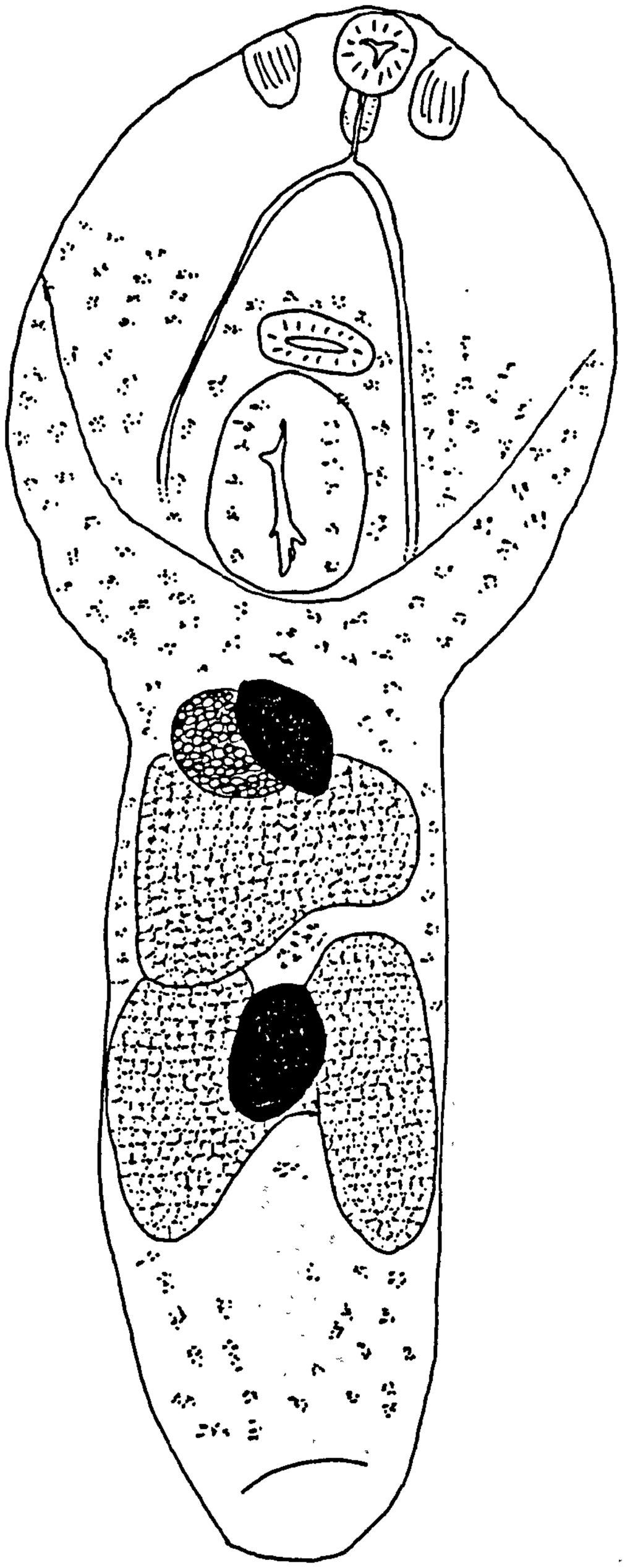


Fig. 4.18. A Type 3 adult.

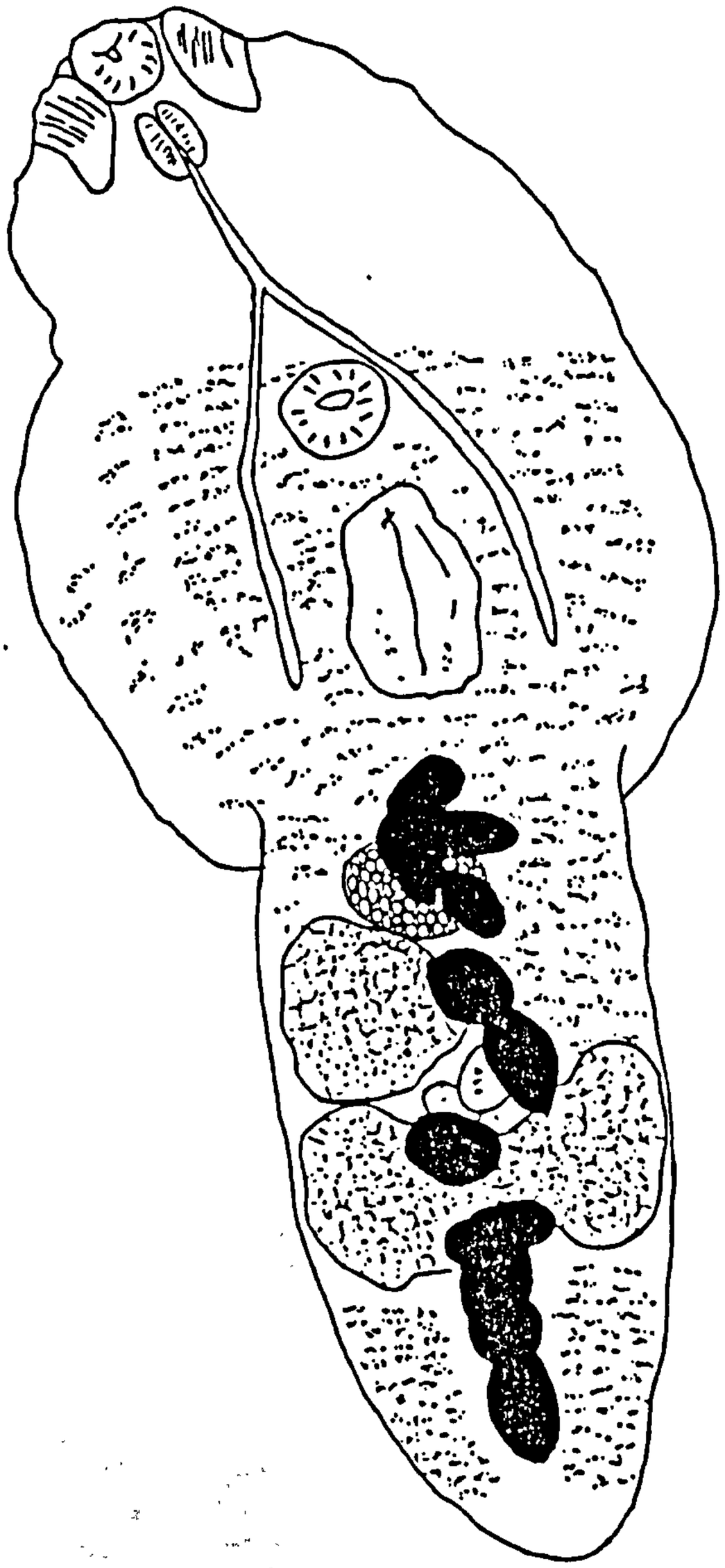


Fig. 4.19. A Type 4 adult.

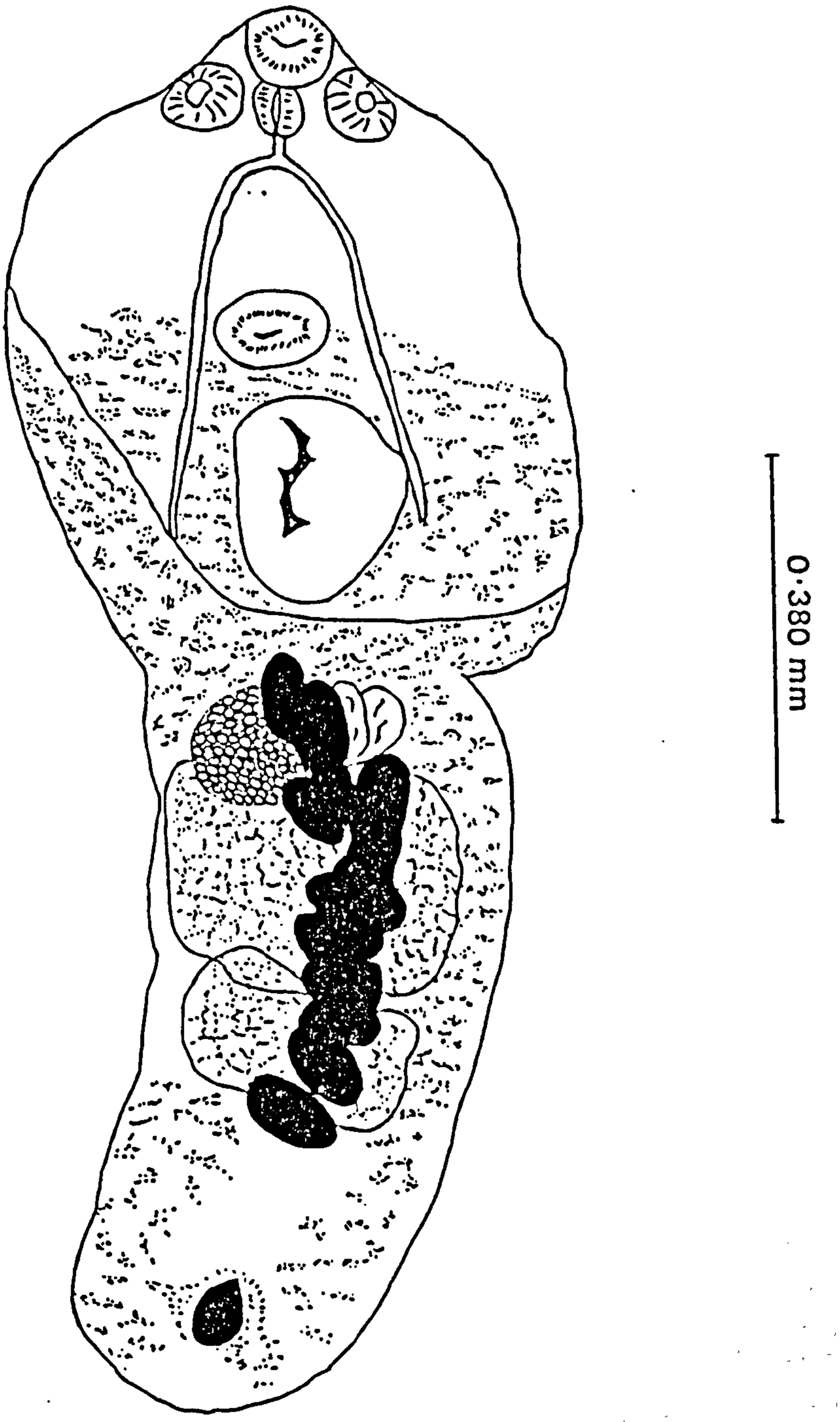


PLATE 4.1. Photomicrographs of the four types of adults.

Scale-bar: 200*um*.

a: Type 1 adult

b: Type 2 adult

c: Type 3 adult

d: Type 4 adult

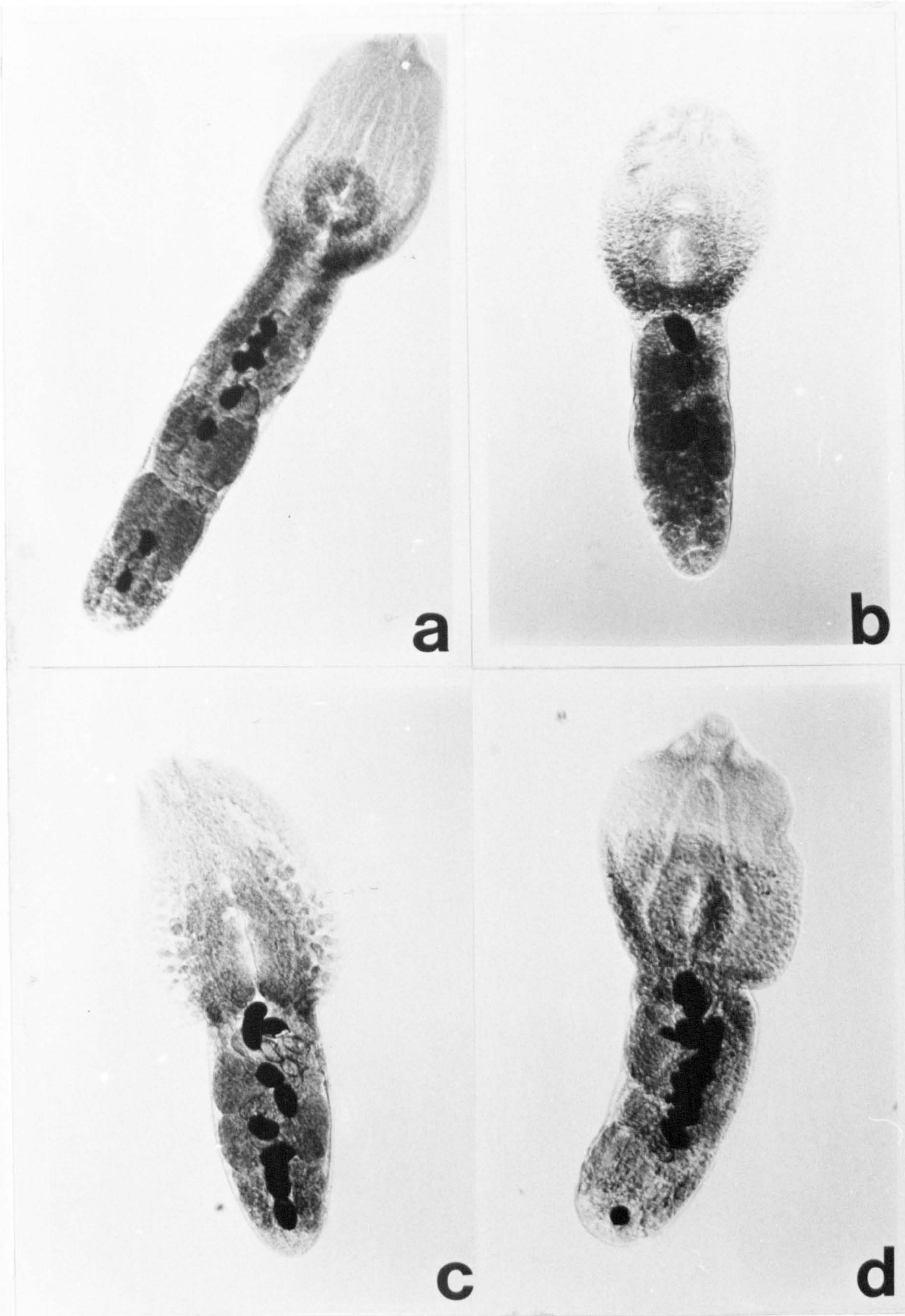
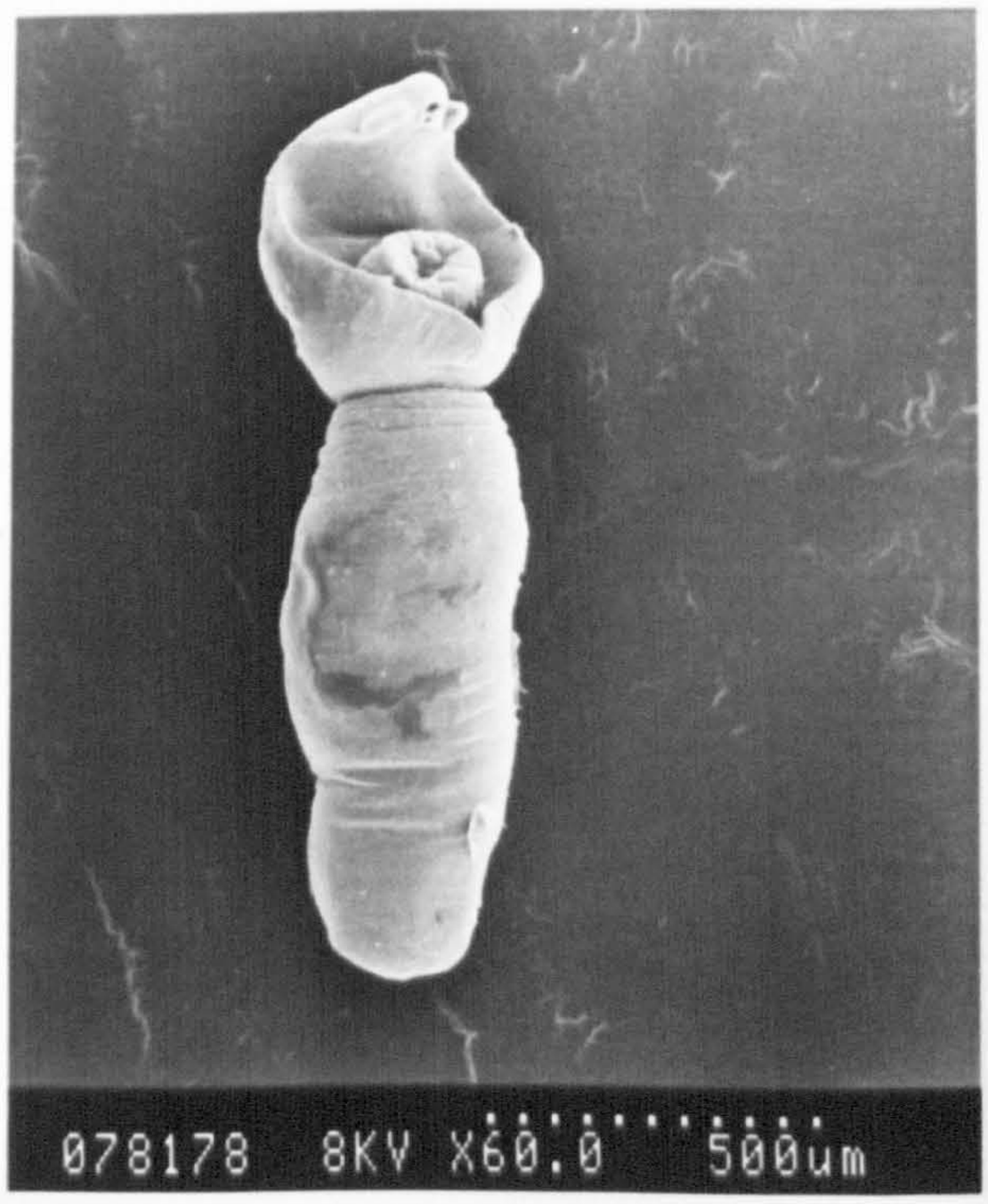

a
b
c
d

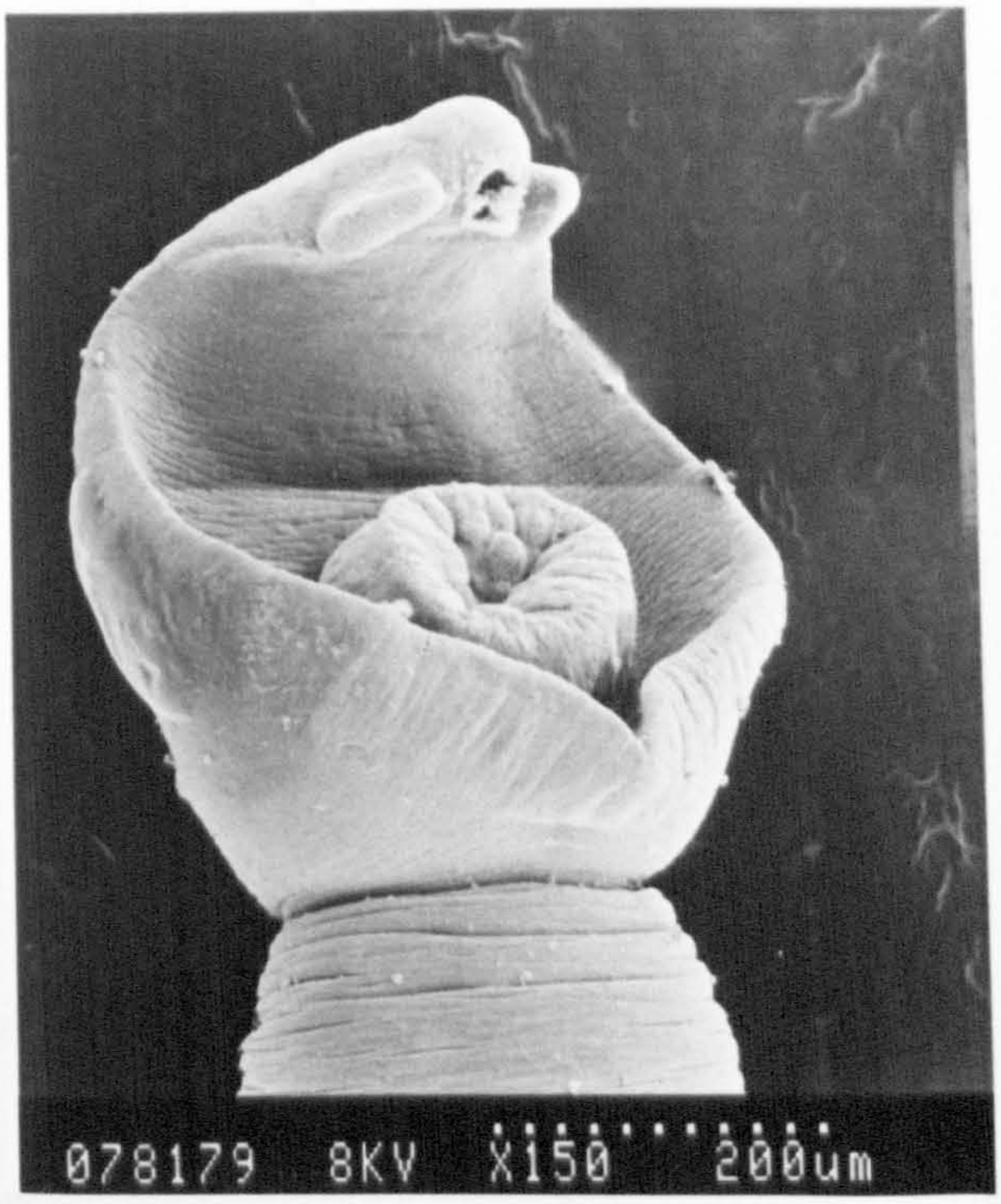
PLATE 4.2. Scanning electron micrographs of lens (Type 1) and humour (Type 2) of rainbow trout adults.

a,b: Type 1 adult

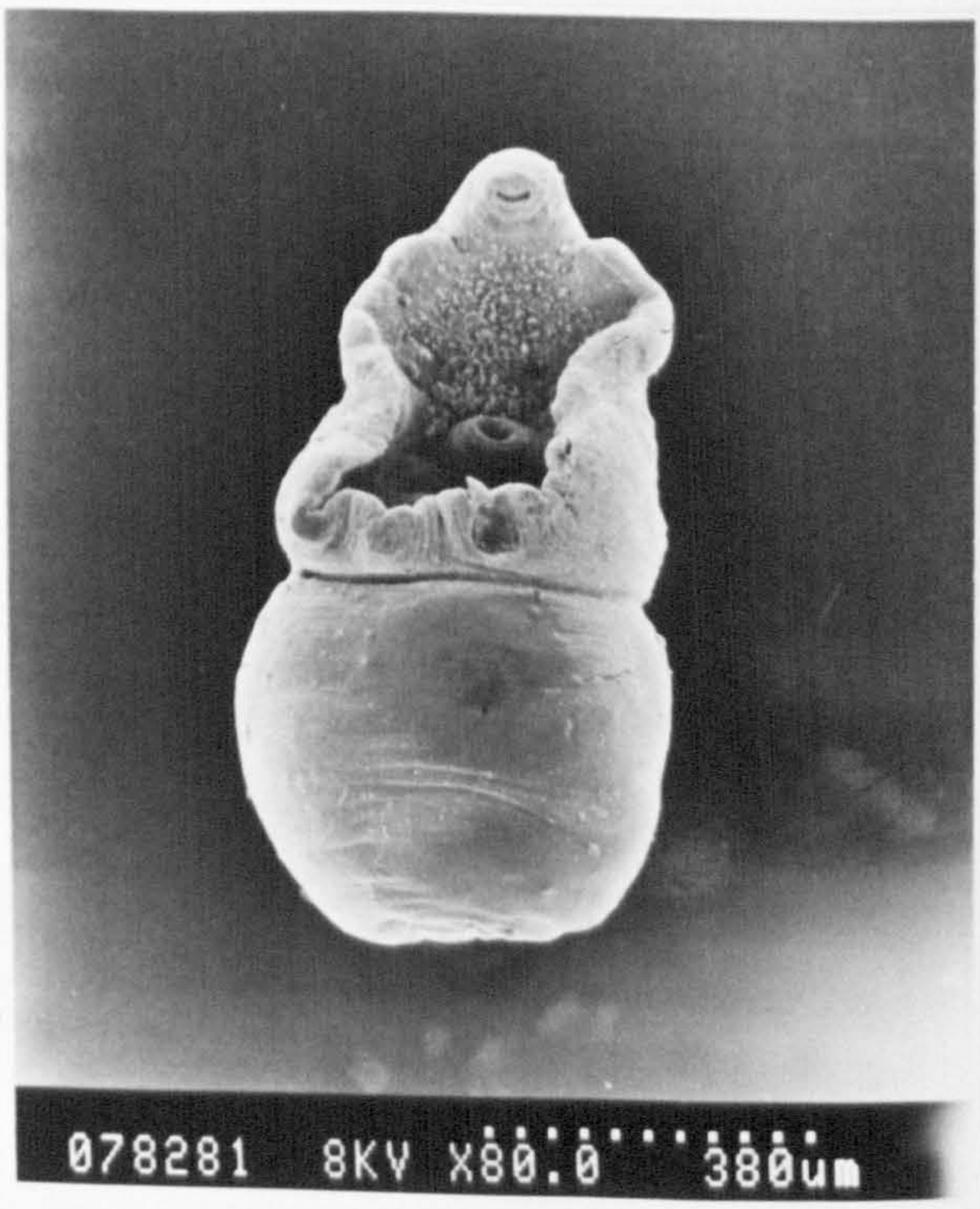
c,d: Type 2 adult


078178 8KV X60.0 500um

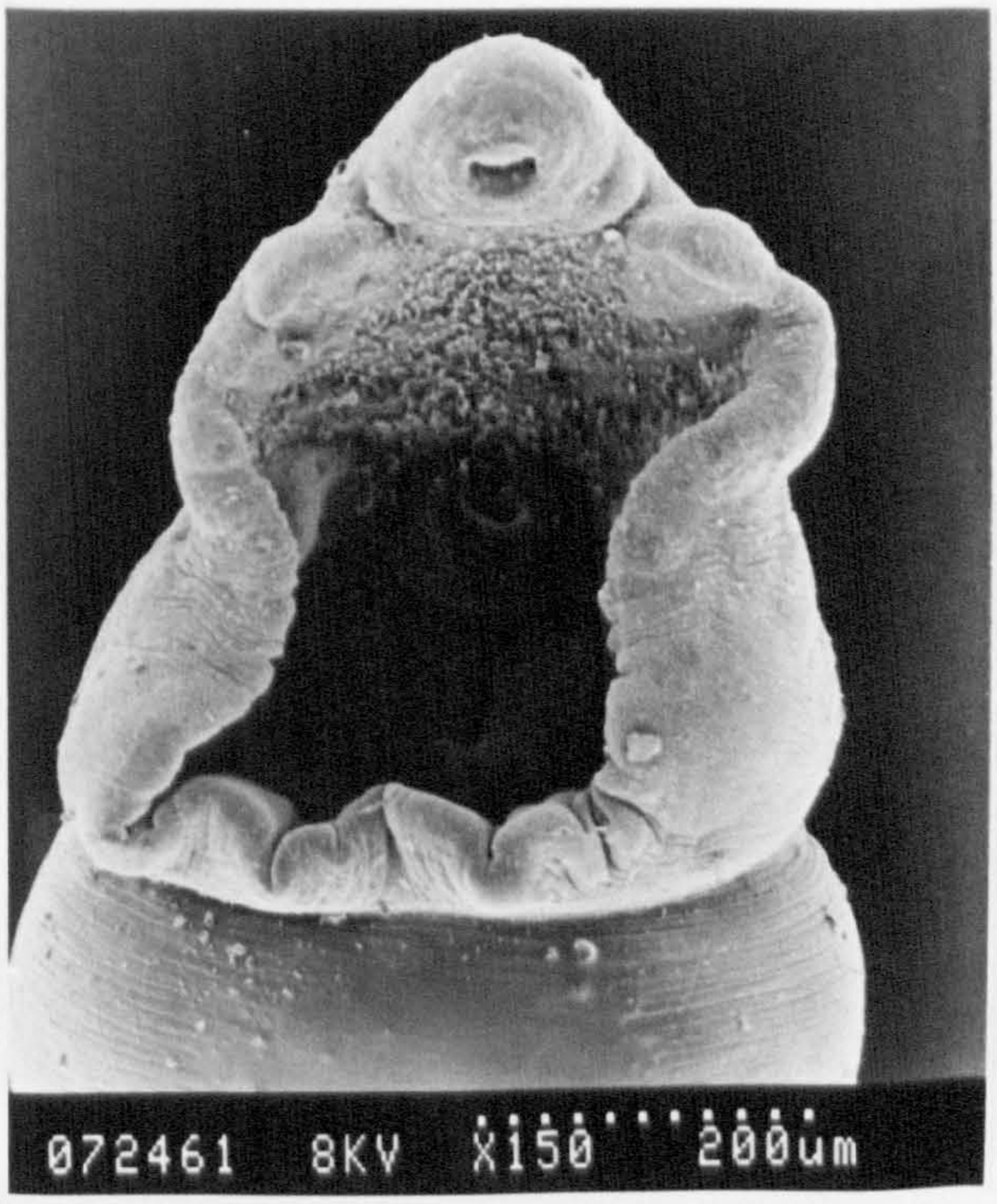
a


078179 8KV X150 200um

b


078281 8KV X80.0 380um

c

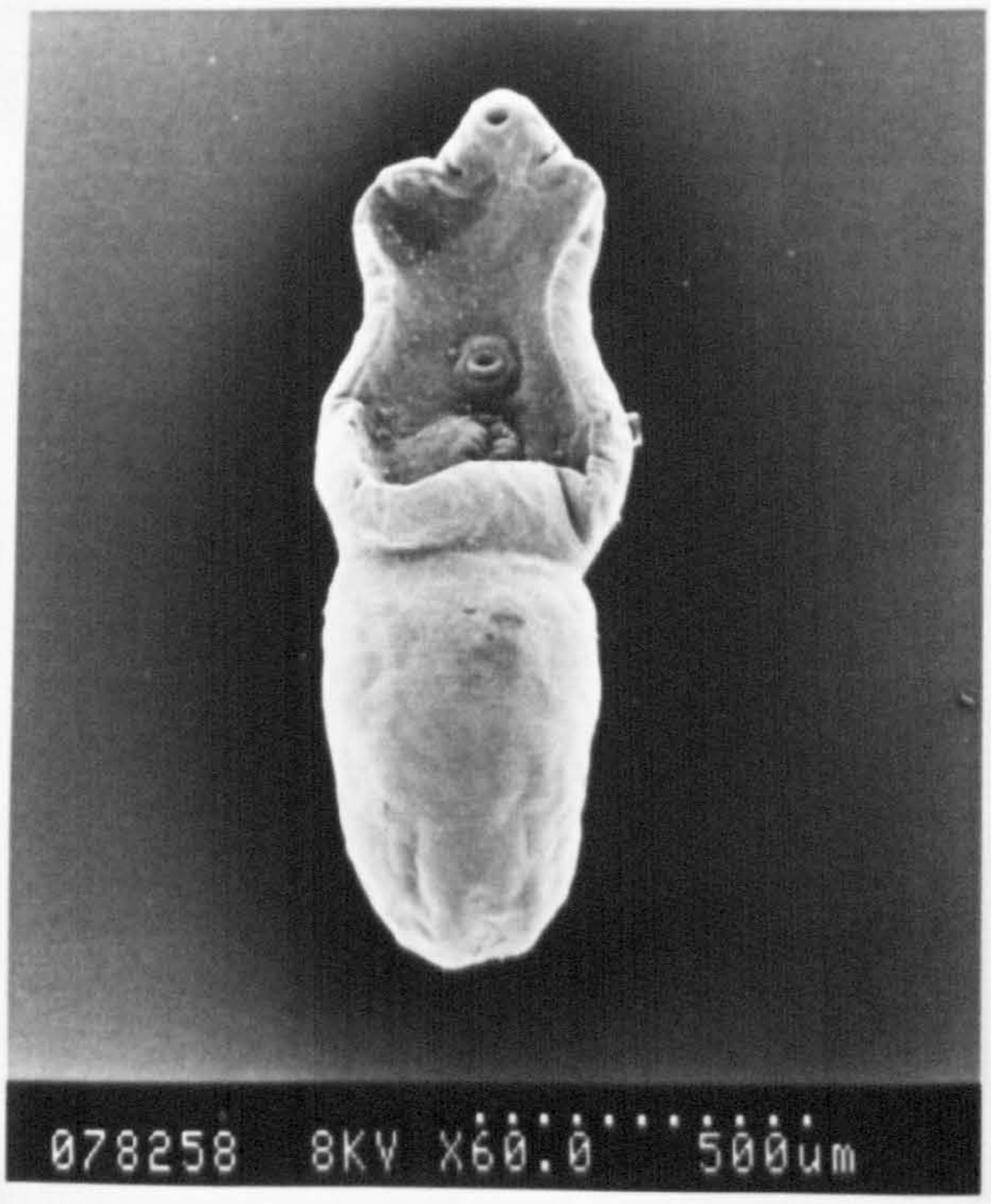

072461 8KV X150 200um

d

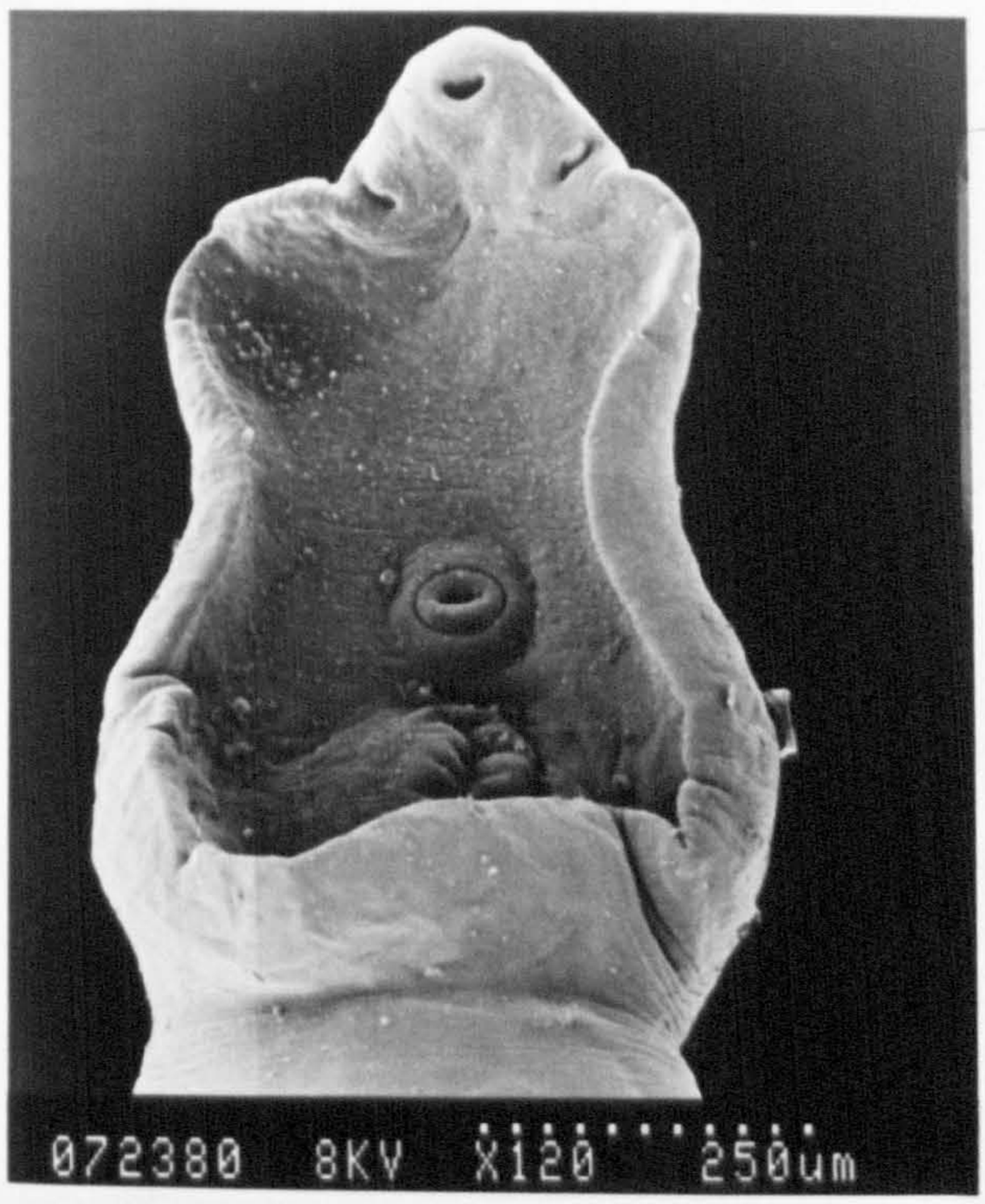
PLATE 4.3. Scanning electron micrographs of retina of rainbow trout (Type 3) and retina of perch (Type 4) adults.

a,b: Type 3 adult

c,d: Type 4 adult


078258 8KV X60.0 500um

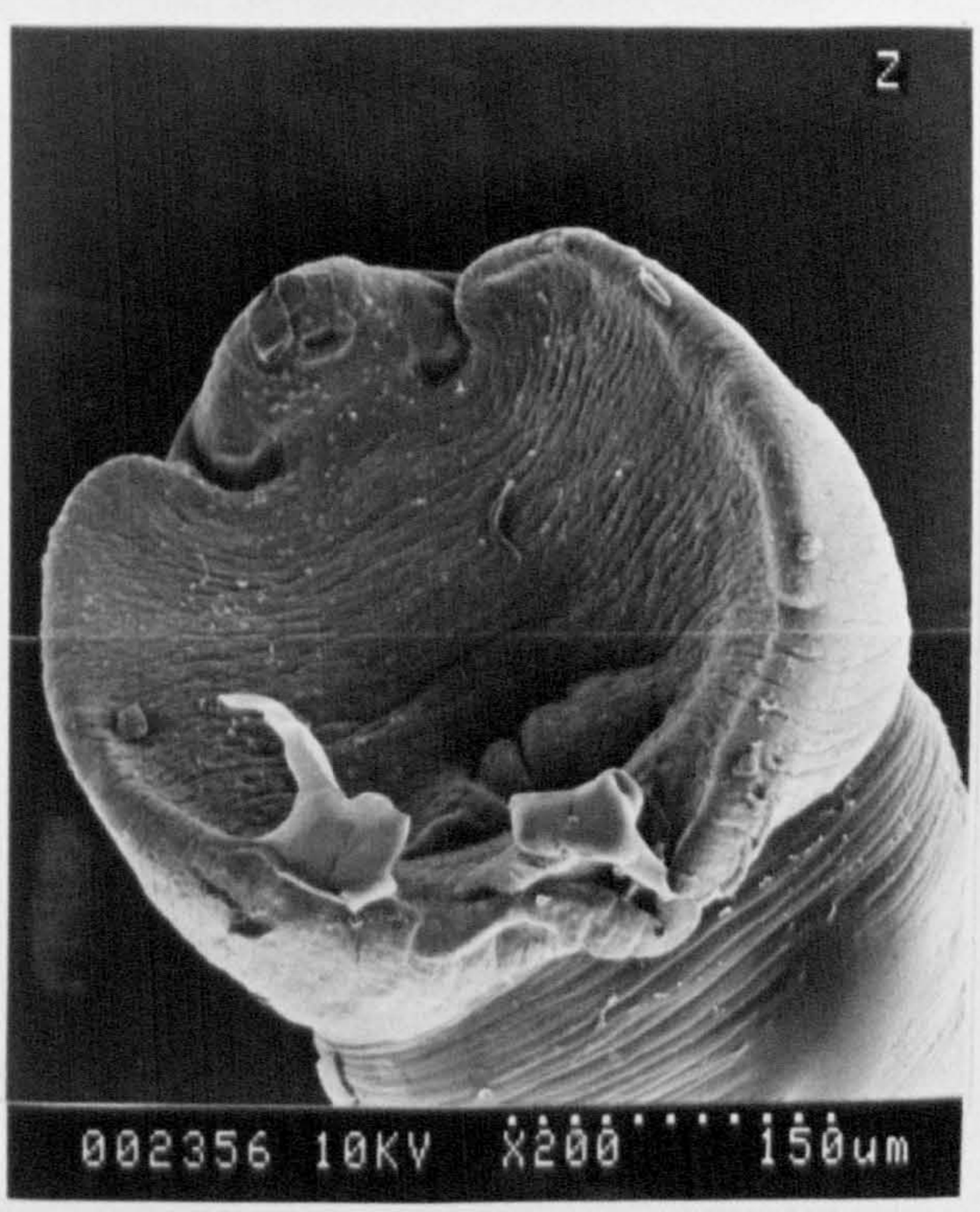
a


072380 8KV X120 250um

b


Z
002360 10KV X150 200um

c


Z
002356 10KV X200 150um

d

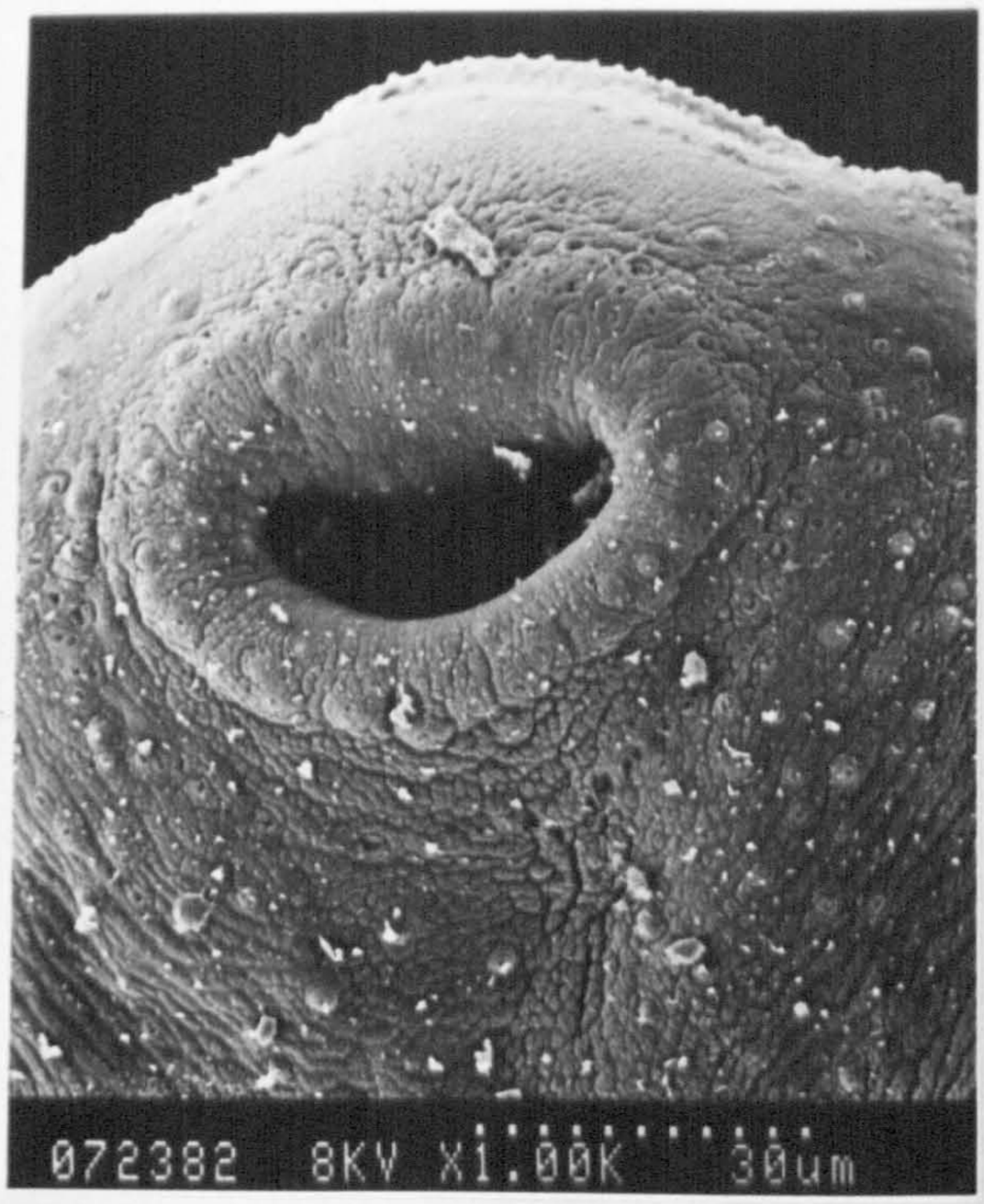
PLATE 4.4. Scanning electron micrographs of the sensory papillae around the oral sucker of adult worms.

a: Type 3 adult

b: Type 4 adult

c: Type B papillae

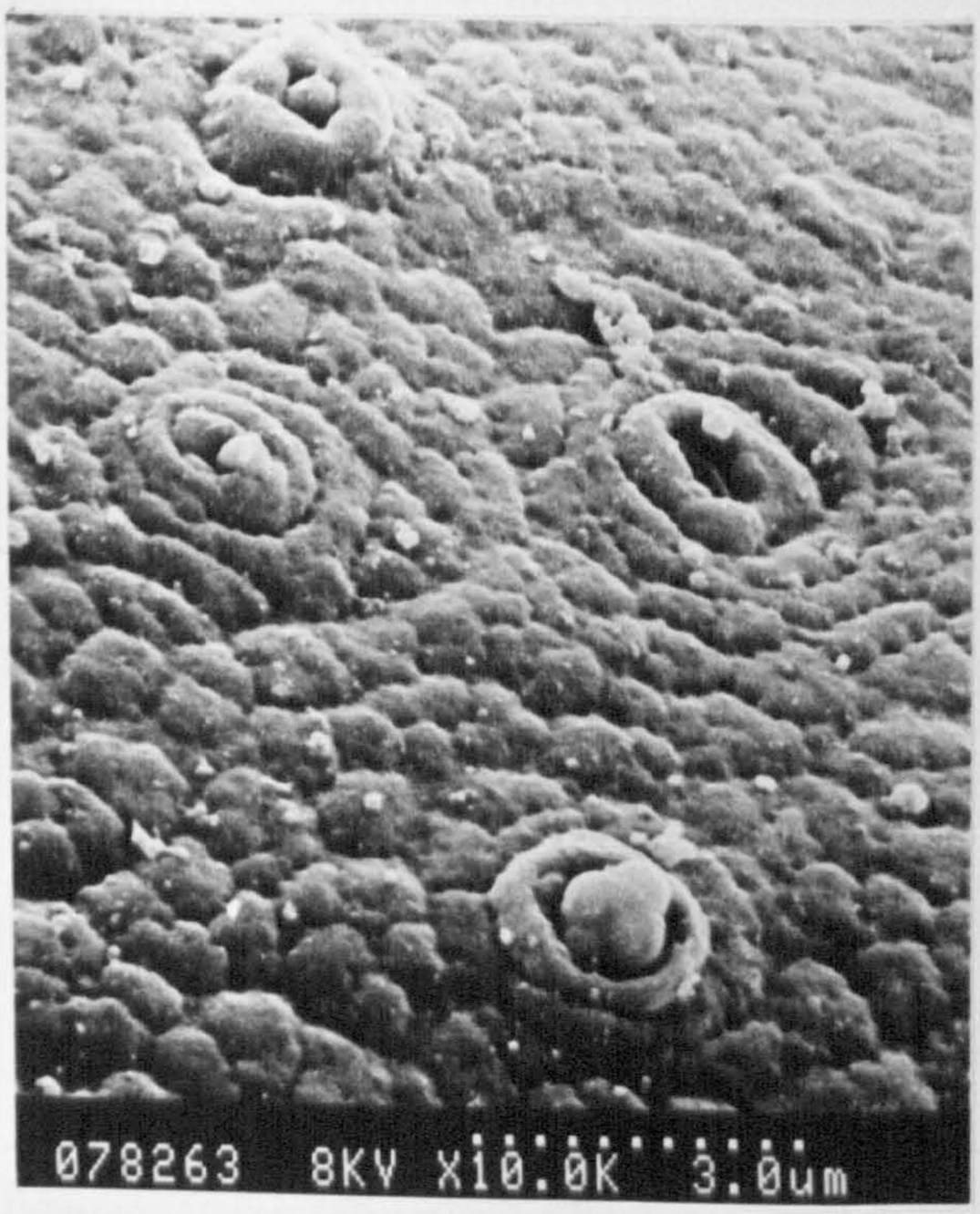
d: Type A papillae


072382 8KV X1.00K 30um

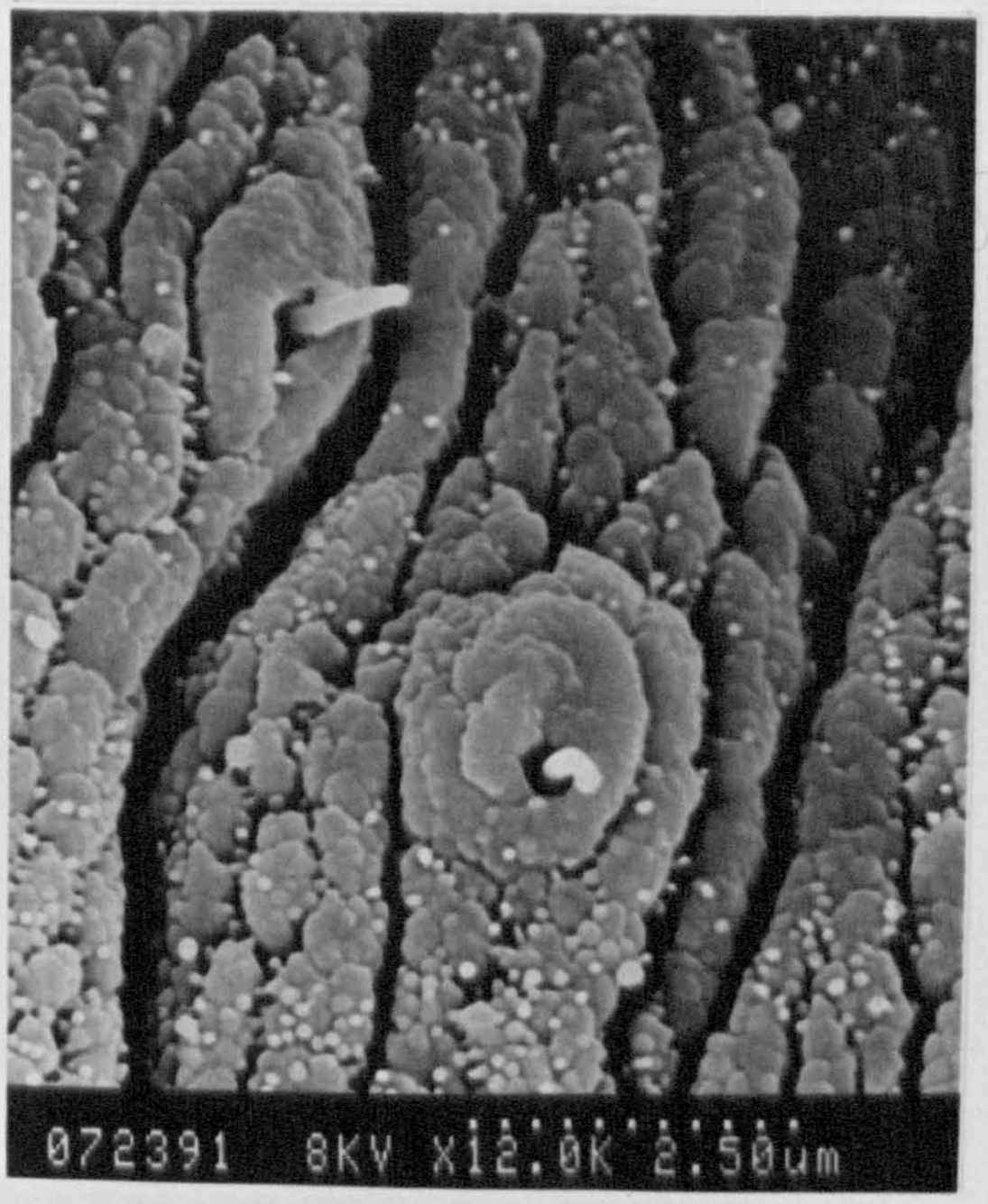
a


Z
002358 10KV X1.00K 30um

b


078263 8KV X10.0K 3.0um

c


072391 8KV X12.0K 2.50um

d

DISCUSSION

Analysis of adult Diplostomum from five different sources using Principal Components Analysis (PCA) supplemented with Analysis of Variance (ANOVA) revealed that four different types were present. This confirms the distinction found between the four types of worms established from investigations of the metacercariae (Chapter 2). PCA is a useful tool for the purpose of highlighting the characters which separate different forms, hence revealing the characters which make each type distinctive. With the removal of the size effect, all differences are allometric growth effects and are, therefore, not obscured by simple geometric differences. ANOVA can be used as a method of enhancing the results of the PCA, in that, from this analysis, it is possible to determine if characters highlighted in the PCA are in fact significantly different compared to one another.

Type 1 adults were obtained when metacercariae from the lens of rainbow trout were cultured in herring gull chicks. They were differentiated from the other three types in that they were much larger in size, the ovary was positioned one third of the way down the posterior segment, and the lappets were very close together.

Type 2 adults were obtained when metacercariae from the humour of rainbow trout were cultured in domestic chickens. These metacercariae did not grow very well in herring gulls and so this feature separates them from the others. Morphologically they can also be distinguished from the others since they have a very small ovary which is positioned at the intersegmental region, whereas in Types 3 and 4 the ovary is positioned further into the posterior segment.

Type 3 adults were obtained from retinal metacercariae from rainbow trout. Types 1 and 2 both grew in domestic chickens but Type 3 failed to grow at all in this host (Chapter 3). Morphologically, this type of adult lay somewhere in between the previou two types.

Type 4 adults were obtained from retinal metacercariae from perch cultured in herring gulls. Like Type 3 adults they would not grow at all in domestic chickens (Chapter 3). These adults differed from the others mainly because the vitellarium extends 60% along the anterior segment, often further than the anterior margin of the ventral sucker, which in turn is also positioned more anteriorly than in the other types. In addition, studies under the scanning electron microscope revealed that the ciliated sensory papillae (Type A) were significantly larger in size than those in the other adults.

The evidence from the analyses suggest that there are four different types of adults. The difficult task remained in deciding to what species they belong. Studying the key of Dubois (1970) Type 1 adults almost certainly keyed down to Diplostomum spathaceum Rudolphi, 1819. Comparing Type 1 adults with measurements given by Niewiadomska (1984), they appeared to be smaller in size. However, they did appear to be proportionally smaller. Characters of Type 1 adults closely resembling those of D. spathaceum sensu Niewiadomska (1984) are: the position of the ventral sucker in relation to Brandes' organ (near to Brandes' organ or partially covered by it), and the proportion of the anterior segment length to total body length (37% in Type 1 adults, mean of 39% for D. spathaceum). A major difference between the two adults was also evident, i.e. in D. spathaceum the vitellarium rarely extends anterior to Brandes'

organ, whereas in Type 1 worms the vitellarium sometimes extends past the ventral sucker. Despite this difference Type 1 worms closely resemble D. spathaceum.

Types 2-4 worms were not so easily identified. Using Dubois' 'Synopsis' (1970) Type 2 worms appeared similar to D. mergi Dubois, 1932, although in terms of length D. mergi is larger (1.6 v 0.938mm). Nevertheless, it does appear to have a very small ovary like Type 2 adults (0.058-0.110 x 0.063-0.140 v 0.056-0.081 x 0.054-0.078mm). Also, the ovary is situated at the intersegmental junction, similar to its position in Type 2. Another similar feature is the extent of the vitellarium which extends to the anterior margin of the ventral sucker and sometimes further anterior in both worms. Differences were also evident between the two worms in that the oral sucker in D. mergi is smaller than the ventral sucker, whereas the opposite is true for Type 2 worms. In this feature D. mergi is more similar to Types 3 and 4. However, the small dimensions of the pharynx in D. mergi relate more closely to Type 2 worms.

Adults of Types 3 and 4 were similar to D. parviventosum Dubois, 1932. The mean length of 1.5mm given by Dubois (1970) is well within the range of Types 3 and 4. The oral sucker is markedly smaller than the ventral sucker of D. parviventosum, but this feature was not so obvious in Types 3 and 4. The dimensions of the pharynx were also somewhat larger in Types 3 and 4 compared to D. parviventosum. Dimensions of the ovary in D. parviventosum are more similar to those of Type 3 worms than Type 4, but the pharynx is smaller in than either Types 3 or 4. The extent of the vitellarium is, however, similar in all three worms.

Diplostomum mahonae Dubois, 1953 is also similar to Types 3 and 4. This worm is slightly smaller than D. parviventosum, but is still well within the ranges of Types 3 and 4. Dimensions of the ovary are similar in all specimens. More importantly the position of the ovary is similar in that it is found at the intersegmental junction or just inside the posterior segment in all worms. The extent of the vitellarium into 60-70% of the anterior segment of D. mahonae makes this worm more similar to Type 4 worms than Type 3. Dimensions of the pharynx are similar in all three worms.

Identification of these four types of worms is not clear cut. However, it can be tentatively said that, on adult morphology alone, excluding metacercarial site data, Type 1 appears to be very similar to D. spathaceum, Type 2 to D. mergi, Type 3 to D. parviventosum and Type 4 to D. mahonae. More conclusive identification does not seem possible until more detailed comparison is carried out on material available from wild-caught hosts fixed in the same manner and until detailed analysis of other aspects of the biology of these worm is available.

CHAPTER 5:

THE LIFE-CYCLE OF DIPLOSTOMUM.

INTRODUCTION

The life-cycle of digenean trematodes typically consists of a molluscan primary intermediate host in which asexual multiplication takes place, an invertebrate or vertebrate second intermediate host and a vertebrate definitive host. In the case of Diplostomum species, these are in general lymnaeid snails, freshwater fish and piscivorous birds, respectively (Fig. 5.1).

Adult Diplostomum species inhabit the intestine of many gulls and other piscivorous birds (Ferguson & Hayford, 1941; Bell & Hopkins, 1956;). Gravid adults release eggs which are expelled in the bird's faeces. An Egg which is released into water develops into a miracidium. Blair (1974) found that eggs of D. spathaceum would develop and hatch under all conditions of pH and salinity tolerated by lymnaeid snails. Various times have been recorded for the development of the miracidia by different authors. Hoffman & Hundley (1957) reported that miracidia of D. baeri eucaliae were first detectable from day 10 in eggs incubated at 23°C, but the free-swimming forms were not seen until day 12. However, Lester & Huizinga (1977) found that development of the miracidium of D. adamsi took 22 days at 22°C and Ferguson & Hayford (1941) had previously found that D. flexicaudum development could take anything from two to three weeks, but they did not state at what temperature. Whyte (1989) found that the miracidium hatched from the eggs of D. spathaceum within 9-11 days at 29°C, although temperatures at this elevation would not normally be encountered in the environment. It is possible that developmental time may vary in different species, but it is perhaps more likely that this variation is due to differing incubation temperatures and other environmental

parameters.

The miracidium is a dispersal stage of the parasite and during the whole of its 24 hour life-span it actively attempts to seek out a snail intermediate host (Blair, 1974). Once the miracidium finds a snail host, it penetrates through the soft part of the body and, within the snail, two parthenogenic generations develop: the miracidium develops into the mother sporocyst which gives rise asexually to numerous daughter sporocysts. These then migrate to the digestive gland where they produce hundreds or thousands of slender furcocercariae. The timing of this development again varies considerably. According to the literature, it takes from 30 days at 22-28°C (Hoffman & Hundley, 1957) through 6 weeks at 25°C (Lester & Huizinga, 1977) to 8 weeks (temperature not given) (Ferguson & Hayford, 1941). As with the miracidial development, the timing largely depends on the environmental parameters.

Once fully developed, the cercariae emerge from the snail by rupturing the body wall. The free-living cercaria is the second transmission stage of the parasite and actively seeks out a freshwater fish host. The life-span of the cercaria is short and temperature dependent. Dubois (1929) demonstrated that the free-swimming existence of Cercaria A of Szidat is 18 hours at 12°C and between 13 and 14 hours at 35°C. Similar times have been documented for other species of cercariae (Miller & McCoy, 1930; Hoffman & Hundley, 1957; Dutt & Srivastava, 1962). The release of Diplostomum cercariae from snails, however, does not occur at temperatures below 10°C (Berrie, 1960b; Bauer, 1962; Brassard, Curtis & Rau, 1982). Epidemiological studies support this since they have revealed that infections of Diplostomum species have all occurred during the warmer

months of the year when the temperature is above 10°C (Bauer, 1962; Chappell, 1969b; Pennycuick, 1971b; Sweeting, 1974; Kennedy & Burrough, 1977). Transmission has, however, also been reported during the winter months, but this was found to be due to fish ingesting precocious metacercariae in molluscs (Becker & Brunson, 1966). Therefore, some transmission may occur at temperatures below 10°C, in cases where the cercarial stage is effectively excluded from exposure to the harsh external environment. The number of cercariae released by one infected snail is extremely high, since it was found that four infected snails can release an average of 9,672 cercariae in a 24 hour period (Hoffman & Hundley, 1957). Brassard, Curtis & Rau (1982) found that Lymnaea arctica infected with D. spathaceum had a maximum output of 30,000 cercariae per 48 hours for an individual snail.

The cercaria, being a disseminating and distributive phase, possesses specialisations, such as a muscular tail for swimming, tactile sense organs and gland cells, which aid in finding and penetrating the next host. The cercariae actively penetrate the second intermediate host, a freshwater fish. Generally it is accepted that penetration takes place at any point on the body. Erasmus (1959) found, however, with Cercariae X of Baylis, that penetration occurred mainly around the head region. Similar results were also found by Whyte (1989) with D. spathaceum. It is possible, therefore, that there is some localisation stimulus attracting the cercariae to the head region. This is likely to be a beneficial adaptation as the cercariae may have a greater chance of survival if they did not have a long distance to migrate within the host. This may also indicate that the cercariae do not merely find the host by chance, but may be attracted by some substance. Whyte (1989) hypothesised, however, that

greater penetration occurred around the head region due to the fact that cercariae were drawn in through the gills in the respiratory current.

There are many factors which may influence the penetration of the cercariae into the fish host, such as the immune response of the host, the thickness of the mucus layer on the skin, the arrangement of the scales and the toughness of the fibrous layer beneath the scales. Also, once inside the host, the cercariae may face a strong inflammatory response and/or cercaricidal chemical agents. These factors could help to explain why some fish are more susceptible to infection than others. Lester & Freeman (1976) found that D. adamsi, which parasitises the retina of the eyes of fish, shows more host specificty than for instance D. spathaceum, which normally resides in the lens of the eye. It is suggested that this may be attributed to the fact that the retina, unlike the lens, is not a completely immumologically privileged site, as macrophages and leucocytes can reach the retina.

Variation in infection levels between different species of fish have been recorded. Bottomley & Woodiwiss (1969) found that roach (Rutilis rutilis (L.)), bream (Abramis brama (L.)), gudgeon (Gobio gobio (L.)), loach (Noemacheilus barbatulus (L.)) and ruffe (Gymnocephalus cernuus L.) were severely affected with diplostomes, whereas tench (Tinca tinca (L.)), brown trout (Salmo trutta L.), pike (Esox lucius L.) and perch (Perca fluviatilis L.) were relatively unaffected. Sweeting (1974) found similar results. He examined the possibility that perhaps the unaffected fish did not share the same habitat as the cercariae and so did not readily come into contact with the infective stage of the parasite. However, this was found not to be the case, and it is more likely, therefore, that the

differing susceptibilities may be attributed to physiological differences between the fish hosts.

Betterton (1974) found that D. spathaceum cercariae caused blindness in rainbow trout (Salmo gairdneri Rich.), whereas brown trout (Salmo trutta) of the same age carried about one tenth of the number of cercariae and were clear-eyed. Experimental evidence revealed that fewer cercariae penetrated the brown trout, and also it took longer for migration to occur in this species. This would suggest, therefore, that brown trout tissues may be less easily penetrated by the cercariae and that the cercariae which achieve penetration may become disorientated. This evidence suggests, therefore, that brown trout are less susceptible to Diplostomum infections than rainbow trout due to physiological differences between the two fish species. It should be noted, however, that S. gairdneri is an exotic species of fish in Britain and Betterton used a British parasite. It may be expected, therefore that native brown trout would be less susceptible to a native parasite than an exotic fish would be.

Migration of the cercariae within the host to the 'preferred site' takes place very rapidly. Hoffman & Hundley (1957) found that within 15 to 35 minutes some D. baeri eucaliae were present in the brain. Wesenberg-Lund (1934) found that D. spathaceum larvae were detectable "creeping in the lens" only 15 minutes after infestation. These early arrivals are most probably cercariae which have penetrated the surface of the fish in the vicinity of the eye itself and, therefore, did not have any great distance to migrate. The cercariae migrate through the body, once the tail is shed, by driving the anterior spines into the host tissue. These spines are backwardly pointing and allow movement in one direction only. Also the

armed ventral sucker forms a very efficient anchorage system allowing the anterior end of the body to be thrust forward into the tissue. Cercariae also possess four gland-cells and there is little doubt that migration is aided by the dissolution of the fish tissues by secretions from these gland-cells (Erasmus, 1958).

The route by which cercariae migrate reflects conflicting views in the literature. Early reports based on dissections (Blochmann, 1910) revealed that there was massive penetration into the blood vessels, heart, brain and nerves. Szidat (1924) noted that fish placed with Cercaria C of Szidat had much haemorrhaging around the eyes, fins and gills, and suggested that the cercariae penetrated the blood vessels and migrated this way. Davis (1936) found in the case of experimental infections of tadpoles and frogs with cercariae of D. flexicaudum that some cercariae migrated through connective tissue but that the majority migrated via the blood vessels. Ferguson (1943) obtained similar results with rainbow trout (Salmo gairdneri) infected with D. flexicaudum. However, investigations into the migratory route of Cercaria X in the three-spined stickleback (Gasterosteus aculeatus) by Erasmus (1959) determined that the cercariae migrated mainly through the connective tissue and few were found to migrate via the bloodstream. Similar results were obtained when D. spathaceum cercariae were studied by Ratanarat-Brockleman (1974) using minnows (Phoxinus phoxinus) as a host, as she found the tail-less cercariae ("diplostomules") in the subcutaneous muscle and connective tissue in serial sections. However, when D. spathaceum cercariae were studied using both brown and rainbow trout as hosts (Betterton, 1973), large numbers were found in the heart and blood, suggesting that the bloodstream is indeed the major migratory route. Other authors have

suggested that migration may also occur via the nervous pathways (Ashton, Brown & Easty, 1969). There is a distinct possibility, therefore, that the migratory route may be different in different species of Diplostomum. Nevertheless, it is also possible that the cercariae burrow into the fish body randomly and then follow the path which offers the least resistence. Whichever the case, it is clear that much more work must be carried out before a definitive answer can be found.

The method by which the cercariae direct themselves towards the eye or brain is as yet undetermined. It would seem initially not to be due to chemotaxis, as experiments have shown that, if the heads of fresh sticklebacks are suspended in water, cercariae do not enter the eye (Szidat, 1924). Ashton et al. (1969) also freed some cercariae from water snails in the presence of human lenses, frogs legs and trout lenses and observed no tendency for the cercariae to be attracted towards them. Ferguson (1943) also found that neither free lenses nor lenses transplanted elsewhere in the body attracted cercariae. However, he still maintained that the eye provided a stimulus for cercarial orientation, and certainly localisation does not occur by chance.

Betterton (1974) proposed that the cercariae, migrating through the blood vessels in response to specific stimuli in the branchial and optic vessels, leave the circulatory system and migrate towards the eye along a chemical gradient. Therefore, the cercariae may indeed localise in the eye using chemotaxis.

It has also been suggested that phototaxis may play a role in the orientation of the cercariae. It has been documented (Dawes, 1956) that

cercariae are greatly influenced by light and that cercariae of the genus Diplostomum show positive phototaxis (Szidat, 1924). However, the effect of the presence of light impinging on the fish body and eyes was examined (Ferguson, 1943a) and none of the combinations affected the migration. Removal of the lens from the eye did, however, impinge on localisation. Also the removal of an entire eye resulted in no cercariae localising in the orbit at all, but large numbers were still found in the intact eye on the other side. Recent studies by Gaten (1987), however, have showed that "diplostomules" do exhibit positive phototaxis and in this way orientate themselves towards the lens. However, once within the lens the metacercariae accumulated in areas of low light intensity, which may be indicative of negative phototaxis.

Ferguson (1943) also carried out a series of experiments in which he ligated optic nerves and blood vessels in a variety of combinations before exposing the fish to cercariae. Results from these experiments strongly suggested that ligation of blood vessels inhibited the migration quite markedly, especially when the blood supply to the eye was ligated. Ferguson suggested that in C. flexicauda this effect may be due to the blocking or elimination of the main route along which entry to the eye occurs. On the other hand it could be that some chemical gradient which attracts the cercariae to the eye is eliminated, resulting in the cercariae merely becoming disorientated. Again, however, more information is needed to give a definitive answer.

Precisely where the cercariae enter the eye is as yet unknown. Many authors believe that penetration may occur at any point on the eye (Blochmann, 1910; Szidat, 1924; Erasmus, 1959, 1972). Others consider,

however, that it takes place via a particular route, for example Ferguson (1943) believed that the cercariae enter the eye via the optic blood vessels.

Whatever method the cercariae use to localise in the eye once there they must also have a method by which they localise in a particular region within the eye itself. From the present study (Chapter 2 and 5) it can be seen that the metacercariae of Diplostomum are very site specific. Perhaps, therefore, the route of localisation to the eye may reflect on the position that the metacercariae reside within the eye. No studies as yet have covered this area of investigation.

Once the cercariae have reached their 'preferred site' development to the infective metacercarial stage begins, which is a fairly lengthy process. Bauer(1954) indicated that Diplostomum species take approximately one and a half months to develop fully. Erasmus (1958) claimed that Cercaria X of Baylis takes around the same length of time. Arvy & Buttner (1954) found, however, that D. phoxini metacercariae were fully developed after only one month. Shorter periods of development were also documented for other Diplostomum species. Sweeting (1974) found that D. spathaceum metacercariae were "mature" after only 18 days at an elevated temperature of 29°C, and Hoffman & Hundley (1957) found that infectivity was achieved after only 13 days of development in the case of D. baeri eucaliae. The developmental process is certainly temperature dependent, and so the fluctuations are likely to be due to differing incubation temperatures. Development may also be influenced by other environmental conditions, such as the nutritional condition of the host and the presence or absence of competition from other parasites and the suitability of the host species.

The developmental time of the metacercariae is lengthy in diplostomes due to the fact that the metacercariae develop anlagens of the adult reproductive system as well as specialised features such as the adhesive organ which is characteristic of the adult strigeoid (Erasmus, 1972). The metacercariae can survive very long periods within the fish host since while they are in the fish eye or brain they are in an immunologically privileged site. The metacercariae, once developed, therefore, may remain safely in the fish host until the fish dies or is eaten or the metacercariae die from senility. This permits the period of infectivity of the life-cycle to be prolonged over a long period of time. Metacercariae have been recorded to survive 544 days post infection in the fish host (Erasmus, 1958) and in some cases even longer (Chubb, 1976; Kennedy & Burrough, 1977). In fact Shigin (1964) reported metacercariae surviving for up to four years in roach (Rutilis rutilis).

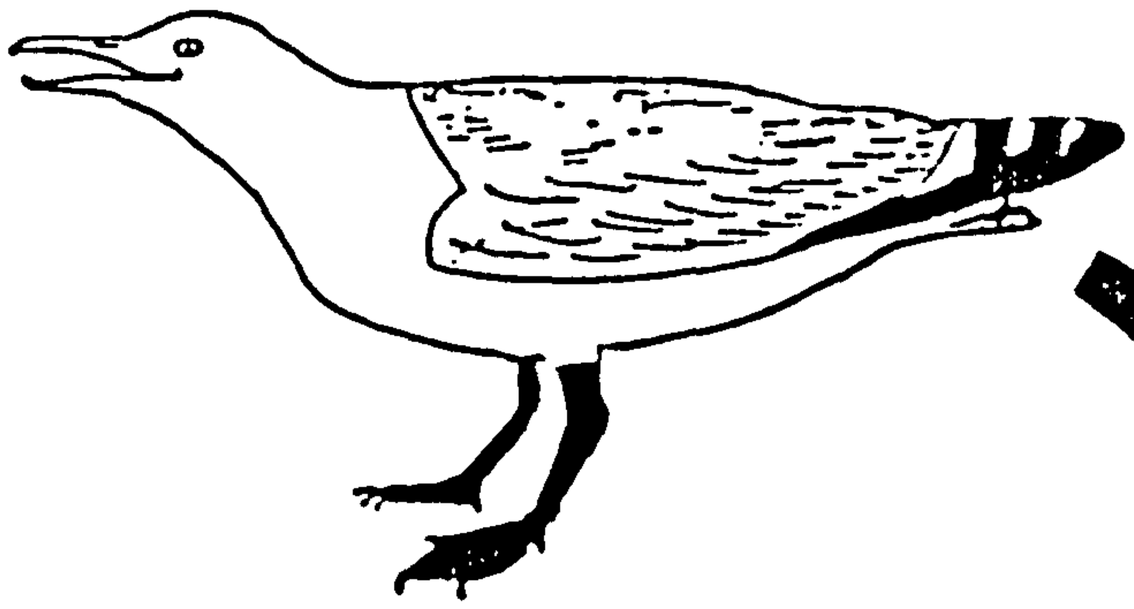
The metacercariae are considered to be fully developed once they become active, possess calcareous corpuscles, have well-developed lappets and no longer show vacuolation of the tissues (Sweeting, 1974). Intermediate stages are distinguished by their cylindrical shape and by the presence of vacuoles in the tissues. The extensive development of the metacercarial stage ensures that the adult strigeoid develops rapidly in the bird intestine once the infected fish is ingested. The pre-developed Brandes' organ ensures that the adult can maintain its position in a microhabitat that provides the most favourable physiological and immunological conditions. Here the adults may very rapidly produce and release eggs thus completing the life-cycle.

The aims of this study were to complete the life-cycles of the four types of metacercariae described in Chapter 2 in order to facilitate the study of the cercarial stage in the life-cycle and also to determine whether the cercariae locate in a specific region within the eye of the fish host, i.e. do the cercariae show site specificity? The opportunity was taken to ascertain whether or not cercariae derived from Type 4 metacercariae would infect rainbow trout as well as perch.

Fig. 5.1.

LIFECYCLE OF DIPLOSTOMUM SPP.

ADULT DIPLOSTOMUM
in S.I. of piscivorous
bird



Larus argentus

Fish eaten
by bird

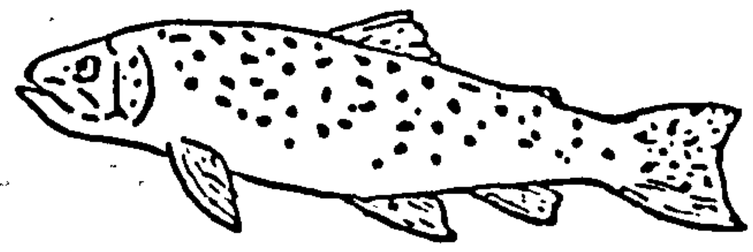
Eggs released
in faeces

80µm

WATER

Hatches to
release
Miracidium

70µm



Salmo gairdneri

Penetrates
snail intermediate
host

penetrates
fish intermediate
host

Lymnaea peregeр

100µm

release of
CERCARIA

sporocyst
↓
mother
sporocyst
↓
cercaria

MATERIALS AND METHODS

1. Collection and Maintenance of Birds.

Herring gull (Larus argentus) chicks were hatched from eggs and hand-reared on a diet of cooked whiting (Merlangius merlangus) supplemented with bran as detailed in Chapter 3, Section 1 of the 'Materials and Methods'.

One-day-old domestic chickens were obtained from Marshall's Newbridge and Whitburn hatcheries and were fed on a diet of commercial chick meal supplemented with some cooked fish as detailed in Chapter 3, Section 1 of the 'Materials and Methods'.

2. Infection of Birds.

All birds were infected using the procedure detailed in Chapter 3, Sections 2 and 3 of the 'Materials and Methods'. Infections were carried out using Types 1-4 metacercariae as detailed in Chapter 2, Section 1 of the 'Materials and Methods'. Herring gull chicks were used as the final host for all Types of metacercariae, but Type 2 metacercariae were cultured in domestic chickens for the collection of eggs.

Eggs, miracidia and cercariae obtained from infections with Type 1 adults will be referred to as Type 1 eggs, miracidia and cercariae. The same nomenclature will be applied to the eggs, miracidia and cercariae of Types 2-4.

3. Collection and Incubation of Eggs.

Eggs were collected from the faeces of infected birds in distilled water as described in Chapter 3, Section 5 of 'Materials and Methods'. This section also details how egg counts were determined. Distilled water was then replaced with filtered artificial pond water (A.P.W.) (Ulmer, 1970). The eggs were then transferred to a large petri-dish and incubated in the dark at:

(a) 25±1°C

(b) 20±1°C

(c) 15±1°C

The A.P.W. was replaced every alternate day. Hatching success of egg batches was determined by calculating the percentage of empty shells at the end of incubation. (All empty shells were removed at the beginning of incubation).

4. Collection and Maintenance of Snails.

A survey of natural infections in wild lymnaied snails was carried out at the following sites:

1. River Earn at the site of a Fish Farm, Comrie, Perthshire.
2. Howietown Fish Farm ponds, Stirling.
3. Loch Thom Reservoir, Renfrewshire.
4. Castle Semple Loch at Loch Winnoch Nature Reserve, Ayrshire.
5. Loch Awe at the site of a Fish Farm, Argyll.
6. River Devon at Crook of Devon, Kinross.

Snails were collected using metal sieves attached to long wooden broom handles. This method was found to be more efficient than using fine mesh

nets, as more debris drained through the metal sieves and, therefore, snails were more easily seen.

Snails were transported back to the laboratory in water from the River or Loch from which they were collected. During transportation the variation in temperature to which the snails were exposed was kept to a minimum by placing them in an insulated box previously cooled with ice-packs. This ensured that any cercariae present in the snails collected were not released during transportation.

Parasite-free colonies of Lymnaea pereger were established using egg masses produced by the wild snails. Egg masses were collected and washed and any containing the oligochaete Chaetogaster limnaei limnaei were discarded since they have been reported to feed on emerging cercariae from infected molluscs (Khalil, 1961) and would, therefore, prevent the establishment of cercariae in the fish intermediate host.

Egg masses and snails were maintained in plastic bowls containing A.P.W. and were fed on porridge oat flakes ad libitum. They were kept at room temperature (18±2°C) until winter and then they were maintained at a constant 15°C. The A.P.W. was replaced with a freshly made-up solution each week.

5. Infection of Snails.

Lymnaea pereger from parasite-free colonies reared in the laboratory were infected when they reached a shell length of 4-6mm. A batch of 10 eggs about to hatch were placed in a small petri-dish with a minimum of A.P.W. with one uninfected snail. The snail was left in the dish until at least four miracidia had hatched from the eggs and were no longer visible in the water. After this the snail was removed and maintained as described in Section 4. A total of 80 snails were exposed in this manner.

6. Collection, Enumeration and Morphological Analysis of Cercariae.

Snails infected by the method described above and snails collected from the wild were tested for cercariae twice every week. Wild snails were placed in groups of five into glass vials containing 30 ml of distilled water and maintained in bright light conditions for 12 hours. Snails infected in the laboratory by the method described above were treated in the same manner but were placed individually in the vials.

The vials were examined for cercariae each hour for the 12 hour period. The number of cercariae released was estimated by taking seven 0.2 ml aliquots and counting the number of cercariae in each and then extrapolating the mean to the total volume of distilled water (Erasmus, 1959).

The resting position of the cercariae was noted from the vials. Morphological analysis was carried out on both live cercariae and cercariae fixed in 5% formalin. All measurements were taken from fixed

material. Mean measurements are taken from batches of 30 cercariae. Drawings of cercariae were taken from approximately four or five live cercariae.

7. Maintenance and Infection of Fish.

Uninfected stocks of fingerling rainbow trout (Salmo gairdneri) and perch (Perca fluviatilis) were obtained from a Perthshire Fish Farm and a commercial Fishery in Yorkshire, respectively. To ensure that none of the fish were infected a sample of 30 fish from each batch obtained were examined for the presence of eye-fluke. The fish were maintained in plastic tanks (length 70cm, width 50cm, depth 40cm) in a flow-through system with continuous aeration. The fish were fed daily on an appropriate grade of commercial fish food.

In order to infect fish a known number of cercariae were collected using the method described above and were suspended in one litre of water, taken from the fish tank, in small round tanks (diameter 19cm, height 15cm). One fish was placed in each tank and left for 15 minutes without aeration. Aeration was then resumed and the fish were left for a further 45 minutes. After this time the fish were returned to a clean tank. Infections were carried out at 15°C and the fish were maintained at 12-14°C after infection. Since reports have shown that metacercariae take approximately 60 days to reach full maturity at 12°C (Sweeting, 1974), the fish were maintained for 70-75 days before being examined.

Groups of five rainbow trout were infected with three different types of

cercariae obtained from laboratory infections of snails, i.e. 15 trout in total. Five perch were infected with Type 4 cercariae. Each fish was exposed to a range of 100-200 cercariae.

Fish infections were also used to determine whether or not cercariae released from wild snails infected the eyes of fish. In this instance the fish were exposed to cercariae by the same method as described above, except that two fish instead of one were normally placed in each suspension of cercariae, and all fish were examined 24-48 hours after exposure.

All fish were killed by severing the spinal cord with a scalpel (this method is listed under Schedule 1 of the Animals (Scientific Procedures) Act, 1986) and examined for metacercariae.

RESULTS

1. Egg Hatching and Incubation.

At 25°C, 20°C and 15°C the majority of egg development and hatching was completed within 12-14 days, 18-20 days and 26-28 days, respectively. From these results, therefore, it is evident that the development of the eggs is very much temperature dependent. Developmental times varied slightly between batches of eggs, but no significant difference was found in development between egg Types 1, 3 and 4 ($P>0.05$). Type 2 eggs obtained from herring gull chicks failed to develop at all. Type 2 eggs from domestic chickens did, however, hatch but the developmental time was on average 4-5 days longer at each incubation temperature and significantly longer than the developmental times for the other eggs ($P<0.05$).

The percentage of eggs hatching at each incubation temperature did not vary significantly ($P>0.05$). Therefore, these temperature ranges do not appear to affect the percentage of eggs developing. The percentage hatch between different types of eggs was, however, significantly different (Table 5.1).

TABLE 5.1. The percentage of Types 1-4 eggs hatching at different incubation temperatures.

Incubation	EGGS			
Temp.	Type 1	Type 2	Type 3	Type 4
15°C	74.3% d	25.8% a	57.9% b	65.3% c
20°C	72.8% d	23.7% a	58.2% b	64.1% c
25°C	76.4% d	20.9% a	54.9% b	68.0% c

Mean percentage hatches are given within rows. Values within columns are not significantly different ($P>0.05$). Values within rows with different letters after them are significantly different ($P<0.05$). The values for Type 2 eggs are obtained from chickens, all others are from herring gull chicks.

Type 1 eggs had the highest percentage hatch with an average of 74.5% of all eggs hatching, whereas Type 2 eggs were the poorest at hatching with a percentage hatch of 23.5% averaged over the three temperature ranges. Type 3 and 4 eggs were in between with a mean hatch of 57.0% and 65.8%, respectvely.

2. Snail Infections.

Infections in snails collected from the wild.

Snails collected from the wild were found to have very little infection with Diplostomum. Table 5.2 shows the number of Lymnaea pereger collected at each site and the number subsequently found to be releasing furcocercariae. From this data it is evident that out of 1,471 snails collected only 41 (2.8%) were found to be releasing furcocercariae. However, when fingerling rainbow trout were exposed to these cercariae, it was found that only one snail was releasing cercariae which went into

the eyes of these fish. Therefore, the percentage infection with Diplostomum in these snails was only 0.07%.

The snail found to be releasing Diplostomum cercariae was collected from Castle Semple Loch. Infection was not evident when the snail was first returned to the laboratory, but it began releasing 3 weeks after arrival. The cercariae penetrated the rainbow trout fingerlings and developed as metacercariae in the lens of the fish. Ten fish were infected using cercariae from this snail and the metacecariae were used to infect herring gull chicks and domestic chickens as detailed in Chapter 3, Sections 2 and 3 of the 'Materials and Methods'.

TABLE 5.2. Infections in Lymnaea pereger collected during the summer months of 1987.

Site 1

River Earn at the site of a Fish Farm.

Month	No. collected	No. releasing furcocercariae
June	110	4 (3.6%)
July	183	11 (6.0%)
August	18	1 (5.6%)

Site 2

Howietown Fish Farm

Month	No. collected	No. releasing furcocercariae
June	360	11 (3.1%)
July	140	0 (0.0%)
August	290	0 (0.0%)

Site 3

Loch Thom reservoir

Month	No. collected	No. releasing furcocercariae
June	50	1 (2.0%)

Site 4

Castle Semple Loch

Month	No. collected	No. releasing furcocercariae
July	160	5 (3.1%)

Site 5

Loch Awe at the site of a Fish Farm.

Month	No. collected	No. releasing furcocercariae
July	60	7 (11.7%)
August	40	0 (0.0%)

Site 6

River Devon at Crook of Devon

Month	No. collected	No. releasing furcocercariae
August	60	1 (1.7%)

Snails infected in the laboratory.

Snails exposed to miracidia in the laboratory were not all found to harbour infection. At room temperature (20±2°C), the developmental period from the miracidial stage to the emergence of cercariae took 6-9 weeks for D. spathaceum (Stables, 1984). The snails infected in this study were maintained at 18±2°C and the cercariae were not found to release until 12-14 weeks after infection with miracidia. During the winter months the temperature decreased to 15°C and at this temperature cercariae were not released until 18-20 weeks p.i. This longer period at a lower temperature supports the fact that the developmental rate within the snail is temperature dependent.

Table 5.3 shows the percentage of snails releasing cercariae after incubation periods at 15°C and 20°C. From this table it is evident that Type 2 miracidia failed to establish infection in this snail host. It may be that the miracidia were not viable or the other possibility is that Lymnaea pereger is not a suitable host. The percentage of hosts in which infection developed was fairly low for the other types of miracidia. Since Stables (1984) obtained 100% infection of Lymnaea pereger with miracidia of Diplostomum spathaceum infection rates of 13-40% would appear to be low. The experimental technique used by Stables is similar to the one used in the present study except that infections were carried out in microtitre plates instead of petri dishes: this may, therefore, be the reason for the difference, since a microtitre plate provides a smaller area for the miracidia to cover.

TABLE 5.3. Results of exposure of Lymnaea pereger to Diplostomum miracidia in the laboratory.

Miracidia Type	No. snails exposed	Incubation temp.	Incubation period	No. releasing cercariae
1	20	18°C	>12-14 wks	6 (30%)
2	10	18°C	>12-14 wks	0
2	10	15°C	>18-20 wks	0
3	15	18°C	>12-14 wks	2 (13%)
3	5	15°C	>18-20 wks	1 (20%)
4	15	18°C	>12-14 wks	2 (13%)
4	5	15°C	>18-20 wks	2 (40%)

Cercarial release.

Snails were tested for cercarial release twice weekly after 6 weeks p.i. over a 12 hour period from 09.00 until 21.00. (Snails were not tested over 24 hours as it was thought that bright light conditions over this period of time would be too stressful. Also it was found that the temperature of the water in the glass vials in which the snails were maintained could reach relatively high levels over a short period of time and, therefore, the risk of mortality was high.) Two peaks of cercarial release were recorded at 11-12.00 and 16.30-17.30, although small numbers were released at intervals between these times. Release was not evident, however, before 10.30 or after 18.00. This pattern of cercarial release was evident in infections with the three types of miracidia, i.e. Types 1,3 and 4. (Infection was not established with Type 2). Snails were found to release cercariae over periods ranging from 14-28 days and then cercarial production would cease. Release of cercariae was not found to occur after this period over the ten weeks tested.

The number of cercariae produced per day by an individual snail was found to be highly variable. Production peaked in most infections 6-8 days after release began. Cercarial counts were not monitored daily and, therefore, a complete picture of cercarial release was not obtained. However, Table 5.4 illustrates some of the results found.

TABLE 5.4. Mean numbers of cercariae released from Lymnaea pereger infected in the laboratory over a twelve hour period (09.00-21.00).

Miracidia	No.	Time interval after release began (days)						
Type	snails	0	4	7	10	14	21	28
1	6	120	256	649	410	357	60	0
3	3	59	197	734	547	325	89	0
4	4	146	365	582	472	393	95	10

Note: Figures within rows are mean numbers of cercariae released per snail per 12 hour period. Types of miracidia are explained in materials and methods section 2.

Snail mortality was found to be very low in both uninfected and infected groups. There was no significant difference between the two groups, therefore, the infections at this low level did not appear to cause death.

3. Cercarial Morphology.

Types 1, 3 and 4 cercariae are illustrated in Figs 5.2-5.4. The drawings were made from live specimens under light cover-glass pressure. The range of measurements for each of these three types are given in Table 5.5. Measurements are taken from specimens fixed in 5% formalin.

Characters common to cercarial Types 1, 3 and 4.

These cercariae possess two pairs of penetration glands posterior to the ventral sucker. They have no eye-spots. The gut caeca are prominent and extend well posterior to the ventral sucker and the penetration glands. There are 16 flame cells in total with two sets of three on either side of the body and two sets of two in the tail stem. The flame-cell formula is 2[(3) + (3) + (2)] = 16. The tail stem is longer than the body as are the furcae. The furcae have a fin-fold along the whole of their ventral and dorsal edges.

Type 1 cercariae (Fig 5.2).

The ranges of measurements are given in Table 5.5. These cercariae have a large body, the length ranging from 0.263-0.306 mm. The anterior organ is very long ranging from 0.068-0.082 mm. The ventral sucker is much larger than in Types 3 and 4 (see Table 5.5).

The body armature consists of: an apical tuft of 12-18 spines, 13-14 rows of spines on the post-oral collar, 7 rows of spines spaced close together on the body, then a further 8-9 rows spaced further apart and reaching midway down the ventral sucker. The body spines become shorter and thicker more posteriorly. The ventral sucker has approximately 100 spines arranged in two rows.

The tail stem has 16-20 pairs of caudal bodies. The flame cells are situated opposite the 5th and 9th pair of caudal bodies.

This cercaria in the resting position hangs down in the water with the furcae spread at an angle of approximately 100° and the tail stem bent at

the base at an angle of 90°.

Type 3 cercariae (Fig. 5.3).

The ranges of measurements are given in Table 5.5. These cercariae have a large body, ranging from 0.271-0.301 mm long. The anterior organ is of average length, measuring 0.064-0.069 mm. The ventral sucker is small in size compared to Type 1 cercariae (see Table 5.5).

The body armature consists of: an apical tuft of 6-8 spines, 7-8 rows of spines on the post-oral collar, 3-4 rows of spines close together on the body, followed by a further 9-10 rows of spines reaching almost to the posterior edge of the ventral sucker. The body spines become shorter and thicker more posterior. The ventral sucker has approximately approximately 80 spines arranged in two rows.

The tail stem has 10-11 pairs of caudal bodies. The flame cells are located opposite the 4th and 6th pair of caudal bodies.

This cercaria in the resting position hangs down in the water with the furcae spread at an angle of approximately 120° and the tail stem bent at the base at an angle of 90°.

Type 4 cercariae (Fig. 5.4).

The ranges of measurements are given in Table 5.5. These cercariae have a shorter body than Types 1 and 3, ranging in length from 0.167-0.174 mm. The anterior organ and ventral sucker are of comparable size to Type 3 cercariae (see Table 3.1).

The body armature consists of: an apical tuft of 6-8 spines, 7-8 rows of spines on the post-oral collar, 3-4 rows of spines closely packed together on the body, and a further 9-10 rows of spines reaching almost to the posterior edge of the ventral sucker. The body spines become shorter but thicker more posteriorly. The ventral sucker has approximately 70-75 spines arranged in two rows.

The tail stem has 10-11 pairs of caudal bodies. The flame cells are located opposite the 5th and 7th pairs.

This cercaria in the resting position hangs down in the water with the furcae spread at an angle of approximately 130° and the tail stem bent at the base at an angle of 90°.

TABLE 5.5. Range of measurements (mm) for cercariae released from Lymnaea pereger infected in the laboratory. Measurements were taken from 20 specimens of each type fixed in 5% formalin.

Character	Type 1	Type 3	Type 4
Body L	0.263-0.306	0.271-0.301	0.167-0.174
Body B	0.047-0.063	0.041-0.050	0.053-0.064
Tail L	0.298-0.318	0.336-0.364	0.210-0.242
Tail B	0.040-0.050	0.035-0.042	0.036-0.051
Furca L	0.267-0.317	0.321-0.342	0.199-0.241
Furca B	0.017-0.025	0.021-0.026	0.022-0.027
Ant. Organ L	0.068-0.082	0.064-0.069	0.061-0.068
Ant. organ B	0.039-0.048	0.035-0.045	0.038-0.046
Ven. sucker L	0.051-0.053	0.031-0.035	0.032-0.038
Ven. sucker B	0.050-0.054	0.032-0.035	0.034-0.041

Note: L = length, B = breadth, Ven. = ventral and Ant. = anterior.

4. Identification of Cercariae.

In the absence of a complete key to Diplostomum cercariae, an attempt was made to identify the three types of cercariae by comparing them to descriptions of cercariae given in the literature by a number of authors (e.g. Blair, 1974; Nasir, 1984; Shigin, 1973a; Shigin et al., 1985; Niewiadomska, 1984, 1986).

Type 1 cercariae appear to be similar to D. spathaceum cercariae, as described by Niewiadomska (1986) (see Table 5.6). There are some size differences between the cercariae found here and those described by Niewiadomska, but these are minimal and are most likely to be due to differences in fixation. Niewiadomska's specimens were fixed in hot water and the specimens examined here were fixed in 5% formalin. The main differences between the two cercarial descriptions is the number of caudal bodies and the position of the flame cells in the tail stem. According to Niewiadomska, D. spathaceum cercariae have 12 pairs of caudal bodies and the tail stem flame cells are situated posteriorly between the 8th and 12th pair of caudal bodies. In this study the cercariae had 16-18 caudal bodies and the flame cells were situated at the 5th and 9th pair of caudal bodies. Despite these differences D. spathaceum appears to be the best match for these cercariae.

Type 3 and 4 cercariae appear to be very similar to one another apart from the fact that Type 3 cercariae are similar in size to Type 1, whereas Type 4 cercariae are much smaller. The morphology and body armature of these two types of cercariae are very close to D. parviventosum, as described by Shigin et al. (1985). However, when comparing the size of the

cercariae, Type 4 most closely resembles D. parviventosum (see Tables 5.5 and 5.6).

TABLE 5.6. Range of measurements (mm) for D. spathaceum cercariae, according to Niewiadomska (1986), and D. parviventosum, according to Shigin et al. (1985).

Character	D. spathaceum	D. parviventosum
Body L	0.222-0.288	0.140-0.170
Body B	0.066-0.081	0.050-0.058
Tail L	0.244-0.303	0.190-0.220
Tail B	0.040-0.044	0.030-0.040
Furca L	0.251-0.296	0.160-0.185
Ant. organ L	0.061-0.095	0.037-0.050
Ant. organ B	0.030-0.044	0.020-0.025
Ven. sucker L	0.047-0.068	0.030-0.035
Ven. sucker B	0.051-0.068	0.035-0.040

Note: L = length, B = breadth, Ant. = anterior and Ven = ventral.

5. Fish Infections.

Fingerling rainbow trout (fork length <14.0cm) and perch (fork length <10.0cm) were exposed to low levels of cercariae (100-200 per fish). Low levels were used to ensure that the fish would survive the invasion of the cercariae and be able to survive at least 75 days p.i. when the metacercariae would be mature. Cercariae not more than 1 hour old were used for the infections.

Table 5.7 shows the number of cercariae to which each fish was exposed plus the site of and the number of metacercariae recovered. From the results it can be seen that the metacercariae are very site specific. Only

one fish had metacercariae localised in two regions of the eye (retina and lens from a Type 3 infection). Why this one fish had 5 metacercariae in the lens cannot be explained. It is highly likely that this one fish may have had an infection before the experiment began since the lens metacercariae possessed hind bodies and were morphologically similar to D. spathaceum. However, a batch of 30 fish had already been examined and no infection found. Type 4 cercariae did not establish in rainbow trout, although they were infective to perch. This suggests that this type of cercariae may be host specific for perch.

The mean percentage parasite establishment for those fish in which infection was achieved was 32.3% (Table 5.7). This is comparable to 29% establishment found by Stables (1984) with D. spathaceum infections in rainbow trout.

TABLE 5.7. Results of rainbow trout and perch exposed to low levels of *Diplostomum* cercariae for 1 hour at 15°C.

No. of Fish	No. and Type of cercariae	Mean no. of metacercariae retrieved per fish	Site of metacercariae
1. Rainbow trout.			
2	100 Type 1	47 (47%)	47 in lens
2	150 Type 1	56 (37%)	56 in lens
1	200 Type 1	79 (39.5%)	79 in lens
3	100 Type 3	27 (27%)	27 in retina
2	200 Type 3	60 (20%)	55 in retina 5 in lens
2	100 Type 4	0	-
3	200 Type 4	0	-
2. Perch			
5	200 Type 4	47 (23.5%)	47 in retina

Note: The types of cercariae are explained in Section 2 of the 'Materials and Methods'.

6. Morphology of Metacercariae.

Metacercariae recovered from fish infected in the laboratory were found to be comparable morphologically to those recovered in the wild ($P>0.05$) (Chapter 2, Section 1 of the 'Results'). The only difference which was evident was that the range of sizes of metacercariae was not as large in those recovered from natural infections. This is most likely to be due to the fact that the infections came from the one snail host and all metacercariae were of comparable age (75 days). Ranges of measurements for Types 1 and 3 metacercariae from rainbow trout and Type 4 metacercariae from perch are given in Table 5.8. Measurements were only taken for length (L), breadth (B), distance from the centre of the ventral sucker to the anterior (VS-ANT) and the distance between the lappets (LAPP), since they

were only used to compare the metacercariae to those from the wild (Chapter 2, Section 1 of the 'Results').

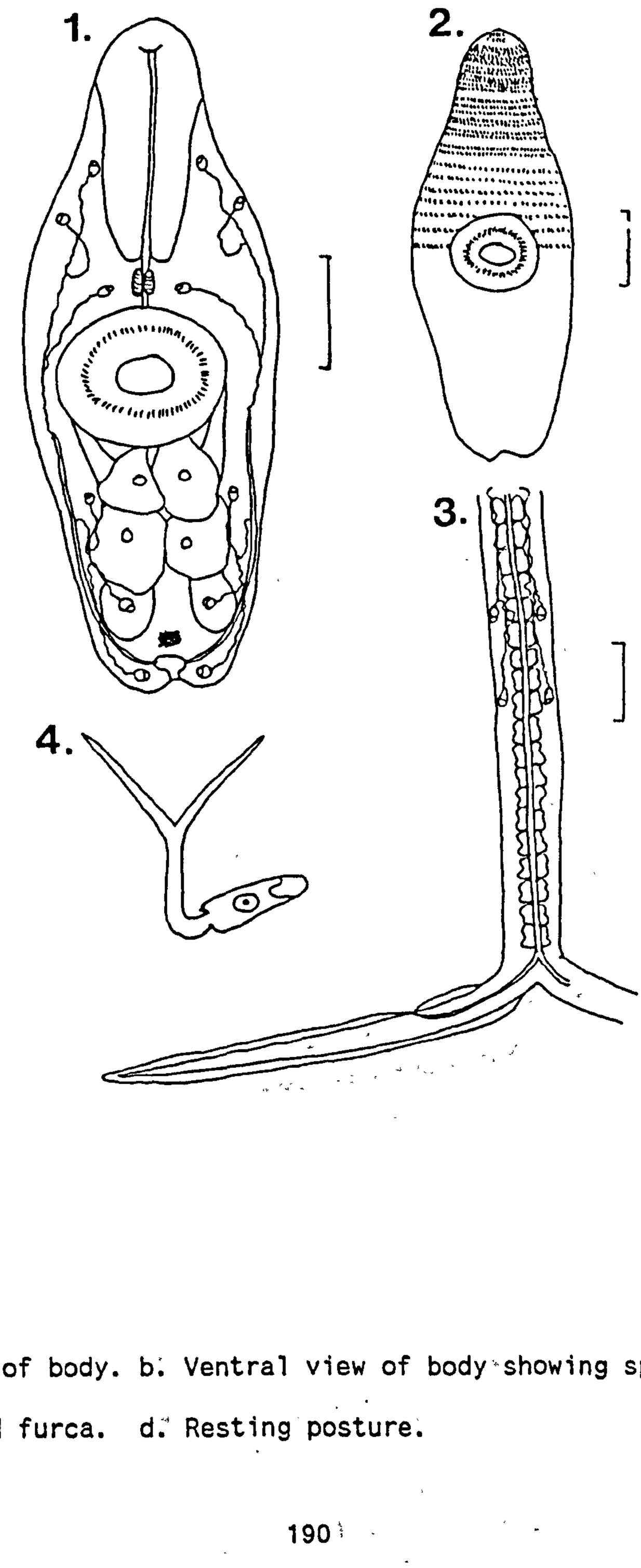
TABLE 5.8. Range of measurements (mm) for metacercariae recovered from fish 75 days after infection in the laboratory. Measurements are taken from 20 metacercariae of each type fixed in Berland's fluid.

Character	Type 1	Type 3	Type 4
L	0.397-0.498	0.386-0.423	0.382-0.511
B	0.200-0.235	0.134-0.152	0.243-0.264
VS-ANT	0.201-0.253	0.187-0.224	0.198-0.241
LAPP	0.050-0.065	0.063-0.079	0.081-0.096

Note: The abbreviations are explained in the text.

Fig. 5.2. A Type 1 cercaria.

Drawings taken from live specimens. Scale-bars: 50 μm.

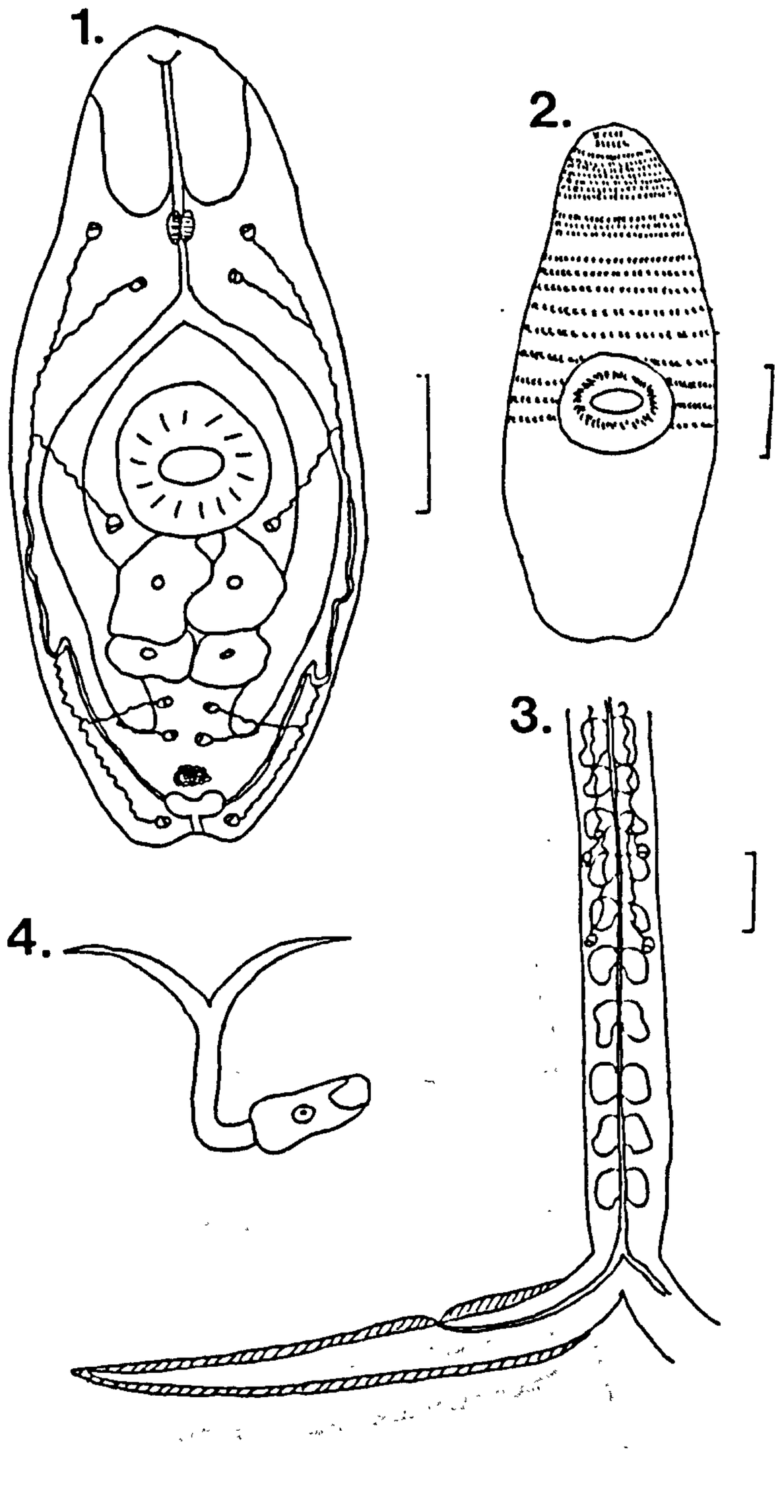


a. Ventral view of body. b. Ventral view of body showing spination.

c. Tail stem and furca. d. Resting posture.

Fig. 5.3. A Type 3 cercaria.

Drawings taken from live specimens. Scale-bars: 50*um*.

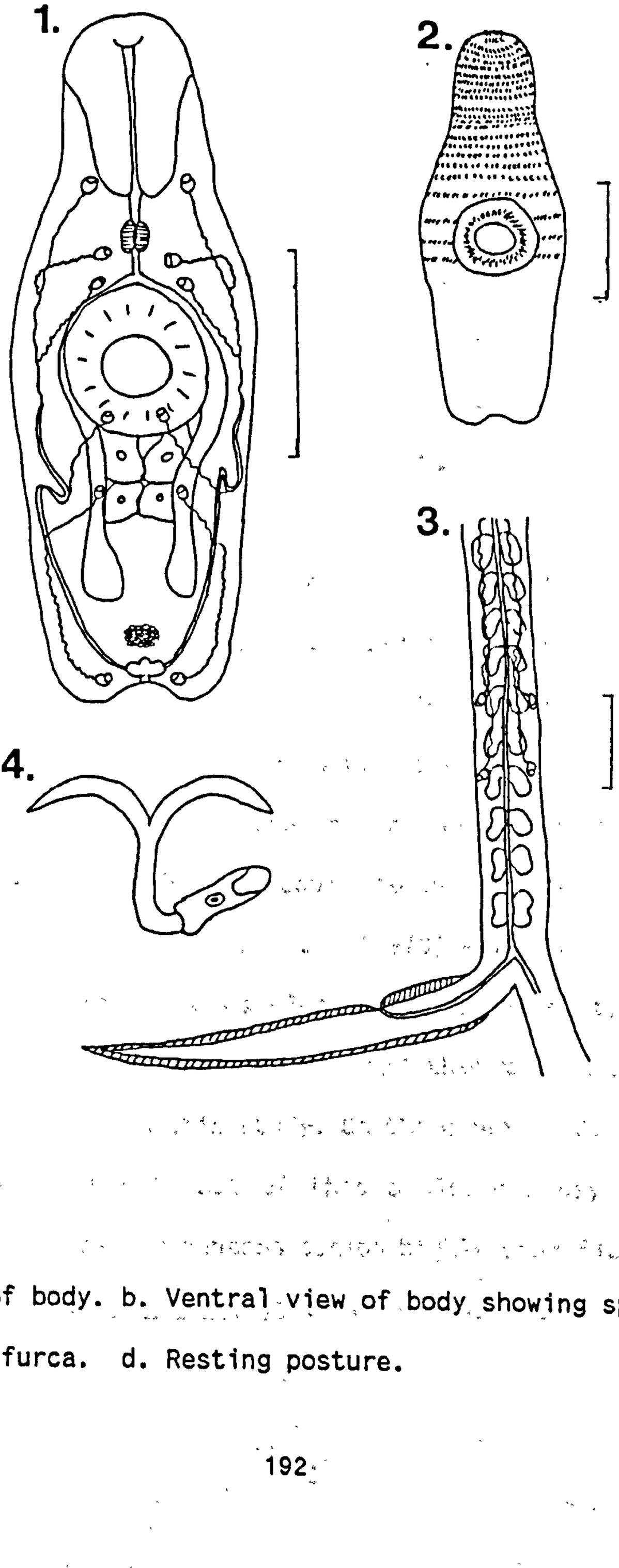


a. Ventral view of body. b. Ventral view of body showing spination.

c. Tail stem and furca. d. Resting posture.

Fig. 5.4. A Type 4 cercaria.

Drawings taken from live specimens. Scale-bars: 50 μm.



a. Ventral view of body. b. Ventral view of body showing spination.

c. Tail stem and furca. d. Resting posture.

DISCUSSION

The life-cycle of three types of metacercariae were successfully completed in the laboratory. The life-cycle of Diplostomum is not easy to complete since it involves three hosts, piscivorous birds, freshwater fish and gastropod molluscs. Completion of the life-cycle, therefore, depends very much on keeping both the parasite and its three hosts alive and well. However, despite the difficulties involved in completion of a life-cycle, the outcome is often worth the initial hard work and perseverance. For many years the advantages of laboratory based studies have been well recognised (Betterton, 1973) and, in the case of a taxonomic study such as this one, the ability to obtain all the stages in the life-cycle is essential in order to provide a complete picture of each parasite.

It is unfortunate that the life-cycle for Type 2 metacercariae could not be completed although the adult and egg stages were produced (Chapter 3). The failure of the snail to become infected by Type 2 miracidia could be due to a variety of reasons. For instance fertile eggs of this parasite were not obtained until later in the season compared to the other types, therefore, the lack of infection could be due to development of age-resistance in the snail host. Betterton (1973) working on the life-cycle of D. spathaceum reported the development of age-resistance in Lymnaea stagnalis and, therefore, it may be assumed that the same could be true for Lymnaea pereger used in this study. On the other hand, the most likely reason is that the natural host of this diplostome may be a different species of lymnaeid snail. Digeneans can be highly specific in the mollusc host. It might be that L. stagnalis a semi-aquatic snail is the natural host.

The life-cycle for Types 1, 3 and 4 metacercariae was completed using herring gulls as definitive hosts, Lymnaea pereger as first intermediate hosts and rainbow trout and perch as second intermediate hosts. The usefulness of herring gulls as definitive hosts is discussed in Chapter 3. L. pereger proved to be an effective host for Diplostomum. Although a very low percentage of snails collected from the wild harboured Diplostomum (0.07%) up to 40% of L. pereger were successfully infected in the laboratory. Rainbow trout proved to be good hosts for Types 1 and 3 cercariae with an establishment rate of up to 47%. Type 4 cercariae did not establish in rainbow trout at all although 23.5% established in perch. These results indicate that some Diplostomum cercariae may show host preference. The fact that in almost all cases the metacercariae were localised in one particular region of the eye also indicates that they show site preference.

It is interesting to note that the metacercariae localised in the area of the eye where the original metacercariae used to initiate the life-cycle were found, i.e. Type 1 metacercariae in the lens of rainbow trout, Type 3 in the retina of rainbow trout and Type 4 in the retina of perch. This would suggest, therefore, that the cercariae may be both host and site specific. This does not necessarily indicate that these are different species, as it could merely be a strain-specific phenomenon, but it does indicate that there are inherent differences between Types 1, 3 and 4.

Close examination of the morphology of the cercariae obtained in this study indicates that Type 1 cercariae are very different to Types 3 and 4. The main difference lies in the number of caudal bodies and the arrangement of the body armature (see Section 3 of the 'Results').

Cercarial Types 3 and 4 are very similar morphologically, although Type 4 are larger.

There are no complete keys to Diplostomum cercariae, although some Diplostomum species are mentioned in those partial keys which do exist. Blair (1977) produced a key to British strigeoids but he only included descriptions for D. spathaceum, D. phoxini, D. gasterostei and Diplostomum sp. No specific name was given for the last species described, but Blair indicated that it had affinities with D. mahonae and D. baeri. His key was based on details of spination, numbers of caudal bodies, resting posture and snail host, but lacked details of measurements of the cercariae, since Blair (1974) had concluded that size was an unreliable character as it could vary, depending on the snail host, locality, temperature of development and method of fixation.

Nasir (1984) also produced a key to British freshwater cercariae, including D. gasterostei, D. phoxini, D. spathaceum, Cercaria breconensis, C. helvetica XIII, and C. paracauda. This key was based on similar categories to that of Blair (1977) and again did not include details on cercarial measurements.

Complete identification of cercarial Types 1, 3 and 4 could not be achieved from these keys although Type 1 cercariae had affinities with D. spathaceum. Identification was, therefore, attempted by comparing these cercariae to existing descriptions in the literature. Type 1 cercariae were very close in size, spination and resting posture to D. spathaceum, as described by Niewiadomska (1986). Some differences were evident in the number of caudal bodies and the arrangement of flame cells in the tail

stem but this appears to be the closest match to these cercariae.

Cercarial Types 3 and 4 are both very similar to D. parviventosum as described by Shigin et al. (1985). The spination details and number of caudal bodies are very similar in D. parviventosum and Types 3 and 4, although Type 3 cercariae were much larger than both D. parviventosum and Type 4 cercariae. Therefore, although cercarial Types 3 and 4 show differences in fish host preference and size they both appear to key down to D. parviventosum. Adults of D. parviventosum have been found in Britain (McDonald, 1969), but the larval stages have not been identified in this location. One or both of these cercariae could, therefore, be the larval stage of this species. Shigin et al. (1985) reported D. parviventosum to be present in lymnaeid snails and many freshwater fish hosts, including perch, but he found that the metacercarial stage localised in the lens of fish when infected in the laboratory. In this study these cercariae developed to metacercariae in the retina of the fish host.In view of this difference, the identification must remain highly questionable.

There still remains a problem in identifying the Diplostomum Types 1-4 in this study. Certainly Type 1 appears to key down to D. spathaceum in all accounts so far, although some differences do exist. Types 2-4 remain un-named even though the metacercariae (Chapter 2), the adults (Chapter 4) and the cercariae (except Type 2) have all been described. Further clarification of the metacercarial and cercarial stages may be achieved by studying the chaetotaxy, since some work using this technique has been carried out by other authors, especially on cercariae.

CHAPTER 6:

CHAETOTAXY

INTRODUCTION

Since the work of Richard (1968, 1971) there has been a growing interest in the study of the patterns of sensory papillae (chaetotaxy) in larval trematodes and their significance to the taxonomy and phylogeny of trematodes. There is also evidence of a relationship between the arrangement of sensory papillae in cercariae and the phylogeny of the Digenea (Bayssade-Dufour, 1979).

Argentophilic papillae (sensilla) have been studied on the cercariae of various species of trematodes, including Diplostomum (Richard, 1971; Niewiadomska & Moczon, 1982; Eklu-Natey et al., 1985; Niewiadomska, 1986) and the schistosomes (Short & Catrett, 1973; Short & Kuntz, 1976). Some authors have shown that differences in papillar patterns in cercariae can be important in differentiating species (e.g. Lie, 1966; Richard & Prevot, 1974).

Studies under the light and electron microscopes have revealed that there are several types of papillae which become stained with silver nitrate (Wagner, 1961; Lie, 1966; Mohandas, 1971; Short & Catrett, 1973). Basically sensory papillae can be divided into two groups: Type A papillae have a smooth surface with a single central cilium projecting from a pit; and Type B are dome-shaped papillae with no cilium (Nollen & Nadakavukaren, 1974; Font & Wittrock, 1980). Short & Catrett (1973), studying schistosome cercariae, found that both of these types of papillae were argentophilic, although Type B papillae in this instance were described as pits rather than dome-shaped. Other structures are also stained with silver nitrate: these include, for example, gland duct apertures, excretory pores and the oral aperture. This can make the

viewing of sensory papillae difficult, especially in the region of the oral cavity where these structures may be clustered. The general pattern can be obtained by viewing many specimens, as no one specimen reveals the complete picture.

The study of sensory papillar patterns in the metacercarial stage of trematodes has been more limited than the cercarial studies. The only study to date is that of Shigin et al. (1985). He recorded the pattern of papillae on the metacercarial stage of Diplostomum parviventosum and he concluded that this bore no relation to the pattern found in the cercarial stage of the same species. However, it would seem likely that, if the pattern of papillae in the cercaria is important taxonomically, the same should be true for the metacercaria.

The aim of this study was to determine the sensory papillae patterns for cercarial Types 1, 3 and 4 (Type 2 cercariae were not available) and their corresponding metacercarial stage, including Type 2 (The types of cercariae and metacercariae are explained in Chapters 2 and 5). It was hoped that this study would shed more light on the identity of Diplostomum Types 1-4 in this present study and, therefore, demonstrate the usefulness of this technique.

MATERIALS AND METHODS

1. Collection of Material.

Cercariae were obtained from laboratory infected Lymnaea pereger (see Chapter 5). Three types of cercariae were used:

1. *Type 1:* these cercariae were derived from laboratory snails exposed to miracidia hatched from eggs from herring gull chicks infected with lens metacercariae obtained from rainbow trout from the Perthshire Fish Farm.
2. *Type 3:* these cercariae were derived from laboratory snails exposed to miracidia hatched from eggs from Marshall's Broiler chickens infected with humoural metacercariae obtained from rainbow trout from the Dumfries Fish Farm.
3. *Type 4:* these cercariae were derived from laboratory snails exposed to miracidia hatched from eggs from herring gull chicks infected with retinal metacercariae obtained from rainbow trout from the Fish Farm on Loch Awe.

Note: a detailed report of the infections is given in Chapters 3 and 5).

In order to collect the cercariae from infected snails, the snails were placed in small glass vials with 30ml of distilled water under bright light conditions. Only cercariae less than one hour old were used.

Metacercariae from natural infections were collected from four sources, as outlined in Chapter 2, Section 1 of the 'Materials and Methods', sources 1-4. Therefore, four types of metacercariae were used:

1. *Type 1:* dissected from the lens of rainbow trout.
2. *Type 2:* dissected from the humour of rainbow trout.
3. *Type 3:* dissected from the retina of rainbow trout.
4. *Type 4:* dissected from the retina of perch.

Metacercariae were dissected from the eye of the fish host using the method outlined in Chapter 2, Section 2 of the 'Materials and Methods'. The only difference in this study was that the metacercariae were always collected into distilled water rather than physiological saline.

2. Silver Nitrate Staining.

In order to view the sensory papillae under the light microscope the cercariae and metacercariae were stained with silver nitrate. The method for staining the papillae was modified from that of Lynch (1933) for miracidia of Heronimus chelydrae. Slight modifications were made to the timing of the procedure since cercariae and metacercariae require more stain than miracidia. The procedure used in this study is outlined below:

1. The parasites were collected into distilled water in an embryo dish.
2. The distilled water was removed and 2-3 mls of hot (60-65°C) 0.5% silver nitrate was pipetted into the embryo dish. The embryo dish was placed in the dark for 4-5 mins.
3. After this incubation period the parasites were washed 5-10 times in

distilled water and placed in direct sunlight for 4-5 mins.

4. Again the parasites were washed 5-10 times in distilled water.
5. The parasites were transferred into an emryo dish containing 10% glycerine in 90% alcohol and placed on a hot plate to allow the alcohol to evaporate.
6. Once the alcohol had completely evaporated the parasites could then be mounted in glycerine or glycerine jelly.

3. Drawings.

Drawings were all drafted with the aid of a microscope drawing apparatus. Patterns for both cercariae and metacercariae were only achieved by examining numbers in excess of 20 for each type, since not all papillae were visible in each specimen. All drawings are, therefore, partly diagrammatic.

RESULTS

1. Cercariae.

Drawings of the sensory papillae patterns on cercarial Types 1, 3 and 4 are illustrated in Figs 6.1-6.3, respectvely. Sensory papillae patterns on Diplostomum cercariae have been described by Richard (1971) and Niewiadomska (1986, 1987) and their terminology was used in this study.

TABLE 6.1. Sensory papillar formula for Type 1 cercariae.

Cephalic region:

Cycle C I comprised 4 papillae in the oral cavity with 3-4 + 1 either side in a latero-ventral position. The rest were as follows;

3 C II V + 6 C II L + 2 C II D

1 + (1+2) C III V + 0 C III L + 1 C III D

Body:

1 + 1 A I V + 6 A I L + 2 A I D

1 + 1 A II V + 2 A II L + 2 A II D

1 + 1 A III V + 2 A III L + 1 A III D

0 M V + 0 M L + 1 M D

2 P I V + 0 P I L + 0 P I D

0 P II V + 2 P II L + 0 P II D

2 P III V + 2 P III L + 0 P III D.

Also at the extremity of the body there were 2 papillae situated ventrally.

Ventral sucker:

3 S I + 5 S II.

Tail stem:

3-3-3-2-2-2-2-2-2-1-2.

This pattern was the same on the ventral and lateral sides.

Furcae:

2-3-2-2-2-2.

Papillae were located laterally on both sides of the furcae.

TABLE 6.2. Sensory papillar formula for Type 3 cercariae.

Cephalic region:

Cycle C I comprised 2 large papillae situated in the oral cavity with approximately 25-30 smaller papillae arranged in pairs around the oral cavity. The rest were as follows;

3 C II V + 0 C II L + 1 C II D

1 C III V + 0 C III L + 1 C II D.

Body:

1 A I V + 6 A I L + 1 A I D

1 A II V + 0 A II L + 1 A II D

4 A III V + 2 A III L + 1 A III D

0 M

2 P I V + 1 P I L + 1 P I D

0 P II

3 P III V + 2 P III L + 0 P III D.

Also at the extremity of the body there were 2 papillae located ventrally.

Ventral sucker:

3 S I + 6 S II.

Tail stem:

3-3-3-2-2-2-1-1.

This pattern was the same on the ventral and lateral sides.

Furcae:

2-2-2-2-2-2.

Papillae were located laterally on both sides on the furcae.

TABLE 6.3. Sensory papillar formula for Type 1 cercariae.

Cephalic region:

Cycle C I comprised 2 large papillae situated in the oral cavity with approximately 30 smaller papillae arranged in pairs around the oral cavity. The rest were as follows;

3 C II V + 0 C II L + 1 C II D

0 C III V + 0 C III L + 1 C II D.

Body:

1 A I V + 5(6) A I L + 0 A I D

2 A II V + 0 A II L + 1 A II D

4 A III V + 2 A III L + 1 A III D

0 M

2 P I V + 1 P I L + 1 P I D

0 P II

2 P III V + 2 P III L + 0 P III D.

Also at the extremity of the body there were 2 papillae located ventrally.

Ventral sucker:

3 S I + 6 S II.

Tail stem:

3-3-2-2-2-2-1-2.

This pattern was the same on the ventral and lateral sides.

Furcae:

2-2-2-2-2-2.

Papillae were located laterally on both sides of the furcae.

Identification.

In Tables 6.1-6.3 it can be seen that Type 1 cercariae have a very different sensory papillar arrangement from Types 3 and 4, which are very similar to one another.

Type 1 cercariae, when compared to descriptions by Eklu-Natey et al. (1985) (Table 6.5) and Niewiadomska (1986) (Table 6.4), are very similar to Diplostomum spathaceum. There are some differences in the papillar pattern, especially around the oral cavity, but this is a very difficult area to examine and so much variation can be recorded. Ignoring the small differences present, these two cercariae have very similar sensory papillar patterns and could, therefore, be one and the same species.

Type 3 and 4 cercariae, as already stated, are very similar to one another. They are both similar to D. parviventosum, as described by Shigin et al. (1985). The papillar pattern is very similar in these three cercariae, although in some areas the number of the body papillae recorded show some variation.

TABLE 6.4. Sensory papillar formula for D. spathaceum cercariae according to Niewiadomska (1986).

Cephalic region:

Cycle C I comprised 4 papillae situated deep inside oral cavity and 12 sensillae in latero-ventral position. Further rows were as follows:

3 C II V + 6(4) + 6(4) C II L +2(1) C II D

I(2) + (1 + 2) C III V + 1 + 1 C III L + 1 C III D

Body:

1 + (1) A I V + 7 A I L + 2 A I D

1 + 1 A II V + 0 A II L + 2 A II D

1 + 2 A III V + 2 A III L + 0 A III D

O M

2 P I V + 0 P I L + 1 P I D

0 P II V + 1(2) P II L + 0 P II D

2 P III V + 1 P III L + 0 P III D

Also at the extremity of the body there were 2 papillae located ventrally.

Ventral sucker:

3 S I + 6 S II

Tail stem:

ventral side: 3-3-2-2-2-3-1(2) = 16(17)

dorsal side: 3-3-2-2-2-3-1(2) = 16(17)

antero-laterally: 2-2

postero-laterally: 2-2-2-2

Furcae:

9 papillae on each edge.

TABLE 6.5. Sensory papillar formula for D. spathaceum cercariae according to Eklu-Natey et al. (1985).

Cephalic end:

16 CI

3 C II V + 8-10 CII L + 2 C II D

1 + 2 C III V + 2 C III L + 1 C III D

Body:

1+ 1 A I V + 6 A I L + 2 A I D

1 + 1 A II V + 2 A II L + 2 A II D

1 A III V + 2 A III L

1 M I V + 1 M I L

2 P I V + 1 P I L + 1 P I D

1 P II L

3 P III V + 1 P III L

Ventral sucker:

3 S I + 6 S II

Tail stem and furcae:

19-22 U V + 16-18 U D + 12 U L

10 F V + 10 F D

2. Metacercariae.

Drawings of the metacercarial Types 1-4 are illustrated in Figs 6.6-6.9, respectively. The sensory apparatus of the metacercariae is very different from that of the cercariae. The papillae are far fewer in number and the pattern of arrangement is different. Papillae were only noted to be present on the ventral surface of the metacercariae.

Shigin et al. (1985) recorded the pattern on the metacercarial stage of D. parviventosum and a comparison with the results in this study reveals that the general pattern is very similar in all the metacercariae. Shigin recorded that the pattern of papillae on the ventral sucker was very variable, but this was not found to be so in this study. On the other hand, the pattern on the oral sucker was found to be highly variable.

Papillar patterns for metacercarial Types 1-4 were all found to be similar on the ventro-lateral surface of the body. Generally there were, on each side of the body, one or two at the base of the lappets, another, one third of the distance to the ventral sucker, one just anterior to the ventral sucker, one at the level of the middle of Brande's organ and one at the posterior extremity of the metacercaria. Type 4 metacercariae differ from the rest in that the papillae level with the middle of Brande's organ were not present.

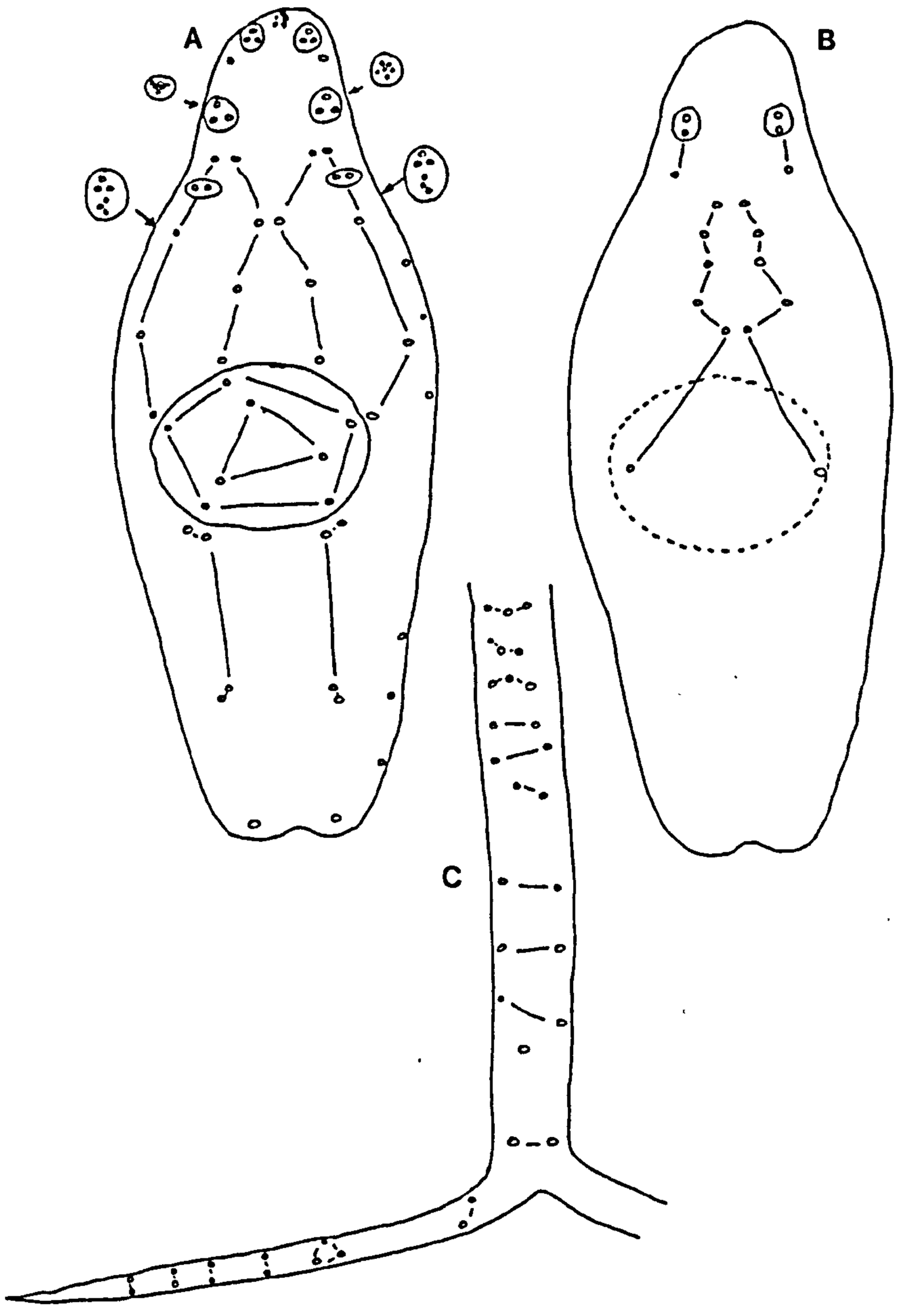
The pattern of papillae was also found to be fairly constant on the lappets of the metacercariae. In all types there was one papilla located at the anterior tip of each lappet and Types 1 and 4 also had an additional papilla in the middle of each lappet. Types 1 and 4

metacercariae were also found to have the same papillar pattern on the ventral sucker, i.e. a circle of eight papillae. Type 2 metacercariae had only six papillae on the ventral sucker, whereas Type 3 had no papillae present here at all. Type 3 were also different from the others in that in six of the 20 specimens there were four papillae situated across the body just posterior to the lappets and the oral sucker. These papillae were not found to be present on any of the other metacercariae.

As mentioned above, the arrangement of papillae on the oral sucker was found to be highly variable. The general pattern found for each type is illustrated in Figs 6.6-6.9. All the metacercariae have a different pattern in this area. This may reflect differences between the metacercariae or it may only reflect how variable the pattern on the oral sucker may be. This question could only be answered by comparing known species, which in this study were not available.

In comparison with the papillar pattern on the metacercariae of D. parviventosum (Shigin et al., 1985), it can be seen that the most similar pattern in this study was on Type 2 metacercariae. The only difference between the two is the arrangement and number of papillae on the oral sucker, but, as noted above, this may be due to natural variation.

Fig. 6.1. Sensory papillar pattern on Type 1 cercariae.

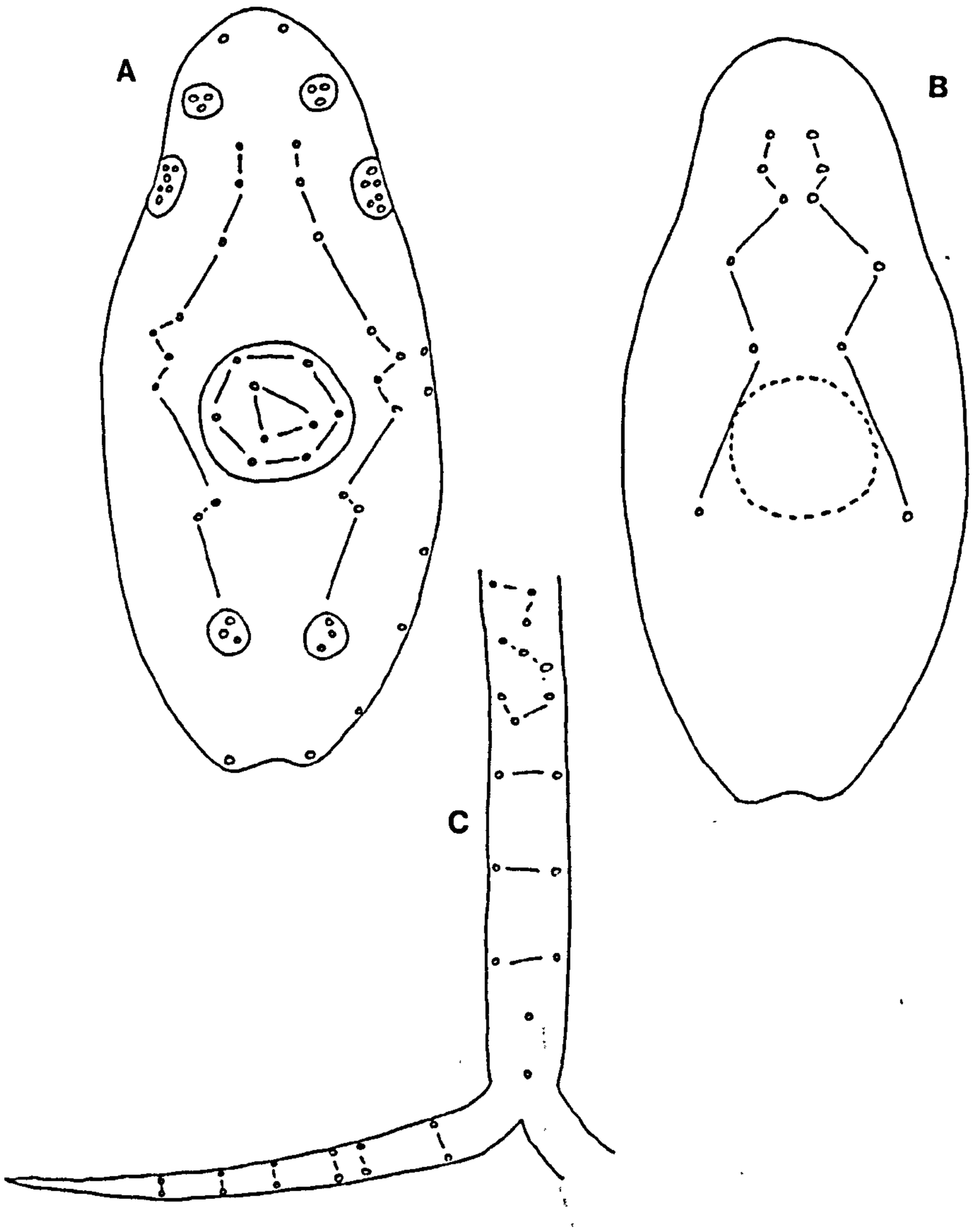


a. Ventral and lateral papillae.

b. Dorsal papillae.

c. Tail stem and furca papillae.

Fig. 6.2. Sensory papillar pattern on Type 3 cercariae.

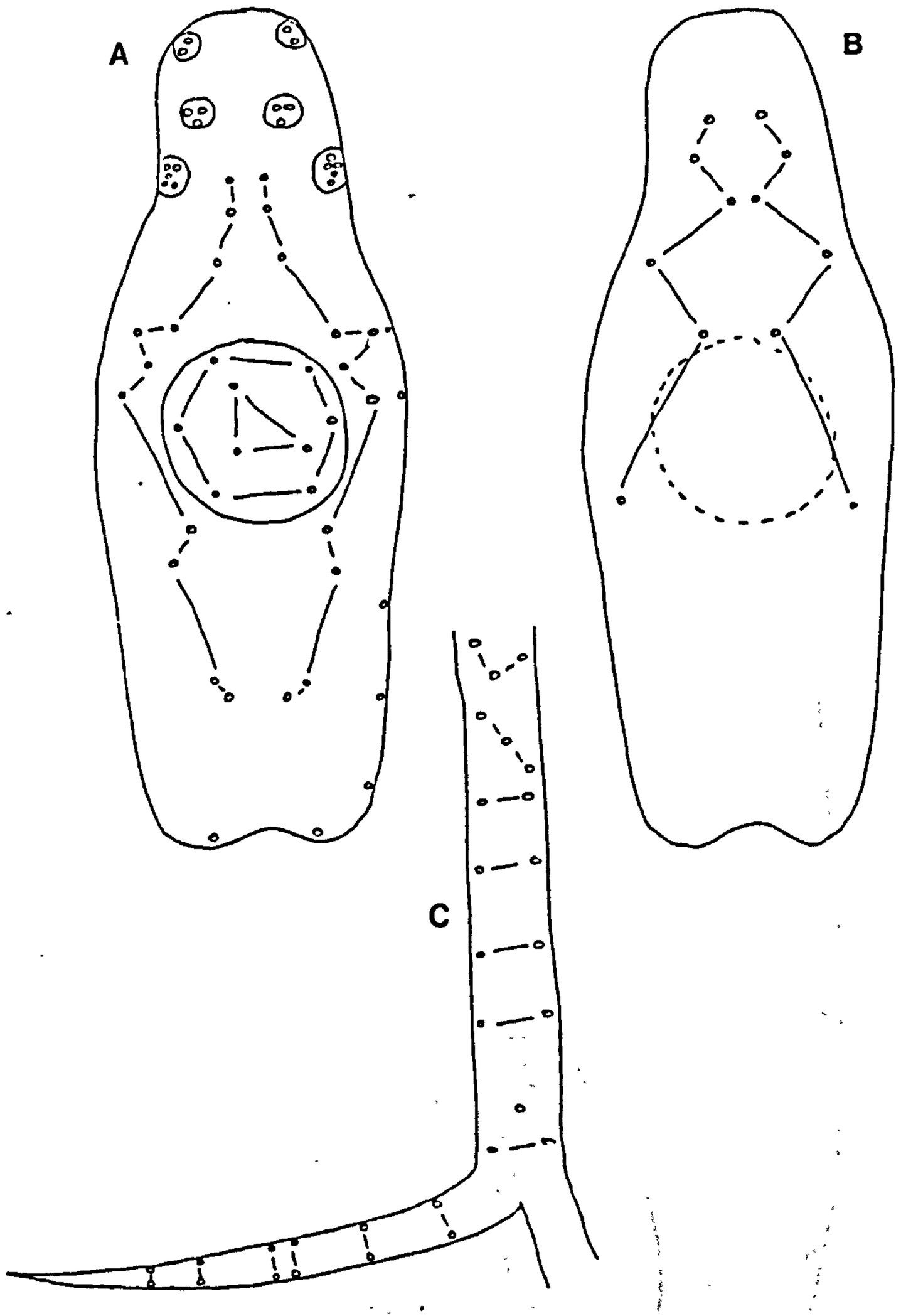


a. Ventral and lateral papillae.

b. Dorsal papillae.

c. Tail stem and furca papillae.

Fig. 6.3. Sensory papillar pattern on Type 4 cercariae.



a. Ventral and lateral papillae.

b. Dorsal papillae.

c. Tail stem and furca papillae.

Fig. 6.4. Sensory papillar pattern on Type 1 metacercariae.

Scale-bar: 50*um*.

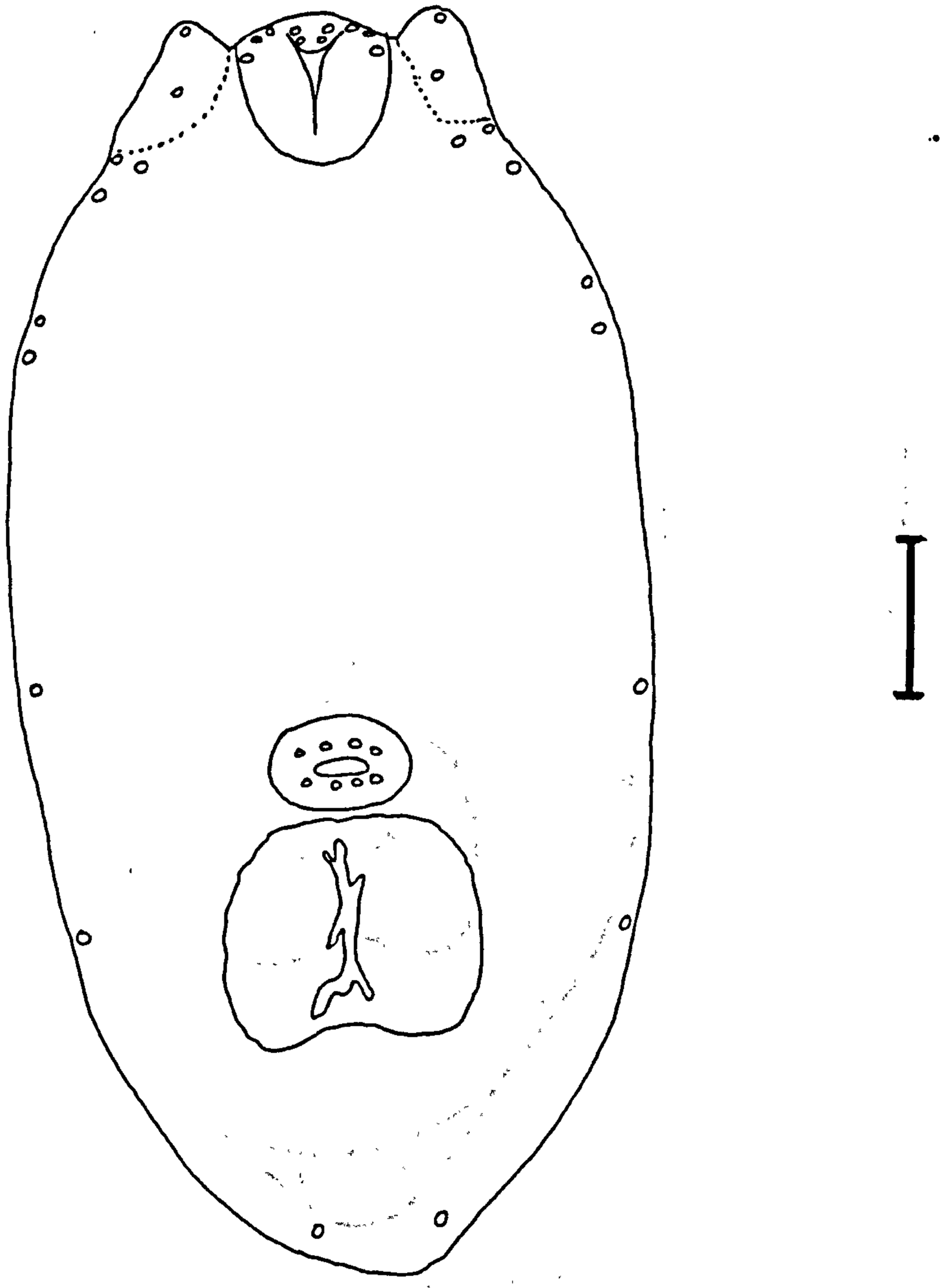


Fig. 6.5. Sensory papillar pattern on Type 2 metacercariae.

Scale-bar: 50 μm.

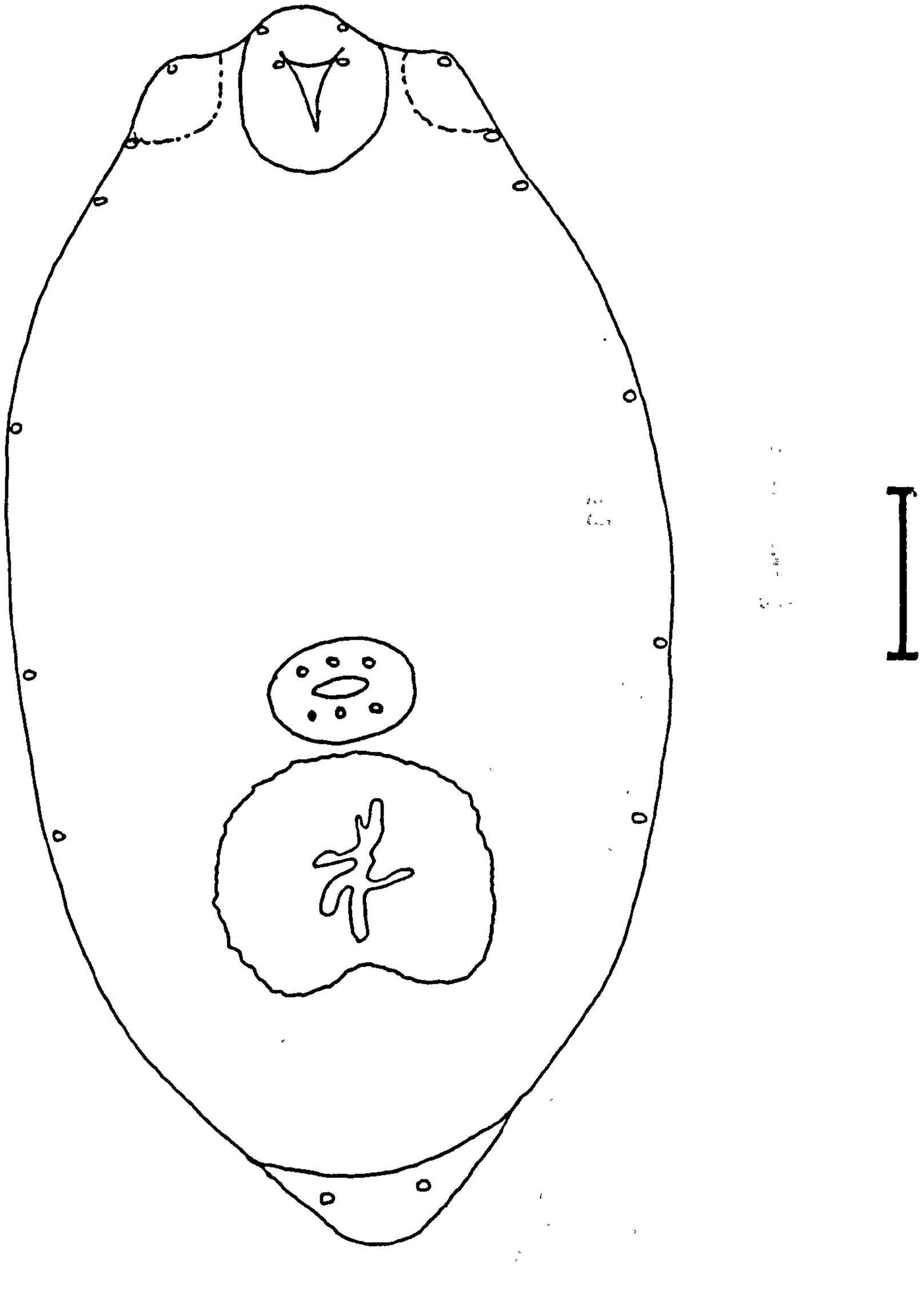


Fig. 6.6. Sensory papillar pattern on Type 3 metacercariae.

Scale-bar: 50 μm. Note:

a. papillae not always present. b. papillae always present.

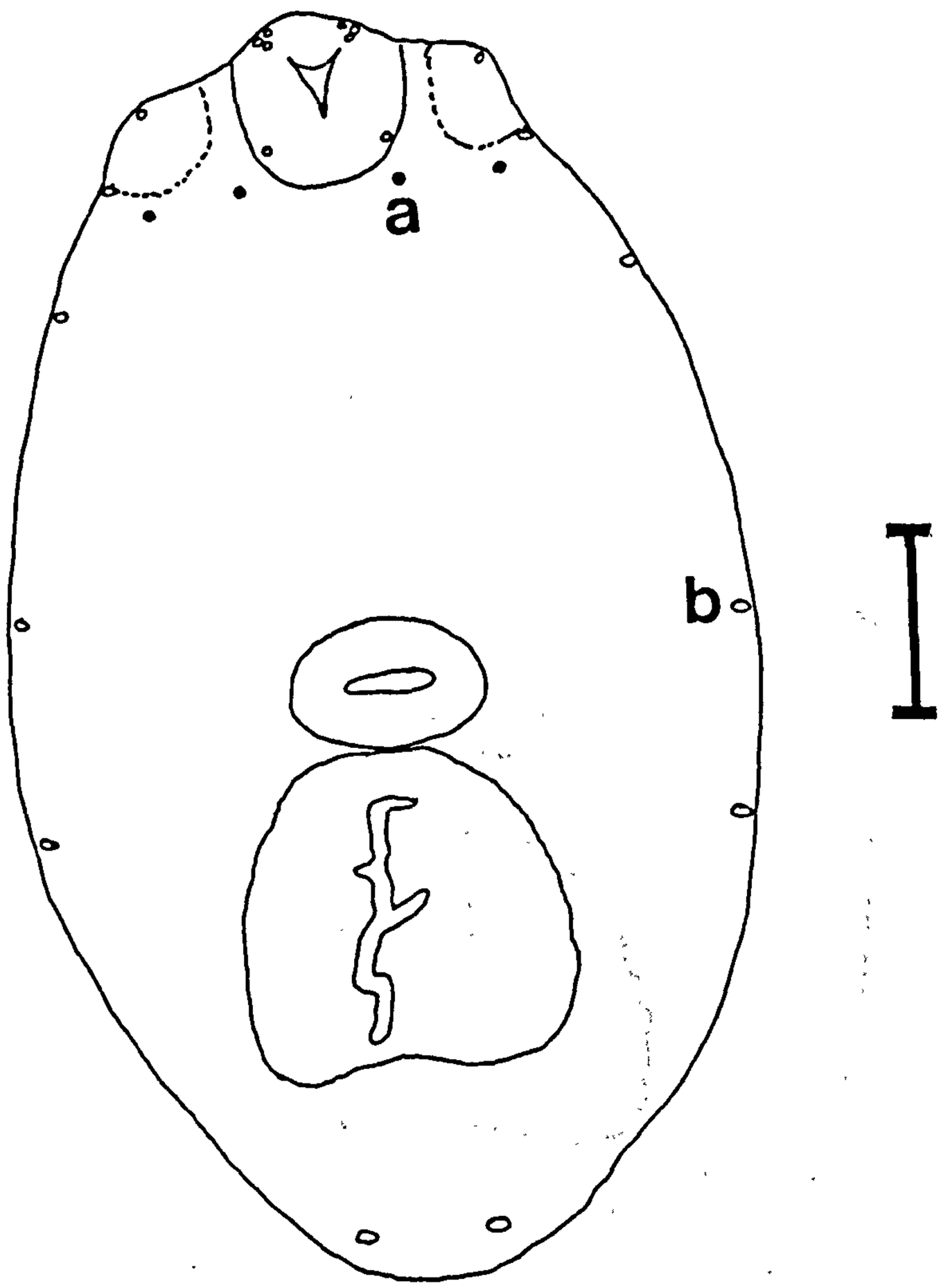
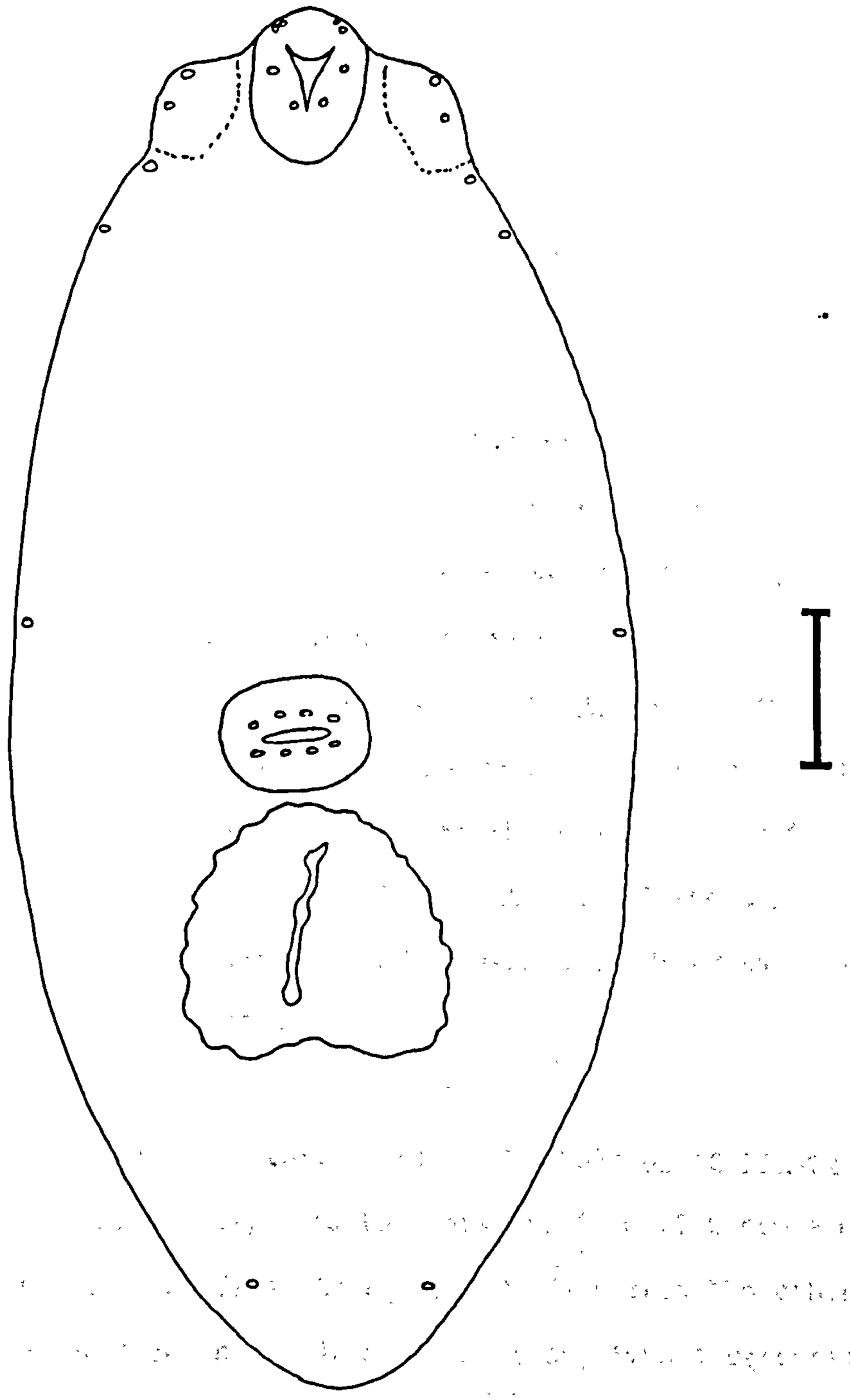


Fig. 6.7. Sensory papillar pattern on Type 4 metacercariae.

Scale-bar: 50*um*.



DISCUSSION

Observations in this study reveal that sensory papillae on Diplostomum cercariae and metacercariae tend to be constant in number and position. Nevertheless, detailed comparisons were not possible since so few species have been studied in this way. Comparison of papillar patterns found in the cercariae in this study with those of Niewiadomska (1986) and Shigin et al. (1985) reveal that Type 1 cercariae are very similar to D. spathaceum and Types 3 and 4 are very similar to D. parviventosum.

There are some differences evident between the patterns of sensory papaillae on D. spathaceum, as reported by Eklu-Natey et al. (1985), and Niewiadomska (1986) when compared to Type 1 cercariae in this study. The first difference is in C I which contains a total of 12-14 papillae in Type 1 cercariae in this study, whereas Eklu-Natey et al. (1985) and Niewiadomska (1986) reported 16. However, this area around the oral cavity is very difficult to examine, as the mouth opening itself is argentophilic, and, therefore, it is possible that not all the papillae can be readily differentiated. Similar problems have been found by other authors (e.g. Short & Catrett, 1973).

C II in Type 1 cercariae matches that of Niewiadomska's D. spathaceum, but Eklu-Natey et al. reported eight to ten papillae in C II L compared to six found in this study. C III in this study differs from the others in that there are no papillae in the lateral position. Type 1 cercariae have the same number of papillae (i.e. two) in the A II L as Eklu-Natey et al., but Niewiadomska reported none. Both Niewiadomska and Eklu-Natey et al. reported no papillae to be present in A III D, whereas Type 1

cercariae had one in this position. The same is true for 1 M D. There is also a difference in the number of papillae on the ventral sucker as Type 1 cercariae only have 5 in S II, whereas the others have six. There are also similar discrepencies between the three patterns in the tail region. Despite these differences Type 1 cercariae do have a similar pattern to the descriptions for D. spathaceum. It is difficult, however, to determine how different two patterns have to be to constitute two different species. Also there is the possibility that different interpretation of 'cycles' may affect the results illustrated by authors.

Cercarial Types 3 and 4 appear to be similar in papillar pattern to D. parviventosum cercariae as described by Shigin et al. (1985). It is difficult to compare Shigin's papillae pattern for D. parviventosum with the cercariae in this study, as he did not use the same nomenclature. However, he did illustrate the patterns and so some comparison was possible. Cercarial Types 3 and 4 in this study differed from each other in that Type 3 cercariae had one extra papilla at C III V, A I D and P III V and Type 4 cercariae had one extra papilla at A II V. Shigin's description of D. parviventosum more closely resembles the Type 3 cercariae pattern, although it differs in that in Shigin's drawings there are three papillae at A I L and two papillae at P II V compared to six(five) and none, respectively, in Type 3 cercariae. Papillar patterns on the ventral sucker are identical in Types 3 and 4 and D. parviventosum, although there are slight differences in the number of papillae in the tail region. Again the differences between these three patterns are minor, but, as discussed above, it is difficult to determine just how different the patterns have to be to represent distict species.

Papillar patterns in the metacercariae are very different from those found in the cercariae and the papillae are much fewer in number. However, the pattern on the body of the metacercariae are fairly constant in number and position, although in this study much variation was found in the oral sucker region. Unfortunately, D. parviventosum is the only species of the genus for which the chaetotaxy of the metacercaria has previously been studied. Shigin et al. (1985), studying the metacercarial stage of this species, reported variation in the ventral sucker pattern. Since the variation occurs in the suckers, it may be that it is due to fixation problems, i.e. silver nitrate may cause the suckers to contract so that the papillae can no longer be viewed properly in some specimens.

On comparing Shigin's results with those in the present study, it was evident that Type 2 metacercariae had an identical papillar pattern to D. parviventosum apart from the highly variable oral sucker region. Types 1 and 4 were also similar to Shigin's description for D. parviventosum, although they had eight papillae on the ventral sucker rather than six. However, Shigin did find some specimens to have eight papillae in this region. Types 1 and 4 also differed, however, in having an extra papilla on the lappets. Type 3 metacercariae differed from all the others in that in all the specimens examined no papillae were observed on the ventral sucker.

From the results in the present study it can be seen that Type 1 cercariae have some affinities with D. spathaceum and the other types are similar to D. parviventosum. Results cannot be properly analysed until the chaetotaxy of the genus is better known. It is evident, however, from this study that it is unlikely that Diplostomum species can be readily

recognised using this technique, although the technique may prove to be a useful aid to identification. The fact that differences are evident in the papillar pattern on the cercariae and the metacercariae of these types of Diplostomum examined in this present study supports the hypothesis that the four types are different from one another.

CHAPTER 7:

ISOELECTRIC FOCUSING

INTRODUCTION

Histochemical staining for isoenzymes shows substantially more specificity than that of general protein staining, where non-specificity can be very confusing, thereby rendering interpretation extremely difficult. Isoenzyme variation can, therefore, be more readily interpreted than that of total protein, since there are fewer variables to be considered.

Isoenzyme analysis has proved to be very useful in studying the taxonomy of schistosomes (Wright & Knowles, 1972; Southgate & Knowles, 1975). Subsequent work by Ross (1976), using starch gell electrophoresis, found that this method was somewhat limiting and for this reason Ross (1976) also tested the use of isoelectric focusing (IEF) in polyacrylamide gels for schistosomes. According to Arbuthnott & Beeley (1975) IEF provides an improved resolution, sensitivity and reproducibility when compared to starch gel electrophoresis. In IEF up to 15 µl of solution may be applied to the gel without detriment to the final pattern, whereas with starch gel electrophoresis a limiting factor has been that of slot dimension related to the quantity of protein in the solution. Also, the final isoenzyme pattern in IEF is related to a pH gradient accross the gel and, therefore, the individual fractions are separated at their isoelectric points (pI, i.e. the pH at which the fraction is neutralised), thereby associating them with an experimentally determined dimension rather than an arbitrary notation as in starch gel electrophoresis.

Results from Ross's study (1976) showed that patterns from individual adult schistosomes using three enzyme systems, namely, LDH, MDH and AcP,

were valuable for comparisons at the intra-population level. Ross (1977) reported that in applying starch gel electrophoresis to the study of larval schistosomes, only one or two often ill-defined isoenzymes of acid phosphatases and malate dehydrogenases were separated, whereas IEF revealed fifteen or more. Since Ross's work many authors have reported that isoenzyme profiles of schistosomes determined by IEF are strain and/or species specific (Ross, Southgate & Knowles, 1978; Wright, Southgate & Ross, 1979; Wright & Ross, 1980, 1983). This analysis has, therefore, much potential in the field of experimental taxonomy.

Although no studies have been carried out on isoenzyme profiles of Diplostomum metacercariae using IEF, there has been one very recent study by Faulkner (1989) (published after most of the work had been carried out for the present study) looking at the total polypeptide distribution profile. In his study, Faulkner determined protein profiles of metacercariae from the eyes of roach (Rutilis rutilis), gywniad (Coregonus laveratus), perch (Perca fluviatilis) and ruffe (Gymnocephalus cernuus) using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This analysis, which has proved to be useful in taxonomic studies in many animal groups, including ascarid worms, insects, snails and salamanders (reviewed by Oxford & Rollinson, 1983), revealed that different morphological forms of Diplostomum metacercariae had key band variations, indicating inter-relationships between known and unknown species of Diplostomum. These variations were found in just three key bands, and the remainder of the polypeptide distributions for the various metacercariae studied were the same. Faulkner suggested that, although the functions of the three key bands were unknown, the expression of the various phenotypes could be related to a single polypeptide variation, or,

as for D. coregonus metacercariae, to reduced concentrations of a group of polypeptides. Total protein analysis could, therefore, be useful in taxonomic studies on Diplostomum metacercariae, since variations were seen between different species.

Due to the small size of Diplostomum metacercariae they must be pooled to provide a sufficient quantity of protein in order to carry out any electrophoretic analysis. This, therefore, rules out the possibility of determining natural variation between individuals, thereby enhancing the complexity of interpreting any variations seen between types of metacercariae. In view of the uncertain nature of the outcome and, as discussed above, since isoenzyme analysis is a more specific approach with fewer variables to be considered than that of total protein analysis, it was decided that this study should concentrate on determining isoenzyme patterns in Diplostomum metacercariae using the IEF technique. Since, at the onset of this study, no literature was available on the application of electrophoretic techniques to the taxonomy of Diplostomum, this study set out to determine whether or not electrophretic analyses could prove useful as a diagnostic tool in the future. Eight isoenzyme systems were chosen to be examined, namely, GPI, PGM, G6PDH, MDH, LDH, AcP, HK and αGDH. These particular systems were chosen as they have proven to be successful in determining strain and species specificity in both schistosomes and amphistomes (Ross, 1976, 1977; Wright, Southgate, Ross & Knowles, 1977; Wright et al. 1979; Ross et al., 1978; Southgate Rollinson, Ross, Knowles & Vercruysse, 1985; Southgate, Brown, Warlow, Knowles & Jones, 1989).

There are some disadvantages to the use of isoenzyme analysis compared

to total protein analysis in that not all enzymes are strongly catalytic and many are particularly labile. Obtaining optimal banding patterns from isoenzymes is, therefore, very difficult to achieve as many experimental variables can affect whether or not the enzymes will stain. With total protein analysis this is not a problem since the proteins are not readily denatured and do not have to remain catalytically active. Also, in carrying out protein analysis on polyacrylamide gels the system can be well buffered, increasing the stablility of the samples, whereas in the use of IEF to determine isoenzyme patterns, the system is buffered only by the ampholines which determine the pH gradient. In addition to the difficulties of the technique, isoenzyme analysis using IEF is also very expensive and time-consuming compared to total protein analysis. Despite the difficulties and cost of the technique, results, when obtained, are certainly more well-defined and easier to interpret than those of total protein analysis.

MATERIALS AND METHODS

1. Collection and Storage of Material.

Metacercariae were collected from the eyes of infected fish as described in Chapter 2, Section 2 of the 'Materials and Methods'. Specimens were washed two or three times in distilled water by flushing with a pipette and stored in cryogenic vials in liquid nitrogen (-196°C) for up to six months (they can, however, be stored indefinitely in liquid nitrogen, but in this study six months was the longest period of time that specimens were maintained). Control host tissue was maintained in exactly the same manner. Lens, humour and retinal material were collected from uninfected rainbow trout and perch as detailed in Chapter 5, Section 7 of the 'Materials and Methods', and were stored in separate vials.

2. The Polyacrylamide Gel.

2mm polyacrylamide gels with a pH range of 3.5-9.5 were prepared according to the method described by Ross et al. (1978) and application of the samples to the plates followed the technique of Ross (1976). The constituents of each gel are documented in the Appendix 5.

3. Sample Preparation.

Metacercariae and control host tissues were removed from liquid nitrogen and allowed to thaw. Metacercariae were used at a concentration of 15-20 per 5 µl of distilled water. This concentration was maintained throughout all the parasite samples in order to standaridise the total amount of

protein applied to the gel. For this reason Type 4 metacercariae were only used on the first four stains as the samples would have been too dilute to use on eight stains. Host tissue was normally diluted with distilled water at a concentration of approximately one host tissue sample per 10μl of distilled water. Standardisation of the total amount of protein applied to the gel for host samples was somewhat arbitrary since it was difficult to assess the amount of protein present in lens, humour and retina and also the size of the fish varied. Samples were then homogenised in plastic precipitin tubes using ground cut glass rods and were centrifuged for 15 minutes at 1800 r.p.m. at 4°C. 10 µl of supernatant from each tube was applied to each track of the gel.

4. Focusing the Gel.

Once the samples were applied (including a human haemoglobin marker which has a major focusing band at a pI of 7.25 which can be used to set the pH meter when measuring the gel) the gels were run for two hours at a constant power of 60W, with upper limits of 1200 volts and 100mA. During isoelectric focusing the gel plate was kept cool by the use of a cooling plate which was maintained at 2°C by circulating chilled water. Maintaining a temperature of 2°C is very important, since many of the enzyme systems used in this study are highly labile.

5. Enzyme Analysis.

Electrophoresis was carried out for two hours after which the gels were stained for eight enzymes:

1. glucosephosphate isomerase (GPI, EC 5.3.1.9)
2. phosphoglucomutase (PGM, EC 2.7.5.1)
3. glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49)
4. malate dehydrogenase (MDH, EC 1.1.1.37)
5. lactate dehydrogenase (LDH, EC 1.1.1.27)
6. acid phosphatase (AcP, EC 3.1.3.2)
7. hexokinase (HK, EC 2.7.1.1)
8. αglycerophosphate dehydrogenase (αGDH, EC 1.1.1.8).

The constituents for the enzyme stains are given in Appendix 5.

6. pH Measurement.

Readings were taken with a Pye Ingold 8-mm membrane surface electrode every 10mm from the anodal end of the gel in the range 20-110mm in order to obtain a pH gradient across the gel. (The pH may be taken at 5mm intervals, but this takes a longer period of time during which the pH gradient of the gel may become unstable.)

7. Enzyme Profiles.

The gels were fixed with 4% glycerine in 5% acetic acid. The background colour was then removed by washing the gel three times per day for two days with 3% glycerine. Washing was carried out by flooding the gel in a large dish with the solution. The separated bands of enzyme activity could then be measured and the pI for each fraction was determined by correlating the position of the band to the pH gradient of the gel as determined by the method above.

RESULTS

Of the eight isoenzyme systems selected for histochemical staining, satisfactory results were obtained from only three, GPI, PGM and MDH. Little information could be obtained from the other five enzyme systems, since few of the samples showed discernable banding patterns. However, some interesting features were evident with LDH and AcP and are noted below. The enzyme profiles for GPI, PGM, MDH, LDH, AcP and G6PD are illustrated in Plate 7.1. G6PD is included to illustrate the point that, although some bands are present, they are not measureable. Although not illustrated, HK and αGDH systems did not reveal measureable banding patterns either.

GPI: As can be seen from Plate 7.1, bands of GPI activity were obtained from extracts of host and parasite material. Although efforts were made to standardise the total amount of protein applied in each sample, there were marked differences in the banding intensities. This suggests, therefore, that differing intensities of staining between parasite samples were due to distinct differences between samples rather than the amount of protein present. This was not the case, however, with host tissue, as standardisation of protein loading was arbitrary.

There was little evidence of contamination with host material in lens and retinal metacercariae, but the bands of activity produced by humoural metacercariae from rainbow trout (Type 2) (track 4) completely match the profile obtained from uninfected humoural material (track 5).

The main banding patterns obtained from metacercariae from the lens of

rainbow trout (Type 1), the retina of rainbow trout (Type 3) and the retina of perch (Type 4) (tracks 3, 7 & 8, respectively) revealed that there was some similarity between the three. The profiles obtained from Types 3 and 4 metacercariae were identical, although the intensity of the stain differed between them. Type 1 metacercariae produced a similar banding pattern, although the pI's of the bands did vary slightly. The main bands in Types 3 and 4 metacercariae are found at pI's of 6.52, 6.62, 6.80. 6.95 and 7.15, whereas in Type 1 they are located at pI's of 6.55, 6.70, 6.85, 7.05 and 7.25.

PGM: In this analysis the extracts of Types 1 and 2 metacercariae merged. Since the profile exactly matched that of uninfected humoural material, it may be assumed that the profile seen is that of Type 2 metacercariae rather than Type 1. If Type 1 bands are present they are probably being masked by those of Type 2. Again, since it was found that the bands of Type 2 metacercariae exactly matched those of control host material, it would appear that the metacercariae were contaminated with host tissue.

G6PD: Only very faint bands of activity were observed with this enzyme system. The control uninfected lens material from rainbow trout and uninfected retinal material from perch, however, showed some banding which indicated that the system was functional, although the parasite material was not active. Similar results were obtained with HK and αGDH, although the results for these are not illustrated.

MDH: Again the results indicate that uninfected humoural material from rainbow trout has exactly the same profile as Type 2 metacercariae. Furthermore, Types 3 and 4 metacercariae had an identical profile to one

another. Type 1 metacercariae, however, showed no banding profile.

LDH and AcP: Apart from Type 2 none of the other types of metacercariae stained for either of these two enzymes. The LDH profile of the control uninfected humoural material again matched the metacercarial profile and, therefore, it was impossible to determine whether or not the metacercarial profile was missing or masked by the host tissue. However, with AcP the uninfected humoural material did not stain, but a very strong band at a pI of 6.1 was obtained for Type 2 metacercariae.

PLATE 7.1. Enzyme profiles obtained from histochemical staining of IEF gels.

a. GPI c. G6PDH

Track	Sample
1	HHM
2	LHT
3	Type 1
4	Type 2
5	HHT
6	RHT(RT)
7	Type 3
8	Type 4
9	RHT(P)
10	HHM

b. PGM d. MDH

Track	Sample
12	HHM
13	LHT
14	Type 1
15	Type 2
16	HHT
17	RHT(RT)
18	Type 3
19	Type 4
20	RHT(P)
21	HHM

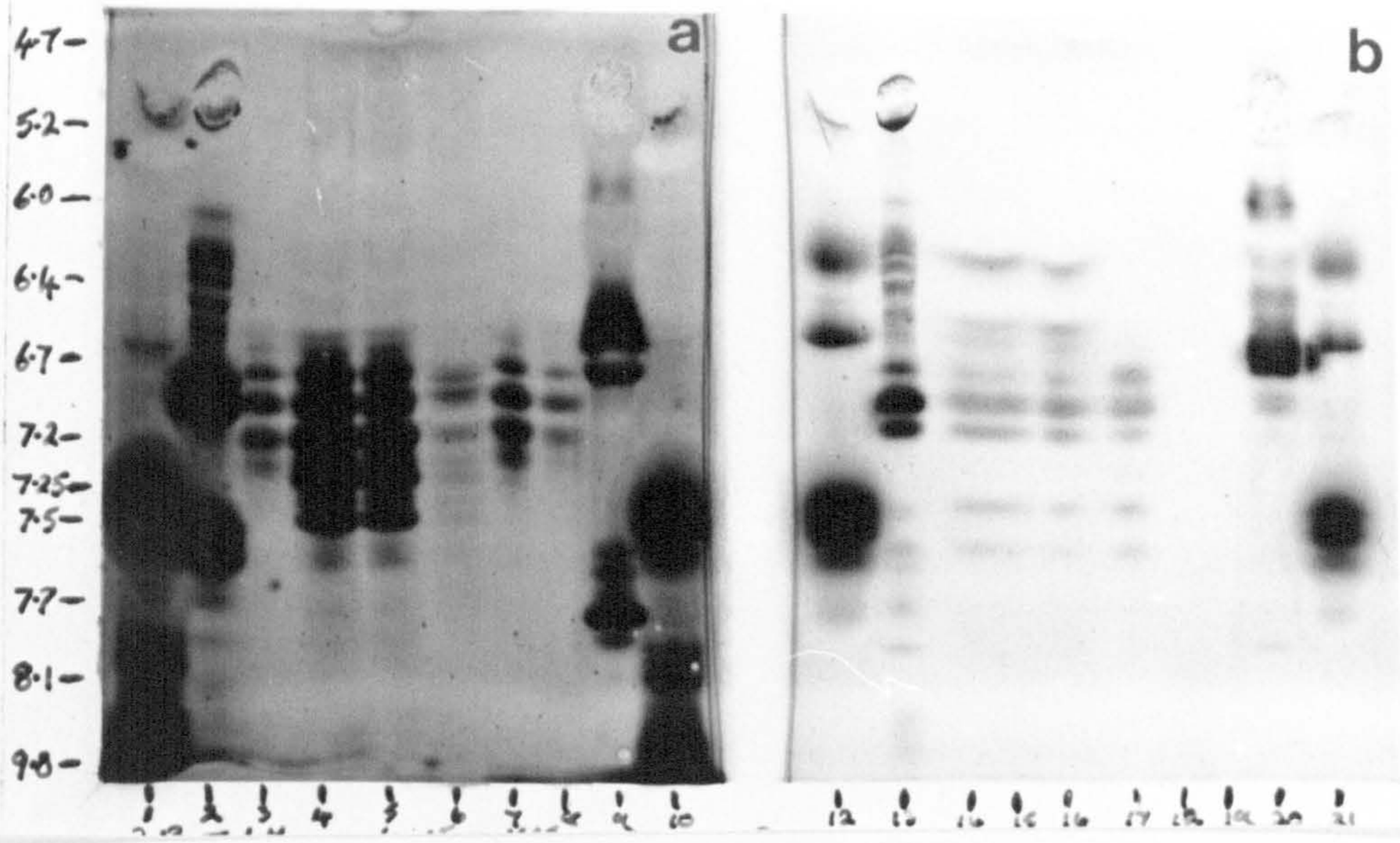
e. LDH

Track	Sample
1	HHM
2	LHT
3	Type 1
4	Type 2
5	Type 3
6	HHT
7	RHT(RT)
8	HHM

f. AcP

Track	Sample
11	HHM
12	LHT
13	Type 1
14	Type 2
15	Type 3
16	HHT
17	RHT(RT)
18	HHM

Note: HHM = human haemoglobin marker, LHT = lens host tissue from r. trout, HHT = humoural host tissue from r. trout, RHT(RT) = retinal host tissue from r. trout, RHT(P) = retinal host tissue from perch, Type 1-4 = Type 1-4 metacercariae.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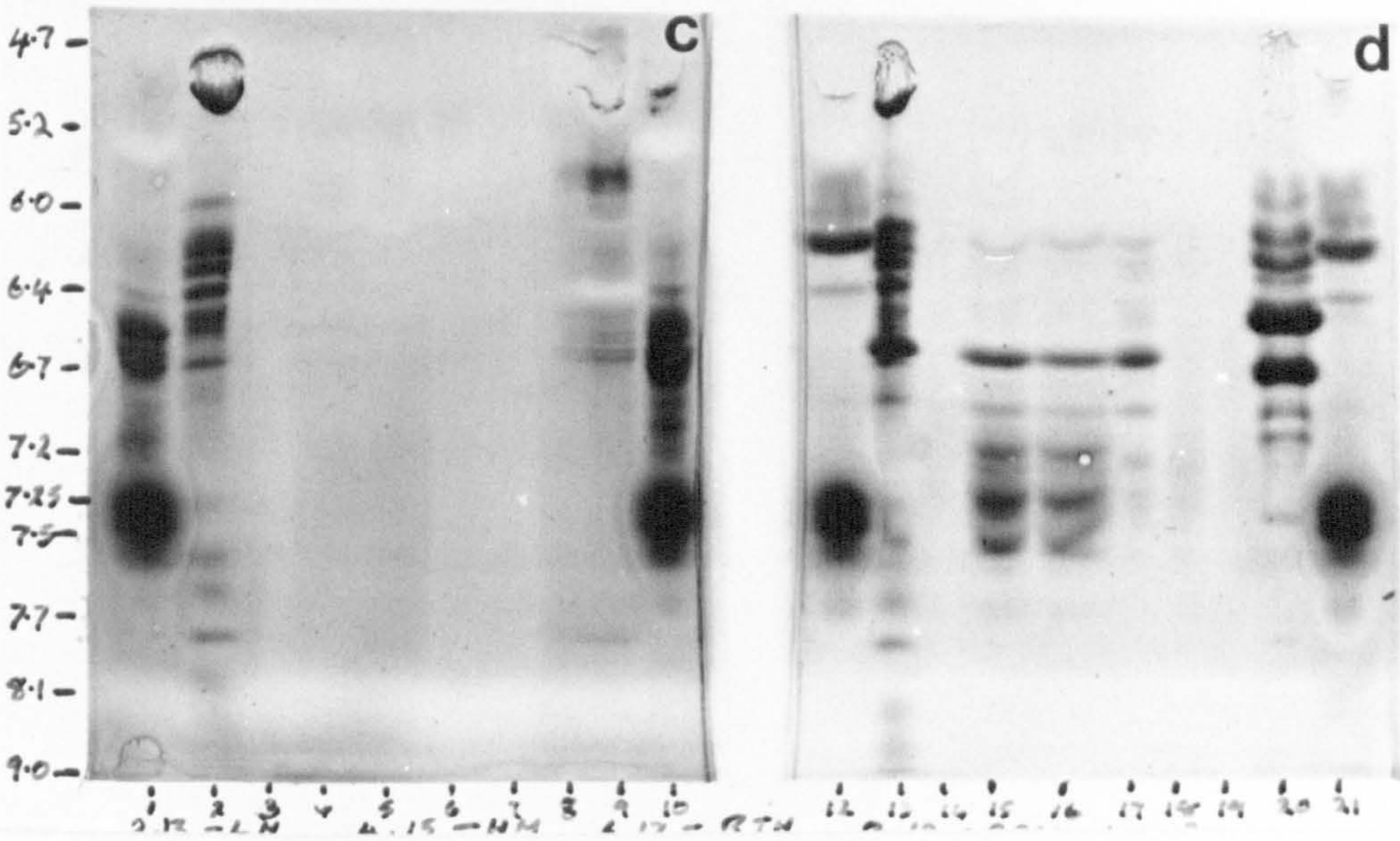
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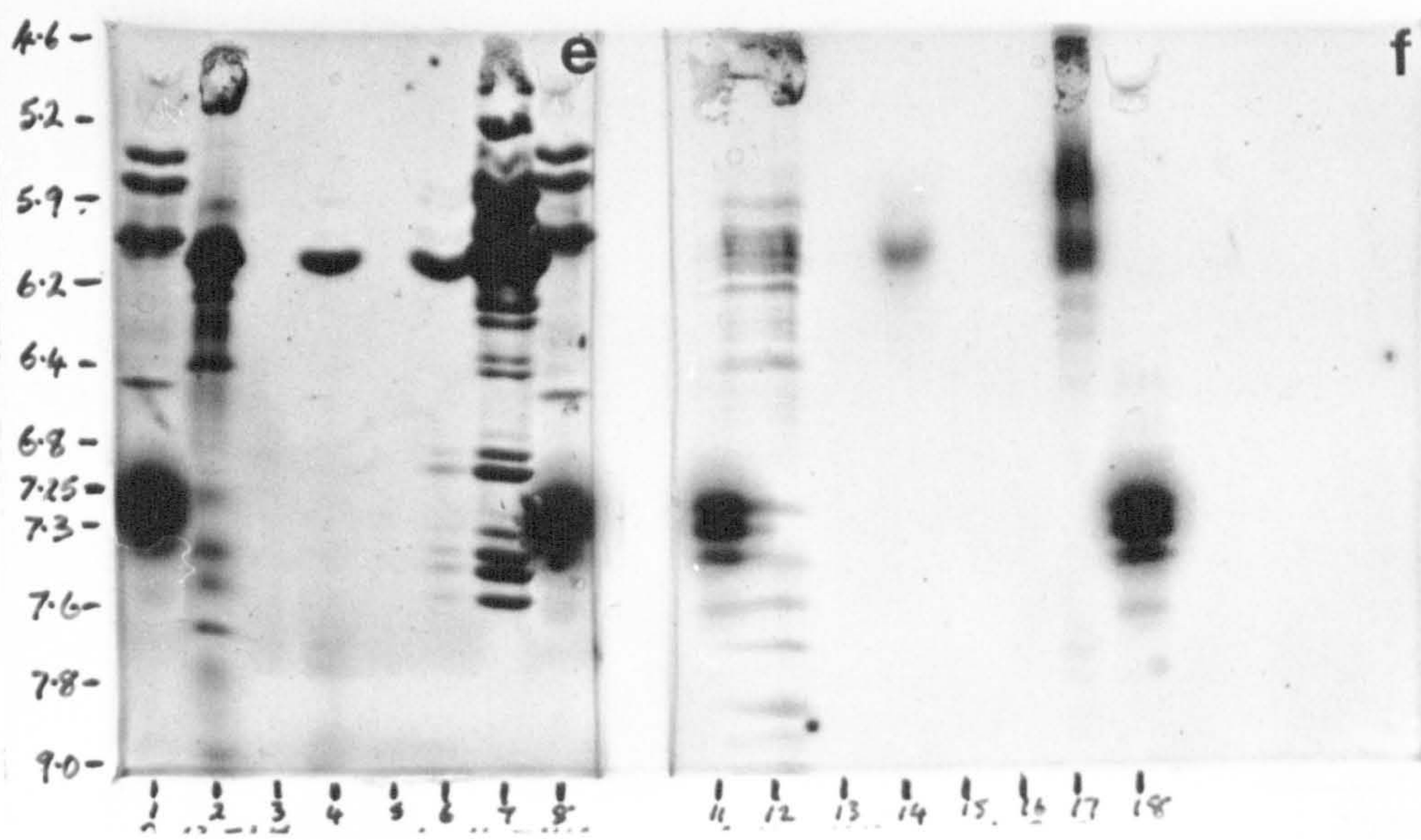
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DISCUSSION

Isoenzyme profiles of Diplostomum metacercariae, as determined using the technique of isoelectric focusing, were apparently very similar. Profiles obtained from the histochemical staining for GPI revealed that there was a general Diplostomum pattern in metacercarial Types 1, 3 and 4. This confirms the results of total protein analysis on Diplostomum metacercariae by Faulkner (1989) which revealed a similar pattern in different types of metacercariae. The profile from Type 1 metacercariae was found to differ from that of Types 3 and 4. However, natural variation was not taken into account, as each sample consisted of more than one metacercaria. Therefore, it is unclear whether the differences seen were due to differences in speciation or merely natural variation between populations. This problem affects the composite picture obtained from all the enzyme profiles and is very difficult to overcome when the samples, i.e. the metacercariae, are of such a small size.

Histochemical staining for the other six enzymes did not provide satisfactory results, since little or no banding was evident. The MDH system also indicated that the profiles for metacercarial Types 3 and 4 were identical. Therefore, it was impossible to distinguish these two metacercariae. AcP and LDH illustrated, however, that Type 2 metacercariae differed from the Types 1 and 3 metacercariae, since only this parasite showed activity with these two systems.

The problem of contamination with host material appears to have been overcome in metacercarial Types 1, 3 and 4 which were obtained from lens and retinal material. On the other hand, Type 2 metacercariae obtained

from humoural material consistently showed the same banding profile as uninfected host humoural material. This indicates that either there were no parasite bands at all or the parasite bands were effectively masked by the host bands. The cause of the contamination may be related to the nature of the humoural material, since this gelatinous structure is very 'sticky'. This is very different to the crystalline nature of the lens and the cellular nature of the retina which would render them less likely to contaminate the parasites. The problem of host material contamination with Type 2 metacercariae may be overcome by more vigorous flushing with distilled water before and after freezing in liquid nitrogen. This could, however, cause more problems since the metacercariae may be damaged if treated more roughly and hence the outer membrane may rupture releasing the body contents.

Poor activity visualised with some of the enzymes systems could be caused by either using a protein concentration which is too dilute or the enzymes may have been denatured. As discussed in the 'Introduction', some enzymes are more labile than others and so treatment for one enzyme may not be suitable for another. However, since great care was taken to ensure that the material was always maintained at or below 4°C during processing, it seems unlikely that the enzymes would be denatured. For all the enzyme systems examined, the protein concentration of the parasite samples was the same. It would appear, therefore, that the protein concentration should not cause problems, since strong activity was visualised with three of the systems. However, these three systems, GPI, MDH and PGM, are strongly catalytic and hence will reveal activity at most protein concentrations. On the other hand, the other enzyme systems examined are not so catalytically active, and, therefore, they perhaps require a larger

amount of protein before activity is seen. However, this would mean more metacercariae per sample and hence the problems associated with the complexity of natural variation would be more acute.

Further analysis of Diplostomum metacercariae using the technique of IEF to look at isoenzyme pattern would involve at least two changes. Firstly, in this study wide-range pH gels (3.5-9.5) were used, as it was uncertain where the parasite bands would focus. However, it was found that the majority of the parasite bands focused in the pH range 5.0-7.5 and thus better separation of these bands may be achieved by using a narrower range of pH. It must be noted, however, that this would eliminate bands with low or high pI's which may be the key bands in differentiating the specimens being examined. Total protein analysis on different types of metacercariae by Faulkner (1989) revealed that differences were evident in only three key bands. The same type of key band variation may, therefore, be expected from this type of analysis. On the other hand, the use of a narrower pH range may give a clearer resolution of the main bands and thus allow for better comparisons. Secondly, it may prove beneficial to compare different concentrations of proteins to obtain the optimal amount of protein to be loaded per sample. This could be achieved by a mass extraction of protein, for example by freeze-drying, and then carry out serial dilution until the optimal protein concentration is achieved.

It is evident from this preliminary study that IEF is a very difficult and time consuming technique. The rewards can be great if results are readily achieved. However, this is not always the case. This present study revealed some promise in the technique being applied to diplostomes in that some differences were evident between the metacercarial types.

However, it must be emphasised that the input of the technique in terms of time and resources can sometimes far outweigh the output.

CHAPTER 8:

FINAL SUMMARY AND CONCLUSIONS.

SUMMARY AND CONCLUSIONS

Collation and integration of the results from this study indicate that there were four different types of Diplostomum present. Type 1 was present in the lens of rainbow trout (Salmo gairdneri), Type 2 in the humour of rainbow trout, Type 3 in the retina of rainbow trout and Type 4 in the retina of perch (Perca fluviatilis).

Morphological analysis of the metacercarial stage revealed that using principal components analysis (PCA) the metacercariae studied separated into four different groups. Type 1 separated from the others due to the fact that the ventral sucker was positioned significantly more anteriorly than all the other types and, more noticeably, the lappets were very close together. This latter feature allowed for easy recognition of Type 1 metacercariae when examined under the microscope even at low power. Type 3 metacercariae separated from the other three types by virtue of its small size. These metacercariae were significantly smaller than all the others. On the other hand Type 4 separated from the other three types due to the fact that they were significantly larger than all the others. In addition, they had a significantly higher B/L ratio, the ventral sucker was positioned more posteriorly than that of the others and the lappets were significantly further apart than those of the other three types. Unfortunately, no significant character could be attributed to the separation of Type 2 metacercariae from the other three types, although they did separate from them by virtue of the fact that they lacked the features which distinguished the others.

Culture of the metacercariae to the adult stage in the bird intestine

also gave supportive evidence of these differences between the four types. Type 1 metacercariae grew in both day-old chickens and herring gull chicks, but better establishment and recovery was obtained in the herring gulls. Type 2 metacercariae also grew in day-old chickens and herring gull chicks, but better establishment and recovery was evident in chickens. Furthermore, although the adult worms of Type 2 produced eggs in gulls, incubation of these eggs revealed that they were not viable, whereas those produced in chickens were viable. Type 3 and 4 metacercariae did not grow in chickens at all, but they both grew well in gulls, producing viable eggs.

Morphological analysis of the adult stage of the Types 1-4 and application of PCA again revealed that separation into four groups was achieved. Type 1 adults separated from the others by their large size and the position of the ovary in the posterior segment. The posterior segment in these worms was much larger than the anterior segment, whereas in the other three types the opposite was found to be the case. Also, the ovary was positioned one third of the way down the posterior segment, whereas in the others it was closer to the intersegmental junction. Type 2 adults separated from the others by the size and position of their ovary. The ovary in these worms was significantly smaller than that of the other three types and it was also positioned closer to the intersegmental junction than in the others. Type 4 adults were distinguished from the others by the fact that the vitellarium extended significantly more anteriorly and the ventral sucker was in a more anterior position. In addition, scanning electron microscopy revealed that the sensory papillae around the anterior extremity of Type 4 adults were significantly larger in size than those of the other three types. Unfortunately, Type 3 adults

did not possess any significant features that distinguished them from the other types, although they separated from the others by virtue of the fact that they lacked the distinguishing features of the other three.

Completion of the life-cycle of these diplostomes also revealed some differences between the four types. Although eggs from Type 2 adults raised in chicks hatched to produce miracidia, it was found that the miracidia would not infect Lymnaea pereger, whereas the miracidia from the other three types did infect this snail host. This could indicate that either the miracidia were not able to infect any snail host, although the fact that they were motile indicated that this was unlikely, or that L. pereger is not the first intermediate host of this type of miracidia. If the latter is true, then perhaps the true host is the semi-aquatic L. stagnalis. It may be significant that Type 2 metacercariae were only found in rainbow trout in pond systems when a survey of various farms was carried out at the beginning of this study. Rainbow trout surveyed in cages in lochs, e.g. Loch Awe near Oban and Loch Fad on the Island of Bute, did not harbour humoural metacercariae. The fact that Type 2 metacercariae also had a preference for a different final host than the other three types indicates that it is possibly biologically distinct from the others employing a different group of hosts which are ecologically distinct.

Eggs from Types 1, 3 and 4 adults hatched to produce miracidia which infected L. pereger. This allowed a morphological study of the cercarial stage to be undertaken. Type 1 cercariae were found to be very different from Types 3 and 4 in both the number of caudal bodies present and the arrangement of the body armature. Morphological features of cercarial

Types 3 and 4 were very similar, but Type 4 cercariae were very much larger than those of Type 3 (Body lengths: Type 3; 0.167-0.174mm, Type 4; 0.271-0.301). Again the different types of diplostomes can, therefore, be distinguished on morphological characters.

Cercariae obtained were also used to complete the life-cycle by infecting a fish second intermediate host. Fingerling rainbow trout were exposed to cercarial Types 1, 3 and 4. At post-mortem it was found that Type 1 cecariae had localised in the lens of the eye, Type 3 in the retina and Type 4 did not establish in the rainbow trout host at all. However, when perch were exposed to Type 4 cercariae, infection established in the retina. This indicates that Types 1, 3 and 4 are very site specific and that Type 4 utilise a different fish host. It is unfortunate that lack of remaining infected snails did not permit exposure of perch to Type 1 and 3 cercariae to determine whether or not these types would establish in the perch host.

Staining of the sensory papillae to reveal the papillar pattern has previously been used with much success by other authors to determine differences between various diplostomes, and, therefore, this method was also used in this study in order to elucidate any differences in this area. Again Type 1 cercariae were very different from Types 3 and 4 in the papillar pattern although it was difficult to determine many differences between Types 3 and 4.

Biochemical analysis of the four types of metacercariae by histochemical staining for isoenzymes failed to show any distinct differences between the metacercariae, but the results showed that further analysis could be

promising.

From all the above information it is clear that there are four different types of diplostomes present which separate from each other both morphologically and biologically. The distinguishing features of each of the four types are summarised in Table 8.1. The next stage of the study was to attempt to identify these diplostomes using the keys and other information available in the literature, albeit in a state of confusion.

Type 1 metacercariae, using Shigin's key (1986), were identified as Diplostomum spathaceum and they also conformed to D. spathaceum sensu Niewiadomska (1986). Using Dubois' key (1970) the adult stage of Type 1 was also identified as D. spathaceum, although there was some discrepency in the extent of the vitellarium. However, it did correspond with Niewiadomska's (1986) description of adult D. spathaceum (1986). The cercarial stage of Type 1 also conformed with Niewiadomska's description of D. spathaceum cercariae, although some differences were evident in the number of caudal bodies and the position of the flame cells in the tail stem. However, in overall size, body armature and chaetotaxy Type 1 cercariae closely resembled her description of D. spathaceum cercariae. D. spathaceum has been previously recorded from the lens of a wide range of fishes, including rainbow trout by British authors (Robertson, 1953; Erasmus, 1958; Berrie, 1960b; Mishra, 1966; Chappell, 1969a; Betterton, 1973; Sweeting, 1974; Wootten, 1974; Crowden, 1976; Stables, 1984; Whyte, 1989) and this study tends to confirm, therefore, their identification of the lens form. Nevertheless, a detailed study of material from the lens of a wide range of fishes in the UK is required before a definitive answer to the problem can be obtained.

Type 2 metacercariae were not so readily identified as those of Type 1. British parasitologists have tended to call all humoural and retinal metacercariae D. gasterostei Williams, 1966, but the range of measurements for Type 2 metacercariae do not correspond to those published for D. gasterostei. Ching (1985) recorded D. baeri bucculentum Dubois & Rausch, 1948 from the humour and retina of chinook salmon (Oncorhynchus tshawytscha Walb.) in Canada by culturing the metacercariae in chickens and identifying the adults. However, in the present study it was found that neither of the two retinal metacercariae would grow in chickens and so it is possible that she only recovered adults from the humoural metacercariae. However, Dick & Rosen (1981), again in Canada, had previously grown retinal and humoural metacercariae from the eyes of lake whitefish (Coregonus clupeaformis Mitch.) in herring gull chicks and identified these as D. baeri bucculentum. Thus, in this case, both types of metacercariae should have grown to produce adults and the resulting description given is likely to be a mixture of both types. Shigin (1977) demonstrated the lack of grounds for discriminating two subspecies within D. baeri Dubois, 1937, and designated D. baeri and D. baeri bucculentum synonymous. When Type 2 metacercariae were run alongside data on specimens of D. baeri sensu Niewiadomska (1988) on the PCA, it was found that they did group closely to this material. Comparison of measurements of Type 2 metacercariae with those given for D. baeri bucculentum sensu Ching (1985) and D. baeri sensu Niewiadomska (1988) revealed that Type 2 were much smaller in size than the others, although the relative proportions were similar. This difference in absolute size might be attributed, however, to either differences in age of the metacercariae or to the method of fixation. In the present study it was found that specimens fixed by Niewiadomska's method using hot water were significantly different from

those fixed in Berland's fluid (the fixative used throughout the present study). Ching (1985) does not mention how she fixed her specimens. If this difference in size can be accounted for by the use of different fixatives then Type 2 metacercariae are otherwise similar to D. baeri.

Using Shigin's (1986) key to identify Type 2 metacercariae was not very successful. They overlapped with characters from both D. volvens Von Nordmann, 1832 and D. pusillum Dubois, 1928. Using PCA on Type 2 metacercariae with data from D. volvens and D. pusillum, Type 2 metacercariae grouped separately from these two species. When measurements of Type 2 metacercariae were compared to those of D. volvens, however, they were very similar, but Shigin (1986) stated that D. volvens was specific to the choroid layer in the retina of the eye and was not present in the humour. It should be noted that Shigin (1986) listed D. baeri as a synonym of D. volvens. Niewiadomska (1988) reported, however, that aside from the similarity in host species, location outside the lens of the eye and geographical distribution, the morphological characters given by Nordmann (1832) for D. volvens cannot justify considering it identical to D. baeri and that they should be considered separate species. For the purpose of the present study, therefore, these two species will be considered to be separate. There is still confusion, as to what species Shigin's (1986) description of D. volvens relates to and indeed it could be a mixture of both D. volvens and D. baeri.

Using Dubois' key on the adults of Type 2, it was found that they were most similar to D. mergi, as the ovary was of comparable size and position. Also, the extent of the vitellarium and size of the pharynx were very similar. The only difference between the two was that in D. mergi,

the oral sucker is smaller than the ventral sucker, whereas in Type 2 adults the opposite was true (although both suckers are of comparable size in Type 2, the ventral sucker is slightly larger). Using Dubois' key, Type 2 adults are also very similar to D. mahonae Dubois, 1953. This species was originally recorded from Northern Ireland. The main difference between the two species is that the ovary in D. mahonae is larger than that of Type 2 adults. In addition, the ventral sucker of D. mahonae is much smaller than the oral sucker, whereas in Type 2 adults they are almost equal in size, and the vitellarium extends much further anteriorly in D. mahonae than in Type 2 adults.

On comparing Type 2 adults with those of D. baeri baeri in Dubois' key, they differ in that the lappets are always posterior to the oral sucker in D. b. baeri, whereas in Type 2 adults the lappets are parallel with the oral sucker, and, in addition, the ovary of D. b. baeri is much larger than that of Type 2 adults.

In summary, it appears that Type 2 adults are similar to D. mergi, D. mahonae and D. baeri, but there are still many differences between them. It is unfortunate that the life-cycle of this diplostome could not be completed, as the morphology of the cercaria may have shed more light on the situation. Chaetotaxy of the metacercarial stage was carried out, but since the pattern for all the metacercariae was fairly similar and only the pattern for D. parviventosum is available in the literature (Shigin, 1985), it would be misleading to assume that these worms are similar to D. parviventosum. Furthermore, since D. parviventosum is a lens diplostome, it is unlikely that these humoural forms would belong to this species. The same holds true for D. mergi as this is also a lens species.

D. baeri, on the otherhand is found outside the lens of the eye according to Niewiadomska (1988), although exact position was not given in her description. Ching (1985) reported them to be present in the humour and retina and, therefore, it would seem more likely that Type 2 diplostomes belong to this species than to any of the others.

Type 3 metacercariae were also very difficult to identify. PCA of these metacercariae alongside Shigin's specimens revealed that they grouped closely to D. volvens. This species of diplostome, as indicated earlier, is specifically located in the retina of the eye, although, it has not been reported to infect salmonids. Using Shigin's (1986) key Type 3 metacercariae keyed down to D. pseudobaeri Razmashkin & Andrejuk, 1978, due mainly to their small size and their location in the humour of the eye. However, since Shigin stated that this species has a double cyst wall around the outside which was not evident in the material in the present study its conspecificity with this species seems very unlikely.

Identification of Type 3 adults using Dubois' key (1970) indicated that they resembled D. parviventosum and D. mahonae most closely. As mentioned earlier, D. parviventosum metacercariae infect the lens of fish, whereas Type 3 adults are derived from retinal metacercariae and were found to locate specifically in this region when the life-cycle was completed. D. mahonae adults also differ from Type 3 adults in that the vitellarium extends further anteriorly in D. mahonae than in Type 3 worms. Comparison of Type 3 adults with adults of D. pseudobaeri also revealed differences in the dimensions of the suckers and the extent of the vitellarium. This evidence, therefore, supports the hypothesis that it is unlikely that Type 3 diplostomes belong to this species.

When the Type 3 cercariae were examined they resembled D. parviventosum both morphologically and in the arrangement of the sensory papillae. Nevertheless, when the chaetotaxy of the metacercarial stage was examined, they were different in that Type 3 metacercariae possessed no argentophilic papillae on the ventral sucker, whereas D. parviventosum metacercariae had 6-8 papillae in this area.

Problems with the identification of Type 4 diplostomes were similar to those of Type 3, since characters for this form did not match well with any named species in the literature. When identification of the metacercarial stage was attempted using Shigin's (1986) key it was found that they resembled D. volvens and D. pusillum. Shigin (1986) stated that D. pusillum was a parasite free in the eye tissues of nemachilids, whereas D. volvens was a parasite of the retina found in salmonids and percids. Therefore, it seems more likely that, since Type 4 metacercariae were found to infect the retina of perch, these metacercariae would resemble D. volvens. D. volvens was reported by Irwin et al. (1989) to be present in the UK and so this further supports this tentative identification.

Examination of the adults of Type 4 using Dubois' key revealed that they resembled D. parviventosum and D. mahonae, although they more closely resembled the latter species. Unfortunately, no specimens of D. mahonae metacercariae or cercariae have been described to make comparisons at these stages. Furthermore, no descriptions for D. volvens adults are available in the literature. It is possible, therefore, that we have one and the same species here, but at different stages in the life-cycle. It would be interesting, therefore, to examine the metacercarial stages in the freshwater fishes of Northern Ireland in order to determine the

morphology of the metacercarial stage of D. mahonae

The cercarial stage of Type 4 diplostomes morphologically resembled those of D. parviventosum both morphologically and in the sensory papillar pattern. However, as detailed earlier, D. parviventosum is a lens diplostome and it is, therefore, unlikely that Type 4 diplostomes belong to this species.

Collation of the information obtained from each of the stages in the life-cycle of these diplostomes is summarised in Table 8.2. In summary, therefore, the identification of Type 1 metacercariae seems most likely to be D. spathaceum, but, no such confident identification can be made for the other three types using the information and descriptions available in the literature. However, Type 2 has most affinities with D. baeri and Type 4 with D. volvens, which they resemble biologically, although there are some morphological discrepencies. Type 3 metacercariae, on the other hand, do not closely resemble any named species of Diplostomum in the literature.

It is very disturbing that, although three stages stages in the life-history of three out of the four diplostomes studied have been described in the present study, a definitive identification still cannot be made using information currently available in the literature. The main problem with identification could be associated with morphological discrepencies caused by differences in fixation. It was shown that two specimens from the same location in the same eye are significantly different morphologically when different fixatives were used. It would seem impossible, therefore, to be able to identify or compare specimens when

material is fixed by different methods. In the present study it was found that allometric growth measurements were affected by fixation techniques rather than absolute measurements. This would tend to render the situation even more complicated, since it is often the relative proportions of characters that are used in identifications. Since fixatives have such a marked effect on specimens, it would be best if all workers used a common method of fixation. In the case of digeneans, the best fixative available is Berland's fluid, which is now widely used throughout the world for a wide range of helminths. It was used to fix all of the specimens in the present study except for cercariae, which were destroyed by this fixative. Berland's fluid produces well-fixed and relaxed specimens since it contains acetic acid to prevent contraction and formalin to control swelling. On the other hand acetic carmine, as used by Shigin, contains a much lower concentration of acetic acid compared to Berland's fluid and thereby allows contraction of the material, and hot water, as used by Niewiadomska, does not contain anything to prevent osmotic swelling if they are killed rapidly or, if the temperature was not hot enough, the specimens would contract due to the slowness of fixation. It is postulated, therefore, that some confusion may be reduced by the universal use of Berland's fluid. On the other hand, ideally one would like to define key characters which were independent of fixation artifact.

Hind-body development, a major taxonomic criterion in Diplostomum, is an area where discrepencies also occur in the literature. Many workers, for example, have removed adult diplostomes from the bird intestine within a matter of five or six days post-infection. In the present study, however, it was found that hind-body development was not fully achieved until day 16 post-infection, although they did produce eggs by day five which

indicated that they were mature. There is a possibility that development in our experimental hosts was unnaturally long, but since chickens and gull chicks have also been used by other workers, then this is unlikely. Furthermore, since Type 1 diplostomes appear to be D. spathaceum and the herring gull is a natural host of this species, it is assumed that the developmental period was normal. It could be, therefore, that other authors have determined that the specimens are fully developed once they have matured rather than looking at full hind-body development. Hind-body development was found to be a very important character in the present study in the case of Type 1 specimens, where it was found that the ovary was situated at the intersegmental junction on day five post-infection, but, by day 16, the ovary was one third of the way down the posterior segment. Since the position of the ovary is such an important taxonomic character, the identification of Type 1 worms would have been incorrect if made with younger worms.

The next step in this study would be to look at the DNA content of the diplostomes. Everything examined in this study has been one or more steps removed from the gene and, therefore, to look at the gene itself would be most likely to determine any differences or similarities in a much clearer fashion. The problem with diplostomes largely surrounds the fact that the species is so difficult to define, and, therefore, looking at the gene pool may clarify the situation. Isoenzyme analysis is just one step from the gene, since activity of the enzymes shows exactly what is coded for by the gene. The preliminary investigation into the isoenzyme profiles of diplostomes did not provide any conclusive evidence in this study partly due to technical difficulties and the shortage of time allocated, but it did produce some promising results that could usefully be pursued.

Examination of the adult stage would reduce the amount of individual variation in the gene pool and perhaps provide a clearer picture. Also, it may prove fruitful to test out more enzyme systems, as often it is only one particular enzyme that will reveal any distinct differences. It should be noted that such an undertaking would be both costly and time consuming and, therefore, should not be tackled without thorough thought.

A survey of all the freshwater fish species in the UK to determine all the types of metacercariae present would also enhance this study. This could be carried out alongside a survey of piscivorous birds and lymnaeid snails in order to establish all the stages and hosts in the life-cycle. From the results in this present study it would appear that the only comprehensive method to study parasites is to look at all the stages in the life-cycle, since this enhances the identification process.

Continuation of experimental work would also help the confused situation as one would be dealing with specimens of a known age, and so variation would be minimised. Experimental work on fish infections of many different species would also clarify host and site specificity of the four types of diplostomes.

In conclusion, from the present study it appears that there are four species of diplostomes in the freshwater fish hosts studied. The metacercariae of these four species are very site specific and there are also some indications of host specificity. Nevertheless, although the cercarial, metacercarial and adult stages in the life-cycle of three out of the four species of diplostomes examined in this study were obtained and characterised, a definite identification still cannot be made This indicates that many more life-cycle studies must be carried out in order

to clarify discrepencies in the literature and make keys more useful. This study has shed much more light on the situation four of the species in the UK, although more information is needed in order to define their identity. Problematic areas have been elucidated, thus clarifying the path of future work.

TABLE 8.1. Distinguishing features of the four types of diplostomes.

Type	Cercaria	Metacercaria
1	No. of caudal bodies and arrangement of body armature.	Lappets positioned very close together. VS-ANT/L small.
2	Not available.	Lacked distinguishing features.
3	Small cercariae (0.167-0.174mm long).	Small metacercariae (0.259-0.443mm long).
4	Large cercariae (0.271-0.301mm long).	Large metacercariae (0.366-0.510mm long). B/L small. VS-ANT/L high.

Type	Adult	Life-cycle
1	Large worms with the ovary located one third into the posterior segment.	Adults developed in both gulls and chickens, but best growth was achieved in the gulls. Metacercariae localised in the lens of rainbow trout.
2	Small ovary situated at the intersegmental junction.	Adults developed in both gulls and chickens, but best growth was achieved in chickens. Cercariae did not establish in *L. peregeer*. Metacercariae localised in the humour of rainbow trout.
3	Lacked distinguishing features.	Adults did not develop in chickens. Metacercariae localised in the retina of rainbow trout.
4	Vitellarium extends significantly more anterior. Ventral sucker located in a more anterior position.	Adults did not develop in chickens. Rainbow trout were refractive to these cercariae, but metacercariae localised in the retina of perch.

TABLE 8.2. Identifications obtained from the various stages in the life-cycle of each of the four diplostomes.

Type	Cercaria	Metacercaria	Adult
1	D. spathaceum	D. spathaceum	D. spathaceum
2	-	D. baeri	D. mergi**
			D. mahonae*
			D. baeri**
3	D. parviventosum	D. volvens*	D. parviventosum**
		D. pseudobaeri*	D. mahonae*
4	D. parviventosum	D. volvens	D. parviventosum*
			D. mahonae**

Note: No. of * designates similarity to the species where more than one species is involved.

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APPENDICES

APPENDIX 1

SPECIES	AUTHORITY	NOTES
abbreviatum	Brandes, 1888	Paradiplostomum La Rue, 1926
adamsi	Lester & Huizinga, 1977	N.American species.
alaroides	Dubois, 1937	Enhydridiplostomum Dubois, 1945
ambystomae	Rankin & Hughes 1937	Didelphdiplostomum Dubois, 1945
americanum	Dubois, 1936	Tylodelphys Diesing, 1850
amygdalum	Dubois & Pearson, 1965	Australian species ADULT ONLY
ardeae	Dubois, 1969	ADULT ONLY
ardeiformium	Odening, 1962	ADULT ONLY
attenuatum	Baryscheva, 1949	Neodiplostomum Raillet, 1919
auriculosum	Dubois & Pearson, 1967	Dolichochis Dubois, 1961
auriflavum	Molin, 1859	No diagram or measurements
auritum	Dujardin, 1845	Neodiplostomum Raillet, 1919
azimi	Gohar, 1933	Cynodiplostomum Dubois, 1936
baeri	Dubois, 1937	Vitreous humour form
baeri bucculentum	Dubois & Rausch, 1948	Humour/retinal form
baeri eucaliae	Hoffman & Hundley, 1958	Synon. of D. scudderi
brevicaudatum	Van Nordmann, 1832	Posthodiplostomum Dubois, 1936
brevis	MacCullum, 1921	Crocodilicola Poche, 1925
brevisegmentum	Perez Vigueras, 1944	ADULT ONLY
browni	Hughes, 1929	Snail species
bufonis	Kaw, 1950	Indian amphibian species
buteii	Vidyarthi, 1937	Dolichorchis Dubois, 1961
capsulare	Diesing, 1858	Very little given information
cardiophilus	Szidat, 1969	Tylodelphys Diesing, 1850
cerebralis	Chakrabarti, 1968	Indian cranial species
chromatophorum	Brown, 1931	Lens form

cinosterni	MacCullum, 1921	Crocodilicola Poche, 1925
clavatum	Van Nordmann, 1832	Tylodelphys Diesing, 1850
cobitidis	Von Linstow, 1890 Filippi 1854	Tetracotyle de
colymbi	Dubois, 1927	ADULT ONLY
compactum	Lutz, 1928	Hysteromorpha Lutz, 1931
commutatum	Diesing, 1850	Lens form
confusus	Krause, 1914	Bolbophorus Dubois, 1935
coniferum	Mehlis, 1846	Tylodelphys Diesing, 1850
coregonus	see Faulkner, 1989	Welsh species
corti	Hughes, 1929	Cranial species
craniarum	Diesing, 1858	Cranial species
crassum	Chandler & Rausch, 1948	American species
cuticola	Van Nordmann, 1832	Posthodiplostomum Dubois, 1936
darteri	Mehra, 1962	Tylodelphys Diesing,1850
desmognathi	Rankin, 1937	American body cavity species
destructor	Szidat & Nani, 1952	Tylodelphys Diesing, 1850
duboisi	Anantaraman & Balosubramaniam, 1953	synon. of D. buteii
duboisilla	Mehra, 1962	Tylodelphys Diesing, 1850
elongata	Lutz, 1928	Tylodelphys Diesing, 1850
ellipticus	Chakrabarti & Baugh, 1973	Indian species
erythropthalmi	Shigin, 1965	synon. of D. paracaudum
eucaliae	Hoffman & Hundley, 1957	synon. of D. baeri eucaliae
excavata	Rudolphi, 1803	Tylodelphys Diesing, 1850
flexicaudum	Cort & Brooks, 1928	synon. of D. spathaceum
fosteri	McIntosh, 1939	Enhydridiplostomum Dubois, 1945
gasterostei	Williams, 1966	Vitreous humour form synon. of D. pungiti
gavium	Guberlet, 1922	Tylodelphys Diesing, 1850

ghanensi	Ukoli, 1968	African species ADULT ONLY
gigas	Hughes & Berkhout, 1929	synon. of D. spathaceum
glossoides	Dubois, 1928	Tylodelphys Diesing, 1850
goborium	Shigin, 1965	Lens form
grande	Diesing, 1850	Posthodiplostomum Dubois, 1936
granulosum	Goss, 1941	synon. of D. triloba
gymnoti	Sudarikov, 1970	S.American cranial species
helveticum	Shigin, 1977	synon. of D. indistinctum
heronei	Srivastava, 1956	synon. of D. ketupanense
heterobranchi	Wedl, 1861	African cranial species
hieraetti	Vidyarthi, 1938	Glossodiplostomoides Bhalerae, 1942
hughesi	Markevich, 1934	Paracoenogonimus Katsurada, 1914
hupehensis	Pan & Wang, 1963	Chinese species
huronense	La Rue, 1926	American species synon. of D. baeri
ictaluri	Haderlie, 1953	American species
immer	Dubois, 1961	Tylodelphys Diesing, 1850
indicum	Thapar, 1967	Indian muscle species
indistinctum	Guberlet, 1923	synon. of D. spathaceum
joyeuxi	Joyeux, 1923	African species
ketupanense	Vidyarthi, 1937	Indian Dolichorchis species
ketupanense vietnamiae	Vidyarthi, 1937	Vietnamese Dolichorchis species
kronshnepi	Bychowskaja Pawlowskaja, 1954	ADULT ONLY
lenticola	Hughes, 1929	synon. of D. spathaceum
lepidosirensis	Jepps, 1929	Pericardial cavity species
leonensis	Williams, 1967	Posthodiplostomoides Williams, 1969
longum	Brandes, 1888	Proterodiplostomum Dubois, 1936
lucknowensi	Chakrabarti & Baugh, 1973	Indian species
macrostomum	Shigin, 1965	synon. of D. paracaudum

mahonae	Dubois, 1953	ADULT ONLY
marahoueense	Baer, 1957	Dolichorchis species
marshalli	Chandler, 1954	ADULT ONLY
mashonense	Beverly & Burton, 1963	Tylodelphys Diesing, 1850
mergi	Dubois, 1932	Lens form
mergi alaskense	Dubois, 1969	Alaskan species ADULT ONLY
metadena	Johnston & Angel, 1942	Australian species
microdenum	Cort & Brackett, 1938	Alaria Schrank,1788
minimum	MacCullum, 1921	Posthodiplostomum Dubois, 1936
minutum	Pandey, 1970	Indian species
mordax	Szidat & Nani, 1951	Austrodiplostomum Szidat & Nani, 1951
murrayense	Johnston & Cleland, 1938	Australian species
musculicola	Waldenburg, 1860 1927	Neascus Hughes,
musculorum percae	Michaloric, 1954	Very little given information
mutadomum	Wallace, 1937	Chinese species
nanus	Stiles & Goldberger, 1908	African species
nassa	Martin, 1945	American cranial species
nemachili	Zhatkanbaeva & Shigin, 1986	Nervous system
niedashui	Pan & Wang, 1963	Chinese species
nordmanni	Shigin & Sharipov, 1986	Lens form
numericum	Niewiadomska, 1988	outside the lens
nurius	Thapar, 1967	Indian species
odeningi	Gupta & Mishra, 1975	Adenodiplostomum Dubois, 1937
odhneri	Travassos, 1925	S.American skin species
oedicnemum	Singh, 1956	Indian species
ophthalmi	Pandey, 1970	Indian species
orientale	Yamaguti, 1934	synon. of D. mergi
paracaudum	Iles, 1959	Lens form

paraspathaceum	Shigin, 1965	Lens form
parviventosum	Dubois, 1932	Lens form
parvulum	Stafford, 1904	Canadian species
pelmatoides	Dubois, 1932	synon. of D. phoxini
petromyzi-fluviatilis	Hughes, 1929	Tylodelphys Diesing, 1850
phoxini	Faust, 1918	cranial species
pigmentata	Singh, 1956	Indian species
pileatum	Brandes, 1888	ADULT ONLY
podicipinum podicipinum	Kozicka & Niewiadoska, 1960	Tylodelphys Diesing, 1850
podicipinum robrauschi	Dubois, 1969	Tylodelphys Diesing, 1850
pseudobaeri	Razmashkin & Andrejuk, 1978	Perch and Coregonidae
pseudomergi	Belopolskaja, 1975	ADULT ONLY
pseudospathaceum	Niewiadomska, 1984	Lens form
pseudostomum	Willemoes-Suhm, 1870	Crocodilicola Poche, 1925
pungiti	Shigin, 1965	Vitreous humour form
pussillum	Dubois, 1928	Vitreous humour form
putorii	Von Linstow, 1877	Mammal oesophagus species
rhachiaeum	Henle, 1833	Tylodelphys Diesing, 1850
ranae	Cort & Brackett, 1938	N.American amphibian species
rauschi	Singh, 1956	Tylodelphys Diesing, 1850
repandum	Dubois & Rausch, 1950	Canadian species ADULT ONLY
rutili	Razmashkin, 1969	Lens form
sabahense	Fischtal & Kuntz, 1973	Malaysian species ADULT ONLY
scheuringi	Hughes, 1929	N.American species
schizothoracis	Faust, 1927	Persian skin species
scudderi	Olivier, 1941	N.American species
shigini	Zhatkanbaeva, 1978	ADULT ONLY

singhi	Pande, Bhatia & Rai, 1964	Indian species
sirensis	Kent, 1940	Pericardial cavity species
sirtali	Hughes, 1929	N.American species
soboleri	Shigin, 1959	ADULT ONLY
spathaceum	Rudolphi, 1819	Lens form Common eyefluke
spathulaeforme	Brandes, 1888	Neodiplostomum Raillet, 1919
splendeus	Manna & Chandhury, 1973	Dolichorchis Dubois, 1961
stahli	Rebecq & Leray, 1950	Cranial species
sterni	Gupta, 1958	Indian species
strigicola	Odening, 1962	Tylodelphys Diesing, 1850
sudarikovi	Shigin & Sudarikov, 1960	ADULT ONLY
thaparia	Lal, 1939	Neoalaria Lal, 1939
tregenna	Nazmi Gohar, 1932	Dolichorchis Dubois, 1961
triangulare	Johnston, 1964	Adenodiplostomum Dubois, 1937
trilobum	Rudolphi, 1819	Hysteromorpha Lutz, 1931
truttae	Lal, 1953	bron trout sp.
trituri	Kelley, 1934	Amphibian species
tulsipurensis	Chakrabarti & Baugh, 1973	Indian species
vancleavei	Agersborg, 1925	Posthodiplostomum Dubois, 1936
vanelli	Yamaguti, 1935	Japanese species
variabile	Chandler, 1932	Didelphdiplostomum Dubois, 1945
vegrandis	La Rue, 1917	Reptile and amphibian species
victorianus	Vercammen-Grandjean, 1960	S.African species
vidyarthi	Gupta & Mishra, 1975	Glossodiplostomoides Bhalerao, 1942
volvens	Van Nordmann, 1832	Retinal sp.
xenopi	Nigrelli Maraventalo, 1944	S.African pericardial species
yogenum	Cort & Brackett, 1937	synon. of D. baeri

APPENDIX 2

Sudarikov's (1964) Key : List of Named Species

	SPECIES	AUTHORITY
1.	D. spathaceum	(Rudolphi, 1819) Braun, 1893
2.	D. baeri	Dubois, 1937
3.	D. baeri baeri	Dubois, 1937
4.	D. baeri bucculentum	Dubois & Rausch, 1948
5.	D. baeri eucaliae	Hoffman & Hundley, 1957
6.	D. brevisegmentatum	Vigueras, 1944
7.	D. buteii	Vidyarthi, 1937
8.	D. colymbi	(Dubois, 1928) Nazmi, 1932
9.	D. commutatum	(Diesing, 1850) Dubois, 1937
10.	D. crassum	Chandler & Rausch, 1948
11.	D. flexicaudum	(Cort & Brooks, 1928) Haitsma, 1931
12.	D. heronei	Srivastava, 1954
13.	D. duboisi	Anantaraman & Balasubramanian, 1953
14	D. indistinctum	(Guberlet, 1923) Hughes, 1929
15.	D. ketupanensis	Vidyarthi, 1937
16.	D. kronschnepi	Bychowskaja-Pawlowskaja, 1953
17.	D. mahonae	Dubois, 1953
18.	D. marahounense	Baer, 1957
19.	D. marshalli	Chandler, 1954
20.	D. mergi	Dubois, 1932
21.	D. murrayense	(Johnston & Cleland, 1938) Johnston & Angel, 1941
22.	D. parviventosum	Dubois, 1932
23.	D. pelmatoides	Dubois, 1932
24.	D. phoxini	(Faust, 1918) Arvy & Buttner, 1954
25.	D. pusillum	(Dunois, 1928) Nazmi, 1932
26.	D. repandum	Dubois & Rausch, 1950
27.	D. soboleri	Shigin, 1959
28.	D. sudarikovi	Shigin, 1960
29.	D. thaparia	(Lal, 1939) Sudarikov, 1960
30.	D. tregenna	Nazmi, 1932
31.	D. vanelli	Yamaguti, 1935

Sudarikov (1971) species added to previous list

1.	D. erythrophthalmi	(Shigin, 1965) Shigin, 1969
2.	D. eucaliae	Hoffman & Hundley, 1957
3.	D. gobiorum	Shigin, 1965
4.	D. huronense	(La Rue, 1927) Hughes, 1929
5.	D. paraspathaceum	Shigin, 1965
6.	D. pungiti	Shigin, 1965
7.	D. rutili	Razmashkin, 1969

APPENDIX 3

Shigin's (1976) Key : Species Described

SPECIES NAME	AUTHORITY
1. D. petromyzifluviatilis	Diesing, 1850
2. D. phoxini	Faust, 1918
3. D. gobiorum	Shigin, 1965
4. D. commutatum	Diesing, 1850
5. D. mergi	Dubois, 1932
6. D. rutili	Razmaschkin, 1969
7. D. indistinctum	Guberlet, 1923
8. D. erythropthalmi	Shigin, 1965
9. D. paraspathaceum	Shigin, 1965
10. D. spathaceum	Rudolphi, 1819
11. D. pungiti	Shigin, 1965
12. D. baeri	Dubois, 1937
13. D. pusillum	Dubois, 1928

Species added in 1986 key.

1. D. nemachili	Zhatkanbaeva & Shigin, 1986
2. D. chromatophorum	(Brown, 1931)
3. D. parviventosum	Dubois, 1932
4. D. helveticum	(Dubois, 1929)
5. D. nordmanni	Shigin & Sharipov, 1986
6. D. pseudobaeri	Razmashkin & Andreyuk, 1978
7. D. volvens	Nordmann, 1832

APPENDIX 4

Dubois' (1970) Key : Species of Diplostomum Described.

	SPECIES NAME	DISTRIBUTION
1.	D. commutatum	Eastern Europe, Siberia and Alaska.
2.	D. sternae	India.
3.	D. crassum	Canada and Alaska.
4.	D. mahonae	Ireland.
5.	D. oedicnemum	India.
6.	D. phoxini	Europe.
7.	D. gavium	U.S.A., Alaska and Europe.
8.	D. pusillum	Europe and Asia.
9.	D. minutum	South America.
10.	D. micradenum	U.S.A.
11.	D. parviventosum	Europe.
12.	D. amygdalum	Australia.
13.	D. ardeae	U.S.A.
14.	D. mergi	
	subspecies;	
	D. mergi mergi	Europe and Asia.
	D. mergi alascense	Alaska.
15.	D. scudderi	U.S.A.
16.	D. baeri baeri	Europe
17.	D. baeri bucculentum	U.S.A.
18.	D. marshalli	U.S.A.
19.	D. repandum	U.S.A., Canada and Alaska.
20.	D. kronschnepi	Siberia.
21.	D. vanelli	Japan.
22.	D. spathaceum	
	subspecies;	
	D. s. spathaceum	Europe and Siberia.
	D. s. indistinctum	U.S.A.
	D. s. huronense	U.S.A., Canada and Alaska.
	D. s. murrayense	Australia.

Dubois also gives a description of;

D. triangulare Hughes, 1929
D. compactum Lutz, 1928
D. amygdalum Dubois & Pearson, 1965
D. gasterostei Williams, 1966
D. sudarikovi Shigin, 1960
D. sobolevi Shigin, 1959

APPENDIX 5

1. Polyacrylamide Gel Constituents.

36ml distilled water
7.5g sucrose
10ml 29.1% acrylamide
10ml 0.9% bis-acrylamide
De-aerate under the pressure of a vacuum
4ml Ampholine mixture (3.3-9.5)
0.4ml 0.005% riboflavin
Polymerise under fluorescent light.

2. Enzyme Development Mixtures.

PGM (EC 2.7.5.1)

50ml tris HCl pH8	100ml tris HCl pH8
3ml 10% $MgCl_2$	1.5g agar
100mg G-1-P	
15mg NADP	
15mg MTT	
1mg G_1-6$(PO_4)_2$	
4 units G6PDH	
10mg PMS	

GPI (EC 5.3.1.9)

50ml tris HCl pH8	100ml tris HCl pH8
4ml $MgCl_2$	1.5g agar
50mg fructose 6 PO_4	
25mg NADP	
25mg MTT	
25 units G6PDH	
10mg PMS	

AcP (EC 3.1.3.2)

100ml Na acetate pH5	50ml Na acetate pH5
4ml 10% $MnCl_2$	1.5g agar
200mg Na_2phenyl phosphate	
100mg Fast Blue 2R at 50°C	
Filter	

MDH (EC 1.1.1.37)

20ml Na malate	100ml tris HCl pH7
30ml tris HCl pH7	1.5g agar
70mg NAD	
30mg MTT	
10mg PMS	

GDH (EC)

50ml tris HCl pH8	100ml tris HCl pH8
1.46g Na_2DL glycerophosphate	
100mg NAD	1.5g agar
25mg MTT	
10mg PMS	

HK (EC 2.7.1.1)

50ml tris HCl pH8
8ml 10% $MgCl_2$
220mg ATP
220mg glucose
15mg NADP
15mg MTT
25 units G6PDH
10mg PMS

100ml tris HCl pH8
1.5g agar

LDH (EC 1.1.1.27)

20ml Na lactate
30ml tris HCl pH7
70mg NAD
30mg MTT
10mg PMS

100ml tris HCl pH7
1.5g agar

G6PDH (EC 1.1.1.49)

50ml tris HCl pH8
1.5ml 10% $MgCl_2$
20mg G6P
15mg NADP
15mg MTT
10mg PMS

100ml tris HCl pH8
1.5g agar